ARTIFICIAL OYSTER REEFS AS A WATER QUALITY REMEDIATION TOOL IN A CONTAMINATED ESTUARY

by

MARY KATHERINE CREWS

(Under the Direction of Erin K. Lipp)

ABSTRACT

Human sewage contamination from faulty septic tanks in coastal environments poses a major risk to public health. Field and controlled laboratory studies were conducted to assess the effectiveness of using the eastern oyster, *Crassostrea virginica,* in an artificial oyster reef as a water quality mitigation tool. Three sites in coastal Georgia were monitored over two years for fecal indicator organisms and enteric pathogens. The site impacted by the highest density of nearby septic systems had significant ($p < 0.05$) levels of fecal coliform bacteria, enterococci, and coliphage, in both water and oyster samples and was 85.7% positive for *Salmonella* (6/7 samples) and 20% positive for enterovirus (1/5 samples) in water samples. Controlled laboratory studies revealed that oysters can quickly reduce the amount of fecal bacteria from water but may reintroduce the contaminant as feces/pseudofeces. Artificial oyster reefs were useful as a sampling medium, but their effectiveness for water quality mitigation were not conclusive.

INDEX WORDS: Shellfish, fecal indicator organisms, enteric pathogens, septic systems

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DEDICATION

To my parents for Dairy Queen on the first day of school and to my grandparents for five dollar bills on every ‘A’.

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CHAPTER 1

Introduction

The health of estuarine environments is at risk due to detrimental effects of anthropogenic pollution associated with urbanization and improper waste management. Continued reliance on septic systems for waste disposal contributes to the introduction of fecal indicators and pathogenic agents to both water and shellfish harvesting grounds (LaPointe et al. 1990; Lipp et al. 2001; Paul et al. 1997). In coastal Georgia, a Geographic Information System (GIS) inventory of septic systems adjacent to coastal waters noted a high incidence of failing septic systems (Walker et al. 2003) that negatively affected nearby water quality (Fong et al. 2005; Gentry et al. 2009). Filter feeding organisms including oysters, mussels, clams, and cockles are of special concern given their ability to concentrate environmental elements into their tissues, including sewage related microbes (Daskin et al. 2008; Alexander 1976; Fukimori et al. 2008; Kovacs et al. 2010). The presence of fecal contaminants in marine waters and shellfish poses a major public health threat to recreational swimmers, shellfish consumers, and residents of coastal communities. Hence monitoring of enteric pathogens and indicators of fecal pollution in marine organisms and water is critical in understanding the effects that poorly sited or ill managed septic systems can have on local ecosystems.

The eastern oyster, *Crassostrea ivrgininca,* is native to coastal Georgia and is a commercially and ecologically valuable species that inhabits near shore areas commonly affected by wastewater inputs (Mackowiak et al. 1976; Gregalis 2006; Cerco and Noel 2007). *C. virginica* has a relatively rapid metabolism and a higher assimilation capacity for foods

compared to other bivalves which makes them an effective indicator of sewage pollution (Hammen 1969; Bayne and Newell 1983). Additionally, oysters may provide a natural bioremediation tool in sewage-affected waters by sequestering contaminants, thereby enhancing water quality and the public's health.

In the literature review (Chapter 2), the effects of human development and use of septic systems on coasts, estuaries, and shellfish harvesting waters are explored in detail. Moreover, the role of *C. virginica* as a filter feeder, detection of fecal contaminants in its tissues, and reef restoration efforts are discussed. Lastly, the effectiveness of fecal indicator organisms and human enteric viruses in assessing the extent of human sewage contamination are examined.

Chapter 3 evaluates the usefulness of artificial oyster reefs in helping to recruit *C. virginica* for water quality mitigation purposes. Additionally, a controlled laboratory experiment examining the fate and retention of a common fecal bacterium, *Enterococcus faecalis*, is outlined in Chapter 3. The fourth chapter provides an overview of the research findings and conclusions of the study.

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CHAPTER 2

Literature Review

Introduction

Burgeoning human populations along coastlines have altered the landscape in profound ways. As development continues, natural environments are covered with impervious surfaces, causing the loading of non-point source pollutants. Further, the misuse of septic systems in coastal regions has contributed to the contamination of water and shellfish harvesting areas through the introduction of fecal bacteria, viruses, and protozoa. Fecal indicator organisms are often utilized to examine the extent of pollution but may not fully encompass the degree of contamination or health risk because they typically fail to predict pathogenic viruses, which are the most disease causing agents in recreational waters and shellfish. Oysters are some of the first organisms affected by an expanding human presence in coastal regions. Their decline in recent years from overfishing, disease, and habitat degradation have demanded the need for restoration efforts. Artificial oyster reefs aim to recruit young oysters in order to establish viable reefs and to improve water quality through the filter feeding role oysters play in estuarine regions. Here, the interplay between human development, water quality, and shellfish contamination from microbial agents are outlined.

Population Growth and Anthropogenic Pollution

During the latter part of the 20th century, the population of United States coastal areas increased significantly (NSCT 1995; Mallin et al. 2001). Coastlines contain only 17% of the contiguous U.S. land area but are home to more than 53% of the nation's population. Further, estimates indicate that coastal populations are increasing by 3,600 people per day, giving a

projected total increase of 27 million people by 2015 (Culliton 1998; Beach 2002). As coastal populations grow concurrent changes in the surrounding landscape can be altered in profound ways (Mallin et al. 2001). Natural area is converted to urban and suburban development, which has major effects on water quality, availability, and movement (Mallin et al. 2001). For example, a study by DiDonato and colleagues (2009) found an increase in the concentration of bacterial and viral indicators with increasing urbanization. Furthermore, an analysis conducted by Mallin and colleagues (2000) found that fecal coliform abundance was significantly correlated with watershed population, and even more strongly correlated with the percentage of developed land within the watershed.

Urbanization alters the coastal landscape by replacing natural ground cover with paved surfaces which results in a net loss of mature vegetation (especially trees), disturbance and compaction of natural soils, and large increases in the amount of impervious surface area (Booth 1991). Even small changes in impervious cover can cause large changes in ecological properties and contributes to the loading of nonpoint source pollutants into local water bodies (Lipp et al. 2001; Holland et al. 2004; DiDonato et al. 2009).

Non-point source pollution includes urban and agricultural runoff, discharges from boats, leaking septic systems, sewerage lines, and combined sewer overflows (US EPA 2001). Non-point source pollutants are often transported to oceans via surface water runoff (Culliton 1998; Bay et al. 2003; Reeves et al. 2004; Warrick et al. 2004), wet and dry deposition of airborne pollutants (Lu et al. 2003), and submarine discharge of contaminated groundwater (Schiff et al. 2000; Boehm et al. 2004). Furthermore, until the late 1990s, effluent from sewage treatment plants, which include nutrients, pathogens, pesticides, and heavy metals were a significant source of urban coastal pollution (Schiff et al. 2000). Presently, there is an emerging consensus that

increasing coastal development is associated with increasing fecal pollution in tidal creeks, estuaries, and bathing beaches (Mallin et al. 2000; Holland et al. 2004; Mallin 2006).

Growing populations require not only management of non-point source pollutants but also effective wastewater treatment and disposal. In areas where sewer systems and wastewater treatment are not available, a dependence on aging and poorly sited septic tanks and drain fields in coastal counties may cause excess loading of nutrients and microbes to estuarine waters (LaPointe et al. 1990; Paul et al. 1997; Lipp et al. 2001a). From a human health perspective, the accumulation of pathogens in water, sediments, and marine organisms may render seafood products unsafe for consumption and water unsafe for bodily contact (Stewart et al. 2008). Many types of diseases may result from exposure to these pathogens including gastroenteritis, ear and respiratory infections, hepatitis, and skin rashes (DiDonato 2009).

Salt Marshes and Estuaries

The Eastern Coast of the United States contains nearly four million acres of salt marshes that stretch from Maine to Florida (Field et al. 1991). Salt marshes are vegetated by herbs, grasses, and low shrubs that border saline water bodies and are recognized for their exceptional ecological value (Eisma 1998; Kennish 2002; Kent 1994; Mitsch and Gosselink 1993; Alongi 1998). Important ecosystem services provided by salt marshes include the catchment of fertile sediments, biogeochemical transformation of carbon, hydrogen, sulfur, and nitrogen, and provision of nursery grounds and habitat of wildlife (Stender and Martore 1990). Additionally, salt marshes and adjacent tidal creeks provide recreational fishing, swimming, and boating areas, natural flood mitigation, and can purify runoff and wastewater (Reimold 1980; Daiber 1986; Tiner 1994; Weinstein 1996).

Unfortunately, more than half of the original salt marsh habitat in the U.S. has been lost, due in large part to development and human activities in coastal regions (Kennish 2002). Human activities potentially threaten the viability of salt marsh systems on local, regional, and global scales (Kennish 2002). In the southeastern United States, coastal uplands adjacent to salt marshes and tidal creeks have become increasingly popular targets for the development of homes, resorts, retirement destinations, and recreational facilities (DiDonato et al. 2009). Direct impacts to salt marshes include those that result from the physical alteration and immediate loss of habitat during construction of bulk- heads, dikes, weirs, levees, piers, docks, pipelines, revetments and other hard structures, as well as the excavation of canals, ditches, and oil drill sites (Deegan et al. 1983; Delaune et al. 1989; Bryant and Chabreck 1998; Kennish 2002; Sasser et al. 1986; Swenson and Turner 1987; Turner and Rao 1990; White and Morton 1997). Dredging, oil spills, marina development, and recreational boating, and point and nonpoint-source inputs of contaminants have also affected the inherent resilience of salt marshes to resist stressors and reduced their capacity to absorb anthropogenic impacts (Weinstein 1996).

Sewage inputs and excess nutrients from land based sources is another issue in estuarine regions since fecal indicator bacteria are the third leading source of impairment of rivers and the second leading cause in estuaries (US EPA 1997; Kennish 2002; Ribaudo 2000). For instance, by 1996 only 62% of U.S. estuaries fully supported their designated uses as fishable and swimmable, under the Clean Water Act (US EPA 1997; Lipp et al. 2001a). Moreover, despite relatively low surveillance, 42% of impaired estuaries nationwide were contaminated with high levels of fecal indicator bacteria (US EPA 1997). Within the first six months of 2012 alone, nine beaches in coastal Georgia were closed due to high levels of enterococci (GA DNR 2012).

A major effect of human development on estuarine systems is the closing of shellfish harvesting grounds due to high levels of fecal coliform bacteria, an indicator of human sewage contamination (Mallin et al. 1998). Of the 20 estuarine areas in Georgia, 19 were closed to shellfish harvesting at one point due to fecal contamination (GA DNR 1998). Ten of these areas were closed due to fecal contamination from non-point sources. Hence, not only is the public at risk to harmful exposures, but detrimental economic impacts can occur through closings in recreational beaches, clam farms, and oyster harvesting grounds (Walker et al. 2003).

The extent of human fecal pollution in coastal waters depends on a multitude of factors, including the density and size of the community, development in the watershed, storm water treatment and detention, waste disposal and treatment systems, and climate and weather (Kurz 1998). If proper waste disposal systems such as sewage treatment plants and separation of storm and sanitary sewer systems are available, the loading of fecal pollutants may decrease (Warrick et al. 2004). Unfortunately, in many areas of the United States and especially in coastal Georgia, septic systems are the only option for waste disposal.

Crassostrea virginica, The Eastern Oyster

The Eastern oyster, *Crassostrea virginica* is a common bivalve in the estuarine regions of the eastern United States. In the southeastern U.S., oyster reefs have the greatest density and biomass of east coast stocks (Dame et al. 1984). They range in size from a few small isolated clumps to massive solid mounds of both living and dead shell (Bahr and Lanier 1981) that may occur for hundreds of meters along creek and river banks. Oyster reefs provide numerous economic and ecological benefits including commercial fisheries value, habitat diversity, and erosion control (Breitbur et al. 2000; Mallin et al. 2000; Meyer et al. 1997; Posey et al. 1999).

Oyster reefs provide structural stability within estuaries and aid in the dispersion of wave energy in a relatively soft mud environment and can shield salt marsh (*Spartina alterniflora*) habitat from erosion associated with heavy boat traffic and winter storms (Coen and Luchenbach 2000). Additionally, filtering by oysters may improve water quality by reducing suspended sediment and nutrients in aquatic systems (Gerritsen et al. 1994; Brumbaugh et al. 2000; Mann 2000). Oyster populations can also influence the surrounding environment through particle depletion, nutrient cycling, and biodeposition (Dame et al. 1984; Jordan 1987; Newell 1988).

