Rapid QPCR-based assay for fecal *Bacteroides* spp. as a tool for assessing fecal contamination in recreational waters

Reagan R. Converse[1], A. Denene Blackwood[1], Marek Kirs[2], Rachel T. Noble[1] and John F. Griffith

Abstract

Concentrations of fecal indicator bacteria (e.g., *Escherichia coli* and *Enterococcus* sp.) can only be used in limited ways for determining the source of fecal contamination in recreational waters because they cannot distinguish human from non-human fecal contamination. Several *Bacteroides* spp. have been suggested as potential alternative indicators. We have developed a rapid, culture-independent method for quantifying fecal *Bacteroides* spp. using quantitative polymerase chain reaction (QPCR) targeting the 16S rRNA gene. The assay specifically targets and quantifies the most common human *Bacteroides* spp. The details of the method are presented, including analyses of a wide range of fecal samples from different organisms. Specificity and performance of the QPCR assay were also tested via a laboratory experiment in which human sewage and gull guano were inoculated into a range of environmental water samples. Concentrations of fecal *Bacteroides* spp., total *Enterococcus* sp., *Enterococcus faecium*, *Enterococcus faecalis*, and *Enterococcus casseliflavus* were measured using QPCR, and total *Enterococcus* sp. and *E. coli* were quantified by membrane filtration (MF). Samples spiked with gull guano were highly concentrated with total *Enterococcus* sp., *E. coli*, E. *faecalis*, and E. *casseliflavus*, demonstrating that these indicators are prominent in animal feces. On the other hand, fecal *Bacteroides* spp. concentrations were high in samples containing sewage and were relatively low in samples spiked with gull guano. Sensitivity and specificity results suggest that the rapid fecal *Bacteroides* spp. QPCR assay may be a useful tool to effectively predict the presence and concentration of human-specific fecal pollution.

Introduction

Monitoring agencies currently employ an indicator-based system for managing recreational waters. Due to the cost and difficulty of measuring a diverse suite of pathogens, fecal indicator bacteria (FIB) are used as proxies for the presence and concentration of fecal contamination and associated pathogens. Specific indicators have been selected based on previously demonstrated, significant relationships with human health outcomes after water contact (Cabelli *et al.* 1982). The United States Environmental Protection Agency (USEPA) currently recommends the use of fecal coliforms (or *E. coli*) and *Enterococcus* sp. as the approved FIB for use in recreational waters.

While several studies have demonstrated a strong correlation between USEPA approved methods for FIB enumeration and rates of human illness after exposure to recreational water, these studies are not applicable to all waters because they have all been conducted only near sewage outfalls containing human fecal material (e.g., Cabelli *et al.* 1979, Dufour 1984, Wade *et al.* 2003). There is a paucity of data relating human health to contact with water contaminated by non-point source (NPS) runoff. This is particularly important because NPS runoff, specifically stormwater runoff, affects a majority of recreational beaches. NPS pollution differs fundamentally from point-source (PS) pollution in both composition and nature. Unlike PS pollution, for which rates of input and composition are typically known, NPS pollution is often diffuse, intermittent, and heterogeneous with agricultural, commercial, residential, and wildlife sources as potential contributors of FIB and pathogens (Schwab 2007). As a

[1] *University of North Carolina at Chapel Hill, Institute of Marine Sciences, Morehead City, NC*
[2] *Cawthron Institute, Nelson, New Zealand*

result, stormwater runoff and other NPS pollution frequently carry a complex mixture of animal fecal material and/or human contamination. This mixture of contamination sources can be highly problematic because *Enterococcus* sp. and *E. coli* are often found in high concentrations in animal feces, and understanding of the public health risk associated with contact or ingestion of animal feces is poor. Additionally, FIB have been documented to survive and grow in sand and other beach sediment, potentially persisting in the environment for longer periods and at greater concentrations than human and animal pathogens of concern (Solo-Gabriele *et al.* 2000, Anderson *et al.* 2005). Levels of *E. coli* and *Enterococcus* sp. have also been shown to have little or no relationship with the presence of human viral pathogens in NPS contaminated waters (Noble and Fuhrman 2001, Jiang and Chu 2004). However, it has been demonstrated that NPS runoff can pose a serious threat to human health (Haile *et al.* 1999). Still, the human health risk associated with exposure to water tainted by NPS pollution is not well understood.

In order to better approximate human health risk and design appropriate best management strategies, the sources of fecal contamination in NPS runoff and recreational waters must be partitioned. The combined use of conventional and alternative indicators of fecal contamination, possibly in combination with a tiered approach, could enhance detection sensitivity and specificity (e.g., Savichtcheva and Okabe 2006). Identification of these new, alternative indicators for source identification is imperative and has received increasing attention in the research community. While there are many potential alternative indicators currently discussed in the literature, this study focused on two QPCR tools for assessing human specific fecal contamination: measurement of *Bacteroides* species concentrations and the determination of the relative ratios of concentrations of three relevant *Enterococcus* species.

