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Monitoring of geosmin producing *Anabaena circinalis* using quantitative PCR

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ARTICLE INFO

Article history:
Received 30 July 2013
Received in revised form
6 October 2013
Accepted 10 October 2013
Available online 18 October 2013

Keywords:
Anabaena circinalis
Geosmin synthesis gene
Monitoring
Quantitative PCR
Reservoir

ABSTRACT

Geosmin is one of the most commonly detected off-flavor chemicals present in reservoirs and drinking water systems. Quantitative real-time PCR (qPCR) is useful for quantifying geosmin-producers by focusing on the gene encoding geosmin synthase, which is responsible for geosmin synthesis. In this study, several primers and probes were designed and evaluated to detect the geosmin synthase gene in cyanobacteria. The specificity of primer and probe sets was tested using 21 strains of laboratory cultured cyanobacteria isolated from surface waters in Australia (18) and Taiwan (2), including 6 strains with geosmin producing ability. The results showed that the primers designed in this study could successfully detect all geosmin producing strains tested. The selected primers were used in a qPCR assay, and the calibration curves were linear from 5×10^1 to 5×10^5 copies mL^{-1}, with a high correlation coefficient ($R^2 = 0.999$). This method was then applied to analyze samples taken from Myponga Reservoir, South Australia, during a cyanobacterial bloom event. The results showed good correlations between qPCR techniques and traditional methods, including cell counts determined by microscopy and geosmin concentration measured using gas chromatography (GC) coupled with a mass selective detector (MSD). Results demonstrate that qPCR could be used for tracking geosmin-producing cyanobacteria in drinking water reservoirs. The qPCR assay may provide water utilities with the ability to properly characterize a taste and odor episode and choose appropriate management and treatment options.

1. Introduction

Taste and odor (T&O) problems in drinking water have caused concerns and complaints about water quality worldwide. Although no reports provide direct evidence of adverse health effects from off-flavors produced by microbes, the T&O compounds create problems for water quality management because people often interpret off-flavors as evidence that the water is unsafe to drink (Smith et al., 2002). Geosmin is one of the most common off-flavor compounds detected in drinking water and is produced by certain cyanobacteria and actinomycetes (Jüttner and Watson, 2007). Humans have a low odor detection threshold for geosmin, as little as 10 ng L^{-1} (Cook et al., 2001), and this sensitivity, combined with

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http://dx.doi.org/10.1016/j.watres.2013.10.028

eutrophication of lakes and reservoirs and the resulting increased incidence of blooms of geosmin-producing cyanobacteria, has led to an increasing number of major water quality incidents caused by the presence of geosmin in drinking water. This is exacerbated by the difficulty of removing taste and odor compounds by conventional water treatment methods such as coagulation-sedimentation and rapid sand filtration (Kim et al., 1997; Nerenberg et al., 2000; Lin et al., 2002). Therefore, quantification of geosmin and geosmin-producing species in water sources is a necessity for proper management of drinking supplies.

Odor problems in drinking water may occur when off-flavor-producing species are present. The growth rate (and double time) of bloom-forming cyanobacteria is about 0.18–1.2 day^{-1} (and 0.58–3.9 day) (Robarts and Zohary, 1987). When the environmental conditions are ideal the numbers of odor producing cyanobacteria can rapidly increase to form blooms. This can occur within a few days. Therefore, methods are required to rapidly measure and characterize the problem. Conventionally, the issue can be understood and quantified by measuring the odor-producing cyanobacteria and the odorants. The former is commonly achieved using microscopy to identify and enumerate odor-producing cyanobacteria (Izaguirre and Taylor, 1998), while the latter may be determined by gas chromatograph (GC) coupled with a mass selective detector (MSD) (Lin et al., 2002). However, these methods either require experienced personnel or sophisticated instrumentation. Most importantly, for multiple samples, the turn-around-time for microscopic cell counting of cyanobacteria and GC/MSD analysis can be long.

Recently, bio-molecular methods, such as quantitative polymerase chain reaction (qPCR), have been developed to detect the genes of harmful cyanobacteria in fresh water bodies (Koskenniemi et al., 2007; Rasmussen et al., 2008; Yen et al., 2012; Marbun et al., 2012; Michinaka et al., 2012). Based on the detection of functional genes for toxins, these studies successfully demonstrated that this method can be used to quantify toxin-producing cyanobacteria in natural waters. A similar approach may be extended to the detection of odor-producing cyanobacteria based on the functional gene involved in geosmin production. A qPCR assay has been developed to quantify a geosmin-producing actinomycete, *Streptomyces* spp., in aquaculture systems (Auffret et al., 2011). The nucleotide sequences of the terpene synthases-encoding gene involved in the geosmin synthesis for cyanobacteria were reported by Giglio et al. (2008), who demonstrated the correlation between odor-producing cyanobacteria and the production of geosmin. However, no field samples were analyzed in the study. Since qPCR can provide multiple sample analysis in the same run, it would be advantageous if this monitoring method of odor-producing genes were available. However, to the best of our knowledge, no previous report has shown the application of qPCR in the monitoring of geosmin-producing cyanobacteria in drinking water reservoirs.

In this study, a qPCR assay was developed to quantify geosmin-producing cyanobacteria and applied to a bloom of *Anabaena circinalis*, a geosmin producer present in Myponga Reservoir in South Australia. To evaluate the applicability of the method developed, qPCR results were compared with microscopic cell enumerations and GC/MSD analysis of geosmin concentrations.

