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Use of an exogenous plasmid standard and quantitative PCR to monitor spatial and temporal distribution of *Enterococcus* spp. in beach sands

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Abstract

Studies using culture-dependent methods have indicated that enterococci, the fecal indicator used to monitor marine waters for the potential of enteric disease risk to swimmers, can be abundant in beach sands and may contribute to water column indicator exceedances. A quantitative PCR (qPCR) method for the *Enterococcus* genus was tested and applied to more rapidly determine the amount of enterococci in beach sands and study their distribution over space and time. The qPCR method amplified a 23S rDNA sequence specific to *Enterococcus* and was used to examine subsamples and composite samples of wet and dry beach sand from Avalon Bay, California, USA. The differences in efficiency of DNA recovery and inhibition in qPCR reactions were accounted for by spiking pairs of duplicate subsamples with a known amount of pGEM plasmid before or after extraction. This study revealed levels of environmental inhibition that were similar in wet and dry sands and efficiency of DNA recovery that was observably lower for wet beach sands and varied between years. Using the correction factors generated by this method to estimate the abundance of *Enterococcus*, we show that wet and dry beach sands both have *Enterococcus* spp. populations that can vary dramatically from day to day, and often are potentially higher than the equivalent health standards mandated for recreational waters.

Coastal managers in the US measure the microbiological quality of water by culturing fecal indicator bacteria (FIB), which are considered proxies for the possible presence of disease-causing pathogens. Epidemiological studies have shown that the density of enterococci in marine waters predicts relative risk of swimming-associated illness (Cabelli et al. 1979, 1982; Haile et al. 1999), and so *Enterococcus* spp. are monitored weekly during the bathing season as mandated by the federal government (USEPA 2000). Although it is well known that sands naturally accumulate cells and organic matter from the overlying water, the contribution of beach sands to ambient water quality is not well understood, and no FIB standards for beach sands exist. On lake beaches, sands have been shown to sustain pop-

ulations of indicator bacteria and act as a diffuse nonpoint source of FIB to the lake (Whitman and Nevers 2003), indicating that sands may not act as a net sink for FIB as previously assumed. Along the California coast, Yamahara et al. (2007) showed that beaches are a diffuse source of FIB to marine water and that seawater can mobilize the loosely attached FIB from sands. Enterococci can also be resuspended by stream and storm water, thereby impacting beach water quality (Le Fevre and Lewis 2003). Furthermore, in the tropics, favorable nutrient concentrations and temperature within sediments allow fecal bacteria to multiply and become a minor population of the sediment microbiome (Roll and Fujioka 1997). Because the abundance of FIB, including those from humans, varies greatly in coastal waters spatially and temporally (Boehm et al. 2003; Boehm 2007), understanding the potentially dynamic relationship between sedimentary cells and the populations measured in the water column may further characterize the variation observed in fecal indicator bacteria in coastal waters.

Many of the studies that have recently addressed the microbial communities of marine recreational beach sands have used culture-based methods (e.g., Elmanama et al. 2005; Ferguson et al. 2005; Lee et al. 2006; de Oliveira et al. 2008). However, culture-based monitoring in beach sands may not adequately characterize populations of *Enterococcus* in sediments,

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because these methods exclude organisms that are dormant or in the viable but nonculturable state. Studies of the viability of *E. faecalis* in artificial seawater microcosms show that at least 80% of the cells are still viable when colony-forming units (CFUs) are no longer recoverable (del Mar Lleo et al. 2006). By using a molecular method, we seek to include viable but nonculturable organisms in our description of the *Enterococcus* spp. population in beach sands, although we recognize that by targeting DNA we will also detect dead cells.

Our primary objective in this study was the development of a flexible quantitative PCR method that can be used for rapid and sensitive quantification of microbes, including *Enterococcus*, in wet and dry beach sands. Expanding on a method developed by Coyne et al. (2005), we incorporated an external plasmid standard to estimate the efficiency of the DNA recovery process after extraction, as well as the impact of sample-specific inhibition of the qPCR assay; in doing this, we address and attempt to correct biases associated with a molecular-based method of detection. A further objective was the application of this method, using the sands of Avalon Bay, California, USA, as a case study, to describe the scale of significant spatial heterogeneity of the *Enterococcus* population in sands over time. By applying our method to individual subsamples and composite samples, we hope to guide future choices for sampling and compositing efforts.

The sands from the recreational beach at Avalon Bay, Catalina Island, California, were sampled for this study (Fig. 1). Avalon Bay has a history of bacteriological exceedances. The source of contamination is the deteriorated municipal sewer infrastructure at this location, and storm drains and storm water runoff are not considered to be major contributors to pollution (Boehm et al. 2003). In 2006, the year before this study's sampling regimen, waters at Avalon Bay violated public health standards 53% of the times sampled (Dorfman and Rosselot 2008), and in the summer of 2007, Avalon Bay had the worst dry-weather water quality in the entire state of California (Heal the Bay 2008).

