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Comparison of Quantitative PCR and Droplet Digital PCR Multiplex Assays for Two Genera of Bloom-Forming Cyanobacteria, *Cylindrospermopsis* and *Microcystis*

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The increasing occurrence of harmful cyanobacterial blooms, often linked to deteriorated water quality and adverse public health effects, has become a worldwide concern in recent decades. The use of molecular techniques such as real-time quantitative PCR (qPCR) has become increasingly popular in the detection and monitoring of harmful cyanobacterial species. Multiplex qPCR assays that quantify several toxigenic cyanobacterial species have been established previously; however, there is no molecular assay that detects several bloom-forming species simultaneously. *Microcystis* and *Cylindrospermopsis* are the two most commonly found genera and are known to be able to produce microcystin and cylindrospermopsin hepatotoxins. In this study, we designed primers and probes which enable quantification of these genera based on the RNA polymerase C1 gene for *Cylindrospermopsis* species and the c-phycocyanin beta subunit-like gene for *Microcystis* species. Duplex assays were developed for two molecular techniques—qPCR and droplet digital PCR (ddPCR). After optimization, both qPCR and ddPCR assays have high linearity and quantitative correlations for standards. Comparisons of the two techniques showed that qPCR has higher sensitivity, a wider linear dynamic range, and shorter analysis time and that it was more cost-effective, making it a suitable method for initial screening. However, the ddPCR approach has lower variability and was able to handle the PCR inhibition and competitive effects found in duplex assays, thus providing more precise and accurate analysis for bloom samples.

Cyanobacteria, also known as blue-green algae, constitute the most notorious phylum of phytoplankton capable of forming harmful blooms in freshwater aquatic ecosystems (1). Their presence in freshwater and brackish and coastal marine waters is of particular interest because of their massive accumulation and proliferation into nuisance blooms in nutrient-enriched water. These cyanobacterial blooms are the cause for a multitude of water quality concerns, due to their potential to produce secondary metabolites, some of which are toxins and compounds that compromise taste and odor. Cyanotoxins have been linked to human and animal illness and death and pose serious health hazards to communities which use surface waters and reservoirs as potable waters (2). Hence, the detection of cyanobacteria is crucial for reliable and prudent water management. The use of efficient detection methods in routine monitoring of waters is necessary to safeguard precious water resources.

Microscopic counting combined with chemical detection of cyanotoxins in water samples is the conventional method to evaluate harmful cyanobacterial blooms (3). However, morphological identification is prone to limitations such as being time-consuming and unable to distinguish between toxic and nontoxic species and prone to misinterpretation when limited morphological differences are available (4, 5). In light of these discrepancies, DNA-based detection methods are becoming increasingly popular because of increasing bioinformatics knowledge and the availability of genetic databases that allow the design of specific, sensitive, and speedy molecular assays (6). These assays were developed to amplify unique nucleotide sequences of a target gene of interest, for example, functional genes responsible for the biosynthesis of cyanotoxins (*mcy*, *cyr*, and *nda*), or housekeeping gene (16S rRNA and RNA polymerase gene) sequences exclusively for certain cyanobacterial species (7, 8). The high sensitivity of molecular methods allows the detection of harmful cyanobacterial species even at low abundance, thus providing rapid evaluation before the occurrence of a cyanobacterial bloom, which cannot be achieved by conventional counting methodology.

Real-time quantitative PCR (qPCR) has been successfully used to monitor harmful cyanobacterial populations in several studies. Most of these assays were designed for a single target, while only a few multiplex assays were developed to detect multiple analytes simultaneously (9–14). Despite the advantages of multiplexing qPCR in identification of taxa (such as offering quicker screening and being less labor-intensive), there is currently no method which can detect and quantify several genera of bloom-forming cyanobacteria in one assay.

Digital PCR (dPCR) is an alternative method of quantitative PCR that derives the target's abundance from the ratio of the number of positive partitions to a large number (hundreds to millions) of total reaction partitions generated from a known volume of PCR mix. The absolute number of copies of the target is calculated from the proportion of positive partitions and statisti-

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TABLE 1 Primer and probe sequences for duplex assay and for cloning of plasmid standards

Primer or probe	Nucleotide sequence (5′–3′)[a]	T_m (°C)	Amplicon size (bp)
CYL rpo F	GCATTCCTAGTTATATTGCCAT	59.0	154
CYL rpo R	TCTAACCATTGGTCTTCTGTTAA	59.7	
CYL probe	FAM-CTAGACATGCCCCTACGAGAYGTTGAGC-BHQ-1	73.5	
MIC cpc F	GYTATGTYACCTACGCTACCTTC	60.5	107
MIC cpc R	TCCWGGTACTCCTAAAGCTACA	59.8	
MIC probe	Yakima yellow/HEX[b]-ACCATTTAAGCAACGATCATCGAGAACAC-BHQ-1	71.7	
Cloning			
CYL rpo F (extended)	TGTAGCCCATGTTTGGTATCTCAAAG	67.8	206
CYL rpo R (extended)	TGAGAATCTTCACTATAAATCCTATCC	61.0	
MIC cpc F (extended)	TTACGCGACATGGAAATCAT	63.3	152
MIC cpc R (extended)	TTGCTTACGCCAGCGGCTA	69.2	

[a] FAM, 6-carboxyfluorescein; BHQ-1, black hole quencher 1.
[b] Yakima Yellow reporter dye was used in the qPCR assay; HEX reporter dye was used in the ddPCR assay.

cally corrected with a Poisson distribution (15, 16). Droplet digital PCR (ddPCR) is one of the commercially available dPCR approaches and generates microfluid droplets in oil, forming tens of thousands of tiny PCRs in an oil-water emulsion. The intrinsic nature of ddPCR allows detection of rare nucleic acids in the presence of a high abundance of standard sequences (15). The applications of this technology have mainly been focused on clinical research and diagnosis, genetic modification, food product screening, and viral surveillance (17–23), while the potential of using ddPCR in environmental studies has yet to be explored.

