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Traditional and Host-Associated Fecal Indicator Bacterial Patterns in Southern California Watersheds: Field Source Identification Studies and Laboratory Microcosms Investigating Presence and Persistence in Water and Sediments

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Traditional and Host-Associated Fecal Indicator Bacterial Patterns in Southern California Watersheds: Field Source Identification Studies and Laboratory Microcosms Investigating Presence and Persistence in Water and Sediments

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy in Civil Engineering

by

Kathryn Beth Mika

2012

ABSTRACT OF THE DISSERTATION

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Professor Jennifer A. Jay, Chair

Overall, recreational beach water quality remains an issue of concern in Southern California and across the globe. Many factors come into play when determining water quality, including physical issues such as the myriad sources that contribute pollution to the site and financial and political issues that control the way water quality is monitored and determined. Current national regulations require the monitoring of fecal indicator bacteria in order to determine recreational water quality. However, it is also important to identify biological and geographical sources of pollution to consistently impaired locations. A commonly applied approach to meet the goals of source identification is to sample sites that have been high in FIB for further study. A tiered approach such as this, however, assumes a correlation between FIB

and the sources of interest in the watershed. The research described in this dissertation tests this assumption in two Southern California watersheds, Santa Monica Canyon and Ventura Harbor. In both cases, a tiered approach to sampling using FIB as a first tier to guide sampling would have failed to identify sources of human fecal pollution (as identified by the presence of the human-associated *Bacteroides* marker HF183).

Every watershed is a distinct environment that has different potential sources of bacteria and many factors contributing to the persistence of the bacteria. Rather than attempting to apply an indicator that has worked as a first tier in other watersheds, it would be better to have as a first tier an in-depth study of the watershed using historical data or local experts to provide information on the most likely sources of pollution in the watershed. Using this information it would be possible to design a study using FIB and one or more source-associated parameters to identify specific sources of pollution in the watershed. In addition, sampling FIB and other parameters such as HF183 allow the application of other microbial source tracking tools including indicator ratios and detection frequencies. Source identification studies do not necessarily have to be long-term to identify consistent sources of pollution. For example, within the first four months of sampling at Ventura, the increased frequency of detection of HF183 at the Marina Dock sample location was apparent, and a dry weather influx of HF183 was seen in the Keys channels.

In addition to the many sources of FIB to the environment such as storm drains, leaking sewers, and wildlife, there are important environmental reservoirs such as sand and seaweed that can foster FIB growth and persistence in the environment. As such, it is important to understand the effect of different factors on the ability of bacteria to survive and persist in these reservoirs.

Microcosm experiments conducted during the course of this dissertation research found that in dry beach sand (0.1% moisture), the addition of moisture was detrimental to the survival of the indicators studied (General *Bacteroidales*, *E. coli*, and enterococci). While increased moisture was not always detrimental to bacterial survival, these results point to the ability of bacteria to persist for long periods of time in beach environments under in-situ conditions (including dry sand). These findings point to the importance of understanding the behavior of indicator bacteria populations that have evolved to survive in environmental conditions so that their potential impact on overlying or adjacent water quality can be better understood.

In summation, results from this research point to the importance of selecting indicators and sample locations that are most relevant to watershed concerns rather than using a first tier such as FIB to preferentially select sites for further analysis. Measuring a marker for human fecal pollution in both watershed studies provided useful information for potential human inputs that would have been missed if sites were chosen based on high FIB levels. In addition it is very important to understand the contribution of different reservoirs, such as sand, in the study area to the observed microbial pollution. Overall, these results point to the need for further examination of the ability of bacteria to survive under various environmental conditions in both water and sand, using both environmental microbial populations and populations from likely sources such as human sewage.

The dissertation of Kathryn Beth Mika is approved.

Richard F. Ambrose

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2012

Dedication Page

To my mom, Cindy, and my dad, Patrick, for supporting me in everything I ever wanted to do and teaching me about the things in life that really matter.

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I. Acknowledgements

I would like to acknowledge my advisor, Professor Jenny Jay, for giving me the opportunity to work in an amazing field, and for her constant support both professionally and personally.

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I would also like to say thank you to my dear friends and family for their constant encouragement, support, and willingness to join me for anything from a quick cup of coffee to a quick weekend to get away from it all.

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CHAPTER 1: Introduction

Currently, the fecal indicator bacteria (FIB) that are used to determine water quality are present in all warm-blooded animals, and thus not specific to any particular animal. In the past 10 years, many molecular methods have been developed in order to detect the presence of bacteria that are specific to an individual source, such as human fecal matter (i.e. sewage or septage), as this can be a very helpful way to identify the location of sources of pollution to a beach that can then be remediated through installation of a treatment plant or repairing sewer lines. However, as the water quality standards currently require the measurement of FIB, an understanding of the relationship between these human-associated bacteria and FIB is very important to make statements about the utility of both of these indicators to improve and monitor water quality. In order to explore this arena, two watersheds (one urban, one urban and agricultural) were studied in order to better understand the relationships between these two indicators, and whether either or both actually helped locate a main source of pollution to the watershed.

In addition to a lack of source specificity, FIB have been shown to be capable of growth and persistence under environmental conditions, particularly in the presence of sediments or aquatic vegetation. Sand provides an ideal growth environment for bacteria – very little sunlight is able to penetrate beneath the surface to kill off bacteria as it can when they are in water, sand provides many surfaces on which bacteria can adhere and form more resilient communities, and sand also provides shelter from predation by larger microorganisms. Therefore, it is important to understand the patterns of microbial survival in sediments in order to understand an additional source of FIB to the environment that may not present a health impact. FIB were originally

selected as a good proxy for pathogens and viruses because they were shown to correlate well with human health effects and illness rates at the beaches studied (all of which had a point source of human fecal pollution such as a sewage discharge). However, more recent studies performed at many beaches that do not have a source of human fecal pollution, but rather are impacted by a large variety of urban sources including urban runoff from washing cars and watering lawns, and environmental regrowth of bacteria in the environment, have shown that FIB do not necessarily correlate well with human health risk after all.

Many studies have been done to better understand the persistence of FIB in sediments using traditional culture-based methods of detection that are currently used to determine recreational water quality. However, the EPA is currently working on new recreational water guidelines which will likely include some new, rapid methods of determining water quality that have been emerging in the last decade, one of which is quantitative Polymerase Chain Reaction (qPCR). As such, it has also become important to understand the persistence of these genetic markers for FIB and other indicators in relation to the persistence of culture-based methods, as qPCR can detect both viable and non-viable cells in the environment.

Chapter 2 Summary: Santa Monica Canyon Channel:

Samples were collected over the course of 1 year, from 2008 to 2009, from 2 creeks in the Santa Monica Canyon watershed that come together and outlet onto Will Rogers Beach in Malibu. All samples were analyzed for a genetic marker from a human-associated species within *Bacteroidales* (HF183), FIB, and a subset of samples was analyzed for physical and chemical parameters such as nitrate and ammonia. Both the relationships between these parameters and

the overall occurrence of these parameters in the watershed were studied in order to determine whether any one of these parameters or some combination could be used to identify likely sources of pollution. However, in this location, the studied parameters were not well correlated, and ubiquitous throughout the watershed. While FIB still provide merit as a first tier to finding problem beaches, this study points to the need for initial site characterization studies that can be used to determine which set of parameters will provide the best tools to locate individual sources of pollution to the watershed.

Chapter 3 Summary: Ventura:

Samples were collected in the Ventura Harbor, Ventura Keys (residential development along canals connected to the harbor), and the Arundell Barranca (large channel draining mostly agricultural land that outputs into the harbor). The Arundell Barranca is suspected to be the main contributor to FIB pollution in the Harbor, and we set out to examine whether this water body was also the source of human pollution to this watershed. Two multi-day rain sampling events were conducted throughout the study to better understand the dynamics of these bacteria during wet weather; additionally, samples were collected once a month to determine whether any one sample location was a hot-spot of HF183. Interestingly, FIB and HF183 were observed to have differing patterns during the rain events. While FIB levels increased at all sample locations and mirrored the rainfall patterns, HF183 levels only increased at a few locations, which varied by sampling time point, throughout the rain event. This difference points to different sources of these two bacterial types as they do not behave the same way in the environment. Although the

Arundell Barranca consistently had high levels of FIB, HF183 was detected more frequently at other locations.

Chapter 4 Summary: Bacterial patterns and persistence under Santa Monica Pier

The water adjacent to the Santa Monica Pier has consistently high levels of FIB, as does the sand under the pier. A diverted storm drain under the pier and occasionally ponded water appeared to be the main sources of this microbial contamination based on historical data collected by the Jay lab from 2006-2009, in part through a service learning outreach project with two Civil and Environmental classes. Accordingly, spatial patterns under the pier were analyzed over the course of a week in spring, summer, and fall of 2010 to determine whether this pattern changed across seasons. As a consistently higher concentration was observed under the pier across all seasons, in sand that tended to be moister, microcosms were set up using sand containing native populations of bacteria from under the pier that were then monitored over time for the effects of different sustained moisture contents. *E. coli* and enterococci were measured by membrane filtration, a culture-based method. Enterococci was also measured by qPCR, a non-culture based method, as was General *Bacteroidales.* Generally, all species tended to survive the best in sands with no additional moisture. Finally, having established the ability of these indicator bacteria to persist under conditions similar to those under the pier, a final source-specific sampling snapshot was conducted in order to further identify potential sources of these indicator bacteria and genetic markers. Sand samples were analyzed using two methods for human – associated markers and one method for gull/pelican specific markers. Although both human – associated

markers used were below detection limits for all samples, the gull / pelican associated markers came up at 8/12 sites.

Chapter 5 Summary: Service Learning Near the Santa Monica Pier

A service learning project was incorporated into the curriculum of a Civil Engineering class as a complementary aspect to better characterize the patterns of FIB in the Santa Monica Pier environs. The convenient location of the pier to the middle schools that we collaborated with, as well as many potential sources of FIB such as flowing storm drains and the shelter provided by the pier, made it the perfect area to facilitate these projects. Undergraduates were trained in lab techniques, then met up with middle school students to design research hypotheses based on what was known about the pier and trained them in the lab techniques. During a sampling field day, all the students chose and processed sand samples to answer their research questions, then analyzed results and created a poster to be presented at UCLA. Overall, the service learning project enhanced the learning of the material and provided the undergraduates with an opportunity to practice their mentoring skills while affording middle school students to apply scientific reasoning and wet laboratory skills in the field. In Chapter 6, overall results and conclusions will be presented.

CHAPTER 2. Widespread and high levels of fecal indicator bacteria do not correspond with incidence of Bacteroides HF183 marker in the Santa Monica Canyon Channel

*Abstract***:** This study is an investigation of fecal pollution patterns in wet and dry weather in two creeks in Santa Monica Canyon, a consistently impaired watershed located in Southern California. The study determines whether fecal indicator bacteria (FIB) levels could be used in this watershed as a first tier to locate areas that were also high in the human associated *Bacteroides* HF183 markers, and, also, if there were consistently present sources of either FIB or HF183. No single sample location proved to be a consistent, identifiable source of either HF183 or FIB to this watershed; FIB levels were generally high throughout all sample sites, exceeding marine water quality standards for *Escherichia coli* and / or enterococci in more than 70% of samples taken. HF183 was detectable at low levels in roughly 58% of all samples (45/78) from the watershed. Interesting patterns emerged looking at the frequency of HF183 detection. Generally, the lower parts of the creeks and confluence had higher frequencies of HF183 detection overall. Although rainfall coincided with a significant increase both in FIB concentrations and water quality exceedance rates, this same pattern was not seen in HF183. In fact, the four samples with the highest levels of HF183 occurred during dry weather. Although FIB have been used in other studies as a first tier, no significant relationship was observed in this study between HF183 and FIB or the other water quality indicators measured. The lack of relationship observed between HF183 and FIB in this study points to the need for either an initial survey of the watershed using a wide array of indicators to determine an appropriate first tier or a more routine use of measuring one or more human associated markers in a watershed.

1. *Introduction*

Coastal urban watersheds have multiple sources of pollution, including leaking sewage pipes and storm drains (Sercu et al., 2009), inadequate onsite waste systems (de Sieyes et al., 2008), behavioral sources such as nuisance runoff from plant-watering and car-washing, and environmental sources such as bacterial regrowth in the environment (Yamahara et al., 2009; Desmarais et al., 2002). While fecal indicator bacteria (FIB) are routinely measured to determine recreational water quality, studies have pointed to their shortcomings as indicators, revealing that FIB can persist and regrow in the environment, particularly in the presence of beach sand or sediment (Yamahara et al., 2009; Ishii et al., 2007; Mika et al., 2009; Lee et al., 2006) or aquatic vegetation (Ksoll et al., 2007; Whitman et al., 2003; Imamura et al., 2011). Furthermore, FIB can originate from non-human sources such as wildlife and waterfowl (Ram et al., 2007) and impact the watershed through various routes.

Tracking high pollution levels to their sources is critical to ensuring that urban beaches stay healthy. Impaired beaches can lead to economic repercussions through both public health costs as the result of beachgoer illness and lost tourism when the beaches are closed due to contamination (Pendleton, 2008). Assembly Bill 538 was passed in California in 2001 and stipulated that storm drains which consistently cause exceedances of water quality standards must be further studied to determine the biological and geographical origins of the contamination (AB538, 2001). One of the ways in which source-identification can be achieved is through the use of molecular methods such as polymerase chain reaction (PCR) (Santo Domingo et al., 2007; Bernhard and Field 2000a) and quantitative polymerase chain

reaction (qPCR), which can be used to detect and measure low levels of species-specific markers from novel indicator organisms in the environment (Layton et al., 2006; Seurinck et al., 2006).

Accordingly, many PCR / qPCR assays have been explored and developed for their specificity and sensitivity to human fecal pollution including *Bacteroides-Prevotella* (Okabe and Shimazu, 2007; Bernhard and Field 2000b), *Bacteroidales* (Kildare et al., 2007), *Bacteroides* spp. (Layton et al., 2006; Converse et al., 2009) including *Bacteroides thetaiotaomicron* (Carson et al., 2005; Yampara-Iquise et al., 2008), enterovirus (Jiang et al., 2007), adenovirus (Jiang et al., 2007), and human polyomavirus (McQuaig et al., 2006). Among the *Bacteroides-Prevotella* assays, the HF183 marker (HF183) has been shown to be specific to human fecal inputs on a global scale (Sercu et al., 2009; Fremaux et al., 2009; Gawler et al., 2007; Dorai-Raj et al., 2009; Ahmed et al., 2009; Ahmed et al., 2010; Seurinck et al., 2005; Kirs et al., 2011). Based upon the previous research showing the robustness of this marker in many environments, HF183 was chosen as the indicator for the presence of human fecal pollution in this study.

A fecal source identification strategy, known as a tiered approach, leverages the simplicity of FIB culture assays (or other water quality measurement parameters) and emerging methods to attempt to assess and identify sources of water pollution. A tiered approach can use many indicators as a first tier to identify hotspots of contamination for further exploration, including rain, historical watershed data, or FIB measurements (Noble et al., 2006; Coulliette and Noble, 2008; Reischer et al., 2008; Boehm et al., 2003). The notion of using FIB or other general water quality parameters as a screen for sites likely

contaminated with human-fecal pollution is appealing due to the specialized nature and higher cost of host-specific assays. However, results from studies using a tiered approach have been mixed, depending largely on the watershed and the parameters being tested; this may be due to the lack of correlation that has been sometimes observed between FIB and human-associated bacteria (Boehm et al., 2003; Bower et al., 2005; Flood et al., 2011). However, other studies have shown promise using FIB to identify locations of interest for human fecal pollution, either because high HF183 was found only at sites with high FIB (Sercu et al., 2009) or because human-associated *Bacteroidetes* and fecal indicators were shown to correlate strongly (Reischer et al., 2008).

The presented work investigates the dynamics of HF183 and FIB in two creeks in Santa Monica Canyon with the overall goal of determining whether FIB are an appropriate first tier to identify hotspots of human contamination. Specific aims of the study were to 1) determine the distribution and frequency of FIB or human pollution in the study site; 2) quantify the effect of rainfall; 3) determine the relationship between FIB and HF183; and 4) examine relationships between physicochemical parameters, FIB, and HF183. Lastly, these findings will be examined in the context of a tiered approach strategy to water monitoring.

2. *Methodology*

2.1. *Site Background.*

Santa Monica Canyon (SMC) begins at the entrance to Mandeville Canyon near the crest of the Santa Monica Mountains and extends eight miles to the southwest to its outlet at the southern end of Will Rogers State Beach (Figure 2-1A). Two to three miles east of the shoreline, lower Rustic and lower Sullivan Canyons merge into the Santa Monica Canyon.

Water drains from Mandeville Canyon, lower Rustic Canyon, and lower Sullivan Canyon to form a broad stream that runs along the base of Santa Monica Canyon. Most of the Rustic / Sullivan Canyon watershed exists in its natural state as it flows through the mountains, draining low-density residential areas in the western reaches. At the Rustic Creek intersection with East and West Rustic Roads, the channel becomes concrete-lined and will be referred to as West Channel. Entrada Channel drains a highly urbanized environment and is lined with concrete along the whole length.

West Channel and Entrada Channel merge east of the Pacific Coast Highway between Entrada Drive and West Channel Road and discharge through an open concrete channel at the southern end of Will Rogers State Beach. The combined watershed drains 10,147 acres, and dry weather flow is roughly 3.5 million gallons / day (Wilson, 2001). Santa Monica Canyon has many sources of both human and non-human pollution to the watershed, including a golf course, horse ranch, waterfowl, pet waste, and storm drain discharges. During high-use season from April to October, dry weather flow is diverted to Hyperion sewage treatment plant to improve water quality at Will Rogers State Beach (Wilson, 2001). Storm drains are present along the length of Entrada Channel studied and along the concrete-lined portion of West Channel. Storm drain flow was observed to be highly variable on dry sampling days both in terms of which storm drains were flowing and the quantity of water being discharged into the channels.

2.2. *Sample collection and analysis.*

*Collection***.** Water samples (n = 130) were collected on 20 days from January 2008 to February 2009 (17/20 dry days, 3/20 wet days). There were a total of eleven sampling sites

through the channel system (Figure 2-1B). A total of eight storm drain samples were collected throughout the study. Six storm drains were sampled on one day in a storm drain survey and two were collected alongside one of the monthly sampling events. One to two liters of water was collected at each site and transported on ice for laboratory analysis.

*Physicochemical parameters***.** When possible, samples were measured for total dissolved solids (TDS), water temperature, electrical conductivity and pH (HANNA instruments #H198130, Smithfield, RI). Selected samples were also analyzed for total suspended solids (TSS) (Environmental Sciences Section Method 340.2) and dissolved oxygen (DO) was measured with a hand-held probe (YSI model 55/12 FT, YSI Inc, Yellow Springs, OH).

*Standard FIB***.** All water samples were analyzed for total coliforms (TC), *E. coli* (EC), and enterococci (ENT) using defined substrate technology (IDEXX Laboratories, Inc, Westbrook, ME). Colilert®-18 (IDEXX) was used to quantify TC and EC, and Enterolert® (IDEXX) was used to quantify ENT. A subset (n = 14) of samples was also analyzed for FIB using an in-field pilot method, covalently-linked immunomagnetic separation/ATP quantification (Lee et al., 2010). In California, single-sample recreational water quality standards as established by Assembly Bill 411 (AB411) use TC (10,000 MPN / 100 mL), FC (400 / 100 mL), and ENT (104 / 100 mL) to determine water quality. EC, because they are a subset of FC, were used as a proxy for the FC in our study as the level of FC would be greater than that of EC. The state of California also uses the ratio of TC : FC as an indicator of recreational water quality (http://www.waterboards.ca.gov/water_issues/programs/beaches/beach_water_quality/beaches_program.shtml).

*HF183***.** Samples collected between August 2008 and February 2009 were further analyzed for HF183 marker (n = 86), processing to be discussed in further detail below.

2.3. *DNA Extraction and Quantification.*

Water samples were vacuum-filtered through 47-mm-diameter, 0.45-□m-pore-size Fisherbrand nitrocellulose filters (Fisher Scientific, Pittsburgh, PA) until the total volume was filtered or sample stopped passing through the filter. DNA was extracted from the filters using the Mobio UltraClean Fecal DNA Kit (MO BIO Laboratories, Inc, Carlsbad, CA), and extracts were stored at -80°C until being processed for HF183. DNA was extracted according to manufacturer's protocol with the addition of 90 seconds of bead-beating in a BioSpec Mini-Beadbeater-8 (BioSpec Products, Bartlesville, OK). Total DNA concentration in the extract was analyzed using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Molecular Probes, Inc, Eugene, OR) with a Stratagene Mx3000P system (Agilent Technologies, Santa Clara, CA). One µL aliquots of sample extract were run concurrently with a standard curve of DNA standard provided in the Quant-iT™ kit ranging from 0.1 ng to 20 ng of DNA μL^{-1} in a total volume of 30 µL.

2.4. *qPCR Analyses.*

Samples were analyzed for HF183 using qPCR. DNA was amplified with primers and master mix as described in Seurinck et al. (2005). Briefly, amplification occurred in 25 µL reaction volumes containing 2 ng of sample DNA, 1X SYBR GreenER PCR Master Mix (Life Technologies, Grand Island, NY), 0.25 mM of each primer, and DNase and RNAse free water (Fisher BioReagents, Pittsburgh, PA). The reactions were performed in 96-well reaction plates using a Stratagene Mx3000P system (Agilent Technologies, Santa Clara, CA).

The qPCR temperature program was set as follows: 2 minutes at 50 °C, 10 min at 95 °C, then 45 cycles of 30 s at 95 °C, 1 min at 53 °C, and 1 min at 60 °C. PCR products were verified through melting curve analysis; a melting temperature between 77 °C and 78 °C indicated positive, correct amplification of HF183. Sample values were calculated using MxPro™ qPCR software. A six-point standard curve using plasmids with HF183 was run in duplicate alongside the environmental samples on every plate. The presence of environmental interference was assessed by running samples spiked with a 2×10^5 copies / μL dilution of a HF183 plasmid alongside the sample duplicates. Plasmids for HF183 were kindly provided by Professor Fuhrman (University of Southern California, CA). If spiked samples values were less than 8×10^4 copies / μL, then samples were diluted by half and reanalyzed. This process was repeated up to three times as needed. If interference was still present after third dilution, samples were designated as interfered and omitted from further analyses. Out of 86 samples, six were eliminated from HF183 analysis due to interference.

2.5. *Statistics and Data Analyses*

Statistical analyses were run using SPSS statistical software (SPSS Inc., Chicago, IL). As FIB results were similar over the entire time period January 2008 – February 2009, only the subset of FIB samples from August 2008 – February 2009 that was also measured for HF183 were used in statistical analyses. Concentrations of bacterial species analyzed and physicochemical parameters measured were log-transformed prior to data analysis. Log-transformed data were not found to be normal using Shapiro-Wilk's test; accordingly, all data analyses were performed using non-parametric tests. Spearman's rank correlation tests were used to determine the relationships between all parameters. Kruskal-Wallis and Mann-

Whitney U tests were used to discern differences between channels and weather types for the parameters analyzed.

The limit of detection (LOD) for the qPCR assay described above was defined as 1 cp / µL; the limit of quantification was defined as 10 cps / µL. Samples in the range of 1 - 10 cps / µL were classified as 'detectable but not quantifiable (DNQ).' All samples that were positive for the HF183 were validated using a melt curve analysis. For the purposes of data analysis, non-detects were assigned a value of 0.1 cps / uL and DNQ's were assigned a value of 1 cps. Marine water standards were used to determine exceedance rates as there is no contact recreation in the regions of SMC sampled and SMC outflow impacts marine water quality at its outlet. Data were further explored for patterns and relationships using the frequency of water quality standard exceedance for FIB and the frequency of detection for HF183.

3. *Results*

3.1. *FIB and physicochemical parameters in SMC.*

Widespread FIB contamination was observed in both channels throughout the sampling period. Marine water quality standards were exceeded in 94% of samples for ENT (maximum values > 62,700 MPN 100 mL^{-1}) and 81% of samples for EC (maximum values up >24,100 MPN 100 mL^{-1}). Entrada Channel exceeded water quality standards for TC and EC more consistently along the full length sampled than did West Channel. Exceedance rates for TC and EC ranged between 50%-80% (n = 4 to 10) at all locations along Entrada as compared to 30%-80% (n = 4 to 10) along West Channel (See Figures 2-2A, 2-2B). ENT exceedance rates were more variable across both channels; exceedance rates ranged from

40%-71% (n = 4 to 10) in West Channel and from 25%-78% in Entrada (See Figure 2-2C). The exceedance rates for TC, EC, and ENT at the confluence are 78%, 89%, and 60%, respectively.

Patterns in concentrations of FIB are similar to those seen in the exceedance rates. ENT concentrations were not found to be significantly different in Entrada Channel compared to West Channel, the confluence, or the subset of storm drains sampled (Kruskal-Wallis, $X^2(3)=3.998$, $p = 0.262$). However, EC was present at significantly different concentrations between the locations (Kruskal-Wallis, $X^2(3)=12.733$, $p = 0.005$). EC was highest at the confluence (5,600 MPN / 100 mL), followed by Entrada Channel (3,500 MPN / 100 mL). West Channel and samples taken from various storm drains throughout the two channels had the lowest levels of EC overall (1,300 MPN / 100 mL).

Patterns in concentrations in the different areas sampled were also explored for all physicochemical parameters measured. Significant differences were only observed in nitrate (Kruskal-Wallis, $X^2(2)=12.862$, $p = 0.002$) and ammonia (Kruskal-Wallis, $X^2(2)=13.191$, $p = 0.001$), which were found to be significantly different among Entrada, West, and their confluence. Entrada Channel was found to be higher than West Channel in both nitrate (Mann-Whitney, $p = 0.001$) and ammonia (Mann-Whitney, $p < 0.001$).

Rainfall also had a significant effect on FIB concentrations. ENT and EC were found to be significantly higher during wet weather sampling events as compared to dry weather sampling events (Mann-Whitney, $p = 0.000$ for both FIB). FIB concentrations ranged from three to ten times higher during wet weather than dry weather. Additionally, exceedance rates were higher during wet weather than dry; wet-weather samples exceeded 100% of the

time (12/12) for both ENT and EC. During dry weather, EC exceeded 73% of the time (47/64) and ENT exceeded 83% of the time (39/47).

3.2. *Levels of and Patterns in HF183 in SMC.*

HF183 was detected in 58% of the water samples collected during this study (45 / 78). HF183 was detected most frequently in stormwater samples (75%, 6/8), followed by West Channel (65%, 17/26), then at the confluence (56%, 5/9), and finally, in Entrada (41%, 16/39) (Figure 2-2). When HF183 was detected in the ocean water (one out of three sample days) at the outlet, it was also detected in the confluence sample. On the other two days HF183 was not detected at the confluence or in the ocean. HF183 was observed slightly more frequently during wet weather (67%, n = 12) compared to dry weather (56%, n = 66).

Overall, HF183 was detected more frequently in the three (most downstream) sample locations in Entrada (50-60%, n = 4 to 10) than in the three most upstream (22-28%, n = 4 to 9). In contrast, HF183 presence was detected most frequently in the two most upstream samples, W1 and W2 in West Channel (See Figure 2-2D). Surprisingly, HF183 was detected 85% of the time in the naturalized portion of the channel at W1 (n = 7). HF183 was detected 100% of the time at W2 (n = 4), and then the frequency of detection dropped off to 20% (n = 5) at W3. Finally, frequency of detection increased to 60% at W4 just before the confluence (n = 10). HF183 was detected roughly 56% of the time (n = 9) at the confluence.