Materials and procedures

Beach sand sampling—Sand samples were collected at 0800 on Thursdays, Fridays, Saturdays, and Sundays beginning on July 26 and ending on September 9, 2007, and at the same sites at 1200 on Fridays, Saturdays, and Sundays from August 1–31, 2008. One hundred sixty-two samples were collected in 2007, and 142 samples were collected in 2008. The beach sand was sampled above and below the water line at sites designated A, B, and C along the beach (see Fig. 1). Triplicate sand cores from wet sand, collected under approximately 10 cm water, and dry sands, collected from the high-tide line, were taken at each site. The sand was cored with sterile 50-mL polypropylene Falcon tubes. Cores were flash-frozen in liquid nitrogen and shipped to the Woods Hole Oceanographic Institution, where they were subsequently stored at -80°C until extraction. In California, the most probable number (MPN)

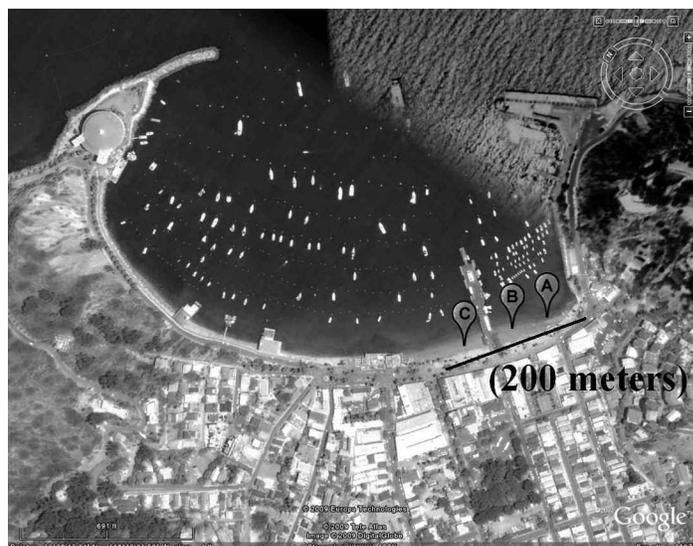


Fig. 1. Sampling sites at Avalon Bay ($33^{\circ}20'N$, $118^{\circ}19'W$), Catalina Island, CA, USA, shown aerially. Google Earth image.

method of *Enterococcus* detection, using Enterolert and 96-well Quantitrays (IDEXX), was used by the Southern California Coastal Water Research Program on sediments collected at the sites.

DNA extraction of environmental samples—Nucleic acids were extracted from 0.25 g sand using the MoBio PowerSoil DNA Kit (MoBio Laboratories). The subsamples selected for extraction were taken from the surface sand of the triplicate sand cores, which were then pooled before extraction. To generate a whole-beach composite sample, surface sand from the triplicates from all three sites along the beach was combined and 0.25 g removed for extraction.

Quantitative real-time PCR detection of Enterococcus—The *Enterococcus* assay uses the forward primer ECST748F (5'-AGAAATTCCAAACGAACTTG) targeting enterococci, lactococci, and some clostridia and the reverse primer ENC854R (5'-CAGTGCTCTACCTCCATCATT), specific for the genus *Enterococcus* (Ludwig and Schliefer, 2000). qPCR reactions were run in triplicate for every environmental extract. Each 25- μL reaction contained 1 μL environmental template DNA, 100 ng ECST748F, 100 ng ENC854R, 12.5 μL SYBR Green Master Mix, and 9.5 μL sterile MilliQ water. The cycling parameters began with a 95°C hold for 3 min, then proceeded through 50 cycles of 95°C (10 s), 52°C (30 s), and 72°C (10 s), with real-time fluorescence detection enabled during extension. This was followed by additional denaturing (95°C for 1 min) and incubation at 52°C (1 min) to ensure that double-stranded product entered the melting curve cycle; the melting curve profile began at 52°C and ended at 95°C , with a temperature change of $+0.5^{\circ}\text{C}$ every 30 s. The extension and annealing steps differ from the single anneal/extension step at 60°C used by Ludwig and Schliefer (2000) because of earlier optimization for product detection with regular PCR. Duplicate dilution series of

purified *E. faecalis* DNA were used to construct the standard curve, and negative duplicates (reactions without template) were run on each plate. Standard curves were based on reactions with starting quantities of 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, and 10 fg *E. faecalis* DNA (Fig. 2A). The qPCR reactions were run with a Bio-Rad myIQ thermal cycler in plates sealed with optically clear flat caps. Plates were prepared on the bench or on ice for qPCR comparison as discussed below.

Melting curves were used as a quality control measure, with *E. faecalis* DNA having a melting point of 80.5°C. Among triplicate qPCR reactions, occasionally there would be a reaction whose amplification was not exponential and whose melting curve did not conform to the profile. In these cases, the particular reaction was excluded from the analysis and the environmental sample quantified from the mean of two, rather than three, qPCR reactions.