Differential partitioning of *Enterococcus* species among hosts has piqued interest in *Enterococcus* speciation as a potential method of bacterial source tracking (Leclerc *et al.* 1996, Pinto *et al.* 1999, Wheeler *et al.* 2002, Ferguson *et al.* 2005). *E. faecalis* and *E. faecium* are the most populous *Enterococcus* species in human feces, and they are much less prevalent in livestock (Leclerc *et al.* 1996). On the other hand, *E. casseliflavus* is generally assumed to be an environmental species, inhabiting plants and soil (Pinto *et al.* 1999, Leclerc *et al.* 1996). The ratio of *E. faecalis* and *E. faecium* to *E. casseliflavus* should be useful in determining how much of the total *Enterococcus* load is from human waste and how much comes from other sources that are of less concern for human health. Until recently, speciation was impractical. For example, *E. casseliflavus* is difficult to differentiate from *E. faecium* using conventional biochemical tests (Devriese *et al.* 1996), but the development of species specific QPCR assays makes *Enterococcus* speciation a practical possibility.

Bacteroides spp. have also received a great deal of attention as an alternative indicator of fecal pollution due to their favorable characteristics over currently used FIB, i.e., high concentrations of the marker in human feces and unlikely persistence or reproduction in aquatic environments. In the past, the utility of *Bacteroides* spp. as an alternative indicator was questioned due to difficulties encountered in culturing anaerobic bacteria. At present, there exist several molecular assays that have been developed for the detection and/or quantification of *Bacteroides* spp. associated markers or genes (Kreader 1995, Bernhard and Field 2000, Blackwood and Noble 2004, Dick and Field 2004, Carson *et al.* 2005, Seurinck *et al.* 2005, Layton *et al.* 2006, Kildare *et al.* 2007, Okabe *et al.* 2007, Reischer *et al.* 2007, Chern *et al.* 2008, Shanks *et al.* 2008, Converse *et al.* In press). Savichtcheva *et al.* (2007) found positive correlations among total coliforms, fecal coliforms, and total and human specific *Bacteroides* spp. genetic markers. Moreover, they also found that the genetic markers for *Bacteroides* spp. were predictive for the occurrence of enteric pathogens such as *E. coli* O-157 and *Salmonella* spp.

The objective of this study was to assess the relationships of several alternative FIB targeting QPCR assays and currently used USEPA approved FIB measurements as tools to distinguish human fecal contamination from animal fecal contamination. The results presented are from a laboratory-based blind experiment that was conducted by spiking sewage influent and gull guano into sterile and environmental samples in various proportions. EPA-approved FIB methods and rapid QPCR assays designed to target fecal *Bacteroides* spp., total *Enterococcus* sp., *E. faecalis*, *E. faecium*, and *E. casseliflavus* were used to analyze the samples, and the data was analyzed to assess the relationships among measured concentrations of the targets and the specificity of the different assays.

METHODS

Sample Preparation

Fourteen samples were created in the laboratory by inoculating various levels of sewage influent (Orange County Sanitation District's Plant #1, Fountain Valley, CA) into seawater collected 11 km offshore, from wavewash at Doheny Beach State Park (Dana Point, CA), and from a pond frequented by large populations of gulls also at Doheny Beach State Park. Sewage was added in various dilutions to achieve final concentrations of approximately 50, 150, 500, 1,000, and 10,000 *Enterococcus* sp. cells per 100 ml.

Four samples were created by inoculating gull guano (Wetland and Wildlife Care Center of Orange County, Huntington Beach, CA) into offshore seawater and Doheny Beach wavewash. For gull samples, approximately 1 gram of gull guano was added to 10 L of seawater. Previous research conducted on similar fecal samples had shown that this inoculation should achieve a total *Enterococcus* sp. concentration of approximately 1,000 cells per 100 ml (Griffith unpublished data).

Environmental water samples were collected from beaches with historically high concentrations of FIB: Imperial Beach, San Diego, CA; Doheny Beach State Park; Surfrider State Beach, Malibu, CA; Malibu Creek, Malibu, CA; Ballona Creek, Culver City, CA; and the Tijuana River, San Diego, CA. Sterile phosphate buffered saline (PBS) and offshore seawater were run as negative controls.

Sample Processing

One-hundred ml of samples were filtered onto 47-mm diameter, 0.4-µm pore-size polycarbonate filters (Millipore, Bedford, MA) using a six-place filtration manifold and vacuum pump assembly with Pall (East Hills, NY) disposable filter funnels. Filters were transferred into sterile 2-ml screw-cap tubes and stored at -80°C until extraction of DNA.

Enumeration of *Enterococcus* sp. and *E. coli* by MF

Total *Enterococcus* sp. and *E. coli* were enumerated by MF, following USEPA Methods 1600 for *Enterococcus* sp. and 1103.1 for *E. coli*. Griffith *et al.* (In press) provides full details of the enumeration process.

Preparation of Calibration Standard

A genomic calibration standard was prepared from *Bacteroides thetaiotaomicron* (ATCC 29148). *B. thetaiotaomicron* was grown anaerobically in an overnight culture at 37°C in Cooked Meat Medium (BD Diagnostic Systems, Sparks, MD). A portion of the cell suspension was removed and centrifuged for 5 minutes at 6,000 x *g*. The supernatant fluid was removed and aliquoted for use as a cell standard. Aliquots were frozen at -20ºC. Cell counts were obtained by removing a portion of the cell suspension, serially diluting, fixing in formalin (1% v/v final) and counting cells using SYBR Green (Invitrogen, Carlsbad, CA) following the protocol of Noble and Fuhrman (1998). A known amount of cells (100,000) was used as a standard for each set of DNA extractions.

