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Sources and persistence of fecal indicator bacteria and *Bacteroidales* in sand as measured by culture-based and culture-independent methods: A case study at Santa Monica Pier, California

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Abstract

This study investigated causes of persistent fecal indicator bacteria (FIB) in beach sand under the pier in Santa Monica, CA. FIB levels were up to 1,000 times higher in sand underneath the pier than that collected from adjacent to the pier, with the highest concentrations under the pier in spring and fall. *Escherichia coli* (EC) and enterococci (ENT) under the pier were significantly positively correlated with moisture ($\rho = 0.61$, $p < 0.001$, $n = 59$; $\rho = 0.43$, $p < 0.001$, $n = 59$, respectively), and ENT levels measured by qPCR (qENT) were much higher than those measured by membrane filtration (cENT). Microcosm experiments tested the ability of EC, qENT, cENT, and general *Bacteroidales* (GenBac) to persist under in-situ moisture conditions (10% and 0.1%). Decay rates of qENT, cENT, and GenBac were not significantly different from zero at either moisture level, while decay rates for EC were relatively rapid during the microcosm at 10% moisture ($k = 0.7 \text{ days}^{-1}$). Gull/pelican marker was detected at eight of 12 sites and no human-associated markers (TaqHF183 and HumM2) were detected at any site during a one-day site survey. Results from this study indicate that the high levels of FIB observed likely stem from environmental sources combined with high persistence of FIB under the pier.

Keywords

Fecal Indicator Bacteria; General *Bacteroidales*; microbial source tracking; sand; moisture content; qPCR; culture-based detection

Introduction

Understanding persistent fecal indicator bacteria (FIB) populations in sand and characterizing their sources is critical to addressing coastal water quality, as FIB levels in sand have been linked to water quality in several studies. For example, enterococci (ENT) concentrations in sand and water correlated at eight South Florida beaches and lower ENT concentrations in sand resulted in fewer ENT exceedances in overlying waters (Phillips et al.

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2011). Further, the amount of ENT entering the water column was roughly matched to that leaving the sand to coastal waters in Lover's Point, CA (Yamahara et al. 2007), and ENT growth in streambed sediments was likely the cause of a substantial fraction of ENT measured in the Santa Ana River (Litton et al. 2010). FIB loads from sand have also been shown to be an important input for modeling FIB values seen in the water under certain weather conditions (Feng et al. 2013; Sanders, Arega et al. 2005; Paul et al. 2004). In addition, ENT levels have been shown to increase in water by being washed out of the sand during storms (Jiang et al. 2007; Abdelzaher et al. 2010; Rogerson et al. 2003) and higher tides (Boehm and Weisberg 2005; Abdelzaher et al. 2011; Yamahara et al. 2007).

Further elucidating the dynamics of bacterial populations in sand is critical for reasons beyond the impact of bacteria in sand on overlying or adjacent water bodies. Sand itself may provide an alternate pathway for beachgoer exposure to FIB and pathogens. Sand has been shown to harbor high concentrations of FIB (Yamahara et al. 2007; Halliday and Gast 2011) as well as pathogens (Shah et al. 2011; Yamahara et al. 2012). An increased risk of gastrointestinal (GI) illnesses with increased exposure to sand has been observed in recent studies (Heaney et al. 2009; Heaney et al. 2012). Identifying the distribution and persistence of FIB and other pathogens in beach sand is also necessary to understand the implications of incorporating beach sand into policy such as monitoring frameworks (Halliday and Gast 2011), as excluding sand from beach monitoring may create a gap in assessing health risks present in beach areas (Sabino et al. 2014).

Moisture content is among the more important factors that impact bacterial survival in natural environments such as sand. In many microcosm studies looking at culturable FIB in sand, the addition of moisture results in either an increase in concentration or no effect (Yamahara et al. 2012; Yamahara et al. 2009; Mika et al. 2009; Desmarais et al. 2002; Eichmiller et al. 2014). Further research into the effects of moisture on bacterial survival is necessary, however, as some beach surveys have found the highest concentration of bacteria in dry sand well above the area wetted by tidal inputs (Feng et al. 2013; Shah et al. 2011; Phillips et al. 2011; Coakley et al. 2016).

