SOURCE AND FATE OF FECAL INDICATOR BACTERIA IN TROPICAL SOIL, SAND, AND SEAWATER ENVIRONMENTS

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAIʻI AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

CIVIL ENGINEERING

MAY 2015

BY

Qian Zhang

Dissertation Committee:

Tao Yan, Chairperson
Roger W. Babcock
Oceana Puananilei Francis
Yuanan Lu
Lin Shen
ACKNOWLEDGEMENTS

I am sincerely grateful to all of my committee members, Dr. Roger Babcock, Dr. Oceana Francis, Dr. Yuanan Lu, Dr. Lin Shen and Dr. Tao Yan for their constructive guidance and invaluable advice.

Many thanks go to many present and past members of the Yan group, including Dr. Xia He, Dr. Eulyn Pagaling, Dr. Kun Yang, Dr. Henglin Cui, Mr. Lavane Kim, Mr. Dustin K. Goto, Ms. Fan Feng, and Ms. Jessica Shelton, and Ms. Bunnie Yoneyama of the Environmental Laboratory for their assistance in the lab.

My utmost thanks go to my advisor Dr. Tao Yan. Without his efforts, this project would not have been possible. Thank you Dr. Tao Yan for everything. Thank you for your encouragement, willingness to help and advice on issues far beyond this degree.
DEDICATION

I dedicate my dissertation work to my father and mother, Fuming Zhang and Xiumin Zhang, who always encourage me to follow my dreams. There is no doubt in my mind that without their continued support and counsel I could not have completed this process. This dissertation is also dedicated to my beloved wife, Miaochan Li, for her selfless support and unconditional love. I could not have completed this effort without her assistance, tolerance, and enthusiasm.
ABSTRACT

Fecal contamination of coastal recreational water can adversely impact public health and economic well-being of many coastal communities. Enterococci and *E. coli* are common fecal indicator bacteria (FIBs) used in water quality monitoring and regulation. This dissertation investigates the source and fate of FIBs in Hawaii’s tropical soil, sand and seawater environments. Since Hawaii’s soils are known to contain high levels of *E. coli* that can serve as alternative sources to waterways, the second chapter of this dissertation investigated the survival of soil *E. coli* strains under desiccation stress. The soil *E. coli* strains showed significantly higher desiccation resistance than a laboratory reference strain and several strains isolated from wastewater, and the *de novo* synthesis and accumulation of trehalose was identified as an important mechanism for soil *E. coli* desiccation resistance. Since beach sand is often reported to contain high levels of FIBs while beach erosion or replenishment activities can abruptly affect beach sand abundance, the third chapter investigated the contribution of beach sand to the decay of FIBs in beach systems. The presence of subtidal sand significantly enhanced the decay of *E. faecalis* in beach microcosms, and the indigenous microbiota of the subtidal sand was largely responsible for the decay enhancement. To further understand the fate of FIBs in beach systems, the fourth chapter determined the decay patterns of FIBs in beach sand and seawater separately and compared them to the overall microbial community dynamics. Biphasic decay patterns of FIBs and other fecal bacteria were observed in both beach sand and seawater, while the decay rates in beach sand were significantly smaller than that in seawater, providing a kinetic explanation to the observed high abundance of FIBs.
in beach sand. In the fifth chapter, microbial communities in beach sand and seawater microcosms contaminated by municipal wastewater were tracked using next-generation sequencing of the 16S rRNA gene amplicons, and the exogenous nutrients in the wastewater appeared to determine the microbial community dynamics to a significant extent. Based on results presented, conclusions and recommendations were also made in the sixth chapter of the dissertation.
Table of Contents

CHAPTER 1. BACKGROUND AND OVERVIEW OF RESEARCH ........................................ 1
  1.1 MONITORING WATER QUALITY ........................................................................... 1
  1.2 THE FATE OF FIBs IN RECREATIONAL BEACH .................................................. 4
  1.3 ENVIRONMENTAL FACTORS AFFECTING FIB SURVIVAL ............................... 5
  1.4 MICROBIAL COMMUNITY .................................................................................. 7
  1.5 OBJECTIVES ........................................................................................................ 8

CHAPTER 2. DESICCATION RESISTANCE OF SOIL ESCHERICHIA COLI POPULATIONS
CORRELATES TO INTRACELLULAR TrehALOSE CONCENTRATION .............................. 15
  2.1 INTRODUCTION .................................................................................................. 16
  2.2 MATERIALS AND METHODS ............................................................................ 18
  2.3 RESULTS .............................................................................................................. 23
  2.4 DISCUSSION ....................................................................................................... 31
  2.5 ACKNOWLEDGEMENT ...................................................................................... 38
  2.6 REFERENCE ........................................................................................................ 39

CHAPTER 3. IMPACT OF INDIGENOUS MICROBIOTA OF SUBTIDAL SAND ON FECAL
INDICATOR BACTERIA DECAY IN BEACH SYSTEMS: A MICROCOSM STUDY .......... 43
  3.1 INTRODUCTION .................................................................................................. 44
  3.2 MATERIALS AND METHODS ............................................................................ 47
  3.3 RESULTS .............................................................................................................. 52
  3.4 DISCUSSION ....................................................................................................... 64
  3.5 CONCLUSIONS .................................................................................................... 68
  3.6 ACKNOWLEDGEMENT ...................................................................................... 69
  3.7 REFERENCE ........................................................................................................ 70

CHAPTER 4. DIFFERENTIAL DECAY OF WASTEWATER BACTERIA IN BEACH SAND AND
SEAWATER MICROCOSMS AND CORRESPONDING MICROBIAL COMMUNITY
DYNAMICS ................................................................................................................ 75
  4.1 INTRODUCTION .................................................................................................. 76
  4.2 MATERIALS AND METHODS ............................................................................ 79
  4.3 RESULTS .............................................................................................................. 83
  4.4 DISCUSSION ....................................................................................................... 98
LIST OF TABLES

Table 2. 1 Desiccation-contributed die-off constant ($k_d$) and the concentration difference of major organic solutes between the desiccated and hydrated cells..................29

Table 2. 2 Isolation patterns of the select *E. coli* strains from soil in Manoa watershed..36

Table 3. 1 Beach sand and seawater sample characteristics and their Pearson’s correlation coefficients with the *E. faecalis* die-off constant ($k_d$) in the beach microcosms. .................................................................................................................................54

Table 4. 1 Spearman’s r and P values (in parentheses) between alpha diversity indices and bacterial decay rates in beach sand and seawater microcosms a ..................97

Table 5. 1 Major OTUs identified in all beach sand and seawater microcosms.............116

Table 5. 2 The correlation of nutrient with Shannon index and total bacterial biomass (Spearman Correlation). ..........................................................................................................................128

Table B. 1 Relative abundance of major wastewater OTUs in the raw wastewater, seawater, and beach sand samples. .................................................................142

Table B. 2 Change rates (unit: day$^{-1}$) of the relative abundance of major wastewater OTUs in the beach sand and seawater microcosms during the first (0-6 days) and second (6-23 days) decay phases. .................................................................142

Table B. 3 Microbial diversity indices in beach sand microcosms and seawater microcosms over time ..................................................................................143

Table B. 4 Pearson’s product moment and P values (in parentheses) between alpha diversity indices and bacterial decay rates in beach sand and seawater microcosms a .................................................................144
LIST OF FIGURES

Figure 2.1 An example $^1$H NMR spectra of intracellular organic solutes of soil $E. coli$ strain B54 under the desiccation stress.................................................................22

Figure 2.2 Reduction of culturable $E. coli$ cells as a result of desiccation in quartz sand microcosms over time. $C_d$: $E. coli$ concentration under the desiccation condition; $C_w$: $E. coli$ concentration under the hydrated control condition. The bar graph at the lower section shows the change of water content in the desiccated microcosms. Error bars are the standard errors of the mean values of triplicate tests.................................................................24

Figure 2.3 Concentration of GB uptaken by the $E. coli$ strains under the desiccated condition (solid bars) and the hydrated condition (open bars). Error bars are the standard errors of the mean values from triplicate tests..................................................26

Figure 2.4 Production of EPS by the $E. coli$ strains under the desiccated condition (solid bars) and the hydrated condition (open bars). Error bars are the standard errors of the mean values from triplicate tests.................................................................27

Figure 2.5 Intracellular concentrations of trehalose in ten soil $E. coli$ strains (designated "S"), four $E. coli$ isolates from municipal wastewater (designated "WW") and the lab strain MG1655 under the desiccation condition (solid bars) or the hydrated condition (open bars).................................................................31

Figure 3.1 Reduction of total number of viable viable $E. faecalis$ cells in beach microcosms for Kailua, Kualoa, and Wailale beaches, Honolulu, HI. $M_t$ and $M_0$ represent total cell counts in the microcosms at time $t$ and time zero, respectively. Error bar indicates the standard deviation of the mean of triplicate microcosms................................................................................55

Figure 3.2 Impact of different levels of indigenous microbiota on the reduction of total number of viable $E. faecalis$ cells in Waialae beach microcosms. $M_t$ and $M_0$ represent total cell counts in the microcosms at time $t$ and time zero, respectively. Error bar indicates the standard deviation of the mean of triplicate microcosms................................................................................57

Figure 3.3 Viable $E. faecalis$ cell reduction (log ($M_t/M_0$)) in regular Waialae beach microcosms that contain both beach sand and seawater or seawater-only microcosms (A) and cell reduction due to the presence of indigenous microbiota (log [$((M_t/M_0)_{a}/ (M_t/M_0))$]) in the beach microcosms (B). $M_t$ and $M_0$ represent total cell counts in the microcosms at time $t$ and time zero, respectively. $(M_t/M_0)_{a}$ indicates the viable cell reduction in the autoclaved beach microcosms. Error bar indicates the standard deviation of the mean of triplicate microcosms.................................................................................59
Figure 3. 4 Impact of sand-to-water ratio on the reduction of culturable *E. faecalis* cells in beach microcosms (A), and culturable *E. faecalis* cell reduction in Waialae beach microcosms containing different sand particle size fractions (B). \( M_t \) and \( M_0 \) represent total cell counts in the microcosms at time \( t \) and time zero, respectively. Error bar indicates the standard deviation of the mean of triplicate microcosms. The red dotted line represents the method detection limit (MDL), and data points below the MDL are for illustration.62

Figure 3. 5 Decay of culturable *E. coli* cells from wastewater in regular Waialae beach microcosms that contain both beach sand and seawater or seawater-only microcosms. \( M_t \) and \( M_0 \) represent total cell counts in the microcosms at time \( t \) and time zero, respectively. Error bar indicates the standard deviation of the mean of triplicate microcosms. The red dotted line represents the method detection limit (MDL), and data points below the MDL are for illustration.63

Figure 4. 1 Reduction of concentration of viable *E. coli*, enterococci, *Clostridium perfringens* and *Salmonella* cells in beach sand microcosms and seawater for Kualoa beaches, Honolulu, HI. \( C_t \) and \( C_0 \) represent cell counts in the microcosms at time \( t \) and time zero, respectively. Error bar indicates the standard deviation of the mean of triplicate microcosms. The dotted lines are linear regression lines, and the vertical red lines demarcate the two phases...85

Figure 4. 2 Concentration of persisting *E. coli*, enterococci (ENT), *C. perfringens* (CP) and *Salmonella* during the second phase (ranging from 6 to 23 day) in the beach sand microcosms (A) and seawater microcosms (B). Error bar indicates the standard deviation of the mean of triplicate microcosms. The red dotted lines indicate the method detection limits........................................87

Figure 4. 3 Decay rates of *E. coli*, enterococci (ENT), *C. perfringens* (CP) and *Salmonella* in the first (A) and second (B) decay phases. Error bar indicates the standard deviation of the mean of triplicate microcosms...............................89

Figure 4. 4 Dynamics of the relative abundance of major wastewater OTUs in the beach sand microcosms (A) and seawater microcosms (B). Error bar indicates the standard deviation of the mean of triplicate microcosms.........................................91

Figure 4. 5 Distance based redundancy ordination (dbRDA) for the fitted model of microbial communities in beach sand microcosms (BS) and seawater microcosms (SW) and environmental parameters (TOC, TN, and HPC). Symbols: the numbers behind BS and SW indicate sampling days (0, 3, 6, 16, 23). 92

Figure 4. 6 Clustering analyses of microbial communities in the beach sand microcosms (BS) and seawater microcosms (SW) using principal coordinates analysis (A) and non-metric multidimensional scaling (B). Symbols: the numbers behind BS and SW indicate sampling days (0, 3, 6, 16, 23)..................................94
Figure 5.1 The variation of top 15 major OTUs in sand microcosm spiked with raw wastewater microcosms. (Raw wastewater was spiked into sand microcosms on Day 1).

Figure 5.2 The variation of top 15 major OTUs in seawater microcosm spiked with raw wastewater microcosms. (Raw wastewater was spiked into seawater microcosms on Day 1).

Figure 5.3 The variation of top 15 major OTUs in sand microcosm spiked with autoclaved wastewater microcosms. (Autoclaved wastewater was spiked into sand microcosms on Day 1).

Figure 5.4 The variation of top 15 major OTUs in seawater microcosm spiked with autoclaved wastewater microcosms. (Autoclaved wastewater was spiked into seawater microcosms on Day 1).

Figure 5.5 Diversity of bacterial communities overtime with exogenous nutrient and/or microbes in beach sand microcosms and seawater microcosms. Error bar indicates the standard deviation of the mean of triplicate microcosms.

Figure 5.6 Clustering analyses of microbial communities in the beach sand microcosms (BS) using non-metric multidimensional scaling. Symbols: red diamond plots indicate initial sand sample at Day 0; solid square in plots indicate sand spiked with raw wastewater; square plots without filled indicate sand spiked with autoclaved wastewater.

Figure 5.7 Clustering analyses of microbial communities in the seawater microcosms using non-metric multidimensional scaling. Symbols: red solid cycle plots indicate initial sand sample at Day 0; solid cycle in plots indicate seawater spiked with raw wastewater; cycle plots without filled indicate sand spiked with autoclaved wastewater.

Figure 5.8 Relative abundance of phyla containing >1% of total sequencing reads with exogenous nutrient and/or microbes in beach sands microcosms and seawater microcosms.

Figure 5.9 The variation of TN and TOC in sand microcosms (A) and in seawater microcosms (B). (wastewater was spiked into sand at Day 1).

Figure 5.10 Distance-based Redundancy Analysis (dbRDA) plot of the distLM model based on total nitrogen and total organic carbon fitted to the variation in taxonomic community composition in beach sand microcosms.

Figure 5.11 Distance-based Redundancy Analysis (dbRDA) plot of the distLM model based on total nitrogen and total organic carbon fitted to the variation in taxonomic community composition in seawater microcosms.
Figure A. 1 Concentrations of viable *E. faecalis* cells in the beach sand (A) and seawater (B) compartments of the beach microcosms for Kailua, Kualoa, and Wailale beaches, Honolulu, HI. Error bar indicates the standard deviation of the mean of triplicate microcosms. .......................................................... 137

Figure A. 2 Impact of different levels of indigenous microbiota on the reduction of viable *E. faecalis* cells the beach sand (A) and seawater (B) compartments of Waialae beach microcosms. Error bar indicates the standard deviation of the mean of triplicate microcosms. .......................................................... 138

Figure A. 3 Viable *E. faecalis* cell concentration in the beach sand (A) and seawater (B) compartments in regular Waialae beach microcosms that contain both beach sand and seawater or seawater-only. Error bar indicates the standard deviation of the mean of triplicate microcosms. .......................................................... 139

Figure A. 4 The impact of sand-to-water ratio on the concentration of viable *E. faecalis* cells in the beach sand (A) and seawater compartments in Waialae beach microcosms. Error bar indicates the standard deviation of the mean of triplicate microcosms. .......................................................... 140

Figure A. 5 The impact of sand size fractions on the concentration of viable *E. faecalis* cells in the beach sand (A) and seawater (B) compartments of Waialae beach microcosms. Error bar indicates the standard deviation of the mean of triplicate microcosms. .......................................................... 141

Figure C. 1 The concentration of total nitrogen and total organic carbon in the beach sand microcosms (A) and seawater microcosms (B) overtime. Error bar indicates the standard deviation of the mean of triplicate microcosms ................................. 145
CHAPTER 1. BACKGROUND AND OVERVIEW OF RESEARCH

1.1 MONITORING WATER QUALITY

Microbial contamination in surface water bodies, especially in the recreational freshwater and marine water, is a major public health issue in the U. S. and worldwide. The U. S. Environmental Protection Agency’s (EPA) criteria in monitoring microorganism contamination is based on fecal indicator bacteria (FIBs) (*Escherichia coli*, enterococci and fecal coliforms) (52, 57), as epidemiology studies have illustrated that the concentration of FIB in recreational water are correlated with human health risk (26, 59). There are a number of sources of FIB, including human wastewater, bird and other animal feces, sewage outflows, leaking septic systems, storm and rainfall events (21), that could contaminate the recreational water. According EPA’s recommended criteria, the geometric mean (GM) should not exceed 35 and 33 CFU/100 mL for enterococci in marine and fresh water, respectively, and 126 CFU/100 mL for *E. coli* in fresh water (53, 57). When recreational water quality exceeds the guideline, there will be probably 36 persons of every 1000 beachgoers under the risk of gastrointestinal ill. In 2013, Natural Resources Defense Council reported that 10 percent of 3485 U. S. coastal beaches exceeded the EPA standards. The State of Hawaii is famous of its beautiful 303 miles recreational marine shoreline and approximately 575 marine water segments. In 2012, the Hawaii State Department of Health (HIDOH) monitored 225 marine segments water quality, and illustrated that 38 of the 225 marine segments were contaminated high concentration of Enterococci (28).
When water bodies are contaminated by fecal pollutants, it is technically unfeasible to directly monitor all waterborne pathogens. Hence, FIBs are used as surrogates based on several fundamental assumptions: a) their high correlation with fecal pathogens, b) similar survival patterns as fecal pathogens, c) the testing methods are simple, cost-effective, and sensitive. Fecal coliforms are a thermo-tolerant subgroup of the coliforms and grow well at 44.5 °C. Most of Escherichia coli strains and some Klebsiella spp. strains are fecal coliform. Klebsiella spp. are generally associated with pulp, textile and paper mill wastes. If these waste sources contaminate water bodies, Klebsiella spp. could be mistaken as fecal coliform and produce false positive results. E. coli is an important and regular facultative inhabitant of the human gastrointestinal tract. E. coli in the environment has been often considered as residual cells from recent fecal inputs (63). The consistent existence of E. coli in the intestine and feces of human and animals suggests that E. coli is a good indicator of fecal contamination. The method of monitoring E. coli in environmental water is basing on that E. coli is capable of hydrolyzing 4-methy-umbelliferyl-β-D-glucuronide (MUG), which separates E. coli from other bacteria (17, 43).

Enterococci have a relatively large tolerance range to environmental conditions, and can grow/persis at large temperate (10-45°C) and pH (4.5-10) ranges, and can also survive under high salinity conditions (e.g., 6.5% (20)). Currently, there are 36 Enterococcus species known under Enterococcus genus. The 36 species were classified into five groups: E. faecalis, E. faecium, E. avium, E. gallinarum, and E. cecorum (10). E. faecalis and E. faecium are the two primary enterococcal species found in human feces. The concentration of enterococci in human feces is approximately $10^4$-$10^6$ CFU per gram
(wet weight). According to Tendolkar et al. (2003), there are approximately 800,000 cases of enterococcal infection in U. S. each year (51). *E. faecalis* is the most frequently identified *Enterococcus* species related to infection.

Enterococci are also a common FIB in water quality monitoring and regulation, especially in marine water. The traditional method of monitoring enterococci in environmental water uses membrane filtration (54). In 2010, USEPA recommended a new qPCR-based method to monitor enterococci in environmental water (56). The qPCR-based method can obtain results in less than 4 hours while the traditional membrane filtration method usually requires 24-48 hours. When compared with the traditional membrane filtration method, qPCR generally produces higher concentrations, most likely due to the presence of viable-but-non-culturable (VBNC) cells or naked environmental DNA (1, 25).

