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Rapid Detection and Quantitation of Microcystin-Producing Microcystis Using Real-Time PCR

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Abstract

This study is to develop and validate a real-time quantitative PCR (rt-QPCR) assay for rapid quantitation of microcystin-producing *Microcystis* using unprocessed surface water samples collected from Alberta lakes. Microcystin synthetase gene E (*mcyE*) was targeted for microcystin-producing *Microcystis* and 16S rRNA was used to measure blooming level of total cyanobacteria. The assay was optimized and validated with 20 reference samples collected in 2011. The limit of detection (LOD) of rt-QPCR was 50 copies/ml for both *mcyE* and 16S rRNA. An excellent precision was observed in 24 replicates [coefficient of variation (cv)=1.12% for 1.0E+05 and = 0.79% for 1.0E+03 copies]. The rt-QPCR assay was applied for detection of *mcyE* in 527 water samples collected from 45 lakes during the open-water season of 2012 in Alberta and 369 samples were *mcyE* positive. Microcystin-producing *Microcystis* was detected in 41 out of the 45 lakes in which, relatively high copy numbers of *mcyE* (≥ 1.0E+05 copies/ml) were determined in 9 lakes. Cyanobacteria were present in all 45 lakes determined by 16S rRNA. The rt-QPCR assay developed with specific target to *mcyE* is sensitive, specific and robust for rapid detection and differentiation of toxic *Microcystis* from non-toxic cyanobacteria in surface water.

Keywords: Real-time quantitative PCR; *mcyE* gene; Microcystin; *Microcystis;* Cyanobacteria

Abbreviations: rt-QPCR: Real-time Quantitative PCR; *mcy*E: Microcystin Synthetase Gene E; PPI: Protein Phosphatase Inhibition; Ct: Cycle Threshold

Introduction

Cyanobacteria, also named blue-green algae, are prokaryotic photosynthetic microorganisms that grow in warm, eutrophic surface waters. In Alberta, Canada, many lakes and reservoirs are eutrophic and support significant blooms of cyanobacteria during summer and early fall. It has been reported that on average 59% of the cyanobacterial blooms contain toxins [1]. Microcystin is the most common and prevalent cyanobacterial toxin and is produced mainly by species of *Microcystis, Planktothrix* and *Anabaena.* Microcystin is a small monocyclic heptapeptide with seven amino acids and is soluble and stable in water [2]. The amino acids D-glutamate and Add a play important roles in interaction with protein phosphatases and thus are critical for the toxicity of microcystin [3,4]. Microcystin poses a potential hazard to humans and animals through its inhibition on eukaryotic protein phosphatase 1 and 2A in hepatocytes, which results in gastrointestinal illness, severe liver damage and dysfunction, and possible carcinogenesis [5-8]. Microcystin is synthesized by microcystin synthetase enzyme complex encoded by *mcy* gene cluster via a non-ribosomal thio-template pathway [9,10]. The *mcy* gene cluster contains 10 genes, namely *mcy* A to *mcy* J, which have been fully sequenced and characterized in species *Microcystis*, *Planktothrix* and *Anabaena* [10-13].

Currently, the most common method for detecting and monitoring cyanobacteria is light microscopy examination of cell morphology and abundance; and methods for quantifying microcystin comprise enzyme inhibition assay, immunological and analytical assays such as the protein phosphatase inhibition (PPI) assay, enzyme-linked immunosorbent assays (ELISA) and high-performance liquid chromatography (HPLC).

Microscopic techniques cannot differentiate toxin- from non-toxin-producing species of cyanobacteria, although potential toxin-producing species can be identified taxonomically using the morphological species concept. Other assays are either time-consuming, requiring sophisticated instruments, or lack of sensitivity and specificity [14]. Therefore, development of a rapid, quantitative, cost-effective and specific method is needed for monitoring the prevailing level of toxin-producing cyanobacteria in environmental water.

Real-time quantitative PCR (rt-QPCR) has been used for a decade in clinical microbiology diagnostics because of its advantages of fast speed, high sensitivity and specificity, and less risk of cross contamination [15]. However, this method hasn't been routinely used in the diagnostics of microorganisms in environmental samples. With the increasing numbers of complete genomic sequences and knowledge of biosynthetic genes involved in toxin production, rt-QPCR has great potential for quantitation of toxin gene levels as well as identification of toxin-producing cyanobacterial species. The *mcy* gene

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clusters have been reported as molecular markers for identification of microcystin-producing cyanobacteria [16-18]. The carboxyl group of the D-glutamate side chain is present in all known variants of microcystin [19,20]. The *mcyE* gene is responsible for the activation and condensation of D-glutamate with Adda moiety [10,13], which can be used as a specific target for microcystin-producing cyanobacteria.