Crassostrea virginica *in Georgia*

C. virginica is native to coastal Georgia and is a commercially and ecologically valuable species that inhabits near shore areas commonly affected by wastewater inputs (Mackowiak et al. 1976; Gregalis 2006; Cerco and Noel 2007). Natural oyster reefs occur throughout the moderate to high salinity areas of estuaries in Georgia. Most reefs in Georgia occur within the intertidal zone starting at the two-hours above mean-low-water mark and may extend to 130 cm above the mean-low-water mark (Bahr and Lanier 1981). Georgia has an extensive oyster population with an overabundance of natural oyster recruits, or spat, each year (Harris 1980). Oyster reefs occur anywhere from small feeder creeks to areas in the open sound and can successfully attach to any hard substrate within the soft mud substrates that dominate coastal Georgia's estuaries. Once established, spat will settle on existing oysters and with each passing year the reef will expand until centuries later massive reefs are in place (Manley et al. 2010). Oysters spawn from March/April to October (Heffernen et al. 1989). Spat that are able to settle in April/May grow rapidly and become sexually mature within two months and spawn throughout the remainder of the summer into fall (O'Beirn et al. 1996a). Oyster recruitment rates are high in Georgia with

record rates as high as 204,700 spat m^{-2} settling within a month (O'Beirn et al. 1995; 1996a; 1997; Thoresen et al. 2005).

Oysters as filter feeders

Oysters are sedentary filter feeders that incorporate environmental elements into their tissues, which in turn reflect environmental influences (Alexander and Young 1976; Fukimori et al. 2008; Kovacs et al. 2010). Oysters can filter several liters of seawater daily in order to obtain food from particulate matter in the water column, including microorganisms or fine dispersed detritus (Galtsoff 1964; Solic et al. 1999). Bivalve molluscan shellfish, such as oysters, hard shell clams, mussels, and cockles can bioaccumulate from >1 to 1000-fold higher levels of microorganisms than overlying waters (Sobsey and Jaykus 1991). Oysters feed by means of cilia, where material held on the gill surfaces is transported to the mouth opening for digestion (Solic et al. 1999). They also have the ability to sort particles, ingesting food and rejecting pseudofeces particles that are non-nutritive or of an unfavorable size (Jorgensen 1966). Moreover, *C. virginica* has a relatively rapid metabolism and higher assimilation capacity for foods compared to other bivalves (Hammen 1969; Bayne and Newell 1983). The innate ability of oysters to filter feed and accumulate contaminants make them good sentinels for sewage related pollution (Carmicheal et al. 2004; Lake et al. 2001; Shriver et al. 2002). The evaluation of the presence and distribution of pathogens among marine bivalves is critical in determining the present and future risk to human health by better understanding the nature of the interaction between pathogens and shellfish (Stewart et al. 2008).

Oysters and other bivalves can present a potential health hazard to consumers who ingest oysters contaminated by pathogens that may be present in marine waters (Solic et al. 1999). The

largest known shellfish-borne disease outbreak caused nearly 300,000 cases of acute hepatitis in China and was linked to oysters contaminated with hepatitis A virus from sewage (Xu et al. 1992; Love et al. 2010). In the United States, infectious disease from raw or undercooked molluscan shellfish has been reported since the 1800s, with reports of more than 400 outbreaks (Rippey 1994). The true incidence of shellfish vectored infectious disease may be underestimated as much as 20 fold or more due to the lack of a federal mandate to report gastrointestinal illness of an unspecified agent (Archer and Kvenberg 1985). Illnesses from oysters are attributed to bacterial or viral agents associated with human wastes or to bacterial pathogens indigenous to coastal marine environments (Rippey 1994).

Shellfish Harvesting Waters

Shellfish waters in the United States are classified as approved, conditional, restricted, or prohibited for shellfish harvesting based on the monitoring of fecal coliform bacteria levels (Oliviera et al. 2011; Lees 2000; Gentry et al. 2009). Shellfish harvesting waters in Georgia are required to have levels of fecal coliform bacteria below 14 MPN (Most Probable Number) 100 ml^{-1} (US FDA 2009). These standards can be effective but they do not indicate the presence of viral pollutants that may persist longer than coliform bacteria (Deng et al. 1994). To prevent illnesses, thousands of hectares of fishing areas in the U.S. are closed to shellfish harvesting due to elevated concentrations of fecal indicator bacteria and other criteria that may not be reflected by fecal coliform indicators (Rippey 1994; Glasoe and Christy 2005).

After several large outbreaks from the consumption of raw molluscan shellfish in the late nineteenth and early twentieth century, the U.S. Public Health Service convened a committee to establish regulations for the sanitary control of shellfish. This committee, a forerunner to the

National Shellfish Sanitation Program, made the following recommendations. First, shellfish should be marketed from areas that are free of any disease-producing organisms or any deleterious or offensive substances. Secondly, after removal from water shellfish should be handled appropriately to safeguard from further contamination from pathogenic microorganisms or toxins, heavy metals, and organics. Lastly, epidemiological studies should be conducted on all outbreaks that implicate shellfish (Frost 1925).

Shellfish Restoration Efforts

Standing stocks of *C. virginica* in the Chesapeake Bay were as high as 188 x 10^6 Kg dry tissue prior to the major harvests of the late 19th Century. Due to the continued effects of overfishing and the effects of disease, the population declined to a level of 1.9 x 10^6 Kg dry tissue by 1988 (Newell 1988). Causes of the decline of oyster populations include overfishing, disease, and habitat destruction (Andrews 1965; Andrews 1988; Rothschild et al. 1994; Jordan and Coakley 2004; Kirby and Miller 2005). The decline in recent years has led to a demand for restoration efforts to not only rehabilitate oyster reefs but also to improve water quality (Calvo et al. 2001; Coen et al. 2007; Luckenbach et al. 2005). For example, *in situ* measurements have demonstrated that oysters reduce the quantity of suspended solids and phytoplankton (Nelson et al. 2004; Grizzle et al. 2006). At the current oyster abundances in Chesapeake Bay, these effects are limited, but significantly enhanced abundances of filter-feeders can significantly improve water quality in shallow, mesohaline regions of estuaries (Newell and Koch 2004; Cerco and Coen 2007). Moreover, researchers in North Carolina that employed transplanted . *C. virginica* beds found a reduction in total suspended solids (TSS) and chlorophyll *a* concentrations downstream of the beds in a small tidal creek system (Nelson et al. 2004). In

terms of using oysters as a water quality sentinel, transplanted oysters were shown to be effective indicators of wastewater contamination over an extended time period (Biancani et al. 2011). While water samples reflect short term variation, or a "snapshot" of local conditions, oysters assimilated N stable isotopes and accumulated microbial indicators through time, which provided integrative information on conditions *in situ* on a scale of days to weeks (Biancani et al. 2011; Burkhardt et al. 1998; Burkhardt and Calci 2000; Daskin et al. 2008).

Artificial oyster reefs can be a useful tool to help promote rapid growth of oyster populations by providing a substrate for natural spat deposition (Manley et al. 2008). A study comparing different types of artificial oyster reefs was conducted in 2006 and found that PVC pipes coated in a cement slurry facilitated the greatest oyster density (Manley et al. 2008). Oysters recruited on these sticks also had the least number of fatally damaged oysters during collection. Additionally, the oyster spat sticks were cost effective, easy to construct, and environmentally friendly.

Septic Systems in Coastal Environments

The U.S. Environmental Protection Agency (2000a) has reported that septic systems were utilized by approximately 25% of the U.S. population and 40% of new development. Further, the U.S. Bureau of the Census (2008) has indicated that at least 10% of septic systems have stopped working, and some communities have reported failure rates as high as 70%. Seepage from failing septic systems is a potential source of pathogens in both coastal and land locked areas. Typically, septic systems are used to treat domestic waste of individual residences in low density suburban and rural areas. Bacteria are removed initially in this type of system through settling in the tank itself, but distribution of tank effluent into unsaturated soils is necessary to

complete the decontamination process (Jones and Holmes 1985). If properly placed in areas with unsaturated soils or with a functioning absorption field, sewage effluents including fecal indicators and pathogenic bacteria will be removed within a few meters of the septic system (Hagedorn et al. 1981). But multiple studies have shown that a large percentage of septic systems are installed in unsuitable soils, including those that are saturated or are on perched or shallow groundwater tables and therefore have the potential to contribute fecal pollutants to groundwater and or surface water (Goehring and Carr 1980; Duda and Cromartie 1982). For example, the Florida Keys are dominated by both sandy soils and karst topography and studies have shown that widespread bacterial and viral contamination of surface waters are often associated with septic systems (Gerba 1984; Anderson et al. 1991; Paul et al. 1995; Griffin et al. 1999). A viral tracer study conducted in 1995 revealed that viruses from septic tanks are able to rapidly move into coastal waters after septic discharge (Paul et al. 1995). More recently, in Shired Island, Florida an area utilizing septic systems consistently failed to meet water quality standards over a five month period in 2006, where 13 tests out of 15 found medium to high levels of enterococci, a fecal indicator (Crabbe 2006). Ensuring adequate soil beneath septic system drain fields is difficult in coastal regions, where tidal influences result in daily fluctuations in groundwater levels (Inouchi et al. 1990; Sun 1997). These factors facilitate the transport of microbes that are able to penetrate the subsurface during saturated conditions (Bicki and Brown 1991; Lipp et al. 2001a).

Appropriate drain fields are also necessary to prevent contamination from septic effluent. Duda and Cromartie (1982) found that bacterial contamination of several North Carolina estuaries and their tributary streams was directly related to the density of drain fields in the watershed. Additionally, Kelsey and colleagues (2004) revealed a positive correlation between

sampling sites with high densities of active septic tanks and high fecal coliform levels. Moreover, viruses, which are much smaller and more mobile than bacterial contaminants, may easily migrate under saturated conditions beyond current setback distances required between the drain field and wet season water table (2 ft or 0.63 m) (Viraraghavan 1978; Potsma et al. 1992; Florida 1993; Nicosia 1998). Without adequate setbacks there may be insufficient unsaturated soil beneath the drain field, particularly during wet seasons when seasonal recharge results in an elevated water table (Cable et al. 1997). Seasonal patterns also play a significant role in the timing and extent of fecal pollution. For instance, fecal pollution in the Charlotte Harbor, FL estuary was concentrated in regions of low salinity, high freshwater input, and dense septic systems. Widespread contamination was seasonal in nature when wet weather events were important in the transport of indicators and human viruses far into the estuary (Lipp et al. 2001b).

Ideally, investments in sewage treatment processes and facilities would reduce the contamination of water and shellfish harvesting grounds. However, investing in adequate sewage treatment systems can be difficult and expensive and has been considered disproportionate in terms of the value of the shellfish industry (Lees 2000). Environmental concerns have contributed in recent years to the increased investment in sewage infrastructure; however, important improvements are still needed, namely appropriate discharge locations for treated water, adequate arrangements for storm water storage and treatment, tertiary treatment of effluents and adequate evaluation methodologies of the effluent microbial quality (Lees 2000). The location of pollution inputs must be well identified in order to assure that quality monitoring programs take them into consideration. This may result in the expansion of sewage infrastructure even to sparsely populated areas or other areas which represent a low sewage input (Lees 2000).

Risk management strategies for shellfish harvesting areas must be improved in other to prevent shellfish contamination (Shumway and Roddick 2009; Oliviera et al. 2011).

Septic Systems in Coastal Georgia

Similar to other coastal regions across the country, McIntosh County, Georgia is experiencing a rapid increase of new residents in developments near marsh front or waterfront properties (Walker et al. 2003). Proper waste management has been an issue in this county because except for the town of Darien, no waste water treatment facilities exist, thus septic systems are utilized for waste management (Walker et al. 2003). This waste disposal method has not been effective due to faulty, aging, or poorly sited systems (Walker et al. 2003). Therefore, there is a much greater likelihood that both chemical and microbial contaminants will infiltrate the adjacent watersheds.

A Geographic Information System (GIS) study was performed in McIntosh County in order to map and inventory those on-site septic systems adjacent to coastal waters (Walker et al. 2003). The study concluded that 1,056 septic tanks were adjacent to salt marshes or coastal waters. Of these septic systems, 5% were visually dysfunctional. Another 100 tanks were found within 1 foot of a body of water even though a drain field of 25 feet is required to service the waste of a septic tank (GA Dept. of Health). Also, 75% of the septic systems were located in 100-year flood plains, and another 18% were found in water recharge areas. Moreover, the combination of the low coastal water table and sandy soil type found in the area contribute to the likelihood that sewage pollution will infiltrate the watershed (Walker et al. 2003). Faulty septic systems can contribute enteric bacteria, protozoa, and viruses to the environment where they can pose a threat to public health through recreational exposure and through contamination of shellfish harvesting areas.

