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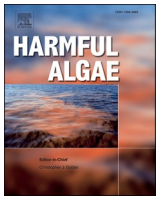

Precision early detection of invasive and toxic cyanobacteria: A case study of *Raphidiopsis raciborskii*

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ABSTRACT

Blooms of the toxic cyanobacterium, *Raphidiopsis raciborskii* (basionym *Cylindrospermopsis raciborskii*), are becoming a major environmental issue in freshwater ecosystems globally. Our precision prevention and early detection of *R. raciborskii* blooms rely upon the accuracy and speed of the monitoring method. A duplex digital PCR (dPCR) monitoring approach was developed and validated to detect the abundance and toxin-producing potential of *R. raciborskii* simultaneously in both laboratory spiked and environmental samples. Results of dPCR were strongly correlated with traditional real time quantitative PCR (qPCR) and microscopy for both laboratory and environmental samples. However, discrepancies between methods were observed when measuring *R. raciborskii* at low abundance ($1 - 10^5$ cells L^{-1}), with dPCR showing a higher precision compared to qPCR at low cell concentration. Furthermore, the dPCR assay had the highest detection rate for over two hundred environmental samples especially under low abundance conditions, followed by microscopy and qPCR. dPCR assay had the advantages of simple operation, time-saving, high sensitivity and excellent reproducibility. Therefore, dPCR would be a fast and precise monitoring method for the early warning of toxic bloom-forming cyanobacterial species and assessment of water quality risks, which can improve prediction and prevention of the impacts of harmful cyanobacterial bloom events in inland waters.

1. Introduction

Harmful cyanobacterial blooms are prevalent in freshwater ecosystems globally and are exacerbated by climate change and human induced global change (Huisman et al., 2018). The occurrence and blooms of the invasive cyanobacterium *Raphidiopsis raciborskii* (formerly *Cylindrospermopsis raciborskii*) have rapidly increased in tropical/subtropical lakes and reservoirs worldwide over the past decades (Haande et al., 2008; Antunes et al., 2015; Yang et al., 2017; Sidelev et al., 2020). The cylindrospermopsins (CYNs) produced by *R. raciborskii* pose adverse health effects on both humans and other animals (Rzymski and Poniedzialek, 2014; Sidelev et al., 2020). Several strains of *R. raciborskii*, from South America, are also able to produce saxitoxins that are harmful to zooplankton (Ferrão-Filho and da Silva, 2020). Many recent studies have centered on the cause and consequence of harmful cyanobacterial blooms (Sinha et al., 2012; O'Neil et al., 2012; Huisman et al., 2018). However, the development of predictive and mechanistic models for *R. raciborskii* blooms is limited by the quality of data from different monitoring methods. Such datasets are an essential tool for better characterization of the formation, development and dynamics of *R. raciborskii* blooms and for deeper understanding into the causes and consequences of blooms. Therefore, there is an urgent need for precise early detection of *R. raciborskii* in water bodies to improve the accuracy of the datasets.

Though there are various monitoring approaches and techniques of cyanobacteria (including *R. raciborskii*), it is not trivial to assess the potential risks of blooms early, rapidly and accurately (Merel et al., 2013). Microscopy is the gold standard method for monitoring harmful

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algae for species identification and cell counts (Yang et al., 2017). However, microscopy-based cell enumeration requires well trained professionals, it is time-consuming and labor intensive. Recently, flow cytometry technology has overcome some of these limitations (Wert et al., 2013; Sanford and Bischel, 2019). However, these methods have relatively low accuracy that only can resolve genus level identification and there is a potential that harmful bloom-forming species will not be detected during the pre-bloom period (Hatfield et al., 2019). In addition, these morphology-based examinations cannot identify and distinguish the toxin-producers among the cyanobacteria (Gaget et al., 2017; Lee et al., 2020). Recently, high-throughput sequencing analysis based on marker genes has led to the detection of cyanobacteria that were not quantified by microscopy and flow cytometry methods (Zamyadi et al., 2019), however, it is a challenge to achieve the absolute quantification of cyanobacteria in waters using such an approach because of the PCR primer preference and variation in rDNA copy number per cell (Louca et al., 2018). The biochemical methods and physicochemical methods such as enzyme linked immunosorbent assay (ELISA), protein phosphatase inhibition assay (PPIA), high performance liquid chromatography (HPLC), liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) are not sufficient to identify the toxins until a high cyanobacterial biomass occurs (Li et al., 2001; Hilborn et al., 2007; Ballot et al., 2010). In this case, the early prevention measures are no longer applicable effectively and efficiently.

Gaps in the aforementioned methods have been addressed through the successful application of quantitative polymerase chain reaction (qPCR) to detect toxin biosynthesis genes and therefore toxin-producing cyanobacteria in freshwater bodies (Rasmussen et al., 2008; Barón-Sola et al., 2016; Pacheco et al., 2016). Previous studies proved that cylindrospermopsin biosynthesis genes (i.e. *cyr* genes) are exclusively present in cyanobacterial producers, in which *cyrJ* gene can be amplified to assess the toxigenic potential of environmental samples with toxin-producing cyanobacteria (Schembri et al., 2001; Mihali et al., 2008; Rzymski and Poniedzialek, 2014). The qPCR assay is able to quantify the genetic target, and it has been used to identify toxic *R. raciborskii* strains and to quantify the copy numbers of the genes responsible for cylindrospermopsins production in reservoirs or lakes (Pearson and Neilan, 2008; Martins and Vasconcelos, 2011; Rodriguez et al., 2017). Although recent studies have suggested there is not a strong correlation between toxin-synthesis gene copies and toxin yield, due to differences in toxin cell quotas between strains (Davis et al., 2009; Orr et al., 2010; Savela et al., 2014; Chiu et al., 2016; Lu et al., 2019), the early detection of toxin synthesis genes is an important bioindicator for risk management of harmful cyanobacterial blooms. A significant limitation of qPCR assays is that it relies on a standard curve that is affected by delayed amplification or variability in threshold cycle (Cq) values, affecting the accuracy of quantification (Sivaganesan et al., 2010). Generally, inhibitions in PCR amplification are especially problematic at very low gene abundance in complex environmental samples (Cao et al., 2013). To overcome these limitations, there is a pressing need to develop a new method that is faster, more accurate, and has lower limits of detection.

Recently, digital PCR (dPCR) technology has been used to detect and quantify the abundance of algal species (Te et al., 2015; Lee et al., 2020). It is an alternative method of quantitative PCR that derives the target DNA abundance from the ratio of the number of positive partitions to a large number of total reaction partitions generated from a known volume of PCR mix. The dPCR method allows absolute quantification that amplifies different genes from single bacterial cells (Ottesen et al., 2006). Furthermore, dPCR results exhibit improved precision, higher repeatability, and higher tolerance for PCR inhibition compared to qPCR assays (Sanders et al., 2011; Whale et al., 2012; Wang et al., 2016). Moreover, duplex dPCR assays were successfully developed in order to reduce the time and cost of sample analysis (Cao et al., 2015; Te et al., 2015). Despite the potential advantages of dPCR in monitoring the abundance of *R. raciborskii*, there is still no study which detected and quantified *R. raciborskii* and its toxin-producing potential in one assay simultaneously.

