SPATIAL AND TEMPORAL VARIABILITY AMONG ENTERIC PATHOGENS AND FECAL INDICATOR BACTERIA IN A TIDALLY MIXED ESTUARY IN SOUTHEAST GEORGIA, USA.

by

MONICA LYNNE GRIFFITH

(Under the direction of Erin K. Lipp)

ABSTRACT

This study sought to evaluate the role of season, location and biotic reservoirs in detection rates of fecal indicator bacteria and human pathogens in shellfish harvesting waters of Georgia. A total of 118 samples were collected from twelve stations over a period of two years. Water quality parameters including pH, salinity, temperature, and dissolved oxygen were measured and water and plankton samples from each station subjected to microbial analysis for fecal coliforms and enterococci. The levels of enteroviruses in the plankton samples were assessed using RT-nested PCR techniques. Fecal coliform bacteria often exceeded standards of ≤ 14 CFU/100 ml for shellfish harvesting waters. Human enteroviruses were detected throughout the year in these areas as well. When fractions were compared, there was significant partitioning of indicator bacteria between the water and plankton fractions, with lowest levels of both enterococci and fecal coliform bacteria in water and highest in the plankton fractions.

INDEX WORDS: enteric viruses, fecal contamination, water quality
SPATIAL AND TEMPORAL VARIABILITY AMONG ENTERIC PATHOGENS AND Fecal Indicator Bacteria in a Tidally Mixed Estuary in Southeast, Georgia USA.

By

MONICA LYNNE GRIFFITH

B.A., California University of Pennsylvania, 2001
B.S., University of Georgia, 2006

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2012
SPATIAL AND TEMPORAL VARIABILITY AMONG ENTERIC PATHOGENS AND Fecal Indicator Bacteria in a Tidally Mixed Estuary in Southeast Georgia, USA.

By

MONICA LYNNE GRIFFITH

Major Professor: Erin K. Lipp
Committee: Marsha Black Dana Cole

Electronic Version Approved:
Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2012
DEDICATION

To my beloved father

Kevin Dodds Griffith (1954-1989)
I would first like to thank my major advisor, Dr. Erin Lipp, for her continuous support and for all the many hours spent reviewing all my work. I also would like to thank my committee members, Dr. Marsha Black and Dr. Dana Cole, for their input; all my fellow lab workers: Carrie, Jeff, Jennifer, Erin and Gordon for all your long hours of helping me process all my samples; my family, especially Cindy, Darrell, Katie, Tyler and Jason and my friends Zack and Jennifer for always supporting me and pushing me to be the best I can be. I would like to thank the staff at the Department of Environmental Health Science and everyone else at the University that made this all possible.
TABLE OF CONTENTS

ACKNOWLEDGEMENTS........................................................................................................iv

CHAPTER

1 INTRODUCTION.................................................................................................................1

References for Chapter 1........................................................................................................4

2 LITERATURE REVIEW

Coastal Water Quality Issues..............................................................................................5

Sewage Contamination and Estuaries.................................................................................11

Fecal Oral Pathogens and Shellfish..................................................................................13

Regulatory Issues and Shellfish Harvesting Waters..........................................................16

Benefits and Problems with using Fecal Indicator Bacteria.............................................17

References for Chapter 2.....................................................................................................22

3 SPATIAL AND TEMPORAL VARIABILITY AMONG ENTERIC PATHOGENS AND FECAL INDICATOR BACTERIA IN A TIDALLY MIXED ESTUARY..................................................................................................................32

Abstract..................................................................................................................................33

Introduction..........................................................................................................................34

Methods...............................................................................................................................36

Results...................................................................................................................................42

Discussion............................................................................................................................48

References for Chapter 3.......................................................................................................52

4 CONCLUSIONS..................................................................................................................63
CHAPTER 1
INTRODUCTION

Pathogens are defined as disease-causing organisms that include viruses, bacteria, and parasites (USEPA 2011). Many of them are found in marine waters and can pose a health risk to swimmers, surfers, divers, and seafood consumers. Estuaries and shellfish harvesting waters may be a reservoir for these disease-causing microorganisms in areas subject to contamination with human waste. Fish and filter feeding organisms such as shellfish have been shown to concentrate pathogens in their tissues and therefore may cause illness or even death in persons consuming them. Contaminated water can transmit many types of enteric viruses (e.g., enteroviruses and noroviruses) through the fecal-oral route and therefore poses a public health risk as well. Current testing methods, which predominately focus on the use of fecal indicator bacteria, have been questioned partly because viral pathogens are not consistently predicted using bacterial indicator standards. Due to the host specificity of viral pathogens they could be a better indicator for human sources of fecal contamination in coastal and marine waters.

In the literature review (Chapter 2), the characteristics, pathogenicity, prevalence, and detection in aquatic environments of both fecal indicator bacteria and enteroviruses are compared for their ability to adequately determine water quality within recreational and shellfish harvesting waters.

In Georgia, point source discharges, which include industrial discharges, sewage treatment plants, marinas, and boats impact water quality and shellfish harvesting in 19 out of 59 sub-watersheds (32.2%) (Fletcher et al. 1999). With the increased rates of urbanization and the
amount of untreated sewage released by boats, enteric viruses become an increasing public health risk for shellfish consumers. In Chapter 3, research is presented on the occurrence of fecal indicator bacteria and enteroviruses, within both the Sapelo and Wassaw Sounds of Georgia, and the effects seasonal, physical, chemical and biological factors have on the fate and persistence of both. Water and plankton samples were collected from six predetermined sites in a bi-monthly pattern for both Sounds from January 2006 through November 2007. Samples were analyzed by Reverse Transcriptase (RT)-nested Polymerase Chain Reaction (PCR) and dot-blot hybridization. Virus presence was correlated with bacterial indicators and environmental and water quality variables such as water temperature, pH, dissolved oxygen (DO), salinity, and 30-day total rainfall using ANOVA, pearson correlation, and binary logistic regression. In Georgia, the approved shellfish harvesting standard for fecal coliform bacteria is ≤ 14 CFU 100 ml\(^{-1}\) (NOAA 1998). For Sapelo Sound this standard was exceeded in 25 out of 58 (43%) water samples collected, but the standard was only exceeded in 8 out of 60 (13%) within Wassaw Sound. Enterococci recreational water quality standards (one time level of 104 CFU 100 ml\(^{-1}\) (USEPA 2011) were exceeded in both sampling areas. For Sapelo Sound the enterococci standard was exceeded in 19/58 (33%) of the water samples, and in Wassaw Sound it was exceeded 13/60 (22%). We also chose to look at two different plankton fractions (63-200 µm and >200 µm) as a possible reservoir for pathogens. When fractions were compared, there was significant partitioning of indicator bacteria between the water and plankton fractions, with lowest levels of both enterococci and fecal coliform bacteria in water and highest in the plankton fractions. A binary logistic regression analysis showed a positive association between enterococci levels and virus presence within the >200 µm plankton fraction.
In Chapter 4, conclusions are made suggesting that viral pathogen detection could be used in conjunction with current fecal indicators in order to better assess water quality and possible human fecal contamination within shellfish harvesting waters. Seasonal, physical, chemical and biological factors affect the fate and persistence of both fecal indicator bacteria and human pathogens. Understanding factors that prolong microbes in estuarine environments is critical to better determining exposure and risk to users.
References for Chapter I


Coastal Water Quality Issues

Nearly half of the U.S. population lives within ~80 km of the coast, and coastal counties are growing three times faster than counties elsewhere in the nation (USEPA 2011b). Human activities in the coastal zone have significantly affected the marine environment resulting in beach and shellfish bed closures, dangerous algal blooms, unproductive fisheries, loss of wildlife habitat, and decimation of fish populations among other human health and natural resource problems (USEPA 2011b). This increase in population could therefore lead to an increase in pathogenic bacteria and viruses within coastal water sources.

Pathogens are defined as disease causing organisms which include viruses, bacteria, and parasites (USEPA 2011a). These organisms are present in marine environments and can pose serious health risks to swimmers, divers, surfers, and seafood consumers (Field 2003). Urban and agricultural runoff, boat and marina waste, faulty or leaky septic systems, sewage treatment plant discharges, combined sewer overflows (CSOs), recreational vehicles or campers, illegal sewer connections, and waste from pets or wildlife are some of the most common sources of pathogens (Field 2003).

Worldwide, 90% of human created wastewater is discharged, without being treated, into marine environments (Crosette 1996; Henrickson et al. 2001). In 2004, 850 billion gallons of untreated wastewater and storm water were released as CSOs and between 3 and 10 billion gallons of untreated wastewater from sanitary sewer overflows (SSOs) are released each year in
the U.S. (USEPA 2004). Sewage pollution has contributed to 20,000 days of closure or advisories at ocean, bay, and Great Lakes beaches in the U.S. in 2008 alone (National Resources Defense Council 2009).

Fong et al. (2005) evaluated the effects of environmental parameters on enteric viruses, resulting from animal and human fecal pollution, in the lower Altamaha River, Georgia. RT-PCR and nested-PCR methods were used to detect human enteroviruses, human adenoviruses (HAdV), and bovine enteroviruses. There was a direct relationship found between the concentrations of these viruses and dissolved oxygen and streamflow. There was an inverse relationship between the water temperature and rainfall. These findings are important in several ways. Increases in the temperature and rainfall can lead to a decrease in viral presence, which is greatly impacted by waste water and urban runoff conditions. This study also points out that contributing factors such as population growth and urbanization can lead to pathogenic microorganisms contaminating coastal rivers and estuaries.

