Comparison of Flow Cytometry and Quantitative PCR Assays for Cyanobacteria Quantification

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***Abstract*—Cyanobacterial blooms deteriorate water quality and some species produce cyanotoxins, posing a potential risk to human health. As such, rapid and accurate quantification of cyanobacteria is of growing interest worldwide. In this study, two conventional methods, flow cytometry (FCM) and real-time quantitative PCR (qPCR) were compared in terms of quantification of the total cyanobacteria species in a lab-cultured *Microcystis aeruginosa* and 10 tropical freshwater samples. Results showed that these two methods have high linearity and strong association ($P<0.01$), although qPCR has higher sensitivity for low concentrations, while FCM approach has much shorter analysis time for a comparable detection range. The 16S rRNA gene copies/cell ranged from 2.31 to 4.07 in natural freshwater samples. The results showed that the FCM method was preferred for routine cyanobacteria monitoring in the picoplankton range.**

Keywords-cyanobacteria; freshwater; flow cytometry; quantitative PCR

I. INTRODUCTION

Cyanobacteria, also known as blue-green algae, are the largest photosynthetic prokaryotes in a wide range of environments [1]. With increasing nutrients pollution (mainly phosphorus and nitrogen), many freshwater and marine ecosystems suffer from cyanobacterial blooms, especially in tropical areas where warm temperatures encourage rapid plankton growth. Cyanobacteria are able to produce a broad range of intracellular toxins called cyanotoxins, including: neurotoxins (anatoxin-a, saxitoxins,), cytotoxins (cylindrospermopsin), dermatotoxins (lyngbyatoxin, aplysiatoxin), and hepatotoxins (microcystins, nodularins) [2]. Therefore their occurrence in reservoirs or catchments, especially those used as drinking water resources, represents a potential health risk for humans and ecosystems.

The traditional method to detect cyanobacteria in aquatic environment samples is microscopic counting, which is very slow, tedious, time-consuming, and always underestimates the number of small cells. In recent years, many sensors have been developed for real-time monitoring of cyanobacteria *via* the detection of their specific photosynthetic pigments: chlorophyll *a* (chl *a*) and phycocyanin (PC). These sensors can provide simple and rapid detection of cyanobacteria, however, accurate and sensitive quantification is still problematic [3, 4]. Many studies showed that the changes of chl *a* and PC contents at different stages of cell cycle could strongly affect the estimation of the cyanobacterial biomass [5]. Furthermore, other factors, such as: photochemical quenching, pigment composition, water turbidity and organic factors concentrations, can also influence the fluorescence signal measurements and consequently biomass estimation [6].

Many molecular methods have been developed to rapidly detect targets of interest in environmental samples, such as qPCR and droplet digital PCR (ddPCR) [1]. These assays amplify the unique nucleotide sequences of the target microbial population, for example the 16S rRNA gene of cyanobacteria, and therefore are able to sensitively detect the total cyanobacterial species even at a very low abundance. On the other hand, FCM has been widely used as an alternative method for cyanobacterial analysis [7]. Basically, FCM is a technique which is able to rapidly and quantitatively characterize individual particles (cells) based on their light scattering and fluorescence properties. Since all cyanobacterial species contain two specific pigments (chl *a* and PC), FCM can easily distinguish them from other bacteria and particles. Despite the advantages of qPCR and FCM, the difference of using these methods for quantification of total cyanobacteria in pure lab-culture and complex environmental water matrices, has not been compared and examined yet. In this study, the performances of qPCR and FCM assays in quantification of cyanobacteria were compared using a lab-cultured *Microcystis aeruginosa* PCC 7806 and 10 environmental freshwater samples collected from three reservoirs in Singapore. Results showed that qPCR has higher sensitivity and linearity at low cyanobacteria concentrations, while the FCM assay has much shorter analysis time and comparable detection range for both pure lab culture and natural freshwater samples. On the basis of the study's findings, FCM is a suitable method for routine monitoring of cyanobacteria dynamics in natural water bodies.

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II. MATERIALS AND METHODS

A. Environmental sample collection and pretreatment

In total 10 water samples were collected from three urbanized reservoirs in Singapore. Two subsamples were taken at each station. The first subsample (100 mL) was fixed with glutaraldehyde (2.5% final concentration) for FCM analysis, while the second subsample (30 L) was used for total DNA extraction as described previously [8]. All samples were stored in the dark at -80 °C until they were used.

To enumerate total microbes, samples were first pre-filtered using 60 µm filtering meshes to remove large particles [9], and stained with SYBR Gold (Molecular Probes, Inc., OR, USA) for 30 min in dark at room temperature before FCM measurement.

