Journal of
Microbial & Biochemical Technology

Goh and Gin, J Microb Biochem Technol 2015, 7:2
DOI: 10.4172/1948-5948.1000193

Research Article Open Access

The Effects of Pretreatments on qPCR Detection of Bacteria – Bacteria Indicator, *Enterococcus* as a Model

Shin Giek Goh and Karina Yew-Hoong Gin*

Department of Civil and Environmental Engineering, National University of Singapore, Singapore

Abstract

Current recreational water guidelines (e.g. USEPA and WHO) recommend enterococci as the indicator for both freshwaters and marine waters. Culture-based method has been used for decades to quantify the presence of enterococci. In year 2012, USEPA guideline for recreational water included qPCR as the alternative method to detect enterococci. qPCR method does not require long incubation time as of culture-based method. In addition, it offers the advantages of high specificity and sensitivity detection. Nevertheless, qPCR hampered by three main limiting factors. There are limited target cell in small volume of sample being analyzed by qPCR, the presence of inhibitors that can easily affect the sensitive detection of qPCR and the persistence of free DNA (DNA released from dead cell) that caused the false-positive signal in qPCR. As such, the upstream treatments are crucial to the accuracy of qPCR detection. Three different pre-concentration methods (i) filtration with nylon membrane; (ii) filtration with polycarbonate membrane and (ii) centrifugation, were examined for their recovery rate. Filtration with polycarbonate membrane was found to give higher recovery efficiency and consistence results regardless of sample matrices. Conventional DNA extraction and two commercially available DNA extraction kits were evaluated according to the purity of extracted DNA and the relative recovery efficiency of extraction. Results show that the commercial kit, QIAamp® DNA Mini Kit (Qiagen), gave the best performance. The application of silica membranes in QIAamp® DNA Mini Kit was proven to enhance the DNA recovery with minimum interference of inhibitors. Ethidium monoazide (EMA) and propidium monoazide (PMA) were evaluated for their performance in reducing the false-positive signal in qPCR. PMA was appear to provide better option to reduce the false-positive detection of DNA in a membrane compromised cell.

Keywords: Bacteria indicator; Enterococci; Quantitative real-time PCR; Pretreatment

Abbreviations

qPCR – Quantitative Real-Time Polymerase Chain Reaction

USEPA – United States Environmental Protection Agency

WHO – World Health Organization

DNA – Deoxyribonucleic Acid

NTU – Nephelometric Turbidity Units

Introduction

The culture-based method is the most common method for enumerating the cell densities of fecal indicators. However, this method typically requires long culturing incubation times from about 18-96 hours. Thus, molecular-based methods such as PCR or qPCR have become suitable alternatives which offer high sensitivity and specificity detection, at the same time giving early warning of potential disease outbreaks. Studies have also shown that the culture-based method could possibly miss the detection of injured cells or viable but non-culturable (VBNC) cells [1,2]. Molecular-based methods which are independent of culturing techniques can help to overcome this problem. In the latest USEPA guideline, qPCR has became one of the recommended methods for *Enterococcus* detection for recreational waters [3]. In addition, there is study showed the use of qPCR tests for fecal indicators to predict the presence and density of waterborne protozoan pathogen [4].

Although molecular-based methods offer many advantages, there are however, three main limiting factors: (i) low sample volume is used in molecular-based methods, thus requiring pre-concentration of larger volume of water prior to sample analysis, particularly for samples with relatively low densities of bacteria, (ii) the presence of interfering substances in environmental waters which can inhibit molecular-based methods and (iii) the persistence of free DNA (DNA released from dead cells) which will subsequently give false positive signals in molecular-based methods [5]. This study aims to evaluate the upstream pretreatments to overcome these three major limitations in qPCR detection and subsequently improve the downstream qPCR detection.

To overcome the problem of low number of target microorganisms in water samples, several concentration methods (i.e. centrifugation and membrane filtration) will be evaluated and optimized for concentrating *Enterococcus* in environmental water samples. The impact of interfering substances (also known as inhibitors) on molecular-based methods can be removed through the purification steps in DNA extraction, addition of bovine serum albumin (BSA) or dilution. This study will examine and evaluate the purity of extracted DNA and the relative recovery efficiency of extraction with different DNA extraction methods.

Another major drawback of DNA-based molecular methods is the inability to differentiate between viable and dead cells. Studies have shown that free or naked DNA (DNA released from dead

***Corresponding author:** Karina Yew-Hoong Gin, Department of Civil and Environmental Engineering, National University of Singapore, Singapore, Tel: +6565168104; E-mail: ceeginyh@nus.edu.sg

Received January 23, 2015; **Accepted** April 09, 2015; **Published** April 16, 2015

Citation: Goh SG, Gin KYH (2015) The Effects of Pretreatments on qPCR Detection of Bacteria–Bacteria Indicator, *Enterococcus* as a Model. J Microb Biochem Technol 7: 124-132. doi:10.4172/1948-5948.1000193

cells) persists in the water over a long period (i.e. days) [6,7]. Under such circumstances, higher false-positive results have been found in the molecular detection methods that are targeting DNA. DNA-intercalating dyes such as ethidium monoazide (EMA) or propidium monoazide (PMA) have been employed to eliminate free DNA prior to downstream molecular analysis. The fundamental concept of EMA and PMA pretreatment is based on the covalent binding of EMA or PMA with DNA. EMA or PMA will only penetrate into dead cells with compromised membranes. In the membrane-compromised cell, EMA or PMA will intercalate with DNA to form a stable covalent bond upon strong photolysis. This permanent DNA modification will not be amplified in the downstream PCR analysis. Previous studies have reported convincing results with respect to the reduction in free DNA in various sample matrices [8-12]. However, it is still a challenging task to develop an effective treatment to reduce free DNA in natural environmental water matrices as there are a number of environmental factors which complicate the issue. For example, turbidity is one of the major parameters that affect the effectiveness of EMA or PMA treatment [13]. The sample with high turbidity level could hinder the penetration of light into the water sample, where the light irradiation is an important source for the photolysis process in the EMA or PMA treatment. In addition, the pre-concentration method was found to influence the effects of the PMA pretreatment [14].

This study will evaluate different pre-concentration methods, purification methods and the application of EMA-qPCR and PMA-qPCR in order to select suitable methods for the pretreatment and subsequently qPCR detection of *Enterococcus*.

