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Escherichia coli, enterococci, and *Bacteroides thetaiotaomicron* qPCR signals through wastewater and septage treatment

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ABSTRACT

Fecal indicators such as *Escherichia coli* and enterococci are used as regulatory tools to monitor water with 24 h cultivation techniques for possible input of sewage or feces and presence of potential enteric pathogens yet their source (human or animal) cannot be determined with routine methods. This critical uncertainty has furthered water pollution science toward new molecular approaches. Members of *Bacteroides* genus, such as *Bacteroides thetaiotaomicron* are found to have features that allow their use as alternative fecal indicators and for Microbial Source Tracking (MST). The overall aim of this study was to evaluate the concentration and fate of *B. thetaiotaomicron*, throughout a wastewater treatment facility and septage treatment facility. A large number of samples were collected and tested for *E. coli* and enterococci by both cultivation and qPCR assays. *B. thetaiotaomicron* qPCR equivalent cells (mean: 1.8×10^7/100 mL) were present in significantly higher concentrations than *E. coli* or enterococci in raw sewage and at the same levels in raw septage. The removal of *B. thetaiotaomicron* target qPCR signals was similar to *E. coli* and enterococci DNA during the treatment of these wastes and ranged from 3 to 5 $\log_{10}$ for wastewater and was 7 $\log_{10}$ for the septage. A significant correlation was found between *B. thetaiotaomicron* marker and each of the conventional indicators throughout the waste treatment process for both raw sewage and septage. A greater variability was found with enterococci when compared to *E. coli*, and CFU and equivalent cells could be contrasted by various treatment processes to examine removal and inactivation via septage and wastewater treatment. These results are compared and contrasted with other qPCR studies and other targets in wastewater samples providing a view of DNA targets in such environments.

1. Introduction

Wastewater is the source of many human enteric pathogens (Nayak and Rose, 2007; Lee et al., 2006; Kamel et al., 2010; Robertson et al., 2006) and often associated with swimming-acquired illnesses in natural waters (Wade et al., 2006). Adequate wastewater treatment prior to effluent discharge plays a critical role in minimizing public health risks. On-site wastewater disposal using septic tanks has also been an issue regarding pathogen entry into and transmission through water particularly groundwater (Fong et al., 2007) and septage treatment and application on land has received little attention in regard to microbial quality. In most states, fecal coliform bacteria are still used to address wastewater treatment using

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the National Pollutant Discharge Elimination System permit programs and are disconnected from ambient water quality monitoring, in which coastal states are moving toward *Escherichia coli* (*E. coli*) and enterococci.

E. coli and enterococci have long been used as indicators of fecal pollution for recreational and drinking waters (USEPA, 2002, 2005a); and cultivation methods are used as the gold standard for the enumeration of these bacteria in water (Messer and Dufour, 1998). Recent advances in molecular biology such as polymerase chain reaction (PCR) and particularly quantitative PCR (qPCR) have revolutionized microbiology. Quantitative PCR has many advantages over standard cultivation methods, such as producing results rapidly (30 min–2 h), the ability to detect viable but non-cultivatable (VNBC) pathogens, and providing quantitative results with a wide detection range (10^0–10^8 copies/reaction). However, it is still recognized that both inactivated and live microbes will be detected, which is a disadvantage when evaluating disinfection processes. Never-the-less, evaluation and application of these qPCR methods for routine monitoring of fecal contamination in recreational waters is ongoing in the US (Haugland et al., 2005; Noble et al., 2006; Wade et al., 2006; Lavender and Kinzelman, 2009) as well as in wastewaters (Silkie and Nelson, 2009; Varma et al., 2009; Wery et al., 2008; Frahm and Obst, 2003).

One of the other disadvantages of using routine bacterial indicators is that the source cannot be identified while the specificity of molecular methods has lead to development of a field known as Microbial source tracking (MST) that has enabled the identification of animal or human sources of fecal contamination (Scott et al., 2002; Simpson et al., 2002; USEPA, 2005b). Many human fecal specific assays to address sewage discharges impacting water quality have targeted species in the *Bacteroides–Prevotella* group directed toward detection of 16S rRNA genes (Seurinck et al., 2005; Layton et al., 2006; Okabe et al., 2007). *Bacteroides* spp. are obligately anerobic, Gram negative, rod shaped, and non-endospore forming bacteria and are normally commensals that constitute the most numerous members of the intestinal flora of all warm blooded animals (Wexler, 2007). There are still concerns regarding cross reactivity of some of these genetic markers with feces from humans and other animals including cats and dogs (Sadowsky et al., 2007; Kildare et al., 2007). Some qPCR assays based on 16S rRNA genes have been reported to cross react with fish DNA (McLain et al., 2009). Moreover, the exact copy number of these 16S rRNA genes present in one cell is not known which makes conversion of qPCR copy number to cell equivalents rather difficult. Recently, Yampara-Iquise et al. (2008) examined a single copy putative mannanase 1-6 gene of *Bacteroides thetaiotaomicron* as a human fecal source tracking marker with good specificity.

The overall aim of this study was to evaluate the concentration and fate of *B. thetaiotaomicron*, throughout a wastewater treatment facility and septage treatment facility in contrast to *E. coli* and enterococci as measure by qPCR and cultivation. In this study, a qPCR assay targeting *uidA* gene for *E. coli* was developed and used, modified from Frahm and Obst (2003). Enterococci qPCR assay focused on the use of primers and probes designed for detection of the 23S rRNA gene sequences (Haugland et al., 2005; Silkie and Nelson, 2009). Samples were collected throughout the wastewater treatment processes as well as from septage before and after treatment. Cultivable counts of *E. coli* and enterococci were compared to the qPCR data generated and an automated DNA extractor was compared to a commercially available QIAmp DNA mini kit (Qiagen, Valencia, CA, USA).

2. Materials and methods

2.1. Samples

a) Wastewater treatment plant samples: Over 200 samples were collected from a municipal wastewater treatment plant, located in East Lansing, Michigan that serves a population of 90 000. The plant receives, on an average basis, a little less than 13.40 MGD (million gallons per day) wastewater inflow. Samples collected from this facility included:
   i) Raw sewage (RS)
   ii) Primary effluent (PE), after the solids have settled
   iii) Secondary effluent (SE pre-chlorination), after activated sludge process and secondary clarification
   iv) Secondary effluent (SE post-chlorination), after disinfection by chlorination, and
   v) Tertiary effluent (TE), effluent from secondary treatment post sodium bi-sulfite dechlorination and filtration through rapid sand filters.