2. Materials and methods

2.1. *Cyanobacteria cultures and cyanobacteria-laden reservoir water samples*

Twenty one cyanobacteria strains were used in this study to test the specificity of the designed primers. Eighteen of the strains were isolated and maintained by the Australian Water

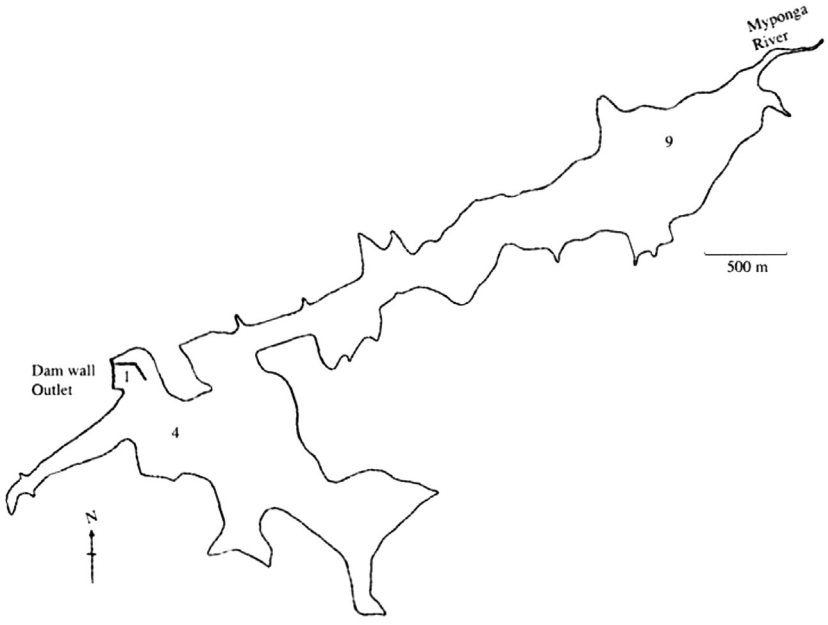

Fig. 1 – Sampling locations in Myponga Reservoir, South Australia.

Table 1 – The list of primers employed in this study for geosmin-producing cyanobacteria.

Primer	Sequence (5′→ 3′)	Position[a]	Product (bp)[b]	Tm (°C)
3139F	CTAGACCMATGCGGGTTTTA	3139–3158		53.5
3245R	CTCAACTACAAGCACACA	3245–3263	106	49.5
3708R	CCATTCTTTRGAATGMTT	3711–3728	569	44.5
SGF1	CATCGAATACATCGAGATGCG	3035–3055		55
JDR1	TCGCCTTCATCTTCCACTTC	3216–3235	200	55
SGP5	AAGGTTGGTGGCGCACCCTGGTCA	3060–3083		70

[a] Corresponding to position of *Phormidium* sp. P2r sesquiterpene synthase 1 (*geo*A1) and sesquiterpene synthase 2 (*geo*A2) genes, complete cds. (EF619621), which are geosmin-producing gene reported by Ludwig et al. (2007).
[b] Product indicated the length of the amplicon (bp) with 3139F/3245R, 3139F/3708R, SGF1/JDR1.

Quality Centre (AWQC), South Australia, including *Anabaena aphanizomeniodes, Anabaena bergii, A. circinalis*, *Anabaena galeata, Anabaena oscilarioides, Anabaena solitaria, Cylindropsermopsis raciborskii, Microcystis aeruginosa*, *Nodularia spumigena* and *Trichodesmium* sp. Two of the cyanobacteria strains, *M. aeruginosa* TWNCKU11 and *Pseudanabaena* sp. TWNCKU12, were isolated from Taiwan. *M. aeruginosa* PCC7820 were obtained from the Pasteur Culture Collection of Cyanobacteria (Paris, France). DNA from the geosmin producer *Anabaena lemmermannii* strain ALGI CA799 (kindly provided by Dr Sue Watson of Environment Canada) was used in the initial development of PCR and qPCR assays and as a standard for qPCR. Cultures were incubated in ASM medium (Rippka, 1988) at 20 °C under 70 μmol photons m^{-2} s^{-1} (measured using a spherical underwater light sensor – LI-COR® LI-193) with a light/dark cycle of 12/12 h for 2 weeks so that cells were in exponential growth phase.

Myponga Reservoir (S 35°24′, E 138°25′), located to the south of Adelaide on the Fleurieu Peninsula, South Australia, was selected as the study site. The reservoir is a water source for approximately 50,000 people in South Australia. Cyanobacteria blooms frequently occur in summer, with *A. circinalis* one of the dominant species (Dixon et al., 2011). Water samples were taken from the surface layer at locations 1, 4, and 9, as well as at a depth of 20 m at locations 1 and 4 (Fig. 1). The sampling was conducted twice a week from 29th November to 23rd December, 2010 during a cyanobacteria bloom episode. The samples were stored in 1.5 L PET bottles for cell counting and DNA extraction. For geosmin analysis, the samples were stored in 355 mL PET bottles at 4 °C before analysis. Detailed procedures employed for cell counting, DNA analysis, and geosmin analysis, are discussed in the following sections.

2.2. Cell counting

The cell numbers of *A. circinalis* were counted using a microscope (Eclipse 50i, Nikon Corporation, Japan) with a Sedgwick-Rafter chamber (Graticules Ltd., UK) according to the Standard Method 10200F (APHA, 1992). After sampling, Lugol's solution was immediately added into the samples, at a concentration of 0.3 mL Lugol's solution per 100 mL of sample for preservation. Then, 1 mL of sample was transferred to the Sedgwick-Rafter chamber, and allowed to settle for 30 min. The number of cells in 50 of the 1000 grids in the Sedgwick-Rafter cell was counted.

