Improved real-time PCR assays for the detection of fecal indicator bacteria in surface waters with different instrument and reagent systems

S. Siefring, M. Varma, E. Atikovic, L. Wymer and R. A. Haugland

ABSTRACT

Previously reported and redesigned primer and probe assays were evaluated for the quantitative analysis of the fecal indicator bacterial groups, *Enterococcus* and *Bacteroidetes* with three real-time PCR instrument and reagent systems. The efficiency and sensitivity of the original assays varied between systems in analyses of DNA extracts from pure cultures of *Enterococcus faecalis* and *Bacteroides fragilis*, whereas the modified assays gave more consistent results. Distinctions between original and modified assays also occurred in analyses of known spike levels of *E. faecalis* and *B. fragilis* cells on filters with diverse surface water retentates. Percentages of samples causing PCR failures due to inhibition were lower using the modified assays. The accuracy and precision of spiked bacteria measurements were also generally higher, although mean measurements of both target organisms were still significantly different between systems ($p < 0.05$). The accuracy and precision of spiked bacteria measurements by both modified assays were further improved using a new sample matrix control spike consisting of cultured *Lactococcus lactis* cells and a reference assay for this organism. Corrections provided by the *L. lactis* assay eliminated significant differences in *E. faecalis* measurements between all three systems and between two of the three systems in *B. fragilis* measurements.

Key words | assays, *Bacteroidetes*, *Enterococcus*, instruments, PCR, real-time

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INTRODUCTION

Monitoring of recreational waters for fecal indicator bacteria groups which occur at high levels in human and other animal feces is widely accepted as a practical substitute for the direct monitoring of diverse and less abundant pathogenic organisms. Previous studies showed that culture-based measurements of one such bacterial fecal indicator group, *Enterococcus*, were correlated with swimming-associated gastro-intestinal illness rates (GIIR) in both fresh and marine recreational beach waters while another indicator species, *Escherichia coli*, was correlated with GIIR in fresh waters only (Cabelli *et al*. 1982; Dufour 1984). These relationships between culturable indicator bacteria concentrations and GIIR currently form the basis for recommendations by the U.S. EPA on the acceptability

of surface waters for recreational use (Dufour & Ballantine 1986). Culture methods require at least 24 hours for results. During this waiting period changing water conditions may result either in unnecessary beach closings or exposures to unsafe conditions (Boehm *et al*. 2002; Leecaster & Weisberg 2001; Wymer *et al*. 2004). Due to its capability of providing results within several hours, a real-time, quantitative polymerase chain reaction (QPCR) method, incorporating a primer and probe assay for the detection of *Enterococcus* DNA and another assay for fecal bacteria in the *Bacteroidetes* class, has been evaluated as an alternative to culture methods for water quality analyses in two of the Great Lakes. Measurements of enterococci by the QPCR method showed positive correlations with enterococci enumeration

results by an approved membrane filtration culture method (Haugland *et al.* 2005) and with swimming-associated GIIR as determined in a concurrent epidemiological study (Wade *et al.* 2006). A positive association was also observed between QPCR-measured *Bacteroidetes* levels and GIIR at one of the two beaches included in this study.

A commercially available, ready to use PCR reagent (TaqMan® Universal Master Mix, Applied Biosystems, Inc. (ABI)) and previously reported primer and probe sets which target the large subunit ribosomal RNA (rRNA) gene of enterococci (Ludwig & Schleifer 2000) and the small subunit rRNA gene of *Bacteroidetes* (Dick & Field 2004) were used in the Great Lakes study. Both of these analyses provided the highest level of detection sensitivity when performed using a two hour thermal cycling protocol (Haugland *et al.* 2005). Since that time, newer PCR reagent systems have been commercially introduced with claims of significantly shorter thermal cycling time requirements. Among these is OmniMix® PCR reagent (Cepheid 2006*a*) and TaqMan® Fast Universal PCR Master Mix (Fast Mix) (Applied Biosystems 2006*a*). Wide-spread acceptance of the real-time QPCR technology for water quality monitoring would be aided by the availability of choices in instruments and also by the newer PCR reagents which offer potential for shorter analysis times. It is important, however, to establish that indicator bacteria measurements by different QPCR systems are consistent.

We report a comparison of three real-time QPCR instrument and reagent systems: ABI standard block model 7900 with TaqMan® Universal Master Mix (analysis time ∼ 2 hr); Cepheid SmartCycler® with OmniMix® (analysis time ∼ 30 min); and ABI fast block model 7900 with TaqMan® Fast Mix (analysis time ∼ 35 min), for the quantitative analysis of known spike levels of two fecal bacteria, *Enterococcus faecalis* and *Bacteroides fragilis*, in the presence of diverse surface water filters. Preliminary analyses of DNA extracts from pure cultures indicated differences in the performance of the previously reported QPCR primer and probe sets for both the *Enterococcus* and *Bacteroidetes* assays on the different instruments. Modifications of the primer and probe assays intended to reduce these differences were therefore also evaluated. A new sample matrix spike consisting of cultured *Lactococcus lactis* cells and an associated quantitative reference and control assay featuring primer and probe sequences targeting the large subunit rRNA gene of this organism at the same sites as those targeted by the modified *Enterococcus* assay primers and probe was also developed. The effects of this new reference assay on the accuracy and precision of measurements of both indicator bacteria in the different types of water samples and with the different instrument systems were compared with those of our previously reported matrix spike reference and control assay targeting salmon DNA (Haugland *et al.* 2005).