For comparison of auto and manual DNA extraction, raw sewage ($n = 9$), and primary effluent samples ($n = 9$), secondary post-chlorinated effluent ($n = 9$), and tertiary effluents ($n = 9$) were used, which is a total of 36 samples from the wastewater environment.

For assessment of conventional indicators and *B. thetaiotaomicron* (α-mannanase gene) RS, PE, SE and TE samples were collected in triplicates during 18 sampling events from January 2009 to January 2010. During six sampling events within this time frame, secondary treated effluent prior to the chlorination step was also collected in triplicates. During each sampling event, one hundred milliliters of RS and PE, 500 mL of pre-chlorinated SE and 2 L of SE and TE were collected in triplicates. Chlorinated SE and TE samples were collected in bottles pre-loaded with sodium thiosulphate (1 mL of 10% solution) to neutralize any residual chlorine present in the effluents. All samples were transported on ice and processed within 2 h after collection.

b) Septage treatment plant samples: Samples were collected from a septage treatment plant located in Charveloix, Michigan. This treatment plant utilizes an aerobic biological treatment system to treat septage wastes (solid waste from septic tanks) and discharges the treated effluent to a municipal sewer system. Samples were collected during eight sampling events between January 2009 and November 2009. During each event, triplicates of 50 mL raw septage and 500 mL of septage effluent were collected, placed on ice and shipped to Water Quality and Health Laboratory at Michigan State University, East Lansing, MI.

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2.2. Comparison of DNA extraction methods

Comparison between automated DNA extraction using Roche MagNaPure LC instrument (Roche Applied Sciences, Indianapolis, Ind.) and manual extraction using QIAmp DNA mini kit (Qiagen, Valencia, CA, USA) was performed. Fifty milliliters RS and PE samples were centrifuged at 8000× *g* for 20 min and for treated sewage, 1 L of SE and TE was filtered through 90 mm, 0.45 μm pore size nitrocellulose membrane filters (Millipore, Billerica, Mass.). The filters were folded and immersed into sterile phosphate buffered saline (PBS) in 50 mL centrifuge tubes. The tubes were then vortexed at high speed to detach the cells from membrane for 2 min. The filters were removed and the tubes were centrifuged at a speed of 8000× *g* for 15 min. Around 48 mL of the supernatant was discarded and the remaining sample was mixed well by vortexing. The volume was recorded and from this, 400 μL of the pellet was used for manual DNA extraction with the QIAmp DNA mini kit (Valencia, CA, USA), another 400 μL of the aliquot was used for extraction by the Roche MagNaPure LC instrument and remainder was stored at −80 °C. These volumes were included in the calculation for concentrations of targets by qPCR.

For DNA extraction using Roche MagNaPure automated instrument, an additional lysis step was performed by mixing 400 μL of concentrated samples with 180 μL of MagNaPure lysis buffer and 20 μL of Proteinase K (20 mg/ml), and incubated at 65 °C for 30 min. The mixture was then centrifuged at 500× *g* for 30 s to settle down the particles. The supernatant was used for DNA extraction by the instrument. Simultaneously, a manual DNA extraction was carried out on the same samples using the QIAmp DNA mini kit. Both extraction methods resulted in 200 μL of DNA suspended in TE buffer. Negative controls (molecular grade water) were used to check for cross contamination in both extraction methods. The concentrations of extracted DNA were determined by using Nanodrop ND-1000 Spectrophotometer. Extracted DNA was stored at −20 °C until further analyses.

2.3. Reproducibility of the auto extraction method

In order to assess the intervariability in extraction during comparison of auto and manual extraction methods, eight replicates of concentrated RS samples were subjected to DNA extraction by both instrument and manual methods following the procedures described above. For all replicates, the same volume of lysis buffer (180 μL) and proteinase K (20 μL) were added to the tubes and subjected to auto extraction. DNA concentrations in all extracts were analyzed by qPCR methods for both *E. coli* and enterococci. The extractions were also carried by two analysts to assess the variability in both instrument and manual methods.

2.4. DNA extraction used for assessment of septage samples

DNA extraction from treated and untreated sewage samples were performed using automated method described previously. Untreated Septage (5 mL) samples were mixed thoroughly by vortexing for 1 min and 600 μL was taken for DNA extraction. For septage effluent samples, 50 mL of the sample

Table 1 – The bacteria, gene targets, primer/probe sequences, and PCR programs used for the assays.

Bacteria/gene	Type of assay	Primer/probe sequence (5′–3′)	Amplicon size (bp)	PCR programs	References
E. coli/uidA[a]	qPCR	CAATGGTGATGTCAGCGTT ACACTCTGTCCGGCTTTTG 6FAM-TTGCAACTGGACAAGGCA CCAGC-BBQ	163	6 s at 95 °C 8 s at 58 °C 8 s at 72 °C	This study
Enterococci/23S rRNA[a]	qPCR	AGAAATTCCAAACGAACTTG CAGTGCTCTACCTCCATCATT 6FAM-TGGTTCTCTCCGAAATAGCTT TAGGGCTA-TAMRA	91	15 s at 95 °C 30 s at 60 °C 15 s at 72 °C	Frahm and Obst (2003)
B. thetaiotaomicron/ α-1-6 *mannanase*[b]	qPCR	CATCGTTCGTCAGCAGTAACA CCAAGAAAAAGGGACAGTGG 6FAM-ACCTGCTG-NFQ	63	15 s at 94 °C 60 s at 60 °C 5 s for 72 °C	Yampara-Iquise et al. (2008)
E. coli/uidA[c]	Regular PCR[d]	GCAGTCTTACTTCCATGATTTCTTTA TAATGCGAGGTACGGTAGG	522	30 s at 95 °C 30 s at 57 °C 60 s at 72 °C	This study
Enterococci/23S rRNA[c]	Regular PCR[d]	ATCTACCCATGTCCAGGTTGAAG CCATCTCGGGTTACCGAATTCAG	223	30 s at 95 °C 30 s at 57 °C 60 s at 72 °C	This study
*B. thetaiotaomicron/*α-1-6 *mannanase*[c]	Regular PCR[d]	GCGGTACACATAACGGG ATCGACTTATATCTACTGGCAAC	306	30 s at 95 °C 30 s at 60 °C 60 s at 72 °C	This study

a PCR programs were repeated for 40 cycles, after an initial cycle of 10 min at 95 °C (For *E. coli* and enterococci).
b PCR programs were repeated for 45 cycles, after an initial cycle of 15 min at 95 °C (For *B. thetaiotaomicron*).
c PCR programs were repeated for 35 cycles, after an initial cycle of 10 min at 95 °C and terminated by a final extension cycle at 72 °C for 8 min.
d Regular PCR was used for producing the amplicon required for the preparation of standards.