In this study, we developed duplex quantification assays targeting two ubiquitous cyanotoxin-producing cyanobacterial genera found in most freshwater systems—*Microcystis* assay (MIC) and *Cylindrospermopsis* assay (CYL)—using both qPCR and ddPCR techniques. The assay performances of the two methods are also compared. The developed assays can be used to detect and quantify the two most widely reported cyanobacterial genera in both laboratory and environmental samples.

MATERIALS AND METHODS

Primer and probe design. Primers and probes were designed based on specific sequences of the RNA polymerase C1 gene (*rpoC1*) for *Cylindrospermopsis* species and the c-phycocyanin beta subunit-like gene (*cpcB*) for *Microcystis* species. Nucleotide sequences (43 *rpoC1* sequences and 73 *cpcB* sequences) from various *Cylindrospermopsis* and *Microcystis* strains were extracted from the National Center for Biotechnology Information (NCBI) database and aligned using Mega 4.0. The aligned sequences were examined manually to determine conserved regions suitable as targets. Potential primer and probe sequences were first generated using IDT PrimerQuest software and then adjusted manually following the instructions of the *TaqMan Multiplex PCR Optimization User Guide* (Life Technologies) for optimum assay efficiency, with the following specifications.

1. The primer melting temperatures (T_m) should be similar for all primers and for the two probes.
2. The T_m of the probe should be ~10°C higher than the T_m of the primers.
3. The sizes of amplicons should be between 50 and 150 bp.
4. The primers and probes should not be self-complementary or complementary to each other.
5. GC content should be between 40% and 60%.
6. The probe length should be 13 to 30 bp, the 5′ end should not be a G residue, and repeating identical nucleotides should be avoided.

Possible primer dimers and hairpins were examined using MFEprimer-2.0 (http://biocompute.bmi.ac.cn/CZlab/MFEprimer-2.0/index.cgi/check_dimer), with a ΔG value of not less than −5 kcal/mole as the recommended value. Specificities of the target regions were initially determined with BLASTN (NCBI).

In addition, qPCR was performed on DNA from laboratory cultures of species of various taxa of cyanobacteria, including *Microcystis*, *Cylindrospermopsis*, *Limnothrix*, *Anabaena*, *Pseudanabaena*, *Planktothrix*, *Hapalosiphon*, and *Synechococcus*, as well as noncyanobacterial species, including *Actinastrum* and *Chlorella* species, to verify the specificity of the assays. qPCR product sizes from the DNAs of the cultures were checked by gel electrophoresis performed with a 1% agarose gel stained with GelRed (Biotium).

Cloning and plasmid standard synthesis. Plasmid DNAs were used to establish the standard curves. Given the known vector size and target qPCR regions (quantified in base pairs), an estimate of gene copy numbers (GCNs) based on the plasmid DNA is more accurate than estimates based on the genomic DNA. To avoid the possibility of mispaired nucleotides at the ends of the PCR product, two sets of extended primers (Table 1) were used to amplify longer sequences flanking the target qPCR regions. PCR amplifications of the DNA of *M. aeruginosa* PCC7806 and *Cylindrospermopsis* strain CS505 were carried out in a Mastercycler Pro PCR system (Eppendorf) subjected to the following steps: polymerase activation at 95°C for 2 min; 35 amplification cycles of 95°C for 20 s, 58°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. Each reaction mixture contained 10 μl of 2× GoTaq Hot Start Green master mix (Promega), 0.5 μM (each) forward and reverse primers, and 2 μl of the DNA template. PCR products were cleaned with a Wizard SV gel and PCR clean-up system (Promega) and cloned into pGEM-T Easy vector (Promega) following the manufacturer's instructions. Successful *Escherichia coli* clones were inoculated in 10 ml of LB broth medium and grown overnight using 37°C incubation. Plasmid DNA was extracted using a QIAprep Spin Miniprep kit (Qiagen), followed by restriction enzyme digestion (SalI; Promega). The quality and concentration of linearized plasmids were determined with a NanoDrop 1000 Spectrophotometer (Bio-Frontier). Plasmid DNAs generated were also sequenced in a ABI 3730xl DNA analyzer using an ABI BigDye Terminator v3.1 cycle sequencing kit (Life Technologies) to verify correct target sequences (see the supplemental material).

qPCR optimization and standard curves. A duplex qPCR assay was developed to enumerate gene copy numbers of CYL *rpoC1* and MIC *cpcB*

TABLE 2 Specificity test of assays using laboratory cultures[a]

Cyanobacterial/algal culture	Assay result	
	MIC qPCR monoplex	CYL qPCR monoplex
Microcystis sp. strain RK18[b]	+	−
Microcystis sp. strain RKC[b]	+	−
Microcystis sp. strain RK1[b]	+	−
Microcystis sp. strain RK7[b]	+	−
Microcystis sp. strain RP1D[b]	+	−
Microcystis aeruginosa PCC7806	+	−
Microcystis aeruginosa CS-573	+	−
Microcystis aeruginosa NIES843	+	−
Cylindrospermopsis sp. strain LPRb[b]	BLOQ	+
Cylindrospermopsis sp. strain CYL1[b]	−	+
Cylindrospermopsis sp. strain CYL2[b]	−	+
Cylindrospermopsis sp. strain CYL8[b]	−	+
Cylindrospermopsis raciborskii CS-505	−	+
Cylindrospermopsis raciborskii CS-509	−	+
Cylindrospermopsis raciborskii CS-511	−	+
Anabaena circinalis CS-337-01	−	−
Anabaena circinalis CS-541-06	−	−
Nodularia spumigena CS-336-05	−	−
Nodularia spumigena CS-588-02	−	−
Limnothrix sp. strain RU5A[b]	BLOQ	−
Pseudanabaena sp. strain RM13A[b]	−	−
Synechococcus sp.[b]	−	−
Chlorella sp. strain RM1[b]	BLOQ	BLOQ
Actinastrum sp. strain PR1[b]	−	−

