Fecal indicator bacteria (FIB) enumeration in beach sand: A comparison study of FIB extraction methods in medium to coarse sands

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Abstract

The absence of standardized methods for quantifying fecal indicator bacteria (FIB) in sand hinders comparison of results across studies. This study aimed to compare methods for extraction of fecal bacteria from sands and recommend a standardized extraction technique. Twenty-two methods for extracting enterococci and *Escherichia coli* from sand were evaluated, including multiple permutations of hand shaking, mechanical shaking, blending, sonication, number of rinses, settling time, eluant to sand ratio, eluant composition, prefiltration, and type of decantation. Tests were performed on sands from California, Florida, and Lake Michigan. Most extraction parameters did not significantly affect bacterial enumeration. ANOVA revealed significant effects of eluant composition and blending, with both sodium metaphosphate buffer and blending producing reduced counts. The simplest extraction method that produced the highest FIB recoveries consisted of 2 minutes of hand shaking, a 30-second settling time, 1 rinse step, and a 10:1 eluant volume to sand weight ratio. This result was consistent across the sand compositions tested in this study, but could vary for other sand types. Method standardization will improve the understanding of how sands affect surface water quality.

Introduction

A number of studies have recognized beach sand as a potentially large reservoir of fecal indicator bacteria (Whitman and Nevers 2003, Alm *et al*. 2003, Lee *et al*. 2006, Beversdorf *et al*. 2007, Yamahara *et al*. 2007). The numbers of FIB in sand can exceed those in the adjacent beach water on a per mass basis, often by orders of magnitude. Concentrations of enterococci (ENT) indicator bacteria have been reported to reach levels over 70 colony forming units (CFU)/g in California and Florida. Concentrations of *E. coli* (EC) indicator bacteria have been found to reach over 2000 CFU/g in Florida dry sand, and 10^5 CFU/g in foreshore sand at a Lake Ontario freshwater beach (Shibata *et al*. 2004, Edge and Hill 2007, Yamahara *et al*. 2007, Goodwin *et al*. 2009).

Fecal indicator bacteria density is used widely to make water quality decisions at beaches, and it is unclear whether their presence in sand is indicative

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of increased human health risks. One complicating factor in understanding their significance is the absence of a widely accepted method for FIB extraction in sand. Published methods range from simply shaking the sample by hand to carrying out complex protocols that involve use of sonication, mechanical shakers, and sophisticated buffers. Methods based on shaking are the most frequently used, but even shaking methods vary in duration/type of shaking, type of eluant, mass of sand used, and volume of eluant.

There have been few studies that compare these method variations, providing little basis for selecting a method or determining if data from different studies are comparable. In the present study, multiple parameters within previously published methods are compared to identify which method combinations (e.g., prefiltration, shaking duration and type, sonication, blending, settling time, volume and type of eluant, number of rinses) produce the highest recovery of ENT and EC.

Methods

Three mixing techniques (shaking, blending, and sonication) were compared by simultaneous application to a common set of sand samples. The following parameters were varied within the shaking technique: type of shaking (hand versus mechanical), shaking duration (1 and 2 minutes), number of rinses (1, 2, and 3), settling time (30, 180, and 600 seconds), eluant to sand ratio (100 ml to 3, 10, or 50 g), eluant composition (phosphate buffered saline (PBS), PBS+ Tween 80, de-ionized water (DI), sodium metaphosphate (DI + $(NaPO_3)_6$), or filtered ambient water), pre-filtration of eluant through a 30-µm net filter, and decantation method (pouring or pipetting). These variations yielded a total of 22 method permutations, hereafter referred to as treatments (Table 1).

Each of the 22 treatments was applied to 3 beach sand samples, hereafter referred to as Sands 1, 2, and 3. Sand 1 was from Doheny Beach, California, USA (33°27'41.35"N, 117°41'2.26"W), a marine beach with fine textured siliceous sand (mean diameter 0.22 mm, moisture content 18%, and organic carbon 0.71%). Sand 2 was from Hobie Cat Beach, Florida, USA (25°44'45.06"N, 80°11'50.06"W), also a marine beach but with coarse calcareous sand (mean diameter 0.77 mm, moisture content 10%, and organic carbon 0.70%). Sand 3 was from a freshwater beach on Lake Michigan, Michigan City, Indiana, USA (41°43'38.16"N, 86°53'39.19"W) and consisted of coarse siliceous sand (mean diameter 0.91 mm, moisture content 12%, and organic carbon 0.25%). Each sample was collected aseptically from the top 2 cm of fore- or backshore sand, except for Sand 3 which was collected to a depth of 10 cm. Out of state sand samples were shipped overnight in a cooler containing ice packs to the Orange County Sanitation District Laboratory, Fountain Valley, California, USA.

Upon arrival at the laboratory, sand samples were homogenized at low speed for a period of 10 minutes using an industrial grade food service mixer (Model 8140, Anvil, Fletcher, NC) with sanitized paddles. After mixing, samples were aseptically transferred into containers and distributed to analysts. Hereafter, "analyst" refers to the researcher processing the sand samples; the term does not refer to the technician performing the microbial enumeration protocols discussed below.

For each Sand, 6 analysts participated in implementing the 22 treatments. Each treatment was processed in duplicate by two analysts, allowing evaluation of both within and between analyst variability. In addition, all analysts performed sample method T1 (Table 1) to further evaluate within-analyst variability. A single sand sample was processed on three separate days, with all analysts beginning processing at the same time.

The first treatment (T1) was the base method that involved placing 10 g of sand into a pre-sterilized 250-ml polypropylene bottle, adding 60 ml of phosphate buffered saline (PBS) eluant and shaking for 2 minutes by hand over an arc of approximately 10 cm. Following a 30 second settling time, the eluant was decanted into a second sterile bottle by pouring, taking care to leave the 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. In summary, this method used 100 ml total of eluant in 2 rinse steps, and a 30 second settling time after each rinse.