Different approaches may potentially lead to different detection results of harmful cyanobacteria. These discrepancies highlight the need for a precise detection approach to better understanding the uncertainties, especially at low cyanobacterial abundance or pre-bloom periods. In this study, we developed and established a new duplex dPCR assay to simultaneously quantify the abundance of total *R. raciborskii* cells and cylindrospermopsin-producing cells. The assay performance of dPCR was compared with traditional qPCR and microscopy. We aimed to: (1) establish a dPCR procedure to simultaneously quantify the abundance and toxin-producing potential of *R. raciborskii*; (2) estimate the accuracy and precision of dPCR for detection of *R. raciborskii* in both laboratory spiked and environmental samples; and (3) compare the results between dPCR, qPCR, microscopy, and high-throughput sequencing approaches for precise early detection of bloom-forming *R. raciborskii*.

2. Materials and methods

2.1. Sampling

We have studied algal community dynamics since 2009 in reservoirs in Xiamen, southeast China (Yang et al., 2012, 2016, 2017; Gao et al., 2021). The water samples (0.5 m depth) were collected from a long-term monitoring program for Xiamen reservoirs. We monitored the plankton communities in Shidou/Bantou, Tingxi and Xinglinwan reservoirs approximately once every 45 days, once every ten days and twice a week, respectively. The *R. raciborskii* cells were observed in Shidou Reservoir and Bantou Reservoir, whereas no *R. raciborskii* cell was observed in Tingxi and Xinglinwan reservoirs after 2016. The details of these reservoirs were described in our previous studies (Gao et al., 2019; Fang et al., 2019).

For field research, both Shidou Reservoir and Bantou Reservoir (Fig. 1) have been the subject of long-term monitoring by members of Aquatic Eco-Health Group, and the algal species (including *R. raciborskii*) were routinely identified and counted by microscopy, the biomass of *R. raciborskii* showed cyclic changes with three bloom or dominance events from 2010 to 2017. Water samples were taken from upstream, midstream and downstream in each reservoir with 37 sampling visits (0.5 m depth) from January 2010 to October 2017 (Table S1). The details of these two reservoirs were described in our previous studies (Yang et al., 2012, 2017).

2.2. Species identification, isolation and cultures

The *R. raciborskii* strain (XM1) was isolated from the Shidou Reservoir in April 2018 (Fig. 1). The culture systems were washed at least three times to remove any microorganisms, then the *R. raciborskii* was maintained in BG11 medium at 27 ± 1 °C under a 12:12-h light: dark photoperiod in a temperature- and light-controlled growth chamber (PGX 250, Safu, Ningbo, China) with light intensity of 10 μmol photons $m^{-2} s^{-1}$.

Enzyme linked immunosorbent assay (ELISA) kit (#522,011; Abraxis, Warminster, PA, USA) was used to check the ability of XM1 strain to produce cylindrospermopsins (CYNs). After the toxin was confirmed, the XM1 strain was used to develop the dPCR method to quantify the abundance and toxin-producing potential of *R. raciborskii*. In addition, one nontoxic *R. raciborskii* strain (N10) (which was kindly provided by Dr. Lamei Lei at Jinan University, China) was used to verify the specificity of primers and probes used in this study.

2.3. DNA extraction

To extract the DNA from the pure cultures of *R. raciborskii*, about 10 mL aliquots were first centrifuged at 12,000 *g* for 5 min to separate the

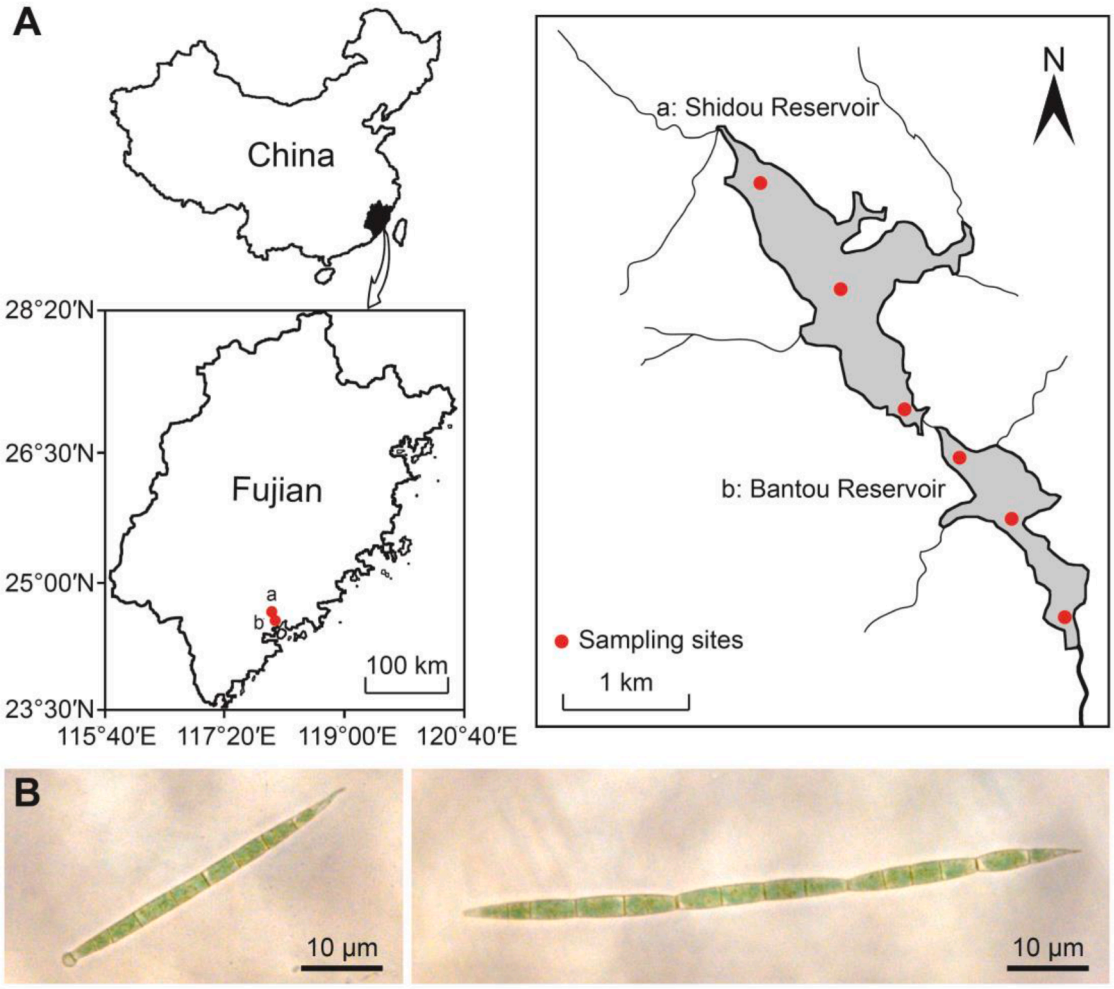

Fig. 1. (**A**) Sampling sites of Shidou and Bantou reservoirs in Fujian province, southeast China (revised from Yang et al., 2017). (**B**) Microscopic photographs of *Raphidiopsis raciborskii* (Scale bar = 10 μm).

cells from the culture medium. For other cyanobacteria-laden samples, including 72 laboratory spiked samples and 222 environment samples, to separate cells, they were filtered through a 0.22 μm pore-size polycarbonate filter (47 mm diameter, Millipore, Billerica, MA, USA). Both the centrifuged samples and cells retained on the filters were stored at −80 °C before DNA extraction. The DNA extractions were operated using the FastDNA spin kit (MP Biomedicals, Santa Ana, CA, USA) following the manufacturer's instructions. The DNA samples were stored at −80 °C until follow-up analyses.

