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Comparison of Rapid Quantitative PCR-Based and Conventional Culture-Based Methods for Enumeration of *Enterococcus* spp. and *Escherichia coli* in Recreational Waters[▽]

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Recreational water quality is currently monitored using culture-based methods that require 18 to 96 h for results. Quantitative PCR (QPCR) methods that can be completed in less than 2 h have been developed, but they could yield different results than the conventional methods. We present two studies in which samples were processed simultaneously for *Enterococcus* spp. and *Escherichia coli* using two culture-based methods (EPA method 1600 and Enterolert/Colilert-18) and QPCR. The proprietary QPCR assays targeted the 23S rRNA (*Enterococcus* spp.) and *uidA* (*E. coli*) genes and were conducted using lyophilized beads containing all reagents. In the first study, the QPCR method developers processed 54 blind samples that were inoculated with sewage or pure cultures or were ambient beach samples. The second study involved 163 samples processed by water quality personnel. The correlation between results of QPCR and EPA 1600 during the first study (r^2) was 0.69 for *Enterococcus* spp., which was less than that observed between the culture-based methods (r^2, 0.87). During the second study, the correlations were similar. No false positives occurred in either study when QPCR-based assays were used with blank samples. Levels of reproducibility measured through coefficients of variation were similar for results by *Enterococcus* QPCR and culture-based methods during both studies but were higher for *E. coli* QPCR results in the first study. Regarding the concentration at which beach management decisions are issued in the State of California, the agreement between results of *Enterococcus* QPCR and EPA method 1600 was 88%, compared to 94% agreement between EPA method 1600 and Enterolert. The beach management decision agreement between *E. coli* QPCR and Colilert-18 was 94%. The samples showing disagreement suggested an underestimation bias for QPCR.

Fecal indicator bacteria (FIB) are presently measured to assess recreational water quality using one of three U.S. Environmental Protection Agency (EPA)-approved method classes: membrane filtration, multiple-tube fermentation, or defined-substrate technology (DST). The membrane filtration approach is based on passing water through a filter that is placed on a medium selective for the bacterial group of interest. Multiple-tube fermentation relies on quantification via most-probable number (MPN) using serial dilutions within replicate tubes incubated with selective media. The DST methods are also typically used in an MPN approach, where water samples are incubated with specific media in a tray with replicate wells. These methods are detailed by the American Public Health Association (1, 2) and in the U.S. Federal Register (29). These culture-based methods are widely accepted because of their relative ease of use, low cost, and demonstrated relationship to health risk (6, 7, 9, 11). However, the time required for sample processing ranges from 18 to 96 h, with confirmation and verification steps taking even longer.

Advances in technology provide new opportunities to measure bacterial water quality more rapidly (4, 16, 19). While currently used methods rely on bacterial growth and metabolic activity, these new methods directly measure DNA, RNA, or surface immunological properties. This is important because FIB concentrations have been shown to change substantially on a time scale of hours (3). Thus, contaminated beaches remain open during the laboratory processing period, but the contamination event has often passed by the time warnings are posted (20). By eliminating the need for a lengthy incubation step, results from rapid methods are available in several hours, enabling managers to take action to protect public health (i.e., post warnings or close beaches) on the same day that water samples are collected. Rapid quantitative PCR (QPCR) methods, such as the *Enterococcus* sp. assay developed by Haugland et al. (16), have also exhibited significant relationships with the risk of gastrointestinal illness in beachgoers (31, 32).

While QPCR-based methods are promising, their results may differ from those of the conventional culture-based methods that they are intended to replace. Since QPCR measures genetic material rather than the viable cells quantified by culture-based methods, it may overestimate FIB concentrations because of the inclusion of target DNA from dead or dying cells in the measurement. Differences may also be related to chemical inhibition of the amplification, assay design, or challenges in technology transfer to personnel with little or no molecular biology-based experience. Acceptance of new meth-

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ods by water quality professionals with a long history of using culture-based methods will depend on understanding the frequency and the underlying causes of these differences. Whereas a number of studies have assessed the relative performance of the three most commonly used culture-based methods (13, 25, 30), there have been few comparisons of QPCR- and culture-based method performance, especially with marine beach samples. Here, quantification of FIB by *Enterococcus* species QPCR (here referred to as simply *Enterococcus* QPCR) and *Escherichia coli* QPCR is compared to that by their respective culture-based assays. We also quantify the effect of two different QPCR sample processing approaches and assess the ability of personnel from a state-certified water quality laboratory to implement the rapid QPCR-based methods.

MATERIALS AND METHODS

The study involved two levels of testing during which water samples were simultaneously processed using QPCR-based and EPA-approved culture-based methods. In the first test, the QPCR assays were conducted by the researchers who developed the method. In the second test, the sample processing and the QPCR analyses were conducted by a state-certified water quality microbiology laboratory with little QPCR experience.

