

Using quantitative real-time polymerase chain reaction (qRT-PCR) for detection microcystin producing cyanobacteria

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ABSTRACT

Introduction: Cyanobacterial blooms (CBs) have become a growing concern worldwide. In the natural environment, potentially toxic (can produce toxins) and non-toxic (can not produce toxins) colonies often co-exist within a bloom. **Methods:** The present study aimed to quantify toxic and non-toxic cells of cyanobacteria in the Tri An Reservoir (TAR) using a quantitative real-time polymerase chain reaction (qRT-PCR). **Results:** Results showed that the *Microcystis* genus dominated the cyanobacterial communities in the TAR. *Microcystis* was also the primary microcystins (MC) producing cyanobacteria in the water. Total cyanobacteria and *Microcystis* cells ranged from 152×10^3 to 27×10^6 copy/L and from 105×10^3 to 19×10^6 copy/L, respectively. The cell number of potentially MC-producing cyanobacteria (corresponding to the *Microcystis mcyD* gene) varied from 27×10^3 to 13×10^6 copy/L. MC concentrations often present in raw water with a concentration up to $4.8 \mu\text{g/L}$. Our results showed that the MC concentration in raw water was positively correlated with the *mcyD* copy number, suggesting that *Microcystis* spp. are the main toxin producers in the TAR's surface water. **Conclusion:** Our study suggested that qRT-PCR techniques and traditional count are comparable and could be used to quantify cyanobacteria. In addition, the qRT-PCR techniques can determine the toxic cyanobacterial cells and could be used as a tool for early monitoring of toxic cyanobacteria in lakes and reservoirs.

Key words: cyanotoxins, microcystins, toxic and non-toxic cyanobacteria, Tri An Reservoir

INTRODUCTION

Toxic cyanobacterial blooms (TCBs) in inland lakes and reservoirs have become a worldwide problem¹. These blooms have resulted in economic loss due to degradation of water quality and increase health risk². Furthermore, TCBs are responsible for several toxic secondary metabolites, namely cyanotoxins, including cyclic peptides, alkaloids, and lipopolysaccharides based on chemical structure. Microcystins (MC), a group of cyclic heptapeptide hepatotoxins, are the most frequently occurring even in eutrophic freshwaters³. The thiotemplate mechanism characteristic synthesizes MC for non-ribosomal peptide synthesis (NRPS), polyketide synthesis (PKS), and fatty acid synthesis in the MC biosynthesis gene cluster (*mcy*), which spans 55 kb and composes of 10 genes structured in two putative operons (*mcyA-C* and *mcyD-J*)⁴. Producing by the toxic cells of different cyanobacteria, including *Microcystis*, *Dolichospermum*, and *Planktothrix*, MC is the largest diverse group of cyanobacterial toxins more than 100 variants reported⁵. TCBs with the dominant of *Microcystis* and MC have been reported worldwide⁶. Microcystins are intracellular toxin and can be released when blooms collapse or as cells die, result-

ing in the contamination of MC in water, sediments and in different aquatic organisms^{1,3}. Because a natural population of cyanobacteria community or a cyanobacterial bloom often consists of two genotypes toxic (can produce toxins) and non-toxic (can not produce toxins) strains, the differentiation of the two genotypes is difficult due to the similar appearance under microscope^{7,8}. Therefore, the discovery of the *mcy* gene cluster has given a potential way for PCR-based detection of MC producers. However, although the conventional PCR could detect the presence or absence of the *mcy* genes in cyanobacteria, it could not quantify the potentially toxic and non-toxic cell number. Therefore, the quantitative real-time PCR (qRT-PCR) techniques based on the detection of the *mcy* genes that only exist in the potentially toxic cells have recently been used for quantification the cell number of toxic and non-toxic within a sample^{9,10}.