[a] +, C_q within quantification range; BLOQ, C_q below limit of quantification (LOQ); −, amplification undetectable. The concentrations of algal culture DNA were 1.25 to 23.90 ng/μl.
[b] Strains isolated from local reservoirs.

genes simultaneously. Amplifications were carried out using a 0.1-ml MicroAmp Fast Reaction 8-tube strip (Life Technologies) in a 20-μl reaction volume using a StepOnePlus real-time PCR system (Life Technologies). Plasmid standards cloned with targets were diluted 10-fold and amplified using primers and dual-label probes designed in this study. To optimize the duplex assay, amplifications were run in monoplex and duplex fashions in parallel. The assay conditions were changed until the monoplex and duplex assays generated comparable quantification cycle (C_q) values for the same samples (C_q difference < 1) with an amplification efficiency of 100% ± 10%. The duplex qPCR assay was optimized in two ways via optimization of (i) amplification conditions and (ii) reagent composition. For the amplification conditions, assay performances for 2-step and 3-step amplifications at various annealing temperatures (from 50 to 60°C) were compared. For the reagent composition, different master mixes, primers, probes, and $MgCl_2$ concentrations were tested. The optimum assay consisted of reagents as follows: 10 μl of 2× QuantiFast multiplex PCR master mix (Qiagen), 0.5 μM (each) MIC cpc F/R primer, 0.75 μM (each) CYL rpo F/R primer, 0.2 μM (each) probe, 1 μM $MgCl_2$, and 2 μl of DNA template in a total reaction volume of 20 μl. The amplification cycles included an enzyme activation step at 95°C for 5 min and 40 cycles of 3-step amplification of 15 s at 95°C, 25 s at 57°C, and 25 s at 72°C.

Standard curves of monoplex and duplex assays were established in a similar way. Serially diluted plasmid samples with 10^0 to 10^6 copies of target were prepared in duplicate or triplicate, and the standard curve was generated as a linear regression between C_q values and the logarithmic target or gene copy number (GCN). The GCN in 1 μl of sample was calculated from the concentration of plasmid used.

ddPCR optimization. The duplex ddPCR assay optimization was similar to the optimization for qPCR, where various thermal cycling conditions (2-step versus 3-step; temperature gradient for annealing; cycle number) and primer-probe concentrations were evaluated. Satisfactory separation of droplets positive for the target from those negative for the target, assay sensitivity comparable to qPCR sensitivity, and linearity were the criteria used in optimization. The same primers and probes were used in ddPCR, except that for the reporter dye for the MIC assay the probe was changed to HEX in ddPCR to allow optimum florescence detection. The optimized PCR mixture contained 10 μl of 2× ddPCR Supermix for probes (Bio-Rad) (no dUTP), 0.9 μM (each) primer, 0.25 μM (each) probe, and 2 μl of the DNA template. Quantification of targets was carried out in a QX200 Droplet Digital PCR system (Bio-Rad Laboratories, Inc.). First, 20 μl of each well-mixed PCR mixture was transferred to a droplet generator cartridge (Bio-Rad). After 70 μl of droplet generation oil (Bio-Rad) was added into the oil wells, the cartridge was covered with a rubber gasket and loaded onto a QX200 droplet generator (Bio-Rad). The emulsions of droplets generated were transferred to a 96-well PCR plate (Eppendorf), sealed with PX1 PCR plate sealer (Bio-Rad), and subjected to amplification in a C1000 Touch thermal cycler (Bio-Rad). The amplifica-

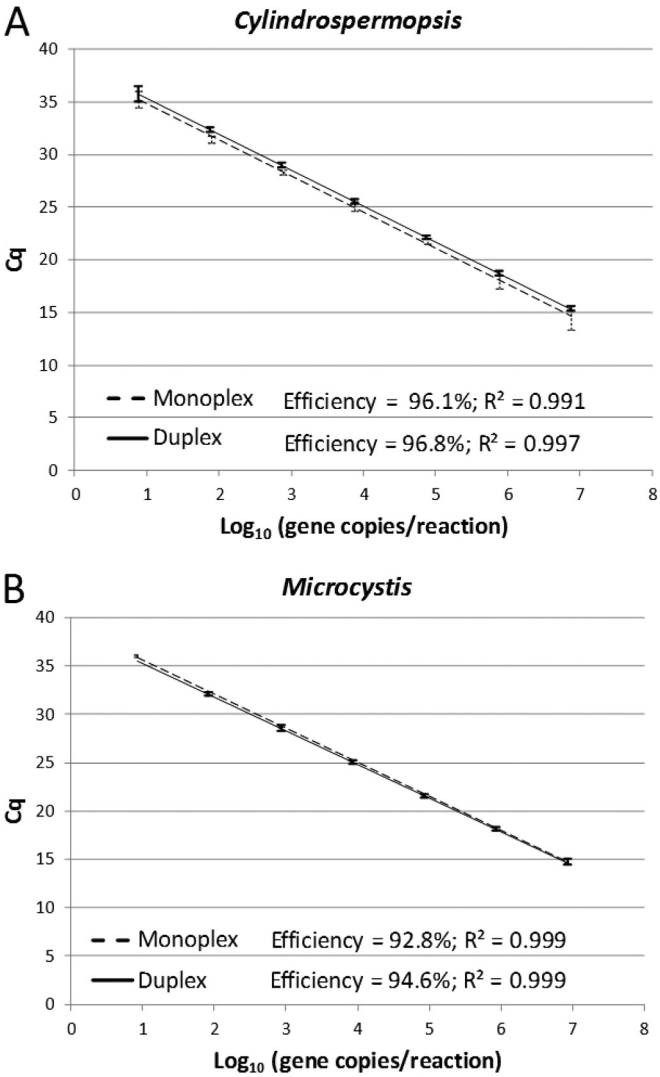

FIG 1 qPCR monoplex and duplex standard curves. C_q values obtained from 10-fold-diluted plasmid standards are plotted against the respective Log_{10} GCNs. (A) Linear regressions for CYL *rpoC1* [monoplex, $C_q = -3.419$ Log_{10} (GCN) + 38.12; duplex, $C_q = -3.422$ Log_{10} (GCN) + 38.88]. (B) Linear regressions for MIC *cpcB* [monoplex, $C_q = -3.508$ Log_{10} (GCN) + 39.05; duplex, $C_q = -3.46$ Log_{10} (GCN) + 38.68].

