Characterization of Fecal Contamination in the Newport River Estuary  
(North Carolina, USA)

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“A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the School of Public Health, Department of Environmental Sciences and Engineering”

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ABSTRACT

Angela D. Coulliette

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(North Carolina, USA)

“Under the direction of Rachel T. Noble, Ph.D.”

Estuaries are valuable habitats (e.g. seagrass meadows, forested wetlands) and are also economically important for various industrial avenues (e.g. shellfish, tourism). They make up only 13% of the nation’s landscape, however, a majority of the human population lives (43%) and works (40%) in coastal and estuarine areas (Restore Americas Estuaries Report 2008). These locations are experiencing increases in land modifications and development (i.e. impervious surfaces) to support such populations. Subsequently, severe water quality issues have resulted due to nonpoint source (NPS) pollution, such as stormwater runoff, contributing fecal contamination to the neighboring waters. Specifically, the Newport River Estuary (NPRE) in North Carolina is listed as impaired for fecal coliforms and an estuarine-wide study was conducted to assess this impairment in relation to stormwater. Major findings regarding the NPRE, a high priority shellfish harvesting area, included (1) stormwater runoff being the main contributor of fecal pollution with concentrations ranging from $1.0 \times 10^2$ to $5.0 \times 10^3$ fecal indicator bacteria (FIB) per 100 ml, (2) four day antecedent (day of sampling and three days prior) rainfall explained 60% of the fecal coliform pollution, and (3) tidal trends played a large role in FIB concentrations. Further research in a tributary
leading to the NPRE showed loading rates to the shellfish harvesting area were approximately $1.0 \times 10^4$ to $1.0 \times 10^5$ FIB per 100 ml every 10 minutes during rainfall (>2.54 cm) when a dilution effect from the shore of 100 FIB per 100 ml per 100 meters was assumed (calculated from data from this study). Partitioning of the fecal signal by using an alternative FIB, *Bacteroides thetaiotaomicron*, illustrated that although a majority of FIB were above acceptable water quality thresholds for shellfish harvesting areas, the fecal contamination was likely to be not of human origin. However, the bacterial pathogen *Campylobacter lari* was detected in the NPRE, raising public health concerns. Overall, the NPRE is experiencing serious water quality issues from NPS pollution via stormwater runoff, and although the contamination is most likely from environmental sources, it will be important to control this runoff to keep this valuable estuary healthy.
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To all those who believed in me…
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<td>Bayesian Maximum Entropy</td>
</tr>
<tr>
<td>BMPs</td>
<td>Best Management Practices</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>B. theta</td>
<td><em>Bacteroides thetaiotaomicron</em></td>
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<tr>
<td>°C</td>
<td>degrees Celsius</td>
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<td>MF</td>
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<tr>
<td>MPN</td>
<td>most probably number</td>
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<td>MR</td>
<td>moderate rain</td>
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<tr>
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<td>MTF</td>
<td>multiple tube fermentation</td>
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<td>NOAA</td>
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<td>NPDES</td>
<td>National Pollutant Discharge Elimination System</td>
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<td>NPRE</td>
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<td>NPS</td>
<td>Nonpoint Source</td>
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<td>NR</td>
<td>no rain</td>
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<td>Nephelometric Turbidity Units</td>
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<td>polymerase chain reaction</td>
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<td>Pearson product</td>
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<tr>
<td>rRNA</td>
<td>ribosomal Ribosomal Nucleic Acid</td>
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<td>S/T</td>
<td>Space/Time</td>
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<td>Space/Time Random Field</td>
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<tr>
<td>TMDL</td>
<td>Total Maximum Daily Load</td>
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<tr>
<td>US</td>
<td>United States</td>
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<td>USEPA</td>
<td>United States Environmental Protection Agency</td>
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<tr>
<td>WC1</td>
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<td>Ware Creek 2</td>
</tr>
<tr>
<td>WC4</td>
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</tr>
<tr>
<td>YD</td>
<td>Yarborough Dock</td>
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LIST OF SYMBOLS

\( \alpha \)  \quad \text{alpha} \\
\( \beta_0 \)  \quad \text{y-intercept} \\
\( \beta_i \)  \quad \text{MPN estimate of FIB concentration for sample } i \\
\( r^2 \)  \quad \text{correlation coefficient} \\
\$  \quad \text{dollar} \\
=  \quad \text{equals} \\
F  \quad \text{F-value} \\
>  \quad \text{greater than} \\
<  \quad \text{less than} \\
\%  \quad \text{percent} \\
\sigma^2  \quad \text{population covariance} \\
p  \quad \text{p-value} \\
\pm  \quad \text{plus minus} \\
\textregistered  \quad \text{registered trademark} \\
i  \quad \text{sample} \\
n  \quad \text{sample size} \\
r  \quad \text{spatial lag} \\
d_i  \quad \text{straight line distance} \\
\tau  \quad \text{temporal lag} \\
t  \quad \text{t-value}
CHAPTER 1

INTRODUCTION

Estuaries are some of the most productive areas on earth due to being the transition zone for fresh and marine environments, thus having vertical and horizontal gradients of nutrients, organic matter, microbes, light penetration, and diverse flora and fauna. These valuable habitats are homes for shellfish beds, seagrass meadows, forested wetlands, and fisheries (Restore Americas Estuaries Report 2008). They also provide a coastal area culture (i.e. recreation, education) and aid in regulating the natural systems and recharge of freshwater (i.e. flooding; Restore Americas Estuaries Report 2008). Estuaries make up only 12.6% of the United States’ (U.S.) landscape, however, a majority of the human population lives (43%) and works (40%) in coastal and estuarine watersheds (Restore Americas Estuaries Report 2008). With the required development to support such populations in a small percentage of area and a lack of attention in minimizing our “human footprint,” many estuaries and coastal areas are experiencing severe water quality degradation. Specifically, North Carolina (NC) estuaries, including shellfish harvesting and recreational waters, are becoming threatened due to the accelerating pollutant inputs.

Pollution can be categorized as either point source (PS) or nonpoint source (NPS) pollution. Point sources, such as wastewater treatment plant effluent, are stringently regulated due to the implementation of the National Pollutant Discharge Elimination System (NPDES). Nonpoint source (NPS) pollution is generally not associated with a discharge
permit or pipe, and can include a mixture of agricultural, residential, industrial, and environmental runoff. Since NPS pollution is not usually associated with a NPDES permit, and commonly diffuse in nature, it can be difficult to control. Stormwater runoff is a form of NPS pollution to receiving waters during periods of precipitation and is difficult to trace back to one point of origin. Several NC studies have illustrated that NPS pollution in the form of stormwater runoff contributed to increased concentrations of pollutants in estuarine and coastal receiving waters (Mallin et al. 2000; Kirby-Smith and White 2006; Coulliette and Noble 2008). The rapid growth of human populations and development in estuarine areas, in combination with land clearing and increased areal extent of impervious surface coverage, removes natural barriers and infiltration mechanisms that would otherwise control and reduce the rate of delivery of runoff entering neighboring waters. Removal of these buffers allows NPS pollution to enter waterbodies at higher concentrations, faster rates, and larger volumes. The U.S. Environmental Protection Agency (U.S. EPA) National Water Quality Inventory mirrors the results of such trends, as stormwater runoff is listed as the leading contributor to surface water impairment.