Treatments T2 through T9 completed a 3 x 3 factorial design that involved varying two factors: shaking duration/type and mass of sand. Sand masses evaluated included 3, 10, and 50 g, which were selected to cover the range of sand masses used in most previously published studies (Baums *et al*.

Table 1. Treatments applied to each sand sample.

Treatment	Mass (g)	Shaking (minutes)	Settling Time (seconds)	Number of Rinses	Eluant	Prefiltration	Eluant Removal
1	10	2	30	2	PBS	no	decant
2	3	1	30	2	PBS	no	decant
3	10	1	30	2	PBS	no	decant
4	50	1	30	2	PBS	no	decant
5	3	2	30	2	PBS	no	decant
6	50	2	30	2	PBS	no	decant
7	3	2 (mechanical)	30	2	PBS	no	decant
8	10	2 (mechanical)	30	2	PBS	no	decant
9	50	2 (mechanical)	30	2	PBS	no	decant
10	10	2	30	2	PBS + Tween 80	no	decant
11	10	2	30	2	DI + $(NaPO_3)_6$	no	decant
12	10	2	30	2	Sea / Lake Water	no	decant
13	10	2	30	2	DI Water	no	decant
14	10	2	180	2	PBS	no	decant
15	10	2	600	2	PBS	no	decant
16	10	2	30	1	PBS	no	decant
17	10	2	30	3	PBS	no	decant
18	3	2	30	2	PBS	yes	decant
19	10	2	30	2	PBS	no	pipette
20	10	1 (blending)	30	2	PBS + Tween 80	no	decant
21	10	1	30	2	PBS + Tween 80	no	decant
22	10	0.5 (sonication)	600	1	DI + $(NaPO_3)_6$	no	decant

2007, Bonilla *et al*. 2007). Shaking duration/type combinations evaluated included one minute of hand shaking, two minutes of hand shaking, and two minutes of mechanical mixing. Samples subjected to mechanical shaking were weighed within Erlenmeyer flasks, sterile eluant added, and then the flasks were sealed and subjected to mechanical shaking using a Burrell Model CC Wrist-Action® shaker set at maximum speed (Burrell Scientific, Pittsburg, PA).

Treatments T10 through T13 varied the eluant type, keeping other parameters the same as the T1 base method. The eluants, in addition to the base eluant of PBS, included PBS + 0.1% Tween 80, sodium metaphosphate (DI + 1% $(NaPO_3)_6$; Fisher #S333-500), filtered ambient water (0.2-µm pore size membrane filtered ambient seawater for Sands 1 and 2 and ambient lake water for Sand 3), and DI. The pH and salinities of the eluants are provided in Table 2.

Treatments T14 and T15 were designed to evaluate the effects of settling time (180 and 600 seconds vs. 30 seconds in the T1 base method). Treatments T16 and T17 were designed to evaluate the effect one and three rinses compared to the T1 base method that uses two rinse steps. Treatment T16 used 100 ml of eluant in a single rinse step, and T17 used a total of 100 ml eluant in three rinse steps of 40, 30, and 30 ml. A 30 second settling time was applied to all rinse steps in T16. Treatment T19 was a modification of the base method T1 to determine if a serological pipette was more effective at removing eluant from the shaking bottle than pouring the eluant into the final container.

Treatment T18 assessed the prefiltration step described by Solo-Gabriele *et al*. (2000). Sixty ml of PBS was placed into a sterile container with 3 g of sand, the mixture shaken for 2 minutes and then the entire contents were passed through a sterilized 30-µm pore size nylon net filter (Type NY30, Millipore, Bedford, MA) into a sterile side-arm flask. An additional 40 ml of PBS was placed into the original container and swirled to gather the remaining sediment. The contents were filtered through the same 30-µm filter, captured in the sterile side-arm flask, and decanted into a second sterile bottle.

Blending (T20) followed a slightly modified version of the protocol described by Edge and Hill (2007). This modification consisted of combining 10 g of sand with 100 ml of PBS + 0.1% Tween 80 and 1 drop of anti-foaming agent (Sigma-Aldrich, St. Louis, MO) in a Model MC-3, 250-ml mini blending container mounted on a Model 70115 blender (Waring, Torrington, CT). The mixture was blended for one minute at maximum speed. The material was allowed to settle for 30 seconds, then the supernatant was decanted into a second sterile bottle. Treatment T21 was used as a comparison to T20; T21 consisted of the base method with one minute hand shaking in PBS + 0.1% Tween 80.

Sonication (T22) followed the method described in Ferguson *et al*. (2005). Ten g of sand were combined with 100 ml of DI + 1% $(NaPO_3)_6$ and sonicated at 30% output for 30 seconds using a Branson Sonifier® Cell Disruptor 450 (Danbury, CT). The material was allowed to settle for 600 seconds, then the supernatant was decanted into a second sterile bottle.

Two different PBS solutions were utilized in the study: a) PBS from PML Microbiologicals (PBS-PML; VWR#29452-140, Wilsonville, OR) that consisted of 4.25% w/v potassium dihydrogen phosphate and 0.05% w/v of magnesium chloride, and b) PBS prepared in the laboratory (PBS-IN) that consisted of 8.5% w/v potassium dihydrogen phosphate and 19% w/v magnesium chloride. For Sands 1 and 2, PBS-PML was used in all treatments requiring PBS. For Sand 3, PBS-PML was used in all treatments requiring PBS except the treatments using PBS + 0.1% Tween 80, which used PBS-IN.