2.3. DNA extraction

DNeasy Blood & Tissue Kit (Qiagen, Maryland, USA) was used to extract DNA from the pure cultures and the cyanobacteria-laden reservoir water samples. For all experiments, sample aliquots (100 mL) were first filtered through a 5 μm pore-size hydrophilic Durapore filter (Millipore, Massachusetts, USA). The filter was placed in a sterile tube, followed by the addition of 180 μL of buffer ATL (contained in the DNeasy kit). Then, the cells were broken up by an ultrasonic peen (Branson Digital Sonifier, model 450, nominal power 400 W; Branson, Connecticut, USA) with a cycle of 15 s on and 10 s off for six repetitions at 20% power output, after which 10 μL of Lysozyme solution (90 mg mL^{-1}, Invitrogen, California, USA) and proteinase K (contained in the DNeasy kit) were added to the tubes and incubated at 56 °C for at least 3 h for cell lysis. Then, DNA extraction was performed following the handbook supplied with the DNeasy Blood & Tissue Kit (as for cell lysates). The extracted DNA was finally eluted in 200 μL of elution buffer for qPCR quantification.

2.4. Detection and quantification of geosmin-producing cyanobacteria by qPCR

The primers used in this study and their target DNA segments are shown in Table 1, including the primers' positions based on the geosmin producing genes reported by Ludwig et al. (2007) for *Phormidium* sp. P2r sesquiterpene synthase (*geo*A2) gene (EF619621).

Quantitative PCR assays were performed on a Smartcycler (Cepheid, California, USA). For the primer set 3139F/3245R, the intercalating dye SYTO9 was incorporated for qPCR quantification (Monis et al., 2005). A 25 μL mixture containing 1.0 U of Platinum® Taq DNA polymerase (Invitrogen, California, USA), 1 × buffer, 5 mM $MgCl_2$, 0.2 mM of dNTP, 0.3 μM of each primer, 3.3 μM of SYTO9 and 2 μL of template DNA solution was used in the PCR reaction. The PCR conditions were denaturation at 95 °C for 300 s, 45 cycles of denaturation at 95 °C for 5 s, annealing at 57 °C for 15 s, and extension at 72 °C for 15 s with fluorescence acquisition (FAM channel, with excitation wavelength = 450–495 nm and emission wavelength = 510–527 nm) at the end of the extension. For the assay using primer set SGF1/JDR1, Taqman probe SGP5 was incorporated for quantification. The PCR mixture was the same as that of the primer set 3139F/3245R, except that 0.1 μM of TaqMan probe was used instead

Table 2 – Results of testing primers with non geosmin-producer and geosmin-producer.

Species/strains[a]	Code	16S sequence ID	Primer set[c]		Geosmin producing capability[b]
			SGF1/JDR1/SGP5	3139F/3245R	
Anabaena aphanizomeniodes	ANA 259	*A. variabilis*	–	–	–
A. bergii	ANA 283 B	*A. bergii*	–	–	–
A. circinalis	ANA 118 AR	nd	+	+	+
A. circinalis	ANA 150 A	*A. circinalis*	–	+	+
A. circinalis	ANA 196	*Anabaena* sp.	+	+	+
A. circinalis	ANA 278 FR	nd	+	+	+
A. circinalis	ANA 335 C	nd	+	+	+
A. circinalis	ANA 118 C	*A. circinalis*	N/A	+	+
A. galeata	PSA-011 C	*Planktothrix* sp.	–	–	–
A. oscilarioides	ANA 025 B	*Anabaena* sp.	–	–	–
A. solitaria	ANA 195 A	*Anabaena* sp.	–	–	–
A. solitaria	ANA 207 A	nd	–	–	–
Cylindropsermopsis raciborskii	CYP 009 A	*C. raciborskii*	–	N/A	–
C. raciborskii	CYP 026J	*C. raciborskii*	N/A	–	–
Microcystis aeruginosa	MIC 021 B	*M. aeruginosa*	–	N/A	–
M. aeruginosa	MIC 042 B	nd	–	N/A	–
M. aeruginosa	PCC7820	*M. aeruginosa*	N/A	–	–
M. aeruginosa	TWNCKU11	*M. aeruginosa*	N/A	–	–
Nodularia spumigena	NOD 009	*N. spumigena*	–	N/A	–
Pseudanabaena sp.	TWNCKU12	*Pseudanabaena* sp.	N/A	–	–
Trichodesmium sp.	TRD 002 A	*Trichormus*	–	–	–

[a] Strains were identified by the sequence of cyanobacterial 16S rDNA.
[b] Measured with GC/MSD: "+" represents geosmin detected in the culture samples, and "–" represents no geosmin detected.
[c] + or – indicates presence or absence of the predicted PCR product after qPCR. N/A indicates no analysis.

of SYTO9. The PCR conditions were denaturation at 95 °C for 300 s, 45 cycles of denaturation at 95 °C for 15 s, annealing at 57 °C for 10 s, and extension at 60 °C for 30 s with fluorescence acquisition (FAM channel) at the end of the extension. All the qPCR results were analyzed using Smart Cycler® Software version 2.0. (Cepheid, California, USA). The extracted DNA from *Anabaena lemmermanii* ALGI CA799 and *M. aeruginosa* PCC7820 were used as the positive and negative controls in qPCR, respectively. DNA standards for calibration curves in qPCR analysis were prepared using amplicons obtained from the standard strain with primer sets 3139F/3708R and SGF1/JDR1. The amplicons were purified with Amicon Ultra-0.5 Centrifugal Filter Devices (Millipore, Massachusetts, USA) according to the manufacturer's protocol. The copy numbers of DNA standards were calculated based on the DNA concentration measured by a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA) (Whelan et al., 2003). The sizes of the amplicons, expected to be 569 bp and 200 bp for the two primer sets, were confirmed by 1.6% (w/v) agarose gel electrophoresis with SYBR Safe DNA stain included in the gels.

