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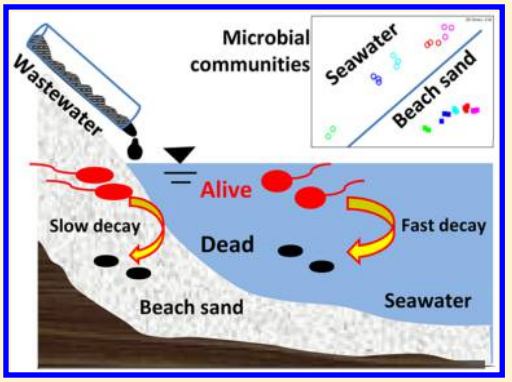
Differential Decay of Wastewater Bacteria and Change of Microbial Communities in Beach Sand and Seawater Microcosms

Qian Zhang, Xia He, and Tao Yan*

Department of Civil and Environmental Engineering, University of Hawaii at Manoa, Honolulu, Hawaii 96822, United States

Supporting Information

ABSTRACT: Laboratory microcosm experiments were conducted to determine the decay kinetics of wastewater bacteria and the change of microbial communities in beach sand and seawater. Cultivation-based methods showed that common fecal indicator bacteria (FIBs; *Escherichia coli*, enterococci, and *Clostridium perfringens*) exhibited biphasic decay patterns in all microcosms. Enterococci and *C. perfringens*, but not *E. coli*, showed significantly smaller decay rates in beach sand than in seawater. Cultivation-independent qPCR quantification of 16S rRNA gene also showed significantly slower decrease of total bacterial densities in beach sand than in seawater. Microbial community analysis by next-generation sequencing (NGS) further illustrated that the decreasing relative abundance of wastewater bacteria was contrasted by the increase in indigenous beach sand and seawater microbiota, and the overall microbial community dynamics corresponded well with the decay of individual FIB populations. In summary, the differential decay of wastewater bacteria in beach sand and in seawater provides a kinetic explanation to the often-observed higher abundance of FIBs in beach sand, and the NGS-based microbial community analysis can provide valuable insights to understanding the fate of wastewater bacteria in the context of indigenous microbial communities in natural environments.



INTRODUCTION

Marine beach environmental quality is important to the health and safety of beach users and the economic prosperity of coastal communities. Currently, beach water quality is routinely monitored using fecal indicator bacteria (FIBs).[1−3] Beach sand, the other integral part of beach systems, has not been included in the current monitoring and regulatory scheme. The consequence of this omission has been increasingly recognized, as studies in the past decade have provided ample evidence supporting the importance of beach sand to public health. Numerous studies have detected high levels of enterococci as FIB in marine beach sand, with concentrations often 10−100 fold higher (on a unit mass basis) than in the corresponding beach water.[4−7] Enterococci-laden beach sand can affect beach water quality by serving as a chronic source of enterococci to beach water due to various interactions between the two beach components.[6,8,9] Contaminated beach sand may also directly cause adverse public health effects via human−sand contact, as indicated by the correlation between exposure to enterococci-laden beach sand and increased enteric illness.[10,11]

Although recent studies have shown that common FIBs (i.e., enterococci and *Escherichia coli*) can have environmental sources,[7,12−14] wastewater pollution is still generally regarded as the most important threat to public health in beach environments.[15] To date, a majority of studies on the decay of wastewater bacteria in marine beach environments have been performed in seawater only.[16−19] Although a few studies have attempted to investigate the decay of wastewater bacteria in marine beach sand,[20] direct comparison of bacterial decay in marine beach sand and seawater has not been reported. Wastewater bacteria in beach sand are expected to primarily interact with sand surfaces where indigenous sand microbiota exists in biofilm.[21] Biofilm offers considerable environmental heterogeneity and various ecological niches that are known to have different effects on bacterial survival.[22] Many important environmental stresses that cause rapid bacterial decay in seawater, including temperature variation,[16] sunlight inactivation,[17,18,23] pH fluctuation,[24] and nutrient depletion,[25,26] are either significantly reduced or completely absent in marine beach sand, which can also cause significantly different bacterial decay behaviors.

Currently, our understanding of bacterial decay in the environment is primarily based on studies on individual bacterial populations using cultivation-based methods. The overall microbial community structure and dynamics were often neglected, primarily due to technical hurdles in characterizing the overwhelming diversity of microbial communities. Recent advancement in next generation sequencing (NGS) have provided unparalleled capabilities in obtaining large numbers of sequence reads to query the microbial community structure and diversity.[27] The application of NGS tools in beach systems

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has revealed tremendous microbial diversity in beach sand and seawater.[7,28] Efforts have also been made to use the NGS tools to address ecological questions in marine beach environments, including the fate and source of enterococci,[7] microbial community response to oil pollutions,[29,30] temporal variation of resident and rare populations,[21] and influence of physicochemical parameters on community structure.[31] However, no study has been reported to investigate how overall microbial community structure change interfaces with the decay of individual wastewater populations in beach sand and seawater environments.

Therefore, the objectives of this study were to (1) compare decay patterns of wastewater bacteria in beach sand and seawater and (2) examine the relationships between the decay of individual populations and the changes in overall microbial community composition. Laboratory beach sand and seawater microcosms were spiked with raw municipal wastewater, and three FIBs (*E. coli*, enterococci, and *Clostridium perfringens*) were enumerated by cultivation-based methods over time to determine their decay patterns. The microcosms were also subjected to qPCR quantification of 16S rRNA genes in order to determine total bacterial density and its change over time. Overall microbial community compositions and dynamics were also revealed by Illumina sequencing of 16S rRNA gene amplicons. The individual FIB decay kinetics, total bacterial density dynamics, major wastewater bacterial populations, and the overall microbial community structures were compared between the beach sand and seawater microcosms.

MATERIALS AND METHODS

Beach Sand, Seawater, and Wastewater Sampling. Backshore sand samples and seawater samples were collected from Kualoa Beach (21.51330°N; 157.8360°W) on the Island of Oahu, Hawaii. Sand samples were collected from multiple locations approximately 0.5 m above the high tide line. The sand samples were collected using an ethanol-cleaned air-dried spatula, and were placed in sterile Whirl-Pak sampling bags. Seawater samples were collected at multiple locations at knee depth using sterile wide-mouth plastic bottles. A grab raw municipal wastewater sample was collected from the headwork of the Sand Island Wastewater Treatment Plant (SIWTP; Honolulu, HI) in sterilized wide-mouth plastic bottles. All samples were placed at 4 °C and in dark during transportation to the laboratory for immediate processing. These samples were used to constitute microcosms and were subjected to microbial analyses described below.

Microcosm Setup. Two sets of microcosms, one for beach sand and one for seawater, were established in one-liter cleaned and autoclaved Mason jars, and each set contained three independent microcosms as biological replicates. The beach sand samples collected from the Kualoa beach were first pooled and mixed thoroughly with a sterile wood tongue depressor, while the beach water samples were pooled and mixed by hand shaking. Each sand microcosm contained 600 g of the pooled beach sand sample, while each seawater microcosm contained 600 mL of the pooled seawater sample. Both types of microcosms were spiked with 30 mL of the raw wastewater sample from SIWTP. After thorough mixing, the microcosms were incubated for 24 days in the dark at room temperature (22−24 °C) without shaking. Samples were collected every day in the first week, and then three times a week during the remaining two and a half weeks. For sand microcosms, 20 g of sand samples were collected from each microcosm after thorough mixing. For seawater microcosms, initially, 20 mL of water samples were collected from each microcosm after thorough shaking in the first 3 weeks, while larger volumes (up to 60 mL) were collected in the last week to compensate for decreased microbial concentration.