Due to potential health risks and biological hazards caused by toxin-producing cyanobacterial blooms, the Government of Alberta commenced monitoring for prevailing levels of microcystin in recreational lakes and reservoirs in 2005. Data collected from 4 open water seasons (2005-2008) revealed that microcystins occur in a majority of Alberta lakes and reservoirs [21]. Since up to 95% of cyanobacterial blooms formed by *Microcystis* sp. were found to be toxic [22], the *mcyE* gene is our primary focus. The purpose of this study is to develop and validate a rapid and simple rt-QPCR assay for detection and quantitation of toxin-producing *Microcystis* in Alberta lakes using specific primers to target *mcyE* gene fragment of *Microcystis*.

Materials and Methods

Reference samples for method development

Twenty water samples collected from 15 Alberta lakes during the summer of 2011 were provided by Alberta Health Services' (AHS') Beach Monitoring Program. These 20 samples (including 17 microcystin positive and 3 negative samples), which were previously analysed for microcystin using PPI assay by the Alberta Centre for Toxicology (ACFT), were used for optimization of sample preparation and the rt-QPCR assay.

Powdered green algae (*Chlorella protothecoids*) were kindly provided by Dr. William McCaffrey (Department of Chemical and Materials Engineering, University of Alberta). The final concentration 100 μg/μl was prepared in water and assayed using rt-QPCR with *mcyE* gene specific primers.

Three strains of *Microcystis aeruginosa* cultured in modified BG-11 medium were purchased from the Canadian Phycological Culture Centre (CPCC, Waterloo, Canada). CPCC124 is a non-toxic strain isolated from Ontario lakes (Canada), while CPCC299 and CPCC300 are toxic strains isolated from Alberta lakes (Canada). The cell numbers of these strains were kindly provided by Dr. Rolf Vinebrooke (University of Alberta). These three strains of *Microcystis* were used in the method development for specificity assessment.

Lake-water samples for method validation

Lake-water samples were collected by AHS' Beach Monitoring Program from 45 lakes/beaches across Alberta during summer/fall (June-Oct) of 2012. Generally, water was collected with a depth integrated sampling tube from the surface to a depth of 20" from several sites along a beach area and combined to create a single composite sample. Some samples were simple grab samples collected by dipping a sampling container several inches below the surface. An aliquot of 50 ml was either frozen at -20°C and then shipped to the lab on dry-ice or sent to the lab fresh on ice within 48 hrs of collection.

Development of Real-time Quantitative PCR

Optimization of sample preparation for amplification

Two sample preparation methods were used for the 20 reference samples to optimize sample volume for DNA extraction as followed: 1) a 2-ml aliquot of water was centrifuged at 8,000 rpm for 6 min and the supernatant was collected. The pellet was dissolved in 200 μl TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0). The supernatant and pellet solution (200 μl/each) were used for DNA extraction; and 2) in order to simplify methods and reduce sample volume, 400 μl of water without any manipulation was directly used for DNA extraction. DNA yields from two different methods were quantified by rt-QPCR assay. After comparison of two methods, method 2 was adopted and applied in all remaining sample preparation for rt-QPCR. DNA was extracted using a Qiagen DNA mini kit according to the manufacturer's instruction (QIAGEN Inc., Ontario, Canada). DNA was eluted with 50 μl elution buffers and stored at -20°C until further processing for rt-QPCR.

Real-time QPCR for detection of *mcyE* gene and *16S rRNA*

rt-QPCR for *mcyE* gene: Primers and TaqMan probe targeting *mcyE* gene of *Microcystis spp* were previously described by Sipari [23]. The forward primer 5'-AAGCAAACTGCTCCCGGTATC-3' and the reverse primer 5'-CAATGGGAGCATAACGAGTCAA-3' were expected to yield a 120bp amplicon. TaqMan probe: 5'-CAATGGTTATCGAATTGACCCCGGAGAAAT-3' with a FAM 5' end label and a TAMARA 3' end label was used for real-time detection during the PCR reaction. Twenty μl of the PCR reaction mixture containing 5 μl extracted DNA, 0.5 μM of each primer, 0.125 μM probe, and 1 × LightCycler TaqMan Master Mix (Roche Diagnostics, Laval, Canada) was added to the capillaries (Roche Diagnostics). The capillaries were mounted onto the carousel, centrifuged and loaded into the Light Cycler 1.0 instrument (Roche, Canada). The thermal cycles were as follows: an initial 10 min at 95°C, followed by 45 cycles of 10 sec denaturing at 95°C, 20 sec annealing at 58°C, and 1 sec extension at 72°C. Data analysis was automatically performed using the Light Cycler software (version 4.0).

