2009

The Importance of Benthic Habitats as Reservoirs of Persistent Fecal Indicator Bacteria

Brian D. Badgley
University of South Florida

The Importance of Benthic Habitats as Reservoirs of Persistent Fecal Indicator Bacteria

by

Brian D. Badgley

A dissertation submitted in partial fulfillment
of the requirements of the degree of
Doctor of Philosophy
Department of Integrative Biology
College of Arts and Sciences
University of South Florida

Co-Major Professor: Valerie J. Harwood, Ph.D.
Co-Major Professor: Florence I. M. Thomas, Ph.D.
Susan S. Bell, Ph.D.
Gordon A. Fox, Ph.D.
Mya Breitbart, Ph.D.

Keywords: *Enterococcus*, clonal structure, population dynamics, microbial ecology, microbial resuspension

To my Mom and Dad – this was made possible by the love, support, and opportunity that you have provided throughout my life. You always have my thanks and my love.

ACKNOWLEDGEMENTS

I would like to extend my sincere gratitude to Drs. Valerie Harwood and Florence Thomas, my co-major professors, for the support, respect, and friendship they have shown to me during my time as a doctoral student. I also thank my other committee members, Drs. Susan Bell and Gordon Fox, for their insight and guidance, and Dr. Mya Breitbart for participating as the Outside Chair of my defense. I am also deeply indebted to the numerous friends and colleagues that have contributed considerable time and effort in assisting me in this research, including Miriam Brownell, Katrina Gordon, Neal Halstead, Gabe Herrick, Phoebe Koch, Shannon McQuaig, Alison Meyers, Bina Nayak, Mythili Penugonda, Chris Staley, Zach Staley, and Stacy Villanueva. This research was made possible in part by awards from the National Water Research Institute, Aylesworth Foundation for the Advancement of Marine Science, and the Old Salt Fishing Club.

Finally, I would like to acknowledge my wife, Andrea, my son, Owen, and my daughter, Annabelle. Their love and laughter have provided endless support over the last five years, and I will be eternally grateful for the sacrifices they made so that I could complete my degree.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

The Importance of Benthic Habitats as Reservoirs of Persistent Fecal Indicator Bacteria

Brian D. Badgley

ABSTRACT

Enterococci are fecal indicator bacteria (FIB) that are used worldwide for water quality assessment. However, evidence of high densities and extended survival of enterococci in sediments and submerged aquatic vegetation (SAV) has caused uncertainty about their reliability in predicting human health risks from recreational activities in environmental waters. To address the concern that sediments and SAV may harbor large reservoirs of enterococci that can affect water column concentrations, aquatic mesocosms and environmental sampling were employed to investigate patterns of enterococci densities and population structure across the Tampa Bay watershed.

In mesocosm experiments and environmental samples, SAV harbored higher densities of enterococci, per mass of substrate, than sediments, and sediments harbored higher densities than water. Population structure assessed by BOX-PCR genotyping was relatively unique in each sample, although slight similarities among samples suggested grouping primarily by location rather than substrate or season. Strain diversity was highly variable, and many samples had low diversity, including nearly monoclonal structure throughout the mesocosm experiments and in several of the environmental samples. Several strains were highly abundant and cosmopolitan (found across sites,

seasons, and substrates), and may represent highly naturalized and reproducing indicator bacteria populations that are not directly related to pollution events.

When the enterococci densities were viewed from the perspective of the entire aquatic system, SAV-associated enterococci did not comprise a major proportion of the total population, due to the typically large differences in volume of each substrate (SAV vs. sediments vs. water). Instead, the largest proportions of enterococci were typically found in the water or the sediments, depending on the relative volume of substrate or the enterococci density associated with each substrate. Modeling results illustrate that the relative importance of each substrate in terms of FIB populations can shift dramatically over time and space due to changes such as vegetation cover, tidal cycles, and bacteria densities. Furthermore, at several sites within the watershed, estimates of sediment and bacteria resuspension from sediments were very low, suggesting that this process rarely, if ever, significantly affects water column concentrations of enterococci in the watershed.

CHAPTER ONE

BACKGROUND AND OVERVIEW OF RESEARCH

Our ability to assess the transmission risk of waterborne pathogens that results from fecal contamination in recreational and fishing waters is an extremely important tool in protecting public health. It is well established that swimmers and bathers in water with known impacts of sewage or other fecal contamination are at a higher risk for gastrointestinal and respiratory illnesses, as well as skin, ear and eye infections (Cabelli et al., 1982; Cheung et al., 1990; Rees et al., 1998; Wade et al., 2003). Although far fewer epidemiological studies have been done concerning contaminated beach sands, it appears that a similar correlation exists (Bonilla et al., 2007; Heaney et al., 2009). In the United States, the Clean Water Act (1972) and the Beaches Environmental Assessment and Coastal Health Act (2002), mandate regular monitoring of recreational water quality and public advisories of risks. Unfortunately, determining an effective standardized means of detecting and quantifying the human health risks that are associated with fecal pollution in all types of environmental waters has been a continuous challenge. One reason for the challenge is that it is difficult for monitoring agencies to conduct assays that directly detect pathogens that are introduced upon sewage contamination. While the direct monitoring for pathogens is ideal in theory, in practice it becomes logistically and financially prohibitive due to the wide diversity of potential pathogens (including viruses,

bacteria, and protists) which would have to be monitored, as well as the facts that many pathogens are difficult and costly to culture, have no reliable molecular assays, or have patchy distributions or low concentrations (Field and Samadpour, 2007).

Traditionally, this problem has been approached by monitoring for fecal indicator bacteria (FIB), or particular bacteria that, although they are not pathogenic themselves, are reliably abundant in feces and sewage. The presence of FIB (at sufficiently high numbers) is assumed to indicate contamination of environmental waters by sewage or other fecal material and the likely presence of human pathogens. The selection of appropriate FIB has a long history, dating back to original work describing *Bacillus coli* (now *Escherichia coli*) as ubiquitous in human feces by its namesake, Theodor Escherich (Griffin et al., 2001). Soon after, other researchers described the coliform group (which contains *E. coli*) as a suitable indicator of fecal pollution and it was recommended for this purpose in the first edition of the America Public Health Association's (1905) *Standard Methods for the Examination of Water and Wastewater*. Afterwards, the coliforms were the dominant indicator of microbial water quality for decades (Tallon et al., 2005). Today, total coliforms are officially characterized as all aerobic and facultative anaerobic, non-spore forming, gram-negative, rod-shaped bacteria that ferment lactose with gas and acid formation within 48 hours at 35.0 ºC (APHA, 1998). A subset of the total coliforms, which are capable of growth at 44.5 ºC and called the fecal, thermotolerant, or thermotrophic coliforms, were later adopted as a more suitable indicator.

Members of the genus *Enterococcus* constitute another important group of FIB that has been used for decades. The enterococci are gram-positive cocci that typically occur singly, in pairs, or in short chains. They have a growth range of 10 to 45 ºC, with an optimum of 35 ºC, and can grow in solutions containing up to 10% NaCl. Metabolically, they are aerotolerant anaerobes that ferment carbohydrates to lactic acid and can hydrolyze esculin in the presence of 40% bile salts (Facklam et al., 2002). Previously, the enterococci were contained within the genus *Streptococcus*, but recent genetic evidence has shown that sufficient difference exists between the two groups to merit a separate genus (Schleifer and Kilpperbalz, 1984). More than 20 species are now included in the genus (Facklam et al., 2002). Enterococci can be found in the feces of most mammals and birds and, in general, they are not host specific. A couple exceptions include *Ent. asini* in donkeys (de Vaux et al., 1998) and *Ent. columbae* in pigeons (Devriese et al., 1990), but it is possible that these may simply be undersampled. Although the same species can be found in many different hosts, there can be distinct differences in the relative abundances of the different species in different hosts, as well as at different ages within the same host (Aarestrup et al., 2002).

Given this wide diversity of potential FIB, including others that had been proposed such as *Clostridium perfringens* and coliphages (Fujioka and Shizumura, 1985; Payment and Franco, 1993; Gantzer et al., 1998), the US Environmental Protection Agency (1986) reviewed the performance of various indicator organisms and developed a standard set of recommendations for regulatory agencies. In their report, they concluded that the best

indicator in fresh water was *E. coli*, specifically, and in estuarine and marine waters, they recommended the enterococci. They cautioned against the use of total or fecal coliforms, as false positives may result because of confusion with other groups (e.g., *Pseudomonas* and *Vibrio*) in media based tests (Griffin et al., 2001) or the ability of some members of the coliform group to survive in the environment (discussed in further detail below). Historically, the successful implementation of water quality monitoring programs based on FIB has led to dramatic reductions in waterborne disease outbreaks in many parts of the world (Leclerc et al., 2001). Furthermore, in a recent meta-analysis of 27 epidemiological studies, Wade et al. (2003) concluded that, among the various potential FIB, the highest associations between specific FIB and risk of waterborne illness were with the EPA-recommended *E. coli* in freshwater and enterococci in marine water.

Despite the successes of water quality monitoring programs, the use of FIB is far from a perfect solution. Decisions regarding the closure of water bodies that may be important for recreation and fisheries have serious economic and public health impacts. False-negative conclusions expose swimmers and bathers to avoidable health risks, and false-positive conclusions result in unnecessary closures that can be economically costly to waterfront communities and industries. One ongoing challenge in the continued use of the indicator approach is searching for and validating the 'ideal' FIB. Typical characteristics of an ideal indicator organism include its occurrence in correlation with human pathogens, an inability to replicate in the environment, ease of identification and

quantification, and a correlation between its density and the degree of hazard to public health (Griffin et al., 2001; Field and Samadpour, 2007; Ishii and Sadowsky, 2008). Unfortunately, recent research has indicated that these assumptions are often false. In fact, many studies have shown that the presence of FIB in environmental waters do not correlate well with the presence of pathogens, including *Salmonella*, *Campylobacter*, *Cryptosporidium*, *Giardia*, or enteroviruses (Lund, 1996; Bonadonna et al., 2002; Lemarchand and Lebaron, 2003; Harwood et al., 2005). While there are many potential reasons for this lack of correlation, and it is likely to be a combination of several factors, one major problem is the assumption that, subsequent to the introduction of fecal contamination into the environment, FIB will exhibit similar survival dynamics as the pathogens they are being used to detect. On the contrary, many recent studies show that FIB – both coliforms and enterococci – are capable of persisting in a culturable form for extended periods of time in a wide variety of environmental matrices after their initial introduction (see below). In contrast, pathogens have often been shown to be very different than FIB in terms of their ability to withstand stressors and survive in the environment (Davies et al., 1995; Lund, 1996; Desmarais et al., 2002; Fujioka and Yoneyama, 2002; Nasser et al., 2007; Englebert et al., 2008). This disconnect between the survival of FIB and pathogens in the environment may undermine one of the key assumptions in the use of water quality indicator bacteria.

Aquatic sediments have been one of the most widely and thoroughly studied environmental substrates for the persistence of FIB. Once introduced to the water, FIB

often quickly attach to suspended particles and then settle into the sediments (Auer and Niehaus, 1993; Davies and Bavor, 2000; Jeng et al., 2005). Ironically, however, the author of one of the earliest published accounts of FIB in the sediments did not find it surprising that FIB were elevated and likely to be persisting in sediments (Savage, 1905). In fact, he theorized that, because of this theoretically expected phenomenon, sediment samples may offer a more uniform and historically accurate record of fecal contamination than single water samples, which, even then, were recognized as being highly variable in time, depending on factors such as tide and weather. In the several decades following Savage's work, a few studies revisited sediments at various locations and reliably showed that FIB concentrations in sediments were well correlated with the level of pollution (Allen et al., 1953; Rittenberg et al., 1958; Bonde, 1967; Vandonsel and Geldreich, 1971). In more recent years, however, numerous studies investigating the persistence of FIB in sediments have been published from a variety of environmental waters, including tropical and temperate streams (Buckley et al., 1998; Byappanahalli et al., 2003b), temperate rivers (Tunnicliff and Brickler, 1984; Obiri-Danso and Jones, 1999), lakes (Doyle et al., 1992; Whitman and Nevers, 2003; Ishii et al., 2007), and subtropical and temperate estuaries (Shiaris et al., 1987; Solo-Gabriele et al., 2000; Desmarais et al., 2002). Much of this recent work has focused on the likelihood that sediments can act as a reservoir of FIB that may represent a significant source of water column concentrations, and several of these studies provide evidence that may be the case at some sites (Crabill et al., 1999; Byappanahalli et al., 2003a; Whitman and Nevers, 2003; Ishii et al., 2007).

Laboratory meso- and microcosms have also been used extensively to study the persistence of FIB in aquatic sediments. Several researchers have found that the addition of sediments to mesocosms containing fresh or saltwater significantly extends the survival of FIB (Vandonsel and Geldreich, 1971; Hood and Ness, 1982; Burton et al., 1987; Craig et al., 2004; Anderson et al., 2005). In addition, although these groups of bacteria have historically been thought to reproduce only in the intestinal tracts of endothermic animals (Savageau, 1983; Leclerc et al., 2001), in some mesocosm experiments using sterilized sediments, culturable FIB have increased in number (Gerba and McLeod, 1976; Laliberte and Grimes, 1982; Davies et al., 1995; Desmarais et al., 2002). This apparent growth of FIB casts doubt on the validity of the assumptions associated with the use of water quality indicator organisms by suggesting the potential for reproducing populations in aquatic sediments, at least in the absence of predation and competition for resources.

One specific type of aquatic sediment that has received particular attention in recent years is shoreline beach sand, specifically that portion which is not constantly submerged, but is periodically wetted due to wave or tidal activity. A number of studies have found that the highest densities of FIB in cross-shore transects of beach sands occur in this shoreline or foreshore section of damp sand (Whitman and Nevers, 2003; Kinzelman et al., 2004; Whitman et al., 2006; Beversdorf et al., 2007; Ishii et al., 2007; Yamahara et al., 2007). Solo-Gabrielle et al. (2000) observed a similar pattern in cross-stream transects of a brackish tidal river. The use of experimentally wetted microcosms has confirmed this

effect, resulting in growth of FIB immediately following the re-wetting event (Solo-Gabriele et al., 2000; Yamahara et al., 2009). While this narrow band of sediment may not provide a significant source of FIB on a large scale, at the small scale it may be sufficient to elevate water column concentrations for short periods of time following changes in water level that result from tidal or wave activity (Whitman and Nevers, 2003; Kinzelman et al., 2004; Whitman et al., 2006; Yamahara et al., 2007).

In addition to aquatic sediments, high densities of FIB can also be associated with submerged aquatic vegetation (SAV). The most well-studied example of this association is the observation of high densities and possible growth of *E. coli* and enterococci on *Cladophora*, a highly abundant green macroalga, in Lake Michigan, USA (Byappanahalli et al., 2003b; Whitman et al., 2003; Olapade et al., 2006; Englebert et al., 2008; Kleinheinz et al., 2009). In addition, FIB have been found to be associated with drifting marine macroalgae washed up on beaches in New Zealand (Anderson et al., 1997) and microalgal periphyton in the Great Lakes, USA (Ksoll et al., 2007). Although FIB have also been shown to be associated with other aquatic biota, such as plankton (Signoretto et al., 2004; 2005) and fish (Del Rio-Rodriquez et al., 2008; Hansen et al., 2008), these sources have not been well studied and it is currently unclear if they have any significant impact on water column concentrations.

Hypotheses explaining increased survival in benthic matrices focus mostly on the availability of increased resources and protection from environmental stress. For

example, increased survival of FIB in sediments has been shown to result from protection of the bacteria from predation by protozoans (Flint, 1987; Marino and Gannon, 1991; Davies et al., 1995), as well as protection from ultraviolet light (Davies and Evison, 1991; Fujioka and Yoneyama, 2002; Sinton et al., 2002). In addition, SAV has been shown to increase available carbon to surrounding sediments both by causing increased settling of suspended particulates (Posey et al., 1993; Fonseca, 1996) and by exudation of photosynthate from the roots (Pollard and Moriarty, 1991). Furthermore, these changes have also been shown to result in increased microbial activity (Lopez et al., 1995; Hansen et al., 2000; Karjalainen et al., 2001), suggesting that the microbial inhabitants of the nearby sediments are able to use the extra resources provided by the SAV.

At the larger watershed scale, several accounts have been published showing significant densities of culturable FIB in terrestrial matrices as well. Much of the early evidence was collected in tropical soils, where it was assumed that the warm temperatures were conducive to the success of FIB (Hardina and Fujioka, 1991; Roll and Fujioka, 1997; Fujioka et al., 1999; Desmarais et al., 2002). More recently, however, similar results have been observed in temperate soils (Zhai et al., 1995; Byappanahalli et al., 2003a), where some strains have even been found to overwinter in the frozen soils (Ishii et al., 2006). Accounts have even been published of enterococci associated with terrestrial plants (Muller et al., 2001; Ott et al., 2001) and insects (Geldreich et al., 1964), although the widespread impacts of this association on our ability to predict human health risks is not well understood. Laboratory mesocosm experiments have provided some evidence

that *E. coli* is capable of growth in terrestrial soils, suggesting that soil may serve not only as a reservoir for persistent bacteria, but even as a source for new cells that may be washed into water bodies during rain events (Byappanahalli and Fujioka, 1998; Topp et al., 2003; Ishii et al., 2006). Although soils typically contain highly variable concentrations of coliforms and enterococci (both within and among studies), some studies have determined that they may represent a non-point source of FIB that is significant enough to affect concentrations in the water column (Hardina and Fujioka, 1991; Roll and Fujioka, 1997; Fujioka et al., 1999).

The extended persistence of FIB in the sediments and SAV of aquatic habitats is one likely cause of the frequently poor correlation between FIB and pathogens that was discussed above. When FIB persist in benthic matrices, the potential for those cells to be resuspended back into the water column may lead to falsely positive conclusions regarding microbiological contamination. Resuspension might occur during any event that generates significant hydrodynamic activity, such as storms, boating and shipping traffic, or high levels of activity by swimmers and bathers. Prokaryotes are typically the most easily resuspended of all benthic organisms, due to their small size and the fact that they are frequently associated with cohesive surficial fluff sediments (Gannon et al., 1983; Auer and Niehaus, 1993; Howell et al., 1996; Shimeta et al., 2002). The potential for resuspension is a major concern about the reliability of the indicator organism concept which has been raised repeatedly in the literature (Solo-Gabriele et al., 2000; Grant et al., 2001; Whitman et al., 2003; Anderson et al., 2005; Ishii and Sadowsky, 2008).

Unfortunately, the importance of long-term environmental persistence and resuspension of FIB has been difficult to quantify, in terms of both their association with human health risks and broader ecological effects. Although recent epidemiological studies have shown that increased exposure to beach sand carries increased risk of disease (Bonilla et al., 2007; Heaney et al., 2009), no correlations have been determined between health risks and concentrations of FIB in sediments or SAV. In fact, no standard method has yet been adopted for their detection and quantification in these matrices. Typically, FIB associated with sediment or SAV are dislodged, in some way, so that they can be resuspended in sterile buffered water. This water can then be subjected to the standard membrane filtration methods used for normal water samples (APHA, 1998; USEPA, 2000). However, the methods of dislodging the cells from their substrate have varied widely, including shaking, sonication, and the use of surfactants (Boehm et al., 2009). Furthermore, there is no widely accepted means of standardizing the densities of benthic FIB, as there is for water column bacteria, which are typically standardized to concentration of colony forming units (CFU)/100 mL. Benthic bacteria are often normalized in some way to the mass of substrate, such as CFU per 1 g or 100 g wet or dry weight of sediments (Solo-Gabriele et al., 2000; Desmarais et al., 2002; Craig et al., 2004; Anderson et al., 2005; Yamahara et al., 2009), but have also been normalized to square centimeter of substrate surface area (Ksoll et al., 2007), volume of interstitial water (Buckley et al., 1998), and square meter of landscape area (Muirhead et al., 2004; Jamieson et al., 2005).

As a result, it is difficult to interpret whether the densities reported in the literature actually represent a large reservoir of FIB that are available to be resuspended and affect water column concentrations and, ultimately, water quality monitoring. The resuspension of FIB has been directly observed to occur in managed streams in the absence of rainfall or groundwater inputs, as a result of both natural (Nagels et al., 2002; Jamieson et al., 2005) and experimentally-induced (McDonald et al., 1982; Wilkinson et al., 1995; Nagels et al., 2002; Muirhead et al., 2004) periods of high flow. However, in other environmental water bodies of recreational importance that are not so easily constrained (such as beaches and lakes) the resuspension of benthic FIB has typically been inferred. For example, observations of relatively high *E. coli* concentrations in the water column have been shown to correlate with factors that cause sediment motion and resuspension, such as wave or tidal activity (Le Fevre and Lewis, 2003; Shibata et al., 2004; Whitman et al., 2006; Yamahara et al., 2007), dredging or boating activity (Grimes, 1975, 1980; Pettibone et al., 1996; An et al., 2002), or recreational activity (Crabill et al., 1999). Water column concentrations have also been directly correlated with sediment densities through the use of time series or structural equation modeling (Whitman and Nevers, 2003; Whitman et al., 2006).

Modeling the fate and transport of FIB is an increasingly popular focus of current research, and these models can also help elucidate the importance of sediments as a potential reservoir of FIB by incorporating terms for settling and resuspension processes

and determining the resulting effect on the model's predictive power. Through this approach, hydrodynamic information and sediment characteristics can be used to predict sediment resuspension and, in turn, offer relatively good approximations of the behavior of benthic FIB in the sediments (Bai and Lung, 2005; Jamieson et al., 2005). Unidirectional (e.g., tidal and stream flows) and oscillatory (e.g., wave action) flow regimes both set up velocity gradients near the bottom of the water column that increase from zero at the sediment-water interface up to the mainstream velocity. The steepness of these gradients, in combination with bottom roughness that results from bedform elements (e.g., sediment grains, sand ripples, rocks, or organisms) establishes a shear stress that acts on the top layer of sediment (Denny, 1988; Soulsby, 1998). If the force of this shear stress is sufficiently strong to overcome the natural settling velocity of individual sediment grains, then some amount of sediment will be resuspended and maintained in suspension (Soulsby, 1998; Le Roux, 2005). Many sets of theoretical and empirical equations exist that allow the prediction of concentrations and transport of suspended sediment under a given set of unidirectional or oscillatory flow regimes, which can then be used to estimate resuspension of the associated FIB (Bai and Lung, 2005; Jamieson et al., 2005).

General terms for resuspension rates (typically based on the shear stresses and sediment qualities described above) have been incorporated into embayment-wide models used to predict net transport of FIB (Steets and Holden, 2003; Sanders et al., 2005). In addition, a much more focused study has been published that uses the Environmental Fluid

Dynamics Code model to specifically predict the impacts of sediment association on the settling, resuspension, transport, and persistence of FIB (Bai and Lung, 2005). These models, however, necessarily rely on many assumptions and generalities with regards to the ecology of FIB and sediment/bacteria resuspension dynamics. It is becoming increasingly clear that improving our understanding of how benthic-pelagic coupling affects the population dynamics of FIB species is an important step towards improving our ability to predict their survival and transport in environmental waters. In fact, additional data and experimentation on the behavior of benthic FIB has been outlined as a distinct need for future model improvement (Bai and Lung, 2005; Pachepsky et al., 2006)

It is important to note here that bacteria attached to SAV would behave very differently than what was described above for sediments. SAV-associated bacteria would only be returned to the water column as a result of some sort of mechanical shearing of the bacterial cells, or the particles to which they are attached (e.g., detritus or epiphytic algae), from the vegetative surface. In either case, these dynamics are probably quite complex and different from sediment resuspension theory. To my knowledge, these processes have never been examined experimentally or modeled. In addition, the structural presence of the macrophytes in vegetated habitats alters the hydrodynamics themselves, by interacting with the overlying flow to create increased turbulence in the water column and in the upper levels of the submerged canopy, but greatly decreased flows within the canopy near the sediment (Gambi et al., 1990; Ikeda and Kanazawa, 1996; Ghisalberti and Nepf, 2002). This altered flow can result in much lower shear

stresses at the sediment-water interface and probably also reduce bacterial resuspension into the water column as compared to similar flows over unvegetated sediments. This effect, coupled with potential for vegetated canopies to supply significant amounts of nutrients to support bacterial growth, suggests that modeling the fate and transport of FIB in highly vegetated habitats may require entirely new model development to accurately predict these processes.

Whether they are attached to sediment or vegetation, one of the major deterrents to easily interpreting the importance of benthic reservoirs of FIB is that indicator densities have typically been normalized per unit volume for water samples, but have been normalized per unit mass of substrate for sediment and SAV samples (Byappanahalli and Fujioka, 1998; Solo-Gabriele et al., 2000; Topp et al., 2003; Whitman et al., 2003; Jeng et al., 2005; Ishii et al., 2006; Ksoll et al., 2007). Normalization by volume makes sense for the water column, as concentration is the most appropriate value to consider in terms of the exposure and health risks posed to swimmers. However, when one tries to compare these values to data that are normalized to mass of sediment or SAV, the two different approaches do not offer an equal measure of water column and benthic densities, nor do they allow for a simple interpretation of the importance of benthic sources of resuspendable bacteria. One approach that bypasses this shortcoming, however, is to use a different method of normalizing bacterial densities and look at aquatic systems on the basis of landscape area (e.g., per m^2). When bacterial densities are integrated vertically (e.g., by depth of the water column or sediments) to obtain total CFU per unit landscape

area, the resulting densities allow for direct comparison of bacterial population sizes in water column and benthic habitats within or between water bodies (Muirhead et al., 2004; Jamieson et al., 2005).

The consequences of persistent FIB in secondary habitats are further complicated by the effects of inherent diversity among, and even within, the various species of FIB. Groups of FIB, such as the enterococci and fecal coliforms, harbor interspecific variability among their member species. Within the coliforms, for example, *E. coli* typically displays a much higher association with sewage and human fecal material, while other members of the group such as *Enterobacter* spp. and *Klebsiella* spp., are widespread in the environment and therefore poorer predictors of fecal contamination (Leclerc et al., 2001). This finding was a major factor in prompting the U.S. Environmental Protection Agency to promote a shift from the use of fecal coliforms to *E. coli* as the preferred water quality indicator in freshwater habitats (USEPA, 1986).

Similar interspecific variability has been seen within the enterococci, although the entire group is still used as the preferred water quality indicator in marine waters. Often, in environmental samples, the dominant species tend to be *Ent. faecalis*, *Ent. faecium*, *Ent. hirae*, and *Ent. mundtii* (Pinto et al., 1999; Harwood et al., 2004; Ferguson et al., 2005; Moore et al., 2008). However, among these species there are wide differences in associations. For example, *Ent. faecalis* and *Ent. faecium* are dominant in human feces and sewage (Ruoff et al., 1990; Manero et al., 2002; Gelsomino et al., 2003), while

pigmented species such as *Ent. casseliflavus* and *Ent. mundtii* are rarely associated with human sources and considered to have environmental sources such as plants and waterfowl (Leclerc et al., 1996; Pinto et al., 1999; Aarestrup et al., 2002). The diversity of each group complicates their survival dynamics and their performance as an indicator, and must be taken into account in order to improve their utility.

In addition to the effects of *inter*specific variability, additional complexity in evaluating indicator performance results from the fact that each individual species can also exhibit considerable *intra*specific variability as a result of the clonal diversity inherent in a given population. In microbiology, the species concept is typically agreed to be functional from an operational perspective, but the lack of sexual reproduction and the existence of many mechanisms for genetic exchange across prokaryotic taxa make it very difficult to firmly ground the concept in theory (Rossello-Mora and Amann, 2001; Oren, 2004). Historically, microbiologists were limited to identifying microbial species by phenotypic characteristics such as morphology, physiology, and culture conditions, usually by working with isolated pure cultures. However, with the advent of molecular techniques, new parameters for species delineation have been based on genomic information, which carries the benefit of being culture independent. The two most common molecular indicators of species delineation are a 70% or greater DNA-DNA hybridization for the total genome and 97% or greater similarity of 16s rDNA gene sequence (largely because it correlates most closely with a 70% hybridization rate). Although the new molecular methods of taxonomy have proven to be very useful, they are not foolproof, and it is now

generally agreed that using a polyphasic approach, where phenotypic characteristics are used in conjunction with genomic information, is the best means of determining prokaryotic relations (Stackebrandt et al., 2002). However, even with a polyphasic approach, it is clear that the criteria delineate a very broad prokaryotic species definition, especially in comparison to eukaryotes, and that intraspecific variation among cells can be quite high for important characteristics such as antibiotic resistance, virulence factors, and physiology (Ward et al., 1998).

For FIB, this inherent variation within species has been a major focus of microbial source tracking (MST), which is an area of active research that attempts to overcome the limitations of using only the concentrations of FIB to determine risks associated with fecal pollution. The goal of MST is to distinguish contamination that originates from various fecal sources (e.g., human, agricultural, or wildlife), thereby offering a means of determining when high concentrations of FIB are truly representative of human fecal pollution and pose increased health risks (Simpson et al., 2002; Field and Samadpour, 2007; Stoeckel and Harwood, 2007). A wide range of methods have been employed to differentiate between contamination sources, including both library-dependent and library-independent approaches that try to identify particular microbial strains or target genes that are specific to, or at least highly associated with, waste from particular host species (Bernhard and Field, 2000; Whitlock et al., 2002; Field et al., 2003; Seurinck et al., 2003; McQuaig et al., 2006; Shanks et al., 2009). Although many of these methods have been successfully used to determine sources of fecal FIB in recent years, the

implementation and interpretation of these methods in the environment may be complicated by the complex population dynamics and variable persistence of FIB in natural environments.

Many methods have been used to differentiate between strains of FIB. Two methods that were widely used in earlier library-dependent source tracking studies include ribotyping (Parveen et al., 1997; 1999; Carson et al., 2001) and antibiotic resistance analysis (ARA) (Wiggins et al., 1999; Harwood et al., 2000; Gaun et al., 2002; Whitlock et al., 2002). By creating libraries of ribotype and antibiotic resistance profiles for numerous strains from known sources, researchers were able to take unknown strains from environmental samples, compare them to the reference library, and classify them according to source with varying degrees of success. In addition to these two methods, a number of others have been suggested, including profiles of carbon source utilization (Hagedorn et al., 2003), sequencing of the 16s-23s intergenic spacer region (Seurinck et al., 2003), or sequencing of the β-glucoronidase gene (Ram et al., 2004).

The typing method that has probably been used the most widely and successfully, however, is repetitive sequence-based PCR (rep-PCR, also known as repetitive extragenic palindromic PCR). This method uses conventional PCR techniques that target repetitive elements in the bacterial genome such as duplicated genes, insertion elements, transposons, and mosaic repetitive elements (Ishii and Sadowsky, 2009). Using total genomic DNA as a template, the amplification of these elements generates multiple

amplicons of various sizes that relate to the distances between each of the repeating elements along the genome, and the separation of those amplicons on a traditional agarose gel creates unique banding 'fingerprints' for each strain. Primers targeting many different types of elements can be used, with REP, BOX, and ERIC elements being commonly used for studies of FIB (Koeuth et al., 1995; Malathum et al., 1998; Dombek et al., 2000; McLellan and Salmore, 2003; Topp et al., 2003). When compared to other techniques, rep-PCR offers clear advantages in terms of higher resolution and sample throughput (Dombek et al., 2000; Ishii and Sadowsky, 2009), and has been shown to be reliably stable over time (Seurinck et al., 2003).

Regardless of the method used to differentiate strains, however, it is becoming increasingly clear that clonal diversity is an important factor in determining the population structure and dynamics of fecal FIB. Clonal diversity has been shown to be lower in environmental samples than source samples (Gordon et al., 2002; Brownell et al., 2007), suggesting that selection and changing clonal structure are occurring over time after the introduction of FIB to the environment. This process has also been observed directly in mesocosm experiments, where different strains of *E. coli* have exhibited differential survival in environmental water and sediment (Anderson et al., 2005) and soils (Topp et al., 2003), with some strains disappearing from the population before others. In addition, high abundances of strains not associated with any known source have been observed at a variety of sites, suggesting that some strains may not only survive longer than others, but may also be adapted to continued persistence or even

growth in environmental habitats. For example, presumably naturalized strains of *E. coli* have been found in temperate soils (Ishii et al., 2006) and freshwater beach sands (McLellan, 2004). Similarly, extremely high density blooms of pelagic *E. coli* in Australia were shown to be mostly comprised of three different strains, even in geographically distant lakes (Power et al., 2005). Evidence for differential survival and naturalized strains implies a high level of intraspecific physiological diversity that affects persistence in natural habitats, resulting in changing clonal structure and complex population dynamics that confound our ability to link FIB to potential sources. Furthermore, naturalized strains have strong potential for decoupling any correlation between FIB concentrations and pathogen presence, raising concerns over the utility of the indicator paradigm. Improving our understanding of these complex population dynamics is a critical need in continuing to improve water quality monitoring and MST efforts, and also to improve our understanding of the species concept in microbiology.

Research Goals and Chapter Objectives

The primary goals of my doctoral work are to determine the extent to which sediments and SAV may be serving as reservoirs of persistent enterococci in the Tampa Bay watershed and to estimate the potential for resuspension of enterococci from these reservoirs to impact their utility as an indicator of water quality. On a broader level, I hope that by investigating the persistence and distribution of various strains of *Enterococcus*, I can contribute additional insight into how intraspecific variability in microbial species effects the population dynamics of the species as a whole. In addition, I hope that by looking at resuspension of bacteria from vegetated habitats I hope to provide more insight into the role that physical forces and benthic-pelagic coupling play in aquatic microbial ecology.

The description, results, and discussion of the original research in my doctoral work are outlined in Chapters Two through Four. In Chapter Two, I employed experimental mesocosms to determine the effects of SAV on enterococci persistence in realistic aquatic habitats that include three different substrates – water, sediment, and SAV – under controlled conditions. Specifically, my three objectives were:

1) Examine the persistence dynamics of environmental enterococci populations (as opposed to inoculated laboratory strains) on different substrates in realistic aquatic mesocosms;

2) Experimentally determine the effect of SAV on the persistence of enterococci by simultaneously comparing enterococci persistence in paired vegetated and unvegetated mesocosms; and

3) Determine the effects of SAV on the population structure of enterococci by examining shifts in species and strain diversity over time.

In Chapter Three, I simultaneously monitored concentrations and population structures of enterococci in water, sediment, and SAV at several sites across the Tampa Bay watershed over an entire year. The goals of the study were four-fold:

1) Determine if high densities of benthic enterococci occurred regularly and consistently across a variety of sites and substrates in the watershed;

2) Investigate the potential for any spatial or temporal patterns in the water column or benthic densities;

3) Employ molecular fingerprinting techniques to investigate how the *Enterococcus* population structure and strain diversity varies over space and time;

4) Look for any evidence of widespread or cosmopolitan strains that appear to be adapted to the environment.

In Chapter Four I revisited the same sites in the Tampa Bay watershed that were sampled in Chapter 3 and I used the concept of landscape area to reexamine the relative population sizes of the enterococci found in the water, sediment, and SAV. Furthermore, I investigated the theoretical potential for benthic substrates, such as sediment and SAV, to serve as important reservoirs of resuspendable FIB, as is often suggested in the literature. Specifically, the study had three important goals:

1) Identify and quantify key habitat characteristics that would allow the normalization of enterococci densities on a landscape basis and directly compare the population sizes in water, sediment, and SAV at each site;

2) Develop a model that predicts shifts in the relative population sizes at a given site that result from theoretical changes in important habitat characteristics such as bacterial densities, water depth, SAV cover, etc.;

3) Use historical wind and flow data at each site, in conjunction with theoretical calculations of sediment resuspension, to determine the likely effect of sediment-associated bacterial resuspension on water quality monitoring at each site.

Significance of Research

Through the objectives outlined above, I aim to improve our understanding of the ecological dynamics of enterococci in the environment. This will have the applied benefit of improving our knowledge of how the enterococci function as indicators of fecal pollution and, hopefully, how their use in this fashion can better predict the human health risks associated with waterborne disease. From the standpoint of basic science, these results offer further insight into the ability of microbial species to adapt to new habitats and environments and the role that clonal diversity plays in determining microbial diversity. In addition, by determining the relative reservoir sizes and potential resuspension of benthic bacteria, we gain a better understanding of the role of physical processes and benthic-pelagic coupling in structuring microbial ecology and affecting the fate and transport of microbial species in the environment.

