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Use of BOX-PCR Subtyping of Escherichia coli and Enterococcus spp. to Determine the Source of Microbial Contamination at a Florida Beach

Miriam J. Brownell
University of South Florida

Use of BOX-PCR Subtyping of *Escherichia coli* and *Enterococcus* spp. to Determine the Source of Microbial Contamination at a Florida Beach

by

Miriam J. Brownell

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Major Professor: Valerie Harwood, Ph. D.
Daniel Lim, Ph.D.
John Lisle, Ph.D.

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Note to Reader: The original of this document contains color that is necessary for understanding the data. The original thesis is on file with the USF library in Tampa, Florida.

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Use of BOX-PCR Subtyping of *Escherichia coli* and *Enterococcus* spp. to Determine the Source of Microbial Contamination at a Florida Beach

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ABSTRACT

Siesta Key Beach, located on the Gulf Coast of Florida, is frequently mentioned among the top ten beaches in the US. In summer 2004, high levels of indicator bacteria caused health warnings to be posted, and a storm drainage system was implicated as a possible source of microbial contamination. A study was initiated to determine whether indicator bacteria that persisted in the stormwater system could contribute to high microbial loads in receiving waters. Two sampling events, one within 48 hours of a rain event and the other during dry conditions, were conducted. Water and sediment samples were taken at various sites from the storm drainage system to the beach. Fecal coliforms and *Enterococcus* spp. were enumerated, and genotypic fingerprints of *E. coli* and *Enterococcus* spp. were generated by BOX-PCR. Diversity of *E. coli* and *Enterococcus* populations was calculated with the Shannon-Weiner diversity index. Similarity of *E. coli* and *Enterococcus* populations was calculated with the population similarity coefficient.

After the rain event, levels of fecal coliforms and *Enterococcus* spp. were high in sediments and exceeded the regulatory standard for all water samples. In dry conditions, levels were lower in water samples, but still high in sediment samples. Significantly greater population diversity was observed in the rain event compared to the dry event for both *E. coli* and *Enterococcus* populations, and greater population similarity was

observed in dry conditions. *Enterococcus* population diversity was significantly higher in untreated sewage and the Siesta Key rain event when compared to dry conditions, and to a site on the Myakka River (no known human input or urban stormwater runoff). Siesta Key populations in dry conditions were most similar to Myakka, and sewage was the least similar to all other populations.

Increased population similarity for *E. coli* and *Enterococcus* spp. during dry conditions suggests that a portion of the population is composed of "survivor" isolates. Persistence of survivor isolates in the storm drainage system, where urban runoff can sit for days, suggests a reservoir for indicator bacteria that can be flushed through the system to the Gulf, causing high levels of indicator bacteria in receiving waters.

INTRODUCTION

Fecal Contamination in Surface Waters

Environmental and recreational waters can be impacted by fecal contamination, leading to the risk of pathogens infecting the public. This can result in closings of recreational water sites and shellfishing beds, and consequently loss of revenue. Sewage from failing infrastructure or onsite septic systems, agricultural runoff, and stormwater discharge can be a potential source of pathogens to a water body, creating a health risk (29, 35, 88). Diseases affecting the respiratory, ocular, gastrointestinal and myocardial processes of the human body are caused by human viruses that are excreted in feces (38, 84). *Cryptosporidium* spp., which are protozoan parasites, can be excreted in the feces of agricultural livestock, domesticated animals, and wildlife (26). *E. coli* O157:H7 has been found in cattle feces (44), and *Campylobacter jejuni* has been found in cattle and poultry feces (2, 78). Thus, the need to protect surface water quality from excessive fecal inputs and remediate impaired watersheds is evident.

Fecal coliforms, *Escherichia coli* and *Enterococcus* spp. are indicator organisms used as surrogates for waterborne pathogens (3). The indicator concept has been used to gauge water quality since the beginning of the 20th century (111). These bacteria normally inhabit the gastrointestinal tract of humans and animals and are excreted in feces; therefore, their presence in environmental and recreational waters indicates the possible presence of pathogens. Characteristics of these indicator organisms should

include the following: 1) non-pathogenic themselves, 2) easy and rapid to detect and enumerate, 3) not native to the environment or able to reproduce in the environment, 4) able to survive as long as pathogens and at least as resistant to environmental stressors, and 5) their presence should correlate with the presence of pathogens and the associated health risk (39).

The Clean Water Act (1972) addressed regulation of water quality to protect surface waters in the United States. The US Environmental Protection Agency (USEPA) utilized this legislation and a series of epidemiological studies to set quality standards based on indicator organism concentrations. The USEPA-recommended indicator for fresh water is *E. coli* or *Enterococcus* spp., while for marine water the recommended indicator is *Enterococcus* spp. (99). The Florida Department of Environmental Protection (DEP) recommends fecal coliforms for fresh and marine waters (31). The Florida Department of Health (DOH), which monitors the beaches of Florida, adopted the USEPA recommendation for *Enterococcus* spp. and the Florida DEP recommendation for fecal coliforms. A water sample of 100 milliliters containing ≥ 104 *Enterococcus* spp. and/or ≥ 400 fecal coliforms would indicate "poor water quality" (http://esetappsdoh.doh.state.fl.us/irm00beachwater/terms.htm). An increase in concentrations of these indicator bacteria in a water body correlates to an increased probability of exposure to pathogens, and therefore indicates an increase in health risk.

Transmission of disease to swimmers via exposure to contaminated recreational waters has been investigated. In 1983 the USEPA published a review of epidemiological-microbiological studies it conducted during the 1970's, which found a positive correlation between density of an indicator (*Enterococcus* spp.) in marine waters and

gastrointestinal symptoms among swimmers (98). A series of epidemiological studies published in the 1980's and 1990's, and reviewed in 1998 (85), examined the link between health risk and indicator organism concentrations in recreational waters by following the health outcomes of groups of people who were exposed to contaminated recreational waters. A majority of the 22 studies were prospective cohort studies, which have the unfortunate drawback that follow up is not always reliable, and subjects being observed can drop from the study (63). Two studies (28, 54) were randomized controlled trials, which are considered to be more reliable than prospective cohort studies because they eliminate many biases and sources of error (8). Subjects were randomly assigned to either an exposed group or a control (unexposed) group, and conditions (exposure time, etc.) were pre-determined. Kay et al. (1994) found a significant difference between reported illnesses of the exposed group versus the control group, and a linear trend between the incidence of gastroenteritis and concentration of *Enterococcus* spp.. In the second study, Fleisher et al. (1996), exposure to water with a concentration of > 50 *Enterococcus* spp.•100 ml^{-1} was predictive of respiratory illness and exposure to water with a concentration of > 100 fecal coliforms•100 ml^{-1} was predictive of ear ailments. Both studies took place in marine waters that were known to be influenced by domestic sewage, thus establishing a link between indicator concentrations and increased health risk.

Sources of Indicator Organisms other than Fecal Contamination

Estimating the extent of fecal contamination in a water body and its relationship to human health risk by indicator organism levels relies on many assumptions, including:

1) there is no source other than feces for these bacteria, 2) all fecal sources pose an equal risk to human health, and 3) persistent survival or regrowth of indicator organisms in the environment does not exist (or mirrors that of pathogens). Fecal coliforms, *E. coli,* and *Enterococcus* spp. have been associated with epiphytic flora (76, 87), insects (37), plankton (69), and green algae (108), as well as effluent from pulp and paper mills. A study by Gauthier and Archibald (2001) measured fecal coliforms and *Enterococcus* spp. densities at Canadian pulp and paper mills, where water used to process the pulp is clarified and aerated before being released as effluent. Water samples taken at various points during this process harbored concentrations as high as 10^5 CFU•100 ml^{-1} of both fecal coliforms and enterococci. Fecal coliforms were detected on wood chips and bark dust, suggesting a possible source of inoculum material whose growth could be supported by either biofilms in machinery and pipes, or conditions conducive to growth in the primary clarifier (36).

Sediment can influence the survival of indicator organisms once they are introduced into the environment by providing nutrients and protection. Previous studies have shown that indicator organisms can survive in water and sediment (6, 16, 17, 33, 56) and can possibly propagate in sediment (17, 20, 94). A study by Byappanahalli and Fujioka (17) demonstrated growth of *E. coli* on 10% soil extract agar. Fecal coliforms and *E. coli* from sewage also increased in numbers after being inoculated into irradiated soil. Anderson et al. (6) examined indicator survival using non-sterile sediment and water in simulated environmental conditions. Separate experiments were conducted using inoculum from contaminated soil, sewage, or dog feces. Decay rates were slower in sediments than in the water columns for fecal coliforms and *Enterococcus* spp.,

indicating greater persistence in sediment. Furthermore, the type of inoculum tended to influence persistence, as the bacteria incubated in mesocosms inoculated with soil inoculum had the lowest decay rate. Bacteria previously exposed to natural conditions like that of the mesocosms would be better adapted than bacteria from fecal matter accustomed to the gastrointestinal tract where conditions (e.g. temperature, nutrient availability) are far different. These studies suggest the ability for *E. coli* and *Enterococcus* spp. to have "survivor" strains.

Stormwater runoff or tidal movement can cause an influx of indicator organisms into surface waters. A study in 2001 (33) compared a group of four Hawaiian beaches receiving discharge from streams or storm drains to a control group of four beaches that did not receive any discharge. *E. coli* and *Enterococcus* spp. concentrations were low (0-2 CFU•100 ml^{-1}) for the beaches in the control group. However, the beaches receiving discharge did exceed the State of Hawaii recreational water quality standard of 7 enterococci•100 ml^{-1}. Solo-Gabriele et al. (94) sampled a tidally influenced river located in an urban south Florida community. *E. coli* concentrations were comparatively elevated during rain events and high tide. Concentrations were also higher in water samples taken close to the river bank when compared to water samples taken in the middle of the river. The authors concluded that the elevated *E. coli* concentrations were not representative of fecal impact alone, but that the growth of *E. coli* in riverbanks soils was a contributing factor.

The persistence of these bacteria in the environment and the association with sources other than the gastrointestinal tract of humans and animals strongly suggests that high numbers are not always correlated to the potential for pathogen presence. Therefore

the ability to determine the source of indicator organisms would be beneficial in establishing the risk to human health of environmental and recreational waters that are classified as "poor quality."