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was centrifuged at 8000*g* for 20 min. The supernatant was discarded and 1 mL of the pellet was left behind. From this, 600 μL was used for DNA extraction. For both treated and untreated sewage and septage samples, DNA extraction was carried out using Roche MagNaPure automated machine following the protocol described above.

2.5. qPCR analyses

In order to prepare the standards, the DNA was extracted from the bacterial strains *E. coli* ATCC 15597, and *Enterococcus faecalis* ATCC 19433, and for *B. thetataiomicron*, genomic DNA from ATCC (number 29148D-5) was used. The *uidA* gene of *E. coli*, 23S rRNA gene of *enterococcus* and α-mannanase gene of *B. thetataiomicron* were amplified separately using primer sets that flanked the qPCR target amplicon sequences (Table 1). These primers were developed using the Roche LightCycler Primer Design Software. Polymerase chain reaction (PCR) was performed in a 25 μL total reaction mix which contained 15 μL Hotstart DNA Polymerase Mastermix, 0.4 μM of each primer, 2 μL of the template DNA and molecular graded water (QIAgen, Valencia, CA, USA) to make up a final volume of 25 μL. The amplified PCR products for all three target genes were cloned into TOPO PCR 2.1 and transformed with the TOPO10 F′ competent cells (Invitrogen Inc., Carlsbad, CA, USA), according to the protocol provided by the manufacturer. Plasmids were extracted with QIAprep Spin MiniPrep kit (Valencia, CA, USA) and were sequenced at the Research Technology Support Facility (RTSF) at Michigan State University that confirmed the insertion of the target inside the vector. The plasmids were quantified using Nano-Drop spectrophotometer and then serially diluted ten-fold to construct qPCR standard curves. Triplicates of dilutions ranging from 10^6 to 10^0 were used for the standard curve.

Targeted bacterial genes, primers/probes and corresponding qPCR programs are described in Table 1. For the *E. coli uidA* gene, satisfactory results with no homology with other targets were noted after checking the primer/probe sequences by BLAST analysis. All isolates of *E. coli* were detected whereas non-*E. coli* bacterial strains and other coliform bacteria were not detected by this assay. The *B. thetataiomicron* α-mannanase assay was tested, in our laboratory, against 226 non-human fecal samples and exhibited excellent specificity (bird, cow, cat, dog, horse and pig feces) (unpublished). DNA extracted from all samples was analyzed by qPCR using Roche LightCycler® 2.0 Instrument (Roche Applied Sciences, Indianapolis, IN). The reaction mixture for amplification of *E. coli uidA* gene consisted of 2 μL of Roche Fast Start LightCycler Mastermix, forward and reverse primers (0.5 μM each), probe (0.2 μM), 3.2 mM $MgCl_2$ and nuclease free water to a final volume of 15 μL. Cycle threshold (Ct) was measured during each amplification and target gene concentration was analyzed automatically by absolute quantification method by the LightCycler® Software 4.0. The enterococci 23S rRNA and *B. thetaiotaomicron* α-mannanase 1-6 (*B. theta* α) qPCR assays were carried out using 10 μL of LightCycler 480 Probes Mastermix (Roche, Indianapolis, IN), forward and reverse primers (0.5 μM each for enterococci and 0.2 μM each for *B. theta* α), probe (0.2 μM for enterococci and 0.1 μM for *B. theta* α {probe number 62 from Roche UPL}), and nuclease free water to make up a final volume of 15 μL. Five microliters of the extracted DNA sample was used as the template and run in duplicates. All sample extracts were diluted (1:5 dilution) and checked for any qPCR inhibition based on the difference in corresponding threshold cycle values.

Every four to five runs, a standard curve was run (using various dilutions in triplicates) and the average efficiency of qPCR assays for the *uidA* gene of *E. coli*, 23S rRNA of enterococci gene and α-mannanase 1-6 gene of *B. thetaiotaomicron* were 102 ± 0.5%, 98 ± 0.3% and 96 ± 1.2%, respectively and r^2 value was always higher than 0.99 for all three assays. A diluted plasmid standard was included in triplicates as a positive control during each qPCR run and the average threshold cycle was compared with the original standard curve. The copies of *uidA* gene of *E. coli*, 23S rRNA of enterococci gene and α-mannanase 1-6 gene of *B. thetaiotaomicron* present in the sample were quantified from the standard curves obtained earlier. The copies of the corresponding genes were converted to cell equivalents; in the case of *E. coli* and *B. thetaiotaomicron*, only one copy of the target gene is present in a cell; thus, one copy number corresponds to one cell. However, in case of enterococci, it has been suggested that there are four copies of 23S rRNA present in a cell; therefore, DNA copies to cell conversions were completed based on four copies of enterococci qPCR targets corresponded to one cell in this study (Viau and Peccia, 2009). All final concentrations for qPCR analyses were reported as qPCR equivalent cells/100 mL.

2.6. Cultivation methods

U.S.EPA methods 1603 and 1600 were used for enumerating *E. coli* and enterococci, respectively by cultivation (USEPA, 2002, 2005a). Serial dilutions of the raw sewage and primary effluents (10^{-1} through 10^{-5}) were made and one mL from these dilutions was filtered through 47 mm diameter, 0.45 μm pore size, membrane filters. For pre-chlorinated secondary effluents, secondary and tertiary treated effluents, one mL and 100 mL, respectively, were filtered. One mL of raw septage samples was serially diluted and these dilutions were used for further bacterial indicator analysis. For septage effluent samples, volumes of 0.1 mL, 1 mL and 10 mL were filtered.

The filters were placed on mTEC agar and mEI agar plates. The mTEC agar plates were incubated 35 ± 0.5 °C for 2 h, followed by incubation in a water bath at 44.5 ± 0.2 °C for 22 h. The mEI plates were incubated for 24 h at 41 °C. The concentrations of *E. coli* and enterococci were reported as colony forming units (CFU) per 100 mL.

2.7. Statistical analysis

All statistical analyses were performed using SAS software 9.2 (SAS Inc, 2002) and significance level was set at $\alpha = 0.05$. The data were $\log_{10}$-transformed to achieve normal distribution and meet the assumptions of a parametric test. Simple *t*-tests were used to compare the means of concentrations of the qPCR and the cultivation method results ($p < 0.05$). The coefficient of variation (CV%) was calculated to evaluate the intervariability in extraction procedures using the formula;

$$CV\% = (\text{standard deviation}/\text{mean}) \times 100$$

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Table 2 – t-Test results for comparison of means between the qPCR cell equivalent (CE) concentrations of *E. coli* and enterococci from DNA extracted by auto and manual methods.