A deeper understanding of the ability of FIB and associated markers currently used to monitor water quality to survive in coastal sediments is critical. Analyzing ENT persistence in coastal sand using both qPCR and culture-based methods is particularly pertinent as the EPA has recommended ENT as the primary FIB for marine environments in its 2012 recreational water quality criteria, with qPCR as an alternative rapid approach to measuring ENT (US EPA Method 1611. 2012). In addition, ENT measured by both qPCR and culture methods has been linked to GI illnesses (Colford et al. 2012). Generally, ENT concentrations detected by qPCR are higher than those detected by culture-based methods in sand (Yamahara et al. 2009; Yamahara et al. 2012; Heaney et al. 2012). While several studies have examined the persistence of ENT in sand (Feng et al. 2010; Mika et al. 2009; Yamahara et al. 2012), limited studies have investigated the persistence of a native population of ENT in sand using qPCR and culture-based methods in tandem (Yamahara, Walters, and Boehm 2009). Shedding light on the correlation between these two detection methods in sand provides valuable information to water quality managers in determining which method is

more appropriate as previous studies in sand and sediment have seen poor correlations (Shah et al. 2011; Rogers et al. 2011).

In addition to including molecular detection technologies such as qPCR, the new EPA recreational water quality guidelines recognize the potential for new indicators that may provide alternative ways to address practical considerations such as sensitivity and specificity (US EPA. 2012; US EPA Method B. 2010). Recent studies have shown *Bacteroidales* to be an effective indicator of both host-associated and general fecal pollution (Okabe and Shimazu 2007; Kildare et al. 2007; Yampara-Iquise et al. 2008; Boehm et al. 2013; Layton et al. 2013). Studies have also linked general *Bacteroidales* (GenBac) to GI illnesses in coastal environments (Wade et al. 2006; Wade et al. 2009; Heaney et al. 2012). Further, sand may be an important factor in GenBac persistence in the environment as the presence of sediment in water was found to enhance the persistence of the *Bacteroidales* markers (Dick et al. 2010). To date, limited studies have looked at GenBac persistence in beach sands (Yamahara et al. 2012; Eichmiller et al. 2014) or sediments (Rogers et al. 2011).

Characterizing sources of FIB pollution in a chronically impaired area such as the Santa Monica pier is also important to provide information to responsible parties that can effectively target and remediate sources [e.g., identifying beaches or sites with a high incidence of human-associated indicators can lead to improved water quality through sewer main repairs and relocation of portable restrooms (Korajkic et al. 2011)]. Other studies using host-associated markers have been useful in identifying potential environmental sources such as stormwater or sand, bird-droppings, and contaminated subsurface waters (Haack et al. 2003; Hansen et al. 2011; Boehm et al. 2003; Kinzelman and McLellan 2009). Ratios of different markers and indicators, ranging from fecal stanol ratios (Jeanneau et al. 2012) to ratios of human-associated markers to GenBac or FIB (Boehm et al. 2013; Layton et al. 2013; McQuaig et al. 2012), can also be used as part of a source identification toolbox to characterize pollutant sources. A better understanding of the relative persistence of relevant indicators of water and sand quality would provide insight on the utility of such ratios to help in identifying the source and age of pollution events (Wang et al. 2013).

The overarching goal of this study was to characterize the dynamics and potential sources of the persistent FIB population observed in the sand under the Santa Monica Pier as well as compare ENT as measured by qPCR and culture methods. As piers provide shading and are often proximal to restaurants and service, they may be frequently visited and thus present an important segment of the beach to understand. The pier structure itself can also provide shelter to birds such as seagulls and pigeons; many birds inhabit the area under and around the Santa Monica pier. The City of Santa Monica has conducted multiple efforts to reduce these bird populations, including installing netting under the pier to reduce bird access to the underside of the pier, placing shiny materials in the netting to further discourage birds, and playing sounds that are a bird deterrent. Despite these efforts, during all sampling trips and also according to anecdotal data from Santa Monica staff, approximately 10 to 30 birds were observed underneath and adjacent to the pier with little seasonal variability. Based on this persistent bird population, a snapshot study to assess the presence of seagull-specific markers was conducted to better understand this potential contributor to the FIB under the pier.