My investigation of enterococci associated with environmental water, sediment, and SAV represents the first account of simultaneously monitoring the role that these three important environmental matrices have on the persistence of FIB. The use of this approach in both mesocosms and field sampling allows direct comparisons of their importance as a substrate, as well as the observation of potential indirect effects among the different substrates from the perspective of the entire aquatic system. It also allows

for a more complete picture of the clonal diversity of environmental enterococci present at each of the sites. In addition, my application of the landscape scale for normalizing bacterial densities greatly improves our ability to directly compare the importance of environmental reservoirs of water quality indicator bacteria. The use of this approach allows targeted focus on only those reservoirs that have the potential to significantly affect water column concentrations, and the model developed in Chapter Four provides a means of estimating how the population sizes in each reservoir may change as habitat characteristics change. Finally, my estimates of resuspension of FIB at select sites illustrates that bacterial densities normalized to mass of substrate are not sufficient to determine when resuspension of benthic sources of FIB may affect water quality, and that additional weather and habitat characteristics must also be known.

CHAPTER TWO

THE EFFECTS OF SUBMERGED AQUATIC VEGETATION ON THE PERSISTENCE OF ENVIRONMENTAL POPULATIONS OF *ENTEROCOCCUS* SPP.

Introduction

Most water quality monitoring strategies are based on the measurement of indicator organisms – microorganisms that indicate fecal pollution and thus the potential for the presence of waterborne pathogens – as opposed to monitoring for individual pathogens. Bacteria belonging to the genus *Enterococcus* are one of the major groups used as such an indicator in many monitoring programs for recreational water quality (USEPA, 2000; WHO, 2001). Recent research, however, has provided evidence that some FIB are capable of persisting in a culturable form for extended periods in the sediments and submerged aquatic vegetation (SAV) of many secondary environmental habitats (Byappanahalli et al., 2003a; Craig et al., 2004; Anderson et al., 2005; Ishii et al., 2006; Englebert et al., 2008). The persistence of benthic FIB (note that we use benthic to describe bacteria associated with the bottom of aquatic habitats, including sediment and vegetation, as opposed to those suspended in the water column) is particularly important because resuspension of those cells back into the water column, such as might occur during storms or high recreational activity, may provide an erroneous signal of recent fecal contamination. If the persistence of FIB exceeds that of most pathogens in secondary habitats, elevated levels of residual FIB can trigger a false alarm of human

health risk, which has been a major concern raised about the reliability of the indicator organism concept (Solo-Gabriele et al., 2000; Grant et al., 2001; Whitman et al., 2003; Anderson et al., 2005; Ishii and Sadowsky, 2008).

Evidence of persistent populations of culturable FIB has been found in terrestrial soils (Topp et al., 2003; Ishii et al., 2006), aquatic sediments (Byappanahalli and Fujioka, 1998; Solo-Gabriele et al., 2000; Jeng et al., 2005), and attached to SAV (Anderson et al., 1997; Whitman et al., 2003; Ksoll et al., 2007; Kleinheinz et al., 2009). Increased environmental persistence of FIB has been experimentally correlated with decreased temperature, salinity, and solar radiation (Davies and Evison, 1991; Howell et al., 1996; Anderson et al., 2005). Field samples and laboratory mesocosms (using inoculated cultures) have been used to show increased persistence of cells associated with benthic sediments or SAV as compared to cells suspended in the water column, presumably due, at least in part, to the increased supply of organic carbon and protection from ultraviolet radiation afforded by these habitats (Sherer et al., 1992; Desmarais et al., 2002; Byappanahalli et al., 2003a; Craig et al., 2004; Anderson et al., 2005; Ksoll et al., 2007). To my knowledge, however, the controlled experimental setting represented by mesocosms has rarely been used to investigate the persistence of unaltered environmental populations of FIB (Desmarais et al., 2002) and has never been used to simultaneously compare the suitability of water, sediment and SAV as refuges for persistent populations of FIB.

The consequences of persistent FIB in secondary habitats are further complicated by the effects of intragroup diversity. This diversity can allow for differential survival of certain members of FIB populations in a given set of habitat conditions (Topp et al., 2003; Anderson et al., 2005; Ishii et al., 2006), which complicates their population dynamics. Furthermore, it confounds our ability to link them to sources and potentially creates a disconnect between FIB concentration and pathogen presence in environmental waters. Many methods have been used to differentiate strains of FIB, including ribotyping and antibiotic resistance patterns (Parveen et al., 1997; Harwood et al., 2000; Anderson et al., 2006) as well as amplification of repetitive DNA sequences (Dombek et al., 2000; Johnson et al., 2004; Ishii et al., 2006), in order to investigate the influence of population structure on the ecology of persistent FIB.

In this study, experimental mesocosms with continuous unidirectional current were employed to investigate the persistence of environmental enterococci populations under controlled conditions over a 14 day period. Specifically, there were three objectives: (1) to simultaneously examine the persistence dynamics of environmental enterococci populations (as opposed to inoculated laboratory or sewage strains) in the water and on sediments and SAV; (2) to experimentally determine the effect of SAV on the persistence of enterococci by simultaneously comparing enterococci persistence in paired vegetated and unvegetated mesocosms; and (3) to determine the effects of SAV on the population structure of enterococci by examining shifts in species and strain diversity over time.

Methods

Experimental Mesocosms

Eight mesocosm experiments were conducted in total and were organized as four paired trials (May, July, and August of 2007 and March of 2008), with a vegetated and unvegetated replicate run side by side. Each pair of mesocosms was maintained and monitored for a two week period in an open-air greenhouse at the University of South Florida Botanical Gardens, allowing exposure to ambient air temperatures and solar radiation, but protection from rainfall. The mesocosms were of a recirculating racetrack flume design, using an electric trolling motor housed in a drop box at one end to power constant water flow under relatively controlled hydrodynamic conditions (*Figure 1*). Such water movement has been found to be important to avoid anoxic conditions in the mesocosms (Harwood, unpublished data), and simulates a flowing water body. Because I originally intended to examine the effect of water velocity on enterococci persistence, the May and August experiments were run at a higher water velocity (12 cm/s) than the April and July experiments (2 cm/s). However, due to the logistical difficulty of achieving high levels of replication with the experimental setup, and the fact that all measured physical conditions (i.e. dissolved oxygen, temperature, pH, and turbidity) in high water velocity vs. low water velocity mesocosms were similar, I pooled data from all experiments for analysis. It is important to note, however, that I am not claiming that hydrodynamics do not have important biological or physicochemical effects – merely that this study, as conducted, was unable to detect any such responses.

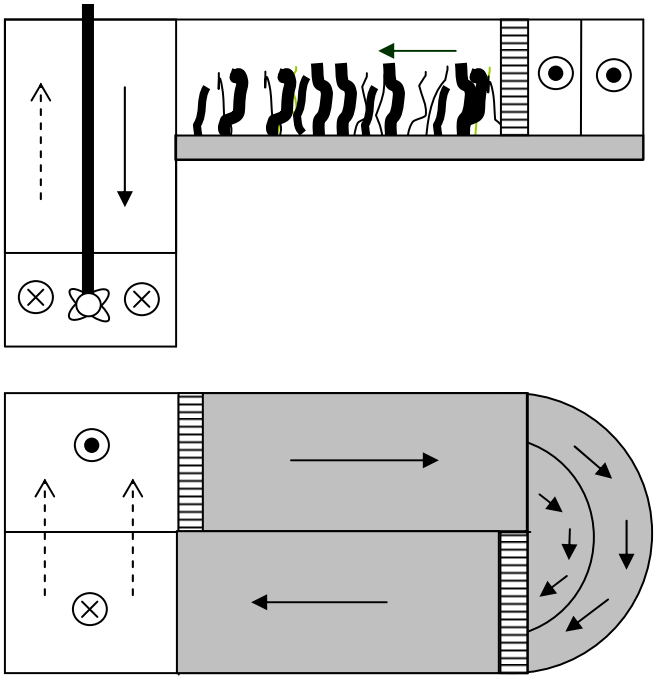

Figure 1. Schematic representation of the recirculating flume (~180L) that was used to created vegetated and unvegetated mesocosms for flow experiments. Top panel is side view and bottom panel is top view; gray shading represents areas with sediment and arrows indicate direction of flow.

Water, sediment, and SAV (mostly *Hydrilla verticillata*) with chronically high enterococci levels were collected from a freshwater lake in Tampa, FL, USA and used to seed the mesocosms without any alteration, so that natural populations of enterococci would be included in the experiments. Each substrate was collected and transported independently from the other in sterile containers to avoid cross-contamination. Sediment and SAV were briefly drained of superficial water at the collection site and transported damp to avoid changing densities due to desiccation or dilution into water during transport. During mesocosm setup, individual shoots of SAV (10-20 cm in length) were placed into two plexiglass plates that had been drilled to accommodate approximately 600 shoots/m^2, and one plate was placed in each side of the vegetate mesocosm to create a thick canopy. Next, sediment was gently added by hand to the working section of each mesocosm to a depth of approximately 2 cm and care was taken to avoid burying the SAV shoots. Water was added to the non-working section of each mesocosm and slowly filled to a depth of about 12cm. Sediment was not resuspended in the mesocosms as a result of filling or the flow generated by the motors, so that mixing of enterococci due to experimental effects would be minimized. The mesocosms were established and the experiments began within two hours of collecting the material from the lake.

Bacterial Concentrations

Water, sediment, and SAV were sampled prior to collection from the lake, approximately two hours after the establishment of the mesocosms, and then 1, 2, 3, 4, 8, and 14 days

from start of the experiment. All samples (except the first set taken after the mesocosm setup) were collected between the times of 0900 and 1100. Triplicate samples of 250 mL water and 25 g sediment were collected aseptically from each mesocosm along with triplicate samples of 25 g SAV from the vegetated mesocosms, and were immediately placed on ice and processed in the laboratory within 4 h. The number of colony forming units (CFU) of enterococci was quantified via membrane filtration. Water samples (1, 10, or 100 mL of each triplicate sample) were concentrated by vacuum filtration directly onto 47 mm nitrocellulose membranes (Fisher Scientific, Inc.) with a 0.45 μm pore size and cultured at 41° C for 24 hours on mEI agar (Difco Laboratories) (USEPA, 2000). For sediment and SAV samples, 10 g (wet weight) of material was diluted in 100 ml of sterile buffered water (0.0425 g/L KH_2PO_4 and 0.4055 g/L $MgCl_2$; pH = 7.2) and sonicated on ice at 14 watts for 30 seconds to dislodge and resuspend attached cells (Anderson *et al*. 2005). Aliquots (10 or 25 mL) of the supernatant were then filtered and cultured as above. Concentrations are presented as CFU/100 mL for water or CFU/100 g wet weight substrate for sediment and SAV samples. For samples in which the analyte was not detectable, one half of the limit of detection (LOD) was used as the concentration for the purpose of presentation and statistical analysis. Limits of detection equaled 0.3 CFU/100 mL in water samples or 13 CFU/100 g in sediment and SAV samples. After counting, well-isolated colonies were picked from the mEI agar (up to a maximum of 32 isolates were saved for each substrate at each time point) and cultured overnight in Enterococcosel broth (EB, Difco Laboratories, enc.) at 37° to confirm esculin hydrolysis.

Glycerol was added (10% v/v) to cultures prior to storage at -80° C for later genetic typing.

Enterococcus Genotyping

Isolates from each substrate at the beginning of every experiment were selected for BOX-PCR genotyping to determine the initial population structure for all of the mesocosms. In addition, one time point was selected near the end of each experiment in the vegetated mesocosm for genotyping to determine if the population structure was changing. These points were day 14 in May and day 5 in July, Aug, and March (only sediment and SAV available from the latter three). For the May experiments, 15 isolates were typed from each substrate at the initial time point. However, after discovering the extremely low strain diversity (see results and discussion), this number was reduced to 6 isolates per substrate per sample to confirm that the population structure had not changed, for a total of 153 isolates across all of the experiments.

Cryopreserved isolates were streaked onto tryptic soy agar to ensure isolation of a pure culture. Isolates were then grown overnight in 1 mL of brain heart infusion broth (BHI, Difco Laboratories) at 37° C and DNA was extracted using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich) per the manufacturer's instructions for Gram-positive bacteria. The DNA of each isolate was then typed by repetitive extragenic palindromic (REP) PCR fingerprinting using the BOX A2R primer (5'-ACG TGG TTT GAA GAG ATT TTC G-3') (Koeuth et al., 1995). Twenty five μL PCR reactions contained 5 μL of

5 × Gitschier Buffer (Kogan et al., 1987), 2.5 µL of 10% dimethyl sulfoxide, 0.4 µL bovine serum albumin (10 mg/mL), 2.0 µL 10 mM dNTPs, 1.0 µL *Taq* polymerase (5000 u/mL), 10.6 µL water, 1.5 µL 10 µM BOXA2R primer; and 2.0 µL of DNA template, containing between 10 and 40 ng/µL of DNA (Versalovic et al., 1991; Malathum et al., 1998). The PCR program included (1) initial denaturation at 95º C for 7 min; (2) 35 cycles of 90º C for 30 s, 40º C for 1 min, and 65º C for 8 min; and (3) final extension at 65º C for 16 min. The amplicons were separated on a 1.5 % agarose gel (90 watts for 4 hrs), stained with ethidium bromide (1% solution) and imaged under UV light. A strain of *Ent. faecalis* isolated from a previous study (Anderson et al., 2005) was chosen as a PCR control because its banding pattern showed even dispersion across the molecular weight range used for analysis (250 – 3000 bp). Banding patterns were analyzed for similarity with BioNumerics 4.0 software (Applied Maths, Inc., Belgium) and confirmed by eye. Similarity was determined from Pearson correlations based upon the densitometric curves (optimization = 1%) for each genetic type and a dendrogram was constructed via UPGMA. Identical reactions of a control strain maintained a similarity of 84%, which was used as a critical value to establish which environmental strains were similar enough to be considered identical, and the results were confirmed by eye.

Taxonomic Identification of Isolates

Two isolates of the dominant BOX-PCR genotype (see results) were identified by sequencing the 16S rRNA gene. A 1,145 bp fragment of the gene was amplified from extracted DNA via PCR using the universal bacterial primers Eco8f (5'-AGA GTT TGA

TCM TGG CTC AG – 3') and ECO1492RC (5'-GGT TAC CTT GTT ACG ACT T-3') (Lane, 1991). Fifty microliter PCR reactions contained 25 µL JumpStart *Taq* polymerase (Sigma, USA), 2 µL each primer, 5 µL water, and 5 µL DNA template. The amplification process included (1) initial denaturation at 94º C for 5 min; and (2) 20 cycles of 94º C for 1 min, 55º C for 1 min, and 72º C for 10 min. Amplicons were frozen and sent to a commercial laboratory for sequencing (Macrogen, Inc., USA). Because the results of the sequencing did not offer resolution between the highly similar *Ent. casseliflavus* and *Ent. gallinarum* species, colonies were examined for the yellow pigmentation that is characteristic of *Ent. casseliflavus.*

Calculations and Statistical Analysis

All CFU data were transformed as $\log_{10}(x + 1)$ to meet normality requirements prior to statistical analysis. Throughout the manuscript, the terms 'concentration' or 'density' are used to define the CFU per unit volume of water or mass of substrate (sediment or SAV), while the term 'total number' is defined as the total number of CFU per mesocosm. Means of either value were calculated as the mean and standard deviations of log CFU for all time points (n = 8) in a given mesocosm experiment. The total number of CFU associated with each substrate in each mesocosm was calculated by multiplying the enterococci density (CFU/100 ml or CFU/100 g) times the total water volume or the total mass of sediment or SAV in each mesocosm at each time point. Water volume and substrate mass in each mesocosm was determined at the end of each set of experiments, and corrections were made to account for the amount of material removed at each time

point for sample analysis. Finally, the total number of CFU per mesocosm at each time point was calculated simply as the sum of the total numbers of CFU per mesocosm associated with each substrate.

Due to the pattern of the survival dynamics observed in our study, which typically included a period of decline in density of enterococci over the first few days, followed by an extended period of persistence, two different types of decay rates were calculated. Initial decay rates for the populations in each substrate were calculated by regressing the log CFU against the sample time for each experiment. Only those data up to the first sample in the time series with a non-detect for enterococci were used. Cumulative decay rates were calculated in the same manner, but using all time points for each experiment. The slope of the regression represents the decay rate and is reported as change in log CFU/d (Davies and Evison, 1991; Craig et al., 2004). A negative decay rate represents a decrease in CFU, while a positive rate represents an increase in CFU. Minimum persistence times are reported as the latest day on which detectable levels of enterococci were found in a particular substrate (these values were converted to categorical data and analyzed non-parametrically with a Kruskal-Wallis *H*-test due to the lack of sample data available on some days). Values were compared statistically using randomized block analyses of variance and paired *t*-tests (SPSS, version 17.0, SPSS, Inc., USA; $\alpha = 0.05$).

Results

Mesocosm Conditions

Water temperature in the mesocosms was measured at each sampling during the experiment and ranged from lows of 18° C in March to highs of 30° C in July and August. The mean temperature at sampling in the March experiments (21.0º C) was significantly lower than the other three months (May = 24.8º C, July = 26.8º C, and August = 26.9º C; p <0.001, ANOVA and Tukey's post-hoc comparisons). Water chemistry stayed relatively constant in all experiments with dissolved oxygen values from 9.5 to 10.5 mg/L, pH values from 7.5 to 8.5, and salinity values from 0.25 to 0.35 ‰. Visible resuspension of sediments did not occur. The mesocosms were of a recirculating flume design, were approximately 1 m x 1.5 m in area, 15 cm deep, and held approximately 180 L (see *Methods* and *Figure 1*). Water current was controlled with submerged trolling motors so that there was a continuous unidirectional current over the substratum.

Population Dynamics

The population dynamics of culturable enterococci obtained from a freshwater lake over a two week period were highly variable among replicate pairs of experiments, each of which included one vegetated and one unvegetated mesocosm. In some form, however, evidence for extended persistence of culturable enterococci was exhibited in each of the four replicates of paired mesocosms. The May 2007 experiments exhibited very high-level, consistent persistence. Enterococci densities were initially high (water = 10^3

CFU/100 mL; sediment = 1.3 x 10^3 CFU/100 g; SAV = 6.3 x 10^4 CFU/100 g), decreased rapidly during the first day, rebounded dramatically at about day 5 and then continued to increase throughout the two week period (*Figure 2*). The July 2007 experiments had much lower initial densities (approximately 10^2 CFU/100 mL in water and sediment and 10^3 CFU/100 g in SAV), and enterococci densities on all substrates varied over 2 – 3 orders of magnitude (*Figure 3*). The August 2007 and March 2008 experiments were in-between these extremes in terms of cell densities and consistency of detection (*Figure 4* and *Figure 5*).

Effects of SAV

Because the enterococci measured in this study were obtained from a lake with a history of a persistent enterococci population, and because the population dynamics in the mesocosms were highly variable, the mean concentrations of enterococci over the entire 14-day experiments were compared by a paired *t*-test. Mean numbers of total enterococci in vegetated vs. unvegetated mesocosms were compared, where each data pair represented the two mesocosms sampled in one month. From the perspective of the entire system, vegetated mesocosms maintained significantly higher mean numbers of total enterococci (SAV+sediments+water) (9.4 x 10^4 CFU per mesocosm) than unvegetated mesocosms (sediments+water) (2.6 x 10^4 CFU per mesocosm; $p = 0.05$, paired *t*-test) when averaged over the 14-day period for each experiment, a 250% increase. It is important to note that this difference was not explained by the extra bacteria initially

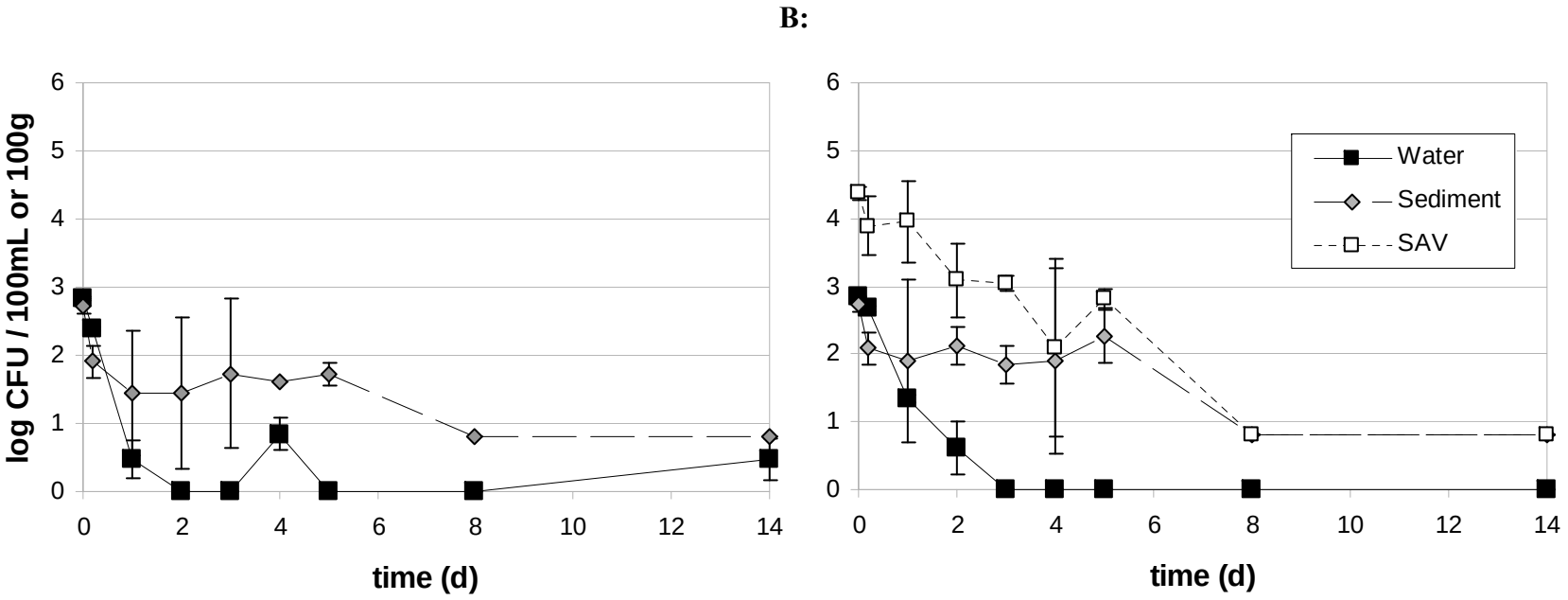

Figure 2. Culturable enterococci concentrations in unvegetated (A) and vegetated (B) mesocosms for the April experiments. Time 0 represents samples from the source lake taken prior to mesocosm setup. Each point is the mean of three replicates ± s.d.

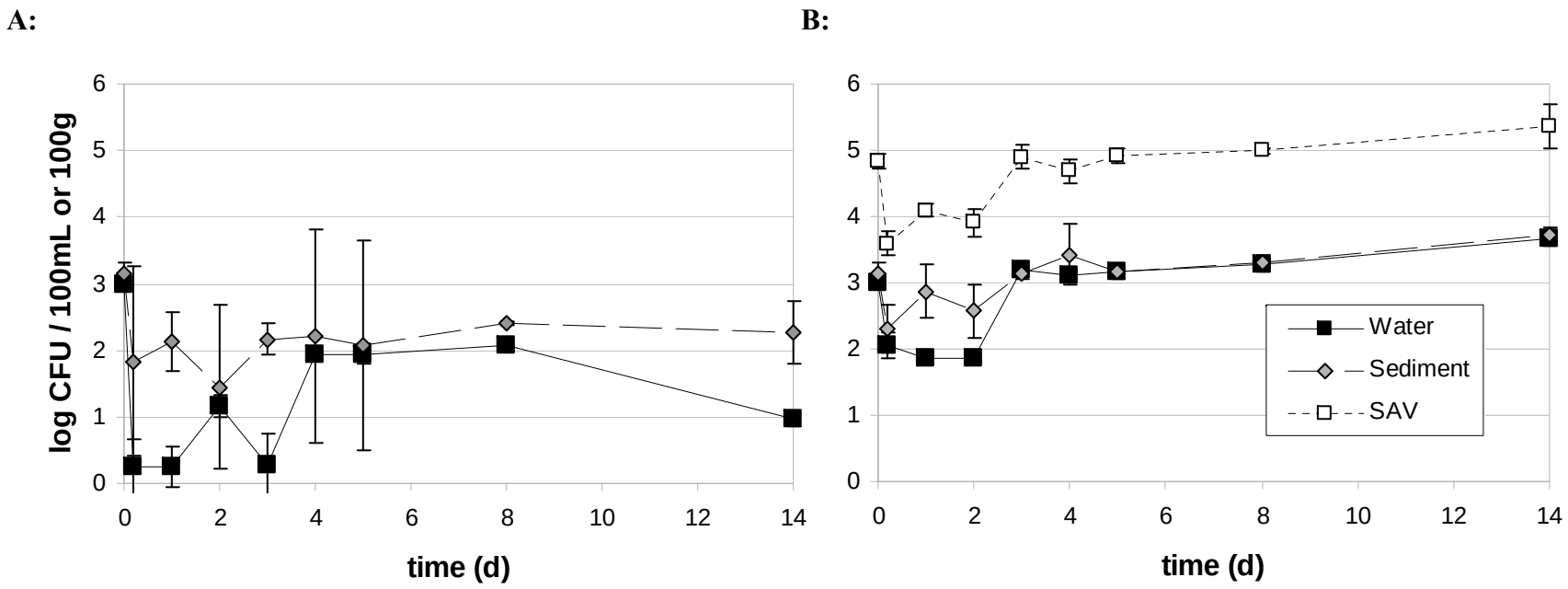

Figure 3. Culturable enterococci concentrations in unvegetated (A) and vegetated (B) mesocosms for the May experiments. Time 0 represents samples from the source lake taken prior to mesocosm setup. Each point is the mean of three replicates ± s.d.

A:

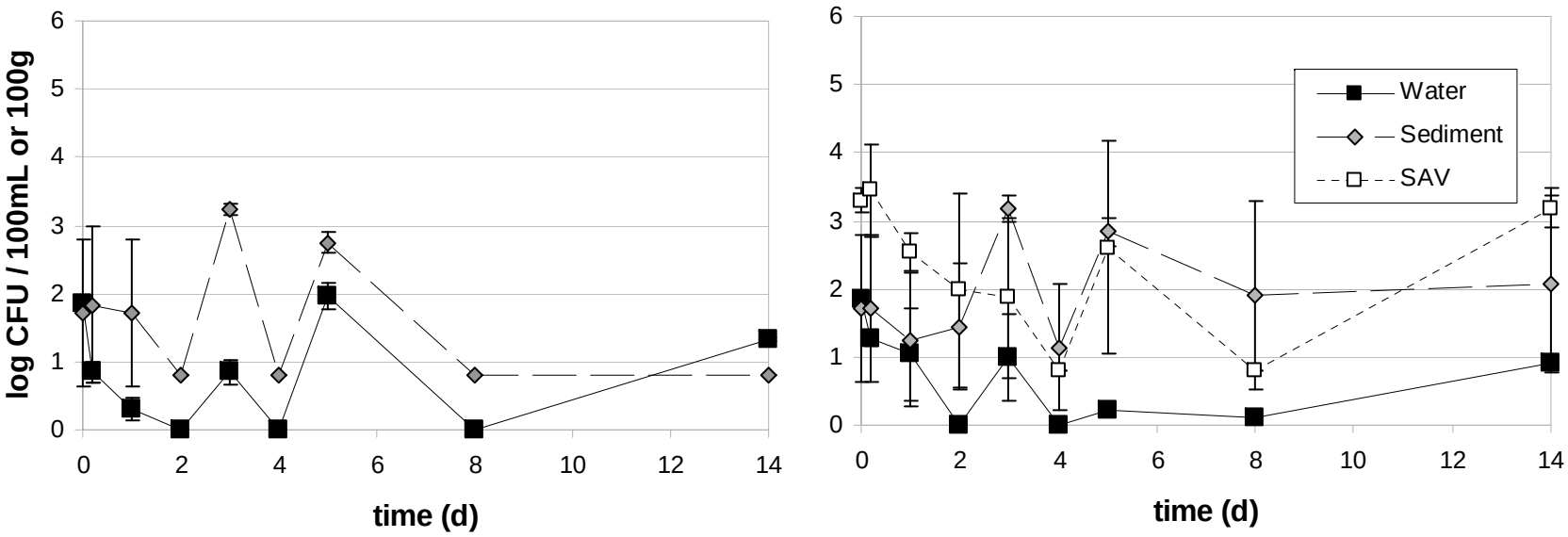

Figure 4. Culturable enterococci concentrations in unvegetated (A) and vegetated (B) mesocosms for the July experiments. Time 0 represents samples from the source lake taken prior to mesocosm setup. Each point is the mean of three replicates ± s.d.

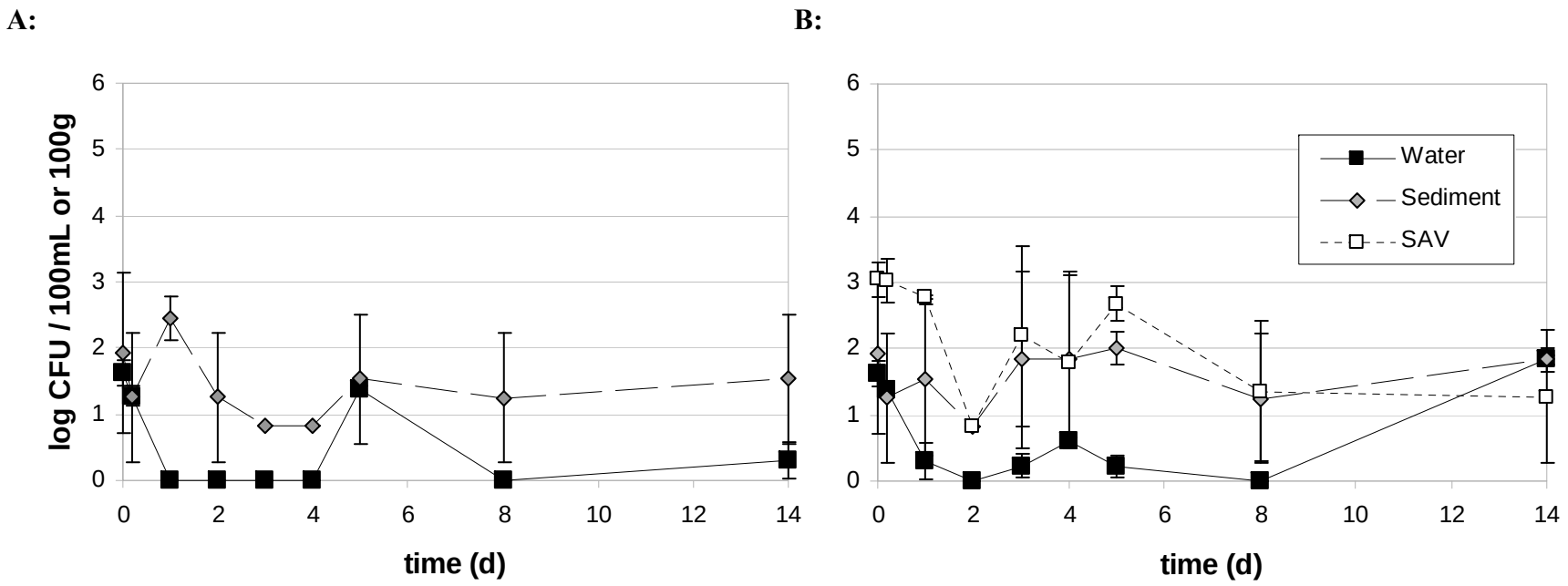

Figure 5. Culturable enterococci concentrations in unvegetated (A) and vegetated (B) mesocosms for the August experiments. Time 0 represents samples from the source lake taken prior to mesocosm setup. Each point is the mean of three replicates ± s.d.

introduced into the vegetated mesocosms with the SAV, which caused only a 10% increase over unvegetated mesocosms. Furthermore, when enterococci concentrations were normalized to the initial concentration measured in the mesocosms, the differences between treatments were still significant. When paired *t*-tests were run on a month by month basis (separate tests run for each month, samples paired by time), significant differences were seen in April, May, and July, but not in August (p = 0.04, 0.001, 0.002, and 0.15, respectively).

When the enterococci densities were compared by substrate, SAV harbored significantly higher mean densities on a per mass basis (8.6 x 10^2 CFU/100 g) than sediments (1.3 x 10^2 CFU/100 g), which, in turn, had significantly higher mean densities than water (18 CFU/100 mL) ($p <$ 0.001, randomized block ANOVA; pair wise comparisons tested with LSD post-hoc tests; p = 0.02 for SAV vs. sediments and p = 0.01 for sediments vs. water; *Table 1*). However, when the data are examined as the total CFU present in or on each substrate in the entire mesocosm (i.e. total on SAV, total in sediment, total in water; see methods for explanation of calculations), the vegetated canopy did not typically harbor the largest proportion of all culturable cells in the system. The proportions varied by experiment; i.e., the population in the water column dominated the May experiment, while in July the sediment population represented the largest proportion of all culturable enterococci in the mesocosm (*Figure 6-Figure* 9). On average, over all experiments, the largest proportion of cells within the mesocosms was in the sediment (50%), followed by

Table 1. Mean concentration and proportional distribution of culturable enterococci in water, sediment and SAV in the vegetated mesocosms for all experiments. Minimum persistence time = latest day in each experiment with detectable levels of enterococci in that substrate. (SD = standard deviation; randomized-block ANOVAs followed by Tukey's post-hoc comparisons; S = sediment, V = SAV, W = water).

	CFU density (log CFU/100 mL or 100 g)		**% Total CFU**		**Initial Decay Rate (log CFU/d)**		**Cumulative Decay Rate (log CFU/d)**		**Minimum Persistence (d)**	
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Water	1.3	1.0	35	18	-0.63	0.50	-0.02	0.12	11	5.5
Sediment	2.1	0.66	50	21	-0.11	0.22	0.02	0.08	10	4.5
SAV	2.9	1.1	15	5	-0.50	0.49	-0.08	0.15	12	4.5
p-value	**0.001**		0.12		0.07		0.13		0.42	
Post-hoc	**S >W; V > W**		N/A		N/A		N/A		N/A	

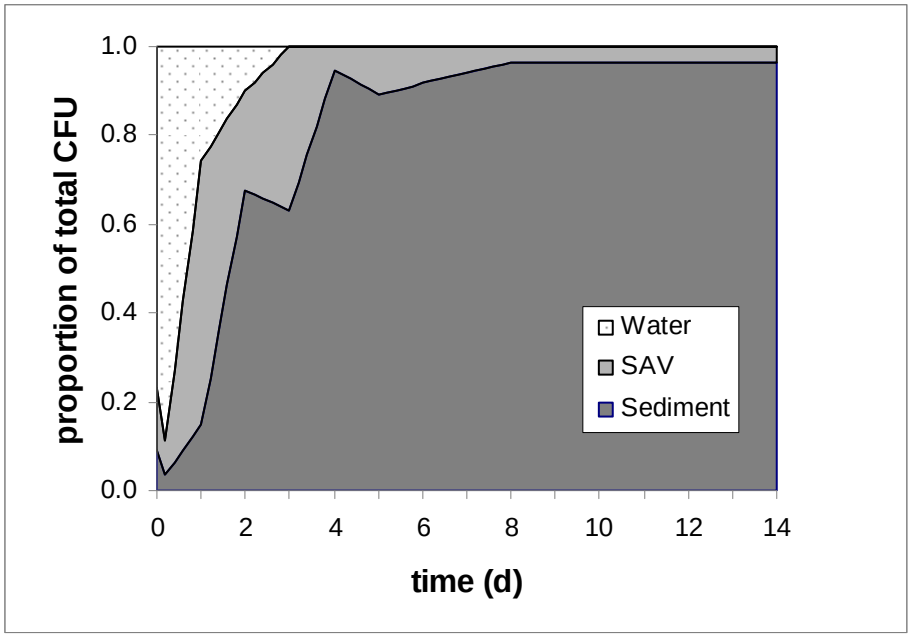

Figure 6. Total number of culturable enterococci associated with each substrate type in the vegetated mesocosms for the April experiments. Time 0 represents samples from the source lake taken prior to mesocosm setup. Data are presented as proportion of the total number of enterococci CFU per mesocosm persisting in each substrate type**.**

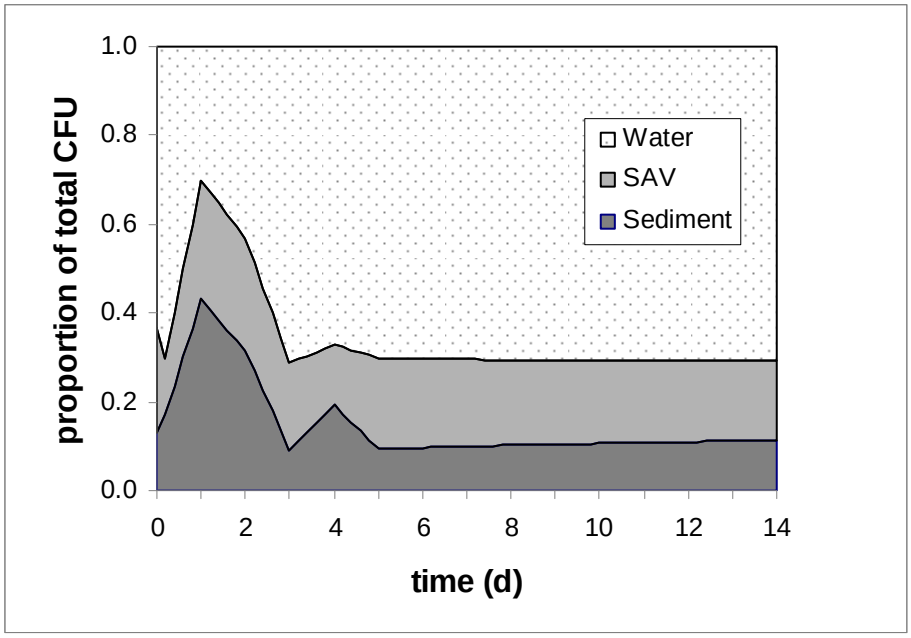

Figure 7. Total number of culturable enterococci associated with each substrate type in the vegetated mesocosms for the May experiments. Time 0 represents samples from the source lake taken prior to mesocosm setup. Data are presented as proportion of the total number of enterococci CFU per mesocosm persisting in each substrate type.