Average CE/100 mL					
Samples[a]	Automated method	Manual method	Mean difference	Standard error for mean difference	*t*-statistic (p)
E. coli					
RS	4.62E+06	2.18E+06	0.31	0.07	4.09 (0.001)
PE	6.79E+06	2.24E+06	0.55	0.16	3.51 (0.004)
SE	3.20E+03	1.22E+03	0.61	0.23	2.60 (0.02)
TE	2.58E+03	1.50E+03	0.42	0.38	1.11 (0.28)
Enterococci					
RS	2.23E+07	2.10E+07	0.26	0.29	0.57 (0.57)
PE	1.99E+07	1.58E+07	0.24	0.23	1.02 (0.32)
SE	1.34E+05	7.16E+04	0.23	0.29	0.80 (0.43)
TE	1.49E+05	7.96E+04	0.09	0.30	0.25 (0.80)

a $n = 9$ for each type of sample. RS (raw sewage) PE (primary effluent-after primary clarification), SE (secondary effluent-after biological treatment and disinfection) TE (Teritiary effluent after dechlorination and filtration).

Analysis of variance (ANOVA) was performed to determine the differences in mean concentrations of target organisms in different treatment groups and if significant, multiple pair-wise comparisons were carried out using Fisher's Least Square Difference (LSD) test. Pearson's correlation coefficient was used to test the relationship between conventional indicators and *B. theta* α. Linear regression analysis was performed using scatter plots of $\log_{10}$ cells/100 mL of *E. coli* and enterococci for comparison between manual and instrument DNA extraction from the samples tested and to estimate the coefficients of the linear equation for conventional indicators that best predicted the concentrations of *B. theta* α after various wastewater treatment processes.

3. Results

3.1. Comparison of manual and automated extraction for *E. coli* and enterococci DNA

Statistical analyses showed that qPCR cell equivalent (CE) concentrations of *E. coli*/100 mL of RS, PE, and SE were statistically higher for the autoextractor as compared to manual extraction ($p < 0.05$) (Table 2). In tertiary effluent samples, both methods returned equivalent cell concentrations. For enterococci, there was no statistically significant difference found between extraction methods in any of the samples (Table 2). The mean difference of qPCR CE concentrations of *E. coli* and enterococci between auto extraction and manual extraction ranged from 0.31 to 0.61, and 0.09 to 0.26 $\log_{10}$ units, respectively, in various samples.

Table 3 shows that total DNA concentration (ng/μL) extracted by the DNA extraction instrument had less variation (CV: 4.50%), than manual DNA extraction (CV: 13.29%). The qPCR CE concentration of *E. coli* per reaction for RS showed a CV (%) of 1.31 for auto DNA extraction and 1.67 for manual DNA extraction whereas cell concentration of enterococci per reaction for RS showed a CV (%) of 1.48 for auto DNA extraction and 1.71 for manual DNA extraction.

Intervariability of total DNA concentration (ng/μl) by auto extraction showed a CV of 5.07% and manual DNA extraction showed a CV (%) of 20.65. The CE concentration of *E. coli* per qPCR reaction for RS showed a CV (%) of 0.86 for auto DNA extraction and 1.88 for manual DNA extraction whereas cell concentration of enterococci per reaction showed a CV (%) of 1.36 for auto DNA extraction and 1.83 for manual DNA extraction by the second individual.

Thus, for further examination of the DNA signals through the treatment processes, the data from the auto DNA extraction were used.

3.2. Assessment of qPCR signals through sewage and septage treatment

3.2.1. Occurrence of bacterial targets in raw sewage and treated effluents

The average concentrations and standard deviations for the 216 samples (54 after each treatment location) collected from the wastewater treatment plant are shown in Table 4. The

Table 3 – Coefficient of variation (CV %) for DNA concentrations and cell equivalent concentrations of *E. coli* and enterococci performed by two individuals for auto and manual extraction methods.

Sample (raw sewage)[a]	Individual 1		Individual 2	
	Instrument	Manual	Instrument	Manual
	CV%	CV%	CV%	CV%
DNA concentration (ng/μL)	4.50	13.29	5.07	20.65
Enterococci (CE/reaction)	1.48	1.71	1.36	1.83
E. coli (CE/reaction)	1.31	1.67	0.86	1.88

a Raw sewage ($n = 9$) from the same sample concentrate was used for the experiment.

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Table 4 – Average log_{10} concentrations for *E. coli*, enterococci and *B. thetaiotaomicron* throughout wastewater treatment by cultivation and qPCR.

Samples[a]		*E. coli* (log_{10} CFU/100 mL)	Enterococci (log_{10} CFU/100 mL)	*E. coli* (log_{10} CE/100 mL)	Enterococci (log_{10} CE/100 mL)	*B. theta* α (log_{10} CE/100 mL)
RS[b]	Mean	6.21 (0.26)	5.72 (0.42)	6.46 (0.59)	6.63 (0.51)	7.26 (0.24)
PE[c]	Mean	6.17 (0.26)	5.58 (0.27)	6.48 (0.72)	6.75 (0.40)	7.31 (0.41)
SE[d]	Mean	1.01 (0.84)	0.64 (0.91)	3.05 (0.95)	4.13 (0.84)	4.19 (0.42)
TE[e]	Mean	1.04 (0.93)	0.75 (1.01)	2.82 (1.19)	3.59 (1.12)	3.67 (0.60)

Number in parentheses represents standard deviation.
a n = 54 for each treatment location.
b RS-Raw sewage.
c PE-Primary effluent.
d SE-secondary effluent.
e TE-Tertiary effluent.

qPCR concentrations of *E. coli* in RS ranged from 1.47×10^5 to 1.48×10^7 cell equivalent/100 mL, for enterococci, ranged from 7.08×10^5 to 5.75×10^7 CE/100 mL, and for *B. theta* α, ranged from 7.76×10^6 to 5.68×10^7 CE/100 mL. The TE had a range of concentrations of 2.16×10^1 to 4.39×10^4 for *E. coli*, 6.35×10^0 to 3.81×10^5 for enterococci and 9.79×10^2 to 1.59×10^5 for *B. theta* α cell equivalent/100 mL.