Identifying the Source of Fecal Contamination: Microbial Source Tracking

Microbial source tracking (MST) is a recently developed concept that includes a group of methodologies that provide information used to identify the dominant source(s) of fecal contamination. Its many methods use phenotypic or genotypic characteristics of an indicator or target organism to differentiate fecal sources. Phenotypic schemes are typically based on characteristics such as antibiotic resistance or carbon source utilization (40, 45). A genotypic characteristic is a specific component of the genome that is identified by a probe or amplified by the polymerase chain reaction (PCR) (77, 97).

Methods of MST can be grouped into two broad categories, library-dependent and library-independent. Library-dependent methods rely upon a database of "fingerprints" or patterns created from the phenotypic or genotypic traits of indicator organisms (e.g., *E. coli* or *Enterococcus* spp.) isolated from feces of specific host sources, i.e. human, cow, dog and seagull (101). This creates a library of patterns from known sources. Fingerprints of the indicator organism found in a contaminated water body are then compared to the library to determine the probable source. An example of a phenotypic library method is antibiotic resistance analysis (ARA), in which a pure culture of a bacterium is grown in the presence of different antibiotics at several concentrations and scored for resistance. The underlying hypothesis behind this method is that different host types are exposed to different antibiotics at different levels, ranging from clinical

treatment to no exposure, which results in variation in ARA patterns. Many studies using this method to identify sources of contamination have been published, showing discrimination between human and non-human sources (41, 45, 75, 107, 109).

An example of a genotypic library-based method is the PCR-mediated amplification of several different genetic repeating elements, collectively known as rep-PCR. Some of the repeating elements targeted are repetitive extragenic palindromes (REP), enterobacterial repetitive intergenic concensus (ERIC), and the Box sequences (BOX) believed to be part of a gene regulatory element (68). Rep-PCR has been primarily used to type pathogen strains (23, 55, 102) and has recently been applied to MST using *E. coli* strains (18, 21, 53, 73). Primers are designed to read outward from the genetic element so that segments of DNA between the repeating elements are amplified, creating amplicons of varying lengths. The amplicons are then electrophoresed, creating a visual fingerprint or pattern. A study in 2000 (21) constructed MST libraries containing human and nonhuman sources generated by REP and BOX primer(s). Using Jackknife analysis, the library generated by the BOX primer was shown to have a higher percentage of isolates correctly assigned to the source groups. A possible reason for the discriminative ability of the BOX primer was the increased number of bands it generated in the pattern when compared to the REP-patterns. A more recent study published in 2005 (46) also compared BOX and REP-generated libraries for *E. coli* and observed both libraries to have the same overall correct classification rate. This MST study was the first peer-reviewed publication to include rep-PCR libraries of *Enterococcus* spp. and demonstrated that BOX-generated patterns for *Enterococcus* spp. had the highest overall

correct classification rate when compared to REP-generated patterns for *Enterococcus* spp. and to both BOX and REP-generated patterns for *E. coli*.

A library-independent method does not require a database of patterns for comparison, but instead has a specific target which, when present, would indicate fecal contamination from a particular source. The target could be a gene, virus, or a bacterium associated with a specific host, and is usually detected by a molecular method such as PCR. An example of a specific gene would be the enterococcal surface protein (*esp*) gene, a putative virulence factor found in human-associated *E. faecium* and *E. faecalis* subtypes (43, 91). Scott et al (89) developed a PCR assay to target the *E. faecium* variant, which was detected in 97% of sewage samples (n=65), and not in bird or livestock fecal samples (n=102). Detection of the *esp* gene is based on absence/presence and has not been modified for quantification.

Host-associated viruses have also been investigated as possible MST markers. Hsu et al (49) developed oligonucleotide probes to differentiate between the four classes (serotypes) of F+ coliphages. A distinction was made between coliphages associated with human feces (class II and III) and coliphages associated with animal feces (class I and IV), but there is a question about the distribution of F+ coliphages in all individuals (47, 79) and serotype cross-specificity between human and animal hosts has been reported (83). Adenoviruses (32, 52, 66, 84) and enteroviruses (32, 62), have been targeted by PCR to detect human and non-human fecal contamination, and more recently polyomaviruses (74) have been used for the detection of human contribution. Polyomaviruses are secreted through the urine of an infected individual in concentrations as high as $10^5 \cdot ml^{-1}$ (14). Serological studies estimate that 27 to 80% of the human

population is infected in early childhood (10, 57) with what is normally an asymptomatic infection unless the individual is immunocompromised. Behzad-Behbahani et al (10) demonstrated that shedding through urine was significantly higher in immunocompromised cohorts than in immunocompetent ones, which would suggest that distribution/contribution would be limited to a portion of the population. However, two studies (13, 14) have detected polyomaviruses in sewage from the US, Europe, and Africa. Bofill-Mas et al (2000) used nested-PCR to target JCV and BKV, two human strains from the genus *Polyomavirus*, in sewage samples collected from Spain, France, Sweden, and South Africa. Ninety-six percent of the samples (n=28) were positive for JCV and 77.8% were positive for BKV. In 2001, the authors detected both strains in all sewage samples (n=15) collected from Egypt, Greece, and Washington, D.C. at concentrations of 10^2 to 10^3 JCV particles•ml^{-1} and 10^1 to 10^2 BKV particles•ml^{-1} (13). The concentrations found would indicate that even though a portion of the human population is secreting the viruses, sewage as a composite sample generally contains the polyomavirus-markers.

An example of a host-specific bacterial group utilized to determine sources of fecal contamination is the *Bacteroides-Prevotella* group (*Bacteroidales*). They are noncoliform, anaerobic bacteria that are highly concentrated in feces. Bernhard et al (11) designed primers to distinguish between human-associated and ruminant-associated species. The PCR assay does not require culturing, but uses DNA extracted from fecal or water samples as template. A study in 2003 (12) tested coastal sites in southern California for human impact using the human-associated primers. No correlation was found between positive reactions (presence of marker) and levels of indicator bacteria (total

coliforms, *E. coli* and *Enterococcus* spp.). There was no exceedance of regulatory standards at the sites testing positive for the human-associated marker, but one site tested negative for the marker and exceeded the enterococci standard. Quantification of the marker would help in determining correlation to enumerated indicator bacteria in contaminated waters. A study in 2005 (90) developed a SYBR Green PCR assay for quantification using a previously published human-specific forward primer (11) and a novel reverse primer. The limit of detection was one nanogram of human feces seeded into one liter of freshwater and the limit of quantification was 10^5 markers per liter of seeded freshwater.

A phylogenic approach used by Dick et al (2005) analyzed *Bacteroidales* 16S rRNA gene sequences from the feces of many animal hosts. Human, cat, dog, and gull sequences clustered together with known culturable species, while ruminant, pig, and horse formed unique clusters of uncultivated bacteria from *Bacteroidales.* Primers were developed for pig and horse that amplified target DNA from the feces of those hosts and not from other species. Such an approach could be useful for identification of other host-specific markers.

Microbial source tracking includes a wide array of phenotypic, genotypic, library-dependent, or library-independent methods that together represent a "toolbox" approach. Currently, no single "tool" or method can predict the source of fecal contamination with great confidence. There are still questions about the distribution of host-specific patterns, fingerprints, and markers; e.g., are they distributed in all individuals of that host, and only for that particular host? As methods continue to develop, and are combined for validation

and robustness, this will aid in identifying sources of fecal contamination and therefore aid in the restoration of impacted recreational and environmental waters.

Diversity/Similarity of Indicator Populations

The eighteenth century biologist, Carolus Linnaeus, created a system to classify all living organisms based on the differences and similarities of the organisms (95). This system, which still exists today, used morphological characteristics to name and separate large, visible organisms into a hierarchy of groups or taxa. To further define species, the smallest unit of the classification system, a biological species concept was first formulated in 1942 by Ernst Mayr (70). According to the concept, species are populations that can reproduce amongst themselves, but not with other groups, therefore keeping their gene pools separate. Applying the classical species concept to prokaryotic organisms is quite problematic. Not only are prokaryotes asexual, but many can participate in lateral transfer of DNA from other species (22, 96).

A molecular approach is used to circumvent the classic species definition for one more accommodating to microorganisms. DNA: DNA hybridization is one method used to determine relatedness between bacterial isolates. There is no set rule, but in general, an outcome of $\geq$ 70% hybridization between the genomic DNA of two isolates would mean they were of the same species (64). Another approach to identifying species is to use a molecular chronometer to measure evolutionary genetic changes. Among prokaryotes the 16S rRNA sequence is considered to be highly conserved and can therefore measure long-term evolutionary relationships. Variable regions within the conserved sequences can be translated into the phylogenic distances that are used to determine genera and

species (110). When comparing isolates, less than 97%similarity in 16S rRNA sequences would infer different species and is usually coupled with less than 70% DNA:DNA hybridization (64).

Genetic differences within bacterial species are also common, and are utilized for library-based MST methods that use molecular typing of bacterial groups (e.g. *E. coli* and *Enterococcus* spp.) from different hosts (46, 53, 101). Various genetic typing methods can be used to generate a "DNA fingerprint" for a given isolate, which can be matched for identity, or a pre-determined level of similarity, to other fingerprints (e.g. unknowns to host sources). These same fingerprints can be compared in terms of their genetic variability to determine how diverse *E. coli* or *Enterococcus* spp. subtypes are in a particular population.

Measuring the diversity of an *E. coli* or *Enterococcus* population by typing the finite number of individuals in that community is an impossible task. Measuring a sample or subset of that population to estimate its diversity is more plausible and can be done with diversity indices such as Shannon-Weiner. This diversity index takes into account the number of subtypes as well as the frequency of those subtypes (9), and has been previously used to measure microbial population diversity in habitats such as rhizospheres, artesian spring sediments, and microbial mats (25, 71, 82). Another method used to measure population diversity is the accumulation curve, which plots the number of new subtypes observed versus sampling effort. This gives information about how well a population has been sampled; as the curve reaches an asymptote a larger portion of the total population has been sampled (50). The accumulation curve has been previously used to estimate diversity in animal populations (15) and more recently applied to *E. coli*

populations in horse, cattle, and human feces (7). Accumulation curves can be useful in comparing relative diversities of populations that have been affected by an environmental change (50).