MATERIALS AND METHODS

Cultured bacterial cells and salmon control DNA

E. faecalis, strain 29212, *L. lactis* strain 19435, and *Sphingobacterium multivorum* strain 51330 from the American Type Culture Collection (ATCC) were grown in brain heart infusion broth (BHIB) for 24 hr, 48 hr and 48 hr, respectively at 37°C. *Myroides (Flavobacterium) odoratus* strain ATCC 4651 was grown in trypticase soy broth for 48 hr at 30°C. *B. fragilis* strain ATCC 25285 was grown anaerobically in BHIB containing 5 g/l yeast extract, 5 mg/l hemin, 120 mg/l MgSO4 and 50 mg/l CaCl2 for 24 hr at 37°C. All cultures were harvested by centrifugation at 8,000 × g for 5 min, washed twice using sterile phosphate buffered saline (PBS) and stored in aliquots at −40°C. Cell concentrations of each organism in the final washed suspensions were determined by bright field microscopy at 40x magnification in disposable hemocytometer chambers (Nexcelcon Bioscience, #CP2-002). Salmon testes DNA was purchased from Sigma-Aldrich (#D1626) and dissolved in AE buffer (Qiagen, Valencia, CA). Concentrations of diluted working stock solutions were determined by spectrophotometric absorbance readings at 260 nm.

Surface water samples

Water samples were obtained from Silver Beach on Lake Michigan near St. Joseph, MI from July 25 through August 1, 2004, from Edgewater Beach on the Gulf of Mexico near Biloxi, MS on May 24, 2005 and from multiple locations on Lake Ponchartrain, LA and its tributaries from

September 20–29, 2005. Collections and determinations of physical and microbiological characteristics of the Lake Michigan and Gulf of Mexico water samples were performed as previously described (Haugland *et al.* 2005). Lake Ponchartrain water samples were collected and characterized by the U.S. Geological Survey using similar methods. A summary of the characteristics of these sets of water samples is provided in Table 1. An additional water sample was collected from the Pacific Ocean, 18 km off of the southern California coast, on June 22, 2006.

Sample processing for QPCR analyses

Water samples were filtered through 47-mm, 0.4-μm pore size polycarbonate filters and the filters extracted by glass bead milling to release total DNA as previously reported (Haugland *et al.* 2005) with some modifications. For the Lake Ponchartrain and Gulf of Mexico samples, only 50 ml of the water samples were filtered as opposed to 100 ml for the Lake Michigan and Pacific Ocean samples. Unlike the Lake Michigan and Gulf of Mexico samples, the Lake Ponchartrain and Pacific Ocean samples were filtered for QPCR analysis after their shipment to the analytical laboratory. All filters were amended with approximately 1×10^5 cells of *L. lactis* in 20 μl of PBS prior to extraction and a replicate filter of each water sample was also amended with 1×10^4 cells of *E. faecalis* and 5×10^4 cells of *B. fragilis* in 10 μl PBS prior to extraction. Calibration standards, used for quantitative analyses, consisted of clean filters amended with 10 μl suspensions of 1×10^4 *E. faecalis* and 5×10^4 *B. fragilis* cells in addition to *L. lactis* cells as described above, and were extracted in the same manner at a frequency of at least two

per each batch of test samples. Three negative control filters were prepared by filtering 40 ml PCR-grade water at the same time as the sample filters and extracted in the same manner. All sample filters, calibration standards and negative controls were extracted in 600 μl of AE buffer containing 0.2 μg/ml salmon testes DNA. After bead milling, the extraction tubes containing the filters were centrifuged at 12,000 $\times$ *g* for 1 min and approximately 400 μl of the resulting supernatants were transferred to clean, low-retention 1.7 ml microcentrifuge tubes (GENE MATE, #C-3228-1) for direct QPCR analysis.

QPCR analyses

Analyses were performed using three different sequence detection instruments, a Cepheid SmartCycler® II (Cepheid, Sunnyvale, CA), an ABI Model 7900 with fast block (ABI, Foster City, CA) and either an ABI Model 7700 or ABI Model 7900 with standard block, using reagents developed specifically for each instrument. Reagent mixes for the SmartCycler® were prepared by dissolving OmniMix® reagent beads (Cepheid) in 35 μl distilled water and adding 5 μl of a mixture of forward and reverse primers (5 μM each) and 400 nM TaqMan® probe. Each of these mixes was sufficient for two reactions. Reagent mixes for the Model 7900 fast block were prepared by combining 12.5 μl of TaqMan® Fast Mix (ABI), 5 μl of primer and probe mix as described above, and 2.5 μl of 2 mg/ml bovine serum albumin (fraction V, GibcoBRL, Gaithersburg, MD) per reaction. Reagent mixes for the Model 7700 or Model 7900 standard block were prepared as described for the Model 7900 fast block except substituting TaqMan® Universal Master Mix (ABI) for the TaqMan® Fast Mix. All reactions

Table 1 | Physical, chemical and microbiological characteristics of water samples

Source of Samples	N	Turbidity (NTU) Mean	Range	Conductivity (μS/cm) Mean	Range	Salinity (ppt) Mean	Range	Enterococci (CFU/100 ml) Mean	Range
Lake Michigan	18	6	2–11	ND	ND	ND	ND	58	3–122
Gulf of Mexico	18	56	18–109	21278	19000 – 22000	12.79	11.55–13.48	79	0–520
Lake Ponchartrain	15	34	10–145	8927	42–16700	3.64	0.03–9.79	1195	11–9300

were prepared by combining 20 µl of the reagent mixes with 5 µl of DNA extracts that were additionally diluted with AE buffer in some cases. Reactions were performed in individual 25 µl optical tubes in the SmartCycler® and in 96-well optical plates in the ABI instruments. The thermal cycling program for most SmartCycler® reactions consisted of 2 min at 95°C, followed by 40 cycles of 5 sec at 95°C and 30 sec at 66°C. The thermal cycling program for the Model 7900 fast block reactions was 2 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 30 sec at 60°C. The thermal cycling program for the Model 7700 and Model 7900 standard block reactions consisted of 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. The 9600 emulator option was used with the Model 7900 standard block program to match the thermal cycling ramping times of the Model 7700. Determinations of cycle threshold (C_T) were performed automatically by the instruments after manually adjusting the threshold fluorescence values to 8 units on the SmartCycler® and 0.03 delta Rn units on the ABI instruments.