2.4. Laboratory experiment and field research

Both dPCR and qPCR assays were performed on the genomic DNA from pure cyanobacterial strains (nontoxic N10 and toxic XM1) at various concentrations to verify the specificity and sensitivity of the primers and probes used. In addition, environmental water samples (which were collected from Shidou, Tingxi and Xinglinwan reservoirs) were used to estimate the effects of water matrix on the performance of these primers and probes.

The experimental design was summarized in Table 1 and described in detail below. Laboratory experiments were used to compare dPCR and qPCR approaches on quantification of the abundance and toxin-producing potential of *R. raciborskii*. First, pure *R. raciborskii* cells were spiked, at concentrations of 0, 10^3, 5×10^3, 10^4, 5×10^4, 10^5, 10^6 cells L^{-1}, into deionized pure water (control group, Group 1), reservoir water without *R. raciborskii* (i.e. waters from Tingxi Reservroir, Group 2), and reservoir water with *R. raciborskii* (i.e. waters from Shidou Reservoir, Group 3), respectively. As *R. raciborskii* was present in Shidou Reservoir samples approximately equal to 4×10^7 cells L^{-1}, the spiked Shidou Reservoir samples were with concentrations in the range of approximately $4 - 5 \times 10^7$ cells L^{-1}. For the comparative study, we artificially defined three abundance levels: the cell concentrations of 0 to 5×10^4 cells L^{-1} indicated the low abundance of *R. raciborskii* (level 1); the cell concentrations between 10^5 and 10^7 cells L^{-1} represented the medium abundance (level 2); the cell concentrations in of $\geq 10^7$ cells L^{-1} represented the high abundance of *R. raciborskii* (level 3). In addition, each group represented an aquatic ecosystem status, each group contained seven cell concentrations with an increasing trend. All samples were performed in triplicate. The purified DNA extracts of 72 samples were run by dPCR and qPCR side by side.

Quantification using dPCR was further compared with qPCR and microscopy using environmental water samples for field research, included 222 samples from Shidou and Bantou reservoirs (Table S1).

2.5. Cell counting

For microscope-based monitoring, 222 water samples collected from Shidou and Bantou reservoirs (Table S1) were preserved with Lugol's solution (1.5% by volume). The cells of *R. raciborskii* were identified and quantified using an inverted microscope and at least 500 algal individuals were enumerated for each sample (Yang et al., 2017). Then at least 50 individuals were investigated to measure the length of the

Table 1
Experimental design and samples for detection of *Raphidiopsis raciborskii*.

Experiment	Groups	Test samples	Approaches
Laboratory samples	Group 1 (pure strain of *R. raciborskii*)	0, 10^3, 5×10^3, 10^4, 5×10^4, 10^5, 10^6 cells L^{-1} of *R. raciborskii*, triplicates	dPCR, qPCR, and microscopy
	Group 2 (pure strain and Tingxi)	0, 10^3, 5×10^3, 10^4, 5×10^4, 10^5, 10^6 cells L^{-1} of *R. raciborskii* mixed with raw water samples from Tingxi Reservoir, triplicates	
	Group 3 (pure strain and Shidou)	0, 10^3, 5×10^3, 10^4, 5×10^4, 10^5, 10^6 cells L^{-1} of *R. raciborskii* mixed with raw water samples from Shidou Reservoir, triplicates	
Field samples	High abundance (bloom stage)	Log cells L^{-1} of *R. raciborskii* ≥ 7	dPCR, qPCR, microscopy, and high-throughput sequencing
	Medium abundance (non-bloom stage)	Log cells L^{-1} of *R. raciborskii* $5-7$	
	Low abundance (non-dominant stage)	Log cells L^{-1} of *R. raciborskii* < 5	

Note: For the laboratory spiked experiment, $0-5 \times 10^4$ cells L^{-1} indicated the low abundance period of *R. raciborskii* XM1 (representing non-dominant stage); 10^5–10^6 cells L^{-1} of *R. raciborskii* XM1 indicated the medium abundance period of *R. raciborskii* XM1 (representing early dominant and non-bloom stage); the Group 3 indicated the high abundance period of *R. raciborskii* XM1 (representing the absolutely dominant or bloom stage).

R. raciborskii filament and cell. Finally, the average cells per filament were calculated by dividing the average filament length by the average cell length (Willis et al., 2015). The environmental samples were artificially defined with three abundance groups to evaluate the applicability and reproducibility of dPCR to quantify the *R. raciborskii* under different abundance conditions (high, medium and low densities of *R. raciborskii*) during the field research. The high abundance group was defined with the log *R. raciborskii* cell number (cells L^{-1}) ≥ 7, medium abundance group was defined with the log *R. raciborskii* cell number (cells L^{-1}) from 5 to 7, and low abundance group was defined with the log *R. raciborskii* cell number (cells L^{-1}) < 5, respectively.

2.6. Plasmid DNA preparation

Two genes targeting *R. raciborskii* (*rpoC1*, a species-specific gene) and specific to target CYN-producing strain (*cyrJ*, a CYN-producing gene) were respectively used to quantify the abundance and toxin-producing potential of *R. raciborskii* (Wilson et al., 2000; Jiang et al., 2012; Gaget et al., 2017). PCR amplification of targeted genes within the toxic *R. raciborskii* XM1 strain was carried out in a S1000TM Thermal Cycler (Bio-Rad Laboratory, Inc., Hercules, CA, USA). The PCR amplification products were purified using Easy Pure Quick Gel Extraction Kit (Transgene Biotech, Beijing, China), cloned into pMD19-T vector (Takara Bio Inc., Otsu, Shiga, Japan) and transformed into *Escherichia coli* DH5α (Takara Bio Inc., Otsu, Shiga, Japan). The plasmid DNA was extracted using a TIANprep Midi Plasmid Kit (Tiangen Biotech, Beijing, China). The concentration and quality of plasmids were assessed with a NanoDrop 1000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). The generated plasmid DNA was sequenced to verify the correct target sequences.

To evaluate the detection limit and detection range of dPCR and qPCR assays for *rpoC1* and *cyrJ* genes, respectively, 10-fold serial dilutions of the plasmid samples with 10^0–10^{10} copies of *rpoC1* and *cyrJ* genes were prepared in triplicate.

2.7. Digital PCR (dPCR)

A duplex dPCR assay was developed to allow simultaneous measurement of abundance (*rpoC1* gene) and toxin-producing potential (*cyrJ* gene) of *R. raciborskii*. The *rpoC1* gene was quantified using a VIC-BHQ1 probe (5′-VIC-TCCTGGTAATGCTGACACACTCG -BHQ1–3′), while the *cyrJ* gene was detected using a FAM-BHQ2 probe (5′-FAM-AGCATTCTCCGCGGATCGTTCAGC-BHQ2–3′). The *rpoC1* gene was amplified by the specific primer cyl2/4 (Wilson et al., 2000), while the *cyrJ* gene was targeted by the primer cyrJF/R (Jiang et al., 2012). The optimized PCR mixture containing 1 × QuantStudio® 3D Digital PCR Master Mix v2 (Life Technologies, ThermoFisher Scientific, Waltham, MA, USA), 0.2 μM (each) probes, 0.9 μM (each) primers, 1.45 μL of DNA and nuclease-free water. A total of 14.5 μL reactions proceed immediately to load the QuantStudio™ 3D Digital PCR Chip v2. The dPCR amplification was performed on an QuantStudio™ 3D digital PCR instrument (Applied Biosystems Inc., Carlsbad, CA, USA) using a ProFlex™ 2 × flat PCR system using the optimized conditions: 10 min at 95 °C, followed by 40 cycles of 2 min at 58 °C and 30 s at 95 °C, followed by 2 min at 58 °C and hold at 10 °C. All samples were run with both non-template negative controls and positive controls, respectively.