Specimen Processing Control and Inhibition Control

A specimen processing control (SPC) was developed in order to measure the amount of sample loss during processing and matrix inhibition by adding a known amount of DNA to each sample, calibration standard, and a blank containing a polycarbonate (PC) filter at the beginning of the extraction step. Salmon Sperm Testes DNA (Sigma, St. Louis, MO) was added to Buffer AE (QIAGEN, Valencia, CA) at a final concentration of 100 ng per 500 µL and is hereafter referred to as the Extraction Buffer. TaqMan® primer and probe (Applied Biosystems, Inc., Foster City, CA) sequences for the SPC were as described by Haugland *et al.* (2005). The primers and probe target a segment of the ribosomal RNA gene operon, internal transcribed spacer region 2 of chum salmon, *Oncorhynchus keta*. Primers and probe were synthesized by MWG Biotech, High Point, NC.

All QPCR sample reactions with a Ct value 0.5 log units higher than that of the calibrator was considered to be inhibited. Inhibited samples were diluted 1:10 and 1:100 with sterile water and reanalyzed.

DNA Extraction

Samples were extracted using a modified version of the GeneRite extraction kit DNA EZ RW04 (Brunswick, NJ). Briefly, the PC filter containing either sample or calibration standard was placed in a 2-ml screw cap tube containing 0.3 gram of 1-mm silica/zirconium beads (BioSpec Corp., Bartlesville,

OK) and 500-µl of Extraction Buffer. The tubes were placed in an eight-place bead beater (BioSpec) and homogenized for two minutes. The tubes were spun at 12, 000 x *g* in an Eppendorf microfuge for 2 minutes to pellet the filter and beads. As much supernatant as possible was removed without disturbing the pellet and added to a 1.5-ml low retention microcentrifuge tube (Genemate, ISC Bioexpress, Kaysville, UT). The supernatant was spun for an additional 5 minutes at 12, 000 x *g* to pellet any debris. The supernatant was removed and added to an equal volume of Binding Buffer and incubated for one minute at room temperature. The solution was added to a DNAsure column and spun at 12, 000 x *g* for 1 minute. The flow through was discarded and 500 µL of Washing Buffer added to the column. The column was spun for 1 minute at 12, 000 x *g*. The flow through liquid was discarded and the column put in a fresh collection tube. The tube was centrifuged at 12,000 x *g* for 1 minute. The collection tube was discarded and the column added to a new collection tube. Fifty µl of Elution Buffer was added directly to the center of the column and allowed to sit for one minute. The tube was spun for 1 minute at 12, 000 x *g* to elute the DNA.

Fecal *Bacteroides* spp. Primer Design and Assay Optimization

Primers (BFDFor) and (BFDRev) and TaqMan® probe (BFDFAM) were constructed to amplify to a 110 bp fragment the 16S rRNA gene of *B. thetaiotaomicron* and other tightly related *Bacteroides* spp. using NCBI GenBank Accession no. 3722642 (Table 1). TaqMan® primers and probe were designed using Primer3 software (Rozen and Skaletsky 2000) and synthesized by MWG Biotech. The developed fecal *Bacteroides* spp. assay was optimized for primer and probe concentrations, as well as dNTP and magnesium concentrations, and cycling parameters. Factorially designed optimization was conducted for all combinations of probe concentrations (varied from 100 to 300 nM, in 100 nM increments), primer concentrations (ranged from 100 to 1000 nM, in 100 nM increments), and magnesium concentrations (varied from 3 mM to 6 µM with an equimolar increase in dNTPs for each increase in magnesium). Optimized reagent concentrations were as follows: 1000 nM of each primer, 100 nM of TaqMan probe, 200µM dNTPs, and 4mM MgCl2.

Table 1. Primer and probe sequences for the fecal *Bacteroides* spp. QPCR assay. Oligonucleotide sequences and corresponding bases based upon GenBank Assession no. 3755642 for *B. thetaiotamicron*.

Primer Name	Sequence	Length	Tm	Corresponding Bases
BFDFor	Cgt tcc att agg cag ttg gt	20 nt	59.99	248-268
BFDRev	Cgt agg agt ttg gac cgt gt	20 nt	60.03	357-337
BFDFAM	Ctg aga gga agg tcc ccc aca ttg ga	26 nt	72.81	306-332

QPCR using TaqMan® Primers and Probes

TaqMan® primers and probes were used to assay for SPC salmon sperm DNA (Haugland *et al.* 2005), fecal *Bacteroides* spp., *E. faecalis* (Santo-Domingo *et al.* 2003), *E. faecium* (Santo-Domingo, unpublished), and *E. casseliflavus* (Santo-Domingo, unpublished). The 25-µL reaction volumes were prepared using OmniMix beads, 1 µM each of forward and reverse primers, 0.1 µM of the TaqMan® probe, and 5 µL of sample DNA extract. Reactions were thermal cycled and monitored in a SmartCycler® II (Cepheid, Sunnyvale, CA). Thermal cycling occurred in two stages: first, 2 minutes at 95°C, followed by 45 cycles of 15 seconds at 94°C and 30 seconds at 60°C. After manually adjusting the threshold, cycle threshold (Ct) was determined automatically by the instrument.