Indicators of Fecal Pollution

If septic systems are in fact poorly placed or dysfunctional and sewage pollution occurs, fecal indicators are employed to define the extent of contamination. Indicator organisms should be non-pathogenic, common inhabitants of the intestinal tract of warm-blooded animals, present whenever enteric pathogens are present, unable to grow in water, and found in high concentrations in fecal material (Wade et al. 2006). Current guidelines for recreational water quality are based upon epidemiological studies of marine and fresh water bathing beaches in the 1970s and 1980s (Cabelli et al. 1975; 1979; 1982). The results of these studies indicated a direct relationship between the density of enterococci and *Escherichia coli* in water and the occurrence of swimming associated gastroenteritis (US EPA 2000). This relationship led to the development of criteria that can be used to establish recreational water standards under the Clean Water Act (US EPA 1986).

Fecal Indicator Bacteria

Enterococci are Gram-positive, catalase negative, non-spore forming cocci that are commonly found in the feces of warm blooded animals and birds (Holt et al. 1994). There are currently 19 species that are recognized within the *Enterococcus* genus, but *Enterococcus faecalis* is the most common species found in the feces of humans (Noble 1978; Manero and Blanch 1999). Enterococci are considered to have certain advantages over the coliform and fecal coliform indicators because they rarely multiply in water, are resistant to environmental stress, and generally persist longer in marine environments (Vasconcelos and Swartz 1976; Gleeson and Gray 1997; Maier et al. 2000). In 2000, the BEACH Act, an amendment to the Clean Water Act, was passed and required states to use enterococci as a standard for marine waters (USA 2000).

Escherichia coli is part of an operationally defined group, the total coliforms, which include *Escherichia*, *Citrobacter*, *Enterobacter*, and *Klebsiella* species (Maier et al. 2000). Total coliform bacteria are characterized as Gram-negative, nonspore forming, rod shaped, facultative anaerobes and were the first fecal indicator due to their presence in feces and the ease of detection (Wolf 1972; Clesceri et al. 1998). A sub-group of total coliforms, the fecal coliforms, were later adopted as a better indicator because of their ability to grow at elevated temperatures (Griffin et al. 2001). Fecal coliform bacteria are still widely used and are currently the only standard for shellfish harvesting waters (FDA 1995). Some disadvantages of using fecal coliform bacteria as an indicator include the inability to distinguish between human and animal contamination and their ability to survive considerable periods of time outside human intestines in tropical waters (Toranzos 1991; Maier et al. 2000).

Even though marine waters and estuaries are not ideal habitats for enterococci and fecal coliform bacteria they are able to persist in sediments and other protective environments for extended periods (LaBelle et al. 1980). In marine sediment, enteric microorganisms have been detected at significantly elevated levels relative to the overlying water column (Lipp et al. 2001a). Environmental factors, including daily temperature, salinity, and UV irradiance, can play a role in the survival of fecal associated microorganisms (Solic and Krstuvoliv 1992). Once introduced into coastal waters, deposition or adsorption in the sediment may further enhance survival of many enteric organisms (Gerba and McLeod 1976).

Indicators have traditionally been used to suggest the presence of enteric pathogens; however it is recognized that there is rarely a direct correlation between bacterial indicators and human pathogens (Ashbolt et al. 2001). The detection of fecal indicator bacteria in aquatic systems can be highly variable because of the influence of temperature, sunlight, tidal stage,

availability of particulates and nutrients, and cell biological factors (Rhodes et al. 1983; Fleisher 1985; Anon 1995). Additionally, fecal coliform bacteria and enterococci are not ideal indicators because they do not meet certain guidelines. For example, they can be found in non-human sources, they may lose culturability in marine waters, and some species of enterococci are able to proliferate in marine waters (Mote et al. 2012; Vasconcelos and Swartz 1976; Fujioka et al. 1981; Perez-Rosas and Hazen 1988; Fujioka et al. 1988; Fujioka et al. 2002; Griffin et al. 2003; Hardina and Fuijioka 1991). Moreover, an incubation time of ≥18 h is required for detection, making these indicators inefficient for public health surveillance and notification. Other types of indicators have been suggested, including viruses such as coliphages and human enteric viruses which may be a more suitable indicator of fecal pollution because they may be present when bacterial indicators are absent (Griffin et al. 1999; Lipp et al. 2002).

Fecal Indicator Bacteria in Oysters

As mentioned above, fecal coliform bacteria are currently used as an indicator bacteriological water quality of shellfish and to classify the waters from which shellfish are harvested (Hunt 1977). Fecal coliform bacteria have exhibited a high positive correlation with fecal contamination from warm-blooded animals in marine environments, therefore, if detected in edible shellfish they may indicate a potential health hazard to consumers (Solic et al. 1999). For instance, in a study in the Gulf Coast, oysters have been shown to accumulate fecal coliform bacteria to levels that averaged 4.4 times greater than levels in the water to which they were exposed (Burkhardt and Calci 2000). The concentration of fecal coliform bacteria in bivalves are a result of complex physiological processes which control rates of filtration, retention, pseudofeces production, as well as environmental factors (Solic et al. 1999).

Coliphages

Coliphages are viruses that infect *E. coli* and have shown promise as an indicator of water quality (Griffin et al. 2001). The structure, morphology, size, and behavior in aquatic environments of coliphages resembles many human enteric viruses, thus they can be indicative of viral fecal pollution (Cole et al. 2003). Additionally, coliphages have been consistently isolated from domestic, hospital and slaughterhouse wastewaters and from treated wastewaters (Gantzer et al. 1998; Funderburg and Sorber 1985). Two important groups of coliphages have been studied, the somatic coliphage and the F-specific (male specific) RNA coliphage (F+RNA). Somatic coliphages infect through receptors on the *E. coli* host cell wall, while F+RNA coliphages infect through the F pili on *E. coli* hosts (Sobsey et al. 2005).

F+RNA coliphages most resemble the size and shape of pathogenic human enteric viruses (Maier et al. 2000). They have been highly correlated with virus concentrations in raw and treated wastewater, raw and partially treated drinking water, and surface and recreational waters. Moreover, grouping of F+RNA coliphages by serotyping or DNA oligoprobing may be used to distinguish between human and non-human fecal contamination sources (Havelaar et al. 1986; Chung 1993; Hsu et al. 1995). Four groups of F+RNA coliphages have been identified. Group I is indicative of cattle, sheep, pigs, and many zoo animals, group II is found in pigs and humans, group III is specifically found in humans and group IV is found in birds and other zoo mammals (Osawa et al. 1981). A study conducted by Brion et al. (2002), revealed that F+RNA coliphage isolation was strongly associated with rainfall events and with land use associated sources of animal fecal pollution. Field isolates were predominately group I compared to raw sewage isolates that were primarily group III. Furthermore, a study in Homosassa Springs,

Florida found that animals rather than human sources were affecting microbial water quality in the study by using F+RNA coliphages (Griffin et al. 2000).

Phages have also been useful as a tracer for fecal pollution. Tracers are excellent tools for tracking contaminants because their transport through the environment closely mimics the microbes and chemicals of interest. Their use is especially informative with regard to abiotic processes that influence the movement of microbes of interest through the subsurface media. Tracers are useful in the field because they can be added to a system in high numbers, their transport can be monitored without introducing a risk of infection, and they are easy to detect (Maier et al. 2000). In a study conducted by Paul et al. (1995), the bacteriophage ΦHSIC-1 was effectively used to trace sewage disposal practices including septic tanks, injection wells, and illegal cesspits in the Florida Keys. Results indicated that ΦHSIC-1 was transported from septic systems in surface canal waters and outstanding marine waters in as little as 11 hours.

A significant advantage of using coliphages as an indicator of fecal pollution is its ability to be detected simply, inexpensively, and within 18 hours (US EPA 2001), although the use of F+RNA coliphages as an alternative indicator does not come without controversy (Allwood et al. 2003). For example, even though a strong correlation has been observed between the seasonal accumulation of F+ RNA coliphages in oysters and the incidence of norovirus associated with oysters, some investigators have reported that F+ RNA coliphages are rarely detected in human feces, suggesting that the presence of these coliphages in water does not necessarily indicate human fecal pollution (Havelaar et al. 1990; Burkhardt and Calci 2000; Schaper et al. 2002).

Coliphage in Oysters

As mentioned previously, F+ RNA bacteriophages are more representative of the pathogenic viruses in shellfish than *E. coli*, either because they are more resistant to environmental stress such as U.V. irradiation, or because they have longer retention time in shellfish (due to the differences in the way they are accumulated and eliminated) or a combination of the two (Richards 1988; Doré et al. 2003). Additionally, seasonal variations in coliphages have been shown to correlate with shellfish-associated disease outbreaks (Doré et al. 2003). Burkhardt and Calci (2000) found that oysters accumulated F+ RNA coliphage to densities averaging 19 times greater than the levels in the contaminated estuarine water; however, accumulation varied, from 1- to 99-fold. From late October through January, the accumulation was significantly greater than during any other time of the year which resembles the seasonal pattern in clinical norovirus cases. When used in conjunction with other source-specific indicators, male specific coliphages have also been shown to be a useful indicator of wastewater effluent exposure in oysters. For instance, a positive correlation between male-specific coliphages and stable isotope ratios provided a viable option for determining oyster exposure to wastewater treatment plant effluent when compared to fecal coliform bacteria (Biancani et al. 2011).

Enteric Pathogens

Human Enteric Viruses

Human enteric viruses represent alternative indicators of fecal pollution, which are specific to human feces. More than 100 viruses are known to occur in human waste and many survive wastewater treatment (LaBelle et al. 1980). Human enteric viruses are host specific, thus their presence not only represents a risk to human health but also indicates a clear sign of

human sewage contamination (Fong et al. 2005; Futch 2010). By nature, enteric viruses need a host for replication, therefore they are unable to proliferate in the environment. Human enteric viruses can be detected in both fresh and marine waters impacted by sewage pollution (Puig et al. 1994; Tani et al. 1995; Castingolles et al. 1998; Pina et al. 1998; Chapron et al. 2000; Jiang et al. 2001). Moreover, enteric viruses are more persistent in marine waters than traditional bacterial indicators (Wetz et al. 2004). Two common types of pathogenic human enteric viruses include human enterovirus (hEV) and human norovirus (NoV).

In oysters, both viruses and bacteria are removed initially by shellfish defecation, following that, a smaller population of viruses may persist in the digestive tract of oysters, and do so for longer than bacteria (DiGirolamo et al. 1975; Power and Collins 1989; Doré and Lees 1995; Schwab et al. 1998; McLeod et al. 2009a). In a study conducted by Love and colleagues (2010), hepatitis A virus and poliovirus were depurated at a slower rate than *E. coli* and *E. faecalis*. Viruses can persist intracellularly and extracellularly in the digestive tract tissues (Le Guyader et al. 2006; McLeod et al. 2009b). Viral persistence relies on interactions among shellfish digestive systems and virus-specific factors, such as histo blood group antigen binding in norovirus (Le Guyader et al. 2006), therefore it is not unreasonable to assume that virus depuration occurs through a different mechanism than bacterial depuration (Power and Collins 1989). The accumulation of viruses by shellfish during feeding is due, in part, to the ionic bonding of viral particles to the mucopolysaccharide moiety of shellfish mucus (DiGirolamo et al. 1975). The level of mucus production, in turn, corresponds generally to the glycogen content of the connective tissue and gonadal development (Galtsoff 1964). Thus, it would appear that enteric bacteria are inappropriate process indicators for depuration by oysters and hard shell clams.

Enterovirus.

The hEV group is part of the Picornaviradae family, and includes poliovirus, echovirus, and Coxsackie A and B viruses. All of these have been found in activated sludge, sewage outfalls, and fresh and marine waters associated with human fecal contamination (Kopecka et al. 1993; Reynolds et al. 1988; Griffin et al. 1999; Jiang et al. 2001; Noble and Fuhrman 2001; Noble et al. 2003). Enteroviruses are characterized as single-stranded RNA viruses with an isocahedral capsid (Maier et al. 2000). Serotyping of enteroviruses can be useful in defining the source of fecal pollution since two bovine, three porcine, one ovine, eleven porcine tescoviruses, and 89 human associated serotypes have been identified (Jimenez-Clavero et al. 2003; Kocwa-Haluch 2001). More recently, Metcalf et al. (1995), first showed that molecular detection of host specific enteroviruses could aid in improved surveillance of watersheds.

The enteroviruses were the first group of enteric viruses to be studied in association with fecal contaminated water (Griffin et al. 2001). Enteroviruses replicate primarily in the gastrointestinal tract and can be shed in high concentrations (10^{10} g^{-1} feces) (Maier et al. 2000). The persistence of hEV in water is affected by both abiotic and biotic factors, including water temperature, UV inactivation, precipitation, host excretion patterns, and streamflow (Tani et al. 1995; Lipp et al. 2001a). hEV is spread through the fecal-oral route and common symptoms of infection include gastroenteritis, acute respiratory illness, and in severe cases, poliomyelitis (Zaoutis and Klein 1998).

