PERSISTENCE AND GROWTH OF THE FECAL INDICATOR BACTERIA, ENTEROCOCCI, IN NATURAL ESTUARINE PLANKTON COMMUNITIES

by

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(Under the Direction of Erin K. Lipp)

ABSTRACT

Enterococci are used as an indicator of fecal pollution to evaluate water quality in marine environments. The presence of enterococci as a group is not only associated with humans, but also has been found in the digestive tracts of other warm-blooded animals, in soil, on plant material and associated with plankton. Given the epiphytic nature of many *Enterococcus* spp., we investigated the contribution of plankton-associated enterococci in estuarine water samples. Our results suggest that *Enterococcus* spp. may be highly concentrated in plankton. Laboratory microcosm experiments showed the ability of *E. faecalis*, a fecal species and *E. casseliflavus*, an epiphytic species, to survive and grow in mixed plankton at 30 and 10^{o}C. Therefore, aquatic biota such as plankton can serve as a reservoir for *Enterococcus* species. Moreover, our findings could have implications for the effectiveness of enterococci as an indicator of coastal water quality.

INDEX WORDS: Enterococci, plankton, fecal indicator, water quality, marine environment

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DEDICATION

To Chad, my family and my students.

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CHAPTER 1

INTRODUCTION

Enterococci are Gram-positive bacteria common in the feces of warm blooded animals including humans. In 1986 the United States Environmental Protection Agency (USEPA) recommended enterococci as the preferred indicator of fecal pollution and health risk in marine water, replacing fecal coliform bacteria. The recommendation was based on epidemiological studies showing enterococci to have a positive correlation with highly credible gastroenteritis at beaches in the United States (USEPA 1986). Enterococci also survive longer in marine environments than fecal coliform bacteria, making the group easier to detect (Vasconcelos and Swartz 1976). Because of their superior survival in marine waters, enterococci also tend to correlate better than fecal coliform bacteria with other human pathogens, such as some enteric viruses (Lipp 2006). Under the federal Beaches Environmental Assessment and Coastal Health (BEACH) Act of 2000 (Public Law 106-284), each state was required to adopt enterococcus standards for recreational water quality criteria by 2004. The BEACH Act also called for the continued evaluation and improvement of fecal indicator standards for recreational marine waters through scientific research. In the 2007 Scientific Experts Report, scientists specifically recognized the importance of an understanding of fecal indicator bacteria ecology when evaluating water quality criteria (USEPA 2007).

Although enterococci are more useful than other historically used indicators in marine environments, there are limitations to using the group to assess fecal pollution and health risk. USEPA recreational water quality indicator recommendations were based on a limited set of studies at six beaches performed in the 1970s (Cabelli et al. 1983). Additionally, these epidemiological studies were specifically focused on beaches affected by publicly owned sewage treatment plants and did not include beaches affected by non-point source pollution such as septic tanks (Cabelli et al. 1983). While efforts are on-going to conduct studies of health effects at beaches not influenced by sewage discharge, it is not clear if enterococci will continue to be viewed as an appropriate indicator. Contributing to the problems is that enterococci have been found to have a range of sources other than humans, including livestock and domestic and wild birds (Devriese et al. 1987; Kuntz et al. 2004). Additionally they have been found in association with soil, plants, zooplankton and algae (Roll and Fujioka 1997; Ulrich and Müller 1998; Signoretto et al. 2004; Whitman et al. 2003). Even *Enterococcus faecalis*, the species in the highest concentration in human feces (Noble 1978), has been found to adhere to zooplankton and to persist in the environment for extended periods of time (Signoretto et al. 2004, 2005). Due to the potential for introduction by other sources and the persistence of enterococci in the environment, high levels may not indicate continuous addition of human fecal wastes in an area, and therefore may present confounding information for regulators charged with assessing water quality.

This thesis will contribute to the knowledge of the ecology of enterococci, which can aide in the evaluation of these bacteria as water quality indicators. This investigation demonstrates the potential for enterococci to associate and proliferate

within the plankton community along Georgia's coast. The second chapter of this thesis provides a review of the relevant literature discussing fecal indicator history and enterococci characteristics, sources and behavior in the environment. The third chapter presents the research and findings of the in situ and in vitro studies. The fourth chapter provides an overview of the findings and conclusions of the study.

References

Cabelli, V.J., A.P. Dufour, L.J. McCabe, and M.A. Levin. 1983. A marine recreational water quality criterion consistent with indicator concepts and risk analysis. J. Water Pollut. Cont. Fed. **55**(10):1306-1314.

Devriese, L.A., A. Van De Kerckhove, R. Kilpper-Bälz, and K.H. Schleifer. 1987. Characterization and identification of *Enterococcus* species isolated from the intestines of animals. Int. J. Syst. Bacteriol. **37**(3):257-259.

Kuntz, R.L., P.G. Hartel, K. Rodgers, and W.I. Segars. 2004. Presence of *Enterococcus faecalis* in broiler litter and wild bird feces for bacterial source tracking. Water Res. **38**(16):3551-3557.

Lipp, E. K. 2006. Assessment of viral pathogen load in Georgia Beaches and relationship to water quality indicators. Final Report to the Georgia Department of Natural Resources.

Noble, C.J. 1978. Carriage of group D streptococci in the human bowel. J. Clin. Pathol. **31**:1182-1186.

Roll B.M., and R.S. Fujioka. 1997. Sources of faecal indicator bacteria in a brackish, tropical stream and their impact on recreational water quality. Water Sci.Technol. **35**(11-12):179-186.

Signoretto, C., G. Burlacchini, M. del Mar Lleò, C. Pruzzo, M. Zampini, L. Pane, G. Franzini, and P. Canepari. 2004. Adhesion of *Enterococcus faecalis* in the nonculturable state to plankton is the main mechanism responsible for persistence of the bacterium in both lake and seawater. Appl. Environ. Microbiol. **70**(11):6892-6896.

Signoretto, C., G. Burlacchini, C. Pruzzo, and P. Canepari. 2005. Persistence of *Enterococcus faecalis* in aquatic environments via surface interactions with copepods. Appl. Environ. Microbiol. **71**(5):2756-2761.

Ulrich, A., and T. Müller. 1998. Heterogeneity of plant associated streptococci as characterized by phenotypic features and restriction analysis of PCR amplified 16S rDNA. J. Appl. Microbiol. **84**(2):293-303.

[USEPA] US Environmental Protection Agency, Office of Water. 1986. Ambient water quality criteria for bacteria-1986. EPA-440/5-84/002.

[USEPA] US Environmental Protection Agency, Office of Water. 2007. Report of the experts scientific workshop on critical research needs for the development of new or revised recreational water quality criteria. EPA 823-R-07-006.

Vasconcelos, G.J., and R. G. Swartz. 1976. Survival of bacteria in seawater using a diffusion chamber apparatus in situ. Appl. Environ. Microbiol. **31**(6):913–920.

Whitman, R.L., D.A. Shively, H. Pawlik, M.B. Nevers, and M.N. Byappanahalli. 2003. Occurrence of *Esherichia coli* and enterococci in *Cladophora (Chlorophyta*) in nearshore water and beach of Lake Michigan. Appl. Environ. Microbiol. **69**(8):4714-4719.

CHAPTER 2

LITERATURE REVIEW

Introduction

The enterococci are a diverse, ubiquitous group of bacteria. Enterococci are Gram-positive, catalase negative, non-spore forming and occur as single cocci, in pairs or in short chains (Holt et al. 1994). Enterococci are facultative anaerobes and can grow under a wide range of temperatures, 10 to 45 ^{o}C, and in the presence of up to 6.5% NaCl; however, their nutritional requirements are complex. Another distinguishing characteristic of enterococci is the ability to hydrolyze esculin in the presence of 40% bile salts. This last phenotypic characteristic is one that commonly sets enterococci apart from the closely related streptococci (Facklam et al. 2002). Enterococci were once classified as Group D streptococci. With the aide of genotypic testing, these Group D streptococci were transferred to a new genus, *Enterococcus* in 1984 (Schleifer and Kilpper-Balz 1984).

Sources of enterococci

Enterococci are commonly found in the feces of warm blooded mammals and birds. Currently, 19 species are recognized within the *Enterococcus* genus (Manero and Blanch 1999). The *Enterococcus* species identified most frequently in domesticated warm-blooded animals (i.e., poultry, cattle, pigs, dogs and horses) are *E. faecalis*, *E. faecium* and *E. hirae* (Devriese et al. 1987). In humans, *E. faecalis* is the species found most commonly and has been found in stool culture at quantities several orders of magnitude higher than that of *E. faecium* (Noble 1978). High percentages (≥ 30% of

enterococci) of *E. faecalis* are also found in association with some wild bird feces (Kuntz et al. 2004).

It was once believed that enterococci found on plants were only temporary residents from other sources such as insects and other wild animals (Mundt 1961, 1963). However certain yellow-pigmented species such as *E. casseliflavus*, *E. mundtii* and *E. flavescens* are commonly found on plants but are not often isolated from animal hosts and thus are considered plant-associated (Devriese et al. 1987; Leclerc et al. 1996). Other enterococcal species, such as *E. faecalis* and *E. faecium,* may be isolated from a plant source but whether or not these organisms are acting as true epiphytes is unclear (Ulrich and Müller 1998).

Human infection and enterococci

Since the early part of the 20th century, enterococci have played an important role as indicators of fecal pollution. More recently, discoveries of the role enterococci play in clinical infections have lead to an increased interest in this group of bacteria. Enterococci, with increasing antibiotic resistance even to third-generation antibiotics like vancomycin, are most often found as pathogens among hospitalized patients (Moellering 1992; Murray 2000). *Enterococcus faecalis* and *E. faceium* are responsible for most infections among this genus, with the latter representing a majority of antibiotic resistant infections (Paterson et al. 1995). Within a hospital setting, environmental factors such as contaminated equipment and linens appear to contribute to the transmission of infectious enterococci. One study showed vancomycin-resistant enterococci surviving on a laboratory bench-top for at least 58 days (Bonilla et al. 1996).

Enterococci as a fecal water quality indicator

Coastal water, including recreational beaches and shellfish harvesting areas, may be contaminated by fecal material due to sewage leaks or wastewater discharges or from non-point sources such as septic tanks. With continued population growth in coastal areas, increased stress will be placed on water resources and water quality. In 2007, in the United States, one third of the advisory and closing days for coastal and Great Lakes beaches were the result of high levels of fecal indicators (NRDC 2008). Contaminated water poses a health risk for all but especially for children and the elderly who are often most affected by changes in recreational water quality due to their activity level and compromised immunity, respectively (USEPA, 1999). Swimming in waters polluted by sewage most often correlates to gastrointestinal symptoms such as vomiting, diarrhea, nausea or stomachache (Cabelli et al. 1979). The ability to ensure good quality water is vital to the safety of the nation's citizens and visitors and to the economy. For example, the Georgia coast alone brings in 1.6 billion dollars through the travel industry annually (TIA, 2007).

Fecal indicators have been used since the early part of the 20th century as markers for fecal pollution to protect wildlife and people in and around water. A fecal indicator does not normally cause illness but can indicate the presence of fecal contamination, which may contain pathogens such as human enteric viruses and protozoa. Total coliform bacteria, a broad group of Gram-negative facultative anaerobes, were the first used fecal indicator based on their presence in feces and ease of detection (Wolf 1972). Later in the 20th century, fecal coliform bacteria, set apart from total coliforms by their growth at elevated temperature, were shown to be better

indicators of fecal contamination (Griffin et al. 2001). The National Technical Advisory Committee (NTAC) to the Federal Water Pollution Control Administration developed a federal fecal coliform guideline in 1968 and by 1979, this fecal coliform standard had been adopted by most states (NTAC 1968; Cabelli et al. 1983).

Due to inherent characteristics of enterococci, the group has also proven useful for indicating fecal pollution for over a century. A study in 1902 found this “sewage streptococci” in sewage, fresh feces, septic tanks and in known polluted river water (Winslow and Hunnewell 1902). Even as far back as the early 20th century, Houston proposed the use of fecal streptococci as a means for detecting human feces in contaminated water based on the consistent detection of this bacteria in human fecal material (Facklam et al. 2002). Houston’s proposal could not be placed into action until detection and enumeration methods were developed and improved later in the century.

