Method repeatability for measuring *Enterococcus* in southern California beach sands

Yiping Cao, Charles D. McGee[1], John F. Griffith and Stephen B. Weisberg

Abstract

A recent study that evaluated 22 methods for enumerating fecal indicator bacteria in sand recommended standardization to a preferred method, but all researchers involved in that study had extensive experience in processing sand samples. The purpose of the present study was to evaluate how well the recommended method can be transferred to laboratories without such experience. Eight southern California laboratories that rarely measure bacteria in sand processed six sand and three water samples in replicate to assess repeatability. Among-laboratory variability was found to be less than within-laboratory variability, with no significant differences in results among any of the laboratories. Moreover, within-laboratory variability was comparable between the sand and water samples, indicating that the elution procedure added little additional method error even when performed by laboratories without prior experience. The simple extraction method for enumerating *Enterococcus* in beach sands was easily transferable to, and repeatable among, laboratories with little or no prior experience. Demonstrated success of technology transfer will further method standardization and adoption, aiding in understanding of how sands affect surface water quality.

Introduction

Beach sand has been found to harbor fecal indicator bacteria (FIB) (Alm *et al.* 2003, Lee *et al.* 2006, Beversdorf *et al.* 2007), which has the potential to affect recreational health management. FIB reservoirs in the sand can affect FIB concentration in overlying waters (Whitman and Nevers 2003, Yamahara *et al.* 2007). Moreover, Whitman et al (2009) have shown the potential for sand bacteria to transfer hand to mouth and Heaney *et al.* (2009) have found elevated rates of gastrointestinal illness for children playing in sand.

There have been a number of studies quantifying bacterial concentrations in the sand, but these have been performed using a variety of methods for removing bacteria from sand, limiting comparability among studies. To address this issue, Boehm *et al.* (2009) compared results from 22 methods for enumerating bacteria in beach sand, including hand-shaking, sonication, mechanical shakers and sophisticated buffers. They found that many of the methods produced comparable results, both in terms of bacterial recovery and repeatability among replicates. They endorsed hand-shaking for two minutes as the preferred method because it is less complex and requires no specialized equipment.

While Boehm *et al.* (2009) was a positive step toward method standardization, the study was conducted by researchers who had extensive

[1] *Orange County Sanitation District, Fountain Valley, CA*

experience processing sand samples. It is unclear whether the suggested method is easily transferable and repeatable among microbiology laboratories whose previous experience was limited to processing water samples. Here we trained eight such laboratories in use of the Boehm *et al*. method for enumerating *Enterococcus* in sand and compared method repeatability between water and sand samples processed by these laboratories.

Methods

Six sand samples and three water samples were provided to eight laboratories that conduct the majority of beach water quality monitoring in southern California, six of which had not processed sand samples prior to this study (Table 1). All laboratories received a 20-minute method demonstration, a brief written operational protocol, and a standard reporting sheet prior to participation in the study.

Water samples were created by inoculating offshore seawater (<2 *Enterococcus* per 100 ml) with a laboratory culture of *Enterococcus faecalis* (ATCC 29212) at three different concentrations (275, 125, 30 CFU ml^{-1}). Following inoculation, water samples were mixed on a stir plate (60 rev $minute^{-1}$, 20 minutes) prior to distributing approximately 200 ml into sterile Nalgene wide mouth bottles. Each laboratory was given one bottle per sample randomly. *Enterococcus* enumeration was conducted on triplicate subsamples from each bottle by each laboratory according to USEPA Method 1600 (USEPA 2002).

Sand samples were obtained from six typical southern California beaches and coded A through F: Doheny State Beach (33.462N, -117.680W, Dana point, CA), Poche Beach (33.440N, -117.644W, San Clemente, CA), Pacific Beach Point (32.808N, -117.265W, San Diego, CA), Baby Beach (33.462N, -117.704W, Dana point, CA), Cabrillo Beach (33.719N, -118.277W, San Pedro, CA), and Newport Dunes (33.615N, -117.891W, Newport Beach, CA), respectively. Samples were collected several days prior to the study to ensure they contained measurable levels of *Enterococcus* and then stored at 4°C until ready for use in the study. Samples with low *Enterococcus* (Sand C and E, <1 CFU g^{-1}) concentration were augmented with slurry of sea gull feces (Doheny State Beach, Dana point, CA) and allowed to incubate for 1 - 2 days at room temperature in the dark. Before distribution to the participating laboratories, all samples were stirred for 10 minutes at low speed with an industrial grade food service mixer with sanitized paddles (Model 8140, Anvil, Fletcher, NC) to homogenize the sample. After homogenization, subsamples of approximately 100 g each were placed into ziploc bags and randomly distributed to each laboratory.