Table 2. Eluant pH and salinity. pH and salinity were measured by an Orion portable pH meter model 290A and an Orion conductivity meter model 125 (Orion Research, Inc., Boston, MA), respectively, according to manufacturer's protocols. Salinity is reported on a practical salinity scale.

Eluant	pH	Salinity
PBS[a]	6.8	0.1
PBS+0.1% Tween 80 (A)[b]	6.8	0.1
PBS+0.1% Tween 80 (B)[c]	7.4	0.3
Filtered seawater (Doheny)	8.3	33.8
Filtered seawater (Hobie Cat)	8.6	36.8
Filtered Lake Michigan water	8.3	0.2
DI water	7.0	7.0
DI + 1% $(NaPO_3)_6$	6.6	2.8

[a] PBS-PML
[b] Used for Sands 1 and 2
[c] Used for Sand 3

Following the various extraction treatments, the eluant was processed using standard methods for FIB enumeration. Enterococci were enumerated by both membrane filtration (ENT-MF) on mEI agar (Method 1600; USEPA 2002) and by the Enterolert defined substrate assay (ENT-DS; (IDEXX, Westbrook, MN; USEPA 2003). *E. coli* were enumerated by the Colilert-18 defined substrate assay (EC; IDEXX, Westbrook, MN; USEPA 2003). Water content of Sands was determined by drying sand at 105°C for 24 hours. Concentrations of FIB are reported as colony forming units (CFU) or most probable number (MPN) per g dry weight of sand for samples processed by membrane filtration or IDEXX, respectively. A single set of experienced technicians from one laboratory carried out these analyses for all samples to eliminate potential confounding of elution and processing variability.

For treatments T4 and T18 (which compared the effect of prefiltration), an additional replicate was processed by one of the two analysts; this eluant was stored at 4°C, then analyzed for suspended solids using laser *in situ* scattering and transmissometry (LISST-100X, Sequoia Scientific, Inc., Bellevue, WA).

Concentrations were $\log_{10}$ transformed for statistical analysis, with concentrations below and above detection limit set to the detection limits. For ENT-MF, 4% of analyses were below the detection limit and 8% were above. For ENT-DS, 8% were below the detection limit and 0 above. For EC, 13% were below the detection limit and 0 above. For each assay, n was 286).

Type III analyses of variance (ANOVA) were used to compare sand extraction treatments, with analyst and interaction terms included as factors and FIB concentration as the quantifiable variable. Post-hoc analyses compared treatment factors pairwise using the Tamhane's T2 test, because the Levene's test indicated that unequal variance between treatments was typical. Post-hoc pairwise comparisons are only possible for factors with three or more levels. Only differences that were significantly different at $p < 0.05$ are discussed. Paired t-tests were used to examine differences in ENT-MF, ENT-DS, and EC-DS within Sands. All analyses were carried out with SPSS (v16.0 for Mac, Chicago, Illinois).

Results

Comparison of EC and ENT among Sands

EC and ENT varied significantly between sands ($p < 0.05$) when results from all treatments were examined in aggregate. Sand quality was ranked based on EC and ENT concentrations, with the highest ranking corresponding to the highest concentration of FIB. Based on EC, Sand quality was ranked as 3>2>1. Ranking based on ENT was not equivalent, with Sand quality ranked as 1>2>3. ENT-MF and ENT-DS provided identical rankings. However, ENT-MF yielded significantly higher mean concentrations than ENT-DS for all three Sands (paired t-test, Sand 1: 0.04 log unit higher, $t = 2.52$, $df = 95$, $p < 0.05$; Sand 2: 0.4 log unit higher, $t = 10.18$, $df = 95$, $p < 0.05$; Sand 3: 0.3 log unit higher, $t = 8.81$, $df = 93$, $p < 0.05$). Using ENT-DS as a proxy for ENT, the concentration of ENT was significantly higher than EC in Sands 1 and 3 (paired t-test, Sand 1: 1.7 log unit higher, $t = 38.5$, $df = 95$; and Sand 3: 0.7 log unit higher, $t = 17.5$, $df = 93$, $p < 0.05$). In contrast, mean ENT concentrations were significantly lower than EC concentrations in Sand 2, but by only 0.1 log unit ($t = 3.57$, $df = 95$, $p < 0.05$). The log-mean FIB concentrations and the standard deviations are shown in Table 3 for each individual treatment (T1 through T22) and all treatments in aggregate (ALL).

Effect of Analyst

Each of six analysts preformed T1 in duplicate for each Sand. Using just this treatment, the variability between and within analyst was evaluated for each indicator analysis (i.e., EC, ENT-MF, ENT-DS). This required nine ANOVAs (three sands x three indicator analyses). There was no analyst effect for Sand 2. For Sand 1, there was an analyst effect for EC ($F = 4.54$, $df = 5$, $p < 0.05$). For Sand 3, there was an analyst effect for ENT-MF ($F = 21.0$, $df = 5$, $p < 0.05$). In both cases where an analyst effect was detected by ANOVA, the post-hoc pairwise comparisons indicated no significant pairwise differences. Based on these results, no single analyst was removed from the analysis; instead, analyst was included as a factor in the ANOVAs described below.

Treatment Comparisons

Comparisons were made between subsets of sand extraction treatments to test whether specific alterations to the base method (T1) significantly increased or decreased the concentration of ENT and

Table 3. Log-mean and standard deviation (in parentheses) of enterococci enumerated using membrane filtration (ENT-MF), enterococci enumerated with defined substrate (ENT-DS), and *E. coli* in the 22 treatments (T) and all treatments in aggregate (ALL). Sand 1 = Doheny Beach; Sand 2 = Hobie Cat Beach; and Sand 3 = a Michigan City beach.