The dPCR data were analyzed using the QuantStudio™ 3D AnalysisSuite™ Software. The data were checked and the samples with filled wells below the 10,000 or the percentage of low quality wells > threshold of 5% were excluded from further analysis. The copy number values of dPCR were generated according to a Poisson maximum-likelihood algorithm (Groth, 1982). The data were $\log10(x+1)$ transformed before statistical analyses.

2.8. Real-time quantitative PCR (qPCR)

The qPCR assay was performed on a Lightcycler 480 instrument (Roche, Basel, Switzerland) using a SYBR® Green approach. The same primers in the dPCR were used in the qPCR analysis. The PCR mixture containing 10 μL 2 × TransSmart® Top Green qPCR SuperMix (Transgene Biotech, Beijing, China), 0.9 μM (each) primers and 2 μL of DNA and nuclease-free water. The qPCR reaction targeted on *rpoC1* gene was amplified with the cycling conditions: 95 °C for 5 min, 40 cycles of 95 °C for 20 s, 58 °C for 30 s, 72 °C for 45 s. The qPCR reaction targeted *cyrJ* gene was amplified at 95 °C for 5 min, 40 cycles of 95 °C for 20 s, 58 °C for 20 s, 72 °C for 20 s. qPCR reactions were tested in triplicate for each sample. Both negative and positive controls were used throughout the experiment.

The qPCR standard curves were established with diluted plasmid DNA with concentration from 10^3 to 10^9 gene copies. The standard curves were generated as a linear regression between the quantification cycles (Cq) values and the logarithmic plasmid copy numbers. The plasmid copy numbers were calculated from the concentration of plasmid DNA used in the assays. The amplification efficiency of qPCR was between 95% and 105%. Only values of the samples within the standard curve range are effective and reliable (data outside of the standard curve or ideal range may be unreliable), and the copy number of each gene was calculated. Finally, the data of qPCR were $\log10(x+1)$ transformed before statistical analyses.

2.9. High-throughput sequencing

Bar-coded fragments of the 16S rRNA gene of the 222 environmental samples were sequenced using Illumina HiSeq platform (Illumina, Inc., San Diego, CA, USA) using a paired-end sequencing strategy. The sequence quality control and operational taxonomic units (OTUs) definition were processed with QIIME as previously described (Liu et al., 2019). Finally, the sequences and OTU of *R. raciborskii* were extracted from the whole bacterial community dataset. All sequence data from this study have been deposited in the public NCBI Sequence Read Archive (SRA) database under the BioProject number PRJNA689332 and the

accession number SRP121028.

2.10. Statistical analysis

The cyanobacterial data were $\log_{10}(x + 1)$ transformed before statistical analyses. Linear correlation, Spearman's correlation and paired *t*-test were used to assess differences between dPCR, qPCR, and microscopy. The linear regressions were carried out using Sigmaplot v14.5 (Systat Software Inc., San Jose, CA, USA). The correlation coefficients and *t*-test were analyzed in SPSS v22.0 (IBM Corp., Armonk, NY, USA). A heatmap was employed to show the *R. raciborskii* cell density, gene abundance and percentage of *R. raciborskii* in the total bacterial community in the environmental samples using the 'pheatmap' package in the R environment (R Core Team, 2020).

3. Results

3.1. Specificity and sensitivity of PCR primer or probe

Probe specificity and primer specificity were confirmed using the toxic *Raphidiopsis raciborskii* strain (XM1), nontoxic *R. raciborskii* strain (N10), and environmental samples (Table 2). The copy numbers of *rpoC1* and *cyrJ* genes measured by dPCR were lower than qPCR approach. However, the copy numbers of the two genes based on dPCR were almost the same as the cell number enumerated by microscopy. Both dPCR and qPCR for toxic XM1 strain and Shidou Reservoir water samples showed positive amplification of *rpoC1* and *cyrJ* genes. Further, the copy numbers of *rpoC1* and *cyrJ* genes confirmed that there was no competition between amplification of two genes in duplex dPCR. The nontoxic N10 strain showed a successful amplification of *rpoC1* gene, but no amplification of *cyrJ* gene in both dPCR and qPCR assays (Table 2), indicating a specific amplification without false positive. Furthermore, both Tingxi Reservoir and Xinglinwan Reservoir water samples showed no amplification of *rpoC1* and *cyrJ* genes, thereby confirming no significant interference of environmental inhibitory factors on dPCR amplification reaction. More importantly, both dPCR and qPCR run on negative controls showed no amplification throughout the study. The probes and primers for *R. raciborskii* were thus considered specific and sensitive to the *rpoC1* and *cyrJ* genes.

The dPCR showed a lower quantification range with lower detection limitation than qPCR (Fig. S1). The serial dilution of plasmid DNA of *rpoC1* and *cyrJ* genes for dPCR was run in triplicate. The dynamic range of *rpoC1* and *cyrJ* genes for dPCR reactions was between 10^0 and 10^4 gene copies per reaction. The limit of detection of dPCR assay was 1.5 and 1.2 gene copies per reaction for *rpoC1* gene and *cyrJ* gene, respectively. For qPCR, the linear dynamic range of *rpoC1* and *cyrJ* genes was between 10^4 and 10^{10} gene copies per reaction, suggesting that the lower and upper limits of qPCR assay was 10^4 and 10^{10} gene copies per reaction, respectively.

Table 2
The *rpoC1* and *cyrJ* gene copies of *Raphidiopsis raciborskii* determined by dPCR and qPCR assays under known cell number.

Sample ID	Cell number (cells L^{-1})	dPCR (copies L^{-1})		qPCR (copies L^{-1})	
		rpoC1	*cyrJ*	*rpoC1*	*cyrJ*
Negative control (pure water)	0	0	0	0	0
N10 (positive control)	1000	1500	0	1.33×10^4	0
XM1 (positive control)	1.08×10^8	1.62×10^8	1.30×10^8	9.44×10^9	8.10×10^9
Shidou	3.32×10^7	4.98×10^7	3.99×10^7	8.58×10^8	8.33×10^8
Tingxi	0	0	0	0	0
Xinglinwan	0	0	0	0	0

Each sample was in triplicate.
N10, *R. raciborskii* N10 strain which cannot produce cylindrospermopsin.
XM1, *R. raciborskii* XM1 strain which can produce cylindrospermopsin.
Negative control, sterilized pure water; Shidou, water samples from Shidou Reservoir; Tingxi, water samples from Tingxi Reservoir; Xinglinwan, water samples from Xinglinwan Reservoir.