Specifically, we set out to 1) understand spatial and temporal patterns of FIB under the pier through three seasonal spatial surveys, 2) characterize the persistence of FIB and general *Bacteroidales* under moisture conditions observed beneath the pier, 3) analyze relationships between ENT concentrations as measured by membrane filtration and by qPCR, and 4) determine potential sources of observed FIB through a site survey using two human-associated markers, Taq HF183 and HumM2, and one gull/pelican-associated marker that detects *Catelliboccus marimammalium*.

Methods

Site Description.

This study was performed at Santa Monica Pier (34°00'31.43" N, 118°29'50.06" W), a popular tourist location in Santa Monica, California (Figure 1). Previous surveys have shown high levels of FIB in sand beneath the Santa Monica Pier (Jay lab data 2006–2009, unpublished). The sample area was a fenced off area beneath the Santa Monica Pier, which included a sealed pipe outlet. A large depression in front of the sealed outlet contained sand that was often wetter than adjacent sand, and sometimes contained ponded water up to 5 inches deep and 20 feet across. Sampling was not conducted during these infrequent ponding events, which were often caused by higher tides depositing water through wave action into this depression.

Seasonal Study.

Sand samples were collected during one week in May 2010, August 2010, and November 2010. For each location, five aliquots of sand were collected from the upper 1.5 cm of sand within one square meter and pooled into a sterile 50 ml Falcon Tubes (BD BioSciences, USA). A second Falcon tube was used to collect a duplicate composite sample within the same square meter in order to achieve a more representative enumeration for the site. The sand was then returned to UCLA and processed within 6 hours. Day 1 sampling sites followed a grid pattern with sites both adjacent to and underneath the pier within the fenced area (Figure 1). Additionally, sites were tested inside the fenced area underneath the pier. Day 2 sample sites were placed in a tighter grid around areas of higher FIB concentrations detected on Day 1. Similarly, Day 3's grid was tightened around the locations of higher FIB levels identified in results from Day 2 (Figure 2).

Microcosm Studies.

Two microcosm studies were conducted to examine the influence of moisture content on FIB persistence in sediments from underneath the Santa Monica Pier. For both microcosms, sand from the top 1.5 to 2 cm was collected using sterilized plastic beakers from 2 to 3 square meter areas under the pier that were identified as having consistently high levels of FIB in the presented seasonal study. All collected sand was then composited into a large sterile bucket, homogenized through 10 minutes of mixing, and transported back to UCLA for partitioning.

Sand was partitioned into 50 sterile quart sized plastic zipper bags (100 g of sediment per bag) for each moisture regime. Initial moisture content of the sand collected was 10% for the

May microcosm and 0.1% for the October microcosm. A 100× diluted phosphate-buffered saline (PBS) with pH 7.0±0.2 was used to increase the moisture content of the sediment in the bags. Each mL of 100× diluted PBS resulted in an increase of 1% moisture to the sediment. The May microcosm examined moisture content of 10% (in-situ conditions) and 20%, and the October microcosm examined 0.1% (in-situ conditions), 7%, and 14% moisture. The bags were sealed and shaken after the addition of diluted PBS to homogenize the moisture throughout the sediment. Each bag was left slightly open to maintain aerobic conditions but minimize evaporation. Four replicate microcosms (bags) were sacrificed for each moisture regime at each sampling timepoint. Moisture contents were monitored over the course of the experiments to ensure that the moisture content was staying consistent for all sampling timepoints.

FIB Measurement.

Sand samples were processed for extraction based on treatment number one as described in (Boehm et al. 2009), with one minute settling time as a modification. Membrane filtration was conducted to enumerate FIB according to EPA method 1600 (US EPA 2006) for ENT (cENT) and EPA method 1603 for *E. coli* (EC) (USEPA 2002).

Moisture Content Determination.

Sand was processed for moisture content using a gravimetric wet-to-dry ratio method (Schmugge, Jackson, and McKim 1980). Briefly, aliquots of sand were placed in tared aluminum boats and oven-dried at 105 degrees centigrade for 12–14 hours until constant weight was obtained. To determine moisture content, the weight of the soil samples was taken before and after drying.

DNA Extraction and Quantification.