Other studies have also focused on environmental conditions as possible determinants of the level of pathogen contamination within different types of water bodies. Increases in the number of predators and environmental competition are often shown to lower the levels of pathogenic microorganisms in the marine environment (Kibbey et al. 1978; Davies et al. 1995; Zaleski et al. 2005). Temperature and salinity have been broadly implicated in the survival times of bacterial indicators in natural waters as well (Goyal et al. 1977; Bogosian et al. 1996; Wang and Doyle 1998). This is because many indicator organisms cannot survive at markedly elevated temperatures and at high salinities due to their explicit range of survival (Shiba et al. 1991; Biosca et al. 1996; Marco-Noales et al. 1999). In fact low temperatures can often increase the
persistence of both bacterial indicators and enteric viruses (Davenport et al. 1976; Anderson et al. 2005; Fong and Lipp 2005; Bosch et al. 2006).

Heavy rainfall has been found to result in significant increases in indicator organism levels in both water and sediment samples, with greater numbers of pathogens found in sediments sometimes than in the overlying water (Gerba and McLeod 1976; Goyal et al. 1977; Smith et al. 1978; Erkenbrecher 1981; Davies et al. 1995; Field 2003). Rising levels in sediments may be due to the natural settling of particles within a water body. Several studies have concluded that sediments can act as possible reservoirs for enteric viruses (Smith et al. 1978; LaBelle et al. 1980; Rao et al. 1984; Bosch 1998). Recreational and environmental activities can cause the dispersion of many microorganisms, which can lead to viruses being re-suspended into the water column over time (Smith et al. 1978; LaBelle et al. 1980; Rao et al. 1984; Field 2003).

To determine the quality of drinking, recreational and shellfish waters, several fecal indicators are commonly utilized. Microbial indicators of fecal pollution should occur with and act as proxies for pathogenic microbes (USEPA 2011a). Their major disadvantage lies in the fact that assays used to detect multiple pathogens in water are difficult and expensive to reliably measure. (Kay and Fawell 2007).

Fecal indicator bacteria (FIB) are utilized in the assessment of water quality (Fong and Lipp 2005). The current water quality criteria derived by the USEPA under the Clean Water Act and the BEACH Act, use Eschericia coli and enterococci as the preferred indicator bacteria for fresh waters and enterococci for marine waters (USEPA 2011a). Additionally, for much of the 20th century fecal coliform bacteria were used worldwide for water quality assessments (Anderson et al. 2005). Fecal coliform bacteria are a broad group of enteric Gram negative
bacteria that includes *E. coli*. They are found in the intestinal tract of humans, livestock, and wildlife and have been traditionally used as indicators of possible sewage contamination (USEPA 2011a). For this reason they were used since the mid 1950s for microbial water quality assessment in recreational waters and remain the standard for assessing the safety of shellfish harvesting waters (Fletcher et al. 1999; USEPA 2011a). Beginning with large epidemiological studies of beach goers in the late 1970s (Cabelli et al. 1982), EPA found that enterococci is a more reliable indicator of gastrointestinal illness in marine waters. Additionally, enterococci are able to persist longer in the environment and are not as susceptible to most sewage treatment methods as are other current indicators (USEPA 2011a).

The concentration of FIB, especially enterococci and *E. coli* (freshwater only) has been shown to correlate to the risk of human gastrointestinal illness when subjects are exposed to waters impacted by point sources of human fecal pollution (Cabelli et al. 1983; Dufour 1984; Wade et al. 2003; Hofer 2008; Wade et al. 2010). While recent health effects studies at some marine beaches suggest that enterococci concentrations are a sufficient predictor of gastroenteritis, this finding is not universal. Many questions remain whether concentrations of FIB are reliably correlated to the presence of human fecal pollution within marine environments because of differences in persistence, regrowth of the indicators in the environment or interference in detection assays (Cabelli 1983; Cabelli et al. 1983; Desmaris et al. 2002; Hofer 2008; EPA 2010). In tropical and warm marine waters, both *E. coli* and enterococci have been observed to persist in the environment outside of the human intestinal tract (Solo-Gabriele et al. 2000; Hofer 2008), and both indicators have been found in areas where there is no apparent sewage contamination (Carrillo et al. 1985; Rivera et al. 1988; Scott et al. 2002; Savichtcheva and Okabe 2006).
The concentration of FIB can be affected by many different contributing factors, including rainfall. In a study of the concentrations of FIB in the Allegheny, Mononaghela, and Ohio Rivers, near Pittsburgh, Pennsylvania in July-September 2001, Fulton and Buckwalter (2005) determined that single-event concentrations of fecal coliform bacteria, enterococci, and \textit{E.coli} during dry weather events exceeded both state and federal water quality standards in 11%, 28%, and 28% of the samples respectively. These organisms exceeded the water quality standards in 56%, 71%, and 81% of samples during wet-weather events. It was also reported that single-event wet weather concentrations were significantly higher for all sampling sites except for the Allegheny River, where it was concluded that lack of upstream sources or dilution during wet weather events contributed to the relatively lower concentrations. It was also found that enterococci levels were correlated with the turbidity of the water in the different sample locations. Many microorganisms are able to adhere to sediment particles within a water source and therefore increase the levels of detected fecal pollution during resuspension events. An increase in turbidity can lead to an increase in enterococci levels, which in turn gives us a view of possible stream water quality or even possible contamination sources (Fulton and Buckwalter 2005).

Another example of pathogens found in water sources are enteric viruses such as enteroviruses, noroviruses, adenoviruses, and rotaviruses, among others. Enteric viruses, as the name implies, spend part of their infection cycle within the intestinal tract of their host and are generally excreted in the feces of infected individuals. Enteroviruses, which are small single stranded RNA, nonenveloped, icosahedral particles with a diameter of about 30 nm belonging to the family Picornaviridae (Hyypia et al. 1997). In addition to three different poliovirus subtypes, there are 62 non-polio enteroviruses that can cause disease in humans: 23 Coxsackie A viruses, 6
Coxsackie B viruses, 28 echoviruses, and 5 other enteroviruses (CDC 2006). They are causative organisms of acute respiratory illness, aseptic meningitis, meningoencephalitis, myocarditis, hand, foot, and mouth disease, neonatal multi-organ failure and acute flaccid paralysis, among other clinically important diseases (Caro et al. 2001). Enteroviruses are usually transmitted through the fecal oral route and primarily infect and replicate in the gastrointestinal tract of the host (Fong and Lipp 2005). They have been reported as being widely distributed in various water environments, which increases the probability of human exposure due to recreational contact (Griffin et al. 2001; Fong and Lipp 2005). Enteroviruses are thought to be one of the most commonly contracted group of enteric viruses among humans but lacking active surveillance, data on case rates are poor (Khetsuranu et al. 2006).

Similar to patterns noted elsewhere for FIB, human enteric viruses also tend to be associated with urban runoff, contributing significantly to pollution of coastal waters (e.g., Jiang et al. 2001; Ahn et al. 2005). However, despite similar sources, there is often little correlation between FIB levels and enteric viruses (e.g., Jiang et al. 2001, Noble and Fuhrman 2001, Jiang and Chu 2004, Fuhrman et al. 2005, Sinderman 2006, Hofer 2008). Whereas fecal indicator bacteria are shown to indicate the presence of various pathogenic bacteria, the lack of correlation between their concentrations and the levels of viruses in these environments suggest water standards should take viruses into account.

Recreational water standards for pathogens have been established in order to protect the public from illnesses (USEPA 2003). These standards have been created based on epidemiological evidence establishing the role of recreational waters in disease causation and are linked to concentration of FIB thresholds to determine beach closure protocols (Cabelli et al. 1982; Alexander et al. 1992; Fleisher et al. 1993; Prüss 1998; Rose et al. 2001; Hunter 2003;
Wade et al. 2010). While FIB are used to determine the safety of recreational water, risk of gastrointestinal illnesses are likely posed by enteric viruses present in recreational water, contaminated drinking water, and shellfish harvesting waters, rather than pathogenic enteric bacteria (Gerba and Rose 1990; Cecuk et al. 1993; Muscillo et al. 1994; Reynolds et al. 1995; Patti et al. 1996; Lipp and Rose 1997). Given this disconnect, improved models may be required to predict the true risk posed to human health by contaminated marine environments (Griffin et al. 2001; Wade et al. 2010). Seasonal, physical, chemical and biological factors affect the fate and persistence of both fecal indicator bacteria and human pathogens. Understanding factors that prolong microbes in estuarine environments is critical to better determining exposure and risk to users.

**Sewage Contamination and Estuaries**

An estuary is a partially enclosed body of water along the coast where freshwater from rivers and streams meet and mix with salt water from the ocean (USEPA 2011b). They form a transition from land to sea, but are still protected from the full force of ocean waves and storm events by barrier islands or peninsulas (USEPA 2011b). The saltwater from the ocean is in part diluted with freshwater from a river, stream, local storm runoff or groundwater (Seabrook 2006). These characteristics make them home to many unique communities of plants and animals that have adapted to life at the margin of the seas (USEPA 2011b). Countless numbers of birds, mammals, fish, and other wildlife depend on these protected waters for places to live, feed, migrate, and reproduce (USEPA 2011b). There is also a direct economic benefit provided by estuaries for tourism, commercial and recreational fishing, boating, and other coastal industries that collectively provide millions of jobs (USEPA 2011b). Due to the accelerated growth rate seen within coastal communities, an increasing concentration of people adds stress to previously
unaffected areas of the estuarine ecosystems (USEPA 2011b). Natural water ways have been rerouted, marshes filled, and shorelines (and beaches) reconstructed to accommodate human housing, transportation, tourism and agricultural needs (USEPA 2011b). These factors have resulted in an increase in pollution resulting in beach and shellfish bed closings, loss of habitat wildlife and plants, and many other human health and natural resource problems (USEPA 2011b).