Materials and Methods

Bacterial strains and cultivation

Enterococcus faecalis ATCC 29212 and *E. faecalis* 56R were grown overnight in brain heart infusion broth (Becton Dickinson, Sparks, MD, USA) at 37°C under constant shaking (120rpm). For long term storage, the bacteria strains were kept at -80°C in the presence of glycerol as the cryoprotective agent.

Pre-concentration methods for environmental water samples

Three different pre-concentration methods were examined for their recovery efficiency: (1) filtration through 0.45 µm pore size nylon membrane (Millipore, MA, USA); (2) filtration through 0.4 µm pore size polycarbonate membrane (Isopore™ Membrane Filters, Millipore, MA, USA); and (3) centrifugation at 11,000 x g for 15 minutes.

Two experiments (in duplicate) were set up to measure the recovery efficiency of each pre-concentration method. The cultures were spiked into phosphate buffer saline (PBS) and environmental water matrices to measure the recovery rate. In measuring the recovery concentration, 1 ml of overnight grown pure culture was spiked separately into 1L of PBS or an environmental water matrix. The seeded water samples were then concentrated according to the different concentration methods and resuspended with PBS to a final volume of 10 ml. For the control concentration, 1 ml of overnight grown pure culture was spiked directly into a 9 ml of PBS without going through any concentration method. Enumeration of bacteria for both recovery concentration and control concentration were done by the membrane filter technique [15]. The percentage of recovery efficiency was calculated by dividing the recovery concentration by the control concentration. Each measurement was performed in duplicate.

Optimization of EMA and PMA pretreatment

EMA was purchased from Molecular Probe (Invitrogen, Carlsbad, CA, USA). EMA powder was dissolved in ultrapure water to a stock concentration of 1 mg/ml. The stock solution was aliquoted and kept at -20°C in the dark. PMA (Biotium, Hayward, CA, USA) was dissolved with 20% of DMSO to a stock solution of 20 mmol l^{-1} and aliquoted to a light-impermeable microcentrifuge tube for storage at -20°C. Optimization experiments were carried out for different EMA concentrations (0, 7.5, 10, 50 and 100 µg ml^{-1}) and PMA concentrations (0, 2, 10, 20, 80 and 100 µmol l^{-1}) with different light exposure times. Direct qPCR (without EMA or PMA pretreatment) was performed in parallel to the experiments as a control to examine the impact of cell losses during the treatment process, such as the cell losses during washing steps and light irradiation. Both live cells and dead cells (heat-killed at 95°C for 10 min) were used for the optimization process. The EMA or PMA treated cells were incubated in the dark, at room temperature for 5 min. Following dark incubation, the cells were exposed to a 500-W halogen light source (Philips) for 1, 3, 5 and 10 min at a distance of 20 cm between the light source and the samples. The cell suspensions were kept on ice when exposed to halogen light to minimize the elevation of temperature in the samples. All samples were prepared in duplicate for each optimization. The optimized PMA treatment was tested on artificial turbid water by adding kaolin (Sigma-Aldrich Corporation, MO, USA) to ultrapure water at turbidity levels of 20 NTU, 50 NTU and 100 NTU.

DNA extraction and purification

Three different DNA extraction methods were tested: (1) commercial DNA extraction kit, Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA); (2) commercial DNA extraction kit, QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany); and (3) conventional DNA extraction method using phenol-chloroform-isoamyl-alcohol. DNA extraction using the commercial kit, Wizard® Genomic DNA Purification Kit and QIAamp® DNA Mini Kit were performed in accordance with manufacturer's instructions. Conventional DNA extraction method was performed based on He and Jiang's study [16]. Briefly, 1 ml to 10 ml of pure culture or environmental water was centrifuged at 6654 x g for 5 minutes. The pellet was resuspended in 100 µl of lysis buffer which consists of 10mM Tris-HCl (pH 8.3) 100 mM NaCl, 1mM EDTA and 1% (vol/vol) Tween 20. Besides lysis buffer, 10 µl of 10 mg/ ml of proteinase K was added. The sample was incubated at 45°C for 3 hours. After 3 hours incubation, the sample was centrifuged and the cell lysates were extracted with 100 µl of phenol-chloroform-isoamyl-alcohol (24:25:1, pH 8). The mixture was centrifuged at top speed for 2 minutes to separate it into phases. The supernatant was transferred to a new microcentrifuge tube, followed by the precipitation of DNA with isopropanol. The precipitated DNA was then washed with 70% ethanol. Finally, the DNA pellet was dissolved in 30 µl of TE buffer.

The purity and concentration of the isolated DNA was examined by a NanoDrop® ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

Quantitative real-time PCR (qPCR)

qPCR was performed using the Applied Biosystems® StepOnePlus™ Real-Time PCR System. Primers and probe sets used for quantification are given in Table 1. The qPCR reagent mixture (20µl) consisted of 10 µl of FastStart Universal Probe Master (Rox), primers and probe at final concentrations given in Table 1 and 5 µl of extracted DNA. The

Target gene	Primer/probe	Sequence (5'-3')	Final concen-tration (μM)	Reference
Enterococcus spp. (23S rRNA gene)	Forward primer	GAGAAATTCCAAACGAACTTG	0.5	[34]
	Reverse primer	CAGTGCTCTACCTCCATCATT	0.5	
	Probe	FAM -TGGTTCTCTCCGAAATAGCTTTAGGGCTA- TAMRA	0.1	
E.faecalis (16S rRNA gene)	Forward primer	CGCTTCTTTCCTCCCGAGT	0.9	[35]
	Reverse primer	GCCATGCGGCATAAACTG	0.9	
	Probe	FAM – CAATTGGAAAGAGGAGTGGCGGACG - TAMRA	0.25	
E.faecalis (*pbp5* gene)	Forward primer	AGGGACATTGAAGCCAGATG	0.5	[18]
	Reverse primer	AAGCGGTACGCAGATTGACT	0.5	
	Probe	FAM - TGTCGCAAAATAGCCGCCCCAAGATT - TAMRA	0.1	
Salmon DNA Sketa	Forward primer	GGTTTCCGCAGCTGGG	0.5	[34]
	Reverse primer	CCGAGCCGTCCTGGTCTA	0.5	
	Probe	FAM – AGTCGCAGGCGGCCACCGT - TAMRA	0.1	

Table 1: Primers and fluorescence probes for the detection of *Enterococcus*, *E. faecalis* and salmon DNA as the internal control.

thermal conditions for amplification were: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Determinations for cycle threshold (Ct) values were performed automatically by the instrument after adjusting the threshold fluorescence values based on the standard curves. Each sample was analyzed in duplicate.

