

# Comparison of *Enterococcus* measurements in freshwater at two recreational beaches by quantitative polymerase chain reaction and membrane filter culture analysis

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## Abstract

Cell densities of the fecal pollution indicator genus, *Enterococcus*, were determined by a rapid (3 h or less) quantitative polymerase chain reaction (QPCR) analysis method in 100 ml water samples collected from recreational beaches on Lake Michigan and Lake Erie during the summer of 2003. Measurements by this method were compared with counts of *Enterococcus* colony-forming units (CFU) determined by Method 1600 membrane filter (MF) analysis using mEI agar. The QPCR method had an estimated 95% confidence, minimum detection limit of 27 *Enterococcus* cells per sample in analyses of undiluted DNA extracts and quantitative analyses of multiple lake water samples, spiked with known numbers of these organisms, gave geometric mean results that were highly consistent with the spike levels. At both beaches, the geometric means of ambient *Enterococcus* concentrations in water samples, determined from multiple collection points during each sampling visit, showed approximately lognormal distributions over the study period using both QPCR and MF analyses. These geometric means ranged from 10 to 8548 cells by QPCR analysis and 1–2499 CFU by MF culture analysis in Lake Michigan ( $N = 56$ ) and from 8 to 8695 cells by QPCR and 3–1941 CFU by MF culture in Lake Erie ( $N = 47$ ). Regression analysis of these results showed a significant positive correlation between the two methods with an overall correlation coefficient ( $r$ ) of 0.68.

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**Keywords:** *Enterococcus*; Beach; Water; Enumeration; PCR; Membrane filtration

## 1. Introduction

Previous health and epidemiological studies by the US Environmental Protection Agency (US EPA) have demonstrated that colony-forming unit (CFU) densities of the bacterial genus *Enterococcus* in both marine and

freshwater samples are directly correlated with gastroenteritis illness rates in swimmers exposed to these waters, Cabelli et al. (1982), Dufour (1984). Based on these data, guidance has been issued on the maximum concentrations of these organisms that may be associated with acceptable health risks, Dufour and Ballantine (1986). Since then an improved, selective culture method has been developed for measuring *Enterococcus* concentrations in recreational water samples, Messer and Dufour (1998), US EPA (2002), however, this

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method still requires at least 24 h to obtain results. Various studies, Leecaster and Weisberg (2001), Boehm et al. (2002), Wymer et al. (2004), have shown that changes in water quality conditions during this delay period can frequently lead to notifications to the public that are not fully protective of swimmer health.

Increasing interest is now being directed towards the possible use of molecular microbial analysis methods with shorter reporting times. One such technology is the quantitative polymerase chain reaction (QPCR). Primer sets and probes, associated with this technology, have now been developed for the specific detection of a number of different fecal indicator organisms and waterborne pathogens, Ludwig and Schleifer (2000), Lyon (2001), Brinkman et al. (2003), Foulds et al. (2002), Blackstone et al. (2003), Frahm and Obst (2003), Guy et al. (2003), Noble et al. (2003). The availability of portable instrumentation that can be operated at or near the site, Depaola (2004) and the development of rapid methods for processing water samples for QPCR analysis, Brinkman et al. (2003) have reduced the potential overall time requirements of this method to a matter of only a few hours, from sampling to results.

In the summer of 2003, the US EPA initiated a new, multi-year health and epidemiological study to evaluate the relationship between illness rates in swimmers and fecal indicator concentrations using a modification of a new water monitoring protocol, Wymer et al. (2004) and several rapid analysis methods, including QPCR. In this report, QPCR analysis results are presented for total enterococci in water samples collected from two freshwater recreational beaches over 8- and 10-week periods in the first year of this study. The sensitivity, accuracy and precision of the method were examined, as well as its ability to measure these organisms over their entire ambient concentration ranges in changing water conditions at the two beaches. Results of these analyses were further compared with corresponding *Enterococcus* CFU counts obtained by a currently accepted membrane filtration and culture method (Method 1600), US EPA (2002). Correlations between the results of the two methods were determined for an initial assessment of the potential applicability of the more rapid QPCR method for monitoring water quality at recreational beaches.

## 2. Materials and methods

### 2.1. Study sites

The beaches examined in this study were West Beach at the Indiana Dunes National Lakeshore in Porter, Indiana on Lake Michigan and Huntington Beach in Bay Village, Ohio on Lake Erie. These beaches were selected based upon several specific criteria including: (1) being

located at an officially designated recreational area near a large population center; (2) generally meeting the state or local water quality standards; (3) potential for contamination by a human source of pollution; (4) having a large attendance (e.g., 300–400 swimmers/day) with a broad range of ages; and (5) having a swimming season of at least 90 days.

