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ORIGINAL ARTICLE

Indicator microbes correlate with pathogenic bacteria, yeasts and helminthes in sand at a subtropical recreational beach site

A.H. Shah[1,2], A.M. Abdelzaher[1,2], M. Phillips[1,2], R. Hernandez[1,2], H.M. Solo-Gabriele[1,2], J. Kish[1,3], G. Scorzetti[1,4], J.W. Fell[1,4], M.R. Diaz[1,4], T.M. Scott[1,5], J. Lukasik[6], V.J. Harwood[7], S. McQuaig[7], C.D. Sinigalliano[1,8], M.L. Gidley[1,8,9], D. Wanless[1,8,9], A. Ager[1,10], J. Lui[1,10], J.R. Stewart[11], L.R.W. Plano[1,12] and L.E. Fleming[1,3,4]

1 University of Miami, NSF NIEHS Oceans and Human Health Center, Miami, FL, USA
2 Department of Civil, Arch., and Environmental Engineering, University of Miami, Coral Gables, FL, USA
3 Department of Epidemiology & Public Health, University of Miami, Miami, FL, USA
4 Rosentiel School of Marine and Atmospheric Science, Division of Marine Biology and Fisheries, University of Miami, Miami, FL, USA
5 BCS Laboratories Inc., Miami, FL, USA
6 BCS Laboratories Inc., Gainesville, FL, USA
7 Department of Biology, University of South Florida, Tampa, FL, USA
8 National Oceanic and Atmospheric Administration, Atlantic Oceanographic and Meteorological Laboratories, Ocean Chemistry Division, Miami, FL, USA
9 Cooperative Institute for Marine and Atmospheric Studies, University of Miami, Miami, FL, USA
10 Department of Microbiology and Immunology, University of Miami, Miami, FL, USA
11 University of North Carolina, Chapel Hill, NC, USA
12 Department of Microbiology and Immunology and Department of Pediatrics, University of Miami, Miami, FL, USA

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Correspondence
Helena Solo-Gabriele, Department of Civil, Arch., and Environmental Engineering, University of Miami, PO Box 248294, Coral Gables, FL 33124-0630, USA.
E-mail: hmsolo@miami.edu

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Abstract

Aims: Research into the relationship between pathogens, faecal indicator microbes and environmental factors in beach sand has been limited, yet vital to the understanding of the microbial relationship between sand and the water column and to the improvement of criteria for better human health protection at beaches. The objectives of this study were to evaluate the presence and distribution of pathogens in various zones of beach sand (subtidal, intertidal and supratidal) and to assess their relationship with environmental parameters and indicator microbes at a non-point source subtropical marine beach.

Methods and Results: In this exploratory study in subtropical Miami (Florida, USA), beach sand samples were collected and analysed over the course of 6 days for several pathogens, microbial source tracking markers and indicator microbes. An inverse correlation between moisture content and most indicator microbes was found. Significant associations were identified between some indicator microbes and pathogens (such as nematode larvae and yeasts in the genus *Candida*), which are from classes of microbes that are rarely evaluated in the context of recreational beach use.

Conclusions: Results indicate that indicator microbes may predict the presence of some of the pathogens, in particular helminthes, yeasts and the bacterial pathogen *Staphylococcus aureus* including methicillin-resistant forms. Indicator microbes may thus be useful for monitoring beach sand and water quality at non-point source beaches.

Significance and Impact of the Study: The presence of both indicator microbes and pathogens in beach sand provides one possible explanation for human health effects reported at non-point sources beaches.

Introduction

Global estimates reveal that bathing in coastal recreational waters polluted by faecal waste have caused over 120 million cases of gastrointestinal illness and 50 million cases of severe respiratory diseases each year (Shuval 2003). These illnesses have been attributed over the course of many studies to known contaminating sources of pathogens (point sources) such as sewage outfalls (Alexander *et al.* 1992), stormwater outflows (Haile *et al.* 1999) and impacts from leaking sewer systems (Boehm *et al.* 2003). These sources not only release pathogens in the water but also release indicator microbes that are used by regulators and beach managers to determine the level of contamination of the water.

Typically, prevention of diseases in recreational waters involves the remediation of the point sources. However, when the source is not directly traceable (i.e. non-point sources), remediating the source and hence preventing illnesses become more complicated. Two studies have been published which evaluate human health of bathers *vs* nonbathers at non-point source beaches (Colford *et al.* 2007; Fleisher *et al.* 2010; Sinigalliano *et al.* 2010). Both of these studies (one in Mission Bay, California, and one in Miami, Florida) found increased illness among bathers relative to nonbathers. In addition, illness rates among bathers were found to correlate with the presence of non-pathogenic indicator microbes in the water (specifically enterococci or F+ coliphage).

This correlation has led researchers to question whether the source of indicator microbes may also be the source of pathogens that are causing the observed illness, as may be assumed for point source (i.e. sewage)-polluted beaches. If such a link was identified, this would open possibilities for source remediation and improved monitoring for bathing water at non-point source-polluted beaches. Determining such a link, however, would require identifying the source or pathway that introduces both indicators and pathogens in the beach water (Boehm *et al.* 2003).

Previous studies have demonstrated that beach sand may serve as a reservoir for pathogens and indicator microbes (Alm *et al.* 2003; Beversdorf *et al.* 2007) that are released into the water through tidal action or run-off. Also, the sands within and near the intertidal zone have been shown to contain nutrients that promote the survival and sometimes the growth of these indicator bacteria and pathogens (Papadakis *et al.* 1997). Numerous studies have been conducted in the Miami study beach showing that sand serves as one of the predominant reservoirs of enterococci (Shibata *et al.* 2004; Wright *et al.* 2011), the indicator microbe used to assess marine beach water safety (USEPA 2004), as well as its avenue of release into the water column through tidal action and run-off (Yamahara *et al.* 2007; Wright *et al.* 2009). Within the sand, different zones of the beach at the study site have also been known to have distinctive differences in enterococci levels, and preliminary studies have documented differences in pathogen levels within different sand zones (Abdelzaher *et al.* 2010).

Determining whether the sand is also the predominant reservoir and avenue of release of pathogens would help to establish a link between these pathogens and indicator microbes, hence explaining the link between indicator microbes and health effects at non-point source beaches seen in the epidemiologic studies (Colford *et al.* 2007; Fleisher *et al.* 2010; Sinigalliano *et al.* 2010; Abdelzaher *et al.* in press). However, a wide variety of pathogens should be screened for in the different sand zones to establish such a link as illness may result from a range of pathogens.

The study beach in Miami, Florida, was the same study beach where a prior epidemiologic study was conducted (Fleisher *et al.* 2010), which found a relationship between levels of indicator bacteria in the water and health effects. The current study is driven by the aim of establishing a causative link between the source of indicator microbes (i.e. beach sand) and pathogens that can potentially cause human illness at recreational beaches, thereby facilitating the identification of suitable methods for improving water quality through source prevention and improved monitoring. The range of microbes evaluated in this study was expanded beyond that measured in earlier studies (Abdelzaher *et al.* 2010) to also include the measurements of yeasts and helminthes, in addition to measurements of viruses, bacteria and protozoa. Measures of yeasts and helminthes are particularly rare in the context of recreational water quality monitoring. Moreover, in contrast to prior studies at this beach, the samples collected during the current study were controlled for solar insolation and tidal period: two factors that may impact microbial levels in sand and water (Abdelzaher *et al.* 2010).