A negative control (nuclease free water) was incorporated in every set of qPCR experiments. Salmon testes DNA (Sigma-Aldrich Corporation, MO, USA) was used as the internal control to measure the matrix inhibition level. The primers and probe sequences for this internal control were obtained from Haugland. Standard curves were generated by 10-fold dilutions of spiked-in salmon testes DNA into environmental water matrices, as well as 10-fold dilutions of spiked-in salmon testes DNA into DNase free water as the calibrator. The standard curves from the spiked environmental water matrices were compared with the calibrator. The standard curve with a difference of more than 3.3 in CT value, which is equivalent to 1 log difference, was concluded as possible inhibition in qPCR. For environmental matrices with high concentration of inhibitor substances, dilutions were performed for each sample.

A whole bacterial genomic DNA was extracted to generate a standard curve. The isolated DNA concentration was measured by utilizing a NanoDrop® ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The concentration of bacterial genomic DNA was converted to a gene copy number (Eq. 1) and a serial dilution was performed. The standard curve was derived from serial dilutions of the genomic DNA and plotted against the CT values. Quantification can be performed by comparing the CT value from the blind sample to the CT value from the standard curve.

$$\frac{\text{Gene copy number}}{\mu l} = \frac{\text{Avogadro's number x DNA concentration}}{\text{genome molecular weight}} \quad (1)$$

where Avogadro's number=6.022 x 10^{23}/ mol

DNA concentration A_{260} reading x dilution factor x 50 μg/ml

Genome molecular weight=Genome size (bp) x 660 g/mol.bp

For quantification purposes and easy comparison with colony forming unit (CFU), the gene copy number was converted to genome equivalent (GE) by multiplying the gene copy number with the normalization factor (ratio of CFU to gene copy number). Although there are multiple copies of 16S rRNA and 23S rRNA genes in each enterococcus genome, this study assumed approximately 4 gene copies equivalent to 1 CFU. This is in agreement with Klappenbach et al.'s study [17] which showed that *E. faecalis* species has an average of 4 copies of 16S rRNA gene in a chromosome ([17]; http://ribosome.mmg.msu.edu/rrndb/index.php,). In the case of pbp5, the ratio of CFU to gene copy number is 1.64 [18].

Laboratory microcosms

Two laboratory microcosms were used for examining the decay kinetics of stressed cells where the cells were stressed with starvation and nutrient depletion. The overnight grown *E. faecalis* was spiked into the two separate laboratory microcosms which contained (a) water from a local reservoir catchment and (b) ultrapure water which served as the control experiment. Both water matrices were autoclaved and filtered with 0.2- μm pore size membrane (Millipore, MA, USA) in order to eliminate the interference of indigenous bacteria in the study. The laboratory microcosms were maintained at room temperature under direct illumination of 1800 lx light intensity. All microcosms were set in duplicate. Culturable *E. faecalis* cells were quantified through the spread plate method on brain heart infusion agar. qPCR and PMA-qPCR were also carried out in parallel to enumerate the cell numbers.

Environmental water sampling

Water samples were collected from five locations in an urban catchment (Marina catchment) in Singapore (Figure 1). Sampling was carried out from July 2011 to June 2013, according to the recommended procedure in Section 9060 of *Standard Methods for the Examination of Water and Wastewater* [15]. The water samples were kept in an ice box during transit to the laboratory. All samples were analyzed for microbiological examination within 6 hours from collection. Enumeration of cultivated *Enterococcus* in environmental water samples were carried out with Enterolert™ (IDEXX Laboratories, Westbrook, Maine) and reported as most probable number (MPN).

For qPCR analysis, 200 ml of environmental water samples were concentrated through 0.4 μm pore size polycarbonate membrane (Millipore, MA, USA). The cells retained on the membrane were eluted in 1x phosphate buffer saline (final volume of 2 ml) through vortexing and scrapping. The cells were subsequently concentrated through centrifugation at 10,000 g for 10 min. The concentrated cells were either treated with PMA prior to DNA extraction or subjected to DNA extraction directly.

Statistical analysis

Statistical analyses were carried out using SPSS software (SPSS.Inc.). The Wilcoxon signed rank test was selected to test the mean difference between the readings for qPCR and PMA-qPCR. The significant level was set at 95% confidence level.

Results

Comparison of recovery efficiency for different pre-concentration methods

Three different pre-concentration methods were tested in PBS seeded samples and environmental water seeded samples. Among the three pre-concentration methods, filtration with polycarbonate membrane achieved the highest recovery efficiency for both seeded PBS water samples (81.6 ± 3.1%) and seeded environmental water samples (65.3 ± 2.4%). However, filtration with nylon membrane gave the least recovery for seeded environmental water samples (15.8 ± 4.3%). Meanwhile, recovery efficiency for filtration with nylon membrane for seeded PBS water samples was 49.5 ± 3.2%. Lower recovery efficiency was found in nylon membrane compared to polycarbonate membrane. This may be attributed to insufficient elution of bacteria from the filter membrane in environmental water matrices where most of the bacterial-attached suspended solids were clogged within the pores in the membrane.

The centrifugation method gave very low recovery efficiency for the seeded experiment in PBS (28.1 ± 1.9%). However, this method was found to achieve high recovery rates in seeded environmental water samples (64.5 ± 16.6%). Nevertheless, the recovery rates were highly variable for different type of environmental water matrices using the centrifugation method. This study found that the water samples which consisted of a high percentage of suspended particles and settle able silt, gave higher rates of recovery. Due to the high variability of recovery efficiency in centrifugation method, environmental water samples (200 ml) were concentrated using polycarbonate membrane in this study.

