Title: Quantitative real-time PCR analysis of total and propidium monoazide-resistant fecal indicator bacteria in wastewater

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ABSTRACT

A real-time quantitative PCR (qPCR) method and a modification of this method incorporating pretreatment of samples with propidium monoazide (PMA) were evaluated for respective analyses of total and presumptively viable *Enterococcus* and *Bacteroidales* fecal indicator bacteria. These methods were used in the analyses of wastewater samples to investigate their feasibility as alternatives to current fecal indicator bacteria culture methods for predicting the efficiency of viral pathogen removal by standard treatment processes. PMA treatment was effective in preventing qPCR detection of target sequences from non-viable cells. Concentrates of small volume, secondary-treated wastewater samples, collected from a publicly owned treatment works (POTW) under normal operating conditions, had little influence on this effectiveness. Higher levels of total suspended solids, such as those associated with normal primary treatment and all treatment stages during storm flow events, appeared to interfere with PMA effectiveness under the sample preparation conditions employed. During normal operating conditions at three different POTWs, greater reductions were observed in PMA-qPCR detectable target sequences of both *Enterococcus* and *Bacteroidales* than in total qPCR detectable sequences. These reductions were not as great as those observed for cultivable fecal indicator bacteria in response to wastewater disinfection. Reductions of PMA-qPCR as well as total qPCR detectable target sequences from enterococci and, to a lesser extent, *Bacteroidales* correlated well with reductions in infectious viruses during both normal and storm flow operating conditions and therefore may have predictive value in determining the efficiency at which these pathogens are removed.

1. INTRODUCTION

The Beaches Environmental Assessment and Coastal Health (BEACH) Act was passed by the U.S. Congress in 2000 as an amendment to the Clean Water Act (CWA) with a directive for the U.S. EPA to establish more expeditious methods for the timely detection of pathogens and pathogen indicators in coastal waters, as well as guidance for State application of new water quality criteria based on these methods. It was further expected that these criteria and methods should demonstrate utility for and be compatible with all CWA 304(a) criteria needs including: water quality assessment for public notification at beaches; assessment of impaired waters listings; development of total daily maximum loads (TMDL); and development of National Pollution Discharge Elimination System (NPDES) permits. Considerable progress has been made in the development of a real-time PCR based method for monitoring DNA target sequence concentrations from enterococci fecal indicator bacteria that meets the CWA 304(a) requirements for water quality assessment at beaches (Haugland et al., 2005; Wade et al., 2006; Wade et al., 2008). Questions remain, however, about the applicability of this method for the other CWA 304(a) criteria needs, most notably for NPDES permitting. These questions stem largely from the recognition that real-time PCR techniques do not distinguish between DNA sequence targets from live and dead microorganisms and thus may not be sensitive to the reductions in fecal indicator bacteria that are achieved by standard wastewater treatment practices. Such practices often include disinfection of the final effluents which may be essential for publicly owned treatment works (POTWs) to meet current effluent

water quality criteria in their NPDES permits. To date, experimental evidence from one study has supported this concern about the difference in reductions of fecal indicator bacteria during wastewater treatment as determined by real-time qPCR and culture analysis (He and Jiang, 2005**)** although it was concluded in another report that measurements of selected bacterial pathogens in treated wastewater by a qPCR method were within governmental guidelines (Shannon et al., 2007). Consequently, there is a need for additional data on the relationship between results of these two methodologies in analyses of wastewaters.

Recent modifications of the qPCR technique involving pretreatment of samples with the intercalating dyes, ethidium monoazide (EMA) or propidium monoazide (PMA) prior to DNA extraction have been reported to allow detection of DNA only from viable microorganisms (Nocker and Camper, 2006; Nocker et al., 2006; Nocker et al., 2007; Rudi et al., 2005). This approach is based on the ability of microorganisms with intact cellular membranes to resist the penetration of these dyes whereas DNA in non-viable microorganisms with permeable membranes or extracellular DNA is available for interaction with the dyes and is unable to be amplified by PCR following light-induced cross-linking. Another study has indicated that DNA from chemically disinfected microorganisms is susceptible to this treatment (Nocker et al., 2007) thus suggesting that this technique might provide accurate assessments of the efficacy of chemically-based wastewater disinfection processes.

We report here on an adaptation of the PMA-qPCR technique into a method for measuring target sequences from enterococci and *Bacteriodales* fecal indicator bacteria and compare its results with those of a conventional real-time qPCR method in analyses

of live and killed laboratory cultured cells in both buffer and wastewater matrices. Previous studies have indicated that while pre-treatment of killed bacterial cells with either EMA or PMA prior to DNA extraction can result in several log reductions in qPCR detectable target sequence levels, PMA is preferable for allowing target sequence detection from live cells of a wider range of bacterial species due the greater ability of EMA to penetrate intact cellular membranes of some organisms (Nocker et al., 2006). For this reason, PMA was used for the analysis of *Enterococcus* and *Bacteroidales* fecal indicator bacteria in this method. These earlier studies also provided guidance in the selection of the dye concentration and light exposure time used in the method.