2.5. Amplification of cyanobacterial 16S rRNA genes

The PCR assay of Shaw et al. (1999), targeting cyanobacterial 16S rRNA gene sequences, was used to verify the quality of extracted DNA and to confirm the identity of isolates. Reactions were conducted in 25 μL volumes containing 0.25 μM each of PF1 (5′-TGTAAAACGACGGCCAGTCCAGACTCCTACG-3′) and PR1 (5′-CGCGTTAGCTACGGCACGGCTC -3′), 1 × Reaction Buffer (Invitrogen, USA), 2 mM $MgCl_2$, 2.5 μM SYTO9 (Invitrogen, USA), 1 U of Platinum *Taq* polymerase (Invitrogen, USA) and 0.2 mM of deoxynucleoside triphosphates (Invitrogen, USA). A real time PCR system (Rotor-Gene 6000, Corbett Life Science, USA) was used for the experiments. Cycling conditions were 5 min 95 °C heat activation, followed by 45 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s (with acquisition to the FAM channel at this temperature). Amplicons were purified using Montage

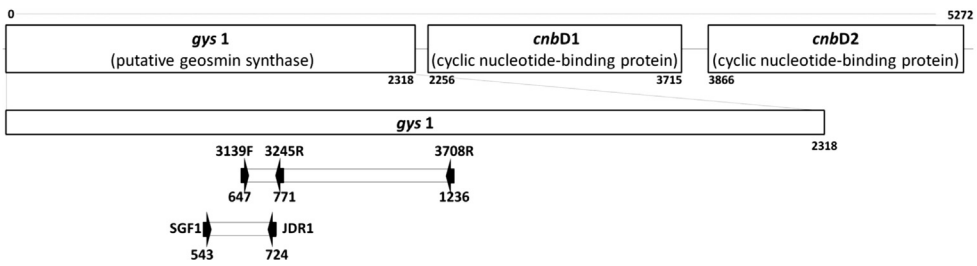

Fig. 2 – Primer positions and alignment of the amplicon from standard strain on *Anabaena ucrainica* CHAB2155 geosmin synthesis operon (HQ404996).

filter devices (Millipore, Australia) following the manufacturer's instructions. Purified amplicons were sequenced utilizing BigDye Terminator 3.1 labeling and an AB3730XL 96-capillary sequencer (performed by the Australian Genome Research Facility).

2.6. Sequence analysis of geosmin synthase gene

Sequencing reactions were performed using a 96-Well C1000™ thermal cycler (BIO-RAD, California, USA) with a PCR mixture containing 5 μL of DNA template solution (10–20 ng DNA), 1 × buffer, 2 mM of $MgCl_2$, 0.2 mM of dNTP, 0.4 μM of each primer (3139F/3708R and SGF1/JDR1) and 0.025 U of *TaKaRa Ex Taq*™ DNA polymerase Hot Start version (Takara Biotechnology, Japan) in 50 μL. The PCR conditions included a pre-incubation step at 95 °C for 5 min, 30 cycles at 95 °C for 30 s, 53 °C for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 10 min. The PCR products were cloned with the TOPO TA cloning kit (pCR II-TOPO vector, Invitrogen, California, USA) according to the method prescribed by the manufacturer. DNA sequencing of cloned inserts was performed by Mission Biotech Company, Taiwan. Similarity searches were performed using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997) with the DNA sequences in the GenBank database.

2.7. Measurement of geosmin

In this study, geosmin was measured for the laboratory cultures and the water samples collected from Myponga Reservoir. Samples were diluted to achieve a geosmin concentration that was within the detection range of the instrument. All the analyses were conducted by AWQC using headspace solid phase micro-extraction (SPME) concentration and GC/MSD. In the analysis, a Gerstell multipurpose sampler (Gerstell Gmbh Co., Germany) was employed to automate the SPME sample concentration, while an Agilent 7980A GC with a 5975 MS and EI source (Agilent Technologies, Australia) was used for the quantification of geosmin. A PDMS fiber (Supelco, USA) was used for the SPME, helium was the carrier gas, and an HP-5MS column, 30 m in length and 0.25 mm in diameter, was used as the separation column. The levels of geosmin were determined via an internal standard present as d5-Geosmin, with a detection range of 4–220 ng L^{-1}.

3. Results and discussion

3.1. Primer selection

A primer set developed by Shaw et al. (1999), targeting cyanobacterial 16S rDNA for sequence analysis, was used to verify the quality of extracted DNA and confirm the identity of the culture collections. Table 2 lists the results for the confirmation of the identities of the isolates in the culture collections. Among the 21 strains tested, six were *A. circinalis* and were geosmin producers based on GC/MSD analysis. For the other cyanobacteria, including *A. aphanizomeniodes, A. bergii, A. galeata, A. oscilarioides, A. solitaria* (2 strains), *C. raciborskii* (2 strains), *M. aeruginosa* (4 strains), *N. spumigena*, *Pseudanabaena* sp. and *Trichodesmium* sp., none showed geosmin producing capability. The PCR results based on the 3139F/3245R primer set show that the presence of geosmin producing genes completely matched the detection of geosmin for the same culture samples, suggesting that the primer set was able to detect the presence of geosmin genes in the samples. PCR data with SGF1/JDR and SGP5 probe of geosmin genes also captured almost all the geosmin producing strains, except for *A. circinalis* 150A.

PCR with the 3139F/3708R primer set gave products with sizes around 570 bp from the *Anabaena circinalas* 118 C. Based on the similarity searches by BLAST, the obtained sequence is similar to the geosmin synthesis gene operon of two *Anabaena ucrainica* strains (CHAB2155 and CHAB1432) with similarities of 97%, and the sesquiterpene synthase (*geo*A) genes of *Phormidium* sp. P2r with similarities of 80%. The alignment of these primers and amplicons with *A. ucrainica* CHAB2155 geosmin synthesis operon are depicted in Fig. 2. The results of cloning and sequencing confirmed the presence of the 3245R primer position in the PCR products obtained, with a complete match. Since a long amplicon may lead to poor amplification efficiency, a shorter primer (3139F/3245R) was developed for quantification by qPCR. This supports the possibility that the amplicon obtained with the 3139F/3245R primer set was from the putative geosmin synthase (gsy1) on the geosmin