In coastal Georgia, human enteroviruses were previously found at a high prevalence at the mouth of the Altamaha River but were not statistically related with either total coliform or fecal coliform bacteria (Fong et al. 2005). Further, both bovine and human enteroviruses were detected, which allowed for the differentiation of human versus animal fecal contamination.

Enterovirus in Oysters

Viruses usually occur in domestic sewage and can survive even after secondary treatment and once discharged they can survive in seawater for a few days to several weeks (Metcalf and Stiles 1967; Gerba et al. 1975). Currently, bacteriological water quality standards are in place to prevent the consumption of contaminated shellfish; however, bacterial standards are inadequate predictors of viral contamination (Goyal et al. 1979). For example, a study in the oyster harvesting waters in Texas detected enteroviruses in water and oyster samples obtained from areas both open and closed to shellfish harvesting. No statistical relationship was demonstrated between virus concentration in oysters and the bacteriological and physiochemical quality of water and shellfish. On five occasions, viruses were found in oysters but not in overlying water (Goyal et al. 1979). Shellfish readily accumulate viruses from polluted water (Atwood et al. 1964; Hedstrom and Lycke 1964; Metcalf and Stiles 1965; Liu et al. 1968). In an experiment on Pacific oysters, *Crassostrea gigas*, the accumulation of poliovirus strain Lsc-2ab was found to reach 46% of the total amount (1.9×10^4 PFU ml^{-1}) of poliovirus added to the system after 12 hours of exposure and 88% after 48 hours of exposure (DiGirolamo et al. 1975). The results of this study also indicated that 67% of the virus was recovered in the digestive area, while the gills, mantle, and palps contained 1.4% of the virus. The findings in this study indicate that these shellfish accumulate virus in approximately the same quantity as reported in Eastern species (*C. virginica*). The uptake of poliovirus type I has been reported by Metcalf and Stiles (1965) to reach 10^5 mean tissue culture infective dose units after 72 hours of contamination. Again, the digestive tract showed the greatest level of viral accumulation, which occurred a few hours after exposure to polluted seawater (Metcalf and Stiles 1965; Liu et al. 1966; Liu 1971).

Currently there is no surveillance for enteroviruses in water or shellfish, thus the true burden of disease for enterovirus-related illness is unknown. Risk assessments can be a valuable tool in estimating adverse effects associated with microbial hazards. In human feeding studies conducted by Rose and Sobsey (1993), individuals consuming raw shellfish from approved waters in the United States may have on the average a 1 in 100 chance of becoming infected with echovirus-12 (an enterovirus). For more infectious viruses such as rotavirus, the risk rose to 5 in 10.

Norovirus

Norovirus is a member of the Caliciviradae family and is characterized as a nonenveloped, single stranded RNA virus that spreads primarily through the fecal oral route (Leon and Moe 2006; Leon et al. 2008; Glass et al. 2009). NoV is the leading cause of nonbacterial gastroenteritis worldwide with an estimated 1.1 million hospitalizations among children in developing countries each year (Glass et al. 2009; Patel et al. 2008; Bosch et al. 2008). The symptoms of NoV infection typically include vomiting, watery non-bloody diarrhea, abdominal cramps, nausea, low-grade fever, and rapid dehydration (Mead et al. 1999). The viruses are highly infectious, only a small inoculum is required to cause illness (<10 viral particles), and attack rates generally range from 50%-90% (Estes et al. 1997).

NoV is genetically grouped into five genogroups (GI – GV), but GI and GII are responsible for the majority of human infections (Lindesmith et al. 2010). Furthermore, GI and GII are subdivided into 25 different genotypes. Most outbreaks are caused by GII.4, while GI strains are associated with fewer outbreaks but are identified in environmental samples and in

children (Bon et al. 1999; Noel et al. 1999; Hewitt et al. 2007; Malasao et al. 2008; Rachakonda et al. 2008; Silva et al. 2008; da Silva et al. 2007; Gentry et al. 2009).

Both food and water are important routes of transmission for NoV (Yori et al. 2009; Bosch et al. 2008). The severity of NoV disease is generally moderate but infection can be fatal in the elderly or immunocompromised (Koopmans et al. 2000; Estes et al. 2006; Okada et al. 2006; Harris et al. 2008; CDC 2007). Norovirus is also environmentally stable and highly contagious, making them very important in terms of oceans and human health (CDC 2006). A study by Seitz et al. (2011) found that NoV can remain detectable for over 3 years and can remain infectious for at least 61 days in groundwater. NoV cannot be cultured via cell lines thus reverse transcriptase-polymerase chain reaction (RT-PCR) is necessary to detect viral RNA from water samples (Le Guyader et al. 1994; Ando et al. 1995; Fankhauser et al. 1998; Green et al. 1998). NoV was shown to be an effective indicator of fecal pollution despite low levels of fecal indicator bacteria in Broward County, Florida, an area impacted by sewage outfalls and inlets (Futch et al. 2011).

Norovirus in Oysters

NoV is excreted in human feces which can lead to considerable amounts in sewage (Formiga-Cruz et al. 2003). Currently septic systems do not properly inactivate NoV thus they can be released into recreational and shellfish harvesting waters (Gentry et al. 2009). Norovirus is the leading cause of food-borne gastroenteritis in the United States (MMWR 2009; Scallan et al. 2011). NoV outbreaks are commonly associated with the consumption of fresh produce and other ready-to-eat foods contaminated by infected food workers or shellfish harvested from waters contaminated by sewage (Parashar et al. 1998; Glass et al. 2000; Glass et al. 2001;

Parashar and Monroe 2001; Fankhauser et al. 2002; Allwood et al. 2003). Outbreaks associated with eating shellfish were first described in 1976 in England and Australia (Adler and Zickl 1969; Appleton and Pereira 1977). In the U.S., the first documented outbreak of NoV from raw oysters occurred in Florida in 1980 (Gunn et al. 1982). From 1993 to 1996 three oyster-related gastroenteritis outbreaks were attributed to NoV in Louisiana, where overboard disposal of sewage was found to be the source (Berg et al. 2000). Between 1994 and 2000, 284 nonbacterial gastroenteritis cases were reported to the Centers for Disease Control and Prevention (CDC), 93 % of these tested positive for NoV (Fankhauser et al. 2002).

In estuarine environments, NoV has been detected in both water and oyster samples in Japan that were most likely contaminated by sewage and treated wastewater input (Ueki et al. 2005). In coastal Georgia, a recent study found that among 225 environmental samples (9 oyster, 72 water, 77 63-200 µm sized plankton, and 72 >200 µm sized plankton), 21 (9.3%) tested positive for NoV. Moreover, 55.0% of the positive samples were found in oysters (Gentry et al. 2009).

Shellfish accumulate NoV in their tissues while filtering contaminated water, oysters are able to depurate some bacteria but have only shown 7% virus removal (Schwab et al. 1998). Moreover, the viruses are able to persist in oyster tissue for at least 10 days as demonstrated by a study conducted by Ueki et al. (2007). Virus particles bind specifically to the digestive ducts (midgut, main and secondary ducts, and tubules) by carbohydrate structures (Le Guyader et al. 2006).

Norovirus is resistant to elevated chlorine levels used during wastewater treatment that usually inactivates vegetative bacterial cells, including the total and fecal coliform group (Keswick et al. 1985). NoV outbreaks show seasonal trends, with a greater proportion of NoV

detected in bivalves during the winter months (Greer et al. 2010). For example, Lowther and colleagues (2008) found that NoV contamination during winter months were approximately 17 times higher in oysters sampled from October to March than during the remainder of the year.

Since 1990, greater than 90% of illnesses associated with shellfish consumption in the U.S. has been attributed to norovirus (Glatzer 1998). Recent advances in real-time RT-PCR have allowed the detection of NoV to be achieved in a rapid and sensitive manner (Kageyama et al. 2003). Moreover, broadly reactive fluorogenic 5' nuclease real-time reverse transcription PCR assays (i.e., quantitative real time PCR assays) have been developed for noroviruses that have been successfully applied to environmentally contaminated shellfish (Jothikumar et al. 2005; Kageyama et al. 2003; Lowther et al. 2008; Loisy et al. 2005). Very low levels of viruses can be detected in about eight hours, compared to several weeks for traditional testing methods. The method has been used to demonstrate the potential health threat from contaminated shellfish (Atmar et al. 1996; Atmar et al. 1995) and has been tested during epidemiological investigations and outbreaks (Shieh et al. 2000).

Salmonella

Salmonella is a non-spore forming, rod-shaped, Gram-negative bacterium that is commonly implicated in food and waterborne outbreaks. The *Salmonellae* represent a large group of bacteria comprising more than 2500 known pathogenic serotypes (Baudart et al. 2000). *Salmonella* spp. infection is the most common source of food-borne gastroenteritis in the western world (McClelland and Pinder 1994). The CDC reports that approximately 40,000 cases of salmonellosis are reported in the United States every year (2010). Typical symptoms of infection by *Salmonella* include diarrhea, fever, and abdominal cramps which last 4 to 7 days.

Salmonella lives in the intestinal tract of humans and other animals, including birds, turtles, lizards, and snakes and is usually transmitted to humans by ingesting food or water contaminated with animal or human feces (CDC 2010). *Salmonella* has long been known to be ubiquitous in fresh and marine environmental surface waters (Cherry 1972; Heintz et al. 2000). *Salmonella*, like many other bacteria in an aquatic environment, are able to persist for an extended period of time (over a month) within the water column under certain conditions (Roszak et al. 1984; Fish and Pettibone 1995; Arnone and Willing 2007).

Salmonella *in Oysters*

Consumption of raw shellfish contaminated with *Salmonella* has a long history in the United States. During the winter of 1924, a widespread typhoid fever (*Salmonella enterica* serotype Typhi) outbreak killed 150 people with cases in New York, Chicago, and Washington D.C. (Frost 1925). Epidemiological investigations pinpointed the consumption of sewage polluted oysters as the source of the outbreak. Local and state public health officials as well as the shellfish industry became sufficiently alarmed over the outbreak and requested that regulatory controls be put in place (FDA 2011). As a result the National Shellfish Sanitation Program was implemented and has been overseeing the classification of shellfish growing areas, the harvesting of shellfish, and post-collection procedures for shellfish ever since (FDA 2009). While *Salmonella* are now less often associated with outbreaks from shellfish consumption, sporadic cases may occur and data suggest that *Salmonella* are detected in commercial shellfish.

Data from temperate regions show that *Salmonella* is not part of the normal microflora in aquaculture environments; therefore, their presence in seafood is therefore seen as a sign of poor standards of process hygiene and sanitation (Dalsgaard 1998). Since the 1970s the FDA advises

using fecal coliform bacteria as an indicator of sewage pollution but fecal coliform bacteria may not be a sufficient indicator of *Salmonella*'s presence. Heintz and colleagues (2000) indicated that *Salmonella* could be present in oysters that did not contain fecal coliforms. Their study tested seafood from sites around the world and found that U.S. shellfish had 1.2% prevalence of *Salmonella*. Moreover, in a study conducted on the East, West, and Gulf coasts of the United States, 7.4% of all oysters tested contained *Salmonella* (Brands et al. 2005). Currently, there are no requirements for states to test oyster harvesting waters for the presence of *Salmonella* (Brands et al. 2005). One study found the concentration of *Salmonella* in oysters may be 50 times that in seawater (UNEP/WHO/IAEA 1998). Conversely, *C. virginica* in Apalachicola Bay and Tampa Bay showed that low fecal coliform bacteria levels in fresh and stored oysters are good indicators of the absence of *Salmonella* spp. but high levels of fecal coliform bacteria are somewhat limited in predicting the presence of *Salmonella* spp. (Hood et al. 1983).

Conclusion

Human sewage from faulty septic systems contains some of the most pervasive contaminants found in coastal regions. The monitoring of indicator organisms and human pathogens in water and oyster tissue, especially in areas impacted by fecal pollution is vital to public health. If coastal communities continue to grow at current rates and concurrent investments in sewage treatment and disposal are not met, then the vitality of recreational water and shellfish quality is at risk. Artificial oyster reefs may be used as a first order approach to evaluate the utility of restored reefs for water quality mitigation. Oysters and other filter feeding organisms can concentrate microbial contaminants in their tissue which may be exploited to

provide a sustainable and cost effective method at removing microbial contaminants from overlying waters.