rt-QPCR for *16S rRNA*: In order to detect cyanobacteria in general, the primer was designed from conserved sequences of 16S rRNA as previously described by Lin S [24]. The forward primer 5'-CGGACGGGTGAGTAACGCGTG-3' and the reverse primer 5'-CCCATTGCGGAAAATTCCCC-3' were expected to yield a 258bp amplicon. SYBR® Green I System was utilized for 16S rRNA. The rt-QPCR reaction was performed in a total volume of 20 μl containing 1 x LightCycler FastStart SYBR Green I Master mix (Roche Diagnostics), 3 mM MgCl2, 0.5 μM of each primer and 5 μl extracted DNA. Amplification for cyanobacteria 16S rRNA consists of the denaturation for 10 min at 95°C; 40 cycles of 10 s at 95°C, 5 s at 58°C, 10 s at 72°C, followed by the melting profile. For data analysis, melting temperature (Tm), fluorescence-d [F1]/dT under melting curve window and cycle threshold (Ct), which is defined as the fractional cycle number where the fluorescence passed the fixed threshold in quantitation window, were selected as the evaluating parameters. Readout of the reaction with Tm of 85 - 87°C, fluorescence-d [F1]/dT above 1.5, and Ct value below 38.00 against a baseline of fluorescence signal at 2.0 was defined as positive.

Development of standard curve for quantitation

The standard curve was established as a correlation between the *mcyE* gene copy numbers and the Ct [25]. A 529 bp fragment was amplified using primers designed from the same *mcyE* gene region covering the full length of targeting *mcyE* sequence. Primer sequences for the *mcyE* fragment were forward: 5'-AACCCGAAATGACTCAAGAAAAA-3', reverse: 5'-TCAAAAATACCGATAGGATGTT-3'. The fragment DNA was purified from the PCR product using a QIA quick PCR purification kit (QIAGEN Inc.) and quantified using NanoDrop 2000 spectrophotometer (Thermo Scientific, Canada). The molecular weight

of fragment DNA was calculated. A series of 10-fold dilutions (2.0E+00 to 2.0E+09 copies) were analyzed by real-time PCR to identify the dynamic range and establish standard curve for quantitation of *mcyE* and 16S rRNA genes. The purified DNA was dispensed in aliquots containing 1.0E+03 or 1.0E+05 copies per µl as positive control and stored at -70°C until use.

Development of an internal control for monitoring PCR inhibition

Salmon testes DNA (Cat # D1626, Sigma, Canada) was dissolved in water at a concentration of 1 mg/ml with stirring at room temperature for 2-4 hours. A real time PCR for detection of salmon testes DNA was previously described [26]. An appropriate amount of salmon DNA identified in Ct = 30 using real time PCR was used for monitoring inhibition of *rt-QPCR*. Briefly, 5 µl salmon DNA (Ct = 30) was added into 400 µl water sample followed by DNA extraction and real-time PCR was performed for detection of salmon DNA. Inhibition was defined as a delay of Ct by 3 cycles as compared to a distilled water control spiked with the salmon DNA.

Evaluation of the sensitivity, specificity and precision of rt-QPCR

PCR efficiency was defined as 100% with the slope value at -3.3 for 10-fold serial dilution [27] and was evaluated in this assay by plotting the Ct values of a series of 10-fold dilution of *mcyE* fragment (529 bp) against the dilution factor. The specificity of real-time PCR was evaluated by three *Microcystis* strains purchased from CPCC and one green algae *Chlorella protothecoids*. The sensitivity of our assay was assessed by serial dilutions (2.0E+00 to 2.0E+09 copies) of the *mcyE* fragment. The precision of rt-QPCR was analyzed using Ct values generated from replicates of positive controls performed in different days. A correlation between the results from the rt-QPCR and PPI assay was analyzed to further validate the reliability of the rt-QPCR method against the well-established method of PPI.

Statistical analysis

Correlation between rt-QPCR and PPI assays for quantitation of microcystin was analyzed using the Pearson Product – Moment test. The precision of the rt-QPCR was expressed as coefficient of variation (CV) and 95% confidence interval.

Results

Evaluation of the rt-QPCR assay

Very good sensitivity of the *mcyE* gene quantitation was revealed in a linear log-range from 2.0E+00 to 2.0E+09 copies per reaction when selected primers/probes were used in the rt-QPCR reaction (Figure 1). The limit of detection was 50 copies/ml and the limit of quantitation range was 5.0E+02 to 5.0E+10 copies/ml for the assay. The rt-QPCR efficiency was observed as 1.994 adjusting from the standard curve. Excellent precision was observed. The coefficient variation of Ct value from 24 replicates of rt-QPCR was 1.12% for 1.0E+05 and 0.79% for 1.0E+03, respectively.

