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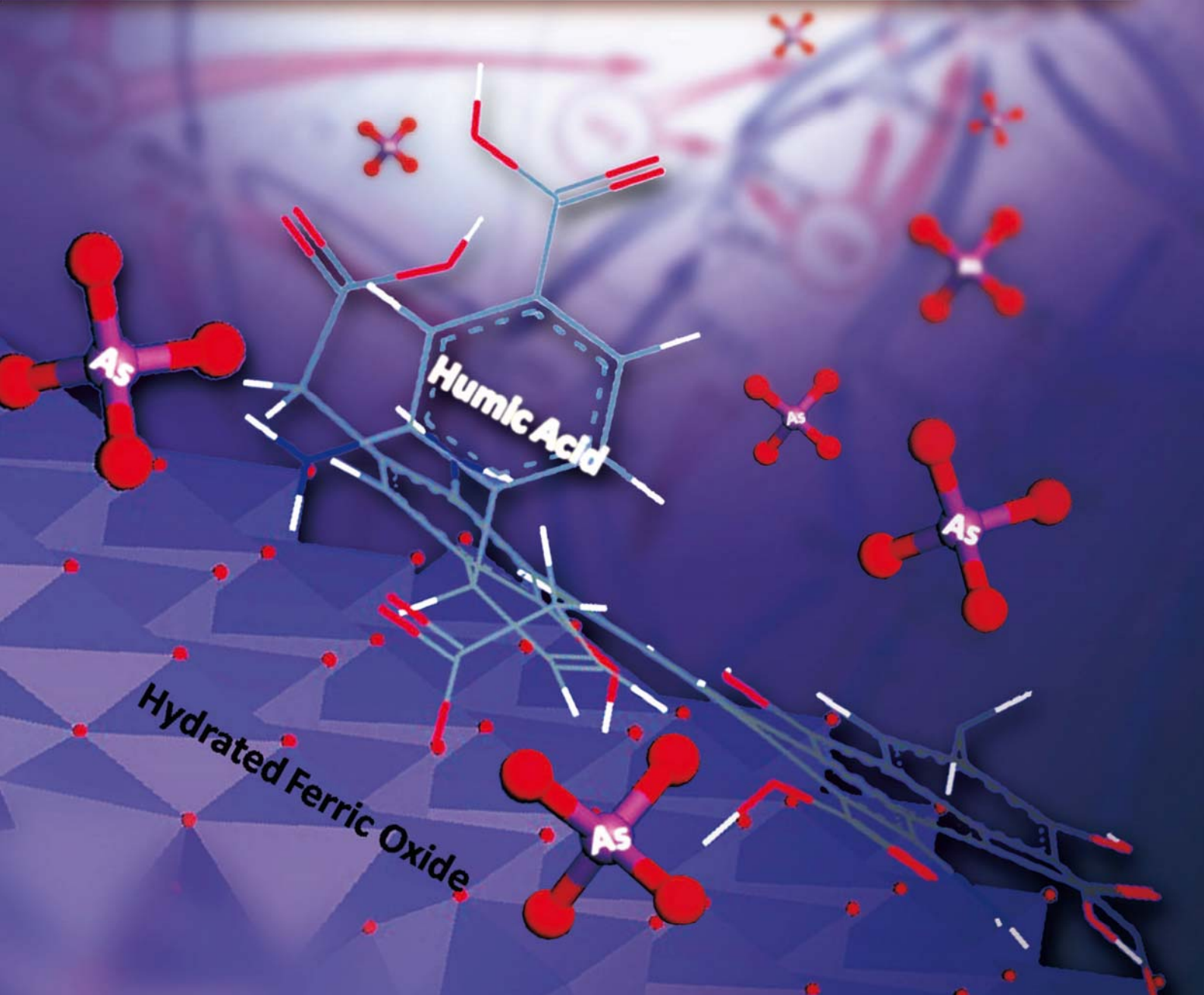

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Quantification of viable bacteria in wastewater treatment plants by using propidium monoazide combined with quantitative PCR (PMA-qPCR)