Located in Southern Vietnam, the Tri An Reservoir (TAR) is one of Vietnam's most important water sources. It provides drinking water for more than 10 million people from Ho Chi Minh City, Dong Nai, and Binh Duong provinces¹¹. The occurrence of toxic cyanobacterial bloom in the TAR has been

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reported during the most ten years. In some case, MC have been found with concentration exceeding the World Health Organization (WHO) provisional guideline concentration of 1.0 $\mu\text{g/L}$ ¹²⁻¹⁴. However, previous studies have focused only on the taxonomic identification of cyanobacterial population or detection of MC concentration in the water. No study contributed to the quantification of the toxic cells within a population. To better understand the variation of cyanobacterial blooms and the toxin production, easy-to-use detection methods for different toxin-producing cyanobacteria are needed. Thus, this study aimed to apply qRT-PCR techniques to quantify toxic and non-toxic *Microcystis* colonies in the water.

MATERIALS AND METHODS

Samples collection

Surface water samples (2L) were collected monthly in a 2L plastic bottle from the TAR in 2017 at one station (Figure 1) and transported to the laboratory with ice. Sub-samples were fixed with Lugol's iodine solution for cell count using a Sedgewick Rafter counting chamber. Cyanobacterial cells in raw water were concentrated by filtering 100 mL through GF/C filters (Whatman, Kent, England). Samples on the filter were divided for DNA extraction and MC measurement. For DNA extraction, the filters were kept at -20°C before further process. For MC analysis, the filters were dried overnight at 45°C and held at -20°C before analysis.

Microcystins extraction and measurement

MC's content in filters samples was extracted with 100% methanol and measured using a high-performance liquid chromatography (HPLC) (Dionex UltiMate 3000, Thermo Scientific, Waltham, MA, USA). The HPLC system is equipped with a reverse-phase C18 column (Acclaim^M 120 C18 5 μm , 4.6×150 mm, Waltham, MA, USA), an autosampler, and a UV-VIS detector. A buffer including methanol and 0.05 M phosphate solution (pH 2.5; 1:1 v/v) at a flow rate of 0.65 mL/min was used as mobile phase. The systems were maintained at 40°C during analysis. Three MC congeners, including (MC-RR, MC-LR, and MC-YR) were distinguished by UV at 238 nm and identified based on retention time and UV spectra. Three MC variants, including MC-LR, MC-RR, and MC-YR from Enzo Lifesciences (Farmingdale, NY, USA) were used as standards. The HPLC system had a detection limit of 0.1 $\mu\text{g/L}$.

DNA extraction and qRT-PCR standard preparation

The frozen GF/C filters were used for DNA extraction. First, cyanobacterial DNA was extracted using the GeneAll^R ExgeneTM Cell SV kit (GeneAll, Seoul, Korea), following the manufacturer's instructions. After extraction, the DNA was purified with the purification kit (Omega Biotek, GA, USA) and quantified with a spectrometer (Eppendorf D30, Hamburg, Germany) to obtain the DNA concentration. The final DNA yields were preserved in a 20 μL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and kept at -20°C before further analysis.

To prepare qRT-PCR standards, the DNA extracted from the two strains, including a toxic *Microcystis aeruginosa* NIES-102 and a non-toxic *M. aeruginosa* NIES-101 (NIES, Tsukuba, Japan) with a serial dilution from 1.0×10^2 to 1.0×10^7 were used. In our study, we aim to quantify three genes, including the cyanobacterial 16S rRNA (responsible for total cyanobacteria cells), the *Microcystis* 16S rRNA (responsible for total *Microcystis* cells), and *Microcystis mcyD* (responsible for a total of *Microcystis* cells with the ability to produce MC). These genes were amplified by using the primers listed in Table 1. The PCR products were collected and purified with the DNA purification kit (Omega Biotek, GA, USA). DNA was then quantified with a spectrophotometer. The copy number of each gene was calculated based on the Avogadro's number ($1 \text{ mol} = 6.02214 \times 10^{23}$ molecules)^{8,15}.

Quantitative real-time polymerase reaction

Total cyanobacteria cells, total *Microcystis* spp. and total toxic *Microcystis* spp. were quantified by using the cyanobacterial 16S rRNA, *Microcystis* 16S rRNA, and *Microcystis mcyD*, respectively. qRT-PCR assay was performed according to the process described by Nübel et al. (1997)¹⁶ and Baxa et al. (2010)¹⁵. Accordingly, all qRT-PCR reactions were run in triplicate with a total of 20 μL that contained 10 μL of SYBR green master mix (Toyobo, Japan), 0.2 μL (10 pmol/ μL) of each forward and reverse primers, 1 μL of DNA template, and MQ water. The reaction was run on a Real-time PCR PikoReal system (Thermo Scientific, MA, USA). The thermal profile of the qRT-PCR reaction was followed from Nübel et al. (1997)¹⁶ and Baxa et al. (2010)¹⁵. For cyanobacterial 16S rRNA the thermal protocol of qRT-PCR was performed as follows: 95°C for 5 min, 45 cycles at 95°C for 15 s, 60°C for 15 s and 72°C for 30 s. The thermal protocol