### 2.2. Water sampling

Unless precluded by extreme weather conditions, water samples were collected on Saturdays, Sundays and holidays from May 31 through August 3, 2003 at West Beach and from July 26 through September 14, 2003 at Huntington Beach. Sampling visits to West Beach occurred three times daily, at 8 AM, 11 AM, and 3 PM in waist-level (1 m deep) and shin-level water (0.3 m deep) along three transects perpendicular to the shoreline (Fig. 1A). Sampling was performed in a similar manner at Huntington Beach with three additional shin-level locations included per visit (Fig. 1B) to properly characterize the beach. Ancillary data were collected at the time of each sampling visit, including pH, turbidity, air and water temperature, cloud cover, rainfall, wind speed and direction, wave height, bather densities and presence of boats, animals and debris in the water and on the beach.

Two 1-l water samples were collected at each location by standard methods as recommended in Section 9060 of *Standard Methods for the Examination of Water and Wastewater*, American Public Health Association (1998). One sample was used for method 1600, pH and turbidity analyses, while the other was used for QPCR. Briefly, 1000-ml presterilized, polypropylene bottles were lowered to the appropriate sampling depth and the lids were removed to fill them. The bottles were then raised out of the water and emptied slightly to allow approximately 1 in of head space before replacing the lids. Samples were taken about 1 ft (0.3 m) under the surface of the water in waist-level water, and 6 in (0.15 m) above the bottom in shin-level water.

Following collection, all samples were placed in coolers and maintained on ice during transport to the laboratory and at 1–4 °C during the time interval before they were filtered for analysis. Processing of all samples for analyses was performed within 6 h of collection. The turbidity and pH of each water sample was determined by standard methods, American Public Health Association (1998).

### 2.3. Microbiological analyses

Viable enterococci were enumerated by EPA Method 1600 on mEI agar plates, US EPA (2002). Volumes of 100, 10, and 1 ml from one of the two bottles of each

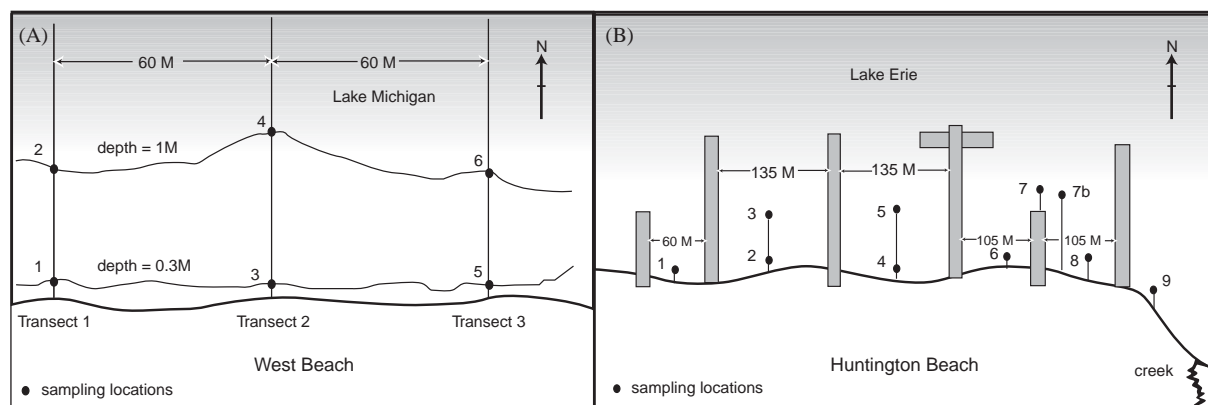


Fig. 1. Diagrams showing water sampling locations at: (A) West Beach, (B) Huntington Beach. Huntington Beach contained several rock breakwaters (shaded areas). Locations 1,2,4,6,8 and 9 at this beach were at a water depth of 0.3 M and locations 3,5 and 7 were at 1 M. Sampling location 7 was moved to location 7b on August 23 for safety reasons.

water sample were filtered on 47-mm diameter, 0.45  $\mu\text{m}$  pore size, membrane filters (Sartorius, Edgewood, NY). The filters were incubated on plates of mEI agar for  $24\text{h} \pm 2\text{h}$  at  $41 \pm 0.5^\circ\text{C}$  before determining colony numbers. Colony counts from the 100 ml sample volumes were reported unless they exceeded 200, in which case counts from one or the other of the smaller volumes were used after multiplying by an appropriate correction factor to express the enterococci CFUs per 100 ml of water. Verification tests on the identities of 5 colonies/sample were performed, as described in the method, for all water samples collected during the first day of the study at each beach site. The overall verification rate for the two beaches was 84% by this method. Each preparation of mEI agar was tested for performance (i.e., correct enzyme reaction) using pure cultures of target and non-target organisms and sterility of the filters and phosphate-buffered water used for rinsing the filtration apparatus were also tested with each batch of samples arriving together at the laboratory.