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ABSTRACT

The detection of viable bacteria in wastewater treatment plants (WWTPs) is very important for public health, as WWTPs are a medium with a high potential for waterborne disease transmission. The aim of this study was to use propidium monoazide (PMA) combined with the quantitative polymerase chain reaction (PMA-qPCR) to selectively detect and quantify viable bacteria cells in full-scale WWTPs in China. PMA was added to the concentrated WWTP samples at a final concentration of 100 μmol/L and the samples were incubated in the dark for 5 min, and then lighted for 4 min prior to DNA extraction and qPCR with specific primers for *Escherichia coli* and *Enterococci*, respectively. The results showed that PMA treatment removed more than 99% of DNA from non-viable cells in all the WWTP samples, while matrices in sludge samples markedly reduced the effectiveness of PMA treatment. Compared to qPCR, PMA-qPCR results were similar and highly linearly correlated to those obtained by culture assay, indicating that DNA from non-viable cells present in WWTP samples can be eliminated by PMA treatment, and that PMA-qPCR is a reliable method for detection of viable bacteria in environmental samples. This study demonstrated that PMA-qPCR is a rapid and selective detection method for viable bacteria in WWTP samples, and that WWTPs have an obvious function in removing both viable and non-viable bacteria. The results proved that PMA-qPCR is a promising detection method that has a high potential for application as a complementary method to the standard culture-based method in the future.

Introduction

Waterborne disease, which is highly contagious and may lead to serious disease outbreaks, is one of the most significant threats to public health all over the world (MacKenzie et al., 1994; Hrudey et al., 2002; Kay et al., 2008; Soller et al., 2010). Considering that untreated fecal polluted water collected by wastewater treatment plants (WWTPs) may contain more than 100 types of pathogens causing a wide range of human diseases and clinical symptoms, WWTPs are a media with high potential for waterborne disease transmission (Ottson et al., 2006; Varma et al., 2009). Knowledge of pathogen concentration variability in the effluents of WWTPs is essential to appropriately quantify and mitigate human health risks.

Conventional monitoring for pathogens in these environments relies on culture-based methods, which have many obvious limitations, such as being time-consuming and laborious, hindering their usefulness as an ideal detection tool. Moreover, pathogens entering a viable but non-culturable (VBNC) state when exposed to environmental stressors cannot be detected by culture-based methods, so that culture-based methods largely underestimate the amount of viable pathogens present in the sample (Oliver et al., 2005). Accordingly, the quantitative polymerase chain reaction (qPCR), a powerful molecular tool, has

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been applied to pathogen detection in samples from surface water (Ahmed et al., 2009b), coastal water (He and Jiang, 2005), and WWTPs (Shannon et al., 2007; Wéry et al., 2008) as a more rapid, sensitive and specific alternative method. However, conventional qPCR suffers from the limitation of an inability to differentiate viable and nonviable cells, because naked DNA can persist in the environment even after cell death (Masters et al., 1994; Varma et al., 2009). This drawback will lead to overestimation of pathogen concentrations by qPCR, resulting in a misleading magnification of the health risk and an inaccurate assessment of pathogen removal efficiency within WWTPs.

Recently, a newly developed detection method combining a novel sample treatment using propidium monoazide and quantitative PCR (PMA-qPCR) has been used to selectively detect viable cells (Nocker et al., 2007, 2009; Varma et al., 2009). PMA is a DNA-intercalating dye that is able to penetrate the compromised membranes of nonviable cells and subsequently combines with extracellular DNA or DNA from nonviable cells via exposure to bright visible light. Once combined with PMA, DNA will be incapable of being amplified in the subsequent PCR reaction, whereas only DNA protected by intact membranes of viable cells will be normally detected by qPCR. Although PMA-qPCR seems to be a promising tool for pathogen monitoring in WWTPs, there are still two issues to be addressed before it is validated as a qualified method for routine monitoring. First, the effectiveness of PMA treatment in diverse environmental samples should be further investigated due to the inactivation effects of frequently-present dark particles and inhibitor substances on PMA cross-linking. Moreover, there are only a few published papers that report pathogen detection results revealed by PMA-qPCR in wastewater treatment processes (Bae and Wuertz, 2009; Varma et al., 2009). Considering that culture-based methods have been used as the "gold standard" for years, a consistent relationship between the results of culture-based methods and PMA-qPCR should be developed in order to validate the effectiveness of PMA-qPCR in pathogen monitoring within WWTPs, indicating that more practical data need to be obtained.

The objective of the present study was to use PMA-qPCR for monitoring of viable bacteria in WWTPs. A broad range of water and sludge sample matrices within WWTPs was investigated to evaluate the effectiveness of PMA treatment in these samples. *E. coli* and *Enterococci*, two typical fecal indicators that are routinely used to evaluate pathogen removal efficiency in WWTPs, were chosen as detection targets. PMA-qPCR and qPCR as well as culture-based methods were simultaneously used to quantify the concentration of these two fecal indicators through different stages in the wastewater treatment process in three full-scale WWTPs in China. Detection results obtained by two PCR-based methods and the culture-based method were compared and the relationships among PMA-qPCR, qPCR and culture-based methods were evaluated for the WWTP samples.