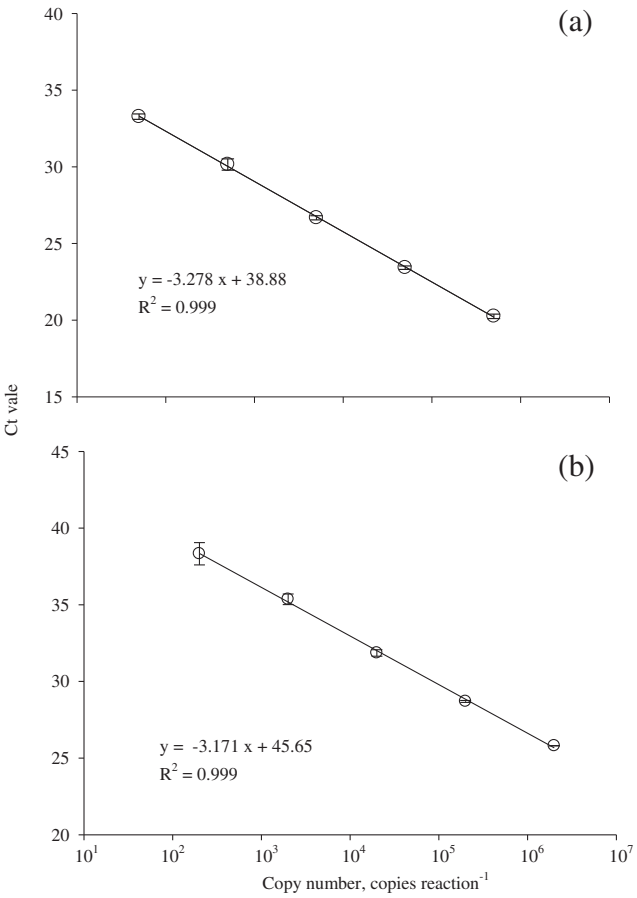

Fig. 3 – qPCR standard curve using primer 3139F/3245R with SYTO9 intercalater (a) and primer SGF1/JDR with probe SGP5 (b).

synthesis operon. Although these strains were not tested in this study, it is theoretically possible to amplify these strains since the DNA sequence includes both primer regions, 3139F and 3245R.

3.2. *Standard curves for qPCR*

Fig. 3 shows that PCR standards were successfully amplified for both primer sets, with the range of 5×10^1–5×10^5 copies μL^{-1} for the primer set 3139F/3245R with SYTO9 intercalator (denoted as 3139F/3245R), and 2×10^2–2×10^6 copies μL^{-1} for the primer set SGF1/JDR and probe SGP5 (denoted as SGF1/JDR/SGP5), respectively. The two standard curves shown in the figures were linear, both with very high correlation coefficients ($R^2 = 0.999$). qPCR efficiency (E) was calculated based on the equation, $E = (10^{-1/s} - 1)$ and s = slope of the standard curve. The E's were found to be 1.01 and 1.09 for primer 3139F/3245R and SGF1/JDR/SGP5, respectively. That both efficiencies were close to 1 suggests that the amplicon quantity nearly doubled every cycle and amplification efficiency was reasonable for quantification. The result of the melting curve analysis for 3139F/3245R showed a sharp melting peak at 82.6 °C, revealing no presence of primer-dimers.

3.3. *Application in the monitoring of Myponga Reservoir water*

The qPCR approach was further applied in quantifying geosmin producers in Myponga Reservoir, South Australia. Water samples from upstream (location 9) and downstream (location 1 and 4) were collected during a cyanobacteria bloom episode, which occurred during the period of the 29th November to 23rd