	Sand 1			Sand 2			Sand 3		
T	ENT-MF log CFU/g	ENT-DS log MPN/g	EC log MPN/g	ENT-MF log CFU/g	ENT-DS log MPN/g	EC log MPN/g	ENT-MF log CFU/g	ENT-DS log MPN/g	EC log MPN/g
1	3.5 (0.1)	3.5 (0.1)	1.7 (0.1)	2.0 (0.1)	1.5 (0.1)	1.7 (0.1)	1.7 (0.2)	1.1 (0.2)	1.9 (0.2)
2	3.4 (0.0)	3.4 (0.1)	1.6 (0.0)	2.1 (0.2)	1.9 (0.4)	1.8 (0.1)	1.6 (0.1)	0.9 (0.3)	1.2 (0.7)
3	3.5 (0.1)	3.5 (0.0)	1.3 (0.2)	2.1 (0.1)	1.6 (0.4)	1.8 (0.1)	1.6 (0.1)	0.9 (0.6)	1.4 (0.9)
4	3.4 (0.0)	3.5 (0.0)	1.8 (0.1)	1.8 (0.1)	1.3 (0.1)	1.8 (0.1)	1.5 (0.1)	1.0 (0.5)	1.6 (0.4)
5	3.5 (0.0)	3.4 (0.1)	1.7 (0.2)	2.0 (0.1)	1.7 (0.2)	1.7 (0.0)	1.6 (0.0)	1.1 (0.5)	1.9 (0.2)
6	3.5 (0.0)	3.4 (0.1)	1.8 (0.1)	2.1 (0.0)	1.4 (0.1)	1.9 (0.1)	1.3 (0.2)	1.2 (0.3)	1.7 (0.5)
7	3.5 (0.1)	3.4 (0.0)	1.7 (0.1)	2.0 (0.1)	1.7 (0.4)	1.7 (0.2)	2.1 (0.8)	1.6 (1.0)	2.4 (0.8)
8	3.6 (0.1)	3.4 (0.0)	1.7 (0.2)	2.0 (0.1)	1.6 (0.1)	1.8 (0.1)	1.3 (0.2)	0.7 (0.3)	1.7 (0.2)
9	3.4 (0.1)	3.4 (0.1)	1.8 (0.2)	1.9 (0.1)	1.5 (0.2)	1.7 (0.1)	1.4 (0.6)	1.0 (0.4)	1.7 (0.6)
10	3.4 (0.2)	3.5 (0.1)	1.9 (0.0)	1.8 (0.3)	1.6 (0.1)	1.8 (0.0)	1.6 (0.0)	1.5 (0.0)	1.9 (0.0)
11	3.5 (0.2)	3.5 (0.1)	1.1 (0.0)	2.1 (0.0)	1.7 (0.2)	1.6 (0.1)	1.6 (0.1)	1.1 (0.1)	1.8 (0.0)
12	3.4 (0.0)	3.4 (0.1)	2.0 (0.1)	2.1 (0.1)	2.0 (0.5)	1.7 (0.1)	1.6 (0.1)	1.3 (0.1)	1.8 (0.1)
13	3.5 (0.2)	3.5 (0.1)	1.8 (0.1)	2.1 (0.1)	1.6 (0.1)	1.7 (0.1)	1.6 (0.1)	1.3 (0.1)	1.9 (0.1)
14	3.5 (0.0)	3.4 (0.0)	1.8 (0.1)	1.9 (0.3)	1.6 (0.3)	1.7 (0.1)	1.6 (0.0)	1.3 (0.2)	1.8 (0.0)
15	3.5 (0.1)	3.5 (0.1)	1.8 (0.3)	2.1 (0.1)	1.7 (0.4)	1.7 (0.0)	1.5 (0.1)	1.3 (0.1)	1.9 (0.1)
16	3.6 (0.1)	3.5 (0.1)	1.8 (0.3)	2.1 (0.1)	1.9 (0.2)	1.7 (0.1)	1.6 (0.1)	1.4 (0.1)	1.9 (0.1)
17	3.5 (0.1)	3.5 (0.1)	1.6 (0.2)	2.0 (0.1)	1.4 (0.0)	1.8 (0.1)	1.5 (0.1)	1.3 (0.1)	1.9 (0.1)
18	3.7 (0.4)	3.4 (0.0)	1.9 (0.3)	2.1 (0.1)	1.9 (0.1)	1.8 (0.1)	1.6 (0.1)	1.2 (0.2)	1.8 (0.1)
19	3.5 (0.0)	3.6 (0.1)	1.6 (0.2)	2.0 (0.0)	1.7 (0.2)	1.8 (0.1)	1.6 (0.1)	1.4 (0.0)	1.9 (0.2)
20	2.4 (1.2)	2.3 (1.2)	1.7 (0.2)	1.3 (0.9)	1.6 (0.2)	1.7 (0.1)	-0.3 (0.0)	0.2 (0.3)	1.8 (0.3)
21	3.6 (0.2)	3.5 (0.1)	1.6 (0.2)	2.0 (0.1)	1.7 (0.1)	1.8 (0.0)	1.7 (0.2)	1.5 (0.1)	1.9 (0.0)
22	3.6 (0.1)	3.6 (0.1)	1.1 (0.0)	2.1 (0.1)	1.6 (0.0)	1.5 (0.2)	1.7 (0.1)	1.1 (0.2)	1.9 (0.1)
ALL	**3.5 (0.3)**	**3.4 (0.3)**	**1.7 (0.3)**	**2.0 (0.3)**	**1.6 (0.3)**	**1.7 (0.1)**	**1.5 (0.5)**	**1.1 (0.5)**	**1.8 (0.4)**

Table 4. Summary table of ANOVAs for ENT-MF. The experiment is given in the first column and factors in the second. F statistic (*F*), degrees of freedom (*df*), and *p* values are given for each sand. The error term is omitted for simplicity. In the case of the last experiment (effect of mass/shaking duration/type) where a 3 way ANOVA was used, 'n/a' indicates that factor was not relevant for the model. Bolded values with * indicate significant factors.

