Applied and Environmental Microbiology, Dec. 2010, p. 7836–7842 Vol. 76, No. 23
0099-2240/10/$12.00 doi:10.1128/AEM.00174-10

Detection of Saxitoxin-Producing Cyanobacteria and *Anabaena circinalis* in Environmental Water Blooms by Quantitative PCR▽†

Jamal Al-Tebrineh, Troco Kaan Mihali, Francesco Pomati,‡ and Brett A. Neilan*

School of Biotechnology and Biomolecular Science, University of New South Wales, Sydney, New South Wales 2052, Australia

Received 24 January 2010/Accepted 23 September 2010

Saxitoxins (STXs) are carbamate alkaloid neurotoxins produced by marine "red tide" dinoflagellates and several species of freshwater filamentous cyanobacteria, including *Anabaena circinalis*, *Aphanizomenon* spp., *Lyngbya wollei*, and *Cylindrospermopsis raciborskii*. A specific quantitative PCR (qPCR) method based on SYBR green chemistry was developed to quantify saxitoxin-producing *Anabaena circinalis* cyanobacteria, which are major bloom-forming freshwater cyanobacteria. The aim of this study was to infer the potential toxigenicity of samples by determining the copy number of a unique and unusual polyketide synthase (PKS) sequence (*sxtA*) in the STX biosynthesis gene cluster identified in cyanobacteria. Our qPCR approach was applied to water samples collected from different Australian lakes, dams, and rivers. The STX concentration and cyanobacterial cell density of these blooms were also determined by high-pressure liquid chromatography (HPLC) and microscopic cell counting, respectively. STX concentrations correlated positively with STX gene copy numbers, indicating that the latter can be used as a measure of potential toxigenicity in *Anabaena circinalis* and possibly other cyanobacterial blooms. The qPCR method targeting STX genes can also be employed for both monitoring and ecophysiological studies of toxic *Anabaena circinalis* blooms and potentially several other STX-producing cyanobacteria.

Saxitoxins are a class of chemically related neurotoxins, also known as paralytic shellfish toxins (PSTs) due to their association with seafood, predominantly filter-feeding shellfish. PSTs are the causative agents of paralytic shellfish poisoning (PSP), which is globally the most widespread alga-derived shellfish poisoning, causing detrimental public health effects and substantial annual damage to the fishing and aquaculture industries. PST toxicity is mediated mainly through inhibition of nerve conduction by blocking neuronal voltage-gated sodium channels in excitable cells (5). Chemically, saxitoxins (STXs) are a group of heterocyclic guanidinium compounds which have the same core molecular structure and comprise more than 30 congeners (21). STX is the most potent cyanobacterial toxin, with a 50% lethal dose of 5 μg/kg (administered intraperitoneally [i.p.]) in mice (3).

The production of PSTs by marine "red tide" dinoflagellates (33) and several species of globally distributed freshwater filamentous cyanobacteria, including *Anabaena circinalis* (13), *Aphanizomenon* spp. (25), *Lyngbya wollei* (4), and *Cylindrospermopsis raciborskii* (19), has been reported. A limited number of bacterial strains have also been reported to produce PSTs, although those findings are not widely accepted due to a lack of reproducibility (10). In Australia, *A. circinalis* has been reported as the sole bloom-forming cyanobacterium producing PSTs in freshwater systems, such as dams, rivers, and reservoirs (30), occasionally causing cases of animal illness and fatalities (31). Furthermore, variability in *sxt* gene sequences of Australian *A. circinalis* isolates is extremely low, and gene sequences are usually identical (15, 28).

The lethal oral dose of STX in humans is between 1 to 4 mg, depending upon the age and physical condition of the patient. There has been only one reported case of human poisoning through consumption of PST-contaminated fish (34). Guidelines for water quality and treatment have suggested that the health alert level expressed in terms of saxitoxin-equivalent concentrations in water for consumption is 3 μg/liter (9). As for phytoplankton monitoring schemes, the health alert level for *A. circinalis* cell counts in the water column has been set at 2×10^7 cells/liter (32).

Continuous monitoring of toxic cyanobacterial blooms raises several issues, including the low availability of laboratories able to undertake toxin analysis, the observation that diverse toxin analysis methods may give varied results, and the difficulty in obtaining biovolume measurements to allow comparison of monitoring results with several guidelines that use biovolume as an alert level criterion. In addition, the traditional method of identifying toxic cyanobacteria by light microscopy does not differentiate PST- from non-PST-producing *A. circinalis* and is also laborious and time-consuming. A widely used method for cyanobacterial toxin detection is high-pressure liquid chromatography (HPLC), although it requires expensive equipment and is equally time-consuming and laborious, with the advantage, however, of low detection limits (8). Screening based on genetic targeting is economically competitive, more precise and sensitive, less time-consuming, and able to distinguish toxic from nontoxic strains (1, 35). The body of work described in this study was aimed at validating a specific, rapid, and sensitive quantitative PCR (qPCR) approach for the detection of PST-producing *A. circinalis* in environmental samples. This

* Corresponding author. Mailing address: University of New South Wales, School of Biotechnology and Biomolecular Sciences, Sydney, New South Wales 2052, Australia. Phone: 61 2 9385 3235. Fax: 61 2 9385 1483. E-mail: b.neilan@unsw.edu.au.

‡ Present address: Eawag, Seestrasse 79, 6047 Kastanienbaum, Switzerland.

† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 8 October 2010.

work was made possible by the recently described unique polyketide synthase (PKS) sequence (*sxtA*), which is an integral part of the cyanobacterial STX biosynthesis gene cluster (*sxt*) (15, 27). This assay can infer toxigenicity and calculate potential sample toxicity by determining the copy number of *sxtA* in cyanobacterial cultures as well as environmental samples and thereby enables monitoring of the formation of toxic *Anabaena circinalis* blooms in Australia. A similar approach may be further applied to other PST-producing cyanobacterial species worldwide.