Study design. The first study involved 54 blind samples consisting of triplicates of each of 18 different test samples. Six of the 18 test samples were natural ambient samples collected at shoreline locations with historically high concentrations of fecal indicator bacteria, including Imperial Beach, San Diego, CA, Doheny State Beach, Dana Point, CA, Cabrillo Beach, Los Angeles, CA, Surfrider State Beach, Malibu, CA, and Paradise Cove, Malibu, CA, and a freshwater sample from the mouth of the Tijuana River, San Diego, CA. Three of the 18 test samples were various types of blanks, consisting of sterile phosphate-buffered saline (PBS; pH 7.2), offshore seawater, and 0.2-μm-filtered offshore seawater. Three of the 18 test samples were laboratory-created samples prepared using seawater collected from 18 km offshore of San Pedro, CA, at a depth of 10 m in an area known to be free from allochthonous fecal contamination and inoculated with three different concentrations of laboratory cultures (*Enterococcus faecium*, *Enterococcus faecalis*, and *E. coli*). The last six samples were created by inoculating 0.2 μm filtered seawater with three dilutions of primary wastewater influent from Orange County Sanitation District Plant No. 1 (OCSD; Fountain Valley, CA) and three dilutions of urban runoff collected from a Dominguez Channel storm drain in Torrance, CA.

Sample processing for the culture-based methods was conducted by five local laboratories: OCSD, Orange County Public Health Laboratory, City of Los Angeles, Los Angeles County Sanitation District, and City of San Diego, using methods employed in their routine water quality monitoring programs. For *Enterococcus* spp., samples were processed using Enterolert (IDEXX Laboratories, Inc., Westbrook, ME) DST and EPA method 1600 membrane filtration utilizing mEI agar (12, 22). For *E. coli*, only Colilert-18 DST was used (IDEXX Laboratories, Inc.).

For the first study, testing took place on 21 to 23 June 2005. Samples were created or collected between 6 and 9 a.m. each day and distributed to all laboratories no later than 11 a.m. Samples were all processed starting at the same time in all laboratories and in numbered order to minimize any concentration differences that might have developed from degradation during sample transport or laboratory holding. Further details and a comparison of the traditional method results among laboratories are available in a report by Griffith and Weisberg (14).

The second study was conducted from February through July 2006 and involved OCSD microbiologists processing 163 samples using both culture-based and QPCR-based methods. Of these samples, 138 were ambient samples collected from 41 locations that are part of their typical weekly monitoring efforts. The remaining 25 samples were seawater spiked with primary sewage influent (19 samples) or secondary effluent (6 samples) (Table 1). Quantitative PCRs were conducted in duplicate from a single original water sample using QPCR for *Enterococcus* spp. and *E. coli*. From the same original water samples, duplicate EPA method 1600, Enterolert, and Colilert-18 analyses were conducted.

For the second study, the ambient water samples were collected from five location types (Table 1): open ocean beaches distant from creeks that drain land-based runoff, open ocean beaches near storm drains, enclosed embayment beaches, locations within storm drains, and wet weather samples from open ocean beaches (further details are available in reference 15). Sewage-spiked samples were created by inoculating clean ocean water with various concentrations of either primary sewage influent or secondary sewage effluent (Table 1). The clean ocean water was collected at a location 11 km offshore of Newport Beach, CA. Sewage was obtained from an OCSD wastewater stream. Following inoculation, sewage-spiked samples were stirred for a minimum of 15 min using a magnetic stirring plate.

TABLE 1. Type and number of samples analyzed during the second study

Sample type	No. of samples		
	Total collected	Included in data analysis	Analyzed using culture-based methods
Ocean beach near drain	18	18	18
Open ocean beach	44	33	36
Enclosed beach	22	22	22
Storm drain	29	29	29
Wet weather	25	25	25
Open beach spiked with sewage influent	19	19	19
Open beach spiked with sewage effluent	6	6	6
Total	163	152	155

Sample filtration and processing for QPCR. Water samples were filtered using a six-place filtration manifold and vacuum pump assembly with Pall disposable filter funnels (Pall Corp., East Hills, NY). The mixed-ester cellulose filters provided by the manufacturer in the filter funnels were replaced with 47-mm diameter, 0.45-μm pore size polycarbonate filters (HTTP; Millipore Corp., Bedford, MA). One hundred milliliters of sample was filtered under vacuum until there was no visible moisture. Filter funnels were subsequently rinsed with ~20 ml of PBS, which was also filtered to visible dryness. Filters were immediately removed from the vacuum manifold using sterile disposable forceps, gently folded in half, and placed into a prelabeled 2.0-ml screw-cap microcentrifuge tube. For each sample, two replicate 100-ml volumes were filtered for processing by (i) bead beating alone and (ii) bead beating followed by an additional DNA purification step.