The PCR primer and hybridization probe sequences for the different assays used in this study are listed in Table 2. All reactions used hydrolysis probe (TaqMan®) chemistry and all probes were labeled with 6-FAM as the 5′-terminal reporter dye and either TAMRA (ABI) or a non-fluorescent (black-hole) quencher (Operon Biotechnologies, Huntsville, AL) at their 3′-terminus.

QPCR data analyses

As previously described (Brinkman *et al.* 2003; Haugland *et al.* 2005), amplification efficiency and per sample detection sensitivity estimates (defined in Table 3) were initially determined for each assay from standard curve-style analyses of serially diluted DNA extracts from at least two separate pure culture cell samples based on the slopes and intercepts, respectively, of linear regressions of C_T values on cell equivalents (CE: cell quantities in original extracted sample / sample extract dilution factor, assuming fixed extract and analyzed sample volumes of 0.6 ml and 5 µl, respectively). C_T results from subsequent analyses of calibration and test samples were used to estimate target organism calibrator cell equivalents (CCE) in the test

Table 2 | QPCR assays

Assay Name	Target Organism(s)	Target gene (Amplicon length)	Primer and probe sequences (5′–3′)	Reference
Entero1	Enterococci	Large subunit rRNA (86 bp)	Forward primer: AGAAATTCCAAACGAACTTG Reverse primer: CAGTGCTCTACCTCCATCATT Probe: TAGCCCTAAAGCTATTTCGGAGAGAACCA	(Ludwig & Schleifer 2000)
Entero2	Enterococci	Large subunit rRNA (139 bp)	Forward primer: GAGGACCGAACCCACGTA Reverse primer: CAGTGCTCTACCTCCATCATT Probe: ACCCACACCTCATCCCCGCACTTTTC	(this study) (Ludwig & Schleifer 2000)
GenBac2	*Bacteroidetes*	Small subunit rRNA (378 bp)	Forward primer: ACGCTAGCTACAGGCTTAACA Reverse primer: ACGCTACTTGGCTGGTTCA Probe: CAATATTCCTCACTGCTGCCTCCCGTA	(Dick & Field 2004)
GenBac3	*Bacteroidetes*	Small subunit rRNA (129 bp)	Forward primer: GGGGTTCTGAGAGGAAGGT Reverse primer: CCGTCATCCTTCACGCTACT Probe: CAATATTCCTCACTGCTGCCTCCCGTA	(this study) (Dick & Field 2004)
Lact3	*L. lactis*	Large subunit rRNA (139 bp)	Forward primer: GGCCCGAACCAGGACA Reverse primer: ACAGTGCTCTACCTCCAATACA Probe: CGCTATCCACAAGTCATCCAAACACTTTTCAA	(this study)
Sketa2	*O. keta* (salmon)	rRNA Internal transcribed spacer region 2 (77 bp)	Forward primer: GGTTTCCGCAGCTGGG Reverse primer: CCGAGCCGTCCTGGTCTA Probe: AGTCGCAGGCGGCGCCACCGT	(Haugland *et al.* 2005)

Table 3 │ QPCR assay amplification efficiency and extrapolated cell equivalent (CE) sensitivity estimates with different instrument and reagent systems from standard curve slope and Y-intercept coefficients

Instrument, Reagent system	Assay[a]	Slope (std error)	Amp. Efficiency[b]	Y-Intercept (std error)	Sensitivity[c]
ABI Model 7700/7900, TaqMan® Mix	Entero1	− 3.41 (0.09)	97%	39.93 (0.34)	0.9 CE
	Entero2	− 3.41 (0.08)	96%	40.27 (0.29)	1.2 CE
	GenBac2	− 4.23 (0.14)	72%	47.62 (0.61)	63.1 CE
	GenBac3	− 3.47 (0.10)	94%	40.61 (0.45)	1.5 CE
	Lact3	− 3.34 (0.02)	99%	39.61 (0.10)	0.8 CE
	Sketa2	− 3.50 (0.10)	93%	23.88 (0.24)	25.0 pg/ml
ABI Model 7900, Fast Mix	Entero1	− 3.71 (0.08)	86%	44.85 (0.28)	20.3 CE
	Entero2	− 3.32 (0.08)	100%	39.65 (0.30)	0.8 CE
	GenBac2	− 3.18 (0.09)	106%	42.58 (0.34)	6.5 CE
	GenBac3	− 3.43 (0.14)	96%	41.24 (0.62)	2.3 CE
	Lact3	− 3.41 (0.06)	96%	40.27 (0.31)	1.2 CE
	Sketa2	− 3.09 (0.19)	111%	25.51 (0.32)	20.4 pg/ml
Cepheid SmartCycler®, OmniMix®	Entero1	− 3.65 (0.09)	88%	42.57 (0.34)	6.5 CE
	Entero2	− 3.34 (0.06)	99%	39.24 (0.22)	0.6 CE
	GenBac2	− 3.51 (0.18)	92%	42.24 (1.01)	4.3 CE
	GenBac3	− 3.47 (0.10)	94%	41.58 (0.50)	2.8 CE
	Lact3	− 3.33 (0.05)	100%	39.54 (0.17)	0.7 CE
	Sketa2	− 3.41 (0.08)	96%	23.12 (0.19)	11.3 pg/ml