Experiment	Factor(s)	Sand 1 *F, df, p*	Sand 2 *F, df, p*	*Sand 3* *F, df, p*
Rinse	Number of Rinses	1.37, 2, 0.32	0.32, 2, 0.74	2.49, 2, 0.16
	Analyst	0.057, 1, 0.82	0.056, 1, 0.82	1.20, 1, 0.32
	Analyst x Number of Rinses	0.29, 2, 0.76	3.98, 2, 0.079	0.23, 2, 0.80
Decanting	Pipette or Pour	0.38, 1, 0.57	1.01, 1, 0.37	0.02, 1, 0.89
	Analyst	6.91, 1, 0.06	0.89, 1, 0.40	0.60, 1, 0.50
	Analyst x Pipette/Pour	1.48, 1, 0.29	0.01, 1, 0.93	n/a
Settling	Settling Time	1.09, 2, 0.39	1.09, 2, 0.40	2.85, 2, 0.14
	Analyst	1.49, 1, 0.27	0.31, 1, 0.60	1.95, 1, 0.21
	Analyst x Settling Time	1.43, 2, 0.31	0.26, 2, 0.78	0.32, 2, 0.74
Eluant	Eluant Composition	0.41, 4, 0.80	**4.97, 4, 0.02***	0.35, 4, 0.84
	Analyst	1.45, 1, 0.26	1.21, 1, 0.30	0.54, 1, 0.48
	Analyst x Eluant Composition	0.97, 4, 0.47	**5.21, 4, 0.02***	0.12, 4, 0.97
Sonication	Sonication	0.19, 1, 0.68	0.28, 1, 0.62	2.93, 1, 0.14
Prefiltration	Prefiltration	0.71, 1, 0.448	0.087, 1, 0.78	1.18, 1, 0.34
	Analyst	1.03, 1, 0.367	1.02, 1, 0.37	0.22, 1, 0.66
	Analyst x Prefiltration	0.74, 1, 0.44	2.21, 1, 0.21	0.90, 1, 0.78
Blending	Blending	**17.11, 1, 0.01***	3.71, 1, 0.13	**593, 1, <0.001***
	Analyst	**9.02, 1, 0.04***	2.79, 1, 0.17	1.22, 1, 0.33
	Analyst x Blending	**16.24, 1, 0.02***	2.13, 1, 0.22	1.13, 1, 0.35
Mass/Shaking	Analyst	0.27, 1, 0.61	0.35, 1, 0.56	3.31, 1, 0.09
	Mass	3.03, 2, 0.73	3.31, 2, 0.06	**5.66, 2, 0.01***
	Shaking	0.26, 2, 0.77	0.49, 2, 0.62	0.22, 2, 0.80
	Analyst x Mass	0.26, 2, 0.77	1.38, 2, 0.28	2.04, 2, 0.16
	Analyst x Shaking	0.32, 2, 0.73	1.56, 2, 0.24	3.01, 2, 0.08
	Mass x Shaking	1.47, 4, 0.25	**3.09, 4, 0.04***	**4.72,4, 0.009***
	Analyst x Mass x Shaking	0.63, 4, 0.65	2.11, 4, 0.12	**5.38, 4,0.005***

EC. The 22 treatments (Table 1) and the variability contributed by the analyst performing the extraction were evaluated by ANOVA for ENT-MF, ENT-DS, and EC. Results are detailed below and provided in Tables 4, 5, and 6.

Effect of sand mass and shaking method

A three-way ANOVA model investigated 1) sand mass, 2) shaking duration/type, 3) analyst, as well as the interactions of these parameters using T1 through T9 for each Sand.

The ANOVA model for ENT-MF produced different results for different Sands. The Sand 1 model revealed no significant factors or interaction terms. For the Sand 2 model, only the interaction term between mass of sand and method of shaking was significant. This result was driven by the fact that the 50-g sand sample produced higher concentrations when hand shaken for 2 minutes relative to other shaking methods. In contrast, the Sand 3 model showed a significant effect of mass, mass x shaking interaction, and a three way interaction between

Table 5. Summary table of ANOVAs for ENT-DS. The experiment is given in the first column and factors in the second. F statistic (*F*), degrees of freedom (*df*), and *p* values are given for each sand. The error term is omitted for simplicity. In the case of the last experiment (effect of mass/shaking duration/type) where a 3 way ANOVA was used, 'n/a' indicates that factor was not relevant for the model. Bolded values with * indicate significant factors.

