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Faecal indicator bacteria enumeration in beach sand: a comparison study of extraction methods in medium to coarse sands

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Abstract

Aims—The absence of standardized methods for quantifying faecal indicator bacteria (FIB) in sand hinders comparison of results across studies. The purpose of the study was to compare methods for extraction of faecal bacteria from sands and recommend a standardized extraction technique.

Methods and Results—Twenty-two methods of 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.

Conclusions—The simplest extraction method that produced the highest FIB recoveries consisted of 2 min of hand shaking in phosphate-buffered saline or deionized water, a 30-s settling time, one-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.

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Significance and Impact of the Study—Method standardization will improve the understanding of how sands affect surface water quality.

Keywords

E. coli; enterococci; faecal bacteria; sand

Introduction

A number of studies have recognized beach sand as a potentially large reservoir of faecal indicator bacteria (FIB) (Alm *et al*. 2003; Whitman and Nevers 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) per gram in California and Florida. Concentrations of *Escherichia coli* (EC) indicator bacteria have been found to reach over 2000 CFU g^{-1} in Florida dry sand and 10^5 CFU g^{-1} 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).

FIB density is used widely to make water quality decisions at beaches, and it is unclear whether their presence in sand is indicative 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 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 are few studies that compare method variations, providing little basis to select a method or to determine whether 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.

Materials and 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 *vs* mechanical), shaking duration (1 and 2 min), number of rinses (1, 2 and 3), settling time (30, 180 and 600 s), eluant-to-sand ratio (100 ml to 3, 10, or 50 g), eluant composition {phosphate-buffered saline (PBS), PBS + Tween, deionized water (DI), sodium metaphos-phate [DI + $(NaPO_3)_6$] or filtered ambient water}, prefiltration of eluant through a 30-μm net filter and decantation method (pouring or pipetting). These variations yielded 22 method permutations, hereafter referred to as treatments (Table 1).

Each of the 22 treatments was applied to three beach sand samples, hereafter referred to as sands 1, 2 and 3. Sand 1 was from Doheny Beach, CA, USA (33°27′41·35″N, 117°41′2·26″W), a marine beach with fine textured siliceous sand (mean diameter of 0·22 mm, moisture content of 18% and 0·71% organic carbon). Sand 2 was from Hobie Cat Beach, FL, USA (25°44′45·06″N, 80°11′50·06″W), also a marine beach but with coarse calcareous sand (0·77 mm, 10% moisture, 0·70% organic carbon). Sand 3 was from a freshwater beach on Lake Michigan

in Michigan City, IN, USA (41°43′38·16″N, 86°53′39·19″W) and consisted of coarse siliceous sand (0·91 mm, 12% moisture, 0·25% organic carbon). 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 samples were shipped overnight in a cooler containing ice packs to the Orange County Sanitation District Laboratory, CA, USA.

Upon arrival at the laboratory, sand samples were homogenized at low speed for 10 min using an industrial grade food service mixer (Model 8140; Anvil, Fletcher, NC, USA) with sanitized paddles. After mixing, samples were aseptically transferred to 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.

For each sand, six 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 between and within-analyst variability. A single sand 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 presterilized 250-ml polypropylene bottle, adding 60 ml of PBS eluant and shaking for 2 min by hand over an arc of about 10 cm. Following a 30-s 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 s, allowed to settle for 30 s and then poured into the same sterile bottle used after the first rinse step. In summary, this method used 100 ml of eluant in two rinse steps with and a 30-s settling time after each rinse.

Treatments T2 to T9 completed a 3 × 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 of the previously published studies (Baums *et al*. 2007; Bonilla *et al*. 2007). Shaking duration/type combinations evaluated included 1 min of hand shaking, 2 min of hand shaking and 2 min 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, USA).

Treatments T10–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% $(NaPO_3)_6$] (S333–500; Fisher Scientific, Fairlawn, NJ), 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 s *vs* 30 s in the T1 base method). T16 and T17 were designed to evaluate the effect of number of rinses compared to the T1 base method that uses two rinse steps. T16 used 100 ml of eluant in a single rinse step and T17 used 100 ml eluant but in three rinse steps of 40, 30 and 30 ml. A 30-s settling time was applied to all rinse steps in T16. T19 was a modification of the base method T1 to determine whether 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 millilitres of PBS was placed into a sterile container with 3 g of sand, the mixture shaken for 2 min, and then the entire contents were passed through a sterilized 30-μm pore size nylon net

filter (Type NY30; Millipore, Bedford, MA, USA) 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 consisted of combining 10 g of sand with 100 ml PBS + 0·1% Tween 80 and one drop anti-foaming agent (Sigma-Aldrich, St Louis, MO, USA) in a Model MC-3, 250 ml mini-blending container mounted on a Model 70115 blender (Waring, Torrington, CT, USA). The mixture was blended for 1 min at maximum speed. The material was allowed to settle for 30 s, and then the supernatant was decanted into a second sterile bottle. T21 was used as a comparison to T20. It consisted of the base method with 1-min hand shaking in PBS + 0·1% Tween 80.

