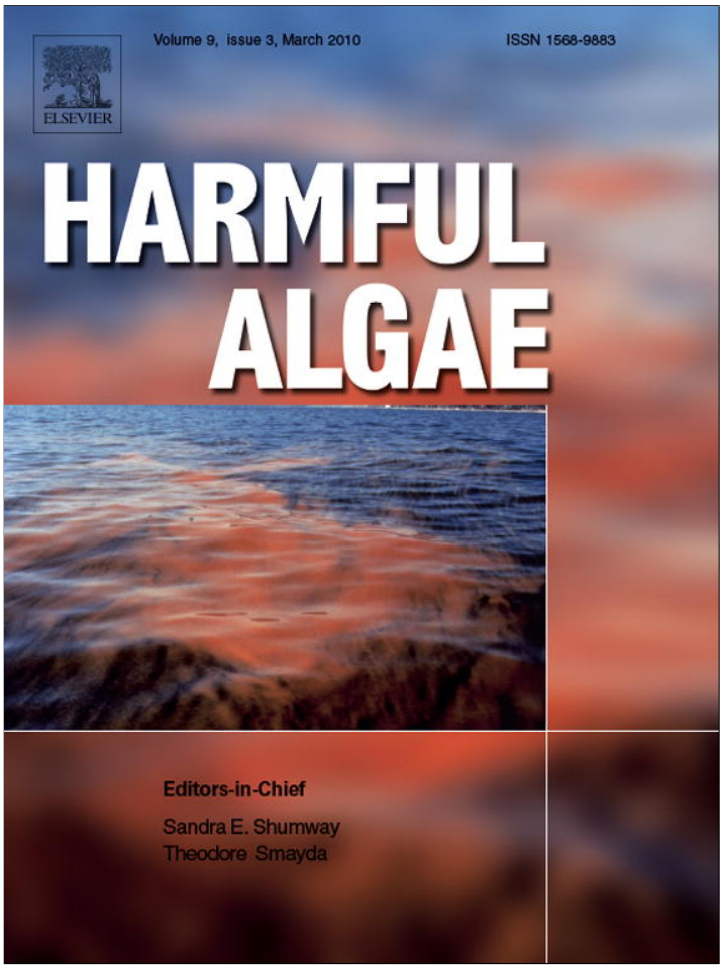

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Estimating the abundance of toxic *Microcystis* in the San Francisco Estuary using quantitative real-time PCR

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ABSTRACT

Developing an effective and rapid method to identify and estimate the abundance of *Microcystis* is warranted in the San Francisco Estuary (SFE) in view of expanding cyanobacterial blooms dominated by *Microcystis* spp. Blooms that occurred in the estuary from July to September 2007 were initially assessed using a standard polymerase chain reaction (PCR) employing primers designed for the conserved *Microcystis*-specific 16S ribosomal DNA (rDNA) region. The presence of microcystin-producing (MC+) toxic *Microcystis* was observed in cyanobacterial and water samples as shown by the amplification of the MC toxin synthetase genes *mcy*B and *mcy*D by standard PCR. The goal of this study was to develop a real-time quantitative PCR (qPCR) based on the 16S rDNA and *mcy*D gene sequences of *Microcystis* found in the SFE to quantify the proportion of toxic *Microcystis* with *mcy*D genes among total *Microcystis* or cyanobacterial population. Cyanobacterial samples collected by diagonal net tows of the water column showed that the ratio of gene copies was dominant for *Microcystis* among cyanobacteria (28–96%), and *Microcystis* carrying *mcy*D genes formed 0.4–20% of the total *Microcystis* spp. Total *Microcystis* was also abundant (7.7×10^4 to 9.9×10^7 cells L^{-1}) in ambient surface waters, and the range of *Microcystis* cell equivalents with *mcy*D genes (4.1×10^2 to 2.2×10^7 cells L^{-1}) indicated a large variation in the ratio of toxic *Microcystis* among total *Microcystis* (0.01–27%). Differences in the proportion of toxic and nontoxic *Microcystis*, as deduced from the cell equivalents of total *Microcystis*, were observed across the sampling locations and seasons where concentrations of total MCs (0.007–10.81 μg/L) also varied. By revealing trends in the sources and magnitude of toxic and nontoxic *Microcystis*, qPCR can contribute to rapid risk assessment and prediction of strategies designed to manage the adverse effects of cyanobacterial blooms in the SFE.

1. Introduction

Cyanobacteria, also known as blue-green algae can form blooms that produce toxins, cause oxygen depletion, alter food webs, and threaten freshwater bodies worldwide that are utilized for drinking, agriculture, fishing, and recreation (Paerl and Huisman, 2009). Toxic blooms in temperate freshwater environments are commonly dominated by *Microcystis* which produce the hepatotoxin, microcystin (Carmichael, 1996; Chorus and Bartram, 1999; Paerl, 2008). Microcystins (MCs) are cyclic heptapeptides produced mainly by cyanobacterial species belonging to the genera *Microcystis*, *Anabaena*, *Nostoc*, and *Oscillatoria* (Sivonen and Jones, 1999; Nishizawa et al., 2000). The peptide synthetase gene cluster is a bidirectional complex of 10 genes, *mcy* (A–J), which control the synthesis of polyketide and peptide synthetases associated with MC synthesis (Tillett et al., 2000; Pearson et al., 2004). Only MC producing (MC+) cyanobacteria carry the *mcy* genes, and genetic differences within this gene cluster determine the production of amounts and congeners of MCs (Pearson et al., 2004). Chronic exposure to cyanotoxins such as MCs causes widespread and serious health problems in animals and humans including liver, digestive and skin diseases, neurological impairment, and death (Carmichael, 2001; Cox et al., 2003). Although the worldwide occurrence of cyanobacterial blooms has increased in recent decades (Chorus and Bartram, 1999; Hudnell and Dortch, 2008), the underlying factors associated with their toxicity and the impact to ecosystems are poorly understood.

Since their initial detection in 1999, cyanobacterial blooms occur annually between June and October in the upper San Francisco Estuary (SFE) across a wide range of salinities (0.1–18 ppt), from the low-flow waters of the central SFE to the western reaches including the Sacramento River (Lehman et al., 2005, 2008). The blooms are composed primarily of colonial forms of *Microcystis aeruginosa* although single cells are also present. Total

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MC concentrations in the estuary have been commonly below the World Health Organization (WHO) recommended limit of 1 μg/l for drinking water (WHO, 1999) in the first half meter of the water column (Lehman et al., 2005, 2008). Various MC congeners are present in the SFE such as LR, LA, and WR that vary in dominance spatially and temporally (Lehman et al., 2005, 2008).