Similarity between *E. coli* or *Enterococcus* populations can be measured with the population similarity coefficient, which measures the proportion of identical subtypes in two populations (60). This has been previously used to compare phenotypic subtypes of coliforms in environmental water samples (60), and phenotypic subtypes of fecal coliforms and enterococci in sewage (67, 104, 105), and in the feces of livestock, seabirds, and dogs (61, 106). Population similarity can be used to explore the hypothesis that physical contribution of indicator bacteria from one environmental compartment to another, such as a storm drainage system to receiving coastal waters, can be a source of indicator bacteria.

Specific Objectives of the Thesis

Siesta Key Beach is located on the Gulf Coast of Florida, south of Tampa, and is frequently mentioned among the top ten beaches in the US. In summer 2004, high levels of fecal coliforms and *Enterococcus* spp. caused health warnings to be posted by the Florida Department of Health. A stormwater drainage system was implicated as a possible source of microbial pollution (Figure 1). Stormwater flows through underground pipes to an underground concrete vault, where it may be retained for many days. Overflow stormwater is delivered to an open retention pond located approximately 100 yards from the landward edge of the beach. Rain events cause movement from this system to a ditch that empties into the Gulf of Mexico at Siesta Key Beach.

The specific objectives of this study were threefold: 1) to assess and compare population diversity of *E. coli* and *Enterococcus* in the drainage system during a rain event and during dry conditions, 2) to observe similarity of the *E. coli* and *Enterococcus* populations between specific sites sampled throughout the storm drainage system to the Gulf, and 3) to compare the *Enterococcus* populations of Siesta Key to that of sewage and of a pristine site (no known human impact, or urban stormwater runoff). These characteristics of the indicator bacteria populations were used to explore the hypothesis that the microbial contamination at Siesta Key Beach originated from the stormwater system.

MATERIALS AND METHODS

Study Site and Sampling Strategy

Siesta Key Beach is located on a barrier island on the west coast of Florida in Sarasota County. A stormwater conveyance system runs parallel to the beach underneath a paved thoroughfare (Figure 1). The stormwater system receives runoff from an urban, residential area of approximately 60 acres. A portion of the stormwater enters a canal on the east side of the road (northeast of the beach), and the majority remains in the underground system, which runs southward to an underground concrete vault on the west side of the road, approximately 100 yards from the beach. Water may be retained in the vault for many days until a rain event causes overflow, which is pumped into an adjacent retention pond. Surface runoff from the road and overflow from the pond enter a ditch, which flows ~100 yards before it empties onto the beach. During heavy rain, the ditch outfall reaches the Gulf waters.

Two sampling events were conducted during this study; one within 48 hours of heavy rainfall (Figure 3), and one during a dry period (Figure 4). Water and sediment samples were taken at various points, i.e., access was obtained via a manhole to sample the stormpipe that feeds the vault, the vault was sampled through a metal-covered access portal, and the ditch and its beach outfall were sampled from the surface (Table 2). The land around the ditch and the ditch itself was heavily vegetated, and therefore shaded, with Brazilian pepper trees and mangroves. More surface sampling sites were added

(retention pond and Gulf of Mexico) for the second sampling (dry period) in order to obtain a more complete picture of the possible sources and sinks of microorganisms in the drainage system.

For genetic diversity studies, *Enterococcus* spp. were also isolated from sewage and a pristine water site. Untreated sewage samples were obtained from lift stations in the Florida counties of Duval and Wakulla. Water samples from a pristine site were collected at Deer Prairie Slough in the Myakka River, Myakka River State Park (Sarasota County; GPS - N Latitude 27° 10.543' and W Longitude 82° 12.705'). This site was chosen due to the absence of known human impact and urban stormwater runoff.

To examine variability in collection of subtypes during the sampling process, a study was conducted using replicate water samples from a pond located on campus at the University of South Florida, Tampa campus (GPS – N Latitude 28° 03.704'and W Longitude 82° 25.060'). One-liter grab samples (triplicate) were collected in a one-meter^2 area, just below the water surface level, close to the shore. The pond covered ~ 3 acres, had little shade, and was inhabited by ducks.

Isolation and Enumeration of Indicator Bacteria

Water and sediment samples were collected in sterile containers, immediately placed on ice, and processed within 4 h of collection at the USF (Tampa, FL) laboratory. Water samples were collected in one-liter containers (in duplicate) and filtered through sterile nitrocellulose membranes (0.45 μm pore-size, 47 mm diameter) to enumerate fecal coliforms and *Enterococcus* spp. Sediment samples were collected (in duplicate) in 50 ml screw-cap conical tubes by scooping the top layer of sediment into the conical tube.

Twenty grams (wet weight) of sediment were added to 200 ml of sterile buffered water (0.0425 g L^{-1} KH_2PO4 and 0.4055 g L^{-1} $MgCl_2$) and sonicated as previously described (6) to release bacteria from soil particles. A range of sample volumes and dilutions for both water and sediment samples were filtered to allow for accurate enumeration of bacterial cells. Fecal coliforms were enumerated on mFC agar (Difco) and incubated for 24 h at 44.5^{o} C in a water bath (4). Blue colonies were counted as fecal coliforms and then inoculated into microtiter plates containing EC broth amended with 4-methylumbelliferyl-β-D-glucuronide (MUG) (50μg/ml) in order to determine the percentage of the colonies that were *E. coli*. After incubation for 24 h at 37^{o} C, the microtiter plates were exposed to ultraviolet (UV) light. Fluorescence indicated strains that had β−glucuronidase activity (MUG +), a characteristic of *E. coli*. For further confirmation, 25% of the MUG + isolates were profiled biochemically using API 20E strips (BioMerieux), and 100% were identified as *E. coli.* MUG + fecal coliforms were therefore designated *E. coli* and fingerprinted by BOX-PCR for the similarity/diversity study.

Enterococcus spp. were enumerated by USEPA Method 1600 (100), in which filters were incubated on mEI agar (base media from Difco; indoxyl β-D glucoside from Sigma Aldrich) at 41^{o}C for 24 h. All resultant colonies with a blue halo were counted as *Enterococcus* spp. Plates with suitable colony numbers (10 – 100 CFU) were counted, and concentrations for each volume were calculated. If indicator bacteria concentrations were low, and no filtration volume contained more than 10 CFU/plate, plates with less than 10 CFU were counted. Concentrations for all indicators were log_{10}-transformed and recorded as CFU·100 ml^{-1} (water samples) or 100 g wet $weight^{-1}$ (sediment samples).

BOX-PCR of *E. coli* and *Enterococcus* spp.

E. coli strains were grown overnight in microcentrifuge tubes containing 750 µl of BHI broth (Becton Dickinson). After centrifugation at 14,000 RPM for one minute, pellets were washed with sterile buffered water two times and resuspended in 500 µl of deionized sterile water. The cell suspension was boiled for 5 minutes to lyse the cells and then centrifuged again at 14,000 RPM for one minute. One µl of supernatant was used as template for each PCR reaction. BOX-PCR fingerprints were generated using the previously published BOXA1R primer (58), which has the following sequence: 5'-CTA CGG CAA GGC GAC GCT GAC G- 3'. Reagents and volumes for each 25 µl reaction were: 2.5 µl 10X Buffer B (Fisher Scientific); 3.0 µl 25mM $MgCl_2$ (Fisher Scientific); 1.0 µl 10mM dNTPs (Fisher Scientific); 2.5 µl 2% bovine serum albumin (Sigma); 1.3 µl 10 µM BOXA1R primer (IDT, Coralville, IA); 1.0 µl Taq polymerase (5000u/ml) (Fisher Scientific); and 12.7 µl PCR-grade water (Fisher Scientific). The thermocycler program contained three steps: 1) initial denaturation at 95ºC for 5 minutes; 2) 35 cycles of 94º C for 1 minute, 60º C for 1 minute, and 72º C for 1 minute; and 3) final extension at 72º C for 10 minutes. The preceding protocol was provided by correspondence with Dr. Cindy Nakatsu, Purdue University, West Lafayette, IN.

Enterococcus spp. were grown overnight in microcentrifuge tubes containing 1.5 ml of BHI broth (Becton Dickinson). DNA was extracted using the DNeasy Tissue Kit (Qiagen, Valencia, CA) and the manufacturer's protocol for Gram-positive bacteria. BOX-PCR fingerprints for enterococci were generated using the BOXA2R primer (58), which has the following sequence: 5'-ACG TGG TTT GAA GAG ATT TTC G- 3'. PCR reagents and conditions used were from previously published protocols with

modifications (65, 103). Each 25 µl PCR reaction contained: 5 µl of 5X Gitschier Buffer (59); 2.5 µl of 10% dimethyl sulfoxide; 0.4 µl bovine serum albumin(10mg/ml); 2.0 µl 10mM dNTPs; 1.0 µl Taq polymerase (5000u/ml); 11.6 µl PCR-grade water; 1.5 µl 10µM BOXA2R primer; and 1.0 µl of DNA template, containing between 30 to 100 ng · μl^{-1}. The thermocycler program contained three steps: 1) initial denaturation at 95ºC for 7 minutes; 2) 35 cycles of 90º C for 30 seconds, 40º C for 1 minute, and 65º C for 8 minute; and 3) final extension at 65º C for 16 minutes.

Fragments were separated by electrophoresis through a 1.5% agarose gel for 4 hours at 90 volts (*E. coli* fingerprints), or 6 hours at 60 volts (*Enterococcus* spp. fingerprints). Gels were stained with ethidium bromide (1% solution). Gels were digitally documented under UV light using a FOTO/Analyst Archiver (Fotodyne, Hartland, WI).

Statistical Analysis

Fingerprint patterns of *E. coli* and *Enterococcus* spp. subtypes generated by BOX-PCR were analyzed with BioNumerics 4.0 software (Applied Maths, Belgium). Dendrograms were created using a densiometric curve-based algorithm (Pearson correlation coefficient, optimization 1%) and UPGMA to cluster patterns by similarity. Repeated runs of the control strains, ATCC 9637 for *E. coli* and ATCC 19433 (*E. faecalis*) for *Enterococcus* spp., were 86% and 93% similar, respectively. Therefore, patterns showing ≥ the similarity value established by the control strains were considered identical. The relationship of patterns considered similar was confirmed by eye.