30 mL of sand wash was vacuum-filtered through 47-mm-diameter, 0.45-µm-pore-size polycarbonate filters. Filters were stored at –20°C until being extracted and processed. DNA was extracted from the filters using the Gene-Rite DNA-EZ Extraction Kit (GeneRite, LLC, North Brunswick, NJ) according to the manufacturer's protocol. DNA was bead-beaten in a BioSpec Mini-Beadbeater-8 (BioSpec Products, Bartlesville, OK). DNA quantity and quality was measured in triplicate 2 uL samples using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc, Wilmington, DE).

qPCR Analyses.

Sand samples from the host-associated marker survey were analyzed through qPCR for GenBac, ENT (qENT), two human genetic markers and one gull/pelican genetic marker. The two human-associated assays consisted of: 1) Taq HF183, which has been shown to be associated with the species *Bacteroides dorei* (Haugland et al. 2010) and 2) HumM2 (Shanks et al. 2009). The gull/pelican fecal marker assay used in this study detects *Catelliboccus marimammalium*, a bacterial species found in feces of gulls and pelicans (Lu et al. 2008; Riedel et al. 2014; Sinigalliano et al. 2013). Sand samples from the microcosms were also analyzed by qPCR for enterococci and for the GenBac species, which detects many of the

Bacteroidales subgroups (US EPA Method B. 2010; US EPA Method A. 2010; Haugland et al. 2005).

DNA was amplified in an Applied Biosystems StepOnePlus Real-Time PCR System (Life Technologies, Carlsbad, CA) using thermal cycles, primers, probes, and master mixes as described in the source papers for each assay. Samples were quantified using the software and algorithm within the StepOnePlus. A six-point standard curve using specific plasmids or genomic DNA, depending on the assay, was run in duplicate alongside samples on every plate. The presence of inhibition was assessed by running samples spiked with 2×10^3 copies of the standard alongside the sample triplicates. Samples were initially run at $5 \times$ dilutions. Samples that showed recovery efficiency less than 30% were rerun at $25 \times$ and $125 \times$ dilution. All microcosm samples were able to be diluted past significant interference. Recovery efficiency (RE) was measured as in (Bell et al. 2009).

Statistical Analyses.

After log 10 or natural log-transforming data (for survey samples and microcosm time series, respectively), microcosm and site survey data were determined to be normal by examining Q-Q plots but seasonal data were not. T-tests or Mann-Whitney U-tests were used in order to determine the difference between parameters measured at different locations or by different methods. Gene marker copy counts for qENT were transformed to cell equivalents (CE) using a factor of four gene markers per genome as described in (US EPA Method A. 2010) for comparisons to cENT concentrations.

ANOVA was used to determine differences between decay rates and indicators in the microcosms. Decay rates were calculated using the Chick-Watson model: $C(t) = C_0 e^{-k(t)}$. In this equation, $C(t)$ is the concentration at a point in time, C_0 is the starting concentration at time zero, and k is the first order decay constant. A linear curve was fit to the natural log transformed normalized concentration $\ln(C/C_0)$ and time to obtain decay rates as in (K. M. Yamahara et al. 2012). T_{90} 's were also calculated using the Chick-Watson model (Chick 1908; Watson 1908). Detection limits for qPCR assays were determined to be 100 copies/filter, corresponding to 4,000–6,700 copies per 100 grams of sand.

Data from the source-specific snapshot were analyzed using binary logistic regression to determine significant relationships between marker presence or absence and concentrations. Pearson's correlation tests were run to assess correlations between microbial indicators and genetic markers. Unpaired t-tests were used to determine relationships between concentrations underneath and outside of the pier using SPSS software.

Results - Seasonal Reconnaissance:

FIB results.

Results over the course of all three seasonal surveys tended to be similar, regardless of the season. FIB concentrations were consistently higher in sand underneath the pier than in sand collected from adjacent to the pier. The highest FIB concentrations under the pier were detected in spring and fall (Mann-Whitney, $p < 0.05$). Generally, ENT and EC concentrations under the pier ranged from 1×10^4 to 1×10^6 CFU 100 grams⁻¹ as compared

to 20 to 320 CFU 100 grams⁻¹ outside the pier. FIB levels were close to FIB concentrations observed in the sand under the pier (10³ to 10⁴ CFU 100 grams⁻¹) at sample location D, located outside of the pier on the south side (Figure 1).