One way *Microcystis* blooms may be detrimental to the SFE is their potential role in the decline of pelagic organisms referred to locally as POD (Sommer et al., 2007; IEP, 2008). Increased levels of MCs during seasonal blooms are hypothesized to cause adverse effects in food resources of pelagic species through direct toxicity or indirectly through impacts on the food web (IEP, 2008; Lehman et al., 2008), which may lead to bioaccumulation of toxins at higher trophic levels particularly fish (Malbrouck and Kestemont, 2006). Variations in the abundance of MC+ *Microcystis* and MC concentrations among locations in the SFE have important ecological implications. For example, liver lesions suggestive of exposure to MCs were observed in striped bass *Morone saxatilis* collected at Antioch in 2005 and 2007 (Lehman et al., 2010). While the potential mechanism of toxin exposure to the striped bass is unknown, fish in the estuary may ingest MC+ phytoplankton and/or zooplankton directly or accidentally while feeding (Vanderploeg et al., 2001; Sedmak and Elersek, 2005; Malbrouck and Kestemont, 2006). In addition, *Microcystis* is an unsuitable food to zooplankton due to its nutritional inadequacy, toxicity, and colonial aggregation (Carmichael, 1996; Hessen et al., 2006; Wilson et al., 2006) that can negatively affect feeding, growth, and reproduction (Kirk and Gilbert, 1992). Survival of dominant zooplankton species in the SFE was reduced following experimental exposures to dissolved MCs and dietary *Microcystis* (Ger et al., 2009, in press). Preliminary *in situ* hybridization analysis also showed the localization of *Microcystis* DNA in the gut and body surface of the zooplankton (Baxa, unpublished data). These findings indicate the need for rapid and accurate diagnosis of *Microcystis* abundance, toxicity and species-specific food web interactions in the SFE using advances in DNA-based molecular tools (Ouellette and Wilhelm, 2003; Nejstgaard et al., 2008).

Although MC concentrations are known to be produced mainly from *Microcystis* populations in the SFE (Lehman et al., 2005, 2008), the proportion of *Microcystis* and other cyanobacterial species that produce the toxin is unknown. It is also unknown how environmental conditions influence MC production in the estuary. The presence of nutrients, light, water temperature and flow, and zooplankton grazing can influence the growth of algae, the onset and development of blooms and the production of MCs (Sivonen and Jones, 1999; Jacoby et al., 2000; Zurawell et al., 2005; Paerl, 2008). For the SFE, research suggests that low water flow is strongly correlated with the production of higher cellular MC concentration (Lehman et al., 2008). Although environmental parameters may affect toxicity by an order of magnitude, the predominance of toxic strains can affect toxicity to 1000 fold (Zurawell et al., 2005). As the proportion of toxic genotypes may determine the overall toxicity of blooms (Kardinaal et al., 2007a), predicting bloom toxicity requires the ability to determine the occurrence and often co-existence of toxic and nontoxic strains of the same species within a genus that are morphologically and taxonomically indistinguishable (Otsuka et al., 1999; Kurmayer et al., 2002).

Real-time quantitative PCR has been successfully applied in toxicity assessments of recurring phytoplankton blooms (Kurmayer et al., 2002; Vaitomaa et al., 2003; Hotto et al., 2008; Pearson and Neilan, 2008; Rinta-Kanto et al., 2005, 2009). While molecular analysis of cyanobacteria in the SFE using the toxin synthetase *mcy*A gene has identified toxic species of *Microcystis* (Moisander et al., 2009), neither the abundance nor the dominance of MC+ *Microcystis* has been quantified in the SFE. The occurrence of cells carrying specific gene targets for cyanobacteria particularly *Microcystis* cells carrying the genes associated with the production of MC toxin was assessed using conventional PCR. Our study focused on developing a qPCR assay to estimate the ratio of MC+ *Microcystis* based on the proportion of *Microcystis* with *mcy*D genes and the abundance of total *Microcystis* to provide an overview of the toxicity of blooms that occurred in the SFE from July to September 2007.

2. Materials and methods

2.1. Study sites and collection of samples

In collaboration with the CA Department of Water Resources, cyanobacterial samples were collected from 7 stations in the SFE including Brannan Island and Chipps Island in the Sacramento River; Antioch, Mildred Island, Old River, San Joaquin, and Venice Cut in the San Joaquin River (Fig. 1). Samples were collected intermittently from these sites between July 24 and September 18, 2007 when *Microcystis* blooms were abundant across the estuary.

Cyanobacterial samples were collected using diagonal tows with a plankton net (153 μm mesh). This collection procedure provided a representative example of large *Microcystis* colonies that may be dispersed in the water column. To include colonies and single cells from ambient surface waters which can escape the plankton net, samples were collected by dipping 2 L amber HDPE bottle (Fisher, PA, USA) on the water surface. Plankton samples from tow nets were stored in acid washed containers while the surface samples were retained in their containers (acid washed). Samples were kept at 4 °C and filtered within 4 h onto a 0.5-μm GF/F Whatman filter (Whatman, Maidstone, UK). For cellular MC analysis, plankton samples from the net tows were filtered onto 0.5-μm GF/F glass fiber filters (Whatman). Filtered plankton samples were wrapped in aluminum foil and frozen at −80 °C until used for DNA extraction and total MC analysis.

2.2. Microcystin (MC) analysis

Toxins from cyanobacterial samples were extracted from the filter by sonication (3 times, 20 s bursts on ice) with 10 ml of 50%

Fig. 1. Map of San Francisco Estuary showing the locations for collecting cyanobacterial samples during the bloom season in 2007.

methanol containing 1% acetic acid, and the extract clarified by centrifugation and used for MC analysis. Recovery of MCs (e.g., LR, RR) using this procedure is greater than 90% as determined using duplicate samples. Concentrations of total MCs in cyanobacterial samples were determined using the protein phosphatase inhibition assay, PPIA (Carmichael and An, 1999). Briefly, assays were run in 96-well plates containing 0.1 mU recombinant phosphatase 1A catalytic subunit (Roche Applied Science, Indianapolis, IN), 1.05 mg para-nitrophenyl phosphate (Sigma, St. Louis, MO) and 10 μl of sample or *Microcystis aeruginosa* MC-LR (Sigma) as previously described (Lehman et al., 2008).