#### 2.4. Sample processing for QPCR analyses

A 100 ml volume from the other bottle of each water sample was filtered through 47-mm, 0.4- $\mu\text{m}$  pore size polycarbonate filters (catalog #K04CP04700, Osmonics Inc., Minnetonka, MN) and the sides of the funnels were rinsed twice with 20 ml of sterile, PCR-grade water. The filters were transferred to a Petri dish with the sample side facing up. Using sterile forceps, each filter was folded into a cylinder with the sample side facing inward, and then inserted into a 2 ml semiconical screw-cap microcentrifuge tube (extraction tube) (catalog #506-636, PGC Scientific, Gaithersburg, MD) containing 0.3 g of acid-washed glass beads (catalog #G-1277,

Sigma, St. Louis, MO). The filters were held at  $-20^\circ\text{C}$  until shipment to the analytical laboratories on dry ice.

DNA was recovered from the organisms retained on the filters by addition of 320  $\mu\text{l}$  of AE buffer (Qiagen, Valencia, CA) containing 0.2  $\mu\text{g}/\text{ml}$  salmon testes DNA (#D-1626, Sigma, St. Louis, MO), added as an exogenous, internal positive control and reference, to each extraction tube and bead milling in an eight-position mini bead beater (Biospec Corp., Bartlesville, OK) for 60 s at maximum rate. The tubes were then centrifuged at  $12,000 \times g$  for 1 min to pellet the glass beads and debris. Resulting supernatants were transferred to sterile 1.6 ml microcentrifuge tubes and, if not analyzed immediately, stored at  $-20^\circ\text{C}$ . Negative controls consisted of two filtrates of 40 ml PCR-grade water, prepared at the same time as the sample filtrates, and six blank filters prepared in the PCR analytical laboratory, that were extracted in the same manner with each batch of samples arriving weekly at the laboratory. Calibration standards, consisting of filters spotted with 10  $\mu\text{l}$  of a suspension of  $1 \times 10^7$  cells/ml *Enterococcus faecalis*, were also extracted in the same manner at a rate of six per each weekly batch of samples. Inoculum for the calibration filters was prepared from a pure culture of *E. faecalis*, ATCC 29212, grown for 18 h with shaking in Tryptic soy broth (Difco, Detroit, MI) at  $37^\circ\text{C}$ . The culture was harvested by centrifugation at  $6000 \times g$  for 5 min and resuspended in phosphate-buffered saline (PBS) prior to aliquoting into smaller volumes for storage at  $-70^\circ\text{C}$ . The cell concentrations in this and additional, similarly prepared, suspensions of other *Enterococcus* strains were determined by mixing appropriate dilutions of these suspensions in PBS at a ratio of 4:1 (v/v) with 0.1% acridine orange in PBS, holding the suspension for several minutes to allow staining, filtering the stained cells on 25 mm, 0.2  $\mu\text{m}$  pore size, black

polycarbonate filters (Whatman #110656, Costar Inc., Cambridge MA) and counting them with epifluorescence illumination under a 100 x oil immersion lens using a Nikon E800 microscope. For each determination, cell counts from four replicate filters and from 10 randomly chosen fields/filter were averaged to determine the cell concentrations in the suspensions as described in Section 9216 B of *Standard Methods for the Examination of Water and Wastewater*, American Public Health Association (1998).

### 2.5. QPCR analyses

QPCR analyses were performed using the previously described fluorogenic 5' nuclease (TaqMan<sup>TM</sup>) system, Heid et al. (1996). Reactions were prepared in 25  $\mu$ l optical tubes (Cepheid, Sunnyvale, CA) by addition of the following components: 12.5  $\mu$ l of TaqMan<sup>TM</sup> Universal Master Mix, a 2  $\times$  concentrated, proprietary mixture of AmpliTaq Gold<sup>TM</sup> DNA polymerase, AmpErase<sup>®</sup> UNG, dNTPs with UTP, passive reference dye and optimized buffer components (Applied Biosystems, Foster City, CA); 5  $\mu$ l of a mixture of forward and reverse primers (5  $\mu$ M each) and 400 nM TaqMan<sup>TM</sup> probe; 2.5  $\mu$ l of 2 mg/ml bovine serum albumin (fraction V, GibcoBRL, Gaithersburg, MD) and 5  $\mu$ l of DNA extracts from the samples, diluted from 10–100 fold in AE buffer. The reactions were monitored in a Smart Cycler I<sup>TM</sup> sequence detection instrument (Cepheid, Sunnyvale, CA). Thermal cycling conditions consisted of 2 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and either 1 or 2 min at 60 °C. Determinations of cycle threshold ( $C_T$ ) were performed automatically by the instrument after manually adjusting the threshold fluorescence value to 8 units.

