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Comparison of Fecal Indicator Bacteria Densities in Marine Recreational Waters by QPCR

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Abstract The US EPA is currently investigating the use of quantitative PCR (qPCR) analysis techniques to estimate densities of fecal indicator bacteria (FIB) in recreational waters. Present water quality guidelines, based on culturable FIB, prevent same day water quality determination, whereas results from qPCR-based approaches are available within several hours. Epidemiological studies at Publicly-Owned Treatment Works (POTW)-impacted freshwater beaches have also indicated correlations between qPCR determined *Enterococcus* densities and swimming-related illness rates. Similar qPCR assays are now available for several other accepted or emerging FIB groups. This study provides an initial assessment of qPCR estimated *Enterococcus*, *Bacteroidales*, *E. coli* and *Clostridium* spp. densities in marine water and sand samples collected over one summer from two POTW-impacted recreational beaches. Relative target sequence densities of these organisms in the samples did not correspond with their relative estimated cell densities. These observations were attributable to differences in target sequences recovered from the calibrator cells of the different types of organisms. Comparative cycle threshold (CT) qPCR analyses of whole cell calibrator samples provide a simple and standardizable approach for estimating both total cell and target sequence densities of different types of FIB in water. Cell density estimates obtained by this approach are subject to uncertainty due to potential variability in absolute numbers of target sequences in the target organisms under different physiological or environmental conditions, but still may allow for informative comparisons with the target sequence estimates.

Keywords Cell equivalents · Fecal indicator bacteria · qPCR · Sequence copies · Marine water

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Introduction

Measurements of fecal indicator bacteria (FIB) levels are routinely used to assess the potential risk of exposure to disease causing pathogens in recreational waters (Anderson et al. 2005; Noble et al. 2003; Shibata et al. 2004). Current US EPA recommended guidelines for acceptable water quality are based on the densities of *Enterococcus* spp. or *Escherichia coli* (*E. coli*) in freshwaters and *Enterococcus* only in marine waters (USEPA 1986). These recommendations are based on findings from previous epidemiological studies that showed positive correlations between the measured densities of these FIB groups and illness rates in recreational waters impacted by publicly owned treatment works (POTW) (Donovan et al. 2008; Dufour 1984; Wade et al. 2003). Measurements of these FIB groups, however, are still performed by culture methods that produce results the next day. During this time, public exposure to waters of unacceptable quality may have already occurred (Kim and Grant 2004). More rapid methods are needed that can alert monitoring authorities of unsafe recreational waters on the same day samples are collected.

Recent advances in molecular techniques such as real-time polymerase chain reaction (PCR) have enabled the quantitative detection of microorganisms in as little as three hours (Guy et al. 2003; Heid et al. 1996). In addition, more recent epidemiological studies at freshwater beaches have indicated that the results of a real-time quantitative

PCR (qPCR) analysis method for *Enterococcus* spp. were a stronger predictor of swimming-associated gastrointestinal illnesses than an accepted culture method for this FIB group (Wade et al. 2006, 2008). Another advantage of the PCR technique is its adaptability to the specific detection of different groups of microorganisms of varying taxonomic and/or phylogenetic breadth (Noble et al. 2006). As a result, numerous PCR-based approaches have been reported for the detection of a variety of other bacterial groups associated with fecal material (Matsuki et al. 2004; Rinttilä et al. 2004; Wang et al. 1996). However, applications of these assays for the quantitative analysis of FIB in surface waters have been somewhat limited to date.

While several conventional PCR assays have been reported for the detection of *E. coli* (Bej et al. 1991; Gyles et al. 1998; Read et al. 1992), to our knowledge only one published qPCR assay, targeting the internal transcribed spacer region of the ribosomal RNA operon, has been applied to the quantitative analysis of this species in surface waters (Khan et al. 2007). Members of the *Bacteroidales* taxonomic order have also received considerable attention as potential indicators of fecal pollution in surface waters. PCR-based approaches are not constrained by the difficulties associated with culturing these anaerobic organisms (Bernhard and Field 2000; Savichtcheva and Okabe 2006). The relatively high abundance of these organisms in feces, as well as their lower potential for re-growth in the environment, has stimulated the development and application of a number of PCR assays for detecting and/or quantifying either this entire order of bacteria (Dick and Field 2004; Kildare et al. 2007; Layton et al. 2006; Siefring et al. 2007) or specific subgroups and gene sequences within this order that may be associated with specific animal sources (Bernhard and Field 2000; Bisson and Cabelli 1979; Fiksdal et al. 1985; Fujioka and Shizumura 1985; Kildare et al. 2007; Kreader 1995; Okabe et al. 2007; Shannon et al. 2007). The huge potential for applying the PCR technique for detecting and quantifying other potentially high abundance anaerobic fecal bacteria groups, such as *Clostridia,* in surface waters has been largely unexplored to date.