2.3. Extraction of genomic DNA

Cyanobacterial biomass showed an even distribution on the filters following filtration of samples from net tows and surface waters. Biomass from the net tows was generally denser of which only half filter was used for genomic DNA extraction. In contrast, cyanobacteria from surface waters were relatively rare in which the entire filter was used for DNA extraction. In both cases, the number of filtered biomass fractions and the volume of filtered water were factored in estimating the DNA concentration. After thawing the frozen filters at room temperature, plankton biomass was removed aseptically from the filter using forceps and processed for genomic DNA extraction using a standard phenol–chloroform procedure (Sambrook and Russell, 2001) combined with a phenol extraction method used in qPCR analysis of plankton (Rinta-Kanto et al., 2005). Briefly, plankton cells were suspended in 100 μl lysis buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.2% sodium dodecyl sulfate), and added proteinase K (Qiagen Inc., Valencia, CA) to a final concentration of 50 μg/ml. The volumes of lysis buffer and proteinase K were doubled in dense plankton biomass from the net tows. The suspension was placed overnight in a shaking incubator at 56 °C. Genomic DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) (Sigma), following to ethanol precipitation. DNA was dissolved in 100 μl of Tris HCl pH 8.0–EDTA (TE) buffer and the concentration was measured with a spectrophotometer (BioPhotometer plus, Eppendorf, NY). Some of the DNA samples were added TE buffer to obtain ≤400 ng/μl of concentrations.

2.4. Conventional PCR

2.4.1. Detection of cyanobacteria, Microcystis and MC synthetase genes

Genomic DNA of cyanobacterial samples were initially examined by conventional PCR to demonstrate the presence of cyanobacteria and *Microcystis* using specific PCR primer sets targeting the 16S rDNA (Neilan et al., 1997; Urbach et al., 2001). To establish cyanobacterial toxicity, primer sets designed for the MC synthetase genes, *mcy*B and *mcy*D, were used to detect MC+ *Microcystis* (Kaebernick et al., 2000; Ouellette et al., 2006). The targets, primer sequences, amplicon size and references are listed in Table 1. The volume of the PCR cocktail was 50 μl containing 200 μM of each dNTP, 1.5 mM of $MgCl_2$, 40 pmol of each primer, 1.5 unit Platinum Taq DNA polymerase (Invitrogen Corporation, Carlsbad, CA) and 10× buffer at 1/10 the volume of the reaction. The PCR conditions for the conventional PCR are as follows: initial denaturation step of 95 °C for 5 min, 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 90 s, followed by a final extension step at 72 °C for 5 min and then held at 4 °C. The PCR product was separated on 2% agarose gels and observed by a transilluminator after staining with 1% ethidium bromide solution for 20 min. Genomic DNA extracted from *M. aeruginosa* producing MC toxin, MC+ (MC-LR UTEX 2385) and non-MC producing *M. aeruginosa*, MC− (MC-LR UTEX 2386) (University of Texas Culture Collection, Austin, TX) were used as controls for the initial screening of mixed cyanobacterial samples to detect the target genes by conventional PCR. Both type strains of *M. aeruginosa* were grown in axenic batch cultures in our laboratory using a modified ASM-1 medium maintained at 22 °C and 16:8 L:D light regime (Ger et al., in press).

2.4.2. Cloning, sequencing and sequence analysis of PCR amplicons

The amplicons were ligated into pGEM-T Easy vector (Promega BioSciences, San Luis Obispo, CA) that was used to transform *Escherichia coli* DH5α competent cells (Invitrogen Corp., Carlsbad, CA). The plasmid containing the inserted DNA fragment was extracted using QIAprep Spin Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. The sequence of the insert was confirmed using M13 forward and reverse primers by fluorescently labeled dideoxy terminator sequencing using an ABI 377 automated DNA sequencer (Applied Biosciences, Foster City, CA). Gene sequences were determined from the plasmid DNA of 10 plankton samples from the 7 locations in the SFE. The sequences were aligned and compared to available sequences of *Microcystis*, gene clusters encoding the biosynthetic enzymes *mcy* (microcystin) and other peptide synthetase genes in GenBank database using Clustal W Program Version 1.83 (Thompson et al., 1994).

2.5. Real-time quantitative PCR (qPCR)

2.5.1. Plasmid preparation for qPCR

The plasmid DNA carrying the target sequence for cyanobacteria, *Microcystis* 16S rDNA, and *mcy*D were prepared as follows: the partial fragment of the 16S rDNA from cyanobacteria (1200 bp), the *Microcystis* 16S rDNA fragment (230 bp), the *mcy*B (320 bp) and *mcy*D (298 bp) synthetase gene fragments were amplified with forward and reverse primer sets listed in Table 1. The genomic DNA of 10 plankton samples from the 7 locations in the SFE was used as template DNA while MC+ *M. aeruginosa* UTEX 2385 and MC− *M. aeruginosa* UTEX 2386 were used as control DNA template. The PCR assay and conditions were conducted as described in Section 2.4.1, the amplicons were cloned into a vector, and the plasmid DNA was extracted as described above. The obtained sequences were deposited in GenBank database (Accession nos. GQ426031, GQ426032). The copy number of the plasmid DNA was calculated according to its molecular weight and concentration, and then converted into the copy number based

Table 1
Primers used for conventional PCR to amplify cyanobacteria, *Microcystis* and microcystin synthetase genes, *mcy*B and *mcy*D, from cyanobacteria in the San Francisco Estuary.

Target	Primer	Sequence (5′–3′)	Target size	Reference
Cyanobacteria	CYA 108 F	ACG GGT GAG TAA CRC GTR A	1200 bp	Urbach et al. (2001)
16S rRNA	CYA16SCYR	CTT CAY GYA GGC GAG TTG CAG C		
Microcystis	MIC 184 F	GCC GCR AGG TGA AAM CTA A	230 bp	Neilan et al. (1997)
16S rRNA	MIC 431 R	AAT CCA AAR ACCT TCC TCC C		
Microcystis	mcyB 2959 F	TGG GAA GAT GTT CTT CAG GTA TCC AA	320 bp	Nonneman and Zimba (2002)
*mcy*B	mcyB 3278 R	AGA GTG GAA ACA ATA TGA TAA GCTA C		
Microcystis	mcyD F2 F	GGT TCG CCT GGT CAA AGT AA	298 bp	Kaebernick et al. (2000)
	*mcy*D R2 R	CCT CGC TAA AGA AGG GTT GA		

on Avogadro's number (1 mol = 6.022×10^{23} molecules). Serial dilutions of the plasmid DNA (1×10^6 through 10 copies/5 μl) were prepared with molecular grade water and used for generation of the standard curve for cyanobacteria, *Microcystis*, and *mcy*D qPCR assays.