PCR primer and hybridization probe sequences for the *Enterococcus* assay consisted of ECST748F: 5'-AGAAATTCCAAACGAACTTG for the forward primer; ENC854R: 5'-CAGTGCTCTACCTCCATCATT for the reverse primer; and GPL813TQ: 5'-6FAM-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA for the probe, Ludwig and Schleifer (2000). These sequences are homologous to the large subunit ribosomal RNA genes of all reported species within this genus. Primer and hybridization probe sequences for the exogenous positive control salmon DNA assay were SketaF2: 5'-GGTTTCCGCAGCTGGG for the forward primer; SketaR3: 5'-CCGAGCCGTCCTGGTCTA for the reverse primer; and SketaP2: 5'-6FAM-AGTCG-CAGGCGGCCACCGT-TAMRA for the probe. These sequences are homologous to internal transcribed spacer region 2 of the ribosomal RNA gene operon of chum salmon, *Oncorhynchus keta*, Domanico et al. (1997). Primers and fluorescently labeled probes were purchased from Integrated DNA Technologies (Coralville, IA).

The amplification efficiencies of the *Enterococcus* and salmon DNA QPCR assays were determined by regression analyses of log<sub>10</sub>-transformed cell equivalents or DNA concentrations, in serially diluted DNA samples, on corresponding assay  $C_T$  results for the samples, Brinkman et al. (2003). The DNA samples, used for evaluation of the *Enterococcus* assay, originated from at least three replicate cell extracts of two strains each of *E. faecalis*, *E. casseliflavus* and *E. faecium*, including the *E. faecalis* calibrator strain. Cell equivalents in the diluted samples were determined from the cell numbers in the original cell extracts divided by the extract dilution factor and spanned a range from approximately 10<sup>7</sup>–10<sup>2</sup>. The regression lines for the dilutions from each cell extract were also extrapolated to estimate the mean sensitivity of the *Enterococcus* QPCR assay, Brinkman et al. (2003). The 95% minimum cell detection limit of the entire method (DNA extraction and *Enterococcus* assay) was estimated from QPCR analyses of independent DNA extracts of serially diluted 10<sup>5</sup>–10<sup>1</sup> cell suspensions of the *E. faecalis* calibrator strain, prepared in triplicate as described above.

Target cells in the beach water filtrate extracts were quantified as calibrator cell equivalents (CCE) by a previously described comparative cycle threshold method, Applied Biosystems (1997), Haugland et al. (1999). Briefly, this method determines the relative quantity of target DNA sequences extracted from an unknown test sample compared to the quantity of target sequences extracted from a known quantity of target organisms in a calibrator sample. This is done after normalizing for the relative recoveries of total DNA in the extraction process from the two samples by comparing the recovered quantities of another reference DNA sequence added in equal amounts to both. For each test sample and corresponding set of calibrator sample reactions, a  $\Delta C_T$  value is obtained by subtracting the  $C_T$  value of the reference sequence assay ( $C_{T,ref}$ ) from the  $C_T$  value of the target sequence assay ( $C_{T,target}$ ). A  $\Delta \Delta C_T$  value is obtained by subtracting the mean  $\Delta C_T$  value of the calibrator samples from the  $\Delta C_T$  value of each test sample. The ratio of target sequences in the test and calibrator samples is described by  $(E + 1)^{-\Delta \Delta C_T}$ , where  $E$  is the amplification efficiency of the target assay. These ratios are multiplied by the known number of target organism cells in the calibrator samples to obtain estimates of the numbers of target cells in each test sample. As described above, salmon testes DNA was used to provide the reference sequences in this study and  $C_T$  values from this assay, were also used as an exogenous positive control to signal potential PCR inhibition, Brinkman et al. (2003). Ten-fold dilutions of the water filtrate and calibration extracts were routinely analyzed and water filtrate samples giving salmon DNA assay  $C_T$  values that were three standard deviations higher than the mean values from the calibration

extracts were reanalyzed at 50- and 100-fold dilutions. *Enterococcus* and salmon DNA assays were performed in separate reaction tubes. Water samples collected at West Beach from May 31 through June 8 were analyzed at the US EPA, National Exposure Research Laboratory in Cincinnati, OH and the remainder of the samples were analyzed at EMSL Analytical, Inc., Westmont, NJ.