We further report on results from analyses of samples collected from different wastewater treatment stages at three POTWs by the qPCR and PMA-qPCR methods and compare them with corresponding levels of cultivable enterococci and fecal coliforms. These analyses and comparisons were made possible by the access of samples and culture data from a joint study conducted by the Interstate Environmental Commission and the U.S. EPA, National Risk Management Research Laboratory to examine the impact of wastewater blending during stormflow events on the efficacy of chlorine disinfection (Rukovets et al., 2008). Corresponding samples and data collected from these facilities during normal dry weather operations were also made available through this study. In addition to cultivable bacterial indicators, data for selected viruses and bacteriophage were collected from two of the POTWs. The availability of these data allowed us to also examine the correlation between reductions of the bacterial indicator DNA target sequences, as determined by both of the qPCR methods, with reductions of cultivable

viral pathogens through each stage of wastewater treatment at these two POTWs under both normal dry weather and storm flow wet weather operating conditions.

2. MATERIALS AND METHODS

2.1. Bacterial cell cultures. *E. faecalis*, strain 29212 from the American Type Culture Collection (ATCC, Manassas, VA) was grown in brain heart infusion broth (Difco, Detroit, MI) for 24 hr at 37EC. *Bacteroides thetaiotaomicron*, strain ATCC 29741 (Microbiologics, Inc., St. Cloud, MN) was grown anaerobically in chopped meat carbohydrate medium (Remel, Lenexa, KS) for 72 hr at 37EC. All cultures were harvested by centrifugation at 6,000 *x g* for 5 min, washed twice using sterile phosphate buffered saline (PBS) and suspended in the same volume of PBS as the original culture volume for immediate use. Aliquots of the cell suspensions were heat-killed by exposure to 85^{o}C in a water bath for 15 minutes. Cultivable *E. faecalis* cell concentrations in the fresh and heat-killed cell suspensions were determined by plating appropriate serial dilutions of the suspensions on brain heart infusion agar and counting colony forming units (CFU) after 24 hr incubation of the plates at 42EC. Cultivable *B. thetaiotaomicron* cell concentrations were similarly determined by plating on 5% defibrinated sheeps blood agar (Becton Dickinson, Franklin Lakes, NJ) and counting CFU after anaerobic incubation of the plates in an EZ gas pak^{TM} chamber system (Becton Dickinson) at 35EC for 48 hr.

2.2. Wastewater samples. The scheme for collection of wastewater samples during normal and stormflow operations at each of the three POTWs investigated in this study is shown in Fig.1. Detailed flow data during the sampling events, procedures used for

sample collection and non-qPCR analyses of these samples and non-qPCR analytical results can be found in a report by the Interstate Environmental Commission (Rukovets et al., 2008). Briefly, during each sampling event, three grab influent (SP1), three grab primary effluent (SP2), three grab pre-chlorinated secondary effluent (SP3) and three grab chlorinated secondary effluent (SP4) samples were collected at 45-minute intervals. A 250 ml portion of each sample from selected sampling events was shipped on ice for next day delivery to the U.S. EPA Laboratory in Cincinnati for qPCR analyses. Total suspended solids (TSS) levels were determined in the samples by Standard Method 2540 D (American Public Health Association, 1998), and mean levels for only those samples that were also examined by qPCR analyses in this study are shown in Table 1.

2.3. PMA treatment and preparation of samples for DNA extraction. PMA (phenanthridium,3-amino-8 azido-5-[3-(diethylmethylammonio)propyl]-6-phenyldichloride (Biotium, Hayward, CA) was dissolved in 0.2% DMSO to make stock solution concentrations of 100 – 300 μM and aliquots were transferred to light-impermeable 1.5 ml micro-centrifuge tubes for storage at -20^{o}C. All manipulations of PMA stock solutions were performed under minimal light. Fresh or heat-killed bacterial cell suspensions were diluted to desired concentrations in PBS and 1 ml aliquots were transferred to 2 ml light transparent microcentrifuge tubes (Costar, Corning, NY # 3213). The cell suspensions were micro-centrifuged at 14K RPM for 5 minutes and the supernatants removed by pipeting. Cell pellets intended for analysis by the direct qPCR method without PMA treatment were suspended in 200 μl AE buffer (Qiagen, Valencia, CA) for subsequent DNA extraction as described below. Cell pellets intended for analysis by the PMA-qPCR method were re-suspended in 100 μl of freshly-thawed PMA stock

solution and held for 5 min in the dark with periodic mixing. The sample tubes were then exposed horizontally on ice for 2-5 minutes to a 650W, 120V tungsten halogen lamp (B & H PhotoVideo, New York, NY, #GBFAD). After light exposures, 100ul of AE buffer was mixed with each sample prior to DNA extraction as described below. Undiluted 1 ml wastewater samples and negative control PBS samples with no cells added were processed in the same manner.