Bacterial Enumeration. The original sand and seawater samples collected from the Kualoa Beach, the raw wastewater sample collected from SIWTP, and the sand and seawater samples collected from the microcosm experiments over time were processed and analyzed to enumerate *E. coli*, enterococci, and *C. perfringens*. Sand samples were first extracted using a procedure described by Boehm et al.,[32] which involved shaking 10 g of sand in 100 mL of sterilized deionized water for 2 min by hand and then collecting supernatants of the sand extracts after settlement for 30 s. The liquid samples (sand extracts, seawater samples, and the wastewater sample) were diluted in 10-fold series, and appropriate dilutions were used in subsequent enumeration using membrane filtration methods. *E. coli* were enumerated on the modified mTEC agar according to EPA method 1603.[33] Enterococci were enumerated using the mEI agar method according to EPA method 1600.[34] *C. perfringens* were enumerated using the mCP agar method.[35]

Total Genomic DNA Extraction. Total genomic DNA extraction was performed on the original beach sand, seawater, wastewater, and a subset of 28 microcosm samples (from Days 0, 3, 6, 16, and 23). All samples (sand extracts, seawater samples, and wastewater sample) were centrifuged at 12 000*g* at 4 °C for 10 min to pellet microbial biomass. After decanting the supernatant, the microbial biomass pellets were stored immediately at −80 °C until DNA extraction. Total genomic DNA extraction and subsequent purification was conducted by using the PowerSoil DNA extraction kit (MoBio, Carlsbad, CA) and following the manufacturer's procedure, which gave 50 μL of DNA elutes for subsequent DNA-based analysis. The quality of DNA and its free of PCR inhibition were verified by successful PCR amplification using the universal bacterial primer set 27*F*/1522R targeting the 16S rRNA gene, following the procedure described in Feng et al.[20]

qPCR Quantification of 16S rRNA Gene. Total bacterial density in the samples was determined by qPCR quantification of 16S rRNA gene copies following the procedure described by Nadkarni et al.[36] The 20 μL qPCR reactions contained 10 μL of 2 × TaqMan Universal PCR Master Mix (Life Technologies; Grand Island, NY), 0.25 μM of PCR primers (Forward: 5′-TCCTACGGGAGGCAGCAGT-3′; Rev: 5′-GGACTACCAGGGTATCTAATCCTGTT-3′), 0.125 μM of fluorescent probe ((6-FAM)-5′-CGTATTACCGCGGCTGCTGGCAC-3′-(TAMRA)) and 0.4 μg/μL of bovine serum albumin (BSA). The qPCR reactions were preformed on an ABI 7300 system (Applied Biosystem; Foster City, California). The thermocycler program included 50 °C for 2 min, 95 °C for 10 min and 45 cycles of 95 °C for 15 s and 60 °C for 1 min. The qPCR calibration standards used *Enterococcus faecalis* ATCC 29212 cells, which were prepared following the procedure described in the EPA Method 1611 for Enterococci.[37] The resulting total bacterial densities were reported as calibrator cell equivalent (CCE) per unit mass (g beach sand or mL seawater) and in copies of 16S rRNA genes per unit mass based on four 4 copies of 16s rRNA gene for one *E. faecalis* cell.[38] The calibration standards were analyzed in duplicate reactions in each 96-well qPCR reaction concurrently with samples, which exhibited acceptable linearity (R^2 = 0.97−0.99) and PCR efficiency (64.7−83.3%).

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Illumina Sequencing of 16S rRNA Gene Amplicons. Preparation of 16S rRNA gene amplicons and subsequent Illumina sequencing were conducted at the DNA Services Facility at the University of Illinois at Chicago. Briefly, triplicate PCR amplification reactions were conducted for each sample using the 515*F*/806R primer set that amplifies the hypervariable V4−V5 region of the 16S rRNA gene, using the PCR reaction conditions provided in Caporaso et al.[27] Barcodes for individual samples were linked to the primer sets to enable multiplexing in the subsequent sequencing. Amplicons of the triplicate PCR reactions were pooled and then sequenced using an Illumina MiSeq instrument. The sequence reads were trimmed using a cutoff quality score of Q15 and read length of larger than 200 bp using the software package CLC Genomics Workbench Version 6.0 (CLC bio, Cambridge, MA). Only the forward reads were used for downstream analysis, as it was shown that including the reverse reads add little additional information.[39]

The QIIME software package (version 1.7.0) was used to analyze the Illumina sequence data by following the general procedure provided by Kuczynski et al.[40] Briefly, the FASTA files for individual samples were combined into a single FASTA file according to the metadata mapping file. The sequence reads were then clustered into operational taxonomic units (OTUs) based on 97% sequence identity using the UCLUST algorithm. The sequence reads of individual OTUs were tabulated in an OTU table for all samples, and the OTU table was subsequently rarified at 7300 sequence reads per sample. The taxonomy of each OTU was determined by comparing a representative sequence of the OTU to the Greengene database (version 12.0). The OTUs with no match in the Greengene database were lumped together as "unassigned". Taxonomic assignments resolved at the genus level were used in subsequent analysis, although some OTUs that could not find match at the genus level were reported at coarser levels (family or class). Alpha diversity indices, including Shannon's H, Chao1, observed species, and phylogenetic diversity (PD) whole tree, were calculated using the rarified OTU table.

Data Analysis. FIB concentration data collected by cultivation-based methods were fitted into either the first-order decay model ($\ln (C_t/C_0) = -k_d t$) or the biphasic decay model ($\ln (C_t/C_0) = -k_{d1} t$ when $t \leq t'$, and $\ln (C_t/C_0) = -k_{d1} t' - k_{d2}(t - t')$ when $t > t'$) using SigmaPlot 10.0 (San Jose, CA). C_t and C_0 are the concentrations of bacterial cells in the microcosms at time t and time zero, respectively. k_d is the decay coefficient of the first-order decay model. The turning point of the biphasic decay model (t') and the decay coefficients of the two phases (k_{d1} and k_{d2}) were identified based on two-segment piecewise regression analysis. The performance of the two kinetic models was evaluated based on the goodness-of-fit (r^2) and residual sum of squares (RSS). Analysis of covariance (ANCOVA) with Tukey's post hoc test was conducted in the R computing environment to test if significant difference exists within the decay rates among the different FIBs, between the two decay phases or between the two types of microcosms. The default significance level is $P \leq 0.05$ for all statistical tests unless stated otherwise.

The relative abundance of an OTU in a sample was calculated by dividing the sequence reads of the OTU with the total sum of reads in the sample, which is the most commonly used normalization method in microbiome studies and gives a detection frequency in percentage.[41] Since a large number of OTUs were detected, a subset of the OTUs were defined as major OTUs if their relative abundances in the microcosm samples were larger than or equal to 1% in at least three samples. These major OTUs were subsequently assigned to the three different sources that were used to establish the microcosms (i.e., wastewater (WW), beach sand (BS), and seawater (SW)) based on the criteria that the same OTUs were also found in the source samples with a relative abundance larger than 1%. Temporal variations of the major OTUs were illustrated using a color-coded heatmap based on the log 10 transformed relative abundance values. To visualize the difference in microbial community composition between different samples, we analyzed the relative abundance values of all OTUs in the microcosm samples by nonmetric multidimensional scaling (NMDS) in the statistical software package PRIMER 6.[42] Permutational multivariate analysis of variance (PERMANOVA) was performed to detect statistically significant difference in microbial communities.

Nucleotide Sequence Accession Number. All data generated from sequencing have been deposited in the NCBI GenBank (SRA) with accession number SRP 051512.

RESULTS

Biphasic Bacterial Decay Patterns. The decay of three FIBs (*E. coli*, enterococci, and *C. perfringens*) from raw municipal wastewater in the beach sand microcosms and the seawater microcosms were determined over a 24 day period (Figure 1). The decay data were fitted with either the biphasic decay model or the first order decay model, and the biphasic model consistently gave better goodness of fit (r^2) and smaller residual sum of squares (RSS) for all FIBs in both beach sand and in seawater microcosms (Table 1). According to the biphasic model, all FIBs in both the beach sand and seawater microcosms exhibited an initial faster decay phase (k_{d1}: 0.20−1.47 day^{-1}) followed by a slower decay phase (k_{d2}: 0.00−0.18 day^{-1}).

Different FIBs exhibited different change of decay rates between the two phases (indicated by the ratio k_{d1}/d_{k2}) and different turning points for the biphasic pattern (t') (Table 1). The difference between k_{d1} and k_{d2} was significant in all six cases (ANCOVA, $P < 0.001$), further supporting the biphasic model being more parsimonious than the first-order decay model. The change of decay rates between the first and the second phases was more obvious for *E. coli* and *C. perfringens* (k_{d1}/k_{d2} >10.5) than for enterococci (k_{d1}/k_{d2} range, 3.5−4.2). Corresponding to its smaller k_{d1}/k_{d2} ratio, enterococci also exhibited later transition from the first to the second phase, which was indicated by larger t' (12.3 days and 7.6 days in the beach sand microcosms and seawater microcosms, respectively), than *E. coli* and *C. perfringens* (t' range, 3.5−6.2 days).