An important threat facing estuaries is contamination by pathogens or fecal indicator bacteria. Pathogens have led to a large number of shellfish bed closures and a decline in recreational water quality as well (USEPA 2011b). Estuaries and shellfish harvesting waters often harbor pathogenic microorganisms in areas where human waste contamination is present (USEPA 2011b). The extent of human fecal pollution can be affected by several factors including a larger population, which results in an increase in volume of wastewater treated and/or disposed (Lipp et al. 2001). Aging and poorly sited septic tanks can lead to direct contamination of estuarine environments through groundwater discharge (Lipp et al. 2001). Human feces can carry various human enteric pathogens, including several types of viruses (Parveen et al. 1999). Sewage treatment processes have even been reported to be inadequate in viral removal (Sorber 1983). It is difficult to study the actual inactivation of gastroenteritis causing viruses following wastewater disinfection due to the fact there are such low and variable levels of enteric viruses in collected effluents (Tree et al. 2003). Many studies have depended on the use of laboratory-adapted strains of enterovirus, such as poliovirus, or indicator viruses, such as F+ specific RNA (FRNA) bacteriophage (Havelaar 1993; Havelaar et al. 1993; Grabow 2001). Tree et al. 2003, found that the inactivation of bacterial indicators, such as Escherichia coli and Enterococcus faecalis, through chlorination was significantly faster than that of the FRNA.
When seeded poliovirus was tested it was in fact significantly more susceptible to chlorine than FRNA, but still more resistant than the bacterial indicators (Tree et al. 1997). Tree et al. 2003, also reported the possibility that organisms naturally present in sewage can become encapsulated in cell debris, aggregated, or adhered to solid particles, which in turn can provide protection from disinfectants. Other studies have shown that enteric viruses within water can become embedded in suspended solids, which can in turn provide protection against disinfectant action (Hejkal et al. 1979; Hejkal et al. 1981; Narkis et al. 1995; Shieh et al. 1997). It was also found that seeded poliovirus was actually less resistant to chlorination than many other indigenous viruses found within sewage effluents (Tree et al. 2003). In the above mentioned studies, dependence on fecal-bacterium based standards used to test water quality could in fact overestimate the actual level of protection we have from human enteric viruses (Havelaar et al. 1993; Tree et al. 1997; Tree et al. 2003).

The rapid increase in urbanization rates coupled with increased quantities of untreated sewage released by boats, aging septic tanks or sewage spills (Lipp et al. 2001) suggest that enteric viruses may be an important public health risk for shellfish consumers and users of recreational facilities (USEPA 2011b). Seasonal, physical, chemical and biological factors affect the fate and persistence of both fecal indicator bacteria and human pathogens.

**Fecal Oral Pathogens and Shellfish**

Once pathogens enter the marine environment, they can be further concentrated by filter feeding organisms such as mussels, clams and oysters (Stewart et al. 2008). Bivalve shellfish are aquatic filter feeding invertebrates, which gather and concentrate microbial organisms, including human pathogens, in their tissues and may transmit illness to persons consuming them (Desenclos et al. 1991; USEPA 2006; Stewart et al. 2008). Various indicators have been used in
order to assess the quality of recreational and shellfish waters, with microbiological indicators being the most widely used (Field 2003).

In the environment, human pathogens, such as enteric viruses, are found routinely and able to survive for extended periods of time at low temperatures and under a wide pH range (Fong and Lipp 2005). Enteric viruses are usually transmitted through recreational contact or direct ingestion (Griffin et al. 2001, 2003). Replication of enteric viruses occurs in the gastrointestinal tract thereby making them effective markers for documenting human sewage contamination in marine and estuarine waters (Fong and Lipp 2005). Enteric viruses in the marine environment include the Adenoviridae (adenovirus strains 3, 7, 40, and 41), Caliciviridae (Norwalk virus, astroviruses, Snow Mountain agent) Picornaviridae (poliovirus, coxsackievirus, hepatitis A virus (Hepatitis A Virus), enteroviruses, Norwalk-like viruses, and echoviruses) and Reoviridae (reoviruses and rotaviruses) (Griffin et al. 2003). They are associated with various disease conditions in humans among them diarrhea, hepatitis, meningitis, exanthema, arthralgias, myalgia, conjunctivitis, otitis, gastroenteritis, and common cold (Griffin et al. 2003).

Human adenoviruses (HAdV) are a major cause of urinary tract infections and have been associated with increased disease in immunosuppressed individuals (Griffin et al. 2001). They are commonly present in high concentrations in feces of infected persons and are in certain instances detected in the urine and feces of healthy subjects, as well (Bofill-Mas et al. 2000). This group of viruses infects bovine, human, avian, and porcine (Hundesa et al. 2006). Human adenoviruses are widely distributed in marine environments and are commonly isolated from shellfish (Gerba et al. 2002). This prevalence is reported to be due to their high resistance to chlorination, filtration and UV radiation (Gerba et al. 2002; Thurston-Enriquez et al. 2003a,b). They are also documented as having longer survival times then other enteric viruses, with the
ability to persist three to five times longer than polioviruses and being more heat stable (Parveen et al. 1999).

Caliciviruses or the Norwalk like viruses (NLV, including noroviruses) are ssRNA viruses that have also been isolated from drinking, recreational, and shellfish waters and have been implicated as a major cause of gastroenteritis (Griffin et al. 2001). Like human adenoviruses, this widely distributed viral group is also more resistant to water treatment processes and to heat than are the fecal indicator bacteria (La Rosa et al. 2010).

High prevalence is also observed with rotaviruses (*reoviridae*), with significant occurrences in surface waters contaminated with untreated effluent (Parveen et al. 1999). This group of viruses has been documented as the leading cause of infantile viral gastroenteritis with numerous disease outbreaks associated with it (Parveen et al. 1999). These outbreaks have been directly linked to water contaminated with feces containing the rotaviruses and water that has not been adequately treated (Parveen et al. 1999).

Human enteroviruses can subsist for extended periods at low temperatures, are widely found in marine environments and estuaries and are largely unaffected by pH changes (pH 3 to 10) (Melnick and Gerba 1989; Griffin et al. 2003; Fong and Lipp 2005). Several studies have reported the ability of these viruses to persist in seawater for up to 130 days at 20°-30°C (Melnick and Gerba 1989). They are also able to survive at these temperatures in freshwater and sewage for periods of up to 120 days and for up to 100 days in soil (Melnick and Gerba 1989).

Formiga-Cruz et al. 2003, studied the prevalence of contamination by human viruses in various shellfish-growing regions of the United Kingdom, Greece, Spain and Sweden by the presence of HAdV, Norwalk-like Virus (NLV), Hepatitis A virus (HAV), and enterovirus levels (Formiga-Cruz et al. 2003). Hepatitis A virus was isolated in all the countries with the exception
of Sweden (Formiga-Cruz et al. 2003). NLV was detected in samples from all the countries sampled (Formiga-Cruz et al. 2003). The most prevalent virus group documented was the Human adenoviruses (Formiga-Cruz et al. 2003). Detection of these human enteric viruses was found to be significantly higher in areas where *Escherichia coli* contamination was highest (Formiga-Cruz et al. 2003). This leads one to believe that the true incidence of pathogen induced shellfish contamination could be greatly underestimated (Muniaín-Mujika et al. 2003).

**Regulatory Issues and Shellfish Harvesting Waters**

Health departments, local counties and regulatory authorities face a difficult task of maintaining water quality. Beneficial uses of coastal areas including beaches, swimming and other recreational areas are negatively affected if high or persistent bacteria levels are observed (Fletcher et al. 1999). Management plans for water quality and development of Total Maximum Daily Loads (TMDLs) is a vital step in attempts to control contamination by identifying sources and measuring levels accurately then setting appropriate regulatory and management standards (Fletcher et al. 1999)

*E. coli* and enterococci are the main indicators for basic studies in testing water quality (USEPA 2010). Health risks to recreational users (such as swimmers, boaters, fishermen, etc.) can be affected by factors such as ingesting, immersing, or wounding themselves in water that contains enteric pathogens from sources such as runoff and sewage discharge treatment levels (USEPA 2010). Recreational water standards have been established in order to protect the public from illnesses.

When waters contain human sewage, animal wastes, disease-producing organisms, or chemicals, then these contaminants can concentrate within the tissue of filter feeding organisms (TCEHD 2001). Within less than 24 hours of exposure, shellfish can take up viruses, following
contact with sewage or seeded contaminated waters (Di Girolamo et al. 1975, Richards 1987, Doré and Lees 1995, Schwab 1998, Burkhardt and Calci 2000, Lees 2000). Apart from FIB and viruses, biological and chemically harmful agents can accumulate in shellfish and be transmitted to humans as well (Georgia Department of Natural Resources 1997).