16S rRNA was detected in all three *Microcystis aeruginosa* strains but not in green algae *Chlorella protothecoids* (Table 1). No amplification of *mcyE* gene was observed in non-toxic *Microcystis* CPCC124 and *Chlorella protothecoids* using the rt-QPCR assay with *mcyE* primers and probe targeting to the *Microcystis,* while 4.1E+07 copies/ml of *mcyE* gene in CPCC299 and 9.78E+05 copies/ml of *mcyE* gene in CPCC300 were detected respectively (Table 1).

Optimization of sample preparation

Comparative results of quantitative *mcyE* gene copy numbers from 19 reference samples assayed parallel using rt-QPCR were observed between two sample preparation methods and showed in figure 2. Direct extraction from water samples without any pre-processing was adopted as the optimized method for monitoring *mcyE* gene of *Microcystis* in Alberta lakes using rt-QPCR assay.

Comparison of rt-QPCR and PPI for detection of toxin-producing *Microcystis*

Overall, rt-QPCR detected 13 out of 17 (76%) microcystin positive samples previously tested by PPI. There was 100% (4/4) agreement in samples containing high concentrations of microcystin (>1.0 µg/L), but only 69% (9/13) in samples with lower toxin levels (< 1.0 µg/L) as assayed by PPI (Table 2). Of 3 samples determined to contain no microcystin by PPI, 2 of these were positive by rt-QPCR (Table 2). There was a correlation between microcystin concentrations by PPI and *mcyE* numbers by *rt-QPCR* from the direct extraction (r^2=0.27, p < 0.05) in 19 reference samples (one sample was excluded due to PCR inhibition).

Detection of *Microcystis mcyE* gene in lake-water samples

A total of 527 water samples were tested for *mcyE* gene using our optimized direct sample preparation method and validated specific *mcyE* primers and probes. Of these, 369 samples (70%) were *mcyE* gene positive and 158 samples were *mcyE* gene negative in which no PCR inhibition was detected using internal control salmon DNA. Among the 45 lakes sampled, *mcyE* gene was not detected in 4 lakes (9%), occasionally detected at a very low level (*mcyE* gene < 500 copies/ml) in 9 lakes (20%), and frequently detected at moderate to high level (500 - 3.4E+06 copies/ml) in 32 lakes (71%) during the sampling period. These results were consistent with previous AESRD report showing that up to 75% of Alberta lakes contained microcystin at least in one occasion during the whole open water (May-Oct) season [21]. In 2012, blue-green algae health risk advisories were issued by AHS for 15 Alberta lakes based on visual inspection of cyanobacterial blooms along public recreation shorelines. Our rt-QPCR results confirmed

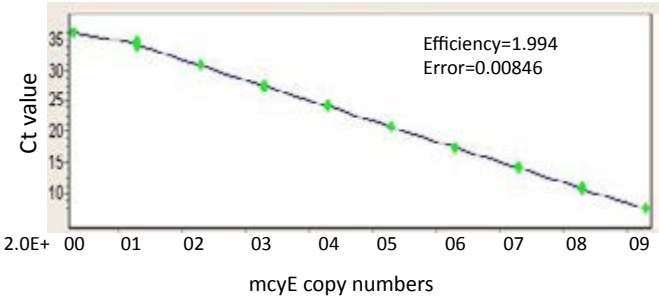

Figure 1: Ct value obtained from real-time PCR for serial dilutions of *mcyE* DNA fragment.

	Microcystis aeruginosa strains			Algae
	CPCC124	CPCC299	CPCC300	Chlorella Protothecoids
Cell count/ml	2.97E+07	2.14E+08	4.2E+07	N/A
mcyE gene copy/ml	negative	4.10E+07	9.78E+05	negative
16S rRNA copy/ml	1.75E+06	7.5E+06	5.78E+05	negative

Table 1: rt-QPCR of three *Microcystis aeruginosa* strains and one algae strain for *mcyE* and 16S rRNA.