Differential Decay between Beach Sand and Seawater. Direct comparison of decay rates between beach sand and seawater microcosms showed that enterococci and *C. perfringens*, but not *E. coli*, decayed significantly faster in the seawater microcosms than in the beach sand microcosms. Enterococci exhibited significantly larger k_{d1} and k_{d2} values in the seawater microcosms than in the beach sand microcosms (ANCOVA; $P < 0.001$). For *C. perfringens* the k_{d1} value in the seawater microcosms was larger than the k_{d1} value in the beach sand microcosms (albeit with no statistical significance; ANCOVA, P = 0.75), and the k_{d2} value in the seawater microcosms was significantly larger than that in the beach sand microcosms (ANCOVA, P = 0.02). *E. coli* was different than enterococci and *C. perfringens* in that it showed significantly

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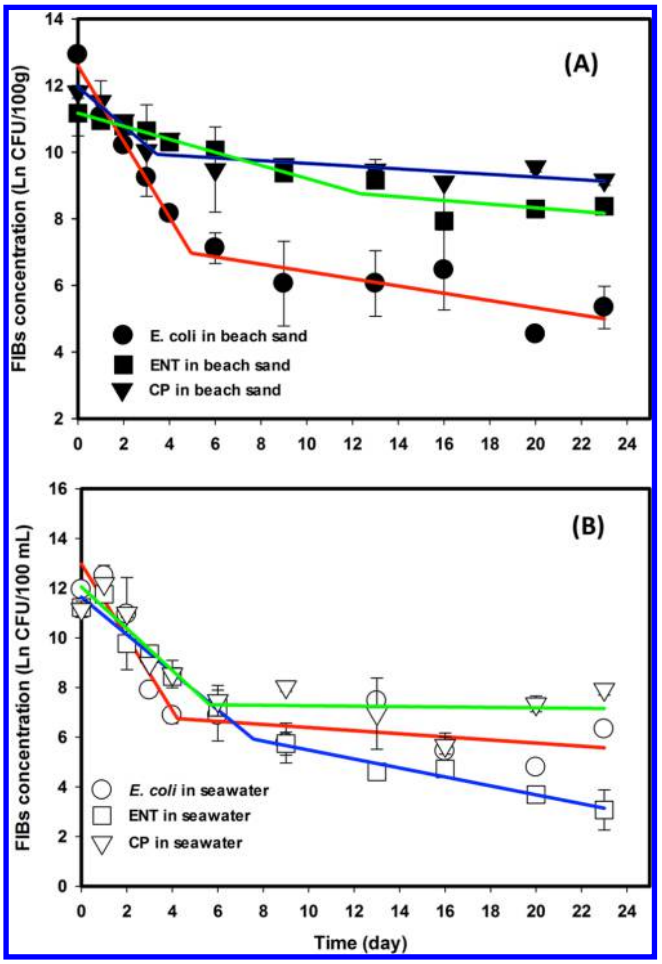

Figure 1. Reduction of concentration of viable *E. coli*, enterococci and *C. perfringens* cells in (A) beach sand microcosms and (B) seawater microcosms. Error bar indicates the standard deviation of the mean of triplicate microcosms. The solid lines were the biphasic regression lines.

faster decay (both k_{d1} and k_{d2}) in the beach sand microcosms than enterococci and *C. perfringens*. Consequently, *E. coli* exhibited no significant difference in either k_{d1} or k_{d2} between the beach sand and seawater microcosms.

The biphasic decay patterns and the significantly slower decay rates in the second phase resulted in higher persisting FIB populations in the beach sand microcosms than in seawater microcosms (Figure S1, SI). *C. perfringens* exhibited the highest persisting population density in both the beach sand and the seawater microcosms, with an average concentrations of 13 500 ± 3822 CFU/100 g sand and 1911 ± 1002 CFU/100 mL seawater over the 17-day period toward the end of the experimental course. Enterococci showed a similar pattern but slightly smaller persisting population (9789 ± 7689 CFU/100 g) than *C. perfringens* in the beach sand microcosms. The levels of persisting *E. coli* in the beach sand microcosms were significantly less than those of *C. perfringens* and enterococci. There was no significant difference between the persisting *E. coli* population in the beach sand microcosms and in the seawater microcosms on a unit mass basis.

qPCR Quantification of Total Bacterial Density. Total bacterial densities in the beach sand and seawater microcosms (28 samples collected on Days 0, 3, 6, 15, and 23) were estimated by qPCR quantification of the 16S rRNA gene, which were reported as CCE per g sand or mL seawater sample (Figure 2). Similar to the biphasic decay patterns of FIBs, the

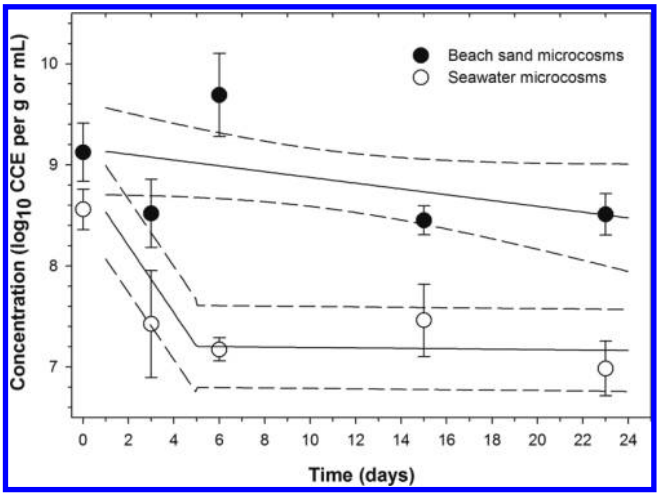

Figure 2. Total bacterial densities in the beach sand microcosms and seawater microcosms estimated by qPCR quantification of 16S rRNA gene. Error bar indicates the standard deviation of the mean of triplicate microcosms. The solid lines were the biphasic or linear regression lines, and the dotted lines represent 95% confidence intervals.

Table 1. Kinetic Model Fitting of the Natural Log Transformed FIB Concentration Data over Time

	beach sand microcosms			seawater microcosms		
	E. coli	Enterococci	*C. perfringens*	*E. coli*	Enterococci	*C. perfringens*
			first-order model			
r^2	0.72	0.82	0.49	0.54	0.89	0.50
RSS[a]	61.8	8.2	18.1	104.3	32.4	63.0
			biphasic model			
r^2	0.93	0.86	0.71	0.86	0.97	0.80
RSS[a]	15.0	6.4	10.3	32.0	8.3	25.0
t' (day)	4.9	12.3	3.5	4.2	7.6	6.2
k_{d1} (day^{-1})	1.14	0.20	0.59	1.47	0.76	0.77
k_{d2} (day^{-1})	0.11	0.06	0.04	0.06	0.18	0
k_{d1}/k_{d2}	10.4	3.5	14.3	23.6	4.2	
[b] $k_{d1} > k_{d2}$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

[a]RSS: Residual sum of squares. [b]ANCOVA comparison of k_{d1} and k_{d2}.