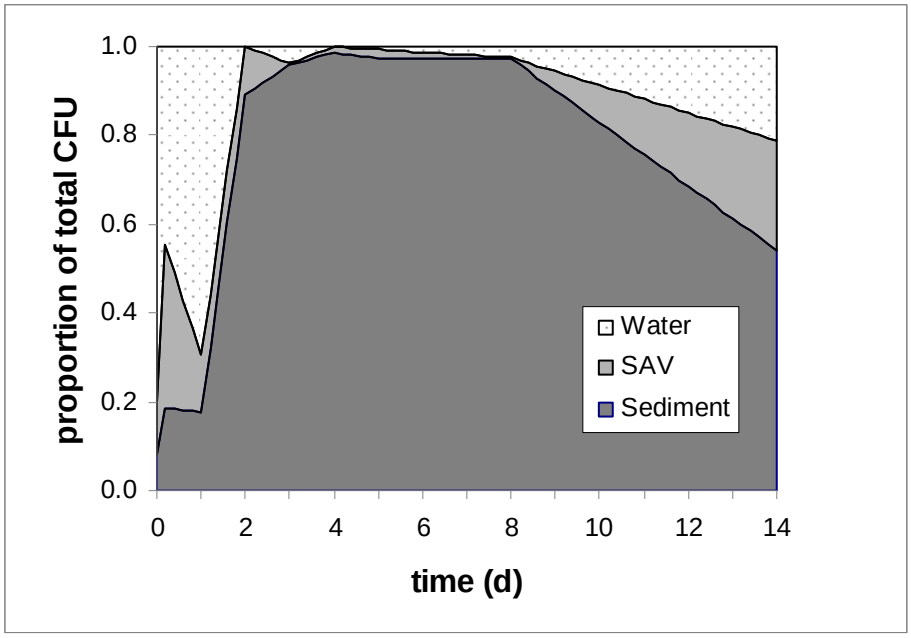

Figure 8. Total number of culturable enterococci associated with each substrate type in the vegetated mesocosms for the July experiments. Time 0 represents samples from the source lake taken prior to mesocosm setup. Data are presented as proportion of the total number of enterococci CFU per mesocosm persisting in each substrate type.

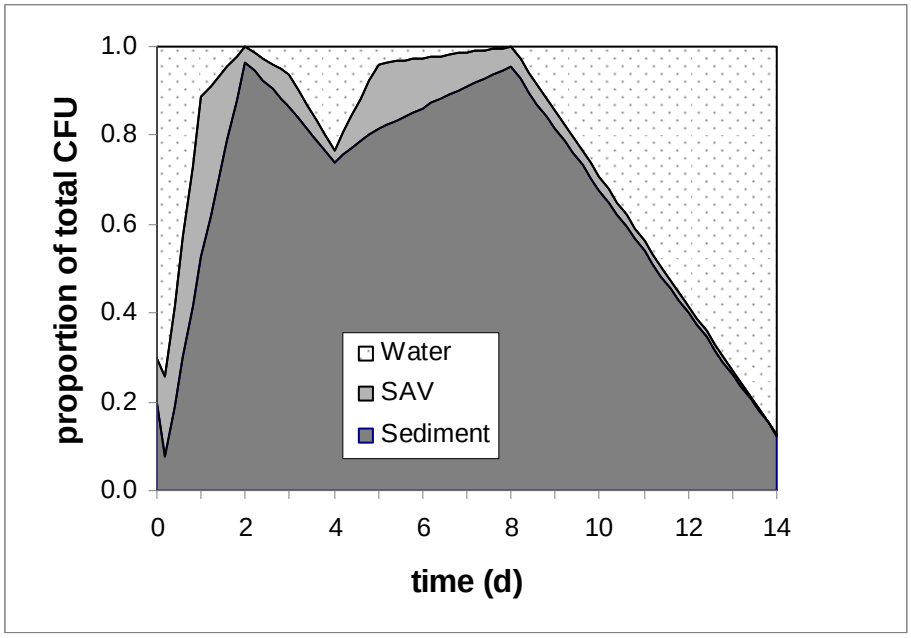

Figure 9. Total number of culturable enterococci associated with each substrate type in the vegetated mesocosms for the August experiments. Time 0 represents samples from the source lake taken prior to mesocosm setup. Data are presented as proportion of the total number of enterococci CFU per mesocosm persisting in each substrate type.

the water column (35%), and finally SAV (15%), although these differences were not significant (*Table 1*).

Indirect effects of SAV were also evident when comparing enterococci densities in the sediment and water of the vegetated mesocosms to those of the unvegetated mesocosms. Across all replicate experiments, mean culturable enterococci densities in the sediment were higher in the vegetated mesocosms than in the unvegetated mesocosms ($p = 0.05$, paired *t*-test). Sediment-associated initial and cumulative decay rates were also lower in vegetated mesocosms, though the differences were not significant at $\alpha = 0.05$ (*Table 2*). In contrast, no significant difference between vegetated and unvegetated treatments was observed in the water column, although similar general trends were observed (*Table 2*). When paired *t*-tests were run for each month (samples paired by time), significant differences in mean enterococci densities were seen in April, May, and nearly in July for sediments ($p = 0.01$, 0.001, and 0.07, respectively), but only in May for water ($p = 0.001$).

Population Structure

BOX-PCR typing of enterococci isolated from the mesocosms showed that these populations were dominated by a single genotype. One particular strain accounted for 96.5% of all isolates recovered from all experiments, regardless of substrate, mesocosm, or month (Figure 10). The dominant strain was identified as *Ent. casseliflavus* by a

Table 2. Comparison of enterococci persistence between vegetated and unvegetated mesocosms. Values for water and sediments are given. Minimum persistence time = latest day in each experiment with detectable levels of enterococci in that substrate. (SD = standard deviation; paired t-tests).

	CFU density (log CFU/100 g)		**Initial Decay Rate (log CFU/d)**		**Cumulative Decay Rate (log CFU/d)**		**Minimum Persistence (d)**	
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Water								
Unvegetated	0.85	0.34	-0.96	-0.74	-0.03	0.06	14	0.0
Vegetated	1.3	1.0	-0.63	0.50	-0.02	0.12	10	5.8
p-value	0.17		0.09		0.40		0.20	
Sediment								
Unvegetated	1.7	0.33	-0.23	-0.20	-0.05	0.05	8	4.2
Vegetated	2.1	0.66	-0.11	0.22	0.02	0.08	10	4.5
p-value	**0.04**		0.17		0.15		0.20	

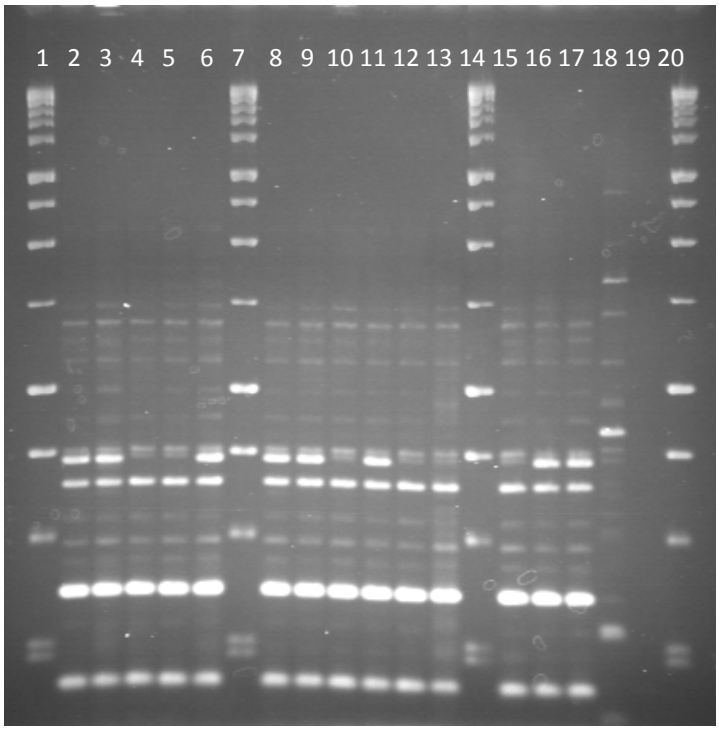

Figure 10. Representative results from *Enterococcus* strain typing showing extremely low strain diversity detected by BOX-PCR for *Enterococcus* isolates recovered from the mesocosm experiments. Lane 2 represents an isolate from a July SAV sample, lanes 3-6, 8, and 9 are from a July water sample, and lanes 10-13, 15, and 16 are from a July sediment sample, and lane 17 is from an August SAV sample. Lanes 1, 7, 14, and 20 are 1kb ladders, and lanes 18-19 are PCR controls (positive and negative, respectively).

combination of 16S rRNA sequencing and the presence of the distinct yellow pigment that is characteristic of the species.

Discussion

Concerns about the relationship between fecal FIB concentrations and the presence of pathogens in aquatic habitats have caused an increased focus on FIB that may persist in terrestrial soils, aquatic sediments, and SAV. In this study, the use of paired mesocosms and enterococci collected directly from an environmental source (a freshwater lake in Florida) allowed us to (1) simultaneously compare the relative importance of water, sediment and SAV as substrates for the persistence of enterococci in environmental waters from the perspective of the substrate and also from that of the entire system; (2) determine indirect effects of the presence of SAV on the persistence of enterococci in an aquatic system, and (3) examine the population structure and dynamics of enterococci from this lake. The results provide evidence that, while SAV can harbor relatively high densities of enterococci per unit mass in relation to other substrates, in some cases it does not harbor a large proportion of the total enterococci present in the entire aquatic system. It can, however, have indirect effects in the system by facilitating higher densities in the sediment and in the habitat as a whole. Furthermore, the fact that the populations collected from the lake over a ten month period were dominated by a single strain of *Enterococcus casseliflavus* strongly suggests that some enterococci strains are highly adapted to secondary environmental habitats and may not be reliable indicators of human health risk in subtropical waters.

Although the persistence of FIB in sediments has been relatively well documented, SAV has been much less extensively studied, with only a few published accounts of the persistence of FIB on submerged macroalgae in temperate climates (Whitman et al., 2003; Byappanahalli et al., 2007; Ksoll et al., 2007; Englebert et al., 2008; Kleinheinz et al., 2009). Our results expand upon previous findings by providing evidence of enterococci persisting on vascular aquatic plants in a subtropical environment and, even more importantly, by allowing the simultaneous comparison of the importance of SAV as a substrate in relation to sediment and water from the same aquatic system. We observed mean densities of enterococci that were comparable to previous work (Solo-Gabriele et al., 2000; Desmarais et al., 2002; Whitman et al., 2003; Ksoll et al., 2007) and were significantly higher on SAV than in the sediment or the water column. While these data certainly highlight the *potential* for SAV to serve as an important substrate for the persistence of enterococci, there are currently no standardized units for normalizing bacterial densities to substrates such as sediment and SAV and normalization methods have varied in the literature (Solo-Gabriele et al., 2000; Whitman et al., 2003; Anderson et al., 2005; Ksoll et al., 2007). As a result, direct comparisons of the relative importance of each substrate have been difficult to make across studies. In our experiments, we were able to make this comparison by calculating the total number of cells associated with each substrate in an entire mesocosm. When viewed in this manner, the apparent importance of each substrate as a reservoir for enterococci shifted dramatically. The high densities observed on SAV were offset by the relatively low vegetative mass, and the

largest proportion on average was in the sediments and the water column. These comparisons, based on the entire mesocosms, illustrate that high densities of FIB per unit mass of vegetative substrates do not *necessarily* indicate cell reservoirs that are large enough to greatly affect water quality upon resuspension into the water column. Thus, the importance of benthic reservoirs (including SAV) is highly dependent on the total mass of resuspendable, bacteria-harboring substrate and its relationship to the volume of water at a specific site.

Even though the enterococci that were directly associated with SAV were not typically numerically dominant, the use of all three substrates in the mesocosms also allowed us to observe important indirect effects that the presence of SAV can have in aquatic systems. The vegetated mesocosms contained significantly elevated total numbers of enterococci as well as significantly elevated mean densities of sediment-associated enterococci, whether or not the densities were normalized to the initial inoculum density to account for the effect of a 10% higher inoculum carried on SAV. These results provide substantial evidence that SAV can, in addition to serving as a substrate for enterococci, also facilitate elevated densities in the surrounding habitat. These results are important from the perspective of the entire system as they illustrate that the presence of vegetation in an aquatic habitat can significantly elevate the number of enterococci that may be able to persist in the entire habitat as a whole. Possible mechanisms for this effect include the growth and efflux (disassociation and entry into the water column) of SAV-associated cells (as suggested in Ksoll *et al*., 2007), increased levels of organic carbon, particularly

in sediments, and protection from solar radiation. Submerged macrophytes have been shown to increase available carbon to surrounding sediments both by causing increased settling of suspended particulates (Posey et al., 1993; Fonseca, 1996) and by exudation of photosynthate from the roots (Pollard and Moriarty, 1991). Furthermore, these changes have also been shown to result in increased microbial activity (Lopez et al., 1995; Hansen et al., 2000; Karjalainen et al., 2001).

Our ability to detect culturable enterococci throughout each 14-day experiment (even if intermittently) strongly suggests that these enterococci populations are capable of extended persistence in secondary habitats. While the initial decay rates observed in this study were comparable to those reported in other studies (Craig et al., 2004; Anderson et al., 2005), the population dynamics observed over the entire course of the experiments were highly variable over both daily and seasonal temporal scales. Unfortunately, the high variability among seasons resulted in a lower than ideal level of reproducibility among the replicates of our experiments, and a high level of replication was difficult with this experimental setup given the limitations of time and resources available for mesocosm sampling and maintenance. However, we do not believe that the relatively low reproducibility is simply a matter of the level of replication we were able to achieve. Instead, we believe that it is actually an interesting finding in its own right, as it illustrates the extremely high level of variability inherent in these systems over time and highlights the need for an increased focus on finer scale temporal dynamics among FIB in the environment. Such variable densities have been previously observed over time in the

field and in laboratory settings (Desmarais et al., 2002; Boehm, 2007), and has been attributed to environmental stresses such as temperature (Stephenson and Street, 1978; Howell et al., 1996; Craig et al., 2004), salinity (Anderson et al., 1979; Anderson et al., 2005), UV radiation (Davies and Evison, 1991; Muela et al., 2000), or grazing (Davies et al., 1995). While some authors have suggested that such variability reflects the death and growth of these bacteria in the environment (Solo-Gabriele et al., 2000; Desmarais et al., 2002; Craig et al., 2004), we have no data to support whether the short-term variability seen in our experiments represents changes in the number of total viable cells or changes in the culturability (Lleo et al., 1998; Muela et al., 2000) of a relatively stable number of cells.

The domination of the environmental populations in these experiments by one particular strain of *Ent. casseliflavus* was highly surprising, especially considering that the experiments and collections from the lake spanned 10 months. This lake is highly impacted from stormwater via man-made structures, but is not known to be directly impacted by sewage or other obvious fecal sources. *Ent. casseliflavus* is commonly associated with waterfowl, which are present at low density around the lake. Most sources of FIB, such as feces, sewage, and stormwater, typically exhibit much higher levels of strain diversity than we observed here (Anderson et al., 2005; Anderson et al., 2006; Brownell et al., 2007). It appears that the chronically elevated levels of enterococci at this site are the result of this particular strain's adaptation and persistence in this particular habitat, rather than the result of a chronic influx of pollution. Power *et*

al. (2005) came to a similar conclusion regarding high densities of *E. coli* in two Australian lakes, where they found that recurring blooms were dominated by only three distinguishable strains. All three of the *E. coli* strains had a group 1 capsule, which would potentially offer an adaptive advantage to environmental strains by ameliorating stresses such as desiccation, irradiance and predation. *Ent. casseliflavus* is characterized by a yellow pigment that may provide similar protection from the stress of UV radiation. Regardless, it is clear that further research on the ecology of persistent fecal FIB in secondary habitats is required to improve our understanding of how FIB populations reflect the fate and ecology of pathogens and how they can best be used as a water quality monitoring tool.

CHAPTER THREE

THE IMPORTANCE OF SEDIMENT AND SUBMERGED AQUATIC VEGETATION AS POTENTIAL HABITATS FOR PERSISTENT STRAINS OF *ENTEROCOCCUS* ACROSS A WATERSHED

Introduction

Most water quality monitoring strategies are based on the measurement of indicator organisms – microorganisms that indicate recent fecal pollution and thus the potential for the presence of waterborne pathogens – as opposed to monitoring for individual pathogens specifically. Bacteria belonging to the genus *Enterococcus* (also termed enterococci) are one of the major groups used as such an indicator in many monitoring programs for recreational water quality (USEPA, 2000; WHO, 2001). Recent research, however, has provided evidence that some FIB are capable of persisting in a culturable form for extended periods in the sediments and submerged aquatic vegetation (SAV) of many secondary environmental habitats (Byappanahalli et al., 2003a; Whitman et al., 2003; Craig et al., 2004; Anderson et al., 2005; Ishii et al., 2006; Ksoll et al., 2007). The persistence of benthic FIB (note that we use benthic to describe bacteria associated with the bottom of aquatic habitats, including sediment and vegetation, as opposed to those suspended in the water column) is particularly important because resuspension of those cells back into the water column, such as might occur during storms or periods of high recreational activity, may lead to false conclusions regarding recent contamination. This

has been a major concern raised about the reliability of the FIB concept (Solo-Gabriele et al., 2000; Grant et al., 2001; Whitman et al., 2003; Anderson et al., 2005; Ishii and Sadowsky, 2008).

The consequences of persistent FIB in secondary habitats are further complicated by the effects of inherent diversity among FIB. Indicator groups, like the enterococci and fecal coliforms, harbor interspecific variability among their member species. For example, within the coliforms, *E. coli* has been found to be much more commonly associated with sewage and human fecal material, while other members of the group, such as *Enterobacter* and *Klebsiella*, are much more ubiquitously found in the environment (Leclerc et al., 2001). The same is true for enterococci, where *Ent. faecalis* and *Ent. faecium* are very abundant in human feces and sewage, while *Ent. casseliflavus* and *Ent. mundtii* are typically associated with environmental sources, such as birds and plants (Leclerc et al., 1996; Muller et al., 2001; Ott et al., 2001; Aarestrup et al., 2002).

However, in addition to this interspecific variability, each species can also exhibit considerable *intra*specific variability as a result of the strain diversity inherent in a given population. This inherent variation has been a major focus of microbial source tracking (MST), which is an area of active research that attempts to overcome the limitations of using solely the concentrations of FIB as the predictor of human health risk from water use. The goal of MST is to distinguish contamination that originates from various fecal sources (e.g., human, agricultural, or wildlife), thereby offering a means of determining

when high concentrations of FIB are truly representative of human or other high-risk types of fecal pollution and pose increased health risks (Simpson et al., 2002; Field and Samadpour, 2007; Stoeckel and Harwood, 2007). Methods include both library-dependent and library-independent approaches that try to identify particular microbial strains and target genes that are specific to, or highly associated with, waste from particular host species.

Although a variety of methods have been successfully employed to discriminate between sources of FIB in recent years (Bernhard and Field, 2000; Dombek et al., 2000; Scott et al., 2005; USEPA, 2005; McQuaig et al., 2006), the implementation and interpretation of these methods in the environment may be complicated by the population dynamics of persistent FIB in natural environments. Many methods have been used to differentiate among strains of FIB, including ribotyping (Carson et al., 2001; Anderson et al., 2005), antibiotic resistance patterns (Parveen et al., 1997; Harwood et al., 2000), and amplification of repetitive DNA sequences (Dombek et al., 2000; Johnson et al., 2004). Regardless of the method employed, however, a substantial body of work indicates that inter- and intraspecific variability can result in differential survival of FIB strains and species in environmental habitats (Topp et al., 2003; Anderson et al., 2005; Ishii et al., 2006). Such evidence implies that strains probably have inherently different physiological capabilities that affect their persistence in natural habitats, resulting in complex population dynamics that confound our ability to link FIB to potential sources.

Low levels of strain diversity have been observed at a variety of environmental sites, particularly for *E. coli*, suggesting that some strains may not only survive longer, but may even be adapted to continued persistence in the environment at high numbers. For example, ubiquitous *E. coli* strains that are common in environmental samples and not related to known fecal sources have been observed in temperate soils (Ishii et al., 2006), freshwater beach sands (Kinzelman et al., 2004; McLellan, 2004), and on the macroalga *Cladophora* (Byappanahalli et al., 2007). Similarly, extremely high density blooms of pelagic *E. coli* in Australia were shown to be mostly comprised of just three different strains, even in geographically distant lakes (Power et al., 2005). Such environmentally adapted strains have strong potential for decoupling FIB concentrations and pathogen presence at these sites, raising concerns over the utility of the indicator paradigm.

In this study, the concentrations and population structures of enterococci were simultaneously monitored in water, sediment, and SAV at several sites across the Tampa Bay watershed over an entire year. The goals of the study were two-fold. Firstly, I wanted to determine if high densities of benthic enterococci occurred regularly and consistently across a variety of sites and substrates in the watershed, and also investigate the potential for any spatial or temporal patterns in the waterborne or benthic densities. Secondly, because strain diversity has been studied much more intensively for *E. coli* than for the enterococci, I wanted to employ molecular genotyping ("fingerprinting") techniques to investigate how the enterococci population structure and strain diversity

varies over space and time in various water bodies and to look for any evidence of widespread or cosmopolitan strains that appear to be adapted to the environment.

Methods

Sampling Sites

Four freshwater sites (two streams, one lake and one river) and two estuarine sites (beaches in upper and lower Tampa Bay) were chosen to represent a typical range of water bodies in the Tampa Bay watershed (*Figure 11*). The small stream site (28º 1.583' N, 82º 11.162' W) was on Spartman Branch, a first order stream surrounded by agricultural lands and subject to complete drying out during the winter dry season. The large stream site (28º 4.583' N, 82º 15.790' W) was on Flint Creek, a third order stream that flows primarily through wooded and rural areas and drains Lake Thonotosassa and empties into the Hillsborough River. The river site (28º 4.260' N, 82º 22.671' W) was at the University of South Florida's Riverfront Park on the Hillsborough River, the main River through the city of Tampa, and downstream of a substantial amount of protected, undeveloped land. The lake site (28º 2.918' N, 82º 29.828' W) was on Lake Carroll, a small residential lake in West Tampa surrounded completely by suburban housing. The two estuarine sites include the upper bay site (27º 58.141' N, 82º 34.522' W) at Ben T Davis Beach, west of the City of Tampa in Old Tampa Bay, and the lower bay site (27º 45.149' N, 82º 37.793' W) at Lassing Park, just south of the city of St. Petersburg in the lower portion of the main stem of Tampa Bay. SAV canopies at the freshwater sites were dominated by *Alternanthera philoxeroides* (alligator weed), *Egaria densa* (Brazilian

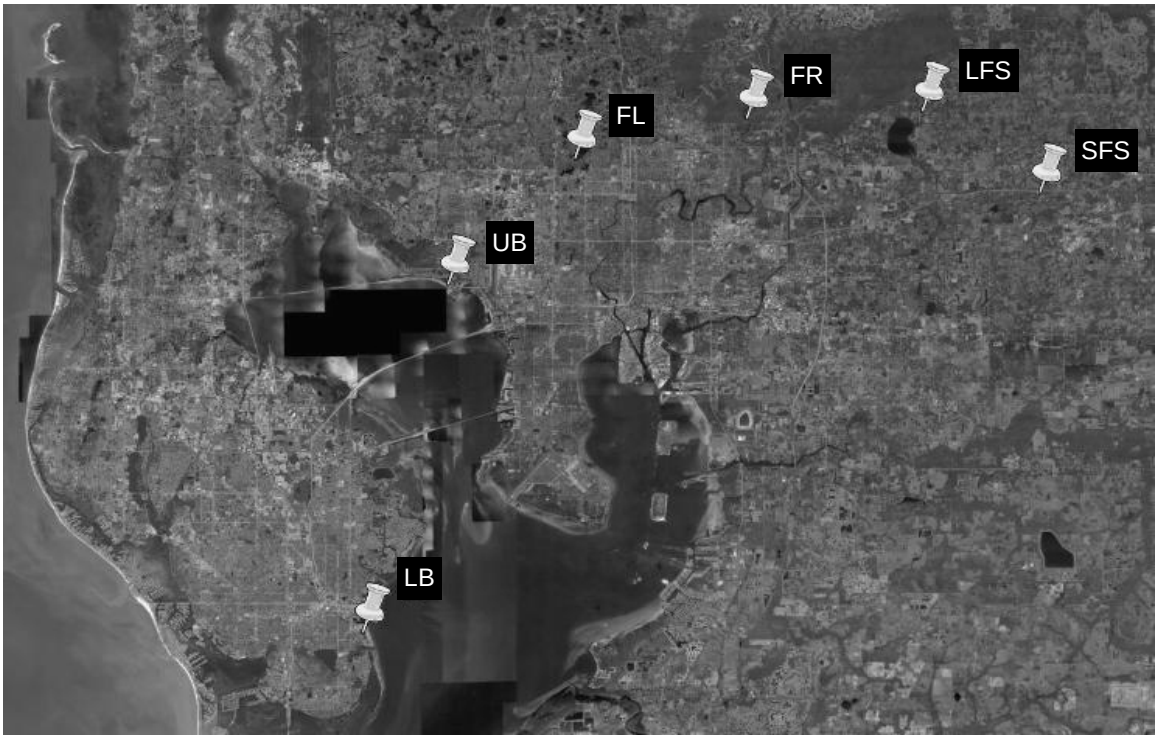

Figure 11. Site locations in the Tampa Bay watershed. Freshwater sites: FL = lake; FR = river; LFS = large stream; SFS = small stream. Estuarine sites: UB = upper bay; LB = lower bay.

waterweed), *Hydrilla verticilata*, *Myriophyllum aquaticum* (parrot feather), and *Vallisenaria Americana* (eel grass). SAV at the marine sites were dominated by *Halodule wrightii* (shoal grass) in the upper bay and a combination of *H. wrightii, Syringodium filiforme* (manatee grass), and *Thalassia testudinum* (turtle grass) in the lower bay. All of these sites range from moderate to heavy SAV coverage and have primarily fine quartz sand bottom sediments.

Environmental Sampling

Water, sediment, and SAV were sampled every month from May 2007 to April 2008. Triplicate samples of 250 mL water, 25 g sediment, and 25g SAV were collected from each site in sterile containers, immediately placed on ice and processed in the laboratory within 4 h. The number of colony forming units (CFU) of enterococci was quantified via membrane filtration. Water samples (1, 10, or 100 mL of each triplicate sample) were concentrated by vacuum filtration directly onto 47 mm nitrocellulose membranes with a 0.45 μm pore size and cultured at 41° C for 24 hours on mEI agar (Difco Laboratories) (USEPA, 2000). For sediment and SAV samples, 10 g (wet weight) of material was diluted in 100 ml of sterile buffered water (0.0425 g/L KH_2PO_4 and 0.4055 g/L $MgCl_2$; pH = 7.2) and sonicated on ice at 14 watts for 30 seconds to dislodge and resuspend attached cells (Anderson et al., 2005). Aliquots (10 or 25 mL) of the supernatant were then filtered and cultured as above. Concentrations are presented as log CFU/100 mL for water samples or log CFU/100 g wet weight substrate for sediment and SAV samples. After counting, well-isolated colonies were picked from the mEI agar (up to a maximum

of 32 isolates were saved each month for all substrates at all sites) and cultured overnight in Enterococcosel broth (EB, Difco Laboratories, enc.) at 37° to confirm esculin hydrolysis. Glycerol was added (10% v/v) to cultures prior to storage at -80° C for later genetic typing.

Genetic Typing

Due to time and cost constraints only a subset of the isolates were genetically typed to investigate population structure. Four sites (large stream, river, lake, and upper bay) were chosen for the comparison and isolates from all substrates in one sample from each season (January, April, July, and October) were selected for BOX-PCR fingerprinting. Initially, up to 16 isolates were typed from each chosen sample, and for those sites with higher diversity ($S > N/2$; S = number of unique strains, and N = number of isolates) all 32 isolates were analyzed. Cryopreserved isolates were streaked onto tryptic soy agar to ensure isolation of a pure culture. Isolates were then grown overnight in 1 mL of brain heart infusion broth (BHI, Difco Laboratories) at 37° C and DNA was extracted using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich) per the manufacturer's instructions for Gram-positive bacteria.

The DNA of each isolate was typed by repetitive extragenic palindromic (REP) PCR fingerprinting using the BOX A2R primer (5'-ACG TGG TTT GAA GAG ATT TTC G-3') (Koeuth *et al*., 1995). 25 µL PCR reactions contained 5 µL of 5 × Gitschier Buffer (Kogan *et al*., 1987), 2.5 µL of 10% dimethyl sulfoxide, 0.4 µL bovine serum albumin

(10 mg/mL), 2.0 µL 10mM dNTPs, 1.0 µL *Taq* polymerase (5000 u/mL), 10.6 µL water, 1.5 µL 10 µM BOXA2R primer; and 2.0 µL of DNA template, containing between 10 and 40 ng/µL of DNA (Versalovic et al., 1991; Malathum et al., 1998). The PCR program included (1) initial denaturation at 95º C for 7min; (2) 35 cycles of 90º C for 30 s, 40º C for 1 min, and 65º C for 8 min; and (3) final extension at 65º C for 16 min. The amplicons were separated on a 1.5 % agarose gel (90 watts for 4 hrs), stained with ethidium bromide (1% solution) and imaged under UV light. Banding patterns were analyzed for similarity with BioNumerics 4.0 software (Applied Maths, Inc., Belgium) and confirmed by eye. Similarity was determined from Pearson correlations based upon the densiometric curves (optimization = 1%) for each genetic type and a dendogram was constructed via UPGMA. Identical reactions of a control strain maintained a similarity of 84%, which was used as a critical value to establish which environmental strains were similar enough to be considered identical, and the results were confirmed by eye.

Taxonomic Identification of Isolates

The species of the fourteen most abundant BOX-PCR genotypes (see results) were identified by sequencing a 1-1.2 kb section of the 16S rRNA gene. The gene was amplified from extracted DNA via PCR using the universal bacterial primers ECO8F (5'-AGA GTT TGA TCM TGG CTC AG – 3') and ECO1492RC (5'-GGT TAC CTT GTT ACG ACT T-3') (Lane 1991). Fifty microliter PCR reactions contained 25 µL JumpStart Taq polymerase (Sigma, USA), 2.5 µL each primer, 5 µL water, and 5 µL DNA template. The amplification process included (1) initial denaturation at 94º C for 5min; and (2) 20

cycles of 94º C for 1 min, 55º C for 1 min, and 72º C for 10 min. Amplicons were frozen and sent to a commercial laboratory for sequencing (Macrogen, Inc., USA). Because the results of the sequencing did not offer resolution between the highly similar *Ent. casseliflavus* and *Ent. gallinarum* species, colonies were examined for the yellow pigmentation that is characteristic of *Ent. casseliflavus*.

Calculations and Statistical Analysis

All CFU data were transformed as $\log_{10}(x + 1)$ to meet normality requirements prior to statistical analysis. Percentage data were transformed as arcsin-square root (x) to meet normality requirements. Values were compared statistically using three-factor ANOVA comparing site, substrate, and season (SPSS, version 17.0, SPSS, Inc., USA; $\alpha = 0.05$). Accumulation curves and rarefaction estimates were conducted using ECOSIM software (Gotelli and Entsminger, 2009). Similarities in clonal structure were compared via ordination. Non-metric multidimensional scaling diagrams were constructed based upon sample by sample matrices of Bray-curtis distances for all samples divided by substrate and each sample of site and season with all substrates combined for comparison (*PRIMER-E* with *PERMANOVA+*, Ivybridge, UK).

Results

Enterococci Densities

Mean enterococci densities, when examined over all months and averaged across all sites, were significantly higher on SAV (2.5×10^3 CFU / 100g) than sediments (1.0×10^3

CFU/100 g), which were in turn significantly higher than those found in water (1.3 x 10^2 CFU/100 mL) (*Table 3* and *Figure 12*; three-way ANOVA, $F = 51.7$, $p < 0.001$). When examined individually by site, densities on both sediments and SAV were significantly elevated over waterborne densities at all six sites (*Figure 12*; post-hoc paired t-tests, *p*-values ranging from 0.013 to < 0.001). At the freshwater sites, mean densities in SAV were also significantly higher than those found in sediments (*Figure 12*; post-hoc paired *t*-tests; *p*-values for the small stream, large stream, river, and lake sites were 0.002, 0.02, 0.03, and 0.05, respectively). At the estuarine sites, however, SAV densities were not significantly different from densities in the sediments (*Figure 12*; post-hoc paired *t*-tests; *p*-values for the upper and lower bay sites were 0.24, and 0.09, respectively). No significant differences were found when densities were compared across seasons with substrates combined in each site or examined individually (*Table 3* and *Figure 13*; three-factor ANOVA, $F = 1.6$, $p = 0.19$).

In addition, mean enterococci densities were also significantly affected by site (*Table 3* and *Figure 12*; three-factor ANOVA, $F = 12.4$, $p < 0.001$). Sites further up in the watershed (such as the stream and river sites) exhibited higher densities than those further downstream in the watershed (such as the bay sites). This was evident from examination of the homogenous subsets of sites generated from post-hoc comparisons (*Table 3*), as well as regression analysis comparing mean site densities against the distance between each site and the mouth of Tampa Bay (considered the most downstream exit from the watershed) (*Figure 14*; $p < 0.001$, $r^2 = 0.98$). When examined by substrate at each site,

Table 3. Results from a three-factor ANOVA showing significant differences in mean enterococci densities across the watershed between sites, matrices, and seasons. Overall model significance: $F = 263.2$; $p < 0.001$.