Materials and methods

Site description

Samples were collected at Hobie Cat Beach located on Key Biscayne within Miami-Dade County, FL, USA. The study site is characterized by a subtropical climate because of rainfall and geographic location with an average temperature of 24·8°C. The study beach is narrow with an approximate average distance between the mean water line and the outer edge of the sand of 5 m. The beach is 1·6 km long, relatively shallow and characterized by weak water circulation (Shibata *et al.* 2004). This beach is the only beach in Miami-Dade County where visitors can bring their pets, particularly dogs. Beach

admission is free, and many bathers frequent the beach, particularly during the summer months (Wang *et al.* 2010). Extensive evaluation of the vicinity of the study beach has not identified point sources of pollution to this beach (such as sewage outfalls, failing lift stations or septic tanks). However, the beach has been placed under Beach Health Advisories periodically (i.e. 4·6 days per year averaged from 2002 to 2009) owing to microbial water quality violations (Florida Dept. of Health 2009).

Sample collection and field measurements

Samples were collected on six different days, 1 h after high tide during two different 3-day periods, one on 2–4 August 2009 and the other on 17–19 August 2009. Sampling occurred over a 1-h sampling period between 8:00 and 10:00 AM (eastern local time) to control for differences in solar insolation. Environmental conditions were obtained from National Ocean and Atmospheric Administration (NOAA, tidal stage), the University of Miami (NSF NIEHS OHH Center Remote Sensing Facilities Core, wind speed and solar radiation) and South Florida Water Management District (SFWMD station SR-27, prior 48-, 24- and 6-h rainfall). In addition, the number of humans and dogs within 100 m during the sampling period was also recorded. *In situ* measurements were taken of both the water [pH, salinity, temperature (600R series sonde; YSI Inc., Yellow Springs, OH, USA)] and turbidity (TD-40 nephelometer; Turner Designs, Sunnyvale, CA, USA) and sand temperature (M, T, & EC-300 Series; Aquaterr, Costa Mesa, CA, USA).

Both water and sand samples were collected for additional processing in the laboratory. One water sample (at an approximate water depth of 30 cm) was collected daily (for a total of six water samples) using 5-l presterilized bottles. Three sand samples (one 'dry' from the supratidal zone, one 'wet' from the intertidal zone and one 'inundated' from the subtidal zone) were also collected along three corresponding transects during each of the six sampling days for a total of 18 sand samples. Each sand sample consisted of a composite of 160 cores, the sand of which was placed into a sterile tray and covered to prevent outside contamination. Sand cores were taken (10 cm apart, 3 cm deep) along transects oriented parallel to the shore line at the beach. The distance between each of the three sampling transects (called 'dry', 'wet' and 'inundated') were determined based on environmental conditions of the particular day (Fig. S1). The dry transect was always 500 cm from a stationary reference point and located within the supratidal zone. The wet sample was collected from the intertidal zone, and the inundated sample was taken submerged in 25-cm-deep water within the subtidal zone.

Sand sample preprocessing

Each sample was homogenized by thoroughly mixing, and the homogenate was split for gravimetric analysis, for helminthes analysis and for the preparation of microbe eluate samples. Gravimetric analysis included measurements in triplicate of moisture content (weighed before and after heating 110°C for 24 h); these same samples were also subject to volatile organic analysis (weighed before and after igniting at 550°C for 24 h). Helminthes were analysed by wet filtration of the sand through a 150-μm sieve and collecting the eluate for centrifugation and microscopy according to procedures detailed in the Data S1. The preparation of the microbe eluate samples (for all microbes except for helminthes as described earlier) was modified from previously published methods (Van Elsas and Smalla 1997; Boehm *et al.* 2009; Wright *et al.* 2009). Two sets of microbe eluates, sample A and sample B, were prepared from each of the 18 sand samples. The 'A' eluate samples were filtered for subsequent virus and protozoa analyses, and thus, a larger eluate volume was needed. The mass of sand used for the 'dry', 'wet' and 'inundated' samples was varied to take into account the differences in moisture content between these sand types, with an effort to maintain a sand mass to volume eluate ratio of 20 g to 1000 ml.

The weight of the sand used for the 'A' samples was 500, 1000 and 1500 g for dry, wet and inundated samples, respectively. For the 'B' samples, the weight of the sand was 80, 160 and 240 g, respectively. The A sand samples were placed into 25-l carboys and filled with sterile phosphate-buffered saline (PBS) solution. The B samples were similarly placed into 4-l containers and filled with sterile PBS solution. All sand eluate samples were thoroughly mixed for 10 min and allowed to settle for 3 min. The supernatant or eluate was then split for subsequent microbe analyses.

The 'A' samples were split into six aliquots: a 1-l sample for enterococci, yeast, faecal coliform, *Escherichia coli*, *Clostridium perfringens*, *Staphylococcus aureus* measurements by membrane filtration (MF); a 1-l sample for qPCR and CS analysis of enterococci and qPCR analysis of Bacteroidales; a 500-ml sample for the analysis of polyomavirus; a 500-ml sample for the analysis of *Vibrio vulnificus*, human-specific enterococci esp gene in *Enterococcus faecium* and coliphage; and two 10-l aliquots (one for enterovirus and one for protozoa filtration and analysis). The 'B' samples were split into four aliquots identical to the 'A' sample, with the exception that the two 10-l aliquots for virus and protozoa analyses were not included. Thus, all sand samples (with the exception of protozoa and enterovirus analysis) were analysed in duplicate within two separate sand aliquots ($n = 36$ for all microbes except protozoa and enterovirus, $n = 18$).

Microbial analysis

Three different methods were used to enumerate enterococci: membrane filtration (MF), chromogenic substrate (CS) and quantitative polymerase chain reaction (qPCR) using the entero1 assay (Haugland *et al.* 2005; Siefring *et al.* 2008). Additional bacterial indicators (faecal coliform, *E. coli* and *C. perfringens*) were evaluated by MF. The viral indicators [somatic (F−) coliphages and male-specific (F+)] were analysed according to the standard single agar layer procedure (USEPA 2001a). Samples for yeast analysis were processed by MF (using Sabouraud dextrose agar, Vogel *et al.* 2007), followed by morphological differentiation from growth on agar plates (red colonies = non-pathogenic, cream or white colonies = potentially pathogenic). The colonies were subsequently identified to determine the presence of the specific potential human pathogens *Candida tropicalis*, *Candida albicans*, *Candida guilliermondii*, *Candida glabrata* and *Candida parapsilosis*. Species detection and identification were assessed with Luminex 100 (Luminex Corp., Austin, TX, USA), a specialized flow cytometer equipped with a dual laser system that allows the simultaneous detection of different target DNA sequences in a multiplex and high-throughput format (Diaz *et al.* 2008). Microbial source tracking (MST) markers analysed by qPCR methods were as previously described and included human-specific Bacteroidales assay BacHum-UCD (Kildare *et al.* 2007), HF8 [modified from Bernhard and Field (2000a), Bernhard and Field (2000b) as described in Sinigalliano *et al.* (2010)] and canine-specific Bacteroidales assay DogBac [modified from Dick *et al.* (2005) as described in Sinigalliano *et al.* (2010)]. MST markers analysed by endpoint PCR were also as previously described and included human-specific esp gene of *Ent. faecium* (Scott *et al.* 2005) and human polyomaviruses (HPyVs) BK and JC (McQuaig *et al.* 2006).

Pathogens evaluated included bacteria [*Staph. aureus* including methicillin-sensitive (MSSA) as described by Mertz *et al.* (2007) and Plano *et al.* (2011), methicillin-resistant (MRSA) strains as described by Shopsin *et al.* (1999) and *V. vulnificus* by nonquantitative MF on VVA agar (Kaysner and DePaola 2004) with confirmation by PCR (Gordon *et al.* 2008)], protozoa [*Cryptosporidium* spp. and *Giardia* spp. via immunomagnetic separation, fluorescent staining, followed by microscopy, (USEPA 2002)] and viruses [enterovirus via culture, (USEPA 2001b) with identification via transmission electron microscopy, TEM]. Helminthes were quantified using microscopic methods (Sloss *et al.* 1994).