Ambient (recreational) and shellfish harvesting water quality are regulated by the U.S. EPA and the Food and Drug Administration/ National Shellfish Sanitation Program (FDA/NSSP), respectively. The U.S. EPA under the collectively known Clean Water Act (CWA), a combination of mandates from the Federal Water Pollution Control Act (1948), Clean Water Act (1977), and Water Quality Act (1987), protects the nation’s surface waters (National Research Council 2004). The CWA, Section 305(d), requires impaired waterbodies to be placed on the 303(d) List, to have a Total Maximum Daily Load (TMDL) developed, and to be defined on how to return the impaired waterbody to healthy status. The
FDA/NSSP regulates shellfish harvesting waters. However, when a harvesting area exceeds the acceptable threshold the area is also placed on the 303(d) List. Each agency has the states and local governments being primarily responsible for determining the health of their waters, as well as establishing appropriate indicators and standards. For example, North Carolina (NC) shellfish harvesting and recreational waters are managed by the North Carolina Department of Environment and Natural Resources (NCDENR) Shellfish Sanitation Section (NCDENR-SSS) and Recreational Water Quality (NCDENR-RWQ) departments. There were 485 fecal coliform (FC) impaired waterbodies in NC based upon the criteria for either recreational contact or use for shellfish harvesting in NC for the 2006 reporting period (USEPA 2006). North Carolina (NC), ranks eighth in tourism in the U.S. with 49 million visitors spending 13.2 billion dollars. Tourism is one of the top-ranked activities in the state (NCDC 2004). There are 24% and 11.9% of NC’s population residing and working in estuarine areas, respectively (Restore Americas Estuaries Report 2008). This southeastern U.S. state was also listed as having the most dramatic shift in development (rural to urban) surrounding estuaries from 1993 to 2003 (Restore Americas Estuaries Report 2008). A NC study revealed that as percent impervious cover increased, bacterial concentrations in the neighboring waters also increased (Mallin et al. 2000). As mentioned previously, additional NC studies show NPS pollution via stormwater runoff to be a main source of fecal pollution (Kirby-Smith and White 2006; Coulliette and Noble 2008). Thus, it’s reasonable to associate FC impairments with the population growth and development in NC estuarine and coastal areas.

The Newport River Estuary (NPRE) is one of the FC impaired waterbodies in NC, and is part of a larger estuarine system within the White Oak River Basin (NCDENR-SSS
The NPRE (35 km$^2$ waterbody, 453 km$^2$ watershed) supports the local economy through commercial fisheries and shellfish areas, as well as being vital for tourism activities (boating, swimming). This shallow estuary (average depth is 1 ml; Kirby-Smith and Costlow 1989) also contains environmentally important natural resources. The NPRE has experienced a population increase of 27.9% from 1986 to 2006 in the main county surrounding the waterbody (NC Office of State Budget and Management), which can be related to a 9% increase of shellfish harvesting area closures since 1986 (NCDENR-Shellfish Sanitation Section, Patricia Fowler person. communication).

Since the NPRE is used for commercial (shellfish) and recreational (boating) purposes, the fecal coliform impairment illustrates the potential presence of human pathogens and public health risk. Waterborne transmission of human enteric bacterial pathogens is linked to those microbes of fecal-oral nature that survive and possibly proliferate when released into surface or drinking water, and have the ability to cause gastrointestinal related illnesses in humans. Waterborne pathogens are also linked to foodborne transmission via filter feeders (i.e. shellfish) or foods that are prepped or washed with contaminated water. Enteric illness includes a range of clinical symptoms not limited to diarrhea, abdominal pain, nausea, and vomiting (Moe 1996). Waterborne bacterial pathogens can be deadly to immunocompromised populations (i.e. infants, elderly, organ transplant recipients, cancer patients).

Recreational waters contaminated with bacterial pathogens pose a health threat to swimmers, skiers, boaters, as well as an occupational hazard to shellfish harvesters. There were 127 waterborne disease and outbreaks reported by the CDC from 2001 to 2004 caused by contaminated recreational water, of which 48.4% (2001 to 2002 period) and 46.2% (2003-
2004 period) caused gastroenteritis (Yoder et al. 2004; Dziuban et al. 2006). In addition, the 2006 National Resources Defense Council (NRDC) reported the highest number of beach closures in the 17 years of surveillance (Dorfman and Stoner 2007). Possible routes of bacterial pathogens entering recreational waters include point source release (i.e. untreated wastewater effluent), nonpoint sources (i.e. runoff) or autochthonous proliferation (i.e. *Vibrio* spp.). Bacterial pathogens that are of concern for recreational waters include *Vibrio* spp., *Campylobacter* spp., and *E. coli* O157.

Shellfish harvesting waters and the harvested shellfish pose a public health risk via waterborne and foodborne routes if contaminated. Most enteric illnesses caused by consumption of raw or undercooked shellfish have no causative agent identified; however, *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Plesiomonas* spp., *Aeromonas* spp., *Staphylococcus aureus*, *Bacillus cereus*, and *E. coli* have been reported as causing an outbreak(s) in the U.S. between the years 1898 to 1990 (Rippey 1994). These pathogens were a larger issue before efforts were made to control point source releases (i.e. wastewater treatment plant effluent). Nonpoint sources of pollution (i.e. stormwater runoff) are now routes of concern for these pathogens entering shellfish harvesting areas. *Vibrio* spp. are also documented as major causative agents of shellfish-associated GI illness (Rippey 1994; Potasman et al. 2002). *V. parahaemolyticus*, *V. cholera*, *V. vulnificus*, *V. mimicus*, and *V. hollisae* are responsible for twelve outbreaks affecting 880 patients in the U.S. in the last 30 years (Potasman et al. 2002). This bacterium is more difficult to control due to being autochthonous to marine and estuarine systems. In addition, *Vibrio* spp. shellfish-associated outbreaks were significantly associated with oysters versus other shellfish (i.e. hard clams, soft clams, mussels, and scallops; Rippey 1994). The other bacterial pathogens related to
shellfish-associated outbreaks were present in all shellfish species listed above, although hard clams and oysters were the main culprits due to social practices of minimal cooking before ingestion (Rippey 1994). A majority of outbreaks occur in late spring and late fall when bioaccumulation rates are high (Rippey 1994).

As mentioned previously, state management (i.e. NCDENR) and federal government (U.S.EPA, FDA/NSSP) efforts aim to prevent exposure of human pathogens to the public by implementing water quality programs that use a fecal indicator bacteria (FIB)-based system. The FIB-based system has been used for over 100 years in the U.S. (National Research Council 2004). Example characteristics of an “ideal” FIB include 1) that the indicator is present when the pathogen is present and at measurable concentrations, 2) the indicator is able to survive for similar periods of time as the pathogen, but does not persist and proliferate in the environment, and 3) that cost effective, and relatively straightforward methods are available to detect the indicator (Bonde 1966). However, as water quality studies have advanced, additional desired attributes include the ability to measure the indicators rapidly, and that the indicator has similar biochemical and physical attributes, and that the indicator demonstrates significant relationships to human health outcomes during epidemiology studies (National Research Council 2004).