*Clostridium perfringens* is a Gram-positive anaerobic bacterium that can cause food poisoning and gas gangrene diseases in humans (46). According to its capability of producing lethal toxins, *C. perfringens* are classified into five types, from A to E (38). *C. perfringens* can exist as either vegetative cells or spores. *C. perfringens* vegetative cells are often identified in fecal samples, while spores are often the result of environmental stresses (6). The concentration of *C. perfringens* in human feces is approximately $10^4$ CFU per gram (wet weight), which is close to that of enterococci. Based on its high concentration in fecal samples, *C. perfringens* was firstly recommend as fecal pollution indicator by Bisson and Cabelli (6). In the following years, similar studies were also supporting and recommending *C. perfringens* as an alternative FIB (22, 35, 40). In the State of Hawaii, in order to clearly identify fecal contamination (e.g. sewage) from other
sources, *C. perfringens* is used as a secondary FIB (50 CFU/100 mL) in conjunction with enterococci (7 CFU/100 mL), and this was approved by EPA (27). The traditional method for monitoring *C. perfringens* (membrane *Clostridium perfringens* (mCP) agar) was developed by Bisoon and Cabelli conducting membrane filtration on (7), and its reliability was shown to be above 91% (7, 22).

### 1.2 The Fate of FIBs in Recreational Beach

Fecal bacteria from external sources, such as sewage or direct fecal deposition by animals (e.g. dogs, birds, cows and etc.) are considered allochthonous to beach systems, which are in contrast to the autochthonous bacteria in the native soil, sand and waterborne environments. When fecal bacteria enter a new ecosystem, most of them, including FIBs (*E. coli* and enterococci) and pathogens, are affected by abiotic factors (e.g. temperature, sunlight, moisture, and nutrient) and biotic factors (indigenous microbial activities, such as competition and predation). The fate of allochthonous microbes, including FIBs, in recreational beach systems would normally follow several routes. First of all, most allochthonous microbes will die within hours of entering into new ecosystems due to the abrupt change of environmental conditions. Secondly, some of the allochthonous microbes are able to resist harsh environmental conditions. For example, *C. perfringens* cells can transition from vegetative cells to spores under environmental stresses, which can then persist in the environment. Thirdly, persistent cells can subsequently replicate when the environmental conditions become appropriate. Some of these fecal populations can even be naturalized into the environment and
become autochthonous microbes, and the environment can become secondary habitats for these naturalized populations (3, 4, 9, 30-33, 60).

1.3 ENVIRONMENTAL FACTORS AFFECTING FIB SURVIVAL

When FIBs (E. coli and enterococci) from external sources, including human wastes, animal feces, sewage leaking, rainfall runoffs, enter recreational beaches, many factors can affect their persistence. Sunlight has been recognized as an important environmental stressor of microorganisms for a long time history (16). There are two major mechanisms (direct photoinactivation and indirect photoinactivation) of sunlight inactivate. When sunlight spectrum is below 400 nm (the ultraviolet range), direct photoinactivation occurs as the UV light directly damage the cellular DNA. Indirect photoinactivation happens when endogenous or exogenous chemicals act as “photosensitizers” by absorbing light and reacting with molecular oxygen to produce reactive oxygen species (ROS) (e.g., $^1$O$_2$, superoxide, hydrogen peroxide, and hydroxyl radicals), which can cause photooxidative damage to membranes, proteins, or enzymes (15, 48, 49, 67). Viau et al. (2011) found that there was higher concentration of enterococci in the morning than in the afternoon when they surveyed 22 streams in Hawaii (58). Similarly, Boehm et al. (2009) also reported higher concentration of enterococci in the morning than at evening at Avalon Beach, CA (8). Similar phenomenon was also demonstrated in E. coli in freshwater lakes (62) and in marine water (23). Although temperature and pH variation are also important factors in affecting the survival of FIBs (for example, elevated temperature and pH had a negative impact on the survival of FIBs (14, 50)), Rijal and Fujioka (2001) demonstrated that when exposure to sunlight, FIB had a more rapid decay rate (44).
Nutrient availability can also affect the survival of FIBs, as FIBs transition from a nutrient rich environment (animal gastrointestinal) to relatively nutrient poor environments (such as water, sand and soil). Generally, when FIBs and other fecal bacteria are released into water bodies, they are subjected to starvation stress, as the amount of inorganic and organic nutrients in freshwater or marine water are obviously much lower than in feces or wastewater. In 1960, Carlucci and Pramer showed that higher concentrations of nutrients (inorganic and organic) correlated positively with the survival of *E. coli* in marine water (13). Similar results were obtained by Byappananhalli *et al.* (2006), who studied the occurrence and persistence of *E. coli* and enterococci in beach sand and found that *E. coli* replicated in sand amended with lake plankton (11). Imamura *et al.* (2011) found greater survival of *E. coli* and enterococci in the sand amended with wrack than in the control, un-amended sand (29).

Osmotic stress is also a major environmental factor affecting the survival of FIBs in soil and sand. Generally, bacteria grow well when the water activity ($a_w$) is 0.97 or above, while some halophilic archaea can grow at $a_w$ as low as 0.75 and some fungi can tolerate $a_w$ as low as 0.6 (61). Under the desiccation stress, bacteria produce organic compounds (e.g., proline, glutamine, acetate, valine, glucose, trehalose, etc.) to mediate resistance to lower $a_w$. Zhang and Yan (2012) demonstrated positive relationship between intracellular trehalose concentration and the desiccation resistance of *E. coli* (65). Conversely, higher moisture may not always be good for FIBs’ survival in the environment. Studies on marine beach sand in Florida showed that higher concentration of *E. coli* or enterococci were detected in supratidal zone than in swash zone (1, 18, 41), which was attributed to
reduced protozoa activities in drier sand as protozoa are also affected by the moisture content.

The impact of competition (for nutrient and other resources) on FIBs has been investigated in numerous studies (19, 36). In recreational beach, Feng et al. (2010) found that the presence of autochthonous bacteria is an important stressor on the survival of E. coli (19). Mitchell et al. conducted the survival of E. coli in different treated seawater ranging from autoclaved seawater to different size fractions of marine microbial inocula, and found that E. coli concentration decreased significantly faster in seawater inoculated with marine microbial inoculum than in autoclaved seawater (36).

1.4 MICROBIAL COMMUNITY

Microbial communities in recreational beaches are highly complex as they are frequently impacted by various exogenous microbial sources (e.g., human activities, animal feces and wastewater spills) and environmental factors (e.g., pH, temperature, salinity, nutrient), which causes high variation in microbial composition and fluctuation in the abundance of individual species. Cultivation-based methods have been traditionally used to study microbial communities. However, these methods are limited in their capability of providing the full information of microbial communities as more than 99% of bacterial species are still not culturable. With the advancement of molecular tools, PCR amplification was used to study the microbial community followed by cloning and sequencing of 16S ribosomal RNA gene. This application has its limitation since it can only assay approximately 100 sequences per sample, and requires intensive labor. The recent development of next generation sequencing (NGS) technologies has shown great
promise in fully characterizing microbial communities. The 454 pyrosequencing technology can generate thousands of 16S rRNA gene amplicons in a single run (45, 64, 66). The more recent Illumina sequencing can generate even more sequence reads with significant less costs, and hence has becoming widely used in microbial community characterization (2, 5, 12, 24, 34, 42).

1.5 OBJECTIVES

The main goals of this dissertation research were 1) to investigate the survival of soil *E. coli* strains under desiccation stress and identify major mechanisms of desiccation resistance; 2) to investigate the survival of *Enterococci faecalis* in submerged beach sand and determine the impact of autochthonous microbial communities in submerged beach sand on *E. faecalis* survival; 3) to exam the persistence of fecal bacteria (*E. coli*, enterococci, *Salmonella*, and *Clostridium perfringens*) in beach sand and seawater by using both cultivation-based and NGS technologies to monitor the composition of major populations of wastewater in beach sand and seawater; and 4) to analyze the variation of microbial communities in beach sand and seawater under the influence of exogenous wastewater nutrients and bacteria.
1.6 REFERENCES


CHAPTER 2. DESICCATION RESISTANCE OF SOIL *ESCHERICHIA COLI* POPULATIONS CORRELATES TO INTRACELLULAR TREHALOSE CONCENTRATION

ABSTRACT

Naturalized soil *E. coli* populations need to resist common soil desiccation stresses in order to inhabit soil environments. In this study, four representative soil *E. coli* strains and one lab strain MG1655 were tested for desiccation resistance via die-off experiments in sterile quartz sand under a potassium acetate-induced desiccation condition. The desiccation stress caused significantly lower die-off rates of the four soil strains (0.17 - 0.40 day\(^{-1}\)) than MG1655 (0.85 day\(^{-1}\)). Cellular responses, including extracellular polymeric substance (EPS) production, exogenous glycine betaine (GB) uptake and intracellular compatible organic solute synthesis, were quantified and compared under the desiccation and hydrated control conditions. GB uptake appeared not to be a specific desiccation response, while EPS production showed considerable variability among the *E. coli* strains. All *E. coli* strains produced more intracellular trehalose, proline, and glutamine under the desiccation condition than the hydrated control, and only did the trehalose concentration exhibit a significant correlation with the desiccation-contributed die-off coefficients (Spearman’s $\rho = -1.0$, $P = 0.02$). *De novo* trehalose synthesis was further determined for 16 *E. coli* strains from both soil and non-soil sources to determine its prevalence as a specific desiccation response. Most *E. coli* strains (15/16) specifically and significantly synthesized trehalose under the desiccation conditions, and, the soil *E. coli* strains, in general, produced more trehalose (106.5±44.9 µmol/mg protein) than the non-soil reference strains (32.5±10.5 µmol/mg protein).
2.1 INTRODUCTION

Traditionally, *E. coli* in the environment was often considered as residual cells from recent fecal inputs (46), which however has been contradicted by recent reports of the ubiquitous presence of high levels of *E. coli* cells in soil samples (5, 18, 23). Studies using molecular tools have detected unique genotypic compositions of *E. coli* populations in soil (6, 19, 23), and the persistence of certain *E. coli* strains over extended periods of time in soils further supports the notion that certain soil *E. coli* populations are naturalized members of indigenous soil microbial communities (23). Since soil-sourced *E. coli* cells may enter nearby waterways in the absence of actual fecal inputs and generate false signals of fecal pollution, intense debate has arisen on the suitability of *E. coli* as a fecal indicator in water quality monitoring.

To understand the survival and inhabitation of *E. coli* in soil, it is important to under how *E. coli* resists soil environmental stresses, in particular the common and unique soil desiccation stress caused by the natural cycles of soil wetting and drying. Studies on traditional soil bacteria have identified numerous cellular mechanisms responsible for enhanced desiccation resistance, including *de novo* trehalose synthesis (10, 29), production of extracellular polymeric substances (EPS) (36, 38), and uptake of exogenous glycine betaine (GB) (2, 4, 44). Although few studies have examined *E. coli* desiccation resistance (45), *E. coli* uses similar mechanisms to resist osmotic shocks caused by increased water salinity in aquatic environments. For example, high water salinity prompted *E. coli* to synthesize trehalose and sucrose (12, 34), to uptake environmental GB (7, 25), and to produce EPS (8).
De novo synthesis of trehalose is a desiccation resistance strategy commonly employed by soil bacteria, including *Bradyrhizobium* spp. (10, 40), *Rhodococcus* spp. (26), *Rhizobium* spp. (29), and *Pseudomonas* spp. (15). During desiccation, intracellular trehalose can help maintain the phospholipid bilayer of cell membrane in the liquid crystalline phase (9) and can also keep protein in hydrated formation by hydrogen bonding and water replacement (27). Consequently, trehalose is believed to be an excellent osmoprotectants under severe water activity reduction (33). The genetic capability of *de novo* trehalose synthesis is widely distributed, and *E. coli* is known to synthesize trehalose for salinity-caused osmotic stress resistance (12, 34). Previous studies have shown that over expression of trehalose in a recombinant *E. coli* strains (30) or osmotically-induced trehalose accumulation in *E. coli* NCIB 9484 (45) can improved *E. coli* desiccation resistance significantly.

Tropical soils in Hawaii have been reported to contain high levels of *E. coli* (5, 16, 20). Recent sampling efforts in Manoa watershed on the Island of Oahu, Hawaii, also detected high levels of *E. coli* (603-1,820,000 CFU/100g soil) during a nine-month sampling period (18). By constructing a soil *E. coli* library for the Manoa watershed, numerous *E. coli* strains were frequently found at different sampling locations and sampling dates (19). These soil *E. coli* strains are thus considered naturalized soil *E. coli* populations and are expected to have higher desiccation resistance than *E. coli* from other sources. In this study, desiccation-contributed die-off of four representative soil strains and the K12 strain MG1655 were investigated under a desiccation condition in sterile quartz sand. The *E. coli* strains’ cellular responses to desiccation, including exogenous GB uptake, EPS production, and synthesis of compatible organic solutes, were quantified.
and correlated with their respective desiccation-contributed die-off patterns. Finally, additional *E. coli* strains from soil and non-soil sources were tested for the production of trehalose as a specific and major desiccation response.

### 2.2 MATERIALS AND METHODS

**E. coli strains.** A previous study isolated 234 unique *E. coli* genotypes from 630 *E. coli* isolates from soil samples collected from Manoa watershed on the island of Oahu, Hawaii (19). Ten *E. coli* strains were selected to represent soil *E. coli* based on their repetitive detection in different sampling sites and at different sampling times (Table 1). Four *E. coli* isolates were isolated from wastewater collected at the Sand Island Wastewater Treatment Plant of Honolulu, Hawaii to represent *E. coli* strain from human fecal sources. The sewage *E. coli* isolates were isolated, verified and characterized using the same procedures as for the soil *E. coli* isolates (19). Additional reference *E. coli* strains include the common lab K12 strain MG1655. A subset of the *E. coli* strains (S-A34, S-B31, S-B32, S-B54, and MG1655) were tested for cell die-off under the desiccation stress and investigated for their specific cellular responses to desiccation, while the whole strains set was tested specifically for the most prevalent desiccation resistance mechanism (i.e., *de novo* trehalose synthesis).

**Incubation of E. coli cells in quartz sand.** Die-off of exponential-phase cells of *E. coli* strains (S-A34, S-B31, S-B32, S-B54, and MG1655) in sterile quartz sand was conducted under a desiccated condition and a fully hydrated condition. The fully hydrated condition was used to determine the natural endogenous decay of the *E. coli* cells. Fresh over-night single colonies of the *E. coli* strains were used to inoculate 5 mL
of mineral salts medium plus glucose (36), which were subsequently incubated in a shaking incubator at 200 rpm and 37 °C to reach mid-exponential stage (OD600 = 0.4 - 0.7). Cells were harvested by centrifugation at 10,000 x g for 10 min, and resuspended in phosphate buffered saline (PBS) to make cell stock solutions. To establish the experimental microcosms, 2.6 mL of the freshly prepared cell stock solutions, which is equivalent to c.a. 10⁹ cells, were spiked into 10 g sterile, acid-washed quartz sand placed in Pyrex petri dishes.

Six identical microcosms were established for each of the five *E. coli* strains; three microcosms for the desiccation experiment and the other three for the hydrated control. The desiccated microcosms were placed in desiccators using a saturated potassium acetate solution (KOAc) as the desiccant, which produces 27% relative humidity at equilibrium (10). The hydrated control microcosms were incubated in desiccators containing sterile double-distilled water to maintain 100% relative humidity. Water content in the microcosms was monitored daily by weighing the microcosms. The microcosms in the desiccators were incubated at room temperature in the dark. Sand samples collected from the microcosms were first extracted using PBS to release *E. coli* cells (3) and then enumerated on tryptic soy agar plates.

**Incubation of *E. coli* cells on membranes.** *E. coli* cells were also incubated on polycarbonate membranes under both the desiccated condition and the hydrated condition to quantify the production of EPS, uptake of exogenous GB, and the production of organic compatible solutes including trehalose. The membrane-based desiccation experiments, in contrast to the sand-based desiccation experiments, facilitate the extraction and quantification of EPS, GB, and intracellular organic solutes by avoiding
sand interferences. The mid-exponential stage cells of the *E. coli* strains were filtered onto polycarbonate membranes (0.2 µm pore size, 47 mm diameter; Whatman, Springfield Mill, UK). The experimental set-up and incubation were otherwise exactly the same as the sand microcosm experiments.

The cell-bearing membranes were incubated for four days for the quantification of EPS production and the synthesis and accumulation of intracellular compatible organic solutes. For the quantification of exogenous GB uptake, the cells were incubated under the desiccated or the hydrated conditions for 48 hours, and then 800 µL glycine betaine (100 µM) was added to each membrane and incubated for 15 min, which allows the uptake of GB by *E. coli* cells to reach a plateau according to Perroud and Le Rudulier (32). The membranes were immediately vacuumed and washed twice with 20 mL 0.9% NaCl solution to remove residual liquid.

**EPS extraction and quantification.** *E. coli* cells on the membranes were washed off with 1mL of 0.15 M NaCl solution and collected in glass test tubes. The test tubes were incubated at 100°C for 10 min, cooled to room temperature, and then centrifuged at 10,000 x g for 15 min. Supernatants of the cell extracts were subjected to the phenol-sulfuric acid test (12). Briefly, cell extracts (500 µL) were first mixed with 12.5 µL of 80% phenol, and then swiftly amended with 1250 µL of concentrated sulfuric acid to initiate reaction at high temperature. The reactions were allowed to progress for 10 min before briefly shaken to mix. After approximately 20 minutes of further incubation at room temperature, absorbance measurements at 485 nm were taken, which were used to calculate EPS concentration based on a five-point calibration curve.
Intracellular organic solute extraction. For the intracellular compatible organic solute synthesis and the exogenous GB uptake experiments, the cell-bearing membranes were extracted following a procedure described by Higo (21). Briefly, the membranes were submerged in 800 µL 80% ethanol, incubated at 65 °C for 3 hours, and finally centrifuged at 10,000 x g for 10 min. The supernatants were separated from the pellets and then vacuum-dried. The pellets were re-suspended in 0.5 mL sterile DI water and centrifuged at 10,000 x g for 10 min, and the supernatants were pooled and vacuum-dried for subsequent solute quantification by different methods.

Quantification of compatible organic solutes using NMR. Nuclear magnetic resonance (NMR) quantification of compatible organic solutes was conducted by the NMR lab at the University of Hawaii at Manoa. The cell extract pellets were first dissolved in 250 µL D2O, and 1H NMR spectra were obtained using a Varian Unity Inova 500 spectrometer. The acquisition parameters used for 1H (500-MHz) NMR were 40.5° pulse width, 3.5 second acquisition time, 0.5 second relaxation delay, and 64 repetitions. Chemical shifts were expressed in parts per million (ppm) downfield from sodium 3-(trimethylsilyl) sulphonate (DSS) (an example proton NMR spectra and the identification of organic solutes based on chemical shifts is shown in Figure 2.1), and quantification was achieved by comparing the integrated peak areas of the organic solutes with that of DSS, which was introduced as the internal quantification standard.
Figure 2.1 An example $^1$H NMR spectra of intracellular organic solutes of soil *E. coli* strain B54 under the desiccation stress.

**Trehalase assay.** The intracellular trehalose in the cell extracts was also quantified using a trehalase assay (21). Briefly, aliquots of cell extracts (100 µL) were mixed with 100 µL of reaction mixture that contained 100 mM morpholinoethanesulfonic acid (MES)-KOH (pH 6.0) and $5 \times 10^{-3}$ U/mL trehalase. The reactions were incubated at 37 °C for one hour to convert trehalose into glucose before being terminated by the addition of 50 µL of 500 mM Tris-HCl (pH 7.5). The resulting glucose was subsequently quantified using a glucose kit (Sigma-Aldrich; St. Louis, MO) following the manufacturer’s procedure.

**Data analysis.** The die-off of *E. coli* cells was modeled using the first-order decay kinetics (Eq. 1 and 2). The desiccation-contributed cell die-off coefficient is $k_d$, and cell natural decay coefficient is $k_w$. The remaining cell concentrations under the
desiccation and the hydrated control conditions are $C_d$ and $C_w$, respectively. Because the initial cell concentration for each strain ($C_0$) is the same for both the desiccated experiment and the hydrated control, the desiccation-contributed cell die-off can be mathematically represented by Eq. 3.

$$\ln C_d - \ln C_0 = -(k_d + k_w) t \quad (1)$$

$$\ln C_w - \ln C_0 = -k_w t \quad (2)$$

$$\ln\left(\frac{C_d}{C_w}\right) = -k_d t \quad (3)$$

The concentrations of EPS, trehalose, and other organic solutes were expressed in reference to cellular protein concentration that was determined using a bicinchoninic acid assay (BCA) kit (Thermo Scientific, MA) (1). The Student’s $t$ test was used to compare the mean values of two different treatments. Spearman’s $\rho$ values were calculated to determine the rank correlation between desiccation-caused die-off coefficients and the accumulated intracellular organic solutes. Unless otherwise stated, the default statistical significance level is $P \leq 0.05$. Statistical analyses were performed using a StatistiXL add-in package in Microsoft Excel.