All microbes analysed by MF methods are reported in units of colony-forming units (CFU) per 100 ml of water or CFU g^{-1} of dry sand. Similarly, enterococci analysed by CS and viruses analysed by cell culture methods were reported in units of most probable number (MPN). All microbes analyzed by qPCR are reported in Genomic Equivalent Units (GEU) per 100 ml with the exception of the canine-specific Bacteroidales assay which is reported in units of target sequence copies, TSC, which utilized a plasmid control as opposed to genomic controls.

The enterococci MF analysis was conducted according to EPA method 1600 (USEPA 2002). Enterococci analysed by CS were based upon the commercial Enterolert™ assay (IDEXX Laboratories, Inc., Westbrook, ME, USA) and followed the manufacturer's instructions with sample volumes of 10 and 2 ml diluted to 100 ml and placed in a tray (Quantitray-2000™; IDEXX Laboratories, Westbrook, ME), which permitted enumeration based upon a MPN.

DNA extraction for enterococci qPCR and for qPCR of *Bacteroidales* markers included amendment with extraction recovery controls and inhibition controls as previously described (Siefring *et al.* 2008; Sinigalliano *et al.* 2010). The microbial population from water samples and sand eluate samples (1000 ml) were collected by filtration through sterile 0·45-μm pore size, 47-mm cellulose nitrate membranes (Whatman, Piscataway, NJ). The filters were then rolled up and placed in bead-beating tubes (FastPrep™ Lysing Matrix 'E'; MP Biomedicals, Solon, OH). Filters were then amended by addition of 1×10^5 cells from an extraction control cell suspension (washed *Lactococcus lactis* cells) for use in the subsequent determination of extraction recovery efficiency and assessment of potential sample inhibition as previously described (Siefring *et al.* 2008; Sinigalliano *et al.* 2010). Total genomic DNA was extracted from the filters using the Fast DNA® Spin Kit (Cat no. 6540-600; MP Biomedicals) as per kit instructions and eluted in a final volume of 100 ul. Purified DNA extracts were separated into replicate aliquots and stored at −80°C until analysis.

Sand samples analysis included all microbes, while water samples included all microbes except coliphage (somatic and F+), esp gene in *Ent. faecium*, HPyVs, helminthes, *V. vulnificus*, *Cryptosporidium* sp., *Giardia* spp. and enterovirus. More details about the microbial analysis methods used (including qPCR primer and probe sequences, extraction recovery controls, inhibition assessment and generation of standard curves) can be found in Data S1.

Statistical tests

The data were tested for normality using Microsoft Excel, XL STAT (Addinsoft USA, New York, NY, USA). Initial analysis indicated that some of the data were not normally distributed; therefore, the nonparametric Mann–Whitney *U*-test was used to evaluate statistical differences between two sets of data. Correlation (*R* values) between

data sets was evaluated using XL STAT using the nonparametric Spearman rank order test. Correlation values were reported for data sets with more than 10% positive samples [$n \geq 4$], and therefore, the following microbes were excluded from the statistical analysis on this basis: MRSA, *Cryptosporidium* spp., *Giardia* spp., enterovirus, human Bacteroidales (UCD) marker, *C. guilliermondii*, *C. glabrata* and *C. parapsilosis*. For statistical tests between environmental parameters and microbes, the central tendency or mean of the microbe concentration in the sand for that given day was tested against that same day's environmental parameter in a 1 : 1 manner. Water samples were not assessed statistically given the low number of total samples ($n = 6$).

Results

Environmental parameters

Hydrometeorological and physical/chemical parameters were relatively consistent between sampling days, with the mean and standard deviations for solar radiation ($28{\cdot}0 \pm 0{\cdot}6$ W m^{-2}), wind speed ($7{\cdot}3 \pm 2{\cdot}2$ m s^{-1}) and tidal height ($1{\cdot}9 \pm 0{\cdot}4$ m) at typical levels for this site. However, the first three sampling days had an average tidal height of 0·7 m lower than the last 3 days. Relatively dry conditions preceded the first three sampling days with only 2 mm of rainfall on 1 August (the day before the first 3-day sampling event) and no additional rain during the following 2 days. The second set of three sampling dates (17–19 August) were wetter, with 74 mm of rainfall 2 days prior to the 17th with 3 mm occurring within 24 h prior and 1·3 mm occurring 6 h prior. Rainfall was also detected shortly after sampling on 17th August (0·8 mm) and 18th August (16 mm), but none of these rainfall events occurred within 6 h prior to sampling the next day. Zero to two swimmers and 0–1 dogs were observed within 100 m of the sampling area during the sampling period on all of the sampling days.

For the water samples, the physical/chemical characteristics were typical of subtropical marine environments (average temperature of $25{\cdot}7 \pm 2{\cdot}5$°C, salinity of $33{\cdot}7 \pm 1{\cdot}2$ psu, turbidity of $5{\cdot}1 \pm 1{\cdot}5$ NTU and pH of $7{\cdot}8 \pm 0{\cdot}2$). For all the sand samples, the average temperature *in situ* was $29{\cdot}6 \pm 0{\cdot}8$°C. The moisture content of the sand increased between the three different sample types: dry ($8{\cdot}4 \pm 5{\cdot}2$%), wet ($20{\cdot}4 \pm 1{\cdot}0$%) and inundated ($24{\cdot}7 \pm 0{\cdot}03$%). The volatile organic content of the sand was $1{\cdot}1 \pm 0{\cdot}7$% and the average grain size, d_{50}, was $0{\cdot}35 \pm 0{\cdot}01$ mm for dry, $0{\cdot}28 \pm 0{\cdot}02$ mm for wet and $0{\cdot}39 \pm 0{\cdot}11$ mm for inundated sand. Twenty-four hour rainfall inversely correlated with temperature (°C) of the sand samples ($r = -0{\cdot}56$, $P = 0{\cdot}001$).

Enterococci analysis methods

For the water samples, enterococci using the MF method ranged from <2 to 290 CFU 100 ml^{-1} with an average of 99 ± 90 CFU 100 ml^{-1}. For the CS method, enterococci levels ranged from <2 to 355 MPN 100 ml^{-1} with an average of 162 ± 145 MPN 100 ml^{-1}. For the qPCR method, enterococci levels ranged from 9 to 167 GEU 100 ml^{-1}, with an average of 57 ± 59 GEU 100 ml^{-1} (Fig. 1).

For sand, results between the different analysis methods and different types of sand, although variable (Fig. 1), were not statistically different between methods ($P = 0{\cdot}46$ for MF and CS, $P = 0{\cdot}06$ for MF, and qPCR, $P = 0{\cdot}19$ for CS and qPCR) when comparing all samples analysed by each method regardless of sampling location. Enterococci, in sand, as measured by MF were strongly and statistically significantly correlated with enterococci measured by CS ($r = 0{\cdot}94$; $P < 0{\cdot}001$); the results from qPCR were not correlated with either MF or CS (Table 1).

Microbial indicators

In the water samples, faecal coliform, *E. coli* and *C. perfringens* data ranged from 50 to 650, 50 to 3410 and 2 to 170 CFU 100ml^{-1}, respectively, for the six sampling days (Fig. 1). In the sand samples, faecal coliform, *E. coli* and *C. perfringens* data ranged from 0·31 to 11 100, 0·32 to 233 and 0·31 to 116 CFU g^{-1}, respectively, for the six sampling days (Fig. 1). Coliphage identification in sand revealed ubiquitous presence of somatic coliphage, but no coliphage with the F+ gene.