Quantification of Target DNA and Statistical Analysis

The target DNA in each sample was quantified using the ΔCt method outlined by Pfaffl (2001). Total *Enterococcus* sp. was quantified using Scorpion® chemistry following the approach of Noble *et al.* (In press). Quantification of the other targets followed a similar approach. Briefly, a duplicate standard curve was conducted during each run using the calibrator (at a concentration of 1 x 10^5 cells) and 3 serial 10-fold dilutions. Using these data, the amplification efficiency was calculated with the given slope from the SmartCycler®II software and the equation: E = 10^(-slope). The ratio of target DNA in the samples to that in the calibrator was calculated following Pfaffl (2001). The ratio was then multiplied by the amount of target DNA in the calibrator to determine the quantity of each unknown.

All analyses of unknowns were run in duplicate. Samples that yielded a "non-detect" QPCR result were assigned a concentration of <5 cells per 100 ml. For regression analyses, the qualified value (<5) was converted to 5. The mean concentrations of each target for all samples were log-transformed. All statistical correlations and differences were tested in SPSS statistical analysis software (SPSS, Inc., Chicago, IL) using Spearman-Rank analysis and the Mann-Whitney U test.

Results

Specificity and Ubiquity of the Fecal *Bacteroides* spp. QPCR Assay

The designed fecal *Bacteroides* spp. QPCR assay specifically amplified four of the six *Bacteroides* spp. tested: *Bacteroides thetaiotaomicron*, *B. uniformis*, *B. distasonis*, and *B. fragilis*. There was no cross-reactivity with any strain of *E. coli* or *Enterococcus* sp. tested: *E. faecalis* (ATCC 29212), *E. faecium* (ATCC 12952), *E. casseliflavus* (ATCC 25758), *E. hirae* (ATCC 8043), *E. gallinarium* (ATCC 700425), *E. pseudoavium* (ATCC 49372), *E. durans* (ATCC 19432), *E. dispar* (ATCC 57266), *E. coli* (ATCC MG1655, ATCC 47076, ATCC 8739, and ATCC JM101, 33876). Amplification occurred over a wide dynamic range. Standard curves were performed in duplicate with each assay in order to measure QPCR amplification efficiency and to obtain a correlation coefficient (R^2). The standard curves showed high reproducibility and consistently performed quantification over 5 logs ranging from 10,000 cells to 1 cell. The average efficiency of the assay as conducted in this study was 99.54% (slope = -3.33, standard deviation = 0.008 and R^2 = 0.995, n = 20 standard curves).

Sequence analyses demonstrated that a significant portion of the amplified products observed for this assay are unculturable Bacteroidales. Sequence analysis for samples taken from human fecal sources, including septic systems and waste water treatment systems, demonstrated a higher diversity of organisms as compared to the animal scat samples. Notably, the fecal *Bacteroides* spp. sequence analyses showed that the opossum had the least organismal diversity, with significant alignment to *B. thetaiotaomicron* strain 12.4, *B. ovatus*, and *B. fragilis*. Sequence data could not be obtained for all organisms tested because of the inability to obtain adequate sequencing reads due to the small amplicon.

As expected, the fecal *Bacteroides* spp. QPCR assay produced positive results in both human and animal fecal source material. Measurements of fecal *Bacteroides* spp. normalized by mass or volume indicated concentrations for human feces 100 to 100,000 times that observed for animal feces (Table 2). For example, dogs average 13-fold lower fecal *Bacteroides* spp. concentration than humans, cats 57-fold lower, geese 1,100-fold lower, and cows at least 5,800-fold lower.

The range of concentration ratios for fecal *Bacteroides* spp. to *Enterococcus* sp. or *E. coli* was 1.08×10^2 to 3.3×10^3 and 2.9×10^1 to 5.9×10^3 (n = 12), respectively, for all human fecal samples (wastewater and septic systems; Figures 1 through 3).

Specificity of Assays in Laboratory Based Blind Experiment

Fecal Bacteroides spp. QPCR

Fecal *Bacteroides* spp. concentrations in the sewage spiked samples were two orders of magnitude greater than those in the guano spiked samples. The average fecal *Bacteroides* spp. concentration in guano spiked samples was 25 cells per 100 ml. The

Table 2. Validation of fecal *Bacteroides* spp. QPCR assay for a range of organisms. Results were normalized as cell equivalents (for *Bacteroides*) or MPN (*E. coli* or *Enterococcus*) per gram per 100 ml for scat and cell equivalents of MPN per 100 ml for wastewater samples. The mean concentration is reported with standard deviation (sd) shown in parentheses.

Sample Type	Fecal *Bacteroides* spp. per 100 ml (sd)	*E. coli* per 100 ml (sd)	*Enterococcus* spp. per 100 ml (sd)
Influent n=4	1.08E+09 (-2.03E+09)	1.12E+08 (-3.26E+08)	3.45E+05 (-6.45E+05)
Septic n=5	3.48E+08 (-3.66E+08)	8.32E+08 (-1.99E+09)	9.26E+02 (-1.75E+03)
Canine n=3	3.62E+03 (-4.99E+03)	1.74E+13 (-3.00E+13)	2.36E+06 (-3.43E+06)
Feline n=1	3.18E+06	7.35E+03	4.05E+02
Bovine n=2	3.91E+04 -3.88E+04	6.01E+10 -6.14E+10	1.51E+03 -1.99E+03
Goose n=1	6.65E+04	2.01E+16	5.25E+02
Pelican n=1	1.21E+06	2.08E+13	4.32E+03