MATERIALS AND METHODS

Cyanobacterial cultures. *A. circinalis* strains used in this study were obtained from the Australian Water Quality Centre (22), while *Cylindrospermopsis raciborskii* AWT205 and T3 were obtained from Australian Water Technologies (12) and from Sandra Azevedo (Federal University of Rio de Janeiro, Brazil) (19), respectively. These cyanobacterial strains were grown in Jaworski's medium (42) static batch cultures. *Nodularia spumigena* NSOR10 (2) was obtained from CSIRO Marine Laboratories (Hobart, Australia) and grown in ASM medium supplemented with 1.5% NaCl (37). *Microcystis aeruginosa* PCC7806 (38), *Nostoc punctiforme* PCC73102 (34), and *Nostoc* sp. strain PCC7120 (24) were obtained from the Pasteur Culture Collection and grown in BG11 medium (39). All cyanobacterial strains were grown at room temperature (25°C) under continuous illumination (10 mmol m^{-2} s^{-1}). *Lyngbya wollei* (4) and *Aphanizomenon* sp. strain NH-5 (25) freeze-dried cells were kindly provided by Wayne Carmichael (Wright State University).

Environmental samples. Water samples were collected from several Australian water bodies in 2007 by the New South Wales (NSW) Department of Natural Resources. Thirteen environmental water samples were collected from dams, rivers, and lakes located in New South Wales, while one sample was collected from the River Torrens, Adelaide, South Australia, Australia (see Table 2). Algal counts were performed using a Lund cell counting chamber (23). All samples were thereafter used for DNA and PST extraction, whereby up to 100 ml of water was concentrated to 1 ml by centrifugation and used for PST extraction and up to 10 ml was concentrated to 1 ml and used for DNA extraction.

DNA extraction. Genomic DNA was extracted as follows. Cells and environmental bloom samples were pelleted and lysed mechanically by ceramic and silica bead beating using a FastPrep FP120 instrument (Savant) at a speed setting of 5.5 for 30 s. DNA was subsequently extracted using a FastDNA Spin kit for soil in accordance with the manufacturer's instructions (Qbiogene MP Biomedicals). Concentrations of DNA were determined with a NanoDrop ND-1000 spectrophotometer (Biolab). The purity of the extracted DNA was assessed by calculating the ratio of the absorbance measured at 260 nm (A_{260}) to the absorbance measured at 280 nm (A_{280}), using the ND-1000 version 3.2.1 software (Biolab). The purity ($A260/A280$) of DNA extracted in this study varied from 1.8 to 1.9.

PCR amplification. DNA extracted from environmental bloom samples, as well as a spiked control containing environmental DNA with the addition of 1.5 ng of control DNA (*A. circinalis* AWQC131C), was tested for the presence of possible PCR inhibitors by PCR amplification of the extracted DNA using the cyanobacterium-specific 16S rRNA gene primers 27F and 809R (14). PCR was performed using 0.2 U of *Taq* DNA polymerase (Bioline, Australia) in a 20-μl reaction mix containing 2.5 mM $MgCl_2$, PCR buffer (Bioline), 0.2 mM deoxynucleoside triphosphates (dNTPs; Bioline), 10 pmol of each of the 16S rRNA gene primer (Sigma-Genosys), and 1 ng of template DNA. Thermal cycling was performed in a GeneAmp PCR system 2400 thermocycler (PerkinElmer Corporation). The initial denaturation step was at 94°C for 4 min, followed by 30 cycles of DNA denaturation at 94°C for 10 s, primer annealing at 55°C for 25 s, strand extension at 72°C for 45 s, and final extension step at 72°C for 5 min. The PCR amplification product was visualized by 1% agarose gel electrophoresis, stained with 0.5 μg/ml ethidium bromide, and documented with a Gel Doc XR camera using the Quantity One (version 4.6.1) software package (Bio-Rad).

Primers design and specificity testing. The software primer express (version 2.0) from Applied Biosystems, Australia, was used to design the saxitoxin (*sxtA*)-specific primers *sxtA*-F (GATGACGGAGTATTTGAAGC)/*sxtA*-R (CTGCAT CTTCTGGACGGTAA) based on the *sxtA* gene sequence alignments from saxitoxin-producing *A. circinalis* (27). Furthermore, the cyanobacterium-specific internal control 16S rRNA gene real-time primers cyano-real16SF (AGCCAC ACTGGGACTGAGACA)/cyano-real16SR (TCGCCCATTGCGGAAA) were designed based on conserved domains for all cyanobacterial species. The *sxtA*-F and -R primers were tested for specificity via BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST) against the nonredundant (nr) database, revealing no close hits from genes other than their designated targets. Real-time 16S rRNA gene and *sxtA* gene primers were tested separately using conventional PCR with cyanobacterial strains and bloom samples for two purposes: first, to check for the presence of *sxtA* and, second, to check the specificity of primers by amplifying single PCR products.

These test PCRs were performed in 20-μl volumes as describe above, with the following modifications: the first step was an initial denaturation for 4 min at 94°C, followed by 45 cycles at 94°C for 10 s, 60°C for 20 s, and 72°C for 20 s, with a final extension step at 72°C for 5 min. The PCR amplification product was determined by using 3% agarose gel electrophoresis, staining, and visualization as stated above.

Sequencing. To further verify that only target genes were amplified, PCR products obtained from strains and environmental bloom samples were sequenced (see Fig. S2 and Table S1 in the supplemental material). Automated sequencing was performed using approximately 50 ng of PCR product and 3.2 pmol of one of the primer pair *sxtA*-F/R using the Prism BigDye cycle sequencing system and the ABI 3730 DNA Analyzer sequencer (Applied Biosystems). Sequence identity was determined by a BLAST search of the National Center for Biotechnology Information (NCBI) database.

Quantitative PCR assay. The PST gene *sxtA* was quantified using *sxtA*-F/R primers and the Rotor Gene instrument with Rotor Gene analysis software 6.0.27 (Corbett Research). Quantitative PCR was performed in 25-μl reaction mixtures containing 12.5 μl of Platinum SYBR Green SuperMix-UDG (Invitrogen), 0.4 pmol of each primer, and 1 μl of template DNA. Cycling was initiated with preheating for 2 min at 50°C and heating for 2 min at 95°C, followed by 45 quantification cycles, each consisting of 15 s at 95°C and 30 s at 60°C. Fluorescence measurement of generated products was obtained at the end of each cycle at 60°C through the 6-carboxyfluorescein (FAM)/Sybr channel (510 nm). The threshold cycle (C_T) values were determined by the second derivative maximum method of Rotor Gene analysis software 6.0.27.