TABLE 3 C_q values of monoplex and duplex assays with serially diluted plasmid DNA

No. of copies of plasmid DNA	C_q for MIC *cpcB*[a]		C_q for CYL *rpoC1*[a]	
	Monoplex	Duplex	Monoplex	Duplex
10^0	36.03 (0.11)	35.39 (0.30)	35.26 (0.78)	35.75 (0.75)
10^1	32.18 (0.27)	32.13 (0.17)	31.43 (0.33)	32.34 (0.21)
10^2	28.70 (0.09)	28.56 (0.31)	28.38 (0.23)	29.00 (0.21)
10^3	25.21 (0.10)	25.06 (0.17)	25.07 (0.47)	25.56 (0.22)
10^4	21.74 (0.08)	21.56 (0.17)	21.76 (0.26)	22.14 (0.17)
10^5	18.20 (0.18)	18.18 (0.16)	17.91 (0.63)	18.68 (0.23)
10^6	14.89 (0.15)	14.75 (0.29)	14.57 (1.18)	15.41 (0.21)

[a] Values shown represent averages of 5 readings from duplicate and triplicate samples analyzed separately. Standard deviations are in parentheses.

tion was carried out at a uniform ramp rate of 2.5°C/s at 95°C for 10 min; 45 cycles of 95°C for 15 s followed by 58°C for 1 min; and a final enzyme deactivation at 98°C for 10 min. Fluorescent signals from amplified droplets were captured individually in the QX200 droplet reader (Bio-Rad) and analyzed with QuantaSoft 1.6.6 software. The distinction between positive and negative droplets (with and without target) was based on the threshold values assigned for all samples (9,000 for CYL and 5,400 for MIC) and on the threshold values automatically obtained from different sample types (i.e., plasmid DNA and DNA from cyanobacterial cultures and environmental sample extracts). The target concentrations were reported as the numbers of copies per microliter of the PCR mixture after correction with the Poisson distribution.

Sensitivity, precision, and competitive effect. Plasmid samples containing 10, 10^2, 10^3, 10^4, or 10^5 target copies per reaction, estimated from previous ddPCR results, were prepared and evaluated with qPCR and ddPCR duplex reactions. To evaluate assay sensitivity, reaction mixtures containing less than 10 target copies were prepared and run for each technique. The limit of detection (LOD) for each assay, defined as the smallest amount of analyte in a sample that could be confidently detected (95th percentile), was calculated following guidelines approved by the NCCLS (24). To examine possible competitive effects in CYL and MIC assays in duplex reactions, samples containing low copy numbers of one target were mixed with a 10-fold-increased level of another target and were then analyzed with both qPCR and ddPCR.

Assay evaluation using laboratory cultures and environmental samples. Once the conditions were optimized, the assay was verified using laboratory cultures and environmental water samples. Six strains of cyanobacteria, three *Microcystis* strains (PCC7806, NIES843, and local isolate RKC), and three *Cylindrospermopsis* strains (CS505, CS509, and local isolate CYL2) grown in MLA medium (temperature, 23 to 25°C; light intensity, 25 μmol quanta/$m^2 \cdot s$) (45) were harvested during the early stationary phase. Concentrations of *Microcystis* strains ranged from 1.9×10^6 to 9.4×10^6 cell/ml, while concentration of *Cylindrospermopsis* strains ranged from 1.4×10^7 to 2.5×10^7 cell/ml. The number of cells was determined by a microscopic count method where 10 μl of sample fixed with Lugol's solution (25) was loaded onto a disposable hemocytometer (C-Chip; iNCYTO) and the cells were counted under an inverted microscope (Leica DM IL light-emitting-diode [LED] fluorescence microscope). Duplicate measurements were made for each culture. For cyanobacterial genomic DNA extraction, 15 ml of each laboratory culture was filtered onto a 0.45-μm-pore-size cellulose nitrate membrane, followed by DNA extraction using a PowerWater DNA isolation kit (MoBio). These samples were also analyzed for total cyanobacterial 16S rRNA gene abundance, using previously reported primers and probe (26) that had been optimized on qPCR and ddPCR platforms.

Environmental water samples were also harvested and extracted with the same method and analyzed with qPCR and ddPCR techniques using the CYL and MIC assays.

Statistical analyses. Significant differences between GCNs and C_q values of different GCNs were determined using the independent-sample *t* test and paired *t* test (Predictive Analytics SoftWare [PASW] version 18).

RESULTS

Assay specificity. The specificities of the primers and probes were evaluated using BLASTN. The combination of CYL primer and probe sequences matched 100% of 35 sequences from *Cylindrospermopsis* species and 2 sequences from *Anabaena sphaerica* var. tenuis, while the combination of MIC *cpcB* primers and probe was specific only to *Microcystis* species (100% identity). Monoplex qPCR conducted on laboratory algal cultures also indicated that the primers and probes were specific to the desired targets (Table 2).

qPCR assay performance. The linear dynamic range of quantification for duplex qPCR was between 10^0 and 10^6 gene copies/reaction (i.e., 7.5×10^0 to 7.5×10^6 gene copies/reaction for *Cylindrospermopsis* and 8.5×10^0 to 8.5×10^6 gene copies/reaction for *Microcystis*). The monoplex and duplex reaction efficiencies and regressions were almost identical (Fig. 1A and B), with differences of less than 1 between the C_q values of the monoplex and duplex assays for each concentration (Table 3). The LOD values for the CYL and MIC assays were 6.88 and 7.36 gene copies/reaction, respectively.

ddPCR assay performance. Using the same dilution series of plasmid DNA for qPCR standard curves, each concentration of plasmid standard was run in triplicate on two separate runs. The dynamic range for ddPCR reactions was between 10^0 and 10^4 gene copies/reaction (Fig. 2), while the LOD values were 4.26 gene copies/reaction for the CYL assay and 3.52 gene copies/reaction for the MIC assay. The linearity decreased for lower target concentrations at 10^0 copies, and reaction saturation was reached at a concentration of 10^5 gene copies/reaction. The GCNs obtained for the monoplex and duplex reactions were indistinguishable (*t* test, $P > 0.05$) for the range from 10^0 to 10^3 gene copies/reaction. Slightly lower amplification efficiency was observed for the duplex assay when both targets reached a concentration of 10^4 gene copies/reaction (*t* test, $P < 0.05$), but the discrepancy between monoplex and duplex assays was below 5%.