Evaluating efficiency of DNA recovery and environmental inhibition—For samples collected on days representing the course of the summer sampling period (July 28, August 11, August 25, and September 8 in 2007 and August 2, 18, and 30 in 2008), an exogenous DNA standard (pGEM Vector, Promega) was added to the MoBio kit's initial extraction buffer (C1, an SDS-based lysis buffer) during the first step of the extraction. Extraction buffer (500 μ L) was prepared with 1 μ g pGEM plasmid, and 60 μ L was added to the 0.25-g sand samples, which were then extracted according to kit directions. The amount of pGEM in the eluted DNA was subsequently quantified with qPCR. The pGEM plasmid primers M13F and pGEMR were used in triplicate qPCR reactions and compared to standard curves constructed with known amounts of pGEM (dilution series of 1 ng to 10⁻⁵ ng plasmid) to determine the recovery rates of pGEM from samples (Fig. 2B). Negative duplicate reactions lacking pGEM were run on each plate. The thermal cycler parameters began with a 95°C hold for 3 min, then proceeded through 50 cycles of 95°C for 15 s and 60°C for 1 min, followed by a melting cycle beginning at 60°C and ending at 95°C, with a temperature change of +0.5°C every 30 s. From these samples, 100% recovery efficiency would be equivalent to recovery, via qPCR, of 1.2 ng pGEM/ μ L template (extraction product) DNA, and thus an average percentage of recovery from the extraction can be derived and used as the correction factor *E*, for efficiency of recovery.

This use of the vector as an exogenous reference standard expands on a method developed by Coyne et al. (2005) for qPCR detection of harmful algal bloom species in water samples but relies on SYBR green for target detection. Because *E* is derived after extraction and qPCR analysis, it is affected by inhibition. Inhibitors present in the qPCR reaction may reduce enzyme activity or interact with DNA and thus prevent complete detection of the target, such that the correction factor (*E*) is likely an underestimation of the true recovery of DNA through the extraction protocol.

To quantify the relative impact of inhibition and its variability in qPCR reactions, PCR reactions using eluted DNA of

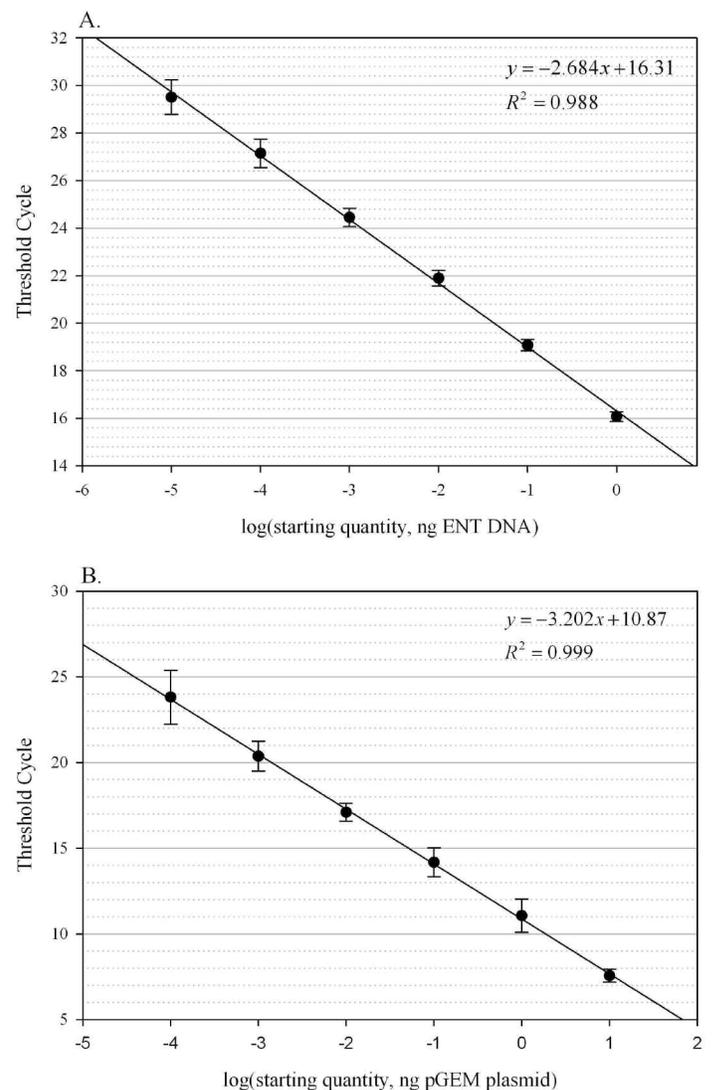


Fig. 2. qPCR standard curve threshold cycles. *E. faecalis* standards (A) and pGEM standards (B). Error bars indicate SEM.

unspiked extracted duplicate samples had an equivalent amount of pGEM (1.2 ng pGEM/ μ L extraction product) added to achieve the same idealized concentration as the samples co-extracted with pGEM. Rate of pGEM recovery from these reactions via qPCR yielded an estimate of environmental inhibition. Thus a correction factor (*I*) could be determined by the qPCR-reported starting quantity of pGEM in the reaction divided by the amount added to the reaction. This factor *I* can be used to correct *E*, such that (*E/I*) is a closer estimation of the recovery of DNA through the extraction protocol. We call this corrected efficiency of recovery *E_c*.