3.2. Accuracy and precision of dPCR assay

The experimental data for the laboratory samples were used to evaluate the accuracy, precision, and the potential inhibitors of dPCR and qPCR assays (Fig. 2). The results from dPCR and qPCR showed a significant linear relationship for both *rpoC1* or *cyrJ* genes in all three experimental groups, with correlation coefficient $R = 0.91$–0.98 ($P < 0.01$) for *rpoC1* gene and with $R = 0.96$–0.98 ($P < 0.01$) for *cyrJ* gene. However, the gene abundance measured by dPCR assay was lower than the qPCR. The magnitude of difference between two methods were 0.00–1.15 log units for *rpoC1* gene and 0.04–1.21 log units for *cyrJ* gene, respectively.

Significant linear correlations were found between the two genes (*rpoC1* and *cyrJ*) and cell number of *R. raciborskii* in the Group 1 and Group 2 samples (Fig. 3), with the $R = 0.95$–0.96 ($P < 0.01$) for dPCR assay and with $R = 0.91$–0.94 ($P < 0.01$) for qPCR assay. The data between gene copies and cell numbers in Group 3 (high abundance) samples failed to fit a linear curve, perhaps due to the high background cell concentration of *R. raciborskii* (Log_{10} cell numbers $L^{-1} = 7.5$).

The dPCR assays exhibited a better accuracy than qPCR, when comparing the results with microscopy, because dPCR assay results were nearly identical with microscopy results (Fig. 3, Table 2). In addition, paired t-test indicated statistically significant difference between *rpoC1* and *cyrJ* genes measured by qPCR and cell numbers. The magnitude of mean difference between qPCR-based gene copies and cell numbers were 0.09–1.02 log units for *rpoC1* gene and 0.01–0.97 log units for *cyrJ* gene, respectively.

The quantification results of the dPCR assay showed a higher precision and reproducibility for the replicates than qPCR, especially in relatively low cell concentration of *R. raciborskii* (Fig. S2). The standard deviations between replicates of *rpoC1* and *cyrJ* genes in dPCR assay were much lower than qPCR in Group 1 and Group 2 samples when the cell abundance higher than 10^3 cells L^{-1}. However, almost equal standard deviation was observed in dPCR and qPCR in high concentration of *R. raciborskii* (i.e. the Group 3 samples).

3.3. Application in monitoring environmental samples

The dPCR and qPCR assays were further applied to quantify the abundance and toxin-producing potential of *R. raciborskii* in 222 environmental samples from Shidou and Bantou reservoirs. dPCR results exhibited a significant correlation with qPCR in three different groups (Fig. 4), when either detected or undetected data in both dPCR and qPCR assays were used in the linear regression analysis. However, the significant linear correlations between dPCR and qPCR assays were relatively weak when all samples were included (Fig. S4).

The results of the dPCR and qPCR assays showed a significant correlation regardless of target genes and distinct cell abundance groups in Shidou and Bantou reservoirs (Table S2). The *rpoC1* and *cyrJ* gene copies measured by dPCR and qPCR were strongly correlated with cell numbers generated by microscopy in all samples ($P < 0.01$). However, different results were observed in the three different cell abundance groups for PCR and microscopy.

In general, the different monitoring approaches (dPCR, qPCR and microscopy) showed similar trends for the abundance dynamics of *R. raciborskii* (Fig. S5), but the cell count was somewhat higher than the dPCR and qPCR measurement in 2010. Furthermore, the *rpoC1* and *cyrJ* gene copies measured by dPCR were generally lower than qPCR. However, the detection rate of *rpoC1* (95%–98%) and *cyrJ* (92%–96%) genes measured by dPCR were significantly higher than that of qPCR (76%–77% for *rpoC1* gene and 55–58% for *cyrJ* gene, respectively), high-

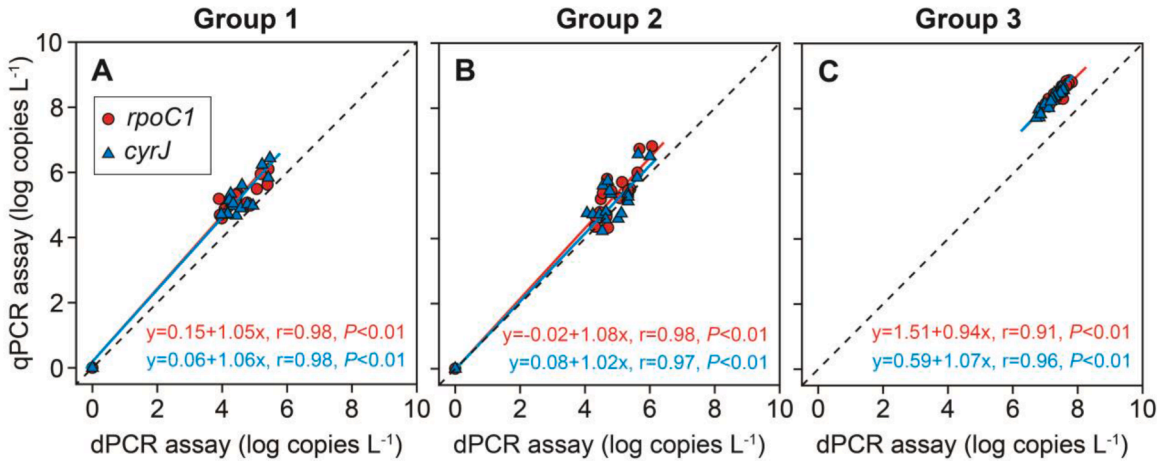

Fig. 2. Correlations between *Raphidiopsis raciborskii* XM1 gene copies measured by dPCR and qPCR assays. (**A**) Group 1, pure strain of *R. raciborskii*; (**B**) Group 2, mixture of pure strain and Tingxi Reservoir waters (without *R. raciborskii*); (**C**) Group 3, mixture of pure strain and Shidou Reservoir waters (with high abundance of *R. raciborskii*). The dash lines in the figures are line of $y = x$.

throughput sequencing (87%–90%) and microscopy (79%–86%) in environmental samples (Fig. 5). These results confirm that dPCR has higher sensitivity than qPCR and microscopy, especially with *R. raciborskii* at low-abundance condition.

Specifically, the dPCR assay exhibited higher sensitivity and reproducibility than qPCR in all distinct cell abundance groups due to higher detection rate (Table 3) and lower standard deviation of replicate samples at cell density $> 10^3$ cells L^{-1} (Fig. S2). The *rpoC1* and *cyrJ* genes were detected by dPCR almost in every sample (97%–100%) in high and medium abundance group. However, *rpoC1* gene was detected by qPCR in 86%–97% high abundance samples, and 73%–91% medium abundance samples; while *cyrJ* gene was detected by qPCR in 73%–84% high abundance samples, and 57%–69% medium abundance samples. For the low abundance samples, the dPCR had the highest sensitivity or detection rate (86%–90% for *rpoC1* gene and 79%–81% for *cyrJ* gene, respectively), followed by qPCR (48%–55% for *rpoC1* gene and 21%–24% for *cyrJ* gene, respectively) and microscopy (29%–45%). Importantly, the detection rate of *cyrJ* gene in all tests was equal to or lower than that of *rpoC1* gene (Table 3). Additionally, our high-throughput sequencing results indicated *R. raciborskii* occurred in 87%–90% of the environmental samples from 2010 to 2017 (Fig. S6).