Comparison between qPCR and ddPCR. The variability of the GCNs measured with the qPCR and ddPCR techniques is shown in the quantitative correlations of qPCR and ddPCR measurements (Fig. 3), where the slopes are 1.1238 for *Cylindrospermopsis* and 1.1436 for *Microcystis* with an R^2 value of ≥0.98. The numbers of target copies measured by the two platforms were well correlated, with qPCR generally providing higher GCNs for both targets. GCNs estimated with qPCR were approximately 1.3-fold to 6.8-fold (average, 2.8-fold) higher than those measured by ddPCR.

A sensitivity test using samples containing small amounts of

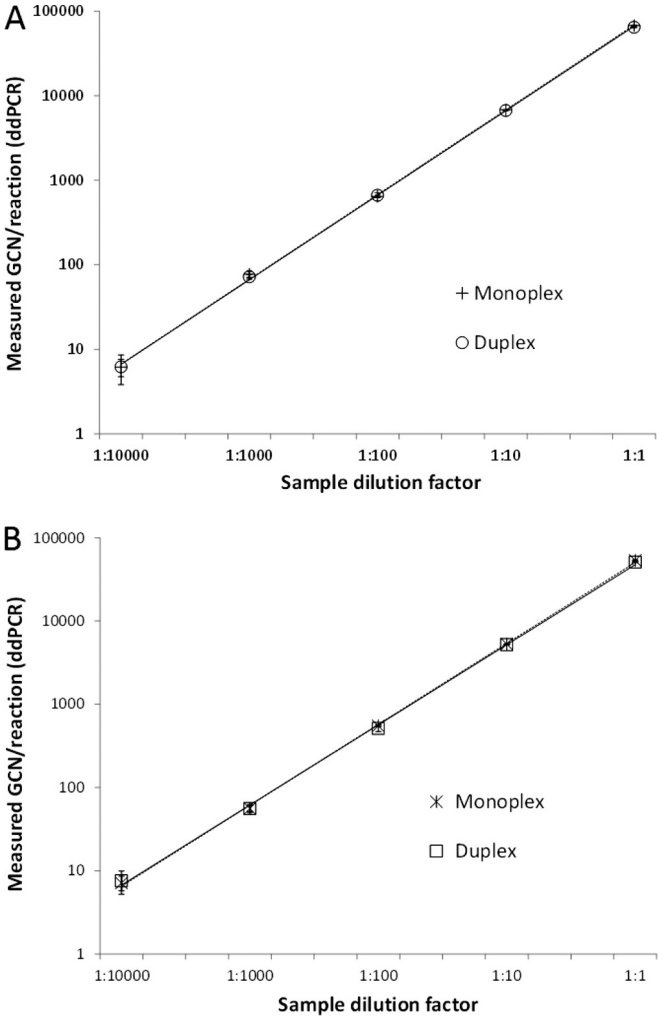

FIG 2 Linear regressions between measured GCN and estimated GCN for ddPCR monoplex and duplex assays. (A) CYL. (B) MIC. GCNs were estimated from concentrations of plasmid DNA estimated by spectrophotometer readings. Each point represents the average of the results from triplicate samples.

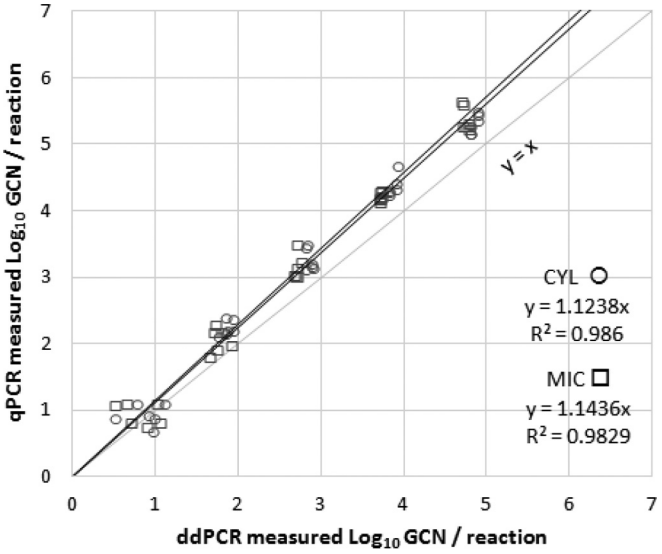

FIG 3 Correlations between measured qPCR and ddPCR GCNs determined using plasmid standards. The correlation equations are $y = 1.1238x$ for the CYL assay and $y = 1.1436x$ for the MIC assay, where y is the Log_{10} GCN measured by qPCR and x is the Log_{10} GCN measured by ddPCR.

targets, where 94.4% of the qPCR samples gave positive amplification compared to 80.5% of the ddPCR samples, indicated that qPCR has greater sensitivity than ddPCR. However, ddPCR showed higher precision, with lower values for standard deviation (SD) and percent coefficient of variation (%CV) (Table 4).