Experiment	Factor(s)	Sand 1 *F, df, p*	Sand 2 *F, df, p*	Sand 3 *F, df, p*
Rinse	Number of Rinses	0.66, 2, 0.55	**8.85, 2, 0.02***	0.45, 2, 0.66
	Analyst	0.066, 1, 0.81	0, 1, 0.99	0.89, 1, 0.38
	Analyst x Number of Rinses	0.72, 2, 0.53	2.91, 2, 0.13	0.48, 2, 0.64
Decanting	Pipette or Pour	2.71, 1, 0.18	0.67, 1, 0.46	4.05, 1, 0.14
	Analyst	2.91, 1, 0.16	0.61, 1, 0.48	1.53, 1, 0.30
	Analyst x Pipette/Pour	0.15, 1, 0.72	0.40, 1, 0.56	n/a
Settling	Settling Time	3.36, 2, 0.10	0.47, 2, 0.65	1.02, 2, 0.41
	Analyst	1.60, 1, 0.25	1.36, 1, 0.29	**24.95,1,0.002***
	Analyst x Settling Time	0.60, 2, 0.58	1.06, 2, 0.40	**15.61,2,0.004***
Eluant	Eluant Composition	2.07, 4, 0.16	2.45, 4, 0.11	**8.86,4, 0.003***
	Analyst	0.36, 1, 0.56	0.31, 1, 0.59	0.26, 1, 0.64
	Analyst x Eluant Composition	0.88, 4, 0.51	**3.53, 4, 0.048***	0.84, 4, 0.53
Sonication	Sonication	0.54, 1, 0.49	1.71, 1, 0.24	0.16, 1, 0.70
Prefiltration	Prefiltration	0.40, 1, 0.56	**13.24, 1, 0.02***	2.08, 1, 0.22
	Analyst	0.01, 1, 0.94	**16.94, 1, 0.02***	0.004, 1, 0.95
	Analyst x Prefiltration	0.12, 1, 0.75	**62.93,1,0.001***	0.036, 1, 0.86
Blending	Blending	**64.47,1,0.001***	2.99, 1, 0.16	**77.4, 1, 0.001***
	Analyst	**53.85,1,0.002***	2.73, 1, 0.17	1.86, 1, 0.24
	Analyst x Blending	**52.91,1,0.002***	1.33, 1, 0.31	0.39, 1, 0.57
Mass/Shaking	Analyst	2.10, 1, 0.16	**4.93, 1, 0.039***	**17.58,1,0.001***
	Mass	2.48, 2, 0.11	**7.44, 2, 0.004***	2.89, 2, 0.081
	Shaking	0.97, 2, 0.40	0.33, 2, 0.72	0.63, 2, 0.54
	Analyst x Mass	0.23, 2, 0.80	2.67, 2, 0.10	1.36, 2, 0.28
	Analyst x Shaking	0.01, 2, 0.99	0.045, 2, 0.96	0.33, 2, 0.72
	Mass x Shaking	1.84, 4, 0.17	0.64, 4, 0.64	1.12, 4, 0.38
	Analyst x Mass x Shaking	0.64, 4, 0.64	2.05, 4, 0.13	1.91, 4, 0.15

mass, shaking method, and analyst. The Sand 3 results were driven by low concentrations obtained by one analyst from the 50-g sample using mechanical mixing.

The ANOVA analysis for ENT-DS revealed no significant factors for the Sand 1 model, but did find significant factors for the Sands 2 and 3 models. For Sand 2, sand mass and analyst were significant factors. In this case, 3 g of sand produced significantly higher ENT-DS than did 50 g (mean difference 0.4 log units, $p < 0.05$), and one of the two analysts produced higher concentrations (average 0.2 log unit) of ENT-DS than the other. For Sand 3, analyst was significant and this was driven by the results of one analyst that were 0.6 log unit (on average) higher than the other.

Table 6. Summary table of ANOVAs for EC. F statistic (*F*), degrees of freedom (*df*), and *p* values are given for each sand. The error term is omitted for simplicity. In the case of the last experiment (effect of mass/shaking duration/type) where a 3 way ANOVA was used, 'n/a' indicates that factor was not relevant for the model. Bolded values with * indicate significant factors.

Experiment	Factor(s)	Sand 1 *F, df, p*	Sand 2 *F, df, p*	Sand 3 *F, df, p*
Rinse	Number of Rinses	1.80, 2, 0.24	0.11, 2, 0.90	3.5, 2, 0.10
	Analyst	1.14, 1, 0.33	0.49, 1, 0.51	4.51, 1, 0.08
	Analyst x Number of Rinses	1.76, 2, 0.25	1.94, 2, 0.22	4.99, 2, 0.053
Decanting	Pipette or Pour	0.79, 1, 0.42	0.73, 1, 0.44	0.42, 1, 0.56
	Analyst	**9.50, 1, 0.04***	0.34, 1, 0.59	0.10, 1, 0.77
	Analyst x Pipette/Pour	0.54, 1, 0.50	0.01, 1, 0.92	n/a
Settling	Settling Time	**5.60, 2, 0.04***	0.19, 2, 0.83	**8.75, 2, 0.02***
	Analyst	**14.98,1,0.008***	0.33, 1, 0.58	4.07, 1, 0.09
	Analyst x Settling Time	2.18, 2, 0.19	0.64, 2, 0.56	2.93, 2, 0.13
Eluant	Eluant Composition	**26.75,4,<0.001***	1.02, 4, 0.44	**3.74, 4, 0.04***
	Analyst	3.93, 1, 0.76	0.41, 1, 0.54	1.14, 1, 0.31
	Analyst x Eluant Composition	2.62, 4, 0.99	0.42, 4, 0.79	0.69, 4, 0.61
Sonication	Sonication	0.60, 1, 0.47	1.96, 1, 0.21	4.98, 1, 0.07
Prefiltration	Prefiltration	0.98, 1, 0.38	7.66, 1, 0.05	0.94, 1, 0.39
	Analyst	0.12, 1, 0.74	0.15, 1, 0.72	0.82, 1, 0.42
	Analyst x Prefiltration	0.10, 1, 0.77	1.35, 1, 0.31	0.14, 1, 0.73
Blending	Blending	0.60, 1, 0.48	**17.64,1,0.01***	0.29, 1, 0.62
	Analyst	0.55, 1, 0.83	0.47, 1, 0.53	0.44, 1, 0.55
	Analyst x Blending	0.01, 1, 0.93	0.31, 1, 0.61	0.93, 1, 0.39
Mass/ Shaking	Analyst	0.75. 1, 0.40	2.52, 1, 0.13	**10.47,1,0.005***
	Mass	**7.00, 2, 0.006***	1.31, 2, 0.30	0.74, 2, 0.49
	Shaking	**7.74, 2, 0.004***	1.48, 2, 0.25	**3.74, 2, 0.04***
	Analyst x Mass	0.32, 2, 0.73	1.52, 2, 0.25	1.30, 2, 0.30
	Analyst x Shaking	1.91, 2, 0.18	0.44, 2, 0.65	0.64, 2, 0.54
	Mass x Shaking	**4.47, 4, 0.011***	2.01, 4, 0.14	1.64, 4, 0.21
	Analyst x Mass x Shaking	0.40, 4, 0.80	0.61, 4, 0.66	1.47, 4, 0.25