2.5.2. Development of real-time quantitative PCR (qPCR) for *Microcystis*

The TaqMan probe and forward and reverse primers for *Microcystis* were designed with the Primer Express 3.0 software (Applied Biosystems) using the 16S rDNA sequences obtained from cyanobacterial samples from the SFE. These sequences were compared against the unique region of *Microcystis* based on alignments with other cyanobacterial species such as *Anabaena* sp. (AJ133160) and *Nostoc* sp. (AB187508). The TaqMan probe was labeled with the fluorescent reporter FAM at the 5′ end and with the quencher minor groove binding (MGB) at the 3′ end. The PCR contained 400 nM of each primer, 80 nM of the TaqMan probe, 2× TaqMan Universal Master Mix with UNG (Applied Biosystems, Foster City, CA), and 5 μl of the extracted DNA samples in a final volume of 12 μl. The samples and plasmid DNA standards were placed in MicroAmp fast optical 48-well reaction plate and sealed with MicroAmp 48-well optical adhesive film (Applied Biosystems). The qPCR assays were run using a StepOne Real-Time PCR System (Applied Biosystems). The PCR program for cyanobacteria and *Microcystis*-specific 16S rDNA was conducted according to conditions suggested in the StepOne qPCR machine: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, and 60 °C for 60 s. For the *mcy*D assay, thermocycling steps were followed from Rinta-Kanto et al. (2005): 50 °C for 3 min, 95 °C for 10 min, followed by 45 cycles of 95 °C for 30 s, 61 °C for 1 min, and 72 °C for 20 s.

Calculations of threshold cycle (*C*t) were conducted using the StepOne Version 2.0 Software (Applied Biosystems), which automatically determined the highest possible correlation coefficient (r^2) for the standard curve. *Microcystis* has two copies of 16S rDNA in its genome (Kaneko et al., 2007) therefore the gene copy number obtained by qPCR based on the plasmid standard curve was divided by 2 to determine the total *Microcystis* MC-LR cell equivalents. Reactions were run in triplicate and results are reported as mean ± SD. The number of MC− *Microcystis* cells were estimated by subtracting the cell number of MC+ *Microcystis* (*mcy*D carriers) from the total number of *Microcystis* cells (MC+ and MC− strains) (Rinta-Kanto et al., 2005).

For samples used in the current study, dense plankton biomass were collected from the tow nets rendering prolonged filtration, from which colonial forms were randomly picked in some samples. As water volumes were not used from these net tow samples, the qPCR values were expressed only as gene copies/μg DNA. In contrast, surface water samples represent ambient concentrations of colonial and single cells with less plankton biomass thus, the *Microcystis* cell density was estimated as the number of cell equivalents L^{-1} based on the volume of samples collected.

2.5.3. Specificity and sensitivity of TaqMan real time qPCR assay for *Microcystis* 16S rDNA and mcyD

The specificity of the qPCR assays was verified by running reactions using genomic DNA from pure cultures of cyanobacterial species including *Synechoccus* sp., *Planktothrix* sp., *Microcystis aeruginosa* type strains: MC+ MC-LR UTEX 2385 and PCC 7806 (from Dr. Steven Wilhelm, University of Tennessee), MC− *M. aeruginosa* (MC-LR UTEX 2386), as well as 10 different *Microcystis* spp. from California (provided by Dr. Pia Moisander, UC Santa Cruz). Approximately 50 ng of genomic DNA was used in the qPCR using procedures described above. The sensitivity of the assays was determined by comparing the standard curve generated from plasmid standards.

3. Results

Cyanobacteria and *Microcystis*-specific 16S rDNA and the MC synthetase genes *mcy*B and *mcy*D were amplified from cyanobacterial samples collected in the SFE (Fig. 2). Using pure cultures of MC+ *M. aeruginosa* (MC-LR UTEX 2385) and MC− *M. aeruginosa* (MC-LR UTEX 2386) as reference strains, *Microcystis* that amplified the *mcy*B and *mcy*D toxin genes was considered toxic and was discriminated from nontoxic *Microcystis* lacking these genes (Fig. 2). Specific primers and probes for the qPCR assay that were designed in this study (Table 2) were able to detect and quantify the 16S rDNA target genes for cyanobacteria and *Microcystis*, and the MC synthetase *mcy*D gene of high analytical sensitivity detecting as few as 10 copies of the target gene/reaction with high specificity against closely related cyanobacteria such as *Synechoccus* sp., *Planktothrix* sp. and 10 different strains of *Microcystis* spp. (data not shown). Analysis by qPCR quantified the gene copies of cyanobacteria, *Microcystis* spp. and *mcy*D from tow net (Table 3) and from ambient surface water samples (Table 5) from which gene copies were expressed as cell equivalents. As mentioned above, the lack of information on filtered biovolumes of some tow net samples precluded the conversion of gene copies to cell equivalents.

Using the qPCR assays developed in this study, variations in the cell equivalents of toxic *Microcystis* (*mcy*D gene carriers) and total *Microcystis* spp. were generally observed across the sites and sampling dates in surface water samples (Table 5). The tow net samples also showed variations in gene copies across sampling sites and dates (Table 3). Based on the estimated percentages of *Microcystis* 16S, cyanobacterial 16S, and *mcy*D gene copies, the

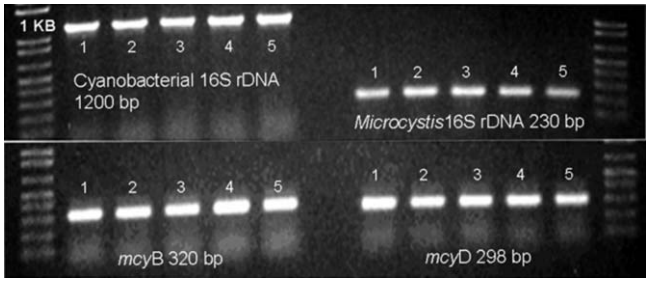

Fig. 2. Gel image of conventional PCR showing amplification of target genes and corresponding lengths (bp): cyanobacterial and *Microcystis* 16S rDNA fragments and the microcystin synthetase genes *mcy*B and *mcy*D from cyanobacterial samples in the San Francisco Estuary.

Table 2
Primers and probes for *Microcystis* spp. and cyanobacteria designed from the 16S rDNA sequences of *Microcystis* present in the San Francisco Estuary.