Processing using bead beating alone. The polycarbonate filter was placed into a 2.0-ml screw-cap tube containing 0.3 g of 1-mm zirconium silica beads (Biospec Corp., Bartlesville, OK). After the filter was added, 600 μl of buffer AE (Qiagen, Valencia, CA) and 10 μl of specimen processing control (SPC; 1×10^5 *Lactococcus lactis* cells) were also added. Tubes were then placed in a 48-position mini bead beater (BioSpec Corp.) for 2 min at the highest speed setting. The tubes were centrifuged at $12{,}000 \times g$ for 1 min to pellet the beads and debris. Resulting supernatants were transferred to sterile 1.6-ml microcentrifuge tubes and spun at $12{,}000 \times g$ for an additional 5 min, and the resulting supernatant fluid was transferred to low-retention microcentrifuge tubes (Genemate C-3360-1) and immediately used for QPCR.

Sample processing using bead beating followed by DNA extraction. After the bead-beating protocol, 490 μl of supernatant fluid was removed from each screw cap tube and placed onto an UltraClean fecal DNA isolation column (MoBio Laboratories, Inc., Carlsbad, CA) following the manufacturer's instructions for maximum yield. In the second study, OCSD personnel processed the first 44 samples using bead beating followed by DNA extraction but encountered difficulty with the extraction process. The resulting DNA was of poor quality, with PCR amplification efficiencies below our data acceptance criteria (amplification efficiency, >90%). These samples were not used as part of this study, and the remaining samples were processed using bead beating alone.

QPCR analysis. The QPCR assays for *Enterococcus* spp. targeted the multiple copy 23S rRNA gene in an approach similar to that outlined by Ludwig and Schleifer (21). The *E. coli* assay targeted the single-copy *uidA* gene as discussed by Frahm and Obst (12). Scorpion QPCR chemistry (34) was used, and the primer-probe complexes were synthesized by Biosearch Technologies, Inc. (Sunnyvale, CA). They were lyophilized along with stabilizing buffers into proprietary beads sold as Total *Enterococcus*, *E. coli*, *and Lactococcus* SmartBeads by Biogx, Inc. (Birmingham, AL). All SmartBeads also contained a propriety PCR positive internal control template (IC; BioGx, Inc.) and a primer/probe set

TABLE 2. Regression analysis results for the first and second testing of rapid QPCR methods for *Enterococcus* spp. and *E. coli*[a]

Organism	Methods being compared	Best linear fit (y value)	No. of samples	r^2	P value
First study					
Enterococcus spp.	EPA 1600 vs. QPCR BB	$0.7571x+0.9206$	54	0.69	<0.0001
	EPA 1600 vs. QPCR BB+DNA	$0.5635x+0.6800$	54	0.62	<0.0001
	Enterolert vs. QPCR BB	$0.8155x+0.6290$	54	0.77	<0.0001
	Enterolert vs. QPCR BB+DNA	$0.6370x+0.3857$	54	0.76	<0.0001
	EPA 1600 vs. Enterolert	$0.9171x+0.3878$	54	0.87	<0.0001
E. coli	Colilert-18 vs. QPCR BB	$1.0346x+0.0324$	53	0.74	<0.001
	Colilert-18 vs. QPCR BB+DNA	$0.9635x+0.0991$	54	0.69	<0.0001
Second study					
Enterococcus spp.	EPA 1600 vs. QPCR	$0.7525x+0.1719$	119	0.74	<0.0001
	Enterolert vs. QPCR	$0.7539x+ 0.256$	119	0.68	<0.0001
	EPA 1600 vs. Enterolert	$0.8817x+0.1124$	152	0.85	<0.0001
E. coli	Colilert-18 vs. QPCR	$0.7064x+0.0565$	119	0.71	<0.001

[a] Regression analysis results for the first and second testing of rapid QPCR methods for *Enterococcus* spp. and *E. coli* detection compared to culture-based methods are shown. In the first study, testing was conducted by the QPCR method developers. Samples were processing using two approaches, bead beating (BB) and bead beating plus DNA purification using a commercial extraction kit (BB+DNA). In the second study, testing was conducted by water quality microbiologists. The QPCR data comparison was conducted only for samples that were processed using bead beating only. vs., versus.

for the IC template, as an extra measure for assessing QPCR failure via inhibition or improper QPCR setup.

For each QPCR run, lyophilized OmniMix (Cepheid, Inc.) and Total *Enterococcus*, *E. coli*, or *Lactococcus* SmartBeads were dissolved in nuclease-free water to create a master mix. For each master mix, 20-μl aliquots were pipetted into reaction tubes, followed by the addition of 5 μl of sample using 25-μl optical tubes (Cepheid, Inc.). For each run, no template controls that included all QPCR reagents plus nuclease-free water were included. The reactions were monitored with a Cepheid Smart Cycler II instrument. Thermal cycling conditions for all reactions (*Enterococcus*/IC, *E. coli*/IC, and *Lactococcus*/IC) were the same, consisting of 1 cycle at 94°C for 2 min (hot start), 45 cycles at 94°C for 5 s, and 62°C for 43 s (optics on). Determinations of the threshold cycle (C_T) were performed automatically by the instrument after manual adjustment of the threshold fluorescence value to 8 units.