The initial aim of this study was to compare the qPCR estimated cell equivalent densities of *E. coli*, *Bacteroidales* and *Clostridium* spp. with *Enterococcus* from water and sand samples collected from two POTW-impacted marine recreational beaches using a previously reported whole cell calibrator based comparative CT quantification method (Haugland et al. 2005; Siefring et al. 2007). These beaches were also included in a recent epidemiology study that will determine the correlations between FIB densities and swimming-associated illness rates (T.J. Wade, personal communication). In addition, we estimated the mean quantities of qPCR target sequence copies recovered per cell from the calibrator strains representing each of these FIB groups by our DNA extraction procedure. These recovery estimates, in turn, were applied to obtain estimates of the total target sequences for each FIB group detected in the marine water and sand samples. The potential merits and limitations of using these comparative CT quantification approaches, as opposed to absolute quantification methods, and some implications of reporting qPCR analysis results for FIB in terms of cell versus target sequence densities are discussed.

Methods

Sample Collection

Fairhope Beach (Fairhope, Alabama, USA) and Goddard Beach (Goddard State Memorial Park, West Warwick, Rhode Island, USA) were sampled on a weekly basis on Saturday, Sunday and holidays from June 2007 through September 2007. On each day, samples were collected at 8 AM, 11 AM and 3 PM from 3 transects, parallel to the shoreline approximately 60 meters apart, at depths of 0.3 and 1 meters. Along with water samples, one sand sample was collected each day from each transect at 8 AM, approximately one meter from the lowest water level using a 2 × 12 inch sterile stainless steel soil auger liner. A total of 15 and 24 days of samples were collected at Fairhope and Goddard Beach, respectively. Upon collection, the water and sand samples were stored on ice during transport to a nearby laboratory and filtered or processed as described below within 6 hours.

DNA Extraction

Fifty milliliters of water samples, collected from Fairhope Beach, and 100 mL from Goddard Beach were filtered through a 0.4 μm pore size (47 mm in diameter) polycarbonate membrane filter (GE Osmonics, Minnetonka, MA), and then filters were rinsed with 20 ml PCR-grade water (OmniPur water, VWR, West Chester, PA). Twenty-five grams (wet weight) of sand samples were mixed with 100 mL of Standard Methods 1 × phosphate buffered saline (PBS), shaken, then centrifuged for 5 min at 600 × g followed by filtration of approximately 20 mL of the supernatant as described above for the water samples. For both water and sand samples, each filter was placed into a 2 mL screw cap tube containing 0.3 g of glass beads followed by the addition of 600 μL of 0.2 μg mL^{-1} salmon DNA in AE buffer (Qiagen,Valencia, CA). Cells were suspended from the filters and lysed in a bead mill for 60 s at maximum speed and the debris were removed by centrifugation as described by Haugland et al. (2005). The supernatant containing extracted DNA was then collected in a sterile 1.7 mL low retention microcentrifuge tube and stored at −80°C until further analysis. One hundred milliliters of 1 × PBS was filtered and extracted in triplicate and used as the negative control.

Calibrator Samples and DNA Standards

Escherichia coli (EPA 206; API# 5-144-572) cells were grown in Typticase Soy Broth at 37°C for 24 hours. *Bacteroides thetaiotaomicron* (ATCC # 29741) and *Clostridium perfringens* (ATCC# 13124) cells were grown in chopped meat carbohydrate broth (Remel #05047) at 37°C for approximately 72 hours. *Enterococcus faecalis* (ATCC# 29212) cells were grown in brain heart infusion broth at 37°C for 24 hours. Cell cultures of each strain were harvested by centrifugation for 5 min at 6000 × g followed by aspiration of the supernatants. Cell pellets were washed twice by suspension in 10 mL of sterile PBS followed by centrifugation and aspiration of the supernatants as described above. Washed cell pellets were suspended in 5 mL sterile PBS. Cell concentrations in these suspensions were determined by counting the cells in either 10^{-2} or 10^{-3} diluted aliquots with the aid of a bright field microscope at 40× magnification in a disposable hemocytometer chamber (Nexcelcon Bioscience, #CP2-002). The remainder of the cell suspensions were transferred with constant stirring in 100 µL aliquots to sterile microcentrifuge tubes and frozen at −40 or −80°C. Calibrator samples were prepared from these frozen, aliquoted stock suspensions each week by first thawing and then combining 10 µL of each organism with 960 µL of AE buffer and then spotting 10 µL of this diluted, combined cell suspension on the same type of polycarbonate membrane filter used for filtering water samples. The filters were placed in tubes containing glass beads and salmon DNA buffer and extracted as described above. Approximate cell quantities in these calibrator samples were 1×10^6 *E. coli*, 4.6×10^4 *C. perfringens*, 1×10^5 *E. faecalis* and 3.0×10^5 *B. thetaiotaomicron*.

Genomic DNA isolated from the cell suspensions were used to prepare qPCR standards for each assay. Cells used for genomic DNA extraction were prepared from 0.5 mL of the washed, undiluted cell suspensions as described above with the addition of a one-hour, 37°C treatment of the crude DNA extracts with 16.6 ng/µL RNase A (Sigma # D-5006). RNase A treated DNA extracts were purified using a commercially available silica column adsorption kit as directed by the vendor (DNA-EZ, GeneRite Inc., North Brunswick, NJ). Total DNA concentrations were spectrophotometrically determined and ribosomal DNA target sequence copy concentrations were determined from reported estimates of the respective genome sizes and rRNA gene copy numbers per genome of these species (Klappenbach et al. 2001). Standards for qPCR analyses consisted of dilutions of these genomic DNA preparations containing from 10^1 to 4×10^4 target sequences per 5 µL. Genomic DNA standard dilutions were stored at 4°C during the course of the study.