There were statistically significant differences in concentrations of all targets either by cultivation (represents cells that are alive and are in dynamic state) or qPCR methods (measure both dead and live cells, presenting a stable data) between samples collected prior to disinfection and after disinfection ($p < 0.05$). Among the different target microorganisms, Fisher's LSD showed that concentrations of *B. theta* α were significantly higher than *E. coli* or enterococci in all samples except in SE (effuent after biological treatment and disinfection) and TE (final effluent after dechlorination and filtration) where their concentrations were not significantly different from that of enterococci ($p < 0.05$). Concentrations of *E. coli* were significantly higher than enterococci in all samples by cultivation methods; however with qPCR enumeration, enterococci concentrations were found to be significantly higher than *E. coli* in SE and TE samples ($p < 0.05$).

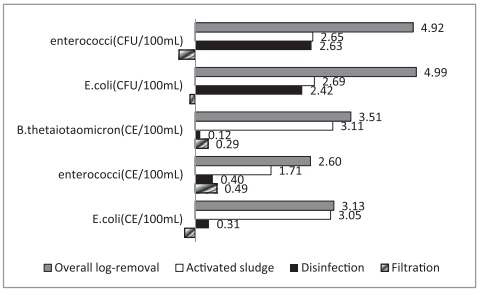
3.2.2. *Removal during wastewater treatment process*

During secondary activated sludge treatment prior to the chlorination step, there was a significant reduction in all target organisms. There was an average log_{10} removal of 2.69 and 3.05 for *E. coli* concentrations, respectively, by cultivation method and qPCR. For enterococci, the log_{10} reduction was 2.65 and 1.71, by cultivation method and qPCR, respectively. The log_{10} removal for *B. theta* α in this process was found to be 3.11, as measured by qPCR. When chlorine was added to the effluent, there was a further significant reduction in the average concentrations of cultivable cells; 2.42 log_{10} for *E. coli* and 2.63 log_{10} for enterococci. But as expected by qPCR methods, this reduction was only 0.31 and 0.40 log_{10} for *E. coli* and enterococci, respectively, and only 0.12 log_{10} for *B. theta* α cells, none of which were found to be significant. Filtration using rapid sand gravity filters did not significantly reduce any of the CFU or qPCR cell equivalents except for enterococci qPCR cells, which were reduced slightly by 0.49 log_{10} units. The overall log_{10} removal, which is the difference in log_{10} concentrations between raw sewage and tertiary effluent (after filtration), for CFU of *E. coli* and enterococci were found to be 4.99 and 4.92 log_{10} units and for qPCR cell equivalent of *E. coli*, enterococci, and *B. theta* α were 3.13, 2.60 and 3.51 log_{10}, respectively (Fig. 1).

3.2.3. *Correlation between conventional indicators and human specific marker in raw sewage and treated effluents*

Table 5 presents Pearson's correlation coefficients between both the conventional indicators and *B. theta* α in treated and untreated group (SE and PE were grouped in this analysis) as well as pooled data (from all locations). The analysis was done on the pooled data sets in order to observe the relationship as the qPCR targets moved through the treatment process. Correlation coefficient (r) between *E. coli* and *B. theta* α was found to be 0.93 for pooled samples. In SE/PE samples and SE/TE samples, the coefficients were 0.33 and 0.66, respectively. Correlation coefficient between enterococci and *B. theta* α was 0.88 for pooled data; in untreated and treated samples, the coefficients were 0.37 and 0.53, respectively. All these correlations were found to be statistically significant ($p < 0.05$).

Regression analysis was used to test the strength of association between conventional indicators and *B. theta* α in the pooled data set. The equation for each regression is displayed on Fig. 2. Fig. 2a shows scatter-plot for *E. coli* and *B. theta*



Overall log-removal= Difference in log_{10} concentrations between raw sewage and tertiary effluent;
Log_{10} removal:
Activated sludge= raw sewage - pre-chlorinated secondary effluent
Disinfection= pre-chlorinated secondary effluent - secondary effluent after chlorination
Filtration= secondary effluent after chlorination - tertiary effluent

Fig. 1 – Average log_{10} reductions of *E. coli*, enterococci (CFU and CE) *and B. thetaiotaomicron* CE by wastewater treatment.

Table 5 – Pearson's correlation between conventional indicators and *B. thetaiotaomicron* in treated, untreated groups of data and pooled data by qPCR.

Pearson's correlation coefficient (R)			
Indicator bacteria	Untreated[a]	Treated[b]	Pooled[c]
E. coli	0.33	0.66	0.93
Enterococci	0.37	0.53	0.88

a Raw sewage and primary effluent data combined (n = 108 samples).
b Secondary and tertiary effluents data combined (n = 108 samples).
c Treated and untreated data combined (n = 216 samples).

α concentrations from pooled data from four treatments which displayed strong correlations with $R^2 = 0.87$. The scatter-plot for enterococci and *B. theta* α from four treatments, which displayed strong correlation with $R^2 = 0.79$ is in Fig. 2b.

3.2.4. *Occurrence of bacterial targets in raw septage and treated effluent*

The average concentrations and standard deviations for the 48 samples (24 each for raw septage and treated effluent) collected from the septage treatment plant are shown in Table 6. The qPCR equivalent concentrations of *E. coli* in raw septage ranged from 6.80×10^6 to 6.23×10^8 cells/100 mL, enterococci ranged from 3.72×10^6 to 6.235×10^7 cells/100 mL and *B. theta* α were in the range of 1.19×10^7 to 1.17×10^8 cells/100 mL. The final septage effluent had a range of concentration of 9.09×10^2 to 5.92×10^4 qPCR equivalent cells/100 mL of *E. coli*, 4.23×10^4 to 4.57×10^5 cells/100 mL of enterococci and 3.83×10^3 to 3.67×10^5 cells/100 mL of *B. theta* α. There was no significant difference between *B. theta* α and conventional indicators as measured by qPCR in raw septage and they were present in concentrations of $>10^7$ cells/100 mL.

The mean $\log_{10}$ difference between concentrations of *E. coli* by cultivation methods and by qPCR in raw septage was 0.85 units whereas for enterococci, the mean $\log_{10}$ difference was 0.95. The mean $\log_{10}$ difference between concentrations of enterococci by cultivation methods and that by qPCR in effluent was 1.25 $\log_{10}$ units. All these differences were statistically significant ($p < 0.05$). However, there was no statistically significant difference found between cultivable levels and qPCR equivalent cells for *E. coli* in septage effluent (there was no disinfection used in the septage treatment process train).