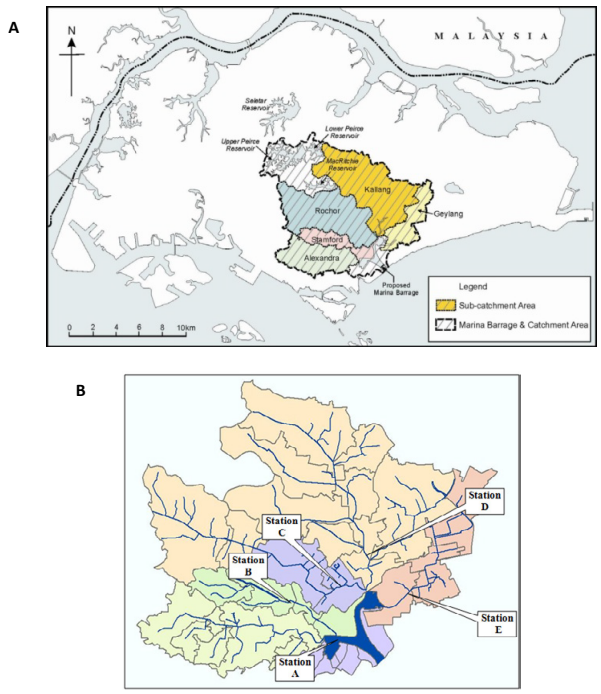

Figure 1: Marina Reservoir catchment plan (1) (A) and sampling sites (B).

Evaluation of DNA extraction methods

The ability to efficiently extract DNA from environmental samples often constrains the utility of any molecular analysis. Therefore, DNA extraction is an extremely important procedure which releases DNA from the microorganisms for downstream molecular analyses. The DNA extraction methods were evaluated according to the purity of extracted DNA and the lowest detection limit achieved in qPCR.

The purity of DNA was analyzed by calculating the ratio of absorbance (OD) at wavelength 260 nm to 280 nm (A_{260}/A_{280}). The A_{260}/A_{280} ratio was measured by a NanoDrop® ND-1000 Spectrophotometer. A ratio ranging from 1.8 to 2 indicates a high purity of DNA being extracted. Among these DNA extraction methods, the commercial kits, Wizard® Genomic DNA Purification Kit and QIAamp® DNA Mini Kit, showed a higher purity of DNA product. The A_{260}/A_{280} ratio was within the range of 1.8 to 2 (Table 2). In contrast, the conventional DNA extraction method gave an A_{260}/A_{280} ratio of 1.54 to 1.56. This indicated that the isolated DNA could be contaminated with protein. Isolated DNA which was contaminated with proteins was shown to inhibit conventional PCR and quantitative real-time PCR (qPCR) reactions [19].

In addition, the DNA extraction methods were evaluated by the detection limit in qPCR. Table 2 shows the lowest detection limit of qPCR using DNA extracted from dilutions of pure culture *E. faecalis* with Wizard® Genomic DNA Purification Kit, QIAamp® DNA Mini Kit and the conventional method. QIAamp® DNA Mini Kit gave the best performance with the lowest detection limit of 9.50 ± 0.71 GE reaction^{-1}. This was followed by Wizard® Genomic DNA Purification Kit with the lowest detection limit of 12.95 ± 0.49 GE reaction^{-1}. The least sensitive detection was found in DNA extracted by the conventional method (13.95 ± 1.77 GE reaction^{-1}).

In summary, the results of the study suggest that QIAamp® DNA Mini kit is the most sensitive DNA extraction method compared to the Wizard® Genomic DNA Purification Kit and the conventional method. Although both QIAamp® DNA Mini Kit and Wizard® Genomic DNA Purification Kit gave high purity in isolated DNA, QIAamp® DNA Mini Kit has the advantage of a higher yield of DNA which can be detected in lower cell numbers.

EMA and PMA optimization on live and heat-killed cells

EMA optimization was carried out under different operating environmental conditions, i.e. light intensity for inactivation, distance between the light source and samples, duration of light irradiation and EMA concentration. A light intensity of 500W at a distance of 20 cm away from the samples gave the best results in terms of highest inhibition in dead cells and minimal inhibition in viable cells (data not shown). This is also in agreement with other studies [20,21]. The optimum EMA concentration was 10 μg ml^{-1}. Figure 2 shows that

Extraction method	DNA purity[a]	Lowest detection limit (GE reaction^{-1}) [a]	qPCR calibration curve [% Efficiency]
Wizard® Genomic DNA Purification Kit	2.01 ± 0.05	12.95 ± 0.49	y = -3.6129x + 36.209 [E=89%]
Conventional	1.56 ± 0.06	13.95 ± 1.77	y = -3.0839x + 36.902 [E=111%]
QIAamp® DNA Mini Kit	1.92 ± 0.03	9.50 ± 0.71	y = -3.5611x + 32.869 [E=91%]

Notes: [a] The values are averages ± standard errors based on two independent extractions

Table 2: Comparison of three DNA extraction methods from PBS spiked with pure cultures of *E. faecalis*.

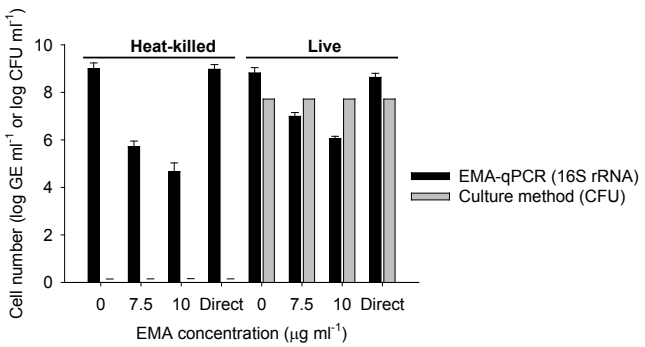

Figure 2: The relationship between EMA concentration and enumeration of *E.faecalis* using qPCR for the 16S rRNA gene and the standard culture method on TSA media for heat-killed cells and live cells. The initial cell concentration was approximately 10^8 CFU ml^{-1}. The detection limit for qPCR is 3 x 10^2 GE ml^{-1} in natural water without pre-concentration.