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CHAPTER 3

Artificial Oyster Reefs as a Water Quality Remediation Tool in a Contaminated Estuary[1]

[1]Crews, M.K. and E. K. Lipp. To be submitted to *Estuaries and Coasts.*

Abstract

Human sewage contamination from faulty septic tanks in coastal environments poses a major risk to public health. Field and controlled laboratory studies were conducted to assess the effectiveness of using the eastern oyster, *Crassostrea virginica*, in an artificial oyster reef as a water quality mitigation tool. Three sites in coastal Georgia were monitored over two years for fecal indicator organisms and enteric pathogens. The site impacted by the highest density of nearby septic systems had significant ($p < 0.05$) levels of fecal coliform bacteria, enterococci, and coliphage, in both water and oyster samples and was 85.7% positive for *Salmonella* (6/7 samples) and 20% positive for enterovirus (1/5 samples) in water samples. Controlled laboratory studies revealed that oysters can quickly reduce the amount of fecal bacteria from water but may reintroduce the contaminant as feces/pseudofeces. Artificial oyster reefs were useful as a sampling medium, but their effectiveness for water quality mitigation were not conclusive.

Keywords Shellfish, fecal indicator organisms, enteric pathogens, septic systems

Introduction

Coastlines contain only 17% of the contiguous U.S. land area but are home to more than 53% of the nation's population (Culliton 1998). An expanding human presence along coasts and subsequent urbanization requires not only management of non-point source pollutants but also effective wastewater treatment and disposal. In areas where municipal sewer systems are not available, a dependence on aging and poorly sited septic tanks and drain fields may cause excess loading of nutrients and microbes to estuarine waters and shellfish harvesting grounds (LaPointe et al. 1990; Lipp et al. 2001a; Paul et al. 1997). Consequently, fecal indicator organisms and enteric pathogens including bacteria, protozoa, and viruses found in high numbers in human feces can contaminate sediment, water, and marine organisms posing a public health risk to seafood consumers, residents, and recreational swimmers (NRC 1993; Ellender et al. 1980; Fattal et al. 1983; Lipp et al. 2001b). Enteric pathogens from untreated sewage range from bacterial contaminants such as *Salmonella* to viral agents such as enterovirus and norovirus and can cause many symptoms including gastroenteritis, ear and respiratory infections, and skin rashes (CDC 2010; DiDonato et al. 2009; MMWR 2009; Scallan et al. 2011; Zaoutis andKlein 1998).

Coastal Georgia has had a rapid increase of new residents in developments near marsh front or waterfront properties and on-site septic systems have been the primary means of waste disposal (Walker et al. 2003). In 2002, a Geographic Information System (GIS) analysis found that in McIntosh County, Georgia alone 1056 on-site septic systems were directly adjacent to the coastal waters. Of these septic systems 63% of septic tanks were found in areas susceptible to high pollution given the combination of a high tidal range, shallow depth to the water table, and the sandy soil type found in the area (Walker et al. 2003). Moreover, previous studies in the area

have already indicated poor water quality based on the incidence of enterovirus and norovirus in oyster harvesting areas (Fong et al. 2005; Gentry et al. 2009).

Commercially harvested bivalve molluscan shellfish including oysters, hard shell clams, mussels, and cockles are of special concern to exposure from enteric pathogens given their ability to filter large amounts of water and concentrate environmental pollutants into their tissues (Alexander and Young 1976; Fukimori et al. 2008; Kovacs et al. 2010). The presence of infectious microbial agents in raw or undercooked molluscan shellfish has been reported in the United States since the 1800s, with evidence of over 400 outbreaks, although this number might not reflect the true incidence of infection since gastroenteritis often goes unreported (Archer and Kvenberg 1985; Dowell et al. 1995; Rippey 1994). The most common bivalve in Georgia, the eastern oyster, *Crassostrea virginica,* is a commercially and ecologically valuable species that inhabits near shore areas commonly affected by wastewater inputs and can be an effective sentinel for sewage pollution (Cerco and Noel 2007; Gregalis 2006; Mackowiak et al. 1976). Eastern oysters have been on the decline in recent years due to habitat degradation, over harvesting, poor water quality, and oyster disease and are therefore in need of restoration efforts (Coen et al. 2007; Luckenbach et al. 2005). Artificial oyster reefs can aid in restoration by helping promote rapid growth of oyster populations by providing a substrate for natural spat deposition. One type of artificial oyster reef, spat sticks, are 1.5m long PVC pipes covered in a cement slurry that have shown to efficiently colonize a large density of oysters within a relatively small time frame (Manley et al. 2008). Oyster spat sticks are cost effective, easy to construct, and environmentally friendly and may help to remediate sewage contaminated water by improving water quality. Additionally, coastal Georgia provides an ideal location for oyster

reef colonization and growth given its temperate climate and long growing season (O'Beirn et al. 1995, 1996a, 1996b, 1997).

The first objective of this study was to determine the effectiveness of experimental oyster spat stick reefs in removing microbial contaminants for water quality mitigation at three sites in coastal Georgia. Additionally, laboratory studies were used to determine the uptake, fate, and retention of a common fecal indicator bacterium, *Enterococcus faecalis*, within a controlled environment. Secondly, we sought to determine the relationship between septic system density and the levels of fecal indicator organisms and pathogens in water and oyster samples from the three study sites.

Methods

Site Selections

Three sites were selected in McIntosh County, Georgia that represent a gradient of septic system density and presumed contamination (Fig 1). Oak Dale Creek is a small tidal creek located downstream of a community served entirely by septic systems, a high incidence of which are failing. Four Mile Island, lies on the Julienton River and was previously used as a shellfish harvesting area but had been threatened by closure due to water quality issues in the past. The reference site, Eagle Creek, is located at the mouth of the Mud River and contains no immediate septic input.

Spat Stick Construction and Deployment

Spat sticks were constructed using 1.5m long , 1.9 diameter, PVC pipes coated in cement slurry as described by Manley and colleagues (2008). Sets of sticks (n = 36) were deployed in

May 2010 during oyster spawning season in triplicate at each site. Each stick was placed about 0.25m into the substrate to secure them in place and were arranged in a 6x6 fashion (108 sticks/site) with approximately 0.2m between each stick, leaving enough space to allow for oyster spat recruitment.

Sampling Methods

Quarterly samples beginning in August 2010 and ending in March 2012 were collected during outgoing tides. Surface water grab samples of 1-L were collected in sterile polypropylene bottles for the detection of fecal coliform bacteria, enterococci, coliphage, and *Salmonella*. Bi-annually, 2-L water samples were collected for the detection of the human enteric viruses, enterovirus and norovirus. Oysters recruited to spat sticks (referred to as "recruited oysters" through the rest of this paper) as well as adult oysters already at each site were collected and placed in sterile plastic bags and used for the detection of each microbial target. Only recruited oysters were used in the detection of human enteric viruses. All water and oyster samples used for microbial analyses were kept on ice until processing which occurred within 8 h of sampling. Water samples for enteric virus detection were held at 4°C until processing which occurred within 72 h. Oyster samples were frozen at -20°C until processing for enterovirus and norovirus. Water quality parameters including pH, salinity, dissolved oxygen, and temperature were measured at each site using a YSI multi-parameter sonde (Yellow Springs, OH). Rainfall in the watershed was recorded using the Brunswick station of The University of Georgia's Automated Environmental Monitoring Network (www.georgiaweather.net).

Microbial Analysis

Fecal Indicator Bacteria

For the detection of fecal coliform bacteria and enterococci in water samples, membrane filtration methods were employed using 0.45µm pore size mixed cellulose ester membranes (47 mm diameter, Millipore, Billerica, MA; APHA 1998; US EPA 2006). Volumes of 5 mL and 50 mL were filtered in triplicate and membranes were placed on membrane-Fecal Coliform agar (mFC) and membrane-Enterococcus Indoxyl-β-D-Glucoside agar (mEI) for fecal coliform bacteria and for enterococci, respectively. Both sets of plates were incubated > 18 hours, at 44.5°C for fecal coliform bacteria in a water bath and 41°C for enterococci. Following incubation, blue colonies on mFC and colonies with a blue halo on mEI were enumerated and colony forming units (CFU) were calculated per 100 mL. For detection in oysters, a modified Most Probable Number (MPN) was used based upon the five tube decimal dilution test (APHA 1970). Briefly, oyster shells were scrubbed with a wire brush and 70% ethanol. A flame-sterilized shucking knife was then used to aseptically remove the oyster tissue. The tissue was wet weighed and an equal amount of 1x phosphate buffered saline (PBS) solution was added. The tissue was then homogenized (Pro 200, ProScientific Inc., Oxford, CT) for 1 min in a sterile 50-mL centrifuge tube and serially diluted (10^{-1} to 10^{-3}) in 1% buffered peptone water in replicates of five. Replicates were enriched overnight at 37°C and streaked onto both mFC and mEI and incubated as described above. After incubation, plates were scored as positive or negative and the MPN for each sample was calculated per 100 g of tissue using FDA's Bacteriological Analytical Manual MPN spreadsheet (Blodgett 2010).

Coliphage

A single agar overlay method (US EPA 2001) was used for the detection of coliphages in both water and oysters. For water, 1 mL of sample water was added to 1 mL of the log-phase host bacteria culture, *Escherichia coli* (ATCC #15597), then mixed with 3 mL of melted and tempered Tryptic Soy Agar (TSA) (0.7% w/v) and poured onto solid TSA plates (1.5% w/v) in replicates of five. Plates were incubated overnight at 37°C and plaques were counted and reported as plaque forming units (PFU) per 100 mL. In oysters, the tissue-buffer homogenate was centrifuged at 3000 x g for 10 min to pellet debris. A 1 ml sample of supernatant fluid was then assayed as described above and reported as PFU per 100 g. MS2 phage (ATCC #15597-B1) was used as a positive control.

Salmonella

The presence of *Salmonella* was determined using a three step process: pre-enrichment, selective enrichment, and differential selection (Haley et al. 2009). For water samples, duplicate volumes of 250 mL were filtered through 0.45-µm pore size mixed cellulose ester membranes (47 mm diameter Millipore, Billerica, MA). Membranes were placed directly in 10 mL of 1% buffered peptone broth for pre-enrichment and incubated at 37 °C with shaking (C24, New Brunswick Scientific, Edison, NJ) at 150 rpm for 18-24 h. Following incubation, enriched samples were vortexed and 100 µL was inoculated into Rappaport-Vassiliadis **(**RV) broth (Becton Dickinson, Franklin Lakes, NJ) for selective enrichment and incubated 18-24h at 43 °C. After incubation, RV enriched broth was then streaked onto xylose lysine deoxycholate (XLD) (Becton Dickinson) agar plates and incubated for 18-24h at 37°C. A representative number of black colonies or yellow colonies with a black halo were picked as presumptive *Salmonella* and

were confirmed using Enterotube II tests (Becton Dickinson). For oysters, 100 µ of the 1% buffered peptone enriched homogenate used for fecal indicator bacteria detection was inoculated into RV broth followed by streaking onto XLD and Enterotube II confirmation.

Human Enteric Viruses

Enterovirus and norovirus were analyzed from water samples using a membrane based adsorption-elution method as described by Kateyama et al. (2002) and Fong et al. (2005). Briefly, viral particles were concentrated from 1 to 2 L of water by reducing the pH to 4 with 10% glacial acetic acid, which allows for adsorption of viruses to negatively charged 0.45 µm pore size HA membrane filters (92 mm diameter, Millipore). After the water had passed through the membrane, 100 mL of 50mM H_2SO_4 was used to rinse the filter. The viruses were then eluted with 10 mL of 1N NaOH and the final eluent was added to a neutralization solution containing 0.1 mL 50mM H_2SO_4 with 0.1 mL 100x Tris EDTA (TE). The samples were then concentrated and rinsed through ultrafiltration using Centriprep 50, which provides a nominal molecular weight cut off of 50 kDa (Millipore). Approximately 2 mL of concentrated samples was recovered, split, and stored at -80°C prior to molecular analysis. Viral RNA (200 µL) was extracted using a Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) eluted into a 30 µL volume of RNase free water and detected using real time reverse transcription (RT)-PCR on a StepOne Plus Real Time PCR System (Life Technologies, Carlsbad, CA). The TaqMan system (sequence specific internal probe) was used to quantify human enterovirus (Donaldson et al. 2002; Fong et al. 2005; Futch et al. 2011), and both genogroups I and II of norovirus (Jothikumar et al. 2005).

An AgPath-ID^{TM} One-Step RT-PCR Kit (Applied Biosystems, Foster City, CA), was used for both enterovirus and norovirus detection. For enterovirus, 2 µL of concentrated and

purified RNA was added to 23 µL of a PCR reaction mixture containing a primer concentration of 600 nM and a probe concentration of 250 nM (Table 1). The RT-PCR reaction was carried out under the following conditions: Reverse Transcription for 10 min at 45 ˚C, 10 min at 95˚C, followed by 45 cycles of 10 s at 95˚C, 30 s at 55˚C and a final extension of 15 s at 72 ˚C. Ten-fold serial dilutions of a known concentration of poliovirus strain Lsc-1 (vaccine strain) cell culture lysates were used to develop standard curves that allow for accurate quantification of enteroviruses and were used for positive controls (provided by Dr. C.P. Gerba, University of Arizona).