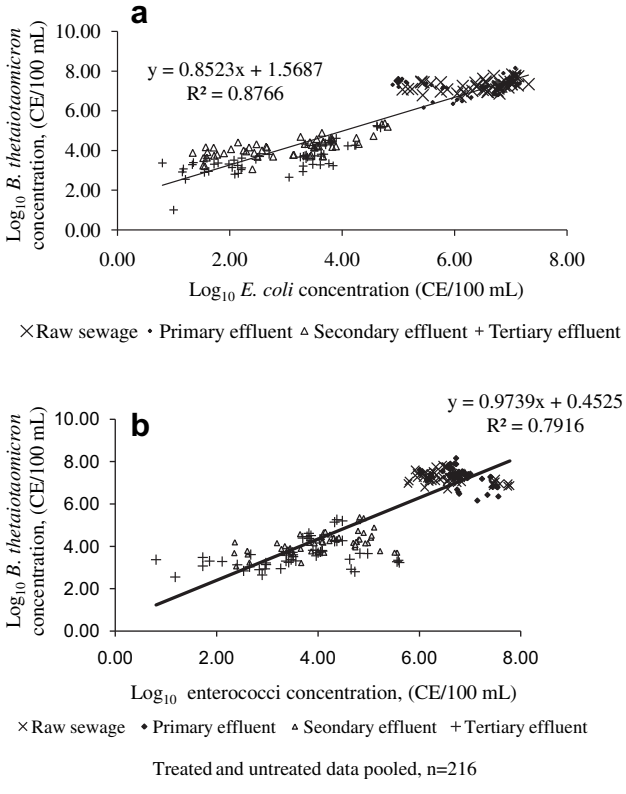


Fig. 2 – Correlation between a) *E. coli* and *B. thetaiotaomicron*, and b) enterococci and *B. thetaiotaomicron* by qPCR from wastewater treatment samples.

3.2.5. *Removal during septage treatment process*

Following the septage treatment process, there was a significant average $\log_{10}$ reduction in all target organisms, shown in Fig. 3. The highest $\log_{10}$ reduction during treatment was found for *E. coli* qPCR equivalent cells with a $\log_{10}$ difference of 3.82 whereas the difference in $\log_{10}$ concentrations for *E. coli* as measured by cultivation methods was 2.52. However, this difference in $\log_{10}$ removals between both methods was due to the higher initial qPCR equivalent concentrations in raw septage. The $\log_{10}$ reduction for enterococci by the cultivation method was 2.29 whereas this difference was only 1.99 by qPCR. The $\log_{10}$ reduction for *B. theta* α was found to be 3.13.

3.2.6. *Correlation between conventional indicators and human specific marker in raw septage and treated septage effluents*

Regression analysis was used to test the strength of association between conventional indicators and *B. theta* α using qPCR (Fig. 4). The equation for each regression is displayed on each chart. *E. coli* and *B. theta* α concentrations displayed strong correlations with $R^2 = 0.91$; and enterococci and *B. theta* α showed correlation of $R^2 = 0.92$.

4. Discussion

Comparison of commercial kits for extraction of DNA from water samples have been undertaken (Lebuhn et al., 2005; Rose et al., 2003), however, in this study, an automated method for extracting DNA was evaluated, and showed significantly higher or equal efficiency compared to manual extraction using a commercially available kit for extracting DNA from treated and untreated sewage. The same instrument had been used for the study of genetic diversity of *Legionella* spp. (Wullings and van der Kooij, 2006), but the auto extraction method was not evaluated or compared with any other existing methods as method evaluation was not a goal of their study. Advantages in using the autoextractor included improved consistency and decreased variability. Based on the DNA concentrations, this appeared to be a recovery issue, and

Table 6 – Average $\log_{10}$ concentrations of bacteria in raw septage and effluent by cultivation and qPCR methods.

Samples[a]	*E. coli* ($\log_{10}$ CFU/100 mL)	Enterococci ($\log_{10}$ CFU/100 mL)	*E. coli* ($\log_{10}$ CE/100 mL)	Enterococci ($\log_{10}$ CE/100 mL)	*B. thetaiotaomicron* ($\log_{10}$ CE/100 mL)
Raw septage	6.47 (0.45)	6.36 (0.82)	7.33 (0.68)	7.31 (0.36)	7.55 (0.34)
Effluent	3.96 (0.86)	4.07 (0.96)	3.51 (0.67)	5.32 (0.28)	4.42 (0.55)

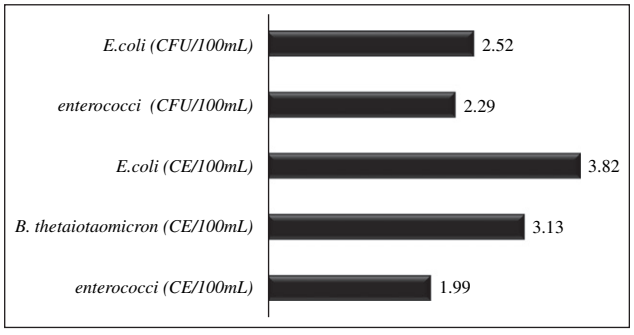
a Total number of samples, $n = 48$ (24 samples for each treatment).

while removal of inhibitors was not evaluated, the available technical information by the manufacturers suggests similar inhibitor removal efficiency as the basic steps of extraction are the same. However, the performance of various extraction methods in removing complex inhibitors from environmental samples should be explored further. With the pre-treatment step included, the time it took for extracting DNA from eight samples was slightly less when using the automated method (1 h) as compared to the manual kit (1.5 h). Previous studies in clinical samples have also compared the cost of DNA extraction using commercially available manual kits and automated methods (Knepp et al., 2003). Roche MagNaPure LC extraction is currently more expensive, with QIAamp kit and the MagNaPure reported to cost $2.64 and $3.58 per specimen, respectively (the price excludes cost of plastics).

Scientific developments such as qPCR will likely yield changes in future regulations and monitoring of drinking and recreational waters. Therefore, efficient and reliable data become important issues for these emerging techniques. These preliminary studies have demonstrated that automation can be used to improve efficiency and reproducibility of DNA extraction and that qPCR can be used to describe bacterial concentrations in wastewater. New instrument configurations for automation, which can handle more samples and a greater diversity of matrices for environmental testing, would be beneficial in analysis of wastewater and recreational waters.