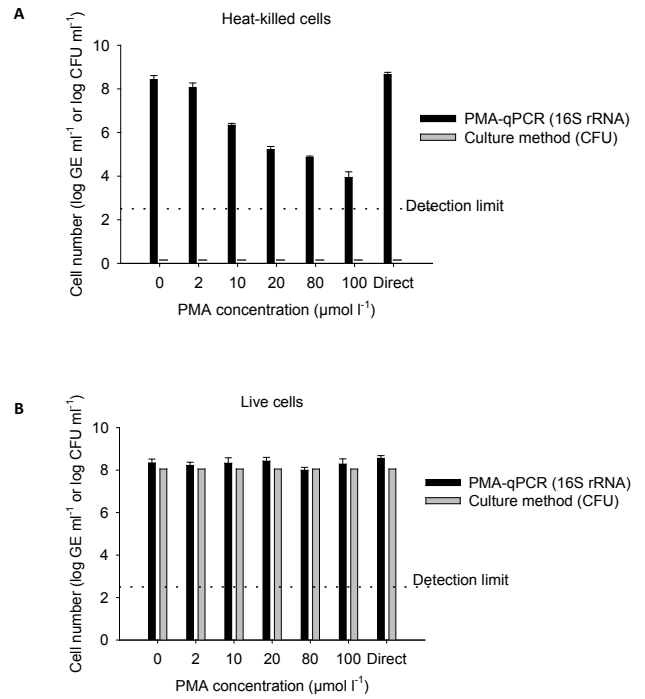

Figure 3: The relationship between PMA concentration and enumeration of *E.faecalis* using qPCR for the 16S rRNA gene and standard culture method on TSA media for A) heat-killed cells; and B) live cells. The initial cell concentration was approximately 10^8 CFU ml^{-1}. The detection limit for qPCR is 3 x 10^2 GE ml^{-1} in natural water without pre-concentration.

the greatest inhibition for dead cells (≈4.5 logs) and ethanol treated cells (≈4 logs) was achieved at an EMA concentration of 10 µg ml^{-1}. Although EMA pretreatment is shown to reduce the false positive signal in heat-killed cells significantly, the influence of EMA on live cells hampers its application in pre-treatment. Approximately 1.5 log units of live cells was inhibited from downstream amplification at an EMA concentration of 10 µg ml^{-1} (Figure 2).

The PMA optimization was performed at two different cell concentrations, i.e. 10^8 CFU ml^{-1} and 10^6 CFU ml^{-1}. At a cell concentration of 10^8 CFU ml^{-1}, the greatest inhibition (≈4 logs) for heat-killed cells was achieved at 100 µmol l^{-1} (Figure 3). The PMA pretreatment had minimal impact on the live cells. Interestingly, when the cell concentration was reduced to 10^6 CFU ml^{-1}, the degree of inhibition was not proportional to the PMA concentration. In fact, the degree of inhibition for dead cells was the same after 20 µmol l^{-1} of PMA pretreatment (Figure 4). The influence of PMA on viable cells increased as the PMA concentration increased beyond 20 µmol l^{-1}. However, the heat-killed cells achieved equilibrium once the PMA concentration reached 20 µmol l^{-1} and further increases in PMA concentration did not cause further inhibition for the heat-killed cells.

Apart from PMA concentration, turbidity is another parameter which can influence the PMA pretreatment method. The previous optimized PMA pretreatment (i.e. PMA concentration of 20 µmol l^{-1}) was shown to give the same reading for both the control (0 NTU) and environmental water samples (20 NTU) for heat-killed cells (Figure 5), although a slight inhibition was observed for samples with live cells (less than 1 log unit). However, for turbid water samples of 50 NTU and 100 NTU, greater inhibition was found for both live cells and heat-killed cells.

Quantification of fecal indicator in stressed aquatic conditions

Two laboratory microcosms were set up to provide stressed growth conditions for the cells (i.e. in ultrapure water and an environmental freshwater matrix). In the first set of experiments, cell enumeration was done by conventional plate count and qPCR. Figure 6A shows a rapid decay in plate count enumeration but consistent results for qPCR thoughout the experiments (i.e. 50 days). With EMA pretreatment in the second set of experiments, a gradual decreasing pattern was observed in the EMA-qPCR results (Figure 6B). However, the initial

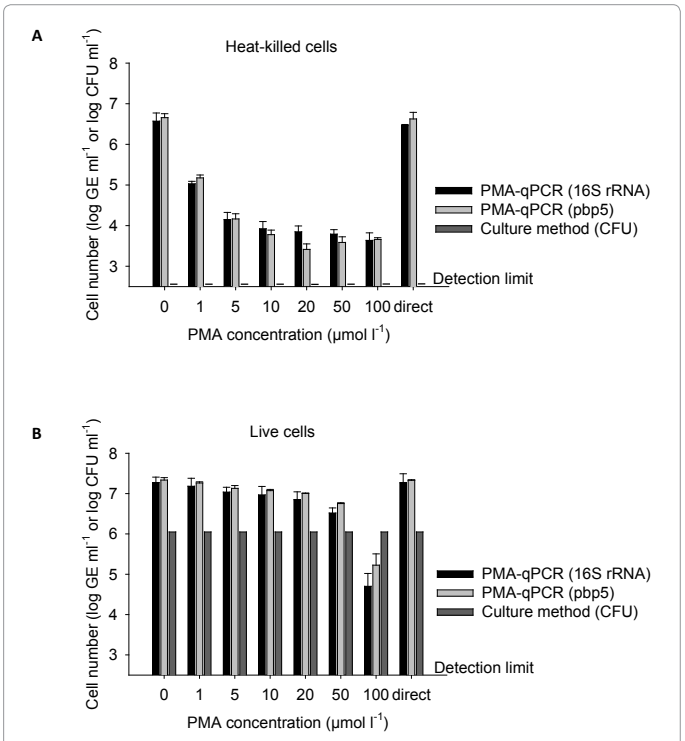

Figure 4: The relationship between PMA concentration and enumeration of (A) heat-killed *E.faecalis*cells and (B) live *E.faecalis* cells, using qPCR for 16S rRNA gene and pbp5 gene, and standard culture method on TSA media. The initial cell concentration was diluted to approximately 10^6 CFU ml^{-1}. The detection limit for qPCR is 3 x 10^2 GE ml^{-1} in natural water without pre-concentration.

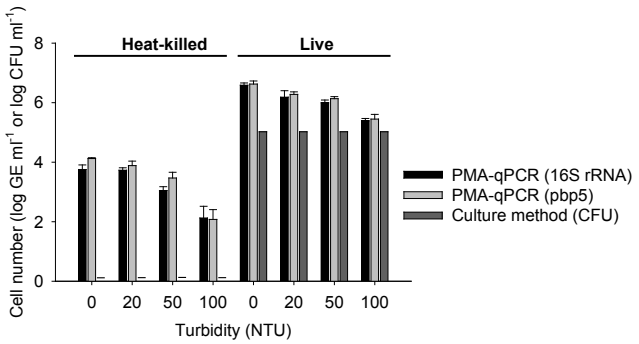

Figure 5: The effect of turbidity in the optimized PMA pretreatment.