The relationships of indicator bacteria populations at the various sites were determined by dendrograms constructed using a population similarity coefficient (S*p*)

(Table 1), previously published by Kuhn et al (1991). The algorithm is based on the proportion of identical isolates between two populations; therefore, if two populations have no identical subtypes Sp = 0, and as the number of identical subtypes increases between two populations, the Sp increases to a maximum of 1.0 (60). The population similarity coefficient was used to compare *E. coli* and *Enterococcus* populations at Siesta Key during a rain event and during dry conditions, and to further compare *Enterococcus* populations at Siesta Key to *Enterococcus* populations in sewage and in a sampled site on Myakka River.

Accumulation curves and the Shannon-Weiner diversity index were calculated using EcoSim 7 software (Acquired Intelligence Inc. & Kesey-Bear, Jericho, VT). An accumulation curve measures the diversity of a sampled population by plotting new subtypes as a function of sampling effort. As the curve approaches an asymptote (slope = 0), the probability of obtaining new subtypes with additional sampling diminishes. The Shannon-Weiner index (H') of diversity considers the frequency of the various subtypes in a population as well as the total number of subtypes (Table 1). Both the accumulation curve and the Shannon-Weiner index were used to compare the relative diversities of *E. coli* and *Enterococcus* populations during a rain event and dry conditions at Siesta Key, and to further compare *Enterococcus* populations at Siesta Key to *Enterococcus* populations in sewage and in a sampled site on Myakka River. Paired t tests, nonparametric tests (Mann-Whitney), and ANOVA were used to determine significant difference in the comparisons. GraphPad Prism version 4.02 (GraphPad Software, San Diego, CA) was used for the statistical analyses.

Figure 1. Sampling locations within the stormwater system draining to Siesta Key Beach (light blue arrows indicate general direction of stormwater flow)

Figure 2. Ditch outfall at Siesta Key Beach

Table 1. Equations for indicator population diversity and similarity

Shannon-Weiner index (H') = $-\Sigma p_i \ln(p_i)$
p_i = # isolates with pattern (i)/total isolates
 	
Population similarity coefficient (Sp) = (Sx + Sy)/2
Sx = $\sum qx_i$ / Nx
Sy = $\sum qy_i$ / Ny
Nx = total # isolates population x
Ny = total # isolates population y
qx_i = proportion of isolates identical to isolate i in population x divided by proportion of isolates identical to isolate i in population y
qy_i = the proportion of isolates identical to isolate i in population y divided by the proportion of isolates identical to isolate i in population x

Figure 3. Rainfall (inches) during wet conditions sampling at Siesta Key Beach

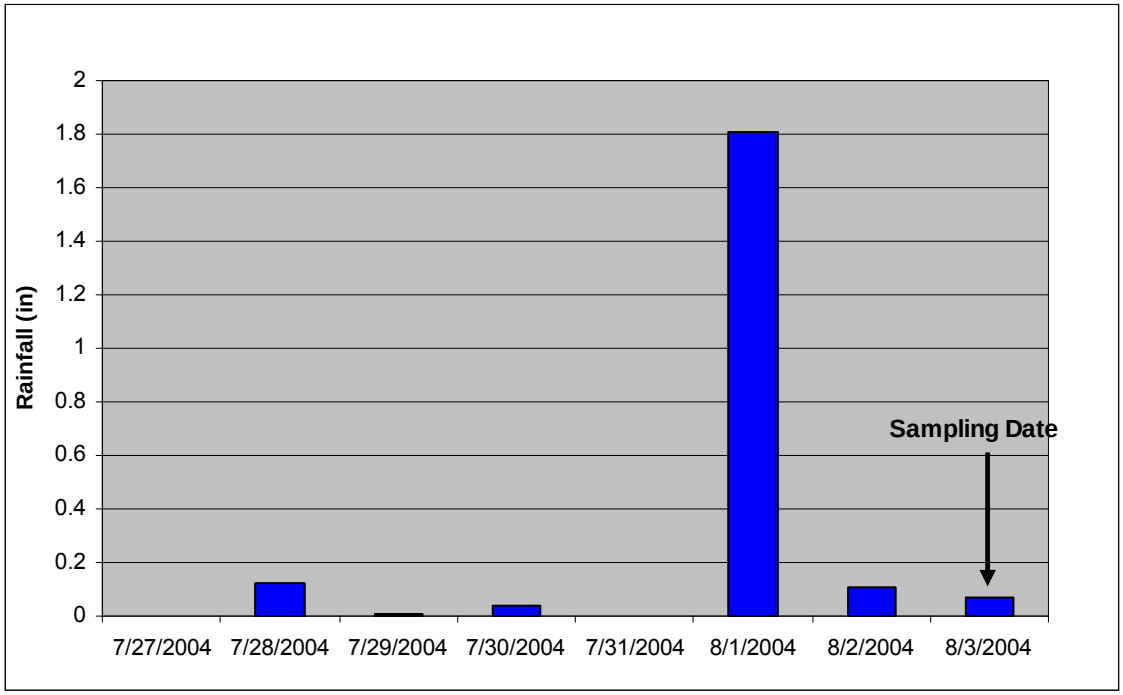


Figure 4. Rainfall (inches) during dry conditions sampling at Siesta Key Beach

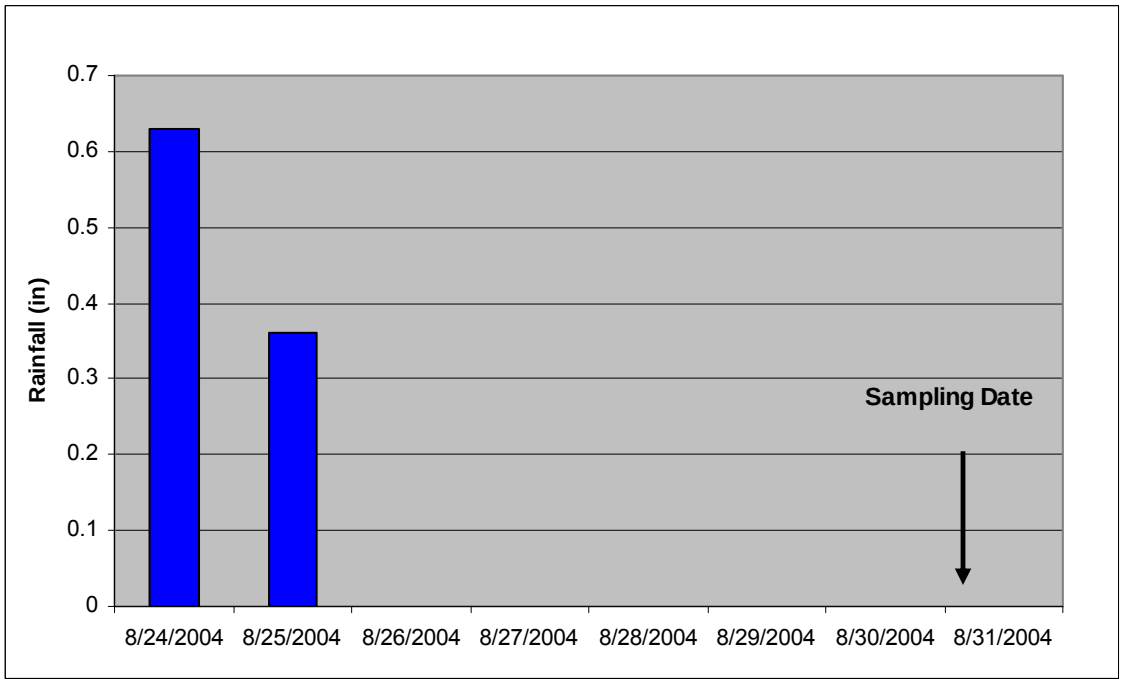


Table 2. Sites sampled at Siesta Key Beach after a rain event (08/03/04) and during dry conditions (08/31/04). Analyses conducted ■, or not conducted □

Site	Sample Date	Analyses Conducted			
		Fecal coliform concentration	*Enterococcus* spp. concentration	BOX-PCR *E. coli*	BOX-PCR *Enterococcus* spp.
Stormpipe water	08/03/04	■	■	■	■
	08/31/04	■	■	□†	■
Stormpipe sediment	08/03/04	□	□	□	□
	08/31/04	■	■	■	■
Vault water	08/03/04	■	■	■	■
	08/31/04	■	■	■	■
Pond water	08/03/04	□	□	□	□
	08/31/04	■	■	□†	□†
Pond sediment	08/03/04	□	□	□	□
	08/31/04	■	■	□†	□†
Ditch water	08/03/04	■	■	■	■
	08/31/04	■	■	■	■
Ditch sediment	08/03/04	■	■	■	■
	08/31/04	■	■	□†	□†
Beach water[1]	08/03/04	■	■	■	■
	08/31/04	■	■	■	■
Beach sediment	08/03/04	■	■	■	■
	08/31/04	■	■	□†	□†
Gulf water[2]	08/03/04	□	□	□	□
	08/31/04	■	■	■	■
Gulf sediment	08/03/04	□	□	□	□
	08/31/04	■	■	□†	□†

[1]Beach water and sediment were collected on the beach, within a few yards of the ditch
[2]Gulf water and sediment were collected in the Gulf of Mexico
†Less than 10 isolates were recovered

RESULTS

Enumeration of Indicator Bacteria

Indicator bacteria were enumerated from water and sediment samples collected from the rain event (Figures 5 and 6) and during dry conditions (Figures 7 and 8). Indicator bacteria (*Enterococcus* spp. and fecal coliform) concentrations exceeded the Florida standards for recreational waters during the rain event in all water samples, including tidal water on the beach (Figure 5). *Enterococcus* spp. concentrations were significantly higher than fecal coliforms ($P = 0.041$, paired t test). The mean concentrations ($\log_{10}$-transformed) were 3.17 ± 0.72 and 4.20 ± 0.37 for fecal coliforms and *Enterococcus* spp., respectively. Indicator bacteria levels were also high in sediments collected during the rain event, at $>10^3$ CFU/100 g (Figure 6), although there are no regulatory standards for indicator concentrations in sediment.