2.3 RESULTS

**Desiccation resistance of soil E. coli strains.** The cell die-off patterns of E. coli strains S-A34, S-B31, S-B32, S-B54 and MG1655 in quartz sand under the desiccated condition and the hydrated control condition were determined. Cell die-off as a result of desiccation ($C_d/C_w$) showed significant difference between the soil E. coli strains and MG1655 (Figure 2.2). Under the hydrated control condition (100% relative humidity), the majority of E. coli strains maintained relatively stable populations without apparent
cell die-off, and cell concentrations of strains MG1655 and S-A34 actually increased by approximately 10 fold, indicating cell growth (data not shown). Under the desiccation condition, the water content in the microcosms gradually decreased from 27% and eventually stabilized at approximately 5% at Day 5. Clear reduction in viable cell counts was observed for MG1655 on Day 2 when the water content was 13%, while for the four soil *E. coli* strains viable cell count reduction only occurred on Day 4 when the water content was 5%. No significant difference in cell die-off under the desiccated condition was detected among the four soil *E. coli* strains towards the end of the experiment.

![Figure 2](image.png)

Figure 2. 2 Reduction of culturable *E. coli* cells as a result of desiccation in quartz sand microcosms over time. $C_d$: *E. coli* concentration under the desiccation condition; $C_w$: *E. coli* concentration under the hydrated control condition. The bar graph at the lower section shows the change of water content in the desiccated microcosms. Error bars are the standard errors of the mean values of triplicate tests.
**Exogenous GB uptake.** To determine whether or not the uptake of exogenous GB was an important mechanism for *E. coli* desiccation resistance, the soil *E. coli* strains and MG1655 were allowed to uptake exogenous GB under both the desiccation stress and the hydrated control condition. The amount of GB uptake by the soil *E. coli* strains was not significantly higher than that MG1655 (Figure 2.3). In fact, one soil *E. coli* strain (S-B32) exhibited significantly less GB (1.28 µmole/mg protein) uptake than MG1655 (4.51 µmole/mg protein), despite having significantly higher desiccation resistance than the latter (Figure 2.2). Furthermore, the amount of exogenous GB taken up by MG1655 and three of the soil *E. coli* strains (S-A34, S-B31, and S-B32) under the hydrated condition were higher than under the desiccated condition, indicating that desiccation stress might actually limit the uptake of exogenous GB by these strains.
EPS production. The production of EPS by the *E. coli* strains under the desiccated condition and the hydrated condition was quantified to determine the involvement of EPS in *E. coli* desiccation resistance. Under the desiccated condition, all four soil *E. coli* strains produced significantly more EPS than MG1655 (Figure 2.4), which corresponds to their higher desiccation resistance than the latter and suggests the involvement of EPS in the high desiccation resistance expressed by the soil *E. coli* strains. Majority of the soil *E. coli* strains, including S-A34, S-B32 and S-B54, produced significantly more EPS under the desiccation condition than under the hydrated condition, while no statistical difference were detected for strain S-B31 and MG1655 under the two experimental conditions.
Major intracellular organic solutes. Major organic solutes present in *E. coli* cells under the desiccation and hydrated conditions were identified and quantified using \(^1\)H NMR. Trehalose, proline, glutamine, acetate, valine and glucose were identified as major organic solutes in the desiccated and hydrated *E. coli* cells by \(^1\)H NMR. The intracellular concentration differences between the desiccated condition and the hydrated control were calculated and correlated with their desiccation-caused die-off coefficients (Table 2.2). Among the major intracellular organic solutes, trehalose, proline, glutamine and glucose showed elevated intracellular concentrations under the desiccated condition.
consistently for all strains. The Spearman’s rank correlation coefficients between the elevated intracellular concentrations of organic solutes under the desiccated condition and the desiccation-contributed die-off coefficients were calculated (Table 2.2). Among the major organic solutes, a strong and significant correlation was observed only for trehalose, suggesting that de novo trehalose synthesis is a universal and specific response to desiccation stress by the *E. coli* strains.
Table 2. Desiccation-contributed die-off constant ($k_d$) and the concentration difference of major organic solutes between the desiccated and hydrated cells.

<table>
<thead>
<tr>
<th>Strains</th>
<th>$k_d$ (day$^{-1}$)</th>
<th>Concentration difference (µmol/mg protein)</th>
<th>Trehalose</th>
<th>Proline</th>
<th>Glutamine</th>
<th>Acetate</th>
<th>Valine</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG1655</td>
<td>0.85</td>
<td></td>
<td>18.9</td>
<td>8.3</td>
<td>24.7</td>
<td>2.4</td>
<td>-9.0</td>
<td>4.5</td>
</tr>
<tr>
<td>S-A34</td>
<td>0.40</td>
<td></td>
<td>27.2</td>
<td>1.0</td>
<td>13.6</td>
<td>11.8</td>
<td>-26.7</td>
<td>9.3</td>
</tr>
<tr>
<td>S-B31</td>
<td>0.35</td>
<td></td>
<td>41.3</td>
<td>6.9</td>
<td>12.3</td>
<td>-0.2</td>
<td>-24.4</td>
<td>8.7</td>
</tr>
<tr>
<td>S-B32</td>
<td>0.27</td>
<td></td>
<td>55.5</td>
<td>12.8</td>
<td>13.9</td>
<td>2.0</td>
<td>-11.1</td>
<td>8.1</td>
</tr>
<tr>
<td>S-B54</td>
<td>0.17</td>
<td></td>
<td>121.5</td>
<td>47.1</td>
<td>7.3</td>
<td>4.7</td>
<td>-6.0</td>
<td>59.3</td>
</tr>
</tbody>
</table>

Spearman’s $\rho^a$ -1.0 (0.02) -0.7 (0.23) 0.7 (0.23) 0.1 (0.95) -0.4 (0.52) -0.6 (0.35)

$^a$ Spearman’s rank correlation coefficients between $k_d$ and the concentration of compatible solutes; $P$ values are in the parentheses.
Prevalence of trehalose as a desiccation response. A total of 16 *E. coli* strains, including ten soil *E. coli* strains, four *E. coli* isolates from municipal wastewater and two lab reference *E. coli* strains, were used to investigate the prevalence of elevated intracellular trehalose production as a specific response to desiccation stress. The intracellular concentrations of trehalose of *E. coli* cells incubated under the desiccated condition and the hydrated condition were determined (Figure 2.5). After four days of incubation under the fully hydrated control condition, the *E. coli* cells accumulated small amounts of trehalose (4.4±5.4 µmol/mg protein), except for soil strain S-A37 (97.1 µmol/mg protein). All *E. coli* strains produced significantly more trehalose under the desiccated condition than under the hydrated control condition (*t* test, *P*≤0.05), except for strain S-A37, indicating that *de novo* trehalose synthesis is a common and specific desiccation response by *E. coli*. Under the desiccation condition, different *E. coli* strains produced different levels of intracellular trehalose (20.9-260.5 µmol/mg protein). The ten soil *E. coli* strains synthesized and accumulated significantly more trehalose (106.5±44.9 µmol/mg protein) than the wastewater *E. coli* isolates and MG1655 (32.5±10.5 µmol/mg protein).
Figure 2. Intracellular concentrations of trehalose in ten soil E. coli strains (designated "S"), four E. coli isolates from municipal wastewater (designated "WW") and the lab strain MG1655 under the desiccation condition (solid bars) or the hydrated condition (open bars).

2.4 DISCUSSION

Recent studies have provided strong evidences indicating that E. coli can exist as an autochthonous component of soil microbial community (5, 6, 16, 20, 23). This recognition questions the reliability of conventional water quality monitoring approach that uses E. coli as a fecal indicator. At geographic locations where environmental conditions favor the inhabitation of E. coli in soil, the soil-sourced E. coli cells can be transported into water under various conditions (e.g. rainfall runoffs), resulting in high E.
coli counts in water in the absence of actual fecal pollution. Even for actual fecal pollutions, the capability of soils to support *E. coli* growth may significantly prolong their survival in the environment, thus making soils lasting secondary reservoirs of fecal *E. coli* cells. The former scenario would invalidate the specificity of water quality monitoring while the latter would undermine the time-sensitiveness of the monitoring efforts.

To inhabit and maintain a population in soil, *E. coli* must be able to resist soil environmental stresses, one of which is the desiccation stress associated with natural soil wetting-drying cycles. Desiccation is a common and unique environmental stress in soil, and soil-dwelling bacteria are generally equipped with mechanisms for desiccation resistance (33, 41). Since habitat stresses affect bacterial adaptation and divergence within the same species (35, 39), one may expect that the *E. coli* strains that inhabit soil should generally exhibit higher desiccation resistance than strains occupying other niches. The present study showed that four soil *E. coli* strains survived better than the common lab strain MG1655 in desiccated sterile quartz sand. The soil strains were observed to maintain relatively stable population size until the water content in quartz sand dropped from 26.8% to 9.3% in three days, exhibiting considerable desiccation resistance (Figure 2.2). It is worth noting that the desiccation die-off experiments were conducted in the absence of other environmental stresses (such as sunlight irradiation, temperature fluctuation and competition/antagonism from indigenous soil microbial communities) that can also affect *E. coli* survival (13, 18). In natural soil environments, the soil desiccation stress works together with the other environmental stresses to determine the fate of soil *E. coli* populations.
The five *E. coli* strains used in the desiccation die-off experiments were further investigated to identify major desiccation resistance mechanisms by the strains, which is important to the understanding of *E. coli*’s ecology and adaptation in soil environments. A reasonable hypothesize is that soil *E. coli* strains utilize the mechanisms used by typical soil bacteria, such as *Rhizobium/Bradyrhizobium* (10, 29) and *Pseudomonas* (36, 38), for desiccation resistance. Numerous mechanisms are employed by typical soil bacteria to resist desiccation (33), with the common ones being exogenous GB uptake (2, 4, 44), EPS production (36, 38), and *de novo* trehalose synthesis (10, 29). In this study, the involvement of certain mechanisms in *E. coli* desiccation was identified by quantifying multiple *E. coli* strains’ specific cellular responses to the desiccation stress, which were then correlated with their respective desiccation resistance levels. This approach differs from the conventional mechanistic studies using single *E. coli* strains, and draw its strength from the involvement of multiple strains.

Among the different mechanisms is the uptake of exogenous GB, which is a common osmoprotectant responsible for desiccation resistance in soil bacteria (33). Although *E. coli* lacks the capability of synthesizing GB *de novo*, *E. coli* was shown to uptake exogenous GB from aquatic environments to enable survival and growth under high salinity conditions (7, 25, 32). However, GB did not appear to be an effective osmoprotectants under desiccation, likely due to the severe water activity reduction associated with desiccation (33). A previous study showed that the salinity-induced intracellular GB accumulation did not increase the desiccation resistance of *E. coli* strain NCIB 9484 (45), which differs from lactic acid bacteria that showed increased desiccation resistance due to salinity-induced GB uptake (24). The non-effectiveness of
GB to *E. coli* desiccation resistance is also supported by the non-specific GB uptake in response to desiccation stress by the five *E. coli* strains in the present study; four out of five *E. coli* strains tested actually accumulated less exogenous GB under the desiccation condition than under the hydrated control condition (Figure 2.3). Furthermore, the amount of GB uptake did not correlate with the desiccation-contributed die-off of the *E. coli* strains.

Comparatively, EPS production appeared to be more involved in soil *E. coli* desiccation resistance than exogenous GB uptake, which is supported by the significantly higher EPS production by the soil *E. coli* strains than MG1655 under the desiccation stress (Figure 2.4). Since EPS is essential to the formation of biofilm that confer desiccation resistance to soil bacteria (14), the elevated EPS concentrations correspond well to the higher desiccation resistance of the soil *E. coli* strains. Among the four soil *E. coli* strains tested, three strains produced more EPS under the desiccation condition than under the hydrated control condition, a pattern often observed in desiccated soil bacteria (36). Strains MG1655 and S-B31 produced slightly less EPS (although not statistically significant) under the desiccation condition than the hydrated control condition, which may be attributed to EPS production being a generic response to numerous environmental cues (11, 37, 42) and the quality of EPS being as important as quantity to desiccation resistance (31), which was not considered in the present study.

Trehalose, on the other hand, exhibited a clear role in the desiccation resistance of the *E. coli* strains. Trehalose is the only major intracellular organic solutes that exhibited significant correlation with the *E. coli* die-off rate under the desiccation condition (Table 2.2). All *E. coli* strains tested responded to the desiccation stress by synthesizing
significantly more trehalose (Table 2.2, Figure 2.5). Furthermore, correlation analyses showed that the amount of trehalose synthesized and accumulated by the different *E. coli* strains corresponded to their respective desiccation resistant capacities. The four soil *E. coli* strains that showed higher desiccation resistant also contained significantly more intracellular trehalose than the reference strain MG1655.

The clear importance of *de novo* trehalose synthesis in *E. coli* desiccation resistance is contrasted and highlighted by the ambiguous effects of exogenous GB uptake, as both trehalose and GB are important osmoprotectants. The different roles played by trehalose and GB was previously demonstrated in *E. coli* strain NCIB 9484, where osmotically-induced intracellular trehalose, but not GB increased cell desiccation resistance (45). The present study provides further evidence by studying the cellular responses of multiple *E. coli* strains under desiccation condition. The difference between trehalose GB in *E. coli* desiccation resistance is attributable to their different protection mechanisms, with trehalose being superior than GB in alleviating severe water activity reduction (33). Desiccation often results in greater loss of water than salinity-induced osmotic stresses, as severe desiccation can result in the loss of the last monolayer of water on biomolecules (33). Trehalose was shown to protect cell membranes by lowering the phase transition temperature of phospholipid membrane and thus maintain the phospholipid bilayer in the liquid crystalline phase when drying (9), and can also stabilize desiccated proteins via hydrogen bonding and water replacement, thus keeping dry proteins in their hydrated conformations (27). However, contribution from GB to *E. coli* desiccation resistance cannot be completely ruled out, as the present study and previous
ones all employed rather severe experimental desiccation conditions while GB may still play a role under relatively mild desiccation conditions.

Both the die-off experiments and the cellular responses under desiccation stress showed that the soil *E. coli* strains are generally more resistant to desiccation stress. The selected soil strains were considered to be naturalized soil *E. coli* populations based on their repeated detection in soil samples from the Manoa watershed (Table 2.1). Given the enormous genotypic diversity of *E. coli* (18), considerable variations in *E. coli* strains’ response to any environmental condition are expected. Therefore, when majority of soil *E. coli* strains exhibit the same trait (i.e., high desiccation resistance), it is reasonable to attribute those traits to specific habitat adaptation and to consider those strains to be naturalized to the soil environments. This is a more logic explanation than the alternative possibility (i.e., these strains were of recent fecal origin but selected by soil because of their desiccation resistance) because of the isolation patterns of the soil *E. coli* strains (i.e., from multiple locations at multiple times) (Table 2.1).

Table 2.2 Isolation patterns of the select *E. coli* strains from soil in Manoa watershed

<table>
<thead>
<tr>
<th>Strains</th>
<th>Detection dates (mm/dd/yy)</th>
<th>Sites a</th>
<th>Frequencies b</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-A34</td>
<td>5/15/09, 6/8/09</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>S-B31</td>
<td>5/15/09, 6/8/09, 8/31/09</td>
<td>3</td>
<td>16</td>
</tr>
<tr>
<td>S-B32</td>
<td>6/8/09, 10/14/09, 12/21/09</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>S-B54</td>
<td>5/15/09, 8/31/09, 12/21/09, 2/24/10</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>S-A18</td>
<td>5/15/09</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>S-A37</td>
<td>2/24/10</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>S-B35</td>
<td>5/15/09</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>S-E52</td>
<td>10/14/09, 2/24/10</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>S-F13</td>
<td>8/31/09</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>S-F18</td>
<td>5/15/09</td>
<td>1</td>
<td>7</td>
</tr>
</tbody>
</table>

a Out of six different sampling sites.  
b Total number of isolates from soil is 630.
The recognition of soils as secondary habitats of *E. coli* not only affects water quality monitoring, but may also have public health implications. Traditionally, unlike other enteric bacteria such as *Salmonella* (46), *E. coli* is not believed to survive extensively once out of its primary habitats. Soils as secondary habitats of *E. coli* imply an *E. coli* life cycle that consists of passage through a host into the environment, adapt and evolve in the environment, and then move back into a new host. This would instigate further scientific inquiries about the adaption and evolution of *E. coli* in natural environments, with particular attention to the emergence of pathogenic *E. coli* strains (43); for example, *E. coli* O157:H7 strains were found to persist in soil for a long period of time (17, 28), and some shiga toxin-producing *E. coli* (STEC) strains exhibited higher desiccation resistance than non-pathogenic *E. coli* strains (22), which will also favor their survival in the environment.

In summary, the present study showed that soil *E. coli* strains are resistant to the desiccation stress common to soil environments, exhibiting a common trait of soil-dwelling bacteria. By quantifying cellular responses to desiccation by multiple *E. coli* strains, *de novo* synthesis and accumulation of trehalose was identified to be a common mechanism for enhanced desiccation resistance, which is also widely shared by typical soil-dwelling bacteria. Comparison between soil *E. coli* strains and reference strains showed that the former group is generally more desiccation resistant, providing further evidences of *E. coli*’s adaptation and naturalization to soil environments. The recognition of soil as a secondary habitat of *E. coli* will not only have significant implications in water quality monitoring, but may also affect how we approach the adaptation and
evolution of *E. coli* in the environment, which is important given recent emergence of various pathogenic *E. coli* strains.

2.5 ACKNOWLEDGEMENT

We thank Ms. Bunnie Yoneyama for her technical support in the laboratory. This material is based upon work supported by the Cooperative State Research, Education, and Extension Service, U.S. Department of Agriculture, under Agreement No. 2009-35102-05212 (to T.Y.)
2.6 REFERENCE


CHAPTER 3. IMPACT OF INDIGENOUS MICROBIOTA OF SUBTIDAL SAND ON Fecal INDICATOR BACTERIA DECAY IN BEACH SYSTEMS: A MICROCOSM STUDY

ABSTRACT

Fecal contamination of coastal recreational water can adversely impact the public health and economic wellbeing of many coastal communities. The current recreational water management practices focus primarily on water itself, while recent studies have identified other beach system components that can impact water quality. The objective of this study was to use microcosms to determine whether subtidal beach sand can enhance the decay of fecal bacteria and identify underlying mechanisms. The decay patterns of exogenous Enterococcus faecalis cells in laboratory beach microcosms for three beaches in Hawaii were determined, and beach sand indigenous microbiota was identified to the major factor correlating to bacterial decay rates. Subsequent experiments observed that higher indigenous microbiota corresponded to faster bacterial decay. Comparison between the two major beach system components (beach sand and seawater) indicated that the indigenous microbiota in beach sand played a significant role in bacterial decay. Manipulating two important beach characteristics (sand-to-water ratio and sand particle size) that relate to indigenous microbiota abundance also resulted in different bacterial decay rates. The significant contribution of beach sand and its indigenous microbiota to fecal bacteria decay identified a positive function of beach sand in beach water quality management, which supports the inclusion of beach sand in beach quality management.
3.1 INTRODUCTION

Coastal beach water quality is important to the public health and economic wellbeing of coastal communities. Recreational water is an important transmission route of waterborne pathogens and can result in large scale disease outbreaks (1, 2); for example, in 2009-2010, U.S. CDC reported 81 recreational water-associated disease outbreaks (32). To protect the public from health risks associated with contaminated recreational water, beach advisories and closures are frequently issued. The National Research Defense Council (NRDC) reported that in 2009 beach water pollutions caused beach closures and advisories to exceed 20,000 for the fourth consecutive year in the U.S. (43). An separate survey by the U.S. EPA indicated that 40% of 3,762 beaches monitored in 2012 experienced at least one advisory or closure (49). Frequent beach advisories and closures can hurt the economy of coastal communities, which increasingly relies upon tourism and recreation (43), and the impact can be very significant because about 75% of the world’s unfrozen shorelines are sandy beaches (15).

The current recreational water quality regulation paradigm is primarily underpinned on beach water itself (17, 18). However, recently a systems view that encompasses all beach system components, including beach sand (55, 56), sediment (5, 10), and aquatic vegetation (9, 16), is developing. In particular, beach sand, the characteristic feature of a beach system, has recently been identified as a potential secondary reservoir of fecal indicator bacteria (FIBs). Higher levels (10- to 100-fold on a unit mass basis) of fecal indicator *Escherichia coli* and enterococci were frequently detected in beach sand than in the corresponding beach water (11, 14, 24, 33, 36, 44, 56-58). Given the significant sand mass in a beach system and its continuous interactions .
with water (42, 53, 54, 57), FIBs-laden beach sands can act as significant secondary reservoirs of FIBs, which can potentially cause chronic water quality deterioration and hamper proper health risk assessment.