Competition between CYL and MIC assays was observed in the duplex qPCR. The effect was considered acceptable ($C_q \leq 1$) when the concentration of one target was <10 times higher than the concentration of the other target. Beyond this range, however, the competitive effect increased, with greater differences in the two target concentrations (Fig. 4A and B) (*t* test, $P < 0.05$). However, measured target copy numbers were still of the same magnitude when the concentration for the second target was 1,000 (CYL assay) or 100 (MIC assay) times higher. Notably, this competitive effect was not detected in the ddPCR reaction when low copy numbers of one target were amplified in the presence of large amounts of the other target. The monoplex and duplex reactions gave identical results as shown in Fig. 4C and D (*t* test, $P > 0.05$), although the latter contained at least 10^4 copies of the other target.

The copy numbers of the CYL *rpoC1*, MIC *cpcB*, and 16S rRNA genes for 6 laboratory cultures were quantified using qPCR and ddPCR. The ratios of the 16S rRNA gene copy numbers to the CYL *rpoC1* gene copy numbers for *Cylindrospermopsis* spp. were 2.33 for qPCR and 3.00 for ddPCR, whereas the ratios of the 16S rRNA gene copy numbers to the MIC *cpcB* gene copy numbers for *Microcystis* spp. were 1.50 for qPCR and 1.84 for ddPCR (Table 5). This result is consistent with the complete genome sequences in NCBI database, showing that *C. raciborskii* CS 505 contains 3 copies of the 16S rRNA gene and 1 copy of the *rpoC1* gene per genome whereas *M. aeruginosa* NIES 843 contains 2 copies of the 16S rRNA gene and 1 copy of the *cpcB* gene per genome (NCBI accession number AP009552; GCA_000175835.1). Our study showed that ddPCR was more accurate in the prediction of the copy numbers of target genes in a genome.

Evaluation of environmental samples. The GCNs measured by qPCR and ddPCR for 17 environmental samples are presented in Fig. 5. Overall, the data generated by the two techniques fell within the same order of magnitude. Several samples showed significant differences in GCNs using the two techniques ($C_q > 1$). For the CYL assay, the mean difference between qPCR and ddPCR was 0.344 $\log_{10}$ (GCN)/ml (SD, 0.210 $\log_{10}$ [GCN]/ml). The MIC assay showed less variability for the two techniques, with a mean difference of 0.264 $\log_{10}$ (GCN)/ml (SD, 0.113 $\log_{10}$ [GCN]/ml). The MIC *cpcB* GCN measured by qPCR was generally higher than that measured by ddPCR (for 15 of 17 samples) (paired *t* test, $P < 0.05$). This is in agreement with the quantitative correlation of qPCR and ddPCR obtained from the plasmid DNA assays (Fig. 3). However, this result was not found in the CYL assay (paired *t* test, $P > 0.05$), where the qPCR assay produced lower GCNs than the ddPCR assay for 11 of 17 samples tested. Of these 11 samples, 9 had a 10-fold-higher MIC *cpcB* GCN than CYL *rpoC1* GCN,

TABLE 4 Comparison of assay sensitivities between qPCR and ddPCR

Parameter[a]	Value[b]							
	CYL *rpoC1*				MIC *cpcB*			
	qPCR		ddPCR		qPCR		ddPCR	
	1	2	1	2	1	2	1	2
Target concn (GCN/reaction)	8.40	2.10	8.40	2.10	5.20	1.30	5.20	1.30
Measured mean concn (GCN/reaction)	7.24	3.34	7.20	2.33	7.54	6.80	7.27	1.80
SD	4.05	4.17	3.51	0.82	4.12	7.11	2.92	0.59
% CV	55.91	125.09	48.75	35.10	54.69	104.65	40.17	32.96
Ratio of no. of positive samples to total no. of samples[c]	9/9	8/9	8/9	6/9	9/9	8/9	9/9	6/9

[a] SD, standard deviation; CV, coefficient of variation.
[b] Columns labeled 1 and 2 represent two different sets of results, based on target concentration.
[c] Positive sample, sample with detectable amplification signal.

suggesting that a competitive effect was present in these samples in analyses performed with duplex qPCR.

DISCUSSION

Freshwater cyanobacterial blooms are of major concern globally due to the natural toxin and off-flavor compounds produced by several genera, causing esthetical and public health issues. *Microcystis* and *Cylindrospermopsis* are two of the most widespread and successful bloom-forming genera across continents (27, 28). Many previous investigations have focused on a single dominant genus during bloom events. However, cooccurrence of these genera has also been reported, with changes in dominance affected by nutrients, light, season, and grazing effects (28–30). The impacts of *Microcystis* and *Cylindrosper-*