Estimation of Enterococcus cell density based on qPCR results—To calculate the total enterococcal DNA (T_{DNA}) in 0.25 g sand, the starting quantity (s_q) of *Enterococcus* DNA (fg/ μ L) reported by qPCR was multiplied by 100, to correct for the fact that only 1 of 100 μ L eluted DNA was quantified. This

Table 1. Assessment of pGEM recovery and amplification.

Treatment	Sand type	Average recovery on bench, %	σ	Average recovery on ice, %	σ
pGEM spiked before extraction (efficiency)	Wet sand ($n = 4$)	4.10	0.36	55.50	5.3
	Dry sand ($n = 4$)	13.3	1.31	66.30	4.2
pGEM added to eluted DNA (inhibition)	All samples ($n = 8$)	63.5	5.20	93	13

adjusted reaction starting quantity was then corrected for inhibition of PCR (I) and the corrected efficiency of DNA recovery by extraction protocol (E_c). We were interested in individually quantifying I and E_c , rather than simply observing total pGEM loss (E), because of the potential variability of I and E_c in wet and dry sands in the environment and over time. Additionally, understanding the differences in loss due to the extraction protocol versus loss due to inhibition provides insight for further optimization and application of the protocol. The correction we used can be described as follows:

$$T_{\text{DNA}} = [100 \times s_p] / (I \times E_c) \quad (1)$$

T_{DNA} was converted to cell equivalents by dividing by the approximate DNA/enterococcal cell. The approximate amount of DNA per cell was calculated using the results of a completed *E. faecalis* genome sequence, which reports the cellular genome as 3,218,031 base pairs (Paulsen et al. 2003). The approximate base pairs per cell (3.2 Mb) was multiplied by the average molecular weight of a nucleotide pair (660 Da), a Dalton being equivalent to $1/(6.022 \times 10^{23})$ grams, yielding about 3.5×10^{-15} g DNA/cell.

Sand extract exchange—Extracted DNA samples were exchanged with the Boehm laboratory at Stanford for comparison of our method of quantification with the method of Yamahara et al. (2009), which accounts for reaction inhibition by spiking with target DNA and uses the same primers (Ludwig and Scheifer 2000) with a TaqMan probe for qPCR detection. Two extracts were obtained from Yamahara and four extracts were sent from our lab. Yamahara ran our extracts as described in Yamahara et al. (2009), and we ran their extracts with our method.

Statistical analysis—Because the data are not normally distributed, but are similarly distributed between sites (skewed right), nonparametric statistical tests (Kruskal-Wallis, Mann-Whitney, and the Wilcoxon signed-rank test) were chosen for data analysis, using $P = 0.05$ for the α -level of significance. All statistics were calculated with SigmaPlot 11.

Assessment

Reliability of pGEM as a measurement of efficiency of DNA recovery and PCR inhibition—The effect of temperature during plate preparation was evaluated. The abundances of pGEM and *Enterococcus* in sand samples were quantified by qPCR using plates that were prepared at room temperature and on ice. For pGEM, a clear difference was observed in the efficiency of recovery for wet sand in comparison to dry sand, with dry

sand having about 10% better extraction efficiency than wet sand (Table 1). However, preparing the plates on ice yielded substantially less-inhibited PCR reactions (Table 1). The environmental inhibition did not significantly differ between wet and dry sand. When the plates were set up in a similar manner for *Enterococcus* quantification, similar results were observed; that is, significantly more enterococcal DNA was detected when the plate had been prepared on ice, indicated by Wilcoxon signed-rank test for correlated samples ($W = 36$, $n = 8$, Z statistic = 2.521, $P = 0.008$). However, by using the pGEM efficiency and inhibition correction factors appropriate to experimental conditions to calculate cell equivalencies from the DNA recovered, we arrived at similar cell equivalencies for each sample. The Wilcoxon signed-rank test showed no significant difference between cell equivalencies from the different treatments after correction ($W = -2.0$, $n = 8$, Z statistic = -0.140 , $P = 0.945$). These results indicated that pGEM accurately reflected PCR reaction conditions, that extraction efficiency is consistently different for wet and dry sands, and that PCR inhibition is relatively constant in these beach sands over time. This result may not apply to all environmental matrices, but increased our confidence in the use of pGEM as a standard for this system.