The State of NC follows FDA/NSSP guidelines and therefore uses FC for management of shellfish harvesting waters: using numerical criteria where the median or geometric mean shall not exceed 14 per 100 ml and the 90th percentile shall not exceed a Most Probably Number (MPN) of 43 per 100 ml (NSSP 2005). The recreational water quality standards recommended by the U.S. EPA for freshwaters are for the E. coli geometric mean to not exceed 126 Colony Forming Unit (CFU) or MPN per 100 ml and Enterococcus
(ENT) to not exceed 33 CFU or MPN per 100 ml, while marine waters ENT can not exceed 35 CFU or MPN per 100 ml. Brief descriptions of the tradition fecal indicator bacteria (FIB) and a proposed alternative molecular FIB, \textit{Bacteroides thetaiotaomicron} are presented below.

Total and fecal coliforms: Total coliforms refer to a wide group of bacteria that are present in the soil, vegetation, and in warm-blooded animal fecal matter. Coliforms are aerobic, gram negative, and lactose fermenting bacteria. This group includes bacteria within the Family \textit{Enterobacteriaceae}, which includes \textit{E. coli}, \textit{Enterobacter}, \textit{Klebsiella}, and \textit{Citrobacter} (Ishii and Sadowsky 2008). Fecal coliforms are thermotolerant coliforms, which grow at the higher temperature of 44.5°C and are understood to be a more specific indication of fecal contamination as compared to Total coliforms. Coliforms were detected at a geometric mean of $2.08 \times 10^3$ (arithmetic mean: $1.90 \times 10^4$) colony forming units per gram of human feces (Slanetz and Bartley 1957).

\textit{Escherichia coli}: \textit{E. coli} (EC) is part of the Domain Bacteria, Phylum Proteobacteria, Class Gamma Proteobacteria, Order Enterobacteriales, Family Enterobacteriaceae, Genus \textit{Escherichia}, and Species \textit{E. coli}. \textit{E. coli} (EC) is a gram negative, rod shaped, and facultative anaerobe (Ishii and Sadowsky 2008). This bacterium is approximated to have $10^6$ cells per gram of colon material (Ishii and Sadowsky 2008). This bacterium is within the fecal coliform group.

\textit{Enterococcus}: \textit{Enterococcus} (ENT) is part of the Domain Bacteria, Division Firmicutes, Class Bacilli, Order Lactobacillales, Family Enterococcaceae, and Genus \textit{Enterococcus}. The genus currently has 26 species with three more being proposed to be species (Klein 2003). \textit{Enterococcus} are gram-positive, cocci shaped, non-spore forming, catalase negative bacteria (Klein 2003). They are also microaerobic but can survive in
anaerobic conditions (Klein 2003). The species present in warm-blooded animal
gastrointestinal (GI) tracts include *E. faecalis*, *E. faecium*, *E. durnas/hirae*, *E. gallinarum*, *E. casseliflavus*, and *E. cecorum/columbae* (Klein 2003). However, *E. faecalis* and *E. faecium*
are the main species within the human GI flora (Klein 2003). This bacterium was detected at
a geometric mean of $1.11 \times 10^3$ (arithmetic mean: $3.01 \times 10^4$) colony forming units per gram
of human feces (Slanetz and Bartley 1957).

**Fecal indicator bacteria survival in surface waters:** The survival of traditional FIB
(coliforms, EC, and ENT) is dependent on several environmental factors. However, it should
be noted that surrounding land-uses (i.e. rural, residential, industrial), native microbial flora,
and study period weather patterns have a large impact on overall dynamics. Studies have
shown that insolation (i.e. solar irradiation) and temperature are major environmental
parameters that impact FIB survival (Noble et. al. 2004; Whitman et al. 2004). Survival can
be specific to bacterial indicator, as research in the coastal waters of North Carolina resulted
in temperature, as part of a seasonal signal, influencing EC survival but not ENT (Coulliette
and Noble 2008). Salinity and attachment to particulate matter also impact FIB survival.
Salinity has been shown to have an inverse relationship with FIB, which has also been
connected to rainfall bringing in freshwater and fecal contamination via stormwater runoff
(Coulliette and Noble 2008; Mallin et al. 2000). Particular matter is often included in runoff
and bacterial attachment will increase survival, protecting the FIB from ultra-violet
irradiation and predation, while often housing valuable nutrients. In general, attachment to
suspended solids increase survival and persistence, especially in tropical and sub-tropical
environments (Fries et al. 2008; Byappanahalli and Fujioka 2004; Shibata et al. 2004; and
Desmarais et al. 2002).
**Bacteroides thetaiotamicron:** *Bacteroides thetaiotamicron (B. theta)* is part of the Kingdom Bacteria, Phylum Bacteroidetes, Sub-Phylum Cytophaga-Flavobacter-Bacteroides, Class Bacteroidetes, Order Bacteroidales, Family Bacteroidaceae, and Genus *Bacteroides*. *Bacteroides* spp. are gram negative, rod shaped, non-endospore forming, and anaerobic (Wexler 2007). The genus *Bacteroides* contains over twenty species (Wexler 2007), including the pathogenic *B. fragilis* and one species believed to be a potential future FIB, *B. thetaiotami*cron. *B. theta* has been reported to be one of the predominant species in human feces (Wang et al. 1996) and related species having approximate concentrations of $10^{10}$ (Holdeman et al. 1976).

Application of this bacterium for water quality purposes has recently peaked with the advent of molecular methods. Thus, published literature on the survival of *Bacteroides* spp. is important to understand its’ environmental dynamics. Temperature has been shown to impact survival of *Bacteroides* spp., as several studies have shown *B. distasonis* and *Bacteroides-Prevotella* group persisting at lower temperatures (Okabe and Shimazu 2007; Seurinck et al. 2004; Kreader 1998). Salinity was suggested to be an indirect factor of survival by controlling predator activity (Okabe and Shimazu 2007). Future studies examining survival in surface waters will most likely follow depending on the success of current epidemiological studies evaluating *Bacteroides* spp. correlation with diarrheal illness.

In summary, the NPRE is under increasing pressure from human impacts and the subsequent FC impairment is reason for serious concern. Although the NPRE is vital to the local and state economy, minimal research has been conducted to characterize the microbial status of the eastern NC estuary. This dissertation revolves around the NPRE and is divided among 6 chapters. This Chapter (Chapter 1) introduces the reader to current issues in water
quality, and the objectives for this dissertation. Chapter 2, “Impacts of rainfall on the water quality of the Newport River Estuary (eastern North Carolina, USA),” provides an estuarine-wide characterization of fecal pollution and environmental dynamics of the NPRE. Chapter 2 has already been published in the Journal of Water and Health (Coulliette and Noble 2008). Specifically, the goals were to characterize microbial water quality of the entire estuary by enumerating FIB and measuring environmental parameters over a large geographic area, to relate FIB findings to rainfall and distance to land to assess impacts of stormwater runoff, and to utilize measurements of FIB to begin to identify hot spots for future work to determine sources of fecal contamination into the estuary. *H₀₁: In the NPRE, there is no statistically significant relationship between fecal indicators and environmental parameters such as rainfall, salinity, temperature, and turbidity.*