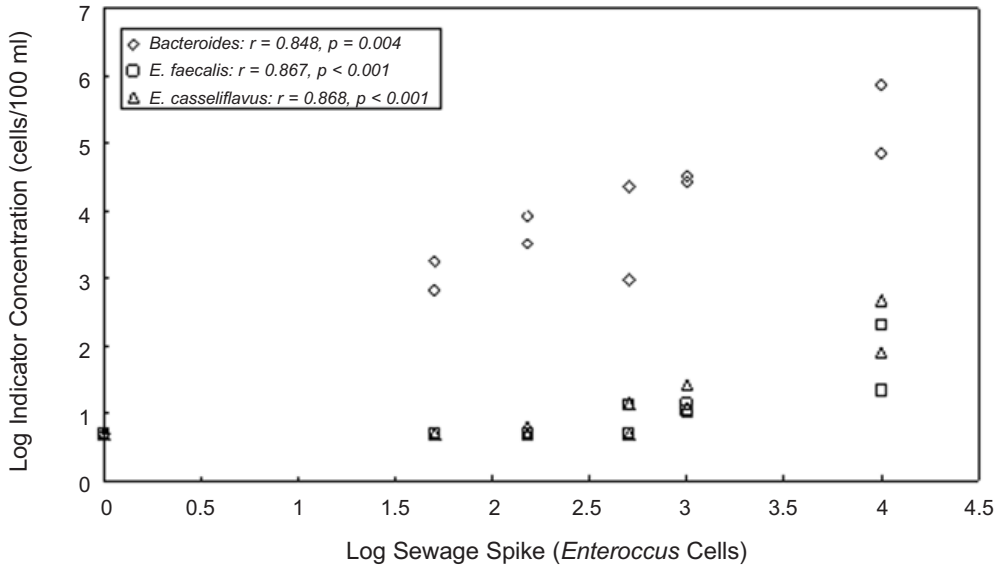

Figure 1. Correlations of *Bacteroides, E. faecalis,* and *E. casseliflavus* to amount of influent spike. Spearman rank coefficients listed.

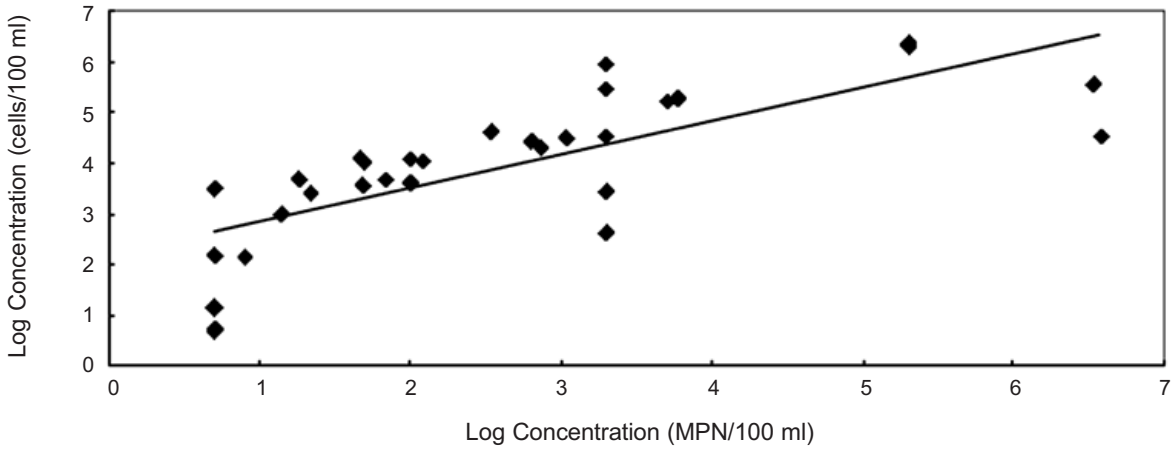

Figure 2. Correlation of *Enterococcus* concentrations as measured by QPCR and MF for all samples excluding those QPCR-inhibited samples from Doheny Pond (r = 0.921, p < 0.001).

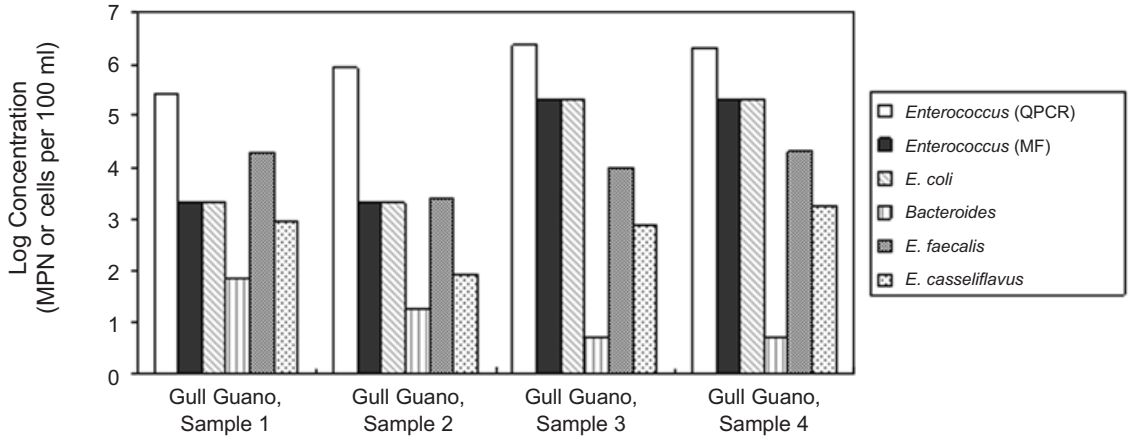

Figure 3. Concentrations of each indicator in samples spiked with full-strength gull guano.

samples that contained the lowest amount of influent spike had an average fecal *Bacteroides* spp. concentration of 1.24 x 10^3 cells per 100 ml. The fecal *Bacteroides* spp.:*E. coli* and fecal *Bacteroides* spp.:total *Enterococcus* sp. ratios ranged from 2.5 x 10^{-5} to 3.5 x 10^{-2} in samples with gull guano. In influent inoculated samples, fecal *Bacteroides* spp.:*E. coli* ranged from 2 to 259, and *Bacteroides*:total *Enterococcus* sp. ranged from 2 to 450.