For norovirus detection, genogroup I and II were detected in two separate assays. Purified RNA (2 µL) were added to two separate PCR mixtures (23 µL each) specific for each genogroup. Unique primers (400 nM) and probes (120 nM) were used for each genogroup (Table 1; Gentry et al. 2009). Each reaction was carried by the following procedure: RT for 10 min at 45 ˚C, 10 min at 95 ˚C, followed by 45 cycles of 10 s at 95 ˚C, 30 s at 55 ˚C, and a final extension of 15 s at 72 ˚C. Previously quantified RNA runoff transcripts for norovirus genogroups I and II, were used for standards and controls. (provided by Dr. J. Vinjé Centers for Disease Control and Prevention National Calicivirus Lab and described in Gentry et al. 2009).

Human enteric viruses from oyster tissue were detected by first removing the digestive gland of the oyster tissue as described by Gentry et al. (2009). Briefly, digestive glands were finely chopped with a sterile razor and digested with an equal amount of PBS plus 100µg ml^{-1} Proteinase K for 1 h with shaking at 320rpm (C24, New Brunswick Scientific, Edison, NJ) at 37°C. Samples were then vortexed and placed in a water bath for 15 min at 65°C to denature the enzyme. The slurry was centrifuged at 3000 x g for 5 min and the supernatant was stored at

-80°C in 1 mL aliquots. An RNeasy Mini Kit was used for extraction of viral RNA using the Plant and Fungi protocol (Qiagen) and real time RT-PCR was completed as described above.

GIS Analysis

Using septic tank inventory data for McIntosh County, GA provided by the University of Georgia's Marine Extension Service (Walker et al. 2003), 1500 and 2500 m buffers, or diameters, were created around each site and the number of septic systems within each of those ranges was enumerated. Subsequently, septic system density near each site and levels of fecal indicator organisms were analyzed to ascertain significant correlations. Maps and analyses were conducted using ArcGIS10 (ESRI, Redlands, CA).

Controlled Laboratory Studies

Collection and Depuration

Naturally existing oysters were collected from Eagle Creek, an area that consistently showed low levels of fecal indicator bacteria. Upon collection, oysters were scrubbed to remove mud and other debris and were placed in a cooler for transportation back to the University of Georgia. Upon arrival (≤ 8 h from collection), oysters were placed in a re-circulating depuration tank equipped with a 2.3 m^2 25-µm biological filter with an 18W UV bulb (Ocean Clear 375) to reduce background levels of indicator bacteria. Oysters were fed 10 mL of Shellfish Diet at a concentration of 2 x 10^9 cells ml^{-1} (Reed Mariculture), an algal food source, once per day and were allowed to depurate 3 days before the experiment began. The depuration system maintained suitable living conditions for oysters (DO, temperature, pH, and salinity).

Experimental Design

A goal of this study was to determine the efficacy of strategically placed oyster reefs as a tool for remediation of tidal creeks impacted by human waste. Sterile bottles (n = 48) each containing 900 ml of sterile Instant Ocean (30 ppt) and a single adult oyster with a length of at least 7.6 cm were seeded with *E. faecalis* (ATCC# 518906) at a final concentration of 8.46×10^3 CFU mL^{-1} and were sampled at 4, 8, 12, and 24 h for detection in water, oyster tissue, and feces/pseudofeces. Depurated oysters were added to vessels and allowed 1 h for acclimation prior to seeding. *E. faecalis* was cultured overnight in a shaking incubator using Brain Heart Infusion Broth (Becton, Dickinson and Company, Sparks, MD). Two negative controls were used; the first contained water only (no oysters and no seeded bacteria), the second contained oysters without bacteria. The positive control contained water with *E. faecalis* (no oysters). Each treatment was run in triplicate, including technical positive and negative controls for each time point.

Detection of E. faecalis

Background levels of *E. faecalis* were accounted for by individually examining three separate oyster samples (as described below) for contamination prior to the start of the experiment. After inoculation, samples of oysters, water, and feces/pseudofeces were taken at each time point. For detection in oysters, a homogenate was made by aseptically shucking each oyster, weighing the tissue, and adding an equal amount of 1xPBS; 100 µL and 10 µL aliquots of the homogenate, in duplicate, were then spread-plated on mE agar. The use of mE agar (without Indoxyl β-D glucoside) was used due to the high cost of the reagent coupled with the large quantity of media needed for the experiment. Water samples were collected from each bottle and

100 µL and 10 µL were spread plated on mE Agar in duplicate. Feces/pseudofeces were collected from the bottom of each sample bottle using a 10 mL serological pipette and transferred to a sterile 15 mL centrifuge tube. Samples were then centrifuged at 3000 x g for 10 min to separate the feces/pseudofeces from water. Water was decanted and the feces/pseudofeces were re-suspended in 250 µL of sterile 1 x PBS. The samples were then vortexed and plated on mE at two volumes, in duplicate. All spread plates were incubated for two days at 41 °C and enumerated as CFU mL^{-1}.

Statistical Analysis

Both field and laboratory data were not normally distributed therefore nonparametric statistics were used. A Kruskal-Wallis test was used to differentiate the levels of indicators at each site in water and oyster samples as well as differences between water, oysters, and feces/pseudofeces from the laboratory studies. To deduce differences between water and oyster samples as well as differences between recruited versus naturally existing adult oysters a Wilcoxon rank sum test was used. Spearman rank correlation aided in finding relationships among environmental parameters, fecal indicator organisms, and septic system density. Lastly, binary logistic regression was used to analyze the relationship between indicator levels and the presence of enteric pathogens. All statistical analyses were considered significant at a p-value of < 0.05 and were conducted using Stata12 (StataCorp LP, College Station, TX) and SAS (version 9.2, SAS Institute Inc., Cary, NC, 2011).

Results

Fecal Indicators

A total of eight water samples and seven oyster samples were collected over a two year period from May 2010 to March 2012; only water samples were collected in May 2010, when spat sticks were deployed in the field. Oak Dale Creek had significantly higher levels of fecal coliform bacteria ($p < 0.001$), enterococci ($p < 0.002$), and coliphage ($p < 0.012$) in water samples over the duration of the study compared to the other two sites (Fig 2). There was no difference between Four Mile Island and Eagle Creek in water samples for fecal coliform bacteria, enterococci, and coliphage. Levels at Oak Dale Creek for fecal coliform bacteria ranged from 194 CFU 100 mL^{-1} (August 2011) to 1113 CFU 100 mL^{-1} (December 2011). At Four Mile Island, fecal coliform bacteria ranged from 2 CFU 100 mL^{-1}(December 2011) to 20 CFU 100 mL^{-1} (May 2010) while Eagle Creek ranged from 0 CFU 100 mL^{-1} (March 2011) to 8 CFU 100 mL^{-1}(December 2011). For enterococci, Oak Dale Creek ranged from 59 CFU 100 mL^{-1} (August 2011) to 2940 CFU 100 mL^{-1} (May 2011). At Four Mile Island, enterococci ranged from 1 CFU 100 mL^{-1} (November 2010) to 80 CFU 100 mL^{-1} (August 2010). Levels at Eagle Creek ranged from 3 CFU 100 mL^{-1} (March 2012) to 82 CFU 100 mL^{-1}(August 2010). Coliphage levels at Oak Dale Creek ranged from 40 PFU 100 mL^{-1} (May 2011) to 620 PFU 100 mL^{-1}(May 2010). Levels of coliphage at Four Mile Island ranged from 0 PFU 100 mL^{-1} (August 2011 and March 2012) to 220 PFU 100 mL^{-1} (August 2010) while at Eagle Creek levels ranged from 0 PFU 100 mL^{-1}(March 2011, and August 2011) to 80 PFU 100 mL^{-1} (May 2010 and November 2010).

There was no difference in indicator levels between oysters recruited to spat sticks and naturally existing oysters at each site; therefore the levels of both sets of oysters were combined for statistical analyses.

Oyster tissue at Oak Dale Creek had significantly higher levels of fecal coliform bacteria ($p < 0.001$), enterococci ($p < 0.009$), and coliphage ($p < 0.01$) than that found at all other sites (Fig 2). There was no difference between Four Mile Island and Eagle Creek for any of the indicators. Fecal coliform bacteria in Oak Dale Creek oysters ranged from 165 MPN 100 g^{-1} (August 2010) to 2913 MPN 100 g^{-1} (March 2012). At Four Mile Island, fecal coliform bacteria in oyster tissue ranged from 0 MPN 100 g^{-1} (August 2010, August 2011, and December 2011) to 308 MPN 100 g^{-1} (November 2011) while Eagle Creek ranged from 0 MPN 100 g^{-1} (December 2011) to 141 MPN 100 g^{-1} (March 2012). Levels of enteroccoci at Oak Dale Creek ranged from 17 MPN 100 g^{-1} (March 2011) to 5130 MPN 100 g^{-1} (December 2011). At Four Mile Island, enterococci ranged from 0 MPN 100 g^{-1} (August 2010 and March 2011) to 214 MPN 100 g^{-1} (March 2011). Enterococci in oyster tissue at Eagle Creek ranged from 0 MPN 100 g^{-1} (August 2011 and March 2011) to 432 MPN 100 g^{-1} (August 2010). Lastly, coliphage concentrations in oyster tissue at Oak Dale Creek ranged from 20 PFU 100 g^{-1} (August 2010) to 540 PFU 100 g^{-1} (November 2010). At Four Mile Island, concentrations ranged from to 0 PFU 100 g^{-1} (March 2011 and May 2011) to 140 PFU 100 g^{-1} (November 2010). Coliphage concentrations at Eagle Creek ranged from 0 PFU 100 g^{-1} (March 2011, December 2011 and March 2012) to 100 PFU 100 g^{-1} (November 2010). There was no statistical difference in the levels of fecal indicator organism between water and oyster samples.

Shellfish waters in the United States are classified as approved, conditional, restricted, or prohibited for shellfish harvesting based on the monitoring of fecal coliform bacteria (FDA

2009). In Georgia, shellfish harvesting waters are required to have levels of fecal coliform bacteria below 14 MPN $100mL^{-1}$ based on the FDA guideline set by the National Shellfish Sanitation Program (GA DNR). Oak Dale Creek exceeded this level at every sampling event in both water and oyster samples. Water samples at Four Mile Island exceeded this limit four times in May 2010, August 2010, May 2011, and August 2011, and in oyster tissue four times in November 2010, March 2011, May 2011, and March 2012. At Eagle Creek, levels in water never exceeded 14 MPN $100mL^{-1}$ but oysters did on five occasions, in November 2010, March 2011, May 2011, August 2011, and March 2012.

Currently, there is no standard for enterococci in shellfish harvesting waters, however, for recreational waters, the EPA statistical threshold value for culturable enterococci is 104 CFU 100 mL^{-1} (US EPA 2011). By this measure, Oak Dale Creek exceeded this limit five times, in August 2010, November 2010, May 2011, December 2011 and March 2012. Eagle Creek and Four Mile Island never exceeded these limits.

Enteric Pathogens

Water collected at Oak Dale Creek consistently had the highest levels of enteric pathogens (Fig 3). *Salmonella* was detected in six out of seven water samples (85.7% positive), although it was never detected in oyster samples. Human enterovirus was detected twice at Oak Dale Creek, once in water samples (38 PFU equivalents L^{-1}) during the initial sampling in May 2010 and once in oyster samples (702 PFU equivalents g^{-1}) in August 2010. Four Mile Island was positive for multiple enteric pathogens in water, including enterovirus (157 PFU equivalents L^{-1}) from May 2010, norovirus genogroup I (1878 genome copies L^{-1}) in August 2011, and *Salmonella* in March 2011, August 2011, and March 2012 (43% positive). The reference site,

Eagle Creek, had the lowest prevalence of *Salmonella* at 14% for water samples (found only in November 2010), but human enterovirus was detected in water in May 2010 (241 PFU L^{-1}) and August 2010 (1208 PFU L^{-1}) and in oysters in August 2010 (11 PFU g^{-1}).

Binary logistic regression analysis showed that fecal coliform bacteria concentrations were predictive of the presence of *Salmonella* and in water samples (odds ratio = 2.87, $p \leq 0.04$; 95% CI = 1.05, 7.86). The presence of enterovirus and norovirus did not show a significant relationship with indicator organism in water and oyster samples.