The same qPCR conditions were employed to amplify and quantify the internal control 16S rRNA gene using the cyano-real16SF/R primers. To quantify the copy number of *sxtA* in environmental samples, standard curves were determined by correlation between *sxtA* gene copy number and the threshold cycle (C_T) values in a 10-fold serial dilution of *A. circinalis* AWQC131C DNA. The genome size of *A. circinalis* is approximately 4.50 Mb (17, 28) and was used as a reference value to calculate *sxtA* gene copy numbers per reaction mixture using the following formula (43): number of gene copies per microliter = [6×10^{23} gene copies mol^{-1} × DNA concentration (g μl^{-1})]/molecular mass of the genome (g mol^{-1}).

These values were obtained assuming that the molecular mass of 1 bp was 660 g mol^{-1} and were expressed as the copy numbers of genes ml^{-1} of originally extracted culture. Amplification efficiencies, e ($e = 10^{-1/S} - 1$, where S is the slope of the linear regression), of *sxtA* and 16S rRNA gene qPCR with standard DNA were calculated as a function of known *sxtA* and 16S rRNA genes copy numbers, determined as described above.

sxtA gene copy numbers in bloom samples were determined by qPCRs, immediately after DNA extraction and purification. DNA samples were amplified in triplicate. A total of 1 μl of diluted DNA was added to 25-μl qPCR mixtures. In all qPCR mixtures that contained DNA extracted from bloom samples, a standard curve was determined by analyzing a triplicate dilution series (10^1 to 10^6 *sxtA* gene copies per 25 μl of reaction mixture) of *A. circinalis* AWQC131C standard DNA. Denaturation temperatures were determined for all amplicons (standard and bloom samples) after ramping from 72 to 95°C, with fluorescence being detected continuously by the Rotor Gene real-time analysis software 6.0.

In order to test the specificity of STX primers in qPCR, different DNA concentrations were analyzed by mixing DNA from *A. circinalis* AWQC131C (toxic) and *A. circinalis* AWQC271C (non-PST producing) in different proportions in triplicate: 100% toxic, 75% toxic plus 25% nontoxic, 50% toxic plus 50% nontoxic, 25% toxic plus 75% nontoxic, and 100% nontoxic. Background DNA concentrations from different cyanobacterial species (containing microcystin, nodularin, and cylindrospermopsin biosynthesis genes) were also mixed with STX-producing *A. circinalis* AWQC131C DNA in different proportions. Triplicates of each combination were analyzed by qPCR as described above (see Tables S2 to S4 in the supplemental material). In addition, bloom samples were spiked with a known number of *sxtA* gene copies to check for any degree of inhibition by cellular components (see Table S4 in the supplemental material). No primer dimers were detected in the *sxtA* and 16S rRNA gene qPCR for all samples tested.

Saxitoxin analysis by HPLC. Cyanobacterial cultures (1 ml) and bloom samples (100 ml) were centrifuged at 7,000 × *g* for 15 min at 4°C, and cell pellets were resuspended in 100 μl 0.5 M acetic acid. Cell pellets were lysed by bead

TABLE 1. Specificity of cyanobacterial 16S rRNA and *sxtA* real-time PCR primers in conventional PCR and qPCR

Species/strain[a]	Result from indicated test							Reference
	Cyanobacterial 16S rRNA gene PCR[b]	Real-time 16S rRNA gene[c]		*sxtA*[d]		HPLC[e]	Toxicity[f]	
		PCR	qPCR	PCR	qPCR			
A. circinalis								
AWQC131C	+	+	D	+	D	+	S-P	22
AWQC118C	+	+	D	+	D	+	S-P	22
AWQC134C	+	+	D	+	D	+	S-P	22
AWQC150A	+	+	D	+	D	+	S-P	22
AWQC150E	+	+	D	+	D	+	S-P	22
AWQC173A	+	+	D	+	D	+	S-P	22
AWQC344B	+	+	D	+	D	+	S-P	22
AWQC271C	+	+	D	−	ND	−	NT	22
AWQC306A	+	+	D	−	ND	−	NT	22
AWQC310F	+	+	D	−	ND	−	NT	22
L. wollei	+	+	D	+	D	+	S-P	4
Aphanizomenon sp. NH-5	+	+	D	+	D	+	S-P	25
C. raciborskii T3	+	+	D	+	D	+	S-P	19
N. spumigena NSOR10	+	+	D	−	ND	−	N-P	2
M. aeruginosa PCC7806	+	+	D	−	ND	−	M-P	38
C. raciborskii AWT205	+	+	D	−	ND	−	C-P	12
Nostoc sp. PCC7120	+	+	D	−	ND	−	NT	24
N. punctiforme PCC73102	+	+	D	−	ND	−	NT	34

[a] Some strain designations include culture collection abbreviations, as follows: AWQC, Australian Water Quality Centre; AWT, Australian Water Technologies; and PCC, Pasteur Culture Collection, Paris, France. Please refer to Materials and Methods for strains without culture collection abbreviations.
[b] Cyanobacterium-specific 16S rRNA gene primers 27F and 809R (31a) were used as controls to ensure the quality of DNA samples.
[c] 16S rRNA gene PCR and qPCR with primers cyano-real16SF and -R.
[d] *sxtA* PCR or qPCR with primers *sxtA*-F/R. +, PCR product in conventional PCR; −, no PCR product in conventional PCR; D, C_T value above detection limit in qPCR; ND, no data.
[e] +, detected; −, not detected.
[f] Toxicity based on the literature. S-P, saxitoxin-producing strain; NT, non-toxin-producing strain; M-P, microcystin-producing strain; N-P, nodularin-producing strain; C-P, cylindrospermopsin-producing strain.

beating with 0.25 g of 0.1-μm glass beads, after which cell debris were removed by centrifugation (14,000 × *g* for 30 min at 4°C). PST analysis was performed by prechromatographic oxidation with H_2O_2, followed by HPLC separation according to the method of Lawrence et al. (20). Briefly, chemical analyses were performed on an HP series 1100 HPLC apparatus (Agilent) coupled to an HP 1040 fluorescence detector using a Zorbax SB-C_{18} column (4.6 mm by 250 mm; Hewlett Packard). The samples and the standard mixture were oxidized prior to injection as previously described (20). Oxidation products (25-μl injection volume) were eluted under isocratic conditions with a mobile phase containing 2% (vol/vol) acetonitrile in 0.1 M ammonium formate, pH 6.0, at a flow rate of 1.0 ml min^{-1}.