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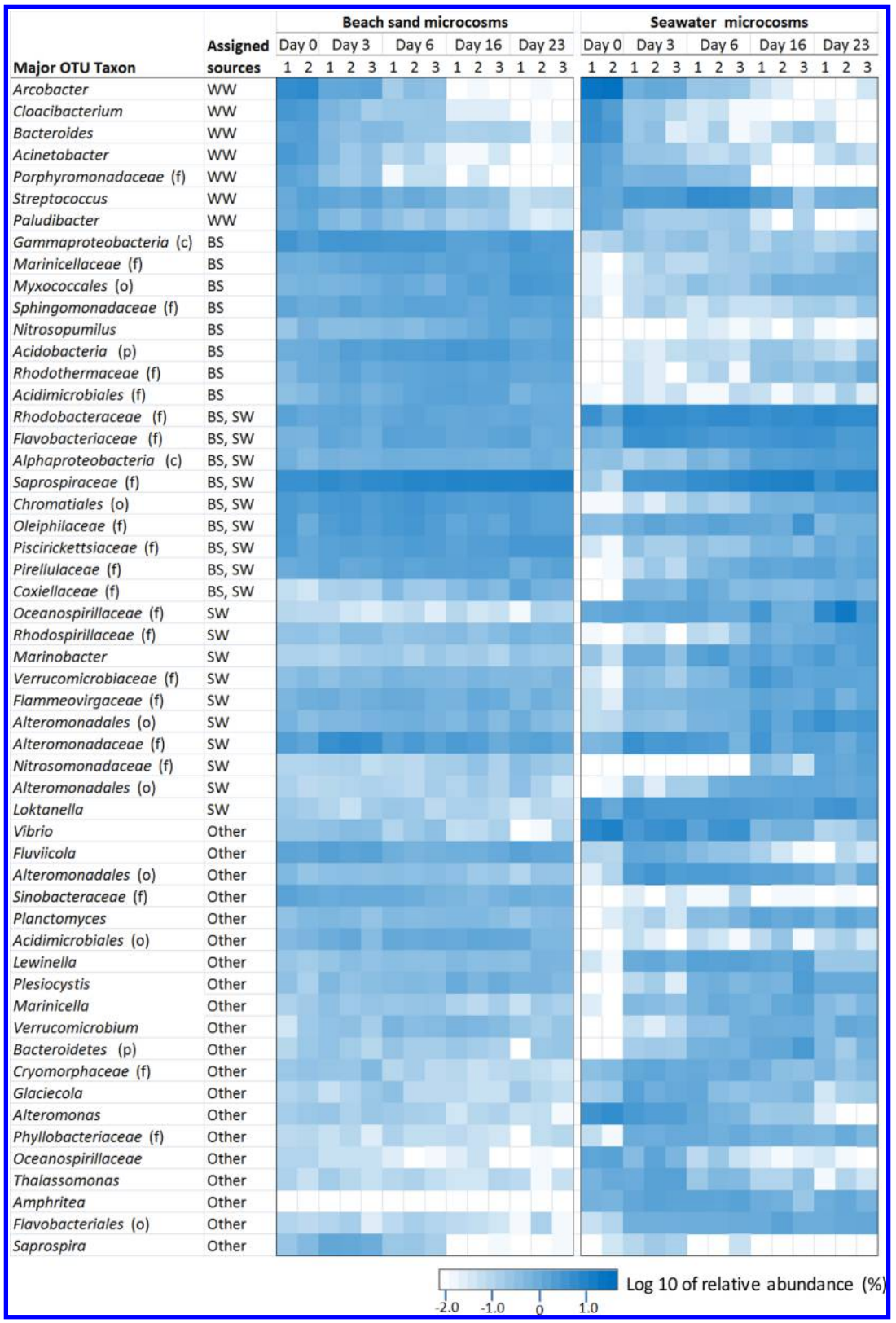

Figure 3. Taxon of major OTUs identified in the beach sand and seawater microcosms, their assigned sources (wastewater, WW; beach sand, BS; seawater, SW), and their relative abundance over time illustrated by a heat map. All OTUs are resolved at the genus level, and taxonomic matches at the closest higher levels (p, phylum; c, class; o, order; f, family) are used for those OTUs with no taxonomic match at the genus level.

total bacterial density change over time in the seawater microcosms was also better fitted to a biphasic model (r^2 = 0.74, RSS = 1.4) than the first-order model (r^2 = 0.37, RSS = 3.5). For the total bacterial density data in the beach sand microcosms, because both the biphasic model (r^2 = 0.27, RSS = 3.2) and the first order model (r^2 = 0.21, RSS = 3.4) exhibited poor goodness-of-fit, the simpler first order model was used in the illustration. Overall, the beach sand microcosms contained

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significantly higher levels of total bacteria (8.2−10.0 $\log_{10}$ CCE/g or 8.8−10.6 $\log_{10}$ 16s rRNA gene copies/g) than the seawater microcosms (6.8−8.7 $\log_{10}$ CCE/g or 7.4−9.1 $\log_{10}$ 16s rRNA gene copies/mL) throughout the experimental course. The total bacterial density also showed slower change in the beach sand microcosms than in the seawater microcosms, which corresponded well to the decay patterns of enterococci and *C. perfringens* in the beach sand and seawater microcosms (Figure 1).

Fate of Major Wastewater OTUs. The overall microbial community structure and its dynamics in the beach sand and seawater microcosms were also revealed by Illumina sequencing of the 16S rRNA gene. The 28 samples from the beach sand and seawater microcosms in different sampling days (Days 0, 3, 6, 16, and 23) and the three source samples (beach sand, seawater, and wastewater) were sequenced to obtain a total of 257 744 sequence reads (7585−9844 per sample) after quality trimming. From these sequence reads, a total of 986 OTUs were identified, and various alpha diversity indices were calculated (Table S1, SI).

To simplify the illustration, we identified 54 of the OTUs detected in the microcosms as major OTUs (Figure 3). These OTUs together accounted for 51−76% of total sequence reads in the samples, with remaining percentages contributed by the other 932 OTUs that are considered minor. These major OTUs were assigned to the three different source samples that were used to establish the microcosms, including wastewater (WW), beach sand (BS), and seawater (SW), based on the criteria that the same OTUs were also found in the source samples with a relative abundance larger than 1%. This source assignment scheme identified 7 major WW OTUs, 17 major BS OTUs, and 19 major SW OTUs. As expected, the 7 major WW OTUs shared no overlap with the major BS and SW OTUs, while the 17 major BS OTUs and 19 major SW OTUs have 9 in common.

Many of the major WW OTUs belong to bacterial genera that were commonly found in fecal or wastewater sources and contain emerging pathogens, including *Arcobacter*,[43] *Cloacibacterium*,[44] and *Bacteroides*.[45] Similar to the FIB decay revealed by cultivation-based methods (Figure 1) and the reduction of total bacterial density shown by qPCR (Figure 2), the seven major WW OTUs also exhibited obvious reduction in their relative abundance over time (Figure 3). The high relative abundance of WW OTUs on Day 0 (1.1−7.6%), which was the result of wastewater introduction, rapidly decreased over time, and reached negligible or no detection levels on Days 16 and 23 (0−0.4%). In contrast, the major BS and SW OTUs in the same microcosms, in general, either maintained their relative abundance or became more abundant over time. Given the total bacterial density decrease and the contrasting dynamics exhibited by the major BS and SW OTUs, the reduction in relative abundance of major wastewater OTUs was clearly the result of cell inactivation followed by subsequent DNA decay.

Correlation between Microbial Community and Bacterial Decay. The overall microbial communities in the beach sand and seawater microcosms were also illustrated using an nMDS plot based on the Bray−Curtis dissimilarity calculated from the relative abundance distribution of all 986 OTUs in the beach sand and seawater microcosm samples (Figure 4). The nMDS plot has a stress value of 0.05, indicating an excellent representation of the dissimilarities within the microbial communities. Microbial communities in the triplicate microcosms of the same type (beach sand or seawater) from

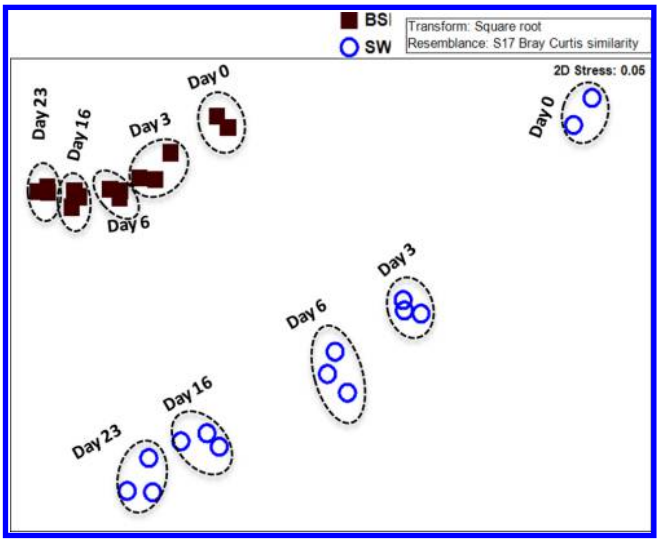

Figure 4. Visualization of the dissimilarities between microbial communities in the beach sand microcosms (BS) and seawater microcosms (SW) using nonmetric multidimensional scaling (nMDS). Triplicate microcosms from the same sampling dates (Days 0, 3, 6, 16, and 23) were grouped in the dotted circles.

the same sampling date were always tightly clustered together, indicating limited variability among the biological replicates. Significant difference in microbial community structure was observed between the beach sand microcosms and seawater microcosms at all sampling dates (PERMANOVA, P = 0.001), indicating strong source and matrix effects and limited influence from the wastewater introduction.