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

Two different PBS solutions were utilized in the study: (i) PBS from PML Microbiologicals (PBS-PML) (VWR#29452–140; Wilsonville, OR, USA) which consisted of 4·25% w/v potassium dihydrogen phosphate and 0·05% w/v of magnesium chloride and (ii) PBS prepared in the laboratory (PBS-IN) which 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.

Following the various extraction treatments, the eluant was processed using standard methods for FIB enumeration. Enterococci were enumerated by both the membrane filtration (ENT–MF) on mEI agar (Method 1600; USEPA 2002) and the Enterolert defined substrate assay (ENT–DS) (IDEXX, Westbrook, MN, USA) (USEPA 2003). *Escherichia coli* were enumerated by the Colilert-18 defined substrate assay (EC) (IDEXX) (USEPA 2003). Water content of sands was determined by drying at 105°C for 24 h. Concentrations of FIB are reported as CFU or most probable number (MPN) per gram 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 4 and 18 (which compared the effect of prefiltration), an additional replicate was processed by one of the two analysts, and the eluant was stored at 4°C and then analysed for suspended solids using laser *in situ* scattering and transmissometry (LISST-100X; Sequoia Scientific Inc., Bellevue, WA, USA).

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 (*n* for each assay 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 *T*2 test, as 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. Differences were considered significantly different if $P < 0{\cdot}05$. Paired *t*-tests were used to examine

differences in ENT–MF, ENT–DS and EC within sands. All analyses were carried out with SPSS (v16.0 for Mac, Chicago, IL, USA).

Results

Comparison of EC and ENT between sands

EC and ENT varied significantly between sands ($P < 0{\cdot}05$) 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, quality for the sands was ranked as 3 > 2 > 1. Ranking based on ENT was not equivalent, with 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, $t = 2{\cdot}52$, $df = 95$, $P < 0{\cdot}05$; sand 2: 0·4 log unit higher, $t = 10{\cdot}18$, $df = 95$, $P < 0{\cdot}05$; sand 3: 0·3 log unit higher, $t = 8{\cdot}81$, $df = 93$, $P < 0{\cdot}05$). 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, $t = 38{\cdot}5$, $df = 95$; sand 3: 0·7 log unit higher, $t = 17{\cdot}5$, $df = 93$ $P < 0{\cdot}05$). In contrast, mean ENT concentrations were significantly lower than EC concentrations in sand 2, but by only 0·1 log unit ($t = 3{\cdot}57$, $df = 95$, $P < 0{\cdot}05$). The log-mean FIB concentrations and the standard deviations are shown in Table 3 for all treatments in aggregate (‘all’) and for each individual treatment (T1–T22).

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 × three indicator analyses). There was no analyst effect for sand 2. For sand 1, there was an analyst effect for EC ($F = 4{\cdot}54$, $df = 5$, $P < 0{\cdot}05$). For sand 3, there was an analyst effect for ENT–MF ($F = 21{\cdot}0$, d.f. = 5, $P < 0{\cdot}05$). 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.

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 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 Table 4–Table 6.

Effect of sand mass and shaking method—A three-way ANOVA model investigated (i) sand mass, (ii) shaking duration/type, (iii) analyst and the interactions of these parameters using T1–T9 for each sand.

The ANOVA model for ENT–MF produced different results for the 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 min relative to other shaking methods. In contrast, the sand 3 model showed a significant effect of mass, mass × shaking interaction, and the three-way interaction between mass, shaking method and analyst. The sand 3 results were driven by low concentrations obtained by one analyst from the 50 g sample by mechanical mixing.

The ANOVA analysis for ENT–DS revealed no significant factors for the sand 1 model, but did find significant factors for the sand 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 higher) 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.

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 2-min hand shake compared to the 1-min hand shake (0·2 log units), but the result was not statistically significant in the *post hoc* pairwise comparison. The interaction term effect arose because the 1-min shake producing lower concentrations (by *c*. 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 1 min produced lower concentrations (by *c*. 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 T1 and T10–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 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. Interestingly, this result was driven by the fact that the treatment using DI + $(NaPO_3)_6$ produced 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.