In Chapter 3 (Space/Time Bayesian Maximum Entropy Analysis of Fecal Pollution and Rainfall in an eastern North Carolina Estuary), a rigorous modeling approach of Space/Time Bayesian Maximum Entropy (S/T BME) was used to estimate the distribution of fecal indicator bacteria in relation to antecedent rainfall and distance to shore in the NPRE. First, a hydrologic model for FIB across the NPRE was developed that accounted for the effect of rainfall and distance to shore. Second, a covariance model integrated the developed hydrologic model, general knowledge, and site-specific knowledge to produce covariance parameters that explained the major fluctuations of FIB concentrations across space and time. Thirdly, S/T BME framework produced informative space/time maps, of which specific dates were chosen to illustrate FIB distribution in the NPRE during extreme seasonal conditions. *H₀₂: In the NPRE, rainfall is not an accurate predictor of water quality.*
Chapter 4 is divided into two sections and revolves around research conducted in Ware Creek, an important tributary to the NPRE. After understanding the estuarine-wide dynamics from Chapter 2 and Chapter 3, the dissertation advanced into quantifying and partitioning the fecal pollution entering the estuary. The first section, Chapter 4.1 (Transport and Partitioning of Fecal Contamination into a High Priority Shellfish Harvesting Area in eastern North Carolina), examines the fate and transport of fecal contamination during a wide spectrum of weather conditions. The FIB signal was also partitioned using a molecular FIB, *Bacteroides thetaiotamicron*, to deduce whether the contamination stems from an environmental or human source. \( H_{03} \): *Stormwater runoff is not responsible for transport of fecal contamination into the NPRE and partitioning of fecal indicators will not show a human signal*. Chapter 4.2 (Comparison of Fecal Coliform and *Escherichia coli* Water Quality Detection Methods) focuses on comparing management (mTEC) and research (Colilert\textsuperscript{®}) methods of choice for FIB. \( H_{04} \): *There is no significant difference between fecal indicator water quality detection methods, mTEC and Colilert\textsuperscript{®}-18*.

In Chapter 5 (Detection of *Campylobacter* spp. in eastern North Carolina Estuarine Waters), a small-scale study of the presence of the bacteria, *Campylobacter* spp., was conducted with respect to traditional FIB. Certain species of *Campylobacter* spp. are human pathogens (e.g. *C. jejuni*, *C. lari*, *C. coli*) and these were investigated in this chapter. It is understood that FIB are proxies for human bacterial pathogens and thus, it was an important component of this dissertation to evaluate the currently used FIB in relation to the potential presence of human bacterial pathogen in eastern NC. Avian species, which are present in large populations along eastern NC, are a main reservoir for *Campylobacter* spp. In addition, the selected *Campylobacter* spp. pathogens are known to survive better in cooler conditions,
which would be when shellfish harvesting season is open. This Chapter is a small-scale study evaluating the presence of *Campylobacter* spp. in comparison to FIB. *H_0*: In the NPRE, the selected human pathogenic species of Campylobacter are not present.
REFERENCES


INTRODUCTION

Estuaries, the transition zone between rivers and oceans, are complex aquatic systems that are utilized globally for commercial (fishing and shellfishing), recreational (swimming and boating), and industrial (transporting goods, mining, and dredging) purposes. In addition to these economically important activities, the 2000 United States (US) Census Bureau Brief reported a growing trend of Americans moving to estuarine areas with 53% of the US population now living in coastal counties (Perry et al. 2001). The surrounding land-water interface of estuaries is undergoing rapid modification due to escalating coastal populations and subsequent development. An increase in impervious surfaces (i.e. parking lots, paved roadways, rooftops, driveways) and the clearing of previously-forested land directly impact estuarine water quality. Receiving waters associated with such modifications are impacted by higher volume and higher transit speed of stormwater runoff. As a result, these waters have degraded water quality and an increase of potential health concerns.

Estuarine water quality is regulated by the Clean Water Act (National Research Council 2004) and a waterbody is designated as impaired when the acceptable level or
concentration of a water quality indicator is exceeded. Fecal indicator bacteria (FIB), such as fecal coliforms (FC), *E. coli* (EC) and/or *Enterococcus* (ENT), are commonly used as proxies of potential pathogenic microorganisms by both recreational and shellfish harvesting water quality management programs. Fecal coliforms are the recommended and commonly applied FIB for managing water quality of shellfish harvesting waters at the state and/or national level (NSSP 2005). According to the US Environmental Protection Agency (USEPA) 303(d) List, of the 726 impaired waterbodies in North Carolina (NC), 341 are listed as impaired based upon FC criteria for either recreational contact or use for shellfish harvesting (USEPA 2004).

The Newport River Estuary (NPRE) is a NC coastal estuarine system (453.25 km$^2$) within the White Oak River Basin (Figure 2.1, NCDENR-SSS 2005). The NPRE is one of the many waterbodies that have been placed on the 303(d) list due to exceedance of the FC standards for shellfish harvesting waters. The degradation of the water quality of the NPRE and subsequent status as an “impaired waterbody” is coincident with increased levels of stormwater runoff due to clearing of land, coastal development, and associated population growth. A reported 13% increase in population from 1990 to 2000 in the NC counties surrounding the NPRE (NCSD 2000) has led to increased levels of anthropogenic influence from coastal development and degraded water quality. Tourism is an additional stressor, as the NC Department of Commerce reported NC 8th in the nation, with coastal activities as a top choice for visiting the state (NCDC 2004). At a local level, the economy is dependent on the NPRE for recreational use, boating, and commercial and recreational shellfish harvesting (responsible for 3.63% of the total NC shellfish profit ($675,537) from 1996 to 2006; NCDMF). Since 1986, the NPRE has experienced a 9% increase in shellfish harvesting area
closures with a total of 32.9% of the areal extent of the estuary being closed (Figure 1b; conditionally approved-closed or prohibited) (NCDENR-SSS, Patricia Fowler pers. communication).

Identifying the cause and understanding the decline of water quality in the NPRE is of fundamental importance, and the microbiological water quality of the NPRE has not been adequately studied. Shellfish harvesting waters are currently managed by determining FC concentrations and by extrapolating weather conditions to establish a classification status (i.e. approved, conditionally approved-open, conditionally approved-closed, prohibited).

Generally, a minimum of six sets of samples are collected randomly each year during ‘open’ status (dry weather or negligible rainfall) and analyzed for FC. Additional sampling efforts are conducted only to reopen shellfish harvesting areas that have been closed due to rainfall and resultant runoff (amounts exceeding 3.81 cm of rainfall occurring within 24 hours). This sampling occurs only when an adequate number of days (3 – 5 d) have passed with dry weather to permit the hydrograph of typical storms to return to baseline. Therefore, with the current sampling program, characterization of estuarine water quality following storm events does not occur. Remediation of degraded water quality can only be initiated after sufficient research has been conducted to characterize and quantify the microbial contaminants in the estuary.

The goal of our research has been to conduct an estuary-wide assessment of FIB concentrations and impacts of stormwater runoff on the NPRE. Specifically, our research objectives were to 1) characterize microbial water quality of the entire estuary by enumerating FIB and measuring environmental parameters over a large geographical area, 2) relate FIB findings to rainfall and distance to land to assess the impact of stormwater runoff,
and 3) utilize measurements of FIB to begin to identify potential hot spots for future work to determine sources of fecal contamination to the estuary.