STX was identified by comparison to chromatograms obtained from an STX standard solution (National Research Council Canada, Halifax, Canada). Quantification of STX was performed by calculating peak area/toxin concentration, as obtained with the injection of certified standard STX solution of known concentrations (36). Average STX concentrations per cell of *A. circinalis* AWQC131C were derived from dividing the amount of STX (mole/liter) obtained from HPLC analysis by the microscopic cell count (cells/liter) and multiplying by the molecular weight of STX (molecular weight = 299.3).

Nucleotide sequence accession numbers. All sequences produced in this study have been deposited in GenBank under accession numbers HQ338473 to HQ338493.

RESULTS

Specificity of *sxtA*-F/R primers. In order to test the specificity of the *sxtA*-F/R primers, genomic DNA from PST-producing and nontoxic cyanobacterial strains was first tested by conventional PCR. A single amplification product was observed (and its specificity confirmed by sequencing) when genomic DNA from PST-producing *A. circinalis* strains (22) and from other PST-producing cyanobacterial species, namely, *L. wollei*, *Aphanizomenon* sp. strain NH-5, and *C. raciborskii* T3, was used as a template in PCR. Furthermore, no amplification product was observed with DNA isolated from several cyanobacterial strains that produce the other major freshwater toxins, microcystin, cylindrospermopsin, and nodularin (Table 1). Toxic and nontoxic cyanobacterial strains were further tested by qPCR, where only PST-producing *A. circinalis* strains gave C_T values over the detection limit of 10^1 gene copies per reaction mixture (Table 1). PST-producing cyanobacterial strains other than *A. circinalis* resulted in qPCR amplification with lower efficiencies and different melt curve profiles (see Fig. S1A in the supplemental material).

Amplification efficiency and quantitation. A standard curve was determined for *sxtA*-F/R and cyano-real16SF/R primers (Fig. 1). The detection range of *sxtA* in qPCR was at least 0.54×10^1 to 3.11×10^6 copies per 25-μl reaction mixture, or conversely, 6.67×10^3 to 3.84×10^9 copies per μg of DNA. For the 16S rRNA qPCR (internal control), the detection range was 0.8×10^1 to 3.95×10^6 copies per 25-μl reaction mixture, or 9.88×10^3 to 4.88×10^9 copies per μg of DNA.

In terms of 16S rRNA gene qPCR data, our results suggested that there are, on average, 4.57 (±0.15) copies of the 16S rRNA gene per cell of *A. circinalis* AWQC131C, derived from the relationship between the experimental 16S rRNA gene copy number and microscopic cell counts of laboratory cultures of *A. circinalis* AWQC131C. The qPCR analysis also indicated that there are, on average, 3.58 (±0.12) copies of the *sxtA* gene per cell of *A. circinalis* AWQC131C.

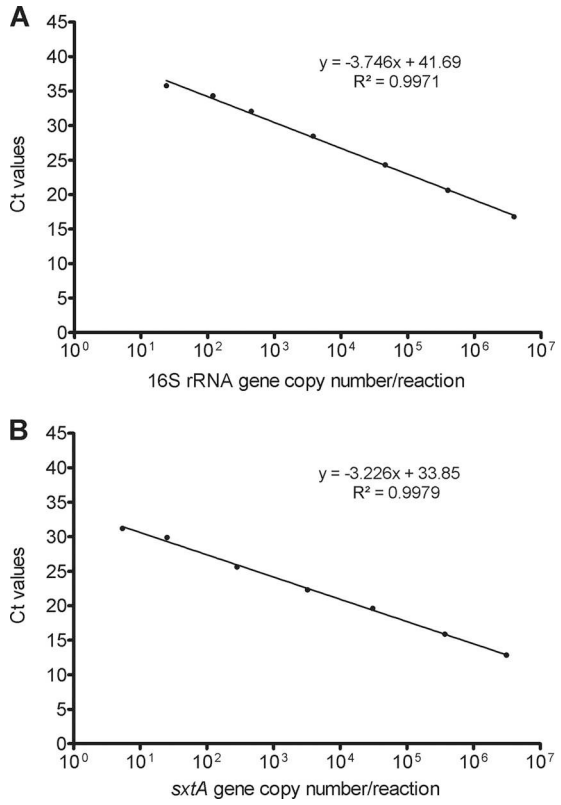

FIG. 1. Standard curves and the linear relation (regression) equation of C_T values obtained by 16S rRNA gene (A) and *sxtA* gene (B) qPCR with an *A. circinalis* AWQC131C dilution series. Error bars, which are hidden by the symbol in all cases, represent the standard deviations from three independent amplifications.

The observed amplification efficiency of both the *sxtA* primers and the 16S rRNA gene primers was 0.99 in both laboratory cultures and bloom samples. The characteristic melting temperature of *sxtA* qPCR products (125 bp) in *A. circinalis* AWQC131C was 83.5°C for all amplifications. In the bloom samples, the range of denaturation peaks was 83 to 83.5°C. For the 16S rRNA qPCR gene products (80 bp), the range of melting temperatures of *A. circinalis* AWQC131C and bloom samples was 85.5 to 86°C.

Effect of background DNA. To further test the specificity of the *sxtA* primers in qPCR, DNA of *A. circinalis* AWQC131C was mixed in different proportions with DNA of *A. circinalis* AWQC271C, a closely related nontoxic strain. The *sxtA* gene primers were able to specifically target toxic *A. circinalis* AWQC131C DNA and differentiate it from the nontoxic *A. circinalis* AWQC271C DNA. There was no amplification in the pure *A. circinalis* AWQC271C DNA sample. In order to test if there was an inhibitory effect of foreign DNA on PCR quantitation, different concentrations of background DNA from *M. aeruginosa* PCC7806, *C. raciborskii* AWT205, and *N. spumigena* NSOR10 (producers of microcystin, cylindrospermopsin, and nodularin, respectively) were added to DNA from *A. circinalis* AWQC131C. No effect on the sensitivity of the qPCR or on the quantification of the *sxtA* gene copy number was observed (see Tables S2 and S3 in the supplemental material).

Furthermore, DNA extracted from bloom samples was spiked with a known amount of *sxtA* gene copies, yielding the expected increase in gene copy number compared to that of nonspiked samples. This indicated that the amplification reactions were not inhibited by cellular components or other unknown compounds carried over from the environmental bloom DNA extraction (see Table S4 in the supplemental material).