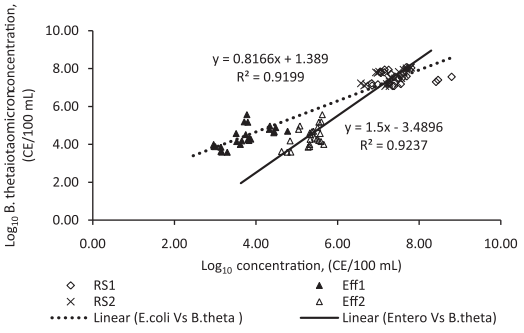
This study characterized human wastewater treatment environments for a novel MST target, *B. thetaiotaomicron*, in comparison with the conventional indicators *E. coli* and enterococci. To our knowledge, this study is the first to contrast raw sewage and septage, and the fate of molecular signals of all these bacterial indicators during waste treatment. The *B. thetaiotaomicron* qPCR equivalent cells were present in significantly higher concentrations than that of *E. coli* or enterococci in raw sewage but similar in septage. Yampara-Iquise et al. (2008) found a wide range of *B. thetaiotaomicron* (6.88×10^2–1.07×10^9 cells/g) in human feces, when stool samples from 10 human subjects were analyzed by qPCR targeting the α-mannanase 1-6 gene. This variation was suggested to be attributed to the variation in DNA extraction efficiency, yet no supporting data were available in the publication. Other human fecal MST markers, such as the host-specific *Bacteroides–Prevotella* 16S rRNA gene markers, have been detected in raw sewage and septage at concentrations of 10^8–10^9 cells/100 mL (Seurinck et al., 2005; Silkie and Nelson, 2009; Sercu et al., 2009) whereas in human feces, concentrations ranging from 10^5–10^{11} cells/gram (wet weight) were observed (Seurinck et al., 2005; Okabe et al., 2007; Sercu et al., 2009). Comparison between *Bacteroides* 16S rRNA human markers, whose exact gene copy number is not known, and *B. thetaiotaomicron* α-mannanase 1-6 gene, where one copy of gene represents one cell, may not be the best approach for determining the sensitivity of these markers.

E. coli was found to occur at concentrations of 10^6 target gene copies/100 mL of raw sewage in this study, which is almost three logs lower than the concentrations reported by another study, where 10^9 copies/100 mL of *lacZ* gene targets for *E. coli* by qPCR were found (Wery et al., 2008). But in five other studies, in which the *uidA* gene was targeted, qPCR cell concentrations of *E. coli* in raw sewage agree with this study (Table 7). One possibility for such high numbers of *E. coli* found by Wery et al. (2008) could be due to the cross amplification of the *E. coli lacZ* qPCR assay with other bacterial strains (coliforms) that harbor this gene, tested only with four bacteria during the assay development (Foulds et al., 2002). Enterococci qPCR assays published before have mostly targeted 23S rRNA



Number of samples, n=48 (24 samples from each treatment location)

Fig. 3 – Average log reduction of *E. coli*, enterococci, *B. thetaiotaomicron* targets by cultivation and qPCR during septage treatment process.



Total number of samples, n=48 (24 samples from raw septage and 24 treated effluents)
RS1= *E. coli* vs *B. theta* α in raw septage
RS2= enterococci vs *B. theta* α in raw septage
Eff1= *E. coli* vs *B. theta* α in treated effluent
Eff2= enterococci vs *B. theta* α in treated effluent

Fig. 4 – Correlation between *E. coli*, enterococci and *B. thetaiotaomicron* by qPCR in septage treatment process.

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Table 7 – Summary of qPCR concentrations for various DNA targets in wastewater and treated effluents.

Treatment	Sampling	Samples, *n*	*E. coli*[a]	Enterococci[a]	*Bacteroides* Human Marker[a]	References
			*uidA**	23S rRNA*	alpha-mannanase [b,*]	
RS	Grab	54	2.88E+06	1.71E+07	1.82E+07	This study
PE	Grab	54	3.02E+06	2.25E+07	2.04E+07	
SE (Pre-cl)	Grab	18	1.01E+03	1.68E+05	1.84E+04	
SE (Post-cl)	Grab	54	1.12E+03	5.40E+04	1.55E+04	
TE	Grab	54	6.61E+02	1.56E+04	4.68E+03	
Septage	Grab	24	2.14E+06	8.17E+07	3.55E+07	
STE	Grab	24	3.24E+03	8.36E+05	2.63E+04	
					16S rRNA[c]	
RS	Grab	9	–	2.76E+07	1.75E+09	Varma et al., 2009
PE		9		3.71E+07	1.59E+09	
SE (Pre-cl)		9		4.12E+05	3.47E+07	
SE (Post-cl)		9		1.58E+05	3.17E+07	
			*uidA**	23S rRNA*	16S rRNA[c]	Silkie and
RS	Grab	12	2.00E+07	1.00E+07	7.94E+08	Nelson 2009
					16S rRNA[c]	
RS	Grab	3	–	–	7.8E+08	Sercu et al., 2009
Septage	Grab	3	–	–	3.9E+08	
			*uidA**			
RS	Grab	5	–	–	–	Lavender and
PE	Grab	5	–	–	–	Kinzelman 2009
TE (UV disinfected)	Grab	5	–	–	–	
			2.85[d]	2.59[d]		
			lacZ*			
RS	composite	6	1.34E+09			Wery et al., 2008
PE		6	1.05E+09			
SE (Pre-cl)		6	2.95E+05	–	–	
					16SrRNA[c]	
RS	Grab	12	–	–	2.10E+05	Savichtcheva
PE	Grab	12			2.90E+05	et al., 2007
					16SrRNA[c]	
RS	Grab	4	–	–	1.72E+10	Seurinck et al., 2005
			*uidA**			
RS	Grab	3	1.66E+07	–	–	Lee et al., 2006
TE (UV disinfected)	Grab	3	6.12E+02			

RS = Raw sewage.
PE = Primary effluent after grit removal.
SE(Pre-cl) = Secondary effluent from secondary clarifier prior to chlorination.
SE(Post-cl) = Secondary effluent after disinfection.
TE = Teritiary effluent after filtration or UV disinfection.
STE = septage treated effluent.
*Target gene.
a qPCR target gene copies/100 mL.
b *B. thetaiotaomicron*.
c Order Bacteriodales.
d Log_{10} difference between raw sewage and tertiary effluent (Initial raw sewage or final effluent concentrations not reported).

gene using the same primer sets as used in this study and have found similar concentrations of target gene copies in raw sewage. Log_{10} removal of enterococci qPCR signals across the wastewater treatment found in this study is also very similar to the study by Varma et al. (2009) (Table 7).