GE obtained from EMA-qPCR was much lower than the CFU obtained from the plate count. This further confirms that the EMA dye tends to penetrate into intact cells as reported in other studies [20,22]. The third set of experiments was carried out using PMA-qPCR. Interestingly, the initial reading from PMA-qPCR was higher than the plate count method (Figure 6C). This could be due to additional injured cells being detected by the PMA-qPCR method or the sudden shock of environmental changes triggering survival mechanisms in the cells such as ceasing replication in the cells and hence, lowering the culture-based count.

Quantification of *Enterococcus* in environmental waters

In addition to spiked samples, PMA-qPCR detection of *Enterococcus* was also performed on environmental waters. Monthly routine water sampling was carried out at a local urbanized reservoir, Marina Reservoir and its catchment. The samples were tested with Enterolert™, qPCR and PMA-qPCR. Due to the small amount of sample used in qPCR analysis and the relatively low cell number in environmental water samples, an additional pre-concentration step with polycarbonate membrane was used to concentrate the target bacteria in environmental water samples. 200 ml of raw water samples were concentrated for qPCR analysis. However, the concentration was still insufficient to capture target microorganisms for qPCR analysis at Stations A, C, D and E (refer to map, Figure 1). Approximately 60% of water samples collected from Stations A, C, D and E were below the detection limit of qPCR (150 GE/100 ml with pre-concentration of 200 ml of raw water samples) and gave undetected signals. Subsequently the sample size was reduced for statistical analysis.

Figure 7 shows the box plots of Enterolert™, qPCR and PMA-qPCR measurement at Station B throughout the sampling period. *Enterococcus* counts reported by Enterolert™ were generally lower than the counts reported by qPCR and PMA-qPCR at Station B. The median value for qPCR was higher than PMA-qPCR. The mean reading for qPCR (27,819 CE/100 ml) was also higher than PMA-qPCR (25,335 CE/100 ml). The additional PMA pretreatment is believed to inhibit the amplification of DNA from dead cells. However, Wilcoxon signed rank test (a non parametric test for paired t-test) showed no significant difference in mean values for both qPCR and PMA-qPCR (P value= 0.110).

Discussion

Pre-concentration

Pre-concentration is often required prior to molecular detection where only small volumes of sample is needed in molecular techniques. Through the pre-concentration step, relatively low densities of bacteria in water are captured and concentrated to a smaller volume. In this study, two main pre-concentration methods, namely membrane filtration and centrifugation, were examined.

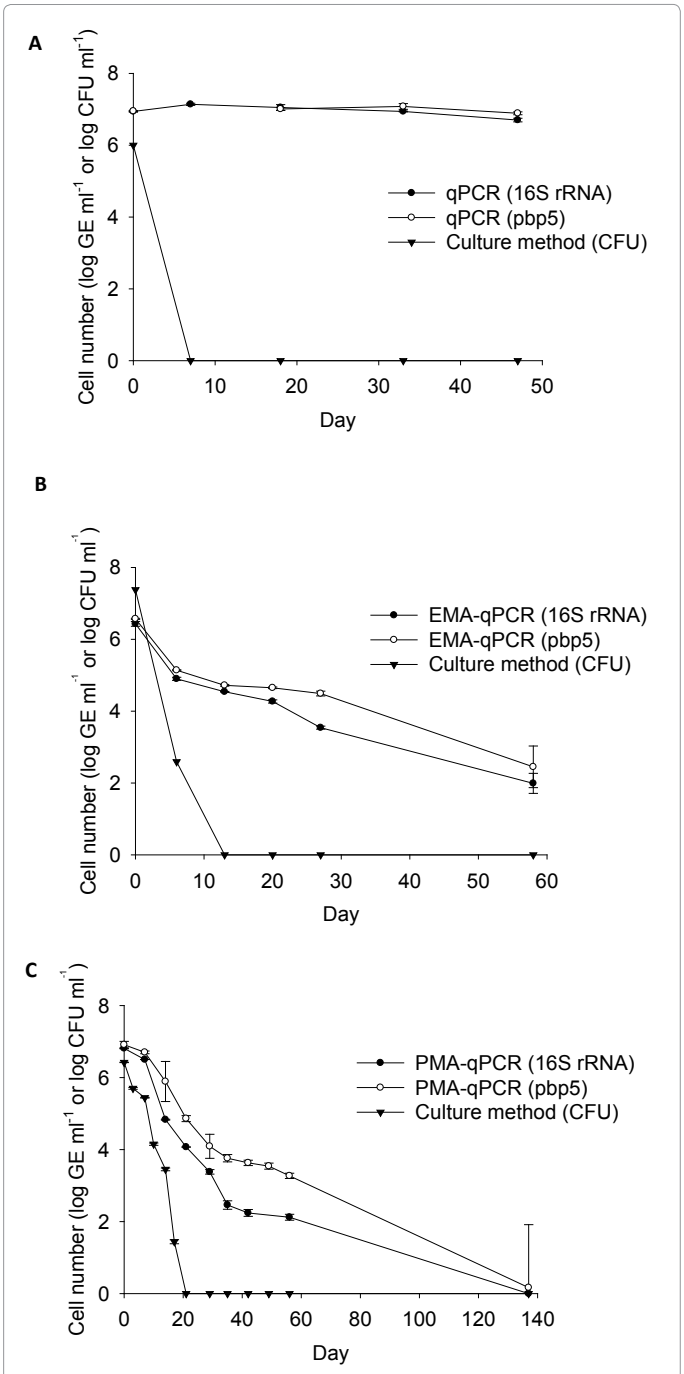

Figure 6: Decay curve plotted with cell number against stress exposure duration in the ultrapure water microcosm. Cell numbers were examined by (i) culture-based plate count; (ii) molecular techniques which targeted the 16S rRNA gene and (iii) molecular techniques which targeted the *pbp5* gene. Molecular techniques included (A) qPCR without pretreatment; (B) qPCR with EMA pretreatment; and (C) qPCR with PMA pretreatment. A similar trend was observed in the environmental freshwater matrix.