Factor	Mean	Std Dev	Std Error	*F*	*p*	Subsets
Site:				**12.39**	**<0.001**	
Small Stream	3.4	0.88	0.13			***a***
Large Stream	3.2	0.83	0.13			***a, b***
River	3.0	0.98	0.13			***b, c***
Lake	2.8	1.14	0.13			***b, c***
Upper bay	2.5	0.86	0.14			***c, d***
Lower bay	2.1	1.03	0.13			***d***
Substrate:				**51.77**	**<0.001**	
Water	2.1	0.79	0.09			***a***
Sediment	3.0	0.90	0.10			***b***
SAV	3.4	0.97	0.09			***c***
Season:				1.60	0.19	
Spring	2.7	1.06	0.11			*a*
Summer	3.0	0.93	0.11			*a*
Fall	2.8	0.96	0.11			*a*
Winter	2.9	1.17	0.11			*a*

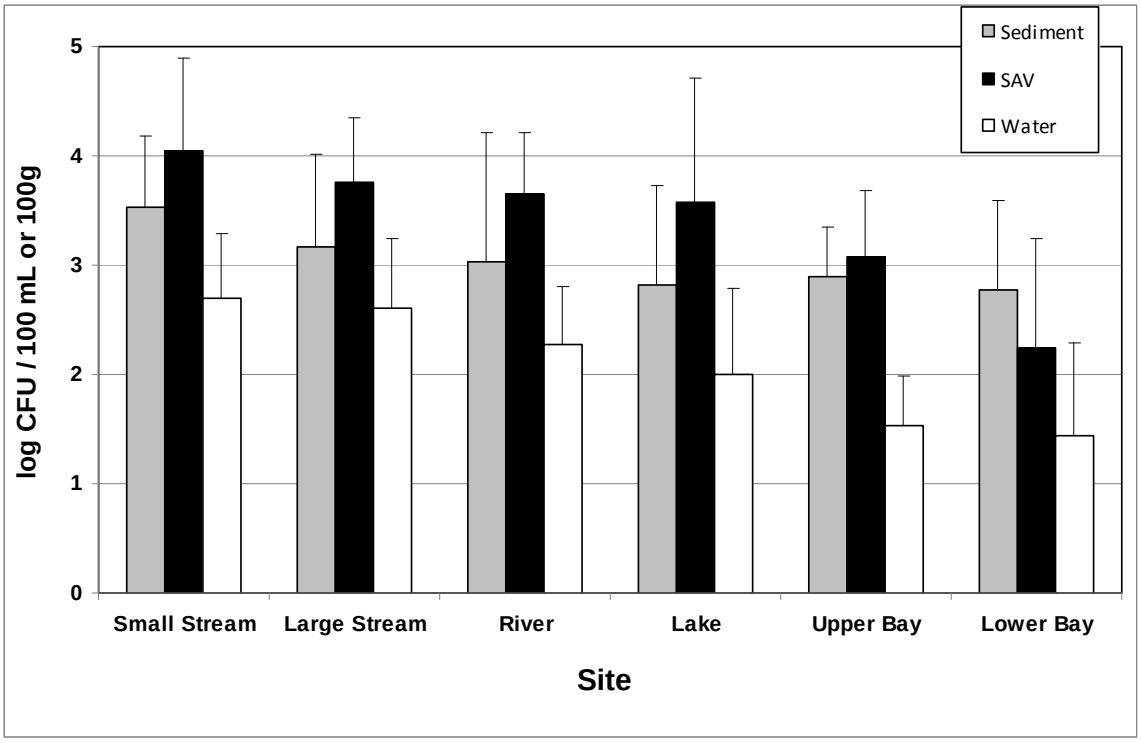

Figure 12. Mean densities of culturable enterococci from water, sediment, and SAV for each site over all sampling dates. (CFU = colony forming units; error bars indicate standard deviations; n=12)

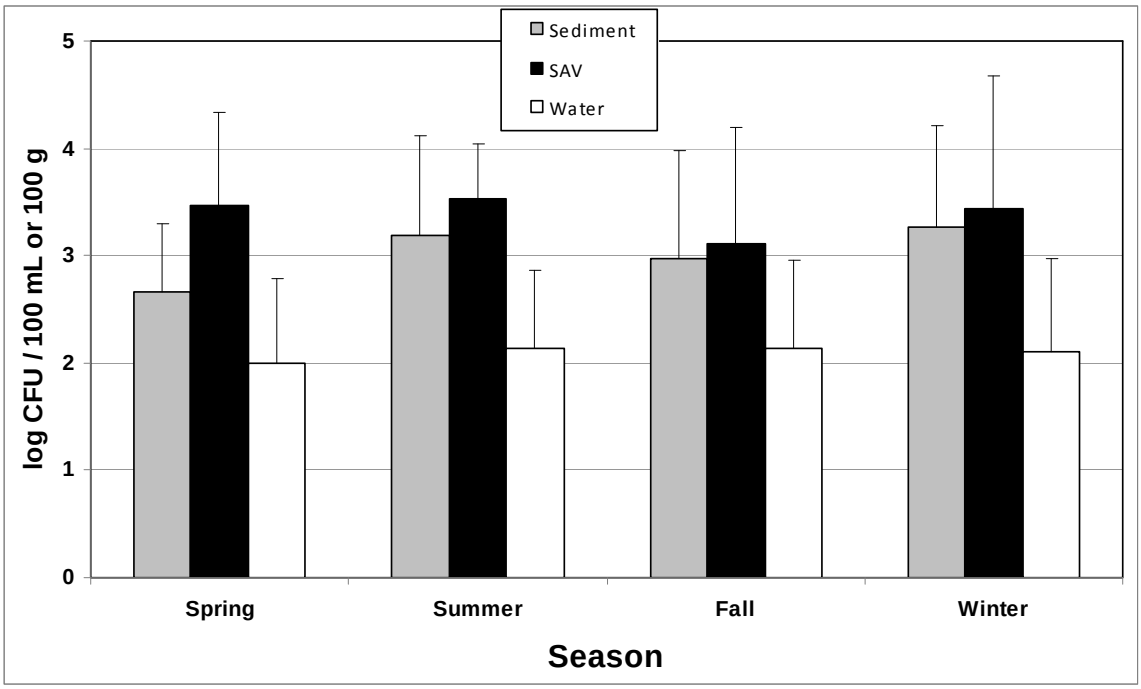

Figure 13. Mean densities of culturable enterococci from water, sediment, and SAV for all sites grouped by each season (CFU = colony forming units; error bars indicate standard deviations; n=18)

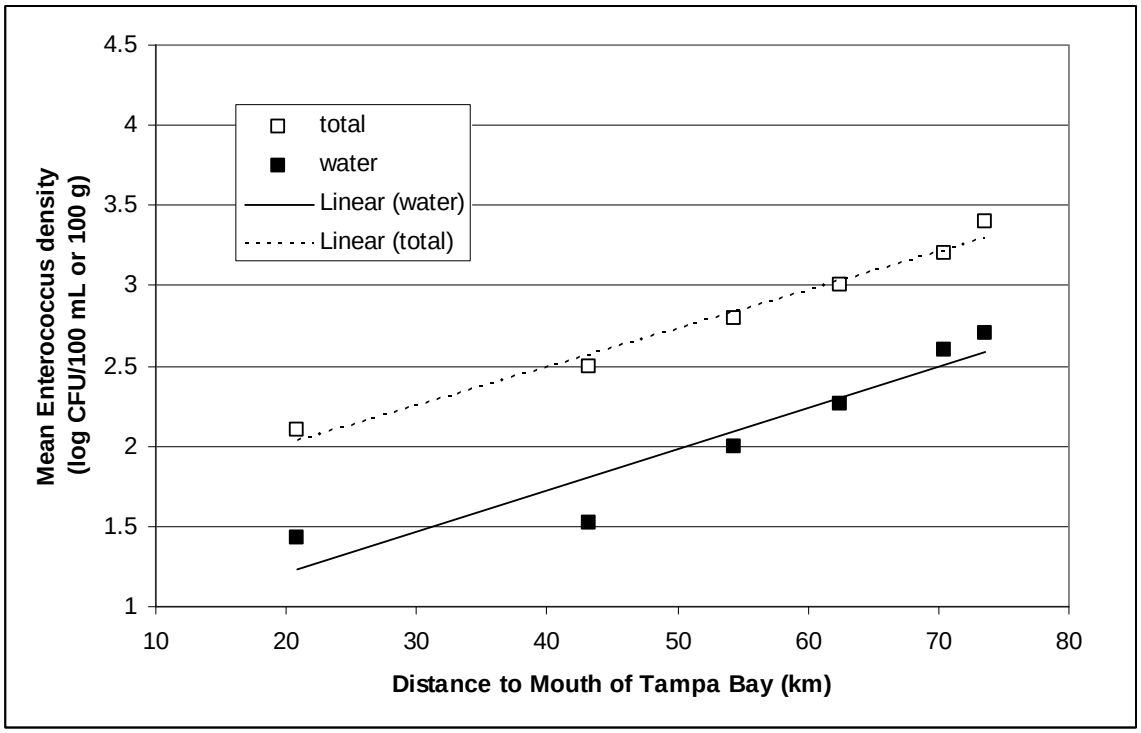

Figure 14. Mean enterococci densities in all substrates combined (total) and in the water at each site compared to direct-line distance of the site from the mouth of Tampa Bay. Regression for total: $F = 197.4$; $p < 0.001$; $r^2 = 0.98$. Regression for water: $F = 33.3$; $p = 0.004$; $r^2 = 0.89$.

the same effect was true for water (*Figure 14*; $p = 0.004$, $r^2 = 0.89$) and SAV (Figure 15; $p < 0.001$, $r^2 = 0.97$), but not for sediments (Figure 15; $p = 0.06$, $r^2 = 0.63$).

Clonal Structure

Clonal structure was highly variable among all samples, regardless of site, substrate, or season. Accumulation curves are shown for individual samples at the river site as an example and illustrate a wide range of values for strain richness across seasons and substrates (*Figure 16*). For individual samples, values for the Shannon-Wiener diversity index ranged from 0 to 2.8, and richness estimates for samples that could be rarified to $N = 14$ ranged from 1 to 13 strains (*Table 4*). When all three substrates were combined for each site, every site displayed a range of diversity throughout the year, as evidence by accumulation curves (*Figure 17*-*Figure* 20). In these samples with substrates combined for each site, values for the Shannon-Weiner diversity index ranged from 0.2 to 3.4, and richness estimates for samples that could be rarified to $N = 35$ ranged from 3 to 26 strains (*Table 5*). However, the patterns were not similar. For example, at the river and upper bay sites, the winter sample showed the highest diversity, whereas fall was the highest at the lake site and summer was the highest at the small stream site. As a result, there were no significant effects of site, season, or substrate in determining the strain richness of a given site, as shown by a three-factor ANOVA conducted on sample richness values rarefied to $N = 14$ ($F = 1.09$; $p = 0.41$).

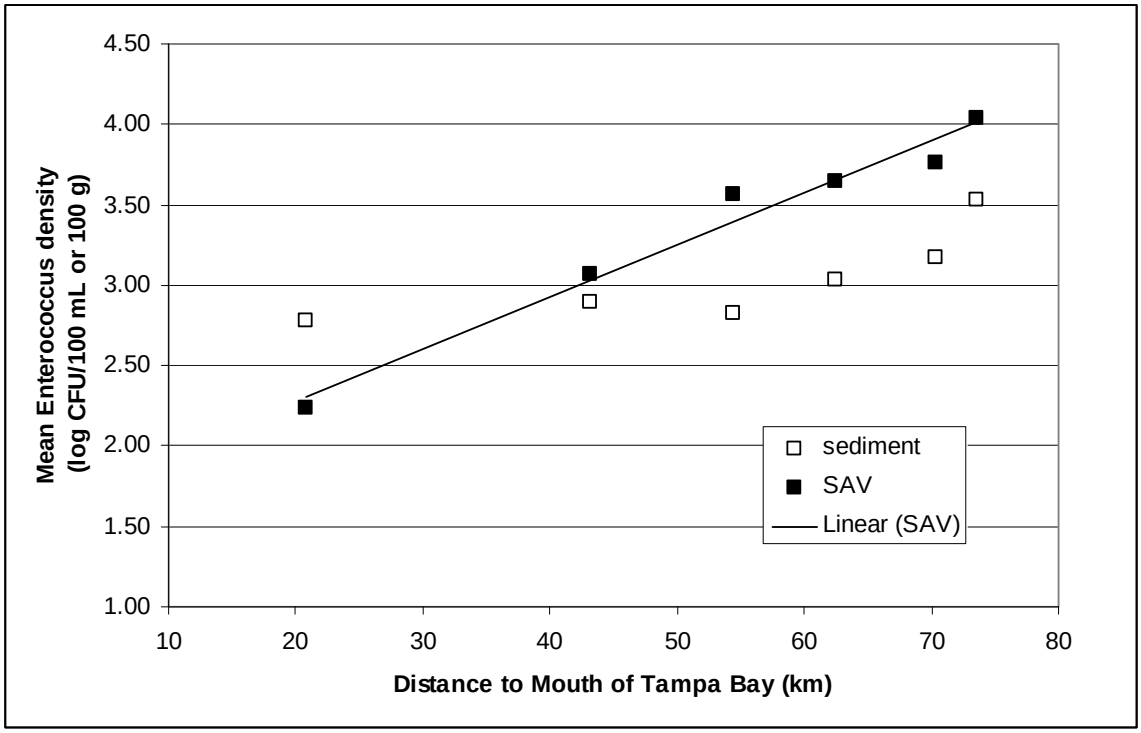

Figure 15. Mean enterococci densities in sediment and SAV at each site compared to direct-line distance of the site from the mouth of Tampa Bay. Regression for sediment: $F = 6.8$; $p = 0.06$; $r^2 = 0.63$. Regression for SAV: $F = 136.2$; $p < 0.001$; $r^2 = 0.97$.

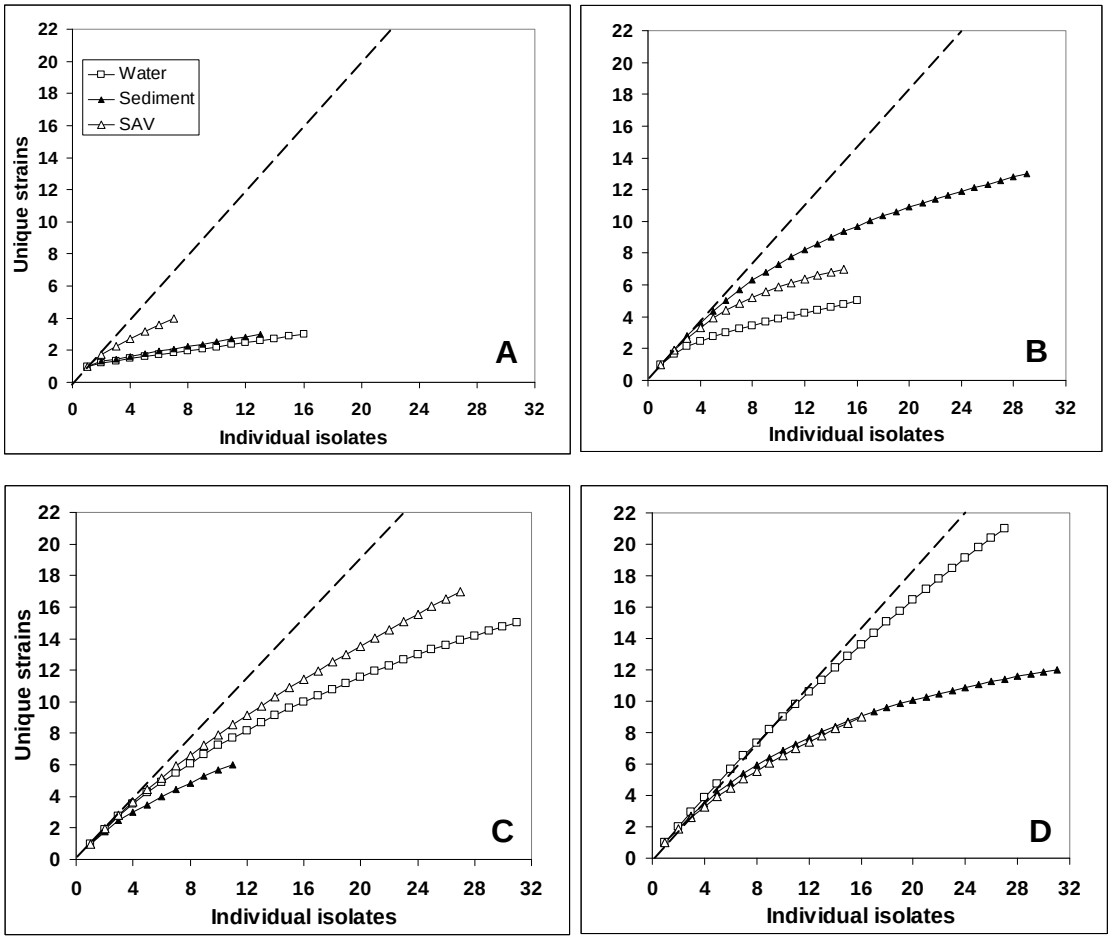

Figure 16. Accumulation curves constructed for unique strains found in each matrix at the river site during the (A) spring, (B) summer, (C) fall, and (D) winter season. The dashed line represents the unity line.

Table 4. Measures of *Enterococcus* strain diversity in each sample of site, season, and substrate. *N* = number of isolates typed; *S* = number of strains observed; % unique = number of strains unique to that sample; Rarefaction (14) = estimated strains after rarefaction to 14 isolates (asterisk indicates less than 14 isolates originally typed for that sample); *J'* = Simpson's diversity index; *H'* = Shannon-Weiner diversity index ($\log_e$).

Site	Season	Substrate	*N*	*S*	% unique	Rarefaction (14)	*J'*	*H'*
Large	Fall	Sediment	13	7	62	*	0.89	1.73
Stream		SAV	10	5	40	*	0.84	1.36
		Water	14	8	57	8.0	0.92	1.91
	Spring	Sediment	7	7	43	*	1.00	1.95
		SAV	15	11	33	10.3	0.91	2.17
		Water	30	6	93	4.1	0.70	1.25
	Summer	Sediment	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		SAV	27	21	48	12.1	0.96	2.94
		Water	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Winter	Sediment	16	3	94	2.8	0.42	0.46
		SAV	16	6	81	5.4	0.63	1.12
		Water	30	19	57	11.3	0.96	2.83
River	Fall	Sediment	11	6	100	*	0.86	1.54
		SAV	27	17	41	10.3	0.92	2.60
		Water	31	15	42	9.2	0.90	2.43
	Spring	Sediment	13	3	0	3.0	0.49	0.54
		SAV	7	4	14	*	0.83	1.15
		Water	16	3	0	2.8	0.42	0.46
	Summer	Sediment	29	13	52	9.0	0.93	2.38
		SAV	15	7	53	6.8	0.93	1.81
		Water	16	5	31	4.6	0.74	1.19
	Winter	Sediment	31	12	65	8.4	0.91	2.27
		SAV	16	9	25	8.2	0.88	1.93
		Water	27	21	41	12.1	0.96	2.94
Lake	Fall	Sediment	15	10	93	9.5	0.94	2.15
		SAV	10	6	40	*	0.84	1.50
		Water	16	15	69	13.2	0.99	2.69
	Spring	Sediment	15	8	33	7.8	0.96	1.99
		SAV	10	5	10	*	0.91	1.47
		Water	13	5	8	*	0.91	1.46
	Summer	Sediment	14	1	0	1.0	0.00	0.00
		SAV	14	2	7	2.0	0.37	0.26
		Water	14	2	0	2.0	0.37	0.26

(cont.)

Table 4 (cont.)

Site	Season	Matrix	*N*	*S*	% unique	Rarefaction (14)	*J′*	*H′*
Lake	Winter	Sediment	29	12	79	8.6	0.93	2.32
		SAV	15	1	0	1.0	0.00	0.00
		Water	12	11	75	*	0.99	2.37
Upper bay	Fall	Sediment	9	7	89	*	0.97	1.89
		SAV	16	8	50	7.3	0.82	1.72
		Water	16	6	0	5.6	0.84	1.51
	Spring	Sediment	5	4	60	*	0.96	1.33
		SAV	30	21	60	10.6	0.89	2.70
		Water	15	4	7	3.8	0.52	0.72
	Summer	Sediment	14	3	100	*	0.60	0.66
		SAV	15	6	13	5.7	0.80	1.43
		Water	16	3	6	2.8	0.42	0.46
	Winter	Sediment	27	23	81	12.8	0.98	3.07
		SAV	7	4	57	*	0.92	1.28
		Water	33	18	42	8.9	0.82	2.38

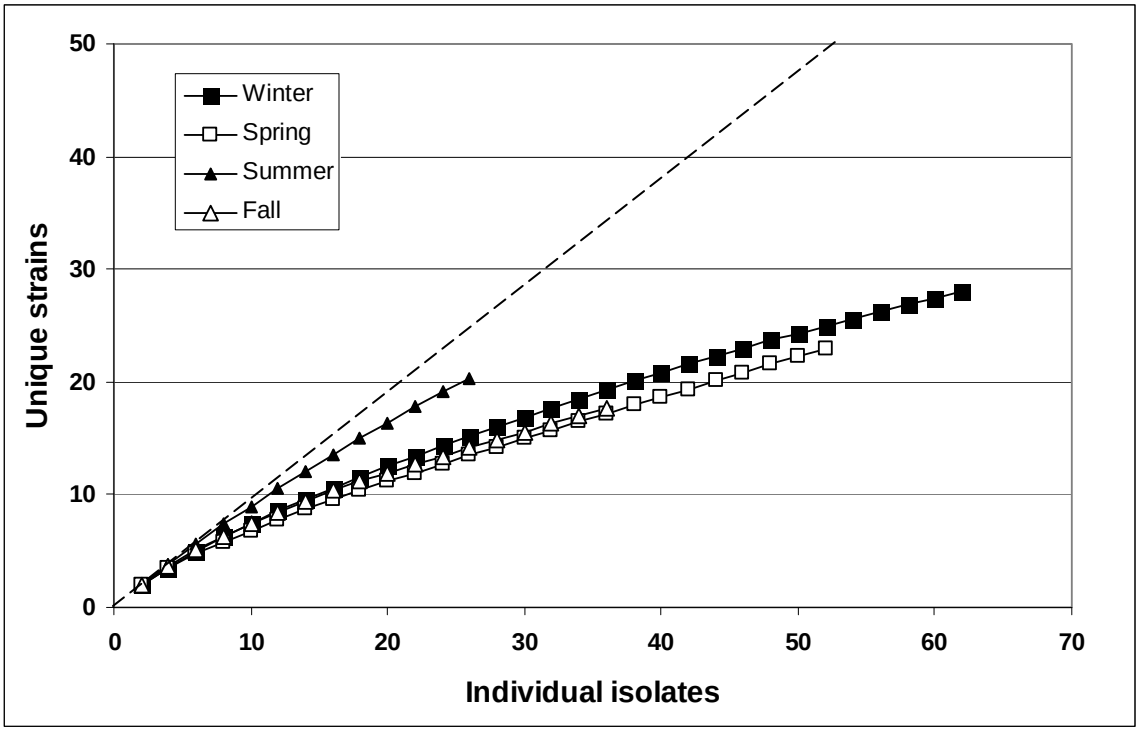

Figure 17. Accumulation curves constructed for unique strains found in all three matrices (water + sediment + SAV) for each season at the large stream site. The dashed line represents the unity line.

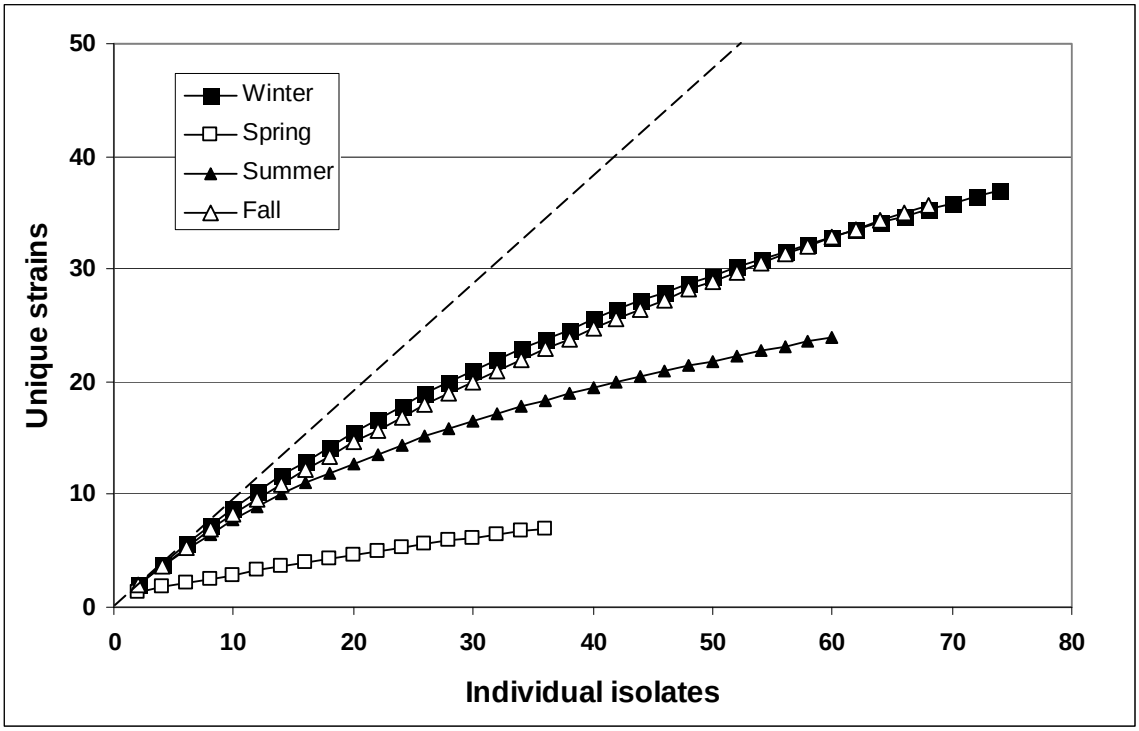

Figure 18. Accumulation curves constructed for unique strains found in all three matrices (water + sediment + SAV) for each season at the river site. The dashed line represents the unity line.

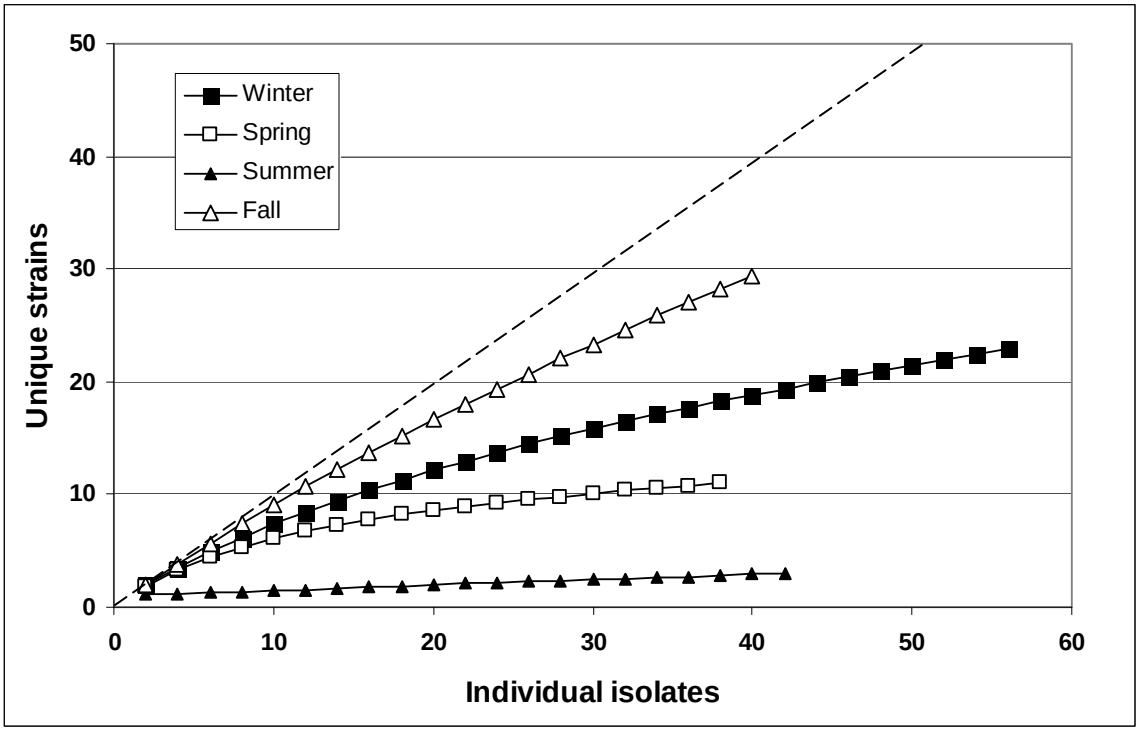

Figure 19. Accumulation curves constructed for unique strains found in all three matrices (water + sediment + SAV) for each season at the lake site. The dashed line represents the unity line.

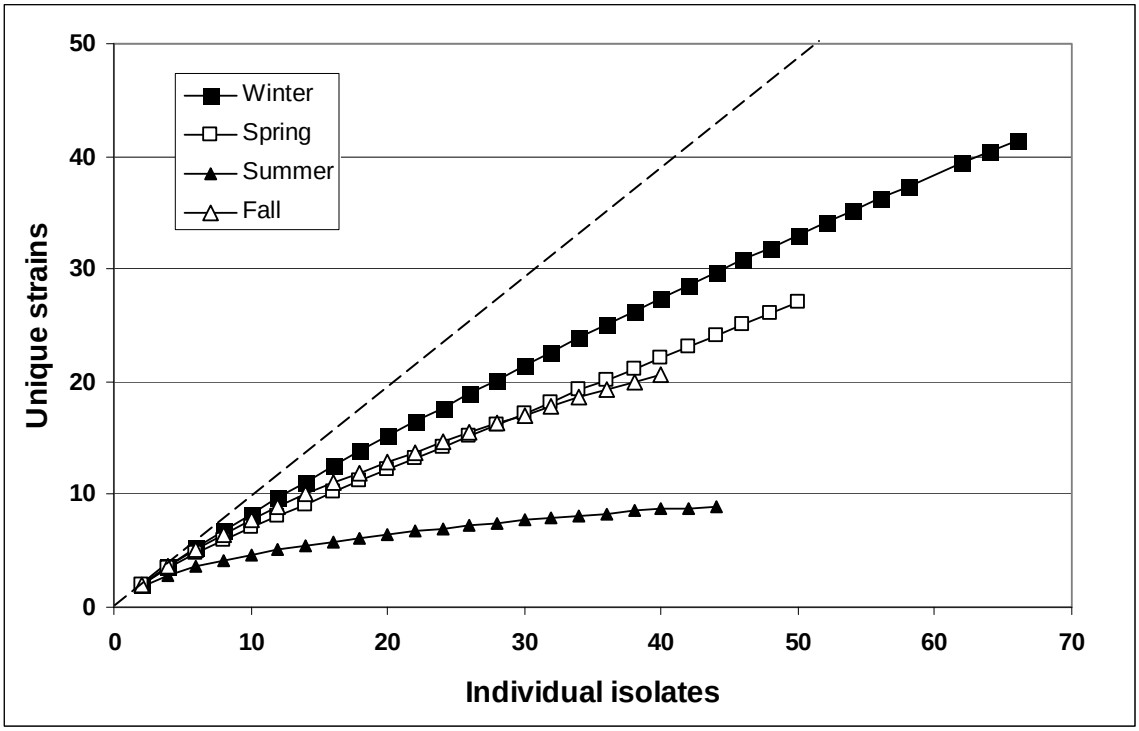

Figure 20. Accumulation curves constructed for unique strains found in all three matrices (water + sediment + SAV) for each season at the upper bay site. The dashed line represents the unity line.

Table 5. Measures of *Enterococcus* strain diversity in each sample of site and season with all three substrates combined (water + sediment + SAV). *N* = number of isolates typed; *S* = number of strains observed; % unique = number of strains unique to that sample; Rarefaction (35) = estimated strains after rarefaction to 15 isolates; *J'* = Simpson's diversity index; *H'* = Shannon-Weiner diversity index ($\log_e$).

Site	**Season**	***N***	***S***	**% unique**	**Rarefaction (35)**	***J'***	***H'***
Large stream	Fall	37	18	54	17.3	0.89	2.58
	Spring	52	23	69	16.8	0.80	2.52
	Summer	27	21	48	21.0	0.96	2.94
	Winter	62	28	73	18.9	0.85	2.83
River	Fall	69	36	51	22.4	0.90	3.21
	Spring	36	7	3	6.9	0.43	0.83
	Summer	60	24	47	18.1	0.89	2.84
	Winter	74	37	47	23.3	0.93	3.37
Lake	Fall	41	30	71	26.4	0.95	3.25
	Spring	38	11	18	10.7	0.87	2.10
	Summer	42	3	2	2.7	0.20	0.22
	Winter	56	23	57	17.4	0.86	2.70
Upper bay	Fall	41	21	39	18.9	0.90	2.74
	Spring	50	27	44	19.7	0.81	2.68
	Summer	45	9	38	8.2	0.76	1.67
	Winter	67	42	60	24.5	0.88	3.31

Dominant Strains and Species

Among all samples, 277 individual strains were identified. Of these, 227 were found in only a single sample (i.e., not found in samples from any other sites, substrates, or seasons). Of the 227 unique strains, the vast majority of them (199) were found as singletons or doubletons. There were a few strains, however, that occurred in higher numbers even if only in one sample. For example, five of the unique strains were highly dominant (recovered more than 10 times) in the sample in which they were found (four samples from the large stream and one from the upper bay). The percentage of unique strains in a given sample (those not found in any other sample) was also highly variable, and was found to range from 0 to 100% across all samples, or from 3 to 73% when the samples were grouped by site (*Table 4 and Table* 5). The percent uniqueness of a sample did vary significantly between the substrates (*Table 6*). On average, sediment samples had a significantly higher percentage of unique strains (65%) than did samples of water or SAV (29% and 33% respectively) (three-way ANOVA on arcsin-square root transformed data; $F = 4.76$, $p = 0.014$.) In addition, the effect of season on percent uniqueness was very nearly significant, driven by higher average percentages of unique strains found in samples from fall and winter (58% and 57% respectively) than in samples from spring and summer (25% and 31% respectively) (three-factor ANOVA on arcsin-square root transformed data; $F = 2.81$, $p = 0.053$).

Overall, however, the large number of unique strains among the samples made the overall similarities between the sites relatively low, and so few, if any patterns, could be

Table 6. Results from a three-factor ANOVA showing significant differences in percentage of strains in each sample that are unique between sites, matrices, and seasons. Overall model significance: F = 3.02; p = 0.01. Data were arcsin-square root transformed prior to analysis, and are back-transformed here.

Factor	Mean	Std Dev	Std Error	*F*	*p*	Subsets
Site:				1.83	0.16	
Large Stream	61	6.2	1.4			*a*
River	36	16.5	1.1			*a*
Lake	27	20.6	1.1			*a*
Upper bay	46	19.4	1.1			*a*
Substrate:				**4.76**	**0.014**	
Water	29	16.3	0.9			***a***
Sediment	65	21.0	0.9			***b***
SAV	33	7.8	0.8			***a***
Season:				**2.81**	**0.053**	
Spring	25.0	14.4	1.1			*a*
Summer	31.2	20.9	1.4			*a*
Fall	58.1	14.8	1.1			*a*
Winter	57.1	11.8	1.1			*a*

discerned. Using non-metric multidimensional scaling, no recognizable groupings of similar clonal structure were observed among the samples with respect to substrate or season (data not shown). There was some slight grouping of clonal structure according to site (*Figure 21* and *Figure 22*), particularly at the river and upper bay sites, which separated slightly. However, the relatively high stress values associated with these analyses make this interpretation weakly supported at best. Among the fifty strains that were found in more than one sample, 14 were found in four or more different samples, and were found on two or more substrates, at two or more sites, and in two or more seasons, suggesting at least some degree of a cosmopolitan nature (*Table 7*). Results of 16s sequencing show that three of these strains are *Ent. casseliflavus*, three are *Ent. faecalis*, three are *Ent. faecium*, three are *Ent. hirae*, and two are *Ent. mundtii*. Beyond these fourteen most abundant strains, nine strains were found in three or more samples, and the remaining 27 strains were found in only two samples. Three strains in particular were found in at least 10 (>20%) samples and appear to represent extremely widespread strains. Strains J35 (*Ent. faecium*) and J8 (*Ent. mundtii*) were particularly abundant, accounting for 7.4% and 9.0% of all isolates, respectively. And although J45 (*Ent. hirae*) was somewhat less abundant (2.3% of isolates), it was the only strain to be found at least once on all three substrates, at each site, and in each season.

Discussion

While a significant number of studies have employed field sampling to investigate enterococci densities in environmental waters, sediments, and SAV, this work is the first

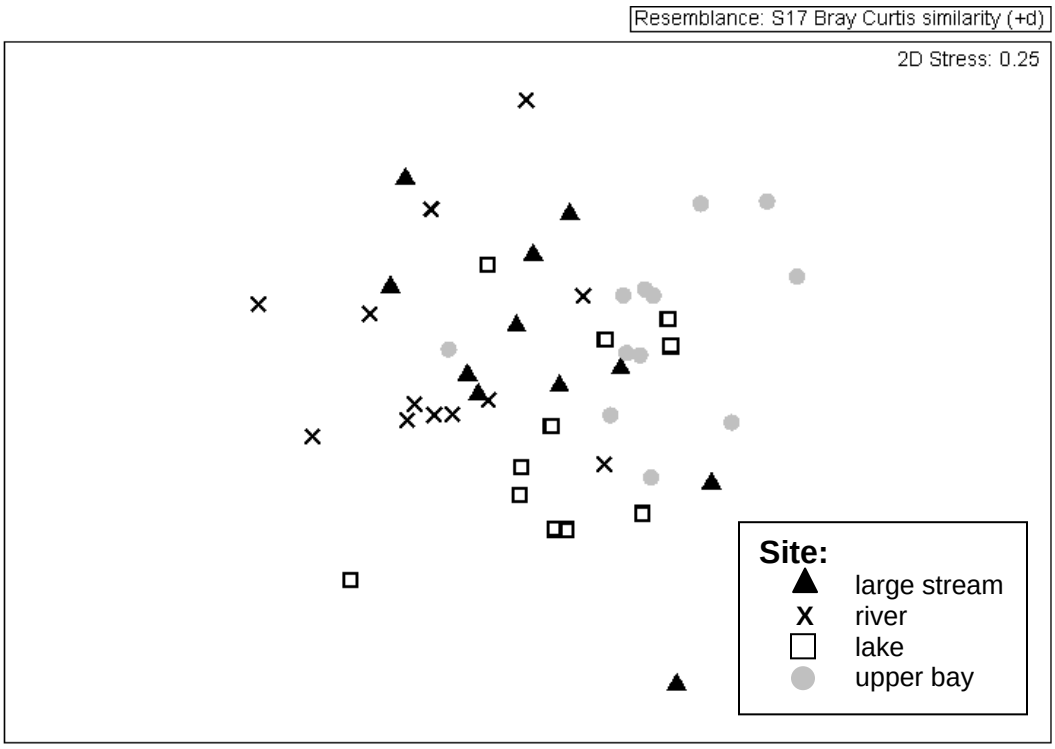

Figure 21. Non-metric multidimensional scaling analysis of similarities in clonal structures from each sample (water, sediment, and SAV plotted separately for each site in each season). Symbols are coded by site. Similarities are based upon the resemblance matrix of Bray Curtis distances.