### 3. Results and discussion

#### 3.1. QPCR assay and method performance testing

A scatter plot of the combined data used for evaluation of the *Enterococcus* DNA QPCR assay is shown in Fig. 2. The slope of the regression line for these combined data was  $-3.34$  (standard error, 0.03), corresponding to an amplification efficiency value of 0.99. Extrapolation of the combined data regression line for the *Enterococcus* assay resulted in an estimated mean detection limit of 0.66 cells per extract, based on 40 amplification cycles. No appreciable differences were seen in either the slopes or intercepts of the regression lines for the data subsets obtained from *E. faecalis*, *E. casseliflavus* and *E. faecium*, nor were the values of these parameters changed appreciably when using 1 or 2 min annealing/extension times per cycle in the thermal cycling protocol. Shorter annealing/extension times per cycle, however, did reduce the performance of the assay, based on these parameters. The slope of the regression line for the salmon DNA QPCR assay was  $-3.75$  (standard error, 0.10), corresponding to an amplification efficiency of 0.85 (data not shown). The lower efficiency of the salmon assay may be attributable, in part, to a

mismatch between the 3' terminal base of the reverse primer in this assay and the target sequence in the commercial salmon DNA preparation used in the study. This lower efficiency may be beneficial, however, since recent results from our laboratory indicate that the salmon DNA assay is more easily inhibited by humic acids than the *Enterococcus* assay (data not shown) and, hence, appears to be a sensitive indicator of PCR inhibition.

The 95% confidence, minimum detection limit of the extraction method and *Enterococcus* assay was estimated to be 27 cells per extract, using 45 amplification cycles and the *E. faecalis* calibrator strain. This value is based on QPCR analyses of undiluted extracts of filters containing pure cell suspensions of this strain and, hence, will not be representative of the method's sensitivity when analyzing various dilutions of the filter extracts, nor does it take into account possible effects of other filter-collected components of surface water samples on DNA recovery by the method.

A preliminary study was conducted in late summer of 2002 to assess the accuracy and precision of *Enterococcus* enumeration results by the QPCR method. In this study, a total of 72 water samples were collected at the West Beach site, processed, and analyzed as described above. An equivalent number of samples were collected in parallel and spiked with  $\sim 1000$  cells of the *E. faecalis* calibrator strain before processing and analysis. The  $\log_{10}$  average of ambient *Enterococcus* CCEs detected in the unspiked samples by QPCR, using direct comparative  $C_T$  analyses of the test and calibrator sample results, was 1.60 (standard deviation, 0.32), corresponding to a geometric mean of 40 CCEs. Normalization of these results for DNA recoveries in the extracts by incorporation of the reference DNA assay results into the comparative  $C_T$  analyses gave a  $\log_{10}$  average of 1.75 (standard deviation, 0.49) or a geometric mean of 57 CCEs per sample. These results suggested that the ambient *Enterococcus* cells in these samples would have a negligible impact on the quantitative measurements of the spiked cells. The  $\log_{10}$  average of *Enterococcus* CCEs detected in the spiked samples using direct comparative  $C_T$  analyses was 2.99 (standard deviation, 0.33), corresponding to a geometric mean of 978 CCEs. Normalization of these results using the reference DNA assay results for the same samples gave a  $\log_{10}$  average of 3.04 or a geometric mean of 1088 CCEs per sample. This procedure slightly improved the precision of the  $\log_{10}$  measurements, resulting in a standard deviation of 0.31% or a 95% occurrence range for individual sample measurements of  $\sim 25\%$  to  $400\%$  of the mean value. Previous studies in our laboratory have indicated a somewhat narrower 95% occurrence range of  $\sim 50\text{--}200\%$  for similar measurements of spore suspensions from various yeast and fungal species,

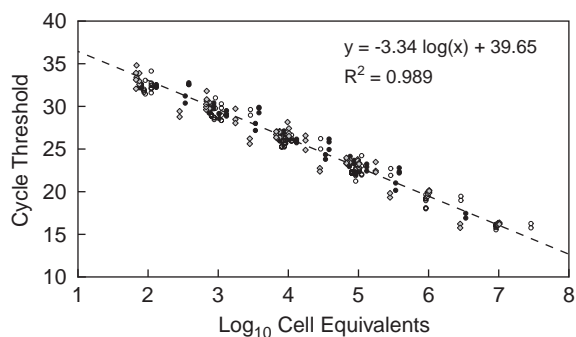


Fig. 2. Scatter plot and regression analysis results of  $\log_{10}$  cell equivalents of *Enterococcus* species on QPCR-measured cycle threshold ( $C_T$ ) values for serially diluted DNA extracts of two strains each of: (●) *E. faecalis*; (○) *E. faecium*; (◆) *E. casseliflavus*. Analyses of at least three replicate DNA extracts of each strain are shown. The regression is represented by the dashed line.

Haugland et al. (1999), Brinkman et al. (2003). The reference DNA in those studies, however, originated from spores of another fungal species that were added to the test and calibration samples prior to extraction. While considerably more convenient, the approach used in this study of adding previously purified DNA to the samples as a reference may therefore be less effective in correcting the results for variations in DNA recovery. Sample-to-sample variations in the addition of *Enterococcus* cells to the water samples and recovery of these cells during the membrane filtration process also may have contributed to the variability in these measurements.