3.3. *Relationships between HF183, FIB and Physicochemical Parameters.*

All water samples collected between August 2008 and February 2009 were tested for FIB and HF183. Only four samples had quantifiable replicates for HF183 detected above 10 cps / uL, so correlations between HF183 and the other parameters were not explored. Three of

these samples were near or at the confluence on different days (W4, E6, C1) and one was from a storm drain sample located near E4. Relationships between the concentrations of any FIB or physicochemical parameters were examined to find significant differences when grouped by the presence of the HF183 marker. Any sample with >1 cp / uL of extract was considered a presence for this analysis. Notably, no statistically significant differences were observed between the FIB species (whether measured by IDEXX or Cov-IMS/ATP) or physicochemical parameters (Mann-Whitney, $p > 0.05$, Table 2-1) when grouped by the presence / absence of HF183 (Figure 2-3).

Relationships between physicochemical parameters and FIB were examined as well. Significant relationships were observed between ENT and TSS (Spearman's, $\rho = 0.000$), ENT and ammonia (Spearman's, $\rho = 0.045$), and EC and nitrate (Spearman's, $\rho = 0.009$) (Figure 2-4). Additional relationships emerge when analyzing the data from Entrada Channel and West Channel individually. Significant relationships were observed between ENT and TSS in Entrada ($n = 15$, $\rho = 0.002$) and between EC and nitrate in West Channel ($n = 9$, $\rho = 0.015$).

Patterns of FIB exceedance rates and HF183 were also examined. Nine samples over the course of the study concurrently exceeded single-sample standards for TC, EC, ENT, and the TC:FC ratio. Although these samples were not much more likely to have HF183 overall (detected in 5 / 9 samples), an interesting spatial pattern emerged on August 15th, when all 6 samples in Entrada exceeded all four standards. HF183 was not detected in the three upstream samples but was detected in the three downstream samples, which matches the overall pattern seen in the frequency of HF183 detection.

Additionally, the ratios of HF183 to ENT and EC were examined. The range of HF183:EC and HF183:ENT were generally quite a bit lower in the environment in our study than that observed in influent-spiked Malibu Lagoon samples (Jay lab, unpublished). Ratios of HF183 to ENT and HF183 to EC from influent-spiked Malibu lagoon samples have been found to be 0.9 to 1.53 and 0.9 to 1.27, respectively (Table 2-2, Jay Lab data, unpublished; units: HF183 cps / MPN). Only one sample (09/24/08, confluence) had ratios of HF183:ENT within the range seen in sewage, and none had ratios of HF183:EC within the range seen in sewage. Data from this date was examined more closely to see if there were other indicators of sewage; although the HF183:EC ratio was among the higher (0.36), it was not in the range previously observed in sewage-spiked samples. Similarly, the TC:EC ratio (16) was not at a level which exceeded the California water quality standard. Overall, the range in ratios in environmental samples for HF183:EC was 9.4×10^{-5} to 0.37 (n = 72) and for HF183:ENT the range was 4.4×10^{-5} to 2.3 (n = 55). The majority of the ratios between HF183 and both EC and ENT were below 0.1 (n = 67 and n=49, respectively).

4. Discussion

Over the course of this study, FIB were observed at varying, albeit fairly high overall, levels at all sample sites in both channels. As a result of this variation within the watershed, no individual sample location was identifiable as a dominant source of FIB to the watershed that could then be studied further for HF183. Similar results were seen in Ballona Creek in a study conducted by Noble et al. (2006); using FIB results, no one tributary or sample location could be used to direct further sampling efforts to a more human-influenced site although enterovirus concentrations were observed to be the highest at the most upstream sample location. In

contrast, in studies done at Avalon Bay (Boehm et al., 2003) and in Santa Barbara (Sercu et al., 2009), certain sample locations were able to be identified as potential sources for human pollution to the watershed based on their high concentrations of FIB.

Notably, HF183 was frequently observed at levels below those allowing quantification throughout all areas sampled. HF183 was detected in 75% of the samples from various storm drains, 65% of the samples in West Channel, 56% of the samples from the confluence, and in roughly 41% of the samples in the Entrada Channel. Frequent occurrence of human-associated *Bacteroidales* species (23% to 86% of samples) has been seen in other studies in Southern California (Santoro and Boehm, 2007), Wisconsin (Bower et al., 2005), and the Pacific Northwest (Shanks et al., 2006). Also, human adenoviruses were observed in 1/3 of samples taken in southern California coastal waters (Jiang et al., 2001).

Measuring HF183 throughout the course of the study allowed an analysis of the frequency of detection at individual sites, which showed some interesting patterns. Frequency of detection of a human marker has been shown to provide useful information toward tracking and then remediating human pollution sources at a Florida beach (Korajkic et al., 2010). In this study, the frequency of HF183 detection was quite high at the two upper most sites in West Channel, while it was lower in the three uppermost sites in Entrada Channel. HF183 was detected between 50-60% of the time at the confluence and nearby sample locations (E4-E6, W4). A potential source to these locations was observed on several sample sampling trips when a couple of people were observed to be camping in the channels near the confluence location.

In addition to frequencies of detection, ratios of microbial indicators have been proposed as a possible tool to help identify sources of pollution in watersheds (Converse et al., 2009; Silkie and

Nelson, 2009). Overall, the ratios of HF183 : ENT and HF183 : EC observed in the presented work were well below the range seen in influent-spiked Malibu lagoon samples (Table 2-2, Jay Lab data, unpublished; units: HF183 cps / MPN). Similar values for influent were observed in a previous study using the fecal *Bacteroides* spp. assay (Table 2-2) (Converse et al., 2009). The higher ratio range seen in influent-spiked samples in the Converse study makes sense as the fecal *Bacteroides* assay is specific to a larger group of *Bacteroides*, including *B. thetaiotaomicron*, *B. uniformis*, *B distasonis*, and *B. fragilis* (Converse et al., 2009), than HF183. Lower ratios of *Bacteroides*:FIB can indicate only environmental sources of FIB to the environment. For example, Converse et al. (Converse et al., 2009) observed low ratios in environmental water samples spiked with gull feces (Table 2-2) (Converse et al., 2009). Since HF183 was observed frequently in the presented work, the lower ratios that were observed indicate the likely presence of environmental sources of FIB in the environment, in addition to the human source that is evidenced by HF183.

Rainfall was observed to have differing effects on the concentrations of FIB and HF183 during the course of this study. During wet weather, the exceedance rate among the samples in the channels was 100% during rain events as compared to the 83% and 73% exceedance rates (for ENT and EC, respectively) observed during dry weather. Wet weather had a weaker effect on HF183 than either species of FIB. Rainfall affecting various indicators differently has been observed before; adenovirus and enterovirus samples were observed to decrease with rain, while FIB levels increased with rain in an earlier study (Jiang et al., 2007). In several other studies, rainfall in Southern California has been shown to lead to increased levels of FIB in local watersheds (Noble et al., 2003; Boehm et al., 2002; Surbeck et al., 2006). In one study, the

prevalence of FIB and F+ coliphage in stormwater throughout the entire storm hydrograph led to a mud-puddle hypothesis in which an almost limitless supply of these microbes are present in the environment to be washed out during rainfall; human viruses, however, were not ubiquitous throughout the hydrograph, which indicates that there may be different watershed sources of these indicator types (Surbeck et al., 2006). Similarly, the difference in behavior between HF183 and FIB observed in this study during storm events in Santa Monica Canyon demonstrates the possibility of different sources for these indicators in the watershed; watershed sources of FIB could be preferentially mobilized during storms as a result of growth while precipitation may have a diluting effect on more source-specific bacteria that may be more limited in number.

Establishing the appropriate first tier parameters is essential in a tiered approach to fecal source tracking. FIB can provide information in a general sense of impacted sites within the watershed, and in some cases they may constitute an effective first tier for locating specific sources of pollution. However, this study illustrates that this is not always possible with FIB. At this site, neither FIB nor any other measured physicochemical parameters would have been an appropriate indicator for a first tier. This study points to the importance of determining a correlation between cheaper, more general water quality parameters such as FIB and physical characteristics (i.e. nutrients, turbidity, pH, DO, etc) and the desired final tier of the study (i.e. HF183) as a preliminary step to developing a tiered approach.

A similar lack in relationship between human indicators and FIB has been observed in other environments, including a study done at Avalon Bay in Catalina where samples positive for the human-associated *Bacteroides/Prevotella* marker or enterovirus did not necessarily correspond with samples exceeding water quality standards for FIB (Boehm et al., 2003). Additionally,

strong associations were not observed between EC and human-associated *Bacteroides* in Lake Michigan (Bower et al., 2005), and between FIB and enterovirus at Ballona Creek in Los Angeles (FIB were ubiquitous in the watershed and enterovirus was only detected in 39% of samples) (Noble et al., 2006). A lack of correlation between FIB and other source-specific or pathogenic microorganisms has been observed in many other studies, (Jiang et al., 2007; Lemarchand and Lebaron, 2003; Lund, 1996; Rajal et al., 2007).

At some locations, however, FIB have been shown to correlate with *Bacteroidales* depending on site conditions, indicating that FIB may still be useful as an element of a watershed-scale analysis. Although no strong correlations between FIB and HF183 were observed in a Santa Barbara study (Sercu et al., 2009) high HF183 concentrations were found exclusively at locations with high FIB concentrations. Reischer and colleagues (2008) found that human-associated *Bacteroidetes* and fecal indicators were shown to correlate strongly ($R^2 = 0.79$ for both fecal coliforms and ENT) along the Danube River.

Results from this study point to the possibility that a differently designed tiered approach that begins with a study of all the available data regarding potential sources of microbial pollution (see Figure 2-5), may be a more appropriate first step than an indicator that has worked well as a proxy in other watersheds. This approach could lead to a shorter-term study looking at all the likely sources in the watershed that would provide much more information to city managers or public officials. In addition, measuring the selected source-associated markers such as HF183 allows the use of techniques such as ratio comparisons or frequencies of detection to provide a more detailed picture of water quality and appropriate remediation actions in the watershed of interest. There may not be a single indicator that can be used as a first step in a source-tracking

study; rather, a multi-parameter study that can be analyzed collectively may be the best way to characterize sources within a watershed (Reischer et al., 2008). If, after this first study, good correlations are observed for parameters in the watershed of interest, then a traditional tiered approach could be used to identify sources if further study is necessary. Alternatively, the first study might provide all the information necessary to remediate the most likely pollution sources. Based on the large variability in watershed characteristics and lack of correlation observed in this study, and others, between FIB and source-associated markers or pathogens, study designs that include monitoring of one or more source-associated markers may be necessary to identify sources of interest.

5. Conclusion

- HF183 was detected in 65% of samples from West Channel and 41% of samples from Entrada Channel. HF183 was detected at all sample locations at various times throughout the study; none of the sample locations was identified as a consistent source of HF183.
- Although levels of HF183 were low throughout the study, interesting patterns emerged when looking at the frequency of detection. The lower parts of the channels sampled and the confluence had high frequencies of HF183 detection. Interestingly, samples taken from the naturalized portion of West Channel had fairly high detection rates of HF183 as well.
- During wet weather, the FIB exceedance rate among the samples in the channels was 100% during rain events as compared to the 83% and 73% exceedance rates (for ENT and EC, respectively) observed during dry weather. Concentrations of

FIB were three to ten times higher in samples taken during rain events than dry. Rainfall had a milder effect on HF183 presence.

- The notion of using FIB or other general water quality parameters as a screen for sites likely contaminated with human-fecal pollution is appealing; however, this study illustrates clearly that this is not always possible. Initial watershed surveys are required to determine the presence of correlations between tiers of a tiered approach before its application in the watershed.
- In the case of this watershed, no other parameter was a satisfactory indicator of human-associated marker, pointing to the need for routine use of human-associated measurements such as HF183.

Table 2-1. Mann-Whitney results of parameters when grouped by presence or absence of HF183 marker.

Parameter	Presence (Mean Rank, n)	Absence (Mean Rank, n)	Mann-Whitney U	p-value
Log (DO)	6.62, 8	9.57, 7	17.000	0.203
Log (TDS)	5.14, 7	9.17, 6	8.000	0.056
Log (Temp)	5.58, 6	6.5, 5	12.500	0.647
Log (pH)	8.06, 8	7.93, 7	28.500	0.954
Log (Nitrate)	11.13, 15	16.73, 11	47.000	0.065
Log (Ammonia)	13.09, 17	18.65, 13	69.500	0.086
Log (TSS)	21.62, 29	28.89, 19	192.000	0.078
Log (Enterococci)	30.72, 37	28.80, 22	433.500	0.678
Log (*E. coli*)	45, 40.31	31, 35.87	779.000	0.389
Log (Total Coliforms)	33.3, 38	37.08, 31	524.500	0.414

Table 2-2. *Bacteroidales* : FIB ratios in different sample matrices

Sample matrix	Microorganisms	Ratio	Reference
Wastewater / septic	Fecal *Bacteroides* : enterococci	108 – 3300	Converse 2009
Influent-spiked sample	Fecal *Bacteroides* : enterococci	2 – 450	Converse 2009
Gull-spiked sample	Fecal *Bacteroides* : enterococci	2.5*10^-5 to 3.5 * 10^-2	Converse 2009
Wastewater / septic	Fecal *Bacteroides* : *E. coli*	29 – 5900	Converse 2009
Influent-spiked sample	Fecal *Bacteroides* : *E. coli*	2 – 259	Converse 2009
Gull-spiked sample	Fecal *Bacteroides* : *E. coli*	2.5*10^-5 to 3.5 * 10^-2	Converse 2009
Influent-spiked sample	HF183 : enterococci	0.9 – 1.53	Jay Lab data
SMC water sample	HF183 : enterococci	4.4*10^-5 to 2.28	Jay Lab data
Influent-spiked sample	HF183 : *E. coli*	0.9 – 1.27	Jay Lab data
SMC water sample	HF183 : *E. coli*	9.4*10^-5 to 0.37	Jay Lab data

FIGURES

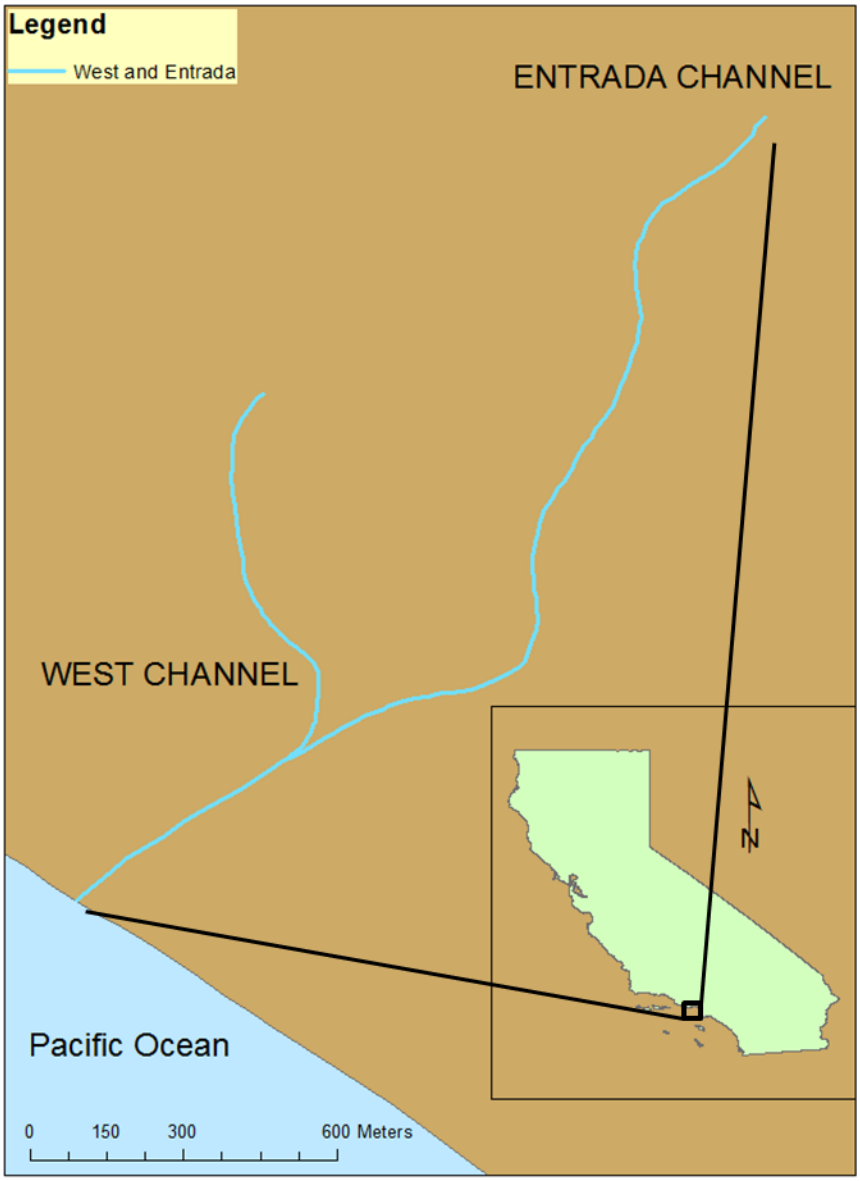


Figure 2-1 GIS map depicting sample location in California context.

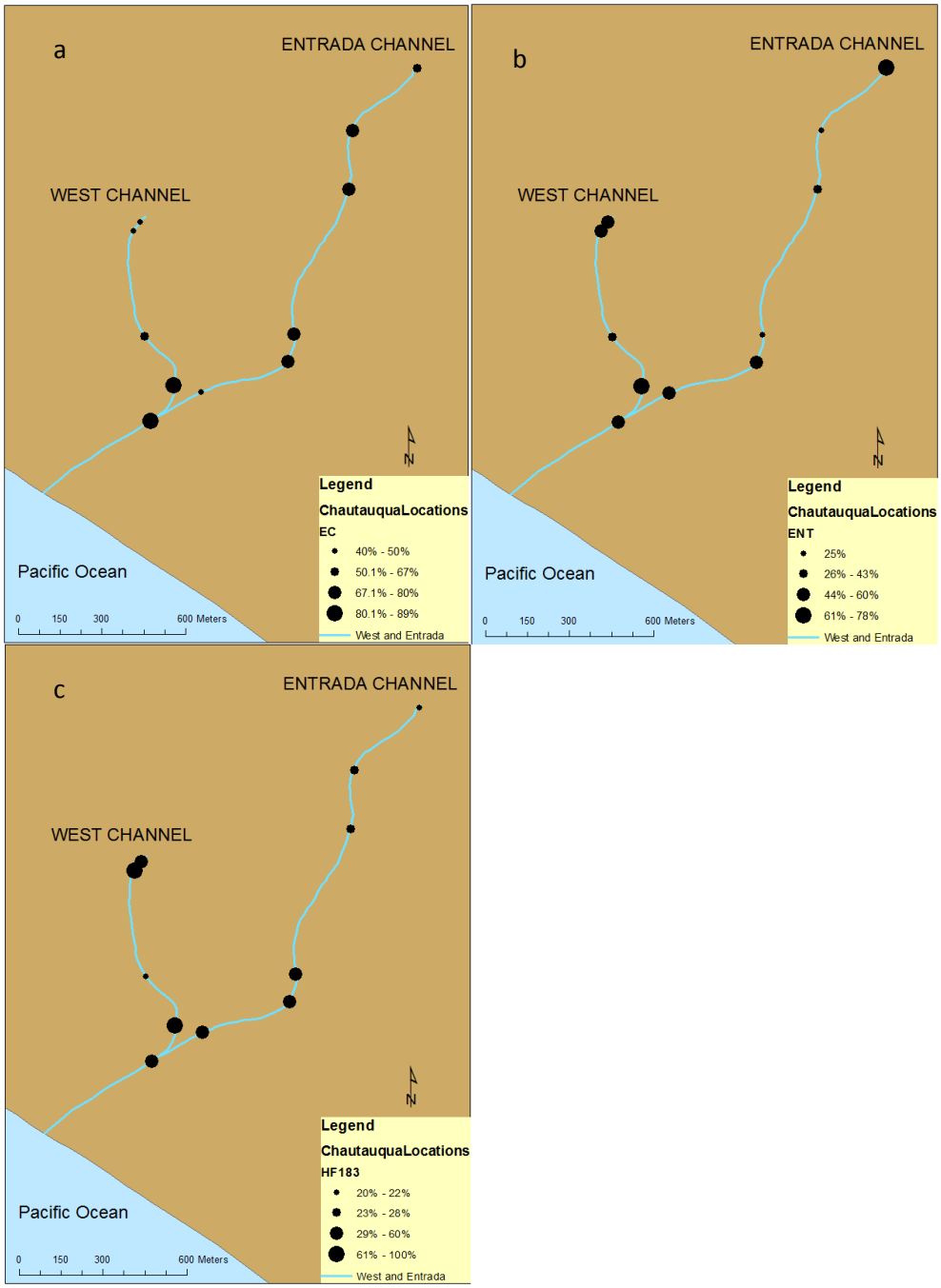
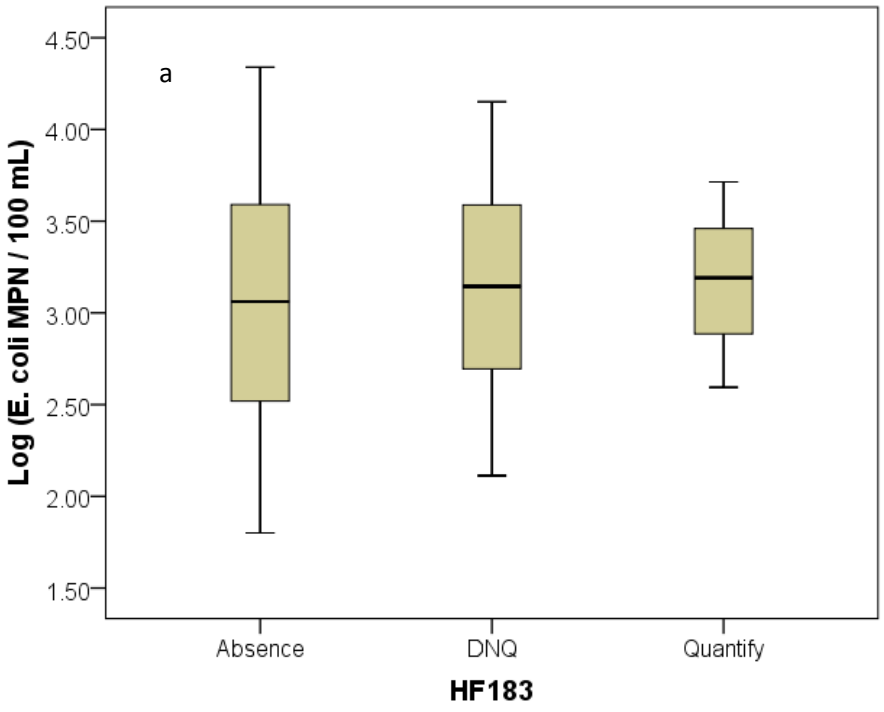


Figure 2-2A, 2-2B, 2-2C Spatial map depicting frequency of exceedance for EC and ENT and frequency of detection for HF183. a) EC b) ENT c) HF183



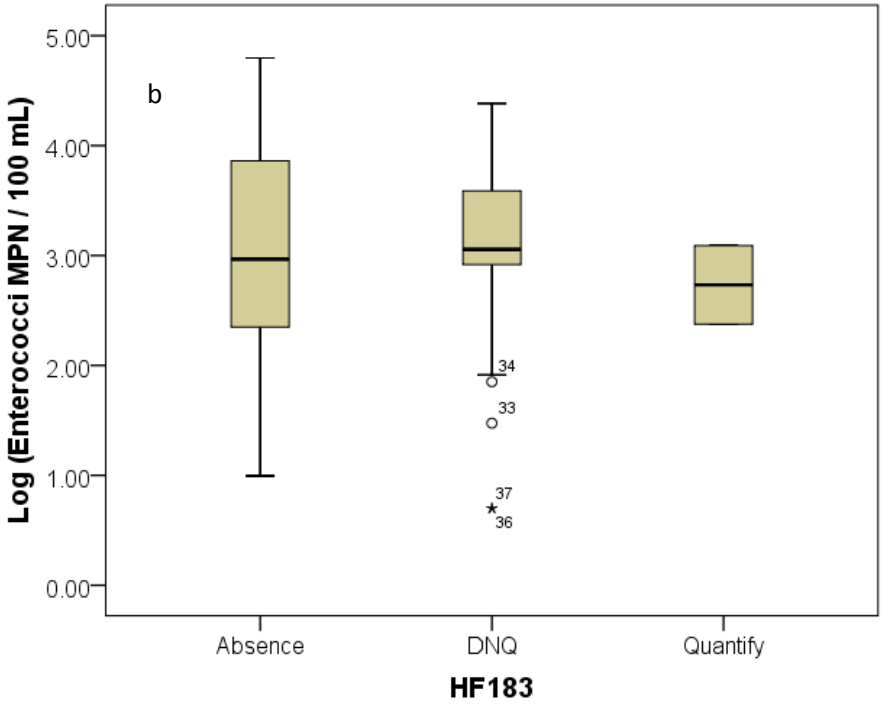


Figure 2-3 Box plots binned by HF183 absence, DNQ, or quantifiable. a) EC concentrations b) ENT concentrations

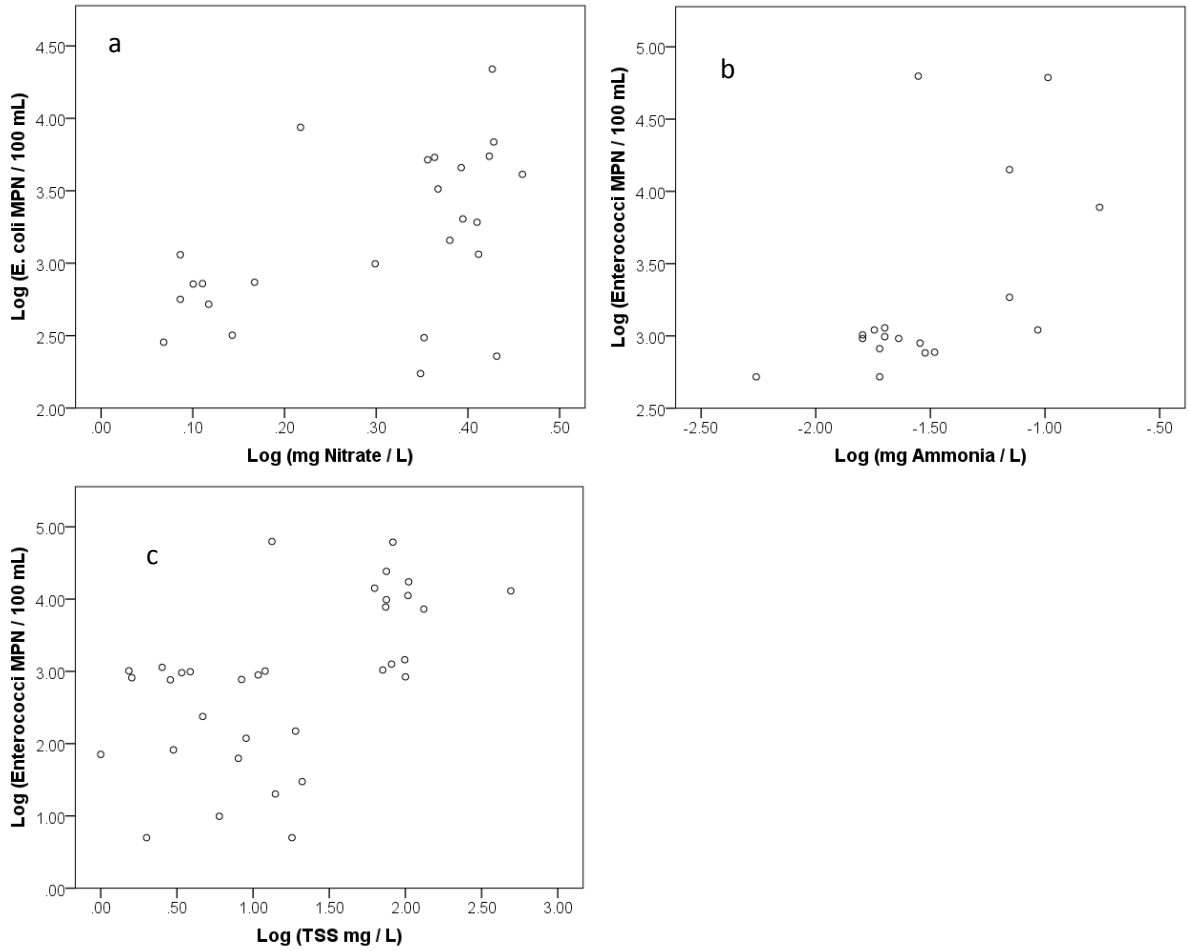


Figure 2-4 Relationships between FIB and physicochemical parameters. a) EC vs. nitrate, Spearman's $\rho = 0.009$ b) ENT vs. ammonia, Spearman's $\rho = 0.045$ c) ENT vs. TSS, Spearman's $\rho < 0.001$