1 Material and methods

1.1 Sample collection and pretreatment

All samples from WWTPs were collected in three full-scale WWTP in Beijing (A and B) and Wuxi (C), China. For practical detection, raw wastewater (after coarse screening), primary effluent, secondary effluent, and sludge from a primary sedimentation tank (sludge 1) and secondary sedimentation tank (sludge 2) were collected using sterile plastic containers. Bacterial pellets were harvested from 15 mL raw wastewater, 15 mL primary effluent, 400 mL secondary effluent, 500 μL sludge 1, and 500 μL sludge 2 by centrifugation at 12,000 r/min for 10 min at 4°C and then re-suspended in a light-transparent 1.5-mL microcentrifuge tube by adding 500 μL sterile phosphate buffered saline (PBS) buffer prior to storage at –20°C for future use.

1.2 PMA treatment and DNA extraction

PMA (Biotium, USA) was dissolved in 20% dimethyl sulfoxide (DMSO) (Ameresco, USA) with the concentration of 20 mmol/L and stored at –20°C in the dark. A volume of PMA stock solution was added to the prepared wastewater samples in order to make a final PMA concentration of 100 μmol/L. All the micro-centrifuge tubes were incubated in the dark for 5 min with occasional thorough mixing and then laid horizontally on ice with the more transparent side facing upwards towards a 650-W halogen light source (GE lighting, USA) for 4 min. The distance between sample tubes and light source was 20 cm. The ice box was shaken during the light exposure in order to ensure that every single droplet received equally good light exposure. After light exposure, the bacteria were harvested by centrifugation at 10,000 r/min for 8 min prior to DNA extraction. Cell lysis was achieved by bead beating using a Mikro-Dismembrator instrument (Sartorius, Germany) at 2500 r/min for 20 sec. Then the DNA was extracted using the FastDNA® Spin Kit for Soil (MP Biomedicals, USA) according to the manufacturer's instructions. Extracted DNA were eluted in 100 μL DES (provided in the kit) and stored at –20°C for future analysis.

1.3 Quantitative PCR assay

Primers and probes used in quantitative PCR were chosen from previous published articles (**Table 1**). SYBR® Green quantitative PCR was used for *E. coli* detection whereas Taqman® quantitative PCR was used for *Enterococci* detection. Primer specificity was validated by

Table 1 Gene targets, qPCR primers and probes used for qPCR detection

Primers or probe	Target gene	Sequence (5′-3′)	Product length (bp)	Reference
E. coli	*uid*A		167	Heijnen et al., 2006
UAL1939b		ATGGAATTTCGCCGATTTTGC		
UAL2105b		ATTGTTTGCCTCCCTGCTGC		
Enterococci	23S rRNA		86	Haugland et al., 2005
ECST748F		AGAAATTCCAAACGAACTTG		
ENC854R		CAGTGCTCTACCTCCATCATT		
GPL813TQ		FAM-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA		

searching for similar microbial genome sequences using the Basic Local Alignment Search Tool (BLAST) program (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/BLAST/).

All the quantitative PCR reactions were performed in a Bio-Rad iQ5 iCycler (Bio-Rad, USA). For the *uidA* gene of *E. coli* , each 20 μL reaction mixture contained 2 μL of template DNA, 10 μL of SYBR® Premix Ex Taq™ (TaKaRa, China), 0.8 μL of each primer (400 nmol/L final concentration), and 6.4 μL of double-distilled H_2O (ddH_2O). The cycling parameters were 10 sec at 95°C for pre-incubation and denaturation of the DNA template, followed by 40 cycles of 95°C for 5 sec for denaturation, 60°C for 20 sec for annealing, and 72°C for 15 sec for amplification. The Taqman® quantitative PCR reaction for *Enterococci* was performed in a 25 μL reaction mixture containing 5 μL DNA template, 12.5 μL of Premix Ex Taq™ (TaKaRa, China), 2.5 μL of each primer (1 μmol/L final concentration), 1 μL probe (400 nmol/L final concentration) and 1.5 μL of dH_2O. The cycling parameters were 30 sec at 95°C for pre-incubation and denaturation of the DNA template, followed by 40 cycles of 95°C for 15 sec for denaturation, 60°C for 120 sec for annealing, and 72°C for 30 sec for amplification. In every quantitative PCR run, negative (no template) controls were processed as a routine quality control of the assay. Whole genomic DNA from *Enterococci* and plasmid DNA from an *E. coli* clone harboring the *uidA* gene were used to generate standard curves for the *Enterococci* and *E. coli* quantitative PCR assays, respectively.