Other characteristics that make enterococci a favorable fecal indicator is the persistence of enterococci in aquatic environments. In situ environmental studies of enteric pathogens such as *E. faecalis* were conducted using an autoclavable diffusion chamber (Vasconcelos and Swartz, 1976). *Enterococcus faecalis* was tested along with other fecal indicators and was found to be the most persistent during the 7-day test period (Vasconcelos and Swartz, 1976). Persistence in the environment provides better tracking with other environmentally persistent pathogens and allows ample time to detect the fecal indicator.

Bacteria such as total coliforms and fecal coliforms have been used as regulatory qualitative measures of water quality for many decades. By incorporating epidemiological studies with microbial measurements, quantitative measures of water

quality can help assess risk to humans. More than 80 years after it was first proposed, the USEPA recommended enterococci as an indicator of fecal pollution and health risk in marine water (USEPA 1986). USEPA's recommendations were made based on a 3-year study in the 1970s comparing gastrointestinal symptoms at two New York beaches and one Louisiana beach with mean densities of several indicators, including enterococci, *E. coli* and fecal coliform bacteria (Cabelli et al. 1983). These epidemiological studies showed enterococci densities to have the strongest positive correlation with highly credible gastroenteritis at these beaches (Cabelli et al. 1983). More recent research from the Great Lakes also supports the EPA's steady state geometric mean maximum guideline level of enterococci at 33 CFU 100 ml^{-1} for five samples taken over a 30-day period as consistent with risks of gastrointestinal illness in fresh water (Wade et al. 2003). For marine recreational water, the maximum steady state geometric mean enterococci density is 35 CFU 100 ml^{-1} for five samples taken over a 30-day period ,and for a single grab sample, the maximum allowable density is 104 CFU 100 ml^{-1} for a designated beach area (USEPA 2004).

The current recreational water quality criteria stated in Section 304 of the Clean Water Act have been used since 1986, but it was only in 2004, following the passage of the BEACH Act (2000), that states were required to use enterococcus standards. Under the BEACH Act, an amendment to the Clean Water Act, the EPA has been given the task of re-evaluating the water quality criteria for recreational marine waters. Although enterococci have provided a useful indication of health risks at some beaches, there is concern with using a "one size fits all" guideline for all marine recreational areas in the United States. In March 2007, the Experts Scientific Workshop met to discuss

development of improved recreational water quality criteria (USEPA 2007). The forum defined specific research that could be accomplished in two to three years to meet the goal of new or revised criteria by 2012. Included in the topics of interest at the workshop were pathogen indicators and indicators of fecal contamination. The workgroup responsible for this topic addressed concern that the 1986 recreational water quality criteria do not take into account “differences in geographical conditions, ecology of microorganisms and varying sources of fecal indicator bacteria” (USEPA, 2007). The report recognized the importance of an understanding of fecal indicator bacterial ecology when evaluating the water quality criteria (USEPA, 2007). In regions such as the Southeastern United States, coastal ecology can have a significant impact on the usefulness of enterococci as a fecal pollution and health risk indicator.

Problems associated with using enterococci as a fecal indicator

Besides limited epidemiological studies correlating enterococci levels in marine water with risk to humans and limited geographic condition considerations, there are other problems associated with using enterococci as a fecal indicator. A critical concern is the lack of correlation between the presence of enterococci and pathogens. A study in an urban estuary in Sydney, Australia found no significant correlation between the presence of enterococci and pathogens such as *Giardia* (Ferguson et al. 1996). A study in the Gulf of Mexico off the coast of Florida found no significant relationship between the presence of enterococci and a broad range of pathogens including *Giardia*, *Cryptosporididum* and enteroviruses (Lipp et al. 2001). For marine recreational sites, mostly impacted by non-point source pollution, a lack of correlation between the levels of pathogens and state and federally recommended indicator threshold levels

demonstrate a need for regionally specific guidelines (Lipp et al. 2001; Ortega et al. 2009).

The method currently recommended and used by many states to enumerate enterococci is EPA method 1600 which uses membrane-Enterococcus Indoxyl-β-D-Glucoside Agar (mEI) and requires 24 hours incubation before enumerating colonies with a blue halo (Messer and Dufour 1998; USEPA 2002). However, evaluating the mEI method for enterococcus enumeration in freshwater samples has shown that colonies with a blue halo may be identified as non-fecal associated enterococci, such as *E. casseliflavus* and *E. mundtii* (Rhodes and Kator 1997). Enumerating enterococci that are plant-associated and not fecal-associated could provide misleading information about water quality and risk to humans, especially in areas with large plant, algae or plankton loads.

The recommended EPA Method 1600 for enterococci enumeration poses another problem because of incubation time. If fecal pollution is suspected in a recreational area, 24 hours is needed to determine indicator levels. Therefore, a 24-hour delay in action occurs. As many as 70% of water quality standard exceedences at a site take place as a single-day event and are over after 24 hours (Leecaster and Weisberg 2001). More rapid tests have been designed to help combat the lengthy 24-hour incubation time of the mEI method. A quantitative polymerase chain reaction (PCR) method has been tested and presents same day results using an *Enterococcus* target (Wade et al. 2006). This study also found a significant correlation between enterococci density and same day gastrointestinal illness.

Environmental enterococci speciation

As previously mentioned, *E. faecalis* and *E. faecium* are the two *Enterococcus* species most often found in human feces. When environmental water samples are evaluated however, these are not the only two species which contribute to the enterococci population (Rhodes and Kator 1997). Regional differences in enterococci populations exist and may impact the reliability of using enterococci as a water quality indicator (Pinto et al. 1999; Moore et al. 2008).

Inclusion of many *Enterococcus* species, especially those that are associated with vegetation, during water quality evaluation may be misleading to regulators as to the true presence of fecal pollution (Geldreich and Kenner, 1969). *Enterococcus* species distribution can vary depending on the characteristics of the site tested. For example, a study in Scotland found the predominant *Enterococcus* species in a stream above a sewage outfall to be *E. casseliflavus*, whereas below the sewer outfall, *E. faecalis* predominated (Bayne et al. 1983). Environmental isolates collected and identified from brackish and marine water studies in Italy and California found not only *E. faecalis* and *E. faecium*, but also similar percentages of plant-associated species including *E. casseliflavus* and *E. mundtii* (Pinto et al. 1999; Moore et al. 2008).

Several studies have been devoted to characterizing enterococci species in water impacted by sewage or runoff, but enterococci have also been identified in epiphytic relationships. Müller et al. (2001) reported that forage grass, in fields where no manure spreading took place, harbored a range of Enterococcus species including *E. faecium, E. mundtii, E. casseliflavus, E. faecalis and E. sulfureus*.

Determining the species distribution among an enterococci population can be used to support fecal source tracking efforts. Bacterial source tracking is based on the idea that certain species, or genes, associated with indicator bacteria are limited to humans and very few other organisms. For example, a combination of *E. faecalis* and *E. faecium* can detect the presence of human fecal pollution (McDonald et al. 2006). *Enterococcus. faecalis* comprises a high percentage ($\geq$ 30%) of enterococci found in humans and some wild birds (Kuntz et al. 2004). Combining the presence of high percentages of *E. faecalis* with the presence of the *esp* gene, a gene detected in *E. faecium* isolates from humans but not in nonhuman animal feces (Scott et al. 2005), enterococci can better be used as an indicator of human fecal contamination (McDonald et al. 2006).

Methods for enterococci speciation in aquatic environments

Enterococci isolated from the environment can be identified to species using phenotypic or genotypic tests. Conventional biochemical testing of isolates, still considered the gold standard for speciation of enterococci, is accurate but is time consuming and often difficult because some species only vary by one phenotypic trait (Moore et al. 2006, Holt et al. 1994). An example of a commercial phenotypic test to identify enterococci is Biolog (Biolog, Hayward, CA, USA). Biolog uses a panel of 95 carbon substrate reactions to test an individual isolate. The reaction results are entered into a database for identification. A phenotypic test such as Biolog correctly identified *Enterococcus* species with 73% overall accuracy with variable accuracy of identification for each species (Moore et al. 2006). A genotypic test, such as 16s rRNA gene sequencing, can provide more accuracy, with up to 92% correct species identification

(Moore et al. 2006). However, 16S rRNA sequences for some closely related species of enterococci can be ≥ 99.9% homologous (Patel et al. 1998), which could also lead to identification errors (Moore et al. 2006).

Behavior of enterococci in aquatic environments

Enterococci can be introduced into aquatic environments from sources other than humans including soil microflora and wild birds (Fujioka and Byappanahalli 2001; Kuntz 2004). Once introduced, enterococci have been found to adhere to both zooplankton and phytoplankton and can persist in the environment for extended periods of time (Signoretto et al. 2004; Whitman et al. 2003). A combination of all of these sources and reservoirs; wild birds, sediments and marine vegetation and plankton, can contribute enough enterococci to significantly impact water quality in areas such as coastal saltwater marshes (Grant et al. 2001). The following provides detail on the association of enterococci with different aspects of the marine environment.

Enterococci survival in water

Laboratory microcosm experiments show the ability of many *Enterococcus* species to survive in artificial seawater without the addition of nutrients under direct illumination at 4^{o}C (Lleó et al. 2005). *Enterococcus faecalis*, for example, survived for 21 $\pm$ 2 days whereas *E. faecium* survived for 48 $\pm$ 4 days in artificial seawater (Lleó et al. 2005). Enterococci can survive in the water column alone, but seemingly vital to the persistence and growth of enterococci in the environment is the protection from sunlight and availability of organic material. In a microcosm experiment using membrane (0.45 µm) diffusion chambers inoculated with sewage effluent, enterococci survival and growth were tested under tropical estuarine conditions (Bordalo et al. 2002).

Enterococci survived approximately twice as long (mean 73.0 hours) in dark conditions compared to light conditions (Bordalo et al. 2002). Organic material was present in the inoculum along with other microorganisms <0.45 μm in size. Turbidity caused by suspended particles such as sediments or plankton can provide protection from UV irradiation. By comparing T_{90} values, the time for bacterial concentrations to decrease by 90%, enterococci survived almost 6 times longer in UV irradiated high-turbidity estuarine waters compared to low-turbidity water (Kay et al. 2005).

Soil, sand and sediment as enterococci reservoirs

Soil and marine sediment can provide enterococci with protection from sunlight and provide a nutrient source. Some *Enterococcus* species are from environmental sources and can be found thriving in soil microflora (Fujioka and Byappanahalli 2001). In tropical locations such as Hawaii, soil rather than fecal contamination can be the major source of enterococci in stream sample sites (Hardina and Fujioka 1991; Roll and Fujioka 1997). Enterococci can be recovered from soil samples as deep as 36 cm below the surface (Hardina and Fujioka 1991). Another study in Hawaii indicates that sand, contaminated with enterococci from sources such as pigeons, contributes to high levels of bacteria in the bay study site (Oshiro and Fujioka, 1995). High concentrations of enterococci also have been reported in intertidal sediments impacted by storm drains (Ferguson et al. 2005). The distribution of species isolated from the water column and those found in the sediments are comparable (Ferguson et al. 2005). Enterococci in marine sediment can survive for more than 50 days as demonstrated in situ (Davies et al. 1995) and has been shown to regrow in desiccated and rewetted sediments (Hartel et al. 2005).

Re-suspension of sediment may contribute to the overall concentration of enterococcus concentrations in water samples. Wave action may re-suspend enterococci from sediment into the water column (Le Fevre and Lewis 2003). Under simulated environmental conditions, enterococci have prolonged proliferation and re-growth in soils influenced by tides in subtropical environments (Desmarais et al. 2002).

Plankton and algae as enterococci reservoirs

Generally plankton is divided into two main categories; phytoplankton, which use photosynthesis to construct carbohydrates, and zooplankton, which are heterotrophic. Planktonic organisms provide a potential reservoir for fecal indicators and human pathogens. For example, the adherence of *Vibrio* species to zooplankton, especially copepods, has been extensively researched. Signoretta et al. (2004, 2005) applied this concept to *Enterococcus faecalis* and found that enterococcal cell walls are also capable of attaching to chitin. Both culturable and viable but not culturable (VBNC) cells are able to use attachment to copepods as a survival strategy in the environment (Signoretto et al. 2004, 2005). Green alga has also been found to harbor enterococci and may serve as another potential reservoir. For example, enterococci survived in sun-dried *Chadophora* mats, a type of green macro-alga, collected from Lake Michigan for over 6 months at 4^{o}C (Whitman et al. 2003).