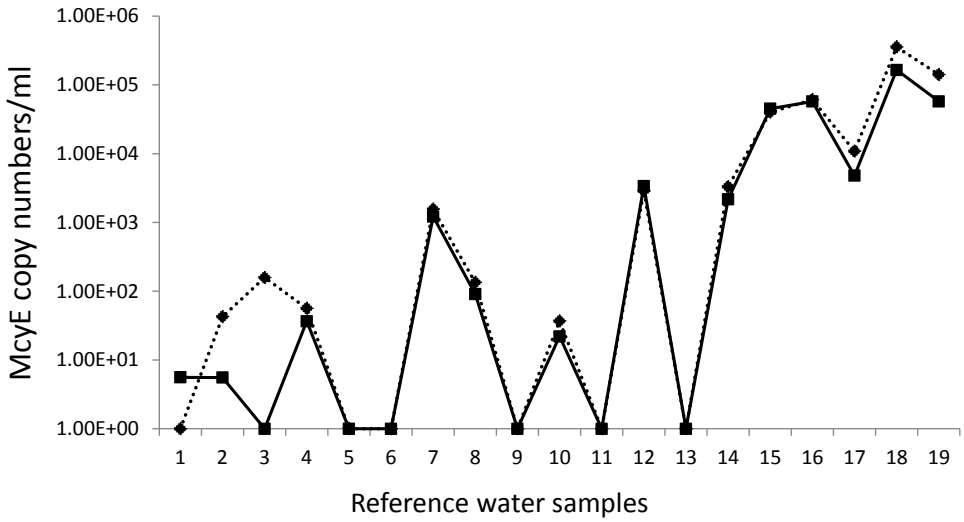

Figure 2: *mcyE* copy numbers from 19 reference water samples of 2011. *Microcystis mcyE* copy numbers were determined by rt-QPCR, which was showed as *mcyE* copy numbers per millilitre. The sum of *mcyE* copy numbers (■) from centrifuge supernatant and centrifuge pellet were compared with *mcyE* copy numbers from direct extraction (♦).

	Microcystin concentration equivalent by PPI assay			
rt-QPCR for *mcyE*	Negative (<0.05 µg/L)	0.05 µg/L ≤ Microcystin < 1 µg/L	Microcystin ≥ 1 µg/L	Total
Negative	1	4	0	5
Positive	2	9	4	15
Total	3	13	4	20

Table 2: rt-QPCR and PPI results of 20 reference samples.

that 10 out of the 15 lakes in the list of advisories had high *mcyE* copy numbers which was greater than 1.0E+05 copies/ml.

Quantitation of toxin-producing *Microcystis* in individual lakes

Two lakes (Pine and Pigeon Lakes) with health risk advisories issued by AHS in 2012 were used to assess the spatial variation of *Microcystis mcyE* gene along different beach areas within the same lake. The *mcyE* gene copy numbers at the Pine Lake's Leisure Camp beach and Green Acre beach (A) and Pigeon Lake's Mission beach and Zeiner Park beach (B) are shown in Figure 3. During the open-water season, the highest *mcyE* copy numbers were 4.0E+05/ml, 2.1E+05/ml, 2.6E+05/ml and 3.0E+04/ml detected in Leisure Camp beach, Green Acre beach, Mission beach and Zeiner Park beach, respectively. The levels of *Microcystis* at both Pine Lake beaches were similar during the season, with peaks in early Aug and mid-Sep. However, observed *Microcystis* levels at the two Pigeon Lake beaches differed during the season, with peaks occurring in late July, early Aug and early Sep at Mission beach compared to early Aug and mid-Sep at Zeiner Park beach.

Cyanobacteria levels in lake-water samples

Total cyanobacteria was determined for all the lake-water samples using rt-QPCR with cyanobacterial specific 16S rRNA primers [24]. All 527 samples detected positive for cyanobacterial 16S rRNA with the highest level at 3.2E+06 copies/ml, suggesting that cyanobacteria were present in all lakes during the sampling period. Moreover, high 16S rRNA copy numbers (>1.0E+05) were detected in samples from all 15 lakes issued with cyanobacteria health risk advisories. Figure 4 shows relatively high levels of cyanobacteria were observed in both Pine (A) and Pigeon Lakes (B) from June to October 2012. The highest 16S rRNA copy numbers were 1.2E+06/ml, 1.5E+06/ml, 4.4E+05/ml and 9.8E+05/ml for Leisure Camp beach, Green Acre beach, Mission beach and Zeiner Park beach, respectively (Figure 4A and 4B). The proportion of microcystin-producing *Microcystis* relative to the total cyanobacteria in these two lakes ranged broadly from 0 to 62%. More abundant cyanobacteria were observed in Pine than Pigeon Lake and both beaches in each lake had similar trend in cyanobacteria levels during sampling period.