There are many different hazards that are associated with harvesting of shellfish, and it has been of major concern to management and regulatory authorities to put control policies in place for consumer safety purposes (Georgia Department of Natural Resources 1997). Harvesters of shellfish face an uphill task to comply with the standards or face permanent closed areas or temporary closures (Georgia Department of Natural Resources 1997). In Georgia, shellfish harvesting is governed by law from the Natural Resources Department to comply with the National Sanitation Program (NSP) (Georgia Department of Natural Resources 1997). The Interstate Shellfish Sanitation Conference (ISSC) is another program that works with the federal and state control agencies to promote shellfish sanitation as well. Testing methods which include fecal coliform bacteria, the preferred indicator for shellfish, and *E. coli* have been shown to have a limited predictive value for many different viral pathogens including enteroviruses (Gerba et al. 1979, Vaughn et al. 1979, Ellender et al. 1980, Jofre 1992).

**Benefits and Problems with Using Fecal Indicator Bacteria**

To assess the quality of drinking, recreational, and shellfish waters, several indicators are commonly utilized. These indicators can include dissolved oxygen to assess the quantity of oxygen available to microorganisms, pH to assess the acidity or alkalinity, conductivity as a measure of the water’s salinity, and suspended solids and turbidity to determine the clarity of water (Reynolds 2003). This, however, does not give us the full picture on water quality. Since
as early as 1880 scientists realized that there was a need to test for microbiological markers, such as fecal contamination, in water (Reynolds 2003). Microbes that serve as indicators of a broad range of pathogens found in human sewage and animal and waste are ideally characterized by certain features. They must be quickly detected, easily quantified, avirulent (non-pathogenic), similar to the pathogens under consideration in terms of survival ability and their occurrence should indicate the presence of pathogenic microbes (Kay and Fawell 2007; USEPA 2011a). Microbial indicators include the total and fecal coliform bacteria, enterococci, and less commonly, enteric viruses (USEPA 2011a). Others are the Bifidobacterium species, Bacteroides fragilis bacteriophage, and F-specific RNA coliphages (USEPA 2011a).

Total coliform bacteria contain several different species which include Enterobacter, Escherichia, Klebsiella, and Citrobacter (Reynolds 2003). Total coliforms are an operationally defined bacterial group which are gram negative, non-sporulating, rod-shaped lactose-fermenting bacteria (Reynolds 2003); they all ferment lactose to acid and gas within 24 h at 35°C. Included as a sub-group of these are fecal coliform bacteria, whose thermotolerance (i.e. ability to grow at 44.5 °C) makes them a better match to microbes found in the intestines and feces of warm blooded animals and in turn a more suitable indicator for sewage contamination of water (Harwood et al. 1999; Reynolds 2003).

Fecal coliform bacteria are found in the intestinal tract of humans, livestock, and wildlife and are used as indicators of possible sewage contamination (USEPA 2011a) in both recreational and shellfish harvesting waters (Fletcher et al. 1999; Scott et al. 2002; USEPA 2011a). Fecal coliform bacteria indicators are also associated with a number of limitations, including the effectiveness of these bacteria as proxies for human enteric pathogens. Desmarais et al. (2002) and Sobsey et al. (1990) demonstrated that the ecology, prevalence and resistance to stress of
fecal coliform bacteria differ in a very fundamental manner from those traits displayed by many enteric microorganisms, which they are supposed to represent (Parveen et al. 1999). Numerous studies have been conducted on the prevalence of fecal coliform bacteria in marine environments (Gerba and McLeod 1976; Xu et al. 1982; Mote et al. 2012) and the health risks involved with them (Cabelli et al. 1982; Cabelli et al. 1983). Harwood et al. (1999) isolated fecal coliform bacteria from the estuarine diamondback terrapin (Malaclemys terrapin centrata) inhabiting brackish-water habitats. Fecal coliform bacteria were isolated in a majority of the samples and E. coli was the predominant species isolated. This was a significant finding with implications on water regulatory standards because of the fact an organism for warm blooded hosts was being expelled by cold-blooded vertebrates, which are not considered as sources of pathogenic contaminants.

Presence of fecal coliform bacteria in both human and animal feces also implies that they are not very reliable indicators to be used as prospective markers for human FIB in the marine environment (Fong et al. 2005). It is also documented that fecal coliform bacteria do not predict presence of numerous other disease-causing pathogens including protozoa such as Giardia and Cryptosporidium, enteric viruses and other disease causing bacteria (Fong et al. 2005). Moreover, viral pathogens have been shown to be present in waters that contained low bacterial indicator concentrations (e.g., Vaughn and Metcalf 1975, Jiang et al. 2000; Noble and Fuhrman 2001; Jiang and Chu 2004; Fuhrman et al. 2005). Studies also demonstrate that indicator bacteria die off faster than viruses and protozoa in marine environments (Noble and Fuhrman 2001). Fecal coliform bacteria (E. coli) are believed to have limited predictive value for viral pathogens such as enteroviruses (Gerba et al. 1979; Vaughn et al. 1979, Ellender et al. 1980; Jofre 1992), Norwalk-like virus (NLV) and infectious hepatitis, caused by the Hepatitis A virus (HAV)
(Wanke and Guerrant 1990; Desenclos et al. 1991). Cabelli et al. 1983 rejected coliforms as the indicator of choice for evaluating sanitary conditions of recreational water because of their variable correlation with fecal contamination. Research from other studies, (Formiga-Cruz et al. 2003) determined that fecal coliform bacteria and \textit{E.coli} organisms are ineffective as indicators of viral pollution in shellfish. In a study performed by Noble and Fuhrman (2001) viral pathogens were shown to be present in waters that contained low bacterial indicator concentrations. There are other indicators of enteric viruses in water including somatic coliphages, F-specific coliphages, \textit{Bacteroides fragilis} phages (Vaughn and Metcalf 1975; Payment and Franco 1993; Jofre et al. 1995; Hot et al. 2003), or even by isolating human and animal adenoviruses and polyomaviruses, which were shown to be effective markers and can be used to assess the presence of viral contamination as well as track environmental contaminant sources (Hundesa et al. 2006).

Enteroviruses in particular have been used as effective markers for documenting human sewage contamination in marine and estuarine waters and are considered to be conservative because they require a human host for replication (Nobel and Fuhrman 2001; Fong and Lipp 2005; Savichtcheva and Okabe 2006; Stewart et al. 2008; Hofer 2008). Due to the host specificity of viral pathogens, they may act as a superior indicator for sources of fecal contamination in coastal and marine waters (Fong and Lipp 2005). Therefore if used in conjunction with the bacterial indicators the combination could improve not only water quality assessment, but public health surveillance as well (Fong and Lipp 2005). Whereas fecal indicator bacteria are shown to indicate the presence of various pathogenic bacteria, the lack of correlation between their concentrations and the levels of viruses in these environments suggest that water standards that take into account viruses need to be formulated (Formiga-Cruz et al. 2003).
The objectives of this study were to assess spatial and seasonal distribution of fecal indicator bacteria and human enteroviruses among two Georgia estuaries, to determine the prevalence, distribution and concentration of fecal indicator bacteria and enteric viruses in water and potential plankton reservoirs, and to relate indicators and pathogens to physical-chemical parameters and seasonal variability such as temperature and rainfall. This could help support The EPA revision of their water quality standards and address needed research on the ecology of fecal indicator bacteria in relation to pathogens
References for Chapter 2


CHAPTER 3

SPATIAL-TEMPORAL VARIABILITY AMONG ENTERIC PATHOGENS AND FECAL INDICATOR BACTERIA IN A TIDALLY MIXED ESTUARY

Monica L. Griffith\textsuperscript{2}, Jeffrey W. Turner\textsuperscript{2,*} and Erin K. Lipp\textsuperscript{2v}

\textsuperscript{1}To be submitted to \textit{Applied and Environmental Microbiology}

\textsuperscript{2}Dept. of Environmental Health Science, University of Georgia, Athens, GA

\textsuperscript{*} Current affiliation: NOAA Northwest Fisheries Science Center, Seattle, WA

RUNNING TITLE: Fecal indicator variability in estuaries

\textsuperscript{v} Corresponding Author:

Dept. of Environmental Health Science
University of Georgia
Room 206 Environmental Health Science Bldg.
Athens, GA 30605
TEL: 706 583 8138 / FAX: 706 542 7472
E-mail: elipp@uga.edu
Abstract

**Aims:** This study sought to evaluate the role of season, location and biotic reservoirs in detection rates of fecal indicators in active shellfish harvesting waters of Georgia.

**Methods and Results:** A total of 118 samples were collected from twelve stations over a period of two years. Environmental parameters including pH, salinity, temperature, and dissolved oxygen were measured. Water and plankton samples from each station were subjected to analysis for fecal coliform bacteria, enterococci, and human enteroviruses (using RT–nested PCR). The fecal coliform bacteria standard for shellfish harvesting waters was exceeded in 28% of the samples collected and the single sample threshold for enterococci in recreational water was exceeded in 27% of the samples. Human enteroviruses were detected (0% to 100%) throughout the year in these areas as well. **Conclusions:** Fecal indicator bacteria showed significant partitioning between bulk water and the two plankton size fractions, with lowest levels of both enterococci and fecal coliform bacteria in water (31 and 5 CFU 100 ml\(^{-1}\)) and highest in the plankton fractions (4.09 \(10^5\) and 1,017 CFU 100 g\(^{-1}\)). There a significant positive correlation between enterococci levels in plankton and temperature and salinity, which suggests that enterococci may grow in association with plankton reservoirs. **Significance and impact of the study:** Results suggest that contamination is common among the studied estuaries in Georgia. Enteric bacteria and viruses may increase their persistence in association with plankton or particles in the water.
Introduction

Nearly half of the U.S. population lives within ~80 km of the coast, and coastal counties are growing three times faster than counties elsewhere in the nation (USEPA 2011b). As the population increases, there is an increased burden on marine and estuarine waters due to an influx of contaminants from waste water treatment and disposal and from nonpoint sources of pollution (Erkenbrecher 1981; Griffin et al. 2001; Lipp et al. 2001; Sinigalliano et al. 2010). Additionally, the population is increasingly exposed to these waters through recreational contact or consumption of fish and shellfish. There remains a critical need for documenting the prevalence, distribution and ecological interactions of microbial contaminants in coastal waters to understand the fate of potential human pathogens and better protect public health.