The distribution of potentially pathogenic bacteria, including enterococci, as free-living and plankton-associated was investigated in a marine coastal area in Italy (Maugeri et al. 2004). Two size fractions were investigated, large plankton greater than 200 µm and small plankton greater than 64 µm but less than 200 µm. Larger size fractions, such as 200 µm and greater, are usually comprised of zooplankton while the

smaller size fractions, such as less than 200 μm, contain a greater proportion of phytoplankton and plant debris. Overall, the study found a greater association of enterococci with the larger plankton fraction (zooplankton) than the smaller plankton fraction (phytoplankton). In the larger plankton fraction, there was also no distinct seasonality for enterococci but for the smaller plankton fraction, there was a drop-off of enterococcus density in the winter months (Maugeri et al. 2004).

It may not be a question of whether or not enterococci attach to zooplankton or phytoplankton more readily, but rather the total particle load. In areas of higher particle load, such as within an estuary with tidal mixing, particle-associated bacterial counts increase relative to free-living bacteria (Bidle and Fletcher 1995).

Growth of enterococci in the environment

Fecal indicator bacteria such as enterococci survive and associate with different components of the marine environment such as sediment and plankton, but do the bacteria grow once introduced into the environment? Studies have shown the potential for enterococci to grow in freshwater environments. In a 1997 study, Medema et al. tested the survival of fecal enterococci, specifically *E. faecium*, in river water. Through analysis of die-off rates at 5 and 15 ^{o}C in a controlled microcosm experiment, a slow die-off rate of 0.005 $\log_{10}$ day^{-1} at 15^{o}C lead researchers to hypothesize that this trend was the net result of both die off and re-growth (Medema et al. 1997). Enterococci associated with aquatic biota also shows potential for growth. Enterococci surviving in sun-dried algal mats from Lake Michigan demonstrated growth after rehydration (Whitman et al. 2003). Moreover, indigenous enterococci demonstrated 100-fold growth for the first 18 hours in *Cladophora* leachate, filtered supernatant from a centrifuged

suspension of *Cladophora* and lake water at 35^{o}C (Byappanahalli et al. 2003). In this investigation, enterococci concentration remained above or at the starting concentration for the 168-hour study duration (Byappanahalli et al. 2003).

Although the above studies were performed in fresh water, evidence supports the potential for enterococci to grow in marine environments as well. By comparing enterococci levels in recreational beaches and seaweed in New Zealand, significantly higher levels in seaweed suggested the possible expansion of enterococci in the environment (Anderson et al. 1997). Further clonal analysis of the isolates from the seaweed and water demonstrated the presence of clonal enterococci populations in seaweed but their absence in water samples (Anderson et al. 1997). With availability of nutrients and protection, as may be found in algae or plankton, enterococci may be able to not only survive, but multiply in marine environments.

Conclusions

Enterococci, a commonly used human fecal indicator bacteria, are ubiquitous in the environment and have a range of sources and associations including other warm blooded animals, plankton and plants (Devreise et al. 1987; Kuntz et al. 2004; Ulrich and Müller 1998; Signoretto et al. 2004). There exists a dynamic relationship between enterococci and both abiotic and biotic factors in the environment such as sediment and plankton (Hardina and Fujioka 1991; Roll and Fujioka 1997; Ferguson et al. 2005; Signoretto et al. 2004, 2005; Maugeri et al. 2004). Their value as a fecal and health risk indicator is in question due to their ability to persist and possibly proliferate once introduced into the environment.

References

Anderson, S. A., S. J. Turner and G. D. Lewis. 1997. Enterococci in the New Zealand environment: implications for water quality monitoring. Water Sci. Tech. **35**(11-12):325-331.

Bayne, S., M. Blankson and D. Thirkell. 1983. Enumeration and speciation of group D streptococci from above and below a sewer outfall, their susceptibilities to six antibiotics and a comparison with clinical isolates. Antonie van Leeuwenhoek. **49**(4-5)399-410.

Bidle, K. D., and M. Fletcher. 1995. Comparison of free-living and particle associated bacterial communities in the Chesapeake Bay by stable low molecular weight RNA analysis. Appl. Environ. Microbiol. **61**(3):944-952.

Bonilla, H. F., M. J. Zervos, M. J. Lyons, S. F. Bradley, S. A. Hedderwick, M. A. Ramsey, L. K. Paul, and C. A. Kauffman. 1996. Long-term survival of vancomycin-resistant *Enterococcus faecium* on a contaminated surface. Infect. Control Hosp. Epidemiol. **17**:770-771.

Bordalo, A. A., R. Onrassami, and C. Dechsakulwatana. 2002. Survival of faecal indicator bacteria in tropical estuarine waters (Bangpakong River, Thailand). J. Appl. Microbiol. **93**:864-871.

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

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

Cabelli, V. J., A. P. Dufour, L. J. McCabe, and M. A. Levin. 1983. A marine recreational water quality criterion consistent with indicator concepts and risk analysis. J. Water Pollut. Cont. Fed. **55**(10):1306-1314.

Davies, C. M., J. A. H. Long, M. Donald, and N. J. Ashbolt. 1995. Survival of fecal microorganisms in marine and freshwater sediments. Appl. Environ. Microbiol. **61**(5):1888-1896.

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

Devriese, L. A., A. Van De Kerckhove, R. Kilpper-Bälz, and K. H. Schleifer. 1987. Characterization and identification of *Enterococcus* species isolated from the intestines of animals. Int. J. Syst. Bacteriol. **37**(3):257-259.

Facklam, R. R., M. G. S. Carvalho, and L. M. Teixeira. 2002. History, taxonomy, biochemical characteristics, and antibiotic susceptibility testing of enterococci, p. 2. *In* M. S. Gilmore, D. B. Clewell, P. Courvalin, G. M. Dunny, B. E. Murray and L. B. Rice (ed.), The enterococci: pathogenesis, molecular biology, and antibiotic resistance, ASM Press, Washington, DC.

Ferguson, C. M., B. G. Coote, N. J. Ashbolt, and I. M. Stevenson. 1996. Relationships between indicators, pathogens and water quality in an estuarine system. Water Res. **30**(9):2045-2054.

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

Fujioka, R. S., and M. N. Byappanahalli. 2001. Microbial ecology controls the establishment of fecal bacteria in tropical soil environment, p. 273–283. *In* K. H. T. Matsuo, S. Takizawa, and H. Satoh (ed.), Advances in water and wastewater treatment technology: molecular technology, nutrient removal, sludge reduction and environmental health. Elsevier, Amsterdam, The Netherlands.

Geldreich, E. E. and B. A. Kenner. 1969. Concepts of fecal streptococci in stream pollution. J. Water Pollut. Cont. Fed. **41**(8):R336-R352.

Grant, S. B., B. F. Sanders, A. B. Boehm, J. A. Redman, J. H. Kim, R. D. Mrše, A. K. Chu, M. Gouldin, C. D. McGee, N. A. Gardiner, B. H. Jones, J. Svejkovsky, G. V. Leipzin, and A. Brown. 2001. Generation of enterococci bacteria in a coastal saltwater marsh and its impact on surf zone water quality. Environ. Sci. Technol. **35**(1):2407-2416.

Griffin, D. W., E. K. Lipp, M. R. McLaughlin, and J. B. Rose. 2001. Marine recreation and public health microbiology: quest for the ideal indicator. Bioscience. **51**(10):817-825.

Hartel, P. G., K. Rodgers, J. A. Fisher, J. L. McDonald, L. C. Gentit, E. Otero, Y. Rivera-Torres, T. L. Bryant, and S. H. Jones. 2005. Survival and regrowth of fecal enterococci in desiccated and rewetted sediments. *In* K. J. Hatcher (ed.) Proceedings of the 2005 Georgia Water Resources Conference, April 25-27, Athens, Georgia.

Hardina, C. M., and R.S. Fujioka. 1991. Soil: The environmental source of Escherichia coil and enterococci in Hawaii's streams. Environ. Toxicol. Water Quality. **6**:185-195.

Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Stanley, and S. T. Williams. 1994. Bergey's Manual of Determinative Bacteriology, ninth ed. Williams and Wilkins Co., Baltimore, MD, p. 528.

Kay, D., C. M. Stapleton, M. D. Wyer, A. T. McDonald, J. Crowther, N. Paul, K. Jones, C. Francis, J. Watkins, J. Wilkinson, N. Humphrey, B. Lin, L. Yang, R. A. Falconer, and S. Gardner. 2005. Decay of intestinal enterococci concentrations in high-energy estuarine and coastal waters: towards real-time T_{90} values for modeling faecal indicators in recreational waters. Water Res. **39**: 655-667.

Kuntz, R. L., P. G. Hartel, K. Rodgers, and W.I. Segars. 2004. Presence of *Enterococcus faecalis* in broiler litter and wild bird feces for bacterial source tracking. Water Res. **38**(16):3551-3557.

LaLiberte, P., and D. J. Grimes. 1982. Survival of *Escherichia coli* in lake bottom sediment. Appl. Environ. Microbiol. **43**(3):623-628.

Leecaster, M. K., and S. B. Weisberg. 2001. Effect of sampling frequency on shoreline microbiology assessments. Mar. Pollut. Bull. **42**(11):1150-1154.

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

Le Fevre, N. M., and G. D. Lewis. 2003. The role of resuspension in enterococci distribution in water at an urban beach. Water Sci. Technol. **47**(3):205-210.

Lipp, E. K., S. A. Farrah, and J. B. Rose. 2001. Assessment and impact of microbial fecal pollution and human enteric pathogens in a coastal community. Mar. Pollut. Bull. **42** (4):286-293.

Lleó, M. M, B. Bonato, D. Benedetti, and P. Canepari. 2005. Survival of enterococcal species in aquatic environments. FEMS Microbiol. Ecol. **54**:189-196.

Manero, A., and A. R. Blanch. Identification of *Enterococcus* spp. with a biochemical key. 1999. Appl. Environ. Microbiol. **65**:4425-4430.

Maugeri, T. L., M. Carbone, M. T. Fera, G. P. Irrera, and C. Gugliandolo. 2004. Distribution of potentially pathogenic bacteria as free living and plankton associated in a marine coastal zone. J. Appl. Microbiol. **97**:354-361.

McDonald, J. L., P. G. Hartel, L. C. Gentit, C. N. Belcher, K. W. Gates, K. Rodgers, J. A. Fisher, K. A. Smith, and K. A. Payne. 2006. Identifying sources of fecal contamination inexpensively with targeted sampling and bacterial source tracking. J. Environ. Qual. **35**:889-897.

Medema, G. J., M. Bahar, and F. M. Schets. Survival of *Cryptosporidium parvum*, *Escherichia coli*, faecal enterococci and *Clostridium perfringens* in river water: influence of temperature and autochthonous microorganisms. Water Sci. Technol. **35**(11-12):249-252.

Messer J. W., and A. P. Dufour. 1998. A rapid, specific membrane filtration procedure for enumeration of enterococci in recreational water. Appl. Environ. Microbiol. **64**(2):678-680.

Moellering, R. C., Jr. 1992. Emergence of enterococcus as a significant pathogen. Clin. Infect. Dis. **14**:1173-1178.

Moore, D. F., M. H. Zhowandai, D. M. Ferguson, C. McGee, J. B. Mott and J. C. Stewart. 2006. Comparison of 16S rRNA sequencing with conventional and commercial phenotypic techniques for identification of enterococci from the marine environment. J. Appl. Microbiol. **100**:1272-1281.

Moore, D. F., J. A. Guzman, and C. McGee. 2008. Species distribution and antimicrobial resistance of enterococci isolated from surface and ocean water. J. Appl. Microbiol. **105**(4):1017-1025.

Moore, D. F., J. A. Guzman, and C. McGee. 2008. Species distribution and antimicrobial resistance of enterococci isolated from surface and ocean water. J. Appl. Microbiol. **105**(4):1017-1025.

Müller, T., A. Ulrich, E.-M. Ott, and M. Müller. 2001. Identification of plant-associated enterococci. Appl. Environ. Microbiol. **91**:268-278.

Mundt, J. O. 1961. Occurrence of enterococci: Bud, blossom, and soil studies. Appl. Microbiol. **9**:541-544.

Mundt, J. O. 1963. Occurrence of enterococci on plants in a wild environment. Appl. Environ. Microbiol. **11**(2):141-144.

Murray, B. E. 2000. Vancomycin-resistant enterococcal infections. N. Engl. J. Med. **342**:710-721.