Temporal variation of microbial community structures was also significant in both beach sand and seawater microcosms (PERMANOVA, P = 0.001), which is illustrated by the clear separation of microbial communities at different sampling dates. First, over the 23-day experimental period, microbial community change was clearly more extensive in the seawater microcosms than in the beach sand microcosms, as illustrated by the larger separation between Day 0 and Day 23 samples in the seawater microcosms than in the beach sand microcosms (Figure 4). This corresponded well to the larger decay rates of enterococci and *C. perfringens* (Figure 1) as well as the faster total bacterial density reduction (Figure 2) in the seawater microcosms than in the beach sand microcosms. Second, the temporal change of microbial communities also exhibited biphasic patterns, which is more so in the beach sand microcosms than in the seawater microcosms. For example, in the seawater microcosms, microbial community dissimilarity between Day 0 and Day 6 is larger than the dissimilarity between Day 6 and Day 23, indicating a faster microbial community change in the first 6 days of incubation.

DISCUSSION

The higher abundance of FIBs often observed in marine beach sand than in beach seawater is likely the result of many processes, such as different contaminant loadings and different levels of indigenous FIB populations.[7,12−14] This study provides direct evidence that different bacterial decay rates between beach sand and seawater are also an important contributing factor. Direct comparison in the well-controlled dark laboratory microcosms in this study showed that in the absence of light effects, enterococci and *C. perfringens* exhibited significantly

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slower decay rates in beach sand than in seawater after the introduction of wastewater bacteria (Figures 1). Because light inactivation of wastewater bacteria is also expected to be more efficient in aqueous phase than in solid matrixes,[18] the cumulative dark and light differential decay of the FIBs in natural marine environments would be even more significant. If the decay behavior of the two FIBs represents that of majority of the wastewater bacteria, which was shown to be the case by qPCR (Figure 2) and NGS analysis (Figures 3 and 4), the same wastewater loading would take longer time to dissipate in beach sand than in seawater, providing a kinetic explanation to the often-observed higher abundance of FIBs in beach sand than in seawater.

However, the significant difference in decay rates observed for enterococci and *C. perfringens* between the beach sand and seawater microcosms was not observed for *E. coli*. This was due to the significantly faster decay of *E. coli* (1.14 day^{-1}) in the beach sand microcosms than enterococci (0.20 day^{-1}) and *C. perfringens* (0.59 day^{-1}). Faster decay of *E. coli* than enterococci was observed previously in marine beach sand microcosms[20] and mesocosms,[46] which likely resulted from *E. coli*'s high susceptibility to salinity stress[24] and/or bactericidal effects of indigenous microbiota.[20,47] In the seawater microcosms during the first phase, *E. coli* was also inactivated at a faster rate (1.47 day^{-1}) than enterococci (0.76 day^{-1}) and *C. perfringens* (0.77 day^{-1}). Previous studies also reported faster decay of *E. coli* (1.13−2.9 day^{-1}) than of enterococci (0.12−1.03 day^{-1}) in seawater.[18,48,49] Although direct comparison between *E. coli* and *C. perfringens* decay in marine beach sand has not been reported in literature, *E. coli* showed faster decay than *C. perfringens* in marine sediments,[50] which corresponds nicely to the preference of enterococci over *E. coli* as the FIB in marine environments.[3]

Although cultivation-based methods remain the standard in bacterial decay studies, they are often limited to a few target populations, usually FIBs, due to method tediousness and technical incapability of cultivating majority of bacterial species. The DNA-based methods, including qPCR and NGS-based microbial community analysis, on the other hand, can target many different bacterial populations at once and provide the essential microbial community context in data interpretation. In particular, the NGS-based microbial community analysis is capable of tracking and contrasting both the exogenous wastewater bacteria as well as the indigenous microbiota, providing essential information on both wastewater populations (some of which are otherwise inaccessible) and the overall microbial communities. In recent years, studies have used qPCR to determine wastewater bacterial decay in waters and soils,[48,51] and NGS-based microbial community analysis has also been used to study microbial community change in wastewater-contaminated seawater,[52] while few have reported their application in directly comparing microbial community changes between beach sand and seawater.

The cultivation-independent, DNA-based methods in this study detected decay patterns of overall wastewater bacteria that are similar to those displayed by enterococci and *C. perfringens* between the beach sand and seawater microcosms. The total bacterial density estimated by qPCR quantification of 16S rRNA gene showed significantly slower reduction in the beach sand microcosms than in the seawater microcosms (Figure 2). The overall microbial community structure revealed by Illumina sequencing also showed considerably smaller changes in the beach sand microcosms than in the seawater microcosms over the same period of time (Figure 4). This shows that the decay patterns exhibited by enterococci and *C. perfringens* were good reflection of the behavior of majority of the wastewater bacteria. The corroboration between the cultivation and cultivation-independent methods further strengthened the observation that slower bacterial decay occurred in the beach sand than in the seawater under the microcosm conditions.

Another great advantage of using an NGS-based method in understanding wastewater bacterial decay in the environment is the capability of tracking numerous wastewater bacteria simultaneously and contrasting their abundance dynamics to those of indigenous microbial populations. For example, in this study numerous major wastewater OTUs were identified, and their continuous decrease in relative abundance over time was distinctively contrasted by the dynamics of bacterial OTUs within the indigenous beach sand and seawater microbiota (Figure 3). Although quantitative interpretation of the NGS data is still challenging due to inherent biases associated with PCR and data normalization,[41,53] this contrasting relative abundance dynamics unequivocally illustrated the diminishment of the wastewater OTUs in the microbial communities.

Many of the major wastewater OTUs identified, such as *Arcobacter*,[43] *Cloacibacterium*,[44] and *Bacteroides*,[45] are more difficult to cultivate than the typical FIBs, and hence were rarely investigated in decay studies in spite of their apparent abundance in wastewater. In fact, none of the FIBs investigated by the cultivation-based methods in this study were detected as major wastewater OTU, which is not surprising since their relative abundance levels in human fecal materials are usually quite low (<1%).[54] Many of the OTUs assigned to the beach sand and seawater sources, such as *Nitrosopumilus*, *Oceanospirillaceae*, and *Marinobacter*, were frequently detected in marine environments. However, majority of these OTUs from the beach sand and seawater sources could not be assigned taxonomy at the genus level (Figure 3), indicating limited availability of cultivated representatives and the capability of NGS methods in revealing previously uncultured microbial diversity.

The slower bacterial decay in the beach sand microcosms than in the seawater microcosms could be attributed to many factors. First of all, the wastewater bacteria could have quickly associated with the sand surfaces and become embedded in sand biofilms. Biofilms are known to provide shelter and enable better resistance to environmental stresses than their planktonic counterparts.[55] For example, the surviving *E. coli* cells in freshwater environments were usually associated with suspended particle surfaces,[56] and the decay of *E. coli* in soil/sediment was often significantly slower than in water.[57,58] Second, different oxygen availability in beach sand and seawater could also have played a role in the observed differential decay. Slower inactivation of FIBs was often observed in anaerobic than in aerobic environments,[59−61] and the beach sand matrix is expected to contain anaerobic microniches within the sand cavities and surface biofilms. Furthermore, many important abiotic environmental stresses that cause rapid bacterial decay in seawater, in particular, light inactivation,[17,18,23] are either significantly reduced or completely absent in marine beach sand due to surface protection. This microcosm study was conducted in the absence of light to remove the complex interactions between light, indigenous microbiota and wastewater bacteria, hence the contribution of light inactivation to the decay rate difference are expected to be minimal.