The correlation of fecal *Bacteroides* spp. QPCR to *Enterococcus* sp. QPCR was 0.417 (p = 0.013) for all samples. In sewage spiked and environmental samples, this correlation was stronger (r = 0.851, p <0.001). The fecal *Bacteroides* spp. concentrations retained their correlation with *Enterococcus* sp. and *E. coli* as measured by MF in environmental samples (r = 0.803, p <0.001; r = 0.720, p = 0.002; respectively). In most offshore seawater and wavewash samples, *E. coli*, *Enterococcus* sp., and fecal *Bacteroides* spp. concentrations were low, most likely due to low inputs of fecal contamination. In Ballona Creek and Malibu Creek, fecal *Bacteroides* spp. concentrations were high while *Enterococcus* sp. and *E. coli* concentrations were low. In Tijuana River samples, fecal *Bacteroides* spp. levels were high (>100,000 cells per 100 ml), but *Enterococcus* sp. and *E. coli* levels were orders of magnitude higher.

Enterococcus speciation QPCR

E. faecalis and *E. casseliflavus* concentrations averaged 1.32 x 10^4 and 881 cells per 100 ml, respectively. The concentrations of both, normalized by the total amount of *Enterococcus* sp. in each sample, were significantly higher in gull spiked samples than in sewage spiked samples.

E. faecalis concentrations were significantly correlated with *Enterococcus* sp. and *E. coli* concentrations (r = 0.615, p <0.001; r = 0.669, p <0.001; respectively). Weaker, but still significant, correlations were found between *E. casseliflavus* concentrations and *Enterococcus* sp. and *E. coli* concentrations (r = 0.459, p = 0.006; r = 0.467, p = 0.005; respectively). *E. faecium* was only detected in one sample, offshore seawater spiked with approximately 1,000 total *Enterococcus* sp. cells.

Sewage spiked samples

Quantitative polymerase chain reaction based measurements of total *Enterococcus* sp., fecal *Bacteroides* spp., *E. faecalis*, and *E. casseliflavus* concentrations were similarly correlated to the relative amount of influent spike (r = 0.891, p <0.001; r = 0.848, p = 0.004; r = 0.867, p <0.001; and r = 0.868, p <0.001; respectively). Total *Enterococcus* sp. and *E. coli* concentrations as measured by MF were also highly correlated with the amount of sewage influent spiked into seawater samples (Table 3).

The QPCR and MF (USEPA 1600) measurements of total *Enterococcus* sp. were significantly correlated with r = 0.888, p <0.001. Total *Enterococcus* sp. as measured by QPCR was also significantly correlated with *E.coli* as measured by MF, and fecal *Bacteroides* spp., *E. faecalis*, and *E. casseliflavus* as measured by QPCR (Table 4).

Environmental samples

Results from the total *Enterococcus* sp. QPCR and MF were significantly correlated in environmental samples (r = 0.561, p = 0.024). It is interesting to note that the correlation was much stronger when the two samples from the Doheny Pond were excluded from the comparison (Table 3). These samples were

Table 3. Correlation between indicators for environmental samples.

Assay	Correlated Assay	Spearman Correlation	P-Value
E. coli (MF)	*Enterococcus* (MF)	0.963	<0.001
	Enterococcus (QPCR)	0.475*	0.073
		0.867**	<0.001
Enterococcus (MF)	*Enterococcus* (QPCR)	0.561*	0.024
		0.921**	<0.001
Bacteroides	*E. coli* (MF)	0.720	0.002
	Enterococcus (MF)	0.803	<0.001
	Enterococcus (QPCR)	0.544*	0.036
		0.794**	0.001
E. faecalis	*E. coli* (MF)	0.614	0.011
	Enterococcus (MF)	0.567	0.022
	Enterococcus (QPCR)	0.735*	0.002
		0.699**	0.008
E. casseliflavus	*E. coli* (MF)	0.157	0.561
	Enterococcus (MF)	0.192	0.476
	Enterococcus (QPCR)	0.236*	0.398
		0.183**	0.549

* Including Doheny Pond samples

** Excluding Doheny Pond samples

Table 4. Correlations of indicators to QPCR results for total *Enterococcus* for samples spiked with sewage influent.