During dry conditions, fecal coliforms exceeded the standard only at the beach, where water pools from ditch outfall and/or at high tide (Figures 2 and 7). *Enterococcus* spp. concentrations exceeded the standard in beach water, stormpipe water, and vault water (Figure 7). Water sampled from the retention pond, ditch, and the Gulf was within the regulatory standard limits for recreational waters for both fecal coliforms and *Enterococcus* spp. (Figure 7). Indicator bacteria concentrations remained high in the sediments during dry conditions with stormpipe sediment the highest at $>10^{3.5}$ CFU/100 g for both fecal coliforms and *Enterococcus* spp. (Figure 8). Overall, *Enterococcus* spp.

concentrations in sediments were significantly higher than fecal coliform concentrations by a paired t-test ($P = 0.020$) during dry conditions. The mean concentrations (log_{10}-transformed) were 1.70 ± 1.38 and 3.21 ± 1.11 for fecal coliforms and *Enterococcus* spp., respectively. Indicator organism concentrations in water samples at sites sampled on both dates (i.e., stormpipe water, vault water, ditch water, and beach water; Table 2) were compared. Mean indicator organism concentrations were significantly higher during the rain event than during dry conditions as assessed by a nonparametric, Mann-Whitney t test. Differences in mean log_{10}-transformed concentrations were statistically significant for *Enterococcus* spp. ($P = 0.028$) and nearly significant for fecal coliforms ($P = 0.057$) at the $\alpha = 0.05$ level. The mean fecal coliform concentration (log_{10}-transformed) on 8/3/04 (rain event) was 3.17 ± 0.72, while it was 1.57 ± 0.91 on 8/31/04 (dry conditions). Corresponding means for *Enterococcus* spp. were 4.20 ± 0.37 on 8/3/04 and 2.55 ± 0.68 on 8/31/04.

Figure 5. Fecal coliform and *Enterococcus* spp. concentrations from water samples collected during the rain event (log_{10} CFU/100ml)

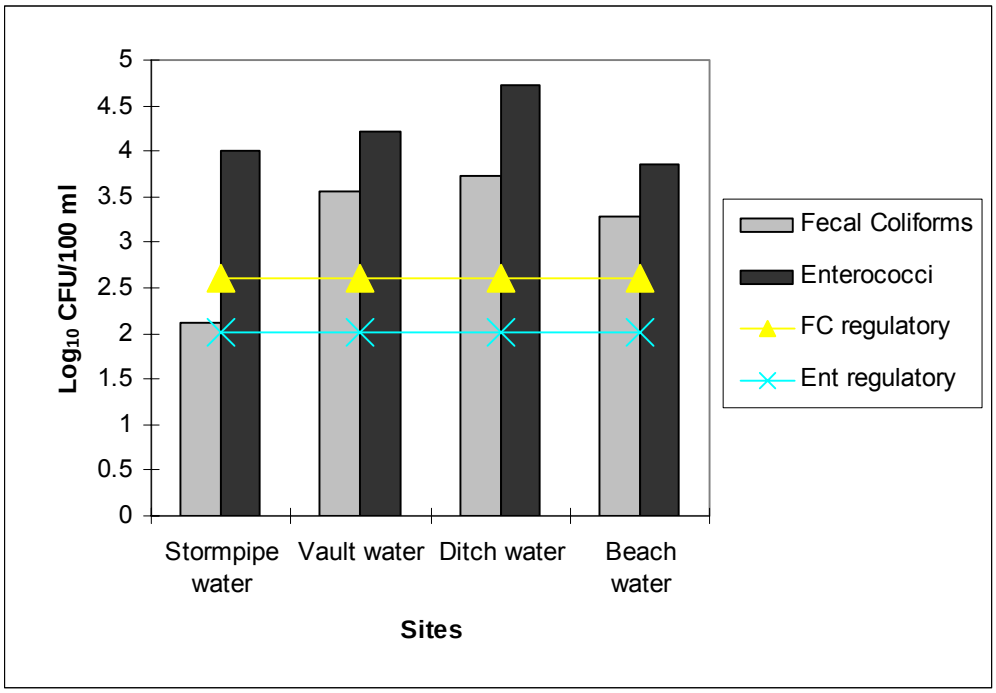


Figure 6. Fecal coliform and *Enterococcus* spp. concentrations from sediment samples collected during the rain event (log_{10}CFU/100g)

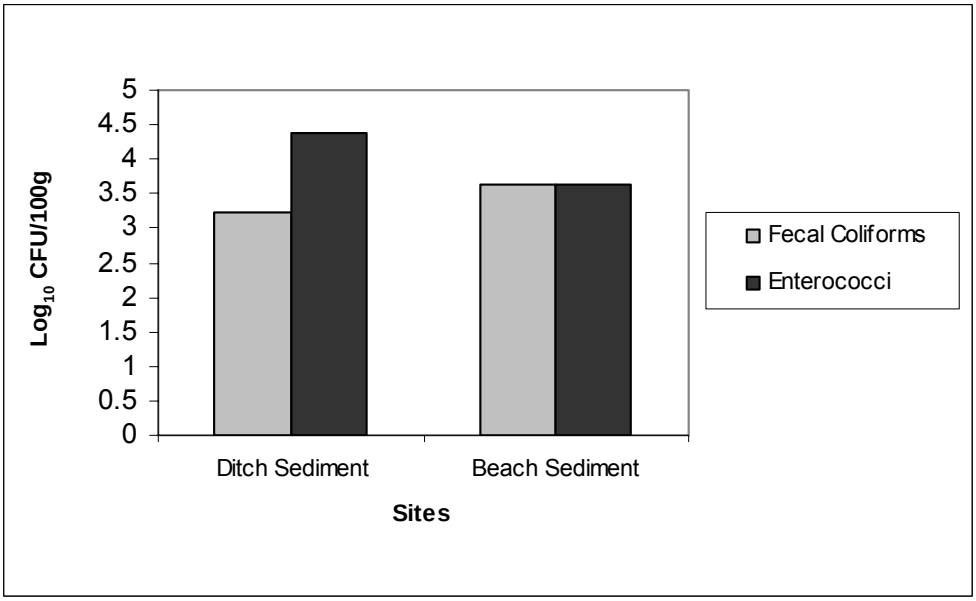


Figure 7. Fecal coliform and *Enterococcus* spp. concentrations from water samples collected during dry conditions (log_{10} CFU/100ml)

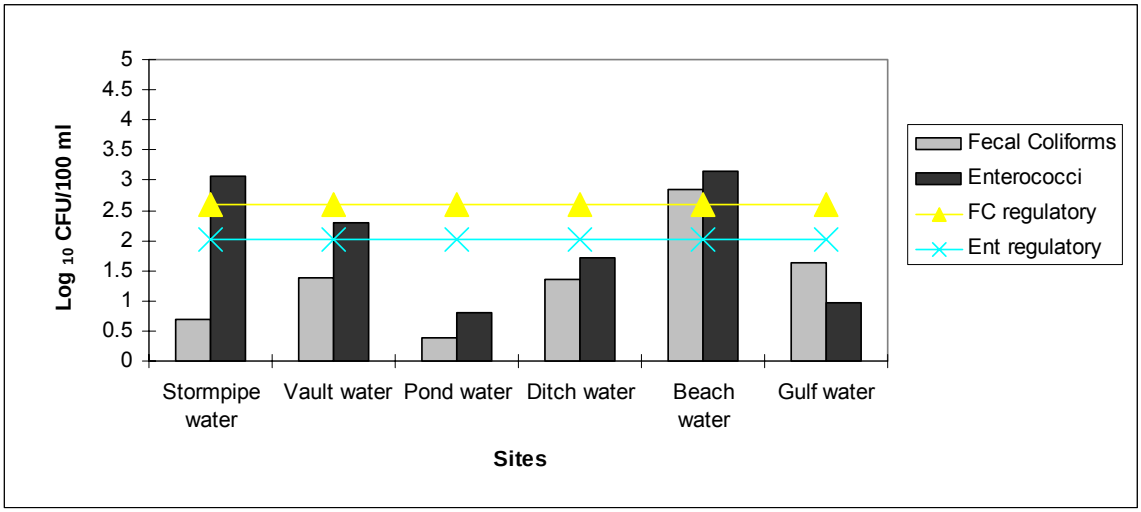
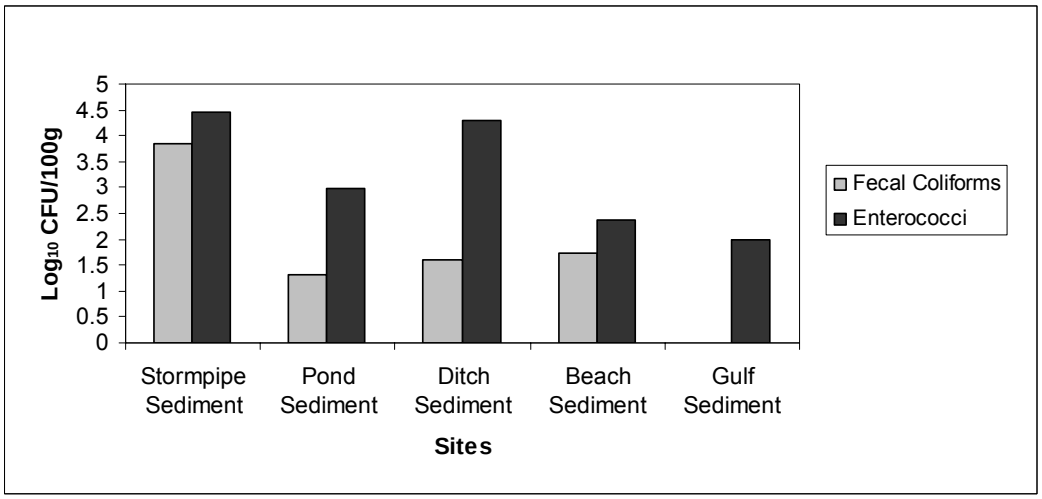


Figure 8. Fecal coliform and *Enterococcus* spp. concentrations from sediment samples collected during dry conditions (log_{10}CFU/100g)



Diversity Measured by Accumulation Curves and the Shannon-Weiner Index

Accumulation curves for the *E. coli* populations (Figure 9) and the *Enterococcus* populations (Figure 10) sampled during the rain event do not reach an asymptote, clearly showing that the population diversity for these sites was not completely captured by the sampling effort. For dry conditions, the accumulation curve for the *E. coli* population sampled from the ditch water and vault water (Figure 11) and the *Enterococcus* population sampled from the Gulf water (Figure 12) reached an asymptote, showing that the population diversity was captured by the sampling effort. Overall, accumulation curves indicated a trend in lower diversity during dry conditions for *E. coli* and *Enterococcus* populations.