EC and ENT were significantly positively correlated with moisture ($\rho = 0.61$, $p < 0.001$, $n = 59$; $\rho = 0.43$, $p < 0.001$, $n = 59$, respectively). Concentrations of EC and ENT were significantly lower in sand with < 10% moisture than those in sand with 10 – 20% moisture (Mann-Whitney, $p=0.002$ and $p=0.025$, respectively; see Figure 3). Large standard deviations were observed across all bins as there was large variability of FIB concentrations within the groups, but the general trend was for higher FIB concentrations to be associated with moister sand.

May Microcosm (10% Moisture Content).

FIB, GenBac, and qENT concentrations were measured for all time-points during the course of this microcosm. Initial concentrations were as follows (Figure 4): 1.3×10^6 copies (cps) GenBac 100 grams⁻¹, 9.26×10^6 cps qENT 100 grams⁻¹, 2.5×10^5 cps cENT 100 grams⁻¹, 5×10^5 CFU EC 100 grams⁻¹. The in-situ moisture content of the sand at time of collection was approximately 10%, with a mean of 9.7% and standard deviation of 0.24% over the course of the microcosm. The interactions of bacteria with time over the course of the microcosm were different (ANOVA, $p < 0.001$) but were not different based on whether they were in sand with 10% or 20% moisture (ANOVA, $p > 0.05$). Slopes of qENT and cENT were not significantly different from zero over the course of the microcosm (Wald's test, $p > 0.05$); slopes of EC and GenBac were significantly different from zero (Wald's test, $p < 0.05$). EC exhibited rapid decay ($k = -0.716$, $T_{90} = 3.2$ days) that was at a significantly faster rate than the other indicators (Wald's test, $p < 0.05$).

October Microcosm (0.1% Moisture Content).

In-situ moisture content at the time of collection was 0.1%; mean moisture content over the course of the microcosm was 0.15% with a standard deviation of 0.06%. Initial bacterial concentrations were (Figure 5): 1.8×10^7 cps GenBac 100 g⁻¹, 4.3×10^4 CFU EC 100 g⁻¹, 1.3×10^4 CFU cENT 100 g⁻¹, 2.4×10^6 cps qENT 100 g⁻¹.

ANOVA results were not able to be processed for this microcosm due to an insufficient number of independent values for T_0 , so trends are discussed. GenBac, qENT, and cENT exhibited similar survival patterns, with both persisting the longest in the driest sand (slopes were not significantly different than zero). EC persistence was comparable to the other indicators in this microcosm ($k = -0.09$). T_{90} 's could not be determined for any of the measured indicators as their slopes were not significantly different from zero.

Comparison of cENT and qENT results.

Over the course of the microcosm, qENT concentrations tended to be higher than cENT concentrations, especially in the drier microcosms. Similar results were observed in the snapshot study; qENT detected significantly higher numbers than cENT ($n=12$, paired t-test, $p < 0.05$). Correlations between ENT concentrations detected by qPCR and membrane filtration were very different across microcosms. In October, cENT and qENT were well

correlated (Pearson's $r^2 = 0.585$, $p < 0.001$), while in May they were uncorrelated (Pearson's $r^2 = -0.034$, $p = 0.517$). Significant but fairly weak positive correlations were observed between qENT and cENT during the snapshot study (Pearson's $r^2 = 0.361$, $p = 0.023$). However, although correlation strength varied between sampling events and microcosms, the correlation between qENT and cENT for all samples was fairly strong (Pearson's $r^2 = 0.64$, $p < 0.001$, Figure 6).

Source-associated Snapshot Study.

FIB results from this snapshot were consistent with seasonal results; FIB levels were generally higher under the pier. Higher levels of FIB were again observed on the south side of the pier. Although EC, cENT, qENT, and GenBac were all higher beneath the pier, only GenBac was significantly higher ($n=12$, unpaired t-test, $p = 0.001$).

Human fecal markers were below the detection limit of both assays for all samples measured and no inhibition was measured in these samples. Therefore it is likely there was no recent human fecal contamination at this study site. Gull/pelican fecal markers were detected at eight of 12 sites despite the presence of significant inhibition with the qPCR assay (Figure 7). Sample location B, which is located outside of the pier but had high levels of FIB in sand, was among the sites with gull markers detected.