Although numerous studies (discussed above) have examined the negative impact that contaminated beach sand can exert (i.e., as chronic sources of FIBs), few studies have investigated the potential positive roles that beach sand may play in beach water quality protection. This is important as various natural phenomena and anthropogenic activities can significantly change the quantity and quality of beach sand in beach systems. Beach erosion, which gradually leads to considerable sand loss, is widespread at present and expected to worsen in the future as a result of global warming (59). Beach nourishment programs, responding to beach erosion, can abruptly change the sand dynamics, affecting both sand quantity and quality. In response to chronic water quality issues implicating contaminated beach sand, sand replacement has become a viable remediation option (30), while some agencies even advocate removing beach sand as a potential solution. Therefore, a full understanding of the various impact (positive and negative) that beach sand can have on beach water quality is essential to the development of a comprehensive, systems approach in beach management.

Beach sand can be expected to positively impact beach water biological quality via various physical processes that can remove fecal bacteria from water, including adsorption, straining and absorption. Although no studies in literature have directly characterized how these physical processes remove fecal bacteria from beach water, these processes have been extensively studied in sand filtration for water treatment, and hence similar functions can be expected of beach sand in principal. Less understood is how
beach sand contributes to the decay of fecal bacteria that are either adsorbed onto the sand particle surfaces, trapped within sand cavities, absorbed into the sand biofilm, or simply present within the interstitial water.

Various abiotic and biotic environmental factors can affect the decay of fecal bacteria in natural environments. Common abiotic environmental stresses include temperature variation (52), sunlight inactivation (27, 47), carbon starvation (48), pH fluctuation (20), and osmotic stress from salinity changes (4), and their effects on the decay of fecal bacteria in the environment are generally well understood. Biotic processes, which typically include protozoa predation (25, 28), phage infection (8, 45) and bacterial antagonism (26, 31, 34, 40, 41), on the other hand, have received limited attention. Surprisingly, recent studies have shown that the biotic processes associated with indigenous microbiota are very important to fecal bacteria decay in the environment. For example, microcosm studies that compared the survival of fecal indicator E. coli and Enterococcus faecalis in sterile and non-sterile soil (7, 13, 38), freshwater (13, 38), seawater (21), and beach sand (26, 29) often reported more than 10-fold faster decay rates in the presence of indigenous microbiota, and the removal of indigenous microbiota even drastically reduced the effect of abiotic stresses, such as sunlight inactivation (37), which were traditionally believed to be the major forces behind bacterial decay.

The objective of this study was to investigate whether subtidal beach sand can enhance the decay of fecal bacteria in beach systems using laboratory microcosms. Initially, the decay patterns of E. faecalis in beach microcosms containing subtidal sand and seawater samples from three natural beaches were determined, and the decay rates were compared with common physicochemical and microbiological parameters of the
beach sand and seawater samples. Then, the impact of indigenous beach microbiota was verified by changing its abundance level in microcosms and comparing the corresponding bacterial decay rates. Subsequently, the relative contributions from beach sand and seawater was isolated and determined by comparing bacterial decay rates in microcosms that contained either both beach sand and seawater or seawater only. Microcosms containing autoclaved beach sand and seawater were used concurrently to quantify the matrix effects in bacterial decay. Finally, the impact of different beach characteristics, including sand particle size and sand-to-water ratio, on bacterial decay was also investigated.

3.2 MATERIALS AND METHODS

**Beach sand and water sampling.** Beach sand samples were collected from three beaches on the Island of Oahu, Hawaii, including Waialae Beach (21.26968° N; 157.77710° W), Kailua Beach (21.40222° N; 157.7394° W), and Kualoa Beach (21.51330° N; 157.8360° W). At each sampling site, four subtidal sand samples were collected at knee depth and from a 0.5m radius of the standing position, and the four samples were pooled together and stored in sterile Whir-Pak sampling bags. Seawater samples were collected at the same sampling locations using sterilized wide-mouth plastic bottles. Grab raw municipal wastewater samples were collected from the headwork of the Sand Island Wastewater Treatment Plant (SIWTP) (Honolulu, HI) in sterilized wide-mouth plastic bottles. All samples were stored at 4°C and in dark during transportation to the laboratory for immediate processing and analysis.

**Sample characterization and analysis.** The beach sand samples were characterized and analyzed to determine various physicochemical and biological
parameters. Total bacterial density in the beach sand samples and seawater were conservatively estimated using heterotrophic plate count (HPC). Sand extraction followed a procedure described by Boehm et al. (12), which involved shaking 10 gram of sand in 100 mL of sterilized deionized water for 2 minutes by hand and collecting supernatants of the sand extracts after settlement for 30 seconds. Supernatants of the sand extracts and the seawater samples were subjected to tenfold dilution, spread plated on tryptic soy agar (TSA), and incubated at 37°C for 24 hours before colony enumeration.

pH and salinity of beach sand extracts and seawater samples were determined using a bench top pH Meter (UltraBasic; Denver Instrument) and Orion 150A plus conductivity meter (Thermo; Waltham, MA), respectively. The total organic carbon (TOC) and total nitrogen (TN) of sand extracts and seawater samples were determined using a TOC analyzer coupled with a TN detector (Shimadzu; Kyoto, Japan). Biochemical oxygen demand (BOD) of sand extracts and seawater were determined using a YSI Model 58 Dissolved Oxygen Meter for 5-day BOD test (BOD5) following the APHA 5210B standard method (3).

**Bacterial strain and enumeration.** *E. faecalis* strain ATCC 29212 was used as the model organism to represent fecal bacteria in this study. The preparation of stationary-stage cell suspensions followed the procedure described previously by Feng et al. (26). In brief, stationary-stage *E. faecalis* cells (OD600nm ≥1.2) were harvested by centrifugation at 10,000 x g for 2 minutes from cultures in tryptic soy broth (TSB) that were cultivated at 37°C with constant shaking (200 rpm). The harvested cell pellets were washed (by resuspension and precipitation) twice with phosphate buffered saline (PBS) (pH=7.0), and then suspended in PBS to prepare working cell suspensions (ca. 10⁵ CFU
mL\textsuperscript{−1}). For the enumeration of \textit{E. faecalis} cells in the lab microcosm experiments, sand samples collected from the beach microcosms (described below) were extracted by following the sand extraction procedure described above. \textit{E. faecalis} cells in the sand extracts and water samples were enumerated using the membrane-\textit{Enterococcus} indoxyl-b-D-glucoside (mEI) agar method (50).

**Beach microcosm setup.** Beach microcosms were constructed in clean and autoclaved Mason jars (500 mL), and contained beach sand (100 g) and seawater (200 mL) collected from the three beaches. Stationary-stage \textit{E. faecalis} cell stock solutions were freshly prepared, and 8 mL of the stock solution, which gave ca. $10^9$ cells in total, was spiked into each beach microcosm. The microcosms were mixed using a sterile stainless steel spatula for two minutes. After thorough mixing, the microcosms were incubated in dark at room temperature (22-24°C) with gentle constant shaking (100 rpm), which facilitated water circulation without slurry formation. During each sampling, the sand and water compartments of the microcosms were temporarily separated by completely draining the water into sterile flasks. Water samples were collected from the completely mixed flasks, while the drained sand in the microcosms was completely mixed and sampled. After sampling, the microcosms were reconstituted, and incubation resumed until the next sampling. This sampling strategy was necessary to avoid extensive mixing that would be required if the sand and water in a microcosm were to be sampled together. All experimental treatments in this study used three replicate microcosms as biological replicates, and the same microcosm setup was used throughout this study unless stated otherwise.
**Manipulation of indigenous microbial communities.** The indigenous microbiota of beach sand and seawater samples collected from the Waialae beach were altered either by antibiotic treatment or by autoclaving. Streptomycin was selected as the antibiotic agent based on a preliminary test, which showed that 500 mg/L of streptomycin reduced the culturable counts of HPC in beach microcosms by more than 10 fold within 24 hours without significantly affect the culturable counts of *E. faecalis* cells (>95% culturable). Microcosms with antibiotic treatment were established as described above, and then amended with streptomycin to reach a final aqueous concentration of 500 mg/L. For the microcosms containing autoclaved beach sand and seawater, the microcosms were autoclaved at 121°C for 20 min on two consecutive days before the amendment of *E. faecalis* cells aseptically.

**Alteration of beach sand and seawater composition in microcosms.** The beach sand and seawater composition in microcosms were also altered. Beach sand and seawater samples collected from the Waialae beach were used. The first set of microcosms used two different sand-to-water mass ratios (0 and 0.5). The seawater-only microcosms (sand-to-water mass ratio = 0) contained zero gram beach sand and 200 mL of seawater, while microcosms with a sand-to-water ratio of 0.5 contained 100 gram beach sand and 200 mL of seawater. Control microcosms for both groups of microcosms were established using autoclaved beach sand and seawater samples. The second set of microcosms used three different sand-to-water mass ratios (0.25, 0.5 and 1.0); the microcosms contained the same volume of seawater (200 mL) and 50 g, 100 g, and 200 g of beach sand, respectively. The third set of microcosms used the same sand-to-water mass ratio (i.e., 100 g sand and 200 mL seawater), but differed in sand particles sizes,
which resulted in different total surface sand area in the beach microcosms. Sand sample collected from the Waialae beach were wet sieved using standard testing sieves (USA Standard Testing Sieve) to collect three different size fractions (particle diameters <1.20 mm, between 1.40-2.00 mm, and between 2.00-2.36 mm). The different total surface areas of the different size fractions were expected to provide different quantities of total biomass of indigenous microbiota, as the finer sand particles provide higher specific surface area and typically contain more organic-rich micro patches.

**Decay of *E. coli* from raw wastewater in beach microcosms.** Beach sand and seawater microcosm were also spiked with 20 mL of raw wastewater collected from the SIWTP. Beach sand and seawater collected from the Waialae beach were used to construct microcosms as described above. Beach sand and seawater samples from the microcosms were processed as described above, and the concentration of *E. coli* in the samples was enumerated by membrane filtration method using the modified mTEC agar according to EPA method 1603 (51).

**Data analysis.** The number of culturable *E. faecalis* cells in beach microcosms over time was fitted into the 1st order decay model \( \ln\left(\frac{M_t}{M_0}\right) = -k_d t \) to determine the 1st-order decay rate decay rate \( k_d \). Because the beach sand microcosms contain two different matrices (i.e., sand and water), it was more convenient to track the total number of culturable *E. faecalis* cells \( M \) in each microcosms, which includes cells in both the beach sand and the seawater compartments. \( M_t \) and \( M_0 \) are the total numbers of culturable *E. faecalis* cells in the microcosms at time \( t \) and time zero, respectively. The number of culturable *E. faecalis* cells in microcosms containing autoclaved beach sand and seawater was fit into the same 1st-order decay modeled \( \ln\left(\frac{M_t}{M_0}\right)_a = -k_{da} t \), where \( k_{da} \) is the
decay rate of *E. faecalis* in the absence of indigenous microbiota. The goodness of fit ($r^2$) and the 95% confidence interval (CI) for the model fitting was obtained and used to determine the suitability of the model. To identify the decay rate solely attributable to the indigenous microbiota, the *E. faecalis* decay data were modeled using this equation:

$$
\ln \left( \frac{M_t}{M_0} \right) = -(k_d - k_{da})t = -k_{dl}t
$$

which assumes that the overall decay rate ($k_d$) is the summation of decay rate in autoclaved microcosms ($k_{da}$) and the decay rate contribution by the indigenous microbiota ($k_{dl}$). Statistical analyses were performed using software packages SigmaPlot 12.0 and SPSS. Pearson’s product moment was calculated to analyze the correlation between the decay rate of *E. faecalis* in beach microcosms and physicochemical and microbiological characteristics of beach sand and seawater samples. Paired $t$-tests were used to compare the time-series data of experimental treatments. One-way ANOVA analysis (with Holm-Sidak post hoc test) was used to determine if significant difference existed between different experimental treatments at given time points. The default significance level is $P \leq 0.05$ unless stated otherwise.

### 3.3 RESULTS

**E. faecalis decay in beach microcosms.** The initial physiochemical and biological parameters of the beach microcosms were determined by characterizing the beach sand and seawater samples from Waialae, Kailua, and Kualoa beaches that were used to construct the beach microcosms (Table 3.1). Since the indigenous enterococci counts were negligible in comparison with the exogenous *E. faecalis* cells amended into the microcosms (Table 3.1), culturable enterococci cells detected in the beach microcosms were all considered to be the exogenous *E. faecalis* cells.
The change of culturable *E. faecalis* cell concentration in the sand and water compartments showed significant difference amongst the three groups of microcosms (Figure A.1). The log reduction of the total number of viable *E. faecalis* cells in each microcosm also exhibited significantly different patterns amongst the three groups of beach microcosms (Figure 3.1). When the total number of culturable *E. faecalis* cells in each microcosm (i.e., combined from the sand and water compartments) was calculated, significantly different decay patterns were observed amongst the three groups of beach microcosms (Figure 3.1). On Day 1, significantly different reduction of culturable cells was observed between Kailua beach microcosms and the Kualoa and Waialae beach microcosms (ANOVA, $P \leq 0.05$) (no statistical difference was observed between the Kualoa and Waialae beach microcosms). Starting from Day three, significant difference was observed amongst all three different beach microcosms (ANOVA, $P \leq 0.05$), except for one comparison between the Waialae and Kualoa on Day 4 ($P=0.06$). Over the entire experimental course, the decay rate of *E. faecalis* cells in the Waialae beach microcosms ($k_d=3.75 \text{ day}^{-1}$; $R^2=0.92$ and CI is $3.17 - 4.31 \text{ day}^{-1}$) was larger than that in the Kualoa beach microcosms ($k_d=2.90 \text{ day}^{-1}$; $R^2=0.92$ and CI is $2.49 - 3.32 \text{ day}^{-1}$). The slowest decay rate was observed in the Kailua beach microcosms ($k_d=1.24 \text{ day}^{-1}$; $R^2=0.91$ and CI is $1.05 - 1.43 \text{ day}^{-1}$). After Day 5, *E. faecalis* cells in the Waialae beach microcosms had decreased to below the method detection limit.
Table 3. Beach sand and seawater sample characteristics and their Pearson’s correlation coefficients with the *E. faecalis* die-off constant (*k_d*) in the beach microcosms.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Waialae</th>
<th>Kailua</th>
<th>Kualoa</th>
<th>Pearson’s r (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterococci in sand (CFU/100 mL)</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Enterococci in seawater (CFU/100 g)</td>
<td>16</td>
<td>7</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>HPC in sand (log CFU/100 gram)</td>
<td>6.39</td>
<td>4.48</td>
<td>5.66</td>
<td>1.00 (0.01)</td>
</tr>
<tr>
<td>HPC in seawater (log CFU/100 mL)</td>
<td>5.57</td>
<td>4.45</td>
<td>5.02</td>
<td>0.99 (0.07)</td>
</tr>
<tr>
<td>Total HPC in microcosm (log CFU)</td>
<td>6.45</td>
<td>4.76</td>
<td>5.75</td>
<td>1.00 (0.02)</td>
</tr>
<tr>
<td>Seawater TOC (mg/L)</td>
<td>1.36</td>
<td>1.28</td>
<td>1.27</td>
<td>0.73 (0.48)</td>
</tr>
<tr>
<td>Seawater TN (mg/L)</td>
<td>0.13</td>
<td>0.11</td>
<td>0.20</td>
<td>0.33 (0.78)</td>
</tr>
<tr>
<td>Seawater BOD₅ (mg/L)</td>
<td>2.46</td>
<td>1.45</td>
<td>2.15</td>
<td>1.00 (0.06)</td>
</tr>
<tr>
<td>Seawater pH</td>
<td>7.63</td>
<td>7.74</td>
<td>7.76</td>
<td>-0.79 (0.42)</td>
</tr>
<tr>
<td>Seawater Salinity (%)</td>
<td>3.36</td>
<td>3.28</td>
<td>3.3</td>
<td>0.92 (0.26)</td>
</tr>
<tr>
<td>Sand TOC (mg/kg)</td>
<td>10.8</td>
<td>6.0</td>
<td>18.9</td>
<td>0.48 (0.68)</td>
</tr>
<tr>
<td>Sand TN (mg/kg)</td>
<td>1.70</td>
<td>0.83</td>
<td>2.5</td>
<td>0.62 (0.57)</td>
</tr>
<tr>
<td>Sand BOD₅ (mg/kg)</td>
<td>15.8</td>
<td>10.6</td>
<td>13.4</td>
<td>1.00 (0.05)</td>
</tr>
<tr>
<td>Sand extraction pH</td>
<td>9.08</td>
<td>9.10</td>
<td>9.07</td>
<td>-0.75 (0.47)</td>
</tr>
</tbody>
</table>
To identify factors contributing to the different decay rates observed in the three groups of beach microcosms, the decay rates were correlated with various initial characteristics of the beach microcosms (Table 3.1). Significant correlation with the decay coefficient ($P \leq 0.05$) was only observed for the bacterial biomass in beach sand, the total bacterial biomass in the microcosm, and the sand BOD$_5$. The decay coefficients also correlated with bacterial biomass in beach water ($P = 0.07$) and BOD$_5$ in seawater ($P = 0.06$) at less significance levels. Since indigenous microbial biomass and BOD$_5$
usually exhibit good correspondence, the observed correlations suggested a strong role of indigenous beach sand microbiota on *E. faecalis* cell decay in the beach microcosms.

**Role of indigenous microbiota.** To further confirm the impact of indigenous beach microbiota on *E. faecalis* cell decay, beach microcosms that contain different levels of indigenous microbiota were constructed using the sand and seawater samples from the Waialae beach. The microcosm experiment included three treatments: (1) unaltered indigenous beach microbiota, (2) reduced indigenous microbial density/activity by antibiotic treatment, and (3) complete removal of indigenous microbiota by autoclaving. The indigenous microbiota strongly affected *E. faecalis* cell decay as indicated by the different reduction patterns of culturable cells in the sand and seawater compartment of the microcosms (Figure A2.1). The total culturable *E. faecalis* cells in each microcosm also exhibited different patterns as a result of the different indigenous microbiota abundance levels (Figure 3.2). At the end of the experimental course (i.e. Day 5), the total culturable *E. faecalis* cells in the three sets of microcosms showed significant difference (ANOVA, P ≤ 0.001). Microcosms with the highest indigenous microbiota level (i.e., unaltered beach sand and seawater samples) showed the fastest decay rate ($k_d=2.23$ day$^{-1}$; $R^2=0.91$ and CI is 1.87 – 2.59 day$^{-1}$). The beach microcosms treated with streptomycin showed significantly slower ($t$-test, P<0.001) *E. faecalis* cell decay ($k_d=1.34$ day$^{-1}$, $R^2=0.92$ and CI is 1.12 – 1.55 day$^{-1}$) than the unaltered microcosms, while the microcosms containing autoclaved sand and seawater exhibited the smallest decay rate among the three treatments ($k_d=0.58$ day$^{-1}$, $R^2=0.77$ and CI is 0.41 - 0.74 day$^{-1}$), which strongly indicate the important role of indigenous beach microbiota on *E. faecalis* cell decay in the beach microcosms.
Figure 3. Impact of different levels of indigenous microbiota on the reduction of total number of viable *E. faecalis* cells in Waialae beach microcosms. $M_t$ and $M_0$ represent total cell counts in the microcosms at time $t$ and time zero, respectively. Error bar indicates the standard deviation of the mean of triplicate microcosms.

**Relative contributions from beach sand and seawater.** Since the contribution of indigenous microbiota on *E. faecalis* cell decay could be from both beach sand and seawater, relative contributions from the two beach components to the decay of exogenous *E. faecalis* cells were determined by comparing regular beach microcosms, which contained both beach sand and seawater, with microcosms containing only seawater. The concentration of culturable *E. faecalis* cells and its change over time in the microcosms are shown in Figure A.3. The total number of culturable cells in each microcosm showed significant difference (paired $t$-test: $P<0.05$) amongst the microcosms.
(Figure 3.3). The beach microcosms containing both sand and seawater exhibited significantly faster \((t\text{-test, } P<0.01)\) \textit{E. faecalis} decay \((k_d=2.19\text{ day}^{-1}; R^2=0.98\text{ and CI is }2.02-2.37\text{ day}^{-1})\) than the beach microcosms that lacked beach sand \((k_d=1.27\text{ day}^{-1}; R^2=0.85\text{ and CI is }1.05-1.49\text{ day}^{-1})\) (Figure 3.3A). Since some of the observed difference in decay rates may be due to the matrix effects that can differ between sand and water, autoclaved controls were established for both types of beach microcosms. Indeed, the autoclaved beach microcosms, which contained both beach sand and water, also exhibited slightly faster decay of \textit{E. faecalis} cells \((k_{da}=0.72\text{ day}^{-1}; R^2=0.81\text{ and CI is }0.59-0.86\text{ day}^{-1})\) than the autoclaved seawater-only microcosms \((k_{da}=0.46\text{ day}^{-1}; R^2=0.62\text{ and CI is }0.32-0.60\text{ day}^{-1})\), which could be attributed to different endogenous decay rates and/or recovery coefficients of the \textit{E. faecalis} cells due to matrix effects.