B. Flow Cytometry

Flow cytometry was performed using a CytoFLEX flow cytometer (Beckman Coulter Inc., CA. USA). All the lab-cultured and environmental water samples were analyzed as described earlier [7]. Briefly, FITC channel (green fluorescence) was firstly used to locate the total microbes from other inorganic and organic particles in the freshwater. After that the APC channel (red fluorescence) was used to distinguish phytoplankton from other bacteria, and finally ECD channel (orange fluorescence) was used to discriminate cyanobacteria from other phytoplankton. 0.1 µm filtered freshwater was used as blank, and 1.0 µm microspheres fluorescent beads were added to each sample as internal reference (Thermo Fisher Scientific Inc., CA. USA). A threshold value of 1000 was applied on the side scatter channel. FCM results were analyzed using FlowJo 7.6.5 (Tree Star, Inc., OR, USA).

C. qPCR

Cyanobacterial 16s rRNA gene was quantified with a StepOnePlus real-time PCR system using the primers and probes as follows [10, 11]: Forward primer 108F: ACGGGTGAGTAACRCGTRA, Reverse primer 377R(2): CCATTGCGGAARATTCCCC, Probe 328R: FAM-CTCAGTCCCAGTGTGGCTGNTC-BHQ-1. Briefly, the 20 µL of reaction mix consisted of 10 µL of FastStart Universal Probe Master (Roche, Mannheim, Germany), 0.8 µM forward and reverse primers, 0.2 µM Taqman probe (FAM) and 2 µL of DNA. Triplicate qPCR amplification was performed under the thermal conditions: 95°C for 10 min and 40 cycles of 15 s at 95 °C, 25 s at 56 °C, and 25 s at 72 °C. The standard curve was established on serial dilutions of plasmid DNA (3 to 3×10^8 gene copies/reaction).

D. Statistical data analysis

All statistical analyses were performed using SPSS version 23 (SPSS Inc., IL, USA).

III. RESULTS

A. Assay set up

By using the plasmid clones of cyanobacterial 16s rRNA gene, a qPCR standard curve was established for the quantification of cyanobacteria. As shown in Fig. 1, the cyanobacteria standard curve equation was y = -3.5661x + 37.449 with an R^2 value of 0.997. The efficiency was calculated as 91% based on the equation ($Efficiency = (10^{-\frac{1}{slope}} - 1) * 100\%$). The dynamic range for cyanobacterial qPCR reactions was between 100 and 108 gene copies/reaction, and the LOD value was 3.16 gene copies/reaction. As mentioned above, in FCM analysis, 1.0 µm microspheres fluorescent beads were added to each sample, therefore, the concentrations of cyanobacteria cells in original samples were calculated by the following formula: *Concentration of cyanobacteria = cyanobacteria events number/beads events number) × beads concentration × dilution factor* (Fig. 2).

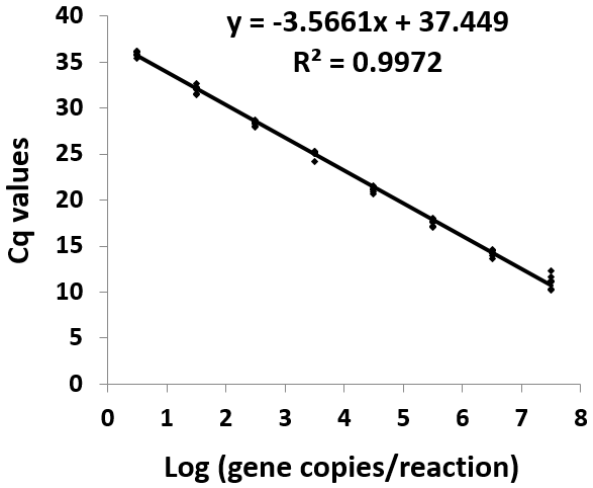

Figure 1 qPCR standard curve for cyanobacteria quantification.