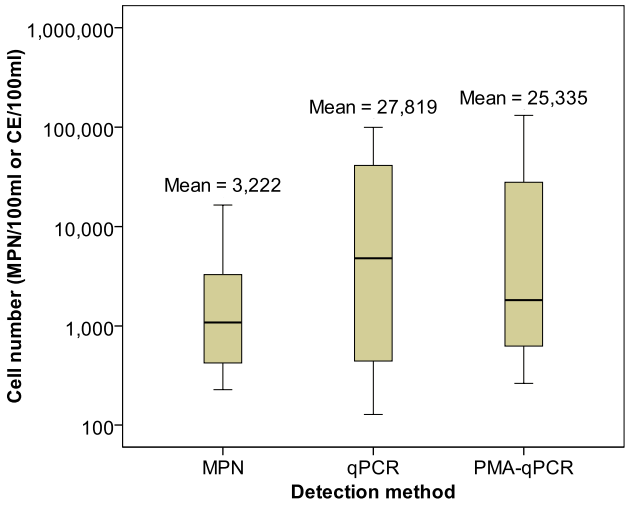

Figure 7: Box plot of enterococci cell number enumerated by (A) Enterolert™, (B) qPCR and (C) PMA-qPCR at Station B.The thick horizontal line in the box indicates the median value. The top and bottom of the box show the 75th and 25th percentile values, while the vertical lines extending from the box represent the largest and smallest values.

Membrane filtration concentrates target microorganisms in water samples based on the physical separation of target microorganisms via a filter membrane. As such, selections of membrane pore size and membrane material are very important to ensure good concentration performance. For instance, membrane filters with pore size of 0.22–0.45 µm are typically selected to concentrate bacteria. In this study, two different membrane filters (polycarbonate membrane filter and nylon membrane) were examined for their recovery efficiency. Polycarbonate membrane filter was found to achieve high recovery rates in both seeded PBS water samples (81.6 ± 3.1%) and seeded environmental water samples (65.3 ± 2.4%). Compared to polycarbonate membrane filters, nylon membrane filters gave much lower recovery rates: 49.5 ± 3.2% and 15.8 ± 4.3% for seeded PBS water samples and seeded environmental water samples, respectively. The great reduction in recovery efficiency for nylon membrane filtration could be attributed to the membrane structure in nylon membranes. A nylon membrane is a depth filter with tortuous paths and the pore structure is the result of stacking layers of porous materials [23]. The retention of cells in nylon membranes is by entrapment within the porous materials and thus difficult to recover. In contrast, the polycarbonate membrane is a screen filter with straight pathways through the membrane [23]. The cells are retained on the surface of the membrane and more easily eluted.

One of the problems with membrane filtration is the clogging of particles on the membrane filter. As such, application of membrane filtration in environmental waters is highly influenced by the presence of particles in the water. Pre-filtration with bigger pore size (i.e. 20 µm) can be applied to remove coarse particles before the membrane filtration pre-concentration step.

The centrifugation method uses centrifugal force to achieve separation of particles from a liquid medium. As a result, this method is not constrained by the turbidity level of the water samples. In addition, the centrifugation method allows larger volumes of water to be concentrated. This can subsequently increase the sensitivity of the downstream detection method. In this study, the centrifugation method gave higher recovery rates in seeded environmental water samples compared to seeded PBS water samples. The attachment of bacteria to particles may enhance the recovery efficiency in centrifugation method. However, high variable in recovery rates were observed for different environmental water matrices. The particle size fractions and different types of clay materials are among the factors that affect the attachment of bacteria in environment [24,25].

The disadvantage of centrifugation is the tendency to simultaneously concentrate particles and background flora, which are not the target microorganisms. This will subsequently complicate the downstream detection particularly in molecular techniques which can be greatly affected by the presence of high background flora and concentrated inhibitors. It also appears that the centrifugation method creates bias towards particle-associated bacteria.

In conclusion, the pre-concentration method is highly dependent on the composition of environmental water matrix. In this study, we found that both filtration with polycarbonate membrane and centrifugation gave the high recoveries, even in environmental water matrices with turbidity levels as high as 50 NTU. However, filtration with polycarbonate membrane offers the advantage of consistency in recovery for different water matrices. The recovery rate for the centrifugation method was found to fluctuate highly for different water matrices. Based on the water matrices tested in this study, pre-concentration with polycarbonate membrane filter offered greater consistency in recovery rates.

EMA and PMA optimization

Numerous studies have been carried out using EMA or PMA as a pretreatment step prior to molecular analysis [5]. However, there is still no universal protocol for its application to different bacteria, as well as different sample matrices (e.g. food samples, wastewater samples, plant samples). This could be due to the different cell wall structures existing in different bacteria, whereas the fundamental theory for EMA and PMA pretreatment assumes penetration of the dye through membrane-compromised cells. The sample matrix is also another important factor to be examined before the application of EMA or PMA.

In this study, the potential application of EMA and PMA to differentiate between viable and dead cells was evaluated. The combination of qPCR and EMA or PMA allows the selective quantification of viable cells in a mixed population of bacteria. This study demonstrates that both EMA and PMA could be used to eliminate dead cells. However, there is a higher tendency for EMA to penetrate into live cells, thus giving false negative results for live cells [22,26].

PMA is an alternative DNA-intercalating dye that has been introduced to overcome the penetration of EMA through intact cell membranes [26]. PMA was shown to work better when compared with EMA due to its higher molecular charge (two positive charges in PMA compared to one positive charge in EMA) [26]. The higher molecular charge provides greater impermeability through intact cell membranes, thus achieving better selective staining of dead cells.

The PMA pretreatment was optimized for analyzing the target bacteria, *E. faecalis*, in natural environmental waters. In the optimization process, we found that cell concentration is one of the main factors determining the optimum PMA concentration. The PMA concentration of 100 µmol l^{-1} has been reported to differentiate viable and dead *Enterococcus* cells [12,14]. In our study, a PMA concentration of 100 µmol l^{-1} gave the optimum treatment for *E. faecalis* at a cell concentration of 10^8 CFU ml^{-1}. However, a cell concentration of 10^8

CFU ml^{-1} is relatively high, when considering environmental surface waters. At a lower cell concentration (i.e. 10^6 CFU ml^{-1}), the optimum PMA concentration can be reduced to 20 µmol l^{-1}.