Quantification during the first study relied on interpolation of cell numbers from the standard curve generated during each QPCR run. All standard curves were made by serial dilution of the calibration standard DNA (generated from either bead beating or bead beating followed by DNA purification) in nuclease-free water. For the first study, the standard curve was generated from a duplicate 4-log dilution series. We compared the results generated by the standard curve and comparative C_T quantification approaches during the first study, and they yielded similar results (data not shown). During the second study, the comparative C_T quantification approach with a duplicate 3-log standard curve was used. The comparative C_T method uses an abridged standard curve of the respective calibrator standard, *Enterococcus* spp. or *E. coli*, to derive the QPCR amplification efficiency (E), which was calculated from the slope (m) of the linear regression generated by the SmartCycler II software: $E = 10^{(-m)}$. The ratio of change between the calibrator C_T for a known cell amount and the unknown C_T was multiplied by E_{QPCR} to arrive at the cell number. Even though the comparative C_T approach was used for enumeration, standard curves were run with every sample batch to assess E, which exceeded 90% for every run in the second study.

Calibration standards, controls, and standard curves. Prior to sample filtration and processing, a range of calibration standards and controls were prepared. All bacterial strains used were derived from the American Type Tissue Culture Collection (ATCC; Manassas, VA) or authorized distributors (Microbiologics, St. Cloud, MN, or Beckton Dickinson, Sparks, MD). *E. coli* (ATCC 25922) was grown in tryptic soy broth, while *Enterococcus faecalis* (ATCC 29212) and *Lactococcus lactis* (ATCC 11454) were grown in brain heart infusion broth, with all cultures incubated overnight at 37°C. The starting concentrations of all three overnight cultures were determined either by using epifluorescence microscopy following the method described by Noble and Fuhrman (24) or by Colilert-18 for *E. coli* and Enterolert for *E. faecalis*. Calibration standards were made by diluting overnight cultures of *E. coli* and *E. faecalis* in PBS, pipetting aliquots into 1.7-ml, low-DNA binding tubes at concentrations of 10^5 cells per 10 μl and storing them at −80°C (this was the calibration standard stock). The SPC was *Lactococcus lactis* overnight cultures that were diluted in PBS into a low-DNA binding 1.7-ml tube, aliquoted in volumes with final concentrations of 10^5 cells per 10 μl, and frozen at −80°C.

Data handling and statistical calculations. To assess inhibition, the SPC (*Lactococcus*) and the IC for each sample were examined. Inhibition was defined as a 1.5 C_T (0.5 log) delay, corresponding to the variability normally observed between replicates from culture-based methods used for southern California beaches (13, 25). Inhibited samples were diluted 10-fold with nuclease-free water and reanalyzed to determine whether dilution relieved all inhibition.

Enterococcus or *E. coli* QPCR results were compared to the culture-based method results using Pearson product-moment analysis and best linear fit. The *Enterococcus* QPCR results were compared to both EPA method 1600 and Enterolert results. For *E. coli*, the only culture-based method used for comparison was Colilert-18. During the first study, the comparison also included the two sample processing approaches, either bead beating alone or bead beating followed by DNA purification (Table 2). Qualified culture-based values were reported as one-half of the detection limit (i.e., <10 became 5), and values greater than the upper limit were deleted from the data pool. Samples that yielded a QPCR result of nondetection were assigned a concentration of 5 cells per 100 ml. All data were log-transformed and tested for normality using the Shapiro-Wilk test. This transformation reduced skewness and led to normality for most, but not all, of the data comparisons (Table 2; also see Table 4). Regarding the concentration at which beach management decisions are issued in the State of California, agreement between the methods was determined by using the current single-sample standard for *Enterococcus* spp. (104 CFU or most-probable number [MPN] per 100 ml) and for *E. coli* (400 CFU or MPN per 100 ml) for marine California beaches (8).

RESULTS

Testing conducted by the QPCR method developers. Concentrations of *Enterococcus* spp. measured using QPCR were significantly correlated with those measured using Enterolert and EPA method 1600 (Table 2) (Fig. 1). The correlation with EPA method 1600 was stronger for samples processed using bead beating alone than for those processed using bead beating plus additional DNA purification (r^2, 0.69 versus 0.62), both of which were weaker than the relationship between QPCR and Enterolert (r^2, 0.77) or between EPA method 1600 and Enterolert (r^2, 0.87) (Table 2). The slope of the regression for both culture-based methods versus *Enterococcus* QPCR-based methods was less than 1.0, indicating relative underestimation by QPCR (Table 2). The slope was higher for samples processed using bead beating only, suggesting some loss of target cells in the DNA extraction.