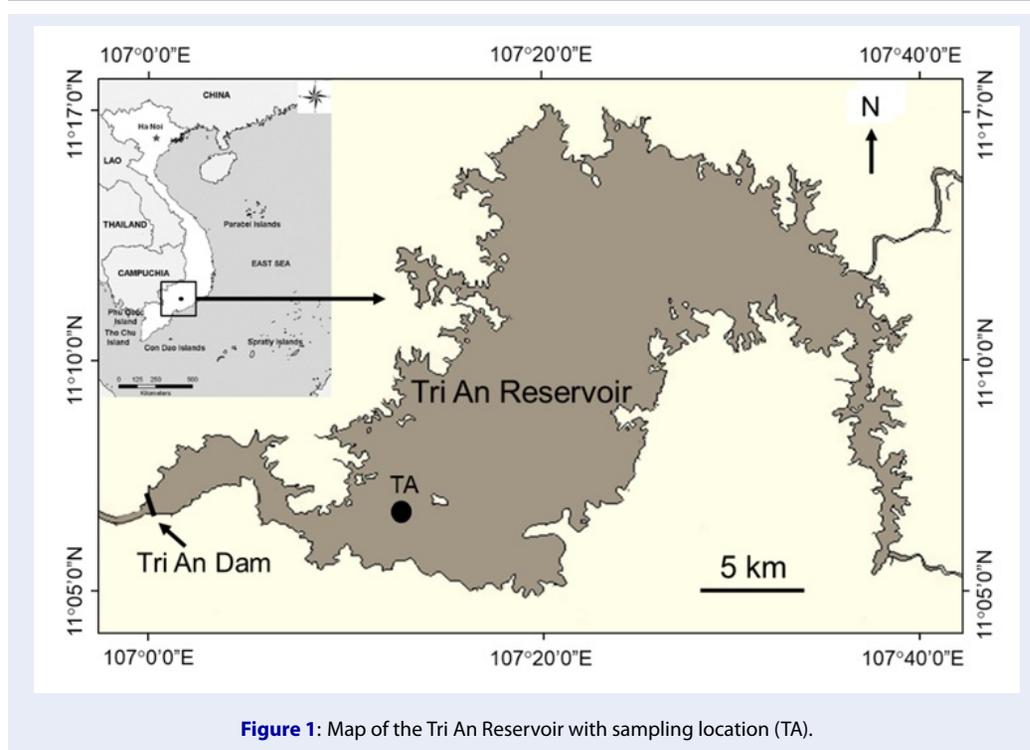


Figure 1: Map of the Tri An Reservoir with sampling location (TA).

Table 1: Primers used for qPCR

Target	Primer	Sequence (5'-3')	Size (bp)	Reference
Cyanobacterial 16S rRNA	Cya 359F	GGGGAATYTTCCGCAATGGG	446	Nübel et al. (1997) ¹⁶
	Cya 781R	GACTACWGGGGTATCTAATCCCWTT		
<i>Microcystis</i> 16S rRNA	Micr 184F	GCCGCRAGGTGAAAMCTAA	220	Baxa et al. (2010) ¹⁵
	Micr 431R	AATCCAAARACCTTCTCTCC		
<i>Microcystis</i> mcyD	mcyD F2 F	GGTTCGCCTGGTCAAAGTAA	298	Baxa et al. (2010) ¹⁵
	mcyD R2 R	CCTCGCTAAAGAAGGGTTGA		

for *Microcystis* 16S rRNA and *Microcystis mcyD* was run as follow: initial at 50 °C for 3 min, then 95 °C for 10 min, followed by 45 cycles at 95 °C for 30 s, 61 °C for 1 min, and finally at 72 °C for 20 s.