Estuaries are unique ecosystems that face many different types of environmental stressors one of which is pathogen contamination (USEPA 2009). Pathogen contamination due to point and non-point sources of human fecal pollution has led to a decline in water quality and an increased risk of human gastrointestinal illness (Cabelli et al. 1983, Dufour 1984; Wade et al. 2003; Harwood et al. 2005; Sindermann 2006; Hofer 2008; McLellan et al. 2010). Microbial water quality in recreational and shellfish harvesting water is determined by levels of fecal indicator bacteria (FIB) for regulatory purposes (NOAA 1998; USEPA 2003; Sinigalliano et al. 2010; USEPA 2011a), which are used to identify possible sewage contamination and associated risks to human health. FIB are not pathogens themselves but tend to occur in feces of warm blooded animals (USEPA 2011a). Since the passage of the Beaches Environmental Assessment and Coastal Health (BEACH) Act, states bordering marine coastlines have been required to use enterococci, as the preferred fecal indicator for health risk associated with recreational exposure to seawater (USEPA 2011a), which have been shown to be significantly associated with the
incidence of gastrointestinal diseases observed among swimmers in a variety of waters (Signoreto et al. 2004; Wade et al. 2006; USEPA 2011c). The National Shellfish and Sanitation Program (NSSP), a division of the Food and Drug Administration (FDA), however, continues to use a fecal coliform index for ensuring water quality for shellfish harvesting (FDA 2009). Monitoring solely for FIB is not always effective for determining when streams and coastal waters are contaminated with sewage because these bacteria can take up residence in the environment and may even multiply under certain conditions (Stewart et al. 2008), or conversely can be subject to a higher decay rate than certain pathogens (e.g., viruses and protozoan parasites) (Anderson et al. 2005; Kay et al. 2005).

The differences in persistence among FIB and enteric viruses is of particular concern given that viral pathogens are believed to cause most cases of waterborne disease (Cloete 2004). Additionally, many enteric viruses have a low infectious dose and can even survive water treatment processes (USEPA 1983). Several studies have shown that viral pathogens are frequently detected in waters with low FIB concentrations; therefore, virus presence can not always be adequately predicted through the use of these indicators alone (Jiang et al. 2001; Noble and Fuhrman 2001; Jiang and Chu 2004; Fuhrman et al. 2005; Sindermann 2006; Hofer 2008). Contaminated water can transmit enteric viruses through the fecal-oral route and therefore poses a public health risk for both recreational exposure and shellfish consumption (Landry et al. 1982; Shieh et al. 2003; Fuhrman et al. 2005; Belguith 2006; Rose et al. 2006). Enteric viruses are also highly host specific, and therefore could act as a conservative marker for human fecal contamination, in particular, in coastal and marine waters (Fong and Lipp 2005).

In many estuaries, contamination trends are often driven by changes in freshwater inflow from large river systems (Lipp et al. 2001; Fong et al. 2005; Fries et al. 2008; Schriewer et al.
2010); however, in the absence of a large riverine input, dynamics of contamination are often less clear and may be dominated by diffuse non-point sources and mixing associated with a large tidal range, as is common along the South Atlantic Bight (including the coastline of Georgia, USA). Additionally, seasonal, physical, chemical and biological factors affect the fate and persistence of both fecal indicator bacteria and human enteric pathogens (including viruses) (Šolić and Krstylović 1992; Griffin et al. 2003; Skrab er et al. 2004; Fong and Lipp 2005; Mill et al. 2006). Understanding factors that increase microbial persistence in estuarine environments, including interactions with particles and plankton, is critical to better determining exposure and risk to users, especially in regions subjected to tidal mixing rather than riverine driven circulation patterns, where there has been little research conducted. The primary goal of this study was to evaluate the spatial and temporal dynamics of FIB and human enteroviruses, and potential reservoirs, in a tidally mixed estuary which supports an active shellfish harvesting program.

Methods

Field Sampling. Samples were collected from twelve stations, six each in Sapelo Sound (near Darien, McIntosh County) and Wassaw Sound (near Savannah, Chatham County) (Figure 1). The coastal area is primarily serviced by septic systems for wastewater disposal (Walker et al. 2003), with the exception of the City of Savannah (population 265,128). The more rural area of Darien has a population of only 14,333 (U.S. Census Bureau 2010). These sites were part of an ongoing shellfish monitoring program of the Georgia Department of Natural Resources (DNR).

Each sound was sampled bi-monthly for a two year period (January 2006 through November 2007) on the full or new moon and coinciding with a falling tide. For each sample, 2 L of water was collected from just below the surface in sterile polypropylene bottles. Temperature, pH, salinity and dissolved oxygen (DO) were measured in situ using an YSI
multiparameter sonde (YSI Environmental, Model 556, Yellow Springs, OH, USA). Two size fractions of plankton were collected via a five minute horizontal tow using plankton nets with 200 µm and 63 µm nylon mesh. The material collected from the 63 µm net was subsequently filtered through a 200 µm mesh to obtain a discrete size fraction of 63 - 200 µm. In both cases, plankton was rinsed from the collection end of the net with sterile Phosphate Buffered Saline (PBS) and resuspended in 1 L of PBS. Both the 63-200 µm fraction (referred to as P63) and the >200 µm (referred to as P200) were washed with an equivalent volume of sterile 1 X PBS. Plankton wet weights were calculated by determining the sample mass and volume and solving for grams of material per 25 ml wet sample. All water and plankton samples were placed on ice in coolers fitted with recording thermometers and transported to the laboratory for processing within 6 hours of the final sample collection. Daily rainfall data for Savannah (for Wassaw Sound) and Brunswick (for Sapelo Sound) were obtained at www.georgiaweather.net.

**Fecal Indicator Bacteria.** Fecal indicator bacteria (fecal coliform bacteria and enterococci) were concentrated from water and the two plankton fractions by membrane filtration and enumerated using EPA method 1600 (USEPA 2002) for enterococci and Standard Methods for fecal coliform bacteria (APHA 1995). Before membrane filtration, plankton samples were first homogenized for 1 min at 12,000 rpm using a PRO homogenizer (PRO Scientific Inc., Model 200, Oxford CT. USA). Up to 50 ml of surface water and 5 ml of homogenized plankton were analyzed in duplicate for each indicator. Samples were filtered through sterile 47 mm, 0.45 µm pore size mixed cellulose ester membranes. The membranes were then placed on selective agar media: mFC for fecal coliform bacteria and mEI for enterococci. mFC plates were incubated at 44.5 °C for ≥ 18 h; blue colonies were counted as fecal coliform bacteria (APHA 1995). mEI
plates were incubated at 41°C for ≥ 18 h and all colonies with a blue halo were recorded as enterococci (USEPA 2002). Bacterial counts were recorded as CFU 100 ml⁻¹ for water or CFU 100 g⁻¹ (wet weight) for plankton.

**Concentration of viruses.** Enteric viruses were concentrated using an adsorption-elution technique modified from Katayama et al. (2002) and described by Fong et al. (2005). Each 1 L water sample was acidified to pH~4.0 by adding 10% acetic acid prior to filtration. Acidified water samples were filtered through a type HA, negatively charged membrane (Millipore, Billerica, MA) with a 90 mm diameter and a 0.45 µm pore size using a sterile glass filter housing. Final volumes filtered were recorded. A volume of 100 ml of 0.5 mM H₂SO₄ was then passed through the membrane, and elution of viral particles was completed by exposing the membrane to 10 ml of 1 mM NaOH for ~1 min. The eluate was then added to a sterile 15 ml tube containing a neutralization solution of 0.1 ml 50 mM H₂SO₄ and 0.1 ml of 100 X Tris-EDTA (TE) buffer. Eluates were stored at -20 °C until further concentrated by ultrafiltration (Centriprep YM-50 concentrator columns (Millipore, Billerica, MA)) to a final volume of ~2 ml, final concentrates were split and stored at -80 °C.

**Extraction of viral RNA.** Commercially available kits (RNeasy Mini Spin kits [Qiagen, Valencia, CA] and UltraClean Tissue RNA Isolation kit [Mo Bio, Carlsbad, CA] were used to extract and purify RNA from water and plankton, respectively, for the detection of enteroviruses. Water extracts were obtained from 200 µl of concentrated eluate, according to the manufacturer’s protocol; purified RNA was eluted in 30 µl of RNase free water. Whole plankton samples were homogenized as described above and RNA was extracted from 30 mg aliquots of
tissue, according to the manufacturer’s protocol. Purified RNA was eluted in 50 µl RNase-free water. Each concentrated and purified sample was serially diluted to a concentration of 10^{-2} prior to analysis. Samples were stored at -20 °C, if not processed immediately. RNA was held less than 24 hours before processing.