Averaged accumulation curves were constructed for *Enterococcus* populations for the rain event (n = 4), dry conditions (n = 4), sewage samples (n = 3), and samples collected at Myakka River (n = 3) (Figure 13). A higher diversity of *Enterococcus* populations in the rain event and sewage and a lower diversity of *Enterococcus* populations in dry conditions and Myakka River reflect the differences in the Shannon-Weiner index for these four groups (see below, and Table 4).

Sites that were sampled for both the rain event and dry conditions and had 14 to 20 isolates per site were chosen for comparison of diversity by using the Shannon-Weiner index (*H*'). In comparing population diversity of *E. coli* versus *Enterococcus* spp., there was no significant difference in either the rain event or dry conditions; however, there was a significant difference in the population diversity of *E. coli* when comparing the rain event versus dry conditions ($P = 0.047$, Table 3), and a significant difference in the

population diversity of *Enterococcus* spp. when comparing the rain event versus dry conditions ($P = 0.008$, Table 3).

Enterococcus populations from sewage samples and from Myakka River (pristine site) samples were measured for diversity using the Shannon-Weiner index. There was a significant difference in the population diversity between sewage (mean $H' = 2.69$) and Myakka River (mean $H' = 1.96$) with the diversity being higher in the sewage samples than in the samples collected from Myakka River ($P = 0.024$). Furthermore, a one-way analysis of variance showed significant difference when comparing the population diversities of the rain event, dry conditions, sewage, and Myakka River (Table 4).

Figure 9. Accumulation curves for *E. coli* populations during the rain event. Subtypes are fingerprint patterns of *E. coli* isolates by BOX-PCR

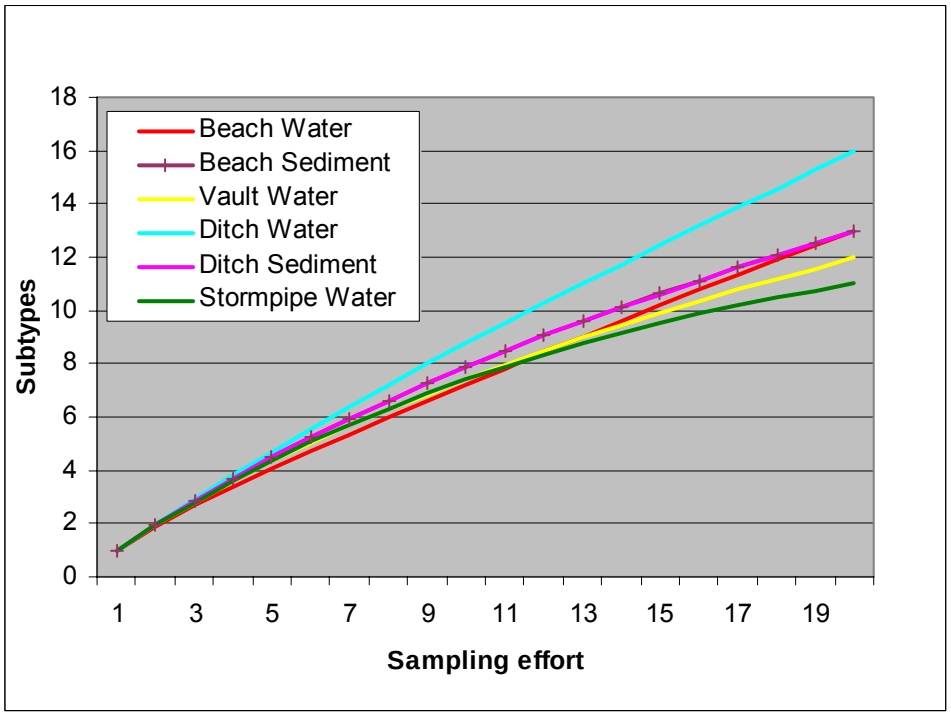


Figure 10. Accumulation curves for *Enterococcus* populations during the rain event. Subtypes are fingerprint patterns of *Enterococcus* spp. isolates by BOX-PCR

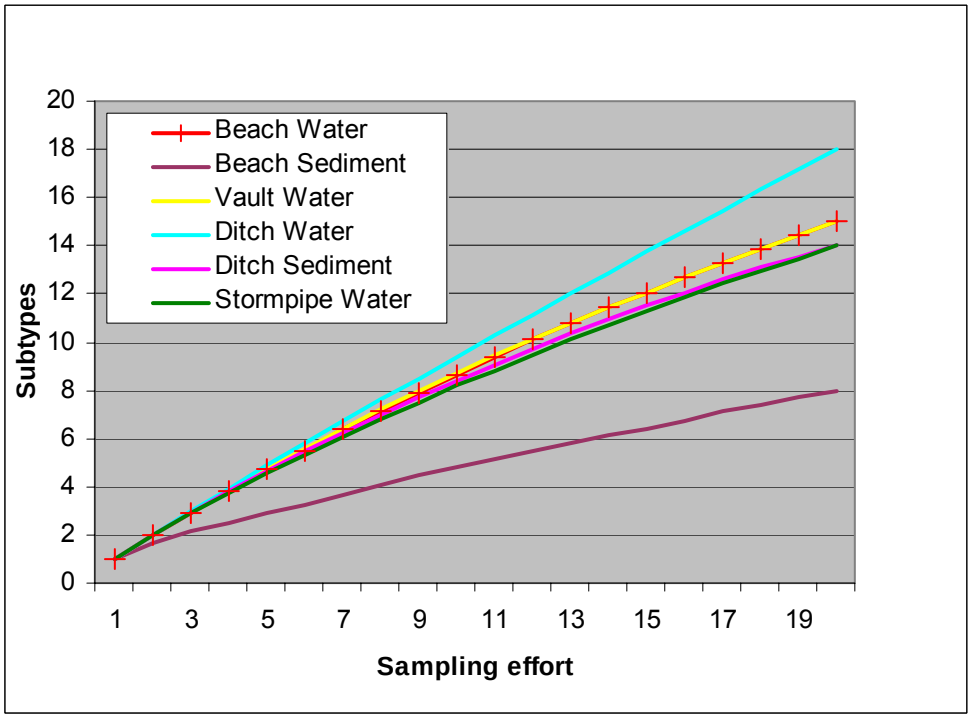


Figure 11. Accumulation curves for *E. coli* populations during dry conditions. Subtypes are fingerprint patterns of *E. coli* isolates by BOX-PCR

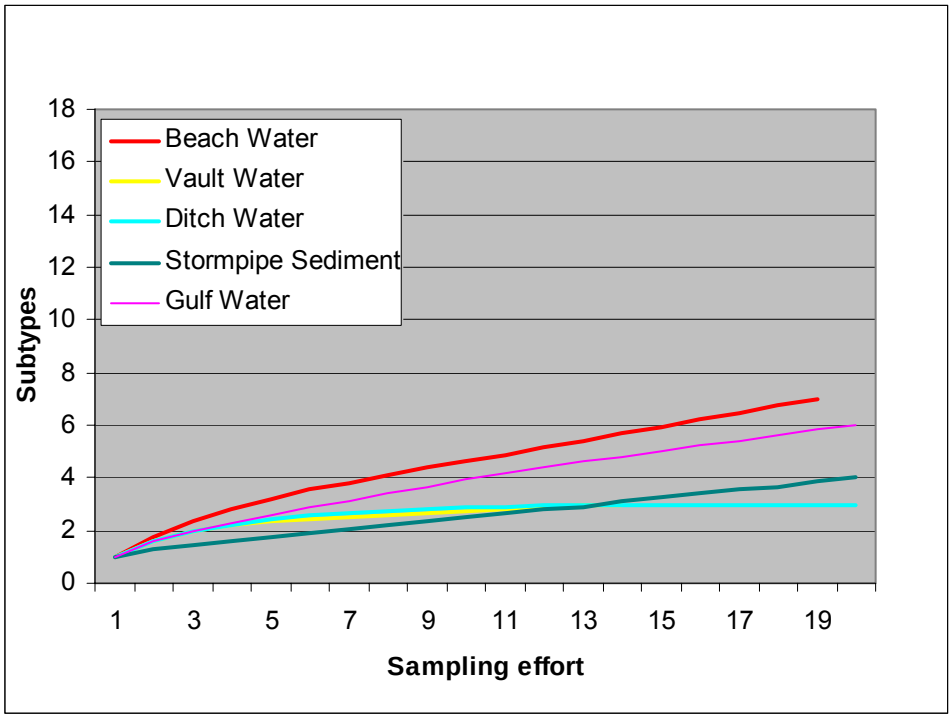


Figure 12. Accumulation curves for *Enterococcus* populations during dry conditions. Subtypes are fingerprint patterns of *Enterococcus* spp. isolates by BOX-PCR

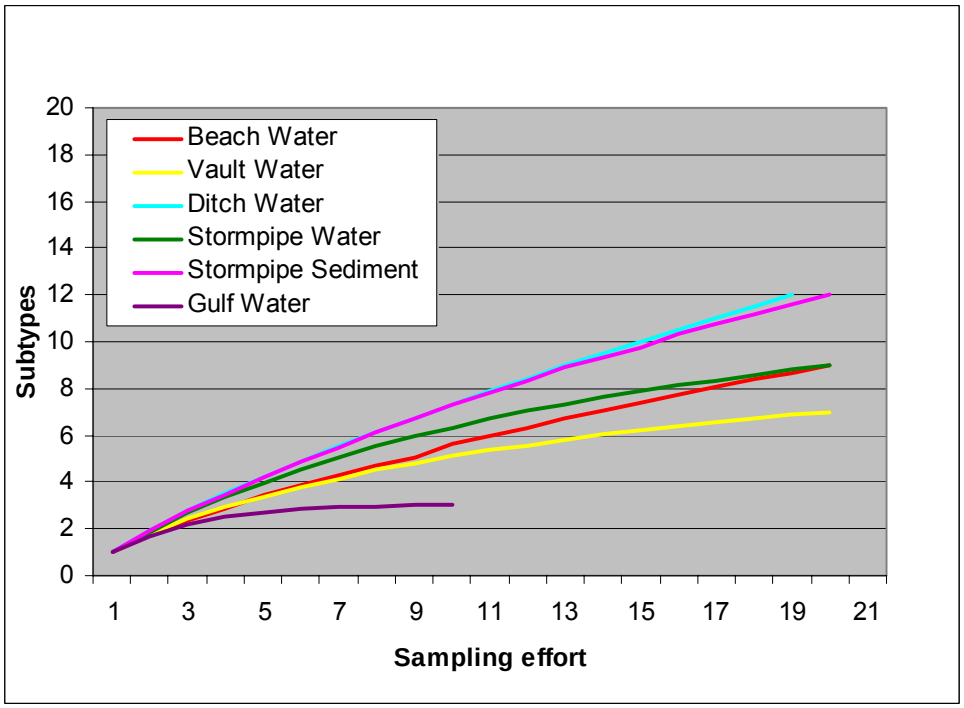


Figure 13. Averaged accumulation curves for *Enterococcus* populations. Sites included for both rain and dry conditions: beach water, ditch water, vault water, and stormpipe water. Subtypes are fingerprint patterns of *Enterococcus* spp. isolates by BOX-PCR