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Biotic stresses are also expected to be different between beach sand and seawater, and hence could also have contributed to the observed difference in decay rates. The starting microbial communities and their change over time between the beach sand and seawater microcosms exhibited considerable overlap but yet distinct difference (Figures 3 and 4, Day 0), which agrees with results from previous studies on natural beach sand and seawater.[7,21,28,31] Although it is not possible from the setup of this study to determine how different community structures affect the decay of exogenous wastewater bacteria, previous studies that manipulated the abundance of indigenous microbiota have clearly illustrated a positive role of indigenous microbiota to wastewater bacterial decay.[20,52,62,63] It would be particularly interesting in future studies to test how microbial diversity affects the decay of exogenous wastewater bacteria, as the NGS approach provides an efficient mean to characterize microbial diversity. In general ecology, higher biodiversity of ecosystem usually provides better resistance to environmental perturbation,[64] and previous studies on microbial communities also showed that higher microbial diversity helped to resist perturbation more effectively than the less diverse ones.[65,66] In this study, the seawater microcosms exhibited lower initial microbial diversity (Table S1, SI) and faster decay rates of exogenous wastewater bacteria (Table 1), although the data points are not sufficient for robust analysis.

The biphasic decay patterns, which were observed for all FIBs monitored by cultivation methods (Figure 1) provide valuable insights to the survival behaviors of wastewater bacteria in beach environments. Several previous studies have also shown biphasic decay pattern of mixed fecal bacteria in soils[51] and in freshwater.[67] Previous studies on enteric bacterial decay in seawater that used cultivated cells of individual strains often observed simple log linear decay curves;[16,20,68] the biphasic curves observed here were likely the result of heterogeneous wastewater bacterial makeup. Cell heterogeneity was often used to explain nonlinear bacterial decay behaviors, including log linear curves with a shoulder, biphasic curves, and sigmoidal curves in previous bacterial decay studies.[69,70]

The biphasic decay patterns and the slower second decay phase resulted in persisting FIB populations in beach sand and seawater (Figure S1, SI), which could have significant implications in water quality monitoring and public health protection. Persisting wastewater populations can also been inferred from the detection of major wastewater OTUs on Days 16 and 23 in the microcosms based NGS-based community analysis (Figure 3). However, the NGS-based microbial community often reported no detection even for these highly abundant major wastewater OTUs, due to the masking effects of the indigenous microbiota compounded by the diminishing wastewater populations. This is where cultivation-based methods are better fitted because of their selectivity to target wastewater populations.

The persisting wastewater bacterial populations may be the result of wastewater bacterial cells with higher stress resistance; for example, bacterial cells collected at the stationary growth phase usually are more resistant to environmental stresses than cells collected at the exponential growth phase.[71] The genetic diversity of wastewater bacteria is another potential factor that could have more profound impact on public health. Single bacterial species (such as *E. coli* and *C. perfringens*) can have certain strains with better capabilities in resisting the environmental stresses. For example, some *E. coli* strains are capable of adapting to high osmotic stresses imposed by seawater salinity[72] or soil desiccation.[73] Different species of the same genus (such as *Enterococcus*) often exhibit different stress resistance and different decay behaviors in the environment.[74] These persisting wastewater bacterial species or strains can clearly prolong the natural cleanup process and hence pose public health risks post wastewater contamination events. It is yet to be determined whether these persisting populations possess proportionally higher public health risks, which is suggested by the often-observed links between stress resistance and virulence in bacterial pathogens.[75] Future research is needed to test if biphasic decay patterns result in disproportionate survival of wastewater bacteria with higher public health threats, which could affect the existing microbial risk assessment regime.

In summary, the different decay rates of wastewater bacteria in beach sand and seawater indicate that beach sand needs to be considered carefully in assessing its impact on water quality monitoring and public health. The significantly slower bacterial decay rates in beach sand than in seawater provide a kinetic explanation to the often-observed higher abundance of FIBs in beach sand, supporting the notion that wastewater-contaminated marine beach sand may act as a chronic source of wastewater bacteria to the beach seawater. The biphasic decay pattern of wastewater bacteria, in particular, the second slower decay phase and the resulting persisting bacterial populations, requires further investigation to elucidate its public health impact. The application of cultivation-independent methods, particularly NGS-based microbial community analysis, can provide valuable insights to understanding the dynamics of wastewater bacteria in the context of indigenous microbial communities in marine beach and other environments.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional information on persisting FIB concentrations and microbial diversity indices in the beach sand and seawater microcossms. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b01879.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 808-956-6024. Fax: 808-956-5014. E-mail: taoyan@hawaii.edu.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Cabelli, V. J.; Dufour, A. P.; McCabe, L. J.; Levin, M. A. Swimming-associated gastroenteritis and water quality. *Am. J. Epidemiol* **1982**, *115* (4), 606−616.

(2) Cabelli, V. J.; Dufour, A. P.; Levin, M. A.; McCabe, L. J.; Haberman, P. W. Relationship of microbial indicators to health effects at marine bathing beaches. *Am. J. Public Health* **1979**, *69* (7), 690−696.

(3) USEPA, Recreational water quality criteria. [820-F-12-058], Office of Water, U.S. Environmental Protection Agency, Washington, D.C. 2012.

DOI: 10.1021/acs.est.5b01879
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