In order to remove the matrix effects, the decay of \textit{E. faecalis} cells in microcosms containing intact beach sand and/or seawater were divided by the decay of \textit{E. faecalis} cells in the microcosms containing autoclaved beach sand and/or seawater. The resulting decay patterns still exhibited significantly faster rates \((t\text{-test, } P=0.001)\) in the beach microcosms containing both sand and seawater than in the microcosms containing only seawater (Figure 3.3B). The corresponding decay rates \(k_{dl}\), which quantify bacterial decay contributed by the indigenous microbiota, were 1.78 day\(^{-1}\) \((R^2=0.90\text{ and CI is }1.47-2.09\text{ day}^{-1})\) and 0.87 day\(^{-1}\) \((R^2=0.81\text{ and CI is }0.70-1.05\text{ day}^{-1})\) for the microcosms containing both sand and seawater and the microcosms containing seawater only, respectively. The difference in decay rates \(k_{dl}\) clearly indicates that the indigenous beach sand microbiota contributed significantly to \textit{E. faecalis} cell decay in the beach microcosms.
Figure 3. Viable *E. faecalis* cell reduction (log \((M_t/M_0)\)) in regular Waialae beach microcosms that contain both beach sand and seawater or seawater-only microcosms (A) and cell reduction due to the presence of indigenous microbiota (log \([((M_t/M_0)/ (M_t/M_0)_a)]\)) in the beach microcosms (B). \(M_t\) and \(M_0\) represent total cell counts in the microcosms at time \(t\) and time zero, respectively. \((M_t/M_0)_a\) indicates the viable cell reduction in the autoclaved beach microcosms. Error bar indicates the standard deviation of the mean of triplicate microcosms.
Impact of beach characteristics. The impact of two important beach characteristics, sand-to-water ratio and sand particle size distribution, on the decay of exogenous *E. faecalis* cells was also investigated in beach microcosms. The concentration of culturable *E. faecalis* cells in the sand and water compartments showed different reduction patterns over time in respect to different sand-to-water ratios (Figure A.4). The total number of culturable cells in each microcosm also exhibited different bacterial decay patterns (Figure 3.4A). On Day 5, the total number of culturable cells in the different microcosms showed significant difference (ANOVA, P<0.05). The beach microcosms containing the smallest amount of beach sand (i.e. sand-to-water ratio=0.25 g/mL) showed the slowest cell decay (*k_d*=2.20 day\(^{-1}\); \(R^2=0.95\) and CI is 1.96 – 2.48 day\(^{-1}\)), while the microcosms containing higher ratios of sand exhibited significantly faster cell decay. The beach microcosms with a sand-to-water ratio of 0.50 g/mL exhibited a *k_d* of 2.37 day\(^{-1}\) (\(R^2=0.89\) and CI is 1.86 – 2.88 day\(^{-1}\)), and the beach microcosms with a sand-to-water ratio of 1.00 g/mL exhibited a *k_d* of 2.49 day\(^{-1}\) (\(R^2=0.95\) and CI is 2.15 - 2.84 day\(^{-1}\)).

When different size fractions of the Waialae beach sand (particle size <1.2 mm, 1.4-2.0 mm, and 2.0-2.4 mm) were used to establish three experimental treatments that had the same total mass but different total surface areas (>893 cm\(^2\), 536-765 cm\(^2\), 450-536 cm\(^2\)), significantly different decay patterns were also observed (Figure A.5). The total number of culturable *E. faecalis* cells in each microcosm also showed clearly different reduction patterns (Figure 3.4B). The beach microcosms containing the finest sand fraction (i.e. particle size <1.2 mm) exhibited significantly faster decay rate (*k_d*=2.05 day\(^{-1}\); \(R^2=0.88\) and CI is 1.52 – 2.58 day\(^{-1}\)) than the two sets of microcosms containing larger
sand size fractions (i.e. particle size: 1.4-2.0 mm and 2.0-2.4 mm). Beach microcosms containing the two larger size fractions did not exhibit statistically distinct decay patterns ($k_d=2.53 \text{ day}^{-1}; R^2=0.93$ and CI is 2.12 – 2.93 day$^{-1}$, which was likely due to their close total sand surface areas.
Figure 3. 4 Impact of sand-to-water ratio on the reduction of culturable *E. faecalis* cells in beach microcosms (A), and culturable *E. faecalis* cell reduction in Waialae beach microcosms containing different sand particle size fractions (B). $M_t$ and $M_0$ represent total cell counts in the microcosms at time $t$ and time zero, respectively. Error bar indicates the standard deviation of the mean of triplicate microcosms. The red dotted line represents the method detection limit (MDL), and data points below the MDL are for illustration.
**Decay of wastewater *E. coli* in beach microcosms.** To investigate if indigenous microbiota of subtidal sand also contribute to the decay of the other common FIB *E. coli*, raw municipal wastewater was introduced to beach microcosms and the concentration dynamics of wastewater *E. coli* was monitored (Figure 3.5). The microcosms containing both beach sand and seawater showed a significantly faster *E. coli* decay than the microcosms containing only seawater. The decay rate of *E. coli* in the beach sand and seawater microcosms ($k_d=3.68\ day^{-1}$) was 3.9 times higher than that in seawater microcosms ($k_d=0.95\ day^{-1}$).

![Graph showing decay of culturable E. coli cells from wastewater in regular Waialae beach microcosms that contain both beach sand and seawater or seawater-only microcosms. M_t and M_0 represent total cell counts in the microcosms at time t and time zero, respectively. Error bar indicates the standard deviation of the mean of triplicate microcosms. The red dotted line represents the method detection limit (MDL), and data points below the MDL are for illustration.](image)

Figure 3. 5 Decay of culturable *E. coli* cells from wastewater in regular Waialae beach microcosms that contain both beach sand and seawater or seawater-only microcosms. M_t and M_0 represent total cell counts in the microcosms at time t and time zero, respectively. Error bar indicates the standard deviation of the mean of triplicate microcosms. The red dotted line represents the method detection limit (MDL), and data points below the MDL are for illustration.
3.4 DISCUSSION

The impact of beach sand and its indigenous microbiota on the decay of fecal bacteria was investigated using laboratory beach microcosms and a model indicator organism *E. faecalis*. Since the bacterial decay was quantified by enumerating culturable *E. faecalis* cells recovered from beach microcosms that contained both beach sand and seawater, the presence of two different types of matrices could result in different cell recovery efficiencies, which needs to be carefully considered in data interpretation. The use of 1st-order decay kinetic model in calculating the decay rate based on the ratio $M_t$ over $M_0$ partially alleviated the potential bias associated with the matrices, assuming the same recovery efficiency for the same matrix. This treatment however introduced an additional assumption, which is that bacterial cell recovery efficiency does not change over time (i.e., lack of a temporal component). The spiked *E. faecalis* cells can adsorb to the sand surfaces (39), which usually occurs either instantaneously or within time frames significantly shorter than the duration of this study (22) and therefore were not expected to contribute a significant temporal component to the cell recovery efficiency. The *E. faecalis* cells may also gradually absorb into and integrate with sand surface biofilms, which could decrease cell recovery efficiency over time. Although few studies have examined this effect directly, sand extraction procedures have often reported good bacterial recovery sufficient for enumeration (12) and transport studies often observed quick mobilization of enterococci in beach sand (46, 56), suggesting limited temporal component of the recovery efficiency. Nevertheless, the potential temporal component of cell recovery efficiency needs to be considered during the interpretation of bacterial decay data.
The first microcosm experiment showed distinct decay patterns of exogenous *E. faecalis* cells in microcosms containing beach sand and seawater samples from three natural beaches (Figure 3.1). Correlation analysis between the bacterial decay rates and various beach sand and seawater sample characteristics only detected significant correlations with parameters related to the indigenous microbiota in beach sand, namely HPC and BOD$_5$ (Table 3.1), with higher abundances of indigenous microbiota corresponding to faster bacterial decay rates. HPC is a very conservative estimator of microbial biomass because majority of microorganisms cannot be enumerated by cultivation-based methods, such as HPC. Furthermore, the use of DI water in sand extraction, which was designed specifically for recovering enterococci (12), apply hypotonic osmotic pressure to the indigenous marine microorganisms and may cause cell lysis. These limitations, however, should not hamper cross sample comparison, assuming all samples experienced the same level of biases. Since beach systems can differ at numerous aspects, the observed exclusive correlation between the decay rate of exogenous *E. faecalis* cells and the abundance of beach sand indigenous microbiota provided strong evidence (albeit indirect) for the study hypothesis that indigenous sand microbiota can enhance the decay of exogenous fecal bacteria. However, it should be noted that the experimental design of this particular experiment could not quantify or qualitatively exclude the contribution of varying cell recovery efficiency, especially if it had decreased over time.

To further determine the contribution of indigenous microbiota on bacterial decay and to identify the impact of cell recovery efficiency, beach microcosms that contained different levels of indigenous microbiota from the Waialae beach (unaltered, antibiotic-
treated, and autoclaved) were established. A positive correspondence between the abundance of indigenous microbiota and bacterial decay rate was observed (Figure 3.2), which strongly indicated a causal relationship between the two parameters. Since microcosms in this experiment were constructed of the same materials, it was possible to deduce from the bacterial decay pattern in the autoclaved treatment that the decreasing of cell recovery efficiency over time in the beach microcosms was fairly limited, and hence the observed cell decay in the microcosms was primarily caused by the presence of indigenous microbiota.

Given that beach sand typically contains significant indigenous microbiota (many studies reported more than 10-fold higher bacterial biomass in beach sand than in seawater on a unit mass basis (23, 35)), beach sand can be expected to contribute significantly to the removal of fecal bacteria in beach systems. This was clearly demonstrated in the microcosm experiment by comparing regular beach microcosms (containing both beach sand and seawater) and seawater-only microcosms, where the former exhibited significantly faster bacterial decay \( (k_d=2.19 \text{ day}^{-1}) \) than the latter \( (k_d=1.27 \text{ day}^{-1}) \) (Figure 3.3A). The inclusion of autoclaved regular beach microcosms and seawater-only microcosms in this experiment further supported the observations made in Figures 3.1 and 3.2 regarding the importance of indigenous microbiota to bacterial decay. More importantly, the decay data in the autoclaved microcosms allowed for the estimation of the temporal component of cell recovery in the different beach microcosms. The significantly faster bacterial decay rate in the autoclaved regular beach microcosms than in the autoclaved seawater-only microcosms indicated the presence of a temporal component in cell recovery efficiency (i.e., decreasing cell recovery from beach sand
over time). However, even when this temporal component of cell recovery efficiency were removed by subtracting the live microcosms with the autoclaved control microcosms, significantly faster decay rate was still observed in the microcosms containing beach sand (Figure 3.3B), clearly indicating the contribution of indigenous microbiota in beach sand to the decay of exogenous *E. faecalis* cells.

Since various beach sand characteristics can affect the abundance of indigenous microbiota, changes in such characteristics can be expected to enhance the decay of fecal bacteria. Two beach sand characteristics, sand-to-water ratio and sand particle size distribution, which are parameters controllable during beach restoration and sand nourishment efforts, were further investigated. Higher sand-to-water ratios resulted in faster *E. faecalis* decay (Figure 3.4A), which is in agreement with the higher decay rate observed in regular beach microcosms than in seawater-only microcosms (Figure 3.2). The sand/water ratios used in this study (0.25 to 1.0) are relative high for experimental purposes, and may only approximate actual sand/water ratios commonly found in the shallow portion of a beach system (e.g. knee to thigh water depth). The intensive interaction between sand and seawater in the shallow portion of beach systems deserve particular attention in recreational water management, as these areas usually see majority of recreational activities yet higher FIB concentrations are often observed in these shallow areas than in the deeper portion of beach systems. Similarly, beach microcosms containing the smallest size fraction (<1.20 mm) of the Waialae beach sand, which were expected to contain more indigenous microbiota due to the largest total surface area and the tendency of indigenous microbiota to accumulate on finer particles, exhibited faster *E. faecalis* decay than the beach microcosms containing larger size fractions (Figure
3.4B). The two sets of beach microcosms containing larger size fractions (1.40-2.00 mm and 2.00-2.36 mm) exhibited very similar *E. faecalis* decay rates, which was probably due to their similar total sand surface areas and hence total abundances of indigenous microbiota.

Indigenous microbiota of natural environments represents a major factor in the decay of fecal bacteria in the environments. Many previous studies have focused on the effects of various abiotic environmental factors on the decay of fecal bacteria in aquatic environments (6, 19, 27, 47, 48, 52), while other studies have suggested that the indigenous microbiota may play a significantly more important role than abiotic stresses (7, 13, 21, 26, 29, 38). If the contributions of abiotic stresses and indigenous microbiota were independent and superimposable, the different decay rates would theoretically suggest dominant role of indigenous microbiota over abiotic stresses (such as temperature fluctuation, sunlight inactivation, osmotic stress, etc). In reality, it is more likely that indigenous microbiota interacts with abiotic stresses, which enhances each other’s effects on the decay of fecal bacteria. Although the complex ecological interactions between indigenous microbiota, other environmental stresses, and exogenous fecal bacteria remain poorly characterized, which was not the main objective of this study, it should be clear that indigenous microbiota plays an important role in the decay of fecal bacteria in environment.

**3.5 CONCLUSIONS**

The significant contribution of beach sand and its indigenous microbiota to the decay of *E. faecalis* in the beach microcosms indicates that beach sand, as the characteristic component of a beach system, not only provides recreational values to
bathers, but also contributes significantly to beach water quality. The contribution from beach sand goes beyond the traditional perception of contaminant adsorption and retention, and involves actively facilitating the removal of fecal bacteria. This newly recognized function of beach sand should be of particular relevance during the early stages of fecal pollutions when large numbers of fecal bacteria are discharged into beach systems. Since other recent studies have also reported beach sand harboring considerable amount of FIBs persistently, which are more likely to occur towards the end of contamination events, the impact of beach sand to water quality is not only substantial but may also have contrasting effects under different circumstances. Therefore, it appears appropriate for beach managers to take a systems approach that recognize beach sand as a critical component of beach water quality management.

3.6 ACKNOWLEDGEMENT

This material is based upon work supported by the Kualoa Supplemental Environmental Project Fund from the Hawaii Department of Health to T.Y. (11-093).
3.7 REFERENCE


CHAPTER 4. DIFFERENTIAL DECAY OF WASTEWATER BACTERIA IN BEACH SAND AND SEAWATER MICRO COSMS AND CORRESPONDING MICROBIAL COMMUNITY DYNAMICS

ABSTRACT

Laboratory microcosm experiments were conducted to compare the decay patterns of wastewater bacteria in beach sand and seawater and to examine the decay kinetics of individual populations in the context of overall microbial community composition and diversity. The dynamics of individual wastewater populations (E. coli, enterococci, Clostridium perfringens and Salmonella) were monitored using cultivation methods, and microbial community compositions were determined by Illumina sequencing of 16S rRNA gene amplicons. Biphasic decay patterns were observed for all bacteria in all microcosms, and significantly slower decay rates were generally observed in beach sand than in seawater. Many major wastewater OTUs identified by sequencing also exhibited similar biphasic decay patterns. The microbial community dynamics corresponded well with the decay of individual wastewater bacterial populations. Higher microbial diversity often correlated significantly with smaller bacterial decay rates. In summary, the different decay behavior of wastewater bacteria in beach sand than in seawater indicate that beach sand needs to be carefully considered in assessing their respective impacts to water quality and public health. The close association between microbial community structure and dynamics highlight the importance of understanding the fate of individual bacterial populations in the context of microbial community dynamics.
4.1 INTRODUCTION

Marine beach environmental quality is important to the health and safety of beach users as well as the economic prosperity of coastal communities. Currently, only is beach water quality routinely monitored using fecal indicator bacteria (FIBs) (10, 11, 53). 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 evidences supporting the importance of beach sand to public health. Numerous studies have detected high levels of fecal indicator enterococci in marine beach sand, with concentrations often being 10 to 100 fold higher (on a unit mass basis) than in the corresponding beach water (8, 17, 45, 56). Enterococci-laden beach sand can affect beach water quality by serving as a chronic source of enterococci to beach water due to interactions between the two beach system components (33, 46, 56). Contaminated beach sand may also directly cause adverse public health effects via human-sand contact, which is supported by the significant correlation between exposure to enterococci-laden beach sand and increased enteric illness (34, 35).

Although recent studies have shown that common FIBs (i.e. enterococci and *Escherichia coli*) can have environmental sources (3, 17, 22, 43), wastewater contaminations still pose the highest public health threat to beach environments. To date, majority of studies on the decay of wastewater bacteria in marine beach environments have been performed in seawater (14, 23, 49, 54). Although a few studies have attempted to investigate the decay of wastewater bacteria in marine beach sand (19), 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 (28). The biofilm life style in beach sand is different from the planktonic life style in seawater, and the different life styles are known to have different effects on bacterial decay (31). Furthermore, many important environmental stresses that cause rapid bacterial decay in seawater, including temperature variation (54), sunlight inactivation (23, 48, 49), pH fluctuation (13), and nutrient depletion (24, 37), 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 microbial community structure and dynamics were often neglected, primarily due to technical hurdles in characterizing the overwhelming diversity of microbial communities. Recent studies have shown that indigenous microbial communities can significantly affect and often rapidly expedite the decay of exogenous bacterial populations (4, 19, 47). General ecology theories suggest that higher biodiversity usually corresponds to better ecosystem stability responding to environmental perturbation (50). Microbial communities with higher diversity also appeared to resist perturbation more effectively than the less diverse ones (1, 27), highlighting the necessity of understanding bacterial decay in the context of microbial community dynamics.

Recent advancements in next generation sequencing (NGS) and bioinformatics tools have provided unparalleled capabilities in obtaining millions of sequence reads to query the microbial community structure and diversity (12). The application of NGS tools in beach systems has revealed tremendous microbial diversity in beach sand and
seawater (17, 32). Efforts have also been made to use the NGS tools to address ecological questions in marine beach environments, including the fate and source of fecal indicator enterococci (17), microbial community response to oil pollutions (39, 44), temporal variation of resident and rare populations (28), and influence of physicochemical parameters on community structure (7). However, no study has been reported to investigate how microbial community structure and biodiversity interface with the decay of individual bacterial populations.

Therefore, the objectives of this study were to compare decay patterns of wastewater bacteria in beach sand and seawater and to examine the relationships between the decay of individual populations and the changes in overall microbial community composition and diversity. Laboratory beach sand and seawater microcosms were spiked with raw municipal wastewater, and the concentrations of four individual wastewater populations (E. coli, enterococci, Clostridium perfringens and Salmonella) were quantified over time to determine their decay patterns. Microbial community compositions in the microcosms were determined by Illumina sequencing of 16S rRNA gene amplicons. The dynamics of major wastewater OTUs were determined based on their relative abundance change over time, and the evolvement of overall microbial communities were illustrated using clustering analyses, both of which were compared with the bacterial decay patterns. Microbial diversity indices were also calculated and used in correlation analyses to infer their relationship with the decay rates of individual bacterial populations.
4.2 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 Whir-Pak sampling bags. Seawater samples were collected at multiple locations at knee depth using sterile wide-mouth plastic bottles. Grab raw municipal wastewater samples were 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 and transported to the laboratory for immediate processing.

**Sample characterization and analysis.** Sand samples were first extracted using a procedure described by Boehm *et al.* (6), which involved shaking 10 gram of sand in 100 mL of sterilized deionized water for 2 minutes by hand and then collecting supernatants of the sand extracts after settlement for 30 seconds. The beach sand extracts, seawater samples, and wastewater samples were analyzed to determine various physicochemical and biological parameters. The pH and salinity of the samples were determined using a bench top pH Meter (UltraBasic; Denver Instrument) and Orion 150A plus conductivity meter (Thermo; Waltham, MA), respectively. Total organic carbon (TOC) and total nitrogen (TN) were quantified using a TOC analyzer coupled with a TN detector (Shimadzu; Kyoto, Japan). The samples were also tested for 5-day biochemical oxygen demand test (BOD₅) following the APHA 5210B standard method using a YSI Model 58 Dissolved Oxygen Meter (2).
Total bacterial density in the beach sand extracts, seawater and wastewater samples were estimated using heterotrophic plate count (HPC). Briefly, the samples were diluted in tenfold series and plated on tryptic soy agar (TSA) and incubated at 37°C for 24 hours before colony enumeration. Individual bacterial populations, including two common fecal indicator bacteria (FIBs) (E. coli and enterococci), one pathogen and optional FIB (Clostridium perfringens), and one group of bacterial pathogen (Salmonella), were also enumerated using the same dilution and plating approach but with selective agar media. E. coli were enumerated on the modified mTEC agar according to EPA method 1603 (52). Enterococci were enumerated using the mEI agar method according to EPA method 1600 (51). C. perfringens were enumerated using the mCP agar method (5). Salmonella were enumerated using the Salmonella Shigella (SS) agar (36).