Strong and significant inverse correlations were observed between sand moisture content and indicator microbe levels [faecal coliforms ($r = -0{\cdot}82$; $P < 0{\cdot}001$), enterococci by MF ($r = -0{\cdot}82$; $P < 0{\cdot}001$), enterococci by CS ($r = -0{\cdot}76$; $P < 0{\cdot}001$) and enterococci by qPCR ($r = -0{\cdot}56$; $P = 0{\cdot}001$)], reflecting the significant differences between the sampling locations (dry, wet and inundated) (Fig. 1). The microbial indicator, *C. perfringens* showed the opposite trend with a positive and significant correlation with moisture content ($r = 0{\cdot}50$; $P = 0{\cdot}002$) (Fig. 1). Analysing relationships between indicator microbes, showed that enterococci measured by MF strongly and significantly correlated with faecal coliforms ($r = 0{\cdot}83$; $P < 0{\cdot}001$) (Table 1).

Over the course of the last three sampling days, water samples exceeded the EPA recommended regulatory guideline (104 CFU 100 ml^{-1}), with an average of 169 CFU 100 ml^{-1} in comparison with 29 CFU 100 ml^{-1} during the first 3 days for enterococci by MF; comparable observations were made for enterococci by CS and qPCR. For sand, values were statistically greater by qPCR (for

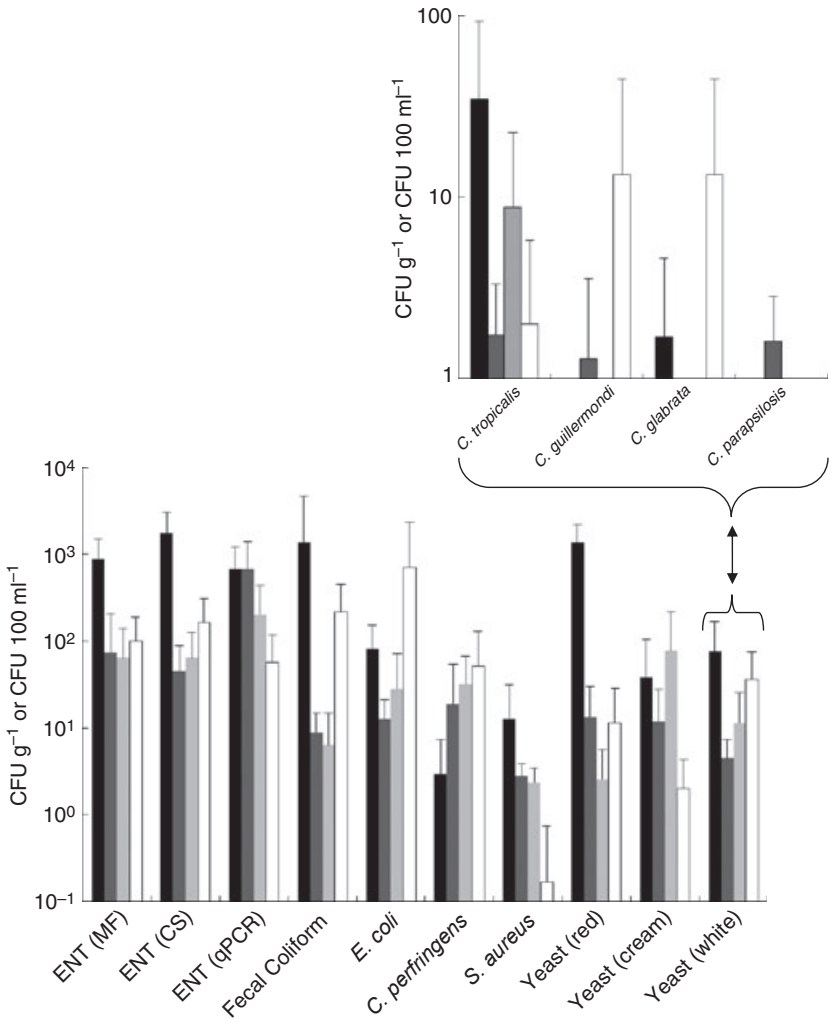

Figure 1 Average CFU g^{-1} or CFU 100 ml^{-1} for microbial indicators and pathogens in water samples and in dry, wet, and inundated sands. Error bars correspond to the standard deviation of duplicate measures. (■) Dry; (■) wet; (■) inundated and (□) water.

enterococci) during the last 3 days when compared to the first 3 days for dry ($P = 0{\cdot}04$), wet ($P = 0{\cdot}002$) and inundated ($P = 0{\cdot}002$) sand samples, but the same statistical significance was not observed in the sand samples for MF and CS ($P > 0{\cdot}05$).

Yeasts

The yeast colonies on each growth plate were counted in groups based on colony colour: red, which is indicative of the basidiomycetous yeast genus *Rhodotorula*, and cream and white, which may include both ascomycetous and basidiomycetous yeasts. The focal points for identifications were specific human pathogens (*C. tropicalis*, *C. albicans*, *C. guilliermondii*, *C. parapsilosis* and *C. glabrata*) which sampling demonstrated were restricted to the white colonies.

The results of the water samples showed means of 12, 2 and 36 CFU 100 ml^{-1} for, red, cream and white yeasts, respectively (Fig. 1). *Candida tropicalis* was found in two water samples, one collected on Day 1 (10 CFU 100 ml^{-1}) and one on Day 5 (2 CFU 100 ml^{-1}), with an average of 2 ± 4 over the six sampling days (Fig. 1). *Candida guilliermondii* was found on Day 2 (80 CFU 100 ml^{-1}), whereas *C. glabrata* was recorded from Day 6 (80 CFU 100 ml^{-1}), both with averages of 13 ± 31 over the six sampling days (Fig. 1).

The yeast cell counts were generally more elevated in the sand samples as compared with water samples; however, in the sand samples, the majority of the yeasts were represented by red yeasts, unlike water where white yeasts were the majority (Fig. 1). *Candida tropicalis* showed the highest frequency among white yeasts with an average of 15 CFU g^{-1} (values averaged across sand types) (Fig. 1). Other pathogenic yeasts yielded averages of 1·1, 1·3 and 1·3 CFU g^{-1} (*C. guilliermondii*, *C. glabrata* and *C. parapsilosis*, respectively) in sand (values averaged across sand types) (Fig. 1).

Table 1 Significant correlations in sand samples observed between different microbes and between microbes and environmental parameters using the nonparametric Spearman rank order method. Statistically significant correlations were characterized by *P*-values <0·05. Correlation values were computed for data sets with more than 10% positive samples [$n \geq 4$]. MRSA, *Cryptosporidium*, enterovirus, human Bacteroidales (UCD) marker, *Candida glabrata*, *Candida guilliermondii* and *Candida parapsilosis* were excluded on this basis

Organism	Correlated with	*r*	*P*
Enterococci (MF)	Moisture content (%)	−0·822	<0·001
	Enterococci (CS)	0·939	<0·001
	Faecal coliform	0·831	<0·001
	Red yeasts	0·758	<0·001
	White yeasts	0·748	<0·001
	Nematode larvae	0·800	<0·001
Enterococci (CS)	Moisture content (%)	−0·755	<0·001
	White yeasts	0·819	<0·001
	Nematode larvae	0·740	<0·001
Enterococci (qPCR)	Moisture content (%)	−0·558	0·001
	Total Bacteroidales (AllBac)	0·822	<0·001
Faecal coliform	Moisture content	−0·816	<0·001
	Red yeasts	0·852	<0·001
	White yeasts	0·782	<0·001
	Nematode larvae	0·789	<0·001
C. perfringens	Moisture content (%)	0·504	0·002
Red yeast	Moisture content	−0·815	0·025
	White yeasts	0·690	<0·001
	Nematode larvae	0·701	<0·001
White yeast	Moisture content (%)	−0·543	0·001
	48-h rainfall	0·503	0·002
	Nematode larvae	0·607	<0·001
Nematode larvae	Moisture content (%)	−0·761	<0·001
	Nematode eggs	0·619	<0·001
	Temperature (sand)	−0·651	<0·001
	48-h rainfall	0·620	<0·001

MF, membrane filtration; CS, chromogenic substrate.