### 3.2. Analyses of beach water samples

Fig. 3 shows the geometric means of *Enterococcus* densities at all sampling locations for each visit to the two beaches over the course of the study as determined by the QPCR and MF methods. Enterococci were detected by the MF method in 89% of the 336 individual water samples collected from West Beach and 95% of the 420 water samples collected from Huntington Beach, whereas *Enterococcus* DNA was detected by the QPCR method in 98% of the samples from West Beach and 97% of the samples from Huntington Beach. Samples with no detectable CFUs or target DNA were assigned

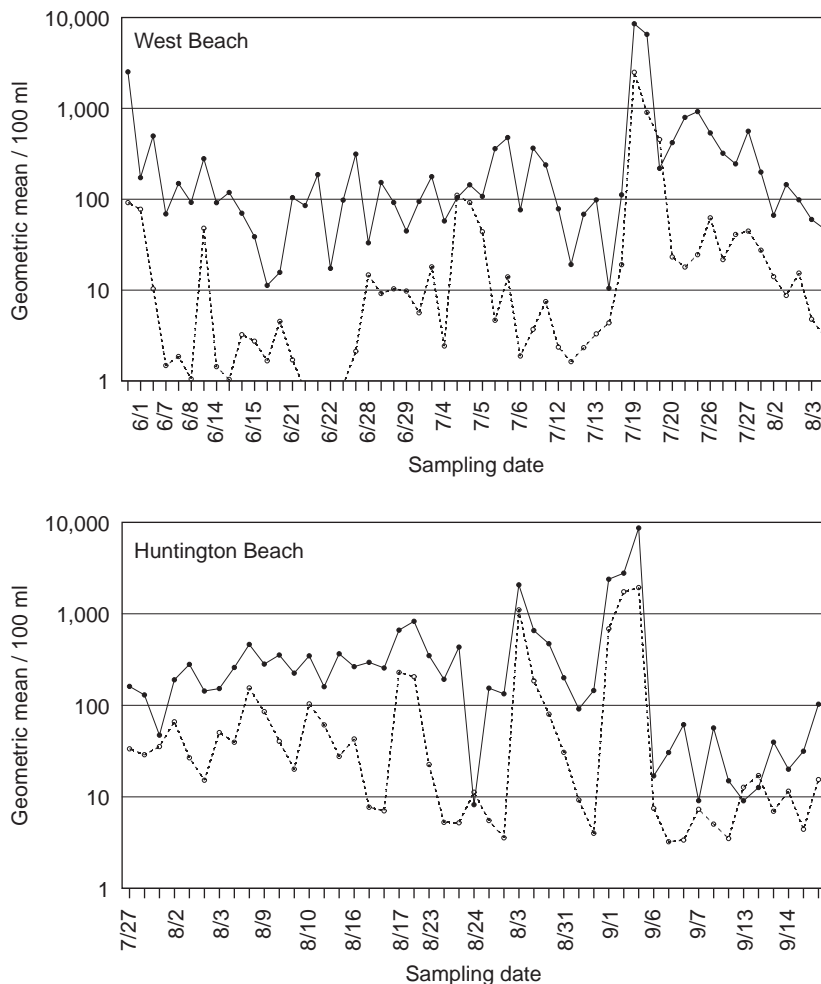


Fig. 3. Geometric means of *Enterococcus* densities per 100 ml of water from all sampling locations at West Beach and Huntington Beach: (●) determined by QPCR method as calibrator cell equivalents; (○) determined by MF method (Method 1600) as CFU. Unless otherwise specified, the three pairs of data points for each of the sampling dates shown in the graphs are for 8 AM, 11 AM and 3 PM sampling visits, in that order. On the West Beach graph, the left-most data points are from a sampling visit on May 31, 11 AM. June 1–8 data points are from 11 AM and 3 PM visits only, July 27 data points are from 8AM and 11AM visits only and August 17 data points are from 8AM and 11AM visits only. Missing data resulted from adverse weather conditions that precluded sampling.

values of 0.5 CFU (equivalent to one-half the detection limit of the MF method) and 0.5 CCE respectively, for calculating the geometric means and for determining the standard deviations of individual sample results within the sampling visits.

Our preliminary studies with the QPCR analysis method had indicated that PCR inhibition was frequently encountered in analyses of undiluted water sample extracts (data not shown). Analyses of 10-fold diluted extracts were therefore routinely performed. Under these conditions, only 2% of all sample extracts from both beaches failed to meet the salmon DNA assay based acceptance criterion, established in this study, for demonstrating the absence of significant PCR inhibition (i.e. sample  $C_T$  value within three standard deviations of the mean of the calibration sample results). All but two of these extracts gave acceptable results upon reanalysis of a further 5-fold dilution of the already 10-fold diluted extracts. While similar studies at additional sites are needed, these observations suggest that the rapid DNA extraction method used in this study may be sufficient for QPCR analysis of freshwater samples from many recreational beaches without the need for additional time-consuming and potentially expensive DNA purification methods. Based on comparative  $C_T$  analyses of the reference assay results for all sample extracts initially meeting the criterion for absence of PCR inhibition, the mean DNA recoveries in the extracts from the West Beach and Huntington Beach samples were 51% and 82% of the calibration samples, respectively.