RESULTS

Cyanobacterial cell count and microcystins in water

The monthly cyanobacteria cells, total *Microcystis* cells, and MC concentration in surface water were shown in Figure 2. Total cyanobacteria cell count ranged from 144×10^3 to 26×10^6 cell/L, with a peak

in July, in which the total *Microcystis* cells ranged from 100×10^3 to 23.6×10^6 cell/L (accounting for 68–91%). MC was detected from March to November, with the concentration ranged from under detection limit (UDL) to 4.8 µg/L, with a peak in June. The maximum concentrations of cyanobacteria, *Microcystis*, and MC concentrations were measured in June and July as the occurrence of heavy blooms of *M. aeruginosa* on surface water (Figure 3).

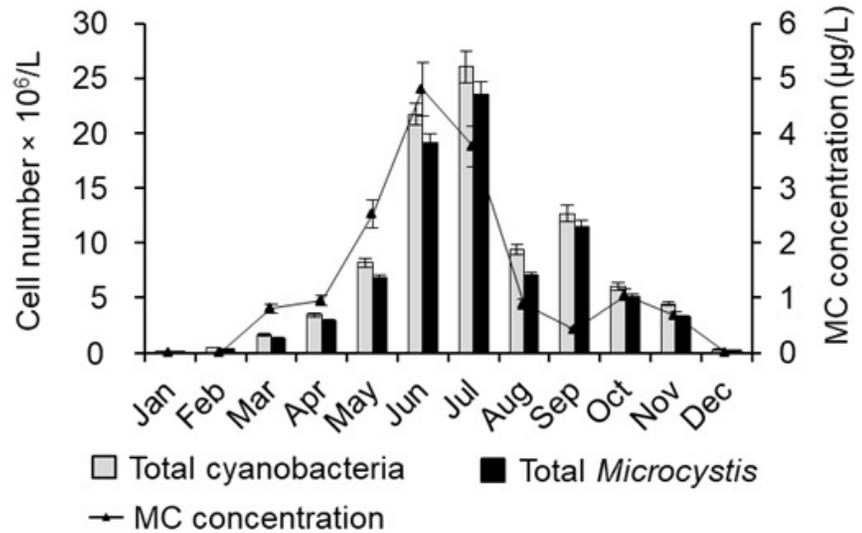


Figure 2: Cell number of cyanobacteria, *Microcystis* and MC concentration

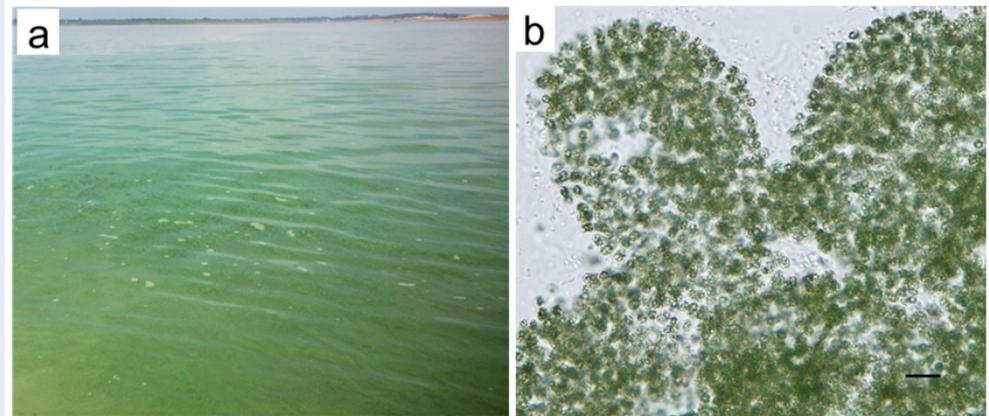


Figure 3: Surface bloom of cyanobacteria (a) with the dominant *Microcystis aeruginosa* (b) in the Tri An Reservoir. Scale bar: 10 µm.