Real Time PCR Amplification of FIB Sequences

All primer and TaqMan®hydrolysis probe sequences are shown in Table 1. Primers and probes used for amplification of *Enterococcus* and *Bacteroidales* target sequences were previously reported by Haugland et al. (2005) and Siefring et al. (2007), respectively. Primers used for the amplification of *Clostridium* spp. were those of the "*Clostridium perfringens* group" assay targeting about 34 *Clostridium* species as reported by Rinttilä et al. (2004). A corresponding *Clostridium* spp. TaqMan® probe was designed from an alignment of previously reported *Clostridium* 16S rRNA gene sequences. The *Clostridium* spp. probe demonstrated 100% identity with 93% of *Clostridium* sequences with greater than 1200 base pairs in the Ribosomal RNA Database Project (Cole et al. 2007). *E. coli* primer and TaqMan® probe sequences were designed from 131 *uid*A gene sequences from *E. coli* and *Shigella* isolates (GenBank accession numbers: S69414; AY698409-AY698519; and AY723315-AY723334). Alignment of all *uid*A sequences indicated that >98% of all sequences were identical to the proposed primer and probe sequences. Probes were all 5′-labeled with 6-FAM reporter dye and 3′-labeled with a TAMRA quencher. QPCR amplification of water sample and calibrator sample DNA extracts was performed by using 5 µL of 5-fold diluted extracts in a total reaction volume of 25 µL. Reagent mixes were prepared by combining 12.5 µL of TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 2.5 µL of 2 mg/mL bovine serum albumin, 1 µM of each primer, and 80 nM of probe for each reaction. Amplification occurred with an initial start at 50°C for 2 min followed by 95°C for 10 min, then forty-five PCR cycles of 95°C for 15 s and 60°C for 1 min. All amplification reactions were carried out in an ABI Model 7900 DNA thermal cycler (Applied Biosystems, Foster City, CA). No template PCR controls were also run with our samples.

Calculation of FIB Cell and Target Sequence Density Estimates

One set of genomic DNA standards from each of the cultured strains and a minimum of three calibrator samples were analyzed in duplicate with each week's batch of beach water and sand samples. The cell density of each FIB group in environmental samples was estimated as equivalents of the corresponding cultured strain cells in the calibrator samples by a previously described method (Haugland et al. 2005) based on comparative cycle threshold (CT) calculations (Applied Biosystems 1997). This method includes normalization for minor matrix-related effects on total DNA recoveries and/or amplification efficiencies in each sample DNA extract provided by CT results of the Sketa2 salmon DNA reference assay. Also, as previously described, sample

Table 1 Real-time PCR primers and probes

Assay name	Target organism	Sequences (5′ to 3′)	Reference
Entero1	*Enterococcus* (large subunit rRNA; 92 bp)	F: AGAAATTCCAAACGAACTTG R: CAGTGCTCTACCTCCATCATT P: TGGTTCTCTCCGAAATAGCTTTAGGGCTA	Ludwig and Schleifer (2000)
GenBac3	*Bacteroidales* (small subunit rRNA; 129 bp)	F: GGGGTTCTGAGAGGAAGGT R: CCGTCATCCTTCACGCTACT P: CAATATTCCTCACTGCTGCCTCCCGTA	Siefring et al. (2007); Dick and Field (2004)
E. coli	*E. coli* (*uid*A gene; 130 bp)	F: CAACGAACTGAACTGGCAGA R: CATTACGCTGCGATGGAT P: CCCGCCGGGAATGGTGATTAC	This study
Cperf	*Clostridium* spp. (small subunit rRNA; 123 bp)	F: CATGCAAGTCGAGCGAKG R: TATGCGGTATTAATCTYCCTTT P: CCCACGTGTTACTCACCCGTCCG	Rinttilä et al. (2004); This study
Sketa2	*O. keta* (salmon) (rRNA ITS region 2; 77 bp)	F: GGTTTCCGCAGCTGGG R: CCGAGCCGTCCTGGTCTA P: AGTCGCAGGCGGCCACCGT	Haugland et al. (2005)