**Microcosm experiments.** Two sets of microcosms, one for beach sand and one for seawater, were established in triplicate in one-liter clean and autoclaved Mason jars. The beach sand samples collected from the Kualoa beach were first pooled and mixed thoroughly with a sterile wood tongue depressor. 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 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 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 three weeks, while larger volumes (up to 60 mL) were collected in the last week to compensate for decreased microbial concentration. The samples from microcosms were subjected to bacterial enumeration following the procedures described above.

**Total genomic DNA extraction.** All samples, including the original beach sand, seawater, and wastewater samples and the microcosm samples collected over time, were immediately centrifuged at 12,000 x g at 4°C for 10 min to pellet microbial biomass. After decanting the supernatant, the cell pellets were stored immediately at -80°C until DNA extraction. Total genomic DNA extraction was conducted by using the PowerSoil DNA extraction kit (MoBio, Carlsbad, CA) and following the manufacturer’s procedure.

**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 515f/806r primer set that amplifies the hypervariable V4-V5 region of the 16S rRNA gene (12). Amplicons of the triplicate PCR reactions were pooled and then sequenced using an Illumina HiSeq 2000 instrument. *De novo* assemble of paired-end reads was performed with the software package CLC Genomics Workbench Version 6.0 (CLC bio, Cambridge, MA), and the resulting fastq files were trimmed using a cutoff quality score of Q15 and read length of larger than 200 bp (20). Only the forward reads were used for downstream analysis, as it was shown that including the reverse reads add little additional information (20).

The QIIME software package (version 1.7.0) was used to analyze Illumina sequence data following the procedure of Kuczynski et al. (40). Operational taxonomic
units (OTUs) were identified based on 97% sequence identity, and the relative abundance of individual OTUs were determined based on the detection frequency and reported in an OTU table. Phylogenetic information of the OTUs was obtained by alignment with reference sequences in the Greengenes Database. Majority of the OTUs were assigned phylogeny at the genus level, while a small number of OTUs were resolved at the species level or at coarser levels (family or class). The genus level was the most resolved level used in subsequent analysis. Alpha diversity indices were calculated using the sequence reads rarified at the median level of all samples.

**Data analysis.** Bacterial concentration data over time were fitted into the 1st order decay model \((\ln \left( \frac{C_t}{C_0} \right) = -k_d t)\) to calculate decay rates. \(C_t\) and \(C_0\) are the concentrations of bacterial cells in the microcosms at time \(t\) and time zero, respectively, and \(k_d\) is the 1st-order decay rate. The turning points of biphasic decay curves were identified as the date that gave the best goodness of fit for the linear regression curves of the two phases. The decay rates for the first and second phases of the biphasic decay pattern were designated as \(k_{d1}\) and \(k_{d2}\), respectively. The instantaneous decay rates of *E. coli*, enterococci, *C. perfringens*, and *Salmonella* on Days 0, 3, 6, 16 and 23 were calculated as described in the following. For any given sampling date, bacterial concentrations from two preceding and two following sampling dates (if available) were used to conduct five-point linear regression. For Days 0 and 23, only three data points were used in the calculation of instantaneous decay rates.

Relative abundances of OTUs identified by Illumina sequencing were calculated as the detection frequencies of the unique sequences in each sample. Major OTUs in the wastewater sample were identified as those whose relative abundance in the wastewater
sample was at least ten times higher than that in the initial beach sand and seawater samples. Nine such populations were identified (Table B.1), and their relative abundance change over time were also fitted into the 1st order decay model and analyzed to determine biphasic patterns and decay rates as described above.

The relative abundance data of the OTUs identified were analyzed using the statistical software package PRIMER 6 (16). Principal coordinate analysis and non-metric multi-dimensional scaling (NMDS) were conducted to visualize the difference in microbial community composition between different samples. Distance-based redundancy analysis (dbRDA) was used to quantify the contribution of chemical and microbiological parameters, including TN, TOC, and HPC, to microbial community variation. Statistical analyses were performed using software packages SigmaPlot 12.0 and SPSS. Linear regression was used to calculate the decay rates of individual bacterial populations and major wastewater OTUs. The Student's t test was used to determine if significant difference exists between the decay rates of different bacterial populations. Pearson’s product moment and Spearman’s rank correlation were calculated to determine the correlation between alpha diversity indices and bacterial decay rates. The default significance level is P≤0.05 unless stated otherwise.

4.3 RESULTS

Biphasic bacterial decay patterns. The decay of four fecal bacteria (E. coli, enterococci, C. perfringens, and Salmonella) from raw municipal wastewater in the beach sand microcosms and the seawater microcosms were determined over a 24-days period (Figure 4.1). All bacteria exhibited biphasic decay patterns either in beach sand (Figure 4.1A) or in seawater (Figure 4.1B). The turning points of the biphasic patterns were
identified as Day 6 based on the goodness of fit of the 1st order decay model. Bacterial decay in the first phase (0-6 days) was significantly faster than in the second phase (6-23 days). The bacterial populations generally exhibited similar decay patterns, except for *E. coli* in the beach sand microcosms, which showed significantly faster decay rate in the first phase than other bacteria.
Figure 4. Reduction of concentration of viable *E. coli*, enterococci, *Clostridium perfringens* and *Salmonella* cells in beach sand microcosms and seawater for Kualoa beaches, Honolulu, HI. $C_t$ and $C_0$ represent cell counts in the microcosms at time $t$ and time zero, respectively. Error bar indicates the standard deviation of the mean of triplicate microcosms. The dotted lines are linear regression lines, and the vertical red lines demarcate the two phases.
The significantly slower rates in the second decay phase corresponded to persisting bacterial populations within the beach sand and seawater microcosms (Figure 4.2). Overall, there was significantly higher abundance of persisting populations in the sand microcosms than in the seawater microcosms. *C. perfringens* exhibited the highest persisting population density in both the beach sand and the seawater microcosms, with an average concentrations of 13,500 ± 3,822 CFU/100 g sand and 1,911 ± 1,002 CFU/100 mL water over the 17-day incubation period in the second phase. Enterococci showed a similar but slightly smaller persisting population (9,789 ± 7,689 CFU/100 g) than *C. perfringens* in the beach sand microcosms. However, the enterococci population exhibited a continuous decrease over time in the seawater microcosms. The levels of persisting *E. coli* and *Salmonella* in the beach sand microcosms were significantly less than those of *C. perfringens* and enterococci. There was no significantly difference between the persisting *E. coli* population in the beach sand microcosms and in the seawater microcosms on a unit mass basis. The persisting *Salmonella* population in the beach sand microcosms was significantly higher than that in the seawater microcosms, but both were very low and became intermittently detectable after Day 13.
Figure 4. Concentration of persisting *E. coli*, enterococci (ENT), *C. perfringens* (CP) and *Salmonella* during the second phase (ranging from 6 to 23 day) in the beach sand microcosms (A) and seawater microcosms (B). Error bar indicates the standard deviation of the mean of triplicate microcosms. The red dotted lines indicate the method detection limits.
**Different decay rates in beach sand and seawater.** Bacterial decay rates were calculated for the two phases and compared between the beach sand and seawater microcosms (Figure 4.3). In the initial fast decay phase (Figure 4.3A), all bacteria, except for *E. coli*, showed significantly faster decay rates in the seawater microcosms than in the beach sand microcosms. For enterococci, the decay rate in the seawater microcosm (0.76 ± 0.07 day\(^{-1}\)) was 4.5 times of the decay rate in the beach sand microcosm (0.17 ± 0.05 day\(^{-1}\)), which was the largest difference amongst the four fecal bacteria. The decay rates of *Salmonella* (1.15 ± 0.09 day\(^{-1}\) in seawater and 0.30 ± 0.11 day\(^{-1}\) in sand) and *C. perfringens* (0.77 ± 0.09 day\(^{-1}\) in seawater and 0.39 ± 0.09 day\(^{-1}\) in sand) also showed 3.8 and 1.9 folds of difference, respectively. To the contrary, *E. coli* actually exhibited slightly higher decay rate in seawater microcosms (1.08 ± 0.15 day\(^{-1}\)) than in the beach sand microcosms (\(k_{dl}=0.94 ± 0.05\) day\(^{-1}\)), although the difference was not statistically significant (P>0.05).

The decay rates for the second decay phase were significantly smaller than those observed in the first phase (Figure 4.3B). Among the four fecal bacteria, *E. coli*, enterococci, *C. perfringens* and *Salmonella* showed no significant difference in decay rates between seawater and beach sand microcosms during the second phase.
Figure 4. 3 Decay rates of *E. coli*, enterococci (ENT), *C. perfringens* (CP) and *Salmonella* in the first (A) and second (B) decay phases. Error bar indicates the standard deviation of the mean of triplicate microcosms.
Fate of major wastewater OTUs. The fate of major wastewater OTUs identified by Illumina sequencing in the beach sand and seawater microcosms were determined by comparing their relative abundances over time. The major wastewater OTUs were identified as those OTUs whose detection frequencies in the wastewater were at least tenfold higher than in the original beach sand or seawater samples (Table B.1). The major wastewater OTUs did not include the four bacteria monitored using cultivation methods, hence providing a broader understanding of the fate of wastewater bacteria. Different major wastewater OTUs exhibited different dynamics, with some showing significant decrease over time and others showing relative stability (Figure 4.4). Many of the major wastewater OTUs that showed decrease of relative abundance over time also exhibited biphasic decay patterns in both the beach sand microcosms and the seawater microcosms, including *Acinetobacter*, *Arcobacter*, *Bacteroides*, and *Parabacteroides*. The two wastewater bacterial OTUs affiliated with *Streptococcus* and *Falvobacteriaceae* exhibited no distinct biphasic decay pattern and significantly slower decay in both the beach sand and seawater microcosms than the other major wastewater OTUs. These observations are supported by the rates of relative abundance change for the major wastewater OTUs (Table B.2).
Figure 4. Dynamics of the relative abundance of major wastewater OTUs in the beach sand microcosms (A) and seawater microcosms (B). Error bar indicates the standard deviation of the mean of triplicate microcosms.

**Microbial community structures.** The microbial community structures in beach sand and seawater microcosms revealed by Illumina sequencing were compared with three chemical and biological parameters (TN, TOC and HPC) using distance based
redundancy analysis (dbRDA) (Figure 4.5). The model shows that 44.6% of the overall variability in microbial communities of beach sand microcosms and in seawater microcosms can be attributed to the three parameters. TN and TOC were identified as the two most important environmental determinants, explaining 25.5% and 18.7% of the total variation, respectively. Total bacterial density quantified by HPC only made marginal contribution (1.6%) to the variation.

![Distance based redundancy ordination (dbRDA) for the fitted model of microbial communities in beach sand microcosms (BS) and seawater microcosms (SW) and environmental parameters (TOC, TN, and HPC). Symbols: the numbers behind BS and SW indicate sampling days (0, 3, 6, 16, 23).](image)

Figure 4.5 Distance based redundancy ordination (dbRDA) for the fitted model of microbial communities in beach sand microcosms (BS) and seawater microcosms (SW) and environmental parameters (TOC, TN, and HPC). Symbols: the numbers behind BS and SW indicate sampling days (0, 3, 6, 16, 23).
The microbial communities in the beach sand and seawater microcosms and their change over time were also analyzed using PCoA analysis (Figure 4.6A) and nMDS (Figure 4.6B). Both analyses showed similar microbial community dynamics over time, with the PCoA plot capturing 68.5% of the total variation while the nMDS plot exhibiting an excellent stress value (0.05). Microbial communities in triplicate microcosms dates were always tightly clustered together regardless of the matrix (either beach sand or seawater), indicating limited variance amongst the experimental replicates. Significant difference in microbial community structure was observed between the beach sand microcosms and seawater microcosms at all sampling dates (P=0.001), indicating strong source and matrix effects and limited influence from wastewater introduction. Temporal variation of microbial community structures was also significant in both beach sand and seawater microcosms, as indicated by clear separation of communities over time (P=0.001).
Figure 4. 6 Clustering analyses of microbial communities in the beach sand microcosms (BS) and seawater microcosms (SW) using principal coordinates analysis (A) and non-metric multidimensional scaling (B). Symbols: the numbers behind BS and SW indicate sampling days (0, 3, 6, 16, 23).
**Relationship between microbial community and bacterial decay.** The microbial community dynamics exhibited patterns similar to those of bacterial decay (Figure 4.6). First of all, over the 23-days experimental period, microbial community change was clearly more extensive in the seawater microcosms than in the beach sand microcosms. This corresponded nicely to the generally faster bacterial decay rates in the seawater microcosms than in the beach sand microcosms (Figure 4.1). Secondly, the microbial community dynamics also exhibited biphasic patterns in both the beach sand and the seawater microcosms, with larger separation of microbial communities observed in the initial phase (0-6 days) than in the second phase (6-23 days). This indicates faster microbial community evolvement in the first phase than in the second phase, which agrees with the faster bacterial decay observed in the first phase than in the second phase.

Microbial diversity for the beach sand and seawater microcosms at different sampling dates were calculated (Table B.3) and compared with the bacterial decay rates at the same sampling dates using either Spearman’s rank correlation (Table 4.1) or Pearson’s product moment (Table B.4). Spearman’s rank correlation exhibited better correlations and hence are described here. The strongest correlation was observed between bacterial decay rates and Shannon’s H in seawater microcosms, where the decay rates of *E. coli*, enterococci, and *Salmonella* all correlated significantly (P<0.05) with Shannon’s H, while the decay rate of *C. perfringens* correlated marginally significant (P<0.10) with Shannon’s H. Significant correlations (P<0.05) were also observed between phylogenetic diversity and the decay rates of *E. coli* and enterococci, but not those of *Salmonella* and *C. perfringens* in the seawater microcosms. In the beach sand microcosms, the only significant correlation (P<0.05) was observed between the decay rate of *E. coli* and Shannon’s H,
and two marginally significant correlations (P<0.10) were observed between Shannon’s H and decay rate of C. perfringens and between phylogenetic diversity and the decay rate of enterococci. Species richness of the microbial communities (Chao1) exhibited no significant correlation to bacterial decay rate in either the seawater or the beach sand microcosms. All the significant and marginally significant correlations had negative coefficients (from -0.48 to -0.81), indicating that higher microbial diversity corresponds to slower bacterial decay.
Table 4.1 Spearman’s r and P values (in parentheses) between alpha diversity indices and bacterial decay rates in beach sand and seawater microcosms.

<table>
<thead>
<tr>
<th>Alpha Diversity</th>
<th>Beach sand</th>
<th>Seawater</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. coli</td>
<td>ENT&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Shannon’s H</td>
<td>0.61 (0.02)</td>
<td>-0.17 (0.54)</td>
</tr>
<tr>
<td>Phylogenetic diversity</td>
<td>-0.02 (0.94)</td>
<td>-0.48 (0.08)</td>
</tr>
<tr>
<td>Chao1</td>
<td>0.02 (0.95)</td>
<td>-0.19 (0.50)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significant correlations (P<0.05) are bolded, marginally significant correlations (P<0.10) are underlined.

<sup>b</sup> Abbreviation: ENT: enterococci, CP: C. perfringens.
4.4 DISCUSSION

The higher abundance of FIBs often observed in marine beach sand than in beach seawater is likely the result of many processes, including different contaminant loadings and different levels of indigenous FIB populations (3, 17, 22, 43). This study provides direct evidence that different bacterial decay patterns between beach sand and seawater is also an important factor. Direct comparison in well-controlled laboratory microcosms showed that three of the four fecal bacteria (enterococci, C. perfringens, and Salmonella) exhibited significantly slower decay rates in beach sand than in seawater after the introduction of wastewater bacteria (Figures 4.1 and 4.3). The decay of E. coli was generally fast and showed no significant difference in decay rates between beach sand and seawater, which is likely caused by its high susceptibility to salinity stress that was present in both the beach sand and seawater microcosms (13). The slower bacterial decay rates in beach sand than in seawater indicate that the same fecal bacteria 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.

The slower bacterial decay in beach sand than in seawater is likely the result of surface-impacted processes. First of all, the wastewater bacteria could have quickly associated with the sand surface and become embedded in sand biofilms. Biofilms are known to provide shelter and enable higher resistance to environmental stresses than their planktonic counterparts (30). For example, the surviving E. coli cells in freshwater environments were usually associated with suspended particle surfaces (9), and the decay of E. coli in soil/sediment was often significantly slower than in water (18, 26). Secondly,
many important abiotic environmental stresses that cause rapid bacterial decay in seawater, in particular light inactivation (23, 48, 49), are either significantly reduced or completely absent in marine beach sand due to surface protection. However, the contribution of light inactivation to the decay rate difference observed in this study should be minimal due to the removal of light in the laboratory microcosms. Thirdly, the biotic stresses, which are very important to fecal bacteria decay in the environment (19, 38, 42), are expected to be different, and hence can also contribute to the observed difference in decay rates between beach sand and seawater.

The biphasic decay patterns, which were observed for all four individual wastewater bacteria monitored by cultivation methods (Figure 4.1) and many major wastewater OTUs by Illumina sequencing (Figure 4.4) in both the beach sand and seawater microcosms, provide valuable insights to the survival behaviors of wastewater bacteria in beach environments. Previous studies on enteric bacterial decay in seawater that used cultivated cells of individual strains often observed log linear decay curves (47, 54), which fitted nicely in the first order decay model. Other non-linear decay curves, such as log linear curves with a shoulder, log linear curves with a tailing (or biphasic curves) and sigmoidal curves, were frequently attributed to different physiological state of the cells and stress variations in the environment (55). Since the laboratory microcosm experiments are expected to maintain constant condition and minimal stress variation, the biphasic curves observed most likely resulted from the bacterial cell heterogeneity in the raw wastewater (15).

The slower second decay phase resulted in persisting wastewater bacterial populations in beach sand and seawater (Figure 4.2), which have significant implications
in water quality monitoring and public health protection. These persisting wastewater bacterial populations may be the result of wastewater bacterial cells with different physiological states; 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 (41). Another potential factor concerns the genetic diversity of wastewater bacteria, which could have more profound impact on public health. Single bacterial species (such as *E. coli* and *C. perfringens*) can have persister 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 (25) or soil desiccation (57). Different species of the same genus (such as *Enterococcus*) often exhibit different stress resistance and different decay behaviors in the environment (21). These persisting wastewater bacterial species or strains can clearly prolong the self-cleaning process and hence pose public health risks post wastewater contamination events. It is yet to determine 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 (29).

The Illumina sequencing also revealed significantly different microbial communities in the beach sand and seawater microcosms (Figure 4.6), which agrees with the different microbial life styles (i.e. biofilm versus planktonic) that are expected of beach sand and seawater. Previous studies using NGS also reported significantly different microbial communities in natural beach sand and seawater samples (7, 17, 28, 32). It is interesting to note that the microbial community structures were only slightly affected by total bacterial density measured by HPC, while TN and TOC were associated with 25.5%
and 18.7% of the microbial community variability, respectively (Figure 4.5). This suggests close associations of microbial communities and their changes to nutrient availability and consumption, which warrants further investigation.

This study, for the first time, tracked microbial community compositions and their change over time in beach sand and seawater after the introduction of wastewater, which allowed direct comparison with the decay of individual bacterial populations. The slower microbial community structure change in beach sand than in seawater (Figure 4.6) corresponded well to the similar pattern of bacterial decay in the two sets of microcosms (Figures 4.1 and 4.3). The rate of microbial community change in both the beach sand and seawater microcosms also appeared to follow a biphasic pattern, with faster changes observed in the first phase (Days 0-6) than in the second phase (Days 6-23). These similarities suggest a close connection between overall microbial community evolvement and the fate of individual bacterial populations.