For EC, the Sand 2 model had no significant factors while models for Sands 1 and 3 did. In the Sand 1 model, sand mass, shaking method, and the two-way interaction term were statistically significant factors. The mass effect arose because using 50 g of sand produced significantly higher, by 0.2 log unit (p <0.05), concentrations of EC than using 3 g of sand, based on post-hoc pairwise analyses. The shaking effect resulted from a marginally higher concentration of EC from the two-minute hand shaking compared to the one-minute hand shaking (0.2 log units), but the result was not statistically significant in the post-hoc pairwise comparison. The interaction term effect arose because the one-minute shaking producing lower concentrations (by ~0.3 log unit) for 10 g of sand than for the other sand masses. For the Sand 3 model, shaking method and analyst were both significant factors. Here, shaking by hand for

one minute produced lower concentrations (by ~0.4 log units) than the other shaking methods, but the differences were not significant in post-hoc comparisons. The analyst result is driven by one of the analysts producing higher EC (by 0.5 log unit) than the other.

Effect of eluant composition

The effect of eluant composition was tested using treatments T1 and T10 through T13. Analyst and interaction with eluant composition were included in each ANOVA. Only the ENT-MF model for Sand 2 revealed any significant factors. These were eluant composition and the interaction between analyst and eluant composition. However, post-hoc analyses revealed no significant difference in pairwise comparisons between eluants. The interaction term was significant in the ANOVA because one analyst produced lower results with PBS + Tween 80 than the other analyst in the pair.

For ENT-DS, the sand 1 model had no significant factors, but models for Sands 2 and 3 did. In the Sand 2 model, the interaction between analyst and eluant composition was statistically significant. This arose because one analyst produced higher ENT-DS concentrations than the other with the filtered seawater eluant. In the Sand 3 model, eluant was a significant factor. Notably, this result was driven by the treatment that used DI + $(NaPO_3)_6$ producing consistently lower concentrations compared to the other eluants. Post-hoc pairwise comparisons indicated that DI + $(NaPO_3)_6$ produced significantly lower (by 0.3 log unit, p <0.05) ENT concentrations by ENT-DS than did PBS + Tween 80.

For EC, eluant composition was a significant factor for the Sands 1 and 3 models; no factors were significant in the Sand 2 model. In Sand 1, DI + $(NaPO_3)_6$ yielded lower concentrations than using filtered seawater, DI water, and PBS + Tween 80 (by 0.9 to 0.6 log unit, p <0.05). Similarly, pairwise comparisons for Sand 3 eluants revealed that DI + $(NaPO_3)_6$ yielded lower concentrations than using PBS by 0.2 log unit (p <0.05).

Effect of settling time

The effect of settling time was investigated using treatments T1, T14, and T15. For ENT-MF, analyst, settling time, and interaction were not significant factors in any of the Sand models. For ENT-DS, settling time was not a significant factor in any of the sand models; however analyst and the interaction with settling time was a significant factor in Sand 3. This was driven by one of the two analysts producing lower ENT-DS concentrations than the other using 180- and 600-second settling times, while producing higher concentrations using a 30-second settling time.

For EC, no factors were significant in the Sand 2 ANOVA model. In the Sand 1 model, settling time and analyst were significant factors. The settling time effect was a consequence of the 30-secind settling time yielding lower EC concentrations relative to the other settling times; however, this comparison was not statistically significant in post-hoc comparisons. The analyst effect could be explained by one analyst producing EC concentrations ~0.4 log units lower than the other. In the Sand 3 ANOVA model, settling time was a significant factor; a 30-second settling time produced significantly higher log-EC (by ~0.1 log units) than a 180-second settling time.

Effect of number of rinses

The number of rinses was investigated using treatments T1, T16, and T17. The only sand-indicator combination that showed a 'number of rinse' effect was ENT-DS in Sand 2. Post-hoc pairwise comparisons indicated that one rinse produced 0.4 log unit higher ENT-DS than three rinses (p <0.05).

The effect of pipetting

The effect of pipetting was tested using treatments T1 (base method) and T19 (base method with pipetting in place of pouring). Pipetting was not a significant factor in any of the models indicating, that the eluant decanting method did not impact ENT or EC enumeration. Only for one model (the Sand 1 EC model) did analyst effect explain a significant fraction of the variance; one analyst produced EC concentrations 0.4 log unit higher, on average, than the other.

The effect of sonication

The effect of sonication was determined by considering treatments T11 and T22. Analyst effects could not be included in this model because treatments T11 and T22 were completed by different sets of paired analysts. Sonication was not a significant factor in any of the nine models, indicating that sonication in DI + $(NaPO_3)_6$ (T22) and the hand-shaking base method performed with DI + $(NaPO_3)_6$(T11) did not produce significantly different FIB concentrations.

The effect of blending

The effect of blending was assessed by comparing treatment T21 (hand shaking for one minute with PBS + Tween 80) to treatment T20 (blending using equivalent parameters). For the ENT-MF models, blending was not a significant factor in the Sand 2 model, but it was a significant factor in the models for Sands 1 and 3. In both Sands 1 and 3, blending produced lower ENT-MF than one-minute shaking by ~1 log unit in Sand 1 and ~2 log units in Sand 3. There were also analyst and analyst x blending effects in Sand 1 caused by one analyst producing lower blending results than the other.