Laboratories processed duplicate or triplicate subsamples (10 g each) from each sand sample following the method described by Boehm *et al.* (2009), which involves placing 10 g of sand into a pre-sterilized 250 ml polypropylene bottle, adding 60 ml of phosphate buffered saline (PBS, prepared as per USEPA 2002) and shaking for 2 minutes by hand over an arc of about 10 cm. Following a 30-second settling time, the eluant was decanted into a sterile bottle by pouring, taking care to leave the

Table 1. Laboratories participating in the study. Laboratories with asterisks had previous experience using the sand measurement method.

Lab ID	Lab Full Name	Location
Asso	Associated Laboratories	Orange, CA
CSD	City of San Diego	San Diego, CA
EWA	Encina Wastewater Authority	Encina, CA
LACSD	Los Angeles County Sanitation District	Whittier, CA
LAEMD	Los Angeles City Public Works Environmental Monitoring Division	Los Angeles, CA
OCPHL	Orange County Public Health Laboratory*	Newport Beach, CA
OCSD	Orange County Sanitation District*	Fountain Valley, CA
VPHL	Ventura County Public Health Laboratory	Ventura, CA

sand behind. An additional 40 ml of PBS was then added to the sand, the bottle gently swirled for 10 seconds, allowed to settle for 30 seconds, and then poured into the same sterile bottle used after the first rinse step. The combined eluant from the two rinse steps was then filtered per USEPA Method 1600 for *Enterococcus* enumeration (USEPA 2002) also used for the water samples. Three different volumes, depending on the sample, were filtered to obtain a countable plate.

Water content of sand samples was determined by drying at 105°C for 16 hours. Particle size distribution was determined using four sieves with pore size of 5 mm, 2 mm, 0.25 mm, and 0.063 mm after drying 50 g of sand at 105°C for 16 hours and cooling in a desiccator. Weight of sand particles from each size fraction was divided by the total dry weight of all size fractions to obtain percentage of sand mass in each size category (Table 2).

The three water samples and the first three sand samples were processed on November 19, 2008. The second set of three sand samples was processed on March 17, 2009. Study participants picked up samples from the Orange County Sanitation District Laboratory in Fountain Valley at 10:00 a.m. and transported samples back to their laboratories on ice. All participants were asked to begin processing at noon to avoid any differences in holding time among samples.

Comparison of method repeatability between- and within-laboratory was conducted, for sand and water separately, using the variance components estimation procedure in SAS (SAS Institute Inc., Cary, USA). *Enterococcus* concentrations were $\log_{10}$-transformed before analysis and results (four data points) below detection limit were set to detection limit. To compare method repeatability between sand and water, coefficients of variation (CV) were calculated for each lab for each sand and water sample. Because CV is by definition not normally distributed, a non-parametric approach was used for the comparison: the CVs were ranked and the ranks compared using the GLM procedure in SAS.

Results

Average *Enterococcus* concentrations (of replicates within each laboratory) for the six sand samples ranged from 1.1 to 5.1 log CFU g^{-1} dry weight of sand (Table 3). Standard deviations of the concentrations for each sample across all labs ranged from 0.08 to 0.30 log CFU g^{-1} dry weight. Standard deviation for replicates within each lab ranged from 0.0 to 0.29 log CFU g^{-1} dry weight.

Average concentrations (of replicates within each laboratory) for the three water samples ranged from 1.5 to 2.4 log CFU per 100 ml (Table 3). Standard deviations of the concentrations for each sample across all labs ranged from 0.09 to 0.16 log CFU per 100 ml. Standard deviation for each sample within each lab ranged from 0.0 to 0.31 log CFU per 100 ml.

Among-lab variability was smaller than within-lab variability for both sand and water (Table 4). For sand, the variance estimate for the between-lab variability was 0.006 compared to 0.010 for the within-lab variability. For water, the between laboratory variability was negligible compared to variability within the laboratories.

Table 2. Sand characteristics. For particle size distribution, % indicates the percentage of sand mass in each size category.

			Particle Size (mm) Category				
Sand	Color	Odor	>5	2 - 5	0.25 - 2	0.063 - 0.25	<0.063
Sand A	Medium brown	none	0.00%	1.50%	64.30%	30.90%	3.30%
Sand B	Medium brown	none	0.60%	1.90%	84.30%	11.40%	1.70%
Sand C	Black	sulfur	0.00%	0.00%	12.00%	83.70%	4.20%
Sand D	Grey brown	none	0.30%	0.10%	9.40%	87.60%	2.60%
Sand E	Medium brown	none	0.00%	0.30%	78.50%	19.20%	2.10%
Sand F	Dark Brown	none	3.70%	2.40%	49.10%	42.30%	2.50%

Table 3. *Enterococcus* concentrations (and standard deviations in parentheses) in the sand (log CFU g^{-1} dry sand) and water (log CFU per 100 ml) samples.