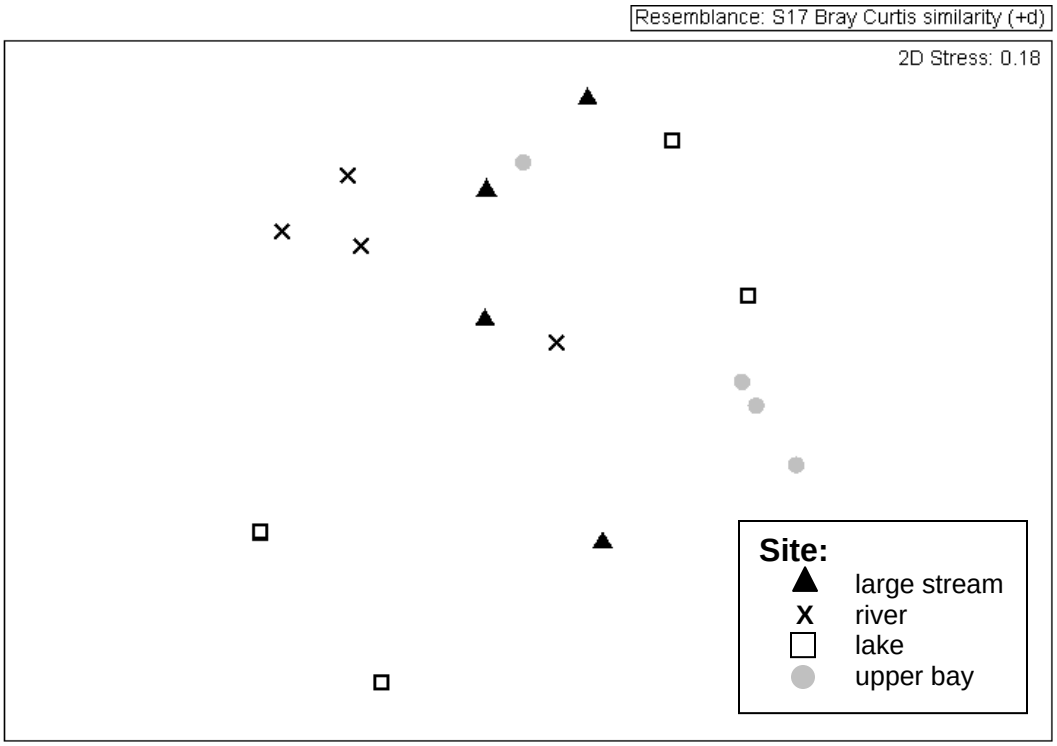

Figure 22. Non-metric multidimensional scaling analysis of similarities in clonal structures from each site (water, sediment, and SAV are combined and plotted for each site in each season). Symbols are coded by site. Similarities are based upon the resemblance matrix of Bray Curtis distances.

Table 7. List of strains that occurred in four or more samples, with species results from 16s sequencing and a summary of their distribution among the samples. Strains are presented in order of descending number of samples in which they were found. Isolates = number of isolates of each strain recovered and percent of total isolates typed; Samples = number of samples in which the strain was found and the percent of total samples typed.

Strain	**Species**	**Isolates**		**Samples**		**Seasons**	**Sites**	**Substrates**
		n	**%**	**n**	**%**			
J35	*faecium*	59	7.4	13	28.3	3	4	3
J8	*mundtii*	71	9.0	11	23.9	4	3	3
J45	*hirae*	18	2.3	10	21.7	4	4	3
J41	*faecium*	12	1.5	6	13.0	4	3	3
J43	*hirae*	7	0.9	6	13.0	3	3	3
J3	*casseliflavus*	8	1.0	5	10.9	3	3	2
J18	*faecalis*	6	0.8	5	10.9	3	2	2
J4	*casseliflavus*	10	1.3	4	8.7	3	2	3
J9	*mundtii*	8	1.0	4	8.7	2	3	2
J20	*faecalis*	8	1.0	4	8.7	3	3	3
J6	*faecalis*	5	0.6	4	8.7	3	2	3
J31	*casseliflavus*	5	0.6	4	8.7	3	2	2
J42	*faecium*	4	0.5	4	8.7	2	2	2
J49	*hirae*	4	0.5	4	8.7	3	3	2

to directly compare all three matrices via simultaneous measurements across a watershed. In terms of clonal structure, much of the previous work has been conducted solely on *E. coli* in a limited geographic range (mostly in the US Great Lakes region), and this work provides much needed data on clonal structure in *Enterococcus*, from a variety of water bodies and substrates in a distinctly different watershed. Several important conclusions are evident from this study. Firstly, on average across the watershed, SAV harbored the highest densities of enterococci, followed by sediments and then the water column. Secondly, mean enterococci densities changed significantly as a function of relative location within the watershed; the highest densities occurred at the sites furthest upstream in the watershed, and decreased as the location of the site moved further downstream in the watershed (i.e., closer to the mouth of Tampa Bay). Thirdly, clonal richness and diversity of *Enterococcus* populations varied widely and grouped slightly by site, but with no clear relationships to substrate or season. And finally, several strains were recovered from multiple samples, with three strains in particular (one each of *Ent. faecium*, *Ent. mundtii*, and *Ent. hirae*) that were highly abundant and cosmopolitan.

While elevated densities of FIB have been previously observed in sediments (Solo-Gabriele et al., 2000; Craig et al., 2004; Anderson et al., 2005) and SAV (Anderson et al., 1997; Whitman et al., 2003; Ksoll et al., 2007) relative to the water column, this study is the first to directly compare all three substrates simultaneously. Averaged across the watershed, SAV harbored significantly higher densities of enterococci than sediment or water, suggesting that it serves as a highly suitable substrate for the persistence of

enterococci in these habitats. Theoretically, this is not surprising that enterococci introduced into the water column would associate with SAV, given the intimate contact between SAV and the water column as well as the increased access to resources and the protection from UV radiation. In *E. coli*, rapid association with SAV has been observed in mesocosms (Englebert et al., 2008; Kleinheinz et al., 2009), and Byappanahali *et al.* (2003b) observed growth of E. coli on algal exudate, offering additional evidence of its suitability as a substrate for persistence and possible growth. There is some evidence in this study that this trend may not be as strong in the estuarine sites as in the freshwater sites, as the mean enterococci densities in sediments were nearly equal to SAV in the upper bay site and greater than SAV in the lower bay site. Possible mechanisms for this difference include the effects of salinity or differences between marine and freshwater aquatic plants, but the data in this study do not offer any conclusions on these theories. Regardless, in highly vegetated sites, it is clear that SAV can be an important matrix for the persistence of water quality FIB. Furthermore, as the major bulk of work investigating this phenomenon has been limited geographically to the Great lakes region of the United States (Byappanahalli et al., 2003b; Whitman et al., 2003; Ksoll et al., 2007; Englebert et al., 2008; Kleinheinz et al., 2009), these data help extend these findings to a broader scale and to a wider variety of habitats.

Across the watershed, sites further upstream had significantly higher mean enterococci densities (all substrates and seasons combined) than those downstream (i.e., closer to the mouth of Tampa Bay). Although a variety of studies have examined densities of FIB in a

variety of habitats in the typical watershed, including soils (Hardina and Fujioka, 1991; Desmarais et al., 2002), streams (Buckley et al., 1998; Byappanahalli et al., 2003a), rivers (Tunnicliff and Brickler, 1984; Obiri-Danso and Jones, 1999), lakes (Doyle et al., 1992; Whitman and Nevers, 2003), estuaries (Shiaris et al., 1987; Solo-Gabriele et al., 2000), and open ocean beaches (Anderson et al., 1997; Boehm, 2007), very few have simultaneously monitored multiple habitat types throughout a watershed. Roll and Fujioka (1997) investigated the potential for surrounding soils to serve as a non-point source of FIB in freshwater streams. There also have been a few studies on smaller watersheds (e.g., coastal streams with outfalls near a recreational beach) that have taken a watershed approach and found higher densities of FIB in upstream or downstream sites (Byappanahalli et al., 2003a; Boehm et al., 2004; Whitman et al., 2006). In one of these studies, Byappanahali *et al.* (2003a) observed an opposite trend, with waterborne *E. coli* concentrations generally increasing as stream order increased. Steets and Holden (2003) used a modeling approach to compare a coastal wetland to a nearby beach, concluding that elevated densities in the wetland constituted a source of contamination for the beach.

In this study, however, by covering such a wide diversity of habitat types and substrates over such a broad of a geographic range, I was able to observe a strikingly consistent increase in mean enterococci densities as sites are situated upstream in the watershed, regardless of substrate. Although I used distance from a fixed point in the watershed (i.e., the mouth of Tampa Bay) as a determination of location in the watershed, this is only a convenient proxy for watershed location. The true causes of the relationship are

most likely to be other factors that vary along the same gradient. For example, sites further upstream in a watershed tend to have of much smaller ratio of water volume to shoreline influence, which allows little potential for dilution and makes these waters the most intimately connected and easily affect by terrestrial sources of FIB. Furthermore, the small, narrow waterways typical of the upstream sites tend to have more shading from riparian vegetation, offering protection from harmful UV radiation, which has been shown to quickly inactivate waterborne FIB (Davies and Evison, 1991; Muela et al., 2000; Sinton et al., 2002; Schultz-Fademrecht et al., 2008).

In terms of population structure, there seems to be little association between the clonal diversity or structure of the *Enterococcus* populations sampled in this study and cofactors such as the season or substrate from which the sample was taken. Diversity was highly variable, from almost zero in some samples to Shannon-Weiner diversity values of just below three, which is similar to values found in another study of enterococci in Florida (Brownell et al., 2007), but much lower than values observed for *E. coli* in lake sediments and algae (McLellan, 2004; Byappanahalli et al., 2007). While some of these differences may be due to differences between the two taxonomic groups, they are also likely a function of sampling effort. In this study, typing effort at the individual sample level was limited to allow for increased sample coverage, and it is clear from the accumulation curves that considerable increases in richness and diversity could be expected in some of the samples from increased sampling effort. Therefore, it is impossible to say whether

clonal diversity was actually significantly lower than that found by McLellan (2004) and Byappanahali et al. (2007).

Ordination showed a slight tendency for some of the sites, particularly the river and upper bay sites, to cluster separately based upon clonal structure. Environmental *E. coli* isolates were found to group very strongly by site and matrix in a study of temperate soils and lake water (Ishii et al., 2006), and by year in sampled patches of *Cladophora* algae (Byappanahalli et al., 2007). In both of these studies, groupings of clonal structure were much more highly separated, but this is likely due to the fact that these groupings resulted from principal component analyses based upon the densiometric curves of individual isolate fingerprints. In contrast, the NMDS analyses in this study are based upon Bray-Curtis distances of the overall clonal structure in each sample, which is entirely dependent on the presence and absence of each strain, and does not account for high degrees of relatedness between two different strains. Kinzelman *et al.* (2004) found very little clustering of *E. coli* in Lake Michigan between sites or substrates and concluded that accumulation of strains, rather than long-term persistence and replication of particular strains were the main cause of the observed population structure. In this study, given the wide range of diversity values and the broad spatial and temporal coverage, it may be that samples represented a mix of some population structures that are dominated by recently accumulated strains and others that represent long-term, persistent strains.

A fair number of strains were observed that were fairly cosmopolitan in nature and may represent naturalized strains that are widespread in the environment. The species mixture of these strains, including *Ent. casseliflavus*, *faecalis*, *faecium*, *hirae*, and *mundtii* are very similar to other studies of species structure of environmental enterococci (Pinto et al., 1999; Harwood et al., 2004; Ferguson et al., 2005). The species indicate a mix of those typically associated with humans (*faecalis* and *faecium*) and those that are typically considered more environmental in origin (*casseliflavus* and *mundtii*). The relatively high abundance of the 'environmental' species of *Enterococcus* compared to that found in human sewage adds weight to the theory that many of these environmental enterococci can not be traced to recent influxes of fecal contamination into the environment. Three strains (J35, J8, and J45) were particularly abundant and cosmopolitan compared to all the others, and appear likely to be naturalized strains that are persisting throughout the watershed. The recovery of these strains from such a wide variety of sites, substrates, and seasons was highly surprising and indicates the need for increased research into the ecology of persistent FIB. Understanding the factors that allow these strains to persist and probably replicate in the environment will greatly improve our ability to discriminate between instances where bacteria such as E. coli and the enterococci are acting reliably as indicators of fecal pollution and instances where they are merely abundant in the environment and other measures of human health risks must be employed.

CHAPTER FOUR

INVESTIGATING THE IMPORTANCE OF SEDIMENT AND SUBMERGED AQUATIC VEGETATION AS ENVIRONMENTAL RESERVOIRS FOR WATER QUALITY INDICATOR BACTERIA

Introduction

Fecal contamination in natural water bodies that are used for shell fishing and recreation poses a significant risk to public health. It is well established that swimmers and bathers who use beaches, lakes, and rivers with known sewage contamination are at a higher risk for gastrointestinal and respiratory illnesses as well as skin, ear and eye infections (Cabelli et al., 1982; Cheung et al., 1990; Rees et al., 1998; Wade et al., 2003). The ongoing challenge has been the development of a suitable means of detecting fecal contamination, as well as quantifying its magnitude and extent, and then accurately gauging the health risks associated with contacting contaminated waters. Ideally, managing agencies would directly monitor for pathogens that are known to result from contamination by sewage or other fecal sources. In practice, however, directly monitoring water bodies for the presence of human pathogens is prohibitively difficult because there is such a wide diversity of potential pathogens (including viruses, bacteria, and protists), many are difficult and costly to culture, many have no reliable molecular

assays, and many have patchy distributions or continue to pose significant health risks at low concentrations (Field and Samadpour, 2007).

The historical approach to this problem has been the use of FIB. FIB include specific (or specific groups of) bacteria that, although they are not pathogenic themselves, reliably occur in high numbers in feces and sewage. Their presence in recreational waters, therefore, is used to indicate contamination by sewage or other fecal material and the likely presence of human pathogens. The main groups of FIB used today in developed areas such as the United States and the European Union include fecal coliforms, or a specific member of that group, *Escherichia coli*, in fresh water and the genus *Enterococcus* in estuarine and marine waters (USEPA, 1986, 2000; WHO, 2001). While these indicators have shown good correlation with sewage contamination and risks of waterborne illness (Wade et al., 2003; Zmirou et al., 2003), there are many assumptions that must hold true for the indicator concept to work optimally. While various published review articles provide extended discussion regarding these assumptions (Griffin et al., 2001; Field and Samadpour, 2007; Ishii and Sadowsky, 2008), one assumption is of particular importance with regard to this study: FIB must co-occur with human pathogens in order to accurately indicate a human health risk. Unfortunately, recent research has indicated that this assumption is often false. Many studies have shown that the presence of FIB do not correlate well with the presence of pathogens, including *Salmonella*, *Campylobacter*, *Cryptosporidium*, *Giardia*, or enteroviruses (Lund, 1996; Bonadonna et al., 2002; Lemarchand and Lebaron, 2003; Harwood et al., 2005).

There are many possible reasons for this lack of correlation, but one major problem is the assumption that, subsequent to sewage contamination in the environment, FIB will exhibit survival dynamics that are similar to those of the pathogens they are being used to detect. In truth, FIB – including coliforms, *E. coli*, and enterococci – are capable of persisting in a culturable form for extended periods in a wide variety of environmental matrices after their initial introduction. Such matrices include terrestrial soils (Topp et al., 2003; Ishii et al., 2006), aquatic sediments (Byappanahalli and Fujioka, 1998; Solo-Gabriele et al., 2000; Jeng et al., 2005), and attached to submerged aquatic vegetation (SAV) (Byappanahalli et al., 2003b; Whitman et al., 2003; Ksoll et al., 2007). The extended persistence of FIB, particularly in the sediments and SAV of recreational waters, is one highly likely cause of the detection of FIB in the absence of pathogens and the poor correlation between the two. Persistent FIB in benthic matrices, such as sediments and SAV, may be reintroduced into the water column whenever sediments get resuspended, such as during storms or high recreational activity. Bacteria are typically the most easily resuspended of benthic organisms due to their small size and association with cohesive surficial fluff sediments (Auer and Niehaus, 1993; Howell et al., 1996; Shimeta et al., 2002; Jeng et al., 2005). And because we do not yet have a good understanding of whether many pathogens are similarly able to persist in the environment or are regularly resuspended from sediments, it is difficult to predict whether these resuspended FIB reflect a real human health risk. This has been a major concern about the reliability of the indicator organism concept that has been raised repeatedly in the

literature (Solo-Gabriele et al., 2000; Grant et al., 2001; Whitman et al., 2003; Anderson et al., 2005; Ishii and Sadowsky, 2008).

Unfortunately, the importance of extended persistence and resuspension of FIB has been difficult to quantify in terms of its effects on human health risks. Although there have been recent epidemiological studies showing that increased exposure to beach sand carries increased risk of disease (Bonilla et al., 2007; Heaney et al., 2009), no correlations have been determined between health risks and concentrations of FIB in sediments or SAV. Nor is there a standard method that has been adopted for their detection and quantification. In managed streams, resuspension of FIB has been observed to occur as a result of both natural (Nagels et al., 2002; Jamieson et al., 2005) and experimentally-induced (McDonald et al., 1982; Wilkinson et al., 1995; Nagels et al., 2002; Muirhead et al., 2004) periods of high flow, in the absence of rainfall and (presumably) groundwater inputs. However, in the vast majority of other recreational water bodies that are not so easily constrained (such as beaches and lakes) the resuspension of benthic FIB has typically been inferred. For example, observations of relatively high water column *E. coli* counts have been shown to correlate with factors that cause sediment erosion and resuspension, such as wave or tidal activity (Le Fevre and Lewis, 2003; Shibata et al., 2004; Whitman et al., 2006; Yamahara et al., 2007) and increased boating activity (An et al., 2002), or have been correlated with sediment densities through the use of time series or structural equation modeling (Whitman and Nevers, 2003; Whitman et al., 2006).

Another approach to elucidating the importance of sediments as a potential reservoir of FIB has been to incorporate resuspension processes into the modeling of FIB fate and transport. Because benthic bacteria are typically adhered to sediment particles (Auer and Niehaus, 1993; Howell et al., 1996; Davies and Bavor, 2000), hydrodynamic information and sediment characteristics can be used to predict sediment resuspension and offer relatively good approximations of the behavior of benthic FIB in the sediments (Bai and Lung, 2005; Jamieson et al., 2005). Both unidirectional (e.g., tidal and stream flows) and oscillatory (e.g., wave action) flow regimes set up velocity gradients along the bottom that increase from zero at the sea floor up to the mainstream velocity. The steepness of these gradients, in combination with bottom roughness that results from bedform elements (e.g., sediment grains and sand ripples) establishes a shear stress that acts on the sediment water interface (Denny, 1988; Soulsby, 1998). If the force of this shear stress is sufficiently strong to overcome the natural settling velocity of individual sediment grains, some amount of sediment will be maintained in suspension (Soulsby, 1998; Le Roux, 2005). As a result, sets of theoretical and empirical equations allow the prediction of concentrations and transport of suspended sediment under unidirectional and oscillatory flow patterns, which can then be used to estimate similar processes for the associated FIB (Bai and Lung, 2005; Jamieson et al., 2005).

General terms for resuspension rates (based on critical shear stresses resulting in sediment resuspension) have been incorporated into embayment-wide models used to predict net transport of FIB (Steets and Holden, 2003; Sanders et al., 2005), and a much

more detailed model has been published that uses the Environmental Fluid Dynamics Code model to specifically predict the fate and persistence of sediment associated fecal bacteria (Bai and Lung, 2005). These models, however, necessarily use very broad brush approaches with regards to resuspension dynamics, and it is becoming increasingly clear that understanding how benthic-pelagic coupling affects the population dynamics of species of FIB is very important to predicting their survival and transport in receiving waters. In fact, continued data and experimentation on the behavior of benthic FIB has been outlined as a distinct need for future model improvement (Bai and Lung, 2005; Pachepsky et al., 2006).

I believe that one of the major deterrents to our ability to readily interpret the importance of benthic reservoirs of FIB is that their densities have typically been normalized per mass or volume of substrate (CFU/100 mL for water or CFU/g for solid substrates such as sediments and SAV) (Byappanahalli and Fujioka, 1998; Solo-Gabriele et al., 2000; Topp et al., 2003; Whitman et al., 2003; Anderson et al., 2005; Jeng et al., 2005; Ishii et al., 2006). Such normalization makes sense for the water column, as the concentration is the most appropriate value to consider in terms of human health risk. However, FIB concentrations that are normalized to volume of water do not represent a direct comparison to concentrations normalized to mass of sediment or SAV, and this discrepancy does not allow for a simple interpretation of the importance of benthic sources of resuspendable bacteria. It is possible, however, to use a different method of normalizing bacterial densities, and analyze data from aquatic systems on the basis of

landscape area (e.g., per m^2), which allows direct comparison bacterial population sizes in waterborne and benthic samples (Muirhead et al., 2004; Jamieson et al., 2005).

In this study I revisited the same sites in the Tampa Bay watershed that were sampled in Chapter 3 and used the concept of landscape area to reexamine the relative population sizes of the enterococci found in the water, sediment, and SAV samples to determine their relative magnitude. Furthermore, I wanted to further investigate the potential for benthic substrates, such as sediment and SAV, to serve as important reservoirs of resuspendable FIB, as is often suggested in the literature.

The study had three specific objectives: (1) To identify and quantify key habitat characteristics that would allow the normalization of enterococci densities on a landscape basis and allow direct comparison of the population sizes in water, sediment, and SAV at each site; (2) develop a simple model that will predict shifts in the relative population sizes at a given site that result from changes in important habitat characteristics such as bacterial densities, water depth, SAV cover, etc.; (3) use historical wind and flow data at each site, in conjunction with theoretical calculations of sediment resuspension, to determine the likely effect of sediment-associated bacterial resuspension on water quality monitoring at each site. This research will contribute to a better understanding of the effects of sediment resuspension on the fate, transport, and performance of FIB within the Tampa Bay watershed, based on realistic, time-averaged values for data collected on enterococci densities.

Methods

Environmental Sampling

Data for bacterial concentrations are from Chapter 3. Briefly, water, sediment, and SAV at four freshwater and two estuarine sites in the Tampa Bay watershed were sampled monthly from May 2007 to April 2008 (*Figure 11*). Samples were placed on ice immediately after collection and processed in the laboratory the same day. Water samples were vacuum filtered directly onto 0.45 μm nitrocellulose membranes and cultured at 41° C for 24 hours on mEI agar (Difco Laboratories) supplemented with nalidixic acid (USEPA, 2000). Sediment and vegetation samples were diluted 1:10 (w/v) in phosphate buffered water and sonicated at 19 watts for 30 seconds to dislodge and resuspend attached cells (Anderson et al., 2005). Aliquots of the water were then filtered and cultured by standard membrane filtration methods (USEPA, 2000). Final concentrations are presented as CFU/100 mL water or CFU/100 g wet weight substrate. After counting, colonies were picked from the mEI agar and cultured overnight in Enterococcosel broth (EB, Difco Laboratories, enc.) at 37.5° to confirm identification.

Habitat Characterization

In order to convert the densities measured in Chapter 3 to a landscape scale, the following habitat characteristics were measured: water depth, sediment density, the depth of sediment containing FIB, the biomass density of SAV in a vegetated bed, and the percent coverage of SAV beds over the entire aquatic bottom. Habitat characterization for all sites was conducted in July of 2008. Although some values, such as SAV biomass,

cover, and water depth, are expected to vary over seasonal cycles, midsummer in the Tampa Bay watershed typically represents the period with the most rainfall and the highest amount of SAV growth. As a result, these values were taken in July under the assumption that they would represent relatively maximal values for SAV biomass and water depth over an annual cycle. However, additional water depth data were obtained at the stream sites in July 2009 during a period of exceptionally heavy rainfall and high water levels, and are presented as an extreme case in terms of water depth.

Water depth at each site was determined as the mean of 10 random measurements. At stream and river sites, these ten values were obtained from a transect across the channel to characterize the entire water body at the location of sampling. At the lake site, ten locations were selected randomly on a bathymetric map averaged to get a mean depth for the entire lake. Finally, at the bay sites, only the local depth was used, which was characterized by ten random measurements that were taken in the vicinity of the sampling location, during the mean tidal hieght. At the river and lake sites, I also obtained estimates for the local shoreline depth in the same manner in an attempt to characterize the immediate sampling area as a contrast to using the entire water body.

To determine sediment density, five sediment samples from each site were collected in 50 mL centrifuge tubes. The sediment was allowed to settle in each tube during transport, and upon return to the laboratory, the overlying water was poured off prior to analysis. The volume and mass of the remaining sediment in each sample was recorded and the

wet density was calculated, as this was the unit or normalization used in Chapter 3. Next, each sediment sample was dried at 80º C for 24 hours, and the dry mass was measured for each sample so that a dry density could be obtained as well. To determine the depth to which FIB were detectable in the sediments, three replicate sediment cores of approximately 2.5 cm in diameter and 20 cm in length were taken at each site. Upon removal, each core was longitudinally divided into six sections (3 cm long – the deepest 2 cm were discarded due to disturbance from sampling), which were homogenized and analyzed for enterococci densities. The samples were analyzed using the same methods as those described above. The enterococci density in each sediment sample was calculated (CFU / 100 gww), and the cutoff depth was determined to be the last depth at which enterococci densities were within at least one order of magnitude of those found at the surface of the core (e.g., see *Figure 23* and *Figure* 24). Densities at deeper depths, which were beyond an order of magnitude lower than surface densities, were assumed to be numerically insignificant in terms of total population size.

To determine the amount of vegetative biomass in an SAV bed, five quadrats (0.0625 m^2) were thrown haphazardly into SAV patches at each site. The emergent portion of all SAV within the quadrat was removed down to bare sand and all excess water was allowed to drain for approximately 30 s. Next the mass was immediately measured and the mean of all replicates for each site was used as the typical wet biomass of SAV. Finally, the percent coverage of SAV beds (as opposed to bare sediment) over the entire

bottom was determined via visual estimation at each site (Dethier et al., 1993; Fourqurean et al., 2001; McDonald et al., 2006; Bell et al., 2008).

Calculations

The values obtained from the habitat characterizations were used, along with the mean enterococci densities for each substrate at each site observed in Chapter 3 (*Figure 12*), in the following formulas to recalculate enterococci densities at the landscape scale. For waterborne bacterial concentrations, CFU/m^2 was calculated as a function of CFU/100 mL and water depth:

$$C_{WL} = 10^4 * C_{WV} * d_W \quad (1)$$

where C_{WL} = waterborne bacterial concentration normalized to landscape (CFU/m^2); C_{WV} = waterborne bacterial concentration normalized to volume (CFU/100 mL); and d_W = water depth (m).

For sediment-associated bacterial densities, CFU/m^2 was calculated as a function of CFU/100 g, sediment density and sediment depth:

$$C_{SL} = 10^4 * C_{SM} * D_S * d_S \quad (2)$$

where C_{SL} = bacterial concentration in sediment normalized to landscape (CFU/m^2); C_{SM} = bacterial concentration in sediment normalized to sediment mass (CFU/100 g); D_S = sediment density (g wet weight/cm^3); and d_S = sediment depth (m).

For SAV-associated bacterial densities, CFU/m^2 was calculated as a function of CFU/100 g, SAV biomass in vegetated patches, and proportion of bottom with vegetated cover:

$$C_{VL} = 10 * C_{VM} * B_V * P_V \quad (3)$$

where C_{VL} = bacterial concentration in SAV normalized to landscape (CFU/m^2); C_{VM} = bacterial concentration in SAV normalized to mass (CFU/100 g); B_V = SAV biomass in a vegetated patch (kg/m^2); and P_V = proportion of SAV cover over entire bottom. Finally, the total CFU/m^2 for the entire system at each site was simply calculated as the sum of each substrate (eqs. 1-3):

$$C_{TL} = C_{WL} + C_{SL} + C_{VL} \quad (4)$$

where C_{TL} = total bacterial density in the system, normalized to landscape area. After the calculation of total densities, the relative population sizes for each substrate were calculated by dividing that substrate's population size by the total population size.

Modeling and Sediment Resuspension Estimates

Four sites (large stream, river, lake, and upper bay) were chosen to theoretically explore the effect of sediment resuspension on waterborne concentrations of FIB. Resuspension estimates were calculated according to the methods outlined in Soulsby (1998) for determining the concentration of suspended marine sands under currents and waves. Sediments at each of the sites in the Tampa Bay watershed were dominated by non-cohesive sand, allowing the use of these methods. For the stream and river sites, estimates were based upon predictions of resuspension under a unidirectional current

based upon bulk water flow velocity, as these sites were too narrow to have any appreciable wave generation. Alternatively, for the lake and bay sites, estimates were based upon predictions of resuspension under wave-generated oscillatory flow, as the lake site is too small to have any significant unidirectional circulation and the bay site is well sheltered from tidal currents.

For resuspension estimates under both unidirectional and oscillatory flow, input data were needed for water temperature, salinity, depth, and median sediment grain size. Mean values for water temperature and salinity obtained during the monthly sampling at each site (Chapter 3) were used. For sediment grain size, samples were collected from each site during the habitat characterization described above and were sorted with sieves in the laboratory. At each site, five replicate samples of surface sediments (top 3 cm) were collected and homogenized into a single representative sample for each site. Upon return to the laboratory, sediment grains were successively washed through a series of metal sieves with pore sizes of 1.0, 0.5, 0.25, 0.125, and 0.063 mm. Each size fraction was washed into pre-combusted ceramic crucibles, and the filtrate from the smallest sieve was captured and triplicate aliquots were filtered onto pre-combusted glass fiber filters (Whatman GF/F, 0.7 μm pore size). All sediment samples were dried at 60 ºC for 24 hours and then weighed to determine dry mass. Next, the samples were combusted at 450 ºC for 4 hours to burn off the organic portion, and the samples were weighed again to determine the percent organic matter. Finally, the cumulative proportions (by mass) of each size fraction were plotted and values for d_{10}, d_{16}, d_{50}, d_{84}, and d_{90} (corresponding to

the 10^{th}, 16^{th}, 50^{th}, 84^{th}, and 90^{th} percentiles of grain diameter) were extrapolated from the graphs (Soulsby, 1998).

For the river and stream sites, water flow speed was measured directly during periods of extremely high flow in July 2009 to obtain an upper limit for realistic flow speeds. Mainstream current velocity was determined by measuring the time for neutrally buoyant particles suspended in the water to travel a known distance. Three sets of ten replicate particles were timed at different locations within the channel to obtain a mean flow speed. These flow speeds were then compared to and supplemented with archived data for the river site available from the United States Geological Survey (station # 02303330). No USGS station was available for the stream site.

For the lake and upper bay sites, mean wave period (T_m) and significant wave height (H_s) were estimated from historical wind data according to established methods outlined in the *Coastal Engineering Manual* (*CEM*) (USACE, 2002). Wind records were obtained from the National Oceanic and Atmospheric Administration's National Buoy Data Center (station OPTF1 – Old Port, Tampa, FL) for the entire year between May 2007 and April 2008. The complete wind records were then filtered for each site to include only the subset that could be considered onshore (and thus wave-generating). For the lake site, this included wind with directions between 60 and 190 degrees, and for the upper bay site this included records between 180 and 270 degrees. Next, a frequency histogram of wind speeds for each site-specific subset was generated and used to create a table of wind

speeds that represent maximum, 0.2, 0.5, 1st, 2nd, 5th, 10th, 25th, and 50th percentiles during the sampling period. Finally, these wind speeds, in combination with a mean fetch distance (measured from areal photographs) were compared to nomograms in the *CEM* to estimate representative values for T_m and H_s, with consideration of limits for shallow-water wave generation.

Because the calculations for estimating resuspension are too lengthy to cover in detail here (Soulsby, 1998), only a brief overview will be given. For resuspension under currents, the mainstream flow speed, U_b, was used to calculate a depth averaged flow speed, $\bar{U}$. The median sediment grain size (d_{50}) was then used to determine the settling velocity, w_s, the bottom skin-friction shear stress, τ_{os}, and the critical bottom shear stress τ_{cr}. If $\tau_{os} > \tau_{cr}$, meaning that some amount of sediment motion and resuspension was occurring, then a profile of sediment concentration by water depth was calculated. This profile was then integrated over the entire water column to determine the total amount of sediment resuspended per square meter area. By multiplying the resuspended sediment mass by the bacteria density (CFU / 100g) in the sediment for each site, the total number of resuspended bacteria (CFU / m^2) was calculated. Finally, by dividing by the volume of water at each site per m^2, the predicted increase in waterborne bacteria concentrations (in CFU / 100 mL) was determined. The process outlined for resuspension under waves is similar in principle, although the calculations vary somewhat. Firstly, the JONSWAP wave spectrum was used (as opposed to monochromatic waves) for each combination of wave height and period obtained from the wind records. The standard deviation, U_{rms},

and amplitude, U_w, of the bottom orbital velocity in a typical wave cycle were then calculated. Similar to the equations for currents, sediment grain size and density were used to determine w_s and τ_{cr}. The presence of bedforms was determined, as well as their height, Δ_r, and wavelength, λ_r, if present. Next, the total bottom shear stress, τ_b, and the rough bed friction factor, f_{wr}, were determined and compared to τ_{cr} as before to determine if there was sediment motion. Finally, the concentration profile and the resulting amount of suspended sediment and bacteria were calculated as explained above.

Results

Habitat Characterization and Area Normalization

Water depths at the sites ranged from extremely shallow (<50 cm) at the stream sites, to moderately shallow at the bay sites (<1 m with 0.5 – 1.0 m tides), to relatively deeper water at the river and lake sites (2 – 3 m). Sediments were primarily sand and the densities were highly consistent at all of the sites (~ 1.9 gww/cm^3) except for the two stream sites, which included a higher proportion of organic material and were slightly less dense (~ 1.6 gww/cm^3) (*Table 8 and* Table 9). Depth profiles of enterococci densities at the six sites generally showed a decline in density with increasing sediment depth (*Figure 23 and Figure* 24). Cutoff depths (the depth beyond which at least an order of magnitude decline in densities was observed) were highly variable, ranging from 3 cm at the small stream site to 15 cm at the lower bay site (*Figure 23 and Figure* 24, *Table 8 and Table 9*). SAV biomass within vegetated patches was higher at the freshwater sites, with values of about 2.5 kg/m^2 at the bay sites and 2.9 to 10.5 kg/m^2 at

Table 8. Key habitat characteristics measured at each of the freshwater sites, which were used to convert enterococci densities in each substrate from mass-normalized values to those normalized to landscape area. Sediment depth is the depth to which enterococci densities were found to be within one order of magnitude of surface densities. SAV density is the biomass density of SAV within a vegetated patch, and SAV % cover is the total percentage of aquatic bottom covered by vegetated patches as opposed to bare sand.