GenBac was not correlated with either cENT or EC. The strongest correlation was between cENT and EC ($r^2 = 0.649$, $p = 0.002$). qENT had significant but weaker positive correlations with cENT ($r^2 = 0.361$, $p = 0.023$), EC ($r^2 = 0.307$, $p = 0.036$), and GenBac ($r^2 = 0.403$, $p = 0.016$). Binary logistic regression found no relationship between the presence of the gull marker and any other measured indicator, which isn't surprising given the high rate of detection and relatively small number of samples ($n=12$) collected during the snapshot study.

Discussion.

FIB in sand.

Moisture levels in sand collected during the surveys (0.1% to 20%) were consistent with those found in site surveys at other marine beaches (Yamahara et al. 2012; Shah et al. 2011). In our study, FIB levels were significantly higher in sands above 10% moisture than in sands below 10% moisture, which is consistent with a previous study observing higher levels of indicators in sands with higher moisture contents (in a range from 0.08% to 20.4%) (K. M. Yamahara et al. 2012). Wetting events such as wave height and one day prior rainfall were positively associated with cENT density in sand at a beach in RI and AL (Heaney et al. 2014). Both field and laboratory studies have shown higher levels of cENT in dry sand samples after they have been subjected to natural or simulated tidal wetting (Halliday et al. 2015; Yamahara et al. 2009) which points to the possibility that drying and wetting cycles, as opposed to constant wetter conditions, result in rapid FIB growth.

Relationships between FIB and sand moisture content can be complex and difficult to untangle from other environmental conditions. Using the iterative sampling approach during 3 successive sampling days as described in the methods section allowed us to hone in on areas of higher FIB concentrations under the pier and provided higher resolution on areas of

higher FIB concentration. During the seasonal sampling excursions in our study, very high levels of FIB were also observed in the driest sand of the samples collected under the pier (0.01% moisture). Previous studies have observed higher microbe concentrations in drier sand at the beach (Abdelzaher et al. 2010) and strong inverse correlations with moisture (Shah et al. 2011). Concentrations and diversity of indigenous bacteria can be very high. In a recent study of intertidal sands along the CA coast, individual sand samples contained between 639 and 2750 taxa (Boehm et al. 2014). Further, biomass of indigenous bacteria was shown to result in faster FIB decay in microcosm studies done with Hawaiian beach sand and seawater (Zhang et al. 2015); many studies have shown competition with autochthonous bacterial species in the sand to have a detrimental effect on the concentrations of indicator bacteria (Feng et al. 2010; Hartz et al. 2008). A lack of competition is more likely to be the cause of the higher microbe levels sometimes seen in drier sand than predation as beach sand has been shown to generally harbor a fairly limited protozoan population (Feng et al. 2010; Wieltchnig et al. 2003; Kirschner and Velimirov 1999).

Decay rates.

Results from our microcosms indicate that the under-pier populations of ENT, GenBac, and to some degree, EC, are capable of persisting for long periods of time. Decay constants for cENT, qENT, and GenBac were not significantly different from zero, which is consistent with results from studies examining ENT in beach sands (Yamahara et al. 2009; Feng et al. 2010) and GenBac in swine manure-amended soils (Rogers et al. 2011). ENT and GenBac in manure-amended soils remained detectable for greater than 120 days (Rogers et al. 2011). Our study provides further support that FIB habituated to environmental conditions can persist for very long periods of time even in the absence of new inputs. Long persistence times of naturalized bacterial populations can also explain why FIB and other microbes are often found at high levels in drier sand as was observed in this study and has been seen by others (Abdelzaher et al. 2010; Cui et al. 2013; Yamahara et al. 2009; Ran et al. 2013; Halliday et al. 2015). EC may be useful as an indicator of a more recent pollution event under the Santa Monica Pier as EC decayed far more rapidly than any other indicator during the May microcosm ($k=-0.7/\text{day}$). EC has been observed to decay more rapidly than ENT because EC is more susceptible to factors including environmental conditions, predation, and competition (Yamahara et al. 2012; Feng et al. 2010).

Understanding the relative persistence of FIB and genetic markers in the environment is also critical to determining the most appropriate markers to, for example, provide evidence for a recent pollution event or to function as a corollary for water quality indicators or pathogens. Recent health studies in sand have shown GenBac and ENT by both qPCR and membrane filtration to be correlated with increased health risk (Heaney et al. 2012). In our study, qPCR was equally protective or conservative relative to culture based FIB methods as qENT decay rates were not distinguishable from those of cENT and were slower than those of EC in May. Working with broad bacterial groups such as GenBac and qENT, which tend to be present in the environment at high concentrations as opposed to the lower concentrations of source-associated assays (Shah et al. 2011), can address some of the concerns with using qPCR in the environment such as the potential for high detection limits, efficiency problems,

and false negatives due to environmental interference (Rogers et al. 2011; Tambalo et al. 2012; Marti et al. 2013).

qENT and cENT Comparisons.