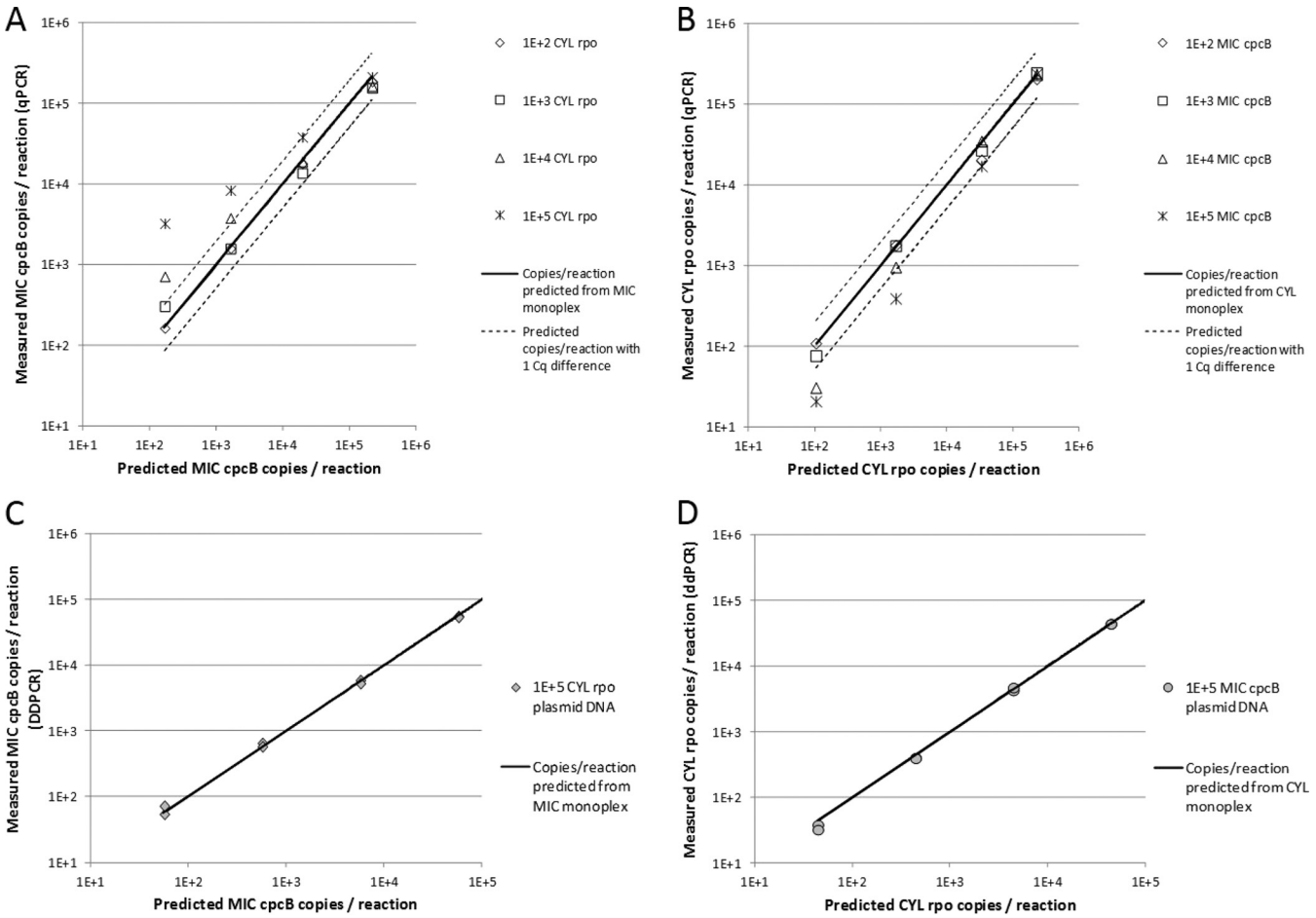

FIG 4 Evaluation of competitive effects in the duplex reaction. (A and B) The measured GCNs of CYL *rpoC1* (A) and MIC *cpcB* (B) in duplex qPCR with the presence of a second target at different concentrations. (C and D) The measured GCNs of CYL *rpoC1* (C) and MIC *cpcB* (D) in duplex ddPCR with and without a second target at a high concentration.

TABLE 5 Ratios of 16S rRNA copy numbers to *rpoC1* copy numbers (*Cylindrospermopsis*) and ratios of 16S rRNA copy numbers to *cpcB* copy numbers (*Microcystis*) predicted by the qPCR and ddPCR techniques

Cylindrospermopsis strain	16S rRNA copy no./*rpoC1* copy no. ratio (SD)[a]		*Microcystis* strain	16S rRNA copy no./*cpcB* copy no. ratio (SD)[a]	
	qPCR	ddPCR		qPCR	ddPCR
CYL2	2.64 (0.02)	3.03 (0.05)	RKC	1.32 (0.01)	1.66 (0.01)
CS505	2.47 (0.01)	2.94 (0.06)	PCC7806	1.82 (0.05)	1.88 (0.02)
CS509	1.88 (0.03)	3.01 (0.07)	NIES843	1.36 (0.01)	1.98 (0.02)
Overall	2.33 (0.36)	3.00 (0.06)		1.50 (0.25)	1.84 (0.15)

[a] Data represent the averages of the results of triplicate determinations, with standard deviations indicated in parentheses.

mopsis blooms are not restricted only to microcystins and cylindrospermopsins but have also recently extended to include compounds such as microsin, microviridin, and β-*N*-methylamino-L-alanine (BMAA), known to cause adverse ecological and health effects (31–33). Thus, monitoring the total abundance of these genera is highly relevant from the perspective of water quality risk assessment and bloom management.

The 16S rRNA gene is by far the most frequently used molecular marker in microbial ecology studies (34). Assays based on the 16S rRNA gene have been designed to determine the total cyanobacterial community members or the members of a particular genus or species (8). However, issues such as inconsistent copy numbers of gene and heterogonous sequences in cyanobacteria make translation from 16S rRNA GCN to cell number difficult. To avoid quantification biases, we selected *rpoC1* and *cpcB* genes as targets for which only a single gene copy is present in a genome. The primers and probes are specific to only the target genera, except for two sequences from *Anabaena sphaerica* var. tenuis that match CYL *rpoC1* primers and probes. In fact, a phylogenetic study of four genes (the 16S rRNA gene and *hetR*, *nifH*, and *rpoC1* genes) of *Anabaena* morphospecies led to the conclusion that *A. sphaerica* is phylogenetically closer to *Cylindrospermopsis* and *Raphidiopsis* and, thus, should be considered a sister group with *Cylindrospermopsis* instead of *Anabaena* (35), thus supporting our assay design.

The qPCR technique has become a popular method in the monitoring of algal blooms over the past decade. However, most of the published assays identify only single targets whereas only a few use multiplex techniques. The multiplex assays available in the literature focus on toxin-producing species. These assays were designed in such a way that they estimate the percentage of toxigenic species within a genus (4, 9, 36, 37) or quantify multiple toxin synthesis genes covering several toxicological groups (hepatotoxins and neurotoxins) (38). The assay developed in this study is the first duplex assay that enumerates abundances of two genera simultaneously, enabling fast and effective assessment of changes in subpopulations, interpopulation competition, and dynamics for an aquatic system.