Assay	Spearman Correlation	P-Value
Bacteroides	0.823	0.001
Enterococcus (MF)	0.888	<0.001
E. faecalis	0.705	0.01
E. casseliflavus	0.749	0.005
E. coli (MF)	0.852	0.001

so inhibited that even with 1:100 dilutions, none of the inoculated salmon sperm testes DNA was amplified. The magnitude of inhibition seen in the Doheny Pond samples was not observed in any of the other environmental samples analyzed and is unlikely to be observed in many beach recreational waters. Doheny Pond is a small, shallow body of water that is visibly contaminated, usually completely disconnected from the ocean, and supports a vast population of gulls. As an essentially closed system, gull waste and sediment are highly concentrated in the pond. Both gulls and sediment bring with them a host of known inhibitors of polymerase chain reaction (PCR), such as humic and fulvic acid, phenolic compounds, complex polysaccharides from algae, urea, and bile salts (Wilson 1997, Watson and Blackwell 2000). Most recreational water bodies experience greater flushing and support much lower densities of gulls, thus it would be unlikely to find PCR inhibitors at the concentrations observed in Doheny Pond at most recreational sites.

Discussion

Fecal *Bacteroides* spp. as an Indicator of Human-Specific Fecal Contamination

The results presented are for quantification of *B. thetaiotamicron* and related *Bacteroides* spp. using a rapid fecal *Bacteroides* spp. QPCR assay that shows specificity for human fecal contamination from a range of fecal, environmental, and laboratory-created samples. With high primer and probe affinity even at low cell number and quantification over a large dynamic range, results from this study demonstrate the usefulness and efficiency of the developed fecal *Bacteroides* spp. assay. The high reproducibility observed in this study was corroborated by the work of Shanks *et al.* (2008), who reported coefficients of variation for this fecal *Bacteroides* spp. QPCR assay of less than 1% across the dynamic range. Shanks *et al.* (2008) also report high precision, even in a multiplex QPCR context. In addition, the assay is more rapid than some other *Bacteroides* QPCR assays, which may require additional monitoring steps during cycling (Stricker *et al.* 2008).

The present study observed a high correlation of fecal *Bacteroides* spp. concentrations respective to sewage influent spikes, surpassing even that of total *Enterococcus* sp. as measured by MF. While also found in the gut and feces of other animals, fecal *Bacteroides* spp. has been previously found to have a high titer in adult human fecal samples and a low titer, if detected, in animal fecal samples (Kreader 1995, Carson *et al.* 2005). In stark contrast to results seen for either *Enterococcus* sp. or *E. coli*, the present study's fecal *Bacteroides* spp. QPCR assay shows (after normalizing cell equivalents per pg DNA) a shift in fecal *Bacteroides* spp. concentrations of at least one order of magnitude when analyzing human feces as compared to animal feces (with the exception of the opossum). The high concentration of fecal *Bacteroides* spp. in opossums has been observed by researchers in New Zealand (Kirs and Gilpin, personal communication) at the same order of magnitude as seen in the North American opossum. Where agricultural waste is a concern, our fecal *Bacteroides* spp. QPCR assay appears to be better suited than two other newly developed QPCR assays targeting human specific *Bacteroides* spp. Assays designed by Layton *et al.* (2006) and Okabe *et al.* (2007) both strongly amplified *Bacteroides* spp. from bovine and swine fecal material. Specifically, the HuBac assay detected up to 1 x 10^4 gene copies from 1 ng of bovine fecal material, only one order of magnitude lower than human samples (Layton *et al.* 2006). The assay designed by Okabe *et al.* (2007) performed slightly better with approximately two orders of magnitude difference between measured *Bacteroides* spp. concentrations in humans and cows. Results from the present study indicate a difference of four or five orders of magnitude (Table 2). This is corroborated by Shanks *et al.* (2008), who found very low copy numbers in bovine fecal samples. An obvious future research need is the direct comparison of all published *Bacteroides* spp. assays for application to aquatic and fecal samples. The present study was unable to complete a direct comparison of this study's newly developed assay with other human specific *Bacteroides* spp. assays due to low and consistent elution volumes for the extraction of DNA and many other QPCR analyses that were included in the present

study, but this work is currently underway.

The presented fecal *Bacteroides* spp. QPCR assay has a strong relationship to *Enterococcus* sp. and *E. coli* in sewage spiked samples, even at relatively low copy numbers. Furthermore, the ratios of fecal *Bacteroides* spp. QPCR results to *Enterococcus* sp. measurements show a higher trend in samples impacted by human feces than in animal scat samples and samples contaminated with gull guano. These results, along with other concurrent assessments, indicate that this assay could be useful, especially if used in a tiered approach, to identify the relative presence of human fecal contamination.

Over the course of this study an attempt was made to derive a set of ratios between fecal *Bacteroides* spp. and either *E. coli* or *Enterococcus* sp. as a preliminary step toward creating a management tool using fecal *Bacteroides* spp. QPCR assay. Ratios of greater than two from human sewage spike samples and less than one from samples inoculated with gull guano were consistently observed. This study involved analysis of a low number of samples, so further work to develop useful fecal typing ratios for animal fecal contamination is necessary. However, preliminary results suggest that the fecal *Bacteroides* spp. assay could be useful in distinguishing runoff containing predominantly human contamination from that with predominantly animal contamination, especially when the animal contamination is predominantly bird feces. That fecal *Bacteroides* spp. concentrations were low in samples spiked with gull guano is particularly advantageous; gulls contribute enormously to fecal contamination at recreational beaches with loadings of 1.77×10^8 fecal coliforms per fecal deposit (Alderisio and DeLuca 1999). In the Great Lakes area, sequencing demonstrates that gulls are sometimes responsible for greater than 50% of *E. coli* in lakes (Ram *et al.* 2007). Additionally, *E. coli* and *Enterococcus* sp. deposited in gull guano are prime candidates for persistence and growth in beach sediments, inoculating beach waters when disturbed by waves or swimmers (Kleinheinz *et al.* 2006).