1.4 Effects of WWTP sample matrix on PMA cross-reaction

In the present study, the effects of the WWTP sample matrix on the PMA cross-reaction were analyzed by spiking non-viable *E. coli and Enterococci* into different WWTP samples. Due to the cost and labor constraints, we chose samples from WWTP A as representatives and the suspended solid concentrations were 85, 5, 21500 mg/L, and 6580 mg/L in the influent, second effluent, sludge 1 and sludge 2, respectively. No suspended solid could be detected in the PBS buffer. The WWTP samples included 1 mL influent, 1 mL secondary effluent, 500 μL sludge 1 and 500 μL sludge 2. Approximately 10^7 CFU/μL *E. coli* and *Enterococci* were thermally inactivated at 95°C for 10 min, and then 1 μL of inactivated bacteria were spiked in the autoclaved WWTP samples as described above and 500 μL sterile PBS buffer as a control matrix. Then all the samples were centrifuged at 10,000 r/min for 8 min at 4°C to harvest bacterial cells, and then re-suspended in a light-transparent 1.5-mL micro-centrifuge tube by 500 μL sterile PBS buffer. The WWTP samples with and without seeding DNA of *E. coli* and *Enterococci* were harvested and then detected by both PMA-qPCR and qPCR in parallel. All the samples were analyzed at least in duplicate.

1.5 Culture-based quantification and detection of *E. coli* and *Enterococci* in water samples

For the culture-based methods, the membrane filtration method was performed to enumerate *E. coli* and *Enterococci* according to EPA Method 1103.1 (U.S. EPA, 2002) and Method 1600 (U.S. EPA, 2002), respectively. Samples were serially diluted and filtered through 0.45 μm pore size (47 mm diameter) nitrocellulose membranes, then the membranes were aseptically removed from the filter base and placed on membrane-thermotolerant *E. coli* (mTEC) agar and membrane-*Enterococcus* indoxyl-D-glucoside (mEI) agar (Becton Dickinson, USA) for quantification of *E. coli* and *Enterococci*, respectively. The mTEC agar plates were incubated at 44.5°C for 24 hr while mEI agar plates were incubated at 41°C for 24 hr. All the samples were tested at least in duplicate.

1.6 Statistical analysis

One-way ANOVA was performed to evaluate the difference between the Cycle threshold (Ct) values of spiked distilled water and those of different samples in WWTP, and a paired-samples *t* test was performed to evaluate the difference between detection results by PMA-qPCR, qPCR and culture-based methods using SPSS 16.0 software (SPSS Inc., USA). Regression analysis was performed to evaluate the linear correlation between PMA-qPCR/qPCR and culture-based methods using Microsoft Excel software (Microsoft Inc., USA).

The reductions of *E. coli* and *Enterococci* in WWTPs by primary and secondary treatments were determined using

the following equation:

$$\log_{10}(\text{reductions}) = \log_{10}\frac{N_0}{N_t}$$

where, N_0 (CFU/L) is the concentration of *E. coli* or *Enterococci* before the water treatment process, and N_t (CFU/L) is the concentration of *E. coli* or *Enterococci* after the water treatment process.

2 Results and discussion

2.1 Quantitative PCR standards

The standard curves in our study have a linear range of quantification from 1.3×10^1 to 1.3×10^7 copies/reaction and from 4.6×10^1 to 4.6×10^6 copies/reaction for *E. coli* and *Enterococci*, respectively (data not shown). The amplification efficiencies were between 90%–110%, with $R^2 > 0.99$. For SYBR quantitative PCR, the peaks of the melting curve were (87 ± 0.5)°C for *E. coli*, indicating correct and specific amplifications of PCR products. Gel electrophoresis also confirmed that DNA of the expected size were amplified using the standard DNA of *E. coli* and *Enterococci*, respectively. To assess the specificity of the primers, 3 other bacteria strains, including *Salmonella, Shigella* and *Aeromonas*, were amplified because of their prevalence in wastewater samples, and no amplification occurred in these reactions (data not shown).