F = Forward primer, R = reverse primer, P = probe

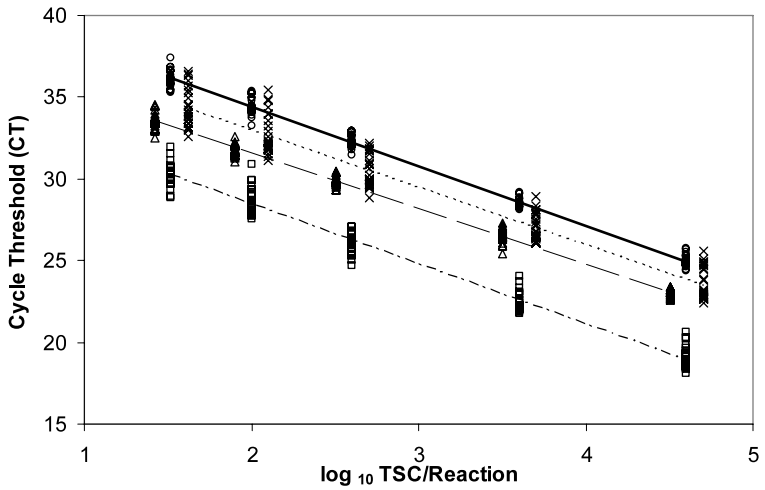

Fig. 1 Master standard curves of $\log_{10}$ target sequence copies per reaction of each indicator based on diluted DNA extracts generated from 13 independent runs. Regression lines in order from top to bottom of figure represent *E. coli* ($y = -3.64x + 41.70$), *Enterococcus* ($y = -3.50x + 39.62$), *Bacteroidales* ($y = -3.40x + 38.70$) and *Clostridium* spp. ($y = -3.66x + 35.77$). *Enterococcus* and *Bacteroidales* concentrations were offset by $+0.1\log_{10}$ and $-0.1\log_{10}$ copy, respectively, for display purposes

extracts giving Sketa2 assay results that were >3 CT units higher than the means of the corresponding calibrator samples were considered as potentially significantly inhibitory and were not included in further analyses. Amplification efficiencies used in these calculations were determined from the slope of a master standard curve for each assay that was produced from all individual standard curves generated over the study period (Fig. 1). The results of the comparative CT calculations were reported as calibrator cell equivalents (CCE). Sample extracts that yielded no CT values (no detection) were assigned CT values of 45 (total number of thermal cycles run) and subjected to the same calculation method. In addition, the resulting values were divided by 2 for final reporting of CCE estimates in these instances.

Mean estimates of CCE detection limits were obtained by performing comparative CT calculations using the intercept

values of respective master standard curves for each FIB group assay combined with the corresponding mean CT values of all calibrator sample results generated over the study period. The intercept value for each respective master standard curve represents the mean CT value expected for a reaction containing a single target sequence copy (TSC). The average effect of the water sample matrices on these detection limits was also addressed by including the mean Sketa2 reference assay CT values for all water and calibrator samples in these calculations.

To estimate the number of target sequences for each FIB group in an unknown sample, the mean TSC recovered per cell of each strain in the calibrator samples was first estimated by averaging all calibrator CT values generated over the study period. The mean calibrator CT value was then used in the following equation based on each respective FIB qPCR master standard curve:

$$\text{Mean TSC per calibrator cell} = \left(10^{(y-b)/m} \cdot v\right)/n$$

where,

y = Mean CT value
b = Intercept from master standard curve
m = Slope from master standard curve
v = Extract volume in PCR reaction
n = Number of calibrator cells

The CCE estimate for each FIB group was then multiplied by the respective mean TSC per calibrator cell value. The product was the estimated total number of target sequences present in the unknown sample, which was reported as calibrator sequence equivalents (CSE). CCE and CSE estimates from the Fairhope beach samples were multiplied by a factor of 2 in order to express them on a per 100 mL sample basis. All CCE and CSE estimates were transformed to log 10 values for further data reductions such as the calculation of geometric means.

Statistical Analysis

Differences in FIB concentrations between the two beaches, water depths, and time of day were evaluated by means of analysis of variance on the logarithms (base ten) of the CCE. All associations among indicator densities were assessed using Pearson's correlation test. All statistical analyses were performed with SPSS (version 16, SPSS, Inc., Chicago, Il.).

Results

Total FIB Cell Densities

Based on qPCR results of the calibrator samples processed along with environmental samples, the 50% limit of detection for the *Enterococcus*, *Bacteroidales, E. coli* and *Clostridium* spp. assays were estimated to be 38, 58, 1278 and 52 cells per sample, respectively. These values increased, by approximately 70% when the Sketa2 reference assay, which is designed to adjust for factors such as low level inhibition or decreases in DNA recovery caused by the environmental samples, was taken into account. The percentage of water samples for which analytical results were excluded from further analyses due to potentially significant inhibition, as defined in Materials and Methods, were 1.13% for Fairhope beach and 4.46% for Goddard Beach. Percentages of sand samples that were excluded were 0% (Fairhope) and 1.39% (Goddard). Contamination of samples was not detected in our negative controls.

In estimating FIB CCE densities in water samples, no statistically significant differences were seen in indicator densities collected at different sampling times with the following exceptions: (1) *Enterococcus* in the 8 AM samples were present at higher levels than in the 3 PM samples at Fairhope beach and lower than the 11 AM samples at Goddard beach ($p < 0.05$); (2) *Bacteroidales* and *Clostridium* spp. densities were significantly higher at the 3 PM sampling than the 8 AM sampling at Goddard beach only ($p < 0.05$). Samples collected at the 1 m depth had significantly lower CCE densities of all FIBs compared to the water samples collected at the 0.3 m depth ($p < 0.05$). Higher densities of each FIB group were generally found at Fairhope Beach. Overall, as summarized in Table 2, there was greater variability between sampling visits than there was within visits for each of the FIB groups. Median densities differed by a factor of about 4 from one sampling visit to the next for *Enterococcus* and *Bacteroidales* at both Fairhope and Goddard Beach and by a factor of about 2 at Fairhope and 3 at Goddard Beach for *E. coli* and *Clostridium* spp. *Clostridium* spp. results revealed the lowest variability both between and within sampling visits at both beaches.