Target	Primer/probe	Sequence (5′ → 3′)
Microcystis 16S rRNA	MIC16S F	AAA GCG TGC TAC TGG GCT GTA
	MIC16S R	CCC TTT CGC TCC CCT AGC T
	MIC16S P[a]	CTG ACA CTC AGG GAC G
*Microcystis mcy*D	*mcy*D F	GGT TCG CCT GGT CAA AGT AA
	*mcy*D R	CCT CGC TAA AGA AGG GTT GA
	*mcy*D P[a]	ATG CTC TAA TGC AGC AAC GGC CAA A
Cyanobacteria 16S rRNA	CYA16S F	TCG CCC ATT GCG GAA A
	CYA16S R	AGA CAC GGC CCA GAC TCC TA
	CYA16S P[a]	TTC CCC ACT GCT GCC

[a] The probes were labeled with 6FAM and MGBNFQ as reporter and quencher, respectively. All primers and probes were developed in the current study except that of *mcy*D from Rinta-Kanto et al. (2005).

Table 3
Mean copy numbers of toxic *Microcystis* (*mcy*D), total *Microcystis* (16S rDNA) and cyanobacterial (16S rDNA) genes, and mean total microcystin concentrations in the San Francisco Estuary. Data refer to cyanobacteria collected by diagonal net tows.

Site, date	Total microcystin (μg/L)	Toxic *Microcystis mcy*D (gene copies/μg DNA)	Total *Microcystis* 16S rDNA (gene copies/μg DNA)	Cyanobacteria 16S rDNA (gene copies/μg DNA)
AT-8/07/07	1.65 ± 0.08	$1.4 \pm 1.24 \times 10^5$	$4.1 \pm 0.28 \times 10^6$	$7.5 \pm 0.32 \times 10^6$
AT-8/21/07	0.28 ± 0.02	$1.2 \pm 0.11 \times 10^6$	$3.5 \pm 0.21 \times 10^7$	$6.1 \pm 0.44 \times 10^7$
AT-9/05/07	0.02 ± 0.001	$6.4 \pm 0.55 \times 10^4$	$5.1 \pm 0.04 \times 10^6$	$1.4 \pm 0.03 \times 10^7$
AT-9/18/07	0.16 ± 0.000	$2.4 \pm 1.78 \times 10^7$	$3.6 \pm 0.31 \times 10^7$	$5.0 \pm 0.16 \times 10^7$
BI-8/07/07	3.06 ± 0.052	$3.8 \pm 1.81 \times 10^4$	$3.3 \pm 0.81 \times 10^5$	$6.3 \pm 0.61 \times 10^5$
BI-8/21/07	0.176 ± 0.03	$1.1 \pm 0.08 \times 10^6$	$4.1 \pm 0.26 \times 10^7$	$5.2 \pm 0.23 \times 10^7$
BI-9/05/07	0.007 ± 0.000	$3.6 \pm 0.07 \times 10^6$	$5.5 \pm 0.58 \times 10^7$	$5.7 \pm 0.38 \times 10^7$
CI-7/25/07	0.046 ± 0.002	$5.0 \pm 2.57 \times 10^5$	$3.9 \pm 0.05 \times 10^6$	$5.5 \pm 0.19 \times 10^6$
MI-7/25/07	0.021 ± 0.001	$1.1 \pm 0.07 \times 10^6$	$2.9 \pm 0.14 \times 10^7$	$5.3 \pm 0.82 \times 10^7$
MI-8/08/07	0.736 ± 0.036	$8.0 \pm 0.42 \times 10^4$	$1.6 \pm 0.05 \times 10^6$	$2.6 \pm 2.77\text{x } 10^6$
MI-8/22/07	0.091 ± 0.005	$6.2 \pm 0.18 \times 10^5$	$1.8 \pm 0.38 \times 10^7$	$1.8 \pm 0.12 \times 10^7$
MI-9/04/07	0.032 ± 0.000	$1.8 \pm 0.11 \times 10^5$	$9.9 \pm 0.76 \times 10^6$	$2.6 \pm 0.78 \times 10^7$
MI-9/18/07	0.016 ± 0.005	$2.2 \pm 0.08 \times 10^6$	$1.1 \pm 0.01 \times 10^7$	$1.8 \pm 0.04 \times 10^7$
OR-8/08/07	0.696 ± 0.000	$2.4 \pm 0.40 \times 10^5$	$1.0 \pm 0.01 \times 10^7$	$1.8 \pm 0.16 \times 10^7$
OR-8/22/07	Not done	$3.8 \pm 0.74 \times 10^5$	$5.9 \pm 0.41 \times 10^6$	$2.0 \pm 0.11 \times 10^7$
OR-9/04/07	0.032 ± 0.000	$5.6 \pm 0.66 \times 10^5$	$1.5 \pm 0.19 \times 10^7$	$2.4 \pm 0.06 \times 10^7$
SJ-7/25/07	Not done	$2.1 \pm 0.26 \times 10^5$	$3.7 \pm 0.11 \times 10^7$	$3.8 \pm 0.09 \times 10^7$
VC-7/25/07	10.807 ± 0.227	$1.4 \pm 0.12 \times 10^5$	$3.4 \pm 0.29 \times 10^7$	$8.6 \pm 0.07 \times 10^7$

Sampling sites: AT = Antioch, BI = Brannan Island, CI = Chipps Island, MI = Mildred Island, OR = Old River, SJ = San Joaquin, VC = Venice Cut.

proportion of MC+ *Microcystis* formed 0.4–20.2% of the total *Microcystis* spp., and from 0.16 to 12.5% of the total cyanobacterial community (as determined by the cyanobacterial 16S; Table 4). The abundance of MC+ *Microcystis* with *mcy*D genes was generally lower when estimated among cyanobacterial 16S rDNA genes. The mean proportion of *Microcystis* spp. with *mcy*D genes over total *Microcystis* spp. was about 2–20 times higher in Antioch, Brannan Island, Chipps Island, and Mildred Island compared to Old River, San Joaquin, and Venice Cut. Based on the calculated percentages of *Microcystis* and cyanobacterial 16S of plankton samples, *Microcystis* dominated (>50%) the cyanobacterial population with the exception of Old River (28.6%, 8/22/07), Venice Cut (40.0%, 7/25/07), Mildred Island (41.7%, 9/04/07), and Antioch (36.1%, 9/05/07) (Table 4).