In the sand samples, red yeasts were found to correlate strongly and significantly with white yeasts ($r = 0{\cdot}69$; $P < 0{\cdot}001$). In addition, red yeasts ($r = -0{\cdot}82$; $P = 0{\cdot}03$) and white yeasts ($r = -0{\cdot}54$; $P = 0{\cdot}001$) were found to significantly and inversely correlate with moisture content (Table 1). Forty-eight hour rainfall positively correlated significantly with white yeasts (0·50; $P = 0{\cdot}002$) (Table 1). In reference to other indicator microbes, red yeasts correlated significantly and positively with enterococci by MF ($r = 0{\cdot}76$; $P < 0{\cdot}001$) and faecal coliforms ($r = 0{\cdot}85$; $P < 0{\cdot}001$) (Table 1). White yeasts also correlated with faecal coliforms ($r = 0{\cdot}78$; $P < 0{\cdot}001$) (Table 1).

No other significant correlations were identified between yeast species and other microbial indicators. There was no statistical difference between dry, wet or inundated sand samples for any of the *Candida* species.

MST markers and bacterial pathogens

All sand samples were below detection limits for polyomavirus, while the human-specific esp gene in *Ent. faecium* was detected in 9 of 36 samples (three detects in dry samples, one in wet sample and five in inundated samples), with no statistical differences found between sand types (Table 2), and no significant correlation with environmental parameters. In addition, six of the nine positive samples for the esp gene in *Ent. faecium* were observed within the last 3 days. Total Bacteroidales levels averaged 427 000 GEU g^{-1} for all samples with high variance between individual days (Table 2) with the highest levels observed for wet sand. The human-specific Bacteroidales marker (BacHum-UCD) was found in low abundance and in only two samples (dry-Day 1 and inundated-Day 4) (Table 2). The human-specific Bacteroidales HF8 marker was not found in any sand sample (Table 2). Relatively high levels of canine-specific Bacteroidales DogBac marker were observed in the sand with the highest levels in dry (904 TSC (Target Sequence Copies) g^{-1}) and lower levels in wet (335 TSC g^{-1}) and inundated sand (352 TSC g^{-1}) (Table 2). Nine of the 11 positive samples for dog Bacteroidales occurred during the last 3 days of sampling, in comparison with the two remaining positive samples on the first 3 days of sampling. No statistical difference was noted between sand types for any Bacteroidales type.

Vibrio vulnificus was found ubiquitously throughout all sand samples (Table 2). *Staphylococcus aureus* levels ranged from 0·5 to 66 CFU g^{-1} in the nine positive sand samples with an average of 6·0 CFU g^{-1} (Table 2). MRSA was found in three of the sand samples, and all MRSA samples were found in the dry sand (Table 2).

Protozoa and viral pathogens

Cryptosporidium spp. was detected at 0·63 oocysts per 100 g sand in a wet sand sample on Day 4. *Giardia* spp. was below the detection limit of 0·2 cysts per 100 g dry sand in all of the samples. Enterovirus was detected at 1·4 infectious units per 100 g in a dry sand sample on Day 5 and at 0·24 infectious units per 100 g in an inundated sand sample on Day 6. Subsequent TEM analysis confirmed the presence of viruses in these samples (See Data S1 for representative images).

Helminthes

Helminthes sand analysis revealed ten positive samples for nematode larvae and four positive samples for nematode eggs. For nematode larvae, values ranged from 0 to 74 larvae per 100 g sand with averages of 27·6 (dry), 3·4

Table 2 Averages of microbial indicators, microbial source tracking markers and pathogens. Samples measuring below detection limit were set at ½ the detection limit value for computational purposes and if all data points within a set (water, dry, wet, inundated) were below detection, then the ½ detection limit value is preceded below by '<'. Yeast levels and remaining microbial indicators are provided in Fig. 1. Expanded data tables are provided in the Data S1

		Sand			
Averages	Water (100 ml^{-1})	Dry	Wet	Inundated	Units
Somatic coliphage	NA	12/12	12/12	12/12	No. positive per total
F+ coliphage	NA	0/12	0/12	0/12	No. positive per total
Total Bacteroidales (AllBac)	18 000	150 000	826 000	304 000	GEU per g dry sand
Bacteroidales (human UCD)	0·9	0·8	<0·6	1·0	GEU per g dry sand
Bacteroidales (human HF8)	<0·5	<0·5	<0·5	<0·5	GEU per g dry sand
Bacteroidales (dog)	71	904	335	352	TSC per g dry sand
esp gene in *Enterococcus faecium* (human)	NA	3/12	1/12	5/12	No. positive per total
Polyomavirus (HPyVs)	NA	0/18	0/18	0/18	No. positive per total
Staphylococcus aureus	<1	12·5	1·9	<1·2	CFU per g dry sand
MRSA	<1	5·0	<1·2	<1·2	CFU per g dry sand
Vibrio vulnificus	NA	12/12	12/12	12/12	No. positive per total
Cryptosporidium spp.	NA	0/12	1/12*	0/12	No. positive per total
Giardia spp.	NA	0/12	0/12	0/12	No. positive per total
Enterovirus	NA	1/12†	0/12	1/12†	No. positive per total
Nematode larvae	NA	27·7	4·0	1·7	Number per 100 g sand
Nematode eggs	NA	6·4	1·2	1·5	Number per 100 g sand

NA, not analysed; MRSA, methicillin-resistant *Staph. aureus*.

*Sample concentration of 0·63 oocysts per 100 g sand for the one positive sample.

†Sample concentration of 1·4 per 100 g sand for the one positive dry sample and 0·24 per 100 g sand for the one positive inundated sample.

(wet) and 1·0 (inundated) larvae per 100 g sand; nematode eggs were only found on Days 4 and 5 (Table 2). Significant correlations were noted between nematode eggs in the sand and environmental factors such as sand temperature ($r = -0{\cdot}65$; $P < 0{\cdot}001$) and 48-h rainfall ($r = 0{\cdot}62$; $P < 0{\cdot}001$) (Table 1). Moisture content significantly and inversely correlated with the number of nematode larvae in the sand ($r = -0{\cdot}76$; $P < 0{\cdot}001$) (Table 1). Nematode larvae were also found to positively and significantly correlate with several indicator microbes and pathogens such as enterococci by MF (r = 0·80; $P < 0{\cdot}001$), enterococci by CS ($r = 0{\cdot}74$; $P < 0{\cdot}001$), faecal coliforms ($r = 0{\cdot}79$; $P < 0{\cdot}001$), red yeasts ($r = 0{\cdot}70$; $P < 0{\cdot}001$) and white yeasts ($r = 0{\cdot}61$; $P < 0{\cdot}001$) (Table 1). The results also indicated a significant positive relationship between nematode eggs and larvae ($r = 0{\cdot}62$; $P < 0{\cdot}001$) (Table 1).

Discussion

As a pilot study at a non-point source subtropical recreational beach, this investigation evaluated the presence and distribution of pathogens in beach sands and assessed their relationship with environmental parameters and indicator microbes. Given prior studies at non-point source beaches that demonstrate human illness, but not necessarily a link between the indicator microbes and human health outcomes (Colford *et al.* 2007; Fleisher *et al.* 2010), the aim of this research was to explore sand as an environmental reservoir of indicator microbes and pathogens, thus representing one step towards identifying a possible cause for the human health outcomes. The uniqueness of this research was in the study of several different classes of indicator microbes and pathogens (including bacteria, viruses, protozoa, yeasts and helminthes) in different sand zones (dry, wet and inundated) as potential sources for water contamination.