(4) Oshiro, R.; Fujioka, R. Sand, soil, and pigeon droppings: Sources of indicator bacteria in the waters of Hanauma Bay, Oahu, Hawaii. *Water Sci. Technol.* **1995**, *31* (5−6), 251−254.
(5) Bonilla, T. D.; Nowosielski, K.; Cuvelier, M.; Hartz, A.; Green, M.; Esiobu, N.; McCorquodale, D. S.; Fleisher, J. M.; Rogerson, A. 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.* **2007**, *54* (9), 1472−1482.
(6) Yamahara, K. M.; Layton, B. A.; Santoro, A. E.; Boehm, A. B. Beach sands along the California coast are diffuse sources of fecal bacteria to coastal waters. *Environ. Sci. Technol.* **2007**, *41* (13), 4515−21.
(7) Cui, H.; Yang, K.; Pagaling, E.; Yan, T. Spatial and temporal variation in enterococcal abundance and its relationship to the microbial community in Hawaii beach sand and water. *Appl. Environ. Microbiol.* **2013**, *79* (12), 3601−3609.
(8) Phillips, M.; Solo-Gabriele, H.; Piggot, A.; Klaus, J.; Zhang, Y. Relationships between sand and water quality at recreational beaches. *Water Res.* **2011**, *45* (20), 6763−6769.
(9) Halliday, E.; Ralston, D. K.; Gast, R. J. Contribution of sand-associated enterococci to dry weather water quality. *Environ. Sci. Technol.* **2014**, *49* (1), 451−458.
(10) Heaney, C. D.; Sams, E.; Dufour, A. P.; Brenner, K. P.; Haugland, R. A.; Chern, E.; Wing, S.; Marshall, S.; Love, D. C.; Serre, M.; Noble, R.; Wade, T. J. Fecal indicators in sand, sand contact, and risk of enteric illness among beachgoers. *Epidemiology* **2012**, *23* (1), 95−106.
(11) Heaney, C. D.; Sams, E.; Wing, S.; Marshall, S.; Brenner, K.; Dufour, A. P.; Wade, T. J. Contact with beach sand among beachgoers and risk of illness. *Am. J. Epidemiol.* **2009**, *170* (2), 164−172.
(12) Fujioka, R.; Sian-Denton, C.; Borja, M.; Castro, J.; Morphew, K. Soil: the environmental source of *Escherichia coli* and Enterococci in Guam's streams. *J. Appl. Microbiol.* **1998**, *85* (Suppl 1), 83S−89S.
(13) Medrek, T. F.; Litsky, W. Comparative Incidence of Coliform Bacteria and Enterococci in Undisturbed Soil. *Appl. Microbiol.* **1960**, *8* (1), 60−3.
(14) Badgley, B. D.; Thomas, F. I.; Harwood, V. J. Quantifying environmental reservoirs of fecal indicator bacteria associated with sediment and submerged aquatic vegetation. *Environ. Microbiol.* **2011**, *13* (4), 932−42.
(15) Wade, T. J.; Sams, E.; Brenner, K. P.; Haugland, R.; Chern, E.; Beach, M.; Wymer, L.; Rankin, C. C.; Love, D.; Li, Q.; Noble, R.; Dufour, A. P. Rapidly measured indicators of recreational water quality and swimming-associated illness at marine beaches: a prospective cohort study. *Environ. Health* **2010**, *9*, 66.
(16) Vasconcelos, G. J.; Swartz, R. G. Survival of bacteria in seawater using a diffusion chamber apparatus in situ. *Appl. Environ. Microbiol.* **1976**, *31* (6), 913−920.
(17) Fujioka, R. S.; Hashimoto, H. H.; Siwak, E. B.; Young, R. H. Effect of sunlight on survival of indicator bacteria in seawater. *Appl. Environ. Microbiol.* **1981**, *41* (3), 690−696.
(18) Sinton, L. W.; Finlay, R. K.; Lynch, P. A. Sunlight inactivation of fecal bacteriophages and bacteria in sewage-polluted seawater. *Appl. Environ. Microbiol.* **1999**, *65* (8), 3605−3613.
(19) Carlucci, A. F.; Pramer, D. Factors affecting the survival of bacteria in sea water. *Appl. Microbiol.* **1959**, *7*, 388−392.
(20) Feng, F.; Goto, D.; Yan, T. Effects of autochthonous microbial community on the die-off of fecal indicators in tropical beach sand. *FEMS Microbiol. Ecol.* **2010**, *74* (1), 214−25.
(21) Gobet, A.; Boer, S. I.; Huse, S. M.; van Beusekom, J. E.; Quince, C.; Sogin, M. L.; Boetius, A.; Ramette, A. Diversity and dynamics of rare and of resident bacterial populations in coastal sands. *ISME J.* **2012**, *6* (3), 542−53.
(22) Hall-Stoodley, L.; Costerton, J. W.; Stoodley, P. Bacterial biofilms: from the natural environment to infectious diseases. *Nat. Rev. Microbiol.* **2004**, *2* (2), 95−108.
(23) Sinton, L. W.; Davies-Colley, R. J.; Bell, R. G. Inactivation of enterococci and fecal coliforms from sewage and meatworks effluents in seawater chambers. *Appl. Environ. Microbiol.* **1994**, *60* (6), 2040−2048.
(24) Carlucci, A. F.; Pramer, D. An evaluation of factors affecting the survival of *Escherichia coli* in sea water. II. Salinity, pH, and nutrients. *Appl. Microbiol.* **1960**, *8*, 247−250.
(25) Garcia-Lara, J.; Martinez, J.; Vilamu, M.; Vives-Rego, J. Effect of previous growth conditions on the starvation-survival of *Escherichia coli* in seawater. *J. Gen. Microbiol.* **1993**, *139* (7), 1425−31.
(26) Joux, F.; Lebaron, P.; Troussellier, M. Succession of cellular states in a *Salmonella typhimurium* population during starvation in artificial seawater microcosms. *FEMS Microbiol. Ecol.* **1997**, *22* (1), 65−76.
(27) Caporaso, J. G.; Lauber, C. L.; Walters, W. A.; Berg-Lyons, D.; Huntley, J.; Fierer, N.; Owens, S. M.; Betley, J.; Fraser, L.; Bauer, M.; Gormley, N.; Gilbert, J. A.; Smith, G.; Knight, R. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* **2012**, *6* (8), 1621−1624.
(28) Halliday, E.; McLellan, S. L.; Amaral-Zettler, L. A.; Sogin, M. L.; Gast, R. J. Comparison of bacterial communities in sands and water at beaches with bacterial water quality violations. *PLoS One* **2014**, *9* (3), e90815.
(29) Newton, R. J.; Huse, S. M.; Morrison, H. G.; Peake, C. S.; Sogin, M. L.; McLellan, S. L. Shifts in the microbial community composition of Gulf Coast beaches following beach oiling. *PLoS One* **2013**, *8* (9), e74265.
(30) Kostka, J. E.; Prakash, O.; Overholt, W. A.; Green, S. J.; Freyer, G.; Canion, A.; Delgardio, J.; Norton, N.; Hazen, T. C.; Huettel, M. Hydrocarbon-degrading bacteria and the bacterial community response in gulf of Mexico beach sands impacted by the deepwater horizon oil spill. *Appl. Environ. Microbiol.* **2011**, *77* (22), 7962−74.
(31) Boehm, A. B.; Yamahara, K. M.; Sassoubre, L. M. Diversity and transport of microorganisms in intertidal sands of the California coast. *Appl. Environ. Microbiol.* **2014**, *80* (13), 3943−51.
(32) Boehm, A. B.; Griffith, J.; McGee, C.; Edge, T. A.; Solo-Gabriele, H. M.; Whitman, R.; Cao, Y.; Getrich, M.; Jay, J. A.; Ferguson, D.; Goodwin, K. D.; Lee, C. M.; Madison, M.; Weisberg, S. B. Faecal indicator bacteria enumeration in beach sand: a comparison study of extraction methods in medium to coarse sands. *J. Appl. Microbiol.* **2009**, *107* (5), 1740−50.
(33) USEPA, Method 1603: *Escherichia coli (E. coli)* in Water by Membrane Filtration Using Modified membrane-Thermotolerant *Escherichia coli* Agar (Modified mTEC). EPA 821-R-02−022. United States Environmental Protection Agency, Office of Water, Washington, D.C., 2009.
(34) USEPA, Method 1600: Enterococci in Water by Membrane Filtration Using membrane-Enterococcus Indoxyl-beta-D-Glucoside Agar (mEI). EPA 821-R-02-022. United States Environmental Protection Agency, Office of Water, Washington, D.C., 2002.
(35) Bisson, J. W.; Cabelli, V. J. Membrane filter enumeration method for *Clostridium perfringens*. *Appl. Environ. Microbiol.* **1979**, *37* (1), 55−66.
(36) Nadkarni, M. A.; Martin, F. E.; Jacques, N. A.; Hunter, N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology* **2002**, *148* (Pt 1), 257−266.
(37) USEPA, Method 1611: Enterococci in Water by TaqMan Quantitative Polymerase Chain Reaction (qPCR) Assay. EPA-821-R-12-008. United States Environmental Protection Agency, Office of Water, Washington, D.C., 2012.
(38) Vetrovsky, T.; Baldrian, P. The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. *PLoS One* **2013**, *8* (2), e57923.
(39) Fierer, N.; Leff, J. W.; Adams, B. J.; Nielsen, U. N.; Bates, S. T.; Lauber, C. L.; Owens, S.; Gilbert, J. A.; Wall, D. H.; Caporaso, J. G. Cross-biome metagenomic analyses of soil microbial communities and their functional attributes. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109* (52), 21390−21395.
(40) Kuczynski, J.; Stombaugh, J.; Walters, W. A.; González, A.; Caporaso, J. G.; Knight, R. Using QIIME to analyze 16S rRNA gene