MATERIALS AND METHODS

Newport River Estuary

The NPRE (Figure 2.1) is located north of Morehead City and Beaufort and is in an area classified as Area E-4 by NC Department of Environment and Natural Resources-Shellfish Sanitation Section (NCDENR-SSS 2005). This estuary has an average depth of 1 m and is a well-mixed system with an average residence time of 6 days or 12 tidal cycles, with flushing stemming from the Atlantic Ocean controlled through the Beaufort Inlet (Kirby-Smith and Costlow 1989). The surrounding land-uses consist of approximately 45% forestland, 38% wetlands, 9% residential, 5% bays/estuaries, and 3% cropland (NCDENR-SSS 2005). There are also two point-source discharges (wastewater treatment plants). Associated with varied land-uses are sources of fecal contamination including wildlife (deer, raccoon, bear, or waterfowl), small farm operations (horse, cow, hog), and agricultural drainage (animal biosolids application). The most likely sources of human contamination are subdivision stormwater runoff, septic tank failure, and treated wastewater from the Morehead City and Newport Wastewater Treatment Plants (NCDENR-SSS 2000, 2000).

Sampling locations

Sampling sites were chosen based on existing NCDENR-SSS stations and NCDENR-SSS sanitary surveys (2000). Our goal was to select sites that (1) were spatial distributed across the NPRE, (2) were in high priority shellfish harvest areas (i.e. areas where commercial and recreational shellfish harvesting is prevalent), and (3) were proximal to runoff from land.
Figure 2.1a shows the location of the sampling sites, while Table 2.1 describes the sampling sites and the land-use proximal to each site. The distance criteria used for the designation of the “close to land” and “distant from land” sites was < 0.25 km and > 0.25 km, respectively.

Sample collection

Between September 2004 and August 2006, a total of 179 surface water samples were collected from the 16 sites listed in Table 2.1. Sampling occurred at least three times a season. Seasons were defined as winter (December 21\textsuperscript{st} to March 20\textsuperscript{th}), spring (March 21\textsuperscript{st} to June 20\textsuperscript{th}), summer (June 21\textsuperscript{st} to September 20\textsuperscript{th}), and fall (September 21\textsuperscript{st} to December 20\textsuperscript{th}). However, additional efforts were made to collect samples across varying weather conditions and a range of storm sizes to produce a robust dataset. One liter samples were collected within 3 hours of low tide in order to collect samples with minimal dilution from marine waters (NPRE is too shallow to navigate at peak low tide). The samples were collected in sterilized containers following sampling techniques outlined in standard methods (APHA 2005). After collection, samples were placed on ice and transported immediately to the University of North Carolina at Chapel Hill, Institute of Marine Sciences in Morehead City, NC for processing.

Fecal indicator bacteria analyses

All samples were tested for EC and ENT using the defined substrate technology test kits, Colilert\textsuperscript{®}-18 and Enterolert\textsuperscript{®} (IDEXX\textsuperscript{®} Laboratories, Westbrook, ME). Conversion of positive wells from these tests to a MPN value was conducted following Hurley and Roscoe (1983). Although literature cites false-positives occurring in tropical and subtropical marine and estuarine waters (Pisciotta et al. 2002), studies conducted in NC coastal estuarine waters
have not demonstrated any measurable rate of false-positive results using Colilert®-18 for *E. coli* enumeration (Noble et al., unpublished). In addition, previous analyses of estuarine water samples taken throughout eastern NC have shown that 93% of the FC are EC (n=3020, Kirby-Smith and Noble, unpublished data). Thus, for the purposes of this study we consider our EC measurements to be conservative representations of FC concentrations.

Sample concentrations were log$_{10}$ transformed prior to all statistical analyses. The percent of samples exceeding the standard was calculated by comparing the number of samples exceeding the limit to the total number of samples. Normality tests were assessed for the datasets (Salkind 2004; Howell 2002). Independent samples t-test was used to examine significant differences (alpha ($\alpha$) = 0.05, two-tailed) between FIB concentrations in comparison to land-use, where Levene’s test for equality of variances determined whether equal variances were or were not assumed ($\alpha$ = 0.05, two-tailed) (Salkind 2004; Howell 2002). Rainfall category comparisons and seasonality regarding FIB was determined using the one-way ANOVA with the post-hoc comparison Bonferroni (Salkind 2004; Howell 2002). A significant relationship was determined with respect to an alpha ($\alpha$) of 0.05 (two-tailed).

As mentioned previously, the goal for this study was to conduct sample collection over a wide range of weather conditions (regardless of ‘open’ or ‘closed’ status of shellfish harvesting waters and independent of shellfish harvesting water management guidelines). For statistical analysis, we applied one of the currently used thresholds for shellfish water quality management; the geometric mean threshold of 14 FC MPN per 100 ml. Although not currently designated for recreational use, the NPRE is actively utilized for boating, sailing, and other forms of recreation. Thus, the “Tier 1” single sample threshold of 104 ENT MPN
per 100 ml was applied as a means to compare this estuarine waterbody with other recreational waters.

Environmental parameter measurements

Turbidity (NTU), salinity (based upon the practical salinity scale), dissolved oxygen (mg/l), and temperature (°C) were measured at each site using a calibrated multi-probe instrument (YSI Inc., Yellow Springs, OH). The relationships between FIB and selected environmental parameters were examined. Normality tests were assessed for each environmental parameter dataset and determined which significance test was conducted (Salkind 2004; Howell 2002). If datasets were normal before or after log_{10} transformation, the Pearson product moment correlation (PP) was used to assess significance. If the datasets did not have normal distributions, then the Spearman Rank (SR) analysis was used to assess significance. Turbidity was log_{10} transformed to achieve normality, while raw scores were used for all other environmental parameters. A significant relationship was determined with respect to an alpha (α) of 0.05 (two-tailed).

Due to the heterogeneous nature of rainfall in coastal NC, daily rainfall data were collected from three rain gauges, situated for full coverage of the NPRE (Figure 1). Rain gauge “A”, located at the Michael J. Smith Field Airport in Beaufort NC, is maintained by the National Ocean and Atmospheric Association (NOAA) National Climatic Data Center (NCDC) and is available online (http://www.ncdc.noaa.gov/oa/ncdc.html). Rain gauge “B”, located in Mill Creek in Newport, NC, is maintained by volunteers for NCDENR-SSS. Rainfall gauge “C”, located in Ware Creek in Beaufort, NC, is maintained by a volunteer from Duke University Marine Laboratory. For comparison of FIB to rainfall levels, data from the closest rain gauge was used for each site. In addition, due to sampling constraints via boating during foul weather, a 48 hour rainfall total was used for analyses comparing rainfall to FIB concentrations.
RESULTS

Rainfall caused a significant increase in FC concentrations at a rain threshold of 2.54 cm (1.00 in; Figure 2.2). Statistical analyses revealed average FC concentrations of 111.8 MPN/100ml and 221.0 MPN/100 ml for the >2.54 cm (general threshold, n=61) and >3.81 cm (1.50 in, management action rainfall threshold, n=15) rainfall categories, respectively. These FC concentrations were significantly higher ($F_{(4, 316)} = 9.4, p<0.001$) than those for the lesser rainfall categories. The threshold categories of >2.54 cm and >3.81 cm exceeded the 14 MPN/100 ml FC limit for shellfish harvesting waters 87% and 93% of the time, respectively. The rainfall categories of <0.25 cm (0.10 in, n=80), 0.25 to 2.54 cm (n=43), and <3.81 cm (n=122) exceeded the FC limit greater than 67% of the time. The average FC concentrations for these categories were 27.0, 36.6, and 43.8 MPN/100 ml, respectively.