The correlation between *E. coli* QPCR and Colilert-18 re-

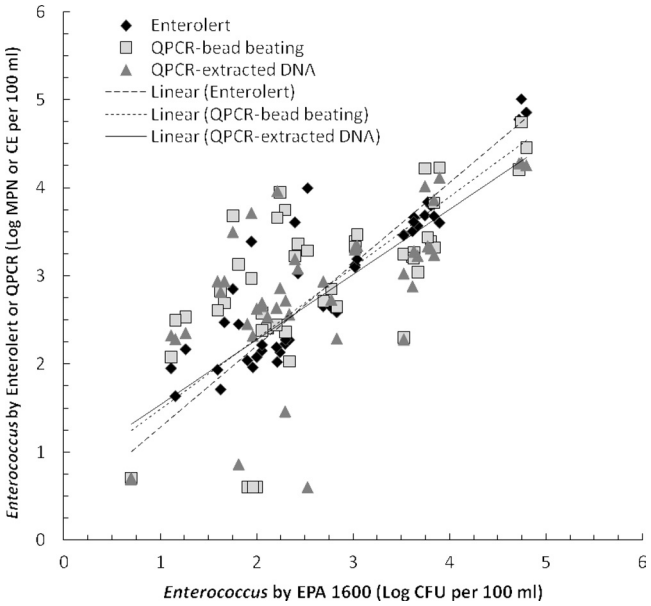

FIG. 1. Comparison among multiple measures of *Enterococcus* sp. concentration, EPA method 1600, and Enterolert (black diamonds), or *Enterococcus* QPCR for a range of water samples. QPCR testing was conducted by the method developers. Log-transformed EPA method 1600 versus log-transformed rapid QPCR for *Enterococcus* results (gray squares) represent samples processed using bead beating only; gray triangles represent samples processed using bead beating followed by a commercial DNA extraction kit. CE, cell equivalents. Best linear fit equations are reported in Table 2.

sults was significant and similar to that for *Enterococcus* QPCR versus culture-based methods (r^2, 0.74) (Table 2) (Fig. 2). However, unlike *Enterococcus* QPCR results, the slope of the relationship between culture-based methods and *E. coli* QPCR

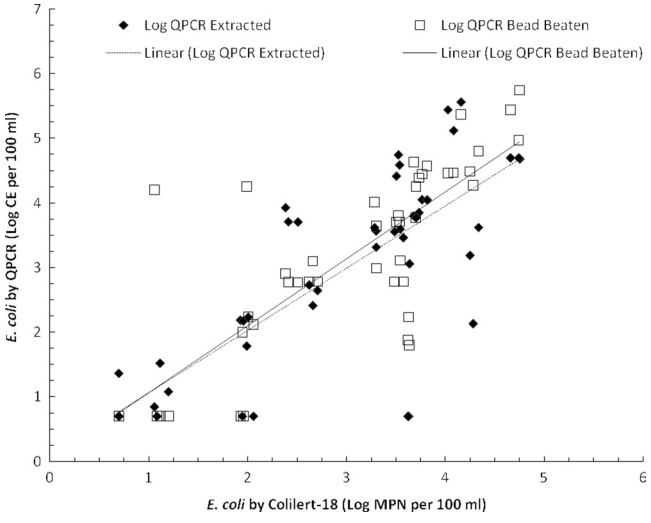

FIG. 2. Comparison between Colilert-18 and QPCR for a range of water samples. The *E. coli* QPCR testing was conducted by the method developers. Log-transformed Colilert-18 results in open squares represent samples processed using bead beating only; log-transformed QPCR results in black diamonds represent samples processed using bead beating followed by a commercial DNA extraction kit. CE, cell equivalents; MPN, most-probable number.

TABLE 3. Average coefficient of variation for testing methods for enumeration of *Enterococcus* spp. and *E. coli* in the two studies[a]

Organism tested when testing was conducted by:	Method(s)	Avg CoV
QPCR method developers		
Enterococcus spp.	QPCR BB	0.27
	QPCR BB+DNA	0.36
	Enterolert	0.31
	EPA method 1600	0.32
E. coli	QPCR BB	0.67
	Colilert-18	0.19
Water quality microbiologists		
Enterococcus spp.	QPCR BB	0.21
	Enterolert	0.21
	EPA method 1600	0.18
E. coli	QPCR BB	0.25
	Colilert-18	0.25

[a] The average coefficient of variation (CoV) was determined for each entire study by calculating the CoV for each sample based on replicate analyses and then calculating the average of those determined values. BB, bead beating; BB+DNA, bead beating plus DNA extraction kit.

results was nearly unity for bead beating alone and bead beating plus DNA extraction (Table 2). A comparison of results from bead beating and from bead beating followed by DNA extraction was conducted in the first study; the DNA extraction step required an additional 40 to 45 min for sample processing.