For EC, eluant composition was a significant factor for sand 1 and 3 models, and 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 (by 0·9–0·6 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 T1, T14 and T15. For ENT–MF, analyst, settling time and the 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 with 180-s and 600-s settling times, but producing higher concentrations than the other analyst using a 30-s 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-s settling time yielding lower EC concentrations relative to the other settling times, but this comparison was not statistically significant in *post hoc* comparisons. The analyst effect could be explained by one analyst producing EC concentrations *c*. 0·4 log units lower than the other. In the sand 3 ANOVA model, settling time was a significant factor. Here, a 30-s settling time produced significantly higher log-EC (by *c*. 0·1 log units) than a 180-s settling time.

Effect of number of rinses—The number of rinses was investigated using 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 ($P < 0·05$).

Effect of pipetting—The effect of pipetting was tested using 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. In only one model (the sand 1 EC model), did analyst explain a significant fraction of the variance. Here, one analyst produced EC concentrations 0·4 log unit higher, on average, than the other.

Effect of sonication—The effect of sonication was determined by considering T11 and T22. Analyst could not be included in the model because 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.

Effect of blending—The effect of blending was assessed by comparing T21 (hand shaking for 1 min with PBS + Tween) to 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 the 1 min shake by *c*. 1 log unit in sand 1 and *c*. 2 log units in sand 3. There were also analyst and analyst × 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 *c*. 2 log units relative to shaking. For sand 3, blending reduced ENT–DS by *c*. 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. Here, it 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 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. Here, prefiltration produced slightly higher ENT–DS concentrations (*c*. 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, on average, by 0·1 log unit, 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, they were generally small and limited to a single sand or to a single bacterial indicator (Table 4–Table 6). 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 s 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.

Another finding was that sodium metaphosphate [DI + $(NaPO_3)_6$] produced lower FIB concentrations than the other eluants, although 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 or interfered with the assay in some way.

The lack of a large eluant effect may provide some insight into the mode of bacterial attachment in these sands. The strength of physicochemical 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 range of salinity and pH among sands and treatments (Table 2), suggests that the attachment between FIB and sand may not be purely physicochemical 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.

DI 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 where 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^{-1} (30%) in sand 1, from 1000 to 700 mg l^{-1} (30%) in sand 2 and from 200 to 21 mg l^{-1} (90%) in sand 3), the absence of much change in FIB concentration with this treatment suggests that FIB are not associated with suspended solids >30 μm. This is consistent with reports of FIB being associated with particles <30 μm in diameter in stormwater (Jeng *et al*. 2005).

It is tempting to conclude from this study that shaking is a preferred method for FIB enumeration because it is simpler than sonication and produced equivalent results, but the study was limited to coarse-to-medium 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 instance, sands or sediments 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 in 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 whether 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 and thus, it is suggested that the simplest method, handshaking for two minutes with one rinse step, a 30-s settling time and a 10 : 1 eluant volume to sand weight ratio with any of the eluants except for sodium metaphosphate, is appropriate for most applications.

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Table 1

Treatments applied to each sand sample

Treatment	Mass (g)	Shaking	Settling time (s)	Number of rinses	Eluant	Prefiltration	Eluant removal
1	10	2 min	30	2	PBS	No	Pour
2	3	1 min	30	2	PBS	No	Pour
3	10	1 min	30	2	PBS	No	Pour
4	50	1 min	30	2	PBS	No	Pour
5	3	2 min	30	2	PBS	No	Pour
6	50	2 min	30	2	PBS	No	Pour
7	3	2 min mechanic	30	2	PBS	No	Pour
8	10	2 min mechanic	30	2	PBS	No	Pour
9	50	2 min mechanic	30	2	PBS	No	Pour
10	10	2 min	30	2	PBS + Tween 80	No	Pour
11	10	2 min	30	2	DI + $(NaPO_3)_6$	No	Pour
12	10	2 min	30	2	Sea/lake water	No	Pour
13	10	2 min	30	2	DI water	No	Pour
14	10	2 min	180	2	PBS	No	Pour
15	10	2 min	600	2	PBS	No	Pour
16	10	2 min	30	1	PBS	No	Pour
17	10	2 min	30	3	PBS	No	Pour
18	3	2 min	30	2	PBS	Yes	Pour
19	10	2 min	30	2	PBS	No	Pipette
20	10	1 min blending	30	2	PBS + Tween 80	No	Pour
21	10	1 min	30	2	PBS + Tween 80	No	Pour
22	10	30 s sonication	600	1	DI + $(NaPO_3)_6$	No	Pour

PBS, phosphate buffered saline; DI, deionized.