**RT-nested PCR.** Human enteroviruses (hEV) were amplified with a conventional reverse transcription-nested-polymerase chain reaction (RT-nested PCR) method using the protocol described by Fong et al. (2005), which was modified to follow the hanging drop method of Ratcliff et al. (2002) to minimize carry over contamination during the second round of PCR.

Briefly, the RT mixture (7.5 µl) and sample RNA (2.5 µl) were placed in the bottom of a 0.2 µl thin-walled reaction tube and overlaid with sterile mineral oil (Sigma-Aldrich, St. Louis, MO). The RT reaction mixture provided 5.0 mM MgCl₂, 1X PCR Buffer II, 0.75 mM deoxynucleoside triphosphates (dNTPs), 2.5 µM Random Hexamer (as provided in the RNA-PCR core kit (Applied Biosystems Inc., Foster City, CA), 2.5 U µl⁻¹ Reverse Transcriptase, and 1.25 U µl⁻¹ RNase inhibitor. The temperature cycle for RT was 22°C for 10 min, 42°C for 15 min, and 99°C for 5 min.

After the RT cycle, samples were cooled to 4°C before the addition of master mix for the first round of PCR (PCR 1). For PCR 1, a reaction mixture of 20 µl was added directly to the RT product and the tubes were centrifuged to mix the amplification reagents below the mineral oil layer. The PCR 1 reaction provided a final concentration of 2.9 mM MgCl₂, 0.2 µM each primer (ENT-up-1 (5´GTAGATCAGGTCGATGAGTC3´) (Fong et al. 2005) and ENT-down-1 (5´ACYGGRTGGCCAATC3´) (DeLeon et al. 1990), 0.4 mM dNTPs, 1X PCR buffer II (Applied Biosystems Inc., Foster City, CA), 1 X TaqMaster (Eppendorf North America,
Westbury, NY), and 0.625 U μl\(^{-1}\) Taq polymerase. Master mix for the second round of PCR (PCR 2) was then suspended in the cap as a hanging drop contained solely by surface tension. The reaction mixture for PCR 2 had a final volume of 50 µl, containing 0.5 mM MgCl\(_2\), 0.2 µM each primer (ENT-up-2 (5´CCTCCGCCCCTGAATG3´) (DeLeon et al. 1990) and ENT-down-2 (5´ATTGTCACCATAAGCAGCC3´) (Fong et al. 2005)), 0.2 mM dNTPs, 1 X PCR Buffer (Eppendorf North America, Westbury, NY), 0.875 X Eppendorf TaqMaster, and 1.25 U of Taq polymerase. After PCR 2 master mix was loaded into the caps, PCR round 1 was performed, with 40 cycles of denaturing at 95 °C for 30 sec, annealing at 57.7 °C for 30 sec, and extension at 72 °C for 45 sec and a final extension at 72 °C for 5 min. The resulting amplicon for PCR 1 was 333 base pairs. Once the cycle was completed, the tubes were then centrifuged to mix in the secondary amplification reagents below the mineral oil layer and the final, PCR 2, cycle was run. PCR 2 consisted of 40 cycles denaturing at 95 °C for 30 sec, annealing at 56.5 °C for 30 sec, and extension at 72 °C for 30 sec and a final extension at 72 °C for 5 min. The resulting amplicon was 154 base pairs. The RT and PCR steps were all performed in a DNA Engine® PTC-0200 thermal cycler (MJ Research, Inc. Waltham, MA), without the use of a heated lid.

For confirmation, all PCR products were hybridized against a biotin-labeled (5´ end) internal probe (5´-TACTTTGGGTGCTCGTGTTTC-3´) (Griffin et al. 1999) specific to the amplified region using a dot-blot platform (Bio-Dot, BioRad, Hercules, CA). All blots were hybridized overnight at 37 °C, with a stringency wash at 47 °C. Labeled and hybridized probes were detected by chemiluminescence using the Southern-Light protocol (Tropix, Inc., Foster City, CA) as described by Fong et al. (2005). Previous work by Griffin et al. (1999) and Fong et al. (2005) has shown that detection by internal probe hybridization improves specificity and limits of detection for human enterovirus when using conventional PCR.
For all runs, Polio virus strain LSC1 (courteously provided by Dr. Charles P. Gerba) was used as a positive control. A no-template negative control was also incorporated into each RT-PCR batch. To minimize the risk of carry-over contamination, separate dedicated labs were used for RNA extraction and PCR preparation and for post-PCR analyses. Additionally, dedicated hoods were used for the preparation of master mix (reagents only) and for RNA extractions. Finally, all labs were located on separate floors with controlled access and individual air handling systems.

**Statistical Analysis.** Enterococci and fecal coliform bacteria counts were log transformed; means were calculated as log means for statistical analysis and reported in the text as geometric means. When counts were below the limit of detection, zeros were used in all statistical analyses. The Pearson correlation test was used to evaluate relationships among fecal indicator bacteria and environmental variables. Analysis of variance (ANOVA) was performed to determine differences in mean levels of bacterial indicators among different sample fractions, stations and dates. Binary logistic regression was used to analyze the relationship between the presence and absence of enteroviruses and levels of bacterial indicators and other environmental variables measured throughout the study. For seasonal analyses, summer was defined as July, August and September, fall as October, November and December, winter as January, February and March and spring as April, May and June. Minitab Release 14.20 (State College, PA) was used for all analyses. In all cases, significance was declared at p< 0.05.
**Results**

**Environmental Parameters**

In total, 354 samples were collected during this 20-month study. The water temperature ranged from 9.6°C in February 2007 to 30.6°C in August 2006. Salinity had a narrow range of 24.7 (Jan. 2006) to 33.3 (Nov. 2007), and averaged 28.5. The pH ranged from 7.5 (July 2006) to 8.0 (Mar. 2006, Feb., April, and Nov. 2007), and averaged 7.8. Dissolved oxygen (DO) ranged from 3.8 (Aug. 2006) to 9.2 (Jan. 2007) mg L⁻¹, and averaged 6.0 mg L⁻¹. The 30-day cumulative rainfall ranged from 0.2 cm in November 2007 to 30.8 cm in September and averaged 9.2 cm, with September showing the highest rainfall amounts for both 2006 and 2007. No significant differences were found in any of these factors between the 12 stations (or between the two estuaries).

Salinity was positively correlated with pH ($r = 0.265$, $p = 0.001$); however, there was an inverse relationship with both DO ($r = -0.181$, $p = 0.001$) and 30-day rainfall ($r = -0.357$, $p = 0.001$). 30-day rainfall was positively correlated with temperature ($r = 0.539$, $p = 0.001$), but an inverse relationship was noted between temperature and pH ($r = -0.615$, $p = 0.001$). pH also had an inverse relationship with 30-day rainfall ($r = -0.499$, $p = 0.001$). DO was positively correlated with pH ($r = 0.394$, $p = 0.001$), and inversely correlated with both temperature ($r = -0.768$, $p = 0.001$) and 30-day rainfall ($r = -0.294$, $p = 0.001$).

**Distribution among Fractions**

Fecal coliform bacteria concentrations for all water samples ($N = 118$) ranged from <1 CFU 100 ml⁻¹ to 123 CFU 100 ml⁻¹ (geometric mean 5 CFU 100 ml⁻¹) (Fig. 2). In the P63 plankton fraction ($N = 117$), fecal coliform bacteria counts ranged from <5 CFU 100 g⁻¹ to 3.77 $\times$ 10⁶ CFU 100 g⁻¹ (geometric mean 534 CFU 100 g⁻¹) and for P200 ($N = 118$) fecal coliform
bacteria averaged 1,678 CFU 100 g⁻¹, ranging from <5 CFU 100 g⁻¹ to 4.17 \times 10^6 CFU 100 g⁻¹. Although levels in the P200 group were higher overall, there was no significant difference between the two plankton fractions (Fig. 2). While water held a significantly lower concentration of fecal coliform bacteria compared to either fraction (p = 0.001).

Enterococci levels in water (N = 100) ranged from <1 CFU 100 ml⁻¹ to 2,110 CFU 100 ml⁻¹ with a geometric mean of 31 CFU 100 ml⁻¹ (Fig. 2). Among P63 fraction (N = 99), enterococci counts ranged from <5 CFU 100 g⁻¹ to 5.82 \times 10^7 CFU 100 g⁻¹ (geometric mean of 3.90 \times 10^5 CFU 100 g⁻¹). In the P200 fraction (N = 100), enterococci counts ranged from <5 CFU 100 g⁻¹ to 4.00 \times 10^8 CFU 100 g⁻¹ (geometric mean of 2.53 \times 10^5 CFU 100 g⁻¹). Similar to the finding for fecal coliform bacteria, enterococci concentrations in the water were significantly lower than that found in either plankton fraction (p = 0.001), but the mean levels were not different between the two plankton fractions (Fig. 2). Unlike fecal coliform bacteria, enterococci were detected at similar frequencies between water and plankton.

Enteroviruses were detected in 54% of all water samples (N = 118). By plankton fraction, 57% (67/118) of the P63 samples and 59% (69/118) P200 samples were positive for enterovirus. The frequency of detection was not significantly different between these fractions or water.

Binary logistic regression revealed that in water fecal coliform bacteria levels were a significant predictor of enterovirus presence (Odds Ratio [OR] = 2.10, 95% Confidence Interval [CI] = 1.06 to 4.16, p = 0.033), but no relationship was found with enterococci levels. There was no significant relationship between fecal coliform levels and enterovirus presence in either plankton fraction, nor between enterococci and enterovirus in the P63 fraction. There was a
positive association between enterococci levels and enterovirus presence within the P200 fraction ([OR] = 1.26, CI = 1.02 to 1.56; p = 0.034).