3.4. Comparison between dPCR, qPCR and microscopy

Different methods have different advantages and disadvantages (Table 4). The primers and probes used for dPCR and qPCR assays achieved high-specificity and high-sensitivity to the target genes. The dPCR assay allowed direct absolute quantification of target genes that realizes a standard curve-free quantification as compared to qPCR. More importantly, dPCR assay was more sensitive at low gene concentrations (Fig. S1), although it had a narrower quantification range as compared to qPCR and microscopy (Table 4). Moreover, dPCR assay had higher accuracy and precision than qPCR in detecting targets of low-abundance genes (Fig. 3, Fig. 4). The economic cost of dPCR was slightly higher than qPCR and microscopy. However, less time was required for dPCR (96 samples per day) compared to qPCR (72 samples per day depending on the equipment) and microscopy (5 samples per day) for large sample sizes. In addition, the dPCR and qPCR assays required higher technical ability, but a well-trained expert was essential for the microscopy method.

4. Discussion

4.1. The performance of dPCR assay

In this study, new duplex dPCR assays were successfully established and applied to simultaneously quantify the abundance and toxin-producing potential of *Raphidiopsis raciborskii* in both laboratory and environmental samples. To better evaluate the quantification of duplex dPCR for abundance and toxicity of *R. raciborskii*, two single copy genetic markers (i.e. *rpoC1* gene for *R. raciborskii* and *cyrJ* for cylindrospermopsin-producing gene) were selected to avoid quantification biases from multiple copy genes per cell. There is no gene copy number variation observed in these two marker genes (Merel et al., 2013). Therefore, both *rpoC1* and *cyrJ* genes in laboratory samples enumerated by duplex dPCR measurement were nearly identical to real cell values, also indicating the accuracy of this technique. Te et al. (2015) first determined the cyanobacterial abundance (*Cylindrospermopsis* and *Microcystis*) based on genus-specific genes using dPCR, but did not consider the toxin-producing gene. The duplex dPCR can provide more measurement than uniplex dPCR when using small amounts of samples or materials (Whale et al., 2013). An example of simultaneous droplet dPCR analyses of a pathogenic bacterium (*Enterococcus*) and human fecal-associated genetic HF183 marker also illustrates the advantage of droplet dPCR in multiplexing measurement (Cao et al., 2015). The isolated *R. raciborskii* N10 (nontoxic) and XM1 (toxic) strains were assayed by dPCR to analyze the presence or absence of the *cyrJ* gene. The production of cylindrospermopsin was in accordance with the presence of *cyrJ*. Consequently, these results further strengthen the evidence for the relationship between *cyrJ* gene and the production of cylindrospermopsin. Therefore, the toxin associated dPCR assay could confirm the toxic potential of a cyanobacterium or cyanobacterial bloom.

The dPCR assay was accurate for quantifying *R. raciborskii* and its toxin-producing potential from both laboratory spiked and environmental samples, but had a narrower quantification range than qPCR, as also reported previously (Te et al., 2015). However, based on the definite quantification range of the dPCR assay used in this study, all samples could be reliably quantified with an acceptable level of precision and accuracy after ten-fold serial dilutions. The dilution combined with Poisson distribution statistics yielded the absolute quantification of the target concentration directly (Sykes et al., 1992; Vogelstein and Kinzler, 1999). Both *rpoC1* and *cyrJ* gene copies measured by dPCR were generally lower than qPCR, which was consistent with the results of previous studies (Nathan et al., 2014; Wang et al., 2016). For the

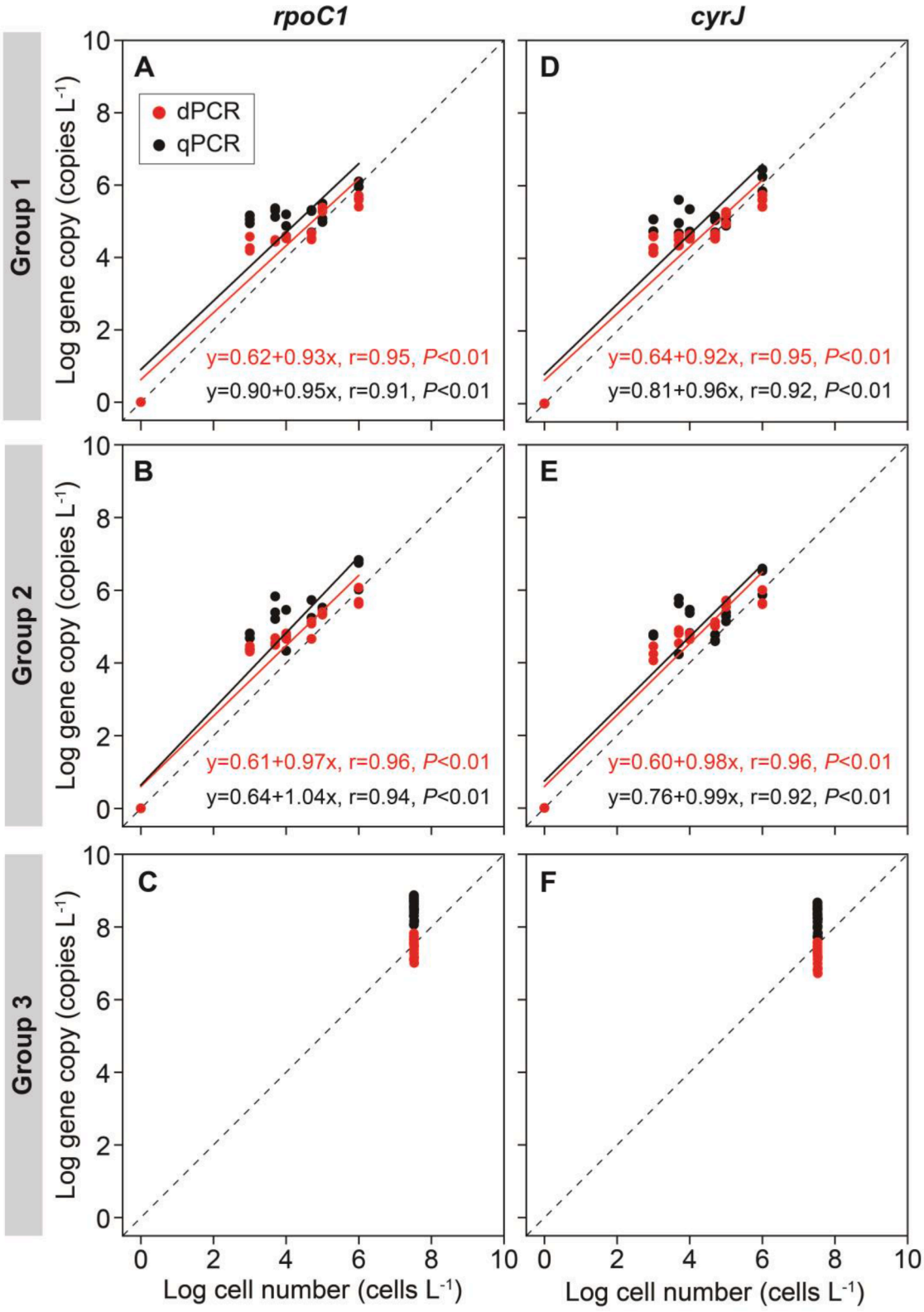

Fig. 3. Correlations between *Raphidiopsis raciborskii* XM1 gene copies (measured by dPCR and qPCR assays) and cell numbers (measured by microscopy). (**A, D**) Group 1, pure strain of *R. raciborskii*; (**B, E**) Group 2, mixture of pure strain and Tingxi Reservoir waters (without *R. raciborskii*); (**C, F**) Group 3, mixture of pure strain and Shidou Reservoir waters (with high abundance of *R. raciborskii*). The dash lines in the figures are line of $y = x$.