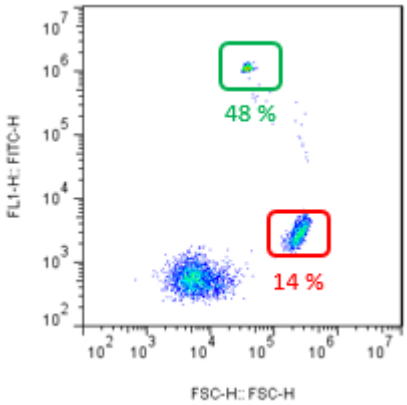

Figure 2 FCM assay for cyanobacteria quantification. Green box: 1.0 µm microspheres fluorescent beads; Red box: *Microcystis aeruginosa* cells

B. Assays validation

The performance, including sensitivity, selectivity, linearity and detection range, of qPCR and FCM assays were carefully compared using the lab-cultured *Microcystis aeruginosa* PCC 7806. As shown in Fig. 3 and Fig. 4, a good linear regression was observed in the 10-fold serial diluted samples for both two assays and the dynamic range of qPCR and FCM were 9 to 101271 gene copies/ml and 14 to 439340 CFU/mL, respectively. Overall, qPCR and FCM have a comparable linearity and detection range. However, compared to qPCR, larger error bars were observed in the FCM assay when measuring low cyanobacteria concentrations. This implies that qPCR assay is a preferable method for measuring low concentrations of cyanobacteria.

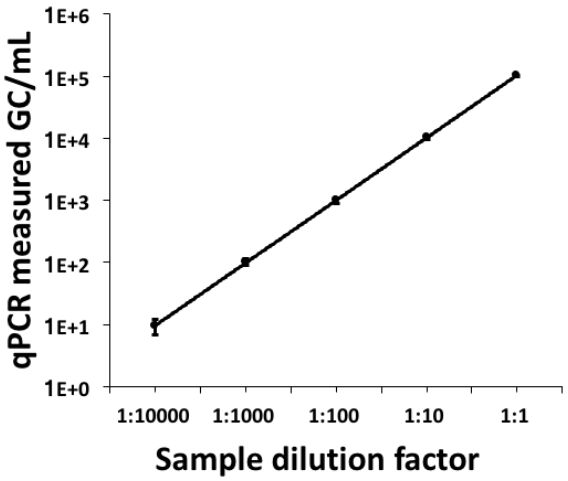

Figure 3 Linear regression between measured GC and estimated GC for qPCR assay

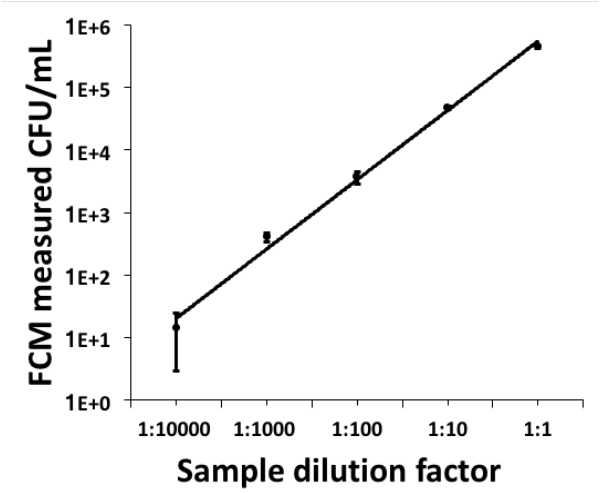

Figure 4 Linear regression between measured CFU and estimated CFU for FCM assay

The correlation between qPCR results (GC/mL) and FCM results (CFU/mL) in measuring *Microcystis aeruginosa* PCC 7806 was also analyzed. A good linear association was observed between different *Microcystis* concentrations, where the slope was 2.3 (Fig. 5). Pearson correlation also suggested a significant linear relationship between these two assays ($r=0.995$, $p<0.01$). However, since each *Microcystis aeruginosa* PCC 7806 cell contains only 2 copies of its 16S rRNA gene, the theoretical slope value between qPCR and FCM should be 2 [12]. The differences between two values can be attributed to the genome changes during the cell cycle, and a higher error rate at low concentrations in FCM assay measurement. Generally, both qPCR and FCM assays are plausible and validated approaches to quantify cyanobacteria and their results are comparable with each other.

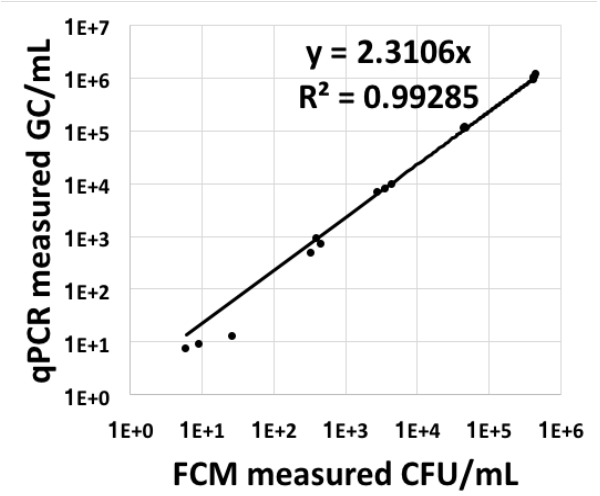

Figure 5 Correlations between qPCR and FCM assays. The correlation equations are $y=2.3106x$ ($R^2=99285$)