**Spatial Distribution**

Among bulk water samples, the geometric mean for fecal coliform bacteria in Sapelo Sound was 9 CFU 100 ml$^{-1}$, with a range of <1 to 78 CFU 100 ml$^{-1}$, which was significantly greater than that found in Wassaw Sound (geometric mean of 3 CFU 100 ml$^{-1}$, with a range of <1 to 123 CFU 100 ml$^{-1}$; p = 0.018). Fecal coliform levels in Sapelo Sound also exceeded the safe shellfish harvesting threshold level of 14 MPN (CFU) 100 ml$^{-1}$ in 25 of 58 samples (43%), while this standard was only exceeded in 13% (8/60) of Wassaw Sound samples (NOAA 1998). When analyzed by individual station the fecal coliform bacteria levels were significantly greater at station B5 (geometric mean of 19.9 CFU 100 ml$^{-1}$) and significantly lower at stations B1 (geometric mean of 4.9 CFU 100 ml$^{-1}$) and B2 (geometric mean of 3.8 CFU 100 ml$^{-1}$) in Sapelo Sound (p = 0.038).

Enterococci within Sapelo Sound water samples showed a geometric mean of 43 CFU 100 ml$^{-1}$, with a range of <1 to 2,100 CFU 100 ml$^{-1}$, which was not significantly different that the levels recorded at Wassaw Sound (geometric mean of 22 CFU 100 ml$^{-1}$ and a range of <1 to 1,330 CFU 100 ml$^{-1}$). Samples exceeded EPA recommended standards for recreational water (USEPA 2003, USEPA 2011a) in 19/58 (33%) of the water samples from Sapelo Sound and in 13/60 (22%) of samples from Wassaw Sound.

For Sapelo Sound, fecal coliforms within the P63 fraction ranged from <5 CFU 100 g$^{-1}$ to 3.77 X 10$^6$ CFU 100 g$^{-1}$ (geometric mean 1,934 CFU 100 g$^{-1}$), which was not significantly different than the levels noted for this fraction collected from Wassaw Sound (<5 CFU 100 g$^{-1}$ to 1.27 X 10$^6$ CFU 100 g$^{-1}$; geometric mean 150 CFU 100 g$^{-1}$). Sapelo Sound levels in the P200
fraction (<5 CFU 100 g$^{-1}$ to 1.04 $\times$ 10$^7$ CFU 100 g$^{-1}$; geometric mean 1.20 $\times$ 10$^4$ CFU 100 g$^{-1}$) were significantly greater than those recorded in Wassaw Sound (p = 0.001; <5 CFU 100 g$^{-1}$ to 3.08 $\times$ 10$^6$ CFU 100 g$^{-1}$; geometric mean 276 CFU 100 g$^{-1}$). When analyzed by individual stations for the P63 fraction, there was a significant difference where the highest fecal coliform bacteria concentrations were found at station B5 (geometric mean 3.67 $\times$ 10$^4$ CFU 100 g$^{-1}$) and the lowest at D3 (geometric mean 78 CFU 100 g$^{-1}$). For the P200 fraction significantly higher fecal coliform bacteria concentrations were noted at B3 (geometric mean 7.81 $\times$ 10$^4$ CFU 100 g$^{-1}$) and the lowest at D1 (geometric mean 38 CFU 100 g$^{-1}$).

Enterococci within the P63 fraction at Sapelo Sound ranged from <5 CFU 100 g$^{-1}$ to 3.49 $\times$ 10$^7$ CFU 100 g$^{-1}$ (geometric mean 2.25 $\times$ 10$^5$ CFU 100 g$^{-1}$) and for the P200 fraction the range was <5 CFU 100 g$^{-1}$ to 4.00 $\times$ 10$^8$ CFU 100 g$^{-1}$ (geometric mean 3.19 $\times$ 10$^5$ CFU 100 g$^{-1}$). Enterococci among the P63 plankton fraction collected at Wassaw Sound ranged between <5 CFU 100 g$^{-1}$ and 7.60 $\times$ 10$^7$ CFU 100 g$^{-1}$ (geometric mean 7.41 $\times$ 10$^5$ CFU 100 g$^{-1}$) and for the P200 fraction, ranged between <5 CFU 100 g$^{-1}$ and 1.10 $\times$ 10$^8$ CFU 100 g$^{-1}$ (geometric mean 1.96 $\times$ 10$^5$ CFU 100 g$^{-1}$). There was no significant difference between the sounds for enterococci levels within either plankton fraction. When analyzed by individual stations, the highest enterococci counts in the P63 fraction were found at station B3 (geometric mean 3.61 $\times$ 10$^6$ CFU 100 g$^{-1}$) with lowest at station B2 (geometric mean 5.34 $\times$ 10$^5$ CFU 100 g$^{-1}$), but were not significantly different. For the P200 fraction there was little difference in the levels with the highest enterococci counts found at station D1 (geometric mean 1.20 $\times$ 10$^6$ CFU 100 g$^{-1}$) and the lowest at station D6 (geometric mean 2.91 $\times$ 10$^4$ CFU 100 g$^{-1}$).

Enterovirus prevalence in water was similar between sounds, with a mean of 55% of samples (32/58) positive in Sapelo Sound and 53% (32/60) positive in Wassaw Sound. In Sapelo
Sound, site B3, had the highest frequency of detection (67%) and the lowest was found at B6 (33%). Wassaw Sound had a similar range with the highest detection rate of 70% (D3) and the lowest 40% (D2 and D4, respectively).

Seasonality and Association with Environmental Factors

By sampling date, fecal coliform bacteria in water ranged from 0.41 CFU 100 ml\(^{-1}\) (Sept. 2007) (N = 6) to 44 CFU 100 ml\(^{-1}\) (Oct. 2006) (N = 6) (Fig. 3). In the P63 samples, fecal coliform bacteria levels ranged from <1 CFU 100 g\(^{-1}\) (July 2007) (N = 6) to a high of 3.69 X 10\(^5\) CFU 100 g\(^{-1}\) (Oct. 2006) (N = 6) (Fig. 3). For the P200 fraction, fecal coliform concentrations ranged between undetectable (<1 CFU 100 g\(^{-1}\)) in July 2007 (N = 6) and 8.42 X 10\(^5\) CFU 100 g\(^{-1}\) (Oct. 2006) (N = 6) (Fig. 3). Fecal coliform bacteria levels for all fractions combined showed significantly higher levels in Jan., May, Oct. 2006 and March 2007 and significantly lower levels in July 2007 (p = 0.001). Periods with high detection levels coincided with collections from Sapelo Sound, an area adjacent to a high density of septic systems.

Enterococci in water ranged between 0.67 CFU 100 ml\(^{-1}\) (April 2007) (N = 6) and 794 CFU 100 ml\(^{-1}\) (Dec. 2006) (N = 6) (Fig. 3). In the P63 fraction, enterococci levels were as low as 114 CFU 100 g\(^{-1}\) (March 2007) (N = 6) and as high as 3.60 X 10\(^7\) CFU 100 g\(^{-1}\) (June 2007) (N = 6) (Fig. 3). The P200 fraction ranged from 4 CFU 100 g\(^{-1}\) (April 2007) (N = 6) to 6.55 X 10\(^6\) CFU 100 g\(^{-1}\) (Sept. 2007) (N = 6) (Fig.3). When all fractions were combined, enterococci levels were significantly lower in March 2007 and April 2007 than any other months in the study (p = 0.001).

There were few significant interactions between FIB levels in water and measured physico-chemical or other environmental parameters. Fecal coliform bacteria levels were inversely related to salinity (r = -0.234, p = 0.011) and temperature (r = -0.239, p = 0.009).
Enterococci levels were negatively correlated with pH \((r = -0.297, p = 0.003)\). Neither enterococci nor fecal coliform bacteria concentrations in water were related to rainfall levels in the watershed. For the P63 and P200 fractions, enterococci levels and 30 day rainfall were positively correlated \((r = 0.325\) and 0.210, respectively, \(p = 0.001)\). Enterococci in the P200 fraction were negatively correlated with pH \((r = -0.281, p = 0.008)\). There were no significant correlations between fecal coliform bacteria levels in plankton and any of the measured parameters.

Enteroviruses in water samples were never detected in June and Sept. 2007 \((N = 6)\) but in March, May, June, July 2006 and Jan. 2007 all samples were positive \((100\%)\) \((N = 6)\). In the P63 fraction, enterovirus prevalence ranged from not detected \((\text{Jan. and Sept. 2006})\) \((N = 6)\) to 100% detection in March, July, Oct., Nov., Dec. 2006 and Jan. 2007. In the P200 fraction, enteroviruses ranged from not detected in Aug. 2006 and June 2007 \((N = 6)\) to 100% in March, July, Nov., Dec. 2006 and Jan. 2007. When all samples were combined \((N = 18)\) the monthly range was 11% positive in June 2007 to 100% positive in March and July 2006 \((\text{Fig. 4})\). When pooled into seasons the highest detection rate \((79\%)\) \((N = 18)\) was noted in the winter months for bulk water. However, the highest prevalence in both plankton fractions \((\text{P63 and P200; 96\% and 92\%, respectively})\) were reported in the fall \((\text{Fig. 4})\).