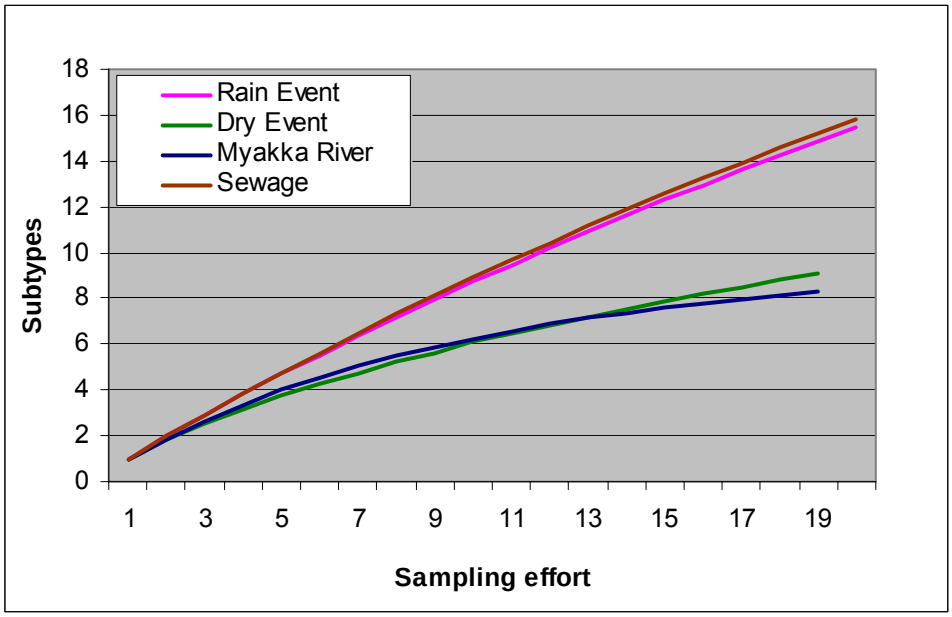


Table 3. Comparison of the population diversity of *E. coli* and *Enterococcus* spp. during the rain event versus dry conditions. Paired t test, (α=0.05, $\pm$ standard deviation)

Indicator (sites)	Mean *H'*	P value
*E. coli (*beach water, ditch water, vault water)	Rain event = 2.39 $\pm$ 0.22 Dry conditions =1.12 $\pm$ 0.34	*P* = 0.047
Enterococcus spp. (beach water, ditch water, stormpipe water, vault water)	Rain event = 2.65 $\pm$ 0.13 Dry conditions =1.88 $\pm$ 0.28	*P* = 0.008

Table 4. Comparison of the population diversity of *Enterococcus* spp. in sewage, Myakka River, rain event, and dry conditions. Values that share the same letter within columns are not significantly different. ANOVA, (*P* = 0.0001, α=0.05, $\pm$ standard deviation)

Sample events	Mean *H'*
Sewage	2.69 $\pm$ 0.09 (a)
Myakka	1.96 $\pm$ 0.22 (b)
Dry conditions	1.88 $\pm$ 0.45 (b)
Rain event	2.66 $\pm$ 0.13 (a)

Similarity Measured by the Population Similarity Coefficient

An estimate of the similarity of *Enterococcus* populations isolated from three replicate samples on the same day was carried out. This experiment was meant to provide a benchmark for population similarity in samples in which the population structure was expected to be very similar, and to examine the variability in observed population structure of *Enterococcus* spp. contributed at the level of replicate samples. The water samples were collected from a pond located on the campus at the University of South Florida. The *Enterococcus* spp. concentration was 40 CFU•100 ml^{-1}, therefore 400 possible (culturable) subtypes were in each one-liter sample. Approximately twenty isolates from each replicate were fingerprinted by BOX-PCR and compared for similarity by using the population similarity coefficient (see Materials and Methods). Among the 58 isolates subtyped from the three replicate samples, only five different BOX-PCR patterns were observed. Samples A and B were 88% similar, while sample C was 52% similar to samples A and B (Figure 14). All three samples (A, B, and C) shared two patterns out of five total patterns. Samples A and B shared one pattern and samples B and C shared another pattern. Sample C had one pattern that was not shared with any other sample.

Fingerprint patterns of indicator isolates (*E. coli* or *Enterococcus* spp.) for sampled sites (rain event and dry conditions) were compared to each other to determine similarity between site populations. The population similarity was calculated by using the population similarity coefficient. Sampled sites included for comparison of *E. coli* populations during the rain event were: beach sediment, stormpipe water, beach water, ditch sediment, ditch water and vault water (Figure 15). During dry conditions, sampled sites for *E. coli* population comparisons were: beach water, ditch water, stormpipe

sediment, vault water, and Gulf water (Figure 16). During the rain event, the highest *E. coli* population similarity was between ditch water and ditch sediment, followed by similarity between stormpipe water and beach sediment. During dry conditions, ditch water and vault water had the highest similarity, followed by beach water and stormpipe sediment. Overall, there was higher similarity between sites during dry conditions when compared to the rain event. The Gulf water population, which was only sampled during dry conditions, had no similarity to any other sites.

Sampled sites included for comparison of *Enterococcus* populations during the rain event were: beach sediment, stormpipe water, beach water, ditch sediment, ditch water and vault water (Figure 17). During dry conditions, sampled sites for *Enterococcus* population comparisons were: beach water, ditch water, stormpipe sediment, stormpipe water, vault water, and Gulf water (Figure 18). During the rain event, beach water and ditch sediment had the highest similarity. During dry conditions, stormpipe water and vault water had the highest similarity followed by beach water and ditch water. The Gulf water population, which was only sampled during dry conditions, had no similarity to any other sites. Overall, population similarities were higher during dry conditions than during the rain event.

Sites that were compared for similarity for the rain event and for dry conditions were grouped together and labeled "Sampling 1" and "Sampling 2", respectively. The two sampling dates were then compared for similarity to sewage and Myakka River (Figure 19). The two populations with the highest similarity were Myakka River and sampling 2, and the population with the least similarity to all other groups was sewage.

Figure 14. Similarity of *Enterococcus* populations from three replicate water samples collected from a pond, based on BOX-PCR fingerprints

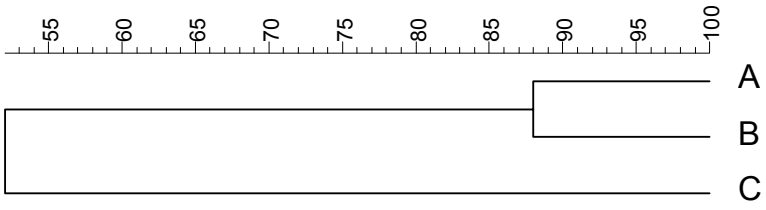


Figure 15. Similarity of *E. coli* populations by site during the rain event, based on BOX-PCR fingerprints

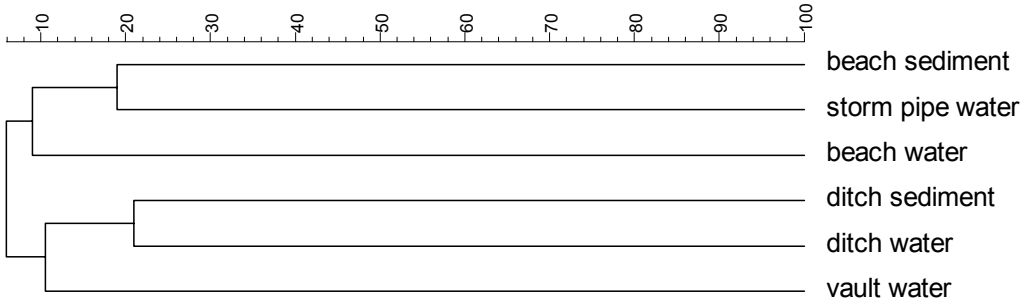


Figure 16. Similarity of *E. coli* populations by site during dry conditions, based on BOX-PCR fingerprints

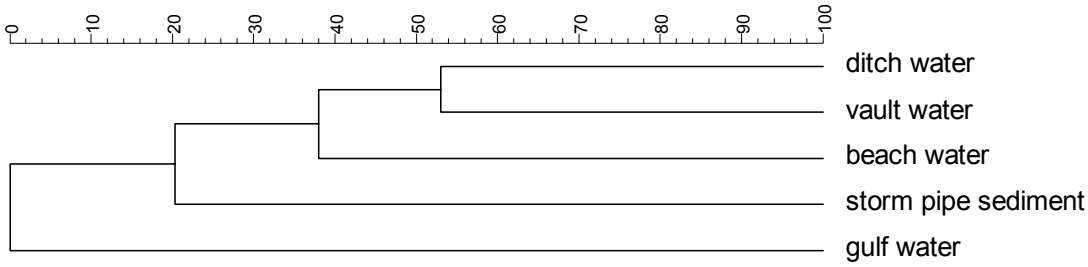


Figure 17. Similarity of *Enterococcus* populations by site during the rain event, based on BOX-PCR fingerprints

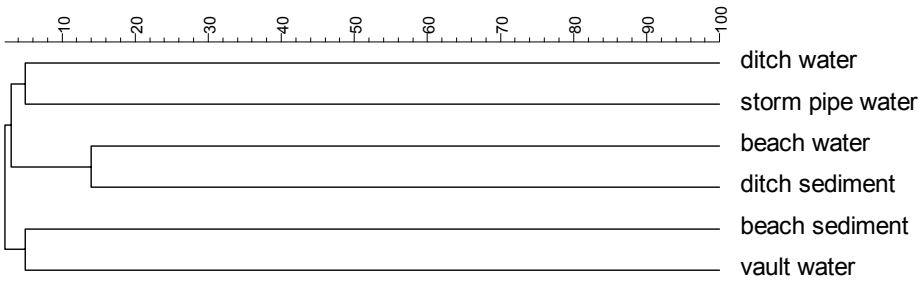


Figure 18. Similarity of *Enterococcus* populations by site during dry conditions, based on BOX-PCR fingerprints