For ENT-DS models, the results were the same as for the ENT-MF models. For Sand 1, blending reduced ENT-DS by ~2 log units relative to shaking. For Sand 3, blending reduced ENT-DS by ~1.5 log units relative to shaking. The analyst and interaction factors were also significant for the Sand 1 model for the same reasons described for ENT-MF.

For the EC models, blending only affected EC enumeration in Sand 2. This blending produced lower EC than hand shaking by 0.2 log units. There were no EC analyst effects.

Effect of prefiltration

The effect of eluant prefiltration through a 30-µm mesh was investigated by comparing treatment T5 (3 g of sand and no prefiltration) to T18 (prefiltration with equivalent parameters). None of the ENT-MF Sand models had significant factors. For the ENT-DS models, prefiltration was a significant factor in the Sand 2 model; prefiltration produced slightly higher ENT-DS concentrations (~0.1 log unit). Analyst and the interaction between analyst and prefiltration were also significant in the Sand 2 model. One analyst had lower ENT-DS by 0.1 log unit on average, and the differences between analyst varied depending on the treatment. There were no significant factors in the EC models.

Discussion

Varying the manner in which FIB were eluted from sands did not result in significantly different FIB concentrations among most treatments (Table 3). Even when there were statistically significant differences, the differences were generally small and limited to a single Sand or a single bacterial indicator (Tables 4, 5, and 6). The one exception was blending, which produced significantly lower numbers than shaking for all FIB. This is consistent with several other studies that have reported blending to be less effective than sonication (Ellery and Schleyer 1984, McDaniel and Capone 1985, Epstein and Rossel 1995). Epstein and Rossel (1995) evaluated blending periods between 30 and 480 seconds and found the highest bacterial recovery was from the shortest blending period. It is possible that longer blending times, particularly with some types of sand particles, may result in an increased chance for cell injury or death.

The present study's second finding was that DI + $(NaPO_3)_6$ produced lower FIB concentrations than the other eluants, though only for the defined substrate assays. One possible explanation is that the sodium metaphosphate was less effective at eluting bacteria from sand grains. Alternatively, bacteria are incubated in a liquid that is 10% eluant in the defined substrate assays, which could affect bacterial growth during incubation. Given that DI + $(NaPO_3)_6$ produced lower counts in the DS assay and not in the MF assay it is likely that the buffer adversely affected bacterial growth during incubation.

The lack of a large eluant effect may provide some insight into the mode of bacterial attachment in the DI + $(NaPO_3)_6$ sand. The strength of physico-chemical interactions between bacterial and sand surfaces, such as electrostatic, hydrophobic, and Van der Waals forces, are modulated by pH and ionic strength, which varied across eluants (Derjaguin and Landau 1941, Verwey and Overbeek 1948, Hijnen *et al*. 2005). The similarity in results among elution methods, particularly with the large range of salinity and pH among sands and treatments (Table 2), suggests that the attachment between FIB and sand may not be purely physico-chemical in nature. Bacteria may be present in thin water films on sand surfaces so that release is controlled by thin film expansion, air-water interface scouring, and shear mobilization (DeNovio *et al*. 2004), all of which are likely to occur during hand shaking of sand and eluant mixtures.

De-ionized water as an eluant might be expected to produce lower FIB concentrations than the other eluants because of the potential for bacteria to be injured or killed by osmotic stress. The absence of detrimental effects observed for ENT enumeration with the use of DI eluant is consistent with reports that Enterococcus spp. are hardy under a variety of stressful conditions including fluctuations in osmotic pressure (Smith *et al*. 1994, del Mar Lleo *et al*.

2005). However, salts present in the sand matrix would have dissolved into the DI eluant, potentially lessening osmotic stress to the bacteria.

Prefiltration of the eluant had a statistically significant effect on ENT-DS enumeration, but only in Sand 2, and it concentrations were elevated by only 0.1 log units. Because prefiltration was effective at lowering total suspended solids in the eluant (from 410 to 290 mg/L (30%) in Sand 1, from 1000 to 700 mg/L (30%) in Sand 2, and from 200 to 21 mg/L (90%) in Sand 3), the absence of significant change in FIB concentration with this treatment suggests that FIB are not associated with suspended solids greater than 30 µm in diameter. This is consistent with reports of FIB being associated with particles less than 30 µm in diameter in stormwater (Jeng *et al*. 2005).

It is tempting to conclude from the present study that shaking is a preferred method for FIB enumeration because it is simpler than sonication and produced equivalent results, but this study was limited to coarse grain sands. McDaniel and Capone (1985) and Craig *et al*. (2002) suggested that method effectiveness for enumerating bacteria in sand is dependent on the sand characteristics. For example, sands containing more organic matter could bind bacteria more tightly and require a more aggressive elution method (Ferguson *et al*. 2005). This may be the reason that Dye (1983) found higher recoveries of bacteria using blending than sonication when working with muddy mangrove sediments.

A successful method was equated with higher bacterial recovery, which may not always be the desired endpoint. Many studies focus on assessing if FIB in the water column originate from reservoirs in the sand, but the mechanism for that transference may be more gentle, particularly in embayment locations, than the shaking used to elute bacteria. In contrast, other studies measure bacteria in sand to assess health implications of children placing sand in their mouths, for which a more aggressive elution method would be desirable. Still, the differences among methods were small. Consequently, it is suggested that the simplest method, handshaking for 2 minutes with 1 rinse step, a 30-second settling time, and a 10:1 eluant volume to sand weight ratio with any of the eluants, except for DI + $(NaPO_3)_6$, is appropriate for most applications.

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