[a]Assays described in Table 2.
[b]Calculated from slope (s) of standard curve as: $(10^{(1/-s)} - 1) \times 100\%$.
[c]CE value or pg/ml at Y = 40.00.

samples by a previously described comparative cycle threshold calculation method (Applied Biosystems 1997) with modifications (Haugland *et al.* 1999, 2005). CCE values were determined using only test sample and mean calibration sample target organism assay C_T values (ΔC_T method) and also after corrections of the data using C_T values from the salmon DNA or *L. lactis* reference assays ($\Delta\Delta C_T$ method). Amplification factors used in the comparative cycle threshold calculations were determined from the slopes of the initial standard curve-style target organism assay regressions described above and confirmed for each set of analyses by combined regression analyses of log-transformed fluorescence measurements taken during the exponential growth phases of the calibration sample reactions. The slopes of these latter regressions provide an estimate of the amplification efficiency which can be

obtained without the need for generating new standard curves (Liu & Saint 2002; Peirson *et al.* 2003).

RESULTS AND DISCUSSION

Determinations of assay efficiencies, sensitivities and specificities with different instrument and reagent systems

A summary of amplification efficiencies and extrapolated CCE sensitivity estimates determined from standard curves for each of the QPCR assays with the different instrument/ reagent systems is shown in Table 3. These results were consistent with our earlier findings for the previously reported *Enterococcus* assay, Entero1, using the TaqMan® Mix (Haugland *et al.* 2005), but suggested that the assay was less efficient and potentially less sensitive in detecting target
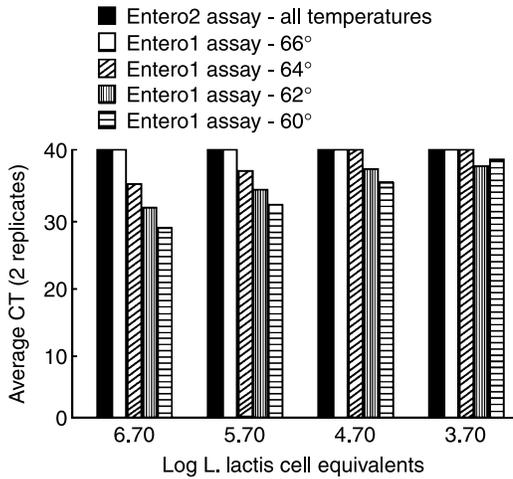
Figure 1 | Cross-detection of *L. lactis* DNA samples by *Enterococcus* QPCR assays at varying annealing/extension temperatures using OmniMix® PCR reagent and 30 min thermal cycling protocol. Cell equivalents are the approximate numbers of cells in the extracted sample divided by the extract dilution factors used for analyses. Cycle threshold (C_T) values of 40 = no detection.

organisms using either of the two newer instrument/reagent systems under the thermal cycling conditions employed. As also shown in Table 3, these differences in performance by the different instrument/reagent systems were largely eliminated using the modified Entero2 assay under the same conditions.

For the SmartCycler®/OmniMix® system a number of thermal cycling programs employing different annealing/ extension times and temperatures were evaluated to characterize optimal conditions. Analyses containing OmniMix®

reagent required more stringent temperatures to maintain comparable levels of specificity to those obtained with the ABI reagents. As shown in Figure 1, cross detection of DNA extracted from 5×10^5 *L. lactis* CE by the OmniMix® Entero1 assay occurred at annealing/extension temperatures below 66°C. Analyses using both ABI systems at 60°C gave detection sensitivities of $\geq 5 \times 10^5$ *L. lactis* CE with the same assay (data not shown). An alignment of large subunit rDNA sequences (Figure 2) was used to design the modified forward primer and probe for the Entero2 assay to reduce cross reactivity with this organism. This alignment also suggested that the Entero2 assay may be less sensitive in detecting DNA from *Lactobacillus* and *Streptococcus* species, but should be similar to the Entero1 assay in its ability to discriminate against other major groups of gram positive bacteria such as *Bacillus* and *Listeria* (Figure 2). The selection of the 66°C annealing/extension temperature for both *Enterococcus* assays with OmniMix® reagent was further dictated by analyses of a pristine, off-shore Pacific Ocean water sample, containing no cultureable enterococci. OminMix® reagents produced consistent detection signals using the Entero2 assay when annealing/extension was performed at 64°C, but yielded no signals at 66°C (data not shown). This water sample extract also gave no signals for enterococci using either the Entero1 or Entero2 assays in analyses with either of the ABI systems at 60°C.

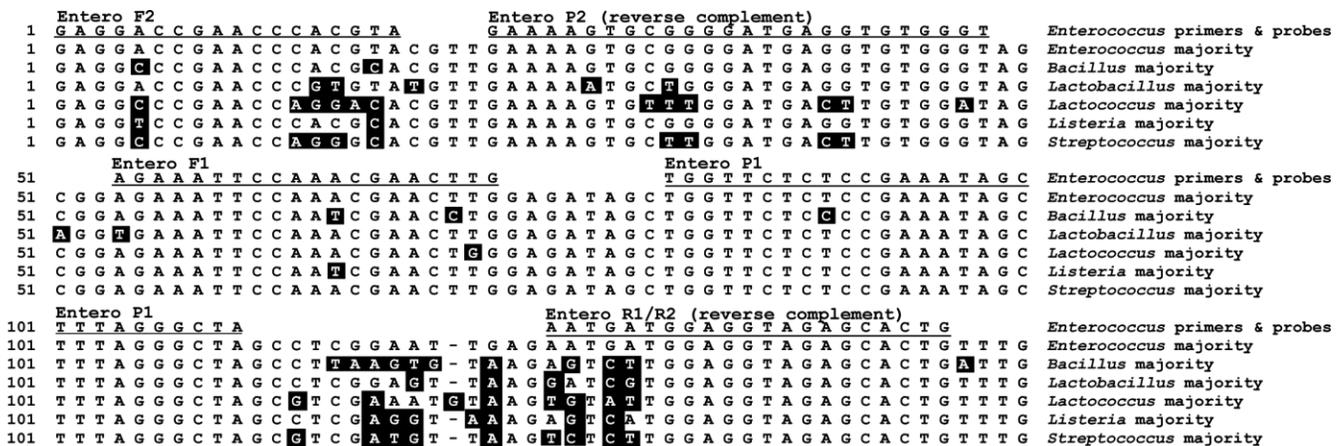The previously reported assay for *Bacteroidetes* class bacteria, referred to here as GenBact2, also showed