Table 2 also shows the log 10 mean CCE densities per 100 mL of the Fairhope and Goddard beach water samples collected during each sampling visit at both depths. These results were equivalent to geometric mean values of 9.33×10^1, 2.95×10^3, 1.00×10^3, and 1.17×10^3 for *Enterococcus*, *Bacteroidales*, *E. coli* and *Clostridium* spp. groups, respectively, at Fairhope beach and 8.71×10^1, 4.79×10^2, 4.47×10^2 and 6.92×10^2, respectively, at Goddard beach. At both beaches, the strongest significant association was detected between *Bacteroidales* and *Clostridium* spp. concentrations across the sampling visits (Pearson's $r = 0.72$ (Fairhope) and $r = 0.53$ (Goddard), $p < 0.01$).

The mean densities of each FIB group found in all sand samples are also shown in Table 2. The geometric mean CCE densities per gram of sand were 1.41, 5.50, 70.8 and 186 for *Enterococcus*, *Bacteroidales*, *E. coli* and *Clostridium* spp. group, respectively, at Fairhope beach and 15.49, 3.47, 437 and 339, respectively, at Goddard Beach. In sand

Table 2 Summary of estimated $\log_{10}$ CCE densities and variability of the different FIB groups in marine beach water and sand

		Fairhope Beach				Goddard Beach			
		Enterococcus spp.	*Bacteroidales* spp.	*E. coli*	*Clostridium* spp.	*Enterococcus* spp.	*Bacteroidales* spp.	*E. coli*	*Clostridium* spp.
Combined depths	Mean	1.97	3.47	3.00	3.07	1.94	2.68	2.65	2.84
	Within visit SD[a]	1.14	0.78	0.61	0.54	0.99	1.43	1.00	0.77
	Between visits SD[a]	1.22	0.80	0.71	0.56	1.07	0.95	0.78	0.73
	Between days SD[a]	1.28	0.97	0.72	0.58	1.23	1.75	1.42	1.23
0.3 M	Mean	2.34	3.71	3.22	3.19	2.21	2.82	2.72	3.14
	Within visit SD[a]	1.18	0.58	0.65	0.43	0.91	0.74	0.74	0.52
	Between visits SD[a]	1.17	0.75	0.81	0.46	1.00	0.90	0.76	0.76
	Between days SD[a]	1.25	0.90	0.84	0.49	1.10	1.14	0.83	0.77
1.0 M	Mean	1.60	3.24	2.79	2.95	1.68	2.54	2.59	2.54
	Within visit SD[a]	1.05	0.74	0.49	0.58	1.01	0.86	0.67	0.68
	Between visits SD[a]	1.24	0.72	0.54	0.61	1.10	0.92	0.79	0.72
	Between days SD[a]	1.26	0.82	0.55	0.64	1.24	1.16	0.79	0.82
Sand	Mean	0.15	0.74	1.85	2.27	1.19	0.54	2.64	2.53
	Within visit SD[a]	1.30	1.25	0.78	0.67	1.15	1.18	0.71	0.47
	Between visits SD[a]	–	–	–	–	–	–	–	–
	Between days SD[a]	1.42	1.45	0.79	0.60	1.51	1.33	1.17	0.93

[a]Standard Deviation

Table 3 Number (Percentage) of samples not detected* by qPCR assays for *Enterococcus*, *Bacteroidales*, *E. coli* and *Clostridium* at Fairhope and Goddard Beach

	Enterococcus spp. (%)	*Bacteroidales* spp. (%)	*E. coli* (%)	*Clostridium* spp. (%)
Fairhope Beach	109 (35.3)	31 (10.0)	218 (70.6)	7 (2.3)
Goddard Beach	104 (20.9)	98 (19.7)	338 (71.9)	28 (5.6)
Total not detected*	213 (26.4)	129 (16.0)	556 (68.9)	35 (4.3)

*Not detected samples were those with CT values >45.00

samples, *Enterococcus* densities were found to be significantly correlated with *E. coli* densities (Pearson's $r = 0.59$, $p < 0.05$) over time at Fairhope, whereas no significant associations were detected among any of the indicators in the sand samples collected from Goddard Beach. In sand and water samples from different sampling depths each day, no significant associations were found in the densities of any of the indicators at Fairhope Beach or in *E. coli* densities at Goddard Beach. However, *Enterococcus*, *Bacteroidales* and *Clostridium* spp. densities at both water depths were significantly associated with those found in sand samples at Goddard Beach (Pearson's $r = 0.58$ at 0.3 m and $r = 0.55$ at 1 m, $p < 0.01$ for *Enterococcus*; Pearson's $r = 0.51$ at 0.3 m and $r = 0.53$ at 1 m; $p \leq 0.01$ for *Bacteroidales*; Pearson's $r = 0.55$ at 1 m only, $p < 0.01$ for *Clostridium* spp.). Ratios of *Bacteroidales* densities in the sand compared to the water samples were consistently lower than those of the other FIB groups at both beaches.