2.4. DNA extraction and purification. PMA treated and non-treated samples, prepared as described above, were mixed with 200 µl AE buffer containing 0.4 µg/ml Salmon testes DNA (Sigma-Aldrich, #D1626, St. Louis, MO) and total DNA was extracted by a previously described bead-milling and centrifugation procedure (Haugland et al., 2005). DNA in the crude samples was purified using a commercially available silica column adsorbtion kit (DNA-EZ, GeneRite Inc, North Brunswick,, NJ). Briefly, this procedure involved mixing 200 µl of supernatants from the bead-milled samples with 600 µl of binding buffer from the kit and transferring these mixtures to the silica columns. Micro-centrifugation for 1 min at 14K RPM was performed to pass the samples through the columns and allow DNA binding. The columns were then washed twice with 500 µl of wash buffer from the kit and the DNA was then eluted twice with 100 µl AE buffer by centrifugation in the same manner and the two eluates from each sample were pooled for qPCR analysis.

2.5. QPCR assays and analyses. Primer and probe sequences for the detection of total enterococci DNA have been previously described (Haugland et al., 2005). A modified primer and probe assay was used for the detection of target sequences from total *Bacteroidales* as previously described (Siefring et al., 2008). All reactions used

hydrolysis probe (TaqMan®) chemistry and all probes were labeled with 6-FAM as the 5'-terminal reporter dye and TAMRA as a quencher dye at their 3'-terminus.

Analyses were performed using an ABI Model 7900 HT sequence detector (ABI, Foster City, CA). Reagent mixes were prepared by combining 12.5 µl of TaqMan® Universal Master Mix (a 2X concentrated, proprietary mixture of AmpliTaq Gold™ DNA polymerase, AmpErase® UNG, dNTPs, passive reference dye and optimized buffer components, ABI) with 2.5 µl of 2 mg/ml bovine serum albumin (fraction V, GibcoBRL, Gaithersburg, MD), 3.5 µl of a mixture of forward and reverse primers (5 µM each) and 400 nM TaqMan® probe and 1.5 µl distilled water per reaction. For each reaction, 20 µl of reagent mix was combined with 5 µl of DNA extract. Reactions were performed in 96-well optical plates, covered with optically transparent sealing tape, with programmed thermal cycling conditions consisting of 2 min at 50EC (to allow inactivation of any potential contaminating carryover PCR amplicons in the reaction by the AmpErase® UNG), 10 min at 95EC (to allow inactivation of the AmpErase® UNG and activation of the hot start DNA polymerase system), followed by 40 cycles of 15 sec at 95EC and 1 min at 60EC. The 9600 emulation option was used to control ramping times between programmed temperatures on the instrument. Determinations of cycle threshold (C_T) were performed automatically by the instrument after manually adjusting the threshold fluorescence values to 0.03 delta Rn units.

2.6. QPCR data analyses. DNA extracts of all samples were tested for acceptable total DNA recovery efficiencies and the absence of PCR inhibitors using a previously described qPCR assay for the salmon DNA that was added to the extraction buffer (Haugland et al., 2005). Quantitative estimates of *Enterococcus* and *Bacteriodales* assay

target sequence copies in the test samples were obtained from master calibration curves, by an approach similar to that described in a previous report (Shanks et al., 2008). *E. faecalis* and *B. thetaiotaomicron* genomic DNAs, used for making the standards, were prepared from washed, undiluted cell suspensions as described above with the addition of a 1 hr, 37^{o} C treatment of the crude DNA extracts with 16.6 ng/ul RNase A (Sigman # D-5006) prior to silica column purification. Total DNA concentrations in these purified stock solutions were spectrophotometrically determined and ribosomal DNA target sequence copy concentrations estimated from reported estimates of the respective genome sizes and rRNA gene copy numbers per genome of these species (Klappenbach et al., 2001). Calibration curve equations used in this study were: $y = -3.37x + 39.0$; and $y = -3.45x + 39.0$, for the *Enterococcus* and *Bacteroidales* assays, respectively. Mean C_T values from analyses of a minimum of two and in most cases three DNA extracts of different test samples per sampling event were used for estimation of target sequence copies from the standard curves.

2.7. Fecal indicator bacteria, bacteriophage and pathogen analyses. Cultivable densities of enterococci and fecal coliform indicator bacteria were determined by Standard Methods 9230 A & B and 9221 A, B, C & D (American Public Health Association, 1998), respectively. Somatic and male-specific coliphages were analyzed by two methods. The first was a modified version of EPA Method 1602 (U.S. EPA 2001b) using E. coli host strain F+amp for male specific and host strain C3000 (ATCC 15597) for both male specific and somatic phage. The second was a version of the large volume presence/absence assay of EPA Method 1601 (U.S. EPA 2001a) was used for analyses of