Generally, qENT was between one and two orders of magnitude higher than cENT in both the microcosms and the source-specific snapshot study (46/64 samples). This is consistent with results seen in other sand studies (Yamahara et al. 2012) although some field studies have not seen a significant difference between qPCR and culture methods (Shah et al. 2011). Correlations were widely different between the May and October microcosms, with a fairly strong correlation in October ($r^2 = 0.585$) and overall ($r^2 = 0.64$) and no correlation in May ($r^2 = -0.03$). Generally, poor correlations have been observed in sand and sediments between cENT and qENT both in the field (Shah et al. 2011; Heaney et al. 2014) and in microcosms (Yamahara et al. 2012; Rogers et al. 2011). Stronger correlations can sometimes be explained by fresher fecal contamination (Haugland et al. 2005). Lack of correlation can result from the presence of dead cells, VBNC, or extracellular DNA (Rogers et al. 2011) but these factors are not always confounding, as can be seen in the stronger correlation when looking at all the data from the study overall as well as in the October microcosm specifically.

In this study at Santa Monica Pier, qENT showed promise as an alternate indicator to cENT since cENT and qENT showed similar decay patterns over time in both microcosms. Further support for using qPCR as an alternate rapid approach to measuring ENT can be found in the fact that health risks have been linked to both qPCR and culture methods (Heaney et al. 2012; Wade et al. 2009; Wade et al. 2006). The more rapid results (~4 hours) available through qPCR can potentially prevent the errors in posting and delayed decisions on beach closures or postings that can result from waiting for results from culture-based methods (~18 to 24 hours) (Boehm et al. 2002; Kim et al. 2004). However, mixed correlation results from our study support the importance of assessing qPCR and culture methods at each beach before using these methods interchangeably.

Ratios of qENT to cENT (qENT:cENT) were also examined, as one would expect measurements based on viable populations to decrease more rapidly than those that measure DNA from both viable and non-viable cell populations. DNA degradation has been shown to be slower than die-off of culture-based measurements (Walters et al. 2009). We did not observe the ratios showing the expected greater decrease in cENT versus qENT that have been observed in prior microcosm studies using sewage-seeded beach sands (Yamahara et al. 2012; Eichmiller et al. 2014). The lack of a definable trend observed in our microcosms could be due to microbial biofilms providing ENT with protection from external stressors (Piggot et al. 2012; Mohamed and Huang 2007). While increasing cENT : qENT ratios can be explained through new influxes from tidal influxes (Converse et al. 2012) there were no new FIB influxes present in the microcosms as designed in this study. Other factors such as, for example, ENT entering and leaving VBNC state could be responsible for the inconsistent ratios observed in our study. The presence of a significant proportion of VBNC ENT available for resuscitation was also observed in a freshwater beach sand microcosm study as there was a lack of difference between the decay rates of ENT markers for total and live

cells (Eichmiller et al. 2014). The long persistence of cENT observed in our study could also be due in part to measuring native ENT populations in unseeded sand that are accustomed to surviving in a dry sand environment, rather than measuring the persistence of an ENT population seeded into sand from sewage.

Source Tracking.

Human-associated sources did not appear to be significant contributors of FIB to the chronic FIB pollution observed under the pier as both human-associated markers were below detection limits at all locations sampled near the pier. However, the bird population under the pier did appear to contribute to the FIB pollution as the gull/pelican marker was detected at eight of 12 sites both under and next to the pier despite significant inhibition with the qPCR assay. Thriving populations of pigeons and seagulls, which are important sources of fecal contamination to surface waters and sand beaches (Wither et al. 2005; Lu et al. 2011; Haack et al. 2003; Hansen et al. 2011), inhabit the area beneath the pier. Starlings roosting on and near piers in Blackpool, England were linked to numbers of fecal coliforms and fecal streptococci in the water (Wither et al. 2005). A previous study showed a significant reduction in both EC and ENT with the removal of a local gull population (Converse et al. 2012).