Noble, C. J. 1978. Carriage of group D streptococci in the human bowel. J. Clin. Pathol. **31**:1182-1186.

[NRDC] National Research Defense Counsel. 2008. Beach Pollution, http://www.nrdc.org/water/oceans/qttw.asp. Accessed on 19 April 2009.

[NTAC] National Technical Advisory Committee. 1968. Water quality and health. Federal Water Pollution Control Administration, Dept. of the Interior, Washington, D.C. 7.

Ortega, C., H. M. Solo-Gabriele, A. Abdelzahar, M. Wright, Y. Deng and L. M. Stark. Correlations between microbial indicators, pathogens and environmental factors in a subtropical estuary. Mar. Pollut. Bull., in press.

Oshiro, R., and R. Fujioka. 1995. Sand, soil, and pigeon droppings: sources of indicator bacteria in the waters of Hanauma Bay, Oahu, Hawaii. Water Sci. Technol. **31**(5-6):251-254.

Patel, R., K. E. Piper, M. S. Rouse, J. M. Steckelberg, J. R. Uhl, P. Kohner, M. K. Hopkins, F. R. Cockerill III, and B. C. Kline. 1998. Determination of 16S rRNA sequences of enterococci and application to species identification of nonmotile *Enterococcus gallinarum* isolates. J. Clin. Microbiol. **36**(11):3399-3407.

Patterson, J. E., A. H. Sweeney, M. Simms, N. Carley, R. Mangi, J. Sabetta and R. W. Lyons. 1995. An analysis of 110 serious enterococcal infections. Medicine (Baltimore). **74**:191-200.

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

Rhodes, M. W., and H. Kator. 1997. Enumeration of *Enterococcus* sp. using a modified mE method. J. Appl. Microbiol. **83**:120-126.

Roll, B. M., and R. S. Fujioka. 1997. Sources of faecal indicator bacteria in a brackish, tropical stream and their impact on recreational water quality. Water Sci. Technol. **35**(11-12):179-186.

Schleifer, K. H., and R. Kilpper-Balz. 1984. Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. Int. J. Syst. Bacteriol. **34**:31-34.

Scott, T. M., T. M. Jenkins, J. Lukasik, and J. B. Rose. 2005. Potential use of a host associated molecular marker in *Enterococcus faecium* as an index of human fecal pollution. Environ. Sci. Technol. **39**:283-287.

Signoretto, C., G. Burlacchini, M. del Mar Lleò, C. Pruzzo, M. Zampini, L. Pane, G. Franzini, and P. Canepari. 2004. Adhesion of *Enterococcus faecalis* in the nonculturable state to plankton is the main mechanism responsible for persistence of the bacterium in both lake and seawater. Appl. Environ. Microbiol. **70**(11):6892-6896.

Signoretto, C., G. Burlacchini, C. Pruzzo, and P. Canepari. 2005. Persistence of *Enterococcus faecalis* in aquatic environments via surface interactions with copepods. Appl. Environ. Microbiol. **71**(5):2756-2761.

Travel Industry Association. 2007. 2006 Travel profile-Georgia state visitors' statistics and travel economic impact: Regional analysis, http://ftp.itt.state.ga.us/georgia/2006_GA_Region_Report_9-10-07.pdf. Accessed on 1 June 2009.

Ulrich, A., and T. Müller. 1998. Heterogeneity of plant associated streptococci as characterized by phenotypic features and restriction analysis of PCR amplified 16S rDNA. J. Appl. Microbiol. **84**:293-303.

[USEPA] US Environmental Protection Agency, Office of Water. 1986. Ambient water quality criteria for bacteria-1986. EPA-440/5-84/002.

[USEPA] US Environmental Protection Agency, Office of Water. 1999. Action Plan for Beaches and Recreational Waters. EPA/600/R-98/079.

[USEPA] US Environmental Protection Agency, Office of Water. 2002. Method 1600: Enterococci in water by membrane filtration using membrane-enterococcus indoxyl-β-D-glucoside agar (mEI). EPA 821-R-02-022.

[USEPA] US Environmental Protection Agency. 2004. Water quality standards for coastal and great lakes recreation waters. Federal Register: **69**(220): 67217-67243

[USEPA] US Environmental Protection Agency, Office of Water. 2007. Recreational Water Quality Criteria. EPA 823-R-07-006.

Vasconcelos, G. J., and R. G. Swartz. 1976. Survival of bacteria in seawater using a diffusion chamber apparatus in situ. Appl. Environ. Microbiol. **31**(6):913-920.

Wade, T. J., N. Pai, J. N. S. Eisenberg, and J. M. Colford. 2003. Do U.S. Environmental Protection Agency water quality guidelines for recreational waters prevent gastrointestinal illness? A systematic review and meta-analysis. Environ. Health Perspect. **111**(8):1102-1109.

Wade, T. J., R. L. Calderon, E. Sams, M. Beach, K. P. Brenner, A. H. Williams, and A. P. Dufour. 2006. Rapidly measured indicators of recreational water quality are predictive of swimming-associated gastrointestinal illness. Environ. Health Perspect. **114**(1):24-28.

Whitman, R. L., D. A. Shively, H. Pawlik, M. B. Nevers, and M. N. Byappanahalli. 2003. Occurrence of *Esherichia coli* and enterococci in *Cladophora (Chlorophyta*) in nearshore water and beach of Lake Michigan. Appl. Environ. Microbiol. **69**(8):4714-4719.

Winslow, C.-E. A., and M. P. Hunnewell. 1902. Streptococci characteristic of sewage and sewage-polluted waters apparently not hitherto reported in America. Science. **15**(386):827-829.

Wolf, H. W. 1972. The coliform count as a measure of water quality, p. 333. *In* R. Mitchell (ed.), Water pollution microbiology, John Wiley and Sons, Inc., New York.

CHAPTER 3

PERSISTENCE AND GROWTH OF THE FECAL INDICATOR BACTERIA, ENTEROCOCCI, IN NATURAL ESTUARINE PLANKTON COMMUNITIES[1]

[1] Mote, B. L., J. W. Turner and E. K. Lipp. To be submitted to *Applied and Environmental Microbiology.*

Abstract

The fecal indicator bacteria, enterococci, are used to evaluate water quality in marine environments. Enterococci are not only associated with humans, but also have been found in the digestive tracts of other warm-blooded animals, in soil, on plant material and associated with plankton. Given the epiphytic nature of many *Enterococcus* spp., we investigated the contribution of plankton-associated enterococci in estuarine water samples. Seven water and size-fractionated plankton samples were collected monthly between April 2008 and January 2009 in the tidal reaches of the Skidaway River (Georgia, U.S.A.). Each size fraction, along with filtered (<30 μm) and bulk estuarine water, was processed according to EPA Method 1600. Presumptive enterococci were saved and species identified using carbon substrate utilization patterns (Biolog). The highest average enterococci densities occurred within the 30-, 63-, 105-, and 150-μm size fractions. These fractions also represented the majority (>99.9%) of the plankton particles within the estuarine water collected. Plankton-associated enterococci accounted for as little as 1% of enterococci in bulk water in April to as much as 95% in July. *Enterococcus casseliflavus* represented 26% of the selected isolates from both plankton (9/34) and water (20/77) and *E. faecalis,* 24% (8/34) and 25% (19/77), respectively. The results of this study suggest that *Enterococcus* spp. may be highly concentrated in plankton, especially during summer and fall months. Laboratory microcosm experiments supported this finding; *E. faecalis* and *E. casseliflavus* were able to survive and grow in mixed plankton at 10 and 30^{o}C. These

findings could have implications for the effectiveness of enterococci as an indicator of coastal water quality.

Introduction

Enterococci are Gram-positive bacteria common in the feces of warm blooded animals, including humans. In 1986, the United States Environmental Protection Agency (USEPA) recommended enterococci as the preferred indicator of fecal pollution and health risk in marine water, replacing fecal coliform bacteria. The recommendation was based on epidemiological studies showing enterococci were positively correlated to highly credible gastroenteritis cases at beaches throughout the United States (USEPA 1986). Enterococci also survive longer in marine environments than fecal coliform bacteria, making the group easier to detect, and they are more closely associated to human fecal matter than animal feces (Vasconcelos and Swartz 1976; Cabelli et al. 1983).

Although enterococci are more useful than other historically used indicators in marine environments, there are limitations to using the group to assess fecal pollution and health risk. USEPA recreational water quality indicator recommendations were based on a limited set of studies performed in the 1970s (Cabelli et al. 1983). Additionally, these studies focused only on beaches impacted by sewage treatment plants (Cabelli et al. 1983), excluding beaches affected by non-point source pollution, such as septic tanks and urban and agricultural run-off. Enterococci also have a range of sources other than humans including livestock and domestic and wild birds (Devriese

et al. 1987; Kuntz et al. 2004). Additionally, they have been found in association with soil, plants, zooplankton and algae (Roll and Fujioka 1997; Ulrich and Müller 1998; Signoretto et al. 2004; Whitman et al. 2003). Even *Enterococcus faecalis*, the species in the highest concentration in human feces (Noble 1978), has been found to adhere to zooplankton and to persist in the environment for extended periods of time (Signoretto et al. 2004, 2005). Due to the potential for introduction by sources other than human feces, and the persistence of enterococci in the environment, high levels may not indicate continuous addition of human fecal wastes in an area, and therefore may present confounding information for regulators charged with assessing water quality.

Under the federal Beaches Environmental Assessment and Coastal Health (BEACH) Act of 2000 (Public Law 106-284), every state with marine or Great Lakes beaches was required to adopt enterococcus standards for recreational water quality criteria by 2004. Furthermore, the BEACH Act also called for the continued evaluation and improvement of fecal indicator standards for recreational marine waters through scientific research. In the 2007 Scientific Experts Report, scientists specifically recognized the importance of an understanding of the ecology of fecal indicator bacteria when evaluating water quality criteria (USEPA 2007).

The overall goal of this research was to investigate the distribution, persistence, and possible growth of the fecal indicator bacteria, enterococci, in the plankton community of coastal Georgia waters. The following hypotheses were tested: (1) enterococci are enriched in estuarine plankton relative to the water column, (2) enterococci are associated with specific groups and sizes of plankton, (3) enterococci species distribution varies between free-living forms found in the water column and

forms found associated with plankton and (4) specific environmental conditions such as temperature and plankton concentration will affect the ability of *Enterococcus* species to grow in association with plankton.

Materials and Methods

Sample collection

Samples were collected seven times between April 2008 and January 2009 at a tidally influenced fixed station on Skidaway Island (GA) along the Intracoastal Waterway (Figure 1). Surface water temperature, pH, salinity, and conductivity were measured with a YSI Model 556 meter (YSI, Inc., Yellow Springs, OH). Rainfall data were obtained for Skidaway Island (www.georgiaweather.net) for the day, week, and month prior to each sampling date. Tidal stage (www.tidesandcurrents.noaa.gov) for each sampling event was recorded. At each collection, 10 sample fractions were obtained, including two water samples and eight plankton fractions. Water samples were collected in 1-liter, sterile polypropylene bottles as grab samples <0.5 m below the surface of the water. Bulk water was filtered through a 30-µm mesh net to provide a final filtered sample. An ISCO 3700 Sampler water pump (ISCO, Inc., Lincoln, NE) was used to deliver water, collected at <0.5 m depth, through a series of mesh nets fixed in PVC housings. The pumping rate of the ISCO water pump was determined at the start of each sample set. Mesh nets were arranged in descending order (500, 335, 250, 200, 150, 105, 63 and 30 µm) and filtered sequentially. Water pumping time was recorded and converted to volume of water pumped. Plankton and particles in each net were collected by rinsing with phosphate buffered saline (PBS) into individual sterile glass beakers to a final

volume of 200 ml. Samples were transported immediately to the laboratory for bacterial processing.

Bacterial analysis

Each concentrated plankton sample was subdivided into four 50-ml aliquots. Prior to membrane filtration, each concentrated plankton sample was homogenized (Pro 200, ProScientific Inc., Oxford, CT) for 2 minutes. Homogenized plankton samples, filtered and bulk water were filtered in duplicate onto 47 mm 0.45-µm mixed cellulose membranes according to USEPA Method 1600 (USEPA 2002). Membranes were placed onto mEI plates and incubated for 24 $\pm$ 2 hours at 41^{o}C. All colonies with a blue halo were considered enterococci, and final counts were recorded as colony forming units (CFU) 100 ml^{-1} of concentrated plankton.