2.2 Effects of WWTP sample matrix on PMA treatment in removing DNA from non-viable cells

The effects of WWTP sample matrix on PMA treatment was analyzed by spiking approximately 10^7 copies of inactivated *E. coli* and *Enterococci* into samples collected from WWTP and 500 μL sterile PBS buffer. As shown in **Fig. 1**, no *E. coli* and *Enterococci* were detected by both direct qPCR and PMA-qPCR assays in secondary effluents, and the initial amounts of these two fecal indicators in the influent, sludge 1 and 2 were about 10^2–10^3 copies per sample, which were less than the spiking numbers of *E. coli* and *Enterococci*.

With about 10^7 copies of thermally inactivated *E. coli* and *Enterococci* (95°C, 10 min) in the WWTP samples and PBS buffer, the numbers of *E. coli* and *Enterococci* cells determined by conventional qPCR were as high as 9.3×10^5–7.5×10^6 copies. In contrast, PMA-qPCR resulted in a varying reduction of this "false positive" detection result for different sample matrices, indicating a significant overestimation of viable cells by qPCR without PMA treatment in these sample matrices. PMA treatment prior to DNA extraction clearly reduced the impact of DNA from non-viable bacteria in samples. Deducting the initial amounts of *E. coli* and *Enterococci* in WWTP samples, the numbers of *E. coli* cells determined by PMA-qPCR were 2.61, 2.56, 2.10, 2.12, and 2.83 $\log_{10}$ units less than those determined by qPCR, and the numbers of *Enterococci* cells determined by PMA-qPCR were 2.34, 2.36, 2.22, 2.05, 2.78 $\log_{10}$ units less than those determined by qPCR, in influent, secondary effluent, sludge1, sludge 2 and PBS buffer, respectively. For sludge from the primary sedimentation tank (sludge 1) and secondary sedimentation tank (sludge 2), the reductions for both *E. coli* and *Enterococci* by PMA-qPCR were obviously less than the bacteria spiking in PBS, indicating that the PMA-qPCR reaction was inhibited in sludge samples, and PMA-qPCR could still lead to overestimation of pathogens in this sample matrix.

The PMA-qPCR method has been validated as an effective molecular tool that was able to selectively detect viable cells in simple matrices like PBS buffer (Nocker et al., 2007, 2009a). For environmental samples such as those collected from WWTPs, however, the primary concern

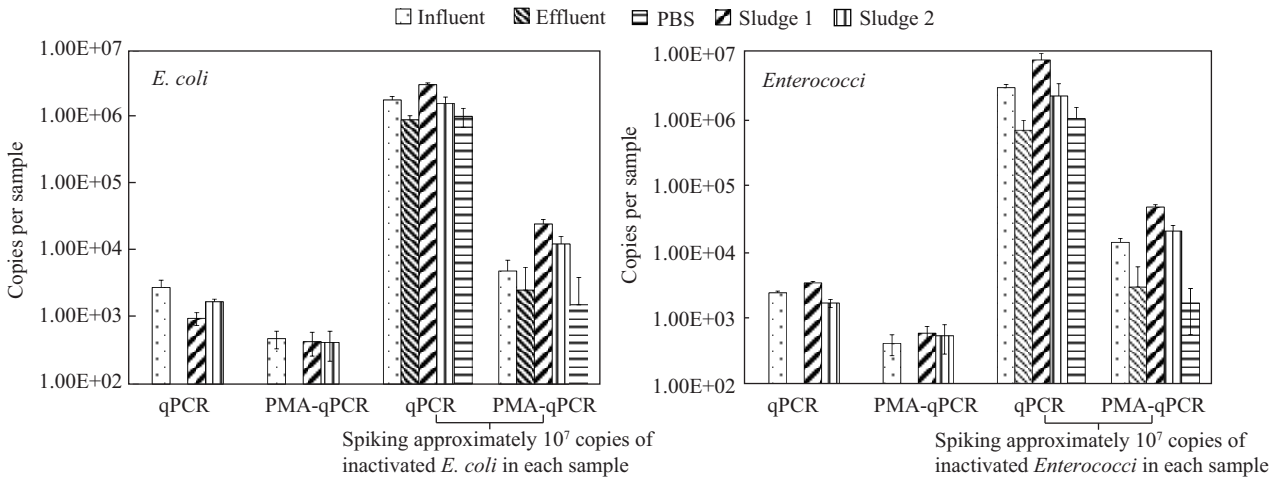

Fig. 1 Detection of *E. coli and Enterococci* by qPCR and PMA-qPCR in WWTP samples and samples with approximately 10^7 copies of non-viable *E. coli and Enterococci* spiked. No *E. coli and Enterococci* were detected in secondary effluent and PBS buffer. All the samples were analyzed at least in duplicate.