DOI: 10.1021/acs.est.5b01879
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

sequences from microbial communities. *Current Protocols in Bioinformatics* **2011**, *36*, 10.7.1−10.7.20.
(41) Paulson, J. N.; Stine, O. C.; Bravo, H. C.; Pop, M. Differential abundance analysis for microbial marker-gene surveys. *Nat. Methods* **2013**, *10* (12), 1200−2.
(42) Clarke, K. R.; Gorley, R. N., PRIMER v6: User Manual/Tutorial. PRIMER-E, Plymouth. 2006.
(43) Stampi, S.; De Luca, G.; Varoli, O.; Zanetti, F. Occurrence, removal and seasonal variation of thermophilic campylobacters and Arcobacter in sewage sludge. *Zentralblatt fur Hygiene und Umweltmedizin = International journal of hygiene and environmental medicine* **1999**, *202* (1), 19−27.
(44) Allen, T. D.; Lawson, P. A.; Collins, M. D.; Falsen, E.; Tanner, R. S. Cloacibacterium normanense gen. nov., sp. nov., a novel bacterium in the family Flavobacteriaceae isolated from municipal wastewater. *Int. J. Syst. Evol. Microbiol.* **2006**, *56* (Pt 6), 1311−1316.
(45) Wexler, H. M. Bacteroides: the good, the bad, and the nitty-gritty. *Clin. Microbiol. Rev.* **2007**, *20* (4), 593−621.
(46) Hartz, A.; Cuvelier, M.; Nowosielski, K.; Bonilla, T. D.; Green, M.; Esiobu, N.; McCorquodale, D. S.; Rogerson, A. Survival potential of *Escherichia coli* and Enterococci in subtropical beach sand: implications for water quality managers. *J. Environ. Qual.* **2008**, *37* (3), 898−905.
(47) Korajkic, A.; McMinn, B. R.; Harwood, V. J.; Shanks, O. C.; Fout, G. S.; Ashbolt, N. J. Differential decay of enterococci and *Escherichia coli* originating from two fecal pollution sources. *Appl. Environ. Microbiol.* **2013**, *79* (7), 2488−92.
(48) Jeanneau, L.; Solecki, O.; Wery, N.; Jarde, E.; Gourmelon, M.; Communal, P. Y.; Jadas-Hecart, A.; Caprais, M. P.; Gruau, G.; Pourcher, A. M. Relative decay of fecal indicator bacteria and human-associated markers: a microcosm study simulating wastewater input into seawater and freshwater. *Environ. Sci. Technol.* **2012**, *46* (4), 2375−82.
(49) Garcia-Lara, J.; Menon, P.; Servais, P.; Billen, G. Mortality of fecal bacteria in seawater. *Appl. Environ. Microbiol.* **1991**, *57* (3), 885−888.
(50) Davies, C. M.; Long, J. A.; Donald, M.; Ashbolt, N. J. Survival of fecal microorganisms in marine and freshwater sediments. *Appl. Environ. Microbiol.* **1995**, *61* (5), 1888−1896.
(51) Rogers, S. W.; Donnelly, M.; Peed, L.; Kelty, C. A.; Mondal, S.; Zhong, Z.; Shanks, O. C. Decay of bacterial pathogens, fecal indicators, and real-time quantitative PCR genetic markers in manure-amended soils. *Appl. Environ. Microbiol.* **2011**, *77* (14), 4839−48.
(52) Sassoubre, L. M.; Yamahara, K. M.; Boehm, A. B. Temporal stability of the microbial community in sewage-polluted seawater exposed to natural sunlight cycles and marine microbiota. *Appl. Environ. Microbiol.* **2015**, *81* (6), 2107−16.
(53) Kennedy, K.; Hall, M. W.; Lynch, M. D.; Moreno-Hagelsieb, G.; Neufeld, J. D. Evaluating bias of illumina-based bacterial 16S rRNA gene profiles. *Appl. Environ. Microbiol.* **2014**, *80* (18), 5717−22.
(54) Yatsunenko, T.; Rey, F. E.; Manary, M. J.; Trehan, I.; Dominguez-Bello, M. G.; Contreras, M.; Magris, M.; Hidalgo, G.; Baldassano, R. N.; Anokhin, A. P.; Heath, A. C.; Warner, B.; Reeder, J.; Kuczynski, J.; Caporaso, J. G.; Lozupone, C. A.; Lauber, C.; Clemente, J. C.; Knights, D.; Knight, R.; Gordon, J. I. Human gut microbiome viewed across age and geography. *Nature* **2012**, *486* (7402), 222−227.
(55) Hall-Stoodley, L.; Costerton, J. W.; Stoodley, P. Bacterial biofilms: From the natural environment to infectious diseases. *Nat. Rev. Microbiol.* **2004**, *2* (2), 95−108.
(56) Brettar, I.; Hofle, M. G. Influence of ecosystematic factors on survival of *Escherichia coli* after large-scale release into lake water mesocosms. *Appl. Environ. Microbiol.* **1992**, *58* (7), 2201−2210.
(57) Faust, M. A.; Aotaky, A. E.; Hargadon, M. T. Effect of physical parameters on the in situ survival of *Escherichia coli* MC-6 in an estuarine environment. *Appl. Microbiol.* **1975**, *30* (5), 800−806.
(58) Gerba, C. P.; McLeod, J. S. Effect of sediments on the survival of *Escherichia coli* in marine waters. *Appl. Environ. Microbiol.* **1976**, *32* (1), 114−120.
(59) Roslev, P.; Bjergbaek, L. A.; Hesselsoe, M. Effect of oxygen on survival of faecal pollution indicators in drinking water. *J. Appl. Microbiol.* **2004**, *96* (5), 938−45.
(60) Eichmiller, J. J.; Borchert, A. J.; Sadowsky, M. J.; Hicks, R. E. Decay of genetic markers for fecal bacterial indicators and pathogens in sand from Lake Superior. *Water Res.* **2014**, *59*, 99−111.
(61) Marti, R.; Mieszkin, S.; Solecki, O.; Pourcher, A. M.; Hervio-Heath, D.; Gourmelon, M. Effect of oxygen and temperature on the dynamic of the dominant bacterial populations of pig manure and on the persistence of pig-associated genetic markers, assessed in river water microcosms. *J. Appl. Microbiol.* **2011**, *111* (5), 1159−75.
(62) Medema, G. J.; Bahar, M.; Schets, F. M. Survival of *Cryptosporidium parvum*, *Escherichia coli*, fecal Enterococci and *Clostridium perfringens* in river water: influence of temperature and autochthonous microorganisms. *Water Sci. Technol.* **1997**, *35* (11−12), 249−252.
(63) Korajkic, A.; Wanjugi, P.; Harwood, V. J. Indigenous Microbiota and Habitat Influence *Escherichia coli* Survival More than Sunlight in Simulated Aquatic Environments. *Appl. Environ. Microbiol.* **2013**, *79* (17), 5329−37.
(64) Tilman, D.; Lehman, C. L.; Thomson, K. T. Plant diversity and ecosystem productivity: theoretical considerations. *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94* (5), 1857−61.
(65) Allison, S. D.; Martiny, J. B. H. Resistance, resilience, and redundancy in microbial communities. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 11512−11519.
(66) Girvan, M. S.; Campbell, C. D.; Killham, K.; Prosser, J. I.; Glover, L. A. Bacterial diversity promotes community stability and functional resilience after perburbation. *Environ. Microbiol.* **2005**, *7*, 301−313.
(67) Hellweger, F. L.; Bucci, V.; Litman, M. R.; Gu, A. Z.; Onnis-Hayden, A. Biphasic Decay Kinetics of Fecal Bacteria in Surface Water Not a Density Effect. *J. Environ. Eng.* **2009**, *135* (5), 372−376.
(68) Rozen, Y.; Belkin, S. Survival of enteric bacteria in seawater. *FEMS Microbiol. Rev.* **2001**, *25* (5), 513−529.
(69) Cerf, O. Tailing of survival curves of bacterial spores. *J. Appl. Bacteriol.* **1977**, *42* (1), 1−19.
(70) Xiong, R.; Xie, G.; Edmondson, A. E.; Sheard, M. A. A mathematical model for bacterial inactivation. *Int. J. Food Microbiol.* **1999**, *46* (1), 45−55.
(71) Lange, R.; Hengge-Aronis, R. Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. *Mol. Microbiol.* **1991**, *5* (1), 49−59.
(72) Gauthier, M. J.; Munro, P. M.; Mohajer, S. Influence of salts and sodium chloride on the recovery of *Escherichia coli* from seawater. *Curr. Microbiol.* **1987**, *15* (1), 5−10.
(73) Zhang, Q.; Yan, T. Correlation of intracellular trehalose concentration with desiccation resistance of soil *Escherichia coli* populations. *Appl. Environ. Microbiol.* **2012**, *78* (20), 7407−13.
(74) Fisher, K.; Phillips, C. The ecology, epidemiology and virulence of Enterococcus. *Microbiology* **2009**, *155* (Pt 6), 1749−1757.
(75) Griffiths, M. *Understand Pathogen Behavior: Virulence, Stress Response and Resistance*. CRC Press: Boca Raton, FL, 2005.

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