Indicator microbes and MST markers

The choice of analytical methods for all indicator microbes may be a significant source of variance that should be considered in beach monitoring and research. However, the juxtaposition of enterococci detection methods revealed no statistical difference between MF, CS and qPCR methods in sand, although previous data had noted that qPCR measurements were generally higher in sand (Abdelzaher *et al.* 2010) and water (Haugland *et al.* 2005; Sinigalliano *et al.* 2007). This lack of statistical difference may have been attributed to the relatively large range of concentration values between the different sampling zones and the relatively few numbers of samples. Significant correlations for enterococci in sand were noted between MF and CS; however, correlations were

not noted between qPCR and other methods; this has been shown in prior research and might be explained by the fact that qPCR can also detect target DNA from non-viable and nonculturable cells, whereas the culture-based methods only detect viable cells (Abdelzaher *et al.* 2010).

Spatial differences were demonstrated for indicator microbes (enterococci, faecal coliforms, *E. coli*), because 3–10 times more indicator microbes were detected in the dry sand *vs* the water samples. Additionally, indicator microbes in the dry sand always resulted in the highest CFU g^{-1} compared with the wet and inundated sand samples (with the exception of *C. perfringens*). Negative correlations were identified between indicator microbes and moisture content except for *C. perfringens* which had a positive correlation with moisture content. This confirms the fact that indicator microbes are found in sand and are found in higher concentrations in dry sand *vs* water samples and in both wet and inundated sand (Alm *et al.* 2003; Bonilla *et al.* 2007; Abdelzaher *et al.* 2010; Wright *et al.* 2011).

Temporal differences were demonstrated for indicator microbes in water samples specifically between the first and last 3 days. Over the course of the last 3 days, water samples exceeded the EPA recommended regulatory guideline for enterococci (104 CFU 100 ml^{-1}) with an average of 169 CFU 100 ml^{-1} in comparison with 29 CFU 100 ml^{-1} over the first 3 days for enterococci by MF and comparable values by CS and qPCR. For sand, statistical tests demonstrated that enterococci measurements by qPCR were significantly greater during the last 3 days when compared with the first 3 days for dry, wet and inundated samples, but the same statistical significance was not observed for MF and CS measurements.

The reason for these temporal differences in enterococci values in water samples by all three analysis methods and sand samples by qPCR may be related to changes in tide and precipitation. Although all sampling occurred approx. 1 h after peak high tide, the first three sampling days had an average tidal height of 0·7 m lower than the last 3 days. The effects of tidal height may be a natural contributor to the detachment of indicator microbes in sand and subsequent release in the water. The last 3 days were characterized by wetter conditions relative to the first 3 days, which suggests 'wash in' of enterococci during rain events. Both run-off during rain events (Rogerson *et al.* 2003; Jiang *et al.* 2007; Abdelzaher *et al.* 2010) and elevated tide (Shibata *et al.* 2004; Boehm and Weisberg 2005; Abdelzaher *et al.* 2010; Wright *et al.* 2011) are known to increase enterococci values in the water column via washing in from the sand.

MST markers also displayed temporal and spatial differences throughout the sampling zones. Specifically, the average of the canine-specific MST marker samples in dry sand was 2–3 times more than wet and inundated sand. Some MST markers also revealed increased temporal variability similar to the indicator microbes. Nine of the 11 positive samples for canine-specific Bacteroidales and six of the nine positive samples for the esp gene in *Ent. faecium* were observed within the last 3 days of sampling, possibly also because of the increased rainfall and tidal height on the last three sampling days. However, other MST markers did not show similar spatial or temporal trends possibly because of the rarity of detects.

Pathogens

In a study conducted by WHO/UNEP (1994), sand sediments were identified as a necessary component of microbiological studies to evaluate health risks. Some studies have shown that beach sand does not pose a significant health risk because of the lack of infectious hazards (Chabasse *et al.* 1986), while other studies have corroborated the relationship of beach sand to health factors. For example, a study published by Heaney *et al.* (2009) indicated a positive association between persons digging in the sand and the development of reported gastrointestinal illnesses, with an even stronger correlation noticed for those who reported burial in sand.

A variety of pathogens were identified and quantified in this study including pathogenic yeasts (*C. tropicalis*, *C. guilliermondii*, *C. glabrata*, *C. parapsilosis*), pathogenic bacteria (*Staph. aureus* including MRSA and *V. vulnificus*), protozoa (*Cryptosporidium* spp.), viruses (enterovirus) and helminthes. Spatial and temporal differences similar to those seen for indicator microbes were noted for some of the pathogens detected in this study. *Staphylococcus aureus*, yeasts and nematode larvae were more abundant in the dry sand compared with the water and other sand samples, while MRSA was only observed within the dry sand. *Staphylococcus aureus* was 3–4 times higher in dry sand than in wet and inundated sand. Nematode larvae were observed ten times more in dry sand than in wet or inundated sand. Positive correlations were noted between indicator microbes and both white yeasts and nematode larvae. *Vibrio vulnificus* was found ubiquitously in all sand samples, which is consistent with previous studies (Sinigalliano *et al.* 2007).

Evaluating temporal trends for the pathogens, the one *Cryptosporidium* spp. detect (in wet sand), two enterovirus detects (in dry sand and inundated sand) and seven of nine samples of *Staph. aureus* (two of three MRSA) samples occurred on the last 3 days of sampling when the tide was the highest and higher precipitation occurred. These observations are consistent with the hypothesis that indicator microbes indirectly monitor several pathogens through common factors, at least in sand. However, for

pathogens such as *Cryptosporidium* spp., *Giardia* spp., enterovirus, generalizations about the predictive ability of indicator microbes are made with caution owing to the sparseness and rarity of detects.

Other studies have also detected many of the pathogens detected in this study in beach sand. Enterovirus was found in beach sand and seawater at low, variable levels depending on holiday season (Nestor *et al.* 1984). Vogel *et al.* (2007) found 21 different species of yeasts at three different beaches in South Florida in 17 collections distributed over 1 year's period. Previous studies have also shown the presence of pathogenic helminthes such as stronglydia, *Toxocaris leonina* and *Toxocaris* spp. in beach sand (Paquet-Durand *et al.* 2007). These helminthes have been shown to cause mild to severe forms of dermatitis, skin ulcers and a variety of GI conditions including bloody diarrhoea and GI inflammation (Warrington 2001).

Overall, the results indicate that the sand may be a potential reservoir as well as the avenue of release to the water column (Oshiro and Fujioka 1995; Ferguson *et al.* 2005; Yamahara *et al.* 2007), for both indicator bacteria and pathogens at non-point source marine beach waters. This suggestion provides a route of possible exposure for human health effects at non-point source beaches and suggests that indicator organisms may be useful in predicting this risk at non-point source beaches.

Recommendations

The presence of the pathogens found in this study can potentially represent a significant health risk to beach goers, and a risk analysis should be conducted to analyse the risk to sand exposure. For example, for enterovirus in particular, a dose as low as 1–2 infectious units could produce infection as observed by previous studies (Koporski 1955; Suptel 1963), and for *Cryptosporidium* spp., 2–10 oocysts in water have been observed to cause infections (Jenkins *et al.* 1997; Chen *et al.* 2003). Ultimately, the data from the current study may also be used in risk assessment models to estimate the human health implications of activities such as playing in the sand or water bathing.

The rare incidence of pathogen isolation does not necessarily equate to a lower risk for bathers. Differences in actual presence of pathogens and detected pathogens may be significant because of technical inabilities to accurately measure all pathogens in the sand. Pathogens may reside on the sand surface, in crevices, or may also reside in biofilms that adhere to sand, which would complicate pathogen quantification (Wang *et al.* 2010). To avoid these complications, a multiple method study to explore pathogenic presence in sand may be helpful in determining the most effective measurement protocol.