Enterovirus presence in water samples was positively associated with DO \((\text{OR} = 1.31, \text{CI} = 1.02 \text{ to } 1.67, p = 0.032)\) and negatively associated with temperature \((\text{OR} = 0.91, \text{CI} = 0.86 \text{ to } 0.97, p = 0.002)\) and 30 day total rainfall \((\text{OR} = 0.77, \text{CI} = 0.77 \text{ to } 0.66, p = 0.001)\). For the P63 samples, there was an inverse relationship \((\text{OR} = 0.82, \text{CI} = 0.70 \text{ to } 0.96)\) between 30 day rainfall and virus presence \((p = 0.011)\). For the P200 samples there was an inverse relationship \((\text{OR} = 0.93, \text{CI} = 0.88 \text{ to } 0.99)\) between temperature and virus presence \((p = 0.016)\).
Discussion

The goals of this study were to assess the spatial and temporal dynamics of fecal indicators and pathogens in tidally mixed and particle-dominated estuaries. We hypothesized that without direct riverine discharge, which is commonly a main contributor to contamination loading (Lipp et al. 2001; Fong et al. 2005) other environmental factors would emerge as correlates to describe the distribution of enteric bacteria and viruses. Such environmental parameters may include direct overland run off following rainfall events, seasonal temperature fluctuations and seasonal shifts in particle and plankton abundance. Understanding these dynamics are important to better describe and predict periods of poor water quality that may impact recreational bathers or shellfish harvesting. Results suggest that contamination is common among the studied estuaries in Georgia, enteric bacteria and viruses may increase their persistence in association with plankton or particles in the water and that complex circulation in these estuaries may allow for resuspension and recirculation over time.

While Georgia’s coastline is relatively small at ~160 km, it provides approximately one-third of the salt marsh habitat on the east coast of the US (Wiegert and Freeman 1990). The coastal area is comprised of a complex system of river dominated estuaries, barrier islands and tidal-marsh lagoons. The circulation among these lagoons, including the Sapelo and Wassaw Sounds, are driven by a large tidal amplitude causing water to move in and out of multiple marsh inlets and tributaries (Atkinson et al. 1978; Oertel and Dunstan 1981; Gross and Werner 1997) but receive little to no input from major freshwater streams (Erkenbrecher 1981). These fast running tidal currents flow in and out of sounds, carrying nutrients, sediments (Oertel and Dunstan 1981; Seabrook 2006) and potential enteric pathogens. Terrestrial nutrients or microbes...
can be transported to tidal creeks by groundwater flow, overland surface flow or tidal inundation (Burnett et al. 2003; Kleppel 2006).

When looking at Sounds there were significantly higher levels of fecal coliform bacteria found in Sapelo (p=0.000). In Georgia, the approved shellfish harvesting standard for fecal coliform bacteria is $\leq 14$ CFU 100 ml$^{-1}$, which was exceeded in 43% (25/58) of samples within Sapelo Sound. Communities near Sapelo Sound and to a lesser extent Wassaw Sound are primarily serviced by septic systems which are often adjacent to tidal creeks and estuaries. In-ground disposal of waste has been documented to contribute to the spreading of sewage associated nutrient and microbes in many coastal areas (Kümmerer 2004) including Georgia (Meile et al. 2010). Meile et al. (2010) found that septic systems in coastal Georgia contributed a significant addition of nitrogen to these waters. Additionally work by Gentry et al. (2009) showed a high prevalence of human norovirus in waters near high densities of septic systems in Sapelo Sound. This confirms work in other coastal areas that also suggests a high probability of enteric viral contamination in coastal waters contaminated by septic systems (Paul et al. 1995; Griffin et al. 1999, Lipp et al. 2001; Futch et al. 2010; Futch et al. 2011). Likewise, in this two year survey of tidal mixed estuaries, enteric viruses were common with 57% of sample positives for enterovirus RNA. Although not significantly different, higher indicator levels and higher viral prevalence was noted in Sapelo Sound, considered to be more directly influenced by septic systems (Meile et al. 2010). Furthermore, highest concentrations and prevalence of both fecal indicator bacteria and enteric viruses were noted most frequently at station B3, in Sapelo Sound. This station, in a public shellfish picking area, is adjacent to an area known to have faulty septic systems (Walker et al. 2003) and was previously noted for containing a high prevalence of human noroviruses (Gentry et al. 2009)
Results indicate that rainfall may have had a small contribution to overall contamination in these estuaries, suggesting that overland flow or groundwater discharge may carry contaminants, but in general seasonal fluctuations in temperature and shifts in potential ‘reservoir’ particulates may have played a larger role in the distribution of microbial contaminants. Plankton, detritus and marine aggregates, particles ranging in size from a few micrometers to more than a centimeter, can serve as environmental reservoirs for pathogens and aid in their survival and transport (e.g., Mote et al. 2012, Turner et al. 2009, Lyons et al. 2006). The estuaries of Georgia are known for their high level of productivity and abundance of particles which manifest themselves in distinct highly turbid zones (Seabrook 2006; Day 1989). Under conditions of plankton blooms or suspension of large amounts of detritus and particulates, enteric bacteria and viruses may find refuge from sunlight (and UV degradation) and other deleterious environmental conditions (Gentry et al. 2009). In this study, indicator bacteria were more abundant in the plankton fractions (both sizes) relative to water and enteric viruses were more commonly detected in those fractions.

Changes in plankton population dynamics or particle loading are seasonal and may interact with seasonal changes in temperature. For example, Mote et al. (2012) found that in the late summer enterococci attached to particles were the major contributor to the total enterococci detected in bulk water samples, with 95% of the enterococci in bulk water found in the particulate fractions. Similar interactions may explain the higher levels of viral detection in both the small and large plankton fractions, especially during the fall season (which were positive for enteroviruses 96% and 92% of the time, respectively). Additionally, shedding rates of enteroviruses are also reported to peak in the late summer and early fall and their increased loading at this time may also contribute to these findings (Khetsurani et al. 2006).
Fecal coliform bacteria were shown to be a significant predictor of enterovirus presence (p=0.033), which would correlate well with current shellfish harvesting standards. However, within the P200 fraction there was shown to be a significant relationship between enterococci and enterovirus presence (p=0.034). This calls into questions whether new shellfish standards need to be created in order to include other indicators. The distribution of particles within and around the coastal zone (Oertel and Dunstan 1981) and increased persistence of bacteria and viruses in association with plankton (Lyons et al. 2006; Signoretto et al. 2004; Haines 1979) could suggest the potential for widespread contamination with these enteric pathogens, especially given the complex water circulation noted in this area (Oertel and Dunstan 1981). Given that enteroviruses may survive for 20 weeks at 15°C in marine water (Lo et al. 1976), circulation patterns and particulate loading should be strongly considered in attempting to understand potential exposure risks for humans.

Acknowledgements

NOAA Oceans and Human Health Initiative (NA04OAR460020 and S0867882 [Georgia Oceans and Health Graduate Training Consortium]) with special thanks to the Georgia Department of Natural Resources (Brooks Good and Dominic Gualdognoli).
Reference Chapter 3


Figure 1. Map of Wassaw and Sapelo Sounds
Figure 2 Indicator Bacteria present in each fraction

Figure 3 Monthly mean temperature (a), rainfall (30-day cumulative) (b), fecal coliform bacteria (c) and enterococci for each of the three fractions (water, plankton 63 – 200 µm and plankton >200 µm.

61
Figure 4 Seasonality of enterovirus prevalence by fraction
CHAPTER 4

CONCLUSIONS

Nearly half of the U.S. population lives within ~80 km of the coast and coastal counties continue to grow in size. With this increase we see a rise in urbanization and therefore an increase in runoff and sewage treatment processes. Estuaries face many different problems, which include pathogen contamination. FIB is the current regulatory standard for recreational and shellfish harvesting waters. However monitoring solely for FIB is not always effective for determining the true conditions of coastal waters since bacteria can survive and even multiply under certain conditions. Several studies have shown that viral pathogens are frequently detected in waters with low FIB concentrations; therefore, virus presence can not always be adequately predicted through the use of these indicators alone. Contaminated water can transmit enteric viruses to humans and therefore pose a major public health risk both recreationally and through shellfish consumption. Many factors including freshwater inflow, seasonal, physical, chemical and biological factors affect the fate and persistence of both FIB and human enteric pathogens (including viruses). Understanding these factors that increase microbial persistence in estuarine environments, including interactions with particles and plankton, is crucial to better determining exposure to users, especially in regions where tidal mixing is the dominant force.

Fecal coliform bacteria were a significant predictor of enterovirus presence in bulk water samples (p=0.033), however in the P200 fraction enterococci were shown to be a significant predictor of its presence (p=0.034). This shows that there is a need to sample more than just fecal coliforms within shellfish harvesting waters. Finally, the results indicate that human
enteric viruses may increase their persistence in association with plankton, on a seasonal basis. Enterovirus prevalence in the P63 fraction was 96% and 92% in the P200 fraction during the fall months. Plankton may be acting as a reservoir to protect viruses and fecal indicator bacteria from environmental stressors, such as UV degradation. The distribution of particles within and around the coastal zone and increased persistence of bacteria and viruses in association with plankton could suggest the potential for widespread contamination with these enteric pathogens, especially with the complex water circulation within this area.

In conclusion, fecal coliform bacteria were only marginally predictive of enteric virus presence in the shellfish harvesting waters of Georgia; however, both enterococci and fecal coliform bacteria followed seasonal patterns and fraction partitioning which may be indicative of key factors in the ultimate fate and transport of enteric pathogens. Finally, enterococci standards which already exist for recreational waters should be explored for use in shellfish harvesting water as well