FC concentrations were significantly higher (average of 78.0 MPN/100 ml) when there was some rainfall (>0.25 cm) in comparison to negligible amounts of rain (<0.25 cm, average of 25.8 MPN/100 ml) ($t_{177} = -4.4, p<0.001$) (Figure 2.3). Regardless of rainfall category, the average FC values exceeded one of the currently used thresholds for shellfish harvesting waters. ENT demonstrated a similar relationship with rainfall where >0.25 cm resulted in a significantly higher average of 33.9 MPN/100 ml, while <0.25 cm averaged 13.1 MPN/100 ml ($t_{165.9} = -3.3, p=0.001$). Unlike FC, all average ENT concentrations for each category were below the single-sample recreational water quality standard (ENT: 104 MPN/100 ml).

There was no significant difference between FIB concentrations at those sites close to land (<0.25 km) versus those sites distant from land based upon rainfall (>0.25 km, Figure 2.3). When data was separated according to the general categories of “developed”
(residential and industrial, n = 76) and “undeveloped” (forested, n = 103), there was no significant different between FC concentrations ($t_{177} = 0.763$, $p>0.05$) (see Table 2.3 for sites designated as “developed” and “undeveloped”). However, ENT concentrations did reveal significantly higher concentrations in “undeveloped” areas (n = 103; $t_{177} = 3.04$, $p<0.005$).

Observations from all stations represented a wide range of turbidity (0 to 87.1 NTU; n =146), salinity (2.0 to 35.4; n = 170), DO (3.6 to 14.4 mg/l; n = 169), rainfall (0 to 7.0 cm in 24 hours and 0 to 14.6 cm in 48 hours; n=179). FC concentrations exhibited positive significant relationships with turbidity and rainfall (48 hours), while also having significant inverse correlations with salinity and DO (Table 2.2). ENT had a similar positive correlation with turbidity, and negative relationships with salinity and DO. Although temperature was measured (n=173, range: 9.9 to 32.0°C), a large portion of sampling events occurred in the summer months (52.9%) and any observed correlations would be biased. Seasonality was therefore examined, considering temperature to be a major factor. The analysis showed that FC concentrations were significantly lower in the winter (8.4 MPN/ 100 ml) and significantly higher in the summer (91.4 MPN/ 100 ml) ($p < 0.001$, $F_{(3,175)} = 17.6$), as compared to all other seasons (Figure 2.4). Seasonally, ENT concentrations showed no statistical differences with the concentrations ranging from 11.0 to 30.0 MPN/ 100 ml.

Environmental parameters were further examined for confounding relationships, and turbidity appeared to be correlated with most other parameters (Table 2.2). Turbidity had significant inverse relationships with salinity and DO, while having a significant positive relationship with rainfall (24 and 48 hours).
DISCUSSION

Non-point source pollution, such as stormwater runoff, is a serious issue for NC coastal water quality. Alterations to the land-water interface (i.e. deforestation, impervious surface coverage) are major contributors to this problem. The gradual increase in NC populations is associated with the degrading water quality, although the problem has not reached the magnitude of other coastal states (CA, FL; Perry et al. 2001; Shehane et al. 2005, Ackerman and Weisberg 2003, Lipp et al.2001, and Noble et al. 2000). The 13% population growth in neighboring watersheds surrounding the NPRE in the past decade (NCSD 2000) and associated changes to the land-water interface are likely impacting the water quality of this ecologically and economically important estuary, especially during times of heavy rainfall.

Results indicate that rainfall is a significant factor in the contribution of fecal contamination via stormwater runoff to the NPRE. After 2.54 cm (1 inch) there are significantly higher EC and ENT concentrations as compared to no rainfall (Fig. 2.2 and 2.3). Our findings agree with other research reports from coastal NC, which describe the increasing impacts of stormwater runoff in the context of land and hydrological modifications, as well as impervious surface coverage (Kirby-Smith and White 2006; Mallin et al. 2000). Further evidence demonstrating the impact of stormwater runoff is provided by the significant relationships between FIB concentrations and the freshwater-input related environmental parameters (Table 2.2). Fecal indicator bacteria had significant correlations with turbidity, salinity, and DO. In addition, these parameters had strong relationships with each other. Stormwater runoff, while introducing FIB into the NPRE, also causes increasing turbidity due to the transport of particulate matter from land sources (scouring) and the resuspension of bottom sediments that occur with high flow or strong winds during rainfall.
events. Runoff (i.e. freshwater input) also creates a decrease in salinity and an increase in DO due to freshwater inputs and associated biological activity, respectively. Similar correlations with turbidity and salinity were found in coastal NC (Mallin et al. 2000). One Florida (FL) study showed similar salinity relationships (Lipp et al. 2001), while another FL study did not show the same trend (Shibata et al. 2004). The contradicting study (Shibata et al. 2004) was in a beach location where salinity did not fluctuate with the tides.

In addition to the observed relationship between rainfall and fecal contamination, there were also unexpectedly high concentrations of FIB during periods of negligible rainfall (<0.25 cm (0.10 in), Figure 2.2 and 2.3). This observation indicates a background signal, most likely due to a reservoir population in the sediment, and suggests that FIB may be persisting in the benthos of the NPRE. This phenomenon has been documented in similar sub-tropical and tropical watersheds (Desmarais et al. 2002; Shibata et al. 2004; Byappanahalli and Fujioka 2004; Fries et al. 2008). Studies conducted in northern temperate regions also reveal the persistence and survival of EC through freezing winters with subsequent growth in the warmer months (Whitman and Nevers 2003; Whitman et al. 2006; Ishii et al. 2006). Ongoing work analyzing reservoir EC and ENT populations in sediments of the NPRE show a contribution of 2.45 to 762.71 and 2.45 to 1072.67 MPN per gram (n=4; dry weight), respectively (Coulliette and Noble, unpublished data). Similar results were observed in the nearby Neuse River Estuary with particle attached ENT (Fries et al. 2007). Given the shallow, well-mixed nature of the NPRE and the turbidity values observed over the course of this study, sediment-attached FIB may be partially responsible for the baseline signal during dry weather.
Distance to land was not found to be a significant factor in determining FIB concentrations in this study. Sampling sites for this study were primarily chosen from historically sampled locations, secondarily to have spatially representative sampling locations in shellfish harvesting areas, and finally, based on distance to land. Thus, the lack of statistically significant difference between the FIB concentrations at sites close and distant from land was a likely product of sites being too far from land. In addition, this lack of distinction was further confounded by the fact that sampling via boat did not allow for immediate sampling after rain events for safety. Samples were taken within 48 hours of rain events but tidal flushing and wind mixing may have dispersed FIB signals by that time. Future examinations of land-based runoff will include sampling in tributaries, sampling throughout the duration of storms, and measuring flow to quantify microbial contaminant loading rates.