Figure 2 | Comparison primer and probes sequences used in Entero1 and Entero2 QPCR assays with corresponding target and non-target bacteria large subunit rRNA gene sequences from the National Center for Biotechnology Information GenBank database. Primer and probe sequences are shown on the top line of each alignment and target organism rDNA sequences are shown on the second line. Bases in non-target organism rDNA sequences that are not homologous with the primer and probe sequences are shaded. Only the majority sequences for each bacterial genus are shown and sequences from some species within these genera may vary slightly.

substantial differences in amplification efficiency and sensitivity with the different instrument/reagent systems (Table 3). Differences were again largely eliminated with the modified assay (GenBact3) under the same reaction conditions. The two assays performed comparably with the SmartCycler®/ OmniMix® system, however, the GenBact3 assay was considerably more efficient and provided a lower extrapolated detection sensitivity than the GenBact2 assay on the ABI 7700 and 7900 instruments with TaqMan® Mix. As indicated in Table 2, the primary difference between these assays is in their amplicon lengths. The target sites of the reverse primers in the two assays partially overlap, while the forward primer in the GenBact3 assay is directed to a sequence approximately 250 bases closer to the reverse primer site. We hypothesize that the lower amplification efficiency of the GenBact2 assay in association with the ABI TaqMan® reagent may be caused by a slower polymerization rate associated with this reagent than by OmniMix® which makes it less efficient in generating long amplicons. This notion is supported by assay development guidelines provided in the manuals for ABI real-time PCR instrument systems which specify that amplicons should be no longer than 150 bp.

Standard curves generated by the GenBact2 and Sketa2 assays with TaqMan® Fast Mix reagent gave mean amplification efficiencies of greater than 100%, however, efficiencies determined from fluorescence growth curves of calibrator sample reactions using these assays indicated much lower mean efficiencies of only 82% and 90%, respectively. Mean efficiencies determined from the individual fluorescence growth curves of all other assays with the different systems were within the 95% confidence ranges determined from the standard curve regression analyses.

Specificities for GenBact2 and GenBact3 assays were estimated *in silico* using Probe Match software (Cole *et al.* 2005). The forward primer sequence of the GenBact2 assay showed complete conservation in 61.6% of the full length *Bacteroidetes* sequences ($n = 8,412$), and no such conservation in any of the *Flavobacteria* ($n = 1,595$) and *Sphingobacteria* ($n = 940$) full length sequences, as opposed to 86%, 1.7% and 5.3%, respectively for the GenBact3 assay forward primer. Allowing for 1 mismatch, the reverse primer sequence of the GenBact2 assay was

conserved in 85%, 0.06% and 0% full length sequences in the *Bacteroidetes*, *Flavobacteria* and *Sphingobacteria* classes, respectively as opposed to 63%, 0% and 0%, for the GenBact3 assay reverse primer. A common probe is used in the two assays which showed complete conservation in 98%, 93% and 5% full length sequences in the *Bacteroidetes*, *Flavobacteria* and *Sphingobacteria* classes, respectively. This type of analysis is not fully predictive of the target group inclusiveness or non-target group discriminatory ability of the assays since a limited number of mismatches can still allow efficient detection in QPCR analyses depending largely on their positions within the probe and primer oligonucleotides (Haugland *et al.* 2004). A limited experimental assessment of the specificity of these assays was performed by analyzing DNA extracts from cultured cells of *M. odoratus* and *S. multivorum* which represent the *Flavobacteria* and *Sphingobacteria* classes, respectively. Neither assay detected *S. multivorum* DNA in samples of up to 5×10^6 CE with any of the reagent/instrument systems. In contrast, both assays detected *M. odoratus* DNA using OmniMix® reagent at the 66°C annealing/extension temperature, however, the GenBact 3 assay exhibited a substantially lower sensitivity of approximately 10^5 CE as compared to 10^2 CE for the GenBact2 assay. Correspondingly, only the GenBact2 assay detected this organism using the ABI system with TaqMan® Mix, although at a sensitivity of only approximately 10^5 CE. Neither assay detected this species in samples of up to 5×10^6 CE with the TaqMan® Fast Mix system.

Analyses of spiked surface water filters

Each group of surface water samples which was examined had different physical and chemical properties which might differentially impair QPCR analysis performance (Table 1). The Lake Michigan samples were fresh water with low turbidity, the Lake Ponchartrain samples were generally brackish water with higher turbidity, while the Gulf of Mexico samples were marine water with the highest average turbidity. The main goal of this study was to compare the relative performances of the different QPCR assays and controls with the different instrument and reagent systems, both in terms of their sensitivity to PCR inhibition, i.e. robustness, and also in terms of the accuracy and precision