Reliability of the standard curve and error associated with environmental samples—Over the course of many individual runs ($n > 20$), the standard curve dilution series of both pGEM and *E. faecalis* DNA amplified consistently, and each dilution had low standard error in threshold cycle (Fig. 2). We noted that as the amount of *E. faecalis* DNA in the qPCR reaction decreased, the standard deviation between qPCR replicates increased. Our environmental samples, which usually fell near the low end of our standard curve dilution series (femtograms), also reflect this observation. Maximizing the amount of starting quantity DNA, and maximizing the reaction efficiency, therefore minimizes the error associated with regression on the standard curve.

Composite versus grab and pooled sampling at Avalon Bay Beach in 2007—Duplicate whole-beach composite subsamples that were extracted for the pGEM experiments were compared to an average daily value calculated from sites A, B, and C. Additionally, on August 11 and September 8, subsamples from each of the triplicate samples from site C were extracted and analyzed to determine the variability within a pooled triplicate site sample. Results indicated a high level of reproducibility between composite samples. The Wilcoxon signed-rank test showed no significant difference between the amount of

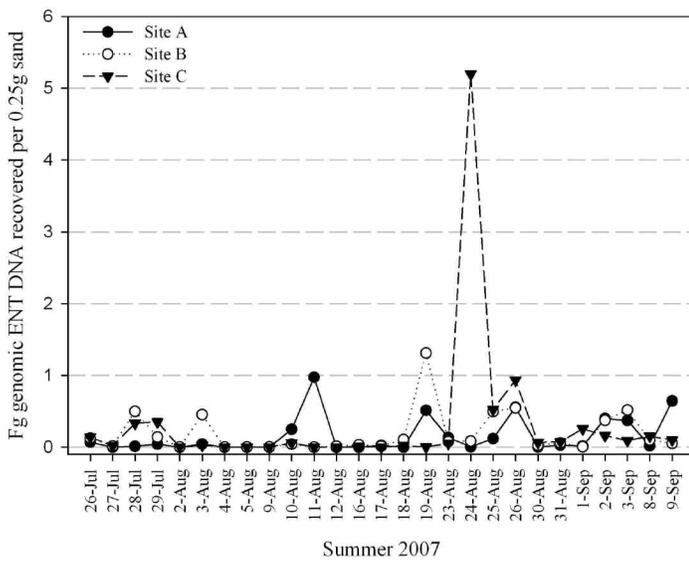


Fig. 3. Recovery of *Enterococcus* spp. DNA in wet sand at Avalon Bay sampling sites.

Enterococcus at sites when calculated by averaging triplicate values or by composite sampling ($W = -2.0$, $n = 4$, Wilcoxon sign-ranked Z statistic = -0.365 , $P = 0.875$).

Spatial differences in *Enterococcus* in wet sand at Avalon Bay Beach—The pooled triplicates for wet sites A, B, and C were extracted and quantified for every day of the study, and the amount of *Enterococcus* in wet sand by site is shown in Fig. 3. Although sites did show some individual variation over the summer, no significant difference was observed in the average amount of *Enterococcus* between sites A, B, and C (Kruskal-Wallis test, $H = 1.029$, $df = 2$, $P = 0.598$).

Wet sands compared to dry sands—For samples collected in 2007, the daily amount of *Enterococcus* in wet sand was estimated by averaging the site values, and because no significant differences were observed spatially, the daily amount of *Enterococcus* in dry sand was measured from whole-beach composite samples. In 2008, all samples were whole-beach composites. In 2007, the wet sand daily averages showed distinct peaks in the amount *Enterococcus* DNA recovered, which occurred roughly every 2 weeks over the entire course of the summer (Fig. 4). In the dry sands, there were consistently low levels of enterococci until August 11. The amount of *Enterococcus* DNA recovered from the dry sand exceeded the amount of *Enterococcus* DNA in the wet sand for the rest of the summer.

The conversion of DNA to cell equivalents (Fig. 5) highlights the importance of measuring the difference in efficiency of recovery, because a slightly different, and interesting, pattern emerges. For example, some local maximums in dry sand *Enterococcus* DNA are, after correction, no longer so different from the wet sand (e.g., 215 cells and 243 cells in wet and dry sand, respectively, on September 2). Most interestingly, the biggest spike recorded for the summer in the wet sands (1935 cells/0.25 g on August 24) was followed by a precipitous

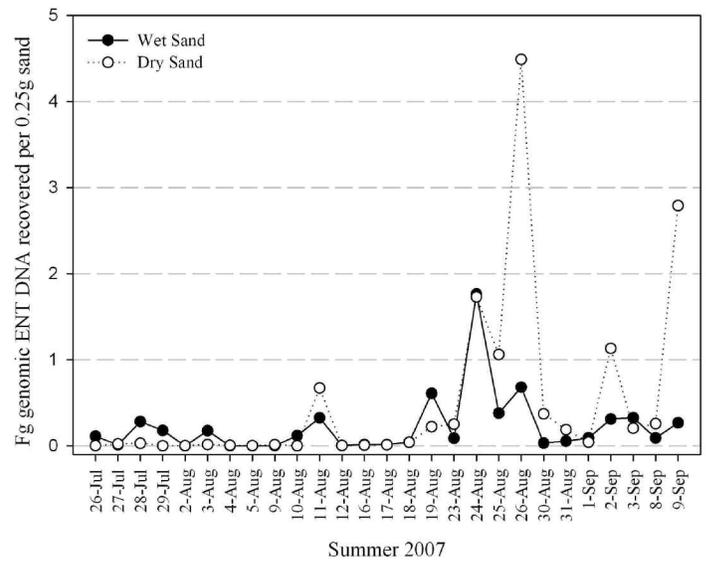


Fig. 4. *Enterococcus* spp. DNA recovered from Avalon Bay beach sand.