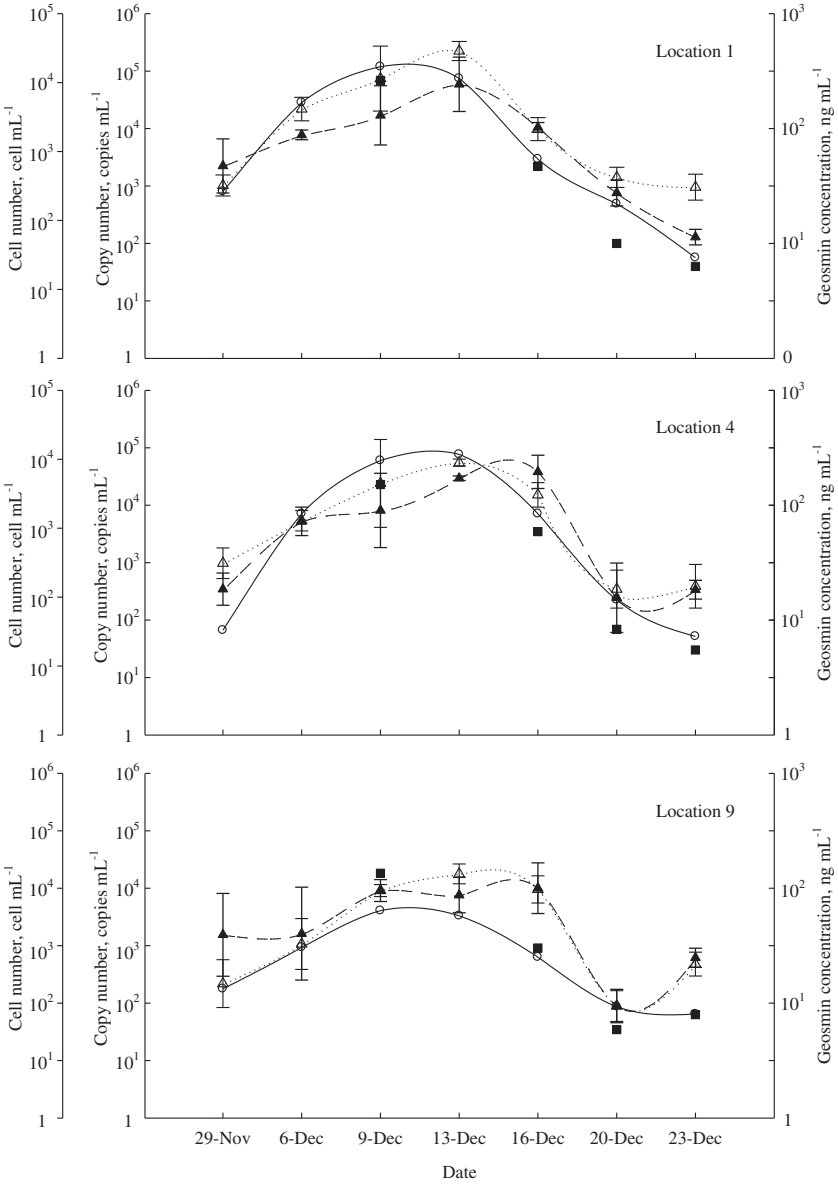

Fig. 4 – Time course of *Anabaena* cells, geosmin synthesis gene, and geosmin concentrations for Myponga reservoir, where cell numbers were measured by microscope (○), copy numbers of geosmin synthesis gene by qPCR with 3139F/3245R (triplicate) (△) and with SGF1/JDR1/SGP5 (triplicate) (▲), and geosmin concentration determined by HSSPME/GC/MS (■).

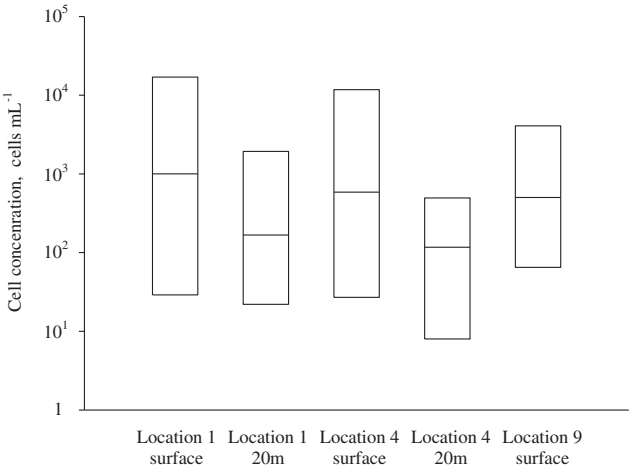

Fig. 5 – Comparison of *A. circinalis* cell concentrations for different sampling locations and depths. The horizontal line within each box shows the average.

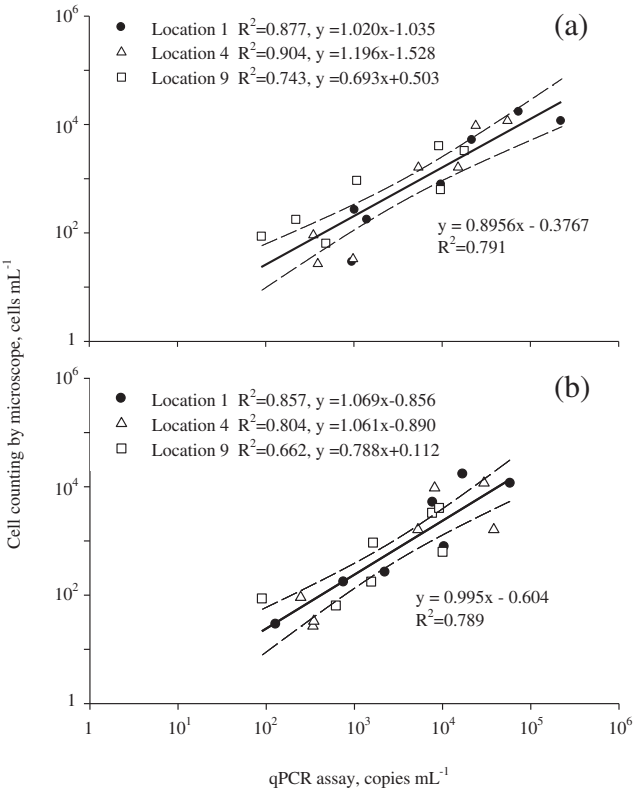

Fig. 6 – Correlation between *Anabaena* cell concentrations measured by microscope and copy number measured by qPCR with 3139F/3245R (a), and with SGF1/JDR1/SGP5 (b).

December, 2010. After the 13th of December, 2010, the bloom collapsed in all locations. Besides qPCR, the samples were also analyzed for geosmin (using HS-SPME/GC/MSD) and cyanobacteria cell concentrations (using microscopy). Morphological identification using microscopy indicated that the dominant species was *A. circinalis*, which is in accordance with the historical data for the reservoir (Lewis et al., 2003). Fig. 4 shows the time course of *Anabaena* cell numbers determined by microscope, copy numbers of geosmin synthesis gene quantified by qPCR with 3139F/3245R and SGF1/JDR1/SGP5, and geosmin concentrations measured by HSSPME/GC/MSD for the surface samples collected at locations 1, 4, and 9. The figure shows that the three parameters associated with *Anabaena* cells, including cell number, gene copies, and geosmin concentration, all increased from November 29, 2010, reaching their highest concentration on December 13, followed by a reduction. Although measured with different methods, the time courses for the three *Anabaena* cell-relevant parameters followed one another closely. In addition, Fig. 4 also indicates that for all three sampling locations, the trends of the three parameters were similar, which implies that the time course of the bloom did not vary between locations in the reservoir.