of their quantitative measurements, against the backgrounds of filter retentates from these diverse waters. To facilitate these comparisons, analyses were performed on filter samples which were spiked with known numbers of *Enterococcus* and *Bacteroides* cells. For determinations of quantitative accuracy, it was first necessary to establish that ambient quantities of target DNA from both of these groups of organisms were not sufficiently high in any of the samples to significantly bias the spiked sample results. Table 4 summarizes the background levels of these organisms in the water sample filters based on representative results from only one reagent and instrument system for each assay. Only one of the samples showed an ambient enterococci DNA level which approached the levels recovered from the 10^4 cells used for spiking and thus all spiked sample results were considered valid for this organism. In contrast, seven of the 15 Lake Ponchartrain samples were found to contain ambient *Bacteroidetes* DNA levels greater than the levels recovered from the 5×10^4 cells used for spiking and these samples were therefore excluded from further analyses for these organisms. Somewhat higher ambient target DNA levels were detected by the Entero1 assay compared with the Entero2 assay in the Lake Ponchartrain samples and similarly, higher target DNA levels were detected by the GenBac2 assay compared with the GenBac3 assay in these same samples as well as the Gulf of Mexico samples. These results were consistent with the predicted relative specificities of the respective assays described above. The Entero2 assay is also predicted to be relatively insensitive in the detection of a few relatively uncommon species of *Enterococcus*, including *E. columbae* and *E. cecorum*.

The GenBact2 assay has been reported to detect members of the non-fecal *Cytophaga-Flavobacterium* group (Dick & Field 2004) which is known be ubiquitous in marine and estuarine waters (Kirchman *et al.* 2003). The GenBact3 assay incorporates the same, more discriminatory reverse primer sequence as an alternative reported assay for total *Bacteroidetes* (Dick & Field 2004 (addendum in proof)). This alternative assay has also been reported to confer greater specificity for *Bacteroidetes* but should have similar limitations in sensitivity associated with its amplicon length as the GenBact2 assay.

Table 5 shows the percentage of filtered sample extracts from each of the water source groups that fully inhibited the detection of the different target organism and control spikes by their respective QPCR assays. Both undiluted and five-fold diluted extracts were analyzed to examine the effects of extract dilution on this inhibition. As a group, the Gulf of Mexico water filters caused the highest frequency of detection failures, followed by the Lake Ponchartrain filters with the Lake Michigan filters causing detection failures only rarely. This ranking order coincided with average levels of turbidity, specific conductivity, and salinity among the samples from the three locations, however, no clear correlations were observed between detection failure and any of these measurements in individual samples within each group (data not shown). In nearly all instances where high percentages of the undiluted filter extracts caused detection failures, these percentages were dramatically reduced by a five-fold dilution of the extracts. The most notable exceptions occurred in analyses involving the Entero1 assay with the TaqMan® Fast Mix and OmniMix®

Table 4 | Ambient Enterococci and *Bacteroidetes* CCE estimates in unspiked surface water filters as determined by QPCR analyses using different primer and probe assays and ΔCT calculation method

Source of samples	Entero1[a]		Entero2[a]		GenBac2[b]		GenBac3[b]	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Lake Michigan	47	<3–246	51	<5–210	67	<12–281	74	<14–627
Gulf of Mexico	104	<11–390	106	<8–514	7493	879–28624	4777	29–17897
Lake Ponchartrain	1001	<4–9616	833	<4–6953	2.6e5	655–1.7e6	1.8e5	204–1.2e6

[a]Analyses performed on ABI Model 7900 instrument with TaqMan® Universal Master Mix reagent.
[b]Analyses performed on SmartCycler® instrument with OmniMix® reagent.

Table 5 | Inhibitory effects of undiluted and diluted filter extracts from different surface waters on QPCR assays as indicated by percentages of samples showing no detection of fecal indicator and control matrix spikes

Instrument, Reagent System	Assay	Lake Michigan			Gulf of Mexico			Lake Ponchartrain		
		N	No Diln.	5 × Diln.	N	No Diln.	5 × Diln	N	No Diln.	5 × Diln.
ABI Model 7900, TaqMan® Mix	Entero1	18	0%	0%	18	39%	0%	15	0%	0%
	Entero2	18	0%	0%	18	33%	0%	15	7%	0%
	GenBac2	18	78%	17%	12	92%	17%	8	50%	12%
	GenBac3	18	0%	0%	12	0%	0%	8	0%	0%
	Lact3	18	6%	0%	18	22%	0%	15	7%	0%
	Sketa2	18	0%	0%	18	28%	6%	15	0%	0%
ABI Model 7900, Fast Mix	Entero1	18	0%	0%	18	94%	89%	15	73%	20%
	Entero2	18	0%	0%	18	72%	17%	15	20%	0%
	GenBac2	18	50%	17%	18	89%	72%	8	100%	25%
	GenBac3	18	0%	0%	18	33%	0%	8	37%	0%
	Lact3	18	0%	0%	18	78%	17%	15	27%	0%
	Sketa2	18	0%	0%	18	72%	6%	15	20%	0%
Cepheid SmartCycler®, OmniMix®	Entero1	18	0%	0%	18	89%	50%	15	20%	0%
	Entero2	18	0%	0%	18	39%	6%	15	7%	0%
	GenBac2	18	6%	0%	18	44%	6%	8	25%	0%
	GenBac3	18	0%	0%	18	78%	6%	8	12%	0%
	Lact3	18	0%	0%	18	78%	6%	15	13%	0%
	Sketa2	18	0%	0%	18	78%	6%	15	13%	0%

reagent systems and the GenBac2 assay with the Fast Mix and TaqMan® Mix systems where a significant number of extracts were fully inhibitory even after undergoing these dilutions.