The contribution of each plankton fraction (and all fractions collectively) to the enterococci load in a bulk water samples was determined. Briefly, the equivalent volume of bulk water represented in the concentrated 200 ml plankton samples was determined and the associated enterococci levels were back calculated. This number was compared to enterococci concentrations in bulk water and a percent contribution was determined.

Bacterial speciation

Following incubation on mEI, up to 10 presumptive enterococci colonies were picked from each membrane filter with a sterile toothpick. Selected colonies were re-isolated three times on mEI or mE agar. Final isolates were grown on Biolog Universal Growth (BUG) media with 5% sheep's blood overnight and inoculated into Biolog GP2 MicroPlates (Biolog, Inc., Hayward, CA, USA) for phenotypic identification based on

carbon substrate utilization patterns. Isolates with similarity index value (SIM) ratings of greater than 0.5 were included for analysis (Solit 2001). Biolog GP2 Microplates correctly identify common enterococci species with 73% overall accuracy and 100, 90, 64, 100, and 100% accuracy for *E. casseliflavus*, *E. faecalis*, *E. faecium*, *E. gallinarum*, and *E. mundtii*, respectively (Moore et al. 2006). All identified isolates were stored in 20% glycerol at -80^{o}C.

Plankton identification

A 25-ml aliquot of each plankton sample (30-63, 63-105, 105-150, 150-200, 200-250, 250-335 and 335-500 µm) was fixed (4% v/v, formalin, final concentration) and stored at 5^{o}C. Samples were preserved for long-term storage in 70% ethanol (v/v) and stored at room temperature. A stereoscope (Olympus SZX9) (Olympus America Inc., Center Valley, PA) was used to determine the actual concentration of plankton and detritus (including plant matter, fecal pellets, and pieces of plankton and exuviae) in each sample. For the larger size fractions (200-250, 250-335, and 335-500 µm), the entire 25 ml volume was counted. For the smaller size fractions (30-63, 63-105, 105-150, and 150-200 µm), a Hensen-Stemple pipette (Wildco Wildlife Supply Company, Buffalo, NY) was used to obtain a countable subsample (100-400 organisms). Briefly, samples were poured or pipetted onto square Petri dishes divided by 5 mm grids (Fisher Scientific Inc., Pittsburg, PA) and immobilized with 3-5 drops of Protoslo (Carolina Biological Supply Company, Burlington, NC). The concentration of plankton or detritus ml^{-1} was determined after categorizing particles into general taxonomical groups or a detritus group. Taxonomical groups consisted of diatoms, cyanobacteria, dinoflagellates, ciliates, cnidarians, rotifers, cladocerans, ostracods, polychaetes,

bivalve nauplii, copepods, copepod nauplii, crab zoea, amphipods, annelids and sessilia. Unless otherwise noted, use of the word “plankton” for all particles greater than 30 µm may include detritus.

Microcosm

Plankton collection: Plankton for use in laboratory microcosm studies was collected in the Intracoastal Waterway in Brunswick, GA in December 2008 during three 10-minute tows by boat performed using 63-µm and 200-µm mesh nets. The contents of each tow (all sizes) were combined into one 2-L bottle and allowed to settle out to the bottom. Water was pipetted from the top of the plankton slurry and the wet weight of the plankton was used to create a 1% plankton suspension in artificial seawater (ASW) (Instant Ocean, Aquarium Solutions, Mentor, OH) and a 5% plankton suspension in ASW with a salinity of 30. All plankton samples were stored at 5^{o}C.

Bacterial Cultures: Environmental strains of *E. faecalis* (representing likely fecal-source) and *E. casseliflavus* (representing likely epiphytic source) were obtained during this study. Each strain was identified using Biolog and confirmed by sequencing of the full 16S-RNA gene (Macrogen, Rockville, MD, USA). Sequences were searched using BLAST in GenBank (Altschul et al. 1990). A series of eight microcosms were used to compare the persistence and/or growth of the two species within 1% plankton, 5% (W/V) plankton and artificial seawater at 10 and 30^{o}C (Figure 2).

Overnight cultures of *E. faecalis* and *E. casseliflavus* were grown in 5 ml Brain Heart Infusion Broth at 35^{o}C. Cultures were centrifuged at 2450 x g for 10 minutes at 4^{o}C. Cells washed by re-suspending in 5 ml 1X PBS and vortexing, were centrifuged

again and re-suspended in a final volume of 5 ml 1X PBS. Cells were inoculated into each microcosm for an estimated final concentration of 10^3 to 10^4 CFU mL^{-1}.

Plankton preparation: A 60-mL sample of prepared plankton mixtures, 1% and 5%, were pipetted into each of six 200-ml flasks and placed into a boiling water bath for 2 minutes to heat-kill bacteria present in the natural plankton. Flasks were allowed to cool overnight to 5°C. The following day, each of three replicate 1% and 5% plankton flasks was inoculated with one of two test *Enterococcus* species (*E. faecalis* or *E. casseliflavus*). The remaining flasks (three each of 1% and 5% plankton) were not inoculated and served as negative plankton controls. Triplicate flasks with 60 ml ASW were also inoculated with each species, and triplicate flasks of 60 ml uninoculated ASW served as negative seawater controls. After inoculation, the contents of each flask were kept in suspension by mixing on a stirplate and pipetted into ten 15-ml sterile polypropylene tubes in 5.5-mL aliquots. Tubes were placed on an end-over-end rotator (Rugged Rotator Model 099A RD4512, Glas-Col, Terre Haute, IN, USA) and held at 10 or 30°C in the dark. These temperatures represented the typical maximum and minimum temperatures found during this and previous studies in coastal Georgia (e.g., Turner et al. 2009). Tubes were removed at 0, 4, 8, 12, 24 and 48 hours, and Days 4, 7, 11, 16, 22. Once removed, the contents of each tube were homogenized, diluted with 1X PBS (as needed), and spread-plated onto mE agar in duplicate. The tubes with homogenized contents from Day 22 were placed back onto the end-over-end rotator and aliquots were taken for spread-plating on Day 28, 35, 42 and 65. After 42 $\pm$ 6 hours incubation at 41°C, colonies (brick-red) were counted and recorded as CFU mL^{-1}. For each time point, change from initial conditions was defined as counts at time n divided

by counts at time 0 (T_n/T_0), where $T_n/T_0 > 1$ represented growth and $T_n/T_0 < 1$ represented die off.

Statistical Analysis

All enterococci counts were log-transformed to approximate a normal distribution. Normality was tested with the Shapiro-Wilk normality test (alpha = 0.05). Log values were used in statistical tests and means are reported as geometric means in the text. Associations between environmental characteristics (e.g., temperature, salinity, dissolved oxygen and rainfall) and enterococci counts (CFU 100 mL^{-1}) in plankton fractions and bulk water were analyzed using the Pearson correlation coefficient (r). When counts were below the limit of detection, a value of zero was used in all statistical calculations. In all cases, significance was declared at $p \leq 0.05$.

For microcosm experiments, timepoints when maximum growth was reached for each flask (highest T_n/T_0 value) was determined. Analysis of variance (ANOVA) was used to determine if this maximum density was significantly different among the treatments. Tukey's posthoc test was used to evaluate pairwise differences. Significance was declared at $p \leq 0.05$.

Results

Sampling occurred on an incoming tide in April, June, early July, November and January and an outgoing tide in late July and September. The temperature ranged from 11.5^{o}C in January to 29.7^{o}C in late July with a mean of 22.8^{o}C for all dates. Salinity ranged from 24.3 to 30.6 with a mean of 28.6 for all dates. Dissolved oxygen (DO) ranged from 3.4 mg mL^{-1} in early July to 8.5 mg mL^{-1} in November. The mean DO for

the sampling dates was 5.8 mg mL^{-1}. The pH ranged from 7.6 in June, early July and January to 7.9 in September and November with a mean of 7.7 for all sampling dates (Table 1). The mean rainfall for the week prior to each sampling date was 0.56 cm and for the month (30 days) prior to each sampling date was 7.06 cm. There was a significant positive correlation ($p = 0.05$) between enterococci concentration in the >500-µm plankton fraction and salinity and a significant negative correlation between enterococci in the 250-335- and 335-500-µm plankton fractions and rainfall the week prior to sampling dates ($p = 0.04$ and 0.02 respectively).

85% of all plankton fraction particles were classified as detritus, which was found in all plankton fractions ranging from an average of 42% of particles in the 105-150-µm fraction to 99% of particles in the >500-µm fraction. Of the total particles, 13.5% were classified as diatoms (found in the four plankton fractions <200-µm) and 1% copepod nauplii (found in the 63-105-, 105-150-, and 150-200-µm fractions). All other plankton categories combined made up less than 1% of the plankton particles. Over 99% of all plankton particles were from the 30-63-, 63-105- and 105-150-µm fractions.

Enterococci density in environmental samples

Enterococci densities in bulk water ranged from 4 CFU 100 ml^{-1} in early July to 938 CFU 100 ml^{-1} in late April (mean 71 CFU 100 ml^{-1}). In filtered water, enterococci densities ranged from 3 CFU 100 ml^{-1} in June to 102 CFU 100 ml^{-1} in April (mean 20 CFU 100 ml^{-1}). There was not a significant difference between mean bulk and filtered water enterococci densities. Enterococci counts from bulk water exceeded EPA's single sample maximum limit (104 CFU 100 ml^{-1}) in April, June, and November.

Among the concentrated plankton fractions, enterococci were detected in all months only in the two smallest fractions (30-63 and 63-105 μm). The abundance of enterococci in the 30-63-μm fraction ranged from 10 to 3.3 x 10^4 CFU 100 ml^{-1} (mean 1.4 x 10^3 CFU 100 ml^{-1}), with the largest abundance in November (Figure 3). Within the 63-105-μm fraction, which averaged 230 CFU 100 ml^{-1}, the range was 60 CFU 100 ml^{-1} in January to the largest abundance in September (1.4 x 10^3 CFU 100 ml^{-1}). The abundance of enterococci in the 105-150-μm fraction averaged 72 CFU 100 ml^{-1} with the largest abundance in April (5.8 x 10^3 CFU 100 ml^{-1}) and was below the detection limit (<2 CFU 100 ml^{-1}) in June. The abundance of enterococci in the 150-200-μm fraction averaged 118 CFU 100 ml^{-1}, with the largest abundance in November (3.9 x 10^3 CFU 100 ml^{-1}) and enterococci recovery below the detection limit in January. The abundance of enterococci in the 200-250-, 250-335-, 335-500- and >500-μm fractions averaged 10, 48, 25, and 13 CFU 100 ml^{-1} respectively, with enterococci recovery below the detection limit for all of these plankton fractions in January. Additionally, enterococci density was below the detection limit for the 200-250- and >500-μm plankton fractions in June and the 250-335-, 335-500-, and >500-μm plankton fractions in April (Figure 3). Significant differences in enterococci concentration between the plankton fractions were found, with mean levels in the 30-63-μm fraction significantly greater ($p = 0.03$) than those in the 200-250- and >500-μm fractions.

The percent contribution of enterococci by each plankton fraction to bulk water was determined (Figure 4). Enterococci from all plankton combined contributed as much as 95% of the enterococci in bulk water in early July and as little as 1.5% in April. The 30-63-μm plankton fraction contributed the most enterococci to bulk water (mean 22%)

on all dates except in April and January. In April, the 105-150-µm plankton fraction contributed the most enterococci (4%) and averaged 1% contribution across the sampling dates. In January, the >500-µm fraction contributed the most enterococci (2.4%). The 63-105-µm plankton fraction averaged 3% contribution across the sampling dates. The 150-220-, 200-250-, 250-335-, 335-500-, and >500-µm fractions each averaged less than 1.5% of the enterococci contribution to bulk water. Each plankton fraction contributed between 2 and 7.6% of enterococci density in bulk water in September.

Enterococcus *species distribution*

A total of 68 isolates were speciated, 51 from water and 17 from plankton (all fractions combined). Growth of non-target bacteria, including *Staphylococcus* species, on the mEI agar plates, made proper isolation of enterococci colonies difficult from plankton samples, even after multiple rounds of subculture. The most common species isolated from both water and plankton (all fractions combined) was *E. faecalis* (16/51 [31%] and 16/17 [35%], respectively). 8/51 (16%) of water isolates and 5/17 (29%) of plankton isolates were identified as *E. casseliflavus.* 16/51 (31%) of water isolates and 4/17 (24%) of plankton isolates were identified as *E. mundtii*. Between the water and plankton samples, *E. faecium* was found less frequently but with similar percentages between water and plankton (Table 2). *Enterococcus gallinarum* and *E. flavescens* isolates were identified in water but not plankton.