Another interesting finding was obtained during the course of PMA optimization. With PMA concentration of 20 µmol l^{-1}, the enumeration of viable cells consistently showed higher counts in PMA-qPCR when compared to the conventional culture-based method which was carried out in parallel. This could be due to the detection of stressed cells, persistent cells, or the viable but non culturable (VBNC) cells. The difference between the PMA-qPCR and culture-based methods is more obvious when the initial cell concentration was diluted 100 times with phosphate buffer. The sudden change in media may have caused stress or injury to the cells, which subsequently affected their growth on nutrient rich agar (i.e. TSA or BHIA). In contrast, the cell count for PMA-qPCR was lower than the plate count with the 100 µmol l^{-1} PMA pretreatment. This clearly shows that a higher concentration of PMA may not be suitable for lower cell concentrations. This may be attributed to residual levels of PMA, which were not successfully inactivated by the photo-inactivation process and subsequently bound to lysed DNA during the DNA extraction step. The gap between the culture-based method and PMA-qPCR was suggested as the VBNC subpopulation where this subpopulation can escape from culture-based detection [18]. In their study, the reading from PMA-qPCR was significantly correlated with live cells including both culturable and VBNC cells.

The application of PMA pretreatment in environmental water samples did not show significant difference between the qPCR and PMA-qPCR readings compared to the laboratory microcosms study. One possible reason could be due to the impact of pre-concentration. In the laboratory microcosm study, cells were subjected to DNA extraction directly without pre-concentration. However, in environmental water samples, cells were concentrated through 0.4 µm pore-size polycarbonate membranes. The pores within the membrane and the external vacuum force applied could have allowed free or naked DNA to filter through. This could subsequently reduce the gap between the qPCR and PMA-qPCR measurements. In other words, there is a higher possibility of false positive detection (due to free DNA) using qPCR, particularly for direct water sampling without pre-concentration.

In addition, the abundance of endogenous nucleases, and the physical and chemical abrasions in the natural environment encourages the degradation of free or naked DNA. It is believed that the degradation of extracellular DNA in the soil environment is mainly by microbial DNases although DNA can be adsorbed on soil and protected from degradation by DNases [27]. The enzymatic degradation of extracellular DNA was found to increase with an increase in temperature [28]. The degraded extracellular material is consumed as nutrients for microbial growth or be incorporated into a bacterial genome through transformation [29,30].

Although PMA-qPCR can significantly suppress the detection of dead cells, this method is still unable to totally discriminate the dead cells from qPCR detection. This is currently the main obstacle for the PMA-qPCR method. Studies have suggested that the presence of high numbers of membrane compromised cells which have exceeded the dye's capacity, may cause insufficient modification to the DNA in the membrane compromised cells [31]. Other studies have suggested that the short amplicon size in PCR or qPCR cannot be suppressed completely by PMA pretreatment [32]. In the latter case, Luo et al. [32] overcame this problem through a combination of two-step nested PCR. Nevertheless, PMA-qPCR is currently the most straight forward method to exclude the false positive detection from membrane compromised cells.

Evaluate the performance of DNA extraction

In this study, two commercial DNA extraction kits and one conventional DNA extraction method were compared. The performance was initially judged based on DNA purity and DNA yield. By using NanoDrop® ND-1000 Spectrophotometer, the purity and yield of DNA can be measured directly. This method has been widely used due to its fast (less than 30 seconds) and easy (no other reagents or accessories required) application. In addition, a small amount (0.5 - 2µl) is required for the analysis. However, there are some disadvantages brought by this spectrophotometric method. For instance, this method relies on the absorbance reading at 260 nm to represent the total amount of nucleic acid present in the sample. As such, this method cannot distinguish between dsDNA, ssDNA and RNA. This method also tends to overestimate the nucleic acid concentration due to some contaminants may absorb at or around 260 nm. In addition, the lowest detection range for NanoDrop® ND-1000 Spectrophotometer is 2 ng $µl^{-1}$ which is equivalent to approximately 5 x 10^5 copies of *Enterococcus*. Some environmental samples may not able to achieve this detection range. Moreover, the results from spectrophotometric method cannot give much information on the specific cell concentration in the complex environment water samples. The qPCR which measures the amplifiable target DNA, was used to examine the performance of the DNA extraction kits. Nevertheless, spectrophotometric method is still a reliable, rapid and easy method to examine the DNA purity and yield in pure culture samples.

Among the DNA extraction methods tested, QIAamp® DNA Mini Kit gave the most satisfactory results in term of DNA purity and DNA yield, which can give the lowest detection limit in qPCR. The silica membrane provided in QIAamp® DNA Mini Kit played the main role in removing the inhibitors. The principle of the silica membrane is based on the adsorption of nucleic acids on the silica membrane in the presence of chaotropic salts. Under these conditions, the major inhibitors (such as polysaccharides and proteins) are not able to adsorb onto the membrane. In other words, the membrane will filter and remove these inhibitor substances [33].

Conclusions

Currently, many advanced molecular analyses have been developed to identify, detect and quantify microorganisms in various sample matrices. Unfortunately, the developments in upstream sample preparation have often been neglected. Sample preparation is the most important step to ensure that the downstream advance molecular analysis can achieve the best performance.

This study was carried out by examined current available sample preparation methods and subsequently, selected the most suitable method for downstream molecular analyses. Filtration with polycarbonate membrane was found to be the most suitable pre-concentration method for this study, particularly for local catchment water samples. However, centrifugation could be a good alternative for water samples with high turbidity. The application of silica membranes in DNA extraction was also proven to enhance the DNA recovery with minimum interference of inhibitors.

Both EMA-qPCR and PMA-qPCR were shown to reduce the detection of heat-killed *E. faecalis* cells efficiently. The major draw-back for EMA-qPCR is the penetration of EMA into intact cells which can inhibit the amplification of live cells. This draw-back can be overcome

by using PMA-qPCR. PMA-qPCR signifies an alternative technique that offers two main advantages: (i) minimize false positive results in conventional qPCR method and (ii) provide rapid results when compared to the time-consuming, yet non-species-specific culture-based method.

Pre-concentration was found to reduce false-positive signals to a certain extent. The external suction force from the vacuum pump during filtration, as well as the pores within the membrane may allow the removal of free or naked DNA. However, DNA inside dead cells and adsorbed on particles will not be removed through filtration. Hence, this study strongly advocates that PMA pretreatment be applied to environmental water samples. Application of PMA pretreatment was found to be particularly necessary for samples without pre-concentration, such as the microcosm experiments in this study.

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