The seasonal analyses revealed atypical FC concentrations (Figure 2.4), as historical data shows higher concentrations of FC during the winter versus the summer months. This anomaly may be due to the abnormally high rainfall levels during the study period, as compared to the State Climate Office of North Carolina measurements from a nearby monitoring station (Station: 315830 Morehead City 2 WNW, rainfall levels being measured since 1948). The winter months during the study period had 2.13 cm less rainfall as compared to normal levels. The summer months during the study had 5.77 cm more rainfall as compared to normal levels. Spring and fall also had higher levels of rainfall as compared to normal levels with 2.83 cm and 13.04 cm more rainfall, respectively. Collectively, the study period represents FIB concentrations during ‘wet’ conditions.
This study did not address potential sources of fecal contamination. Previous sanitary surveys conducted by NCDENR-SSS indicate that animals and humans are both likely contributors of fecal contamination to the NPRE (NCDENR-SSS 2005). The large avian community, in addition to livestock and wildlife, may be responsible for contributing various fecal pathogens, such as Cryptosporidium spp. (Jellison et al. 2007) and Campylobacter spp. (Dixon 2001; Waldenstrom et al. 2007). Neighboring wastewater treatment plants can also become overburdened during times of heavy rainfall or during disinfection failure, and can release human fecal contamination into the estuary. However, during the study period, the treatment plants did not become overburdened to our knowledge.

The NCDENR-SSS has the complex task of managing the NPRE by taking into account poor estuarine water quality (often associated with rainfall), livelihoods of local shellfish harvesters, and overall public safety. Through the NCDENR-SSS program, only FC sample concentrations during ‘open’ status are used to classify shellfish harvesting waters, and this historical data indirectly aids in determining the rainfall threshold used for managing shellfish harvesting areas in the NPRE. The current rainfall threshold (management action threshold) of 1.5 in (3.81 cm) of rainfall in 24 hours, established in 1994 for the NPRE, is used to close shellfish harvesting waters. Our research demonstrates that the current rainfall limit may not be adequately protective of human health, and that a more stringent limit should be considered.

This is the first intensive study conducted on the water quality of the NPRE. Future work will incorporate this data into ongoing modeling efforts intended to assist TMDL development, as well as determine sources of fecal pollution through molecular approaches and sediment studies. Data from the TMDL models are being utilized for the development of
probabilistic models of fecal contamination transport. Molecular techniques will be used to identify and quantify human versus non-human sources (Noble et al. 2006; Boehm et al. 2003). Resulting data will be used to partition the sources of fecal contamination and to determine microbial contaminant loading rates. The incorporation of modeling efforts, the partitioning of fecal contamination, and the quantification of microbial loading are integral to understanding and characterizing the major sources of fecal pollution to the NPRE. Hopefully these efforts will lead to the design of effective Best Management Practices (BMPs) for future restoration of the estuary.

CONCLUSIONS

Based on our fecal contamination data, the NPRE is experiencing water quality degradation. Stormwater runoff appears to be a main contributor of fecal pollution; however the existence of a persisting bacterial indicator population deserves further investigation. Application of molecular fecal indicator tools (i.e. Bacteroides) and pathogen testing will be needed to determine whether the high concentrations of FIB are indicative of a human health threat. The combination of research and governmental efforts will hopefully allow the remediation of this estuary by identifying problem areas and utilizing BMPs for restoration.
FIGURES

Figure 2.1. Newport River Estuary (NPRE)

(a) NPRE sampling stations and rain gauges during the study period August 2004 to September 2006

(b) NPRE closed areas to shellfish harvesting according to decade
Figure 2.2. Fecal coliform (FC) concentrations found in the NPRE. The rainfall categories are according to general rainfall categories of <0.25 cm, >0.25 to <2.54 cm, and >2.54 cm; and then in regards to the management action plan of <3.81 cm and >3.81 cm. The percent of samples exceeding the FC standard limit of 14 MPN or CFU per 100 ml (horizontal dotted line) within that rainfall category are marked above its respective column. Asterisks over the bar indicators a significant difference as compared to the other categories and the error bars are ±1 standard error.
Figure 2.3. Mean FIB concentrations at NPRE sampling sites. The sites are categorized by distance from land (close = <0.25 km; distant = >0.25 km). The geometric mean threshold for FC (14 MPN/100 ml) is shown by the bar over the left pairs of columns, while the single-sample threshold for ENT (104 MPN/100 ml) is shown by the bar of the right pair of columns. The error bars are ±1 standard error.
Figure 2.4. Seasonal concentrations of FIB in the NPRE. Asterisks over the bar indicators a significant difference as compared to the other categories and the error bars are ±1 standard error.
Table 2.1. NPRE sampling stations and neighboring land-uses. The land-uses noted are general descriptions relative to the NPRE. Those noted with an asterisk (*) indicate a higher density of development for residential or industrial purposes (i.e. “developed”) while all other sites are referred to as “undeveloped.”

<table>
<thead>
<tr>
<th>Close in Proximity to Land (&lt;0.25 km)</th>
<th>Approximate Distance (km)</th>
<th>Land-use</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 West Crab Point</td>
<td>0.18</td>
<td>low density residential, forested</td>
</tr>
<tr>
<td>5A Closure Line</td>
<td>0.15</td>
<td>low density residential, forested</td>
</tr>
<tr>
<td>29 Ware Creek</td>
<td>0.24</td>
<td>residential</td>
</tr>
<tr>
<td>56 Closure Line Core Creek</td>
<td>0.16</td>
<td>low density residential</td>
</tr>
<tr>
<td>85 Calico Creek</td>
<td>0.11</td>
<td>light industrial (including marina), low density residential</td>
</tr>
<tr>
<td>Brickyard</td>
<td>0.16</td>
<td>low density residential</td>
</tr>
<tr>
<td>Recreation Site</td>
<td>0.08</td>
<td>light industrial, low density residential</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Distant Proximity to Land (&gt;0.25 km)</th>
<th>Approximate Distance (km)</th>
<th>Land-use</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 Turtle Rock</td>
<td>0.74</td>
<td>low density residential, forested</td>
</tr>
<tr>
<td>4A Telephone/Cable Crossing</td>
<td>0.66</td>
<td>low density residential, forested</td>
</tr>
<tr>
<td>7 Harlowe Creek</td>
<td>0.32</td>
<td>low density residential, forested</td>
</tr>
<tr>
<td>10 Marker #36</td>
<td>1.46</td>
<td>light industrial</td>
</tr>
<tr>
<td>24 Marker #30</td>
<td>1.30</td>
<td>low density residential</td>
</tr>
<tr>
<td>28 Core Creek</td>
<td>0.32</td>
<td>low density residential</td>
</tr>
<tr>
<td>35 Middle River</td>
<td>1.26</td>
<td>no nearby land use, in center of estuary</td>
</tr>
<tr>
<td>Between 35/55</td>
<td>0.80</td>
<td>no nearby land use, in center of estuary</td>
</tr>
<tr>
<td>55 White Rock</td>
<td>0.66</td>
<td>low density residential, forested</td>
</tr>
</tbody>
</table>
Table 2.2. Correlations of environmental parameters and FIB in the NPRE. Statistically significant values were determined for all correlations ($\alpha = 0.05$, two-tailed) using the Pearson product moment correlation (PP), except for rainfall correlations (24 and 48 hours) when Spearman rank correlation coefficient was used.