Microcosms

At 10^{o}C, there were notable differences between *E. faecalis* and *E. casseliflavus* among the seawater and plankton treatments (Figure 5). In both cases, bacteria

declined in ASW alone; however, *E. casseliflavus* persisted for 7 days while *E. faecalis* died off by 24 hours. Persistence was greatly enhanced for both species by the presence of plankton. At both 1% and 5% treatments, both species showed viable (culturable) populations through Day 65. At 1% plankton, *E. casseliflavus* showed static population density with a T_n/T_0 of 1.21 at Day 65. The maximum growth was reached at Day 16 with a T_n/T_0 of 1.67. *Enterococcus faecalis* showed a greater decay than *E. casseliflavus* with a T_n/T_0 of 0.06 on Day 65 and no growth (T_n/T_0 always ≤1) during the course of the experiment. At 5% plankton, *E. casseliflavus* showed logarithmic growth early in the treatment, resulting in a maximum T_n/T_0 of 58.09 on Day 11 and persisting near this level through Day 65. *Enterococcus faecalis* also showed growth, although minimal, reaching a T_n/T_0 of 1.24 (Day 11), but also persisting near this level through the end of the experiment. All non-inoculated controls (1%, 5%, and ASW) remained negative throughout the experiment.

Persistence at 30°C was less than that observed for 10°C in all experimental treatments. For both species, populations declined rapidly in ASW with *E. faecalis* losing culturability by 4 hours and *E. casseliflavus* by 12 hours. In 1% plankton, *E. casseliflavus* reached a maximum growth of 4.49 (T_n/T_0) at Day 2 but lost culturability by Day 16. For *E. faecalis*, growth was observed, albeit moderate, (T_n/T_0 = 1.19) but cells persisted to day 16. In 5% plankton, *E. casseliflavus* grew to a maximum T_n/T_0 of 39.09 at Day 2 and persisted to Day 22, after which it could no longer be cultured. *Enterococcus faecalis* also grew in the 5% treatment, reaching 7.19 (T_n/T_0) at Day 2 and persisting to Day 28. Controls remained negative throughout the experiment, with the

exception of the 5% plankton in which 1 CFU was noted at the 4-h time-point (negative at all other times).

At both 10°C and 30°C, a significant difference was observed in the maximum growth achieved between *E. faecalis* and *E. casseliflavus* in the 5% plankton treatment. *Enterococcus casseliflavus* T_n/T_0 levels were significantly greater than those of *E. faecalis* at both temperatures ($p < 0.0001$). *Enterococcus casseliflavus* growth was also significantly greater in 5% plankton than1% plankton at both temperatures ($p < 0.0001$).

Discussion

This study compared the density of the fecal indicator bacteria, enterococci, in water and size-fractionated plankton samples from an estuary in Georgia over the course of seven months. We found that plankton-associated enterococci contributed up to 95% of the enterococci in bulk water samples and in vitro studies indicate that plankton may serve as a reservoir for growth and persistence of this fecal indicator. Enterococci have previously been found in association with specific types of plankton such as copepods, green algae and seaweed (Signoretta et al. 2004, 2005; Whitman et al. 2003; Anderson et al. 1997). In this study, we compared the contribution of groups of plankton, separated by size, to enterococci densities. The density of enterococci greatly varied between plankton fractions and between sampling dates; however, the highest densities were found consistently in the 30-63, 63-105, and 105-150 µm plankton fractions. These plankton fractions also represented the majority (>99.9%) of the plankton particles in bulk water, which was comprised of an average of 85% plant detritus, 13.5% diatoms and 1% copepod nauplii. Contrary to what was observed in this study, a coastal marine study in Italy found enterococci densities in greater numbers in

larger plankton (>200 µm and mostly zooplankton) when compared to smaller plankton (<64 µm and mostly phytoplankton) (Maugeri et al. 2004). The large tidal range and particle load in the estuary where our study was conducted could explain the differences observed between our study and a coastal study performed 50 m from shore.

Data suggest that, unlike some *Vibrio* spp. which tend to attach to specific types of organisms (Kaneko et al. 1975; Huq et al. 1983), enterococci levels may be more related to the general particle load present in the environment. In areas of higher particle concentrations, such as within an estuary with high levels of tidal mixing, particle-associated bacterial counts increase relative to free-living bacteria (Bidle and Fletcher 1995). This result is consistent with results found here where the smaller plankton fractions (mostly plant detritus) made up the majority of particles collected and therefore provided more opportunity for bacterial association. Enterococci, acting as detritivores, may be able to colonize the organic material in the marine environment and persist. Enterococci may not only find necessary nutrients in plankton but also find shelter from sunlight inactivation (Kay et al. 2005).

While we hypothesized that the recognized epiphytic *Enterococcus* species would be more common among the plankton fractions, we found that species distribution was actually similar between plankton and water. *Enterococcus faecalis* represented the majority of the isolates identified in both categories. *Enterococcus faecalis* is commonly isolated from the intestines of warm-blooded animals including humans (Noble 1978; Devriese et al. 1987) and has been found in large concentrations (>30% of enterococci population) in estuarine areas along the Georgia coast (McDonald et al. 2006). *Enterococcus casseliflavus* and *E. mundtii* were identified frequently in both the

plankton and water sample isolates. Unlike *E. faecalis*, *E. casseliflavus* and *E. mundtii* are considered plant-associated species found on plant matter and in soil but not often isolated from the intestines of animals (Devriese et al. 1987; Leclerc et al. 1996). Our results are consistent with a study in Orange County, California that found 31% of presumptive *Enterococcus* isolates from ocean samples to be *E. casseliflavus* and 24% of isolates were *E. faecalis* (Moore et al. 2008). In Greece, the majority of marine recreational water enterococci isolates were human fecal-associated: *E. faecium* (11/94 18%) and *E. faecalis* (10/94 17%) (Grammenou et al. 2006). These studies demonstrate regional variation in the composition of the bacterial community. It also illustrates that non-human strains are a common component of the bulk enterococci densities and species level differences in growth or persistence in coastal environments may impact the utility of this group as a fecal indicator.

Enterococci survival in the environment depends upon several biotic and abiotic factors, including temperature, sunlight and presence of organic matter, among others. Our microcosm results show persistence and growth of both *E. casseliflavus* and *E. faecalis* at 10^{o}C and 30^{o}C in the presence of plankton, however, at 10^{o}C, both species persisted at near inoculum levels (~10^{3} CFU ml^{-1}) for greater than 2 months whereas culturability was lost by 28 days at 30^{o}C. Although enterococci can survive and even grow at higher temperatures with sufficient nutrients and organic matter, our findings in artificial seawater (without plankton) for both *E. faecalis* and *E. casseliflavus* were consistent with previous studies on the survival of total enterococci from natural sewage in aquatic environments with survival reduced to only several hours at 30^{o}C compared

to lower temperatures (Lessard and Sieburth 1983, Bordalo et al. 2002; Lleó et al. 2002).

With availability of nutrients and protection, as may be found in algae or plankton, enterococci may be able not only to survive, but multiply in marine environments. In our study, growth was enhanced by the presence of plankton compared to artificial seawater. Relative to seawater, both 1% and 5% plankton concentrations resulted in significant growth and enhanced persistence of both *E. casseliflavus* and *E. faecalis*. Similarly, indigenous enterococci demonstrated 100-fold growth for the first 18 hours in *Cladophora* leachate, filtered supernatant from a centrifuged suspension of *Cladophora* (a green alga) and lake water at 35^{o}C (Byappanahalli et al. 2003). In this investigation, enterococci concentration remained above or at the starting concentration for the 168-hour study duration (Byappanahalli et al. 2003). Moreover, in situ evidence also supports the potential for enterococci to grow in marine environments. By comparing total enterococci levels in recreational beaches and seaweed in New Zealand, significantly higher levels in seaweed suggested the possible expansion of enterococci in the environment (Anderson et al. 1997). Further clonal analysis of the isolates from the seaweed and water demonstrated the presence of clonal enterococci populations in seaweed but their absence in water samples (Anderson et al. 1997). Results from the present study confirm these previous results, but also indicate the potential for growth among individual *Enterococcus* species. While *E. casseliflavus*, a plant-associated species, grew to significantly higher densities than *E. faecalis*, *E. faecalis*, a fecal-associated species, exhibited measurable growth in association with plankton, especially with increasing plankton concentrations and temperature. These results are

also consistent with our field survey, which showed an increased contribution of plankton-associated enterococci during the warmest months of the year, with 95% of the enterococci in bulk water attributed to plankton in early July.

In addition to growth in association with plankton, these organic-rich particulates could also protect enterococci from damage by UV irradiance, which has been cited as an important driver for enterococci survival in the environment (Kay et al. 2005). Estuarine waters with high turbidity due to suspended solids such as plankton provide protection from sunlight inactivation. In microcosm experiments testing enterococci survival in estuarine water at 15^{o}C, enterococci persisted longer in high-turbidity waters compared to low turbidity waters in both irradiated and dark experiments (Kay et al. 2005).

While this and other studies suggest the importance of plankton and particulate organic matter on enterococci growth and persistence (i.e., Anderson et al. 1997; Byappanahalli et al. 2003; Kay et al. 2005), there is evidence that plankton may also control enterococci abundance (Hartke et al. 2002). Although not accounted for in the size-fractions studied here (all > 30 μm), protozoan predators (i.e., flagellates < 20 μm) have been shown to graze on enterococci in laboratory controlled studies (Hartke et al. 2002). Furthermore, once introduced into the environment, enterococci may be temporarily sequestered in sediments, where they remain in high concentrations but are not accounted for in routine water monitoring during calm conditions (McDonald et al. 2006). Storm events could resuspend these populations leading to artificially high levels (McDonald et al. 2006). Together, these studies suggest that enterococci, once introduced in the natural aquatic environment, are subject to multiple factors which may

result in net growth or loss, independent of those human activities that these bacteria are meant to represent such as loading from sewage discharge or other wastewater disposal.

This study contributes to the knowledge of the ecology of enterococci within estuarine plankton communities, which can aide in the evaluation of these bacteria as water quality indicators. Our investigation demonstrated the potential for enterococci to associate and proliferate within the plankton community along Georgia's coast. In estuaries, which are typically high particle-load environments, particle-associated bacterial counts increase relative to free-living bacteria (Bidle and Fletcher 1995). By providing nutrients and protection from sunlight, particles such as plankton are potential reservoirs for enterococci. Whereas enterococci may serve as a reliable indicator in some regions of the world, in particle rich marine environments, enterococci density analysis may provide misleading information for regulators assessing water quality.

References

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. **215**:403-410.

Anderson, S. A., S. J. Turner and G. D. Lewis. 1997. Enterococci in the New Zealand environment: implications for water quality monitoring. Water Sci. Tech. **35**(11-12):325-331.

Bidle, K. D., and M. Fletcher. 1995. Comparison of free-living and particle associated bacterial communities in the Chesapeake Bay by stable low molecular weight RNA analysis. Appl. Environ. Microbiol. **61**(3):944-952.

Bordalo, A. A., R. Onrassami, and C. Dechsakulwatana. 2002. Survival of faecal indicator bacteria in tropical estuarine waters (Bangpakong River, Thailand). J. Appl. Microbiol. **93**:864-871.

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

Cabelli, V. J., A. P. Dufour, L. J. McCabe, and M. A. Levin. 1983. A marine recreational water quality criterion consistent with indicator concepts and risk analysis. J. Water Pollut. Cont. Fed. **55**(10):1306-1314.

Devriese, L. A., A. Van De Kerckhove, R. Kilpper-Bälz, and K. H. Schleifer. 1987. Characterization and identification of *Enterococcus* species isolated from the intestines of animals. Int. J. Syst. Bacteriol. **37**(3):257-259.

Grammenou, P., I. Spiliopoulou, E. Sazakli, and M. Papapetropoulou. 2006. PFGE analysis of enterococci isolates from recreational and drinking water in Greece. J. Water Health. **4**:263-269.

Hartke, A., S. Lemarinier, V. Pichereau, and Y. Auffray. 2002. Survival of *Enterococcus faecalis* in seawater experiments is limited in the presence of bacterivorous zooflagellates. Current Microbiology. **44**:329-335.