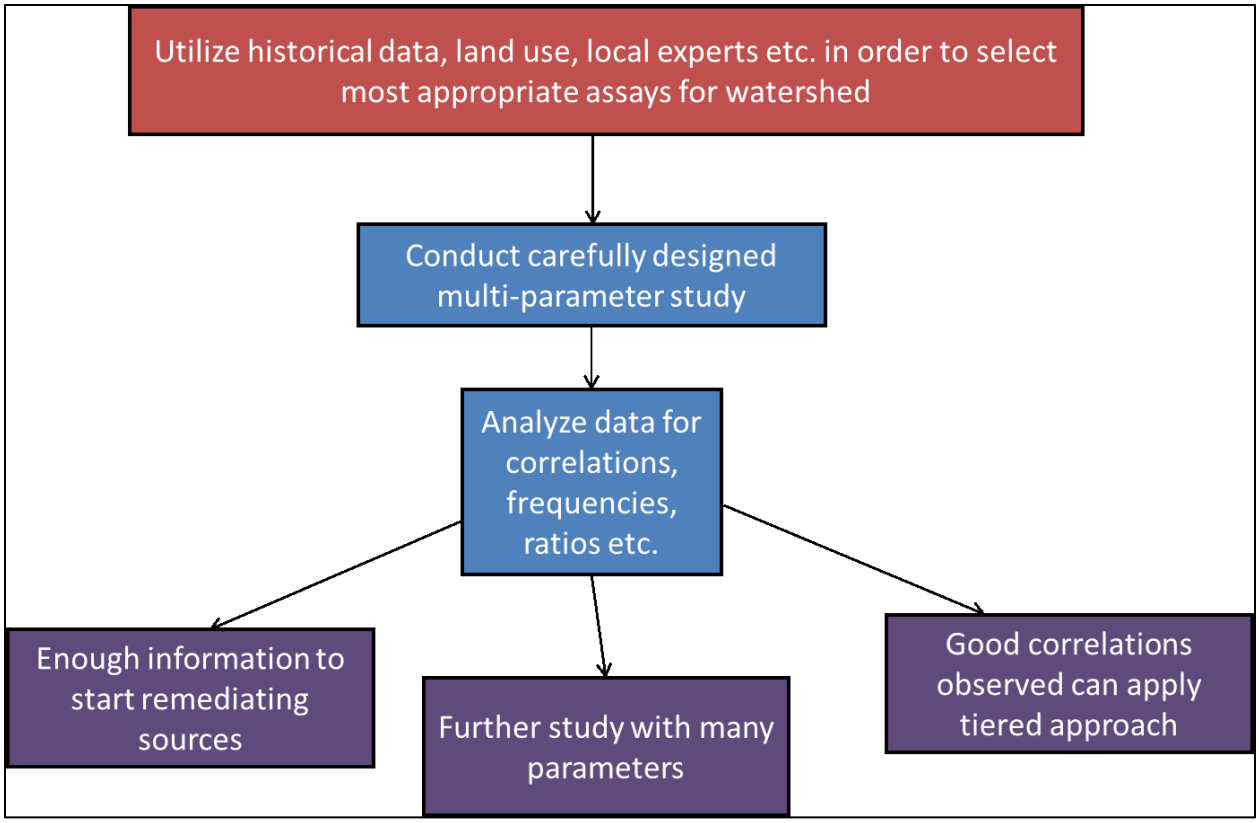


Figure 2-5. Schematic of a different tiered approach to microbial source tracking studies.

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CHAPTER 3. Patterns of fecal indicator bacteria (FIB) and a human-associated marker (HF183) in Ventura Harbor, Marina, and Arundell Barranca: Does the source of FIB to the watershed correspond with the source of the human-associated marker HF183?

Abstract

Ventura Harbor is a mixed use harbor that includes residential and commercial developments. A large channel, the Arundell Barranca (AB), drains into the harbor near the Ventura Keys. The AB watershed includes mainly agricultural, residential, and commercial land uses, and is a potential source of the persistent microbial contamination seen at this site. Levels of fecal indicator bacteria (FIB) and HF183, a genetic marker from a human associated member of *Bacteroidales*, were measured monthly between May 2008 and April 2009 and more frequently during a rain event in February 2009 in order to understand the hotspots of contamination for both types of indicators as well as any relationships between them. AB was found to have significantly higher concentrations of both nitrate and FIB but not of HF183 compared to the other sample locations. HF183 was detected at a frequency of 14% throughout the study period and was detected at least once at the majority of sites. While HF183 was detected more frequently (19%) during rain events (12% during dry weather), there was not a systematic pattern consistent with large amounts of HF183 washing into the study area, as was seen with FIB. The sample location by Marina Dock appeared to be a hotspot of human pollution based on the frequency of detection of the HF183 marker at this site (53%) relative to other sites. Finally, HF183 was detected at both sample locations within one of the Keys channels in the residential development on one dry day in November, which points to the possibility of a localized influx of human waste during that time period.

1. Introduction

Persistent, long-term FIB contamination of urban coastal areas is a common problem along both ocean coastlines and lakeshores. Microbial contamination can lead to costs through public health risks (Given et al. 2006, Dwight et al. 2005), loss of tourism (Wiley et al. 2006), or loss of economic functionality such as shellfishery closures (Conn et al. 2011). The relatively calm waters in harbor and marina areas, although not as commonly used as high frequency swimming areas, provide space for many activities such as learning to kayak or sail. A recent study has shown an increase in health risk even when partaking in limited-contact recreational activities such as kayaking (Dorevitch et al. 2012). Furthermore, harbors and marinas have been shown to have higher concentrations of fecal indicator bacteria (FIB) in general (Sobsey et al. 2003, Guillon-Cotard et al. 1998, Ho et al. 2011). Enclosed areas such as harbors and marinas

tend also tend to be areas of lower flow and circulation, conditions which have been shown to harbor more bacteria in water (Clarke et al., 2007) and sand (Lee et al., 2006). Generally, frequent boat activity has also been shown to be a potential source of bacteria and able to affect patterns of bacteria in the area (Ho et al., 2011; Faust et al., 1982)

This study examines Ventura Harbor, which is a mixed-use developed harbor consisting of a marina, a residential development called the Ventura Keys, and some parks and commercial areas. Ventura Harbor sees a great deal of tourism, both from the local population and travelers from other states or abroad, and is the recipient urban and agricultural runoff, much of it from the Arundell Barranca (AB). Based on chronic water quality issues, a TMDL for bacteria was established at the jetty-sheltered Harbor Cove Beach in order to decrease bacterial levels there.

Other studies have identified many different sources of fecal microbial contamination to urban coastal locations including faulty sewer infrastructure (Korajkic et al., 2010; Dickerson et al., 2007), sand (Kinzelman), agricultural practices (Hagedorn et al., 1999), and non-point sources such as urban runoff or stormwater runoff (Parker et al., 2010; Reeves et al., 2004; Sercu et al., 2011; Jeong et al., 2005). In a study of the Talbert watershed in dry season runoff from many locations; residential land contributes the largest FIB concentration, followed by agricultural land, commercial land, industrial land, and parks (Reeves et al. 2004). While rainfall has been shown to create an increase in the concentrations of indicator bacteria, this pattern has been mixed in more specific source-associated markers and pathogens (Parker et al., 2010; Jokinen et al.; 2011; Newton et al., 2011).

In the field of microbial source tracking it has become highly useful to apply source-associated markers of pollution as an effective means of identifying potential sources of different microbial contaminations (Mallin et al., 2010; Korajkic et al., 2010). This information can help guide remediation efforts both spatially and in targeting the most likely contributor to the microbial contamination.

As the currently used standards for water quality (FIB) are not source-specific, both the scientific community and policy-makers are looking to molecular techniques and bacteria that can be linked to specific populations (i.e. seagulls, humans, or livestock) as a potentially more

useful method of identifying the sources of persistent water quality issues (Roslev et al., 2011). Members of the order *Bacteroidales* are a very good candidate for this new type of indicator, as they are found exclusively in endothermic organisms, and reside within feces, the digestive tract, and other body cavities (Paster et al., 1994). In addition, *Bacteroidales* levels in human sewage are orders of magnitude higher than levels of fecal coliform bacteria (Gerba 2000) and could thus be indicative of a recent sewage event at a higher dilution in the environment. Finally, these organisms are obligate anaerobes, and thus do not have the potential for regrowth in the environment that confounds the use of *E. coli* and enterococci as indicators (Walters and Field, 2006). Among the *Bacteroides-Prevotella* assays, the HF183 marker (HF183) has been shown to be specific to human fecal inputs on a global scale (Sercu et al., 2009; Fremaux et al., 2009; Gawler et al., 2007; Dorai-Raj et al., 2009; Ahmed et al., 2009; Ahmed et al., 2010; Seurinck et al., 2005). Based upon the previous research showing the robustness of this marker in many environments, HF183 was chosen as the indicator for the presence of human fecal pollution in this study.

The goal of this study was to better understand the chronic water quality issues in the Ventura Harbor watershed and whether the dynamics of FIB in both wet and dry weather were related to those of HF183. FIB and HF183 levels were measured monthly between May 2008 and April 2009, and daily to twice-daily during a rainstorm in February 2009 in order to understand the hotspots of contamination for both types of bacteria as well as any relationships between them.

2. Methods.

Site Description.

Ventura Harbor is located one half mile north of the Santa Clara river estuary, with the harbor opening out in the midwest portion of the harbor (Figure 3-1). Ventura Harbor is protected by a breakwater perpendicular to the main entrance of the harbor as well as three jetties; one is north of the opening, two are south of the opening. Once inside, the harbor splits into two channels, with the southern channel veering down into the mixed-use Ventura Marina and the northern channel veering up to branch out into three individual fingers of the Ventura Keys (Figure 1). The AB, which drains mostly agricultural land (Figure 3-2) (although the

watershed includes agricultural, municipal, and urban uses) empties out at the entrance to the Ventura Keys. The Los Angeles Regional water quality board has identified the Harbor Cove beach, located inside the harbor after the second jetty, as impaired based on the elevated bacterial densities and has imposed TMDLs for enterococci based on the current EPA water quality standards.

Historical data compiled by Heal the Bay (www.healthebay.org/brc) spanning from April 2002 to the present were also used to determine appropriate sample locations. Four sample locations near the mouth of Ventura Harbor were examined to determine areas of interest (i.e. sites with consistently high levels of FIB) as well as potential "unimpacted" sites with consistently low levels of FIB. Based on these data, all four sample locations show few dry weather exceedances of FIB. Sites 42, 13, and 49 (which corresponds to our "Open" sample location) show consistently "clean" records during most rainy seasons as well. Site 49 was selected as our "unimpacted" site, as that sample location had only had one grade lower than a B+ (a "C") since November 30, 2004 including both wet and dry sample days. Before that date, site 49 showed sporadic exceedances during wet weather. Site 28 (which corresponds to our "Enclosed" sample location) showed consistently good grades during dry weather, but was consistently given a poor rating during the wet season. We chose this sample location as it represents an important mixing zone between the "unimpacted" water coming in from the open ocean and the more impacted water coming out of the harbor, which can be seen in the higher number of exceedances during wet weather than at site 49.

Sample collection and analysis.

Collection. Monthly samples were collected on the following dates: May 15, 2008; June 19, 2008; July 21, 2008; August 6, 2008; September 23, 2008; October 30, 2008; November 11, 2008; December 19, 2008; January 23, 2009; February 11, 2009; March 31, 2009; and April 17th, 2009. Water and sediment samples were collected throughout the Ventura Keys, Marina, and at the outlet of the AB (Figure 2). Water samples were collected at six locations throughout the keys (D1-D6), with D5 and D6 being sampling sites located just east and west of the mouth of AB. Two enclosed beaches within the Ventura Keys at Beachmont Rd and Sailor Ave, were

also sampled for water and sediment. Samples were also collected from Marina Park (MP), off the docks in the Ventura Harbor (Marina Dock (MD)), and from a jetty-enclosed beach, Harbor Cove (ENC), and open beach, Surfers' Knoll (OPEN).

In addition to the monthly surveys, rainy day intensive surveys were performed on April 2-4, 2008 (FIB only) and February 4-6, 2009 (FIB and HF183). Rainfall in April 2008 was very minimal, with only 0.1 inches falling on 4/2/08 and 0.07 inches on 4/3/08. Most of the samples were below the detection limit for E. coli and enterococci during this storm; accordingly, data for total coliforms was analyzed. A larger storm was sampled in February 2009; 1.6 inches of rain fell over Feb 5-6 and an additional 1.36 inches of rain fell through February 9th. One set of samples was collected on February 4th, the day before the storm began. Samples were again colelcted at 10 am and 4 pm on February 5th, the first day of the storm, and at 10 am on February 6th on the second day of the storm. After an interceding dry day, samples were taken on February 11 to determine whether the FIB levels were still elevated two days after a large storm. One to two liters of water was collected at each site and transported on ice for laboratory analysis.

Physicochemical parameters. When possible, samples were measured for total dissolved solids (TDS), water temperature, electrical conductivity and pH (HANNA instruments #H198130, Smithfield, RI). Selected samples were also analyzed for total suspended solids (TSS) (Environmental Sciences Section Method 340.2); dissolved oxygen (DO) was measured with a hand-held probe (YSI model 55/12 FT, YSI Inc, Yellow Springs, OH); and flow was estimated.

*Standard FIB***.** All water samples were analyzed for total coliforms (TC), *E. coli* (EC), and enterococci (ENT) using defined substrate technology (IDEXX Laboratories, Inc, Westbrook, ME). Colilert®-18 (IDEXX) was used to quantify TC and EC, and Enterolert® (IDEXX) was used to quantify ENT. In California, single-sample recreational water quality standards as established by Assembly Bill 411 (AB411) use TC (10,000 MPN / 100 mL), FC (400 / 100 mL), and ENT (104 / 100 mL) to determine water quality. EC, because they are a subset of FC, were used as a proxy for the FC in our study as the level of FC would be greater than that of EC. The state of California also uses the ratio of TC : FC as an indicator of recreational water quality

(http://www.waterboards.ca.gov/water_issues/programs/beaches/beach_water_quality/beaches_program.shtml).

*HF183***.** Samples collected between May 2008 and April 2009 were further analyzed for HF183 marker (n = 152), processing to be discussed in further detail.

DNA Extraction and Quantification. Water samples were vacuum-filtered through 47-mm-diameter, 0.45-µm-pore-size Fisherbrand nitrocellulose filters (Fisher Scientific, Pittsburgh, PA) until the total volume was filtered or sample stopped passing through the filter. DNA was extracted from the filters using the Mobio UltraClean Fecal DNA Kit (MO BIO Laboratories, Inc, Carlsbad, CA), and extracts were stored at -80°C until being processed for HF183. DNA was extracted according to manufacturer's protocol with the addition of 90 seconds of bead-beating in a BioSpec Mini-Beadbeater-8 (BioSpec Products, Bartlesville, OK). Total DNA concentration in the extract was analyzed using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Molecular Probes, Inc, Eugene, OR) with a Stratagene Mx3000P system (Agilent Technologies, Santa Clara, CA). One µL aliquots of sample extract were run concurrently with a standard curve of DNA standard provided in the Quant-iT™ kit ranging from 0.1 ng to 20 ng of DNA μL^{-1} in a total volume of 30 µL.

qPCR Analyses. Samples were analyzed for HF183 using qPCR. DNA was amplified with primers and master mix as described in Seurinck et al. (2005). Briefly, amplification occurred in 25 µL reaction volumes containing 2 ng of sample DNA, 1X SYBR GreenER PCR Master Mix (Life Technologies, Grand Island, NY), 0.25 mM of each primer, and DNase and RNAse free water (Fisher BioReagents, Pittsburgh, PA). The reactions were performed in 96-well reaction plates using a Stratagene Mx3000P system (Agilent Technologies, Santa Clara, CA). The qPCR temperature program was set as follows: 2 minutes at 50 °C, 10 min at 95 °C, then 45 cycles of 30 s at 95 °C, 1 min at 53 °C, and 1 min at 60 °C. PCR products were verified through melting curve analysis; a melting temperature between 77 °C and 78 °C indicated positive, correct amplification of HF183. Sample values were calculated using MxPro™ qPCR software. A six-point standard curve using plasmids with HF183 was run in duplicate alongside the environmental samples on every plate. The presence of environmental interference was assessed

by running samples spiked with a 2×10^5 copies / µL dilution of a HF183 plasmid alongside the sample duplicates. Plasmids for HF183 were obtained from Professor Fuhrman (University of Southern California, CA). If spiked samples values were less than 8×10^4 copies / µL, then samples were diluted by half and reanalyzed. This process was repeated up to three times as needed. If interference was still present after third dilution, samples were designated as interfered and omitted from further analyses.

Statistics and Data Analyses. Statistical analyses were run using SPSS statistical software (SPSS Inc., Chicago, IL). Concentrations of bacterial species analyzed and physicochemical parameters measured were log-transformed prior to data analysis. Log-transformed data were found to be not normal using Shapiro-Wilk's test; accordingly, all data analyses were performed using non-parametric tests. Spearman's rank correlation tests were used to determine the relationships between all parameters. Kruskal-Wallis and Mann-Whitney U tests were used to discern differences between channels and weather types for the parameters analyzed.

The limit of detection (LOD) for the qPCR assay described above was defined as 1 cp / µL; the limit of quantification was defined as 10 cps / µL. Samples in the range of 1 to 10 cps / µL were classified as 'detectable but not quantifiable (DNQ).' All samples that were positive for HF183 were validated using a melt curve analysis. For the purposes of data analysis, non-detects were assigned a value of 0 cps / uL and DNQ's were assigned a value of 1 cps. Marine water standards were used to determine exceedance rates as the majority of sample sites were in marine waters. Data were further explored for patterns and relationships using the frequency of water quality standard exceedance for FIB, the frequency of detection for HF183, and the ratio of HF183:FIB.

3. Results

Spatial and Temporal patterns in FIB and physicochemical parameters. The three types of FIB currently used to determine water quality were measured throughout this study, and observed at many of the sampled locations. During the summer months, *E. coli* and enterococci were often non-detects at sample locations with the exception of AB and the enclosed beaches. The highest concentrations of TC and enterococci were consistently detected at AB, D5, and D6 compared to

the water samples taken elsewhere in the Ventura Keys, Harbor, or Marina areas. Although *E. coli* was also generally detected with a similar frequency to enterococci and TC, *E. coli* concentrations were consistently lower.

In general, all FIB levels were consistently higher in the Keys (D1-D6, Beachmont, and Sailor) and AB and lower in the Harbor (MD, MP, Enc, Open) (Figure 3-3), although only TC was almost statistically significantly lower (Mann-Whitney, $p = 0.055$). Concentrations of FIB appeared to experience a seasonal decrease, in which samples had the highest levels of FIB during January and February (also corresponding with wetter weather) and faded to non-detects by April. FIB concentrations stayed low through August, and then increased in September through the winter.

Patterns of FIB in sediment. Patterns in enterococci in sand were similar to patterns observed in FIB in water at the beaches. Enterococcus concentration in sediments were consistently higher at Keys beaches compared to other beaches, with the concentrations decreasing in the following order: Beachmont> Sailor> Enclosed> Open. Enterococci was detected in 60% (6/10) of sediment samples at Keys beaches compared to 30% (3/12) water samples. In addition to higher frequency of detection, enterococci was detected at higher levels in sediment (10^3 - 10^7 MPN / 100 grams versus 0 to 10 MPN / 100 mL). Interestingly, *E. coli* concentrations were low or non-detect at most locations. Finally, sand collected at Beachmont had higher levels of TC and enterococci than the other beaches within the keys, which may be caused in part by the fact that the rising tide pushes bacteria-laden AB water towards the beach at Beachmont. TC, EC, and ENT levels in Keys sands were higher than levels at the open beaches (Mann-Whitney, $p < 0.001$) (Figure 3-4).

Spatial and Temporal patterns in HF183. HF183 was detected in 14% of samples analyzed over the course of this study (22/152), and quantifiable in 4.6% (7/22). HF183 was not detected at the open beach location or at D6, near the mouth of AB (Table 3-1). At eight sites (MP, SF, D1, D3, D4, D5, AB, and ABO), HF183 was only detected one time during the study. Of these eight, seven occurred during wet weather, with all but one of these HF183 detections during the February 2009 storm. HF183 was detected twice at BM during the rain and then on 2/11, after

one day of dry weather post-storm. The enclosed beach was found to have HF183 three times over the course of the study, twice between 2/5/09 and 2/11/09 and then again on 4/17/2009. Interestingly, the only time that HF183 was detected at D3 was on a dry day in November, and it was present at quantifiable levels (1200 cps / 100 mL). During the same sampling event, the other sample in that channel (D2) also had quantifiable levels of HF183 (1500 cps / 100 mL), which points to the possibility of an isolated event in the channel that day.

The marina dock (MD) location had the highest frequency of both detection and quantification of HF183. HF183 was detected in 54% (7/13) of the samples and quantifiable at 4/13 samples across all times of year and types of weather. HF183 was significantly different across sample locations (Kruskal-Wallis, $p=.009$). All significant differences were between MD and other sample locations (Open, D1, D3, D4, D5, D6, MP, AB, SF, $.010<p<.029$). BM, ABO, D2, and ENC were not significantly different from MD according to the post-hoc pairwise comparisons.

Arundell Barranca. AB appears to be a dry weather source of TC and enterococci in the Ventura Keys, as the highest levels of these two indicator bacteria were detected near the outlet of AB (D5 and D6). Exceedance rates were the highest at the AB sample locations compared to all other locations. The AB and AB outlet samples were significantly higher than the other locations in all three FIB ($p<.001$), nitrate ($p<.001$), and temperature ($p=.029$). The AB sites were also significantly less saline than the remainder of the sites which were in marine waters ($p<.001$). However, there was no statistically significant difference between the AB sites and the other locations in DO, pH, Ammonia, DNA, or TSS.

HF183 was detected once at each of the two locations sampled at the AB, and both were during rain events. Interestingly, the positive results were seen on different days – despite a detection of HF183 at AB, which was upstream of ABO, no HF183 was detected at ABO during the same sampling event. HF183 levels, when grouped by whether or not they were AB samples, were not significantly different (Mann-Whitney, $p=0.568$).

Rainfall effects on FIB and HF183. Rainfall had a significant effect on the concentrations of FIB as well as the water quality standard exceedance rates. FIB concentrations increased at most

sample locations throughout the watershed during the storm. All three species of FIB were significantly higher during wet weather than dry weather, as was nitrate. The FIB storm signal decayed rapidly; both enterococci and *E. coli* concentrations receded to pre-storm levels by 2 days after the storm ended on February 9th. Salinity was significantly lower during wet weather than dry during the February 2009 storm. TSS and ammonia were not significantly different between wet and dry weather. Interestingly, FIB levels during the first storm monitored for FIB levels in April 2008 were not significantly different between wet and dry weather. This may be because the amount of rainfall that fell in that storm (0.17") was not enough to flush the FIB out of the watershed, as opposed to the 1.6" of rain that fell between 2/5/2009 and 2/6/2009. Salinity was not significantly different during the April 2008 rainstorm compared to dry weather values either. Exceedance rates for all FIB were between 8 and 24 times higher during wet weather than during dry weather (Table 3-2).

Although HF183 was significantly higher during wet weather (Mann-Whitney, $p = 0.012$) than dry and was detected more frequently during wet weather than dry weather (Table 3-1), the pattern was not constant over the course of the rain storm as was seen in FIB. At least one sample location had HF183 during each sampling event during a rainstorm (1/23/2009, 2/5/2009, or 2/6/2009), but they were scattered throughout the sample area without any one site having a consistent HF183 presence throughout the storm. While the afternoon of 2/5 was the sample date that had the highest number of sites positive for HF183 of any day during the course of the study (5 sites out of 13), the positives were scattered throughout the Keys and at MD. Although both samples taken from the easternmost channel (BM and SF) were both positive for HF183, this pattern was not seen in the other two channels. Only one of the three samples (D2) taken in the middle channels was positive while the other two were negative (D3 and SF). Furthermore, HF183 was not significantly different across the dates of the study (Kruskal Wallis, $p > 0.05$).

Relationships between HF183, FIB, and physicochemical parameters. EC and ENT were both significantly higher in samples with HF183 (Mann-Whitney, $p=.004$ and $p=.029$, respectively) but TC was not (Table 3-3) (See Figure 3-5). Salinity was also significantly lower in samples with HF183 compared to those without, which may be related to the higher detection rates during rainy weather when salinity at the sampling sites was significantly lower than during dry.

Additionally, the ratios of HF183 to ENT and EC were examined. Notably, five samples out of the seven which had quantifiable levels of HF183 had both EC and ENT in or above the range seen in sewage. The exceptions were AB during the rainstorm, in which ENT was within the range of sewage and EC was not, and MD on 10/31/08, when there was no information for EC to determine the ratio. D2 and D3 on 11/11/08 were also included in this group, which provides further indication that a human waste discharge event may have occurred on this day.

Ratios of HF183 to ENT and HF183 to EC from influent-spiked Malibu lagoon samples have been found to be 0.9 to 1.53 and 0.9 to 1.27, respectively (Jay Lab data, unpublished; units: (HF183 cps / MPN). Similar values were observed in a previous study using the fecal *Bacteroides* spp. assay: the ratio range in influent-spiked samples for fecal *Bacteroides*:EC and fecal *Bacteroides*:ENT were 2 to 259 and 2 to 450, respectively (Converse et al. 2009). The higher ratio range seen in influent-spiked samples in the Converse study makes sense as the fecal *Bacteroides* assay is specific to a larger group of *Bacteroides*, including *B. thetaiotaomicron*, *B. uniformis*, *B distasonis*, and *B. fragilis* (Converse et al. 2009), than HF183. Additionally, Converse et al. (2009) observed low ratios in environmental samples spiked with gull feces, with the ratio range in influent-spiked samples for fecal *Bacteroides*:EC and fecal *Bacteroides*:ENT being 2.5×10^{-5} : 3.5×10^{-2}. Overall, the range in ratios seen in environmental samples from the presented work was 6.8×10^{-3} to 248 for HF183:EC (with 11 samples in or above the range of sewage and 11 below sewage range) and the range was 2.1×10^{-3} to 445 for HF183:ENT (with 13 samples in or above the range of sewage and nine below sewage range). Thus, roughly half of the samples with DNQ or quantifiable HF183 in this study were in the range seen in sewage, while the other half was more in the range seen in environmental waters (Converse et al., 2009; Mika et al., 20xx, submitted).