Results from the one day survey generally agreed with seasonal survey results; qENT, GenBac, EC, and cENT were all higher on average under the pier, but only GenBac was significantly higher. qENT showed significant correlations with all other indicators measured ($R^2 = 0.31$ to 0.43), while GenBac did not correlate with EC or cENT. Lack of correlation between indicators may indicate there is no single source of pollution to this study area (Shibata et al. 2004), which is consistent with the many potential sources under the pier such as tidal influx, stormwater, birds, and environmental regrowth. The lack of correlation between total *Bacteroidales* and FIB has also been observed during a multi-year study of fecal sources using *Bacteroidales* markers (Marti et al. 2013), and mixed correlation results have been reported in other studies (Yamahara et al. 2012; Shah et al. 2011).

Examining the ratio of qENT : cENT in the field provided information regarding the potential age of the ENT pollution. qENT : cENT varied at each site; overall, qENT was one to two orders of magnitude higher than cENT. The ratio of cENT (CFU 100 grams⁻¹) : qENT (cps 100 grams⁻¹) was highest, 0.14 CFU cps⁻¹, at Site B (See Figure 7 for location of B) of the twelve sample locations assessed during the one-day snapshot study. Site B had unusually high FIB levels for a location outside of the pier. While these ratios do not provide any source-associated information, they may provide evidence of the occurrence of a more recent pollution event (e.g. previously observed stormwater flooding at this location Site B). It is important to note that this is a small sample size and thus an area that needs future research to further assess the potential information that can be obtained through measuring water quality with both culture-based and qPCR methods. If similar patterns in this ratio are observed elsewhere, then using a ratio of cENT and qENT, which are both included in the new standards for recreational water quality (USEPA 2012), potentially offers a cost-effective way to obtain information about the percent of the ENT population that is viable at

the sample location while also assessing the use of qPCR to measure water quality at a location.

Conclusions

During the course of three seasonal surveys, FIB levels were found to be significantly higher under the pier than outside the pier as well as significantly higher in moister sand than drier sand. Microcosm experiments demonstrated that native populations of ENT (from both moisture levels), and *Bacteroidales* (from both moisture levels), and EC (from drier conditions only) under the pier were capable of persisting for long periods of time in sand without any new inputs. Based on the results from our study, the most likely cause of FIB contamination under and adjacent to the Santa Monica Pier appears to be environmental, related to the long persistence of the indicators in the sand under the pier and to the thriving populations of pigeons and seagulls that inhabit the region around the pier.

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Highlights

- FIB levels were consistently higher in sand underneath the pier than that collected from adjacent to the pier, showing highest concentrations under the pier in spring and fall.
- EC and ENT were significantly positively correlated with moisture ($\rho = 0.61$, $p < 0.001$, $n = 59$; $\rho = 0.43$, $p < 0.001$, $n = 59$, respectively).
- Minimal decay was observed throughout the course of two microcosms at various moisture levels for ENT and GenBac.
- GenBac was not correlated with either cENT or EC. The strongest correlation was between cENT and EC ($r^2 = 0.649$, $p = 0.002$).
- Human markers were not detected, but gull/pelican fecal markers were detected at eight of 12 sites.

Figure 1.
Example day 1 sample locations

Figure 2.

- Example day 2 sample locations, tightened around areas identified with higher FIB concentrations in day 1.
- Example day 3 sample locations, tightened around areas identified with higher FIB concentrations in day 2

E coli moisture

Ent moisture

Figure 3.

EC and ENT concentrations vs. sand moisture contents. EC and ENT levels are higher in sands with moisture levels greater than 10%. *Vertical black line* denotes moisture level of 10%

May, 10% moisture microcosm.

Figure 4.
May microcosm results, 10% moisture

Oct, 0.1% microcosm.

Figure 5.
October microcosm results, 0.1% moisture

unchanged

Figure 6. qENT/cENT correlations. Overall parametric correlation ~ 0.64 , $p < 0.001$. Calibrator cell equivalent (CCE)

Figure 7.

Gull presence under the pier. *Xs* are positive for seagull/pelican marker. *Plus* are negative for seagull/pelican marker. *Solid black* represents a water sample; *black outline* represents a sand sample