Huq, A., E. B. Small, P. A. West, M. I. Huq, R. Rahman, and R. R. Colwell. 1983. Ecological relationships between *Vibrio cholerae* and planktonic crustacean copepods. Appl. Environ. Microbiol. **45**(1):275-283.

Kaneko, T., and R. R. Colwell. 1975. Adsorption of *Vibrio parahaemolyticus* onto chitin and copepods. Appl. Environ. Microbiol. **29**(2):269-274.

Kay, D., C. M. Stapleton, M. D. Wyer, A. T. McDonald, J. Crowther, N. Paul, K. Jones, C. Francis, J. Watkins, J. Wilkinson, N. Humphrey, B. Lin, L. Yang, R. A. Falconer, and S. Gardner. 2005. Decay of intestinal enterococci concentrations in high-energy estuarine and coastal waters: towards real-time T_{90} values for modeling faecal indicators in recreational waters. Water Res. **39**:655-667.

Kuntz, R. L., P. G. Hartel, K. Rodgers, and W.I. Segars. 2004. Presence of *Enterococcus faecalis* in broiler litter and wild bird feces for bacterial source tracking. Water Res. **38**(16):3551-3557.

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

Lessard, E. J., and J. McN. Sieburth. 1983. Survival of natural sewage populations of enteric bacteria in diffusion and batch chambers in the marine environment. Appl. Environ. Microbiol. **45**(3):950-959.

Lleó M. M., B. Bonato, D. Benedetti, and P. Canepari. 2002. Survival of enterococcal species in aquatic environments. FEMS Microbiol. Ecol. **54**:189-196.

Maugeri, T. L., M. Carbone, M. T. Fera, G. P. Irrera, and C. Gugliandolo. 2004. Distribution of potentially pathogenic bacteria as free living and plankton associated in a marine coastal zone. J. Appl. Microbiol. **97**:354-361.

McDonald, J. L., P. G. Hartel, L. C. Gentit, C. N. Belcher, K. W. Gates, K. Rodgers, J. A. Fisher, K. A. Smith, and K. A. Payne. 2006. Identifying sources of fecal contamination inexpensively with targeted sampling and bacterial source tracking. J. Environ. Qual. **35**:889-897.

Moore, D. F., M. H. Zhowandai, D. M. Ferguson, C. McGee, J. B. Mott and J. C. Stewart. 2006. Comparison of 16S rRNA sequencing with conventional and commercial phenotypic techniques for identification of enterococci from the marine environment. J. Appl. Microbiol. **100**:1272-1281.

Moore, D. F., J. A. Guzman, and C. McGee. 2008. Species distribution and antimicrobial resistance of enterococci isolated from surface and ocean water. J. Appl. Microbiol. **105**(4):1017-1025.

Noble, C. J. 1978. Carriage of group D streptococci in the human bowel. J. Clin. Pathol. **31**:1182-1186.

Roll, B. M., and R. S. Fujioka. 1997. Sources of faecal indicator bacteria in a brackish, tropical stream and their impact on recreational water quality. Water Sci. Technol. **35**(11-12):179-186.

Signoretto, C., G. Burlacchini, M. del Mar Lleò, C. Pruzzo, M. Zampini, L. Pane, G. Franzini, and P. Canepari. 2004. Adhesion of *Enterococcus faecalis* in the nonculturable state to plankton is the main mechanism responsible for persistence of the bacterium in both lake and seawater. Appl. Environ. Microbiol. **70**(11):6892-6896.

Signoretto, C., G. Burlacchini, C. Pruzzo, and P. Canepari. 2005. Persistence of *Enterococcus faecalis* in aquatic environments via surface interactions with copepods. Appl. Environ. Microbiol. **71**(5):2756-2761.

Solit, R. 2001. MicroLog System, Release 4.2 User Guide. Biolog, Inc., Hayward, CA.

Turner, J. W., B. Good, D. Cole, and E. K. Lipp. 2009. Environmental factors affect the status of plankton as a reservoir for *Vibrio* species. ISME J. doi: 10.1038/ismej.2009.50

Ulrich, A., and T. Müller. 1998. Heterogeneity of plant associated streptococci as characterized by phenotypic features and restriction analysis of PCR amplified 16S rDNA. J. Appl. Microbiol. **84**:293-303.

[USEPA] US Environmental Protection Agency, Office of Water. 1986. Ambient water quality criteria for bacteria-1986. EPA-440/5-84/002.

[USEPA] US Environmental Protection Agency, Office of Water. 2002. Method 1600: Enterococci in water by membrane filtration using membrane-enterococcus indoxyl-β-D-glucoside agar (mEI). EPA 821-R-02-022.

[USEPA] US Environmental Protection Agency, Office of Water. 2007. Recreational Water Quality Criteria. EPA 823-R-07-006.

Vasconcelos, G. J., and R. G. Swartz. 1976. Survival of bacteria in seawater using a diffusion chamber apparatus in situ. Appl. Environ. Microbiol. **31**(6):913-920.

Whitman, R. L., D. A. Shively, H. Pawlik, M. B. Nevers, and M. N. Byappanahalli. 2003. Occurrence of *Esherichia coli* and enterococci in *Cladophora (Chlorophyta*) in nearshore water and beach of Lake Michigan. Appl. Environ. Microbiol. **69**(8):4714-4719

Tables

Table 1. Physiochemical parameters at time of sample collection.

Sampling date	Temperature (^{o}C)	Salinity	Dissolved oxygen (mg mL^{-1})	pH
29 Apr 2008	22.1	24.3	7.1	7.7
10 June 2008	29.7	29.2	4.4	7.6
8 July 2008	28.4	30.6	3.4	7.6
29 July 2008	29.7	30.5	3.7	7.7
24 Sept 2008	22.0	30.6	5.9	7.9
12 Nov 2008	16.2	27.0	8.5	7.9
15 Jan 2009	11.6	28.2	7.6	7.6

Table 2. Summary of speciation results for water and plankton samples.

Rank	Water	Percent (n=51)	Plankton	Percent (n=17)
1	*E. faecalis*	31%	*E. faecalis*	35%
2	*E. mundtii*	31%	*E. casseliflavus*	29%
3	*E. casseliflavus*	16%	*E. mundtii*	24%
4	*E. faecium*	10%	*E. faecium*	12%
5	*E. flavescens*	8%	*E. flavescens*	0%
6	*E. gallinarum*	4%	*E. gallinarum*	0%

Figures

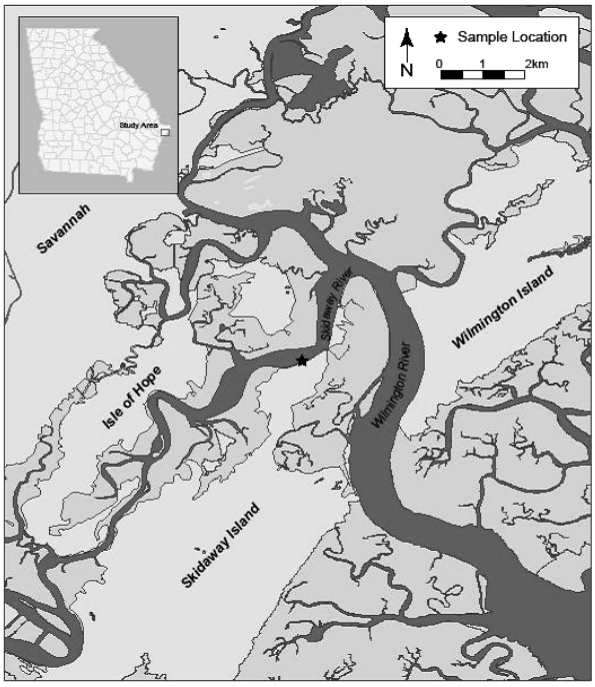

Figure 1. Water and plankton samples were collected between April 2008 and January 2009 at a fixed station on Skidaway Island (GA) along the Intracoastal Waterway.

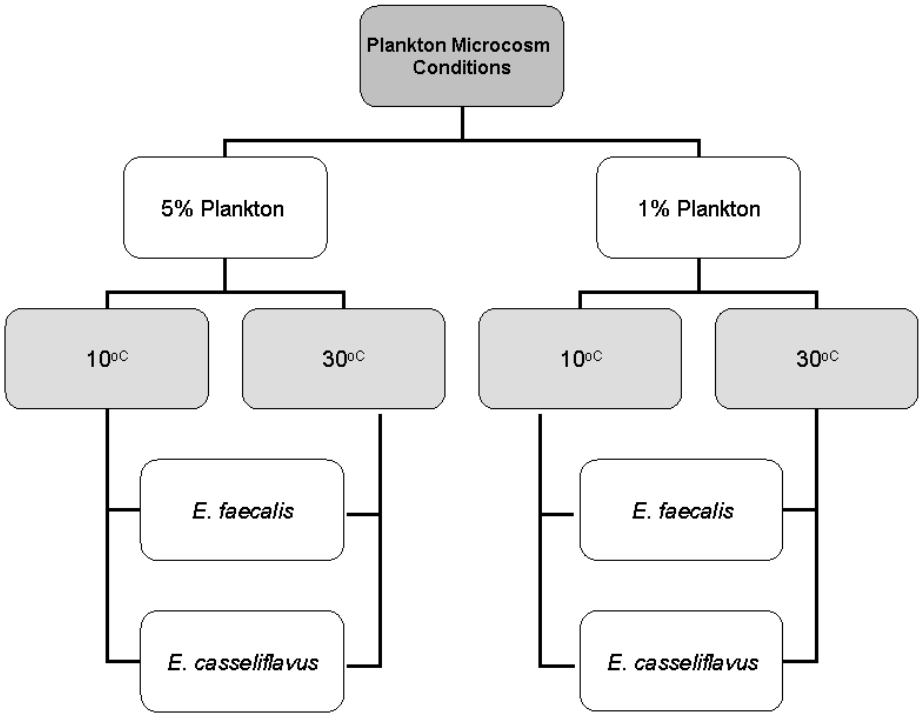

Figure 2. Plankton microcosm conditions.

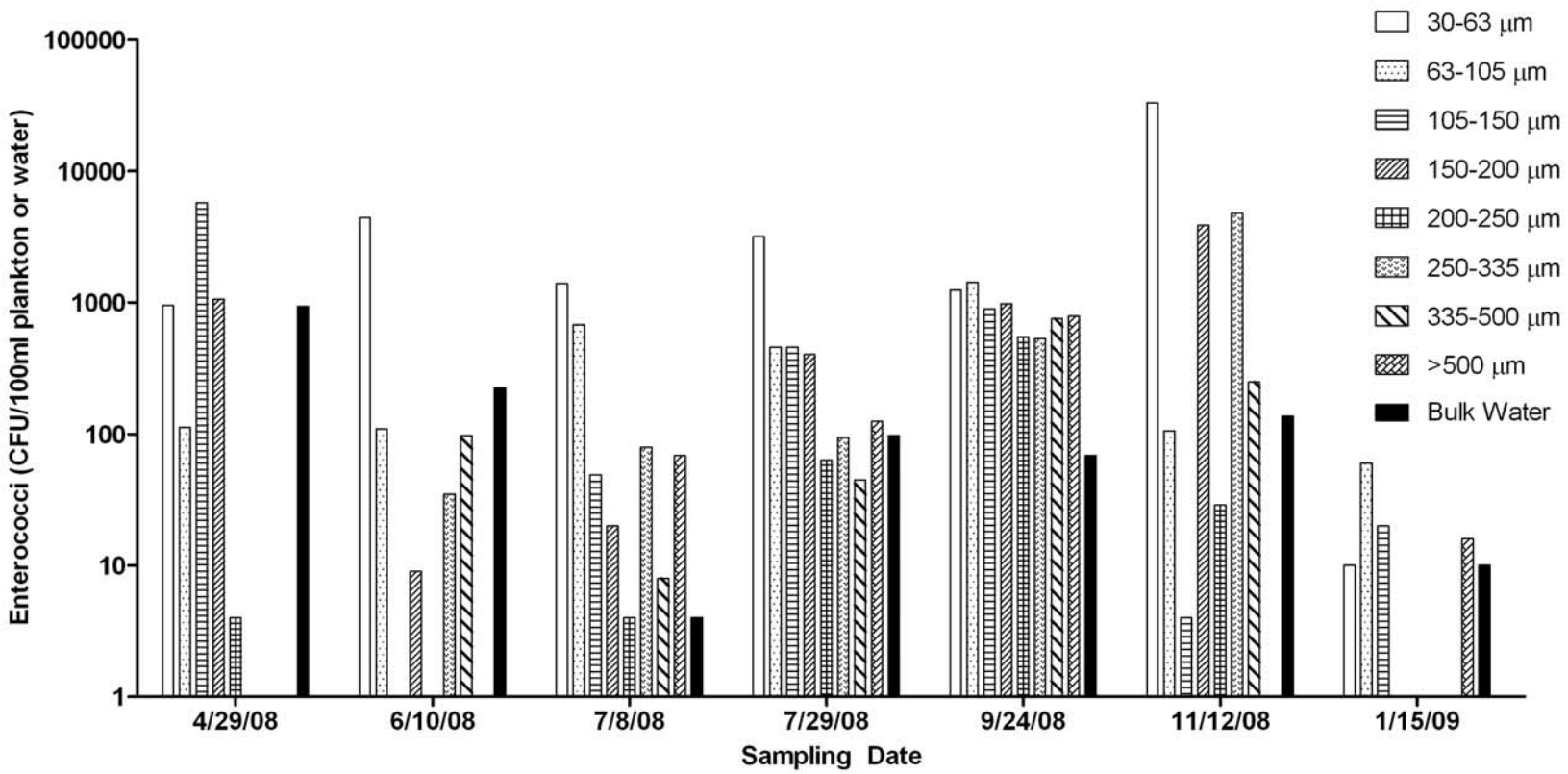

Figure 3. Geometric mean enterococci densities in plankton fractions and bulk water (n=2).