4. Discussion

Generally, TC and enterococci were detected at higher levels and with more frequency than EC overall. Enterococci has been shown to persist longer in seawater (Jeanneau et al., 2012), and to persist and grow in beach sands (Yamahara et al., 2009; Mika et al., 2009). A recent study in a wastewater effluent dominated stream showed that enterococcus grows in streambed sediments,

and that 73% of the enterococci species detected in the water and sediment were *E. casseliflavus*, which is not a species typically associated with fecal material. Furthermore, although solar inactivation has been shown to decrease the survival of enterococci in some cases, a recent study of enterococci composition in a marine harbor on Catalina Island found a highly diverse community of enterococci in the waters (Maraccini et al., 2012). Some of the enterococci, particularly the strains more commonly associated with environmental sources, were found to be pigmented and thus more resistant to solar inactivation (Maraccini et al., 2012), which may also be the case in the Ventura Harbor and marina areas.

Rainfall events had different effects on FIB and HF183 over the course of the storms. FIB levels increased consistently over the entire study site for the duration of the storm. While HF183 was detected more frequently and at slightly higher levels at several sites within the watershed, there was not a consistent pattern over the course of the storm. These results are consistent with many studies that have shown FIB levels to increase greatly during storm events (Noble et al., 2003; Boehm et al., 2002) and less consistent effects on source-associated bacteria or pathogens (Jiang et al., 2007; Walters et al., 2011)). Although an increased incidence of HF183 was observed during the February 2009 storm, this was not the case in the January 2009 storm; thus, not all storms have an effect on the prevalence of HF183, which has also been seen in other studies (Parker et al., 2010). Aside from the increased frequency of detection during some rain events, no seasonal variation of HF183 was seen, which is also consistent with other studies (Tambalo et al., 2011; Schriewer et al., 2010).

HF183 was detected in roughly 15% of the samples taken in this study, which is generally in the range of the rates of detection seen in recent environmental studies (Santoro and Boehm, 2007; Ahmed et al., 2012; Walters et al., 2011; Tambalo et al., 2011). However, one location, MD, was identified as a site with potential frequent human inputs, as the frequency of detection of HF183 was far higher than at any other site. Frequency of detection has led other studies to potential sources of microbial contamination as well (Jokinen et al., 2011; Korajkic et al., 2010).

An additional input of HF183 at quantifiable levels was observed on a dry day in November in the middle Keys Channel. As HF183 was not observed on any other day at D3 and only once

during wet weather at D2, and ratios of HF183 : FIB were in the range seen in sewage, this occurrence was examined more closely to see if an illicit dumping of boat waste into this channel of the Keys could have resulted in the observed concentrations. As there is very slow flow into or out of the channels, a static control volume was assumed for the channel with dimensions of 7 m for depth, 50 m for width, and 670 m for length. A ten-gallon waste tank was assumed for the volume of waste held on the boat, and initially (Scenario 1) concentrations for FIB and HF183 were assumed to be at levels observed in wastewater (Silkie and Nelson, 2009). Calculated levels in scenario 1 were actually lower than those observed in the channel (Table 3-4). As human waste in boat systems is likely to be more concentrated than that in urban wastewater influent, Scenario 2 assumed concentrations of FIB and HF183 at 10 times that seen in wastewater influent. At these levels, calculations (1600 cps / 100mL) were quite similar to observed values (1200 and 1500 cps /100 mL). Additionally, TC and EC were present at higher concentrations in the sample with higher concentrations of HF183 (Table 3-4), which lends further support to the theory that the source of FIB and HF183 in these samples was the same.

Additional patterns emerged on days that HF183 was present at quantifiable levels (>10 cps/ uL). 71% of those sampled (5/7) had a range of HF183:FIB consistent with that seen in samples spiked with raw influent (Jay lab, unpublished). Those samples were located at MD on a few occasions, the Enclosed beach sample location, and at D2 & D3 during the dry weather November sampling event. These results may provide evidence of a recent influx of human waste, as host-associated markers have been shown to decay rapidly relative to FIB (Schulz and Childers 2011) although the persistence is slightly enhanced by salinity(Okabe and Shimazu 2007). In addition, effects of solar radiation on Bacteroidales marker persistence have been shown to be mixed; in some studies solar radiation is detrimental (Walters et al. 2009) and in others not much effect has been observed (Walters and Field, 2009; Bae and Wuertz, 2009).

5. Conclusion

- Marina Dock was identified as a potential hot spot for HF183 based on a more frequent detection rate.

- Arundell Barranca was consistently higher in FIB but not in HF183 than the other sites sampled.
- FIB levels increased across all locations during a rainstorm. Although HF183 was detected at a slightly higher frequency during wet weather, the spatial pattern was still sporadic across the sites, which may indicate different sources of these two indicators in the environment.
- Although EC and ENT were at higher levels over the course of the study when HF183 was detected, incidence of HF183 was not predictive of one of the main sources of FIB to the watershed, the Arundell Barranca.
- HF183 was detected at both sample locations in one of the Keys channels on a dry sampling day at quantifiable levels with a ratio of HF183:FIB comparable to that seen in sewage, which may be indicative of a recent influx of human sewage.

Table 3-1. Overall HF183 detection. Pink denotes DNQ, Yellow denotes Quantifiable.

	AB	ABO	BM	D1	D2	D3	D4	D5	D6	ENC	MD	MP	OP	SF
July	A	X	A	A	A	A	A	A	A	A	850	A	A	X
Aug	A	X	A	A	A	A	A	A	X	A	D	A	A	A
Sept	A	X	A	A	A	A	A	A	A	A	A	A	X	A
Oct	A	A	A	A	X	A	A	X	X	A	1800	A	A	A
Nov	A	A	A	A	1500	1200	A	A	A	A	4400	A	X	A
Dec	X	X	X	A	X	A	A	A	A	A	A	X	X	A
Jan	A	D	A	A	A	A	A	A	A	A	D	D	A	X
Feb 4	A	A	A	A	A	A	A	A	A	A	A	A	X	A
Feb 5 am	3100	A	A	A	A	A	A	D	A	A	A	A	A	A
Feb 5 pm	A	A	D	D	D	A	D	A	A	A	4700	X	A	A
Feb 6	A	A	A	A	A	A	A	A	A	D	A	A	A	D
Feb11	X	A	D	D	A	A	A	A	A	D	A	A	A	A
Mar	A	A	A	A	A	A	A	A	A	A	D	A	A	A
Apr	A	A	A	A	A	A	A	A	A	D	X	A	X	A
Frequency	8%	10%	15 %	14 %	16%	7%	7%	8%	0%	21%	54%	8%	0%	8%

Table 3-2. Exceedance rates of FIB and frequency of detection of HF183 in wet and dry weather.

Bacteria	Dry rate (%)	N	Wet rate (%)	N
TC	5	6/122	56	31 / 55
EC	2.5	3 / 122	60	33 / 55
ENT	7.4	9 / 122	60	33 / 55
HF183	12	12/100	19	10/52

Table 3-3. Mann-Whitney results for FIB grouped by presence absence HF183.

Parameter	**Presence (Mean Rank, n)**	**Absence (Mean Rank, n)**	**Mann-Whitney U**	**p-value**
Log (DO)	5.5, 4	8.91, 11	12	.192
Log (TDS)	30.6, 12	46.1, 75	289.5	.048
Log (Temp)	11.92, 6	17.56, 26	50.5	.184
Log (pH)	10.7, 5	13.6, 20	38.5	.435
Log (Nitrate)	27.8, 9	23.1, 38	205	.358
Log (Ammonia)	32.61, 9	28.93, 49	248.5	.547
Log (TSS)	37.6, 10	40.9, 7	321	.673
Log (Enterococci)	107.11, 22	83.48, 150	2103	.029
Log (*E. coli*)	108.57, 21	77.4, 141	2049	.004
Log (Total Coliforms)	96.2, 20	78.84, 141	1714	.118
Log (Total DNA)	101.57, 22	81.33, 145	1981	.067

Table 3-4. Calculated vs. Observed levels of HF183, TC, EC, and ENT in D2 and D3 in November samples

Scenario	Location	TC*	EC*	ENT*	HF183*
Observed	D2	450	10	<10	1510
Observed	D3	96	<10	<10	1240
Scenario 1	D2, D3	13	0.1	0.8	160
Scenario 2	D2, D3	130	1	8	1600

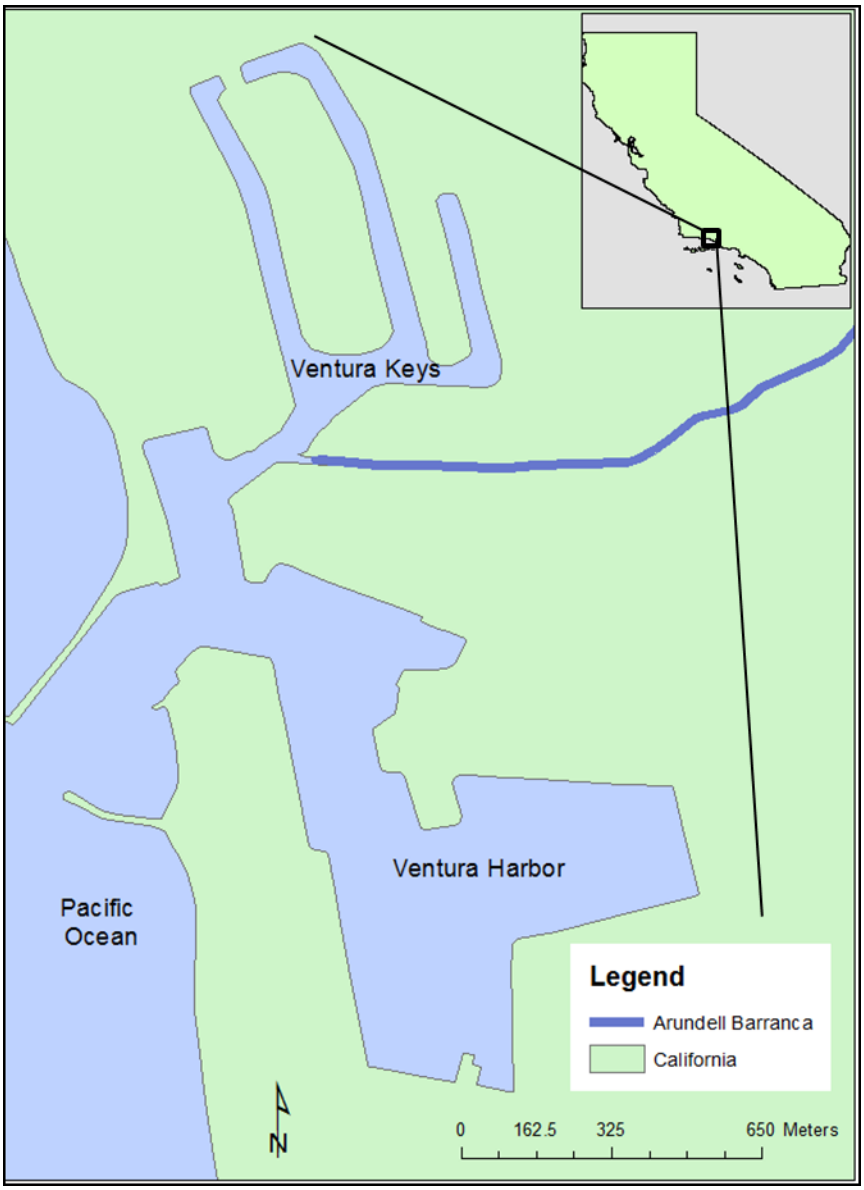

Figure 3-1. Context map of study area within California.

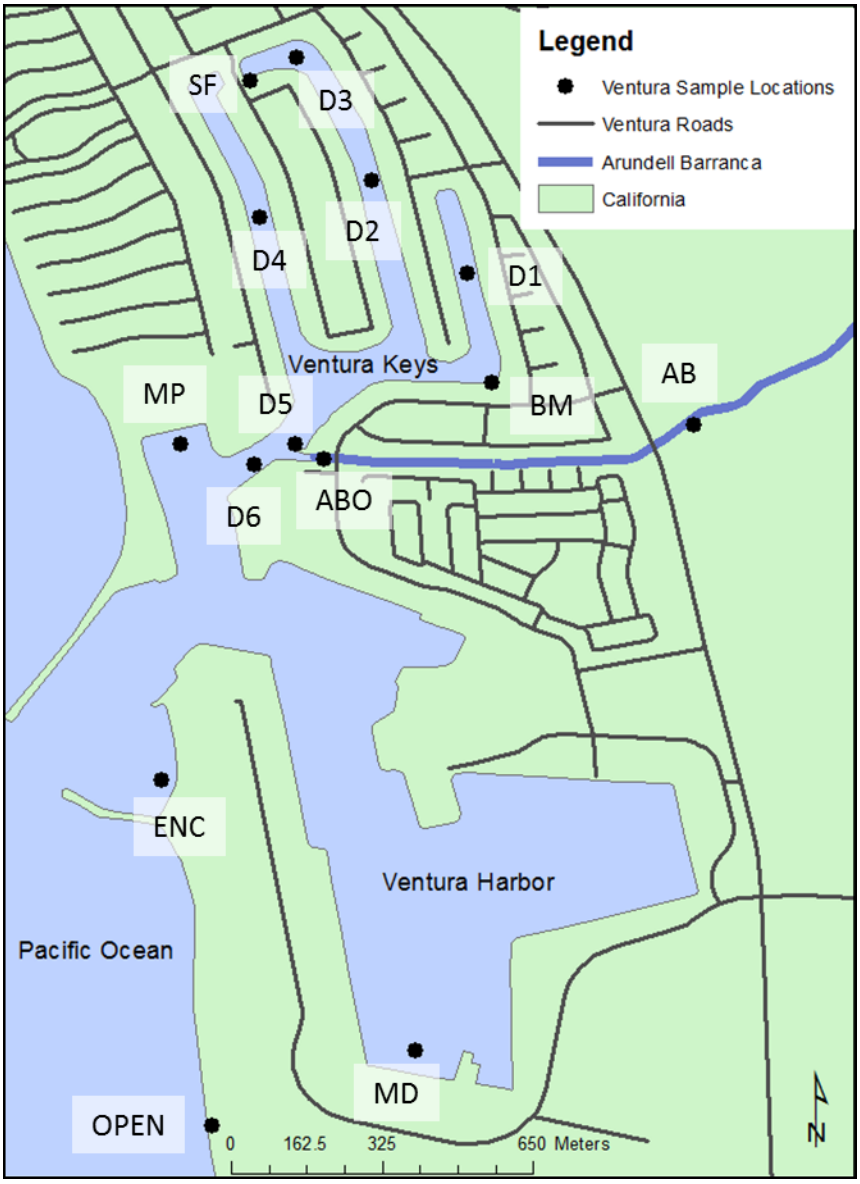

Figure 3-2. Map of sampling locations. D1-D6 = Dinghy Sample Sites; AB = Arundell Barranca; ABO = Arundell Barranca Outlet MP = Marina Park; MD = Marina Dock; SF = Keys Beach 1, @Surfrider; BM = Keys Beach 2, @Beachmont; ENC = Harbor Cove Beach; OPEN = Surfers' Knoll Beach

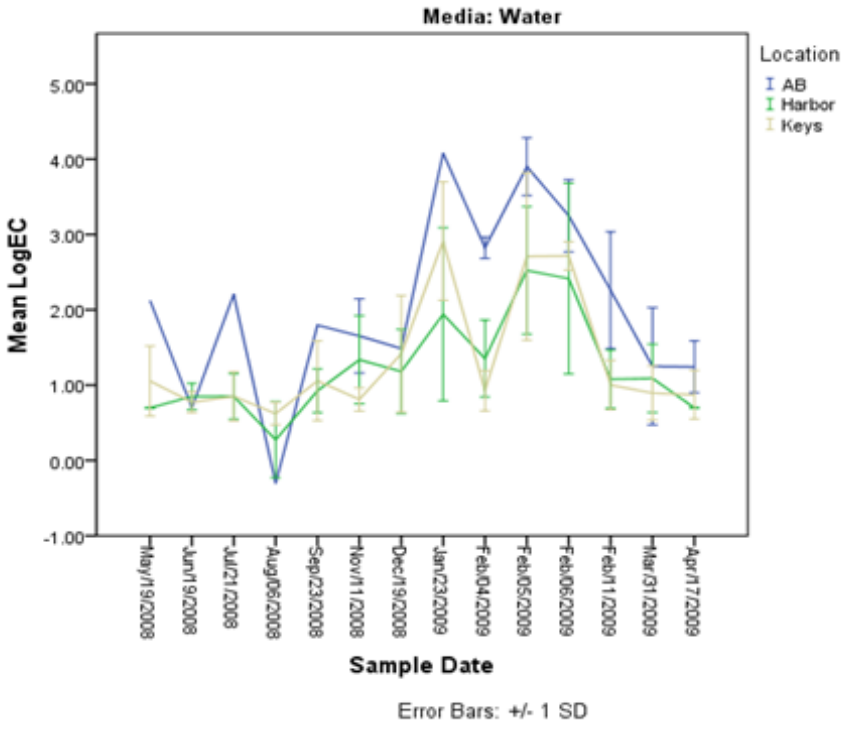

Figure 3-3. An increase in both *E. coli* and enterococci was observed during the storm time and both receded to pre-storm concentrations by the dry day. *E. coli* is shown here.

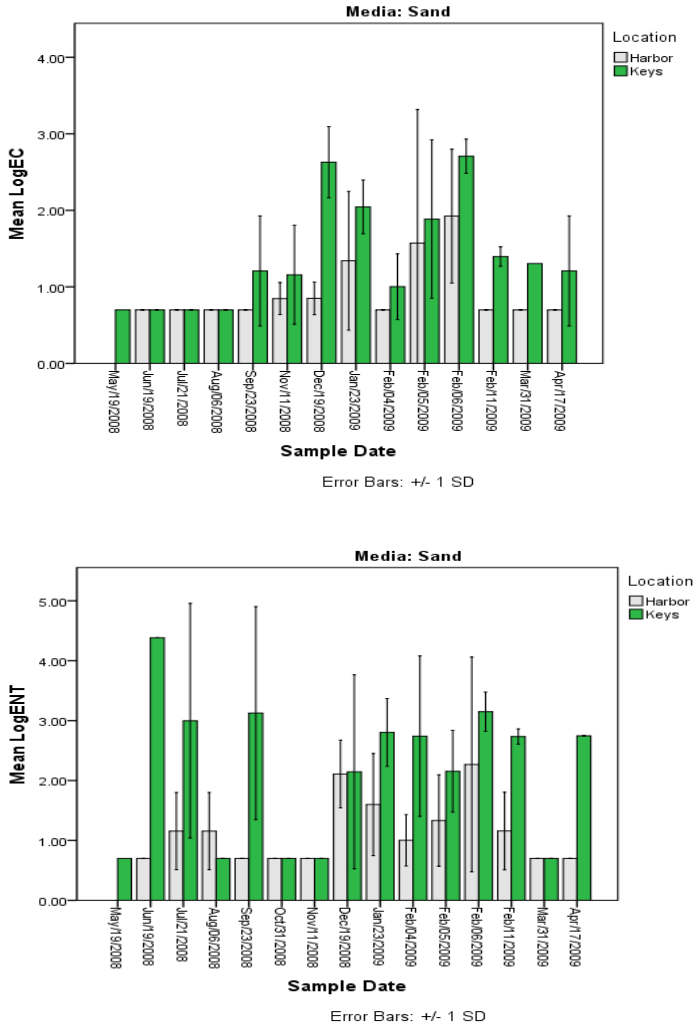

Figure 3-4. FIB levels are higher in Keys sediments than in those at Open or Enclosed beaches. A) EC, B) Enterococci.

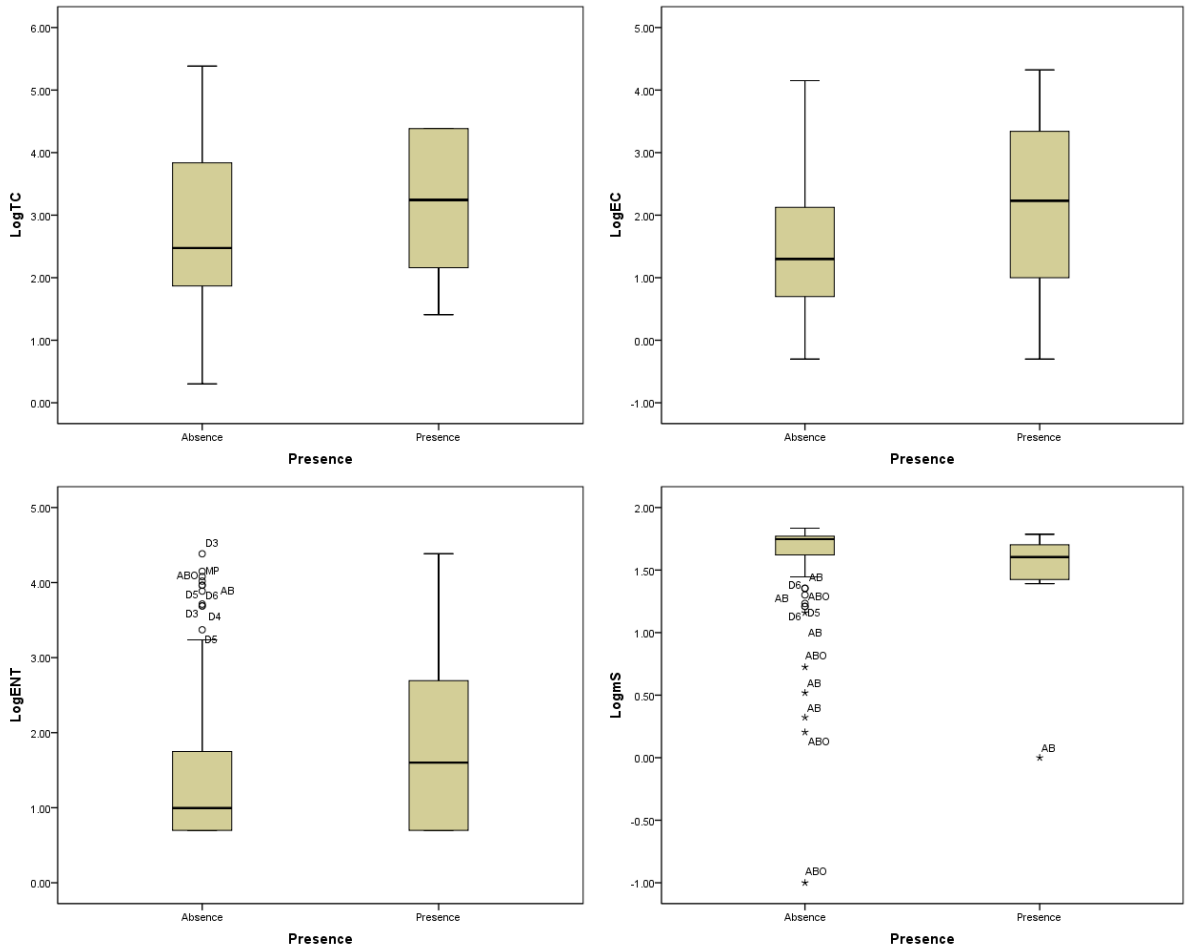

Figure 3-5. Boxplots of concentrations grouped by presence/absence of HF183 marker for A) TC, B) EC, C) ENT, and D) mS.

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CHAPTER 4. Patterns in presence of FIB and persistence of FIB and general Bacteroidales by culture-based and culture-independent methods in beach sand under a pier: A case study at Santa Monica Beach, California

Abstract

This study set out to better understand the persistent population of fecal indicator bacteria (FIB) in the sand under and around the Santa Monica pier, located in Santa Monica, CA. Three-day quarterly sampling excursions were conducted to characterize FIB concentration spatially in three different seasons. As FIB levels tended to be higher in moister sand under the pier, two microcosms were set up spanning a moisture range of 0.1% to 20% moisture to study the effects of different moisture regimes on native populations of bacteria. *E. coli* and enterococci by membrane filtration (cENT), enterococci by qPCR (qENT), and General *Bacteroidales* (GenBac) by qPCR were all measured over the course of the experiment. Interestingly, native populations habituated to sand at a moisture content of 0.1% persisted the longest at that moisture content; the addition of moisture to 7% and 14% was detrimental to their persistence. This pattern was also observed in qENT and GenBac. Finally, having observed the ability of the FIB in pier sand to persist under in-situ conditions we conducted a one-day snapshot survey under and around the pier to identify potential sources of the FIB contamination. Two human-associated markers, TaqHF183 and M2 were below detection limits, but the gull/pelican associated marker was detected at 8/12 locations despite inhibition of the assay. In this study, the more likely cause of FIB contamination appears to be environmental, related to the long persistence of the indicators in the sand under the pier and the thriving populations of birds that inhabit the region.

1. Introduction

One of the most important sources of fecal indicator bacteria (FIB) to coastal environments is the sand (Badgley et al., 2011), which has been shown to foster growth (See Appendix A for a discussion of factors affecting microbial persistence in sand) and allow FIB to persist over long periods of time. Although mechanisms controlling the exchange between the sediment and water column, such as resuspension of particles (Craig et al., 2004; An et al., 2002; Puleo et al., 2003; Grant et al., 2005) and "through-beach transport" (Boehm, 2006), have not been fully established (Boehm and Weisberg, 2005; Desmarais et al., 2002b), many studies have seen links between FIB levels in sand and water in the environment. Enterococci levels have been shown to increase in water levels by being washed out of the sand during storms (Jiang et al., 2007; Abdelzaher et al., 2010; Rogerson et al., 2003) and higher tides (Boehm et al., 2005; Abdelzaher et al., 2011; Yamahara et al., 2007). Column studies have shown that at least a percentage of enterococci in sand from the site could be mobilized by inundation with seawater (Yamahara et al., 2007; Phillips et al., 2011a). Further, sediment FIB loads have been shown to be an important input for modelling FIB values seen in the water under certain weather conditions (Sanders et al., 2005; Paul et al., 2002).

Therefore, a more developed awareness of the survival of FIB as well as the survival of other indicators in sand has become very relevant to monitoring water quality. Recent studies have seen an increased risk of gastrointestinal illness (GI) with increased exposure to sand (Heaney et al., 2012; Heaney et al., 2009). Additional studies have found evidence that sediments provide a favorable environment for pathogenic microorganisms including viruses

(Green and Lewis, 1999; Gantzer et al., 1998; Meschke and Sobsey, 1998; Ferguson et al., 1996; Gerba and Schaiberger, 1975), and bacteria such as Salmonella (Burton et al., 1987). It is very important to understand the how markers persist or decay under various environmental conditions (Santo Domingo et al., 2007; Field and Samadpour, 2007). Recent studies have detected pathogens in environmental beach sand samples (Abdelzaher et al., 2010; Yamahara et al., 2012). Some beach surveys have found the highest concentration of bacteria in dry sand well above the area wetted by tidal inputs (Shah et al., 2011; Phillips et al., 2011).