1 treated effluents. Bacteriophage were enumerated as plaque forming units (PFU) per 100
2 ml of wastewater or by most probable number (MPN) using the presence/absence assay.
3 For analyses of enteric viruses, 1 liter (SP1 and SP2) or 5 liter (SP3 and SP4) of
4 wastewater was passed through 1MDS filters (Cuno, Inc., Meriden, CT). The 1MDS
5 filters were shipped on ice to a commercial laboratory (BCS Laboratories, Inc.,
6 Gainesville, FL) and processed immediately upon receipt. Filters were eluted with 1 liter
7 of 3% BBL beef extract V/Glycine (pH 9.2, 25°C), and the eluates were concentrated by
8 organic flocculation. Infectious viruses were assayed in a portion of the concentrated
9 eluates by passage on Buffalo Green Monkey (BGM), Caco-2, PLC/PRF-5, and MA-104
10 cells. Viral groups reported to be detected by each of these cell lines are as follows:
11 BGM Enterovirus, Reovirus; Caco-2 Enterovirus, Astrovirus; PLC/PRF-5 Adenovirus;
12 MA-104 Enterovirus, Rotavirus, Reovirus. Infectious viruses were identified by
13 observation of cytopathic effects (CPE) and quantified using U.S. EPA's most probable
14 number (MPN) calculator (www.epa.gov/microbes). Positive controls were performed in
15 a designated area using poliovirus serotype 1. Samples were also assayed using an
16 integrated cell culture-PCR method to detect viruses that do not cause CPE in cell
17 culture. These viruses were detected by PCR or RT-PCR according to published methods
18 (Grimm et al., 2004; Oberste et al., 2006; Reynolds 2004; Spinner and DiGiovanni, 2001;
19 Van Heerden et al., 2005).
20 **2.8. Regression and statistical analyses.** The degree to which each potential indicator
21 corresponded to virus removal/inactivation efficacy along the wastewater treatment train
22 was assessed via partial correlation between the respective indicator and virus
23 concentration as determined by the average recovery among the four plaque assays.

Results for each individual plaque assay were determined as well. Inter-POTW variability was first eliminated by centering observed recoveries on the mean for the indicator or virus at each POTW. Analyses were performed on $\log_{10}$-transformed data for normal and storm flow operating conditions individually, given that differences between these operating conditions were anticipated to influence relative concentrations of targets. This indicates how well each indicator may be expected to perform as a surrogate for virus removal in wastewater treatment. Additionally, partial correlations among alternative culture and PCR methods for different indicators were assessed in a similar fashion. Because results are representative of only two POTWs from which virus data were obtained, this was viewed as a screening procedure to identify potential relationships.

3. RESULTS

3.1. QPCR and PMA-qPCR analyses of viable and heat-killed culture cells. As shown in Fig. 2, pretreatment of heat-killed, cultured *E. faecalis* and *B. thetaiotaomicron* cells with 100 μM PMA for 5 min followed by light exposure for 2 min resulted in up to a 3-4 log reduction in detectable target sequences compared to qPCR estimated levels from DNA extracts of the same numbers of cells that were not exposed to PMA. PMA pretreatments of fresh cultured cell samples that were not subject to heat inactivation also resulted in slight mean reductions of approximately 50% in target sequence measurements compared to those of the corresponding untreated cell samples. Treatment of the cells with 300 μM PMA under the same conditions substantially reduced the sensitivity of the qPCR assays. QPCR analyses for the positive control salmon DNA,

added to the samples after the PMA treatments and exposure to light, showed approximately a 70-fold reduction in sensitivity compared to non-PMA-treated samples, suggesting an inhibitory effect by residual PMA in the samples. Longer light exposures of 5 minutes still showed approximately a 60-fold reduction in the sensitivity of the salmon DNA qPCR analyses and hence did not relieve this inhibition. Cell-free, negative control samples that were subjected to the same treatments and DNA extraction method occasionally gave positive signals in the *Enterococcus* and particularly in the *Bacteroides* qPCR analyses. When observed, these signals corresponded to target sequence levels of less than 10 copies per reaction and appeared to result from sporadic contamination of the reactions by aerosolized DNA.

3.2. Comparison of sample concentration techniques in the PMA-qPCR method. As part of the development of a routine method for wastewater sample processing and analysis, the effects of two alternative cell concentration approaches were investigated for their relative effects on the efficiency of target sequence recoveries from 5×10^4 freshly cultured *E. faecalis* cells. As shown in Fig. 3, estimated target sequence levels were not significantly different with or without PMA treatment from *E. faecalis* cell suspensions that were concentrated by centrifugation. Similar target sequence levels without PMA treatment were determined from cells harvested by membrane filtration followed by washing the cells from the membranes and centrifugation. However, the levels of target sequences estimated with PMA treatment from cells harvested in this manner were approximately 5-fold lower. These results suggested that the procedures of filtering the cells or recovering them from the filters reduced their percent viability as assessed by resistance to PMA uptake and qPCR template inactivation. Consequently, the direct

centrifugation procedure, as described in Materials and Methods, was routinely used for concentrating samples prior to PMA treatment and/or DNA extraction throughout the remainder of the study.