Analysis of bloom samples. Several naturally occurring cyanobacterial blooms were sampled and analyzed for PST production via qPCR and HPLC (Table 2). In all but one of the samples, the 16S rRNA gene copy number exceeds the lower detection limit, while *sxtA* was not detected in three samples. The Murray River bloom sample used in this study did not give amplification in PCR or qPCR for either the cyanobacterial 16S rRNA or *sxtA* genes, most probably due to PCR inhibitors in the extracted DNA, as evident from a spiked control analysis (see Table S4 in the supplemental material). The Glenning Green Dam sample gave amplification only in the 16S rRNA gene conventional PCR and its corresponding qPCR. This result is in agreement with the HPLC data, which failed to

TABLE 2. Environmental bloom sample analysis by *sxtA* qPCR and HPLC

Sample[a]	16S rRNA gene amplification by qPCR	*sxtA* copy no./ml (±SD)	Potential toxicity (μg of STX/ml)	HPLC result (μg of STX/ml)[b]	No. of cells/ml[c]
Murray River	−	ND		ND	4.00×10^4
Glenning Green Dam	+	ND		ND	2.50×10^6
Lake Cargelligo	+	8.44 (±3.10)	5.0×10^{-9}	ND	6.60×10^5
Menindee Lake	+	8.32 (±2.70)	4.9×10^{-9}	ND	5.40×10^4
Chaffey Dam	+	9.75 (±4.60)	5.7×10^{-9}	ND	3.30×10^5
Windamere Dam STN 4	+	5.30×10^2 (±4.20)	3.1×10^{-7}	ND	1.20×10^5
Manly River	+	6.72×10^1 (±2.50)	3.9×10^{-8}	ND	1.50×10^5
Macintyre River, Boggabilla	+	2.94×10^4 (±3.70)	1.7×10^{-5}	ND	1.90×10^5
Ainsworth Lake	+	7.61×10^3 (±2.80)	4.5×10^{-6}	8.57×10^{-6}	6.70×10^4
Pindari Dam, site 1	+	2.91×10^9 (±1.60)	1.71	1.56×10^{-2}	1.20×10^9
Pindari Dam, site 2	+	1.14×10^8 (±2.80)	6.7×10^{-2}	1.34×10^{-2}	5.50×10^7
Homebush River	+	7.51×10^6 (±3.10)	4.4×10^{-3}	8.45×10^{-3}	6.30×10^6
Adelaide KV/GL Torrens River	+	2.61×10^8 (±2.70)	1.5×10^{-1}	1.45×10^{-2}	7.70×10^8

[a] Environmental water samples.
[b] ND, not detected.
[c] Number of cyanobacterial cells per ml of bloom, derived from microscopic cell counts.

detect any PSTs in this bloom sample. In addition, in five of the analyzed bloom samples (Lake Cargelligo, Menindee Lake, Chaffey Dam, Manly River, and Windamere Dam station [STN] 4), *sxtA* copy numbers were below or near the lower detection limit (Table 2). The low *sxtA* copy numbers detected in these environmental samples were in agreement with HPLC results, where concentrations of SXT were below the HPLC detection limits. The high *sxtA* copy numbers detected in samples obtained from Ainsworth Lake, Homebush River, Adelaide KV/GL Torrens River, and Pindari Dam sites 1 and 2 were positively correlated with HPLC-determined STX levels (Table 2). In contrast to this general trend, the sample obtained from the Macintyre River, Boggabilla contained a high copy number for *sxtA*, although no PSTs were detected by HPLC.

Determination of the toxigenicity and potential toxicity of bloom samples. It is possible to infer the potential toxicity of a water sample by determining the quantity of STX biosynthesis genes per ml, which can then be related to the number of toxic cells and the average amount of STX produced by 1 cell of *A. circinalis*.

We determined that the average amount of STX produced by a cell of *A. circinalis* AWQC131C was 2.1×10^{-9} μg (see Materials and Methods). This amount was subsequently used to estimate the potential toxicity of environmental samples in STX equivalents, assuming that all STX derivatives are synthesized by the same gene cluster. The potential STX concentration inferred by qPCR was consistently higher than the levels measured by HPLC in each sample (Table 2).

Comparing the qPCR-inferred potential STX concentrations to the Australian guidelines for water quality and treatment (9), some of the tested water samples (Table 2) should be considered above the alert level for STX (3 μg/liter).

DISCUSSION

The primers *sxtA*-F/R developed in this study were demonstrated to be specific to the unique PKS gene *sxtA* and to saxitoxin-producing *A. circinalis* strains, based on conventional PCR, qPCR, sequencing, and BLAST searching. The *sxtA*-F/R primers also amplified the *sxtA* gene from other saxitoxin-producing cyanobacterial species, albeit with lower efficiency and different melt curve profiles. This can be ascribed to mismatches in primer alignment to their target *sxtA* sequences in other species (see Fig. S1B in the supplemental material). This observation suggests the possibility to develop more PST-specific qPCR assays targeting other cyanobacterial species in the future. Since the *sxtA*-F/R primers amplified *sxtA* from several other PST-producing cyanobacteria with different characteristic melt curves, it might be further possible to determine which PST-producing cyanobacterial species are present in a given sample, based on the melt curve profiles. This approach would surely be of aid to water authorities in monitoring bloom formation as well as for the development and implementation of management and treatment strategies. In our assay, however, *sxtA*-F/R primers were regarded as specific to *A. circinalis* in Australian water samples, as to date it is the only PST-producing cyanobacterial species detected in Australian environments (30). In addition, the *sxtA*-F/R primers were also tested against the effect of background DNA from other cyanobacteria, thereby indicating that our qPCR assay seems not to be affected by background DNA, contrary to what was previously reported by Koskenniemi et al. using a similar qPCR method (16).

As with any genetic analysis of only part of a toxin gene cluster, it cannot be excluded that these samples could contain species that have lost several other key genes in the toxin biosynthetic pathway, rendering them nontoxic, while still retaining some loci of this gene cluster. This scenario would produce false-positive results. To minimize the occurrence of false-positive and negative results, the first step in the biosynthesis of PSTs (*sxtA*) was chosen as the target of our assay. Targeting this key enzyme in the biosynthetic pathway increases the reliability of this method, since variation in subsequent biosynthetic steps, such as tailoring and accessory genes, which are more common, may lead to false-positive results. A previous study has in fact reported the detection of partial, inactive microcystin (*mcy*) gene clusters in *Planktothrix* isolates obtained from alpine lakes (6).