Lab	Sand						Water		
	Sand A	Sand B	Sand C	Sand D	Sand E	Sand F	Water 1	Water 2	Water 3
Asso	5.2 (0.1)	4.9 (0.0)	1.4 (0.1)	3.5 (0.0)	1.8 (0.1)	1.4 (0.1)	2.4 (0.0)	2.1 (0.1)	1.7 (0.1)
CSD	5.2 (0.1)	4.7 (0.1)	1.0 (0.0)	3.5 (0.1)	1.7 (0.1)	1.2 (0.1)	2.4 (0.1)	2.2 (0.2)	1.5 (0.1)
EWA	4.9 (0.0)	4.5 (0.1)	1.2 (0.1)	3.5 (0.1)	1.6 (0.1)	1.3 (0.1)	2.4 (0.2)	2.0 (0.2)	1.5 (0.1)
LACSD	5.1 (0.2)	4.4 (0.2)	1.3 (0.2)	3.4 (0.1)	1.7 (0.0)	1.4 (0.2)	2.5 (0.1)	2.1 (0.2)	1.5 (0.1)
LAEMD	5.3 (0.2)	4.8 (0.1)	1.2 (0.0)	3.5 (0.1)	1.7 (0.0)	1.5 (0.1)	2.4 (0.1)	2.1 (0.1)	1.4 (0.1)
OCPHL	5.1 (0.1)	4.6 (0.1)	0.9 (0.1)	3.5 (0.1)	1.7 (0.0)	0.7 (0.0)[a]	2.5 (0.1)	2.0 (0.3)	1.4 (0.1)
OCSD	5.2 (0.1)	4.7 (0.1)	0.7 (0.1)	3.5 (0.0)	1.7 (0.1)	1.1(0.0)[a]	2.5 (0.1)	2.1 (0.3)	1.5 (0.1)
VPHL	5.1 (0.1)	4.6 (0.1)	0.9 (0.1)	3.7 (0.1)	1.7 (0.1)	0.8 (0.3)	2.4 (0.1)	2.2 (0.0)	1.4 (0.3)
All	5.1 (0.2)	4.6 (0.2)	1.1 (0.2)	3.5 (0.1)	1.7 (0.1)	1.1 (0.3)	2.4 (0.1)	2.1 (0.2)	1.5 (0.1)

[a] Data below detection limits and reported as detection limits

Within-lab variability for sand measurements was smaller than that for water measurements. The coefficient of variation (CV) ranged from 0.022 to 0.097 with a median of 0.033 when the labs processed sand samples; the CV ranged from 0.021 to 0.083 with a median of 0.063 when the labs processed water samples. GLM analysis of CV ranks indicated that CV for water measurements was significantly higher than that for sand measurements (p value = 0.0125).

Discussion

Lack of prior experience with the sand method recommended by Boehm *et al*. (2009) was not an impediment to its successful implementation. Six of the labs had never used the method previously, yet there was no difference in the mean or variance from the two labs that had previous experience with the method. While it is difficult to conduct a direct comparison because different sands were tested, we also found that the coefficients of variation in this study were comparable to that of Boehm *et al*. (2009) when the method experts employed the method (p value = 0.85, GLM analysis of ranks of CVs).

Processing of the sand sample includes several additional elution steps before yielding a water sample for *Enterococcus* enumeration, yet we found no additional variability for the sand method compared to the water method. This is consistent with Boehm *et al.* (2009), who found that the elution method was robust to minor operational deviations. For instance, they found that shaking intensity (hand shaking vs. mechanical shaking) or variation in settling time (30, 180, or 600 seconds) did not have a significant effect on the resulting *Enterococcus* concentrations. While different laboratories may differ slightly in how they perform these additional steps to process sand samples, the method appears robust to such operational deviations.

The present study expanded application of the method by studying fine sands (0.05 - 0.25 mm), whereas Boehm et al. (2009) focused on medium-to-coarse sands. However, the method has yet to be tested on sediments with high silt-clay (<50 µm) or organic content. Silt has the potential to affect colony formation and enumeration on mEI agar (Boehm *et al.* 2009), and to increase sediment organic content due to preferential absorption of organic matter to fine particles (Hedges and Keil 1995). While bacteria in sands or sediments may exist in several forms (Marshall 1999): free "floating" in the interstitial space, reversible attachment to particles via physicochemical forces, or irreversible attachment as biofilm via biological exopolymeric substance, high organic content promotes biofilm formation (Costerton *et al.* 1994) that leads to patchy distribution and measurement variability (Parkin 1987).

It is also important to recognize that high method repeatability in the laboratory procedures does not

guarantee low variability in field collections. Whereas we used a commercial food mixer to homogenize our samples, spatial variability in the field may be related to frequency of tidal inundation and/or beach usage. Therefore it is important to consider field replicates and sampling variability when applying this method to study *Enterococcus* in sands.

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