C. Evaluation of environmental samples

The cyanobacteria concentrations in 10 freshwater samples were measured by qPCR (GC/mL) and FCM (CFU/mL) respectively. As shown in Table. 1, the cyanobacteria concentrations vary from 41 to 18112 GC/mL by qPCR and 32 to 5270 CFU/mL by FCM. In terms of detection frequency, all of the samples were detected positively by qPCR whereas 1 of the environmental samples (RC-3) could not be detected by FCM. This is reasonable as FCM detection limit is slightly higher than qPCR, rendering FCM unable to enumerate cyanobacteria at a low concentration range. Different cyanobacteria species carries different 16S rRNA gene copies/cell, and the range could vary from 1 to 4 [12]. For the 10 freshwater samples, the ratio between qPCR results and FCM results ranged from 2.31 to 4.07, indicating a mixture of different cyanobacteria species in the collected environmental samples. Most of the cyanobacteria species have 2 gene copies whereas 4 rRNA copies could only be observed in several species (e.g., *Anabaena variabilis* ATCC 29413, *Nostoc azollae* 0708, *Nostoc punctiforme* PCC 73102, *Nostoc sp. PCC 7120*) [12]. It could be deduced that the samples with higher ratio value (>3) might exhibit the species with 4 rRNA copies.

IV. Discussion

Freshwater cyanobacterial blooms have become a worldwide environmental problem. Thus, rapid detection of the total abundance of cyanobacteria in natural water bodies is highly important for water quality risk assessment and algal bloom control.

qPCR based 16S rRNA gene quantification has become the most popular method to quantify cyanobacteria during the past decade. With the increasing bioinformatics knowledge, this assay could detect the cyanobacteria number, the total bacteria community number, different cyanotoxins abundance, and even distinguish between toxic and nontoxic cyanobacterial species. Although powerful, there are two recognized shortcomings with this method, (1) it is labor-intensive, time-consuming, and requires sample enrichment; (2) it cannot directly detect at single cell level, and thus cannot measure cell size and morphological properties.

In contrast, FCM is a rapid automated method. The application of FCM for freshwater phytoplankton and cyanobacteria study has been carried out since the 1980s. This method can provide rapid quantification of total cyanobacteria counts based on autofluorescence emitted by intracellular pigments: chl *a* and PC. However, the common use of FCM is to analyze mammalian cells, and the application of FCM for environmental studies is limited mainly because of the difficulty in interpreting complex signals from various objects. In recent years, advances in fluorescent dye technology offer more realistic multicolor detections and a better application of this technique. Thus, the total microorganism counts (DNA dye), fluorescent bacteria (such as cyanobacteria) number, other target pathogens (specific biomolecular dyes) counts can be assessed simultaneously by FCM, and interesting cells can also be isolated for further study. As a result, FCM analysis can provide complementary information for qPCR in cyanobacteria quantification, and can also provide new insights into the relationship between cyanobacterial blooms and other microbes in natural water systems.

TABLE I. Correolation between measured qPCR and FCM in 10 environmental samples

Sample name	***qPCR (GC/mL)***	***FCM (counts/mL)***	***Ratio (qPCR/FCM)***
RA-1	18112.41±1945.44	5270.02±799.04	3.44
RA-2	574.52±15.42	184.86±71.81	3.11
RA-3	2630.29±61.8	922.43±131.70	2.85
RA-4	2612.97±133.06	642.12±40.12	4.07
RB-1	2199.75±55.06	569.35±36.63	3.86
RB-2	6255.99±221.67	1785.01±219.28	3.50
RB-3	72.89±0.53	31.54±14.95	2.31
RC-1	4181.61±110.39	1496.00±122.48	2.80
RC-2	242.32±1.83	96.79±39.13	2.50
RC-3	40.53±1.91	ND	NA

V. Conclusion

In this study, two conventional methods were used to quantify the cyanobacteria concentrations in a lab-cultured *Microcystis aeruginosa* and 10 different freshwater samples collected in Singapore. qPCR based 16S rRNA gene quantification showed higher sensitivity and wider accurate detection range of cyanobacteria numbers, while FCM, which directly detects individual cyanobacteria cells, provided much more rapid detection of cyanobacteria with similar linearity, making it a suitable method for routine cyanobacterial monitoring. To the best of our knowledge, this is the first methodology comparison study investigating the performance of qPCR and FCM in quantification of cyanobacteria.

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