Discussion

PCR based methods for detection of *Microcystis* in environmental water samples were first described using mcy gene clusters [28,29]. A positive correlation between *Microcystis mcyE* gene copy numbers and microcystin concentrations was reported using the samples from two Finnish lakes, indicating that *mcyE* gene could be used as an alternative biomarker for toxin-producing *Microcystis* [17]. In this study, the rt-QPCR method targeting *mcyE* gene has been further evaluated and validated in comparison with classic method of PPI assay.

To measure the prevailing levels of *Microcystis* and its fluctuation during the open-water season, it is important to know the proportion of *Microcystis* relative to total cyanobacteria in individual lakes. Detection and quantitation of 16S rRNA gene was developed and added to the rt-QPCR assay for estimation of total cyanobacterial population. Using the relative level of *mcyE* gene versus 16S rRNA, a trend of toxin-producing *Microcystis* population in the cyanobacterial bloom in individual lakes can be estimated, allowing for further assessment of risks caused by *Microcystis*.

The rt-QPCR assay developed and validated in this study is capable of detecting and quantifying microcystin-producing *Microcystis*

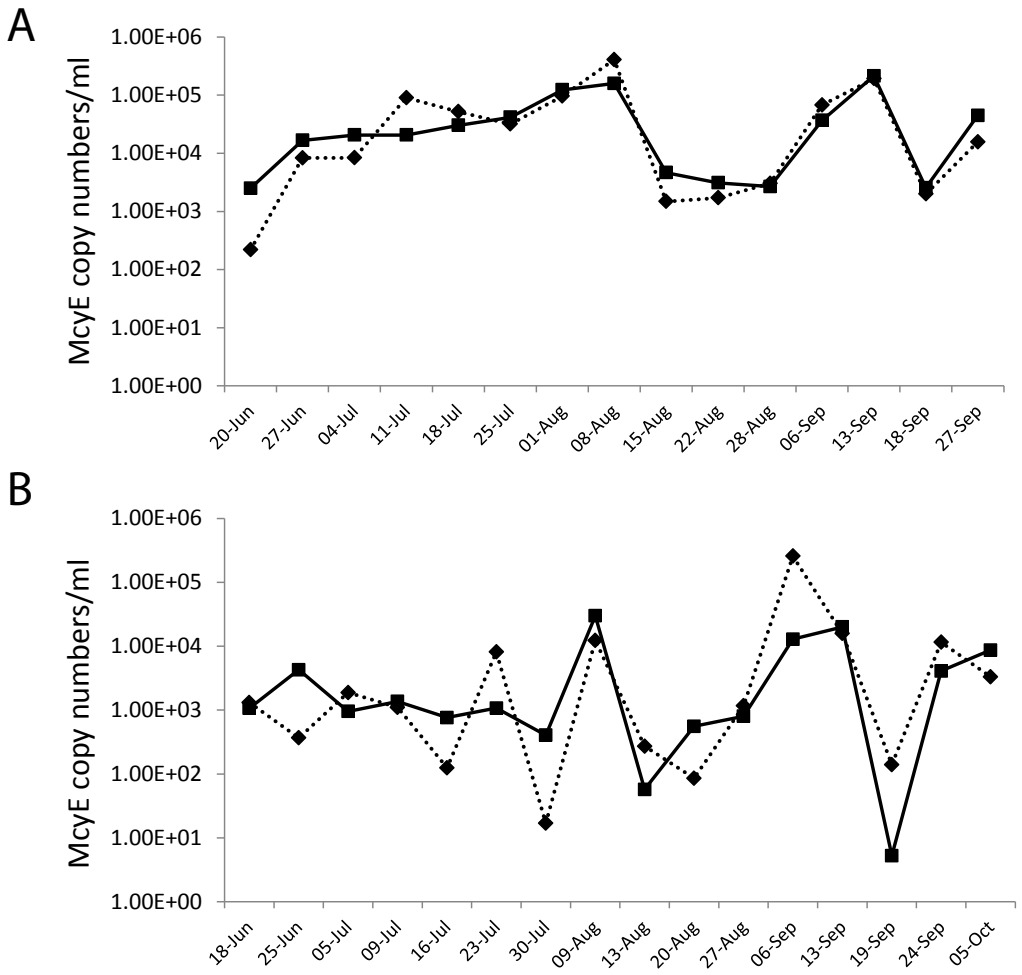

Figure 3: *McyE* copy numbers of Pine Lake (A) and Pigeon Lake (B) during the sampling period. *Microcystis mcyE* copy numbers were determined by rt-QPCR, which was showed as *mcyE* copy numbers per millilitre. Pine lake: Leisure Camp beach (♦) and Green Acre beach (■); Pigeon Lake: Mission beach (♦) and Zeiner Park beach (■).

and total cyanobacteria with high sensitivity and specificity. The discordance observed between the rt-QPCR and PPI results from the 4 reference samples with low microcystin concentration (<1 µg/L) (Table 2) could potentially be attribute to enzyme inhibition with other protein phosphatase inhibitors such as okadaic acid and tautomycin in the PPI assay [14]. A more plausible explanation however, is that microcystin detected by PPI assay was produced by microcystin-producing cyanobacteria species other than *Microcystis* including *Planktothrix and Anabaena*. Further development of the rt-QPCR assay including other potential toxin-producing cyanobacteria will make it a useful and cost-effective method for measurement and surveillance of spatiotemporal changes in the population of toxin-producing cyanobacteria in individual lake.