In addition to colony forming cyanobacteria throughout the water column, single cells were sampled in the surface layer. The estimated cell equivalents carrying the gene targets in surface water showed that MC+ *Microcystis* and total *Microcystis* varied across the sites and phases of the bloom development (Table 5). On the average, the lowest cell equivalents of toxic *Microcystis* spp. was observed at Chipps Island ($8.5 \pm 6.7 \times 10^3$ cells L^{-1}), Brannan Island ($3.04 \pm 5.1 \times 10^4$ cells L^{-1}), and San Joaquin ($3.4 \pm 3.3 \times 10^4$ cells L^{-1}). The mean highest cell equivalents of toxic *Microcystis* spp. occurred in Antioch ($4.8 \pm 9.5 \times 10^6$ cells L^{-1}) and Franks Tract (mean $5.1 \pm 7.1 \times 10^6$ cells L^{-1}) with the rest of the sites at one magnitude lower (Table 5). While the cell equivalents of total *Microcystis* cells was generally high in all sampling sites and dates

Table 4
Percentages of gene copy numbers of toxic *Microcystis* (*mcy*D) in the San Francisco Estuary. Data refer to cyanobacteria collected by diagonal net tows.

Site	Toxic *Microcystis*	Toxic *Microcystis*	Total *Microcystis*
Date	Total *Microcystis*	Total cyanobacteria	Total cyanobacteria
AT-7/24/07	11.49 ± 4.0	6.96 ± 0.7	64.27 ± 16.9
AT-8/07/07	3.12 ± 2.6	1.76 ± 1.4	55.02 ± 3.2
AT-8/21/07	3.58 ± 0.6	2.05 ± 0.2	57.86 ± 7.4
AT-9/05/07	1.24 ± 0.1	0.31 ± 0.2	36.19 ± 2.1
AT-9/18/07	17.87 ± 3.6	12.56 ± 0.6	72.12 ± 13.9
BI-8/07/07	17.89 ± 16.4	5.51 ± 1.8	56.17 ± 29.1
BI-8/21/07	2.48 ± 0.1	2.07 ± 0.3	75.76 ± 0.01
BI-9/05/07	6.60 ± 0.7	6.32 ± 0.4	96.17 ± 5.1
CI-7/25/07	15.93 ± 14.1	8.64 ± 5.9	72.49 ± 21.9
MI-7/25/07	3.93 ± 0.9	2.14 ± 0.4	55.11 ± 8.8
MI-8/08/07	5.05 ± 1.2	2.87 ± 0.3	66.11 ± 12.1
MI-8/22/07	3.56 ± 1.6	3.38 ± 0.04	73.31 ± 11.6
MI-9/04/07	1.89 ± 0.1	0.78 ± 0.3	41.76 ± 19.7
MI-9/18/07	20.25 ± 1.2	12.02 ± 1.1	59.32 ± 3.2
OR-8/08/07	2.49 ± 0.1	1.35 ± 0.1	54.32 ± 1.7
OR-8/22/07	6.37 ± 1.3	1.83 ± 0.4	28.60 ± 0.9
OR-9/04/07	3.81 ± 1.3	2.38 ± 0.3	64.49 ± 12.2
SJ-7/25/07	0.59 ± 0.1	0.57 ± 0.09	93.28 ± 2.7
VC-7/25/07	0.37 ± 0.1	0.16 ± 0.02	40.00 ± 6.1

Sampling sites: AT = Antioch, BI = Brannan Island, CI = Chipps Island, MI = Mildred Island, OR = Old River, SJ = San Joaquin, VC = Venice Cut.

Table 5
Cell equivalents of toxic *Microcystis* (*mcy*D) and total *Microcystis* from ambient surface water samples in the San Francisco Estuary.

Site	Toxic *Microcystis*	Total *Microcystis*	Toxic *Microcystis*
Date	(Cell equivalents L^{-1})[a]	(Cell equivalents L^{-1})	Total *Microcystis*
AT-7/24/07	$8.1 \pm 2.55 \times 10^2$	$7.7 \pm 0.04 \times 10^4$	1.03 ± 0.32
AT-8/07/07	$4.1 \pm 0.15 \times 10^3$	$6.1 \pm 0.42 \times 10^6$	0.06 ± 0.005
AT-8/21/07	$2.7 \pm 0.88 \times 10^3$	$8.8 \pm 0.90 \times 10^6$	0.03 ± 0.007
AT-9/05/07	$2.2 \pm 0.46 \times 10^7$	$9.9 \pm 0.28 \times 10^7$	23.08 ± 4.84
AT-9/18/07	$1.2 \pm 0.06 \times 10^6$	$4.6 \pm 0.36 \times 10^6$	27.67 ± 2.20
BI-7/24/07	$4.1 \pm 0.16 \times 10^2$	$8.9 \pm 1.68 \times 10^6$	0.01 ± 0.009
BI-8/07/07	$4.0 \pm 0.02 \times 10^3$	$7.3 \pm 1.20 \times 10^5$	0.55 ± 0.10
BI-8/21/07	$7.7 \pm 5.60 \times 10^2$	$1.9 \pm 0.06 \times 10^6$	0.03 ± 0.02
BI-9/04/07	$1.1 \pm 0.01 \times 10^5$	$8.9 \pm 6.73 \times 10^6$	3.89 ± 5.16
CI-7/24/07	$2.6 \pm 0.55 \times 10^3$	$1.9 \pm 0.10 \times 10^6$	0.13 ± 0.02
CI-8/07/07	$1.4 \pm 0.35 \times 10^4$	$5.9 \pm 0.06 \times 10^5$	2.43 ± 0.62
FT-8/01/07	$7.7 \pm 0.16 \times 10^5$	$3.1 \pm 0.20 \times 10^7$	2.48 ± 0.11
FT-8/08/07	$1.4 \pm 0.008 \times 10^7$	$5.5 \pm 0.35 \times 10^7$	26.46 ± 1.85
FT-8/21/07	$1.9 \pm 0.49 \times 10^4$	$2.2 \pm 0.07 \times 10^7$	0.08 ± 0.02
MI-7/25/07	$8.3 \pm 1.46 \times 10^3$	$3.8 \pm 0.37 \times 10^7$	0.02 ± 0.002
MI-8/08/07	$1.0 \pm 0.11 \times 10^4$	$6.9 \pm 1.30 \times 10^6$	0.15 ± 0.02
MI-8/22/07	$1.4 \pm 0.39 \times 10^3$	$2.9 \pm 0.01 \times 10^7$	0.005 ± 0.001
MI-9/04/07	$8.6 \pm 0.77 \times 10^5$	$3.4 \pm 0.25 \times 10^7$	2.48 ± 0.22
MI-9/18/07	$3.7 \pm 0.78 \times 10^5$	$9.0 \pm 0.34 \times 10^6$	4.14 ± 0.74
OR-8/08/07	$1.6 \pm 0.12 \times 10^5$	$1.0 \pm 0.04 \times 10^7$	1.55 ± 0.16
OR-8/22/07	$6.4 \pm 0.98 \times 10^3$	$2.8 \pm 0.37 \times 10^7$	0.02 ± 0.005
OR-9/04/07	$9.8 \pm 1.07 \times 10^4$	$1.0 \pm 0.07 \times 10^7$	0.94 ± 0.13
OR-9/18/07	$4.4 \pm 0.46 \times 10^5$	$7.5 \pm 0.48 \times 10^6$	5.84 ± 0.59
SJ-7/25/07	$6.3 \pm 1.27 \times 10^4$	$1.7 \pm 1.33 \times 10^7$	0.98 ± 1.21
SJ-8/22/07	$4.3 \pm 0.11 \times 10^3$	$3.2 \pm 0.08 \times 10^7$	0.01 ± 0.003
VC-7/25/07	$1.2 \pm 0.08 \times 10^6$	$1.0 \pm 0.06 \times 10^7$	12.03 ± 0.87
VC-8/22/07	$3.9 \pm 0.71 \times 10^5$	$3.3 \pm 0.21 \times 10^6$	11.91 ± 2.45