Fig. 5 shows the concentration ranges for *A. circinalis* collected at different locations and depths during the sampling period. Interestingly, the cell concentrations for the three sampling locations were in the same range, all between 0.8×10^1 and 1.7×10^4 cells mL^{-1}. However, the cell concentrations for the samples taken at the depth of 20 m were much lower if compared to the corresponding surface water samples. This may be attributed to the fact that the buoyant *A. circinalis* cells were floating to the surface and grew well under sunlight condition or the reservoir was stratified. To understand the geosmin concentrations and its association with *Anabaena* cells, Table 3 lists the measured geosmin concentrations and related cell quotas. Throughout the life of the bloom, the average geosmin concentrations were in the range of 13–82 ng L^{-1}, with a maximum concentration of 264 ng L^{-1}. Table 3 also shows the geosmin cell quotas for the samples,

Table 3 – Geosmin concentrations and cell quotas in samples collected from Myponga Reservoir, South Australia.

Location		Total geosmin concentration (ng L^{-1})			Geosmin quota (pg $cell^{-1}$)		
		Average	max	min	Average	max	min
1	(Surface)	81.77	264.33	6.33	0.088	0.218	0.015
4	(Surface)	56.10	152.20	5.50	0.087	0.204	0.016
9	(Surface)	44.87	135.50	5.87	0.068	0.124	0.034
1	(20 m)	26.13	75.83	5.27	0.156	0.333	0.039
4	(20 m)	12.93	31.40	5.77	0.248	0.838	0.030

*Average geosmin concentration and geosmin quota represent the mathematic means of 4 samples collected at each location except 6 samples at Location 1 (20 m).

which were calculated based on microscopic cell count and GC/MSD analysis. The geosmin cell quota falls in the range of 0.015–0.838 pg cell^{-1}, with averages of between 0.068 and 0.248 pg cell^{-1} for different locations and depths. Jones and Korth (1995) conducted a study to monitor volatile odorants and phytoplankton populations for Hay Weir pool on the Murrumbidgee River and Carcoar Dam, near Blayney, both in New South Wales, Australia. During their sampling period, *Anabaena* sp. was one of the dominant cyanobacteria for both sites. The geosmin cell quota was estimated to be 0.01 pg cell^{-1}. Our measurements of geosmin cell quota ranged from 0.015 to 0.248 pg cell^{-1}, which is similar to that observed by Li et al. (2010) and Hobson et al. (2010). Hobson et al. (2010) analyzed the samples of natural blooms of *A. circinalis* in Myponga, Happy Valley and Mount Bold Reservoirs in South Australia and Yan Yean Reservoir in Victoria, Australia. The geosmin content ranged from approximately 0.02–0.05 pg cell^{-1}, with the lowest value being 0.02 pg cell^{-1} for the samples collected from Myponga Reservoir. Li et al. (2010) investigated the occurrence of geosmin during a cyanobacterial bloom episode in the Yanghe Reservoir in north China in 2007. The geosmin concentration profile was closely related to the cell density of *Anabaena spiroides*, with an average geosmin cell quota of about 0.1 pg cell^{-1}.

The geosmin cell quotas for samples collected in surface waters was lower than in cells deeper in the water column, i.e., at 20 m depth. Giglio et al. (2011) investigated geosmin synthase gene expression in *A. circinalis*, and found that dark incubation did not affect the geosmin expression for 2 days. In this period, intercellular geosmin was increasing but cell number remained constant. Only after cultured in dark for more than 2 days, the expression of geosmin synthase started to decrease. It is speculated that the living cells at 20 m depth kept producing geosmin, but stopped growing due to