Quantitative analysis of cyanobacteria by qPCR

We first run the standard curves using the DNA extracted from the toxic *M. aeruginosa* NIES-102, then applied for the samples collected from the TAR. Our results showed that all three genes (cyanobacterial 16S rRNA gene, *Microcystis* 16S rRNA gene, and the *Microcystis mcyD* gene) generated good efficiencies and high R-square value. In addition, significant linear curves between the concentration of DNA and the threshold cycle values (Ct) were obtained for all genes (Figure 4 and Table 2). Table 2 showed the efficiencies

and other parameters obtained for different standard curves. The efficiencies of the qRT-PCR assays ranged from 0.997 to 1.050, demonstrating the high reliability of the qRT-PCR amplification.

The mean copy number of cyanobacterial 16S rRNA, *Microcystis* 16S rRNA, and *Microcystis mcyD* as determined by qRT-PCR were shown in Figure 5. Our results indicated that the copy number of the cyanobacterial 16S rRNA and *Microcystis* 16S rRNA showed almost the same trend and ranged from 152×10^3 to 27×10^6 copy/L and from 105×10^3 to 19×10^6 copy/L, respectively. They gradually increased from Mar and

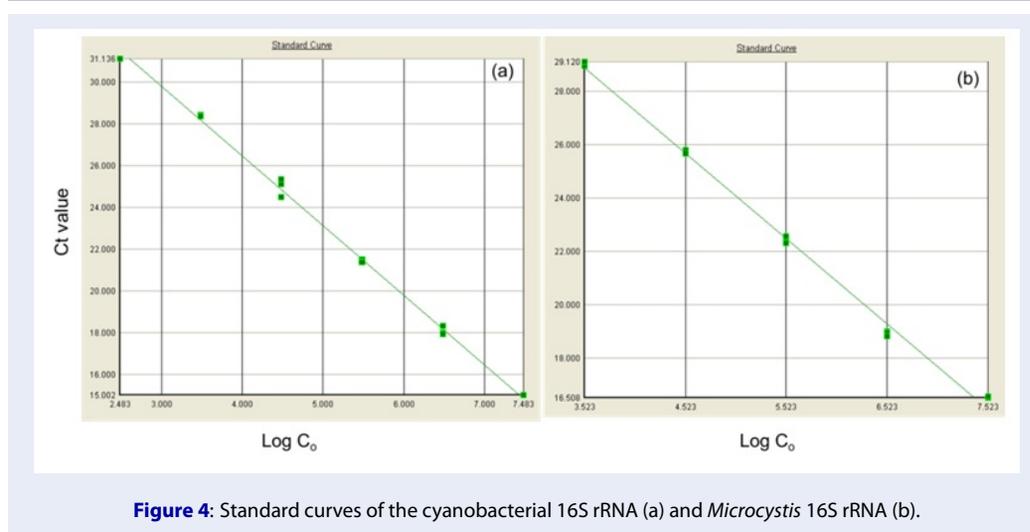


Table 2: The efficiencies and other standard curve parameters were obtained by real-time qPCR analysis for (a) the cyanobacterial 16S rRNA and (b) the *Microcystis* 16S rRNA specific primer sets. The amplification efficiency (e) was calculated by $e = 10^{-1/S} - 1$, where S is the slope.

Target gene	Standard	Efficiency	Slope	Y-intercept	R2
Cyanobacterial 16S rRNA	<i>M. aeruginosa</i> NIES-102 DNA	1.007	-3.305	44.328	0.995
<i>Microcystis</i> 16S rRNA	<i>M. aeruginosa</i> NIES-102 DNA	1.050	-3.208	40.167	0.996
<i>Microcystis mcyD</i>	<i>M. aeruginosa</i> NIES-102 DNA	0.997	-3.33	40.683	0.993

often got peaks during Jun or Jul and remained at high values to October before declining during December to April. The *Microcystis* 16S rRNA gene was dominant, accounting for 60.3–91.7% of the total cyanobacteria abundance. The number of the *Microcystis mcyD* gene corresponding to the number of cells with the ability to produce MC) changed similarly with the cyanobacterial 16S rRNA and *Microcystis* 16S rRNA genes. It got the maximum value in June (13×10^6 copy/L) and the minimum value in January (27×10^3 copy/L). The proportion of potentially MC-producing cyanobacteria varied from 16.0% to 95.7%, with the highest value recorded in October and the lowest value recorded in March. Our results confirmed this method could be applied to detect cyanobacteria in environmental samples with a wide range of cyanobacterial abundance (from 1.0×10^2 to 1.0×10^7 cells/L).