The coefficient of variation (CoV) for *Enterococcus* QPCR results using bead beating was less than that for either of the culture-based methods, though the CoV for samples processed using DNA extraction was higher (Table 3). Culture-based methods for *E. coli* had a substantially lower CoV than that observed for QPCR (Table 3).

Testing conducted by a state-certified water quality laboratory. The correlation between results of QPCR- and culture-based methods for *Enterococcus* spp. was nearly the same from the first study to the second study (r^2, 0.69 versus 0.74) (Table 2) (Fig. 1 to 4). When the results were examined with respect to whether they exceeded 104 CFU per 100 ml, the concentration at which beach management decisions are issued in the State of California, *Enterococcus* QPCR agreed with EPA method 1600 and Enterolert for 88% and 87% of the samples, respectively. This was less than the 94% agreement rate between the two culture-based methods.

The correlation between *E. coli* QPCR and Colilert-18 was also similar between the first and second studies (Table 2) (Fig. 2 and 4). When assessed relative to the beach warning decision criterion using the State of California *E. coli* single sample standard of 400 CFU per 100 ml, the agreement rate between QPCR and Colilert-18 was 94%.

The reproducibility of the *Enterococcus* QPCR assay was nearly the same as that for the culture-based methods when testing was conducted by water quality agency personnel (CoV, 0.18 versus 0.21) (Table 3). Unlike the testing conducted by the QPCR method developers, for which we observed a large difference in CoV between results of the *E. coli* QPCR assay and Colilert-18, the CoV for the two methods was identical when samples were processed by the conventional water quality laboratory.

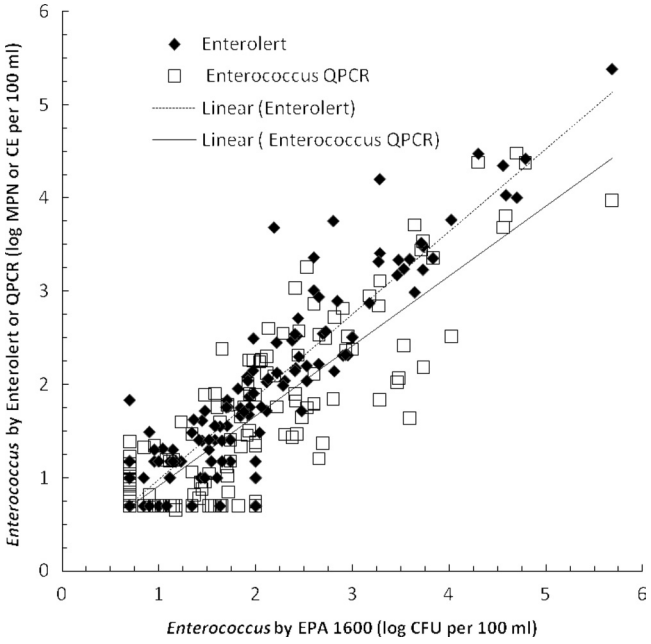

FIG. 3. Comparison among multiple measures of *Enterococcus* sp. concentration; EPA method 1600 versus Enterolert (black diamonds) or *Enterococcus* QPCR (open squares) for a range of ambient southern California marine water samples. The QPCR testing was conducted by water quality personnel at Orange County Sanitation District. All QPCR analyses were conducted with samples processed using bead beating only. CE, cell equivalents; MPN, most-probable number. Best linear fit equations are reported in Table 2.

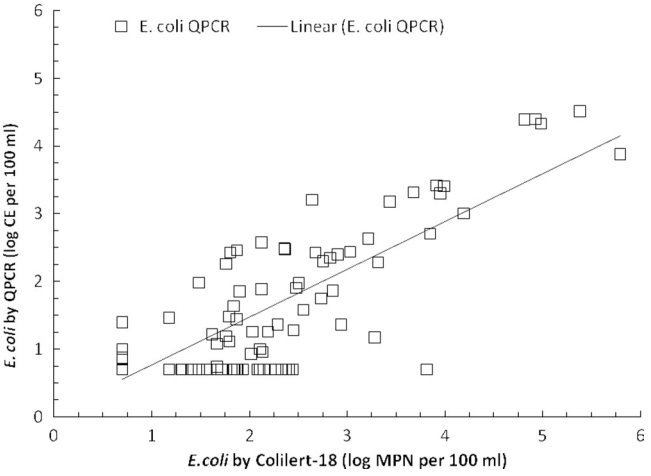

FIG. 4. Comparison between concentrations of *E. coli* measured using Colilert-18 and *E. coli* QPCR for a range of ambient southern California marine water samples. The QPCR testing was conducted by water quality personnel at Orange County Sanitation District. All QPCR analyses were conducted with samples processed using bead beating only. CE, cell equivalents; MPN, most-probable number. Best linear fit equations are reported in Table 2.