decrease in the *Enterococcus* population in the wet sand, and only 2 days later we observed the summer peak for dry sands (1519 cells/0.25 g on August 26). We saw a similar pattern again in 2008 (Fig. 6), when a tremendous spike was observed in the enterococcal population of the wet sand on August 29, followed the next day by a precipitous decline of cells in the wet sand and corresponding increase of cells in the dry sand. Over the entire 2007 sampling period ($n = 27$ days) wet sand had a higher, though not significantly higher, total amount of enterococci than dry sand (Mann-Whitney test, $U = 268.5$, $P = 0.098$). In 2008, there was no significant difference in the amount of *Enterococcus* in the wet and dry sands (Mann-Whitney test, $U = 90$, $P = 0.730$), although the wet sand had the highest numbers recorded all summer on August 15 and 17.

Tidal impact—All of the daily composite dry sand samples and the daily average wet sand samples were ranked by amount of *Enterococcus* DNA recovered. One significant tidally associated difference emerged, in that the average rank of samples taken in 2007 on days when the tide was high (sampled within 1.5 h before and after true high tide, $n = 18$) were of higher ranking than the average of samples taken on days when the tide was at a low or flood stage ($n = 36$; Mann-Whitney test, $U = 213.0$, $P = 0.043$). A similar analysis for 2008 was untenable because overall there were fewer data points, and all samples were taken during the ebb or flood tide and none at the high or low tide marks. However, it is worth noting that in 2008, the largest peak in enterococci concentration in the wet sand occurred during the spring tidal period (August 15, 16; full moon in 2008 on August 16, new moon on August 1 and 30). Likewise, in 2007 the peaks in enterococci occur during spring tidal periods, directly before new (August 12, September 11) and full (July 30, August 28) moons. This is purely observational, as there were no statistically significant correlations between the

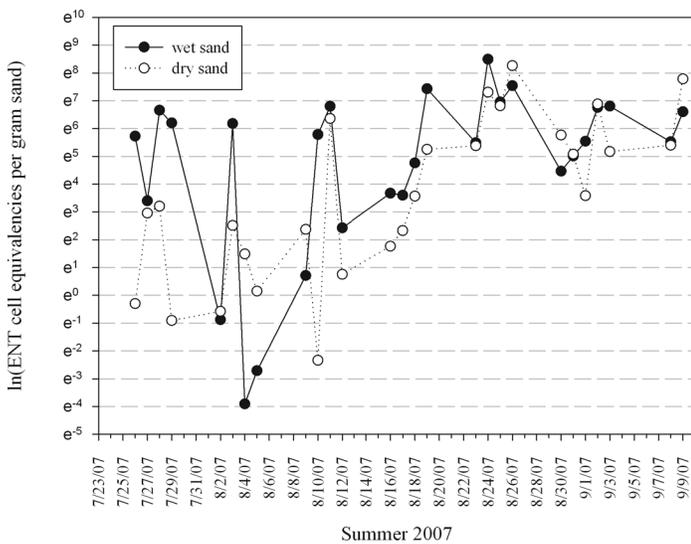


Fig. 5. *Enterococcus* spp. in Avalon Bay beach sand, 2007.

amount of enterococci in the sand and the tidal range of that day or with the height of the water when sampled.

Discrepancy in detection of *Enterococcus* via qPCR versus MPN—In the 2007 data, there was no correlation between the number of cells detected using qPCR and the number of cells detected from the sediments using MPN. The wet sand MPN measurements taken at sites A, B, and C collectively averaged 3.1 cells/g and ranged from 0 to 42 cells, whereas all the wet sand qPCR measurements had an average detection of 612 cells/g, with a range of 1–4880 cells/gram. There was some agreement between peaks in both data sets (peaks around August 11/12 and September 8/9). The ratio of the averages for MPN versus qPCR in this study is 0.005, which is on the lower end of those reported by other studies (Yamahara et al. 2009; Haugland et al. 2005; He and Jiang 2005).