Sampling sites: AT = Antioch, BI = Brannan Island, CI = Chipps Island, FT = Franks Tract, OR = Old River, SJ = San Joaquin, VC = Venice Cut.
[a]Cell equivalents of total and toxic *Microcystis* was calculated from the copy number of *Microcystis* 16S rDNA (two copies per genome, Kaneko et al., 2007) or *mcy*D gene (one copy per genome, Kaebernick et al., 2002), respectively.

($7.7 \pm 0.02 \times 10^4$ to $9.9 \pm 0.16 \times 10^7$ cells L^{-1}), the proportion of toxic among total *Microcystis* spp. was highest on the average in Antioch (10.37%), Franks Tract (9.67%), and Venice Cut (11.97%) and generally lower (mean ranges of 0.49–2.09%) in Brannan Island, Chipps Island, San Joaquin, Mildred Island, and Old River (Table 5).

Total MC concentrations from the tow net samples varied across locations and sampling times (0.007–10.8 μg/L) with the highest level in one sampling date in Venice Cut on 7/25/07 (10.8 μg/L), followed by Brannan Island (mean 1.08 μg/L) and Antioch (mean 0.51 μg/L) (Table 3). The estimated mean cell equivalents of *Microcystis* with *mcy*D genes in plankton samples from these sites were 1.4×10^5, 1.4×10^6, and 2.2×10^6 gene copies/μg DNA for Venice Cut, Brannan Island, and Antioch, respectively (Table 3). In ambient surface waters, the estimated mean cell equivalents of *Microcystis* with *mcy*D genes were 8.2×10^5, 3.0×10^4, and 4.8×10^6 cells L^{-1}, respectively for Venice Cut, Brannan Island and Antioch (Table 5).

4. Discussion

Microcystis was dominant among cyanobacterial populations collected in the SFE with variations in cell equivalents of toxic *Microcystis* carrying *mcy*D genes. The proportion of toxic and nontoxic *Microcystis*, which represent the difference in the frequency of toxic and total number of *Microcystis* cells (Rinta-Kanto et al., 2005), varied between sites and phases of the bloom development. Toxic *Microcystis* with *mcy*D genes formed up to 27% of the total *Microcystis* population in the SFE (cell equivalents of ambient surface water), which co-existed with approximately 73% nontoxic (MC−) *Microcystis*. Our findings concur with previous investigations in Lake Erie where toxic genotypes are generally lower than the abundance of total *Microcystis* in natural algal populations (Rinta-Kanto et al., 2009). In other cyanobacterial-prone bodies of water, the percentage of MC+ among nontoxic *Microcystis* assemblages ranged from 1.7 to 71% in Lake Wannsee, Germany (Kurmayer and Kutzenberger, 2003) and from 0.5 to 35% in Lake Mikata, Japan (Yoshida et al., 2007).

A combination of factors may influence variations in the abundance of MC+ and MC− *Microcystis* in the SFE. Streamflow was shown to affect the spread of plankton blooms across the estuary (Lehman et al., 2008). Alternately, wind and tide can enhance the aggregation of *Microcystis* biomass in shallow or low-flow water reaches such as Brannan Island and Antioch. Mixing processes in the ecosystem can affect the development and distribution of blooms (Huisman et al., 2004; Jöhnk et al., 2008) in addition to water temperature, nutrient loading, and light (Zurawell et al., 2005). Furthermore, the role of climate change has been suggested as a potent catalyst for the further expansion of cyanobacterial blooms in eutrophic waters on a global scale (Paerl and Huisman, 2009). How variations in water temperature can affect the magnitude of recurrence and toxicity of plankton blooms in the SFE is unknown and is an enormous task that remains to be determined.

The presence or absence of MC− and MC+ *Microcystis* was established in the current study using the *Microcystis*-specific 16S rRNA gene (16S rDNA) and the *mcyD* gene. The *Microcystis* 16S rRNA gene is specific to the *Microcystis* genus, which was used to estimate total *Microcystis* cell equivalents. As the MC biosynthesis gene cluster, *mcy*, is present only in toxic cyanobacterial (MC+) strains (Tillett et al., 2000), the ability to detect and quantify the *mcy*D gene among mixed populations of *Microcystis* spp. provided an evidence of the relative abundance and frequency of MC+ *Microcystis* strains in the SFE. Estimating the proportion of cells carrying *Microcystis*-specific 16S rRNA and *mcy*D gene targets in the current study provided, to the best of our knowledge, the first description on the abundance of MC+ *Microcystis* strains among cyanobacterial populations in the SFE. Our results enhance recent research on the description of toxic *Microcystis* in the estuary based on the *mcy*A gene, which partly encodes the peptide synthetase cluster for MC production (Moisander et al., 2009).

The genomic DNA of MC+ strain of *M. aeruginosa* (MC-LR UTEX 2385) was used to estimate the cell equivalents of *Microcystis* in the SFE carrying the specific 16S rDNA target genes. Although a local *Microcystis* strain would have been an ideal standard, the MC-LR is one of the dominant toxin congeners in the estuary (Lehman et al., 2005) making this strain an appropriate standard surrogate in the current study. Furthermore, the 16S rDNA gene has two copies in each *Microcystis* genome (Kaneko et al., 2007) that would account for the different *Microcystis* spp. producing different MC+ congeners in the SFE.