Current EPA regulations for water quality involve measuring three FIB: enterococci, *E. coli* and total coliform by either Defined Substrate Technology or membrane filtration (EPA). In early studies, FIB were shown to correlate well with the incidence of human health effects and the presence of pathogens, and this is still true in some regions. However, at many locations, in the absence of a point source, FIB have not been shown to correlate with either of these effects due to many factors, including their ability to regrow in the environment (Ksoll et al., 2007; Imamura et al., 2011), persist in sand (Mika et al., 2009; Yamahara et al., 2007) and their presence in the feces of many warm blooded animals (Ram et al., 2007).

The EPA is in the process of drafting and establishing new recreational water quality guidelines, which will include rapid, non-culture based methods such as quantitative Polymerase Chain Reaction as supplementary method options to characterize pollution at recreational beaches (EPA 2012). Currently proposed draft standards have acceptable levels of enterococci detection at 1 log higher than culture-based standards, and a recommendation

that both culture-based and culture-independent methods should be tested against each other at each location before using the more rapid culture-independent methods in lieu of culture-based (EPA 2012). Therefore, in addition to better understanding the efficacy of traditional FIB, it has become important to understand the relationships of different indicators and different methods of detecting the same FIB to find indicators that best protect human health (EPA 2000). Quantitative Polymerase Chain Reaction (qPCR) methods are rapid, getting from sample to results in less than 5 hours (Bernhard and Field, 2000a; Bernhard and Field, 2000b) and very specific. Additionally, EPA methods already exist for some, including general *Bacteroidales* (EPA Method B) and enterococci (Haugland et al., 2005, EPA Method A).

However, one potential limitation for rapid, culture-independent methods such as qPCR is that qPCR is capable of detecting DNA from all cells present, including non-viable or dead cells (Abdelzaher, 2011). Although not much is known about the fraction of live versus inactive bacteria in sediments (Barcina et al., 1997), a limited number of studies have shown non-living bacteria to be a significant portion of the biomass as live bacteria only represents between 40%-63% of the total bacterial biomass in freshwater and marine sediments (Haglund et al., 2003; Manini and Danovaro, 2006). Interestingly, enterococci as detected by both qPCR and membrane filtration and General *Bacteroidales* as detected by qPCR have both been shown to correlate with increased incidence of GI and diarrhea in recent studies (Heaney et al., 2012; Wade et al., 2008; Wade et al., 2006). Therefore both methods show potential for being good water quality indicators and as such it is important to monitor their persistence patterns in sand.

A better understanding of the persistence of enterococci qPCR in sand relative to the culture-based methods would be very helpful given the varied relationships observed in previous field studies. Furthermore, enterococci have been shown to be a better indicator of water quality in marine waters than *E. coli* (Cabelli et al., 1982), and so its persistence in sand in a marine coastal environment is even more important to explore (Jeanneau et al., 2012).

Several studies have looked at the concentrations of enterococci detected by qPCR alongside culture-based methods and found levels detected by qPCR to be higher (Heaney et al., 2012; Yamahara et al., 2012; Ferretti et al., 2011). Although levels varied, enterococci trends as detected by qPCR tended to be consistent with those detected by culture-based methods, i.e., an increase seen from one week to the next would be seen in the results from both methods (Ferretti et al., 2011). Interestingly, correlations between actual enterococci concentrations detected by these methods have been shown to be inconsistent, agreeing highly in some studies (Ferretti et al., 2011) and not in others (Shah et al., 2011; Rogers et al., 2011). Limited work has been done looking at the persistence of enterococci in beach sand. One study examined decay rates of qENT among other pathogens and indicators after spiking beach sand with influent as detected by both qPCR and membrane filtration, and found that decay is slower as detected by qPCR (Yamahara et al., 2012). However, no studies have been done to date looking at the persistence of a native population of enterococci in sand using these methods in tandem.

A group of bacteria that has shown promise as a new set of indicators for both source-associated and general fecal pollution is *Bacteroidales* (Okabe and Shimazu, 2007; Kildare et

al., 2007; Yampara-Iquise et al., 2008). Many studies recently have begun exploring the survival kinetics of *Bacteroidales* in water, and shown temperature and salinity to have a significant effect on persistence (Okabe and Shimazu, 2007) and mixed results with regard to solar insolance (Walters et al., 2009; Walters and Field, 2009; Bae and Wuertz, 2009a). However, while some survival studies have found the presence of sediment in water to enhance the persistence of the *Bacteroidales* markers, very few have measured the persistence in sediment and none have looked at the persistence of general *Bacteroidales* in unseeded sediments. Two studies have examined the persistence of several species in sediment; one measured general *Bacteroidales* and qENT alongside host-specific bacteroides in manure-amended soils (Rogers et al., 2011) and the other examined the persistence of qENT and cENT alongside several host-specific markers and culturable microorganisms in sewage-amended sand microcosms (Yamahara et al., 2012). Although some studies have shown general *Bacteroidales* to be present in environmental sediments even in pristine areas (Vierheilig et al., 2012), studies linking GenBac to GI in a coastal environment (Wade et al., 2006; Wade et al., 2008; Heaney et al., 2012) and an existent EPA method (EPA Method B) indicate that this is an important group for which to study survival kinetics at marine environments.

Identifying sources of pollution to a watershed also provides information to responsible parties that can help to more effectively target and remediate sources [i.e. identifying beaches or sites with high incidence of human associated indicators can lead to improved water quality through sewer main repairs and relocation of portable restrooms (Korajkic et al., 2010)]. Other studies using source associated markers have been useful in identifying

potential sources such as stormwater or sand (Kinzelman and McLellan, 2009), faulty sewers (Dickerson et al., 2007), land-based sources including bird-droppings and contaminated subsurface waters (Boehm et al., 2003) or cattle (Hagedorn et al., 1999). In addition to indicators which can help identify a specific source of pollution to the watershed, ratios of different markers and indicators ranging from fecal stanol ratios (Jeanneau et al., 2012) to ratios of human-associated markers to general bacteroides or FIB (Converse et al., 2009; Mika et al., submitted) have been proposed as part of a source identification toolbox to characterize pollutant source at the beach. A better understanding of the relative decay of relevant indicators of water and sand quality would provide insight on the utility of such ratios to help in source identification.

In this study, two assays for human fecal markers and one specific to seagulls and pelicans was applied on a single day snapshot to narrow down potential sources of the persistent FIB presence under the pier. The first human fecal marker assay used was Taq HF183, which has been shown to be associated with the species *Bacteroides dorei* (Haugland et al., 2010). *Bacteroides dorei* has been detected in 40% of non-human hosts (chickens and dogs) but at levels an order of magnitude lower than in human hosts (Haugland et al., 2010). The second human assay was HumM2, which demonstrated 99.2% specificity when tested against 265 fecal DNA extracts from 22 different animal species (2 false positives with chicken feces) (Shanks et al., 2009). Additionally, HumM2 detected human fecal markers in all samples of human feces and effluent tested. Finally, the gull/pelican fecal marker assay used detects *Catellicoccus marimammalium,* a bacterial species found in feces of gulls

(tested on samples from California, Georgia, Ohio, Wisconsin, and Toronto, Canada) and pelicans (Lu et al., 2008).

This study set out to understand the dynamics of FIB in the pier environs, and then examine the persistence of general *Bacteroidales* and qENT alongside culture-based methods for FIB in sediments from under the Santa Monica Pier. Spatial surveys were conducted under and adjacent to the pier in spring, summer, and fall to determine whether any seasonal factors affected the presence of FIB at this site. FIB were consistently detected at higher levels underneath the pier, so two microcosms were conducted to understand the effects of moisture on the FIB populations under the pier to see if this was a contributing factor to their persistence. In addition to culture-based methods for FIB, microcosm samples were analyzed using qPCR for general *Bacteroidales* and enterococci. To our knowledge, these are the first studies examining the decay of *Bacteroidales* in unamended beach sands. Further, we examined potential sources of the persistent FIB presence under the pier through a spatial survey using three qPCR assays, two human-associated assays and one gull/pelican-associated assay.

2. Methods

Site Description. This study was performed at Santa Monica Pier (34˚00'31.43" N, 118˚29'50.06" W), a popular tourist location in Santa Monica, California. Previous studies conducted by Jay Lab group at University of California, Los Angeles (UCLA) have shown high levels of FIB concentrations in sand underneath the Santa Monica Pier. The sample area

was a fenced off area beneath the Santa Monica Pier, which includes a pipe outlet that has been sealed shut. West of the sealed outlet was a large indented area in the sediment with moist sand, and sometimes a ponded channel of water. The indented area spans from the west gate of the enclosure to the sealed outlet. On occasion, the ponded channel has been observed to rise as high as 5 inches above the surface sediment. Additionally, it has been observed to expand as wide as 15-20 feet in some locations. It should be noted that the reconnaissance phase was not conducted during any of these infrequent ponding events.

Phases

Seasonal. The seasonal sampling plan consisted of taking sand samples over the course of one week each season. On day one sampling sites were patterned as a grid with sites outside and north of the pier and sites outside and south. Additionally, sites were tested inside the fenced area underneath the pier. The second day of sampling was at least two days later because results were unavailable for 24 hours (the incubation time for enumerations). Day two's grid pattern was tightened around areas of higher FIB concentrations from day 1; this resulted in a sampling grid that was fully underneath the Santa Monica Pier. Again, results required a 24 hour incubation period before a third day of sampling could be conducted. Day three's grid was tightened around the locations of higher FIB levels in day two. The reconnaissance phase revealed locations of higher FIB concentrations in the study area, to better understand the dynamics of the site over time.

Microcosms. Two moisture content microcosms were designed to specifically test the influence of moisture content on FIB persistence in sediments from underneath the Santa Monica Pier. Sediment was collected using a small spade that had previously been rinsed with a 70% ethanol solution, wiped with a paper towel, and allowed to dry. For the first microcosm, a composite was formed from sediment samples collected from various sites underneath the pier where reconnaissance had seen consistently higher levels of FIB concentrations. As these sites were generally near the channel, well underneath the pier and wetted intermittently from the channel, the in-situ moisture content of the sand composit was 10%. For the second microcosm, sand was collected from a drier region under the pier with consistently high levels of FIB in order to examine the effect of moisture on FIB accustomed to drier conditions.

The sediment was then returned to UCLA for initial resting and held on ice overnight to allow for the processing of the initial test results. The following day, the sediment was partitioned into sterile quart sized plastic zipper bags (100g of sediment per bag). A 100X diluted phosphate-buffered saline (PBS) with pH 7.0±0.2 was used to increase the moisture content of the sediment in the bags. Each mL of 100X diluted PBS was resulted in an increase of 1% moisture to the sediment. The initial microcosm moisture content examined 10% (in-situ conditions) and 20%, and the second microcosm examined 0.1% (in-situ conditions), 7%, and 14% moisture. After adding the appropriate amount of 100X diluted PBS (i.e. 10mL of 100% diluted PBS to attain 20% moisture content), the bags were shaken to homogenize the moisture throughout the sediment. Each bag was left slightly open to

maintain aerobic conditions but minimize evaporation. At each time-point, FIB was measured in four replicates for each moisture content. In order to preserve undisturbed conditions, each bag was sacrificed after sampling. Fifty bags were prepared for each moisture regime. Moisture contents were monitored over the course of the experiments to ensure that the moisture content was staying fairly consistent for all sampling timepoints. Moisture contents stayed consistent with less than 0.4% variation for all moisture contents measured in May (Table 4-1).

*Sand Sampling***.** Sediment was extracted at sites during the seasonal sampling using sterile 50 ml Falcon Tubes (BD BioSciences, USA) by sterile gloved hands. The upper 1.5 cm of sediment from the surface of the site was sampled with this method. Each site was sampled in duplicate in order to achieve a more representative enumeration for the site. The sediment was then returned to UCLA and processed within 6 hours.

FIB Measurement. The reconnaissance and conditions study microcosm sediment samples were brought back to UCLA to be processed and analyzed. The moisture study microcosm sediment was already present in lab and was analyzed on location. An aliquot of 10 g of sediment was weighed into sterile 120 mL Nalgene bottles and 50 mL of PBS (pH 7.0+0.2) was dispensed into the bottle. The sediment and PBS mixture was shaken vigorously by hand for two minutes and allowed to rest and settle for one minute. Supernatant from the bottle was then transferred into a separate sterile 120 mL Nalgene bottle. Another 50 mL of PBS was poured into the same 10 g aliquot of sediment and the mixture was shaken for an

additional two minutes. Another minute was given to settle before the second 50 mL of supernatant was decanted into the same secondary bottle. The result is a "wash" of 100 mL from 10 g of sediment and was used as a proxy for FIB in water enumeration.

Membrane filtration was conducted according to EPA method 1600 (US EPA 2006) for enterococci in groundwater using the wash proxy for sediment enumeration. For reconnaissance phase in groundwater, each sample was filtered at 2 different volumes (0.5 mL and 10 mL); sediment wash volumes filtered were 0.2 mL and 5 mL. During the moisture content microcosm phase, the sediment samples were sampled at four volumes for day zero (5 mL, 1 mL, 0.1 mL, and 0.01 mL); only two volumes were sampled on subsequent days which were changed according to the die-off rates of the two bacterial groups.

The water and wash were filtered through 47 mm FisherBrand polycarbonate membranes with pore size of 45 μm. Filter membranes were then placed on membrane-Enterococcus Indoxyl-ß-D-Glucoside Agar (mEI) in 15x60 mm plastic Petri dishes. The plates with filters were then incubated for 24 hours at 41°C and all colonies with visible blue halos were considered as one CFU of enterococci. *E. coli* were enumerated in a similar fashion, in agreement with EPA method 1603 (US EPA 2002). Volumes filtered and filter types were the same as for enterococci. Filters were placed on membrane-Thermotolerant *E. coli* Agar (mTEC) in 15x60 mm plastic Petri dishes. The filters and plates were incubated at 35°C for 2 hours and moved to a 45°C incubator for 22-24 hours. All colonies with visible magenta halos were enumerated as *E. coli.* Both the *Enterococci* and *E. coli* plates were counted by hand. FIB concentrations (per 100 g dry sediment) were calculated in log10 form according to the following:

$$FIB\ Concentration = \log_{10}\left(\frac{CFU}{100\ mL\ wash} \times \frac{100mL\ wash}{10\ g\ wet\ sediment} \times WtD \times \frac{100}{100}\right)$$

Moisture Content Determination. Sediment was also processed for moisture content using a wet-to-dry ratio method. An aluminum weigh boat was first weighed to obtain the "Boat Weight". Sampled sediment was then added to the aluminum boat for a total of between 6.0 – 10.0 grams (Boat Weight and moist sediment weight). The aluminum boat with sediment was then placed in a 100°C furnace for 24 hours for the moisture to evaporate. The dried sediment and aluminum boat were then cooled in a desiccation vessel for 5 – 10 minutes. After cooling, the boat and dried sediment were then weighed. The wet-to-dry ratio was obtained through the three weights: (WtD) = (Wb+w - Wb) / (Wb+d - Wb). In this equation, WtD is the wet-to-dry ratio, Wb is the aluminum boat weight, Wb+w is the weight of the aluminum boat and wet sediment, and Wb+d is the weight of the aluminum boat and dried sediment.

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

qPCR Analyses. Sediment samples from the source-specific survey were analyzed through qPCR for two human fecal markers and one gull/pelican fecal marker. The two human assays consisted of: 1) Taq HF183, which has been shown to be associated with the species *Bacteroides dorei* (Haugland et al., 2010) and 2) HumM2, which demonstrated 99.2% specificity when tested against 265 fecal DNA extracts from 22 different animal species (2 false positives with chicken feces) (Shanks et al., 2009). The gull/pelican fecal marker assay used detects *Catellicoccus marimammalium,* a bacterial species found in feces of gulls (tested on samples from California, Georgia, Ohio, Wisconsin, and Toronto, Canada) and pelicans (Lu et al., 2008). Sediment samples from the microcosm were analyzed for qPCR for general *Bacteroidales* species, which detects many of the *Bacteroidales* subgroups (EPA Method B, 2010).

DNA was amplified with the primers, probes, and master mixes as described in the source papers for each assay. Thermal cycles were also inputted as in the source papers for each assay. Samples were quantified using the machine's software and algorithm. A six-point standard curve using specific plasmids or genomic DNA, depending on the assay, was run in duplicate alongside samples on every plate. The presence of environmental interference was assessed by running samples spiked with 2×10^3 copies of the standard alongside the sample triplicates. Samples were initially run at 5x dilutions. Samples that showed a recovery efficiency less than 30% were rerun at 25x and 125x dilution. All microcosm samples were able to be diluted past significant interference. Recovery efficiency was measured as in Bell 2009 according to the following equation:

$$RE = [(\text{measured copies in sample spiked with } 2 \times 10^3 \text{ copies} - \text{measured copies in unspiked sediment sample})/(2 \times 10^3)] \times 100.$$

Statistical Analyses. After log 10 or natural log-transforming data (for survey samples and microcosms, respectively) data were determined to be normal by examining Q-Q plots. Accordingly, parametric tests were run to analyze results. T-tests were used in order to determine the difference between parameters measured at different locations or by different methods.

ANOVA analyses were run in order to determine significant differences among moisture contents during the microcosms. Decay rates were calculated using the Chick-Watson model: $C(t) = C_0e^k(t)$. In this equation, C(t) is the concentration at a point in time, C_0 is the starting concentration at time zero, and k is the first order decay constant. A linear curve was fit to the natural log transformed normalized concentration $\ln(C/ C_0)$ and time to obtain decay rates as in (Yamahara et al., 2012). T_{90}'s were also calculated using the Chick-Watson model.

During the source-specific snapshot, binary logistic regression was used in order to determine significant relationships between presence/absences and concentrations. Pearson's correlation tests were run to assess correlations between microbial indicators and genetic markers. Unpaired t-tests were used to determine relationships between concentrations underneath and outside of the pier.

3. Results - Seasonal Reconnaissance:

FIB results. Results over the course of all three seasonal surveys tended to be similar regardless of the season. FIB was consistently higher in sand underneath the pier than that collected from adjacent to the pier (Mann-Whitney, $p<0.05$) on the first sampling events, so later events focused under the pier. Enterococci and *E. coli* concentrations under the pier ranged from 10^4 to 10^6

CFU / 100 grams as compared to $10^{1.2}$ to $10^{2.5}$ CFU / 100 grams with one exception. In both samples collected in dry sand outside of the pier on the south side, FIB levels were quite high, from 10^3 to 10^4 CFU / 100 grams. This area was a known issue, as stormwater occasionally overflows and ponds in this area. Another possible explanation for the high numbers observed were the numerous sea gulls and pigeons that were observed around the pier.

Overall, concentrations of FIB tended to be higher when sand was moister (See Figure 4-1). Up through 7% moisture, FIB concentrations were at or below around 10,000 CFU / 100 grams of dry sand. Above 7% moisture, the FIB levels in sand were between 15,000 CFU / 100 grams of dry sand and 30,000 CFU / 100 grams of dry sand. Large standard deviations were observed across all these binned groups, as there was a large variability of FIB concentrations within the groups, but the general trend was for higher FIB concentrations to be associated with moister sand. Above 20% moisture, sand was fully saturated.

Correlations between the wet/dry ratio, or percent moisture, were examined to further develop the connection between moisture and FIB content. The data plotted is from all seasonal samplings. Similar moisture contents (1%-60%) and FIB concentrations were present across all seasons. Wetter sand tended to have higher levels of FIB (>10^4 CFU / 100 grams of dry sand). Drier sand tended to have lower FIB levels, but occasionally had high levels.

May Microcosm 10% - 20% Moisture Content. FIB concentrations, general *Bacteroidales* concentrations, and enterococci by qPCR were measured for all time-points during the course of this microcosm. Initial concentrations of GenBac were at 1.3×10^6 cps /100 grams, qENT was at 9.26×10^6 cps / 100 grams, cENT was at 2.5×10^5 cps /100 grams, and *E. coli* were at 5×10^5 CFU

/100 grams. The in-situ moisture content of the sand at time of collection was 10% (Table 4-1). Slopes of GenBac, qENT and cENT were not significantly different from zero over the course of the microcosm ($p>0.05$), nor were they significantly different between moisture contents (Figure 4-2A, 4-2C). *E. coli* behaved very differently (Figure 4-2B, 4-2D) from GenBac, qENT or cENT, exhibiting rapid decay in both moisture contents (k=-0.716 in 10% and -0.656 in 20%) (Table 4-2), but they were not significantly different from each other.

October Microcosm: *0.1%-14% Moisture Content.* A second microcosm was done to examine the effects of moisture on the survival of a native population of bacteria accustomed to living in dry sand (0.1% moisture). Moisture contents studied in this microcosm were 0.1%, 7%, and 14% moisture; variation in moisture content over the course of the study was at or below 1.1% (Table 4-1). Initial GenBac concentrations were 1.8×10^7 cps /100 grams in this study, which was slightly higher than those seen in May. FIB levels by way of contrast started slightly lower, with EC at 4.3×10^4 CFU / 100 grams and cENT at 1.3×10^4 CFU / 100 grams. qENT initial concentration was 2.4×10^ cps /100 grams, which was similar to the May study. Interestingly, T_{90}'s were longer for *E. coli* for all three moisture contents (range from 13.9 to 32.9 days for *E. coli* in October as compared to 3.2 to 3.5 days in May) (Table 4-2).

GenBac, qENT, and cENT exhibited similar survival patterns in this microcosm, with both persisting the longest in the driest sand (slopes were not significantly different than 0 for either one) and decaying more quickly in the wetter sediments (Figure 4-3A, 4-3C). Although more decay in both the GenBac and qENT markers was visible in the 7% and 14% moisture contents (See Figure 4-3B, 4-3D), the slopes were not significantly different from zero. Decay

patterns in GenBac and qENT were also similar; patterns were different between 0.1% and both wetter sand types ($p<0.05$), but 7% and 14% were not different from each other. For cENT, significant decay rates were observed in both 7% and 14% moisture contents (k=-.304 and k = -.219, respectively) (Table 4-2). Decay patterns in cENT were only significantly different between 0.1% and 14% moisture contents. *E. coli* persistence was less clearly affected by moisture contents in this range than the others. *E. coli* decayed the fastest, at the only statistically significant rate, in the 7% moisture microcosm (k= -0.176, p=.005) (Figure 4-3B, 4-3D). Although slopes were not statistically significant, *E. coli* appeared to persist the best in the driest sand, but better in the 14% moisture microcosm than the 7%. This is further seen in that *E. coli* is only significantly different between the 0.1% and the 7% moisture conditions ($p<0.05$). In addition T_{90}'s were the longest in the 0.1% moisture followed by the 14% then the 7% (T_{90}'s of 32.9, 24.5. and 13.1, respectively).

Results across both microcosms. Although in both microcosms, qENT tended to be higher than cENT, this difference was more pronounced in the drier microcosms than wetter. Initial ratios of cENT:GenBac and EC:GB varied widely across both microcosms. In the first, initial ratios were 3.5e-1 and 1.9e-1, respectively. In the second microcosm, taken from a location with dry sand, cENT:GenBac and EC:GenBac ratios were orders of magnitude lower at the starting point of the October microcosm, at 2.4e-3 and 7.8e-4, respectively. Interestingly, although trends in qENT and cENT were similar in both microcosms the correlation between measured concentrations detected by the two methods were very different across microcosms. In October, cENT and

qENT were very well correlated (adj. r2=.585, p=.000, Figure 4-4A), while in May they were uncorrelated (adj r-sq = -.034, p=.517, Figure 4-4B).

Source-specific Snapshot Study. One other important piece of information regarding the bacterial population under the pier, in addition to the spatial component, is what the potential sources of this contamination may be, and whether there is an area that contains high levels of source-specific bacteria. FIB results from this sampling excursion were similar to those observed over the course of the seasonal studies, with FIB levels generally being higher under the pier than outside of the pier, with one exception: Site B, which is located on the south side of the pier near the drop-off to the water level, was also very high in FIB (Figure 4-5). This is a known, occasional issue that occurs due to some pooling of stormwater there after a rain storm.

Although EC, cENT, qENT, and GenBac were all higher beneath the pier than outside of it during this source survey, only GenBac was significantly different (unpaired t-test, p=0.001). GenBac was not correlated with either cENT or EC. The strongest correlation was between cENT and EC (r2=.649, p=.002). qENT had significant but weaker positive correlations with cENT (r2=.361,p=.023), EC (r2=.307, p=.036), and GenBac (r2=.403, p=.016). Binary logistic regression found no relationship between the presence of the gull marker and any other measured indicator, which isn't surprising given the high rate of detection and the relatively small n. Although cENT and qENT were correlated, qENT detected significantly higher numbers than cENT (paired t-test, $p<0.05$).

Interestingly, human fecal markers were below the detection limit of both human assays for all samples measured. However, despite the presence of significant inhibition with the qPCR assay, gull fecal markers were detected at 8/12 sites both underneath and adjacent to the pier

(Figure 4-6). Due to the presence of inhibition, negative results do not necessarily preclude the presence of gull feces. Notably, sample location B, which is located outside of the pier but had high levels of FIB in sand also had gull markers. Since human fecal markers were below detection limits under the pier and gull fecal markers were detected under the pier it is likely that gull/pelican fecal matter is a potential source of FIB pollution to this area.

4. Discussion.

Survival kinetics and persistence. These microcosms studied the survival of native bacterial populations across a range of moistures (0.1%-20%) that is consistent with moisture levels found in site surveys at different beaches (Yamahara et al., 2012; Shah et al., 2011). Sustained moisture negatively impacted the survival of both the culture-based and qPCR measurements in the microcosm starting with native populations starting at 0.1%, while effects were less pronounced between 10% and 20% in a population that started at 10%. These results are different than those observed in other sediment microcosms in which moisture has a beneficial effect on the survival of many of the bacterial populations (Mika et al., 2009; Desmarais et al., 2002b; Yamahara et al., 2012). However, these studies were studying the survival of bacterial populations spiked into sand or sediment, either through pure culture or sewage, which may explain the difference in behavior observed. Different populations of bacteria survive differently based on their sources. If the bacteria originate from a nutrient-rich environment such as wastewater they often maintain at a constant population for a short period of time before decay begins; if they come from a nutrient-poor environment decay can begin faster.

Working with native populations of bacteria that have adapted to their environs can potentially provide more information about their ability to survive without the additional parameters such as nutrients and different bacterial composition that adding influent adds. However, it is not then always possible to study the survival of source-specific bacteria that may not be naturally present in the native population at high enough levels to understand their decay rates. Decay constants for many of the species measured were not significantly different from zero under the conditions represented in both microcosms. Similar results have been seen for enterococci in cow manure-amended soils that were detectable for greater than 120 days (Rogers et al., 2011) and enterococci in beach sands (Feng et al., 2010). This indicates an ability to persist for very long periods of time even in the absence of new inputs, which shows that bacteria are capable of adapting well to in-situ conditions. Long persistence times of naturalized bacterial populations may explain in part why FIB and other microbes are often found at the highest levels in drier sand at the beach then in wetter sand (Abdelzaher et al., 2010). In one field study, ENT detected by IDEXX, membrane filtration, and qPCR as well as fecal coliforms were seen to have a strong inverse correlation with moisture(Shah et al., 2011). However, this pattern can vary among different dryness levels and different indicators. Among drier sands (in a range from 0.08-20.4%), those with higher moisture contents had higher levels of indicators (Yamahara et al., 2012). This relationship with is not always observed for pathogens; for example, C. perfringens was observed to have higher concentrations in the wetter sands in the same paper which saw the opposite result for many other microbes (Shah et al., 2011).