The ratio of Cyanobacteria 16S rRNA/total cyanobacteria and *Microcystis* 16S rRNA/total *Microcystis* as determined by qRT-PCR and traditional count were shown in Table 3. Both the ratio range from 0.81–1.18, and in most cases, these numbers higher than 1, suggesting that the number of cells is determined by

qRT-PCR is a little higher than the traditional count. However, both methods are comparable and could be used as optional tools for cyanobacteria quantification. The qRT-PCR methods could be quantified the toxic cell number of cyanobacteria while the other could not.

DISCUSSION

In natural environments, toxic and non-toxic genotypes are often found within a bloom¹⁷. Globally, 20–75% of cyanobacterial bloom cases reported being toxic³. In most cases the *Microcystis* spp. blooms have been reported the bloom-forming and toxin producer⁶. The bloom of *Microcystis* spp. with toxin production has been reported in many lakes and reservoirs, including Lake Thanh Cong, Hoan Kiem Lake, Huong River, and Dau Tieng Reservoir, Tri An Reservoir, Tuyen Lam Reservoir. However, earlier reports have not yet employed molecular techniques^{12,14,18}. Especially, the qRT-PCR technique has not been used to quantify the toxic and non-toxic cyanobacteria from Vietnam's water. The traditional count is the common methods used to quantify the total cyanobacteria number but did not quantify

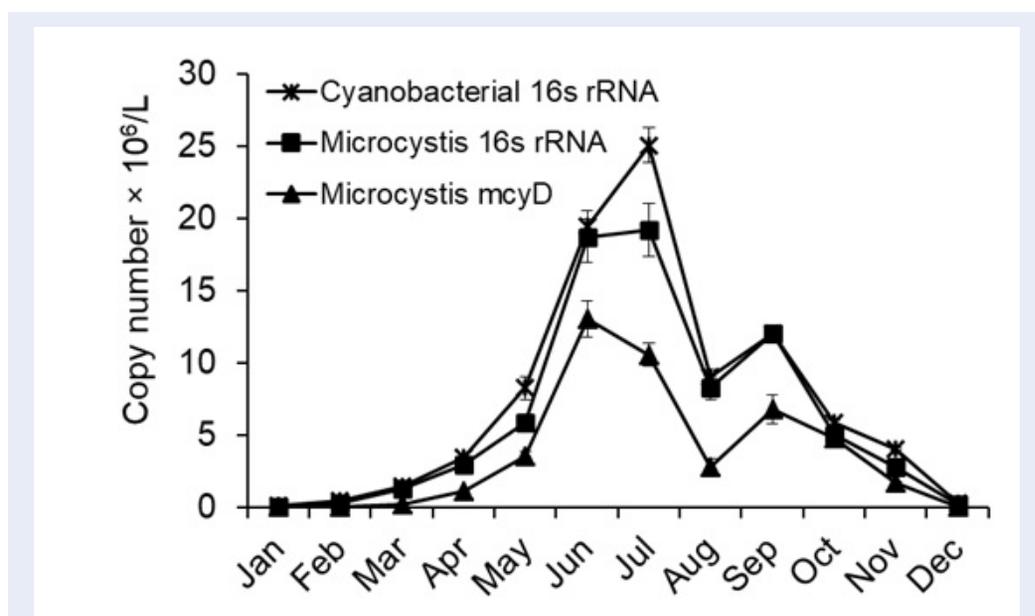


Figure 5: Mean copy number of cyanobacterial 16S rRNA, *Microcystis* 16S rRNA, and *Microcystis mcyD* as determined by qRT-PCR.