Our *sxtA* qPCR appeared to be more sensitive than a comparable qPCR method for the detection of microcystin, a frequently occurring hepatotoxin. qPCR targeting the microcystin biosynthetic gene *mcyE* resulted in detection ranges of 6.6×10^2 to 6.6×10^6 and 6.6×10^2 to 6.6×10^5 gene copies when DNA from the microcystin producers *Microcystis* spp. and *Anabaena* spp. was used, respectively (43). The *sxtA* qPCR described here was 1 order of magnitude more sensitive and more similar in detection limits to the qPCR assay targeting the hepatotoxin nodularin biosynthesis gene *ndaF* (10^1 to 10^6 gene copies) (15). Both of the above-mentioned studies, however, did not include an internal control gene (16S rRNA) in the qPCR assay, as incorporated in this study. However, the inclusion of internal controls in microcystin qPCR analysis has been previously reported (11).

Cell counts and our qPCR analysis of cultured *A. circinalis* AWQC131C suggested that 1 cell of *A. circinalis* AWQC131C contains 4.57 and 3.58 copies of the 16S rRNA and *sxtA* genes, respectively. This observed ribosomal 16S RNA copy number is in agreement with those found in the rRNA database (http://rrndb.cme.msu.edu) for *Anabaena variabilis* and *Anabaena flosaquae*, which have an average of 4.75 copies of the 16S rRNA gene per genome. Our results, however, can be confounded by the fact that filamentous cyanobacteria are known to possess multiple copies (up to 10) of their genome in each cell, thereby hindering the elucidation of an exact copy number of ribosomal or PST biosynthesis genes per cell. Additionally, during rapid growth, cyanobacteria may have multiple replication origins and, hence, more than one copy of parts of the bacterial chromosome (29). As there is no publically available *A. circinalis* genome sequence, it is not possible at this time to ascertain the exact copy number of the 16S rRNA and *sxtA* genes per genome. However, due to the results of the 16S rRNA qPCR matching the predicted copy number of the 16S rRNA gene per cell, we assume that the *sxtA* copy number also represents a close match to the actual copy number in *A. circinalis*. In all calculations of toxicity, we therefore normalized data, assuming 3.58 copies of *sxtA* per cell.

In our environmental bloom samples, the potential production of PST identified by qPCR was supported by HPLC analysis. The Murray River bloom sample did not provide ampli-

fication of the 16S rRNA and *sxtA* genes by PCR or qPCR. Furthermore, the spiked control PCR of this sample resulted in no amplification, indicating that the Murray River sample contained PCR inhibitors, thereby preventing the complete analysis of this sample. This result highlights the importance of the incorporated internal positive control. The Glenning Green Dam sample gave amplification only for the 16S rRNA gene, thereby suggesting that this sample did not contain any toxic *A. circinalis* strains, in agreement with the lack of PST detection by HPLC. In Lake Cargelligo, Menindee Lake, and Chaffey Dam samples, the *sxtA* copy number was below or near the detection limit. These results were in agreement with HPLC data for which STX was not detected. This suggested that in these samples, there was approximately 1 PST-producing cell per ml, in agreement with the trend observed in the microscopic cell counts, where 5% of total cells were identified as *A. circinalis* (with unknown toxicity). Manly River and Windamere Dam STN 4 bloom sample cell counts also contained *A. circinalis* cells, although they were dominated by *M. aeruginosa* species (90%). The low abundance of *sxtA* detected by qPCR at these sites was in agreement with the low levels of STX measured by HPLC.

Samples obtained from Ainsworth Lake, Homebush River, Adelaide KV/GL Torrens River, and Pindari Dam sites 1 and 2 contained high copy numbers of *sxtA* and were positively correlated to STX concentrations detected by HPLC. Microscopic cell counts indicated that 50 to 80% of cyanobacteria were *A. circinalis*. The exception was the sample obtained from Macintyre River, Boggabilla, which had a measurable amount of *sxtA* genes, although no PSTs were detected by HPLC. This could have been caused by STX concentrations at the lower end of the HPLC detection limit or due to the degradation of PSTs in this sample.

Interestingly, few bloom samples had higher cell densities, as derived from *sxtA* gene copy numbers, than microscopic cell counts and differed by a factor not greater than 4-fold. These results were close to the data obtained by Kurmayer and Kutzenberger (18) in terms of copy numbers of target genes compared to cell counts. There have been some proposed explanations for the high copy numbers of target genes compared to cell counts using *mcyE* as a target (43), which included multiple copies of genes per genome and errors in cell counts. In addition, other studies have reported that multiple copies of the genome or large extrachromosomal elements might be present in the environmental strains tested (26, 40, 41).

Cyanobacterial blooms frequently contain more than a single species, while natural bloom progression and dynamics result in changes in the relative abundance of the cyanobacterial species present and/or changes in bloom toxicity. Chemical analysis of bloom samples provides only information regarding the abundance of the toxin analyzed at a set time point and can frequently reliably detect only one toxin group at a time. Nonetheless, cell-regulated changes in toxin biosynthesis often occur and may result in varied toxin concentrations or isoforms in the water column. This study affords the determination of an *A. circinalis* bloom sample's potential toxicity by quantifying the *sxtA* copy number per ml and relating that to a previously measured average concentration of STX per cell. We used *A. circinalis* AWQC131C as a model to calculate the STX per cell quota. Our calculated value of 2.1×10^{-9} μg/cell is comparable to those reported by Llewellyn et al. (22) for several *A. circinalis* strains, including *A. circinalis* AWQC131C (3.67×10^{-8} μg of STX per cell). Other strains of *A. circinalis* reported in the above-mentioned study differed in PST production scales, with a maximum of 0.45×10^{-6} μg per cell. Our results were also comparable with data reported by Velzeboer et al. (44), in which *A. circinalis* cells contained between 0.02 and 0.08 pg of STX equivalent (2 to 8×10^{-8} μg), and by Dias et al. (7), which reports 1.5×10^{-7} or 1.7×10^{-7} μg of PST per cell in *Aphanizomenon* spp.