A relatively large volume of environmental water samples was required by harvesting the cells via filtration or centrifugation in order to increase detection rate of toxin-producing cyanobacteria [1,17,24]. However, the filtration and centrifugation could eliminate or reduce free nucleic acids released from natural degradation of cyanobacteria, resulting in low yields of total DNA. It may also alter the ratio between free DNA and intracellular DNA existing in natural environment. Kumar et al. [30] reported that the presence of free DNA in pond water was detected and *mcyE* gene from fextracellular DNA was amplified successfully by PCR. They suggested that amplification of *mcyE* gene directly from the extracellular DNA present in the water was feasible for monitoring the microcystin-producing *Microcystis*. Based on these findings, the sample preparation for best yield and quality of DNA was optimized during our method development and validation in this study. A better correlation between microcystin concentrations and *mcyE* gene copy numbers was found in the lake-water sample without manipulation (correlation coefficient: 0.52) than that from the supernatant and pellet after centrifugation of the sample (correlation coefficient: 0.45), suggesting that DNA extracted directly from the lake-water was suitable for downstream amplification using rt-QPCR. A possible explanation for this outcome is that a mixed ratio between intracellular genomic DNA and extracellular free DNA may accurately reflect a natural metabolic and synthetic status of *Microcystis* in lake-water samples. In addition, the volume of samples required for our rt-QPCR assay was only 400 µl, which was much less than that in other studies (50 ml to 1L of water for processing) [1,17,31]. Taken together, optimized DNA extraction directly from lake-water for the rt-QPCR assay will shorten the time of sample preparation, increase efficiency of the assay, and reduce the cost related. It allows a practical application of this method for rapid screening of large quantity of samples during open-water season.