This was the first study comparing qPCR and ddPCR as methods to quantify harmful cyanobacterial species in laboratory and freshwater systems. The designed primers and probes are suitable for duplex detection on both PCR platforms, with the assays achieving satisfactory specificity, sensitivity, and efficiency. Although both techniques required substantial optimization efforts to achieve the desired assay performance and sensitivity, once optimization was achieved, multiplexing reduced the number of re-

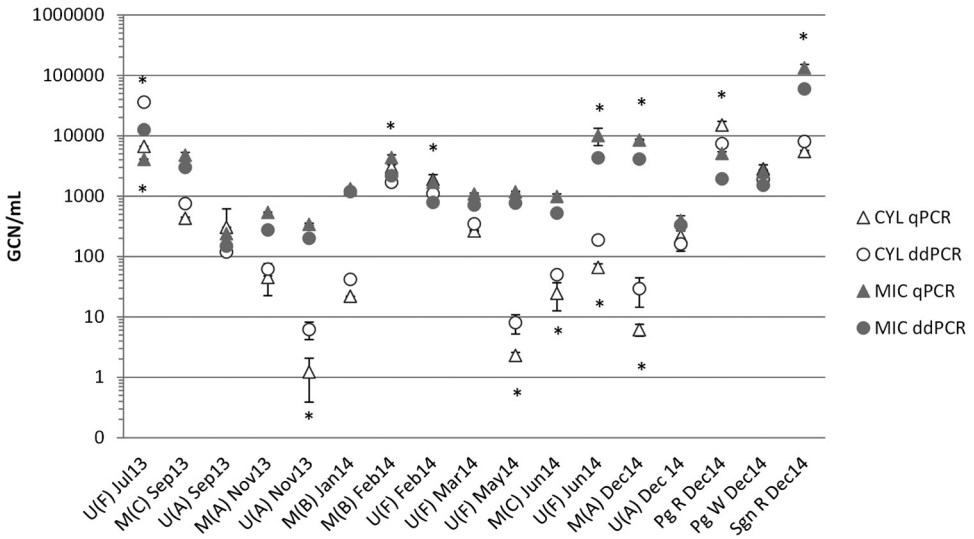

FIG 5 *Microcystis* and *Cylindrospermopsis* abundances in reservoirs measured with qPCR and ddPCR. Each point represents the average of the results from duplicate samples. Error bars denote standard deviations; significant differences between qPCR and ddPCR are indicated with asterisks above the sample points for *Microcystis* and below the sample points for *Cylindrospermopsis*.

actions required to generate data sets as well as sample requirements and costs of reagents and other laboratory consumables. The optimized qPCR assay developed in this study has a larger quantification dynamic range and is more sensitive in detecting targets of low concentrations. While the ddPCR assay was lower in sensitivity, other criteria required for operation are comparable to or even surpass those of the qPCR assay.

ddPCR is known to provide several advantages over qPCR: it is less susceptible to PCR inhibition and high background DNA levels; it gives absolute quantification without relying on an external standard reference; and it is more precise than qPCR (20–22). Our findings are in agreement with those previous observations showing that the ddPCR method was indeed higher in precision for either plasmid standard or environmental sample analysis.

The accurate prediction of ratios of *rpoC1* and *cpcB* gene copy numbers to 16S rRNA gene copy numbers in *Cylindrospermopsis* and *Microcystis* strains indicated that ddPCR is excellent in predicting gene copy number variations in organisms. Multiple gene copy numbers found in human eukaryotic organisms and bacteria have been shown to be associated with human diseases, a faster cellular response to resource availability, and a higher cell growth rate (39). Variation in 16S rRNA gene copy numbers for cyanobacterial genera has been described previously (40, 41). It is believed that gene copy number variation at a specific gene is a result of positive selection, contributing to phenotypic differences in adaptive traits, and it could be phylogenetically informative (39, 42). Therefore, ddPCR is useful in shedding light on natural selection by providing valuable information on copy number variation.

Optimization is required in multiplex assays due to competition between individual assays as well as an increased probability of unwanted cross-oligonucleotide interactions. Inhibition and an effect of competition between targets are major limitations for multiplex qPCR, especially for environmental samples, where the targets may be present in low quantities with a large amount of nontarget background DNA, leading to under- or overestimation of (43, 44) of target gene copy numbers. Quantification bias due to the competitive effect, which was evident in this study, needs to be addressed carefully, as many natural bloom samples are dominated by a single cyanobacterial species. Our results showed that the ddPCR technique was able to overcome this problem, providing accurate quantification for at least two targets with a 1,000-fold difference in concentration.

Nevertheless, the qPCR assay has several advantages in environmental sample analysis. Its higher sensitivity and larger quantification dynamic range allow more flexibility in evaluating environmental samples, where the target could be of a very low concentration (nonbloom/nondominant species) or a very high concentration (bloom occurrence). In addition, qPCR was generally the cheaper of the two techniques. Based on duplex assays developed in this study, the cost to analyze a full 96-well plate was $156.00 for qPCR (~$1.60 per reaction) and $470.00 for ddPCR (~$4.90 per reaction). The qPCR assay was also less laborious than the ddPCR assay. For 96 samples, the preparation time needed for qPCR was 45 min, the amplification time was 80 min, and the data processing time was 15 min; for ddPCR, the preparation time was 150 min, the amplification time was 100 min, and the plate reading time was 120 min. Therefore, for routine water resource monitoring and screening involving large numbers of samples and a quick operational response, the qPCR technique is more cost-effective and able to generate results more rapidly. ddPCR, however, is a better technique when accuracy and precision are of major importance or when PCR inhibition and competitive effects are likely, such as when analyzing bloom samples with a duplex qPCR assay.

Knowledge about molecular techniques and genetic understanding of cyanobacteria have increased tremendously over the past decade, and yet the application of molecular methods in the detection of cyanobacteria for water management and risk assessment is still in its infancy. The potential of molecular quantification assays to monitor cyanobacteria can be fully realized if these assays are robust and reliable and possess high efficiency. In this study, development of duplex assays based on the *rpoC1* and *cpcB* genes shows promise to detect *Cylindrospermopsis* and *Microcystis* on two molecular quantification platforms, qPCR and ddPCR. To the best of our knowledge, this is the first duplex ddPCR assay developed to quantify cyanobacterial species abundance in natural environments.

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