However, our calculated potential STX concentration inferred by qPCR was slightly higher than corresponding levels obtained by HPLC (Table 2). This may have been due to higher sensitivity of the qPCR assay than the HPLC method or, conversely, could be due to natural variability in cellular STX accumulation. Furthermore, the *sxtA* qPCR-derived STX concentration represents the potential to produce STX, and concentration outcomes are based on an average population and cell quota value, which may vary between cells and environmental conditions, while the HPLC analysis determines the actual STX concentration at a particular time point.

In this study, we were able to estimate the toxigenicity of cyanobacterial blooms based on the *sxtA* gene copy number per ml of sample. This method will be useful for water managers in terms of being able to monitor the formation and progression of PST-producing cyanobacterial blooms while also enabling scientists to further study toxic cyanobacterial bloom dynamics. Presently, it is hard to monitor and almost impossible to predict STX-producing *A. circinalis* blooms in lakes and rivers. It is also laborious and time-consuming to assess the toxicity of a sample by common methods such as HPLC, liquid chromatography-mass spectrometry (LC-MS), and microscopic analysis, while a rapid first estimation of potential toxicity would strongly improve real-time risk assessment and afford fast application of mitigation measures. The ability to precisely and sensitively quantify the presence of toxic strains in the environment is the first step toward understanding the dynamics and risks associated with a PST-producing freshwater bloom.

REFERENCES

1. **Beltran, E. C., and B. A. Neilan.** 2000. Geographical segregation of the neurotoxin-producing cyanobacterium *Anabaena circinalis*. Appl. Environ. Microbiol. **66:**4468–4474.
2. **Bolch, C. J. S., P. T. Orr, G. Jones, and S. Blackburn.** 1999. Genetic, morphological, and toxicological variation among globally distributed strains of *Nodularia* (Cyanobacteria). J. Phycol. **35:**339–355.
3. **Carmichael, W. W.** 1992. Cyanobacteria secondary metabolites—the cyanotoxins. J. Appl. Bacteriol. **72:**445–459.
4. **Carmichael, W. W., W. R. Evans, Q. Q. Yin, P. Bell, and E. Moczydlowski.** 1997. Evidence for paralytic shellfish poisons in the freshwater cyanobacterium *Lyngbya wollei* (Farlow ex Gomont) comb. nov. Appl. Environ. Microbiol. **63:**3104–3110.
5. **Catterall, W. A.** 1980. Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. Annu. Rev. Pharmacol. Toxicol. **20:**15–43.
6. **Christiansen, G., R. Kurmayer, Q. Liu, and T. Borner.** 2006. Transposons inactivate biosynthesis of the nonribosomal peptide microcystin in naturally occurring *Planktothrix* spp. Appl. Environ. Microbiol. **72:**117–123.
7. **Dias, E., P. Pereira, and S. Franca.** 2002. Production of paralytic shellfish toxins by *Aphanizomenon* sp. LMECYA 31 (Cyanobacteria). J. Phycol. **38:** 705–712.
8. **Etheridge, S., J. Deeds, S. Hall, K. White, L. Flewelling, J. Abbott, J. Landsberg, S. Conrad, D. Bodager, and G. Jackow.** 2006. Detection methods and their limitations: PSP toxins in the southern puffer fish *Sphoeroides nephelus* responsible for human poisoning events in Florida in 2004. S. Afr. J. Mar. Sci. **28:**383–387.
9. **Fitzgerald, D. J., and M. D. Burch.** 1999. Development of health alerts for cyanobacteria and related toxins in drinking water in South Australia. Environ. Toxicol. **14:**203–209.