	Water depth (cm)	Sediment Depth (cm)	Sediment Density (gww / cm^3)	SAV density (kg / m^2)	SAV % cover	Enterococci densities (CFU / m^2)			
						Water	Sediment	SAV	Total
Small Stream	36	3	1.63	2.9	80	$1.84 \cdot 10^6$	$1.65 \cdot 10^6$	$2.52 \cdot 10^5$	$3.74 \cdot 10^6$
Small Stream, high	98	3	1.63	2.9	80	$4.95 \cdot 10^6$	$1.65 \cdot 10^6$	$2.52 \cdot 10^5$	$6.85 \cdot 10^6$
Large Stream	17	12	1.66	10.5	60	$6.90 \cdot 10^5$	$2.92 \cdot 10^6$	$3.66 \cdot 10^5$	$3.97 \cdot 10^6$
Large Stream, high	140	12	1.66	10.5	60	$5.58 \cdot 10^6$	$2.92 \cdot 10^6$	$3.66 \cdot 10^5$	$8.86 \cdot 10^6$
River	190	6	1.92	5.4	10	$3.70 \cdot 10^6$	$1.06 \cdot 10^6$	$2.37 \cdot 10^4$	$4.79 \cdot 10^6$
River Bank	36	6	1.92	5.4	95	$6.74 \cdot 10^5$	$1.06 \cdot 10^6$	$2.37 \cdot 10^4$	$1.43 \cdot 10^6$
Lake	320	6	1.90	5.4	95	$2.97 \cdot 10^6$	$7.61 \cdot 10^5$	$1.62 \cdot 10^5$	$3.89 \cdot 10^6$
Lake Shore	53	6	1.90	5.4	90	$5.22 \cdot 10^5$	$7.61 \cdot 10^5$	$1.62 \cdot 10^5$	$1.44 \cdot 10^6$

Table 9. Key habitat characteristics measured at each of the freshwater sites, which were used to convert enterococci densities in each substrate from mass-normalized values to those normalized to landscape area. Sediment depth is the depth to which enterococci densities were found to be within one order of magnitude of surface densities. SAV density is the biomass density of SAV within a vegetated patch, and SAV % cover is the total percentage of aquatic bottom covered by vegetated patches as opposed to bare sand.

						Enterococci densities (CFU / m^2)			
	Water depth (cm)	**Sediment Depth (cm)**	**Sediment Density (gww / cm^3)**	**SAV density (kg / m^2)**	**SAV % cover**	**Water**	**Sediment**	**SAV**	**Total**
Upper Bay, mid-tide	80	9	1.91	2.4	40	$2.72 \cdot 10^5$	$1.61 \cdot 10^6$	$6.40 \cdot 10^3$	$1.84 \cdot 10^6$
Upper Bay high tide	30	9	1.91	2.4	40	$4.42 \cdot 10^5$	$1.61 \cdot 10^6$	$6.40 \cdot 10^3$	$2.06 \cdot 10^6$
Upper Bay, low tide	130	9	1.91	2.4	40	$1.02 \cdot 10^5$	$1.61 \cdot 10^6$	$6.40 \cdot 10^3$	$1.72 \cdot 10^6$
Lower Bay, mid-tidle	75	15	1.92	2.6	80	$2.01 \cdot 10^5$	$1.45 \cdot 10^6$	$3.24 \cdot 10^3$	$1.66 \cdot 10^6$
Lower Bay, high tide	125	15	1.92	2.6	80	$6.7 \cdot 10^5$	$1.45 \cdot 10^6$	$3.24 \cdot 10^3$	$1.79 \cdot 10^6$
Lower Bay, low tide	25	15	1.92	2.6	80	$3.35 \cdot 10^5$	$1.45 \cdot 10^6$	$3.24 \cdot 10^3$	$1.52 \cdot 10^6$

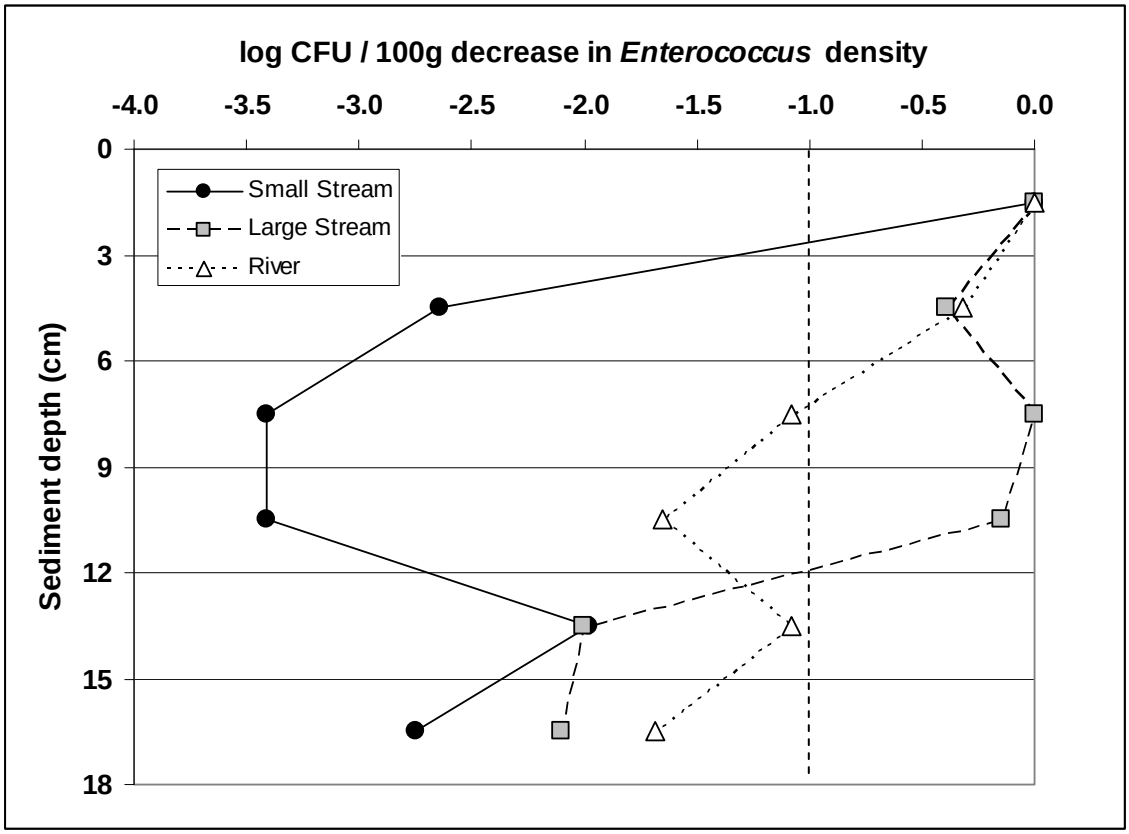

Figure 23. Depth profiles of mean enterococci densities (presented as decrease in log CFU / 100g from the shallowest depth) in from sediment cores at the small stream, large stream, and river sites; n = 3 at each depth. The vertical dashed line represents a one order of magnitude decrease from surface levels, which was used as the cutoff depth for the calculations of sediment depth in the models.

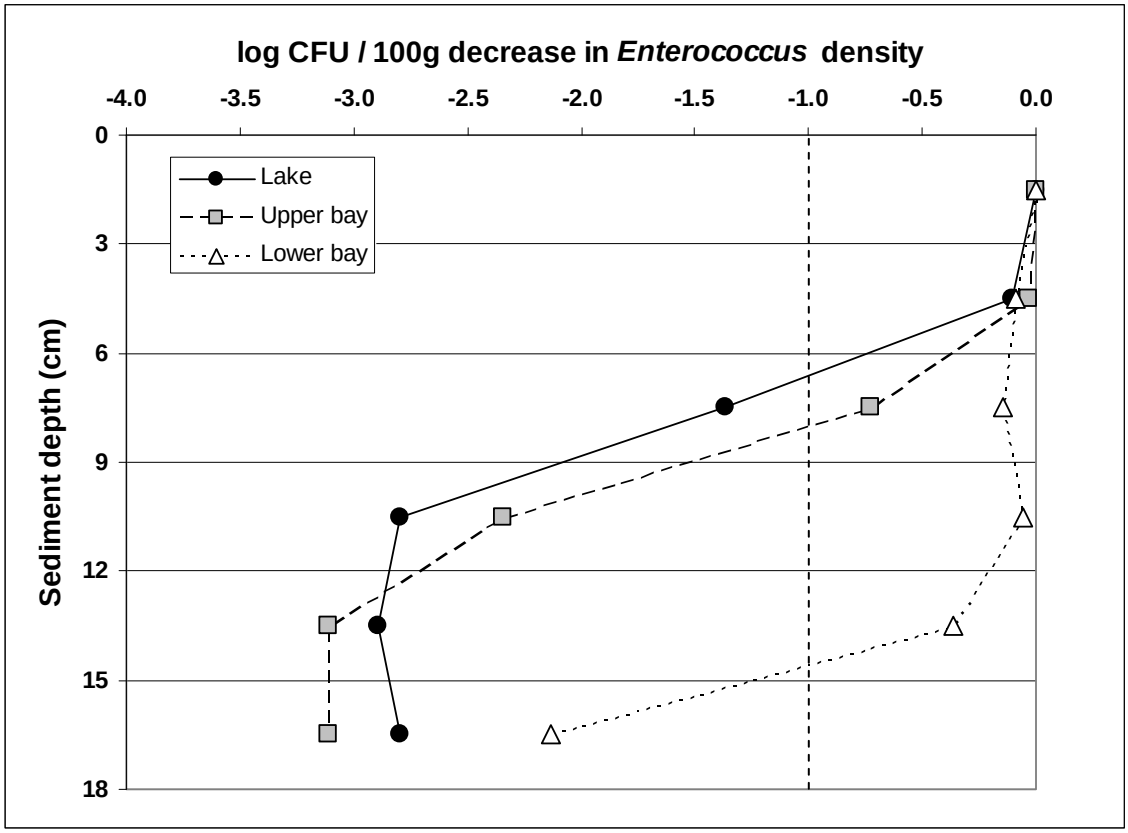

Figure 24. Depth profiles of mean enterococci densities (presented as decrease in log CFU / 100g from the shallowest depth) in from sediment cores at the lake, upper bay, and lowe bay sites. Each core was 18 cm deep and divided into 6 sections that were each 3 cm long; n = 3 at each depth. The vertical dashed line represents a one order of magnitude decrease from surface levels, which was used as the cutoff depth for the calculations of sediment depth in the models.

the freshwater sites. SAV cover over the entire bottom was also highly variable, ranging from 10% at the river site to almost complete cover at the lake site (*Table 8 and* Table 9).

Total enterococci densities normalized to landscape area at all of the sites were surprisingly consistent, on the order of 10^6 CFU/m^2. Generally, sites with lower sediment enterococci densities normalized to mass (e.g., bay sites), were compensated by having a relatively deeper depth of colonization in the sediment, resulting in total population sizes that were comparable to sites with higher sediment enterococci densities per mass (e.g., stream sites). The landscape-normalized densities at each site were highest in water and sediments, ranging from 10^5 - 10^6 CFU/m^2, while the densities in SAV were consistently lower, ranging from 10^5 at the stream sites to 10^3 at the bay sites (*Table 8 and Table 9*). When the numbers of enterococci on each substrate were reexamined as relative proportions of the total population of enterococci at a given site, the results differed between the freshwater and estuarine sites (*Figure 25 and Figure* 26). At the freshwater sites, water depth seemed to be the major factor in determining relative population sizes of enterococci in each substrate. In situations with shallow water depths (such as during periods of low stream flow, or when considering only the shoreline at the lake or river site), the sediment population was similar to or even greater than the waterborne population. However, during periods of high water in the streams, or when accounting for the entire volume of water at the lake or river site, the waterborne population became the dominant portion (*Figure 25*). Conversely, at the estuarine sites, the populations in the sediments were consistently dominant, typically accounting for 80-90% of the total

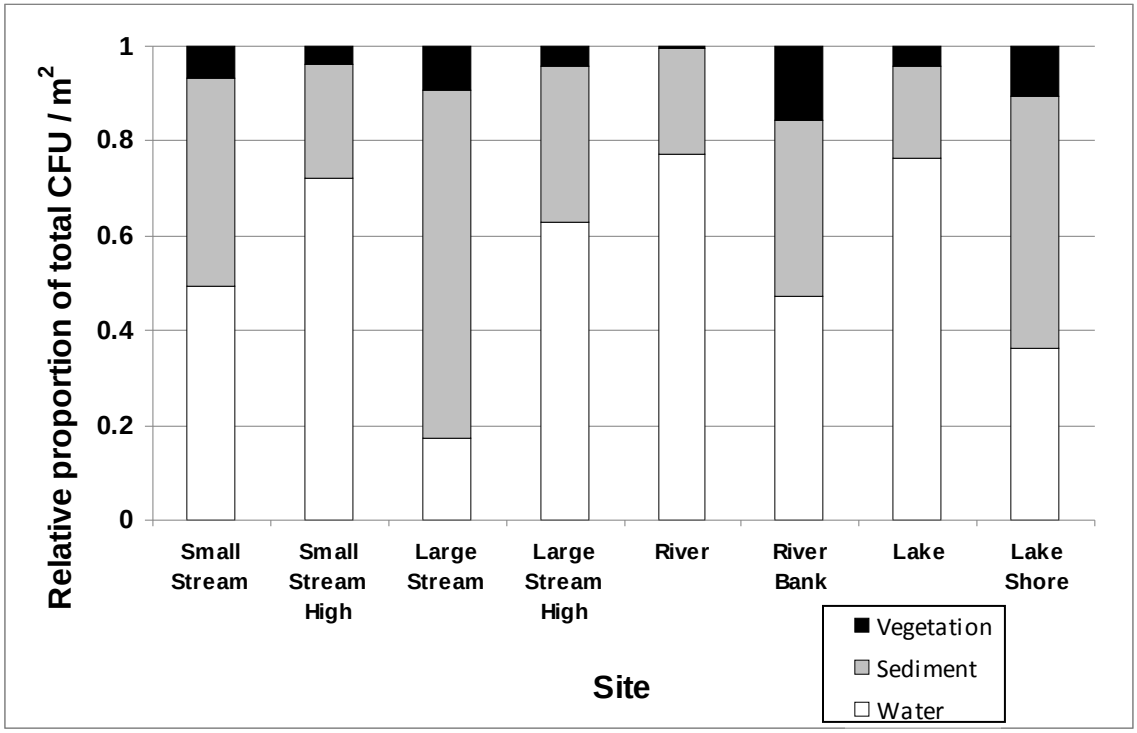

Figure 25. Mean proportion of total number of enterococci per square meter landscape area found in water, sediment, and SAV samples at three freshwater sites around the Tampa Bay watershed obtained from monthly samples between May 2007 and April 2008. The two columns for each stream site represent normal and extreme high water depths. The two columns for the river and lake sites represent values for the entire water body vs. those if consideration is constrained to the nearshore banks only. CFU = colony forming unites.

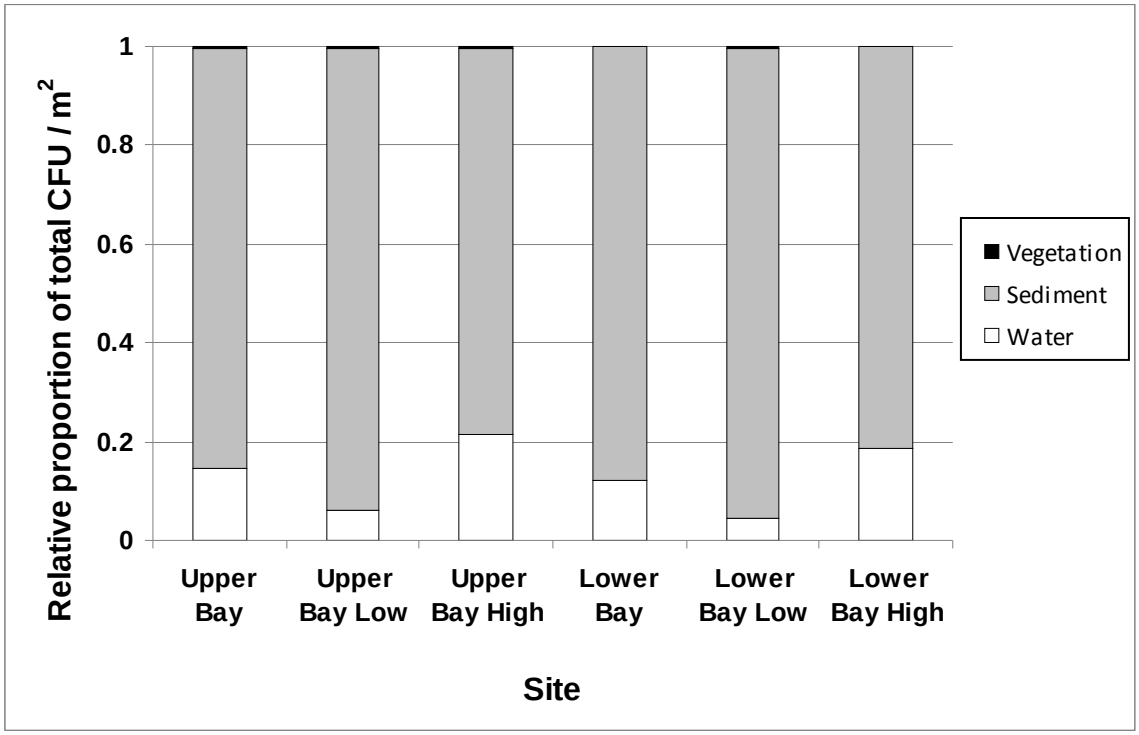

Figure 26. Mean proportion of total number of enterococci per square meter landscape area found in water, sediment, and SAV samples at two estuarine sites in Tampa Bay obtained from monthly samples between May 2007 and April 2008. The three columns for each site represent changing proportions for varying water depths at mid-, low, and high tide leves, respectively. CFU = colony forming unites.

enterococci load regardless of changing water levels during the tidal cycle (*Figure 26*). SAV was consistently found to harbor small to negligible fractions of the total enterococci, ranging from a maximum of 9.2% at the large stream site down to 0.2% at the lower bay site (*Figure 25* and *Figure 26*).

Modeling Theoretical Habitat Changes

After examining the relative population size of enterococci in each substrate at each site, the model was used to theoretically vary key habitat characteristics along a realistic continuum to determine how much the relative population sizes may potentially shift. As a result of my initial interest in the importance of SAV as a substrate for enterococci, I chose to use the model to investigate the large stream site, which had the highest proportion of total enterococci associated with SAV. *Figure 27* shows the results of modeling changes in the relative population size of enterococci in the sediments vs. the water column, as a result of theoretically varying water depth and SAV coverage. The relative proportion of total enterococci predicted to be on SAV ranged from 0% to approximately 18% at the shallowest depths and full coverage. The proportion in the water column along the same gradient was predicted to range from approximately 58% to 0%, respectively. So at the shallow depths sometimes found in this stream (15-20 cm), the proportion of enterococci found in SAV was predicted to exceed that found in the water if the SAV coverage were to approach 100%. However, due to high densities of enterococci in the sediment, SAV was never predicted to harbor a dominant proportion of total enterococci under any conditions. At all levels of water depth and SAV coverage,

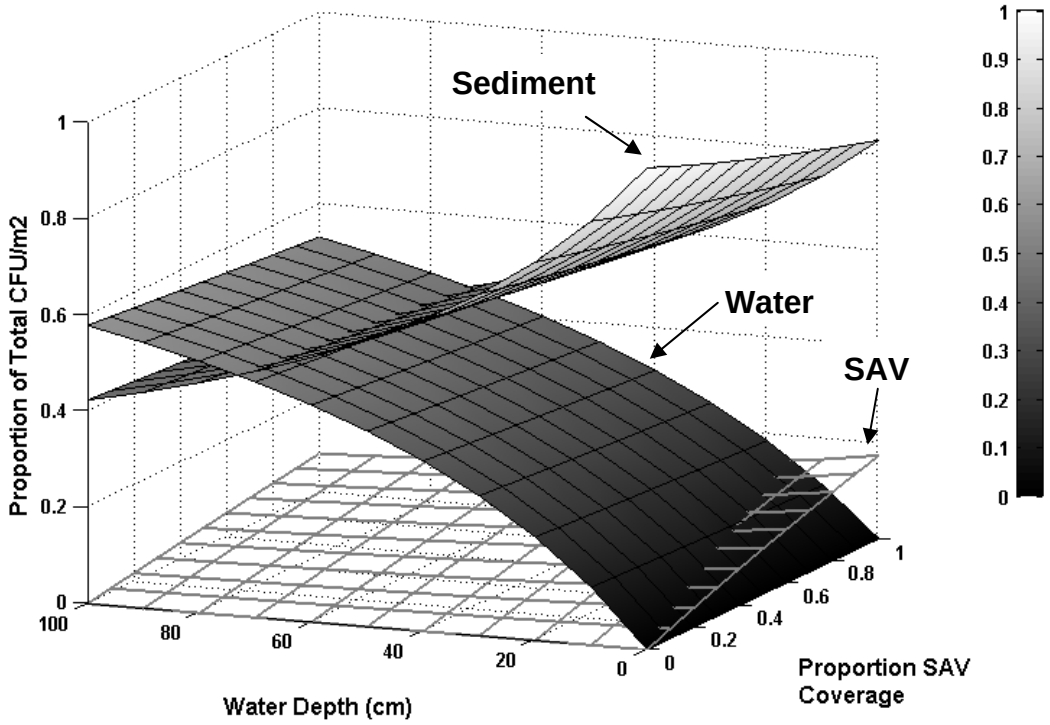

Figure 27. The relative proportion of total enterococci found in water, sediment, and SAV in response to theoretically varying values for water depth and SAV bottom cover at the large stream site. Shades of gray and associated sidebar indicate corresponding proportion value on the z-axis. CFU = colony forming units; waterborne bacteria = 4.0 x 10^2 CFU/100 mL; sediment bacteria = 1.5 x 10^3 CFU/100 g; sediment depth = 12 cm; sediment density = 1.66 g/cm^3; SAV bacteria = 5.8 x 10^3 CFU / 100 mL; SAV biomass = 10.5 kg / m^2.

the SAV-associated population was always considerably smaller than the size of the sediment-associated population, even though the mass-normalized densities in SAV at this site averaged nearly 10^4 CFU/100 g.

The model was also used to predict how relative enterococci population sizes would change at the upper bay site as a result of normal tidal fluctuations in water depth. Even though the vast majority of enterococci were found in the sediments on average at the bay sites, *Figure 28* represents one particular sample (April 2008) in which the proportions of enterococci found in water and sediment were relatively equal. As a result, tidal fluctuations were predicted to cause dramatic shifts between states where the water and sediment alternated as the dominant proportion of total enterococci. As expected, SAV consistently harbored a very small fraction of the total enterococci. As a result, the degree to which the resuspension of sediments could potentially effect waterborne concentrations of enterococci was predicted to be highly dependent on the tidal cycle, and therefore shift rapidly in time.

Finally, the idea of sediment resuspension at the upper bay site was explored more thoroughly in *Figure 29*. The model was used to predict the increase in waterborne enterococci concentrations as a result of the resuspension of various amounts of sediment under a range of sediment enterococci densities. At low sediment densities (10^2 CFU/100 g) there were never enough enterococci resuspended to exceed the waterborne regulatory limit of 104 CFU/ 100 mL with the model limited to 10 cm of total sediment depth. At

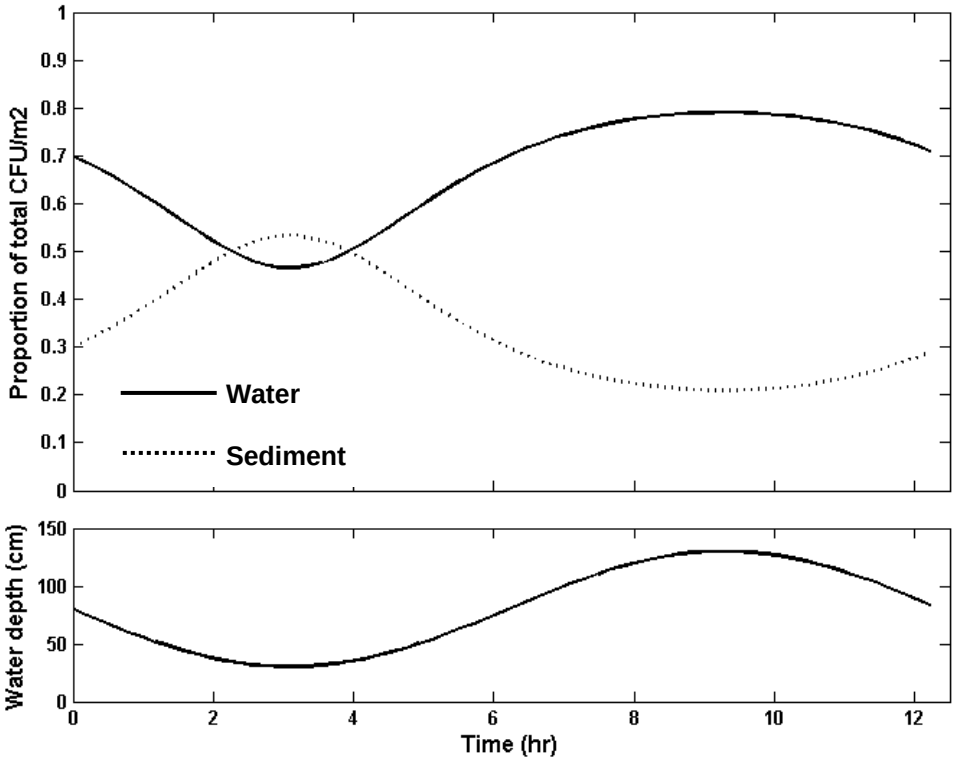

Figure 28. The relative proportion of total enterococci found in water and sediment in response to theoretically varying water depth as a result of tidal fluctuations at the upper bay site during the April 2008 sampling event. Waterborne bacteria = 1.1 x 10^2 CFU/100 mL; sediment bacteria = 2.2 x 10^2 CFU/100 g; sediment depth = 9 cm; sediment density = 1.92 g/cm^3; SAV bacteria = 1.2 x 10^3 CFU/100 g; SAV biomass = 2.4 kg / m^2; SAV cover = 40%.

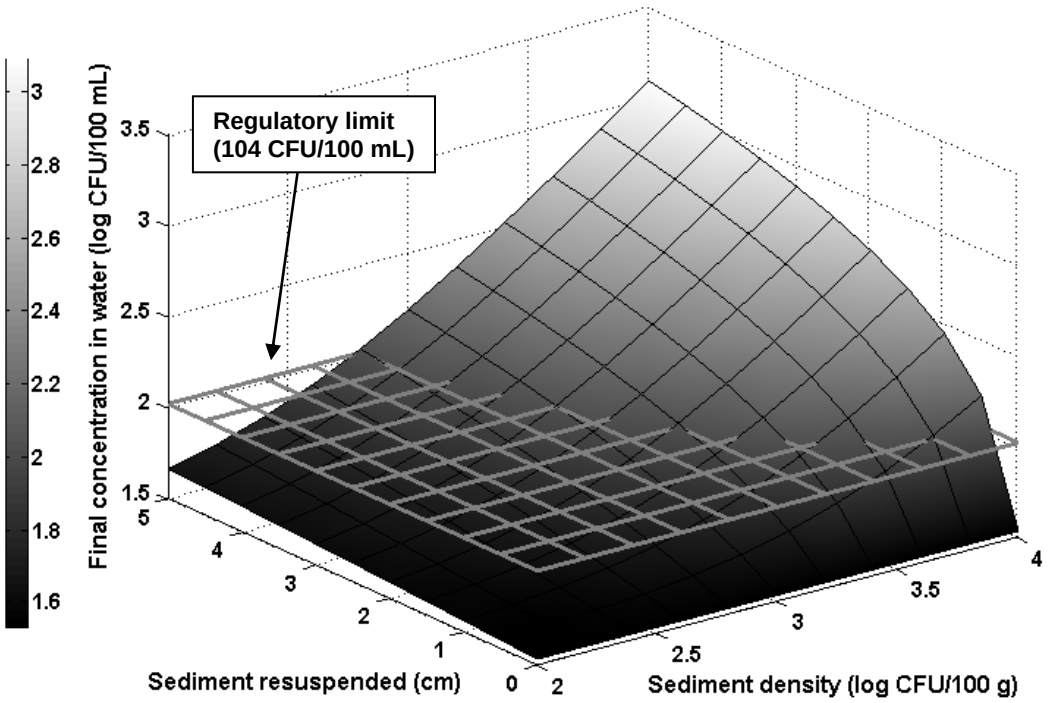

Figure 29. Predicted increases in waterborne enterococci concentrations at the upper bay site resulting from theoretically varying values for sediment enterococci densities and the amount of sediment resuspended. (SAV negligible and not shown.) CFU = colony forming units; initial waterborne bacteria = 1.5 log CFU/100 mL; water depth = 80 cm; sediment density = 1.92 g/cm^3; regulatory limit = 104 CFU/100 mL.

moderate sediment densities (10^3 CFU/100 g), resuspension of approximately 3 cm of sediment was required to cause an exceedance, while at relatively high densities (10^4 CFU/100 g), an exceedance was predicted after resuspension of only 0.5 cm of sediment.

Resuspension Estimates

Sediment characteristics at all of the sites were very similar. Estimated values for d_{50} ranged from 0.18 to 0.26 mm, with corresponding φ values of 1.95 to 2.5. Sediments at the river, lake, and upper bay sites were characterized as fine sand, while the sediment at the large stream site was characterized as medium sand (*Table 10*). The sediments were relatively homogenous and well sorted, with d_{10} values ranging from 0.09 to 0.13 mm and d_{90} values ranging from 0.24 to 0.45 mm. The sediments contained a very low amount of organic material at all sites, ranging from 1-2% at the river and stream sites to a fraction of a percent at the lake and bay sites. Settling velocities were all similar, at approximately 0.03 m/s (*Table 10*).

Current velocities at the stream and river sites were very low. Direct measurements taken at the two sites during the high flow period of July 2009 averaged 0.32 m/s at the stream site and 0.25 m/s at the river site. Examination of historic data for the river site revealed that the site typically experienced much lower flows during the sampling period, less than 0.1 m/s (*Figure 30*). This flow rate was only exceeded a few times during the summer of 2007, and then once during a period of extremely high flow in the later winter of 2008 when the flow reached approximately 0.4 m/s. Resuspension estimates for these flows at

Table 10. Sediment characteristics at the large stream, river, lake, and upper bay sites. Diameters are reported in millimeters, and values for φ are based on d_{50}.

	d_{10}	d_{16}	d_{50}	d_{84}	d_{90}	φ	% organic	Classification	Settling velocity (m/s)
Large Stream	0.12	0.15	0.26	0.42	0.45	1.95	2.1	medium sand	0.039
River	0.09	0.09	0.18	0.27	0.36	2.5	1.2	fine sand	0.024
Lake	0.09	0.12	0.18	0.23	0.24	2.5	0.03	fine sand	0.024
Upper Bay	0.13	0.15	0.23	0.40	0.44	2.1	0.2	fine sand	0.028

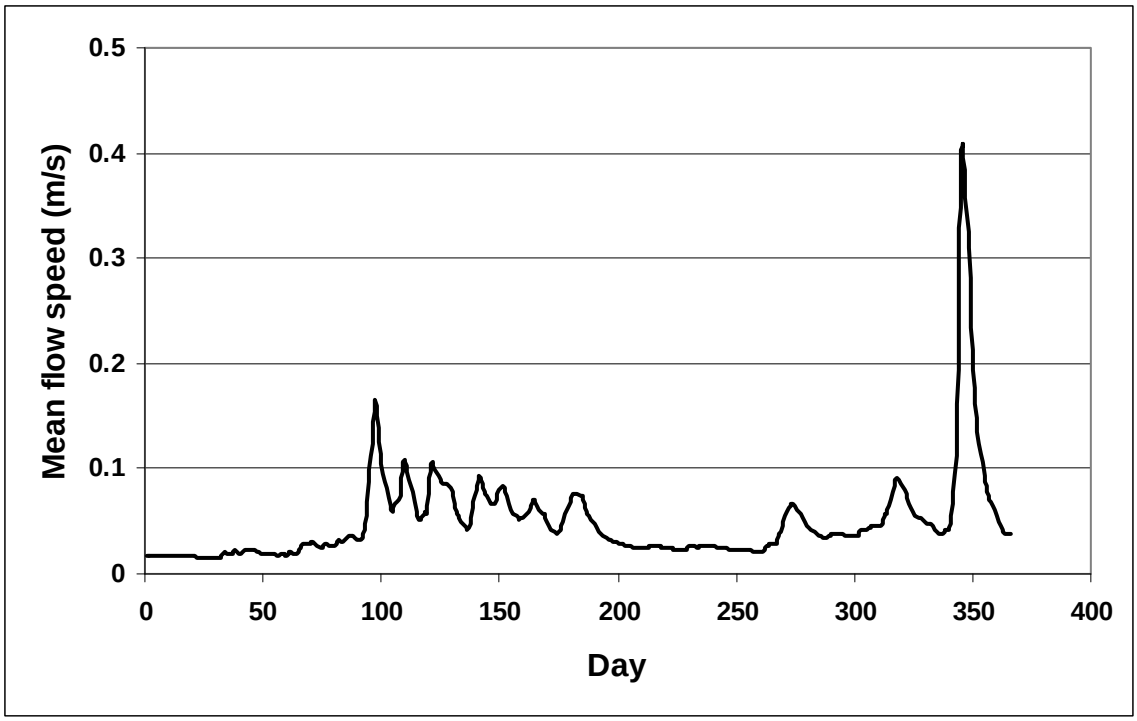

Figure 30. Mean flow speed at the river site (the only site for which historical data were available) for all days between May 2007 and April 2008.

the two sites were extremely low. Bottom shear stress (τ_b) only exceeded the critical stress for threshold motion at the absolute highest flows of 0.4 m/s and above (*Table 11*). Even in these cases, however, resuspenion of both sediment and associated bacteria were minimal. If the mean values for enterococci densities in sediment were used for each site, resuspenion was negligible at all flow speeds, resulting in suspended bacterial loads that were several orders of magnitude below what would be necessary to affect water quality monitoring. Even if the maximum observed densities were used at each site, the highest bacterial load that was predicted from resuspension was an increase of 1 CFU/100 mL at the river site, and this only occurred under an extremely high theoretical flow speed that was never actually observed (*Table 11*).

Examination of the wind records for the entire year showed wind from all directions during some part of the year, with the most dominant directions being easterly and northeasterly, and smaller peaks in frequency coinciding with northwesterly and southerly winds (*Figure 31*). In total, winds were considered wave-generating approximately 45% of the time for the lake site and 16% of the time for the upper bay site. In general, wind speeds were predominantly below 20-25 km/hr, with occasional records of higher speeds, up to maxima at each site of approximately 60 km/hr. On average, the lake site received slightly higher onshore wind speeds than the upper bay site, as judged by its relatively broader histogram shape and higher percentile values (*Figure 32 and Figure* 33, *Table 12*). The resulting predictions for wave generation, however, varied between the two sites due to the different fetch lengths, which were

Table 11. Values for key hydrodynamic parameters and resuspension of sediment and associated bacteria estimated from stream flow data at the large stream and river sites. U = mainstream water flow speed; τ_{cr} = threshold bottom shear stress for sediment motion; τ_b = estimated bottom shear stress; sediment resuspended = dry weight of sediment in suspension per m^2 area; sediment bacteria = FIB density in the sediment (the two different values used represent the mean and the maximum observed at each site); and bacteria resuspended = concentration of FIB added to water column through resuspension.