Over the entire study, 19% of the total of 217 negative control samples that were analyzed by the *Enterococcus* QPCR assay gave positive results. These false positives appeared to result primarily from low levels of aerosolized DNA contamination in the analytical laboratories. The mean  $C_T$  value of these negative controls, however, was only 43.65 which translated to

less than 2 *Enterococcus* CCEs per sample based on the mean reference assay  $C_T$  values for all samples. This observation, combined with the fairly random occurrence of false positives in the negative control samples and the use of mean results of multiple samples from each beach visit, would suggest that the low background of DNA contamination-related measurements had a minimal effect on the QPCR analysis results obtained in this study. Independent QPCR analyses of samples collected on June 14 and 15 at West Beach by the US EPA and EMSL laboratories gave nearly identical mean *Enterococcus* measurements for both days.

As summarized in Table 1, the geometric mean *Enterococcus* CFU concentrations at Huntington Beach averaged ~3-fold higher than at West Beach, as determined by the MF method. Conversely, the mean QPCR results at Huntington Beach were fairly similar at the two sites and were higher than the MF results by a factor of ~16-fold at West Beach and 6-fold at Huntington. The higher *Enterococcus* measurements obtained by QPCR were not surprising given the ability of this method to detect DNA from both culturable and non-culturable or dead organisms. Previous studies have demonstrated that PCR-amplifiable DNA can persist in dead bacterial cells and as free molecules in natural waters, Duprey et al. (1997), Kreader (1998). This difference in the nature of the targets of the two methods may also contribute to the differences seen in their between-visit variability in *Enterococcus* measurements for all sampling locations, shown in Table 1. The somewhat lower overall variability seen in the QPCR method results can be largely attributed to its relatively high measurements at the lower end of the concentration distribution ranges at the two beaches (Fig. 4). This, in turn, may be attributable to the greater persistence of non-culturable organisms and/or free DNA. Conversely, the higher-end measurements of the two methods, that

Table 1  
Summary of *Enterococcus* beach water analysis results from MF and QPCR methods

Enterococci/100 ml	West Beach		Huntington Beach	
	MF	QPCR	MF	QPCR
Geometric mean of all sampling visits <sup>a</sup>	9	143	27	159
Log <sub>10</sub> mean of all sampling visits	0.95	2.16	1.44	2.20
Log <sub>10</sub> SD <sup>b</sup> between sampling visits	0.79	0.58	0.73	0.67
C.V. <sup>c</sup> between sampling visits	0.93	0.63	0.84	0.75
Log <sub>10</sub> SD within sampling visits <sup>d</sup>	0.35	0.76	0.60	0.73
C.V. within sampling visits	0.36	0.88	0.66	0.84

<sup>a</sup>One sampling visit corresponds to the geometric mean of results from all sampling locations ( $N = 6$  at West Beach and 9 at Huntington Beach, respectively) for a particular time and day.

<sup>b</sup>Standard deviation.

<sup>c</sup>Coefficient of variation (= SD in original units/mean).

<sup>d</sup>Calculated from the variance in results within each sampling location and sampling visit pooled over all sampling locations and all sampling visits.

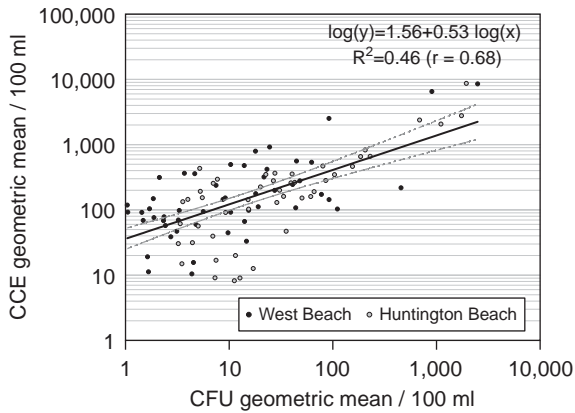


Fig. 4. Scatter plot and regression analysis results of geometric mean *Enterococcus* CFU densities, determined by MF method (Method 1600), vs. *Enterococcus* CCE, determined by QPCR method, for all sampling visits to both West Beach and Huntington Beach.

presumably arose from more recent pollution incursions, were more similar. Previous studies have also demonstrated that the QPCR method can overestimate viable cell quantities in the logarithmic phase of growth, Ludwig and Schleifer (2000). We do not expect, however, that this phenomenon had a significant impact on the measurements obtained in this study because of the low probability that organisms collected from ambient surface waters will be undergoing rapid growth and because the calibrator samples used for quantification in this study were derived from early stationary phase cultures that were also no longer undergoing rapid growth. The median change in CFU concentration from one visit to the next was by a factor of 2.25 at Huntington and 2 at West Beach, as determined by the MF method, while similar corresponding change factors of 2.15 and 2.30 were determined from the QPCR results.