3.3. Effects of wastewater matrices on the PMA-qPCR method. Additional preliminary experiments were conducted to examine the effects of concentrating different volumes of wastewater samples on the results of the qPCR and PMA-qPCR analysis methods. In these experiments, 1 and 10 ml aliquots of wastewater samples, collected from POTW 1 at each of the treatment stages specified in Fig 1 during normal dry weather operation, were concentrated by centrifugation and processed with and without PMA pretreatment for qPCR analysis. The overall ratio of *Enterococcus* target sequences recovered from all 10 ml samples compared to all 1 ml samples was 12 to 1 as determined by qPCR analysis without PMA treatment, indicating that the larger sample volumes did not adversely affect the efficiency of total DNA recovery or amplification. As shown in Fig. 4, however, the ratios of target sequences from total and presumptively viable organisms, as determined by the qPCR and PMA-qPCR methods, respectively, remained fairly constant in the 10 ml samples from the different treatment stages, whereas these ratios increased going from the raw wastewater and primary treatment stages to the secondary and disinfection stages in the 1 ml samples. The latter results were more consistent with the cultivable levels of enterococci determined in these samples which decreased from 7×10^3 CFU/ml in the influent samples to 0.4 CFU/ml in the disinfected samples. The unexpectedly low ratios observed for the 10 ml samples suggested that the larger amounts of biomass collected from these volumes of sample may have interfered with the effectiveness of PMA treatment in inactivating target

sequences from nonviable organisms. Consequently, wastewater sample volumes of only 1 ml were routinely processed for qPCR analysis through the remainder of the study.

The effects of the different wastewater sample matrices on target sequence recovery efficiencies and on the effectiveness of PMA treatment in discriminating target sequences from viable cells were further investigated in a series of spiking experiments. In these experiments, 5×10^5 freshly cultured or heat-killed *E. faecalis* cells were added to 1 ml volumes of the POTW 1 wastewater samples described above after the samples had been stored for ~4 weeks to reduce their ambient levels of cultivable enterococci to below detection. These samples, as well as one ml buffer samples spiked in the same manner with fresh or heat-killed *E. faecalis* cells as controls, were processed for qPCR analysis with and without PMA treatment. As shown in Fig. 5, estimated levels of total target sequences, determined without PMA treatment, in the spiked wastewater samples were not substantially different from those obtained from the spiked buffer control samples indicating that the residual levels of sequences from ambient organisms in these wastewater samples did not substantially influence the results. With the exception of the primary treatment samples, these wastewater matrices also did not substantially influence the effectiveness of PMA treatment on inactivating target sequences from the killed spike organisms relative to the buffer controls.

3.4. Fecal indicator and pathogen levels in POTWs during normal operations. Figure 6 and Table 1 in supplementary materials shows the mean densities of *Enterococcus* and *Bacteroidales* target sequences estimated by the qPCR and PMA-qPCR analysis methods at the four stages of wastewater treatment in the three POTWs during normal dry weather operations. Also shown are the corresponding mean densities of *Enterococcus* and fecal

coliform CFU's, different categories of coliphage and viral infectious units (see Materials and Methods for definitions of virus categories), which were determined from measurement results provided by other collaborators in the study (Rukovets et al. 2008). Virus and phage densities were only determined in POTWs 1 and 2. The density estimates or measurements of all indicators and viruses generally declined through the successive treatment stages and showed similar sampling related variability (coefficients of variation provided in supplementary materials). At POTWs 1 and 2, the largest overall reductions occurred in the densities of *Enterococcus* and fecal coliform CFU's which declined by approximately 4-5 $\log_{10}$ units from raw wastewater (SP1) to disinfected effluent (SP4). Somewhat lower reductions of approximately 3 $\log_{10}$ units occurred in *Enterococcus* CFU densities in POTW 3. The smallest overall reductions occurred in coliphage infectious units which dropped by approximately 1-2 $\log_{10}$ units in POTWs 1 and 2. Estimated qPCR target sequence densities of both *Enterococcus* and *Bacteroidales* showed reductions of approximately 2-3 $\log_{10}$ units in POTWs 1 and 2 and slightly lower reductions of approximately 1.5 $\log_{10}$ units in POTW 3, whereas PMA-qPCR determined target sequence densities showed higher reductions of approximately 3-4 $\log_{10}$ units in POTWs 1 and 2 and 2 $\log_{10}$ units in POTW 3. Reductions in infectious unit densities of the different categories of viruses varied somewhat but ranged from approximately 2-4 $\log_{10}$ units in POTWs 1 and 2.

3.5. Fecal indicator and pathogen levels in POTWs during storm flow operations. Figure 7 and Table 1 in supplementary materials shows the indicator and pathogen densities that were determined during wet weather operating conditions at the three POTWs. Under these conditions, primary (SP2) and secondary treated effluents were

blended prior to collection of the SP3 samples (Fig. 1) and then disinfected. Again, viral cell culture group and phage densities were only determined in POTWs 1 and 2. Overall reductions of all indicator and virus densities from raw wastewater to disinfected effluents were lower under these conditions than under normal dry weather operating conditions and in a few instances increases in densities were observed in primary treated wastewaters compared to the raw wastewaters which could indicate interferences in the measurements of the raw samples. All viable indicator (bacteria and phage) and viral densities dropped from raw wastewater (SP1) to disinfected effluent (SP4) by approximately 1.5-2.5 $\log_{10}$ units in POTWs 1 and 2, whereas reductions in *Enterococcus* and *Bacteroidales* target sequence densities by both the qPCR and PMA-qPCR were comparatively lower at approximately 0.5-1.5 $\log_{10}$ units. Similarly, smaller reductions of the viable counts of both bacterial indicator groups as well as the nucleic acid target reductions for both of the indicators by the qPCR and PMA-qPCR methods were observed in POTW 3 in comparison to those seen under normal dry weather operations.