laboratory samples, the difference between the dPCR and qPCR assays was 0.00–1.15 log units for *rpoC1* gene and 0.04–1.21 log units for *cyrJ* gene, respectively. For the environmental samples, the difference between the dPCR and qPCR assays was 0.05–1.59 log units for *rpoC1* gene and 0.20–1.87 log units for *cyrJ* gene, respectively. Therefore, we conclude that the data of dPCR and qPCR were at an acceptable level. As expected, the quantification and detection rate of dPCR for *cyrJ* gene in some environmental samples were lower than *rpoC1* gene, perhaps due

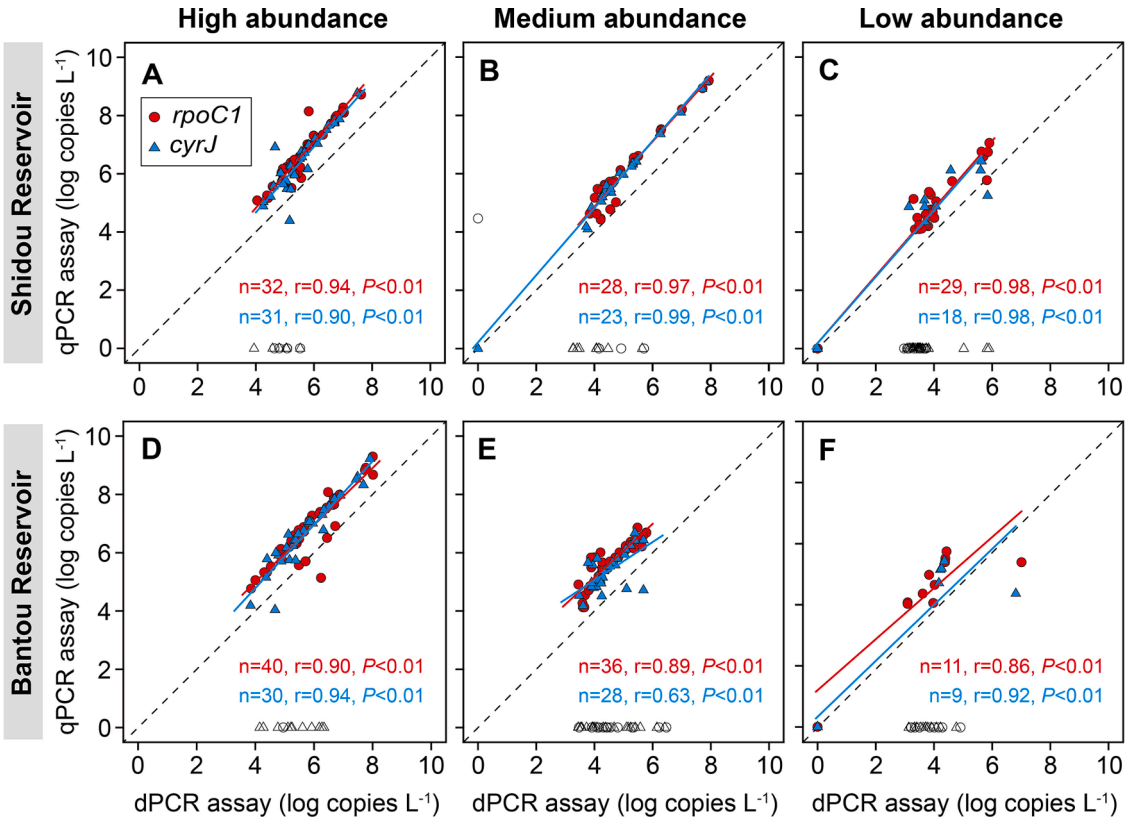

Fig. 4. Correlations between *Raphidiopsis raciborskii* gene copies measured by dPCR and qPCR assays of the environmental samples from two reservoirs. (**A, D**) Environmental samples with high abundance of *R. raciborskii*; (**B, E**) Environmental samples with medium abundance of *R. raciborskii*; (**C, F**) Environmental samples with low abundance of *R. raciborskii*. The three-density levels were defined based on the cell abundance of *R. raciborskii* (For details see Fig. S3). The *rpoC1* and *cyrJ* gene levels below the detection limit of dPCR or qPCR are indicated by open circles or open triangles, respectively. The data below the detection limit of qPCR were not included in the correlation analyses. The dash lines in the figures are line of $y = x$.

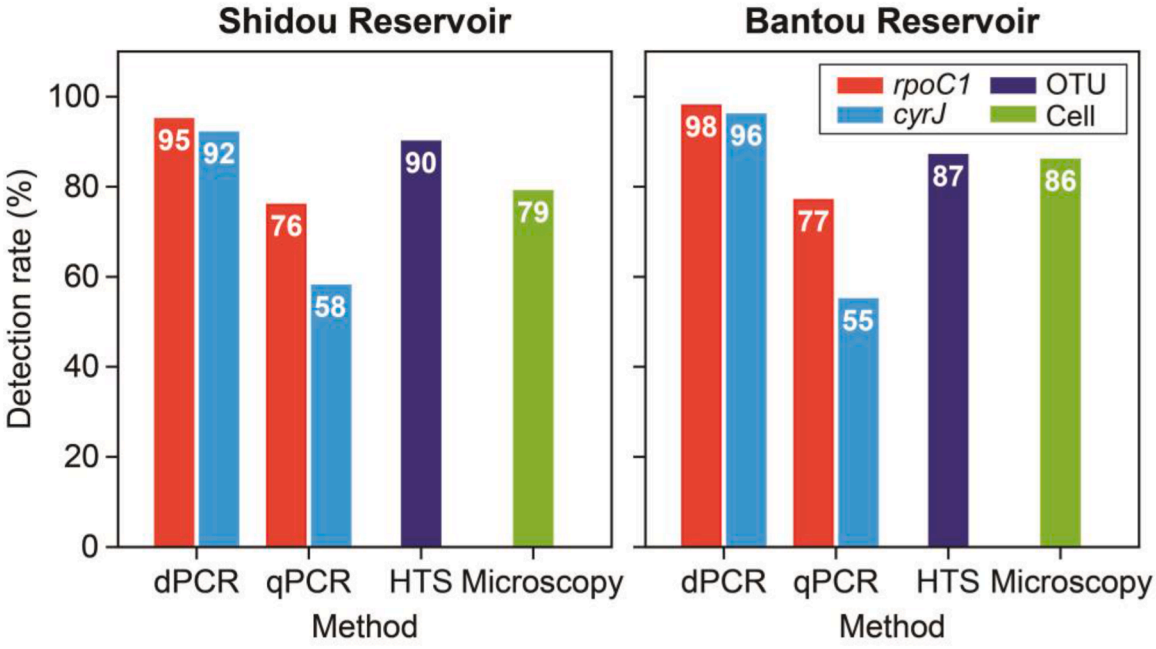

Fig. 5. The detection rate of dPCR assay, qPCR assay, high-throughput sequencing (HTS) and microscopy for *Raphidiopsis raciborskii* of all 222 environment samples.