Because of the broad nature of this study, fiscal limitations set hurdles for the definitive modelling of pathogen relations and interactions in the sand. A more complete and extensive study of pathogens in the sand at other non-point source beaches would help explore the nature of pathogens in subtropical environments and would support the need for monitoring beach sand. Another study, similar to the one conducted by Hartz *et al.* (2008) on *E. coli*, investigating the survivability of pathogens in a controlled beach environment would also provide insight into the general trends of pathogen survival in sand.

Although indicator bacteria did not correlate consistently with pathogens in this study, the fact that both can originate from sand and that correlations were noted between certain pathogens and indicator microbes provides value to measurements of indicator bacteria as predictors of health effects from pathogens at non-point source subtropical marine beaches.

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References

Abdelzaher, A., Wright, M.E., Ortega, C., Solo-Gabriele, H.M., Miller, G., Elmir, S., Newman, X., Shih, P. *et al.* (2010) Presence of pathogens and indicator microbes at a non-point source subtropical recreational marine beach. *Appl Environ Microbiol* **76**, 724–732.

Abdelzaher, A.M., Wright, M.E., Ortega, C., Hasan, A.R., Shibata, T., Solo-Gabriele, H.M., Kish, J., Withum, K. *et al.* (2011) Daily measures of microbes and human

health at a non-point source marine beach. *J Water Health*, in press.

Alexander, L.M., Heaven, A., Tennant, A. and Morris, R. (1992) Symptomatology of children in contact with sea water contaminated by sewage. *J Epidemiol Community Health* **46**, 340–344.

Alm, E.W., Burke, J. and Spain, A. (2003) Fecal indicator bacteria are abundant in wet sand at freshwater beaches. *Water Res* **37**, 3978–3982.

Bernhard, A.E. and Field, K.G. (2000a) A PCR assay to discriminate human and ruminant feces on the basis of host differences in Bacteroides–Prevotella genes encoding 16S rRNA. *Appl Environ Microbiol* **66**, 4571–4574.

Bernhard, A.E. and Field, K.G. (2000b) Identification of non-point sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes. *Appl Environ Microbiol* **66**, 1587–1594.

Beversdorf, L.J., Bornstein-Forst, S.M. and McLellan, S.L. (2007) The potential for beach sand to serve as a reservoir for *Escherichia coli* and the physical influences on cell die-off. *J Appl Microbiol* **102**, 1372–1381.

Boehm, A.B. and Weisberg, S.B. (2005) Tidal forcing of enterococci at marine recreational beaches at fortnightly and semidiurnal frequencies. *Environ Sci Technol* **39**, 5575–5583.

Boehm, A.B., Fuhrman, J.A., Morse, R.B. and Grant, S.B. (2003) Tiered approach for identification of a human fecal pollution source at a recreational beach: case study at Avalon Bay, Catalina Island, California. *Environ Sci Technol* **37**, 673–680.

Boehm, A.B., Griffith, J., McGee, C., Edge, T.A., Solo-Gabriele, H.M., Whitman, R., Cao, Y., Getrich, M. *et al.* (2009) Faecal indicator bacteria enumeration in beach sand: a comparison study of extraction methods in medium to coarse sands. *J Appl Microbiol* **107**, 1740–1750.

Bonilla, T.D., Nowosielski, K., Cuvelier, M., Hartz, A., Green, M., Esiobu, N., McCorquodale, D.S., Fleisher, J.M. *et al.* (2007) Prevalence and distribution of fecal indicator organisms in South Florida beach sand and preliminary assessment of health effects associated with beach sand exposure. *Mar Pollut Bull* **54**, 1472–1482.

Chabasse, D., Laine, P., Simitzis-Le-Flohic, A.M., Martineau, B., el Hourch, M. and Becaud, J.P. (1986) Sanitary study of surface water and of the beach of a water sports and leisure complex. *J Hyg* **96**, 393–401.

Chen, W., Harp, J.A. and Harmsen, A.G. (2003) *Cryptosporidium parvum* infection in gene-targeted B cell-deficient mice. *J Parasitol* **89**, 391–393.

Colford, J.M., Wade, T.J., Schiff, K.C., Wright, C.C., Griffith, J.F., Sandhu, S.K., Burns, S., Sobsey, M. *et al.* (2007) Water quality indicators and the risk of illness at non-point source beaches in Mission Bay, California. *Epidemiology* **18**, 27–35.

Diaz, M.R., Dunbar, S. and Jacobson, J.W. (2008) Multiplexed detection of fungal nucleic acid signatures. *Curr Protoc Cytom* **44**, 13.9.1–13.9.20.

Dick, L.K., Simonich, M.D. and Field, K.G. (2005) Microplate subtractive hybridization to enrich for *Bacteroidales* genetic markers for fecal source identification. *Appl Environ Microbiol* **71**, 3179–3183.

Ferguson, D.M., Moore, D.F., Getrich, M.A. and Zhowandi, M.H. (2005) Enumeration and speciation of enterococci found in marine and intertidal sediments and coastal water in southern California. *J Appl Microbiol* **99**, 598–608.

Fleisher, J.M., Fleming, L.E., Solo-Gabriele, H.M., Kish, J., Sinigalliano, C.D., Plano, L.R.W., Elmir, S.M., Wang, J.D. *et al.* (2010) BEACHES study: health effects and exposures from non-point source microbial contaminants in subtropical recreational marine waters. *Int J Epidemiol* **3**, 1–8.

Florida Department of Health (2009) Florida Healthy Beaches Program. Florida Dept. of Health. Available at: http://esetappsdoh.doh.state.fl.us/irm00beachwater/default.aspx (accessed 5 October 2009).

Gordon, K.V., Vickery, M.C., DePaola, A., Staley, C. and Harwood, V.J. (2008) Real-time PCR assays for quantification and differentiation of *V. vulnificus* strains in oysters and water. *Appl Environ Microbiol* **74**, 1704–1709.

Haile, R.W., Witte, J.S., Gold, M., Cressey, R., McGee, C., Millikan, R.C., Glasser, A., Harawa, N. *et al.* (1999) The health effects of swimming in ocean water contaminated by storm drain runoff. *Epidemiology* **10**, 355–363.

Hartz, A., Cuvelier, M., Nowosielski, K., Bonilla, T.D., Green, M., Esiobu, N., McCorquodale, D.S. and Rogerson, A. (2008) Survival potential of *E. coli* and enterococci in subtropical beach sand: implications for water quality managers. *J Environ Qual* **37**, 898–905.

Haugland, R.A., Seifring, S.C., Wymer, L.J., Brenner, K.P. and Dufour, A.P. (2005) Comparison of *Enterococcus* measurements in freshwater at two recreational beaches by quantitative polymerase chain reaction and membrane filter culture analysis. *Water Res* **39**, 559–568.

Heaney, C.D., Sams, E., Wing, S., Marshall, S., Brenner, K., Dufour, A.P. and Wade, T.J. (2009) Contact with beach sand among beachgoers and risk of illnesses. *Am J Epidemiol* **170**, 164–172.

Jenkins, M.B., Anguish, L.J., Bowman, D.D., Walker, M.J. and Ghiorse, W.C. (1997) Assessment of a dye permeability assay for determination of inactivation rates of *Cryptosporidium parvum* oocysts. *Appl Environ Microbiol*, **63**, 1.

Jiang, S., Noble, R., Chu, W. and He, J. (2007) Seasonal detection of human viruses and coliphage in Newport Bay, California. *Appl Environ Microbiol* **73**, 6468–6474.

Kaysner, C.A. and DePaola, A. (2004) Bacteriological Analytical Manual Online: Chapter 9 Vibrio. U.S. FDA. Available at: http://www.cfsan.fda.gov/~ebam/bam-9.html (accessed 21 May 2008).