3.6. Correlations between reductions in qPCR target sequence and cultivable fecal indicator densities during wastewater treatment. Table 2 summarizes the correlations between qPCR and PMA-qPCR determined *Enterococcus* and *Bacteroidales* target sequence densities and those of cultivable enterococci and fecal coliforms counts over the four sampling points at POTWs 1 and 2 during normal and storm flow wastewater treatment conditions. Reductions in enterococci target sequences estimated by both qPCR and PMA-qPCR were significantly correlated ($r > 0.7$, $P < 0.05$) with the reductions of each of the two fecal indicator groups through the stages of treatment under both operating conditions. Reductions in *Bacteroidales* target sequences estimated by both

qPCR methods similarly showed significant levels of correlation with reductions of the two fecal indicator groups under normal operating conditions, however, lower and less significant ($P > 0.05$) correlations were observed under wet weather conditions.

3.7. Correlations in reductions of cultivable fecal indicator and qPCR target sequence densities with virus levels during wastewater treatment. Table 3 summarizes the correlations of density changes of the cultivable bacterial indicators, infectious bacteriophage and qPCR and PMA-qPCR detectable bacterial indicator target sequences determined in this study with those of infectious viruses over the four sampling points of POTWs 1 and 2 during normal and storm flow wastewater treatment conditions. Under normal dry weather operating conditions, reductions in each of the indicator densities were highly and significantly correlated ($r > 0.8$, $P < 0.05$) with the reductions in mean viral densities. This significant relationship with mean viral reductions through the treatment stages was maintained by all indicators in the pooled data sets from wet weather operating conditions although *Bacteroidales* PMA-treated target sequences showed somewhat lower correlations with several of the individual viral groups under these conditions.

4. DISCUSSION

4.1. Performance of the PMA-qPCR method. The effects of PMA pretreatment on recoveries of PCR-detectable target sequences from cultured cells of the fecal indicator bacteria *E. faecalis* and *B. thetaiotaomicron* observed in this study were consistent with those previously reported for a number of other bacterial species (Nocker et al., 2006). We observed that pretreatment of live cells from fresh cultures of these organisms with

PMA had minimal effects on estimated levels of target sequences compared with parallel results obtained for untreated cells. Slight losses of target sequences that were observed in the PMA-treated samples as compared with the untreated samples may easily have been related to the presence of subpopulations of dead cells in the stationary phase cultures and/or the retention of extra-cellular DNA adsorbed onto the cells, even after extensive washing of the harvested cell pellets. In contrast, pretreatment of heat-killed cell suspensions of both organisms with PMA resulted in relative reductions of approximately 3-4 $\log_{10}$ units of detectable target sequences. The ~ 1 log lower reductions seen for *B. thetaiotaomicron* may have been related to the observation from culture analysis that a small fraction of these cells, $\sim 10^{-7}$, survived the heat treatment process or possibly from the fact that higher densities of these organisms were analyzed. For both organisms, these reduction levels appeared to be maximum values based on the observation that PMA treatment of heat-killed cells in increasing quantities over this threshold resulted in the detection of corresponding increasing levels of target sequences by both methods. This observation, coupled with the fact that culture plating of even the highest concentration heat-killed *Enterococcus* cell suspensions gave no colony forming units, suggested that the effectiveness of PMA activity may be saturated by increasing cell numbers, at least under the treatment conditions employed in this study.

Evidence for inhibition of PMA activity by increasing biomass was also observed in our preliminary analyses of concentrates from different volumes of wastewater samples from POTW 1. The use of relatively small volume (i.e. 1 ml) wastewater samples for concentration and analysis appeared to minimize this problem, at least in the analysis of secondary and disinfection treatment effluents. This conclusion was supported

by our experiments in which 1 ml samples from the primary, secondary and disinfection treatment effluents of POTW 1 during normal operation were spiked with heat killed *E. faecalis* cells. With the exception of the primary sample which initially contained ~ 6 times higher levels of suspended solids, PMA treatment of killed cells spiked in an effluent sample background resulted in nearly the same reductions as those observed for controls where killed cells were spiked into a simple buffer matrix.