The importance of microbial community structure in the dynamics of individual bacterial populations was further illustrated by significant correlations between alpha diversity indices and bacterial decay rates (Table 4.1 and Table B.4). The significant negative Spearman’s correlations observed between the alpha diversity indices (i.e., Shannon’s H and phylogenetic diversity) and the decay rates of certain bacterial populations indicate that higher microbial diversity were associated with slower bacterial decay in these comparisons. This is in agreement with the general ecological proposition that higher biodiversity of ecosystem usually provides better resistance to sudden environmental perturbation (50), and is supported by previous studies on microbial communities where higher microbial diversity helped to resist perturbation more
effectively than the less diverse ones (1, 27). These also corroborate the common observation that indigenous microbial communities can significantly affect and often rapidly expedite the decay of exogenous bacterial populations (4, 19, 47). Taken together, these results highlight the necessity of understanding the fate of individual bacterial populations in the context of microbial community dynamics.

In summary, the different decay behavior of wastewater bacteria in beach sand and seawater indicate that beach sand needs to be considered differently than seawater in assessing their respective impacts to 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. 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 close association between microbial community structure and dynamics highlighted the importance of understanding the fate of individual bacterial populations in the context of microbial community dynamics.

4.5 ACKNOWLEDGEMENT

This material is based upon work supported by the Kualoa Supplemental Environmental Project Fund from the Hawaii Department of Health to T.Y. (11-093).
4.6 REFERENCE


CHAPTER 5. THE EFFECT OF EXOGENOUS NUTRIENTS AND/OR MICROBES ON MICROBIAL COMMUNITY IN BEACH SAND AND SEAWATER

ABSTRACT

Coastal ecosystem health is threatened by the release of untreated sewage wastewater. In this study, the relationship between microbial community diversity and nutrient availability in beach sand or seawater was investigated by spiking raw wastewater into beach microcosms. High throughout Illumina sequencing of 16S rRNA gene amplicons was conducted to determine the microbial community diversity and composition. Proteobacteria, Bacteroidetes, Euryarchaeota, Planctomycetes, and Acidobacteria are the major populations that together accounted for more than 80% of the beach sand microbial community, while Proteobacteria and Bacteroidetes were the dominant phyla that together accounted for more than 80% of the microbial community in seawater. There is no significant difference in microbial community diversity between beach sand microcosms spiked either with raw wastewater or with autoclaved wastewater. Similar observation was made in the seawater microcosms. Moreover, the TOC/TN ratio showed significant correlation with microbial community diversity in most microcosms, indicating that the TOC/TN ratio is an important factor controlling microbial communities.
5.1 INTRODUCTION

Annually, more than 4 trillion liters of untreated sewage wastewater are released into waterways in the United States (22), introducing exogenous nutrients including pharmaceuticals (19) and BOD (20). In addition to these pollutants, fecal pathogens can impose high risk to public health, causing respiratory, skin, eye and ear illness (18). In the U.S., approximately 39% of population (i.e., over 123 million people) are exposed to this risk every year in coastal areas (17). For example, a 50 million gallons of raw sewage wastewater spill in Hawaii severely damaged the coastal ecosystem health, resulting in the closure of Waikiki beach for one week in 2006. More attention has been paid to study the impact of untreated wastewater on coastal ecosystem and to reduce its threat to public health at coastal areas.

Currently, water quality monitoring relies on fecal indicator bacteria such as *E. coli*, enterococci, and *Clostridium perfringens*. Most of studies boost on fecal bacteria at recreational area(8, 23-25). Recently, several studies have characterized microbial communities in beach sand and in seawater (2, 7, 16), while only a few studies have investigated the correlation between FIBs and overall microbial community (5). The aim of this study was to study the variation of indigenous microbiota diversity and composition using NGS approach in beach sand or seawater when raw wastewater (wastewater nutrients plus microorganisms) or autoclaved wastewater (only wastewater nutrients) was spiked into beach sand microcosms and seawater microcosms. Our primary objective is to gain insight into the temporal variation of microbial community diversity and composition as a result of wastewater pollution to beach systems.
5.2 MATERIALS AND METHODS

Sample collection. 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 Whir-Pak sampling bags. Seawater samples were collected at multiple locations at knee depth using sterile wide-mouth plastic bottles. Grab raw municipal wastewater samples were 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 and transported to the laboratory for immediate processing.

Microcosm set-up. Four sets of microcosms, each in triplicate, were established to investigate the impact of wastewater on indigenous microbiota in beach sand and seawater. The two sets of beach sand microcosms include beach sand spiked with either raw municipal wastewater (BSRW) or beach sand spiked with autoclaved municipal wastewater (BSAW). The two sets of seawater microcosms included either seawater spiked with raw wastewater (SWRW) or seawater spiked with autoclaved wastewater (SWAW). The microcosms were constructed in one-liter clean and sterile Mason jars. The beach sand samples collected from the Kualoa beach were first pooled into one aggregated sample and mixed thoroughly with a sterile wood tongue depressor. The seawater samples were also pooled into one aggregated sample and thoroughly 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. The
BSRW and SWRW microcosms were spiked with 30 mL of the raw wastewater sample from SIWTP, while the BSAW and SWAW microcosm were spiked with 30 mL of the autoclaved wastewater sample from SIWTP. After thorough mixing, the microcosms were incubated for 24 days in dark at room temperature (22-24°C) without shaking.

**Sample collection and characterization.** Samples were collected on Days 1, 4, 7, 17, and 24 (the original sand, seawater and wastewater samples were called Day 0 samples for simplification). 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 three weeks, while larger volumes (up to 60 mL) were collected in the last week to compensate for decreased microbial concentration. All sand samples were first extracted using a procedure described by Boehm *et al.* (1), which involved shaking 10 gram of sand in 100 mL of sterilized deionized water for 2 minutes by hand and collecting supernatants of the sand extracts after settlement for 30 seconds.

Sand extracts from the original aggregated beach sand sample and samples collected during the microcosms experiments, as well as the original seawater sample, wastewater sample and seawater samples collected during the microcosm experiments were analyzed to determine chemical parameters (TOC and TN), total bacterial biomass by qPCR, and microbial communities by Illumina sequencing. The total organic carbon (TOC) and total nitrogen (TN) of sand extracts, seawater and wastewater samples were determined using a TOC analyzer coupled with a TN detector (Shimadzu; Kyoto, Japan).

**Total genomic DNA extraction.** All samples, including the original beach sand, seawater, and wastewater samples and the microcosm samples collected over time, were
immediately centrifuged at 12,000 x g at 4°C for 10 minutes to pellet microbial biomass. After decanting the supernatant, the cell pellets were stored immediately at -80°C until DNA extraction. Total genomic DNA extraction was conducted by using the PowerSoil DNA extraction kit (MoBio, Carlsbad, CA) and following the manufacturer’s procedure. The total genomic DNA samples were used for 16S rRNA gene qPCR quantification.

**qPCR quantification of 16S rRNA gene.** The total bacterial biomass in the samples were estimated using qPCR quantification of 16S rRNA gene copies using the procedure described by Nadkarni et al. (15). The 20 μL qPCR reactions contained 10 μL of 2 × TaqMan Universal PCR Master mix (Life technologies; ), 0.25 μM of PCR primers (Forward: 5′-TCCTACGGAGGCAGCAGT-3′; Rev: 5′-GGACTACCAGGGTGATCTAATCTGTGTT-3′), 0.125 μM of fluorescent probe ((6-FAM)-5′-CGTATTACCGCGGTCTGCTGAC-3′-(TAMRA)) and 0.4 μg/μL of bovine serum albumin (BSA). The qPCR reactions were reformed 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 and followed the procedure described in the EPA Method A for Enterococci (21). The calibration standards were analyzed in duplicate reactions in each 96-well qPCR reaction concurrently with samples, and the R², PCR efficiency.

**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 515f/806r primer set.
that amplifies the hypervariable V4-V5 region of the 16S rRNA gene (3). Amplicons of the triplicate PCR reactions were pooled and then sequenced using an Illumina HiSeq 2000 instrument. De novo assemble of paired-end reads was performed with the software package CLC Genomics Workbench Version 6.0 (CLC bio, Cambridge, MA), and the resulting fastq files were trimmed using a cutoff quality score of Q15 and read length of larger than 200 bp (6). Only the forward reads were used for downstream analysis, as it was shown that including the reverse reads add little additional information (6).

The QIIME software package (version 1.7.0) was used to analyze Illumina sequence data following the procedure of Kuczynski et al. (9). Operational taxonomic units (OTUs) were identified based on 97% sequence identity, and the relative abundance of individual OTUs were determined based on the detection frequency and reported in an OTU table. Phylogenetic information of the OTUs was obtained by alignment with reference sequences in the Greengenes Database. Majority of the OTUs were assigned phylogeny at the genus level, while a small number of OTUs were resolved at the species level or at coarser levels (family or class). The genus level was the most resolved level used in subsequent analysis. Alpha diversity indices were calculated using the sequence reads rarified at the median level of all samples.

**Data analysis.** Relative abundances of OTUs identified by Illumina sequencing were calculated as the detection frequencies of the unique sequences in each sample. Major OTUs in the sand or seawater samples were defined as those whose relative abundance in the initial sand or seawater samples were at least ten times higher than that in the initial wastewater sample. The relative abundance data of the OTUs identified were analyzed using the statistical software package PRIMER 6 (4). Principal coordinates
analysis and non-metric multi-dimensional scaling (NMDS) were conducted to visualize the difference in microbial community composition between different samples. Distance-based redundancy analysis (dbRDA) was used to quantify the contribution of chemical and microbiological parameters, including TN, TOC, and total bacterial biomass, to microbial community variation. Statistical analyses were performed using software packages SigmaPlot 12.0 and SPSS. Pearson’ product moment and Spearman’s rank correlation were calculated to determine the correlation between alpha diversity indices and environmental parameters. The default significance level is $P \leq 0.05$ unless stated otherwise.

5.3 RESULTS

Dynamics of indigenous microbial populations in the presence of raw wastewater. The top 15 major indigenous OTUs in the beach sand and seawater microcosm spiked with raw wastewater were identified (Table 5.1). Their relative abundance change (as determined by Illumina sequencing) over time were plotted to illustrate their dynamics in response to the introduction of wastewater (Figures 5.1 and 5.2). Upon the introduction of raw wastewater (i.e. on Day 1), the relative abundance of the top 15 major indigenous microbial OTUs in beach sand microcosms (Figure 5.1) and the seawater microcosms (Figure 5.2) experienced sudden decrease due to the dilution effect. The change in relative abundance was much more distinct in the seawater microcosms than in the beach sand microcosms, which can be explained by the higher bacterial biomass observed in beach sand than in seawater. Over time, many of the major OTUs gradually recovered in relative abundance, and appeared to reach their original abundance levels toward the end of the experimental course. Within the microcosms
spiked with raw wastewater (i.e., BSRW and SWRW), OTU_229 (identified at the family-level *Saprospiraceae*) and OTU_818 (identified at the family-level *Oleiphilacea*) were the common major OTUs identified in these two sets of microcosms.

Figure 5. The variation of top 15 major OTUs in sand microcosm spiked with raw wastewater microcosms. (Raw wastewater was spiked into sand microcosms on Day 1).
Figure 5.2 The variation of top 15 major OTUs in seawater microcosm spiked with raw wastewater microcosms. (Raw wastewater was spiked into seawater microcosms on Day 1).
Table 5. Major OTUs identified in all beach sand and seawater microcosms.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Pearson r (P)</th>
<th>Spearman Rho (P)</th>
<th>Taxon</th>
<th>Pearson r (P)</th>
<th>Spearman Rho (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTU_008</td>
<td>0.92(&lt;0.01)</td>
<td>0.89(&lt;0.01)</td>
<td>OTU_229</td>
<td>0.93(&lt;0.01)</td>
<td>0.94(&lt;0.01)</td>
</tr>
<tr>
<td>OTU_015</td>
<td>0.76(&lt;0.01)</td>
<td>0.58(0.02)</td>
<td>OTU_514</td>
<td>0.92(&lt;0.01)</td>
<td>0.90(&lt;0.01)</td>
</tr>
<tr>
<td>OTU_047</td>
<td>0.71(&lt;0.01)</td>
<td>0.76(&lt;0.01)</td>
<td>OTU_564</td>
<td>-0.01(0.96)</td>
<td>0.20(0.46)</td>
</tr>
<tr>
<td>OTU_061</td>
<td>0.73(&lt;0.01)</td>
<td>0.67(&lt;0.01)</td>
<td>OTU_588</td>
<td>0.93(&lt;0.01)</td>
<td>0.90(&lt;0.01)</td>
</tr>
<tr>
<td>OTU_166</td>
<td>0.67(&lt;0.01)</td>
<td>0.65(&lt;0.01)</td>
<td>OTU_734</td>
<td>0.72(&lt;0.01)</td>
<td>0.56(0.03)</td>
</tr>
<tr>
<td>OTU_229</td>
<td>0.74(&lt;0.01)</td>
<td>0.81(&lt;0.01)</td>
<td>OTU_744</td>
<td>0.79(&lt;0.01)</td>
<td>0.70(&lt;0.01)</td>
</tr>
<tr>
<td>OTU_505</td>
<td>-0.20(0.48)</td>
<td>-0.26(0.34)</td>
<td>OTU_759</td>
<td>0.78(&lt;0.01)</td>
<td>0.84(&lt;0.01)</td>
</tr>
<tr>
<td>OTU_724</td>
<td>0.45(0.09)</td>
<td>0.40(0.13)</td>
<td>OTU_810</td>
<td>0.12(0.68)</td>
<td>0.24(0.38)</td>
</tr>
<tr>
<td>OTU_725</td>
<td>0.84(&lt;0.01)</td>
<td>0.76(&lt;0.01)</td>
<td>OTU_818</td>
<td>0.83(&lt;0.01)</td>
<td>0.52(0.04)</td>
</tr>
<tr>
<td>OTU_734</td>
<td>0.71(&lt;0.01)</td>
<td>0.41(0.12)</td>
<td>OTU_846</td>
<td>0.86(&lt;0.01)</td>
<td>0.90(&lt;0.01)</td>
</tr>
<tr>
<td>OTU_818</td>
<td>0.02(0.94)</td>
<td>0.11(0.69)</td>
<td>OTU_909</td>
<td>0.86(&lt;0.01)</td>
<td>0.82(&lt;0.01)</td>
</tr>
<tr>
<td>OTU_848</td>
<td>0.58(0.02)</td>
<td>0.47(0.07)</td>
<td>OTU_168</td>
<td>SWRW</td>
<td>SWRW</td>
</tr>
<tr>
<td>OTU_016</td>
<td>BSRW</td>
<td>BSRW</td>
<td>OTU_170</td>
<td>SWRW</td>
<td>SWRW</td>
</tr>
<tr>
<td>OTU_598</td>
<td>BSRW</td>
<td>BSRW</td>
<td>OTU_209</td>
<td>SWRW</td>
<td>SWRW</td>
</tr>
<tr>
<td>OTU_682</td>
<td>BSRW</td>
<td>BSRW</td>
<td>OTU_733</td>
<td>SWRW</td>
<td>SWRW</td>
</tr>
<tr>
<td>OTU_047</td>
<td>BSAW</td>
<td>BSAW</td>
<td>OTU_231</td>
<td>SWAW</td>
<td>SWAW</td>
</tr>
<tr>
<td>OTU_227</td>
<td>BSAW</td>
<td>BSAW</td>
<td>OTU_732</td>
<td>SWAW</td>
<td>SWAW</td>
</tr>
<tr>
<td>OTU_564</td>
<td>BSAW</td>
<td>BSAW</td>
<td>OTU_784</td>
<td>SWAW</td>
<td>SWAW</td>
</tr>
</tbody>
</table>
Similar dynamics of the major indigenous microbiota OTUs were also observed when autoclaved wastewater was spiked into the beach sand (Figure 5.3) and seawater microcosms (Figure 5.4). The relative abundance levels of many major indigenous OTUs in the beach sand and seawater microcosms (spiked with raw or autoclaved wastewater) exhibited a strong and significant correlation (Table 5.1), indicating that the presence of live wastewater bacteria did not significantly affect the dynamics of indigenous microbiota. Twelve common major OTUs were identified in top 15 majors between the BSRW microcosms and BSAR microcosms. Nine of the twelve common major OTUs showed significant correlation between the BSRW microcosms and the BSAR microcosms (Pearson correlation, P<0.05) (Table 5.1). In the seawater microcosms (SWRW and SWAW), eleven major OTUs were detected in both sets of microcosms, while nine out of the eleven common OTUs showed significant correlation (Pearson correlation, P<0.05) (Table 5.1).
Figure 5. 3 The variation of top 15 major OTUs in sand microcosm spiked with autoclaved wastewater microcosms. (Autoclaved wastewater was spiked into sand microcosms on Day 1).
Microbial diversity. Microbial diversity in the beach sand and seawater microcosms was calculated using all OTUs (major and rare) identified by Illumina sequencing of the 16S rRNA gene amplicons. After removing low quality sequence (limit = 0.01), the number of sequence reads varied from 7309 and 9844 among fifty-seven sand and seawater samples in the four types of microcosms. In the rarified data set (7,300 reads), a total of fifty phyla were identified out of the 416,100 sequence reads. The Shannon-Weaver diversity index was computed for each sample (Figure 5.5), and the average indices of BSAW, BSRW, SWAW, and SWRW elucidated the variation of microbial
community diversity in beach sand microcosms and in seawater microcosms over time. Beach sand microcosms (BSAW and BSRW) consistently exhibited significantly higher Shannon-Weaver diversity index than the seawater microcosms (SWAW and SWRW). Meanwhile, significant diversity differences were also observed when introducing wastewater (either raw wastewater or autoclaved wastewater) into sand microcosms (P =0.001 for BSRW and BSAW, Pared t-test) or seawater microcosms (P<0.001 for SWRW and SWAW, Pared t-test).

Figure 5. Diversity of bacterial communities overtime with exogenous nutrient and/or microbes in beach sand microcosms and seawater microcosms. Error bar indicates the standard deviation of the mean of triplicate microcosms.
**Microbial community dynamics.** Clustering analyses were conducted using non-metric multi-dimensional scaling (nMDS) (Figure 5.6 and 5.7), which showed the dynamics of bacterial community over time. In the beach sand microcosms (Figure 5.6), when wastewater (either raw or autoclaved) was introduced on Day 1, the initial microbial communities in the sand microcosms were significantly disturbed due to the introduction of live wastewater bacteria or their DNA (in the case of autoclaved wastewater). The distance between the BSRW and BSAW microcosms decreased continuously over time, as the bacterial community compositions were significantly different on Day 1 but became highly similar on Day 24. Similar phenomenon was also observed in seawater microcosms (Figure 5.7)

![Cluster analysis of microbial communities](image)

**Figure 5.6** Clustering analyses of microbial communities in the beach sand microcosms (BS) using non-metric multidimensional scaling. Symbols: red diamond plots indicate initial sand sample at Day 0; solid square in plots indicate sand spiked with raw wastewater; square plots without filled indicate sand spiked with autoclaved wastewater.
Figure 5. Clustering analyses of microbial communities in the seawater microcosms using non-metric multidimensional scaling. Symbols: red solid cycle plots indicate initial sand sample at Day 0; solid cycle in plots indicate seawater spiked with raw wastewater; cycle plots without filled indicate sand spiked with autoclaved wastewater.

Despite the fact that the bacterial phyla dominating in BSRW and BSAW (Global R=0.22<0.75, P=0.001, permutation test =1000, ANOSIM) or SWRW and SWAW (Global R=0.12<0.75, P=0.001, permutation test =1000, ANOSIM) were mostly similar (Figure 5.8), bacterial community composition provided insight into the distribution of specific OTUs among samples on the temporal scale. In beach sand microcosms, significant difference (Global R=0.87>0.75, P=0.001, permutation test =1000, ANOSIM) was observed between BSRW and BSAW when introducing the temporal factor along with the treatment factor (autoclaved wastewater vs. wastewater). Similarly, in the seawater microcosms, the Global R became 0.91 (P=0.001) while combining two factors of treatment and time.
Influence of TN and TOC on the variability of community composition.