Primary treatment in the wastewater treatment process train (primary sedimentation) does not reduce fecal bacteria and this has been shown with CFU and is similar when monitoring DNA targets as shown in this study and by others (Lucena et al., 2004; Puig et al., 2010). Significant reduction in all targets (both cultivable and qPCR equivalent cells) were observed during the secondary treatment process comprising of biological process (activated sludge), sedimentation and disinfection (chlorination). However there was a difference between enterococci (gram positive cells) and the gram negative cells of *E. coli* and *B. thetaiotaomicron*. Enterococci were the least removed during secondary treatment in both sewage and septage but were better removed by filtration by rapid sand filters. Loss during secondary treatment before disinfection may be due to biological and sedimentation

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processes (e.g. attachment to solids, sedimentation, predation by protozoa), thus a difference in attachment to activated sludge versus sand of various types of bacterial cells with different cell-wall properties needs further exploration. Sludge was not monitored for in this study; however, others have observed that in liquid sludge, 3.7 $\log_{10}$ higher concentrations of *E. coli* were found by qPCR compared to cultivation (Wery et al., 2008). This study observed that the rapid sand filtration used in this wastewater treatment facility did not adequately remove the cells. In the absence of coagulants, microorganisms may not be retained efficiently in the filters thus increasing their concentrations in final effluents (Koivunen et al., 2003).

There is currently a disconnect between sewage treatment monitoring requirements and recreational standards or criteria. Under the Clean Water Act of 1972, wastewater treatment plant discharge into streams and the effluent has to be monitored to meet the permitted levels of pollutants specified in NPDES. Wastewater treatment plants use mostly fecal coliforms to monitor the treatment efficacy (primarily disinfection). Total and fecal coliforms were used for monitoring recreational water quality after epidemiological studies conducted by U.S. Public Health Service (PHS) found that any increase in the levels of total coliforms could possibly be used as a "warning signal" (Stevenson, 1953) for public health risk and related this to the numbers of fecal coliform bacteria in the water. Later, U. S. EPA adapted *E. coli* and enterococci as new water quality criteria based on other epidemiological studies conducted in marine and fresh water beaches USEPA (1986).

A qPCR method is an effective tool that gives quicker results as compared to cultivation, and is being explored for same day analysis to address beach closures. One key limitation of qPCR methods is the inability to differentiate between live and dead cells and we found significant difference between qPCR and cultivable levels of *E. coli* and enterococci following disinfection (chlorination). However, it is not clear if this difference in number is solely due to the dead cells or if some of the cells had transformed into a viable but non-cultivable stressed state, which prevents their growth in culture media. Cell death upon chlorination is attributed to different mechanisms such as membrane damage, intracellular or extracellular enzyme inactivation, or uncoupling of the electron chain (Virto et al., 2005). Where low concentrations of chlorine may alter the *E. coli* cytoplasmic membrane and injure the cells without necessarily causing the cell inactivation, a higher dosage is required to cause the damage to nucleic acids (Lisle et al., 1998; Phe et al., 2007). Resistance to chlorine by different cell-wall types in the presence of organic matter has also been suggested (Virto et al., 2005). Viable qPCR methods using dyes such as Ethidium Monoazide (EMA) and Propidium Monoazide (PMA) distinguish live cells and dead cells (heat killed) but when applied to untreated and treated sewage samples in this study as well as by Varma et al. (2009) there was no observed reduction in the qPCR signals. After disinfection, the qPCR signal was, definitely not comparable to colony counts suggesting EMA was not able to penetrate some of the cells present in the disinfected effluent samples (data not shown). It is not clear if this is due to the effect of turbidity reducing the photolysis of the dyes and binding with DNA, no change in membrane permeability, or if it is due to the presence of viable but non-culturable cells present in the disinfected effluents. Thus currently qPCR can be used a tool to monitor loading and physical removal or dilution but cannot be used to address viability.

The Beaches Environmental Assessment and Coastal Health (BEACH) Act of 2000 is supporting science for qPCR to monitor recreation beaches (EPA, 2000). A recent study found qPCR gave higher values than cultivation based methods for recreational water samples from sites closer to treated effluent discharge points (Byappanahalli et al., 2010). In contrast, another study suggested underestimation bias for qPCR (Noble et al., 2010) and the sampling sites for this study were far from such outfalls. These contradictory results suggest that qPCR tool may underestimate the public health risks or cause unnecessary economic losses due to beach closures when used for regulatory purposes. The levels of qPCR signals detected from wastewater effluents (as in this current study) may be very useful in mapping the fate and transport of these signals from discharge points to exposure sites, getting a better understanding of wastewater impacts on recreational sites. These signals should also be explored in relationship to virus and parasite pathogens as well.

The discharged effluents from wastewater treatment plant in this study had a $\log_{10}$ qPCR cell concentration of 3.59 per 100 mL of enterococci. The *B. thetaiotaomicron* qPCR equivalent cell levels ($\log_{10}$ average of 3.67 cells/100 mL) were almost at the same levels as that of enterococci in 100 mL of treated final effluents. Following a CSO or SSO event the levels of these signals would be much higher. Wade et al. (2006) found a strong correlation between enterococci qPCR daily average concentrations and swimming related gastrointestinal illnesses at two of the Great Lakes beaches they studied by applying the 23S rRNA qPCR assay for enterococci. According to their study, a $\log_{10}$ increase in the daily average of qPCR cell equivalents was associated with 1.30 (95% CI, 1.08–1.57) increase in the odds of gastrointestinal illness for any contact with water. The enterococci concentrations found at the beaches ranged from 1.90 to 2.04 $\log_{10}$ qPCR cell equivalents per 100 mL. Wastewater effluents were suspected as impacting these beaches. These correlations between qPCR signals and public health risks, and the high qPCR target signals detected in the wastewater effluents in the current study suggest that in spite of the viability issues; qPCR may be a valuable tool in monitoring water environment for both enterococci and *Bacteroides* to protect public health risk.

5. Conclusions

- Automated methods can be effectively used to extract DNA from water and wastewater samples, thus reducing the risks of cross contamination and human errors.
- *B. thetaiotaomicron* qPCR equivalent cells based on the α-mannanase target were present at statistically significantly higher concentrations (~7 $\log_{10}$) than that of *E. coli* or enterococci (~6 $\log_{10}$) in raw sewage ($p < 0.05$) and at the same levels in raw septage (7.3–7.5 $\log_{10}$).
- There was a significant correlation between this MST marker and each of the conventional indicators throughout the waste treatment process for both wastewater and

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septage ($p < 0.05$). A clear difference was observed between indicators and their removal rates in different treatment processes; enterococci were better removed by filtration and *E. coli* and *B. thetaiotaomicron* were better removed by secondary treatment.

- Effluents discharged from wastewater treatment plants have concentrations of 2–3 $\log_{10}$ cell equivalents/100 mL of both the conventional indicators and *B. thetaiotaomicron* human marker.

Acknowledgments

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