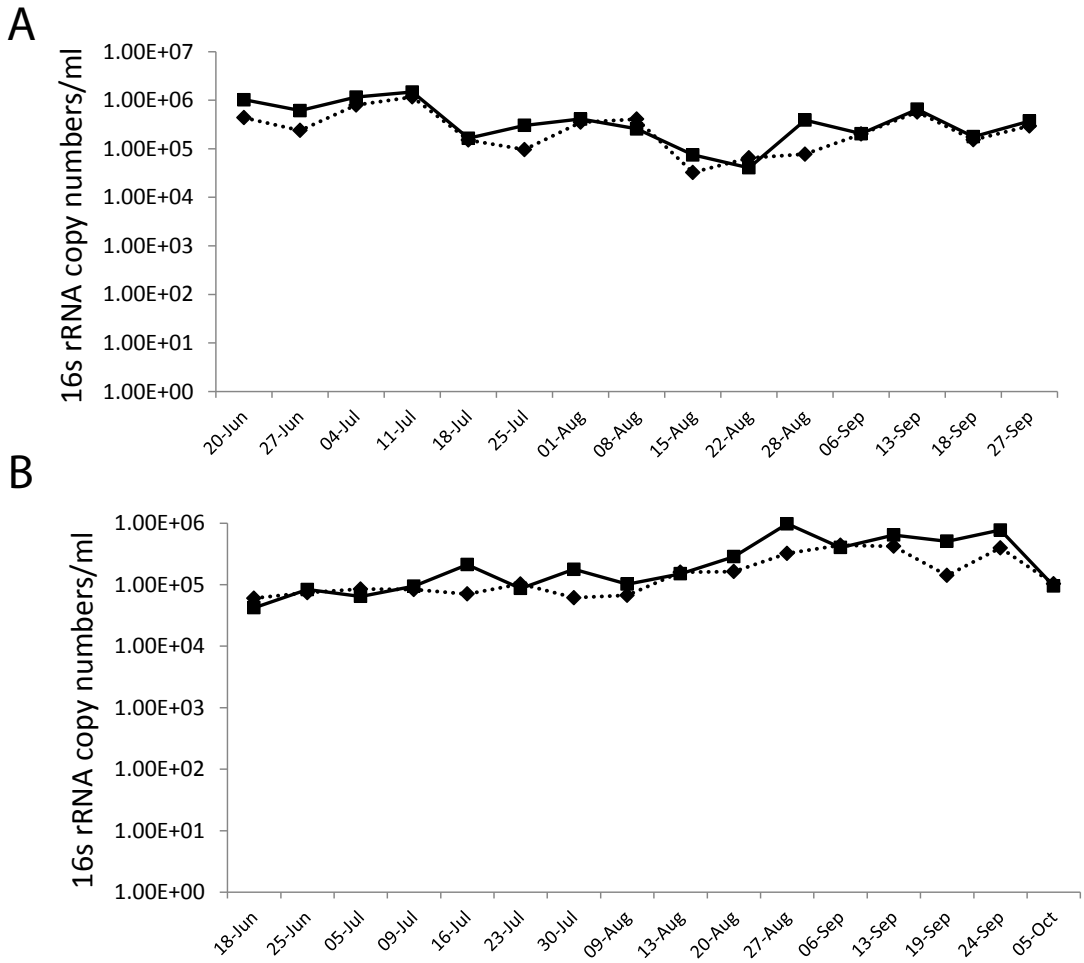

Figure 4: 16S rRNA copy numbers of Pine Lake (A) and Pigeon Lake (B) during the sampling period. Cyanobacterial 16s rRNA copy numbers were determined by rt-QPCR, which was showed as 16S rRNA copy numbers per millilitre. Pine Lake: Leisure Camp beach (♦) and Green Acre beach (■); Pigeon Lake: Mission beach (♦) and Zeiner Park beach (■).

One of the challenges of amplification-based PCR method for environmental water samples is the presence of inhibitors, which may cause false negative results. The presence and level of inhibitory effect on PCR reaction are largely unpredictable. To overcome this difficulty and better control the quality of PCR assay, an internal control has to be integrated into the rt-QPCR assay. Most of studies on cyanobacteria used 16S rRNA as an internal control to monitor PCR inhibition [1,17,25,32]. However, the wide range of variations of 16S rRNA level derived from total cyanobacteria made it difficult to assess the end-point of PCR results, suggesting that 16S rRNA may not be an appropriate quality control (QC) for detection of inhibitory effect in lake-water samples. Haugland et al. [26] used salmon testes DNA to evaluate PCR inhibition in the QPCR reaction for *Enterococcus* measurements. The similar approach was adapted in our rt-QPCR method in order to evaluate inhibitory substances in water samples. Among the 200 samples tested, one reference sample with high microcystin concentration (> 500 μg/L) was detected with complete PCR inhibition and no inhibition was detected in other samples, indicating that salmon DNA as an internal control for PCR inhibition is sensitive and reliable.

In this study, the population of microcystin-producing *Microcystis* alone was examined in selected Alberta lakes for the purpose of method development and validation. Our results indicated that this major microcystin-producing genus was present in 71% of the lakes with a relatively high level during open-water season, which was in accordance with previous report showing that 25% to 92% of the cyanobacterial blooms were hepatotoxic [1]. In addition, among the 15 Alberta lakes that issued with health advisory due to heavy cyanobacterial blooms in 2012, 10 lakes (67%) were detected with high level of microcystin-producing *Microcystis* by our rt-QPCR method, suggesting that *Microcystis* is the main microcystin producer in majority of Alberta lakes. Although *Microcystis* is the most important and worldwide distributing microcystin-producing species, *Planktothrix* and *Anabaena* are also major microcystin-producing cyanobacterial genera. Previous phylogenetic study showed that *mcyE* gene sequences from different cyanobacteria have their own clusters and maintain exclusion from horizontal gene transfer [33]. Therefore, different microcystin-producing genera can be covered and identified by targeting different regions of *mcyE* gene using the PCR approach [1,23,34,35].

In conclusion, the rt-QPCR assay with integrated internal quality control and optimized sample preparation for lake water were developed and validated in current study. With selected specific primers/probes targeting *mcyE* gene and 16S rRNA gene, the rt-QPCR assay was

applied to detect and quantify microcystin-producing *Microcystis* population and its proportion to total cyanobacteria in 45 Alberta lakes during open-water season in 2012. Newly developed rt-QPCR assay was proven to be a simple, rapid and cost-saving assay with high sensitivity and specificity, as well as integrated internal control. It can be used in surveillance program for monitoring microcystin-producing *Microcystis* during summer season. Further comparative data analyses on the correlation between *mcyE* copy numbers by rt-QPCR assay and microcystin concentrations measured by other assays (e.g. PPI and LC/MS) under Alberta health agent will be carried out in near future.

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