Site	**Water depth (m)**	**Temp (°C)**	U **(m/s)**	**Bedforms**	C_D	τ_{cr} **(N/m^2)**	τ_b **(N/m^2)**	**Sediment Resuspended (kg/m^2)**	**Sediment bacteria (CFU/100 g)**	**Bacteria resuspended (CFU/100 mL)**
Large Stream	1.4	23	0.32	ripples	$8.07 \cdot 10^{-3}$	0.16	0.16	0.0	0.0	0.0
			0.40	ripples	$8.07 \cdot 10^{-3}$	0.16	0.24	$2.4 \cdot 10^{-4}$	$1.5 \cdot 10^{3}$	$3.4 \cdot 10^{-4}$
									$6.8 \cdot 10^{4}$	$1.6 \cdot 10^{-2}$
			0.50	ripples	$8.07 \cdot 10^{-3}$	0.16	0.38	$6.5 \cdot 10^{-3}$	$1.5 \cdot 10^{3}$	$9.3 \cdot 10^{-3}$
									$6.8 \cdot 10^{4}$	$4.3 \cdot 10^{-1}$
River	1.9	23	0.25	ripples	$7.07 \cdot 10^{-3}$	0.14	0.08	0.0	0.0	0.0
			0.40	ripples	$7.07 \cdot 10^{-3}$	0.14	0.20	$6.3 \cdot 10^{-5}$	$9.1 \cdot 10^{2}$	$4.2 \cdot 10^{-5}$
									$1.3 \cdot 10^{5}$	$5.9 \cdot 10^{-3}$
			0.50	ripples	$7.07 \cdot 10^{-3}$	0.14	0.32	$2.3 \cdot 10^{-3}$	$9.1 \cdot 10^{2}$	$1.5 \cdot 10^{-3}$
									$1.3 \cdot 10^{5}$	$2.1 \cdot 10^{-1}$

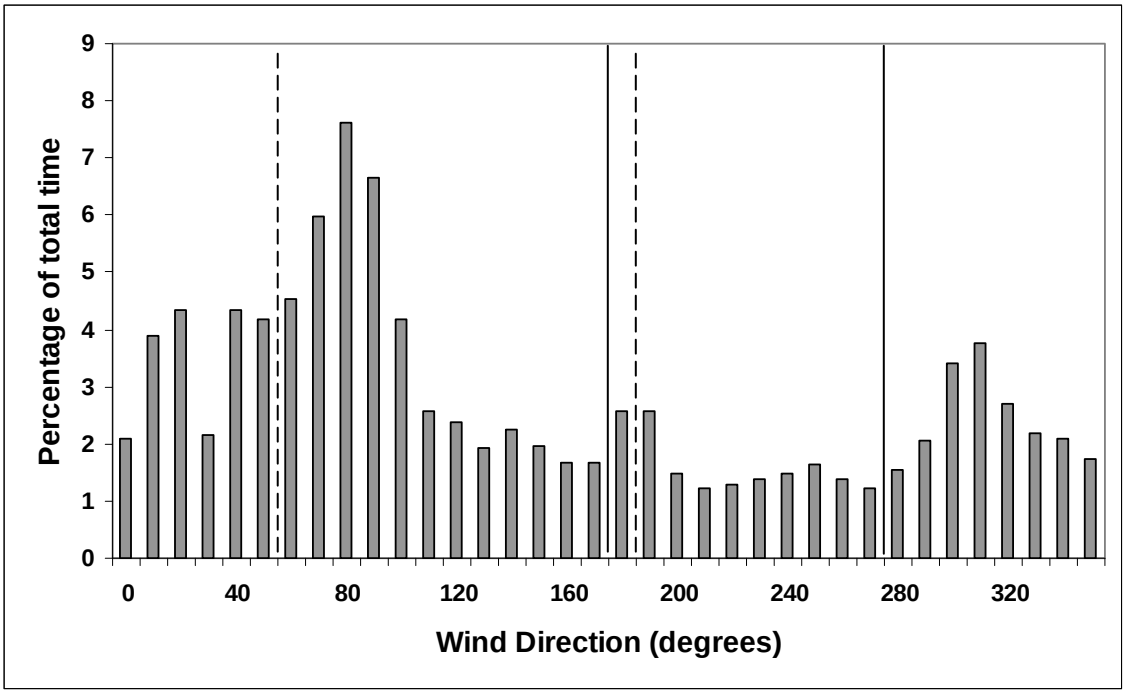

Figure 31. Frequency histogram of wind direction (grouped in bins of 10 degrees) for all available wind records at the port of Tampa between May 2007 and April 2008. The solid vertical lines represent the records considered to be onshore winds for the upper bay site (between 180 and 270 degrees) and the dashed lines represent the limits for the lake site (between 60 and 190 degrees).

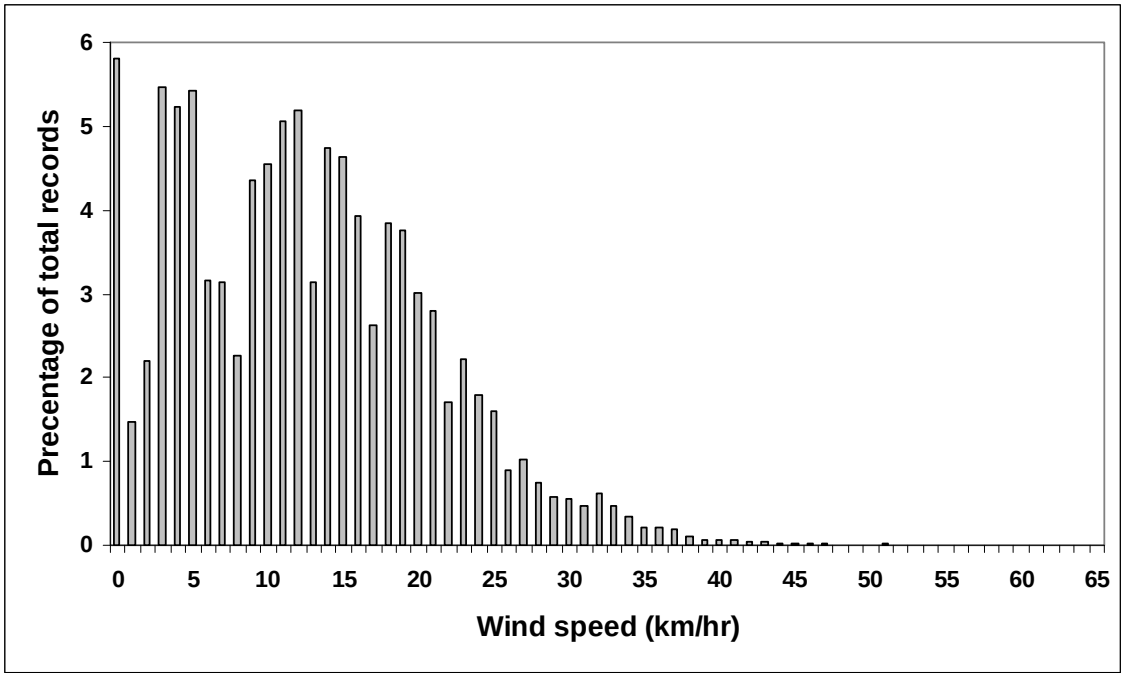

Figure 32. Frequency histogram of wind speed (grouped in 1 km/hr categories) for all wind records that were onshore for the upper bay site (between 180 and 270 degrees) between May 2007 and April 2008.

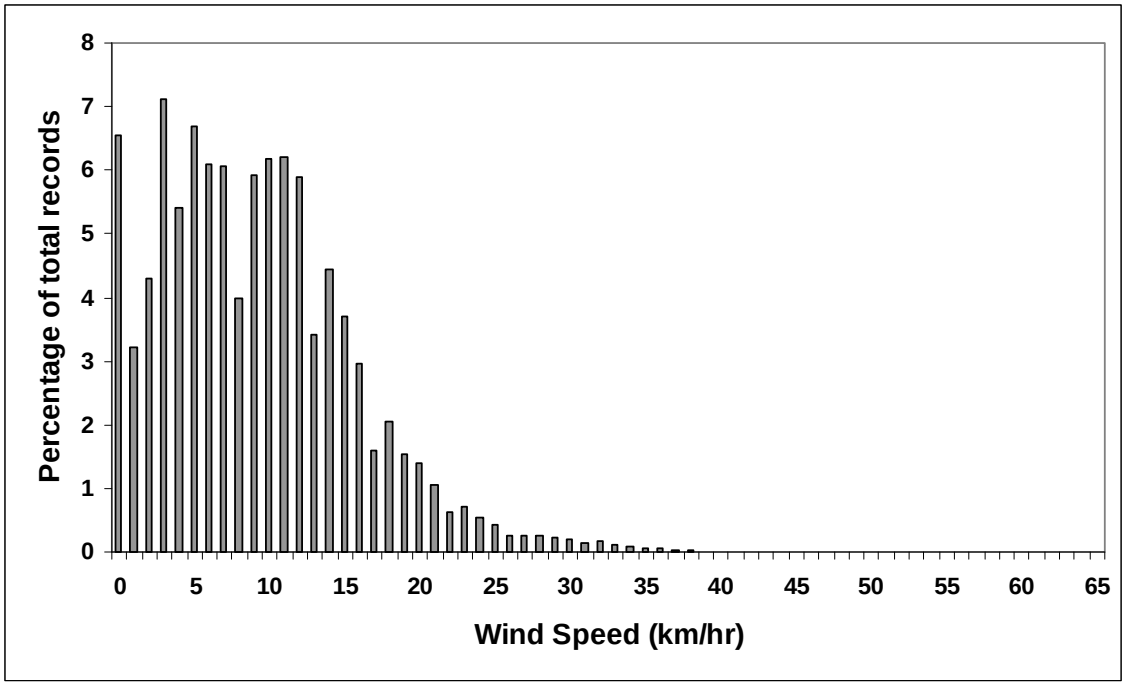

Figure 33. Frequency histogram of wind speed (grouped in 1 km/hr categories) for all wind records that were onshore for the lake site (between 60 and 190 degrees) between May 2007 and April 2008.

Table 12. Values for key hydrodynamic and bedform parameters, and estimates for resuspension of sediment and associated bacteria at the lake and upper bay sites. FIB densities (CFU/100 g) at the lake site: mean = $6.6 \cdot 10^2$ and max = $1.5 \cdot 10^4$. FIB densities (CFU/100 g) at the upper bay site: mean = $9.3 \cdot 10^2$ and max = $2.9 \cdot 10^3$. τ_{cr} = 0.14 N/m^2 at the lake site and 0.15 N/m^2 at the upper bay site; Δ_r = amplitude of sand ripples; λ_r = wavelength of sand ripples; τ_b = estimated bottom shear stress; sediment resuspended = dry weight of sediment in suspension per m^2 area; and bacteria resuspended = concentration of FIB added to water column through resuspension.

	Wind frequency (%)	**Wind Speed (km/hr)**	**Δ_r (cm)**	**λ_r (cm)**	**τ_b (N/m^2)**	**Sediment suspended (kg/m^2)**	**Bacteria resuspended (CFU/100 mL)**	
							mean	**max**
Lake	max	61	1.2	7.3	0.61	$1.6 \cdot 10^{-1}$	$1.5 \cdot 10^{-1}$	3.3
	0.2	35	0.7	3.7	0.24	$8.4 \cdot 10^{-3}$	$7.7 \cdot 10^{-3}$	$1.7 \cdot 10^{-1}$
	0.5	32	0.6	3.5	0.22	$6.0 \cdot 10^{-3}$	$5.5 \cdot 10^{-3}$	$1.2 \cdot 10^{-1}$
	1	29	0.5	2.7	0.19	$2.9 \cdot 10^{-3}$	$2.6 \cdot 10^{-3}$	$5.8 \cdot 10^{-2}$
	2	26	0.4	2.3	0.17	$1.9 \cdot 10^{-3}$	$1.7 \cdot 10^{-3}$	$3.8 \cdot 10^{-2}$
	5	21	0.3	1.8	0.13	0.0	0.0	0.0
	10	18	0.3	1.5	0.12	0.0	0.0	0.0
	25	13	0.1	0.8	0.05	0.0	0.0	0.0
	50	9	0.1	0.5	0.04	0.0	0.0	0.0
Upper bay	max	59	2.7	19.8	1.22	1.37	1.8	5.5
	2	33	2.7	19.8	1.22	1.37	1.8	5.5
	5	28	2.4	18.3	1.29	1.19	1.6	4.8
	10	24	2.7	18.6	1.00	0.95	1.3	3.8
	25	19	2.7	16.9	0.74	0.51	0.7	2.7
	50	12	1.9	11.1	0.40	0.07	0.1	0.3

found to average approximately 12 km at the upper bay site and less than 1 km at the lake site (*Table 12*). As a result, even the strongest winds resulted in waves of only 0.3 m in height with a period of 1.3 s at the lake site, generating orbital velocities of 0.27 m/s. Once the wind speed dropped below the second percentile (< 26 km/hr) vales for wave height and orbital velocity were both below 0.1 m and m/s, respectively. In contrast, all of the wind records at the upper bay site down to the second percentile (> 33 km/hr, occurring on 27 different days in the year) were predicted to generate waves of the maximum possible height and period possible for the 1 m depth , resulting in relatively high orbital velocities of 0.53 m/s. Even wind speeds down to 12 km/hr (occurring 50% of the total time, at least once on 159 days of the year) generated modest orbital velocities of 0.22 m/s and above (*Table 12*).

Regardless of the wind speed or orbital velocity, however, predicted bacterial resuspension due to wind-generated waves at the lake site was found to be negligible. Wind speeds below 26 km/hr (which occurred 98% of the time) failed to even initiate sediment motion. Even at the highest wind speed, and assuming the highest observed sediment densities of enterococci, an increased bacterial load of only 3 CFU/100 mL was predicted (*Table 12*). Resuspension at lower wind speeds was several orders of magnitude below any level that might have a significant impact on concentrations in the water column. At the bay site, the wave action was found to generate much more sediment resuspension on a regular basis, often one to two orders of magnitude more than the lake site at a given wind speed. Significant resuspension of sediments even occurred

water column. At the bay site, the wave action was found to generate much more sediment resuspension on a regular basis, often one to two orders of magnitude more than the lake site at a given wind speed. Significant resuspension of sediments even occurred at wind speeds as low as 19 km/hr, which occurred on approximately 30% of the total days during the year. However, the relatively low mean and maximum densities of enterococci observed in the sediments at that site still caused predictions of resuspended bacteria to be very low. The resuspension of bacteria at the upper bay site was predicted to typically result in an increase of only 1-5 CFU/100mL at this site (*Table 12*).

Discussion

Much attention has been paid to the potential for FIB that persist in aquatic sediments and vegetation to be resuspended into the water column and thereby negatively impact their reliable use as an indicator of fecal pollution (Solo-Gabriele et al., 2000; Grant et al., 2001; Whitman et al., 2003; Anderson et al., 2005; Ishii and Sadowsky, 2008). Unfortunately, the historical tendencies for collecting and reporting data on densities of FIB in water, sediment, and SAV have made the analysis of their impact on water quality difficult. By quantifying key habitat characteristics at my research sites, and utilizing historical data and theoretical equations for sediment resuspension, in this study I was able to directly examine the relative size of benthic reservoirs of enterococci in the Tampa Bay watershed and determine their potential to significantly affect water column concentrations. The results can be summarized in four key findings. Firstly, SAV, even at highly vegetated sites, always harbored the smallest percentage (between 0% and 18%)

of the total number enterococci in the system and is predicted to have minimal potential to affect concentrations in the water column. Secondly, sediment can harbor a relatively large fraction of the total reservoir of enterococci, depending on the water depth, FIB densities, and the depth of sediment containing FIB at each site. Thirdly, a simple modeling exercise shows the potential for the relative size of sediment and water column enterococci populations to shift dramatically as key habitat characteristics, such as water depth and SAV bottom coverage vary in space and time. And finally, realistic estimates of sediment resuspension illustrate that, even though the reservoir of sediment-associated enterococci may be numerically dominant, current and wave conditions in Tampa Bay and the surrounding watershed would rarely, if ever, cause an amount of sediment resuspension sufficient to significantly affect water column concentrations of FIB.

SAV as a Reservoir

One of the most interesting results of the habitat characterization was to discover the relatively small and unimportant (at least numerically) proportion of total enterococci in the habitat that were found in SAV. This result was unexpected considering that the sites in this study were specifically chosen for their high vegetated biomass and that high FIB densities in SAV on a per gram basis have been consistently reported in this study (*Figure 12*) and others (Whitman et al., 2003; Olapade et al., 2006). This discrepancy illustrates the importance of properly considering how FIB densities are normalized and compared before judging the relative importance of environmental reservoirs of FIB. Even though the traditional mass unit (1 g or 100 g) of SAV typically contains orders of

magnitude greater densities of enterococci than the traditional volume unit (100 mL) of water, there are typically orders of magnitude more volume units of water than mass units of SAV in a square meter of aquatic habitat. At all of the sites in this study, the sheer volume of water in the entire system, relative to the mass of SAV, more than offset the high enterococci densities in SAV, making it a minor reservoir in the vast majority of samples.

It is important to note, however, that I am discussing *numerical* importance – the potential for a large number of FIB associated with one substrate to shift to another substrate and significantly change the density in the new substrate (e.g., bacteria being released from SAV into the water and affecting water column concentrations.) I am not implying that SAV are generally unimportant as a refuge for FIB. As others have suggested, SAV may also serve an important role as a substrate for FIB growth, not just persistence (Byappanahalli et al., 2003b; Ksoll et al., 2007). In this case, we would need to be able to compare the rates of FIB growth on SAV and flux to the water column to rates of mixing and dilution within the water column. If the rates are sufficiently high, FIB in SAV could have significant effects on FIB concentrations in the water column, even though SAV is a proportionally minor reservoir at any given instant. Unfortunately, although the above studies have reported growth of FIB on SAV, a lack of detailed estimates of growth kinetics and flux rates between SAV and the water column prohibit any realistic estimates of the importance of this process. More data on FIB growth in SAV will be highly valuable in predicting the importance of SAV as a reservoir of FIB

and the impact of SAV on the success of using FIB to determine risks to human health in environmental waters.

Sediment as a Reservoir

My calculations show that sediment is potentially a much more important reservoir of enterococci than SAV at these sites. Depending on the site conditions, the relative proportion of enterococci in the sediments might be much higher than, approximately equal to, or much lower than that found in the water column. The outcome at a given site was largely driven by the relative FIB densities in sediments and water, as well as the depths of the water column and the cutoff depth of the sediments that harbor FIB. In other words, the relative volume of water and habitable sediments in an aquatic system, combined with the relative densities of FIB contained in each, typically determined which substrate held the largest population. This was particularly true at the freshwater sites, where changes in water depth could quickly alter the relative volumes such that a shift in dominance between the sediment and waterborne populations could be observed. At depths approaching 1m and above, the volume of the water column typically became large enough to cause it to hold the dominant proportion of enterococci in a given freshwater site.

It is important to consider that changing water depths at a given site will also probably be accompanied by changes in many of these other values, depending on the time scale. Longer term changes in water depth (weeks to seasons) at a given site would be

accompanied by long-term changes in FIB densities, SAV biomass, and possibly the depth of FIB colonization in the sediments. But even short-term (hours to days) changes in water depth at freshwater sites that occur as a result of recent rain events would likely be accompanied by changes in FIB concentrations in the water column. For example, FIB concentrations in the water tend to increase after a rain event as a result of runoff from non-point sources on land or stormwater systems (Reeves et al., 2004; Ahn et al., 2005; Brownell et al., 2007). However, because these sources are finite, other studies have observed increased FIB concentrations only during the early periods of rain event (i.e, the rising limb of the storm hydrograph), followed by relatively lower concentrations once the system has been flushed for some time (McDonald et al., 1982; Nagels et al., 2002; Muirhead et al., 2004; Jamieson et al., 2005). In either case, it is clear that estimating changes in FIB concentration that correlate with water depth are highly dependent on several spatial and temporal factors. In my approach, I believe that using the mean values for such estimates gives at least a good first order approximation of the importance of sediments as FIB reservoirs.

At the estuarine sites, sediments were consistently found to contain the numerically dominant enterococci population, even when water depth was allowed to vary over the entire tidal cycle, up to the deepest depths of ~ 1.5 m. This dominance seems to be driven by the relatively deep colonization of the sediments (9 – 15 cm before a log unit decay in density) and the low concentrations found in the water column (typically well below 10^2 CFU/100 mL). Data regarding the depth distribution of FIB in sediments is

sparse and highly variable. Whitman et al. (2006) reported significant colonization (defined similarly as in this study) of *E. coli* in Lake Michigan beach sands to deeper depths, down to about 30 cm. Meanwhile, enterococci concentrations in marsh sediments along the California coastline were significant only in the top 1 cm (Grant et al., 2001). While many factors may affect depth distributions of culturable FIB (e.g., wave or current action, organic content, sediment grain size), it is clear that such estimates are critical to improving attempts at modeling fate and transport of FIB in receiving waters. For example, in areas where FIB are limited only to the shallowest sediment depths (such as the small stream site in this study) care must be taken not to overestimate the total number of FIB present in the sediments and available for resuspension and transport.

Predicting Reservoir Shifts

The model developed for predicting shifts in relative population sizes at a given site that result from changing habitat or microbial conditions proved to be a valuable and illustrative tool. It is important to note that sufficient data are not available to use this model to precisely predict water quality conditions at a particular point in space and time, nor was that the intent behind its development. Rather, my intent was to use it as a tool to look at broad shifts in relative sizes of FIB populations among the substrates. By doing so, it helped to determine particular ranges of conditions at a given site where the numbers of FIB bacteria in the benthos are large relative to those in the water column, and thus represent a potentially important reservoir in the system that could affect water quality monitoring. For example, as explained above, one of the initial conclusions that

became evident from the habitat characterization was that SAV rarely, if ever, was predicted to be a numerically dominant reservoir of FIB at any of the sites. Through the use of the model at the large stream site, I was able to determine that, at the typical FIB densities for this site, SAV-associated populations of FIB would only be significantly larger than water column populations at the very shallowest depths (<15 cm) and with a high percentage of vegetated bottom cover. Outside of these conditions, and barring a drastic change in relative FIB densities, SAV-associated enterococci would simply not be present in dominant enough numbers in the system to drastically affect water column concentrations, no matter what proportion might be resuspended. Furthermore, even when the population in SAV exceeds that in the water, both of them are dwarfed by the proportion found in the sediments, suggesting that the role of SAV as a reservoir is still relatively minor in comparison.

In terms of sediments, the model offers a similarly valuable perspective on determining conditions under which bacteria-laden sediments will at least have the potential to affect waterborne concentrations at a given site. As stated above, the relative sizes of FIB populations in water and sediments at many of the sites were highly dependent on the depths of water and sediment available to FIB as substrate. And as was just discussed, the use of the model allowed a rough approximation of where this transition takes place (dominance shifting from sediments to the water) at a given site under a given set of conditions. Such determination gives a much better idea of what percentage of the time bacteria-laden sediments may potentially impact water quality monitoring at a particular

site of concern. Regardless of the densities that may be present in sediments, if there are not sufficient numbers to dramatically affect the concentration in water, they may be of little concern, at least in terms of determining the fate and transport of FIB. (The presence of pathogens also possibly persisting in sediments is, of course, another question altogether.)

Even at the upper bay site, where the sediment-associated population was always dominant under average conditions, the model was used to show that, in certain conditions, this may not always hold true. However, even more interestingly, the model illustrates that there are times when a shift in dominance may occur not only on longer time scales (as relative concentrations of FIB change), but even on very short time scales (as tidal depth changes). Although short-term variability (i.e., hours) has been shown to occur in populations of FIB both in experimental chambers (Desmarais et al., 2002; Chapter 2) and in the field (Boehm, 2007), it is not at all well understood. Short-term variability in habitat conditions will also affect the relative importance of various FIB populations in environmental waters, and these dynamics associated with habitat variability may even exacerbate the inherent population dynamics of the FIB themselves.

Resuspension of FIB

The lack of significant resuspension of bacteria predicted for any of the sites using flow and wind data was a surprising and important conclusion of this study. The theoretical approach to sediment suspension worked well at these sites for at least two reasons.

Firstly, all of the sites in this study, even the inland freshwater sites, were dominated by well sorted quartz sand grains with very little organic content. The mechanics of resuspension of non-cohesive sediments are much better understood and theoretical predictions tend to be simpler and more accurate than for the organic rich, cohesive sediments found in many other areas. Secondly, the hydrodynamics at each site is predominantly driven by only one process (either unidirectional current or wind-generated waves), which could be estimated from available historical data with relative ease. As a result, we were able to obtain realistic estimates for suspended sediment concentrations at four of the sites over an entire range of hydrodynamic conditions during the sampling year. By coupling these estimates with the frequency with which they occurred during the year, I was able to generate an in-depth picture of how the resuspension of sediments laden with FIB might affect the apparent microbial water quality at a range of sites around the Tampa Bay watershed.

There are several reasons that may explain the discrepancy between our results and those of other studies in which resuspension from bottom sediments was found to be a significant source of FIB in the water column. Firstly, sediment resuspension is highly dependent on bottom shear stresses and sediment grain size, which can vary widely between different habitats and different hydrodynamic regimes (Soulsby, 1998). For example, the upper bay and lake sites, like most inshore waters along the Florida Gulf coast, are highly fetch and depth limited, which prohibits the generation of large waves. As a result, bottom shear stresses are more limited at these sites than at sites from other

studies, such as Lake Michigan and California beaches, that may experience much steeper bottom slopes and larger wave action (Whitman and Nevers, 2003; Whitman et al., 2006; Yamahara et al., 2007). And similarly, the non-cohesive quartz sand found at all of the sites in this study responds very differently to hydrodynamic forces than the much finer, cohesive silt and mud found beneath many freshwater streams and estuarine marshes (Grant et al., 2001; Jamieson et al., 2005; Sanders et al., 2005), which may also account for much of the difference.

Secondly, based upon the data from our previous sampling efforts, we were able to use realistic data regarding CFU densities in water and sediments at all of our sites. On average, the values in the sediment (CFU/100 g) were only about 1 to 1.5 orders of magnitude higher than those in the water column (CFU/100 mL) (Chapter 3). As a result, our sites exhibited a relatively less concentrated reservoir of sediment-associated bacteria than other modeling efforts have assumed. For example, in what is probably the most intensive modeling effort looking at the role of sediments in affecting the fate and transport of FIB, Bai and Lung (2005) apply their model to a case study conducted by Muirhead et al. (2004) looking at resuspension in a stream bed during experimental flood stages. In their model, they assume an initial FIB concentration of 10^8 CFU / m^2 in the sediments and 0 CFU in the water. While these input conditions result in their model fitting that particular case study very well, this represents densities of sediment-associated bacteria that were approximately two orders of magnitude greater than what was found on average at the sites in this study. And while sediment densities did

approach these levels during three different sampling events in our study (once at each of the stream sites and once at the river site), they occurred when water column concentrations were already well in exceedance of the regulatory limit, suggesting that further resuspension was no longer as great a concern as it would be at less-polluted sites. In order for a water quality exceedance to be caused by sediment resuspension alone at any of the sites in this study, I calculated that, even at the maximum predicted water flows, the sediment concentrations would have to be at least 5×10^4 or 5×10^5 CFU/100 g at the upper bay and lake sites, respectively, and approximately 10^7 CFU/100 g at the stream and river sites. These densities were never seen at any of these sites, regardless of the accompanying water column concentration.

A final factor that is important to consider regarding conclusions about the importance of FIB resuspension is the water depth upon which the study focuses. In this study, I chose to model the site with water depths of 1-2 m because that is the true nature of the sites from which the original CFU data were collected. Furthermore, predicting sediment resuspension at significantly shallower depths, primarily where wave breaking and wave swash are involved, introduce an entirely new level of hydrodynamic complexity which is beyond the scope of this study. However, a very rough estimation of FIB resuspension in shallow water can be made from the data in this study, illustrating that there is potential for sediment-associated FIB to dominate water column concentrations, particularly at the upper bay site. If we assume suspended sediment loads that are similar to those predicted in *Table 12*, but constrain the water to a much shallower depth that is

typical of the shoreline swash zone (e.g., 10-20 cm), the resulting bacterial load would be much more highly concentrated and becomes significant when compared to the regulatory standard of 104 CFU/100 mL (e.g., concentrating the same number of bacteria for the highest wind speeds at the upper bay site into 10 cm water depth results in an increase in enterococci concentrations of approximately 55 CFU / 100 mL). Therefore, in shallow depths along the extreme shoreline (which is where water quality samples are often taken in practice), we can not rule out the possibility that resuspension from sediments may cause elevated waterborne concentrations of FIB and potentially incorrect conclusions regarding microbial water quality. However, it is important to remember that such concentration of bacteria-laden sediments in extremely shallow depths is likely to be quickly mixed by wave action into the deeper water column and diluted to insignificant levels (Boehm, 2003; Boehm et al., 2005; Yamahara et al., 2007).

Although I found SAV to be an insignificant reservoir of FIB in this study and omitted it from my resuspension estimates, that may not be the case at all sites. There are some key differences that the presence of SAV would have on resuspension. Firstly, SAV-associated bacteria would not be easily modeled by sediment resuspension, as they could only be resuspended as a result of mechanical shearing of the bacteria or the particles to which they are attached (e.g., detritus or epiphytic algae) from the surface of the SAV. Obviously, the physics of this process are likely to be quite different from sediment resuspension and, to my knowledge, have never been examined theoretically or experimentally. Furthermore, in vegetated habitats the macrophytes interact with the

overlying flow to create increased turbulence in the water column and in the upper levels of the submerged canopy (Gambi et al., 1990; Ikeda and Kanazawa, 1996; Ghisalberti and Nepf, 2002), which can alter the shear stress and likely also bacterial resuspension into the water column as compared to sediment alone. This effect would make the hydrodynamics that affect the SAV, and also any sediment beneath it, much more complex and difficult to model.

Finally, it is important to note that weather and hydrology are not the only drivers of sediment resuspension, especially in waters of recreational importance. Recreational activities themselves can cause increased suspended sediment loads in the water column, particularly at the local scale. Swimming and wading activity, for example, has been found to correlate with periods of high FIB concentrations in recreational waters (Cheung et al., 1991; Crabill et al., 1999; Phillip et al., 2009). While the users themselves may act as a source (Elmir et al., 2007), it is also likely that they are disturbing FIB-laden sediments and causing resuspension into the water column, as was found experimentally by Phillip et al. (2009). Furthermore, it is widely known that recreational boating, particularly in shallow waters, can resuspend sediment from both the bottom (Garrad and Hey, 1987; Beachler and Hill, 2003; Lenzi et al., 2005) and the shoreline (Bauer et al., 2002) of water bodies, although it may not always be a significant amount relative to natural mechanisms of resuspension such as waves and currents (Anthony and Downing, 2003). However, if areas of high boat traffic overly sediments with high concentrations of FIB, this may constitute a significant source of FIB in the water column (An et al.,

2002). Resuspension of sediments from boats is most likely to be a bigger factor in areas with fine, cohesive sediments (< 60 μM) and with larger boats traveling below planing speeds (Gucinski, 1982; Beachler and Hill, 2003).

LITERATURE CITED

Aarestrup, F.M., Butaye, P., and Witte, W. (2002) Nonhuman reservoirs of enterococci. In *The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance*. Gilmore, M.S., Clewell, D.B., Courvalin, P., Dunny, G.M., Murray, B.E., and Rice, L.B. (eds). Washington, D. C.: ASM Press, pp. 55-99.

Ahn, J.H., Grant, S.B., Surbeck, C.Q., Digiacomo, P.M., Nezlin, N.P., and Jiang, S. (2005) Coastal water quality impact of stormwater runoff from an urban watershed in southern California. *Environmental Science & Technology* **39**: 5940-5953.

Allen, L.A., Grindley, J., and Brooks, E. (1953) Some chemical and bacterial characteristics of bottom deposits from lakes and estuaries. *Journal of Hygiene* **51**: 185-194.

An, Y.J., Kampbell, D.H., and Breidenbach, G.P. (2002) Escherichia coli and total coliforms in water and sediments at lake marinas. *Environmental Pollution* **120**: 771-778.

Anderson, I.C., Rhodes, M., and Kator, H. (1979) Sublethal Stress in *Escherichia coli* - function of salinity. *Applied and Environmental Microbiology* **38**: 1147-1152.

Anderson, K.L., Whitlock, J.E., Harwood, V.J., and K (2005) Persistence and differential survival of fecal indicator bacteria in subtropical waters and sediments. *Applied and Environmental Microbiology* **71**: 3041-3048.

Anderson, M.A., Whitlock, J.E., and Harwood, V.J. (2006) Diversity and distribution of *Escherichia coli* genotypes and antibiotic resistance phenotypes in feces of humans, cattle, and horses. *Applied and Environmental Microbiology* **72**: 6914-6922.

Anderson, S.A., Turner, S.J., and Lewis, G.D. (1997) Enterococci in the New Zealand environment: Implications for water quality monitoring. *Water Science and Technology* **35**: 325-331.

Anthony, J.L., and Downing, J.A. (2003) Physical impacts of wind and boat traffic on Clear Lake, Iowa, USA. *Lake and Reservoir Management* **19**: 1-14.

APHA (1905) *Standard Methods for the Examination of Water and Wastewater*. Washington, D. C.: American Public Health Association.

APHA (1998) *Standard Methods for the Examination of Water and Wastewater*. Washington, D. C.: American Public Health Association.

Auer, M.T., and Niehaus, S.L. (1993) Modeling Fecal-Coliform Bacteria .1. Field and Laboratory Determination of Loss Kinetics. *Water Research* **27**: 693-701.

Bai, S., and Lung, W.S. (2005) Modeling sediment impact on the transport of fecal bacteria. *Water Research* **39**: 5232-5240.

Bauer, B.O., Lorang, M.S., and Sherman, D.J. (2002) Estimating boat-wake-induced levee erosion using sediment suspension measurements. *Journal of Waterway Port Coastal and Ocean Engineering-Asce* **128**: 152-162.

Beachler, M.M., and Hill, D.F. (2003) Stirring up trouble? Resuspension of bottom sediments by recreational watercraft. *Lake and Reservoir Management* **19**: 15-25.

Bell, S.S., Tewfik, A., Hall, M.O., and Fonseca, M.S. (2008) Evaluation of seagrass planting and monitoring techniques: Implications for assessing restoration success and habitat equivalency. *Restoration Ecology* **16**: 407-416.

Bernhard, A.E., and Field, K.G. (2000) A PCR assay to discriminate human and ruminant feces on the basis of host differences in *Bacteroides-Prevotella* genes encoding 16S rRNA. *Applied and Environmental Microbiology* **66**: 4571-4574.

Beversdorf, L.J., Bornstein-Forst, S.M., and McLellan, S.L. (2007) The potential for beach sand to serve as a reservoir for *Escherichia coli* and the physical influences on cell die-off. *Journal of Applied Microbiology* **102**: 1372-1381.

Boehm, A.B. (2003) Model of microbial transport and inactivation in the surf zone and application to field measurements of total coliform in Northern Orange County, California. *Environmental Science & Technology* **37**: 5511-5517.

Boehm, A.B. (2007) Enterococci concentrations in diverse coastal environments exhibit extreme variability. *Environmental Science & Technology* **41**: 8227-8232.

Boehm, A.B., Shellenbarger, G.G., and Paytan, A. (2004) Groundwater discharge: potential association with fecal indicator bacteria in the surf zone. *Environmental Science & Technology* **38**: 3558-3566.

Boehm, A.B., Keymer, D.P., and Shellenbarger, G.G. (2005) An analytical model of enterococci inactivation, grazing, and transport in the surf zone of a marine beach. *Water Research* **39**: 3565-3578.

Boehm, A.B., Griffith, J.F., Mcgee, C., Edge, T.A., Solo-Gabriele, H.M., Whitman, R.L. et al. (2009) Fecal indicator bacteria enumeration in beach sand: a comparison study of extraction methods in medium to coarse sands. *Journal of Applied Microbiology*.

Bonadonna, L., Briancesco, R., Ottaviani, M., and Veschetti, E. (2002) Occurrence of Cryptosporidium oocysts in sewage effluents and correlation with microbial, chemical and physical water variables. *Environmental Monitoring and Assessment* **75**: 241-252.

Bonde, G.J. (1967) Pollution of a marine environment. *Journal Water Pollution Control Federation* **39**: R45-&-R45-&.

Bonilla, T.D., Nowosielski, K., Cuvelier, M., Hartz, A., Green, M., Esiobu, N. et al. (2007) Prevalence and distribution of fecal indicator organisms in South Florida beach sand and preliminary assessment of health effects associated with beach sand exposure. *Marine Pollution Bulletin* **54**: 1472-1482.

Brownell, M.J., Harwood, V.J., Kurz, R.C., McQuaig, S.M., Lukasik, J., and Scott, T.M. (2007) Confirmation of putative stormwater impact on water quality at a Florida beach by microbial source tracking methods and structure of indicator organism populations. *Water Research* **41**: 3747-3757.

Buckley, R., Clough, E., Warnken, W., and Wild, C. (1998) Coliform bacteria in streambed sediments in a subtropical rainforest conservation reserve. *Water Research* **32**: 1852-1856.

Burton, G.A., Gunnison, D., and Lanza, G.R. (1987) Survival of pathogenic bacteria in various fresh-water sediments. *Applied and Environmental Microbiology* **53**: 633-638.

Byappanahalli, M., Fowler, M., Shively, D., and Whitman, R. (2003a) Ubiquity and persistence of *Escherichia coli* in a Midwestern coastal stream. *Applied and Environmental Microbiology* **69**: 4549-4555.

Byappanahalli, M.N., and Fujioka, R.S. (1998) Evidence that tropical soil environment can support the growth of *Escherichia coli*. *Water Science and Technology* **38**: 171-174.

Byappanahalli, M.N., Shively, D.A., Nevers, M.B., Sadowsky, M.J., and Whitman, R.L. (2003b) Growth and survival of *Escherichia coli* and enterococci populations in the macro-alga Cladophora (Chlorophyta). *FEMS Microbiology Ecology* **46**: 203-211.

Byappanahalli, M.N., Richard, L.W., Shivelya, D.A., John, F., Ishii, S., and Sadowsky, M.J. (2007) Population structure of cladophora-borne *Escherichia coli* in nearshore water of lake Michigan. *Water Research* **41**: 3649-3654.