Even using the smaller sample volumes for analysis, the effects of PMA treatment on qPCR detection of target sequences from ambient bacteria in the wastewater samples were not as pronounced as seen with heat-killed pure culture cells. As expected, the highest ratio of target sequences detected by qPCR analysis in untreated over PMA-treated samples was observed in the disinfected samples. However, this ratio still only averaged approximately 10 to 1 in our initial experiment with disinfected samples from POTW 1 and remained fairly consistent in subsequent analyses of the disinfected samples obtained during normal operation of this as well as the other POTWs (Fig 6 and supplementary materials). Furthermore, in analyses of the blended and disinfected samples obtained during wet weather operations at the three POTWs, almost no differences were seen in the target sequences densities detected by qPCR in the untreated and PMA-treated samples (Fig. 7 and supplementary materials). These samples contained, on average, approximately 3-fold higher levels of total suspended solids than the corresponding disinfected samples from normal operations (Table 1) and so an inhibitory influence of increased biomass on PMA activity in these samples can not be precluded. An alternative explanation for these results, particularly in the disinfected samples, could relate to the effectiveness of the POTW disinfection treatments in

rendering bacterial cells in these samples susceptible to PMA-induced DNA inactivation. Previous studies have suggested that treatment with chlorine levels commonly used for wastewater disinfection can stress conventional fecal indicator bacteria in a sub-lethal manner, leading to a non-cultivable state than may be reversible in an appropriate environment (Blatchley et al., 2007; McFeters and Camper, 1983). The cell membranes of these organisms may still be impermeable to penetration by PMA and thus not allow inactivation of their genomic DNA. This scenario is consistent with previously reported culture and PMA-qPCR analysis results of *Salmonella* cells that were treated with different chlorine concentrations (Nocker et al., 2007). These results showed that treatments of the cells with chlorine concentrations that totally eliminated culture counts caused only partial losses of PMA-qPCR detectable target sequences while further increases in chlorine treatment concentrations caused further reductions in detectable target sequences.

4.2. Relationships of qPCR and PMA-qPCR target sequence reductions with reductions of cultivable fecal indicators and pathogens during wastewater treatment and implications for water quality monitoring. Results from this study of three different POTWs are inconsistent with the results previously reported by He and Jiang (2005) in that densities of qPCR detectable target sequences of both *Enterococcus* and *Bacteroidales* as well as densities of the cultivable fecal indicators all showed substantial reductions through the initial, physical waste removal stages of wastewater treatment, i.e. primary and particularly secondary treatment. Our results were more consistent with the previous report, however, in showing that levels of qPCR detectable target sequences

generally were not reduced by the final disinfection processes at these facilities. A PMA-qPCR method was developed and evaluated in this study primarily to determine if this technique would be more responsive in demonstrating the efficacy of wastewater disinfection. A trend toward greater reductions in PMA-qPCR detectable target sequences than total qPCR detectable target sequences was observed at the POTWs under normal dry weather operating conditions. However, these differences were not specifically associated with disinfection and in no instances were reductions in qPCR detectable target sequences of the same magnitude as seen for the cultivable bacterial indicators. While some potential may exist for improving the efficiency of PMA treatment in the presence of suspended solids through methodological improvements (Bae and Wuertz, 2009, Vesper et al., 2008; Yang et al., 2007), there is a strong indication, from the results of this study and others (Nocker et al., 2007), that standard wastewater chlorine disinfection treatments are not as effective in rendering bacteria susceptible to PMA-induced DNA inactivation as they are in reducing the cultivable numbers of these organisms. Ultraviolet light treatment is also growing in popularity at POTWs as a wastewater disinfection procedure and the discrepancy between responses of PMA-qPCR and culture-based enumeration methods may also occur in association with this treatment (Nocker et al., 2007).

Previous studies have suggested that the reductions of cultivable bacterial indicators such as enterococci and fecal coliforms during standard wastewater treatment procedures may be somewhat over-representive of the efficiency of eliminating viral and protozoan pathogens (Blatchley et al., 2007; Bonadonna et al., 2002; Crockett, 2007; Van Heerden et al., 2007). In terms of average total reductions of these cultivable bacterial

indicators compared to viruses, the results from two normally operated POTWs in the present study support this conclusion. Bacteriophage levels have been suggested as a more conservative indicator of viral reductions in treated wastewaters (Lucena et al., 2004; Tree et al., 2003) and, again, this conclusion was generally supported by the results of the present study. Our results also indicated, however, that the reductions of PMA-qPCR as well as total qPCR detectable fecal indicator bacteria target sequences compared favorably with both cultivable bacteria and bacteriophage indicators in terms of their correlations with reductions of viruses during wastewater treatment at these two facilities, particularly during normal operations. These results suggest that measurements of fecal indicator bacteria target sequences by either the PMA-qPCR method or the easier to perform standard qPCR method in wastewater influents and treated effluents at POTWs may have predictive value in determining the efficiency of virus removal. Such a predictive relationship was not apparent between enterococci qPCR target sequences levels and adenovirus levels in the study by He and Jiang (2005). In view of this discrepancy, further studies at additional POTWs and under different operating conditions are needed to determine the consistency of the relationships between qPCR target sequence and pathogen reductions during wastewater treatment and the factors that may affect these relationships.

The results of this study are also consistent with previous epidemiological studies which have shown a strong correlation between qPCR determined levels of bacterial fecal indicators and bather illness rates at POTW impacted beaches (Wade et al., 2006; Wade et al., 2008). Further studies examining the relative persistence of qPCR detectable target sequences from these fecal indicators and pathogenic viruses in surface waters are also

needed to confirm this evidence for a possible positive association between qPCR monitoring results, pathogen levels and swimmer illness rates at POTW-impacted recreational beaches.