Environmental Parameters

At all sites,water temperature ranged between 13.8 °C (December 2011) and 33.1 °C (August 2011). Spearman rank correlations indicated a positive relationship between temperature and coliphage concentrations in water ($p < 0.003$) and a negative correlation with dissolved oxygen ($p \leq 0.004$) (Table 2). Salinity ranged from 25.23 (March 2011) to 35.62 (August 2011). Rainfall in the watershed varied from 0.38 cm (March 2011) to 3.4 cm (August 2010). There was no correlation between the levels of fecal indicators for salinity or rainfall. Fecal coliform bacteria and enterococci in water and oyster tissue were negatively correlated with pH ($p < 0.05$), where it ranged from 6.75 (May 2010) to 7.68 (November 2010). Changes in pH were also correlated with salinity ($p < 0.05$) and dissolved oxygen ($p < 0.001$). Dissolved oxygen was also negatively correlated with fecal coliform bacteria and enterococci in water samples ($p \leq 0.027$). The lowest levels of dissolved oxygen were seen in August 2010 (1.47 mg L^{-1}), the highest levels were noted in March 2011 (8.28 mg L^{-1}).

GIS Analysis

The 1500 m buffer around the Oak Dale Creek sample site included 11 septic systems, followed by one at Four Mile Island, and none at Eagle Creek. At the 2500 m buffer, 37 septic systems were found around sample sites at Oak Dale Creek, 25 around 4 Mile Island, and 1 around Eagle Creek, however, 1,056 septic systems directly adjacent to coastal waters were previously identified in McIntosh County (Walker et al. 2003). The septic system density at the 1500 m range was significantly correlated with the concentration of fecal coliform bacteria in water ($p < 0.001$), and oysters ($p < 0.001$). For enterococci, water ($p < 0.001$) and oyster tissue ($p < 0.01$) were also significantly correlated as were coliphage in water ($p \leq 0.014$) and oyster tissue ($p \leq 0.0104$). The 2500 m buffer yielded similar results, with significant correlations with fecal coliform bacteria in water ($p < 0.001$), and oysters ($p < 0.001$). Enterococci and coliphage concentrations were significantly correlated with septic systems for both water ($p \leq 0.0043$, < 0.002) and oysters ($p < 0.001$, < 0.01), respectively.

Controlled Laboratory Studies

Oysters were able to quickly remove *Enterococcus faecalis* from the water column; within 8 h of seeding, *E. faecalis* concentrations were reduced by 92%. Over all time points, feces/pseudofeces contained a significantly greater amount of *E. faecalis* compared to levels in water and oyster tissue ($p \leq 0.026$) (Fig 4). Positive controls containing only water and *E. faecalis* showed a decline in viable *E. faecalis* after 8 h, with 46% remaining at 24h, signifying natural die off of the organism. *E. faecalis* were never detected in negative (water and oyster) controls, therefore the *E. faecalis* supplied to the system was assumed to be the only source and no contamination occurred over the course of the study.

Discussion

Septic System Density

The U.S. EPA (2000) estimates that septic systems are used by 25% of the U.S. population and in 40% of new development. Further, the U.S. Bureau of the Census has indicated that at least 10% of septic systems have stopped working, and some communities have reported failure rates as high as 70% (2008). Properly placed and maintained septic systems can be a viable option for waste disposal but in areas where soil type, appropriate drainfields, or proximity to coastlines are unfavorable the likelihood for contamination increases. In coastal Georgia, and elsewhere in the U.S., a high incidence of septic systems may contribute to poor water quality. In 2011, 28 beaches in coastal Georgia were closed due to high levels of enterococci (GA DNR 2012). The correlation we found in this study between septic system density and fecal indicator organisms supports the notion that poorly placed septic systems can have a negative impact on water quality. Oak Dale Creek, the site impacted by the highest density of nearby septic systems, repeatedly exhibited high levels of fecal coliform bacteria (mean = 495 CFU 100 ml^{-1}) enterococci (595 CFU 100 ml^{-1}) and coliphage (291 PFU 100 ml^{-1}) in both water and oyster samples. Our results corroborate with other studies; for instance, septic system densities were associated with endemic diarrheal illness in central Wisconsin (Borchardt et al. 2003), with groundwater contamination (Yates 1985), and poor microbial water quality in Florida (Lipp et al. 2001a; Futch et al. 2010; Futch et al. 2011; Griffin et al. 1999).

Enteric Pathogens

The fecal indicators employed in this study were only partially effective at predicting the presence of enteric viruses. At Four Mile Island the presence of enterovirus in May 2010 and

norovirus in August 2011 coincided with an exceedance of the FDA threshold of 14 MPN $100mL^{-1}$ for fecal coliform bacteria. Conversely, Eagle Creek, the reference site with no immediate septic input, had consistently low levels of fecal coliform bacteria, enterococci, and coliphage but human enteroviruses were still detected two out of five times. Furthermore, while the binary logistic regression was effective at predicting the relationship between fecal coliform bacteria and the occurrence of *Salmonella* (odds ratio = 2.87; $p \leq 0.04$, 95% CI = 1.05, 7.86) it did not hold true for enterovirus (odds ratio = 0.72, $p < 0.61$, 95% CI = 0.215, 2.45) or norovirus (odds ratio = 0.86; $p < 0.90$, 95% CI = 0.097, 7.59). Numerous studies have suggested that fecal coliform levels are not effective at predicting the presence of human enteric viruses and our results align with these findings (Wyer et al. 1995; McQuaig et al. 2006; Pina et al. 1998; Noble and Fuhrman 2001; Jiang and Chu 2006; Fong et al. 2005).

Although the GIS analysis revealed few nearby septic systems at Four Mile Island and Eagle Creek, the sheer density of septic systems within the whole county coupled with the vast amount of mixing in the estuary may influence the presence of viruses detected even when indicators were low (Verity et al. 1993; Verity et al. 2006). Other likely contributors to fecal contamination within the estuary could be from animals, boat discharge, or non-point source pollution.

Artificial Oyster Reefs

The artificial oyster spat stick reefs deployed at the start of this study proved to be an excellent sampling medium. They were able to quickly and efficiently recruit oysters, allowing the sampling of the same group of oysters over the duration of the study. However, the volume of water present at each of these sites coupled with the relatively small size of the artificial oyster

reefs did not allow an effective way to measure water quality mitigation. Hydrological models completed by Gerritsen et al. (1994) suggested that intertidal reefs located in shallow tributaries may have a greater potential for filtration due to an increased ratio of water column to reef surface area. Out of all three sites, Oak Dale Creek most resembled these characteristics; however, the artificial oyster reef did not encompass enough of the tidal creek to effectively measure changes in indicator or pathogen concentrations. Going forward, a higher density of oysters (in an artificial oyster reef or otherwise) in a small tidal creek system would be needed to fully test their efficacy as a mitigation tool for sewage affected waters.

Our results showed that oyster tissue failed to accumulate a higher proportion of fecal indicators compared to the surrounding water column; therefore they may not remove contaminants as efficiently as hypothesized. These results confirm findings of two studies in North Carolina that aimed to measure reductions in fecal coliform bacteria and total suspended solids (TSS) downstream of oyster reefs (Cressman et al. 2003; Nelson et al. 2004). Cressman et al. (2003) found minimal statistically significant reductions of fecal coliform bacteria downstream of an intertidal oyster reefs. Nelson et al. (2004) used transplanted *C. virginica* reefs to measure water quality, and found reductions in TSS downstream but the findings were not statistically significant. TSS has been found to correlate with fecal indicator bacteria, including *E. coli*, enterococci, and fecal coliform bacteria in the water column because suspended solids can provide a protective environment (Simon and Makarewicz 2009; Davies-Colley et al. 2004; Sayler et al. 1975; Pommepuy et al. 1992; Gerba and McLeod 1976; Mallin et al. 2000).

Results from the controlled laboratory studies may help to explain why we did not observe accumulation of bacterial and viral contaminants in oysters at our three field sites.

Oysters have the ability to sort particles, ingesting food and rejecting particles that are non-nutritive or of an unfavorable size as pseudofeces (Jorgensen, 1966; Newell and Jordan 1983; Haven and Morales-Alamo 1970; Wetz et al. 2002; Loosanoff 1949). The fact that a statistically significant portion ($p \leq 0.026$) of *E. faecalis* was discharged as feces/pseudofeces may indicate that oysters can expel undesirable bacteria as feces/pseudofeces and reintroduce them to sediments or overlying waters (Haven and Morales-Alamo 1966). The concentration of *E. faecalis* supplied in the microcosm may exceed environmentally relevant concentrations of enterococci by approximately 10-fold but still presents the notion that the production of feces/pseudofeces laden with bacteria might negate the use of oysters as a water quality remediation tool. More experiments would be useful to determine the fate, retention, and expulsion of other fecal contaminants such as *E. coli* or coliphages.

Our results also indicated that oysters are an effective sentinel species for sewage pollution, because they reflect concentrations found in surrounding water (Biancani et al. 2011). On the other hand, higher accumulation, or sequestration of fecal indicator organisms within oyster tissue compared to water was not observed. Perhaps the ongoing introduction of human sewage from septic systems is constantly filtered by nearby oysters; contaminants are then distributed in tissue (potentially causing outbreaks), digested and released as feces, or rejected as pseudofeces.

To conclude, the density of nearby septic systems in this study significantly influenced the levels of fecal coliform bacteria, enterococci, and coliphage detected in both water and oyster tissue. Hence, increased surveillance of water and molluscan shellfish coupled with better management of existing septic systems and improved placement of septic systems in new developments is critical in reducing the contamination of surrounding water and oysters.

Artificial oyster reefs aided in the recruitment of oysters, but their use as a bioremediation tool was inconclusive because the volume of water present at each site dwarfed our ability to effectively measure changes in fecal indicator concentrations. Controlled laboratory studies revealed that the fate of *E. faecalis* was primarily concentrated in feces/pseudofeces; in a field setting this may reduce the viability of oysters as a water quality mitigation tool because they may reintroduce fecal bacteria into surrounding water and sediment.

Tables

Table 1 Primers and Probe sequences for detection of human enteric viruses, enterovirus and norovirus genogroup I and II

Primer/Probe	Sequence (5' to 3')[a]	Target/Location	Reference
Enterovirus			
EV-U	GGCCCCTGAATGCGGCTAAT	192 base pair region	Donaldson et al. 2002
EV-D	CACCGGATGGCCAATCCAA 5'	of untranslated region	
EV-Pr	FAM-CGGACACCCAAAGTAGTCGGTTCCG-BHQ	(UTR)	
Norovirus			
Genogroup I			
JJVIF	GCCATGTTCCGITGGATG	5282–5299	Jothikumar et al. 2005
JJVIR	TCCTTAGACGCCATCATCAT	5377–5358	Jothikumar et al. 2005
JJVIP	FAM-TGTGGACAGGAGATCGCAATCTC-BHQ	5319–5341	Jothikumar et al. 2005
Ring1b	FAM-AGATCGCGGTCTCCTGTCCA-BHQ	5340–5321	Kageyama et al. 2003
Norovirus			
Genogroup II			
JJV2F	CAAGAGTCAATGTTTAGGTGGATGAG	5003–5028	Jothikumar et al. 2005
COG2R	TCGACGCCATCTTCATTCACA	5100–5080	Kageyama et al. 2003
Ring2	FAM-TGGGAGGGCGATCGCAATCT-BHQ	5048–5067	Kageyama et al.2003

[a]FAM- 6 carboxyfluorescein, fluorescence reporter dye; BHQ- Black Hole Quencher

Table 2 Spearman Rank Correlation of fecal indicator organisms, fecal coliform bacteria (FC), enterococci (Ent), and coliphage (Phage) with each other and with environmental parameters and septic system density. Correlation coefficients (r) in bold type were statistically significant.