Difficulties have been encountered in the development of qPCR for cyanobacterial diagnostics in field samples due to variations in the copy number of 16S rDNA genes that may affect estimates of cell density (Rinta-Kanto et al., 2005). In our study, two copies of 16S rDNA genes per genome (Kaneko et al., 2007) and one copy of *mcy*D gene per genome (Kaebernick et al., 2002) were used to estimate the number of cell equivalents. This ratio was effectively used to determine the percentage of toxic *Microcystis* and the total *Microcystis* cell equivalents in the cyanobacterial samples. In some surface water samples however, the cell equivalents were lower for cyanobacteria compared to *Microcystis* (data not shown). While the cause of this anomalous result is unknown in the current study, previous studies attributed this finding to using the 16S rDNA as a target for quantifying both cyanobacteria and *Microcystis* with variable copy number of 16S rDNA operons (Rinta-Kanto et al., 2009), to sequence heterogeneity in natural populations of cyanobacterial cells (Crosby and Criddle, 2003), or an error in estimating the genome size of reference strains (Kardinaal and Visser, 2005). Although estimates of total cyanobacterial cell equivalents were not accurate in some samples in the current study, assessing the genome ratio of the *Microcystis* 16S rDNA and *mcy*D genes of plankton and surface waters demonstrated the variations in the frequency and distribution of MC+ and MC− *Microcystis* during the bloom development in the SFE, which was the main focus of this investigation.

Microcystin concentrations did not correspond to the frequency of toxic *Microcystis* cell equivalents in our study. One possible explanation is the potential occurrence of other MC+ genera, in addition to *Microcystis*, that may contribute to bloom toxicity in the SFE (Lehman et al., 2010). Other types of MC toxin congeners with varying toxicity were probably present in the estuary at the time of the study (Lehman et al., 2005, 2008) that potentially amplified the variations of total MC concentrations. In addition, the MC+ MC-LR UTEX strain used as a standard in the qPCR assay specifically targets MC+ (MC-LR) *Microcystis* precluding the different MC composition present in the SFE. Although the presence of other MC+ cyanobacteria (e.g., *Anabaena*, *Planktothrix* sp.) was not detected from samples examined in the current study using *mcy*A and E primers using conventional PCR assays (Hotto et al., 2008), other toxin-producing cyanobacterial species have been previously reported from the estuary including *Planktothrix* sp., *Anabaena* sp., and *Cylindrospermopsis* sp. (phytoplankton data files, www.iep.water.ca.gov). Microscopic analysis of samples was not conducted in the current study to verify the presence of other species of MC+ cyanobacteria.

Some studies indicate that MC levels do not necessarily correlate to the frequency of MC+ *Microcystis*. For example, qPCR analysis targeting the toxin synthetase *mcy*E gene in *Microcystis* and *Anabaena* from most locations examined showed relatively weak to no correlation between MC concentrations and *Microcystis* *mcy*E gene copies due to seasonal differences in *Microcystis* genome copies and toxin concentrations (Vaitomaa et al., 2003). Further

investigation is needed to elucidate the potential gap between the proportion of toxic *Microcystis* including other MC+ cyanobacteria and MC levels during bloom development in the SFE. Lastly, the few number of samples tested in a geographically large and dynamic ecosystem such as the SFE may have resulted in the lack of correlation between MC concentrations and qPCR-based cell equivalents of MC+ *Microcystis*.

Factors that promote or exacerbate the growth and survival of MC+ and MC− strains of *Microcystis* spp. in the SFE are unknown. The role of nutrients (e.g., iron or phosphorus) in altering *Microcystis* toxicity as shown in other watersheds (Sevilla et al., 2008; Davis et al., 2009), has only been initially examined in the SFE (Lehman, unpublished data). Environmental parameters including high water temperature, eutrophication, nutrients, and light affect the ability of MC+ genotypes to produce MCs (Kardinaal et al., 2007b; Davis et al., 2009). The length of spring–summer period and light availability can alter interactions between toxic and nontoxic strains (Kardinaal et al., 2007b) including the seasonal succession of different *Microcystis* genotypes as a key mechanism affecting MC concentrations in *Microcystis*-dominated waters (Via-Ordorika et al., 2004; Kardinaal et al., 2007a; Welker et al., 2007).

The management implications of this study demonstrate that the qPCR technique can facilitate rapid and specific determination of the relative proportion of MC+ strains among the total cyanobacterial population within the SFE based on the quantity of target genes from mixed cyanobacterial samples. The qPCR technique will be useful alone or in combination with other currently used methods such as the enzyme-linked immunosorbent assay (ELISA) or the protein phosphatase inhibition assay (PPIA) (Lehman et al., 2005, 2008) to enhance the analysis and interpretation of *Microcystis* toxicity in the SFE. While ELISA can demonstrate the presence and abundance of MCs, it cannot quantify the relative abundance of MC+ cells within a cyanobacterial sample. The PPIA can detect relative toxicity due to enzymatic activity but it cannot identify the MCs involved in the toxicity. While both ELISA and PPIA have their advantages, they do not offer any information on the species composition of a cyanobacterial bloom. In contrast, qPCR can quickly identify and quantify the presence and proportion of cyanobacterial species, particularly *Microcystis*, that are toxic (MC+) or nontoxic (MC−). Quantifying the relationship between total *Microcystis* biomass and MC+ *Microcystis* biomass using qPCR in combination with chemical methods such as PPIA can offer greater accuracy for understanding the source and variability of different MC producers and bloom toxicity. Past methods of monitoring the toxicity of *Microcystis* blooms in the SFE have relied on assessment of cell density and chemical detection of MC concentrations in algal samples using PPIA (Lehman et al., 2005, 2008). As measurements such as cell density do not correlate well with toxicity (Sivonen and Jones, 1999; Baker et al., 2002; Ouellette and Wilhelm, 2003), generally as well as in the SFE (Lehman et al., 2005, 2008), the qPCR assay can circumvent the limitation of this technique. The cost and the length of time needed for analysis of chemical assays such as PPIA may limit large-scale application and rapid management decisions needed to protect humans and wildlife. By revealing trends in the proportion of MC producing cells, qPCR can identify the sources and magnitude of MC producers among mixed populations of *Microcystis* or cyanobacterial species present in the bloom. As such, qPCR can contribute to rapid risk assessment and prediction of strategies designed to manage the adverse effects of blooms in this ecosystem, which provides drinking water to 22 million people in California (IEP, 2008).

In conclusion, the qPCR for *Microcystis* described in our study provided a specific and rapid approach for estimating the spatial and temporal variations in the proportion of toxic *Microcystis* strains among mixed cyanobacterial populations that co-exist in blooms in the SFE. As local *Microcysis* strains were recently shown to be unique in the estuary (Moisander et al., 2009), qPCR will be an invaluable method to further analyze the relationship between environmental factors and MC synthesis, and the potential threats of blooms to the food web by using cultures of local *Microcystis* strains currently being established in our laboratory.

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