The relative accuracy and precision of quantitative results from the different real-time PCR assays, instruments and reagent systems were also compared using results from the same samples and the comparative cycle threshold calculation method - both without (ΔC_T calculation) and with ($\Delta\Delta C_T$ calculation) corrections from either the Sketa2 or Lact3 reference assays. Tables 6 and 7 show the mean log-transformed measurements and standard deviations of *Enterococcus* and *Bacteroides* CCEs, respectively, for all samples which did not show complete PCR inhibition of the reference control assays. The relatively poor performances of the Entero1 assay with the Fast Mix and OmniMix® reagent systems and the GenBac2 assay with the Fast Mix

and TaqMan® Mix systems were further demonstrated by their target organism CCE quantitation results which were consistently well below the spiked cell numbers and highly variable using the ΔC_T calculation method without reference assay corrections. Particularly in analyses of the undiluted extracts, the mean ΔC_T calculation-based CCE quantitation results were also lower for the modified Entero2 and GenBac3 assays than the spiked cell numbers suggesting that the filter extracts also partially inhibited these assays in some instances where total PCR failure was not observed.

The Sketa2 reference assay for salmon DNA often over-corrected the CCE quantitation results of the Entero2 and GenBac3 assays, particularly with the OmniMix® and Fast Mix systems. We hypothesize that this is caused by a higher degree of sensitivity by the Sketa2 assay to sample matrix inhibitory effects than the corresponding target organism

Table 6 | Real-time PCR analysis measurements of spiked enterococci in diverse surface water filters using different target and reference assays with TaqMan® Fast Mix (Fast Mix), OmniMix® and TaqMan® Mix in corresponding instrument systems

Extract	Assay (calculation)	Log-transformed mean (std. deviation) CCE[a] by system in filters spiked with 10^4 cells		
		Fast Mix	OmniMix®	TaqMan® Mix
No Dilution	Entero1 (ΔC_T)	2.35 (0.99)	2.86 (1.32)	3.19 (0.85)
	Entero1 ($\Delta\Delta C_T$ - Salmon DNA ref.)	3.71 (0.76)	4.24 (0.94)	4.06 (0.48)
	Entero1 ($\Delta\Delta C_T$ - Lactococcus ref.)	3.05 (1.05)	3.30 (1.05)	3.97 (0.41)
	Entero2 (ΔC_T)	3.16 (1.24)	3.53 (1.08)	3.41 (0.69)
	Entero2 ($\Delta\Delta C_T$ - Salmon DNA ref.)	4.89 (0.73)	5.04 (0.59)	4.33 (0.32)
	Entero2 ($\Delta\Delta C_T$ - Lactococcus ref.)	4.08 (0.54)	3.96 (0.42)	4.06 (0.34)
5 × Dilution	Entero1 (ΔC_T)	2.98 (0.64)	3.41 (0.87)	3.89 (0.31)
	Entero1 ($\Delta\Delta C_T$ - Salmon DNA ref.)	3.82 (0.71)	4.18 (0.49)	4.13 (0.24)
	Entero1 ($\Delta\Delta C_T$ - Lactococcus ref.)	3.42 (0.87)	3.56 (0.74)	4.06 (0.17)
	Entero2 (ΔC_T)	3.54 (0.80)	3.89 (0.50)	3.86 (0.35)
	Entero2 ($\Delta\Delta C_T$ - Salmon DNA ref.)	4.38 (0.47)	4.72 (0.44)	4.10 (0.25)
	Entero2 ($\Delta\Delta C_T$ - Lactococcus ref.)	4.03 (0.42)[b]	4.05 (0.19)[b]	4.02 (0.18)[b]

[a]calibrator cell equivalents.
[b]No significant difference in results between systems ($P > 0.05$).

Table 7 | Real-time PCR analysis measurements of spiked *Bacteroides* in diverse surface water filters using different target and reference assays with TaqMan® Fast Mix (Fast Mix), OmniMix® and TaqMan® Mix in corresponding instrument systems

Extract	Assay (calculation)	Log-transformed mean (std. deviation) CCE[a] by system in filters spiked with 5×10^4 cells		
		Fast Mix	Omni Mix®	TaqMan Mix®
No Dilution	GenBac2 (ΔC_T)	1.78 (0.83)	3.85 (1.03)	2.61 (0.45)
	GenBac2 ($\Delta\Delta C_T$ - Salmon DNA ref.)	3.49 (1.00)	5.24 (1.17)	3.34 (0.39)
	GenBac2 ($\Delta\Delta C_T$ - Lactococcus ref.)	2.56 (1.05)	4.42 (1.24)	3.29 (0.77)
	GenBac3 (ΔC_T)	4.45 (0.62)[b]	4.32 (1.15)[b]	4.24 (0.45)[b]
	GenBac3 ($\Delta\Delta C_T$ - Salmon DNA ref.)	5.78 (0.50)	5.78 (0.67)	4.96 (0.27)
	GenBac3 ($\Delta\Delta C_T$ - Lactococcus ref.)	4.97 (0.48)	4.80 (0.70)	4.84 (0.33)
5 × Dilution	GenBac2 (ΔC_T)	3.35 (1.07)	4.79 (0.28)	3.89 (0.61)
	GenBac2 ($\Delta\Delta C_T$ - Salmon DNA ref.)	4.17 (1.02)	5.47 (0.48)	4.15 (0.49)
	GenBac2 ($\Delta\Delta C_T$ - Lactococcus ref.)	3.74 (1.07)	4.85 (0.21)	4.28 (0.55)
	GenBac3 (ΔC_T)	4.59 (0.46)	4.67 (0.28)	4.38 (0.41)
	GenBac3 ($\Delta\Delta C_T$ - Salmon DNA ref.)	5.31 (0.41)	5.34 (0.38)	4.66 (0.20)
	GenBac3 ($\Delta\Delta C_T$ - Lactococcus ref.)	4.90 (0.37)	4.72 (0.13)	4.72 (0.11)

[a]calibrator cell equivalents.
[b]No significant difference in results between systems (P > 0.05).

assays in these systems. The Sketa2 reference did allow relatively accurate target organism CCE quantitation results from both the Entero2 and GenBac3 assays with the TaqMan® reagent system. This observation suggests that the Sketa2 assay is relatively insensitive to matrix inhibitory effects with the TaqMan® reagent system, possibly due to the longer thermal cycling times it employs.