	FC Water	Ent Water	Phage Water	FC Oyster	Ent Oyster	Phage Oyster	Temp	pH	Salinity	DO	Rainfall	Septic 1500m
Ent Water	**0.6706**											
Phage Water	**0.5866**	0.403										
FC Oyster	**0.8304**	**0.5232**	**0.6683**									
Ent Oyster	**0.652**	0.4106	0.4146	**0.5756**								
Phage Oyster	**0.4998**	0.2121	**0.7412**	**0.5493**	**0.5587**							
Temp	0.0255	0.2994	**0.9385**	-0.0883	-0.0021	-0.1274						
pH	**-0.5141**	**-0.4573**	-0.3484	**-0.5354**	**-0.3915**	-0.1984	-0.0261					
Salinity	-0.0947	-0.1918	-0.1777	-0.3346	-0.0879	0.3244	0.0268	**0.4048**				
DO	**-0.4811**	**-0.7212**	-0.1328	-0.3144	-0.3052	-0.0209	**-0.461**	**0.5525**	0.2282			
Rainfall	0.0325	0.1944	0.2381	0.1501	0.2314	0.2716	-0.1496	0.2053	-0.1549	**-0.3719**		
Septic 1500m	**0.8684**	**0.787**	**0.5988**	**0.816**	**0.5042**	**0.5235**	-0.027	**-0.5595**	-0.1957	**-0.4757**	0.0468	
Septic 2500m	**0.8903**	**0.561**	**0.6**	**0.7471**	**0.4771**	**0.4509**	0.034	**-0.5155**	-0.0004	**-0.4242**	0.0543	**1**

Figures

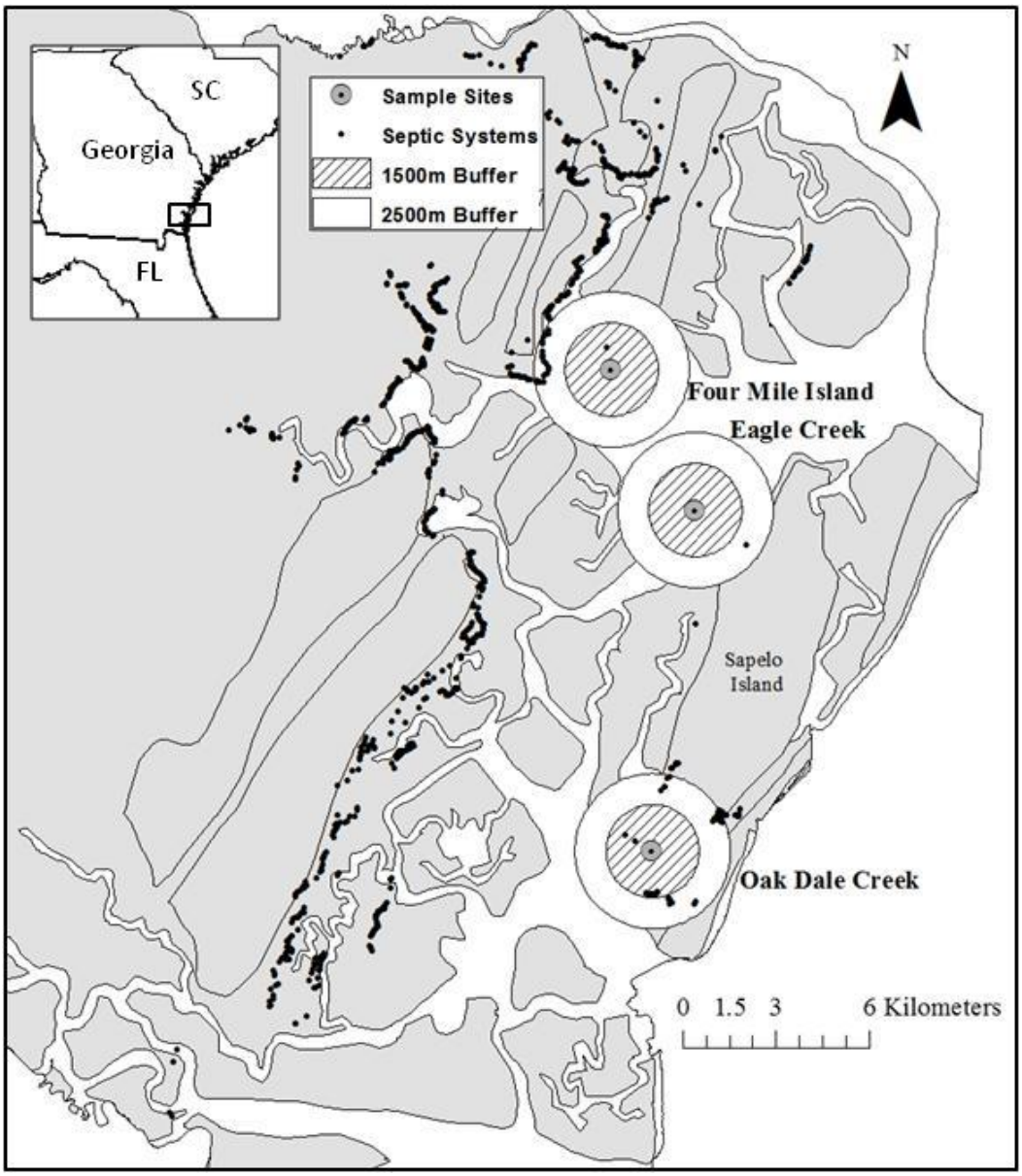

Fig 1 Sampling sites and near-marsh septic systems in McIntosh County, Georgia. The circular buffers around each site are part of a GIS analysis of septic systems located with 1500m and 2500m of each sampling site.

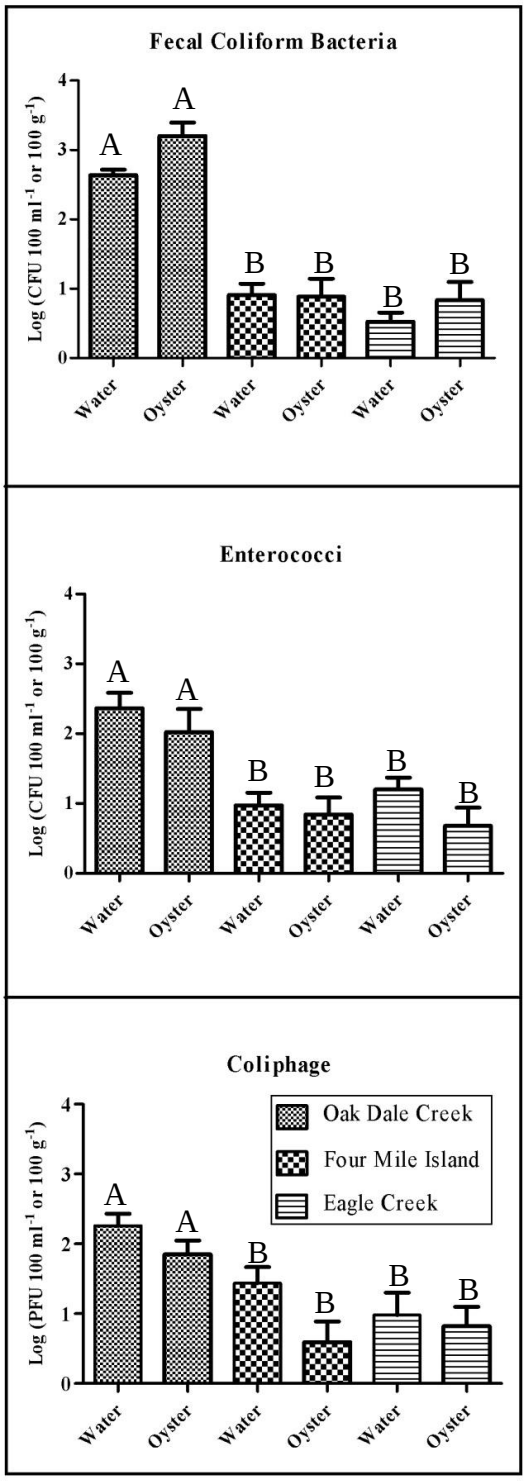

Fig 2 Mean levels of fecal coliform bacteria, enterococci, and coliphage in water (n=8), and oysters(recruited and naturally existing) (n=14) collected over the two year sampling period, by site. The letters denote signiifcant differences between sites and sample type.

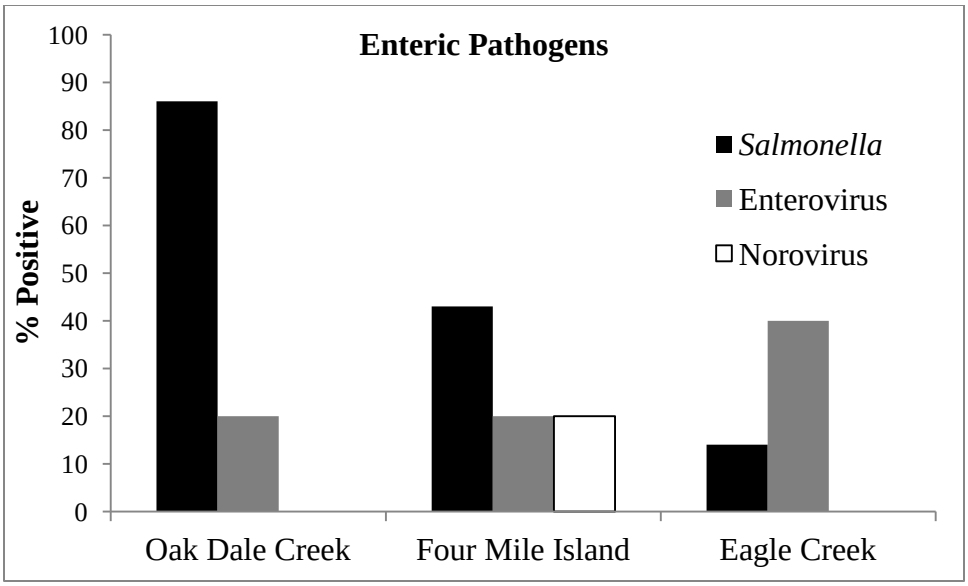

Fig 3 Detection of enteric pathogens, *Salmonella* (n=7), enterovirus (n=5), and norovirus (n=5) in water samples collected over the two year field study, grouped by sampling site.

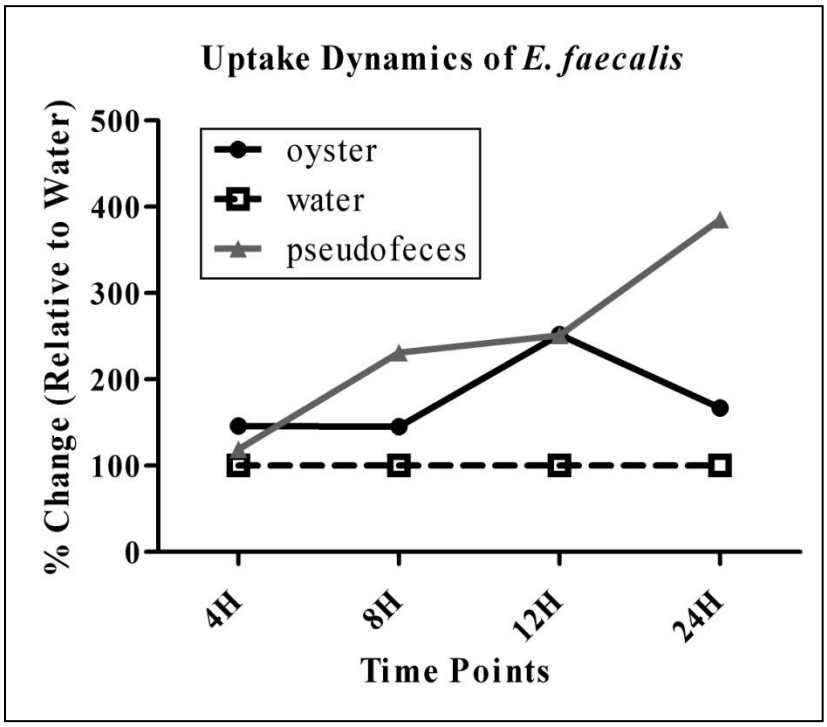

Fig 4 The fate of *E. faecalis* in water (n=3), oyster tissue (n=3), and pseudofeces (n=3) during a 24 hour laboratory exposure.

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CHAPTER 4
Conclusion

Field and controlled laboratory studies were conducted to assess the effectiveness of using the eastern oyster, *Crassostrea virginica,* in an artificial oyster reef as a water quality mitigation tool at three sites in coastal Georgia. Water and oyster samples were collected over a two year period and were monitored for fecal coliform bacteria, enterococci, and coliphage as well as *Salmonella*, enterovirus, and norovirus. Results showed that Oak Dale Creek, the site impacted by the highest density of nearby septic systems had significantly higher levels of fecal coliform bacteria, enterococci, and coliphage, in both water and oyster samples ($p < 0.05$ for all) and was 85.7% positive for *Salmonella* (6/7 samples) and 20% positive for enterovirus (1/5 samples) in water samples. Both Four Mile Island and Eagle Creek consistently showed low levels of fecal indicator organisms but were positive for human enteric viruses on multiple occasions. Septic system density showed a strong positive correlation with the levels of fecal indicator organisms, including fecal coliform bacteria, enterococci, and coliphage in both water and oysters.

Artificial oyster reefs were an excellent experimental medium because they allowed the sampling of the same group of oysters over the course of the study. But the usefulness of artificial oyster reefs as a bioremediation tool is still in question because a more appropriate ratio of water volume to oyster density is necessary to fully test this hypothesis.

Controlled laboratory studies indicated that oysters can efficiently reduce the concentration of fecal bacteria from the water column; in 8 h 92% reduction of *E. faecalis* was

observed. The fate of *E. faecalis* in the lab studies also showed that a significant amount of the bacterium was concentrated as feces/pseudofeces. In the field, this may indicate that rather than acting only to remove fecal contaminants from overlying waters, oysters may reintroduce contaminants to the environment thereby negating their use as a remediation tool.