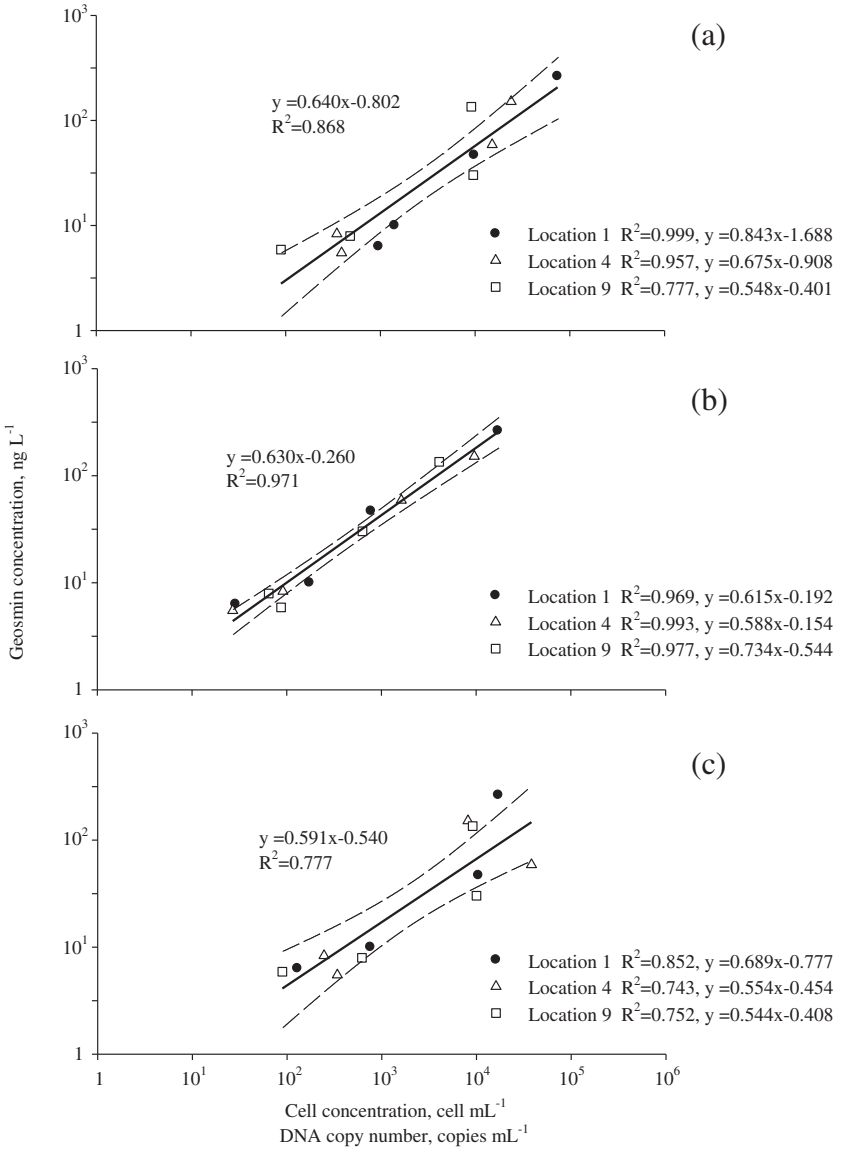

Fig. 7 – Relationships between geosmin concentration and cell number estimated by qPCR with 3139F/3245R (a), microscopic cell count (b), and DNA copy number by qPCR with SGF1/JDR1/SGP5(c), with 95% confidence interval (broken line).

insufficient light intensity. Several researchers have reported the connection between geosmin and pigment synthesis. They assumed that geosmin production might be lowest at the surface and increase with water column depth, because geosmin was proposed to have the same precursor as chlorophyll and carotenoids through the isoprenoid pathway (Naes et al., 1989; Utkilen and Frøshaug 1992, Saadoun et al., 2001). Zhang et al. (2009) reported that the effect of light intensity on geosmin production in *Lyngbya kuetzingi*, where the highest geosmin production was observed at 10 μmol m^{-2} s^{-1} compared with 20 or 75 μmol m^{-2} s^{-1}. This suggests that a low light intensity environment promotes geosmin production. Similarly, in this study cells at the 20 m depth showed a higher geosmin cell quota than those at the surface. Although the cell quotas were higher for *Anabaena* cells located in the deeper water, the total amount of geosmin was higher at the surface due to higher biomass density. Therefore, cell density is an important variable for estimating total geosmin levels present in a reservoir.

Cell density measured by microscopy and gene copy number measured by qPCR are compared in Fig. 6, with good correlation between the two methods (R^2 = 0.79 for both primer sets). Calculation of the ratio of genes/cell showed that for all samples across the 3 locations, there was on average 10.3 gene copies/cell (range 1.1–35.6 gene copies/cell) for primer set 3139F/3245R and 8.5 gene copies/cell (range 1.2–27.4 gene copies/cell) for primer set SGF1/JDR1. Fig. 7 displays the relationship between geosmin concentration and cell density of *A. circinalis* measured by both microscope and qPCR. Correlation was good for both microscope (R^2 = 0.97) and qPCR (R^2 = 0.87 and 0.78).

In view of the quick turnover time property compared to conventional GC/MSD analysis and taxonomical identification, qPCR has been recently proposed to quantify geosmin producers present in different food and water matrix, including liquors (Du et al., 2013), aquaculture systems (Auffret et al., 2011), laboratory culture systems (Giglio et al., 2008; Su et al., 2013), and reservoirs (Lylloff et al., 2012). While most of the studies focused on monitoring geosmin-producing *Streptomyces* cells through quantification of either *GeoA* gene (Du et al., 2013; Auffret et al., 2011) or 23S rRNA gene (Lylloff et al. 2012), only few are relevant to geosmin-producing cyanobacteria. Although Giglio et al. (2008) and Su et al. (2013) have successfully developed qPCR assays to measure geosmin synthase gene and demonstrated the correlation with the production of geosmin, only laboratory cultures fortified into reservoir water were tested in the studies. The current study provides the first field results for the monitoring of geosmin-producing cyanobacteria during a natural bloom episode in a drinking water reservoir. It demonstrates the applicability of qPCR assay in quantifying geosmin producing cyanobacteria as well as the potential of geosmin production.

Currently, microscopic enumeration and GC/MSD analysis are commonly used in the monitoring of cyanobacteria cell density and geosmin concentrations for drinking water systems, respectively. Considering the nature of the methods available for the measurement of *Anabaena* cell density and geosmin production potential, qPCR is the only one that offers multiple sample analysis in the same run. In view of the aforementioned, this qPCR assay offers a rapid and high-throughput method for detection of geosmin-producing *A. circinalis*. Analysis of multiple samples provides water utilities with the ability to properly characterize a T&O episode and make the best decision when choosing control and treatment options. Compared with enumeration by microscopy and GC/MSD analysis, qPCR has the potential to give a much faster turnover time for multiple samples and hence the ability to rapidly quantify geosmin-producing *A. circinalis* cells in reservoirs.

4. Conclusions

A PCR assay was successfully developed for the quantification of geosmin-producing *A. circinalis*. The project developed two primer sets to target the geosmin producing genes (*geo*A2). After testing the two primers against 21 geosmin and non-geosmin producer cyanobacteria species, the primer sets were further tested using field samples collected during an *A. circinalis* bloom at Myponga Reservoir, South Australia. The analysis of reservoir samples indicated good correlation between cell counts by microscope, geosmin gene copies by qPCR, and geosmin concentration by GC/MSD, suggesting that the qPCR based approach may be used to quantify geosmin producers and geosmin concentrations during an *A. circinalis* bloom. Compared with GC/MSD analysis and microscopic enumeration, qPCR assay offers a rapid and high-throughput method for detection of geosmin-producing *A. circinalis*. The approach may allow water utilities to better and faster characterize a geosmin episode in their source water so that appropriate management and treatment options can be selected.

Acknowledgments

This research is supported in part by the Water Research Foundation (Project # 4210), the South Australian Water Corporation, and the Taiwan National Science Council under project NSC 98-2221-E-006-022-MY3, and in part by the Landmark Project of National Cheng Kung University, Taiwan. The Foundation assumes no responsibility for the content of the research study reported in this publication or for the opinions or statements of fact expressed. The mention of trade names for commercial products does not represent or imply the approval or endorsement of the Foundation

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