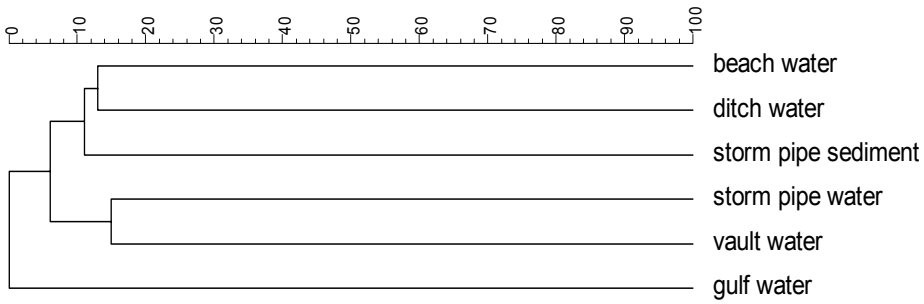
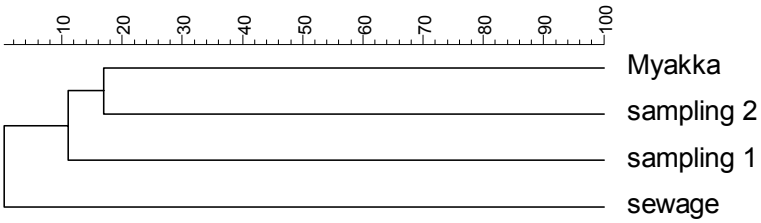


Figure 19. Similarity of *Enterococcus* populations sampled during the rain event (sampling 1), dry conditions (sampling 2), from sewage, and from Myakka River, based on BOX-PCR fingerprints



DISCUSSION

The stormwater drainage system at Siesta Key Beach was sampled within 48 hours of a rain event (~2 inches) and during dry conditions (no precipitation 6 days prior). During the rain event, stormwater flowed through an underground stormpipe to an underground vault. Because of the high volume during the rain event, the stormwater was pumped into a retention pond. Stormwater from the retention pond and surface runoff from the main road flowed through a ditch to the beach, where it flowed into the Gulf of Mexico. At that time (rain event), levels of indicator bacteria were above the regulatory standards at all sites sampled throughout the drainage system (stormpipe to beach). In contrast, during dry conditions, no water was observed flowing through the system except for a trickle from the ditch to the beach, where the water pooled and did not reach the Gulf. During this time, levels of indicator bacteria were much lower in the water column samples; however, the stormpipe, vault, and beach sites still exceeded the regulatory standards. The stormpipe and vault are enclosed structures that could provide protection to indicator bacteria from stressors (discussed below) and the water pooled onto the beach could be directly impacted by another source such as seagulls (Figure 2).

The stormwater and surface runoff are from an approximately 60-acre area of the residential community of Siesta Key. High levels of indicator bacteria in the stormwater drainage system initially suggested a possible sewage influence. However, prior to the study, the wastewater collection system was examined for any leaks into the stormwater

conveyance system by the Siesta Key Utilities Authority. Furthermore, as part of this study, another laboratory (Biological Consulting Services of North Florida) conducted tests for human polyomaviruses and the enterococcal surface protein gene (*esp*) for *Enterococcus faecium*. Both tests have previously been used to determine the presence of human sewage in environmental waters (74, 89) and produced negative results for this study, which suggests that human sewage input was not involved.

Previous studies have shown that stormwater runoff can elevate levels of indicator bacteria (1, 27, 51, 81, 86). A study conducted in a coastal urban watershed in southern California (2004) observed that during dry conditions, total coliforms, *E. coli* and *Enterococcus* spp. were highly concentrated in runoff from forebays (underground storage tanks), and that indicator bacteria concentrations were higher in residential runoff when compared to other land-uses, including channels, parks, agricultural, and commercial (86).

Underground storage of urban runoff may well provide favorable conditions for bacterial persistence, allowing it to act as a source of indicator bacteria. Two conditions known to affect the survival of *E. coli* and *Enterococcus* spp. are temperature and sunlight. Increased die-off rates were observed with an increase in temperature (5, 30, 80) and exposure to sunlight (19, 34, 93). The underground system provides protection from these abiotic influences, and supplies nutrients such as nitrogen and phosphate from residential fertilizers, promoting survival and possible regrowth.

High concentrations of both fecal coliforms and *Enterococcus* spp. were found in sediments for both sampling events. *Enterococcus* spp. concentrations remained high during dry conditions even when the overlaying water column (retention pond, ditch, and

Gulf) had concentrations below the regulatory standard. This implies that the dynamics of indicator populations differ between the water column and sediments. Both *E. coli* and *Enterococcus* spp. are known to persist in a culturable state in sediments (6, 17, 20, 51). Studies conducted have shown lower decay rates of indicator bacteria in sediment than in water (6, 48, 92), indicating that sediments provide protection from harmful stressors (e.g. high temperatures and sunlight). Two studies (17, 20) suggest that soil contains the nutrients needed for regrowth of indicator bacteria. Byappanahalli and Fujioka (1998) observed an increase in fecal coliforms and *E. coli* when adding sewage to cobalt-irradiated soil, and Desmarais et al (2002) observed an increase in *E. coli* and *Enterococcus* spp. after adding sterile sediment to river water. This supports the premise that sediments are a possible reservoir for indicator organisms once introduced into the environment.

In comparing the Siesta Key indicator bacteria populations originating from the rain event and from dry conditions, not only were the levels of indicator bacteria different, but also the genotypic makeup of the indicator bacteria populations. Increased population diversity for *E. coli* and *Enterococcus* spp. during the rain event indicates a trend for greater diversity during conditions that result in stormwater influence on surface water quality. Higher diversity would implicate recent inputs, possibly from multiple sources. The diversity of *Enterococcus* populations during the rain event and during dry conditions was compared to the diversity of *Enterococcus* populations found in sewage and in water samples collected from Myakka River, considered to be a pristine site with no known human input or urban runoff. Similar diversity levels were observed in Siesta Key *Enterococcus* populations during the rain event and *Enterococcus* populations in

sewage samples collected from lift stations in two Florida counties. Previous studies have shown that the *Enterococcus* population in domestic sewage has a higher diversity when compared to river water (105), and animal feces (61, 67). In contrast, significantly lower diversity was found in *Enterococcus* populations during dry conditions and at a pristine site (Myakka River). This suggests that stormwater and urban runoff can influence the diversity of indicator bacteria populations in the environment to mimic that of sewage input, although the subtypes represented in these two environments were dissimilar (see below).

Increased population similarity for *E. coli* and *Enterococcus* spp. during dry conditions suggests that a substantial portion of the population is composed of "survivor" isolates (6). Both a diversity decrease and a similarity increase were observed in the stormpipe and vault *Enterococcus* populations as well as the beach and ditch under dry conditions compared to wet conditions. For *E. coli*, a diversity decrease and a similarity increase were observed in the vault, ditch, and beach populations. These populations also shared similarity with the stormpipe sediment. During dry conditions, both *E. coli* and *Enterococcus* populations had similarity between all sites with the exception of Gulf water. During this time, the water from the ditch pooled onto the beach and did not reach the Gulf.

Studies on the population similarity of an indicator bacterium in environmental waters are relatively rare in the literature (60, 72, 105). To demonstrate similarity in indicator bacteria populations considered to be similar, three water samples were collected from the same pond on the same day. *Enterococcus* spp. from each sample were typed by BOX-PCR and compared, showing high similarity among samples. The

number of isolates per sample was 19 or 20 and the total number of subtypes was 5. This data represents one end of the spectrum with low diversity and high similarity from samples collected at one site. When comparing populations with a much higher diversity and a broader area of sample collection, such as the *Enterococcus* population at Siesta Key in the rain event, percent similarity is greatly reduced. An inverse relationship was observed during dry conditions; as the population diversity decreased, the population similarity increased. Sewage isolates, which displayed the highest diversity, was the group least related to populations isolated during the rain event, dry conditions, and from the Myakka River.

The fate of the two indicator groups in the environmental habitat probably contributed to observed differences in their population similarity, in that *E. coli* populations displayed greater population similarity than the *Enterococcus* spp. populations. Since concentrations of *Enterococcus* spp. were higher than fecal coliforms, this could be a contributing factor and has been previously reported in estuarine sites (27, 51, 81), suggesting that *Enterococcus* spp. are better survivors in estuarine-type waters. Moreover, it is plausible that *Enterococcus* spp. as a genetic group provides more variability and possible candidates for survival when compared to the available genetic variability of the one *Escherichia* species.

Even though human sewage input is not evident at Siesta Key Beach, it cannot be definitively stated that there is less risk to human health when indicator bacteria concentrations exceed the regulatory standard. The health risks associated with exposure to recreational waters impacted by stormwater runoff have not been as well studied as the risks associated with sewage impacted waters. In one study, Haile et al (42) observed

that respiratory and gastrointestinal symptoms increased as the distance decreased between swimmers and a stormwater outlet in Santa Monica Bay, CA. Dwight et al (24) observed that during an El Nino year, surfers in Orange County, CA reported twice as many symptoms as surfers in Santa Cruz County, considered to be less impacted by urban runoff. These studies show that adverse heath outcomes are associated with stormwater impact of recreational waters.

The population dynamics of indicator bacteria in the storm drainage system at Siesta Key Beach are evidently affected by rain events. A change in concentrations and diversity, as well as similarity, of the populations extending from the stormpipe to the Gulf was observed. The transport of urban runoff collecting for days in the stormpipe and vault, and the persistence of survivor isolates in the sediments, suggests a reservoir for indicator bacteria that can be flushed through the system to the Gulf during a rain event, causing high levels of indicator bacteria. Such environmental reservoirs of indicator bacteria further complicate the already questionable relationship between indicator organisms and human pathogens (6, 17) , and call for a better understanding of the ecology, fate and persistence of indicator bacteria in water.

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