Sand extract exchange—To test the difference between our qPCR method and an alternative, that of Yamahara et al. (2009), we exchanged a total of six extracted samples (two from Yamahara, four from Halliday). The results are given in Table 2. Whereas our method consistently estimated the number of enterococci as higher, there was a clear relationship between our two methods of quantification for the Avalon samples exchanged (Fig. 7). Considering the potential difference in sensitivity of the two methods (TaqMan versus SYBR), different machines, and different personnel, the similarity between the results seems to be quite good. Furthermore, the standard deviation of the results generated with the different methods were within the range that we observe when comparing the results of different cyclor runs for the same samples (between 20 and 60 cell equivalents), with the exception of one sample for which we had a very high result. If the highest outlier was removed from each comparison, then the average standard deviation between detection methods would be

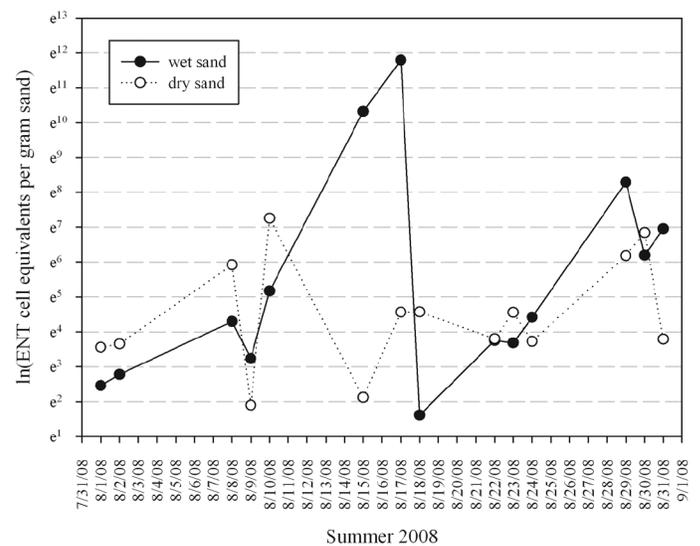


Fig. 6. *Enterococcus* spp. in Avalon Bay beach sand, 2008.

reduced from 49.6 to 23.0 cell equivalents, and the average standard deviation between two different runs would be reduced from 30.6 to 25.5 cell equivalents.

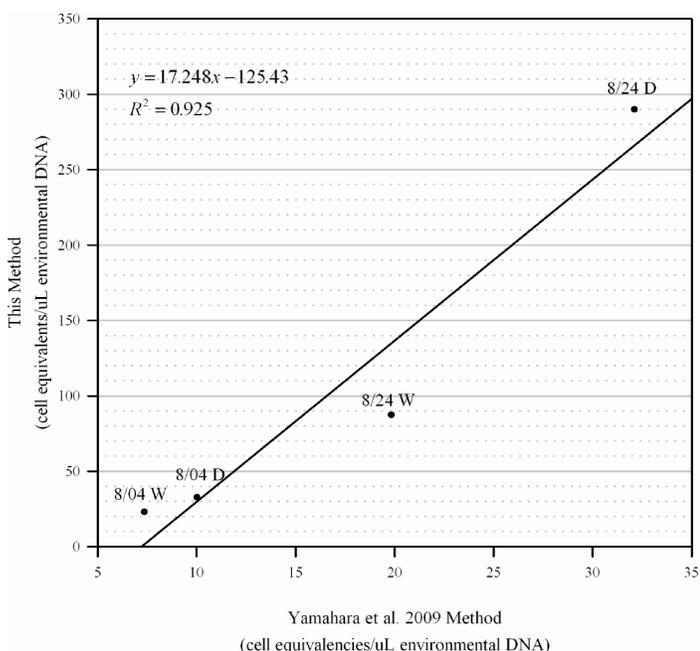
Discussion

Efficiency of DNA recovery and inhibition of PCR are two biases that can differ between sample type and potentially skew quantitative PCR results. In this study, the pGEM plasmid proved to be a reliable way to characterize these biases and correct for them, and to reconcile differences in the methods different investigators may use in qPCR plate preparation.

There are multiple examples of different approaches that have been applied to account for inhibition in quantitative PCR. They generally use either nontarget DNA or some version of the target sequence as the mechanism to judge whether inhibition is occurring in the samples. We limit our comparison to several that have been used for the detection of indicator organisms in natural samples (Lebuhn et al. 2004; Siefring et al. 2008; Shanks et al. 2009; Yamahara et al. 2009). Yamahara et al., Shanks et al., and Lebuhn et al. all employ some aspect of the desired target sequence as the internal control, which is considered more likely to reflect the same amplification biases as the actual target. Shanks et al. (2009) took the innovative approach of creating an artificial sequence of a similar size to the target that contained the same primer sites, but a different TaqMan probe target. In the case of Lebuhn et al. (2004), samples were spiked before extraction with known numbers of the cell type being detected. Yamahara et al. (2009) directly spiked their qPCR reactions with genomic DNA from their target organism. They also determined the extraction efficiencies for their samples by spiking with a known number of enterococci, and then correcting the qPCR values for inhibition. Siefring et al. (2008) have taken probably the

Table 2. Sand sample exchange qPCR data, presented in units of ENT cells/uL.