Cabelli, V.J., Dufour, A.P., McCabe, L.J., and Levin, M.A. (1982) Swimming-associated gastroenteritis and water quality. *American Journal of Epidemiology* **115**: 606-616.

Carson, C.A., Shear, B.L., Ellersieck, M.R., and Asfaw, A. (2001) Identification of fecal *Escherichia coli* from humans and animals by ribotyping. *Applied and Environmental Microbiology* **67**: 1503-1507.

Cheung, W.H., Chang, K.C., and Hung, R.P. (1991) Variations in microbial indicator densities in beach waters and health-related assessment of bathing water quality. *Epidemiology and Infection* **106**: 329-344.

Cheung, W.H., Chang, K.C., Hung, R.P., and Kleevens, J.W. (1990) Health effects of beach water pollution in Hong Kong. *Epidemiology and Infection* **105**: 139-162.

Crabill, C., Donald, R., Snelling, J., Foust, R., and Southam, G. (1999) The impact of sediment fecal coliform reservoirs on seasonal water quality in Oak Creek, Arizona. *Water Research* **33**: 2163-2171.

Craig, D.L., Fallowfield, H.J., and Cromar, N.J. (2004) Use of macrocosms to determine persistence of *Escherichia coli* in recreational coastal water and sediment and validation with *in situ* measurements. *Journal of Applied Bacteriology* **96**: 922-930.

Davies, C.M., and Evison, L.M. (1991) Sunlight and the survival of enteric bacteria in natural waters. *Journal of Applied Bacteriology* **70**: 265-274.

Davies, C.M., and Bavor, H.J. (2000) The fate of stormwater-associated bacteria in constructed wetland and water pollution control pond systems. *Journal of Applied Microbiology* **89**: 349-360.

Davies, C.M., Long, J.A.H., Donald, M., and Ashbolt, N.J. (1995) Survival of fecal microorganisms in marine and fresh-water sediments. *Applied and Environmental Microbiology* **61**: 1888-1896.

de Vaux, A., Laguerre, G., Divies, C., and Prevost, H. (1998) Enterococcus asini sp. nov. isolated from the caecum of donkeys (Equus asinus). *International Journal of Systematic Bacteriology* **48**: 383-387.

Del Rio-Rodriquez, R.E., Inglis, V., and Millar, S.D. (2008) Survival of *Escherichia coli* in the intestine of fish. *Aquaculture Research* **28**: 257-264.

Denny, M.W. (1988) *Biology and the Mechanics of the Wave-Swept Environment*. Princeton, NJ: Princeton University Press.

Desmarais, T.R., Solo-Gabriele, H.M., and Palmer, C.J. (2002) Influence of soil on fecal indicator organisms in a tidally influenced subtropical environment. *Applied and Environmental Microbiology* **68**: 1165-1172.

Dethier, M.N., Graham, E.S., Cohen, S., and Tear, L.M. (1993) Visual Versus Random-Point Percent Cover Estimations - Objective Is Not Always Better. *Marine Ecology-Progress Series* **96**: 93-100.

Devriese, L.A., Ceyssens, K., Rodrigues, U.M., and Collins, M.D. (1990) Enterococcus-Columbae, a Species from Pigeon Intestines. *Fems Microbiology Letters* **71**: 247-252.

Dombek, P.E., Johnson, L.K., Zimmerley, S.T., and Sadowsky, M.J. (2000) Use of repetitive DNA sequences and the PCR to differentiate *Escherichia coli* isolates from human and animal sources. *Applied and Environmental Microbiology* **66**: 2572-2577.

Doyle, J.D., Tunnicliff, B., Kramer, R., Kuehl, R., and Brickler, S.K. (1992) Instability of fecal-coliform populations in waters and bottom sediments at recreational beaches in Arizona. *Water Research* **26**: 979-988.

Elmir, S.M., Wright, M.E., Abdelzaher, A., Solo-Gabriele, H.M., Fleming, L.E., Miller, G. et al. (2007) Quantitative evaluation of bacteria released by bathers in a marine water. *Water Research* **41**: 3-10.

Englebert, E.T., McDermott, C., and Kleinheinz, G.T. (2008) Impact of the alga *Cladophora* on the survival of *E. coli*, *Salmonella*, and *Shigella* in a laboratory microcosm. *Journal of Great Lakes Research* **34**: 377-382.

Facklam, R.R., Carvahallo, M.S., and Teixeira, L.M. (2002) History, taxonomy, biochemical characteristics, biochemical charasteristics, and antibiotic susceptibility testing of enterococci. In *The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance*. Gilmore, M.S., Clewell, D.B., Courvalin, P., Dunny, G.M., Murray, B.E., and Rice, L.B. (eds). Washington, D. C.: ASM Press, pp. 1-54.

Ferguson, D.M., Moore, D.F., Getrich, M.A., and Zhowandai, M.H. (2005) Enumeration and speciation of enterococci found in marine and intertidal sediments and coastal water in southern California. *Journal of Applied Bacteriology* **99**: 598-608.

Field, K.G., and Samadpour, M. (2007) Fecal source tracking, the indicator paradigm, and managing water quality. *Water Research* **41**: 3517-3538.

Field, K.G., Bernhard, A.E., and Brodeur, T.J. (2003) Molecular approaches to microbiological monitoring: fecal source detection. *Environmental Monitoring and Assessment* **81**: 313-326.

Flint, K.P. (1987) The long-term survival of *Escherichia coli* in river water. *Journal of Applied Bacteriology* **63**: 261-270.

Fonseca, M. (1996) The role of seagrasses in nearshore sedimentary processes: a review. In *Estuarine Shores: Evolution, Environments and Human Alterations*. Nordstrom, K., and Roman, C.T. (eds). London: Wiley, pp. 261-286.

Fourqurean, J.W., Willsie, A., Rose, C.D., and Rutten, L.M. (2001) Spatial and temporal pattern in seagrass community composition and productivity in south Florida. *Marine Biology* **138**: 341-354.

Fujioka, R., Sian-Denton, C., Borja, M., Castro, J., and Morphew, K. (1999) Soil: the environmental source of *Escherichia coli* and enterococci in Guam's streams. *Journal of Applied Microbiology* **85**: 83S-89S-83S-89S.

Fujioka, R.S., and Shizumura, L.K. (1985) Clostridium-Perfringens, a Reliable Indicator of Stream Water-Quality. *Journal Water Pollution Control Federation* **57**: 986-992.

Fujioka, R.S., and Yoneyama, B.S. (2002) Sunlight inactivation of human enteric viruses and fecal bacteria. *Water Science and Technology* **46**: 291-295.

Gambi, M.C., Nowell, A.R.M., and Jumars, P.A. (1990) Flume observations on flow dynamics in *Zostera marina* (eelgrass) beds. *Marine Ecology Progress Series* **61**: 159-169.

Gannon, J.J., Busse, M.K., and Schillinger, J.E. (1983) Fecal coliform disappearance in a river impoundment. *Water Research* **17**: 1595-1601.

Gantzer, C., Maul, A., Audic, J.M., and Schwartzbrod, L. (1998) Detection of infectious enteroviruses, enterovirus genomes, somatic coliphages, and Bacteroides fragilis phages in treated wastewater. *Applied and Environmental Microbiology* **64**: 4307-4312.

Garrad, P.N., and Hey, R.D. (1987) Boat Traffic, Sediment Resuspension and Turbidity in a Broadland River. *Journal of Hydrology* **95**: 289-297.

Gaun, S.K., Xu, R.L., Chen, S., Odumeru, J., and Gyles, C. (2002) Development of a procedure for discriminating among *Escherichia coli* isolates from animal and human sources. *Applied and Environmental Microbiology* **68**: 2690-2698.

Geldreich, E.E., Clark, H.F., and Huff, C.B. (1964) A study of pollution indicators in a waste stabilization pond. *Journal Water Pollution Control Federation* **36**: 1372-1379.

Gelsomino, R., Vancanneyt, M., Cogan, T.M., and Swings, J. (2003) Effect of raw-milk cheese consumption on the enterococcal flora of human feces. *Applied and Environmental Microbiology* **69**: 312-319.

Gerba, C.P., and McLeod, J.S. (1976) Effects of sediments on survival of *Escherichia coli* in marine waters. *Applied and Environmental Microbiology* **32**: 114-120.

Ghisalberti, M., and Nepf, H.M. (2002) Mixing layers and coherent structures in vegetated aquatic flows. *Journal of Geophysical Research - Oceans* **107**.

Gordon, D.M., Bauer, S., and Johnson, J.R. (2002) The genetic structure of *Escherichia coli* populations in primary and secondary habitats. *Microbiology-Sgm* **148**: 1513-1522.

Gotelli, N.J., and Entsminger, G.L. (2009). EcoSim: null models software for ecology, version 7. URL http://garyentsminger.com/ecosim.htm.

Grant, S.B., Sanders, B.F., Boehm, A.B., Redman, J.A., Kim, J.H., Mrse, R.D. et al. (2001) Generation of enterococci bacteria in a coastal saltwater marsh and its impact on surf zone water quality. *Environmental Science & Technology* **35**: 2407-2416.

Griffin, D.W., Lipp, E.K., McLaughlin, M.R., and Rose, J.B. (2001) Marine recreation and public health microbiology: Quest for the ideal indicator. *Bioscience* **51**: 817-825.

Grimes, D.J. (1975) Release of sediment-bound fecal coliforms by dredging. *Applied Microbiology* **29**: 109-111.

Grimes, D.J. (1980) Bacteriological water quality effects of hydraulically dredging contaminated upper mississippi river bottom sediment. *Applied and Environmental Microbiology* **39**: 782-789.

Gucinski, H. (1982) Sediment Suspension and Resuspensoion From Small Craft Induced Turbulence. In. Washington, D. C.: U. S. Environmental Protection Agency, p. 61.

Hagedorn, C., Crozier, J.B., Mentz, K.A., Booth, A.M., Graves, A.K., Nelson, N.J., and Reneau, R.B. (2003) Carbon source utilization profiles as a method to identify sources of faecal pollution in water. *Journal of Applied Microbiology* **94**: 792-799.

Hansen, D.L., Clark, J.J., Ishii, S., Sadowsky, M.J., and Hicks, R.E. (2008) Sources and sinks of Escherichia coli in benthic and pelagic fish. *Journal of Great Lakes Research* **34**: 228-234.

Hansen, J.W., Udy, J.W., Perry, C.J., Dennison, W.C., and Lomstein, B.A. (2000) Effect of the seagrass *Zostera capricorni* on sediment microbial processes. *Marine Ecology Progress Series* **199**: 83-96.

Hardina, C.M., and Fujioka, R.S. (1991) Soil -- the environmental source of *Escherichia coli* and enterococci in Hawaii streams. *Environmental Toxicology and Water Quality* **6**: 185-195.

Harwood, V.J., Whitlock, J., and Withington, V. (2000) Classification of antibiotic resistance patterns of indicator bacteria by discriminant analysis: Use in predicting the source of fecal contamination in subtropical waters. *Applied and Environmental Microbiology* **66**: 3698-3704.

Harwood, V.J., Delahoya, N.C., Ulrich, R.M., Kramer, M.F., Whitlock, J.E., Garey, J.R., and Lim, D.V. (2004) Molecular confirmation of *Enterococcus faecalis* and *E. faecium* from clinical, faecal and environmental sources. *Letters in Applied Microbiology* **38**: 476-482.

Harwood, V.J., Levine, A.D., Scott, T.M., Chivukula, V., Lukasik, J., Farrah, S.R., and Rose, J.B. (2005) Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. *Applied and Environmental Microbiology* **71**: 3163-3170.

Heaney, C.D., Sams, E., Wing, S., Marshall, S., Brenner, K., Dufour, A.P., and Wade, T.J. (2009) Contact with beach sand among beachgoers and risk of illness. *American Journal of Epidemiology* **170**: 164-172.

Hood, M.A., and Ness, G.E. (1982) Survival of *Vibrio cholerae* and *Escherichia coli* in estuarine waters and sediments. *Applied and Environmental Microbiology* **43**: 578-584.

Howell, J.M., Coyne, M.S., and Cornelius, P.L. (1996) Effect of sediment particle size and temperature on fecal bacteria mortality rates and the fecal coliform/fecal streptococci ratio. *Journal of Environmental Quality* **25**: 1216-1220.

Ikeda, S., and Kanazawa, M. (1996) Three-dimensional organized vortices above flexible water plants. *Journal of Hydraulic Engineering* **122**: 634-640.

Ishii, S., and Sadowsky, M.J. (2008) *Escherichia coli* in the environment: Implications for water quality and human health. *Microbes and Environments* **23**: 101-108.

Ishii, S., and Sadowsky, M.J. (2009) Applications of the rep-PCR DNA fingerprinting technique to study microbial diversity, ecology and evolution. *Environmental Microbiology* **11**: 733-740.

Ishii, S., Ksoll, W.B., Hicks, R.E., and Sadowsky, M.J. (2006) Presence and growth of naturalized *Escherichia coli* in temperate soils from lake superior watersheds. *Applied and Environmental Microbiology* **72**: 612-621.

Ishii, S., Hansen, D.L., Hicks, R.E., and Sadowsky, M.J. (2007) Beach sand and sediments are temporal sinks and sources of *Escherichia coli* in lake superior. *Environmental Science & Technology* **41**: 2203-2209.

Jamieson, R.C., Joy, D.M., Lee, H., Kostaschuk, R., and Gordon, R.J. (2005) Resuspension of sediment-associated *Escherichia coli* in a natural stream. *Journal of Environmental Quality* **34**: 581-589.

Jeng, H.W.C., England, A.J., and Bradford, H.B. (2005) Indicator organisms associated with stormwater suspended particles and estuarine sediment. *Journal of Environmental Science and Health Part A-Toxic/Hazardous* **40**: 779-791.

Johnson, L.K., Brown, M.B., Carruthers, E.A., Ferguson, J.A., Dombek, P.E., and Sadowsky, M.J. (2004) Sample size, library composition, and genotypic diversity among natural populations of *Escherichia coli* from different animals influence accuracy of determining sources of fecal pollution. *Applied and Environmental Microbiology* **70**: 4478-4485.

Karjalainen, H., Stefansdottir, G., Tuominen, L., and Kairesalo, T. (2001) Do submersed plants enhance microbial activity in sediment? *Aquatic Botany* **69**: 1-13.

Kinzelman, J., McLellan, S.L., Daniels, A.D., Cashin, S., Singh, A., Gradus, S., and Bagley, R. (2004) Non-point source pollution: Determination of replication versus persistence of Escherichia coli in surface water and sediments with correlation of levels to readily measurable environmental parameters. *Journal of Water and Health* **2**: 103-114.

Kleinheinz, G.T., Coenan, A., Zehms, T., Preedit, J., Leewis, M.C., Becker, D., and McDermott, C. (2009) Effect of aquatic macrophytes on the survival of *Escherichia coli* in a laboratory microcosm. *Lake and Reservoir Management* **25**: 149-154.

Koeuth, T., Versalovic, J., and Lupski, J.R. (1995) Differential subsequence conservation of interspersed repetitive *Streptococcus pneumoniae* BOX elements in diverse bacteria. *Genome Research* **5**: 408-418.

Kogan, S.C., Doherty, M., and Gitschier, J. (1987) An improved method for prenatal diagnosis of genetic diseases by analysis of amplified DNA sequences -- Application to Hemophilia-A. *New England Journal of Medicine* **317**: 985-990.

Ksoll, W.B., Ishii, S., Sadowsky, M.J., and Hicks, R.E. (2007) Presence and sources of fecal coliform bacteria in epilithic periphyton communities of lake superior. *Applied and Environmental Microbiology* **73**: 3771-3778.

Laliberte, P., and Grimes, D.J. (1982) Survival of *Escherichia coli* in lake bottom sediment. *Applied and Environmental Microbiology* **43**: 623-628.

Lane, D.J. (1991) 16S/23S rRNA sequencing. In *Nucleic Acie Techniques in Bacterial Systematics*. Stackebrandt, E., and Goodfellow, M. (eds). Chichester, United Kingdom: Wiley, pp. 115-148.

Le Fevre, N.M., and Lewis, G.D. (2003) The role of resuspension in enterococci distribution in water at an urban beach. *Water Science and Technology* **47**: 205-210.

Le Roux, J.P. (2005) Grains in motion: A review. *Sedimentary Geology* **178**: 285-313.

Leclerc, H., Devriese, L.A., and Mossel, D.A.A. (1996) Taxonomical changes in intestinal (faecal) enterococci and streptococci: Consequences on their use as indicators of faecal contamination in drinking water. *Journal of Applied Bacteriology* **81**: 459-466.

Leclerc, H., Mossel, D.A.A., Edberg, S.C., and Struijk, C.B. (2001) Advances in the bacteriology of the Coliform Group: Their suitability as markers of microbial water safety. *Annual review of Microbiology* **55**: 201-234.

Lemarchand, K., and Lebaron, P. (2003) Occurrence of Salmonella spp. and Cryptosporidium spp. in a French coastal watershed: relationship with fecal indicators. *Fems Microbiology Letters* **218**: 203-209.

Lenzi, M., Finoia, M.G., Persia, E., Comandi, S., Gargiulo, V., Solari, D. et al. (2005) Biogeochemical effects of disturbance in shallow water sediment by macroalgae harvesting boats. *Marine Pollution Bulletin* **50**: 512-519.

Lleo, M.D., Tafi, M.C., and Canepari, P. (1998) Nonculturable *Enterococcus faecalis* cells are metabolically active and capable of resuming active growth. *Systematic and Applied Microbiology* **21**: 333-339.

Lopez, N.I., Duarte, C.M., Vallespinos, F., Romero, J., and Alcoverro, T. (1995) Bacterial activity in NW Mediterranean seagrass (*Posidonia oceanica*) sediments. *Journal of Experimental Marine Biology and Ecology* **187**: 39-49.

Lund, V. (1996) Evaluation of E-coli as an indicator for the presence of Campylobacter jejuni and Yersinia enterocolitica in chlorinated and untreated oligotrophic lake water. *Water Research* **30**: 1528-1534.

Malathum, K., Singh, K.V., Weinstock, G.M., and Murray, B.E. (1998) Repetitive sequence-based PCR versus pulsed-field gel electrophoresis for typing of *Enterococcus faecalis* at the subspecies level. *Journal of Clinical Microbiology* **36**: 211-215.

Manero, A., Vilanova, X., Cerda-Cuellar, M., and Blanch, A.R. (2002) Characterization of sewage waters by biochemical fingerprinting of Enterococci. *Water Research* **36**: 2831-2835.

Marino, R.P., and Gannon, J.J. (1991) Survival of Fecal-Coliforms and Fecal Streptococci in Storm-Drain Sediment. *Water Research* **25**: 1089-1098.

McDonald, A., Kay, D., and Jenkins, A. (1982) Generation of fecal and total coliform surges by stream flow manipulation in the absence of normal hydrometerological stimuli. *Applied and Environmental Microbiology* **44**: 292-300.

McDonald, J.I., Coupland, G.T., and Kendrick, G.A. (2006) Underwater video as a monitoring tool to detect change in seagrass cover. *Journal of Environmental Management* **80**: 148-155.

McLellan, S.L. (2004) Genetic diversity of *Escherichia coli* isolated from urban rivers and beach water. *Applied and Environmental Microbiology* **70**: 4658-4665.

McLellan, S.L., and Salmore, A.K. (2003) Evidence for localized bacterial loading as the cause of chronic beach closings in a freshwater marina. *Water Research* **37**: 2700-2708.

McQuaig, S.M., Scott, T.M., Harwood, V.J., Farrah, S.R., and Lukasik, J.O. (2006) Detection of human-derived fecal pollution in environmental waters by use of a PCR-based human polyomavirus assay. *Applied and Environmental Microbiology* **72**: 7567-7574.

Moore, D.F., Guzman, J.A., and Mcgee, C. (2008) Species distribution and antimicrobial resistance of enterococci isolated from surface and ocean water. *Journal of Applied Microbiology* **105**: 1017-1025.

Muela, A., Garcia-Bringas, J.M., Arana, I., and Barcina, I. (2000) The effect of simulated solar radiation on *Escherichia coli*: The relative roles of UV-B, UV-A, and photosynthetically active radiation. *Microbial Ecology* **39**: 65-71.

Muirhead, R.W., Davies-Colley, R.J., Donnison, A.M., and Nagels, J.W. (2004) Faecal bacteria yields in artificial flood events: quantifying in-stream stores. *Water Research* **38**: 1215-1224.

Muller, T., Ulrich, A., Ott, E.M., and Muller, M. (2001) Identification of plant-associated enterococci. *Journal of Applied Microbiology* **91**: 268-278.

Nagels, J.W., Davies-Colley, R.J., Donnison, A.M., and Muirhead, R.W. (2002) Faecal contamination over flood events in a pastoral agricultural stream in New Zealand. *Water Science and Technology* **45**: 45-52.

Nasser, A.M., Telser, L., and Nitzan, Y. (2007) Effect of sunlight on the infectivity of Cryptosporidium parvum in seawater. *Canadian Journal of Microbiology* **53**: 1101-1105.

Obiri-Danso, K., and Jones, K. (1999) Distribution and seasonality of microbial indicators and thermophilic campylobacters in two freshwater bathing sites on the River Lune in northwest England. *Journal of Applied Microbiology* **87**: 822-832.

Olapade, O.A., Depas, M.M., Jensen, E.T., and McLellan, S.L. (2006) Microbial communities and fecal indicator bacteria associated with Cladophora mats on beach sites along Lake Michigan shores. *Applied and Environmental Microbiology* **72**: 1932-1938.

Oren, A. (2004) Prokaryote diversity and taxonomy: current status and future challenges. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* **359**: 623-638.

Ott, E.M., Muller, T., Muller, M., Franz, C., Ulrich, A., Gabel, M., and Seyfarth, W. (2001) Population dynamics and antagonistic potential of enterococci colonizing the phyllosphere of grasses. *Journal of Applied Microbiology* **91**: 54-66.

Pachepsky, Y.A., Sadeghi, A.M., Bradford, S.A., Shelton, D.R., Guber, A.K., and Dao, T. (2006) Transport and fate of manure-borne pathogens: Modeling perspective. *Agricultural Water Management* **86**: 81-92.

Parveen, S., Portier, K.M., Robinson, K., Edmiston, L., and Tamplin, M.L. (1999) Discriminant analysis of ribotype profiles of *Escherichia coli* for differentiating human and nonhuman sources of fecal pollution. *Applied and Environmental Microbiology* **65**: 3142-3147.

Parveen, S., Murphree, R.L., Edmiston, L., Kaspar, C.W., Portier, K.M., and Tamplin, M.L. (1997) Association of multiple-antibiotic-resistance profiles with point and nonpoint sources of *Escherichia coli* in Apalachicola bay. *Applied and Environmental Microbiology* **63**: 2607-2612.

Payment, P., and Franco, E. (1993) Clostridium-Perfringens and Somatic Coliphages as Indicators of the Efficiency of Drinking-Water Treatment for Viruses and Protozoan Cysts. *Applied and Environmental Microbiology* **59**: 2418-2424.

Pettibone, G.W., Irvine, K.N., and Monahan, K.M. (1996) Impact of a ship passage on bacteria levels and suspended sediment characteristics in the Buffalo River, New York. *Water Research* **30**: 2517-2521.

Phillip, D.A.T., Antoine, P., Cooper, V., Francis, L., Mangal, E., Seepersad, N. et al. (2009) Impact of recreation on recreational water quality of a small tropical stream. *Journal of Environmental Monitoring* **11**: 1192-1198.

Pinto, B., Pierotti, R., Canale, G., and Reali, D. (1999) Characterization of 'faecal streptococci' as indicators of faecal pollution and distribution in the environment. *Letters in Applied Microbiology* **29**: 258-263.

Pollard, P.C., and Moriarty, D.J.W. (1991) Organic-Carbon Decomposition, Primary and Bacterial Productivity, and Sulfate Reduction, in Tropical Seagrass Beds of the Gulf of Carpentaria, Australia. *Marine Ecology-Progress Series* **69**: 149-159.

Posey, M.H., Wigand, C., and Stevenson, J.C. (1993) Effects of an Introduced Aquatic Plant, Hydrilla-Verticillata, on Benthic Communities in the Upper Chesapeake Bay. *Estuarine Coastal and Shelf Science* **37**: 539-555.

Power, M.L., Littlefield-Wyer, J., Gordon, D.M., Veal, D.A., and Slade, M.B. (2005) Phenotypic and genotypic characterization of encapsulated *Escherichia coli* isolated from blooms in two Australian lakes. *Environmental Microbiology* **7**: 631-640.

Ram, J.L., Ritchie, R.P., Fang, J.W., Gonzales, F.S., and Selegean, J.P. (2004) Sequence-based source tracking of *Escherichia coli* based on genetic diversity of beta-glucuronidase. *Journal of Environmental Quality* **33**: 1024-1032.

Rees, G., Pond, K., Johal, K., Pedley, S., and Rickards, A. (1998) Microbiological analysis of selected coastal bathing waters in the UK, Greece, Italy and Spain. *Water Research* **32**: 2335-2340.

Reeves, R.L., Grant, S.B., Mrse, R.D., Oancea, C.M.C., Sanders, B.F., and Boehm, A.B. (2004) Scaling and management of fecal indicator bacteria in runoff from a coastal urban watershed in southern California. *Environmental Science & Technology* **38**: 2637-2648.

Rittenberg, S.C., Mittwer, T., and Ivler, D. (1958) Coliform bacteria in sediments around three marine sewage outfalls. *Limnology and Oceanography* **3**: 101-108.

Roll, B.M., and Fujioka, R.S. (1997) Sources of faecal indicator bacteria in a brackish, tropical stream and their impact on recreational water quality. *Water Science and Technology* **35**: 179-186.

Rossello-Mora, R., and Amann, R. (2001) The species concept for prokaryotes. *FEMS Microbiology Reviews* **25**: 39-67.

Ruoff, K.L., Delamaza, L., Murtagh, M.J., Spargo, J.D., and Ferraro, M.J. (1990) Species identities of enterococci isolated from clinical specimens. *Journal of Clinical Microbiology* **28**: 435-437.

Sanders, B.F., Arega, F., and Sutula, M. (2005) Modeling the dry-weather tidal cycling of fecal indicator bacteria in surface waters of an intertidal wetland. *Water Research* **39**: 3394-3408.

Savage, W.G. (1905) Bacteriological examination of tidal mud as an index of pollution of the river. *Journal of Hygiene* **5**: 146-174.

Savageau, M.A. (1983) *Escherichia coli* habitats, cell types, and molecular methods of gene control. *American Naturalist* **122**: 732-744.

Schleifer, K.H., and Kilpperbalz, R. (1984) Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus *Enterococcus* Nom Rev as *Enterococcus faecalis* Comb-Nov and *Enterococcus faecium* Comb-Nov. *International Journal of Systematic Bacteriology* **34**: 31-34.

Schultz-Fademrecht, C., Wichern, M., and Horn, H. (2008) The impact of sunlight on inactivation of indicator microorganisms both in river water and benthic biofilms. *Water Research* **42**: 4771-4779.

Scott, T.M., Jenkins, T.M., Lukasik, J., and Rose, J.B. (2005) Potential use of a host associated molecular marker in Enterococcus faecium as an index of human fecal pollution. *Environmental Science & Technology* **39**: 283-287.

Seurinck, S., Verstraete, W., and Siciliano, S.D. (2003) Use of 16S-23S rRNA intergenic spacer region PCR and repetitive extragenic palindromic PCR analyses of *Escherichia coli* isolates to identify nonpoint fecal sources. *Applied and Environmental Microbiology* **69**: 4942-4950.

Shanks, O.C., Kelty, C.A., Sivaganesan, M., Varma, M., and Haugland, R.A. (2009) Quantitative PCR for genetic markers of human fecal pollution. *Applied and Environmental Microbiology*.

Sherer, B.M., Miner, J.R., Moore, J.A., and Buckhouse, J.C. (1992) Indicator bacterial survival in stream sediments. *Journal of Environmental Quality* **21**: 591-595.

Shiaris, M.P., Rex, A.C., Pettibone, G.W., Keay, K., McManus, P., Rex, M.A. et al. (1987) Distribution of indicator bacteria and *Vibrio parahaemolyticus* in sewage-pollluted intertidal sediments. *Applied and Environmental Microbiology* **53**: 1756-1761.

Shibata, T., Solo-Gabriele, H.M., Fleming, L.E., and Elmir, S. (2004) Monitoring marine recreational water quality using multiple microbial indicators in an urban tropical environment. *Water Research* **38**: 3119-3131.

Shimeta, J., Amos, C.L., Beaulieu, S.E., and Ashiru, O.M. (2002) Sequential resuspension of protists by accelerating tidal flow: Implications for community structure in the benthic boundary layer. *Limnology and Oceanography* **47**: 1152-1164.

Signoretto, C., Burlacchini, G., Pruzzo, C., and Canepari, P. (2005) Persistence of *Enterococcus faecalis* in aquatic environments via surface interactions with copepods. *Applied and Environmental Microbiology* **71**: 2756-2761.

Signoretto, C., Burlacchini, G., Lleo, M.D., Pruzzo, C., Zampini, M., Pane, L. et al. (2004) Adhesion of *Enterococcus faecalis* in the nonculturable state to plankton is the main mechanism responsible for persistence of this bacterium in both lake and seawater. *Applied and Environmental Microbiology* **70**: 6892-6896.

Simpson, J.M., Santo Domingo, J.W., and Reasoner, D.J. (2002) Microbial source tracking: state of the science. *Environmental Science & Technology* **36**: 5279-5288.

Sinton, L.W., Hall, C.H., Lynch, P.A., and Davies-Colley, R.J. (2002) Sunlight inactivation of fecal indicator bacteria and bacteriophages from waste stabilization pond effluent in fresh and saline waters. *Applied and Environmental Microbiology* **68**: 1122-1131.

Solo-Gabriele, H.M., Wolfert, M.A., Desmarais, T.R., and Palmer, C.J. (2000) Sources of *Escherichia coli* in a coastal subtropical environment. *Applied and Environmental Microbiology* **66**: 230-237.

Soulsby, R. (1998) *Dynamics of Marine Sands*. London: Thomas Telford, Ltd.

Stackebrandt, E., Frederiksen, W., Garrity, G.M., Grimont, P.A.D., Kampfer, P., Maiden, M.C.J. et al. (2002) Report of the *ad hoc* committee for the re-evaluation of the species definition in bacteriology. *International Journal of Systematic and Evolutionary Microbiology* **52**: 1043-1047.

Steets, B.M., and Holden, P.A. (2003) A mechanistic model of runoff-associated fecal coliform fate and transport through a coastal lagoon. *Water Research* **37**: 589-608.

Stephenson, G.R., and Street, L.V. (1978) Bacterial variations in streams from a southwest Idaho rangeland watershed. *Journal of Environmental Quality* **7**: 150-156.

Stoeckel, D.M., and Harwood, V.J. (2007) Performance, design, and analysis in microbial source tracking studies. *Applied and Environmental Microbiology* **73**: 2405-2415.

Tallon, P., Magajna, B., Lofranco, C., and Leung, K.T. (2005) Microbial indicators of faecal contamination in water: A current perspective. *Water Air and Soil Pollution* **166**: 139-166.

Topp, E., Welsh, M., Tien, Y.C., Dang, A., Lazarovits, G., Conn, K., and Zhu, H. (2003) Strain-dependent variability in growth and survival of *Escherichia coli* in agricultural soil. *FEMS Microbiology Ecology* **44**: 303-308.

Tunnicliff, B., and Brickler, S.K. (1984) Recreational water-quality analyses of the Colorado River in Grand Canyon. *Applied and Environmental Microbiology* **48**: 909-917.

USACE (2002) Coastal Engineering Manual. In. Washington, D. C.: U. S. Army Corps of Engineers.

USEPA (1986) Ambient Water Quality Criteria for Bacteria - 1986. In: Office of Water, U. S. Environmental Protection Agency.

USEPA (2000) Improved Enumeration Methods for the Recreational Water Quality Indicators: Enterococci and *Escheria coli*. In. Washington, D. C.: Office of Water, U. S. Environmental Protection Agency.

USEPA (2005) Microbial Source Tracking Guide Document. In. Washington, D. C.: National Risk Management Laboratory, United States Environmental Protection Agency.

Vandonsel, D.J., and Geldreich, E.E. (1971) Relationships of *Salmonella* to fecal coliforms in bottom sediments. *Water Research* **5**: 1079-+-1079-+.

Versalovic, J., Koeuth, T., and Lupski, J.R. (1991) Distribution of repetitive DNA sequences in Eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Research* **19**: 6823-6831.

Wade, T.J., Pai, N., Eisenberg, J.N.S., and Colford, J.M. (2003) Do U.S. Environmental Protection Agency water quality guidelines for recreational waters prevent gastrointestinal illness? A systematic review and meta-analysis. *Environmental Health Perspectives* **111**: 1102-1109.

Ward, D.M., Ferris, M.J., Nold, S.C., and Bateson, M.M. (1998) A natural view of microbial biodiversity within hot spring cyanobacterial mat communities. *Microbiology and Molecular Biology Reviews* **62**: 1353-+.

Whitlock, J.E., Jones, D.T., and Harwood, V.J. (2002) Identification of the sources of fecal coliforms in an urban watershed using antibiotic resistance analysis. *Water Research* **36**: 4273-4282.

Whitman, R.L., and Nevers, M.B. (2003) Foreshore sand as a source of *Escherichia coli* in nearshore water of a Lake Michigan beach. *Applied and Environmental Microbiology* **69**: 5555-5562.

Whitman, R.L., Nevers, M.B., and Byappanahalli, M.N. (2006) Examination of the watershed-wide distribution of *Escherichia coli* along southern Lake Michigan: an integrated approach. *Applied and Environmental Microbiology* **72**: 7301-7310.

Whitman, R.L., Shively, D.A., Pawlik, H., Nevers, M.B., and Byappanahalli, M.N. (2003) Occurrence of *Escherichia coli* and enterococci in *Cladophora* (*Chlorophyta*) in nearshore water and beach sand of Lake Michigan. *Applied and Environmental Microbiology* **69**: 4714-4719.

WHO (2001) Bathing Water Quality and Human Health: Faecal Pollution. In. Geneva, Switzerland: World Health Organization, p. 58.

Wiggins, B.A., Andrews, R.W., Conway, R.A., Corr, C.L., Dobratz, E.J., Dougherty, D.P. et al. (1999) Use of antibiotic resistance analysis to identify nonpoint sources of fecal pollution. *Applied and Environmental Microbiology* **65**: 3483-3486.

Wilkinson, J., Jenkins, A., Wyer, M., and Kay, D. (1995) Modeling fecal coliform dynamics in streams and rivers. *Water Research* **29**: 847-855.

Yamahara, K.M., Walters, S.P., and Boehm, A.B. (2009) Growth of Enterococci in Unaltered, Unseeded Beach Sands Subjected to Tidal Wetting. *Applied and Environmental Microbiology* **75**: 1517-1524.

Yamahara, K.M., Layton, B.A., Santoro, A.E., and Boehm, A.B. (2007) Beach sands along the California coast are diffuse sources of fecal bacteria to coastal waters. *Environmental Science & Technology* **41**: 4515-4521.

Zhai, Q., Coyne, M.S., and Barnhisel, R.I. (1995) Mortality rates of fecal bacteria in subsoil amended with poultry manure. *Bioresource Technology* **54**: 165-169.

Zmirou, D., Pena, L., Ledrans, M., and Letertre, A. (2003) Risks associated with the microbiological quality of bodies of fresh and marine water used for recreational purposes: summary estimates based on published epidemiological studies. *Archives of Environmental Health* **58**: 703-711.

ABOUT THE AUTHOR

Brian Douglas Badgley was born in Hilliard, Ohio. In 1995, he received his B.S. in zoology from the University of Georgia, in Athens, Georgia. Afterward, he taught environmental education for a year at the Jekyll Island 4-H Center in Jekyll Island, Georgia, before later returning to obtain his M.S. degree in Marine, Estuarine, and Environmental Science from the University of Maryland in College Park, Maryland, which he received in 2001. His thesis focused on the uptake of dissolved nitrate as a source of nitrogen for symbiotic corals, which he researched at the Bermuda Biological Station in St. George's, Bermuda. During his final year at the University of Maryland, Brian was awarded a one-year Knauss Marine Policy Fellowship from NOAA's Sea Grant Program, which he spent with the National Estuarine Research Reserve System serving as a program officer for the mid-Atlantic Reserves and working on other policy initiatives such as expanding the Reserve System. Following this, Brian worked as the Coastal Training Program Coordinator at the Rookery Bay National Estuarine Reserve in Naples, Florida, where he developed professional training programs for local environmental professionals that focus on coastal resources. Finally, in 2004 Brian began the Ph.D. program in Biology at the University of South Florida, under the advisement of Drs. Florence Thomas and Valerie Harwood, where he then completed his doctoral degree.