Decay rates. Additionally, it is important to understand the ability of a potential water quality marker to persist in the environment relative to pathogens or indicators that have been shown to correlate with health risks (Santo Domingo et al., 2007). An organism that persists longer than its corollary is conservative, as it can be detected after the indicator it represents has disappeared from the environment. An organism that persists less time is not conservative for the presence of its corollary but can be very useful if it is highly source-specific to provide information about recent inputs to the system. The relationship of GenBac to currently used FIB indicators changed slightly depending on the moisture contents but tended to be a conservative marker. For example, in the 7% sand, GenBac is conservative as the T_{90} for *E. coli* was 22.4 days and the decay rate for GenBac was not significantly different from zero. In other cases, GenBac was consistent with the patterns of FIB, particularly with cENT and qENT.

Generally, decay rates for EC, cENT, qENT, and GenBac were either unaffected or increased by the addition of moisture to the beach sand, which is different than the effects observed in other studies using sediments spiked with pure cultures or sewage. In many of those studies looking at culturable FIB, the addition of moisture results in either an increase in concentration or no effect (Yamahara et al., 2012; Yamahara et al., 2009; Mika et al., 2009; Desmarais et al., 2002). Limited studies have been done looking at survival in sediments using qPCR methods but decay rates in qENT for 10%, 20%, and 0.1% moisture contents were 0±0.05, which is similar to those seen in sewage-amended beach sands Yamahara (2012) and Rogers (2011) in swine-amended soils. Decay rates for GenBac were not statistically significant from zero (range from -0.15 to 0.10), which is within the rates observed in Rogers (2011) for swine manure amended soil (-.04 to -.09) but different than those observed in bovine manure amended

soil. In addition to differences in decay rates among various sources of fecal pollution, differences can arise from the impacts of heterogeneous populations that inhabit sand as well. One possible explanation for the decreased survival observed with the addition of moisture to this microcosm is that the addition of moisture enhanced growth conditions for competing bacterial populations in the sand or increased the motility of protozoa in the beach sand. However, the former is more likely, as beach sand has been shown to generally harbor a fairly limited protozoan population (Feng et al., 2010; Wieltschnig et al., 2003; Kirschner and Velimirov, 1999) and a recent study found predation to have minimal impact on the decay rates of *E. coli* and enterococci(Feng et al., 2010). Many studies have shown competition with autochthonous bacterial species in the sand to have a detrimental effect on the concentrations of indicator bacteria (Hartz et al., 2008; Medema et al., 1997; Andrews et al., 2004).

qPCR/culture-based correlations. Although patterns in persistence were similar between cENT and qENT, a wide range in correlations between the concentrations measured by qENT and cENT was observed in both the microcosms and the source-specific survey (adj r-sq from -.03 to .585). qPCR decay rates were always observed to be slower in the microcosms than culture-based but the behavior was similar. Correlations comparing results from enterococci culture and qPCR-based methods in the field (Shah et al., 2011; Ferretti et al., 2011) as well as in in microcosms (Yamahara et al., 2012; Rogers et al., 2011) have been mixed. However, as both health risks (Heaney et al., 2012; Wade et al., 2008; Wade et al., 2006) and relative information about beaches' contamination are fairly well related for both method types (Ferretti et al., 2011), qPCR offers information on a much faster time frame for posting beaches which is more

beneficial to protect public health. One concern with using qPCR can be with the potential for high detection limits, efficiency problems, or interference that could result in many false negatives, especially for pathogens with low infectivity doses (Rogers et al., 2011). However, working with bacterial groups such as GenBac and qENT minimizes this problem, as they tend to be present at high concentrations as opposed to the lower concentrations of very source-specific assays (Shah et al., 2011). Interestingly, recent health studies in sand have shown GenBac and enterococci by both qPCR and membrane filtration to be better correlated with increased health risk than with fecal Bacteroidales (Heaney et al., 2012), a qPCR assay which detects a tightly related group of *Bacteroides* that are more human-associated (Converse et al., 2009).

Generally, qENT was between 1 and 2 orders of magnitude higher than cENT in both our microcosm measurements and source-specific study, which is also consistent with that seen in other studies (Yamahara et al., 2012) although other field studies have not seen a significant difference between enterococci detection in sand as detected by qPCR, mf, or IDEXX (Shah et al., 2011). Ratios of culture-based : culture-independent assays were also examined, as one would expect measurements based on viable populations to decrease more rapidly than those that measure both viable and non-viable cell populations. DNA degradation has been shown to be slower than die-off of culture-based measurements (Walters et al., 2009). One microcosm experiment looking at survival of different indicators found that the ratio of tENT : cENT steadily increased over time during the microcosm, which provided evidence of the more rapid decay of the culture-based measurements relative to non-culture based (Yamahara et al., 2012). This is not the case in either microcosm in this study although generally the lower numbers of

cENT were in the later days of microcosm (Figure 4A, 4B). However, in some instances the ratio of culture-based:qPCR was greater at the end of the experiment than at the start (i.e. day 14, EC:GB = 1.3e-2 compared to initial ratios of 2.4e-3) or spiked upward at certain time points (i.e. day 3, cENT:GB is 8.1 vs. 1.9e-1 at the initial time point. This could be explained by part by the shorter duration of our experiments – perhaps after 20 or 30 days we would start to see these trends as well, or by the increased ability of native bacteria to enter and exit a VBNC state as conditions permit, or just a generally increased ability to persist in the environment relative to that of microorganisms acclimated to living in influent.

Source Tracking. Microbial source tracking is a rapidly expanding field of research that seeks to better characterize and remediate persistently impaired beaches by tracking pollution to specific sources, whether they be point or non-point, if they are present. Many types of information have been proposed as part of a microbial source tracking toolbox, including recently developed molecular assays such as qPCR that are highly source specific and the ratios of different indicators in the environment (Converse et al., 2009)(cite cite). In this study, after establishing a persistent spatial pattern of FIB under and around the pier across seasons, a one day snapshot sampling excursion was conducted to examine whether there were any source-specific hotspots that might further explain potential sources of this contamination.

Both human associated markers were below detection limits at all locations sampled near the pier, but despite significant inhibition with the qPCR assay the gull/pelican marker was detected at 8 / 12 sites both under and next to the pier. qENT, GenBac, *E. coli,* and cENT were all higher on average under the pier, but only GenBac was significantly higher. qENT generally

correlated with all other indicators measured (R^2 = 0.31 to 0.43), while GenBac didn't correlate with either culture-based FIB measurements. Mixed correlation results between indicators have been seen in other studies as well (Yamahara et al., 2012; Shah et al., 2011).

Although the concentrations of qENT were 1-2 orders of magnitude higher than those of cENT on average, the ratio of cENT to qENT at each site provides some further information. At all but one of the locations tested (water samples under pier were not used for qENT analysis due to poor recovery efficiency), the ratio of cENT:qENT was less than 10^{-3} CFU / cps. However, at Site B, which had unusually high FIB levels for a location outside of the pier, the ratio was 0.75. While these ratios do not provide any source specific information, they may provide evidence of the occurrence of more recent pollution event (i.e. stormwater flooding at Site B). Although there are alternative methods being developed to incorporate viability into qPCR assays such as detecting rRNA or PMA/qPCR (Bae and Wuertz, 2009a; Nocker et al., 2007; Bae and Wuertz, 2009b), using a ratio of cENT and qENT, both of which are likely to be included in the new standards for recreational water quality, potentially offers a more cost-effective way to obtain similar information. In this study, the more likely cause of FIB contamination appears to be environmental, related to the long persistence of the indicators in the sand under the pier and the thriving populations of pigeons and seagulls that inhabit the region around the pier.

5. Conclusions

- FIB concentrations are extremely variable in sediment both spatially and temporally, but influenced by the presence of moisture and sunlight based on results from spatial surveys.

Due to shading, moisture, or wetting/drying cycles, FIB levels under the Pier tend to be higher than outside of the pier in both sand and water.

- Seagulls and pelicans appear to be a likely source of FIB contamination under the pier as no genetic markers for human feces were detectable in samples under and next to the pier but genetic markers for gull feces were found at 8/12 locations.
- Native populations of qENT, cENT, and GenBac decay similarly to each other in moisture ranges from 0.1%-14%
- The addition of moisture to very dry sand (0.1%) tended to decrease the persistence of EC, cENT, qENT, and GenBac as compared to those that stayed at 0.1% moisture.

Table 4-1. Moisture content variation over the course of the microcosm experiment.

Date	Predicted Moisture (%)	Experiment Length (Days)	Mean Actual Moisture Content (%)	Standard Deviation (%)
May	10	5	9.7	.24
May	20	5	20.2	.39
October	0.1	15	0.15	.06
October	7	15	6.4	1.6
October	14	15	14.1	1.1

Table 4-2. Microbial indicator and genetic marker decay rates and T_{90}

Month	Moisture	Microbe	Co, mean (SD)	T90	K1 (day-1)	R2
MAY	10%	EC	475,000 (54,000)	3.2	-.716	.964
		GenBac	1.34E6 (1E6)	N/A	0.108	.215
		cEnt	251000 (28600)	31	-.074	.100
		qEnt	9,182,214 (2,000,744)	N/A	-.0269	.025
	20%	EC	475,000	3.5	-.656	.978
		GenBac	1.34E6 (1E6)	N/A	0.30	.403
		cEnt	251000 (28600)	N/A	0.098	.057
		qEnt	9,182,214 (2,000,744)	N/A	.0482	.059
OCT	0.1%	EC	43,100 (39,000)	32.9	-.070	.631
		GenBac	1.79E7 (2.45E6)	24.3	-.0947	.970
		cEnt	13,900 (12,500)	43.6	-.0528	.210
		qEnt	2,444,526 (354,659)	60.3	-.0382	.385
	7%	EC	43,100 (39,000)	13.09	-.1758	.521
		GenBac	1.79E7 (2.45E6)	22.3	-.1032	.6306
		cEnt	13,900 (12,500)	7.57	-.304	.9278
		qEnt	2,444,526 (354,659)	15.76	-.1461	.9501
	14%	EC	43,100 (39,000)	24.5	-.0941	.4703
		GenBac	1.79E7 (2.45E6)	15.2	-.1515	.7418
		cEnt	13,900 (12,500)	10.5	-.2192	.9246
		qEnt	2,444,526 (354,659)	14.18	-.1624	.9366

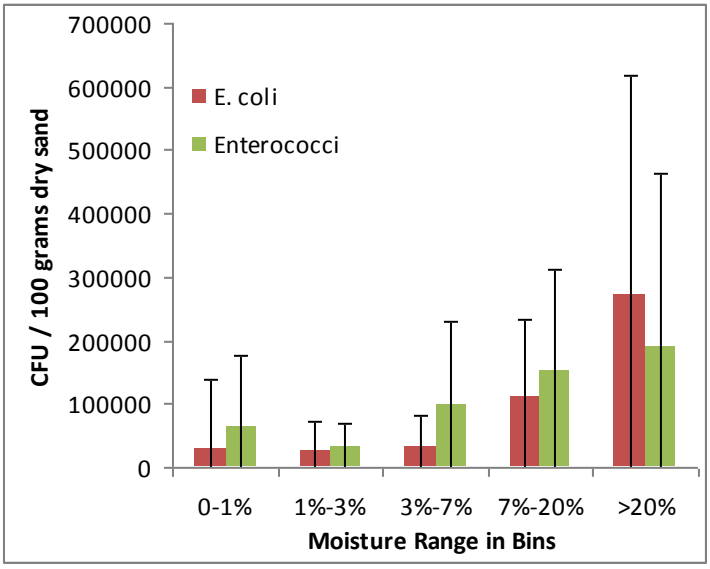

Figure 4-1. Moisture percentage and FIB concentration. Error bars show standard deviation.

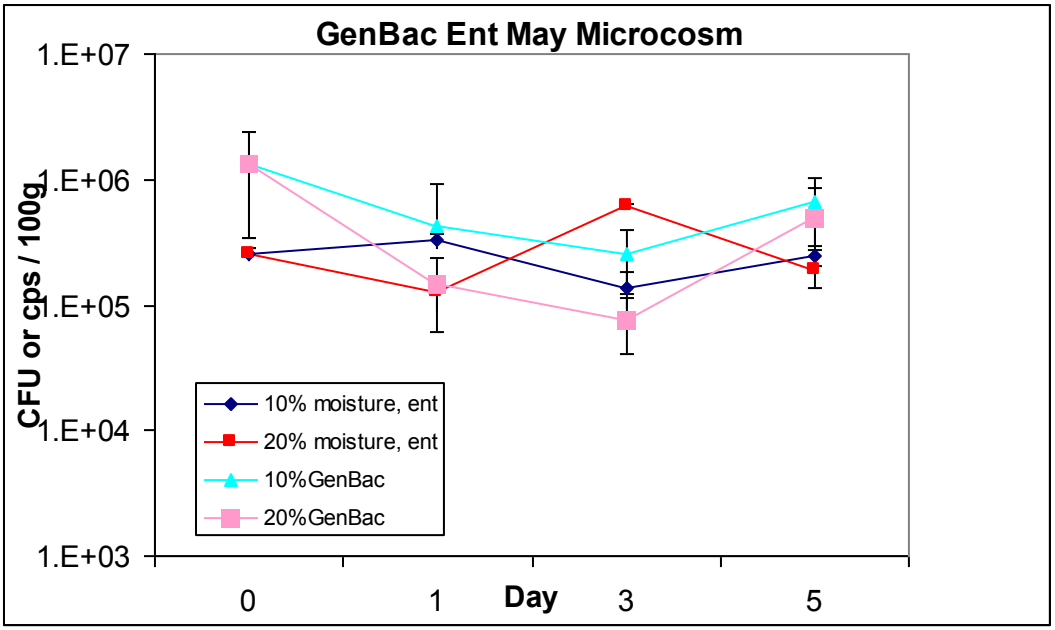

Figure 4-2A. Survival of enterococci and general *Bacteroidales* marker over course of May microcosm.

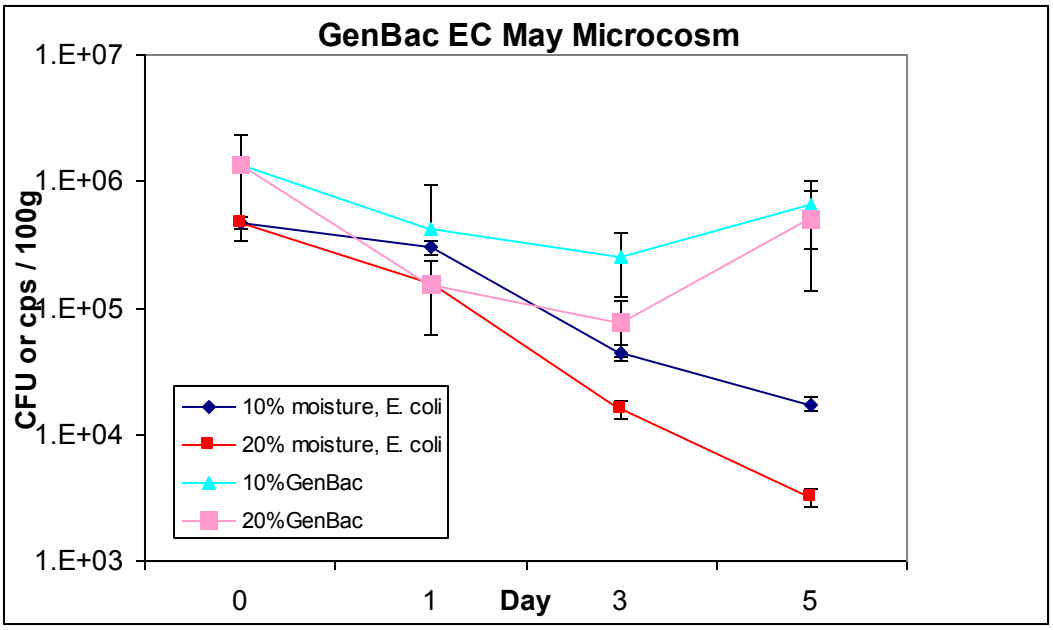

Figure 4-2B. Survival of *E. coli* and general *Bacteroidales* marker over time.

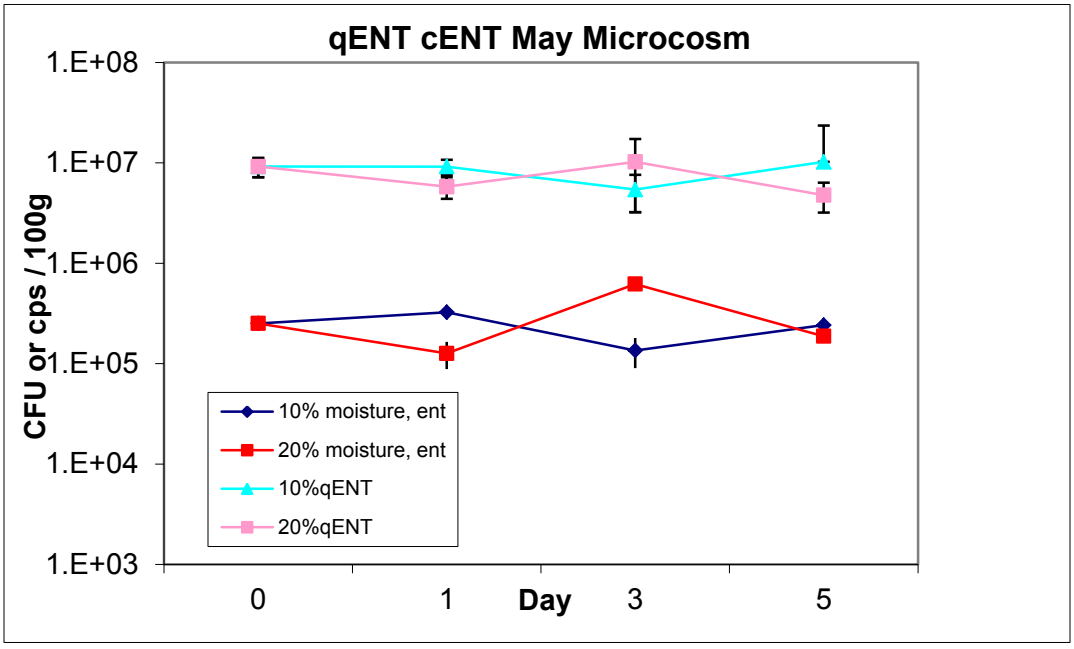

Figure 4-2C. Levels of enterococci by qPCR and membrane filtration over time.

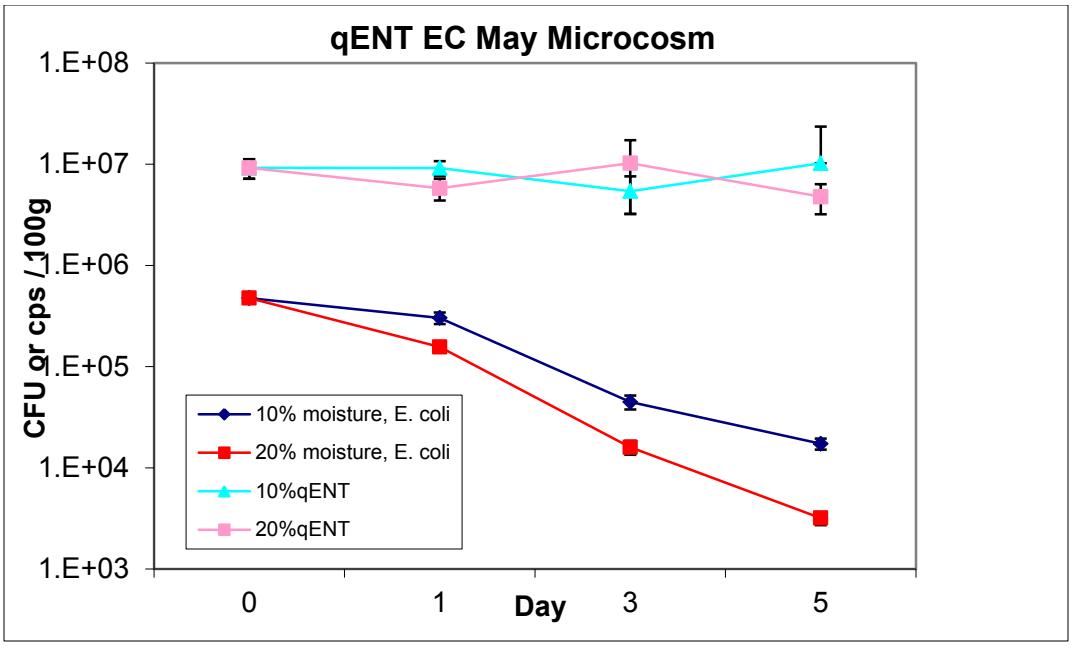

Figure 4-2D. Levels of enterococci by qPCR and *E. coli* over time.

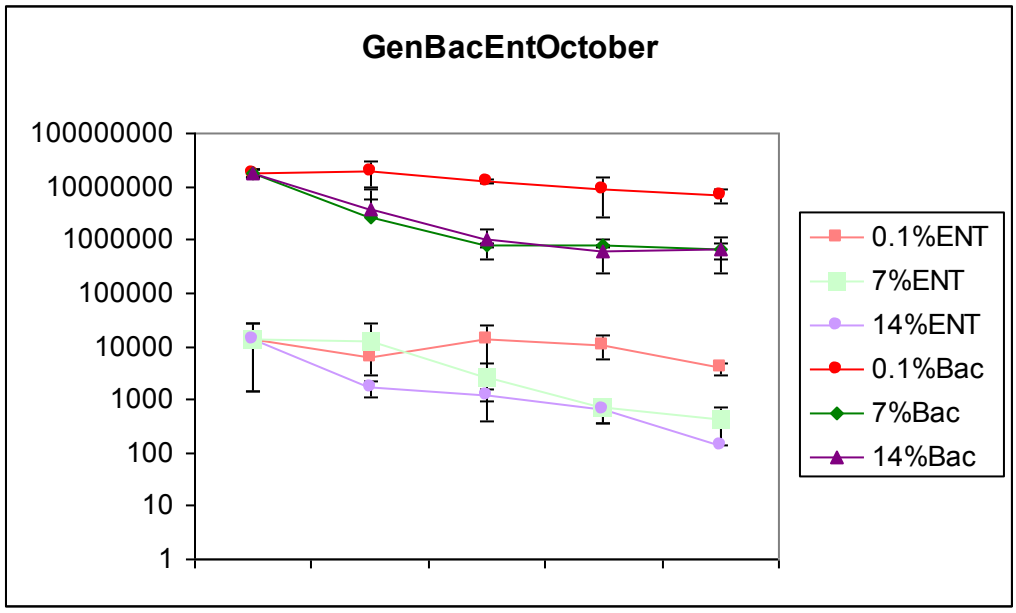

Figure 4-3A. Levels of enterococci and general *Bacteroidales* over time.

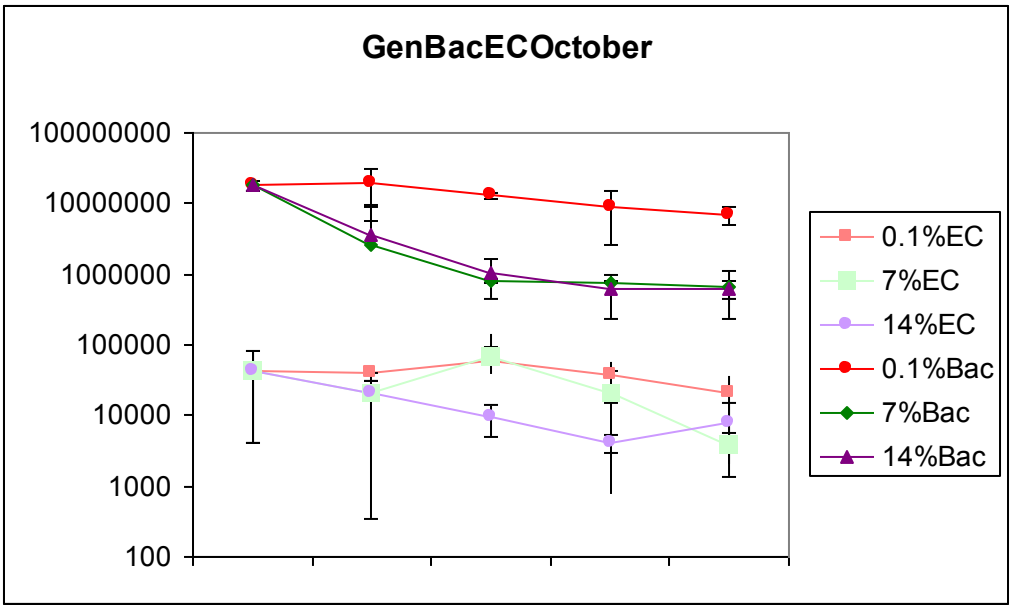

Figure 4-3B. *E. coli* and general *Bacteroidales* levels over time.

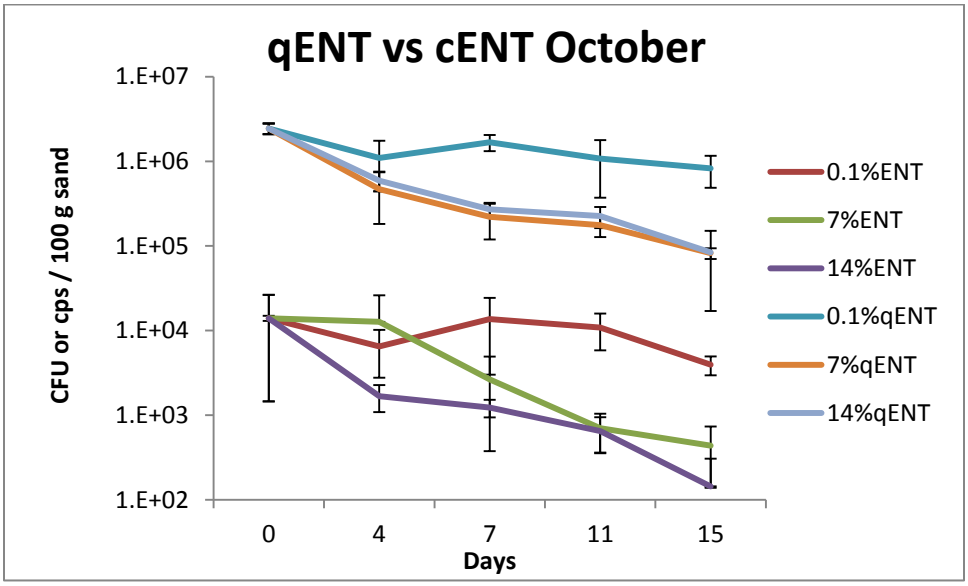

Figure 4-3C. Levels of enterococci by qPCR and membrane filtration over time.

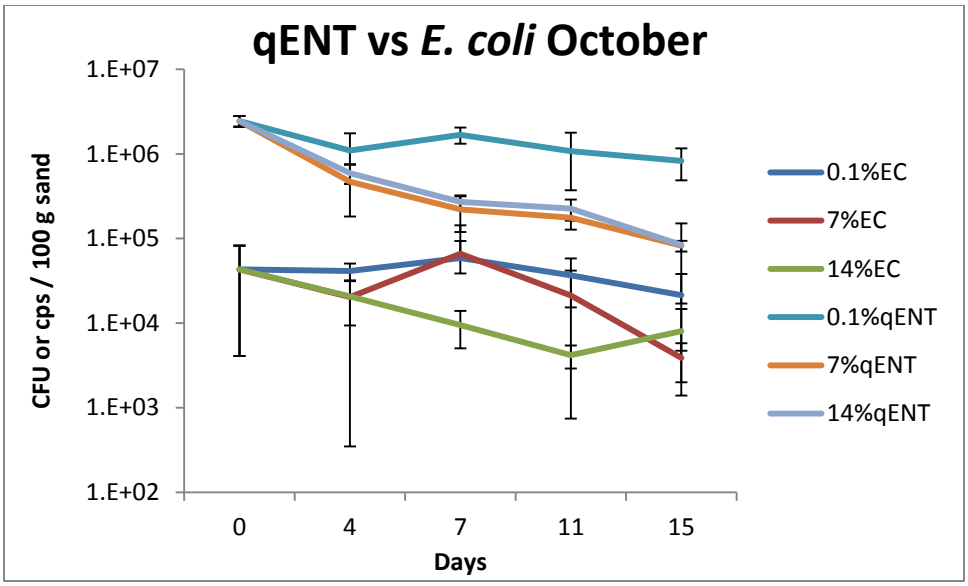

Figure 4-3D. Levels of enterococci by qPCR and *E. coli* over time.