It has been suggested previously that fecal *Bacteroides* spp. and other anaerobes could be useful tools in water quality management because the anaerobic bacteria would not be expected to survive in recreational waters and sediments for long periods of time (for example, Wang *et al.* 1996, Carson *et al.* 2005). At warm temperatures (≥24°C), PCR-detectable *Bacteroides* persists for only 1 to 2 days (Kreader 1998), suggesting that for some enteric pathogens, they could be an improved indicator for human health. Some pathogens may not be well represented by *Enterococcus* sp. and *E. coli*, which have been documented to persist and grow in the marine environment (Solo-Gabriele *et al.* 2000, Anderson *et al.* 2005). Poliovirus, for instance, can be inactivated in as few as one to three days in warm temperatures (Wait and Sobsey 2001). The potentially short survival of *Bacteroides* spp. in environmental waters may improve estimations of the presence of fresh fecal contamination, and may therefore more accurately predict the presence of viral pathogens that are not capable of persisting or replicating in the environment. This study did not focus on the persistence of fecal *Bacteroides* spp. in water samples, but persistence is a necessary area for further research.

Because no single indicator may be a good proxy for all pathogens, a tiered approach may be best for quantification and characterization of human health risk. *E. coli* and *Enterococcus* sp. serve as good indicators of overall fecal contamination over sources and time. When there are exceedances of current FIB standards, the fecal *Bacteroides* spp. assay used in this study may be useful in apportioning human from non-human sources. Also because the *Bacteroides* spp. members are found in higher concentrations than either *E. coli* or *Enterococcus* sp. in gut flora, it may be that the ratio of *Bacteroides* spp. to either *E. coli* or *Enterococcus* sp. could be a useful tool for water quality management. With the exception of Tijuana River samples, influent spiked seawater and environmental samples in this study had ratios of fecal *Bacteroides* spp.:*Enterococcus* sp. (as measured by MF) ranging between 6.26 and 152 when *Enterococcus* sp. concentrations exceeded single sample standards. Samples from the Doheny Pond (known to be contaminated with gull guano) or with gull guano spikes had much smaller ratios: 2.5×10^{-5} to 0.16. The Mann-Whitney U test shows that these two ranges of ratios are significantly different, suggesting that a ratio between fecal *Bacteroides* spp. and *Enterococcus* sp. could indicate the presence of human fecal material. This ratio will likely be most useful only when *Enterococcus* sp. concentrations exceed the standards; i.e., in a tiered approach context. In cases for which the *Enterococcus* sp. or *E. coli* concentrations are very low and when it is likely that fecal *Bacteroides* spp. concentrations would be low, it would be difficult to

determine whether the low concentrations are due to lack of human input, methodological variability, or dilution of contamination.

Enterococcus Speciation as an Indicator of Human-Specific Fecal Contamination

An addition component of our study has been the analysis of laboratory-created samples using specific primer sets for three *Enterococcus* species. Until recently, speciation was impractical given that *E. casseliflavus* is difficult to differentiate from *E. faecium* using conventional biochemical tests (Devriese *et al.* 1996). The development of QPCR assays for each species allows *Enterococcus* speciation to become a practical possibility for bacterial source tracking. In fact, Scott *et al.* (2005) have effectively designed an *E. faecium* based assay for the *esp* gene that is often specific to human fecal contamination. Unfortunately, the assays tested in the present study provide only inconclusive results. Concentrations of *E. faecalis* and *E. casseliflavus* were each correlated with amounts of sewage additions. Concentration of both species were nearly equal in most sewage samples, suggesting that *E. casseliflavus* may be a more important component of human sewage than previously thought. The strong detection of *E. faecalis* in samples spiked with gull guano also indicates that *E. faecalis* is no more strongly related to human fecal contamination than to bird contamination; consequently, at least in this context detection of *E. faecalis* would have no source predictive value. The *Enterococcus* speciation QPCR may have promise in another arena. The *E. faecalis* and *E. casseliflavus* QPCR assays related strongly to total *Enterococcus* sp. QPCR and MF methods in samples containing fresh contamination (i.e., those samples inoculated with fresh sewage or guano). It may be that specific species of *Enterococcus* could be used to assess the presence of fresh contamination, thereby permitting partitioning of *Enterococcus* sp. stemming from reservoir populations (in sand and sediment) from fresh fecal contamination sources (like fresh sewage). This concept will require further refinement and testing, but could represent an advancement in water quality management.

QPCR versus MF for Total *Enterococcus* sp.

The correlation between total *Enterococcus* sp. concentrations as measured by QPCR and MF show that the QPCR assay yielded results similar to existing methods, corroborating findings from a previous study conducted in southern California waters by Noble *et al.* (In press).

Total *Enterococcus* sp. as measured by QPCR were often orders of magnitude higher than those for *E. faecalis* and *E. casseliflavus*. This is likely the result of a combination of several factors. First, *Enterococcus* sp. cells may carry multiple copies of the gene targeted by the *Enterococcus* primers and probe. Second, QPCR could be measuring DNA from dead cells. Third, cells may be in a viable but non-culturable state due to environmental stress, leading to underestimations by culture based MF.

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