lies in the possible adverse effects of sample matrices on the effectiveness of PMA in removing DNA from non-viable bacteria. Our study confirmed that WWTP sample matrices did reduce the effectiveness of PMA treatment, because PMA performed better in PBS buffer than in all the environmental samples tested. A previous study also noticed a similar phenomenon. Varma et al. (2009) reported that primary treatment samples in WWTPs affected the effectiveness of PMA. Also, Wagner et al. (2008) observed a huge discrepancy between detection results by plate count and PMA-qPCR in fermenter sludge exposed to a heat treatment of 50°C for more than 10 hr. The possible explanation of the adverse effect posed by sample matrices may be the presence of suspended solids that prevent light from penetrating into samples and thus inhibit the key light-induced PMA cross-linking. In our study, the most significant adverse effect on the effectiveness of PMA was found in sludge from the primary tank with the highest suspended solid concentration and the darkest appearance. Bae and Wuertz (2009) systematically analyzed the effects of PMA concentration, exposure time, and suspended solid concentration on PMA treatment and found that suspended solid concentration had the most significant impact on the difference observed between viable and non-viable bacterial cells after PMA treatment.

2.3 Effects of PCR inhibitors in WWTP samples

PCR inhibitors in environmental samples may be a barrier to accurate qPCR detection. In previous studies, PCR inhibitors were present in surface water, animal fecal and sewage samples (Ahmed et al., 2009a, 2009b). Thus, in our study we tested the presence of PCR inhibitors in all three kinds of samples in WWTPs. In the present study, only the *uid* gene for *E. coli* was tested for the effects of PCR inhibitors as a representative. When undiluted DNA was used, the Ct values were 18.4 ± 0.6, 18.3 ± 0.3, 18.0 ± 0.3, 18.0 ± 0.5 for influent, primary effluent, secondary effluent and distilled water, respectively (**Table 2**). For undiluted DNA, there were no significant differences observed between the Ct values of all the tested samples ($p > 0.05$). For serially diluted DNA (10-fold, 100-fold and 1000-fold) of all samples, no significant differences were observed either between their Ct values and that of spiked distilled water ($p > 0.05$). These results indicated that the undiluted DNA from all the samples did not contain inhibitors that could substantially inhibit the following PCR amplification. However, it should be noted that PCR inhibitors may function differently in inhibiting PCR amplification for different primers (Ahmed et al., 2009b).

2.4 Correlations of PMA-qPCR, qPCR and culture-based methods for detection of *E. coli* and *Enterococci* in WWTP waters

The results and correlations of *E. coli* and *Enterococci* detected by PMA-qPCR, conventional qPCR and culture-based methods in WWTPs water samples are shown in **Fig. 2**. Linear relationships were observed between the two PCR-based methods and the culture-based methods. The monitoring results showed that both conventional qPCR and PMA-qPCR lead to a higher fecal indicator concentration than the culture-based methods. However, the PMA-qPCR slightly improved the linear correlation and the results obtained by PMA-qPCR assay were closer to those obtained by culture-based methods, for both *E. coli* (linear slope of 1.1149 and 1.1039 for conventional qPCR and PMA-qPCR, respectively) and *Enterococci* (linear slope of 1.1765 and 1.0747 for conventional qPCR and PMA-qPCR, respectively), indicating that PMA pretreatment did reduce the amount of naked DNA or that from non-viable cells in practical samples in WWTP. The paired-sample *t* test reveals that detection results obtained by qPCR and those by culture-based methods are significantly different ($p < 0.05$ for both *E. coli* and *Enterococci*) while the differences are not obvious between detection results obtained by PMA-qPCR and those by culture-based methods ($p > 0.05$ for both *E. coli* and *Enterococci*).