Comparison of CCE and CSE Densities

Based on the raw CT results from the qPCR analyses, *E. coli* was the only FIB group examined in this study that was present at levels below detection in the majority (68.9%) of the samples collected from both beaches (Table 3). *Enterococcus* spp, *Bacteroidales* spp. and *Clostridium* spp. were not detected in 26.4%, 16.0% and 4.3%, respectively, of the samples analyzed. These results, however, contradicted the relative CCE density estimates that suggested that *E. coli* were among the most abundant organisms in the samples from both beaches.

The mean estimated recoveries of target sequences per cell for the *Enterococcus*, *Bacteroides*, *Clostridium* spp. and *E. coli* strains in calibrator samples were 14, 14, 11 and 0.4, respectively. Multiplying the CCE estimates by these respective values provided estimates for the numbers of CSE from each FIB group recovered from water samples. In addition, sequence equivalent estimates of the 50% limit of detection for the *Enterococcus*, *Bacteroidales*, *E. coli* and *Clostridium* spp. assays per sample were 532, 812, 511 and 572, respectively. The relative $\log_{10}$ mean levels of CSE recovered from *Enterococcus*, *Bacteroidales*, *E. coli* and *Clostridium* spp. groups were 3.12, 4.61, 2.63 and 4.11, respectively, at Fairhope Beach and 3.08, 3.81, 2.27 and 3.88, respectively, at Goddard Beach. CSE relative $\log_{10}$ mean levels differed substantially from their relative CCE levels as shown in Table 2. The most pronounced differences were associated with the relatively low CSE estimates for *E. coli* compared with their respective CCE estimates. Based on the CSE estimates, all four FIB groups were found to be significantly different from each other in water samples with total *Bacteroides* occurring at the highest levels followed by *Clostridium* spp., *Enterococcus* and then *E. coli* ($p \leq 0.05$). Because the conversions to CSE units involved simply the multiplication of CCE estimates by a constant mean target sequence recovery estimate for each FIB group, all relationships described above for CCE estimates at the two beaches were the same as for the CSE results.

Discussion

The results of this study provide an initial assessment of the suitability of our previously described *Enterococcus* assay, as well as three alternative FIB qPCR assays, targeting *Bacteroidales*; *Clostridium* spp; and *E. coli* groups, for monitoring recreational waters and conducting epidemiological studies at marine beaches. The primary factors used to make this assessment included: (1) the relative overall levels of FIB CCE and CSE density estimates, (2) the comparison of spatial and temporal variability of FIB concentration estimates, (3) the frequency of negative results from each beach, and (4) the correlations between FIB density estimates in both water and sand samples at these beaches.

The *Enterococcus* CCE densities in the marine water samples at both beaches were on average almost 2-folds lower than those previously reported at two freshwater recreational beaches (Haugland et al. 2005). These lower mean densities, may be due to the use of frozen DNA extracts in our analysis and also may have contributed to the relatively high spatial or "within visit" variability of the *Enterococcus* estimates at the six sampling locations that was seen in this study, compared with the freshwater study, which incorporated a similar sampling scheme. Also observed was a relatively high degree of temporal or "between visit" variability

in the *Enterococcus* CCE densities at these marine beaches compared to the freshwater beaches. Whereas the amounts of spatial and temporal variability in density estimates were approximately the same for this FIB group in the freshwater study, temporal variability was greater than spatial variability in this study. In general, high spatial variability can be considered to be an undesirable characteristic at a beach since it suggests that multiple samples need to be collected during each sampling visit and analyzed to comprehensively determine the FIB levels present (Boehm 2007). Conversely, high temporal variability, particularly from day to day, may be considered as a desirable characteristic in epidemiological studies since varying FIB levels on different days can be more readily compared with the illness levels on these days to determine the strength of their associations.

The mean CCE density estimates of the other three FIB groups were, in each case, substantially higher than those of *Enterococcus* at both marine beaches. In most cases, these higher densities also corresponded to lower within-visit variability in the estimates at the different sampling locations than *Enterococcus*. As was the case for *Enterococcus*, the CCE density estimates of each of these alternative FIB groups also showed greater temporal than spatial variability at both of the marine beaches. Taken together, these observations suggest that qPCR analysis results for each of these alternative FIB groups, as well as for *Enterococcus*, warrant investigation in current and future epidemiological studies to determine the relationships of their estimated densities with illness rates. Surprisingly, the estimated densities of the different FIB groups were, in many cases, not well-correlated with each other over time. Since both of the beaches in this study were selected on the basis of indications that the primary potential source of fecal pollution impacting them would be from a POTW point source, this lack of correlation between the indicators may either suggest differences in their persistence in the surface water environment or else influences by other, unexpected fecal or non-fecal sources. The relatively low variability in the density estimates of *Clostridium* spp. that were seen could be consistent with the first of these two possibilities since the spore forms of these organisms would be expected to have the greatest persistence in the environment (Medema et al. 1997). These results for *Clostridia* could also be consistent with the second possibility, however, since the qPCR assay employed for analyzing this group detects a wide variety of species (Rinttilä et al. 2004) that may not all be exclusively restricted to fecal sources. Regardless of the reasons for these differences between the FIB groups, they create the potential to determine whether their respective densities differ in association with illness rates at the beaches which could aid in the selection of the most useful indicator.