Figure 5.9 presents the variation of soluble TN and TOC over time in the beach sand microcosms and seawater microcosms. After wastewater was introduced into the sand microcosms on Day 1, TN concentration increased while TOC concentration decreased (Figure 9A). In the seawater microcosms, TN concentration also increased continuously from Day 1 to Day 17, and then started declining to Day 24. The TOC concentration fluctuated over time in the seawater microcosms.
Figure 5. The variation of TN and TOC in sand microcosms (A) and in seawater microcosms (B). (wastewater was spiked into sand at Day 1)
The best distance-based linear model (DisbLM) was used to understand how the exogenous nutrients affected the variation of bacterial community composition (Figure 5.10). In the beach sand microcosms, 17.3% of the total variation was attributed to total nitrogen (TN) and total organic carbon (TOC); TN contributed 14.3% to the explained variation while TOC merely contributed 3.0%. In seawater microcosms (Figure 5.11), TN and TOC were responsible for 21.7% of the total variation, with 18.8% attributed to TN and 2.9% attributed to TOC. Apparently, TN was an important factor that influenced the variation of bacterial communities in both the beach sand microcosms and the seawater microcosms, and the influence of TN was greater in seawater microcosms than in beach sand microcosms.
Figure 5. 10 Distance-based Redundancy Analysis (dbRDA) plot of the distLM model based on total nitrogen and total organic carbon fitted to the variation in taxonomic community composition in beach sand microcosms.
Spearman correlation coefficient was also conducted to explain the bacterial community variations caused by exogenous nutrients (Table 5.2). In BSRW, TN and TOC were not significantly correlative with bacterial community diversity (Shannon index, P>0.05), but the ratio of TOC/TN was significantly negative correlated with Shannon index ($\rho = -0.53$, P=0.05). TOC demonstrated a significant correlation with the total bacterial biomass ($\rho = 0.55$, P=0.04), while TN and the ratio of TOC/TN showed no significant correlation with biomass. In the BSAW microcosms, nutrient parameters
(TN, TOC, and the ratio of TOC/TN) were not significantly correlated with either bacterial community diversity index (Shannon index) or biomass abundance. In the seawater microcosms (SWRW and SWAW), TN showed a significant and positive correlation with bacterial community diversity (Shannon index), while the ratio of TOC/TN showing significantly negative correlative with Shannon index.

Table 5. 2 The correlation of nutrient with Shannon index and total bacterial biomass (Spearman Correlation).

<table>
<thead>
<tr>
<th></th>
<th>Shannon index</th>
<th>Biomass</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BSRW</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TN</td>
<td>0.33(0.24)</td>
<td>-0.38(0.18)</td>
</tr>
<tr>
<td>TOC</td>
<td>-0.33(0.23)</td>
<td>0.55(0.04)*</td>
</tr>
<tr>
<td>TOC/TN</td>
<td>-0.53(0.05)*</td>
<td>0.41(0.14)</td>
</tr>
<tr>
<td><strong>BSAW</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TN</td>
<td>0.63(&lt;0.01)**</td>
<td>-0.22(0.42)</td>
</tr>
<tr>
<td>TOC</td>
<td>-0.50(0.06)</td>
<td>0.07(0.81)</td>
</tr>
<tr>
<td>TOC/TN</td>
<td>-0.43(0.10)</td>
<td>0.30(0.27)</td>
</tr>
<tr>
<td><strong>SWRW</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TN</td>
<td>0.78(&lt;0.01)**</td>
<td>-0.23(0.43)</td>
</tr>
<tr>
<td>TOC</td>
<td>0.06(0.83)</td>
<td>0.54(0.05) *</td>
</tr>
<tr>
<td>TOC/TN</td>
<td>-0.68(&lt;0.01)*</td>
<td>0.51(0.07)</td>
</tr>
<tr>
<td><strong>SWAW</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TN</td>
<td>0.63(0.02)*</td>
<td>-0.30(0.30)</td>
</tr>
<tr>
<td>TOC</td>
<td>0.09(0.77)</td>
<td>-0.04(0.90)</td>
</tr>
<tr>
<td>TOC/TN</td>
<td>-0.70(&lt;0.01)**</td>
<td>0.37(0.19)</td>
</tr>
</tbody>
</table>

Bold values mean significant under the statistic calculation.
* means P<0.05.
** means P<0.01.

5.4 DISCUSSION

With the addition of raw wastewater or autoclaved wastewater as exogenous nutrients, the top 15 major OTUs in beach sand microcosms or in seawater microcosms were found to share 80% of common OTUs in sand microcosms, and 73.3% of common OTUs in seawater microcosms, respectively. Many of these common OTUs showed
similar temporal fluctuation; with 75% showing significant correlation between the BSRW and BSAW microcosms, and 82% showing significant correlation between the SWRW and SWAW microcosms.

Majority of OTUs (>85%) in the BSAW microcosms and the BSRW microcosms were Proteobacteria, Bacteroidetes, Euryarchaeota, Planctomycetes, Acidobacteria and Actinobacteria (Figure 5.8A. and 5.8B), which are also commonly observed soil microbial communities (10). ANOSIM analysis showed that there was no significant difference between the BSRW and BSAW microbial communities (R=0.22<0.75). In spite of similar community composition, there was significant difference in Shannon-Weaver index (P<0.05) between the BSRW and BSAW microbial communities (Figure 5.5). We also used nonmetric multidimensional scaling (nMDS) with Bray-Curtis method to visualize the differences and similarities in the beach sand microcosms over time (Figure 6). From Day 1, the longest distance matrix between the original beach sand community (BS) to BSAW or BSRW was obtained, which clearly showed that the naked DNA in autoclaved wastewater did not influence the result of Illumina sequencing as illustrated by the distinct distance between the BSAW and BSRW microcosms on Day 1. The distance between the BSAW and BSRW microcosms decreased continuously over time, demonstrating that the communities between the BSAW and BSRW microcosms became more and more similar. Thus, the exogenous nutrients, instead of microbes, from wastewater were the major factor driving the evolvement of bacterial communities in the sand microcosms.

In seawater microcosms, Proteobacteria and Bacteroidetes (Figure 5.8C and 5.8D) were the major population. Similar major composition of seawater was also
documented by Halliday *et al.* (7). It is not surprising to find that the microbial community diversity in the SWAW and SWRW microcosms exhibited a sharp increasing trend because the additional nutrients from either untreated wastewater or autoclaved wastewater can affect the bacterial diversity and activity in seawater (11). The difference between the SWAW and SWRW microcosm was also significant based on the Shannon-Weaver index ($P < 0.05$) over time, while the diversity variation pattern was very similar. Comparing to the sharp increasing trend in the seawater microcosms, change in the beach sand microcosms was more stable, which was likely due to the presence of biofilm in the beach sand environment that prevent abrupt variation of bacterial diversity and activity. Figure 5.7 also showed the variation of distance between the SAWW and SWRW microcosms over time. Similar to the variation in the beach sand microcosms, the microbial community composition between the SWAW and SWRW microcosms became more and more similar toward the end of experimental period.

Many environmental factors, including pH, salinity, temperature and nutrient availability, can have significant impact on microbial community composition and diversity (10, 12, 14). In this microcosm study, pH, salinity and temperature were maintained constant, while nutrient availability (characterized by TOC, TN and TOC/TN ratio) were found to be the major force driving the change of microbial community composition and diversity. This is supported by the observation that TN showed a significant and positive correlation with Shannon-Weaver diversity index in some microcosms while the ratio of TOC/TN was significantly negatively correlated with the microbial community diversity. Meanwhile, TOC showed significant correlation with total bacterial biomass.
5.5 ACKNOWLEDGEMENT

This material is based upon work supported by the Kualoa Supplemental Environmental Project Fund from the Hawaii Department of Health to T.Y. (11-093).
5.6 REFERENCE


12. **Li, H., S. Z. Yang, B. Z. Mu, Z. F. Rong, and J. Zhang.** 2007. Molecular phylogenetic diversity of the microbial community associated with a high-
temperature petroleum reservoir at an offshore oilfield. FEMS microbiology ecology 60:74-84.


CHAPTER 6. CONCLUSIONS AND RECOMMENDATIONS

This dissertation conducted laboratory experiments to investigate the source and fate of fecal indicator bacteria in tropical soil, sand, and seawater environments. Chapters Two to Five presented experimental results concerning different aspects of this dissertation. Taken together, the following conclusions and recommendations can be made.

1. **E. coli may not a reliable FIB for tropical fresh water bodies due to its ability to survive in and inhabit tropical soils.**

   We showed that *E. coli* strains that are frequently detected in Hawaii soil have high resistance to soil desiccation stress. In laboratory microcosm experiments, these soil strains were shown to remain viable until the water content in quartz sand dropped to 9.3%, exhibiting considerable desiccation resistance. The *de novo* synthesis and accumulation of trehalose, which is a common mechanism used by many soil bacteria for desiccation resistance, was identified to be a major mechanism for the enhanced desiccation resistance by the soil *E. coli* strains, further supporting the notion that these *E. coli* strains inhabit tropical soils.

   **Recommendations:** Further studies on the ecology of soil *E. coli* and its implication for water quality monitoring and public health are needed.

2. **Beach sand not only provides recreational values to bathers, but can also contribute positively to beach water quality.**

   The contribution from beach sand goes beyond the traditional perception of contaminant adsorption and retention, and involves actively facilitating the
removal of fecal bacteria. This newly recognized function of beach sand should be of particular relevance to recreational water management during the early stages of fecal pollutions when large numbers of fecal bacteria are discharged into beach systems. Since other recent studies have also reported beach sand harboring considerable amount of FIBs persistently, which are more likely to occur towards the end of contamination events, the impact of beach sand to water quality is not only substantial but may also have contrasting effects under different circumstances. Therefore, it appears appropriate for beach managers to take a systems approach that recognize beach sand as a critical component of beach water quality management.

**Recommendations:** This study only provided a qualitative assessment of the contribution of subtidal sand to FIB decay in beach water. Mathematical modeling that accounts for all processes, including advection, diffusion, sedimentation, solar inactivation, is needed to provide a comprehensive understanding of the overall dynamics between beach sand and seawater.

3. **Beach sand needs to be considered separately from seawater in assessing their respective impacts to 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 backshore sand. 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 close association between microbial community structure and dynamics
highlighted the importance of understanding the fate of individual bacterial populations in the context of microbial community dynamics.

**Recommendation:** Further study on the fundamental mechanisms by which indigenous microbiota affect the survival of individual bacterial populations is needed to further elucidate the proposed bacterial inter-species interactions.
Figure A. 1 Concentrations of viable *E. faecalis* cells in the beach sand (A) and seawater (B) compartments of the beach microcosms for Kailua, Kualoa, and Wailale beaches, Honolulu, HI. Error bar indicates the standard deviation of the mean of triplicate microcosms.
Figure A. 2 Impact of different levels of indigenous microbiota on the reduction of viable *E. faecalis* cells the beach sand (A) and seawater (B) compartments of Waialae beach microcosms. Error bar indicates the standard deviation of the mean of triplicate microcosms.
Figure A. 3 Viable *E. faecalis* cell concentration in the beach sand (A) and seawater (B) compartments in regular Waialae beach microcosms that contain both beach sand and seawater or seawater-only. Error bar indicates the standard deviation of the mean of triplicate microcosms.
Figure A. 4 The impact of sand-to-water ratio on the concentration of viable *E. faecalis* cells in the beach sand (A) and seawater compartments in Waialae beach microcosms. Error bar indicates the standard deviation of the mean of triplicate microcosms.
Figure A. 5 The impact of sand size fractions on the concentration of viable *E. faecalis* cells in the beach sand (A) and seawater (B) compartments of Waialae beach microcosms. Error bar indicates the standard deviation of the mean of triplicate microcosms.
## APPENDIX B. SUPPLEMENTAL INFORMATION FOR CHAPTER 4

Table B. 1 Relative abundance of major wastewater OTUs in the raw wastewater, seawater, and beach sand samples.

<table>
<thead>
<tr>
<th>Major wastewater OTUs</th>
<th>Wastewater</th>
<th>Seawater</th>
<th>Beach Sand</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Arcobacter</em> (genus)</td>
<td>20.38</td>
<td>ND</td>
<td>0.11</td>
</tr>
<tr>
<td><em>Flavobacteriaceae</em> (family)</td>
<td>14.94</td>
<td>0.37</td>
<td>1.45</td>
</tr>
<tr>
<td><em>Bacteroides</em> (genus)</td>
<td>9.4</td>
<td>0.01</td>
<td>0.1</td>
</tr>
<tr>
<td><em>Acinetobacter</em> (genus)</td>
<td>5.01</td>
<td>ND</td>
<td>0.07</td>
</tr>
<tr>
<td><em>Parabacteroides</em> (genus)</td>
<td>4.76</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>Streptococcus</em> (genus)</td>
<td>4.63</td>
<td>ND</td>
<td>0.91</td>
</tr>
<tr>
<td><em>Marinilabiaceae</em> (family)</td>
<td>2.74</td>
<td>ND</td>
<td>0.04</td>
</tr>
<tr>
<td><em>Bacteroidia</em> (class)</td>
<td>2.52</td>
<td>0.04</td>
<td>ND</td>
</tr>
<tr>
<td><em>Pseudomonadaceae</em> (family)</td>
<td>1.38</td>
<td>0.01</td>
<td>0.34</td>
</tr>
</tbody>
</table>

Table B. 2 Change rates (unit: day$^{-1}$) of the relative abundance of major wastewater OTUs in the beach sand and seawater microcosms during the first (0-6 days) and second (6-23 days) decay phases.

<table>
<thead>
<tr>
<th>Major wastewater OTUs</th>
<th>Beach sand</th>
<th>Seawater</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-6 day</td>
<td>6-23 day</td>
</tr>
<tr>
<td><em>Acinetobacter</em></td>
<td>0.55</td>
<td>0.34</td>
</tr>
<tr>
<td><em>Arcobacter</em> (genus)</td>
<td>0.52</td>
<td>0.16</td>
</tr>
<tr>
<td><em>Bacteroides</em> (genus)</td>
<td>0.34</td>
<td>0.27</td>
</tr>
<tr>
<td><em>Parabacteroides</em> (genus)</td>
<td>0.61</td>
<td>0.24</td>
</tr>
<tr>
<td><em>Streptococcus</em> (genus)</td>
<td>0.09</td>
<td>0.43</td>
</tr>
<tr>
<td><em>Flavobacteriaceae</em> (family)</td>
<td>0.28</td>
<td>0.10</td>
</tr>
<tr>
<td><em>Bacteroidia</em> (class)</td>
<td>0.21</td>
<td>0.31</td>
</tr>
<tr>
<td><em>Pseudomonadaceae</em> (family)</td>
<td>0.59</td>
<td>-0.14</td>
</tr>
<tr>
<td><em>Marinilabiaceae</em> (family)</td>
<td>0.33</td>
<td>0.54</td>
</tr>
</tbody>
</table>
Table B. 3 Microbial diversity indices in beach sand microcosms and seawater microcosms over time

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Observed Species</th>
<th>Shannon Index</th>
<th>Chao1</th>
<th>Phylogenetic Diversity</th>
<th>Sequence Reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS0-A</td>
<td>2376</td>
<td>10</td>
<td>4930</td>
<td>185</td>
<td>7585</td>
</tr>
<tr>
<td>BS0-C</td>
<td>2289</td>
<td>10</td>
<td>4081</td>
<td>193</td>
<td>8221</td>
</tr>
<tr>
<td>BS3-A</td>
<td>2445</td>
<td>10</td>
<td>4881</td>
<td>194</td>
<td>9067</td>
</tr>
<tr>
<td>BS3-B</td>
<td>2361</td>
<td>10</td>
<td>4172</td>
<td>191</td>
<td>8564</td>
</tr>
<tr>
<td>BS3-C</td>
<td>1318</td>
<td>9</td>
<td>1644</td>
<td>130</td>
<td>9844</td>
</tr>
<tr>
<td>BS6-A</td>
<td>2189</td>
<td>10</td>
<td>3244</td>
<td>183</td>
<td>8210</td>
</tr>
<tr>
<td>BS6-B</td>
<td>2423</td>
<td>10</td>
<td>4706</td>
<td>196</td>
<td>7755</td>
</tr>
<tr>
<td>BS6-C</td>
<td>2203</td>
<td>10</td>
<td>4074</td>
<td>186</td>
<td>7767</td>
</tr>
<tr>
<td>BS16-A</td>
<td>2022</td>
<td>10</td>
<td>3087</td>
<td>171</td>
<td>8848</td>
</tr>
<tr>
<td>BS16-B</td>
<td>2528</td>
<td>10</td>
<td>4456</td>
<td>204</td>
<td>8258</td>
</tr>
<tr>
<td>BS16-C</td>
<td>2290</td>
<td>10</td>
<td>4297</td>
<td>190</td>
<td>8145</td>
</tr>
<tr>
<td>BS23-A</td>
<td>1598</td>
<td>9</td>
<td>2126</td>
<td>135</td>
<td>8254</td>
</tr>
<tr>
<td>BS23-B</td>
<td>2378</td>
<td>10</td>
<td>4517</td>
<td>186</td>
<td>7748</td>
</tr>
<tr>
<td>BS23-C</td>
<td>2387</td>
<td>10</td>
<td>4779</td>
<td>186</td>
<td>7666</td>
</tr>
<tr>
<td>SW0-A</td>
<td>1109</td>
<td>7</td>
<td>2146</td>
<td>78</td>
<td>8063</td>
</tr>
<tr>
<td>SW0-C</td>
<td>875</td>
<td>6</td>
<td>1537</td>
<td>72</td>
<td>8369</td>
</tr>
<tr>
<td>SW3-A</td>
<td>1399</td>
<td>8</td>
<td>2421</td>
<td>115</td>
<td>8737</td>
</tr>
<tr>
<td>SW3-B</td>
<td>1254</td>
<td>8</td>
<td>1988</td>
<td>112</td>
<td>8135</td>
</tr>
<tr>
<td>SW3-C</td>
<td>1008</td>
<td>8</td>
<td>1308</td>
<td>93</td>
<td>8431</td>
</tr>
<tr>
<td>SW6-A</td>
<td>1438</td>
<td>9</td>
<td>2660</td>
<td>121</td>
<td>7730</td>
</tr>
<tr>
<td>SW6-B</td>
<td>1389</td>
<td>9</td>
<td>2288</td>
<td>117</td>
<td>7611</td>
</tr>
<tr>
<td>SW6-C</td>
<td>1052</td>
<td>8</td>
<td>1350</td>
<td>97</td>
<td>8245</td>
</tr>
<tr>
<td>SW16-A</td>
<td>1440</td>
<td>9</td>
<td>1925</td>
<td>123</td>
<td>8878</td>
</tr>
<tr>
<td>SW16-B</td>
<td>1295</td>
<td>9</td>
<td>1653</td>
<td>115</td>
<td>9241</td>
</tr>
<tr>
<td>SW16-C</td>
<td>1520</td>
<td>9</td>
<td>2240</td>
<td>122</td>
<td>9408</td>
</tr>
<tr>
<td>SW23-A</td>
<td>1500</td>
<td>9</td>
<td>2113</td>
<td>116</td>
<td>8170</td>
</tr>
<tr>
<td>SW23-B</td>
<td>1521</td>
<td>9</td>
<td>2024</td>
<td>121</td>
<td>8498</td>
</tr>
<tr>
<td>SW23-C</td>
<td>979</td>
<td>8</td>
<td>1290</td>
<td>87</td>
<td>8529</td>
</tr>
</tbody>
</table>

*BS: beach sand microcosm, SW: seawater microcosm, The numbers (0, 3, 6, 16, 23) indicate sampling days, and letters after hyphen (A, B, C) indicate replicate microcosms.
Table B. 4 Pearson’s product moment and P values (in parentheses) between alpha diversity indices and bacterial decay rates in beach sand and seawater microcosms

<table>
<thead>
<tr>
<th>Alpha Diversity</th>
<th>Beach sand</th>
<th></th>
<th></th>
<th>Seawater</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. coli</td>
<td>ENT&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Salmonell&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>E. coli</td>
<td>ENT&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Shannon index</td>
<td>-0.46 (0.10)</td>
<td>-0.25 (0.40)</td>
<td>-0.37 (0.19)</td>
<td>-0.62 (0.02)</td>
<td>-0.57 (0.04)</td>
<td>-0.74 (0.00)</td>
</tr>
<tr>
<td>Phylogenetic diversity</td>
<td>0.07 (0.82)</td>
<td>-0.11 (0.71)</td>
<td>-0.11 (0.72)</td>
<td>-0.32 (0.27)</td>
<td>-0.48 (0.08)</td>
<td>-0.62 (0.02)</td>
</tr>
<tr>
<td>Observed species</td>
<td>0.03 (0.92)</td>
<td>-0.08 (0.79)</td>
<td>-0.07 (0.80)</td>
<td>-0.40 (0.16)</td>
<td>-0.46 (0.10)</td>
<td>-0.52 (0.05)</td>
</tr>
<tr>
<td>Chao1</td>
<td>0.10 (0.75)</td>
<td>-0.02 (0.94)</td>
<td>0.03 (0.91)</td>
<td>-0.31 (0.29)</td>
<td>-0.14 (0.64)</td>
<td>0.02 (0.96)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significant correlations (P<0.05) are bolded, marginally significant correlations (P<0.10) are underlined.

<sup>b</sup> Abbreviation: ENT: enterococci, CP: C. perfringens.
Figure C. 1 The concentration of total nitrogen and total organic carbon in the beach sand microcosms (A) and seawater microcosms (B) overtime. Error bar indicates the standard deviation of the mean of triplicate microcosms.