In previous studies, many researchers reported that results by qPCR and culture-based methods have an obvious positive correlation but they also point out that DNA from non-viable cells largely accounts for the detection results, making the qPCR incapable of accurately assessing microbial contamination in water samples (Haugland et al., 2005; Lavender et al., 2009). Accordingly, PMA treatments were applied prior to DNA extraction in order to decrease the naked DNA and DNA from non-viable cells extracted from the WWTP samples. Our study showed that the PMA-qPCR assay did result in an obviously lower detection result than qPCR, indicating a fraction

Table 2 Evaluation of PCR inhibitors on qPCR detection in real samples from WWTP

Sample	Threshold cycle (Ct) value in different samples			
	Undiluted DNA	10-fold dilution	100-fold dilution	1000-fold dilution
Distilled water	18.0 ± 0.5	NA	NA	NA
Influent	18.4 ± 0.6	17.8 ± 0.1	17.8 ± 0.1	17.9 ± 0.1
Primary effluent	18.3 ± 0.3	18.0 ± 0.3	18.0 ± 1.1	18.2 ± 0.1
Secondary effluent	18.0 ± 0.3	18.2 ± 0.2	17.9 ± 0.4	17.9 ± 0.4

NA: not available.

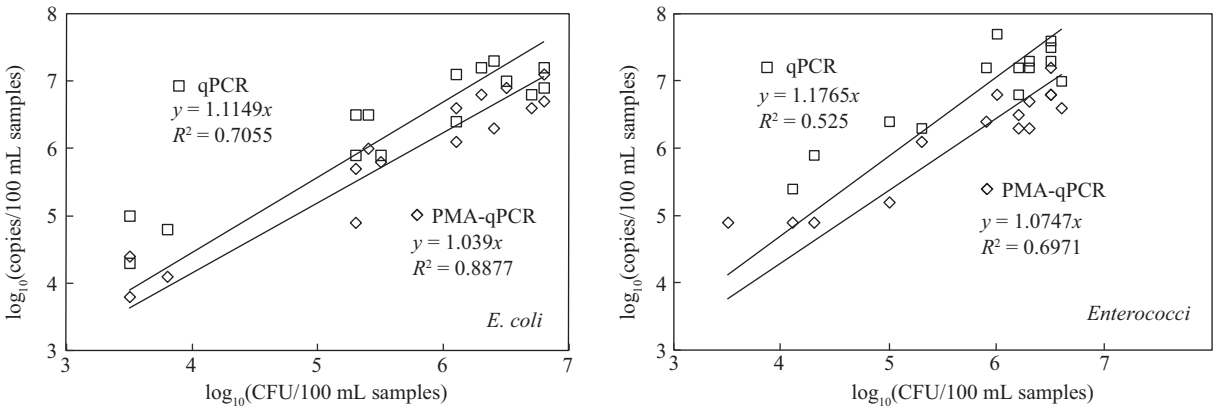

Fig. 2 Correlations between results detected by PMA-qPCR, qPCR and culture-based methods for *E. coli* and *Enterococci* by regression analysis (n = 15) in WWTP waters.

of DNA from non-viable cells was present in WWTP samples. This is contrary to the assumption that wastewater has high metabolic activity that permits rapid cycling of DNA from non-viable cells (Wéry et al., 2008), therefore additional procedures such as PMA treatment prior DNA extraction are necessary in order to accurately assess the fecal indicator or pathogen concentrations within WWTP by qPCR. Also, the monitoring results by PMA-qPCR are highly correlated to, but not obviously different from those by culture-based methods, giving PMA-qPCR a promising potential to be used as a reliable complement to culture-based methods that reflects a more reasonable fecal indicator concentration.

Although PMA-qPCR assay is shown to be more advantageous than qPCR, there is a long way to go for application of PMA-qPCR in routine fecal indicator or pathogen monitoring. Our study shows that some sample matrices such as sludges may largely inhibit the effectiveness of PMA and make PMA-qPCR not able to differentiate viable and non-viable cells in these samples. Thus, PMA-qPCR may not be fit for pathogen detection in some samples (e.g. with high concentrations of dark particles or inhibitor substances) due to the effects posed by sample matrices. Furthermore, removal of DNA from non-viable cells by PMA is based on PMA's ability to penetrate the compromised cell membranes. However, many disinfection methods such as UV radiation rely on directly destroying DNA in bacterial cells instead of the cell membrane. In these cases, PMA treatment may be ineffective in removing DNA from non-viable cells since their cell membranes are still intact (Nocker et al., 2007). Recently, one study (Süß et al., 2009) showed that qPCR could detect the UV-induced reduction of bacterial numbers in wastewater, indicating that qPCR alone may be a more proper alternative for pathogen detection when the mechanism of pathogen activation is based upon DNA break-up induced by UV radiation. The concept of active-labile compound (ALC) was also raised in order to eliminate the drawbacks of PMA (Nocker et al., 2009b).