Our observation that the estimated densities of all FIB groups were significantly greater at the near-shore 0.3 m sampling depth than in the deeper 1 m water samples is consistent with the results of previous studies examining culturable FIB (Wymer et al. 2004). It has been suggested that such observations may be related to the occurrence of relatively high FIB densities in beach sand that may allow these organisms to be continuously resuspended into the water column by wave action (Desmarais et al. 2002; Yamahara et al. 2007). However, the correlation between FIB densities in sand and the 0.3 m water depth samples were not better correlated than densities at the 1 m water depth. Overall, significant correlations between CCE densities in sand and water samples were either not observed or were inconsistent at the two beaches for the different FIB groups. This lack of consistent correlations suggests that the relationships between different FIB densities in sand and in the water column may be complex. The relatively low levels of *Bacteroidales* in the sand compared to the water samples observed in this study could be consistent with a lower persistence of these organisms (Walters and Field 2009). In addition, the efficiency of the technique used in this study for recovering the organisms from the sand samples was not determined. Our results suggest that the recovery efficiency of this technique should be further characterized.

The approaches described in this study for estimating both cell and target sequence densities of FIB based on comparative CT qPCR analyses of whole cell calibrator samples can be rationalized for several reasons. The CT values obtained from analyses of DNA extracts represent the actual measurements of the qPCR technique and comparison of these values with each other would provide the most direct approach for determining relative quantities of the target sequences in different samples. Such comparisons are only valid, however, if it can be assumed that the measurements are obtained under identical qPCR conditions. Due to potential differences in reagents, instruments and operators, this assumption normally cannot be met with certainty. The most commonly used approach to address these sources of variability is by comparing the CT values from DNA extracts with those from DNA standards containing known quantities of target sequences. While this approach also provides estimates of the absolute target sequence quantities in the DNA extracts, it does not necessarily provide accurate estimates of the absolute target sequence quantities in the original samples. For analyses of microorganisms, this determination can only be made with knowledge of the efficiency at which the target sequences are recovered from the cells in the sample by the DNA extraction procedure. The numbers of target sequence copies per genome of a growing number of microbial species and strains have been determined from genome sequencing studies (J. Craig Ventner Institute, http://cmr.jcvi.org/tigr-scripts/CMR/shared/Genomes.cgi) and other sources (Klappenbach et al. 2001), and these

reports demonstrate that genetic variation among different target species and strains could contribute to uncertainty in such recovery estimates. However, the results from this study, as well as others (Jackson et al. 2008; Kurmayer and Kutzenberger 2003; Ludwig and Schleifer 2000; Mazel et al. 1990), indicate that the actual copy numbers per cell may vary even more substantially in response to the physiological status of the organisms. For example, in this study, the mean estimate of the rRNA gene copy numbers recovered per cultured *E. faecalis* cell in the calibrator samples was ~3.5 times greater than the reported copy number per genome based on sequencing studies. While errors in the cell counts of the calibrator samples could potentially explain this discrepancy, we have obtained very similar estimates of target sequence recoveries from extracts of cell preparations prepared by flow cytometry (unpublished data) which is widely considered as one of the most accurate methods currently available for performing these counts. Potential errors in determining the TSC concentrations of the genomic DNA standards used to obtain these TSC to cell ratios could also introduce a bias in the estimates.

Based on these observations it can be argued that it is generally difficult to obtain verifiable absolute estimates of the quantities of target sequences from microorganisms in an environmental sample because of the difficulty in accurately determining the absolute recovery efficiency of the sequences from the cells in these samples. For the whole cell calibrator, comparative CT quantification approach does not necessarily improve such absolute quantitative estimates but it should allow for the standardization of results caused by potential extraction method-related differences in target sequence recovery efficiencies, as well as differences in PCR analytical sensitivity. Unpublished results in our laboratory have indicated that changes in target sequence extraction efficiency from the cells in calibrator samples are representative of similar changes in unknown test samples. The most important requirement for standardization in this approach is that the calibrator sample cells be in a consistent physiological state with similar TSC quantities per cell. A single best estimate of this ratio from a consistent source of calibrator cells could then be universally applied in estimating CSE densities in different water samples. While we have generally obtained similar mean target sequence recovery estimates from multiple cell cultures of *E. faecalis* over several years, it is anticipated that the widespread implementation of this method by different laboratories may require the use of standardized cell preparations that, as mentioned above, have been counted by flow cytometry or a comparably accurate and precise method. Potential commercial sources of such cell preparations already exist, for example in the form of the Bioball® product line (BioMerieux/BTF, Inc., Sydney, Australia).