Our results support the widely-held contention that target organism assays and corresponding reference or control assays should be designed to respond in a similar manner to matrix interferences. This requirement has been most commonly met by designing synthetic DNA templates with the same primer recognition sequences as those occurring in the target organism DNA (Edwards *et al.* 2004; Hoorfar *et al.* 2004). While normally working well as a control for detecting inhibitory effects in the reactions, this approach may present technical problems in providing corrections for matrix interferences in quantitative analyses because of competitive interactions between the control and target assays. Care must be exercised to ensure that the quantity of control templates added to the samples are in a similar range as the quantities of target organism templates in the samples in order to obtain accurate results for both assays (Wang *et al.* 2004). Since the quantities of target assay templates in environmental water samples generally will not be known, control assays of this nature may not be useful as references for making corrections to the quantitative results in some instances. By targeting the same DNA region as the Entero2 assay with similar but discriminatory primers, the Lact3 assay can serve as both a control for detecting gross inhibitory effects and also as a reference for correcting quantitative analysis results for partial matrix interferences. The effectiveness of this assay was empirically illustrated in this study by the mean corrected $\Delta\Delta C_T$ calculation data in combination with the Entero2 assay which closely approximated the spiked cell numbers in the samples with all three analytical systems. Higher precision levels were also generally obtained with this target and reference assay combination.

Fortuitously, the Lact3 reference assay also provided relatively good corrections of the GenBac3 assay in quantifying the *B. fragilis* cell spikes. Our results did suggest, however, that this reference assay may be slightly more sensitive to inhibition than the GenBac3 assay;

particularly with the Fast Mix system, thus leading to slight over-estimations in quantitative analyses for *Bacteroidetes*. Another possible limitation of using *L. lactis* cells for spiking water samples or filters relates to the potential for ambient levels of this species in the samples to influence the results of the reference assay. Like enterococci and *Bacteroidetes*, lactococci are commonly associated with fecal material (Blaut *et al.* 2002) although generally at lower concentrations. At the high levels of 10^5 *L. lactis* cells per sample used for spiking in this study, we do not expect that ambient levels of these organisms in surface waters will normally affect the reference assay results, however, the use of this approach for analyses of highly contaminated water samples may require spiking at even higher concentrations.

While far from comprehensive, the three real-time QPCR systems investigated in this study provide a fairly representative sampling of the different types of instruments and reagents presently available for analyses of surface water samples. The ABI TaqMan® Universal Master Mix reagent has been in use for a number of years now and has an extensive track record of providing reliable results not only on ABI platforms but also on other instruments such as the Cepheid SmartCycler® (Haugland *et al.* 2005; Noble *et al.* 2006). The primary limitation with this reagent resides in its requirement for long primer extension times in the thermal cycling protocol in order to obtain optimal performance. This limitation may be offset in many applications by instruments with 96 well and now even 384 well sample analysis capabilities, however, for applications such as recreational water quality testing for beach notifications, this longer analysis time would still represent a short-coming. Newer generation instruments such as the ABI fast block model 7900 in association with the Fast Mix reagent offer the potential to overcome this time limitation but, due to their size and expense, these instruments are still likely to be restricted to use in larger analytical laboratories. The Cepheid SmartCycler® represents another group of instruments which possess greater flexibility and portability and may be particularly attractive from the standpoint of performing water quality analyses on-site at different beach locations. The Omni-Mix® reagent developed for this instrument complements its rapid thermal cycling capability which can also lead to significantly shorter analysis times. Our results illustrate that care must be exercised in the

transfer of primer and probe assays developed for one platform and reagent system to others. Adherence to assay design guidelines provided by a number of commercial services and published sources (e.g. Edwards *et al.* 2004; Applied Biosystems 2006*b*; Cepheid 2006*b*; DNA Software 2006) probably represents the best approach toward maximizing the likelihood of obtaining comparable results with different analytical systems, however, this does not eliminate the need for experimental testing and comparison of results.

CONCLUSIONS

The quantitative measurement of bacteria or other microorganisms from diverse surface water samples by real-time PCR analysis requires careful attention to the design of both target and reference or control primer and probe assays in order to obtain optimal accuracy, precision, robustness and consistency of results between different instrument and reagent systems. This point is illustrated by the present study results where the redesign of two previously reported real-time PCR assays for the fecal indicator bacteria groups *Enterococcus* and *Bacteroidetes*, and the development of a new reference assay for matrix spikes of the related bacterial species, *L. lactis*, was shown to improve quantitative measurements of these bacteria with respect to all of the above-mentioned performance characteristics as well as apparent target group specificity. Even with optimization of the assays, water samples exhibiting high turbidity and/or conductivity and salinity may cause PCR inhibition and resultant failure to detect both target and control organisms when performing direct analyses of the undiluted DNA samples obtained by our rapid filter extraction method. Similar to previous reports (Haugland *et al.* 2005; Noble *et al.* 2006), the percentage of filter extracts causing this type of inhibition in this study was significantly reduced by diluting the DNA samples as little as five-fold before analysis. This simple dilution technique, while contributing to one of our primary goals in recreational water quality analysis of obtaining results in the shortest amount of time possible, obviously reduces the overall sensitivity of the method. For analyses of relatively abundant fecal indicator organisms this technique still generally allows adequate sensitivity, however, for less abundant pathogenic

organisms and more specific groups of fecal indicator organisms, addition of a DNA concentration and purification technique to the method may need to be considered.

NOTICE

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