2.5 Reductions of *E. coli* and *Enterococci* in WWTPs determined by PMA-qPCR, qPCR and culture-based method

As shown in **Table 4**, the concentrations of both fecal indicators were clearly reduced by the secondary treatments. For *E. coli*, the average reductions through WWTP are 1.63 ± 0.79, 1.68 ± 0.63, and 1.91 ± 1.01 $\log_{10}$ units, obtained by PMA-qPCR, qPCR and culture-based method, respectively. For *Enterococci*, the average reductions through WWTP are 1.32 ± 0.92, 1.51 ± 0.55, and 1.84 ± 0.68 $\log_{10}$ units, obtained by PMA-qPCR, qPCR and culture-based methods, respectively. All three analytical methods demonstrate that the concentrations of fecal indicators of primary effluent are no less or even higher than those of the influent in most cases, indicating that it is secondary treatment but not primary treatment that plays a main role in reducing pathogens in WWTP **(Table 3)**.

Wastewater treatment is mainly designed to remove organic or nutrient chemical pollutants contained in influent but also has the function of reducing the numbers of fecal indicators and pathogens in its effluent. In the present study, *E. coli* and *Enterococci* were obviously reduced though wastewater treatment, revealed by both of two molecular biological tools as well as culture-based methods, but our reductions are less than those reported by Lavender et al. (2009) in which more than 2.5 $\log_{10}$ reductions for both *E. coli* and *Enterococci* were achieved within WWTP, indicating a geographical difference in fecal indicator reduction through WWTP. PMA-qPCR obtained a reduction for both fecal indicators very close to that of a culture-based method, but qPCR still achieved similar reduction results. This could be explained by the fact that DNA from non-viable cells was reduced through various treatment processes in WWTP, which was also observed by Lavender et al. (2009). Since no UV disinfection was applied on the WWTP samples in this study, this reduction of ambient DNA in WWTP samples is probably due to the DNA break-up by exposure to sunlight

Table 3 Reductions of *E. coli* and *Enterococci* in WWTPs determined by PMA-qPCR, qPCR and culture-based method

Detection method	Reductions of *E. coli* (log_{10} units)		Reductions of *Enterococci* (log_{10} units)	
	Primary treatment	Secondary treatment	Primary treatment	Secondary treatment
Culture assay	0.05 ± 0.19	1.91 ± 1.01	0.04 ± 0.25	1.84 ± 0.68
PMA-qPCR	–0.38 ± 0.20	1.68 ± 0.63	0.08 ± 0.23	1.51 ± 0.55
qPCR	–0.23 ± 0.38	1.63 ± 0.79	–0.14 ± 0.36	1.32 ± 0.92

(Bae and Wuertz, 2009) or absorbing to the settleable matters in the secondary sedimentation tank. The primary treatment process has almost no effectiveness in reducing concentrations of fecal indicators. Due to the potential fecal indicator propagation and release from feces during primary treatment, even an increased concentration was observed in many cases. Key et al. (2008) also reported a similar result, that primary settlement produces a negligible reduction in fecal indicator concentration. Thus our result is in agreement with the assumption that primary treatment contributes little to remove bacterial pathogens from wastewater (Asano et al., 1997) and pathogen removal in WWTP mainly occurs in secondary or more advanced treatment processes.

3 Conclusions

In this study, the PMA-qPCR assay was established, and the effects of WWTP sample matrix on PMA treatment were also evaluated for detection of viable bacteria in WWTP samples. PMA-qPCR, qPCR as well as culture-based assays were simultaneously applied to quantify the concentration of two bacteria (*E. coli* and *Enterococci*) through different stages in the wastewater treatment process in three full-scale WWTPs in China.

(1) PMA-treatment removed more than 2 log_{10} units (99%) DNA from non-viable cells in WWTP sample matrices including influent, primary effluent, secondary effluent and sludge from the secondary sedimentation tank under the experimental conditions in our study. Sludge from the primary sedimentation tank largely inactivated the effectiveness of PMA in removing DNA from non-viable bacterial cells.

(2) Compared to conventional qPCR, PMA-qPCR results were closer and highly linearly correlated to those obtained by culture-based methods, indicating that PMA treatment obviously reduced DNA from non-viable cells in full-scale WWTP samples.

(3) *E. coli* and *Enterococci* were both reduced through the wastewater treatment process. PMA-qPCR and qPCR result in a different concentration but similar reduction results of fecal indicators, indicating that DNA from non-viable cells is present in WWTP samples and reduced in the wastewater treatment process.

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