In order to simplify the method and minimize analysis time and costs, the calibration of the CT results obtained in this and in our previous studies was based upon analyses of only one concentration of cells for each FIB group per calibrator sample. Using this approach, a second important requirement in the method is for the amplification efficiency of each assay to be accurately determined and consistent. Previous analyses of standard curve data for other qPCR assays in our laboratory (Brinkman et al. 2003), as well as the standard curve data from the multiple instrument runs performed in this study, have indicated that this requirement can be met. It should be noted that this approach is similar in concept to the analysis of a single standard concentration in each instrument run to adjust the y-intercept together with the use of a fixed slope from multiple, pooled standard curves to obtain TSC estimates. It has been recently reported that the uncertainty in estimating TSC quantities is lower using this "mixed" standard curve approach than when using either a master or an individual standard curve (Sivaganesan et al. 2009). In the event that a water quality criterion level is established on the basis of this method, implementation of the method could be further simplified by the use of calibrator samples with cell numbers that are similar to the criterion level. This would minimize the influence of any uncertainty in amplification efficiency estimates on interpretation of results.

If the above-described requirements for calibrator standardization and accurate estimation of amplification efficiencies can be met and if it can be assumed that potential sample-related PCR inhibitory and DNA recovery effects are adequately controlled for, then target sequence density estimates obtained by different laboratories implementing the CSE quantitative method should be comparable. However, for CCE estimates in water samples from different sources to be compared, it is necessary to assume that the ratios of target sequences to cells in these samples are similar. Further studies would be needed to determine the variability of these ratios in different surface and waste waters. Another possible argument that can be raised against the expression of qPCR results as CCE density estimates is the temptation that this approach creates for comparing the results to those of conventional culture based methods. The most obvious reason why such comparisons may not be valid is that the qPCR results will be influenced not only by the culturable organisms present in the samples but also by non-culturable and dead organisms, and even potentially free DNA in the samples. The expression of results from the qPCR method as target sequence estimates would reduce the inclination to compare its results with those of culture methods.

Nevertheless, the examination of both CCE and CSE density estimates may be informative. This is illustrated by our comparisons of these two sets of results for the different FIB groups in the marine water and sand samples in this study. While the CSE estimates suggested that the numbers of *E. coli* target sequences present in these samples were relatively low compared to those of the other FIB groups, the

CCE estimates for these organisms were among the highest. This discrepancy was attributable to the relatively low recovery of *E. coli* target sequences per calibrator cell. This may in part be due to the late growth stage at which the *E. coli*, as well as our other calibrator cell preparations, was harvested. It has been reported previously that the ratio of *E. coli* target sequences to cells declines significantly while going from early to later phases of growth (Ludwig and Schleifer 2000). In most instances it would seem likely, however, that the physiological status of these microorganisms in the environment would be more similar to that found in the late growth phase cultures that we have used. This discrepancy may also be related to the fact that the target sequence for the *E. coli* qPCR assay used in this study is the single copy *uid*A gene as opposed to multicopy rRNA gene sequence targets of the other assays. Our current results indicate that the major limitation to analyses for this FIB group in epidemiological studies at marine beaches is the high frequency of water samples that are below the qPCR limit of detection. If the relatively high cell densities of *E. coli* that are suggested to be present by the CCE estimates are a reasonable approximation, then the selection of an assay targeting the multicopy rRNA genes of these organisms may increase the frequency of samples giving positive detection of this FIB group to levels that are comparable to those of the other indicator groups. Alternatively, the relatively low recoveries of target sequences from the *E. coli* cells observed in this study could reflect the unsuitability of our DNA extraction method for cells of this type. If this is the case, then alternative DNA extraction methods may need to be employed that are more suited for this species.

Another approach to increasing the sensitivity of our current qPCR analysis method that would reduce the frequency of non-detects obtained by the *E. coli* assay in particular, but also for all indicators, would be to filter larger water volumes or use more of the DNA extracts from the filters for PCR analysis. The water volumes and crude DNA extraction technique used in our current method have the advantages of speed and simplicity but do not readily lend themselves to this approach. Conventional filtration of larger water volumes with flat polycarbonate filters has been found in many instances to result in filter clogging and previous studies in our laboratory have indicated that the use of larger volumes of crude extracts in the PCR analyses results in significantly higher frequencies of inhibited reactions (Haugland et al. 2006). Advances in the development of hollow fiber filtration devices (Smith and Hill 2009) could potentially circumvent the limitation of water volumes that can be filtered, and a number of DNA concentration and purification systems are now available that have been used for analyses of pathogens and lower density fecal indicator organisms in surface waters. Both of these types of modifications could be expected to significantly increase the sensitivity of our current method although with some costs in speed and simplicity. Modifications like these may be particularly important, however, for the accurate monitoring of lower concentration fecal indicators in non-point source impacted surface waters.

One important limitation to the use of whole cell calibrator samples for quantification stems from the fact that some qPCR assays, that may be of interest for monitoring FIB, do not have an identified or culturable microbial strain that can be used in these samples. This is most noticeably true for several assays that are currently being evaluated for the detection of source-specific FIB groups (Kildare et al. 2007; Shanks et al. 2008). In these instances the DNA standards are generally derived from cloned target sequences and quantitative estimations of target sequences in environmental samples are still generally based upon conventional standard curve based approaches. It may be possible, in some instances, to spike environmental samples with a strain or species that is expected to have similar properties as the target organisms of each of these types of assays. Results from a qPCR assay for the surrogate organism could then be used to adjust or control for differences in target sequence recoveries in different sample extracts (Siefring et al. 2007).

Notice

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