10. **Gallacher, S., and E. A. Smith.** 1999. Bacteria and paralytic shellfish toxins. Protist **150:**245–255.
11. **Ha, J. H., T. Hidaka, and H. Tsuno.** 2009. Quantification of toxic Microcystis and evaluation of its dominance ratio in blooms using real-time PCR. Environ. Sci. Technol. **43:**812–818.
12. **Hawkins, P. R., N. R. Chandrasena, G. J. Jones, A. R. Humpage, and I. R. Falconer.** 1997. Isolation and toxicity of *Cylindrospermopsis raciborskii* from an ornamental lake. Toxicon **35:**341–346.
13. **Humpage, A. R., J. Rositano, A. H. Bretag, R. Brown, P. D. Baker, B. C. Nicholson, and D. A. Steffensen.** 1984. Paralytic shellfish poisons from Australian cyanobacterial blooms. Aust. J. Mar. Freshw. Res. **45:**761–771.
14. **Jungblut, A. D., I. Hawes, D. Mountfort, B. Hitzfeld, D. R. Dietrich, B. P. Burns, and B. A. Neilan.** 2005. Diversity within cyanobacterial mat communities in variable salinity meltwater ponds of McMurdo Ice Shelf, Antarctica. Environ. Microbiol. **7:**519–529.
15. **Kellmann, R., T. K. Mihali, Y. J. Jeon, R. Pickford, F. Pomati, and B. A. Neilan.** 2008. Biosynthetic intermediate analysis and functional homology reveal a saxitoxin gene cluster in cyanobacteria. Appl. Environ. Microbiol. **74:**4044–4053.
16. **Koskenniemi, K., C. Lyra, P. Rajaniemi-Wacklin, J. Jokela, and K. Sivonen.** 2007. Quantitative real-time PCR detection of toxic *Nodularia* cyanobacteria in the Baltic Sea. Appl. Environ. Microbiol. **73:**2173–2179.
17. **Krohn, A.** 2005. Genome analysis of a saxitoxin-producing cyanobacterium. M.S. thesis. University of Alaska, Fairbanks, AK.
18. **Kurmayer, R., and T. Kutzenberger.** 2003. Application of real-time PCR for quantification of microcystin genotypes in a population of the toxic cyanobacterium *Microcystis* sp. Appl. Environ. Microbiol. **69:**6723–6730.
19. **Lagos, N., H. Onodera, P. A. Zagatto, D. Andrinolo, S. M. Azevedo, and Y. Oshima.** 1999. The first evidence of paralytic shellfish toxins in the fresh water cyanobacterium *Cylindrospermopsis raciborskii*, isolated from Brazil. Toxicon **37:**1359–1373.
20. **Lawrence, J. F., B. Wong, and C. Menard.** 1996. Determination of decarbamoyl saxitoxin and its analogues in shellfish by prechromatographic oxidation and liquid chromatography with fluorescence detection. J. AOAC Int. **79:**1111–1115.
21. **Llewellyn, L. E.** 2006. Saxitoxin, a toxic marine natural product that targets a multitude of receptors. Nat. Prod. Rep. **23:**200–202.
22. **Llewellyn, L. E., A. P. Negri, J. Doyle, P. D. Baker, E. C. Beltran, and B. A. Neilan.** 2001. Radioreceptor assays for sensitive detection and quantitation of saxitoxin and its analogues from strains of the freshwater cyanobacterium, Anabaena circinalis. Environ. Sci. Technol. **35:**1445–1451.
23. **Lund, J. W. G., C. Kipling, and E. D. LeCren.** 1958. The inverted microscope method of estimating algal numbers and the statistical basis of estimation by counting. Hydrobiologia **11:**143–170.
24. **Lyra, C., S. Suomalainen, M. Gugger, C. Vezie, P. Sundman, L. Paulin, and K. Sivonen.** 2001. Molecular characterization of planktic cyanobacteria of Anabaena, Aphanizomenon, Microcystis and Planktothrix genera. Int. J. Syst. Evol. Microbiol. **51:**513–526.
25. **Mahmood, N. A., and W. W. Carmichael.** 1986. Paralytic shellfish poisons produced by the freshwater cyanobacterium Aphanizomenon flos-aquae NH-5. Toxicon **24:**175–186.
26. **Makarova, K. S., E. V. Koonin, R. Haselkorn, and M. Y. Galperin.** 2006. Cyanobacterial response regulator PatA contains a conserved N-terminal domain (PATAN) with an alpha-helical insertion. Bioinformatics **22:**1297–1301.
27. **Mihali, T. K., R. Kellmann, and B. A. Neilan.** 2009. Characterisation of the paralytic shellfish toxin biosynthesis gene clusters in Anabaena circinalis AWQC131C and Aphanizomenon sp. NH-5. BMC Biochem. **10:**8.
28. **Moustafa, A., J. E. Loram, J. D. Hackett, D. M. Anderson, F. G. Plumley, and D. Bhattacharya.** 2009. Origin of saxitoxin biosynthetic genes in cyanobacteria. PLoS One **4:**e5758.
29. **Nadkarni, M. A., F. E. Martin, N. A. Jacques, and N. Hunter.** 2002. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology **148:**257–266.
30. **Negri, A. P., and G. J. Jones.** 1995. Bioaccumulation of paralytic shellfish poisoning (PSP) toxins from the cyanobacterium *Anabaena circinalis* by the freshwater mussel *Alathyria condola*. Toxicon **33:**667–678.
31. **Negri, A. P., G. J. Jones, and M. Hindmarsh.** 1995. Sheep mortality associated with paralytic shellfish poisons from the cyanobacterium *Anabaena circinalis*. Toxicon **33:**1321–1329.
31a. **Neilan, B. A., D. Jacobs, T. Del Dot, L. Blackall, P. R. Hawkins, P. T. Cox, and A. E. Goodman.** 1997. rRNA sequences and evolutionary relationships among toxic and non-toxic cyanobacteria of the genus *Microcystis*. Int. J. Syst. Bacteriol. **47:**693–697.
32. **NHMRC.** 2001. Australian drinking water guidelines. National Health and Medical Research Council and the Agricultural Resource Management Council of Australia and New Zealand, Artarmon, Australia.
33. **Oshima, Y., M. Hasegawa, T. Yasumoto, G. Hallegraeff, and S. Blackburn.** 1987. Dinoflagellate Gymnodinium catenatum as the source of paralytic shellfish toxins in Tasmanian shellfish. Toxicon **25:**1105–1111.
34. **Oshima, Y., Y. Kotaki, T. Harada, and T. Yasumoto.** 1984. Paralytic shellfish toxins in tropical waters, p. 161–170. *In* E. P. Ragelis (ed.), Seafood toxins. American Chemical Society, Washington, DC.
35. **Pearson, L. A., and B. A. Neilan.** 2008. The molecular genetics of cyanobacterial toxicity as a basis for monitoring water quality and public health risk. Curr. Opin. Biotechnol. **19:**281–288.
36. **Pomati, F., M. C. Moffitt, R. Cavaliere, and B. A. Neilan.** 2004. Evidence for differences in the metabolism of saxitoxin and C1+2 toxins in the freshwater cyanobacterium *Cylindrospermopsis raciborskii* T3. Biochim. Biophys. Acta **1674:**60–67.
37. **Provasoli, L., J. J. McLaughlin, and M. R. Droop.** 1957. The development of artificial media for marine algae. Arch. Microbiol. **25:**392–428.
38. **Rippka, R., and M. Herdman.** 1992. Pasteur culture collection (PCC) of cyanobacterial strains in axenic culture, catalogue of strains, vol. 1. Institute Pasteur, Paris, France.
39. **Rippka, R., J. Deruelles, J. Waterbury, M. Herdman, and R. Stanier.** 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. **111:**1–61.
40. **Rounge, T. B., T. Rohrlack, A. J. Nederbragt, T. Kristensen, and K. S. Jakobsen.** 2009. A genome-wide analysis of nonribosomal peptide synthetase gene clusters and their peptides in a *Planktothrix rubescens* strain. BMC Genomics **10:**396.
41. **Simon, R. D.** 1978. Survey of extrachromosomal DNA found in the filamentous cyanobacteria. J. Bacteriol. **136:**414–418.
42. **Thompson, A. S., J. C. Rhodes, and I. Pettman.** 1988. Culture collection of algae and protozoa: catalogue of strains, p. 22. Natural Environment Research Council, Freshwater Biological Association, Ambleside, United Kingdom.
43. **Vaitomaa, J., A. Rantala, K. Halinen, L. Rouhiainen, P. Tallberg, L. Mokelke, and K. Sivonen.** 2003. Quantitative real-time PCR for determination of microcystin synthetase E copy numbers for *Microcystis* and *Anabaena* in lakes. Appl. Environ. Microbiol. **69:**7289–7297.
44. **Velzeboer, R. M. A., P. D. Baker, and J. Rositano.** 1998. Research report no. 135. Urban Water Research Association of Australia, Melbourne, Australia.