Table 3
The detection rate of dPCR assay, qPCR assay and microscopy for *Raphidiopsis raciborskii* in the environment samples under different abundance levels.

Group	Reservoir	Detection rate (%)				
		dPCR		qPCR		Microscopy
		rpoC1	*cyrJ*	*rpoC1*	*cyrJ*	
High abundance (log cell numbers ≥7)	Shidou	100	100	86	84	100
	Bantou	100	100	97	73	100
Medium abundance (log cell numbers 5 − 7)	Shidou	97	97	91	69	100
	Bantou	100	100	73	57	100
Low abundance (log cell numbers <5)	Shidou	86	79	55	21	45
	Bantou	90	81	48	24	29

The three environmental sample groups were defined based on the log *R. raciborskii* cell numbers (For detail see Fig. S3).

Table 4
Comparison of the advantages and disadvantages of three methods for determination of *Raphidiopsis raciborskii*.

	dPCR	qPCR	Microscopy
Target level	Gene	Gene	Cell
Primer-dependent	High	High	NA
Standard-dependent	Low	High	High
Detection limitation	1.5 copies per reaction	10^3 copies per reaction	10^4 cells L^{-1}
Optimal detection range	1–10^4 copies L^{-1}	10^4–10^{10} copies L^{-1}	10^4–10^{11} cells L^{-1}
Detection cost	High ($15 per sample/one gene)	Moderate ($10 per sample/one gene)	Low ($3 per sample)
Detection speed	Fast (96 samples per day)	Fast (72 samples per day)	Slow (5 samples per day)
Water volume	≥ 100 mL	≥ 100 mL	≥ 1000 mL
Accuracy	High	High	High
Precision	High	Low	Low
Toxic gene detection	Yes	Yes	No
Technical ability requirement	Moderate	High	Low
Experience requirement	Low	Moderate	High

to the coexistence of the toxic and nontoxic strains. Morphologically indistinguishable toxic and nontoxic strains of the same species often co-occur in harmful cyanobacterial blooms, which can only be differentiated by bio-molecular methods (Davis et al., 2009; Burford et al., 2014).

4.2. Comparison of dPCR with other methods

Quantification of the *rpoC1* and *cyrJ* genes using duplex dPCR technique was significantly correlated with qPCR quantification and cell counts for both laboratory and environmental samples. The weak correlations at low concentration (Fig. S4) were mainly due to the discrepancy of sensitivity, accuracy and precision between dPCR and qPCR assays. Unlike qPCR assays, the dPCR reactions were partitioned into 20,000 nanoliter wells for amplification to ensure targeting of zero or a single copy of the gene (Baker et al., 2012), and this direct and absolute quantification contributes to more accurate and precise results. Moreover, the qPCR quantification requires a DNA standard for calibration by a standard curve, and the sensitivity and amplification are affected by PCR inhibitors in the complex environment (Green et al., 2012). Compared to these two bio-molecular detection methods, the relatively low accuracy and precision of the microscopy could result in the uncertainty in the estimation of bloom-forming cyanobacterial species at low abundance condition. Taken together, the major finding is that the accuracy and precision of detection method depend on the detection limit. Although the detection rate of high-throughput sequencing technique is relatively high, the abundance of *R. raciborskii* cannot be quantified accurately, it may be due to the PCR primer preference. In addition, high-throughput sequencing result relies on 16S rRNA gene of microbial community, however, many bacteria have more than one copy of the 16S rRNA gene, which leads to biased cell count estimates when the latter are estimated solely based on 16S rRNA read counts (Louca et al., 2018).

We also found the results of cell count numbers were similar to dPCR and qPCR for environmental samples from 2011 to 2017, although the cell numbers were higher than dPCR and qPCR for the environmental samples in 2010 (Fig. S5). In addition, there were some samples in the high and medium abundance groups that were not detected by qPCR. There are several reasons that may have led to these results. First, we did find that the differences of results between PCR methods (dPCR and qPCR) and microscopy increased with preservation time (Fig. S7). Second, the first and the largest bloom event dominated by *R. raciborskii* was observed in 2010 (Yang et al., 2017), which consisted of an unprecedented number of individuals (10^{11} cells L^{-1}) that may interfere with the accuracy of the cell count (Srivastava et al., 2013). Indeed, inconsistent results between microscopy and the two PCR methods in high and medium abundance groups were occasionally observed. This is not unprecedented, as Ho and Michalak (2015) concluded that different monitoring tools could show different development and persistence of cyanobacterial bloom, and Bertani et al. (2017) found that discrepancies between different monitoring approaches influence the observed relationships between cyanobacterial blooms and the main environmental drivers. Therefore, multiple types of monitoring approaches need to be integrated to better understand the causes and consequences of cyanobacterial blooms.

4.3. Application in monitoring R. raciborskii in the environment

The dPCR assay established in this study enables fast, effective and simultaneous evaluations of abundance and toxin-producing potential of *R. raciborskii* in the environment. The specificity analysis (Table 2) eliminated the false positives or negative of data generated by dPCR. Moreover, we clarified the reliable practical application of the dPCR method by qPCR and microscopy. dPCR can detect a low density of *R. raciborskii* prior to a bloom and can be useful as an early warning signal in risk management. In case of the environmental samples, dPCR data could also estimate the potential toxicity of *R. raciborskii* that demonstrated the potential role of dPCR in the identification of the elevated cylindrospermopsin risk. Recently, Lu et al. (2020) observed that the detection of toxic gene expression is approximately 3 weeks earlier than toxin for *Microcystis*. The specific and sensitive dPCR analysis can be used to monitoring the possible cyanotoxins that provides ample warning time for water quality agencies to implement management strategies to avoid or minimize economic losses and health risks caused by harmful cyanobacteria.

5. Conclusion

We established a dPCR assay for detecting the bloom-forming and toxic cyanobacterium *Raphidiopsis raciborskii* through simultaneously monitoring its abundance and toxin-producing potential. The duplex dPCR assay showed a good correlation with simplex qPCR and microscopy in both laboratory spiked and environmental samples. Compared with qPCR assay and microscopic cell count, the dPCR assay offers a simple and rapid detection of *R. raciborskii*. More importantly, the dPCR assay was highly sensitive and could accurately and precisely quantify low-abundance *R. raciborskii* in complex environmental samples. This approach can allow the early detection of low-abundance and toxin-producing potential of harmful cyanobacteria, thereby it is valuable to

improve the early warning system for water quality protection and harmful cyanobacteria prevention in inland waters.

Author contributions

JY conceived the idea and designed the experiments. JY, JRY, and HC performed the sample collection. FT isolated and cultured the *Raphidiopsis raciborskii*. AW provided advice on techniques for isolating and culturing *R. raciborskii*. FT and PX performed the qPCR and dPCR assays, while JRY, YY and LJ performed the microscopy. FT and PX performed the high-throughput sequencing. FT analyzed the data. FT and JY wrote the first draft of the manuscript, TL and AW reviewed and revised the manuscript, and all authors contributed to and have approved the final manuscript.

Supplementary data

Supplementary data related to this article can be found online.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2021.102125.

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