Sample	Yamahara method	Present method	σ
092807-EX-1	31.6	91.5	42.4
102807-EX-1	11.2	48.3	26.2
A080407DG	7.4	36.1	20.3
A080407WG	10.0	52.5	30.1
A082607DG	19.8	137.3	83.1
A082607WG	32.1	467.8	308.1

**Fig. 7.** Results of sand DNA exchange of Avalon Beach samples. W, wet sand; D, dry sand samples.

most effective steps toward developing an extraction and inhibition standard for enterococcal qPCR. They have redesigned the forward primer for the *Enterococcus* TaqMan assay and designed a *Lactococcus*-specific probe within that region. This allows the simultaneous detection of enterococci and correction for extraction efficiency and PCR inhibition by spiking with *Lactococcus* organisms. The fact that the amplification region is the same for both the control and the target significantly reduces the effect of amplification bias for different fragments.

These methods have all taken different, but valid, approaches to deal with some of the inherent problems associated with qPCR. We believe that our approach also is valid, although there is the potential problem that the pGEM target would not reflect the same amplification efficiency as the target. In an effort to assess whether this was a problem, we participated in the small sample exchange with Yamahara. Although our values tended to be higher, the standard deviation between Yamahara's method results and ours was within

the standard deviation we observed for our method on different cyclor runs of the same samples. Although we are unable to say whether our method is equivalent to the others, we believe that this information does tell us that our method of correcting for DNA recovery and PCR inhibition is likely to have no more of an impact on estimation error than other issues associated with qPCR variation. These other issues include sample degradation due to multiple freeze/thaws, variability in pipetting accuracy, and variability between and within qPCR machines. There is also the fact that we are using SYBR detection rather than the TaqMan probe method of Yamahara, which could contribute to our method giving slightly different values. At this point, we don't have an absolute answer as to why our results differed in the sample exchange, but this is one of the first examples of such a comparison. We will continue to explore the reasons behind the difference, but based on the fact that there is apparently an inherent level of variability for qPCR, we argue that in our hands pGEM has been an appropriate and flexible method for estimating and correcting for DNA recovery and PCR inhibition of environmental samples.

The results of this study show that beach sands have significant *Enterococcus* spp. populations that are patchy spatially and temporally and appear to be influenced by tidal stage. Although the wet sands at Avalon Bay had, overall, higher levels of enterococci, it is interesting to note that the sampling days that took place during higher tidal stages also had higher amounts of enterococci. At a beach like the one at Avalon Bay, which is narrow and does not have a large tidal range, this observation of wet and dry sand taken at higher tidal stages having more enterococci may correspond to common reports of dry beach sands having elevated levels of enterococci, because the sands sampled at high tide represent overall "drier" sands over the course of a day than the sands sampled at low tide. Yamahara et al. (2009) also showed that wetted sands in mesocosm experiments showed an initial decrease in enterococci numbers, but that over time, the numbers increased due to regrowth. The increase in enterococcal abundance in the dry sand over time at Avalon Bay suggests that after tidal wetting, the environmental populations may have regrown.

We have also shown that at Avalon Bay composite samples effectively integrated spatial variability, thereby giving a good estimate of *Enterococcus* prevalence in the sand from day to day. The fact that no significant variability was observed in the *Enterococcus* spp. populations between the three sites may be due to the fact that Avalon Bay is a small beach, making it ideal for composite sampling. This is valuable information because whenever composite samples can be analyzed in lieu of multiple site samples, costs of supplies and time associated with molecular monitoring are drastically reduced.

When our data are converted to equivalent monitoring volumes and analyzed for bacteriological compliance (using the current health standard of 104 CFU/100 mL for marine waters, and assuming one cell is equivalent to one CFU and that

1 g = 1 mL), the sand would rarely be in compliance with the health standard. Only 1 day (August 2, 2007) had compliance in both wet and dry sand; there were two other days when the wet sand would have been in compliance and three other days when the dry sand would have been in compliance. In contrast, many days would exceed the water health standard by several orders of magnitude. Perhaps the discrepancy between CFU detection and DNA-based detection can partially be explained by the large amounts of silicate minerals normally found in beach sand, which concentrate DNA from the overlying seawater (Naviaux et al. 2005). Environmental enterococcal DNA and dead cells also likely contribute a background signal in our monitoring. Further research is needed to understand the relative contributions of active, viable but nonculturable, and dead cells and environmental DNA in the beach sand environment. The presence of viable but nonculturable indicator organisms in sediments could be a significant part of this population, and therefore the degree to which they are indicative of health risk, as well as the conditions that may resuscitate them in the environment, are both questions worthy of further investigation.

From a human health perspective, the sanitary quality of beach sand may be important because beach sand is a common interface of interaction between beachgoers and the marine environment. FIB in beach sands may or may not be correlated to human pathogen presence in sands, but our data suggest that monitoring programs designed to protect human health, as well as studies analyzing the fate and transport of FIB in coastal waters, should consider the potential role of sedimentary *Enterococcus* spp. populations.

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