Kildare, B.J., Leutenegger, C.M., McSwain, S.M., Bambic, D.G., Rajal, V.B. and Wuertz, S. (2007) 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal Bacteroidales: a Bayesian approach. *Water Res* **41**, 3701–3715.

Koporski, H.J. (1955) Living attenuated poliomyelitis virus as immunizing agent in man. *S Afr Med J* **29**, 1134–1142.

McQuaig, S.M., Scott, T.M., Harwood, V.J., Farrah, S.R. and Lukasik, J.O. (2006) Detection of human-derived fecal pollution in environmental waters by use of a PCR based human polyomavirus assay. *Appl Environ Microbiol* **72**, 7567–7574.

Mertz, P.M., Cardenas, T.C., Snyder, R.V., Kinney, M.A., Davis, S.C. and Plano, L.R. (2007) *Staphylococcus aureus* virulence factors associated with infected skin lesions: influence on the local immune response. *Arch Dermatol*, **143**, 1259–1263.

Nestor, I., Costin-Lazar, L., Sovrea, D. and Ionescu, N. (1984) Detection of enterovirus in seawater and beach sand. *Zentralbl Bakteriol Mikrobiol Hyg B* **178**, 527–534.

Oshiro, R. and Fujioka, R. (1995) Sand, soil and pigeon droppings: sources of indicator bacteria in the waters of Hanauma Bay, Hawaii. *Water Sci Technol* **31**, 251–254.

Papadakis, J.A., Mavrido, A., Richardson, S.C., Lambiri, M. and Marcelou, U. (1997) Bather related microbial and yeast populations in sand and seawater. *Water Res* **31**, 799–804.

Paquet-Durand, I., Hernandez, J., Dolz, G., Zuniga, J.J., Schneider, T. and Epe, C. (2007) Prevalence of *Toxicara* spp., *Toxacaris leonina*, and ancylostomidae in public parks and beaches in different climate zones in Costa Rica. *Acta Trop* **104**, 31–37.

Plano, L.R.W., Garza, A.C., Shibata, T., Elmir, S.M., Kish, J., Sinigalliano, C.D., Gidley, M.L., Miller, G. *et al.* (2011) Shedding of *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* from adult and pediatric bathers in marine waters. *BMC Microbiology* **11**, 1–10.

Rogerson, A., Estiobu, N. and McCorquodale, D. (2003) *Final Report: Prevalence and Survival of Microorganisms in Shoreline Interstitial Waters: A Search for Indicators of Health Risks*. Ft. Lauderdale, FL: Environmental Protection Agency. Nova Southeastern University Oceanographic Center. EPA Grant Number: R828830.

Scott, T.M., Jenkins, T.M., Lukasik, J. and Rose, J.B. (2005) Potential use of a host associated molecular marker in *Enterococcus faecium* as an index of human fecal pollution. *Environ Sci Technol* **39**, 283–287.

Shibata, T., Solo-Gabriele, H.M., Fleming, L. and Elmir, S. (2004) Monitoring marine recreational water quality using multiple microbial indicators in an urban tropical environment. *Water Res* **38**, 3119–3131.

Shopsin, B., Gomez, M., Montgomery, S.O., Smith, D.H., Waddington, M., Dodge, D.E., Bost, D.A., Riehman, M. *et al.* (1999) Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. *J Clin Microbiol* **37**, 3556–3563.

Shuval, H. (2003) Estimating the global burden of thalassogenic diseases: human infectious diseases caused by wastewater pollution of the marine environment. *J Water Health* **2**, 53–64.

Siefring, S., Varma, M., Atikovic, E., Wymer, L. and Haugland, R.A. (2008) Improved real-time PCR assays for the detection of fecal indicator bacteria in surface waters with different instrument and reagent systems. *J Water Health* **6**, 225–237.

Sinigalliano, C.D., Gidley, M.L., Shibata, T., Whitman, D., Dixon, T.H., Laws, E., Hon, A., Bachoon, D. *et al.* (2007) Impacts of hurricanes Katrina and Rita on the microbial landscape of the New Orleans area. *PNAS* **104**, 9029–9034.

Sinigalliano, C.D., Fleisher, J.M., Gidley, M.L., Solo-Gabriele, H.M., Shibata, T., Plano, L.R.W., Elmir, S.M., Wanless, D. *et al.* (2010) Traditional and molecular analyses for fecal indicator bacteria in non-point source subtropical recreational waters. *Water Res* **44**, 3763–3772.

Sloss, M.W., Kemp, R.L. and Zajac, A.M. (1994) *Veterinary Clinical Parasitology*, 6th edn. Ames, IA: Iowa State University Press.

Suptel, E.A. (1963) Pathogenesis of experimental coxsackie virus infection. *Acta Virol* **7**, 61–66.

USEPA (2001a) *Method 1602: Detection of Male-specific (F+) and Somatic Coliphage in Water by Single Agar Layer (SAL) Procedure*. EPA 821-R-01-029. Washington, DC: Office of Water, Engineering and Analysis Division, US Environmental Protection Agency.

USEPA (2001b) *USEPA Manual of Methods for Virology*. EPA 600/4-84/013. Washington, DC: US Environmental Protection Agency.

USEPA (2002) *Method 1600: Membrane Filter Test Method for Enterococci in Water*, EPA-821-R-02-022. Washington, DC: US Environmental Protection Agency.

USEPA (2004) Water quality standards for coastal and Great Lakes recreation waters: proposed rule. *Fed Regist* **69**, 41720–41742.

Van Elsas, J.D. and Smalla, K. (1997) *Methods for Sampling Soil Microbes. Manual of Environmental Microbiology*. Washington, DC: ASM Press, pp. 383–390.

Vogel, C., Rogerson, A., Schatzc, S., Laubache, H., Tallman, A. and Fell, J. (2007) Prevalence of yeasts in beach sand at three bathing beaches in South Florida. *Water Res* **41**, 1917–1920.

Wang, J., Solo-Gabriele, H.M., Abdelzaher, A. and Fleming, L. (2010) Estimation of enterococci input from bathers and animals on a recreational beach using camera images. *Mar Pollut Bull* **60**, 1270–1278.

Warrington, P. (2001) Aquatic Pathogens: Helminth Worms. Environmental Protection Division: Water Quality. Government of British Colombia. Available at: http://www.env.gov.bc.ca/wat/wq/reference/helminth.html (accessed 5 May 2010).

WHO/UNEP (1994) Microbiological quality of coastal recreational waters. *Marine Pollution Monitoring and Research Programme* **2**, 5.

Wright, M.E., Solo-Gabriele, H.M., Elmir, S. and Fleming, L. (2009) Microbial load from animal feces at a recreational beach. *Mar Pollut Bull* **58**, 1649–1656.

Wright, M.E., Solo-Gabriele, H.M., Abdelzaher, A.M., Elmir, S. and Fleming, L.E. (2011) The inter-tidal zone is the geographic location of elevated concentrations of enterococci. *Water Sci Technol*, **6303**, 542–549.

Yamahara, K.M., Layton, B.A., Santoro, A.E. and Boehm, A.B. (2007) Beach sands along the California coast are diffuse sources of fecal bacteria to coastal waters. *Environ Sci Technol* **41**, 4515–4521.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1 Illustration of transects, indicator microbes, microbial source tracking markers and pathogenic microbes

Figure S1 Distance between the 'dry', 'wet', and 'inundated' sampling transects during each of the six sampling dates

Figure S2 Virus images taken with transmission electron microscope

Figure S3 Moisture content vs. enterococci (MF) for all sand samples ($R^2 = 0{\cdot}68$ for exponential line of best fit and $r = -0{\cdot}82$ for non parametric correlations)

Table S1 Expanded table: averages of microbial indicators and pathogens

Table S2 Individual values for microbiological parameters for each sampling day

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