5. CONCLUSIONS

- PMA pretreatment of DNA extracts was effective in substantially reducing qPCR detectable target sequences from heat-killed culture cells of *E. faecalis* and *B. thetaiotaomicron* and thus has the potential to allow relatively specific detection of live cells of these organisms by the qPCR technique.
- High levels of suspended solids or biomass in water samples appeared to interfere with the ability of the PMA-qPCR method to specifically detect live cells suggesting that further optimization of the method may be required.
- Standard POTW chlorine disinfection practices resulted in substantially greater reductions in fecal indicator bacteria CFU levels than those observed for PMA-qPCR detectable target sequences. Further studies are needed to determine the potential role of viable but non-cultivable organisms in causing this discrepancy.
- Over all stages of normal wastewater treatment, reductions of both PMA-qPCR and qPCR detectable fecal indicator bacteria target sequences were highly correlated with reductions of infectious viruses suggesting that either of these molecular methods may be applicable for predicting the efficiency of normal wastewater treatment processes in reducing loads of these pathogens.
- The overall efficiency of viral load reductions was reduced during non-standard wastewater treatment processes such as blending during storm flow events.

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NOTICE

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Figure legends

Figure 1. Schematic of wastewater treatment processes at the three POTWs during normal and storm flow operating conditions and locations of sampling points (SP).

Figure 2. Target sequences determined by qPCR analyses in DNA extracts of fresh and heat-killed cell suspensions of *Enterococcus faecalis* and *Bacteroides thetaiotaomicron*. Heat treatment of the *E. faecalis* suspension resulted in no cultivable cells whereas for *B. thetaiotaomicron*, the undiluted, heat-treated cell suspension contained ~ 20 CFU/ml. Cell suspensions were either pre-treated with 100 μM PMA for 5 min followed by light exposure for 2 min (+ PMA) or not treated with PMA (- PMA) prior to DNA extraction and analysis as described in Materials and Methods. Data points represent means and error bars represent standard deviations from analyses of either two or three replicate samples.

Figure 3. QPCR and PMA-qPCR detectable target sequence recoveries from freshly cultured *E. faecalis* cells concentrated by two different approaches. Suspensions containing ~ 5 x 10^4 *E. faecalis* cells were concentrated either by filtration through 47-mm, 0.4-µm pore size polycarbonate filters, washing the cells off the filters in PBS and further concentrating them by centrifugation (Filter) or by direct centrifugation (CF) as described in Materials and Methods prior to qPCR and PMA-qPCR analyses. Columns represent means and error bars represent standard deviations from analyses of three replicate samples.

Figure 4. Ratios of ambient *Enterococcus* qPCR/PMA-qPCR detectable target sequence recoveries from different volumes of wastewater samples. One ml and 10 ml influent, (SP1), primary treatment effluent (SP2), secondary treatment effluent (SP3) and disinfected secondary treatment effluent (SP4) samples, collected from POTW 1 during normal operating conditions, were concentrated by direct centrifugation for qPCR and PMA-qPCR analyses as described in materials and methods. Columns represent mean ratios from duplicate analyses of three replicate samples.

Figure 5. QPCR and PMA-qPCR detectable target sequence recoveries from wastewater samples spiked with live and dead *E. faecalis* cells. Primary treatment effluent (SP2), secondary treatment effluent (SP3) and disinfected secondary treatment effluent (SP4) samples, collected from POTW 1 during normal operating conditions were stored for approximately four weeks to reduce their viable enterococci densities. These samples and PBS control samples were then spiked with 5x 10^5 freshly cultured or heat-killed *E. faecalis* cells. Cells were concentrated by direct centrifugation for qPCR and PMA-qPCR analyses as described in Materials and Methods. Columns represent means and error bars represent standard deviations from analyses of either two or three replicate samples.

Figure 6. Densities of selected indicators and all viruses determined in influent (SP1), primary treatment effluent (SP2), secondary treatment effluent (SP3) and disinfected secondary treatment effluent (SP4) samples during normal dry weather operations at the three POTWs. Enterococcus (Ent) qPCR and PMA-qPCR densities are shown as target sequences per 100 ml. Virus densities are mean values determined from all four host cell lines (see Table 2). Means, indicated by data points, and standard deviations, indicated by error bars, were determined from analyses of multiple samples taken from a single sampling event (see supplementary materials). Virus and phage densities were only determined in POTWs 1 and 2.

Figure 7. Densities of selected indicators and all viruses determined in influent (SP1), primary treatment effluent (SP2), blended primary and secondary treatment effluent (SP3) and disinfected effluent (SP4) samples during storm flow operations at the three POTWs. Enterococcus (Ent) qPCR and PMA-qPCR densities are shown as mean target sequences per 100 ml. Virus densities are mean values determined from all four host cell lines (see Table 2). Means, indicated by data points, and standard deviations, indicated by error bars, were determined from analyses of multiple samples taken from two to four sampling events (see supplementary materials). Virus and phage densities were only determined in POTWs 1 and 2.