DISCUSSION

While we found an 88% level of agreement between beach management decisions that would be made using *Enterococcus* QPCR and EPA 1600, this represents a 12% disagreement rate, which is twice that between EPA 1600 and Enterolert. Moreover, most of the errors were false negatives, in which QPCR missed a warning that would have been given by culture-based methods. A false-negative result is problematic, because there is no subsequent mechanism for determining that a problem exists. In contrast, a false-positive result would lead to an inappropriate warning, but one which could be remedied by additional sampling with alternative methods whose use would be triggered by the positive measurement.

There are several possible explanations for the observed underestimation, one of which is inhibition of DNA amplification during QPCR. Inhibition typically occurs when high-molecular-weight compounds in the source water (e.g., humic acids and other complex carbohydrates) combine with metal ions to sequester nucleic acids from polymerases and prevent amplification (10, 17, 27, 28, 33). Typical approaches to deal with inhibition in analysis of water samples are the use of DNA extraction kits, dilution, or addition of adjuvants (e.g., bovine serum albumin) (17). The use of lenient criteria for identifying inhibition (1.5 C_T delay in the SPC) could also have played a role in relative underestimation. Internal controls, though, indicated that none of the samples in the first study and less than 5% of the samples in the second study exhibited inhibition, and even those were from a small subset of sites. Three were from Newport Dunes, an enclosed beach, and five were storm drain samples, four of which were from the same location: Back Bay Storm Drain in Newport Beach, CA. QPCR provided underestimates relative to traditional methods even when these samples were excluded.

Another possible explanation for underestimation is that the molecular primers may be more specific to the target species than to the wider range of *Enterococcus* species enumerated using EPA method 1600. We observed stronger correlations and ones with slopes closer to unity between QPCR and the culture-based methods when including only samples spiked with pure cultures or sewage in our comparisons (Table 4). For the samples inoculated with pure cultures, we observed some overestimation of QPCR in relation to results of the culture-based methods, but this did not occur with the sewage-spiked samples, possibly due either to clumping of the cultured cells or to the high affinity of our primer-probe set for the organism used to inoculate the samples (Table 4). Our *Enterococcus* primer-probe set was designed for high affinity to *E. faecalis* and *E. faecium*, two species prominent in the human gut, and may not be as effective at capturing *Enterococcus* spp. that originate in other animal sources or species such as *E. casseliflavus* that grow naturally in the environment (26). Some of the QPCR underestimation may also be attributable to growth of nontarget species by EPA method 1600, which has previously been reported to have a 17% to 40% false-positive rate for samples from southern California beach locations, many of which were the same sites sampled for the second study (23).

Beyond specificity of the primer/probe design, another potential cause for discrepancy between results of the *Enterococcus* QPCR and the culture-based methods could be the use of a single species (*E. faecalis*) as the calibrator standard. The target gene (23S rRNA) is a multicopy gene, so it may be possible that other species within the *Enterococcus* genus carry different copies of the gene, depending upon nutrient status,

TABLE 4. Correlation and best linear fit between methods in the first study[a]

Methods being compared for indicated sample type	Best linear fit (y value)	r^2	No. of samples	P value
Seawater inoculated with pure cultures				
EPA method 1600 vs. *Enterococcus* QPCR BB	$1.2586x - 1.4949$	0.84	9	<0.0001
EPA method 1600 vs. *Enterococcus* QPCR BB+DNA	$0.8099x - 0.3814$	0.89	9	<0.0001
Enterolert vs. *Enterococcus* QPCR BB	$1.1419x - 1.0329$	0.87	9	<0.0001
Enterolert vs. *Enterococcus* QPCR BB+DNA	$0.7354x - 0.0866$	0.92	9	<0.0001
EPA method 1600 vs. Enterolert	$1.1101x - 0.4366$	0.99	9	<0.0001
Colilert-18 vs. *E. coli* QPCR BB	$1.2749x - 0.5575$	0.83	9	<0.0001
Colilert-18 vs. *E. coli* QPCR BB+DNA	$0.8458x + 0.7251$	0.96	9	<0.0001
Seawater inoculated with sewage influent				
EPA method 1600 vs. *Enterococcus* QPCR BB	$0.7771x + 1.0022$	0.98	9	<0.0001
EPA method 1600 vs. *Enterococcus* QPCR BB+DNA	$0.7354x + 0.3197$	0.90	9	<0.0001
Enterolert vs. *Enterococcus* QPCR BB	$0.936x + 0.4902$	0.93	9	<0.0001
Enterolert vs. *Enterococcus* QPCR BB+DNA	$0.9244x - 0.2813$	0.94	9	<0.0001
EPA method 1600 vs. Enterolert	$0.8008x + 0.6343$	0.98	9	<0.0001
Colilert-18 vs. *E. coli* QPCR BB	$1.1409x - 0.053$	0.89	9	<0.0001
Colilert-18 vs. *E. coli* QPCR BB+DNA	$0.9294x + 1.464$	0.92	9	<0.0001

[a] Correlation and best linear fit between types of methods during the first study for samples inoculated with only pure cultures or sewage and processed using either bead beating (BB) or bead beating followed by a DNA purification step (BB+DNA).

stress, and replication. This could be especially true in open beach environments, where UV irradiation can place stress on the cells. This problem may make direct calibration to cells inappropriate. In the future, it is possible that a plasmid DNA standard could be used more effectively to quantify only gene copies.