Table 3: The ratio of Cyanobacteria 16S rRNA/Total cyanobacteria and *Microcystis* 16S rRNA/Total *Microcystis* as determined by qRT-PCR and traditional count

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Cyano 16S rRNA/Total cyanobacteria	1.06	1.04	0.91	1.00	1.01	1.04	1.04	1.04	1.14	1.16	1.05	0.94
Micro 16s rRNA/Total <i>Microcystis</i>	1.03	1.00	1.05	1.04	0.86	0.98	0.81	1.18	1.04	0.97	0.86	1.08

the number of the toxic cell of cyanobacteria. Thus, in the present study, we have successfully applied a qRT-PCR technique to quantify different cyanobacterial groups based on molecular approaches. The qRT-PCR could be used for the determination of total cyanobacteria or different toxic genotypes with a population. When applied qRT-PCR for monitoring the dynamics of cyanobacteria and MC production in a tropical reservoir of Singapore, Te and Gin (2011)¹⁹ reported that *Microcystis* and *Anabaena* were present (mean concentrations 4.16×10^6 gene copies/mL and 4.47×10^4 gene copies/mL, respectively) and were well correlated to each other ($P < 0.001$), and that *Microcystis* was the primary microcystin producer. The average percentage of toxigenic *Microcystis* spp. was 55.92%, whereas no *Anabaena*-specific microcystin-producing gene was detected. Our results are consistent with previous observations that the proportion of potentially toxic *Microcystis* genotypes in different water locations can vary widely. In some cases,

the potentially toxic *Microcystis* genotypes reached 100%¹⁵. We suggested to use these techniques for future monitoring of cyanobacteria as well as cyanotoxins in Vietnam waters.

Many studies have documented the contamination of MC in Vietnam's surface waters^{4,7,12,18}. However, the variation of toxin producers has not been investigated to the same extent. In this study, MC was detected in almost all tested samples, including those of raw water and water blooms. In comparison, we determined the variation of toxic *Microcystis* via the *mcyD* copy number. Our results showed that the MC concentration in raw water was positive correlated with the *mcyD* copy number ($R = 0.87$), suggesting that *Microcystis* spp. are the primary toxin producer in the surface water of the TAR. This confirmed again the toxic genotypes are linking with MC concentration in a natural population. Furthermore, our results were consistent with previous reports that *Microcystis* spp. was the bloom-forming species and toxin producer in Vietnam waters^{7,17,18,20}.

The present study showed the MC concentration was often present in raw water. Based on the results, the MC concentration in the surface water sample was up to 4.8 µg/L. This concentration was higher than the concentration reported in the Nui Co reservoir but lower than the number reported in Hoan Kiem Lake and other locations in Southern Vietnam^{18,20}. However, these concentrations sometimes exceeded the WHO guideline value of 1 µg/L for MC in drinking water toxic³. The treated water may be contaminated with MC due to the water treatment plants having no facilities for removing MC from drinking water, nor is monitoring being conducted to detect MC in drinking water. Therefore, during periods of high *Microcystis* spp. in the reservoir, local people may suffer toxic effects via daily exposure to the contaminated water. It is necessary to establish a regularly monitoring program for cyanobacteria and cyanotoxins in lakes and reservoirs used for drinking purposes. In addition, the detection of other cyanotoxins such as anatoxins, saxitoxins, and cylindrospermopsins from the TAR is highly recommended.

CONCLUSIONS

In the present study, a qRT-PCR technique was successfully applied to quantify the potential of MC production in different cyanobacterial genotypes from the TAR. Our results indicated that *Microcystis* main produced MC. The concentration of *Microcystis* spp. contributed from 60–92% of the total cyanobacterial population. Our results indicated that the qRT-PCR techniques and traditional count are comparable and could be used to quantify cyanobacteria. In addition, the qRT-PCR techniques can determine the toxic cell number with a population. They could be used for early monitoring of toxic cyanobacteria in lakes and reservoirs.

LIST OF ABBREVIATIONS

CBs: Cyanobacterial blooms
Ct: cycle values
HPLC: high performance liquid chromatography
MC: microcystins
NRPS: non-ribosomal peptide synthesis
PKS: polyketide synthesis
qRT-PCR: quantitative real-time polymerase chain reaction
TAR: Tri An Reservoir
TCBs: Toxic cyanobacterial blooms
UDL: under detection limit
WHO: World Health Organization

COMPETING INTERESTS

The authors declare that they have no conflicts of interest.

AUTHORS' CONTRIBUTIONS

Pham Thanh Luu carried out the sample collections, analyses, interpretation of data, and writing the manuscript. Tran Thi Hoang Yen carried out the sample collections, analyzed and counted cyanobacteria. Tran Thanh Thai carried out the sample collections. Ngo Xuan Quang supported data analyses and revising the manuscript.

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