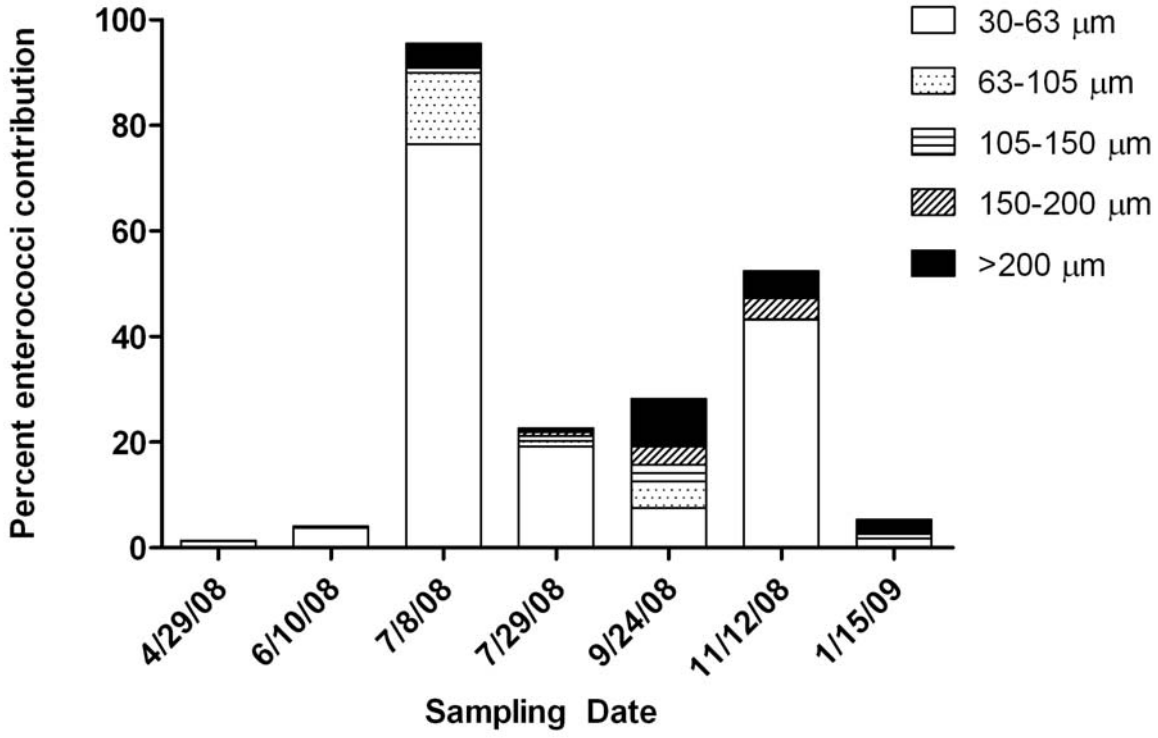

Figure 4. Percent enterococci contribution by plankton to bulk water.

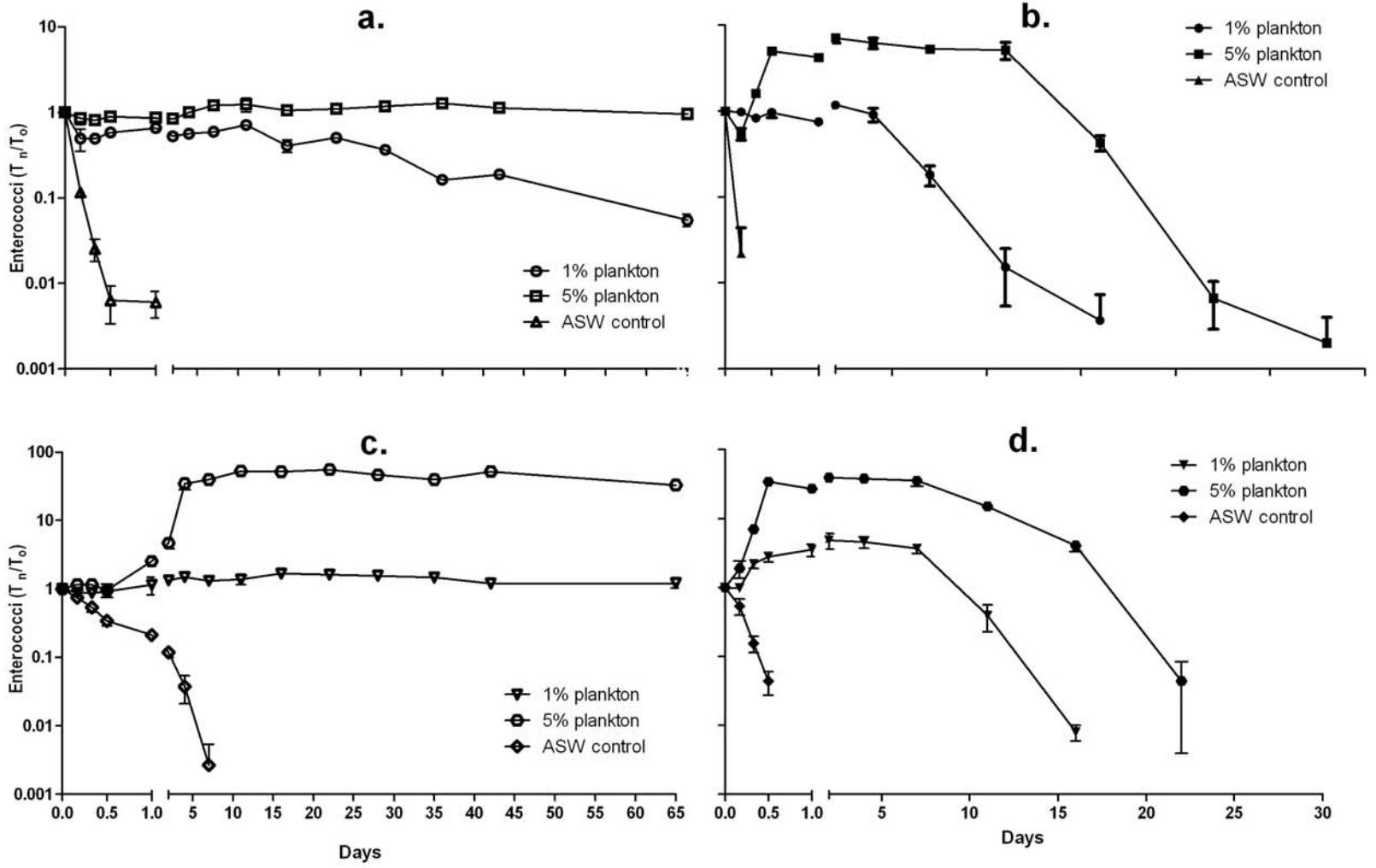

Figure 5. Microcosm results for **a.** *E. faecalis* at 10°C; **b.** *E. faecalis* at 30°C; **c.** *E. casseliflavus* at 10°C; and **d.** *E. casseliflavus* at 30°C.

CHAPTER 4

SUMMARY AND CONCLUSIONS

Enterococci are used as an indicator of human fecal pollution to evaluate water quality in marine environments. The presence of enterococci as a group is not only associated with humans, but also has been found in the digestive tracts of other warm-blooded animals, in soil, on plant material and associated with plankton (Devreise et al. 1987; Roll and Fujioka 1997; Ulrich and Müller 1998; Signoretto et al. 2004; Whitman et al. 2003). By associating with biotic or abiotic factors in the environment, enterococci have been shown to survive for extended periods of time (Signoretto et al. 2004, 2005; Whitman et al. 2003). Due to the potential for introduction by other sources and the persistence of enterococci in the environment, high levels may not indicate continuous addition of human fecal wastes in an area, and therefore may present confounding information for regulators charged with assessing water quality.

Given the epiphytic nature of many *Enterococcus* spp., we investigated the contribution of plankton-associated enterococci in estuarine water samples. To investigate the source, persistence and possible growth of the fecal indicator bacteria, enterococci, in the plankton community of coastal Georgia waters, the following hypotheses were tested: (1) enterococci are enriched in estuarine plankton relative to the water column, (2) enterococci are associated with specific groups and sizes of plankton, (3) enterococci species distribution varies between free-living forms found in the water column and forms found associated with plankton and (4) specific

environmental conditions such as temperature and plankton concentration will affect the ability of *Enterococcus* species to grow in association with plankton.

This study demonstrated that enterococci are enriched in estuarine plankton relative to the water column. Enterococci were also associated with specific sizes of plankton with the size fractions <150-µm contributing the greatest number of enterococci to bulk water. These size fractions were comprised of mostly plant detritus, diatoms, and copepod nauplii. *Enterococcus* species distribution varied little between free-living forms found in the water column and forms found associated with plankton. Two species commonly associated with humans, *E. faecalis* and *E. faecium*, were both isolated from plankton samples. *Enterococcus casseliflavus*, an epiphytic species, was isolated more than other species in both plankton and water samples. Laboratory microcosm experiments showed the ability of *E. faecalis* and *E. casseliflavus* to survive and grow in mixed plankton at 30 and 10^{o}C. More growth was observed for both species in experiments with 5% plankton compared to 1% plankton experiments. These findings demonstrated aquatic biota such as plankton potentially serving as a reservoir for *Enterococcus* species which may limit the group's usefulness as a fecal contamination indicator.

References

Devriese, L. A., A. Van De Kerckhove, R. Kilpper-Bälz, and K. H. Schleifer. 1987. Characterization and identification of *Enterococcus* species isolated from the intestines of animals. Int. J. Syst. Bacteriol. **37**(3):257-259.

Roll, B. M., and R. S. Fujioka. 1997. Sources of faecal indicator bacteria in a brackish, tropical stream and their impact on recreational water quality. Water Sci. Technol. **35**(11-12):179-186.

Signoretto, C., G. Burlacchini, M. del Mar Lleò, C. Pruzzo, M. Zampini, L. Pane, G. Franzini, and P. Canepari. 2004. Adhesion of *Enterococcus faecalis* in the nonculturable state to plankton is the main mechanism responsible for persistence of the bacterium in both lake and seawater. Appl. Environ. Microbiol. **70**(11):6892-6896.

Signoretto, C., G. Burlacchini, C. Pruzzo, and P. Canepari. 2005. Persistence of *Enterococcus faecalis* in aquatic environments via surface interactions with copepods. Appl. Environ. Microbiol. **71**(5):2756-2761.

Ulrich, A., and T. Müller. 1998. Heterogeneity of plant associated streptococci as characterized by phenotypic features and restriction analysis of PCR amplified 16S rDNA. J. Appl. Microbiol. **84**:293-303.

Whitman, R. L., D. A. Shively, H. Pawlik, M. B. Nevers, and M. N. Byappanahalli. 2003. Occurrence of *Esherichia coli* and enterococci in *Cladophora (Chlorophyta*) in nearshore water and beach of Lake Michigan. Appl. Environ. Microbiol. **69**(8):4714-4719