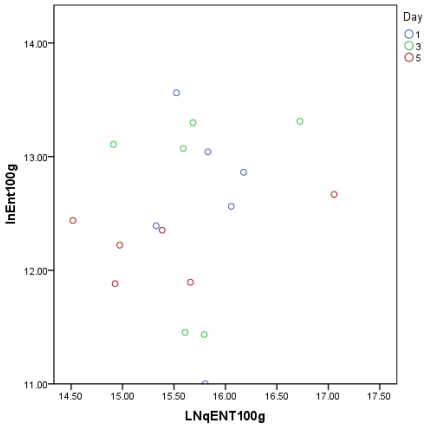

Figure 4-4A. Correlations qENT / cENT, May Microcosm. Colors correspond to timepoint day.

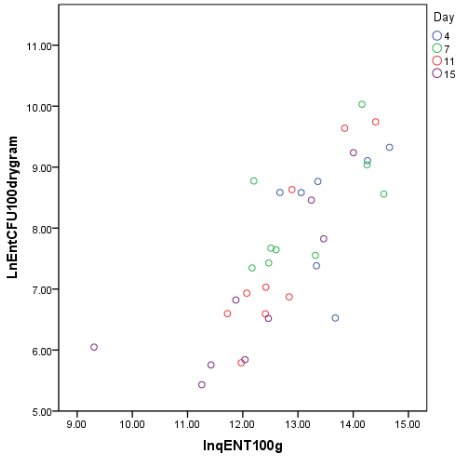

Figure 4-4B Correlations qENT / cENT, October Microcosm. Colors correspond to timepoint day

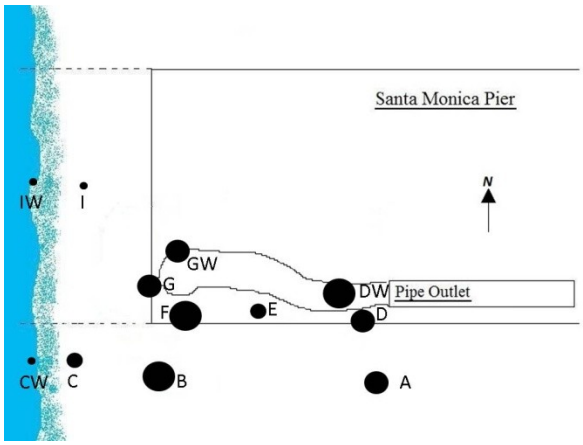

Figure 4-5A. Enterococci levels, Nov 30, 2011.

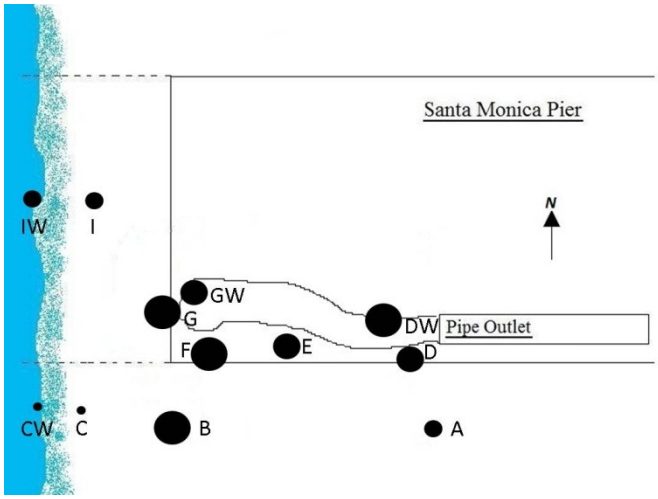

Figure 4-5B. *E. coli* levels, Nov 30, 2011.

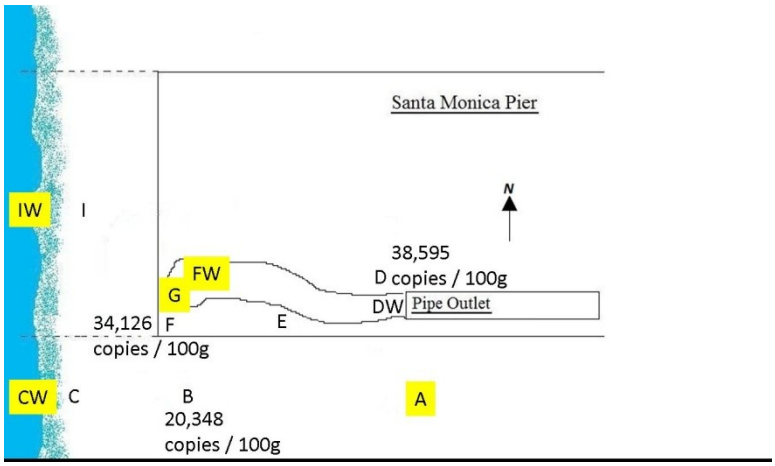

Figure 4-6. Gull fecal marker levels, Nov 30, 2011. Despite significant inhibition with the qPCR reaction, the presence of gull feces was observed at 3 locations (F, B, and D). Highlighted IDs also had gull marker detections but at very low levels.

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Chapter 5. Incorporating Service-Learning in Traditionally Lecture-based Environmental Engineering Courses Through Researching Bacterial Contamination at a Local Beach

Abstract

The objective of this study was to determine the efficacy of an optional 1-2 unit service learning (SL) course added onto two undergraduate Civil and Environmental Engineering classes: C&EE 154: Fate and Transport of Chemicals in the Environment and C&EE 166/266: Environmental Microbiology. The SL add-on aimed to increase participant understanding of and interest in local environmental science issues relevant to the course material and consisted of classroom visits to a partnering middle school class, collaborative environmental field research to test student-generated hypotheses, and presentations of the results at the university. Letter writing about environmental issues was included as a political engagement opportunity for the middle school students some years. While the add-on has been offered for since 2002-2011, pre- and post- surveys were administered to assess the effect of the SL on middle school and university student awareness of different issues in 2008 and 2009. The SL component resulted in self-reported increase in undergraduates feeling more informed about politics, feeling more that they have a say in government, and feeling more interested in management as a career choice. A similar survey was administered to middle school students, whose results were less consistent across both years. In the post-survey in 2008, middle school students' thought that an understanding of science was more important, that they were more interested in the local environment, that they were better informed about politics, and the public officials cared more

about issues than in the pre-surveys. These results show that even the addition of an optional service learning component can provide value to both the community and the undergraduate students through increased understanding of local environmental issues, and how scientific topics such as microbiology or chemical fate and transport can answer questions about broadly important topics such as recreational water quality.

1. Introduction

Over the past ten years (From 2002- 2012), we have offered service-learning (SL) opportunities for undergraduate students in Civil and Environmental Engineering at the University of California, Los Angeles (UCLA) through collaborative research partnerships with local K-12 classrooms. The SL involvement has taken two forms. In the first form, which was offered from 2002 to 2011, SL was offered an optional add-on to a traditional lecture-based course for any enrolled students who were interested in partaking. In two of those years, 2008 and 2009, pre- and post-activity surveys were administered to assess the efficacy of these add-ons as an added benefit to the undergraduate and middle school students. Human Subjects Internal Review Board approved the survey for both university undergraduates and middle school students. The other SL form was begun in Winter Quarter 2012, in which we offered a designated SL course (which, as of 2012, was the available SL course in the Henry Samueli School of Engineering at UCLA) in which the SL was an integral and required part of the course (Jay, 2012, submitted).

Service learning (SL) is a form of experiential learning that integrates academic subject matter with community needs; key elements include reciprocity, reflection, and a community voice in projects (Shastri, 2000; Brandell and Hinck, 1997). SL courses have been shown to improve mastery of technical objectives and critical thinking skills (Shastri, 2000; Cawthorn et al., 2011; Mpofu, 2007; Peters, 2011; Strage, 2000). In addition, students in SL courses have reported a higher course satisfaction (Evangelopoulos et al., 2003) and improved attitudes about the subject matter (Packer, 2009). Having been well-established over the last three decades in other disciplines, SL is now increasingly being adopted in engineering (Coyle et al., 1997; Mehta and Sukumaran, 2007; Beach et al., 2007; Zhang et al., 2007; Riley and Bloomgarden, 2006; Cline and Kroth, 2008). Partnerships between universities and K-12 classrooms offer educational and mentoring benefits for students at all levels, particularly when the SL addresses a community needs and material is integrated into the curricula (Lima, 2004; Moskal et al., 2007; Moskal and Skokan, 2011).

One important benefit of SL is that it helps students navigate complicate novel concepts through educating others and experiencing a real-world context for the course material (Zlotkowski, 2012). In pre- and post- activity surveys, SL components have been shown to increase students' understanding of course concepts (Cawthorn et al., 2011) or to increase their feeling that they made progress toward gaining knowledge (Packer, 2009). In addition to greater understanding of the course material, SL can increase more intangible benefits for students as well. Student ratings of the field of study, course, and instructor all increased after an SL component (Packer,

2009), and SL components show some impact on behavior and appreciation of the environment when that is included as a focus of the SL activity (Cawthorn et al., 2011; Packer, 2009).

One additional aspect that was incorporated into these SL add-ons was that of community-based research (CBR), which involves collaboration on research projects between faculty, students, and community partners. CBR is a growing and significant component of the community-engagement efforts at institutes of higher learning (Stocking and Cutforth, 2006; Polanyi and Cockburn, 2003; Weinberg, 2003). One of the basic principles of CBR is that it is a true collaboration between the involved parties, rather than the community serving as a "lab" for academic research.

In order to bring in the practices of both CBR and SL into our department, the add-on course was structured so that university students would visit middle school classes to develop research ideas in collaboration with the middle school students rather than providing the hypotheses. Under the guidance of university students, middle school students worked together in teams to develop hypotheses, conduct research at local field sites and make posters of the results. Middle school students were then invited to UCLA to tour the campus and present their results at a poster session. The objectives of the SL program were to increase interest in science and environmental issues among the middle school students. There were mentoring benefits as well for both the middle school students and the university students, due to the focus on small group work with consistent mentors. This study focuses on the addition of an optional service learning lab that

can be added to a required course, which is different from other studies that look at a service learning component integrated into a course.

In this paper, we will: 1) give an overview of this add-on model for incorporating SL in traditionally lecture based environmental engineering courses at UCLA; 2) present some of the experimental results obtained at a field site by middle school students involved in this SL project; 3) describe two years of results of an IRB-approved survey of participating middle school students; and 4) present excerpts from reflections from university students working with middle school students.

2. Methods and Program Overview

Partnering schools. For the optional add-on model for SL, we worked with two middle schools: St. Francis X. Cabrini and St. Anne's Schools. Both schools serve a population that is over 95% Latino with 70% of the students qualifying for reduced hot lunch. Schools were chosen based on both demographics, as one goal of the program was to encourage students from minority groups underrepresented in science and engineering to pursue this field, and the need for supplementary resources for science education.

Description of Add-on Model of SL. For 10 years, a subset (between 12-30) of UCLA students in CEE166A Environmental Microbiology or CEE154 Chemical Fate and Transport in Aqueous Environments also enrolled in optional one- or two-unit course to work with middle school

students on an environmental research project. First, these university students were given some background in local coastal water quality and required to read a literature paper on this topic. They were then trained by graduate student researchers in the instructor's lab in the techniques they would be required to teach to the middle school students. The main technique required for this project was to measure fecal indicator bacteria (FIB), which are used to assess the microbial quality of recreational waters. After learning the needed laboratory techniques and background information, university students participated in a series of four weekly sessions with middle school students to share this knowledge with them.

Session 1. At the first meeting, which occurred at the middle school, undergraduate students were joined with small groups of middle school students who would be their students for the remainder of the service-learning component. For the first twenty minutes of the hour-long visit, undergraduates discussed concepts in microbiology with their groups and led the microbe safari, which is a self-directed experiment where middle school students collected bacteria from the environment and cultured (further described below). This 'icebreaker' activity provided a hands-on or tangible opportunity to familiarize middle school students with general microbial techniques prior to the water quality-oriented project of collecting and culturing specific bacteria from the environment to answer a scientific question.

For the microbe safari exercise, each seventh grader was given a Petri dish to grow bacteria from two samples, such as from their hand before and after washing, or from the bottom of their shoes and socks. Middle school students were encouraged to propose simple hypotheses, such as "My hands will have fewer microbes after I wash my hands compared to before" or "the bottom of my shoes will be much dirtier than my hands". Creativity was encouraged and prizes

were sometimes offered for the plates that grew the most bacteria or that showed the most interesting results.

For the remainder of the one-hour session after middle school students collected their microbe safari samples, each group of university and middle school students discussed the beach water quality project, that would be the main focus of the class thereafter. Together, they studied aerial photos of the field sites that would be sampled (locations included Mother's Beach in Marina del Rey, the Santa Monica Pier, and a large storm drain on Santa Monica Beach) and discussed known information about bacteria levels in water and sand at that site. Previous field research in the lab had found the areas underneath Santa Monica Pier and close to the storm drain to be consistently contaminated with FIB, with concentrations generally decreasing with distance from the Pier or storm drain. Undergraduates also presented general information about FIB, including their increased ability to survive in sand over water due to factors such as decreased solar inactivation (Sinton, 1999) or increased protection from predators (Brettar and Holfe, 1992).

Following these presentations, each middle school group brainstormed (with guidance from undergraduate group leaders as needed) to come up with their research question, hypothesis (See Table 5-1), and research approach. University students familiarized middle school students with the materials that would be used during sample measurement at the beach field site (field kits containing measurement materials were brought to the class). Field kits were comprised mainly of easily purchasable items such as small spray bottles to sterilize with ethanol, measuring spoons to add the correct amount of sand for processing, a plastic box with lid to hold kit, and instant hand sanitizing gel to clean up after processing the samples.

Session 2. For the next session, university students met the middle school students at the field site where they spent two hours collecting and analyzing samples. Middle school students gained hands-on experience processing and analyzing samples at the beach. To measure *Escherichia coli* and enterococci in water samples, middle school students diluted water collected from the sites and added a reagent packet (Colilert or Enterolert, IDEXX) to the bottle. Each reagent packet contained a species (or genus) specific formulation of nutrients that would initiate growth, which could then be used to quantify concentration of bacteria in a sample. For sand samples, students weighed and transferred a known quantity of sand into a sample container, and eluted bacteria from the sand by adding a saline buffer to the container and shaking it in a manner similar to that described by Cao et al. (2012). This standardized protocol essentially mobilizes bacteria into the buffer, which can then be mixed with the IDEXX reagent packet and induced to grow. After an overnight incubation step, university students were able to complete the analysis in lab and determine bacterial density for all samples.

Session 3. The following week, university students shared sample results and assisted middle school students with data analysis and poster-making. Middle school students worked with university students to graph the data their group had obtained. In some cases, data were shared among groups for related research questions so data could be analyzed together. Students also chose photos from the field site and sample-processing events, wrote the text to put into the PowerPoint slides, and decided the format and layout of the information and data on the poster. Each group created one PowerPoint slide to be printed as a large (2' by 3') poster.

Session 4. Middle school students visited UCLA to present results from the field day. A typical schedule for the visit was as follows: 9:30-10:30: Panel on college life. University

students served as the panel and middle school students were free to ask questions on any topics, including pathways to college or what life was like as a college student. Even though the students had been getting to know each other informally all quarter, this provided an opportunity for a lively discussion guided by the interests of the middle school students. 10:30-11:30: Students toured campus together in their small groups. Tours were tailored to the students' interests, with locations including the dorms, Powell library, and research laboratories. 11:30-12: Lunch. 12-1: Poster session at which the middle school students were able to present their findings to a broad range of university faculty and students from many departments.

Post-activity letter writing. Middle school students wrote letters to the mayor about the importance of coastal water quality and walked the letters to City Hall. Both the letter writing and the response from the mayor came well after the end of the course; thus, any potential benefit from this engagement activity would not be reflected in the course surveys.

Survey Questions. Pre- and post-activity surveys were developed and administered for two years of the program (See Appendix A). Undergraduate students were asked about the likelihood they would pursue one of the following careers: Engineer for a private company; Engineer in the public sector; K-12 teacher; College/university professor; or management. Middle school students were asked to rate their agreement with the following science-themed statements: I consider myself to be very interested in science; I feel that an understanding of science is important for just about everyone; I feel that clean drinking water (same question was also asked re: recreational water) is an issue that concerns me and that is worth my time to be involved in; I am interested in local environmental issues; I am interested in global environmental issues. Both

undergraduate and middle school students were asked to rate their agreement with the following policy-themed statements: 1) I consider myself to be well qualified to participate in politics and/or community issues; 2) I feel that I have a pretty good understanding of the important political issues facing the country; 3) I feel that I could do as good a job in public office as other people; 4) I think that I am better informed about politics and government than other people; 5) Public officials don't care much about what people like me think; 6) People like me don't have any say about what the government does; 7) Sometimes politics and government seem so complicated that a person like me can't understand what's going on.

Statistics. Survey answers from undergraduate students regarding career choices were transformed into numbers for the purposes of data analyses on a scale from 1-5 (See Table 5-2.) Differences between pre- and post-activity answers were determined through a Repeated Measures ANOVA with a Greenhouse Geisser correction using SPSS statistical software (SPSS Inc., Chicago, IL).

3. Results and Assessment of SL program

Quantitative Evidence: Pre- and Post- Survey Results. Pre- and post- activity survey results were examined in each year to assess the impact of the SL component on the students' opinions of various scientific and political issues as well as impacts on their visions of future careers (Figure 5-1). Of the 12 questions asked of undergraduate students in both years, only four were statistically significantly different, two in each year. Undergraduate students in 2008 felt that

they were better informed about politics and government than most (p=0.056) and that they had more say in the government (p=0.069) after the SL activity. In 2009, undergraduate ratings (n=10) for both their likelihood to go into management ($F(1,9) = 6$, $p= 0.037$) and their feeling that politics was very complicated ($F(1,9) = 9$, $p= 0.015$) increased significantly from the initial to the final survey. It is interesting to note that undergraduate students both felt that they were now better informed than their peers about politics and that politics was more complicated after the SL activity. Often a better understanding of material leads to a feeling that it is more complicated because more of the nuances become visible to a better-informed party. For all four of these categories, the same trends were observed across both years, although they were only statistically significant in the one discussed above.

Interestingly, although trends were consistent across years for the undergraduate students, that was not the case for middle school students. In 2008, middle school student pre- and post-activity survey results (n = 20) were analyzed, and ratings for both the importance of science (p=0.054) and for interest in the local environment (p=0.072) had increased significantly from the initial to the final survey. In 2008, middle school students felt they were better informed about politics (p=0.058) and thought that public officials cared more (p=.061) after the session. In 2009, middle school students (n=25) felt that politics was less complicated after completing this activity (p=0.076).

Qualitative Evidence – Excerpts from Undergraduate Open-Ended Survey Questions 2009. In order to glean a qualitative understanding of the effects of this project on the students, surveys were read through to assess overall student opinions. Although middle-school students were also

asked about future career choices and what, if anything, had changed in their answers, no visible trends emerged in reading through career choices. Additionally, none of the middle-school students addressed the question about changes through the course of the SL experience. The following statements are about the undergraduate responses to the surveys in 2008 and 2009. Overall, students had a positive view of the course and the SL portion. Among other questions, students were asked to read through their pre-survey answers after answering the post-survey and analyze their answers. If anything had changed, they were asked what had changed and what, if anything, they did in the class they thought might have changed their answers. Students who responded to that question on the survey were very pleased with the results of the lab class, regardless of whether their answers had changed over the course of the class or not. One undergraduate wrote, "nothing changed in my answers [to the survey questions], but the lab class was a very rewarding experience and I would do something like it again, even if there was no school credit." In some cases, this course helped students refine their career goals from broadly within the environmental sector to more specifically environmental engineering or water resources. The reflection question in the post-survey also offered undergraduates a chance to think about what had changed in their survey answers after participation in SL, and why they thought their answers might have changed. One student wrote: "My likelihood of being a K-12 teacher increased slightly. Maybe the fact that we worked with students and my interaction with them helped change this. For some students, this course also fostered an understanding and appreciation of the course material because they learned it in an application that was interesting and relevant to an environmental / public health question. "[After this experience] I have a new

appreciation for microbiology because I see a lot of how the environment is affected by microbiological things."

Another interesting facet that emerged from looking through the undergraduate pre- and post-surveys resulted from the question: Do you think you will be a civically engaged citizen in the future (Yes/No)? If yes, what kinds of things will you do as a civically engaged citizen. Most undergraduates responded yes to this question, although a few said no both before and after the SL activity. Undergraduate responses in terms of what they would do reflected their enjoyment of the course. Responses included an increased interest in working with K-12 students from seeing their enthusiasm, a feeling that they understand better how much there is to know about microbiology and water quality, and that working on this project with kids increased their interest in the research and material. The results in politics were mixed, with some students feeling more informed about politics and others feeling like they were less qualified for politics or public office. This mixed result was likely an artifact of less time spent on the political outreach portion of the SL project than the scientific research portion. Nonetheless, that even a few students felt more empowered to engage in local politics and more informed even with such a small element of policy-oriented activity is very promising to further develop this portion of the SL component. It can also be observed that participating in a service learning / community outreach project helped some students clarify and refine the ways in which they wanted to be a civically engaged citizen in their lives (See Table 5-3).

4. Conclusion

This work shows that a SL program can be added as an optional component to a traditionally lecture-based class and that even a modest addition resulted in beneficial impacts on both undergraduates and middle school students. UCLA students were able to gain mentoring experience and develop relationships with middle school students through repeated sessions working in the same small groups. Hypothesis-driven environmental research was the focal point of the work, and students gained experience in analyzing and presenting results. Even a relatively small intervention was able to show statistically significant gains in the level of interest in science of participating middle school students.

Table 5-1. Research hypotheses generated by student groups after a brief background on contamination of the site.

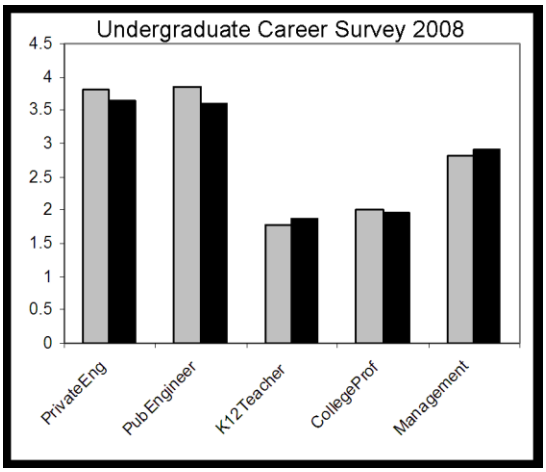
Group	**Hypotheses Created by Student Groups for Research Projects**
1	We investigated the hypothesis that bacterial levels would be higher near the storm drain outlet. We also wanted to find out if bacterial levels in the sand under the pier, since it is always shady, were higher than sand exposed to sunlight.
2	Bacteria levels will decrease with depth because it exposure to the water decreases.
3	We believe that bacteria levels are higher underneath the pier because there is no sunlight and there are bird droppings adding to the contamination.
4	We investigated the hypothesis that bacteria were coming from a small pool under the pier, caused by a storm drain.
5	We investigated the question: are there any bacteria in the sand and how much is in the sand?

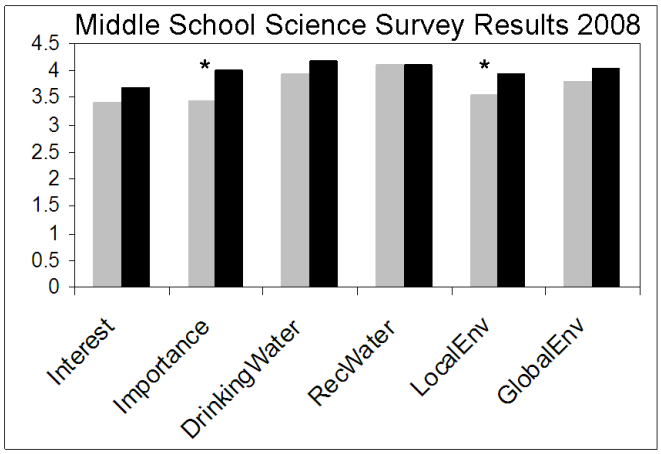
Table 5-2. Numerical ranking of qualitative survey answer options.

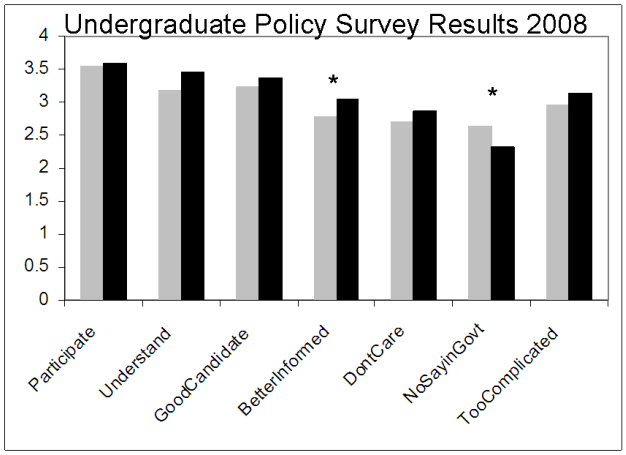
Career answer options	Science/Policy answer options	Number
Highly unlikely	Strongly disagree	1
Unlikely	Somewhat agree	2
Maybe	Neither agree nor disagree	3
Likely	Somewhat disagree	4
Highly likely	Strongly Disagree	5

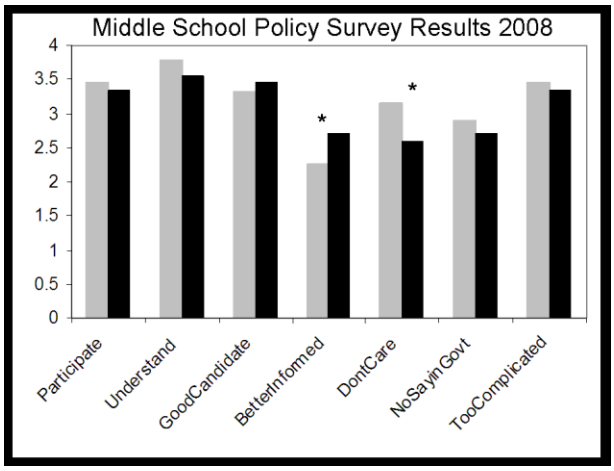
Table 5-3. First and Last Week responses to the Survey Question: What kinds of things will you do as a civically engaged citizen?

Student	**First Week**	**Last Week**
1	Strive to make socially and environmentally responsible choices in both my personal and professional lives.	Try to stay informed on political/social issues. Take action when I can, volunteer to help.
2	Help the underpriveledged (SIC) / those in need.	Help out in the community and vote.
3	I will likely be working with members / leaders of cities to help build infrastructure.	Mentoring students and aware of societal concerns as an engineer.
4	Vote, work on community projects, be involved.	Get involved and work on community projects that benefit everyone, especially environmentally.
5	Health care.	I would help the underserved community of LA or whichever city I end up residing in.