Our finding of underestimation differs from that of other researchers (5, 16), who generally found *Enterococcus* QPCR results that were consistently higher than that for EPA method 1600 at Great Lakes beaches. In a study by Byappanahalli et al. (5), this was attributed partially to the fact that QPCR does not differentiate between dead and viable cells. The sampling conducted by Byappanahalli et al. (5) was focused on sites near disinfected wastewater outfalls, where nonviable cells would be prevalent, whereas our sample sites were distant from such disinfected water sources. Whitman et al. (35) studied variance of culture-and QPCR-based measurements and noted overestimation at most of the sites studied, including marine waters. Haugland et al. (16) reported comparative work at three Lake Michigan locations, with two consistently reporting an overestimation compared to results by EPA method 1600. At the site most suspected to contain wastewater discharge, however, the QPCR-based determinations were generally lower than those observed for EPA method 1600 (16). The trend toward overestimation observed in these three previous studies, however, is likely strongly influenced by their quantification approach, in which they report their QPCR results as "calibrator cell equivalents" (CCE) by assessing relative quantification using both a calibrator (in their case, a known number of *Enterococcus* sequences or cells) and a salmon testes DNA control (reference DNA sequence or cells added in known quantities to unknowns). Thus, their relative overestimation could result from differences in the amplification efficiency of the two assays used for calibration and the increased sensitivity to inhibition of the salmon testes DNA QPCR assay.

For *E. coli*, another recent comparison study confirms the findings observed here. We observed a beach management agreement of 94% when comparing results of *E. coli* QPCR to those of Colilert-18. For Great Lakes waters, very strong agreement (98%) has been reported for *E. coli* detection results between the two classes of methods (29). They used the same lyophilized-bead Scorpions QPCR chemistry-based assay as that employed here. However, in this study, a large change was observed in the slope of the best linear fit between Colilert-18 and *E. coli* QPCR from the first to the second study, with the slope dropping from roughly 1.0 to 0.7. This difference indicates either a loss of *E. coli* cells during sample processing or possibly false positives using the Colilert-18 tests for southern California marine beach and storm drain samples. Lavender and Kinzelman (18) did not observe this type of loss, but they used freshwater samples.

We used the Scorpion primer-probe chemistry (34), whereas others (5, 16, 35) used TaqMan probe chemistry. We chose the Scorpion chemistry because it functions in a hairpin format that allows the template and the probe to hybridize more quickly due to close proximity of the probe region to the target sequence. However, we do not believe this had a material effect on the observed differences, as others (e.g., see reference 16) still focused their primer/probe design on similar regions within the 23rRNA gene. A linear regression of the raw C_T values produced by the TaqMan *Enterococcus* QPCR assay (run on an ABI 7500 platform) compared to those from the Scorpion Enterococcus sp. QPCR assay described here (conducted with a Cepheid SmartCycler II), was conducted during the first study and had a regression (y value) of $0.9636x + 1.4871$ and an r^2 of 0.9816 (in reference 14, see Appendix A). It is likely that the approach chosen for calculation of the final target cell or DNA concentration (i.e., using comparative cell equivalents versus cell equivalents) is a more important consideration.

While our study suggests that a number of intricacies remain to be resolved to better understand differences between conventional and QPCR-based quantification approaches, the second study clearly demonstrates that QPCR technology can be successfully transferred to a local laboratory. The OCSD personnel were able to produce results in less than 3 h and with

performance characteristics similar to those produced by the method developer in the first study. Some of this success may be due to the use of lyophilized beads, which reduced sample manipulation to only two pipetting steps and simplified the workflow.

However, not all aspects of technology transfer were successful. We abandoned the extra DNA purification step because of an observed loss of target DNA (Tables 2 and 4). Moreover, the OCSD staff found the DNA extraction complex and time consuming, with the many additional pipetting steps introducing opportunities for imprecision; this was confirmed by the CoV between replicates being three times higher than that for samples processed using bead beating alone (data not shown). Of additional importance, the OCSD staff felt that the extra DNA purification step disrupted laboratory workflow. For the southern California beaches, removal of the extraction step appeared to be inconsequential, as we saw little indication of inhibition at these open ocean beaches that have high circulation. However, for other locations where inhibition is more prevalent, removal of the extraction might be more problematic and could possibly be remedied by increased automation in the extraction process.

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