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, USA), respectively, according to manufacturer's protocols. Salinity is reported on a practical salinity scale

Eluant	pH	Salinity
PBS*	6·8	0·1
PBS + 0·1% Tween 80 (A)†	6·8	0·1
PBS + 0·1% Tween 80 (B)‡	7·4	0·3
Filtered seawater (Doheny)	8·3	33·8
Filtered seawater (Hobie Cat)	8·6	36·8
Filtered take Michigan water	8·3	0·2
DI water	7·0	0
DI ± 1% $(NaPO_3)_6$	6·6	2·8

PBS, phosphate buffered saline; DI, deionized.

*PBS-PML.

†Used for sands 1 and 2.

‡Used for sand 3.

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 *Escherichia coli* in the 22 treatments (T) and all treatments in aggregate (all in bottom row). Results are provided for sand of the three sands. Sand 1 is from Doheny beach, sand 2 is from Hobie Cat beach and sand 3 is from a beach in Michigan City

	Sand 1			Sand 2			Sand 3		
	ENT–MF	ENT–DS	EC	ENT–MF	ENT–DS	EC	ENT–MF	ENT–DS	EC
T	log CFU g^{-1}	log MPN g^{-1}	log MPN g^{-1}	log CFU g^{-1}	log MPN g^{-1}	log MPN g^{-1}	log CFU g^{-1}	log MPN g^{-1}	log MPN g^{-1}
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)**

MPN, most probable number; CFU, colony-forming units.

Table 4

Summary table of ANOVAS for enterococci enumerated using membrane filtration. The experiment is given in the first column and factors in the second. *F* statistic (*F*), degrees of freedom (d.f.) 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 three-way ANOVA was used, 'n/a' indicates that factor was not relevant for the model.

		Sand 1	Sand 2	Sand 3
Experiment	**Factor(s)**	***F*, d.f., *P***	***F*, d.f., *P***	***F*, d.f., *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 × 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 × 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 × 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 × 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 × 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 × 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 × mass	0·26, 2, 0·77	1·38, 2, 0·28	2·04, 2, 0·16
	Analyst × shaking	0·32, 2, 0·73	1·56, 2, 0·24	3·01, 2, 0·08
	Mass × shaking	1·47, 4, 0·25	**3·09, 4, 0·04***	**4·72, 4, 0·009***
	Analyst × mass × shaking	0·63, 4, 0·65	2·11, 4, 0·12	**5·38, 4, 0·005***

Significant factors are shown in bold with a '*'

Table 5

Summary table of ANOVAS for entero-cocci enumerated with defined substrate. The experiment is given in the first column and factors in the second. *F* statistic (*F*), degrees of freedom (d.f.) 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 three-way anova was used, 'n/a' indicates that factor was not relevant for the model.

		Sand 1	Sand 2	Sand 3
Experiment	**Factor(s)**	***F*, d.f., *P***	***F*, d.f., *P***	***F*, d.f., *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
	Number of rinses × analyst	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 × 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 × 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 × eluant	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 × 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 × 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 × mass	0·23, 2, 0·80	2·67, 2, 0·10	1·36, 2, 0·28
	Analyst × shaking	0·01, 2, 0·99	0·045, 2, 0·96	0·33, 2, 0·72
	Mass × shaking	1·84, 4, 0·17	0·64, 4, 0·64	1·12, 4, 0·38
	Analyst × mass × shaking	0·64, 4, 0·64	2·05, 4, 0·13	1·91, 4, 0·15

Significant factors are shown in bold with a '*'

Table 6

Summary table of ANOVAS for EC. The experiment is given in the first column and factors in the second. *F* statistic (*F*), degrees of freedom (d.f.) 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 three-way anova was used, 'n/a' indicates that factor was not relevant for the model.

		Sand 1	Sand 2	Sand 3
Experiment	**Factor(s)**	***F*, d.f., *P***	***F*, d.f., *P***	***F*, d.f., *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 × 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 × 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 × 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 × eluant	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 × 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 × 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 × mass	0·32, 2, 0·73	1·52, 2, 0·25	1·30, 2, 0·30
	Analyst × shaking	1·91, 2, 0·18	0·44, 2, 0·65	0·64, 2, 0·54
	Mass × shaking	**4·47,4, 0·011***	2·01, 4, 0·14	1·64, 4, 0·21
	Analyst × mass × shaking	0·40, 4, 0·80	0·61, 4, 0·66	1·47, 4, 0·25

Significant factors are shown in bold with a '*'