Undergraduate Career Survey 2008
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
PrivateEng
PubEngineer
K12Teacher
CollegeProf
Management


Middle School Science Survey Results 2008
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
Interest
Importance
DrinkingWater
RecWater
LocalEnv
GlobalEnv


Undergraduate Policy Survey Results 2008
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
Participate
Understand
GoodCandidate
BetterInformed
DontCare
NoSayinGovt
TooComplicated


Middle School Policy Survey Results 2008
4
3.5
3
2.5
2
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0.5
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Participate
Understand
GoodCandidate
BetterInformed
DontCare
NoSayinGovt
TooComplicated

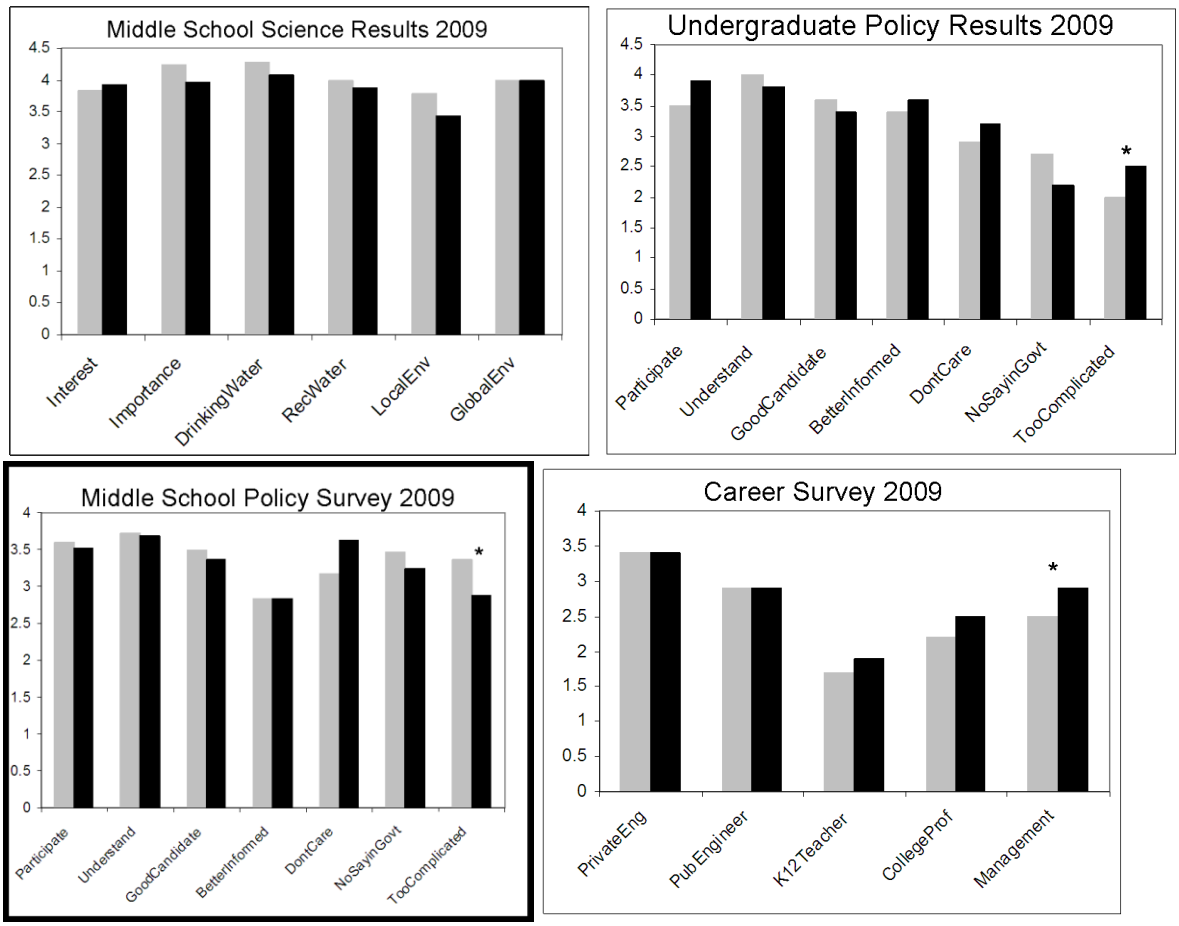

Figure 5-1. Average results from surveys. * denotes statistically significant result. Y-axis is average score (out of 5).

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Chapter 6. Conclusion

Overall, recreational beach water quality remains an issue of concern in Southern California and across the globe. Many factors come into play when determining water quality, including physical issues such as the myriad sources that contribute pollution to the site and financial and political issues that control the way water quality is monitored and determined. Current national regulations require the monitoring of fecal indicator bacteria in order to determine recreational water quality. California Assembly Bill 538 mandates that attempts must be made to identify biological and geographical sources of pollution at locations where storm drains are frequently causing exceedances of water quality standards. FIB are not correlated with human fecal pollution in all watersheds, which is in part due to the ability of FIB to persist and survive in the environment outside of the gut.

A commonly applied approach to meet the goals of source identification is to sample sites that have been high in FIB for further study. A tiered approach such as this, however, assumes a correlation between FIB and the sources of interest in the watershed. The research described in chapters 2 and 3 of this dissertation tests this assumption in two Southern California watersheds, Santa Monica Canyon and Ventura Harbor. In both cases, a tiered approach to sampling using FIB as a first tier to guide sampling would have failed to identify sources of human fecal pollution. The portion of Santa Monica Canyon sampled was highly impacted by both HF183 and FIB. However, no relationship was observed between sites with either higher concentrations of FIB or increased frequency of water quality standards exceeded for FIB and the presence of HF183.

Although Ventura was a very different watershed, with overall lower exceedance rates and rates of detection of HF183, the tiered approach using FIB would again have been unsuccessful in guiding site selection for HF183. HF183 was only detected once at the Arundell Barranca sample location, which consistently had the highest levels of FIB among the sample sites The human marker was detected very frequently at the Marina Dock sample location, which had overall low levels of fecal indicator bacteria and thus a very potential source of human fecal pollution to the watershed would not have been selected for further study based on FIB levels. In both of these studies, for different reasons, the commonly applied tiered approach study design using FIB as a preliminary tier would not have led to the selection of appropriate sites for studying the final tier of interest, HF183.

Additionally, rainfall, physical, and chemical parameters were explored as first tiers to better identify sites of interest for HF183. While rainfall increased the incidence of HF183 detection in both cases, the human marker remained scattered throughout the sample area. Thus, rain did not prove to be an effective first tier to identify a consistent source of HF183, although it did increase the likelihood of detecting HF183 overall during some storms. No relationship was observed between HF183 and any of the physical or chemical parameters examined in the Santa Monica Canyon watershed. While some relationship was observed between the presence / absence of HF183 and increased FIB and nutrient concentrations in the Ventura study, this relationship was likely due to the increased concentrations in all parameters observed during rainfall because, on a site by site basis, good relationships were not observed between these parameters.

Every watershed is a distinct environment that has different potential sources of bacteria and many factors contributing to the persistence of the bacteria. Rather than attempting to apply an indicator that has worked as a first tier in other watersheds, it would be better to have as a first tier an in depth study of the watershed using historical data or local experts to provide information on the most likely sources of pollution in the watershed. Using this information it would be possible to design a study using FIB and several parameters (or even one parameter, if there is only one likely source of concern such as human fecal waste) to get a better understanding of what all the sources of pollution are in the watershed. In addition, sampling FIB and other parameters such as HF183 allow the application of other microbial source tracking tools including indicator ratios and detection frequencies.

These studies do not necessarily have to be long-term to identify consistent sources of pollution. For example, within the first four months of sampling at Ventura, the increased frequency of detection of HF183 at Marina Dock was apparent, and a dry weather influx of HF183 was seen in the Keys channels. A shorter-term study looking at specific parameters of interest in the watershed of concern may provide more useful information about identifying sources in a watershed for a similar cost to a longer-term study monitoring FIB alone.

Additionally, more value is added by studying sites where sources are suspected as well as those that are high in FIB as each site may provide information about different types of sources (e.g. biological or geographical). For example, in Ventura, measuring FIB revealed a geographic source of FIB to the watershed at AB, which could be remedied by a management action such as putting in a dry weather diversion or a small treatment unit to treat the water before it discharges into the Keys. Measuring HF183, however, provided information about very

different areas to focus on for human waste sources. Potential sources near the Marina Dock sample location such as the restroom, nearby boats, or storm drains could be examined to determine the likely source of the frequently observed HF183. Observing HF183 in a Keys channel at levels consistent with those calculated if a boat dumped a 10 gallon waste tank in the channel points to a need to educate residents further on this issue, and perhaps put dye into residential boat waste tanks to monitor illicit discharges. While FIB levels were not elevated on this day, dumping human waste creates a health risk for residents and tourists alike if they use the enclosed beaches within the Keys or capsize while kayaking that would have gone undetected if sites were chosen for study based solely on FIB levels.

In addition to the many sources of FIB to the environment such as storm drains, leaking sewers, and wildlife, there are important environmental reservoirs such as sand and seaweed that can foster FIB growth and persistence in the environment. As such, it is important to understand the effect of different factors on the ability of bacteria to survive and persist in these reservoirs.

Microcosm experiments conducted during the course of this dissertation research found that in dry beach sand (0.1% moisture), the addition of moisture was detrimental to the survival of the indicators studied (General *Bacteroidales*, *E. coli*, and enterococci). Interestingly, compared to many other studies looking at the survival of bacteria in sand, the persistence of the bacterial groups and genetic markers was not enhanced by the addition of moisture. These results point to the ability of bacteria to persist for long periods of time in beach environments under in-situ conditions. This indicates that the addition of moisture to bacterial populations in sand is not necessarily going to result in increased survival if the population has adapted to the dry environmental conditions. The effect of additional moisture was not always detrimental. No

effect was observed on the persistence of these indicators between 10% in-situ moisture conditions and 20%, which may be in part because these sands were occasionally wetted during high tides and so these bacterial populations were accustomed to wetting / drying cycles.

These findings point to the importance of understanding the behavior of indicator bacteria populations that have evolved to survive in environmental conditions so that their potential impact on overlying or adjacent water quality can be better understood. The ability of FIB and general *Bacteroidales* to persist for long periods of time in the environment indicates that these bacteria may not be a good choice as indicators of recent pollution events. By way of contrast, source-associated markers in sewage-amended or manure-amended sediment microcosms were shown to decay much more rapidly (within a week) after the input of fecal matter then the general bacterial groups were (Rogers et al., 2012; Yamahara et al., 2012). As such, source-associated markers can provide more information about recent influxes of pollution then a more general group such as *Bacteroidales* or enterococcus. However, these more general groups can also be useful as they provide information about general fecal or microbial contamination of an area, and are less susceptible to the limitations of qPCR such as bad sample recovery and lower reaction efficiencies.

Furthermore, while all three of the research chapters point to the potential utility of using ratios of bacteria measured to provide further information about the study area, they also point to the need for more research into the ability of bacteria and genetic markers to persist in the environment in order to interpret these ratios. Variable rates of decay will affect the ratios of these indicators as well different sources, so it is important to have a thorough understanding of decay rates in order to correctly interpret the ratios. Examining the ratios of culture- dependent

and culture-independent measurement of the same bacteria can provide a cost-effective way of getting information about the percentage of viable bacteria in the study area. This is particularly true if measuring enterococci as both a culture-dependent and culture-independent method are proposed in the EPA 2012 draft recreational water quality guidelines. Thus, measuring with both methods both helps inform water quality and provides information on viability without the additional cost or time associated with other methods of measuring cell viability such as IMS-ATP (Lee et al., 2010) or PMA-qPCR (Bae and Wuertz, 2009).

In summation, results from this research point to the importance of selecting indicators and sample locations that are most relevant to watershed concerns rather than using a first tier such as FIB to preferentially select sites for further analysis. Measuring a marker for human fecal pollution in both watershed studies provided useful information for potential human inputs that would have been missed if sites were chosen based on high FIB levels. In addition it is very important to understand the contribution of different reservoirs, such as sand, in the study area to the observed microbial pollution. Overall, these results point to the need for further examination of the ability of bacteria to survive under various environmental conditions in both water and sand, using both environmental microbial populations and populations from likely sources such as human sewage.

Appendix A. Literature Overview: Factors Affecting Survival of Microorganisms in Sediment.

Many gaps in knowledge exist around the factors increasing or decreasing bacterial survival in sediments. Depending on conditions in sediment such as nutrient and moisture levels and the level of extant native microbial competition and predation, bacterial survival ranges from dramatic increases to exponential decay. Although some studies have been done that look at the effects of some of these factors on bacterial population, no intensive work has been done to attempt to quantify the effect of these different factors on the survival of bacteria in different environmental frameworks. In general, sediment may be more conducive to FIB survival relative to the water column due to reduced sunlight inactivation (Sinton et al., 1999), protection from predators (Brettar and Holfe, 1992; Davies and Bavor, 2000), nutrient and organic carbon availability (Craig et al., 2004; Blumenroth and Wagner-Dobler, 1998; LaLiberte and Grimes, 1982a; Gerba and McLeod, 1976), and the presence of a surface for the formation of biofilms (Brettar and Holfe, 1992; Davies et al., 1995; Decho, 2000). However, influences of these factors on the prevalence and persistence of FIB in sediment are not understood. In addition, many of the human health effects are thought to be related to the presence of pathogenic viruses in the environment, rather than bacteria so a more developed awareness of viral survival in the environment is also very relevant to monitoring water quality. Additional studies have found evidence that sediments provide a favorable environment for pathogenic microorganisms including viruses (Green and Lewis, 1999; Gantzer et al., 1998; Meschke and Sobsey, 1998; Ferguson et al., 1996; Gerba and Schaiberger, 1975), and bacteria such as Salmonella (Burton et al., 1987). An understanding of the ability of bacteria and viruses to survive in the sediment is

important in order to better characterize potential human health effects from direct contact with sediment.

In addition to a need to understand the survival of bacteria in sediment in order to better understand their potential effects on human health through direct contact with sediment, it is also now recognized that in order to accurately model FIB persistence effects on recreational water quality in the environment, we need to consider FIB dynamics in sediments. Compared to the vast literature modeling FIB survival in pelagic environments, relatively few FIB modeling studies allow for bacterial adsorption to sediments and settling. This is clearly inadequate as up to 80% of FIB are thought to be associated with particles (Auer and Niehaus, 1993; Gannon et al., 1983; Pommepuy et al., 1992; Hunter et al., 1992) with up to 40% of the total FIB on settleable fractions (Krometis et al., 2007). Studies investigating ecological dynamics in sediments in combination with partitioning to sediments are clearly lacking (Surbeck, 2009). Bacterial dynamics in sediment are very complicated based on the myriad factors influencing their survival in the environment. Bacterial survival patterns can range from exponential dieoff in a nutrient-poor environment with exposure to sunlight to a static value when competing processes present at the site are balanced to dramatic increases when the ecological and physicochemical parameters are sufficient to support the bacterial populations. Bacteria can also be resuspended into the water column in the environment through water flowing (either by tidal flows or stream flows) over the sediment surface.

This study aims to better characterize the effects of predation as well as physicochemical parameters such as DOC, nutrients, and salinity on the survival of bacteria and viruses in sediments using a series of microcosms. Sediments will be chosen from a wide variety of

environments which have varying levels of the parameters which have been show to impact bacterial survival in order to quantitatively define the effects of these parameters on bacterial survival.

Background and Justification

Effects of physicochemical parameters on bacterial persistence. Previous studies have shown several factors to be important to the growth or decay of bacteria in water and/or sediment, and will be the focus of our microcosm experiments. The justification for the use of these factors (particle size, temperature, nutrients, and organic carbon) will be presented in the following paragraphs.

One of the factors that has been shown to be significant in affecting the ability of bacteria to persist and grow is sediment particle size. Bacterial species, including E. coli and Salmonella, have been shown to survive and grow better in sediments with higher percentages of small particles (i.e. clays) than larger particles (i.e sands and gravels) (Burton et al., 1987; Garrido-Perez et al., 2008; Craig et al., 2003; Laliberte and Grimes, 1982b). Similar trends have been seen in rates of bacterial carbon production; bacterial carbon production has been found to be higher in muddy soils than sandy soils, and higher in sandy soils than in coarser sediments (Luna et al., 2002); (Marxsen, 2001).

Temperature has a pronounced effect on the ability of bacteria to survive in the environment; in general, cooler temperatures enable increased persistence of bacteria. While persistence is increased by cooler temperatures, growth effects are not observed until temperatures increase above 15-18 degrees C (Hipsey et al., 2007). Many studies have shown temperature to be important to the survival of bacteria, with increased survival occurring in

sediment at lower temperatures ranging from 4C up through 55C (Bogosian et al., 1996; Anderson et al., 2005; Czajkowska et al., 2008; Ogden et al., 2001). Interestingly, protozoa grazing rates increase in the opposite way; protozoa digestion rates were shown to increase exponentially from 12C to 22C (Sherr et al., 1988).

Nutrients are another parameter which can affect the survival of bacteria in sediments, and have been shown to have mixed effects on survival in previous studies. In some studies, nutrients have been shown to have a strong impact on the abundance of bacteria, in particular N and P (Franz et al., 2008; Wu et al., 2007). In other studies, nutrients have been shown to have a smaller effect on survival; nutrients were observed to prolong the length of survival but not affect the initial dieoff rate of E. coli (Brettar and Hofle, 1992) or to have no observable effect on survival (Burton et al., 1987). In many environments, DOC, particularly the fraction assimilable by bacteria, has been shown to be the limiting factor for bacterial survival (Hipsey et al., 2007). Increased levels of DOC have also been shown to increase bacterial survival in sediments (Craig et al., 2003; Franz et al., 2008).

Effects of predation and competition on bacteria in sediment. While predation is well studied in pelagic environments (Pernthaler, 2005; Vaque et al., 1992; Surbeck et al., 2010) little is known about the effects of predation/grazing in sediments, in particular in marine sediments. Flagellates have also been observed to be important in planktonic environments but less significant in the sediments studied (Hamels et al., 2001); (Kirschner and Velimirov, 1999). Although heterotrophic nanoflagellates (HNF) have been observed to be the primary consumers of bacteria in sediments (responsible for up to 90% of the grazing rate on bacteria), they are present at such low concentrations in sediments that they can have a fairly low impact on

bacterial survival ((Kirschner and Velimirov, 1999). A weak, statistically insignificant correlation was observed between HNF and concentrations of bacteria in sediment microcosms ((Kirschner and Velimirov, 1999).

However, other research has indicated that autoclaved sediments show much more growth of inoculated bacteria than unautoclaved sediments, indicating a potentially important role for grazers (Laliberte and Grimes, 1982b, Bogosian et al., 1996). The effects of predation on bacteria in solids have been seen in both natural and engineered systems. The addition of protozoa has been shown to be an effective way to improve bacterial removal in wastewater treatment systems. The addition of ciliated protozoa to activated sludge reduced the half-life of E. coli from 16 hours to 1.8 hours in activated sludge (Curds and Fey, 1969). In addition, the removal efficiency of sand filters was observed to be positively correlated with the addition of protozoa (Bomo, 2004). In microcosms using natural sediments, both predation and potential competition were found to have a strong effect on bacterial survival using cycloheximide as a predation inhibitor. Levels of enteric bacteria have been shown to stabilize at higher levels in sediments in the presence of cycloheximide as a predation inhibitor, (Marino and Gannon, 1991). Further, autoclaved microcosms run in parallel with predation-inhibited microcosms hints at the potential effects of competition as well as predation; FC and FS grew and stabilized at orders of magnitude higher levels (10^7-10^9 /100 mL) in autoclaved sediments than those with only predators inhibited (Marino and Gannon, 1991).

Additional uncertainty around the effects of predation in sediment stems from a lack of knowledge around the effects of physical sediment characteristics on the rates of protozoan grazing and/or its effectiveness at limiting bacterial growth. The increase in bacterial

growth rates fostered by sediments with increased nutrients and DOC (Craig et al., 2003; Franz et al., 2008; Wu et al., 2007) could effectively negate any effects of predation by increasing the growth rate above the grazing rate. Grain size, porosity, and interstitial space have also been shown to limit the occurrence of protozoa, (Hondeveld et al., 1994; Starink et al., 1996). Interestingly, while bacterial density and production have been observed to increase with decreasing median sediment grain size, the opposite pattern has been observed with flagellate biomass (Hamels, 2001). Predation has been shown to affect the survival of bacteria in certain sediments, whether it be by increasing grazing rates in a low-productivity sandy soil (Hamels, 2001) or by allowing bacterial populations to stabilize at higher levels in storm drain sediments (Marino and Gannon, 1991).

Live vs Inactive Bacteria. Further, not much is currently known about the fraction of live versus inactive bacteria in sediments (Barcina et al., 1997; Luna et al., 2002) although this would be important to understand whether the increased persistence of bacteria in sediment is in part due to larger reserves of inactive bacteria than are present in water. Dormancy is thought to be a mechanism used by microorganisms to retain diversity; as the ecosystem health improves, some of these organisms are able to revive. In two types of marine sediments, 26-30% of the total counted bacterial population were observed to be live bacteria, with only 4% containing observable nucleoids and thus actively growing while the remainder of the population (70-74%) consisted of dead cells (Luna et al., 2002). In these sediments, a reactivation of 6-11% of the inactive population was observed with the addition of nutrients to the system (Luna et al., 2002). In productive ecosystems, the percentage of dormant bacteria is fairly low; in nutrient poor ecosystems dormant populations generally comprise 40% of the taxon richness (Jones and

Lennon). Limited numbers of studies have been performed showing live bacteria to represent between 40%-63% of the total bacterial biomass in freshwater and marine sediments (Haglund et al., 2003; Manini and Danovaro, 2006).

Coliphage presence and persistence. In addition to studying the persistence of bacteria in the environment, it is important to characterize the survival of viruses as well since pathogenic viruses are also thought to be an important cause of recreational water illnesses. Viruses exhibit a very different survival pattern from a very basic level, as they are unable to grow and multiply in the environment in the absence of a host in the same way bacteria can persist; accordingly, a model virus will be studied in parallel with multiple bacterial species. Coliphage and other bacteriophages have also been found to have similar behavior to pathogenic viruses, so furthering an understanding of how these viruses survive in different environments is beneficial to determine the extent to which they can be uses as proxies for human viruses (Skraber et al., 2004). F-specific RNA bacteriophages (for salmonella) were found to be highly correlated with viruses in river water, coagulated river water, lake water, and drinking water, but not in raw or biologically treated sewage (Havelaar et al., 1993). In addition to further informing the survival characteristics of viruses in the environment, the ability of coliphage to persist in an urban environment is an interesting question; coliphage are not generally thought to be able to survive and persist well in the environment (Jofre, 2009). However, F+ coliphage were observed to have the same flow-independent storm flow pattern as FIB (Surbeck et al., 2006), which seems to indicate an ubiquitous environmental source of coliphage similar to that of FIB is present on the surface of the urban landscape. Further, coliphage samples were detected at all of 12 beach sites sampled in southern California, with 5/12 sites having F-specific coliphage (Jiang et al., 2001).

Authors also observed a significant correlation between F-specific coliphage and human adenovirus in these water samples (r2=.99) but no such correlation was observed between human adenovirus and general coliphage presence. In addition, although F+ coliphage was shown to only correlate with their host EC in river, somatic coliphage did show up more frequently in areas with more intensive land use (Franke et al., 2009), which indicates that urban sources can affect coliphage levels in the natural environment.

FIB Modeling in or attached to sediment. A deeper knowledge of the levels of bacteria in stream bed sediments is important to both the concentration of bacteria available for resuspension as well as the levels of free bacteria in the stream available to associate with suspended solids in the stream. Various bacterial types have been shown to be associated with sediment particles at varying percentages; 20-40% of fecal coliforms, *E. coli*,and enterococci were shown to be associated with settleable particles (Krometis et al., 2007; Characklis et al., 2005), 65% of *Clostridium perfringens* spores, and only 13% of total coliphage were associated with settleable particles (Krometis et al., 2007). Other studies have observed as much as 80% of fecal indicators to be associated with settleable particles (Sayler et al., 1975), with seasonal variations in the level of attachment (De Souza et al., 2003).

Some studies have shown significant correlations between FIB levels in sediment and those in the water column; Sanders et al. determined that sediment levels of FIB were an important input parameter in a model of FIB levels in a southern CA intertidal wetland (Sanders et al., 2005). Of models that do include effects of sediment deposition and resuspension on FIB concentrations in the water column, most do not account for FIB die-off or growth in sediment. Steets and Holden (Steets and Holden, 2003) present a mechanistic model of FIB dynamics in a

southern CA coastal lagoon. They assume a constant sediment FIB concentration, although they state that it would be advantageous to allow for regrowth in light of field work showing this to be important in some circumstances. Cho further built on the Steets model by incorporating a hydrodynamic model for both temporal and spatial variations in FIB transport and resuspension and dieoff terms for both water (irradiative) and sediment (natural) (Cho et al., 2010). Bacterial levels in the stream were found to be influenced by different factors in wet and dry weather; the dieoff rate in sediment was observed to have a significant effect on bacterial levels in the stream in wet weather but not in dry weather, where solar inactivation and fraction of bacteria sorbed to sediment were the two controlling factors (Cho et al., 2010). (Jamieson et al., 2005a; Jamieson et al., 2005b) modeled *E. coli* attached to sediment, taking into account advection, dispersion, adsorption, dieoff, and bed deposition making several key assumptions. Using this model, 20% of bacteria was observed to be associated with sediment. These numbers are similar to those observed by Schillinger and Gannon, which were 15% of FC within stormwater being associated with particles (Schillinger and Gannon, 1985).

Steady state models of interactions between the water column and sediment of microbial concentrations have shown that the main contributors to uncertainty in the model arise from a lack of characterization of bacterial settling velocity, fraction attached to sediment, resuspension rate, and the net bacterial growth in the sediment (Rehmann and Soupir, 2009). BASINS, a model developed by the EPA Office of Water, found stream temperature and the ability of land to absorb *E.coli* were the most meaningful parameters in the model when attempting to recreate peak values observed in the stream (Paul et al., 2002).

Previously observed kinetic models of bacterial growth/decay. Previous studies have observed many different survival behaviors among bacteria in sediments, and some models have been utilized to begin to describe these behaviors (many exist mainly for water at this point). In some environments, exponential decay has been observed in unmoistened autoclaved sediment microcosms inoculated with wastewater (Mika et al., 2009); interestingly, in microcosms kept moist *E. coli* levels were observed to persist for much longer periods of time and achieve more of a steady state (Mika et al., 2009). While first order decay is frequently used to describe bacterial persistence kinetics in the environment, biphasic decay has also been observed (Dick et al., 2010) which can result from species diversity (Hellweger et al., 2009). Diversity in persistence among different species of bacteria as well as among different strains of the same species of bacteria has been observed as well (Anderson et al., 2005; Dick et al., 2010). Additionally, the effects of predation or competition on maintaining a steady bacterial concentration in sediments has been observed in parallel microcosm studies where bacterial levels in autoclaved sediments were observed to increase while those in unautoclaved sediments were shown to remain the same (Lee et al., 2006). Briefly, expressions incorporating a temperature dependency term and a nutrient dependency term (based upon Michaelis-Menten kinetics) have been developed to define and model bacterial survival in water (Hipsey et al., 2007). These kinetic models will be discussed in greater detail in the experiment design section as background for the development of the microcosm experiments and parameters to be tested throughout.

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