As indicated by the within-visit standard deviation and coefficient of variation values shown in Table 1, the variability of the QPCR results was somewhat higher than for MF. This higher variability is reflected by the standard deviation values seen in our preliminary QPCR analyses of spiked water samples and may be related to the above-described limitations of our current reference assay approach as well as to a lower intrinsic precision of QPCR measurements that can result from analyses of small portions of the total sample extracts by this method. The results obtained by the MF method, however, also differed greatly by sampling location for each of the visits to the two beaches. Similar levels of spatial variability in *Enterococcus* densities to those seen in this study, particularly at Huntington Beach, were reported in a recent 5-beach study conducted by the US

EPA and were primarily related to the water depth (i.e. distance from the shore) at the sampling locations, Wymer et al. (2004). These results further demonstrate the need for a comprehensive water sampling scheme, such as the one used in this study, to perform accurate monitoring of fecal indicators at recreational beaches.

Turbidity measurements in the water samples ranged from approximately 0.5–50 NTU at West Beach and 2–141 NTU at Huntington Beach. Positive correlations were observed between these measurements and enterococci measurements by both MF and QPCR for all samples from both beaches, however, the extent of these correlations was not dramatic ( $r = 0.54$  for MF and  $0.37$  for QPCR). The possibility that high turbidity levels might be correlated with low DNA recoveries from the water filtrates and/or serve as a predictor of interference in the QPCR analyses was also examined. Overall, however, only a weak positive correlation ( $r = 0.25$ ) was observed between salmon DNA reference assay  $C_T$  values and turbidity values in the water samples, suggesting that higher levels of solids had little effect on DNA recoveries. In addition none of the DNA extracts that failed to meet the acceptance criterion for demonstrating the absence of PCR inhibition were from water samples with high turbidity levels.

As a prerequisite to performing regression analyses and statistical comparisons of the MF culture and QPCR results, the geometric means of *Enterococcus* densities per sampling visit, determined by both methods, were first demonstrated to show approximately lognormal distributions (data not shown). Fig. 4 shows the scatter plot and regression analysis results of QPCR- and MF-measured geometric mean densities of enterococci from all sampling visits to both beaches. The regressions of the data sets from the two beaches were not found to be significantly different from one another ( $p$ -value for equality of slopes = 0.35) and therefore are expressed as a single curve. The overall correlation coefficient ( $r$ ) between the results from the two methods was 0.68. Given the strong correlation that has been previously demonstrated between swimming-related illness rates and *Enterococcus* culture results, Cabelli et al. (1982), Dufour (1984), this positive correlation between the results of the two methods offers promise that the QPCR method may also, be a useful tool for determining health risks due to fecal contamination at freshwater recreational beaches. The slope and intercept values of the curve shown in Fig. 4 also suggest, however, that the current guidelines, based on MF results, for beach closings are unlikely to be applicable to QPCR results. Analyses of the correlation between the QPCR analysis results and swimmer illness rates in the current US EPA health and epidemiology study are in progress and could provide the basis for new risk-based guidelines for beach closings based on results of this method. Similar studies will also determine the correlation between QPCR

results and swimming-associated illness rates in marine waters.

#### 4. Conclusions

- Using a rapid method for extracting DNA from membrane-filtered water samples, a previously reported QPCR assay for the genus *Enterococcus*, Ludwig and Schleifer (2000), was found to provide accurate and sensitive measurements of these organisms in 3 h or less.
- A newly developed QPCR assay for commercially available salmon DNA provided a convenient and sensitive test for inhibition of the PCR analyses, as well as at least partial correction of *Enterococcus* measurements for variability in DNA recovery in the extracts.
- Measurements of *Enterococcus* densities by the QPCR method and by the approved MF method both showed high levels of spatial and temporal variability in water samples from two freshwater recreational beaches over 8–10-week periods.
- A significant positive correlation was observed between the QPCR and MF method measurements of *Enterococcus* densities at both beaches, suggesting that the QPCR method has the potential to supplement or replace MF as a means of assessing the levels of fecal contamination at freshwater recreational beaches. The lack of a perfect correlation between the results of these methods, however, suggests that new data on the relationships between QPCR measurements and health outcomes will be required before results of this method can be used to predict potential health risks to bathers.

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