<table>
<thead>
<tr>
<th></th>
<th>Turbidity (NTU)</th>
<th>Salinity (mg/l)</th>
<th>DO (24 hours)</th>
<th>Rainfall (24 hours)</th>
<th>Rainfall (48 hours)</th>
<th>FC MPN/100 ml</th>
<th>ENT MPN/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turbidity (NTU)</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salinity</td>
<td>-0.321</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DO (mg/l)</td>
<td>-0.324</td>
<td>0.178</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rainfall (24 hours)</td>
<td>0.199</td>
<td>-0.084</td>
<td>-0.064</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Rainfall (48 hours)</td>
<td>0.232</td>
<td>-0.042</td>
<td>-0.149</td>
<td>0.640</td>
<td>1.00</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>FC MPN/100 ml</td>
<td>0.127</td>
<td>-0.400</td>
<td>-0.496</td>
<td>0.048</td>
<td>0.179</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>ENT MPN/100 ml</td>
<td>0.208</td>
<td>-0.608</td>
<td>-0.277</td>
<td>0.099</td>
<td>0.117</td>
<td>0.554</td>
<td>1.00</td>
</tr>
</tbody>
</table>
REFERENCES


CHAPTER 3

SPACE/TIME ANALYSIS OF FECAL POLLUTION AND RAINFALL IN AN
EASTERN NORTH CAROLINA ESTUARY

INTRODUCTION

Space/Time Random Field (S/TRF) theory provides a framework to model uncertainty and variability of environmental parameters (i.e. microbial concentrations, rainfall), across space and time in terms of statistical moments such as the covariance model, which models the magnitude at which the parameters are related. The Bayesian Maximum Entropy (S/T BME) is a modeling technique that allows one to incorporate general (e.g. the covariance) and site specific (e.g. measurement from multiple sources) knowledge about the environmental parameter to produce visual maps that represent the distribution of the parameter at any unsampled point of interest in space and time. Similar studies have successfully applied the BME method, resulting in rigorous analysis of water and air quality (Akita et al. 2007; Puangthongthub et al. 2007). Such an application for microbiological water quality data would allow researchers to further the understanding of a waterbody, as traditional water quality research approaches provide limited information on a subset of the waterbodies true dynamics across space and time. Specifically, North Carolina (NC) coastal areas are
experiencing serious water quality issues due to man-made pressures (i.e. development) and the subsequent growing issue of stormwater runoff causing increased fecal pollution in the neighboring waters. The presented research describes a combination of the S/T BME framework with an intensive water quality study regarding a fecal impaired waterbody in North Carolina.

In the State of NC, the growing impact of stormwater runoff with respect to land modifications and increased fecal indicator bacteria (FIB) concentrations has been documented (Mallin et al. 2000; Kirby-Smith and White 2006; Coulliette and Noble 2008). The Newport River Estuary (NPRE), an estuarine system in the White River Oak Basin (approximately 35 km² waterbody within a 453 km² watershed), is one of 341 waterbodies in NC that is listed as impaired for either recreational or shellfish harvesting uses (Figure 3.1). This estuary has experienced a population increase of 27.9% from 1986 to 2006 in Carteret County, which is the county that largely surrounds the NPRE (NC Office of State Budget and Management). Coastal development to support such growth is suspected of being one of the main causes for the 9% increase of shellfish harvesting area closures since 1986 (NCDENR-SSS, Patricia Fowler pers. Commun.). Studies conducted in the county have identified stormwater runoff as a significant cause of microbial contamination in the NPRE (Coulliette and Noble 2008). The NPRE is valuable to the local economy with respect to the shellfish and tourism industry, as well as being a vital environmental sanctuary.

The current water quality management program for the NPRE consists of sampling at least six times a year (random sampling strategy; NSSP 2005) and additional sampling only during dry weather to re-open waters after closure due to more than 3.81 cm of rainfall within 24 hours (NCDENR-SSS, Patricia Fowler pers. communication). These sampling
events occur generally during dry weather, or when the hydrograph has returned to baseline (3-5 days), thus creating a limited and biased water quality dataset. Such datasets do not accurately represent FIB concentrations across the range of meteorological conditions experienced by the NPRE. The objective of this presented work was to capture a range of meteorological conditions using S/T BME modeling approaches. Simultaneously, we have incorporated the variability of FIB concentrations in estuarine waters and captured the significant role of rainfall on estuarine water quality.

The NPRE is a prime candidate for this modeling approach due to the complexity of the system (patchy coastal rainfall, a variety of inputs from tributaries and neighboring rivers, as well as tidal exchange), as no regular water quality sampling approach would capture the estuarine dynamics without a large budget and extensive manpower. The goal of our research was to evaluate a NPRE water quality dataset collected from September 2004 to August 2006, and supplemental historical data from December 2002 to March 2004, using a BME geostatistical framework. This approach was used to thoroughly assess the impacts of stormwater runoff and associated factors on FC impairment of the NPRE. We present (1) a hydrological driven model for FIB across the NPRE that accounts for the effect of rainfall and distance to shore, as well as models the hydrologic-driven trends in microbial concentrations, (2) a covariance model that models space/time dependencies in residual microbial concentrations (i.e. detrended for the hydrological model), and (3) a BME integration of the developed hydrological model and the general and site-specific knowledge bases about concentration residuals that yield informative space/time maps depicting the distribution of microbial concentration in the NPRE during extreme seasonal conditions.
**RESEARCH**

*Study area:* The NPRE (Figure 3.1) is located north of Morehead City and Beaufort in eastern NC and is in an area classified as Area E-4 by NC Department of Environment and Natural Resources-Shellfish Sanitation Section (NCDENR-SSS 2005). This estuary has an average depth of 1 m, is a well-mixed system with an average residence time of 6 days or 12 tidal cycles, with flushing stemming from the Atlantic Ocean controlled through the Beaufort Inlet (Kirby-Smith and Costlow 1989). The surrounding land-uses consist of approximately 45% forestland, 38% wetlands, 9% residential, 5% bays/estuaries, and 3% cropland (NCDENR-SSS 2005). There are also two point-source discharges (wastewater treatment plants). Associated with varied land-uses are sources of fecal contamination including wildlife (deer, raccoon, bear, or waterfowl), small farm operations (horse, cow, hog), and agricultural drainage (animal biosolids application). The most likely sources of human contamination are from stormwater runoff from subdivisions and associated, septic tank failure, and overflows during heavy rainfall from the wastewater systems of Morehead City and Newport Wastewater Treatment Plants (NCDENR-SSS 2005, NCDENR-SSS 2000).

*Sampling locations:* Sampling sites were chosen based on existing NCDENR-SSS stations and NCDENR-SSS sanitary surveys (2005; Figure 3.1a). Our goal was to select sites that (1) were spatially distributed across the NPRE, (2) were in high priority shellfish harvest areas (i.e. areas where commercial and recreational shellfish harvesting is prevalent), and (3) were proximal to runoff from land.

*Sample collection:* Between September 2004 and August 2006, a total of 179 surface water samples were collected. Sampling occurred at least three times a season. Seasons were defined as winter (December 21st to March 20th), spring (March 21st to June 20th),
summer (June 21\textsuperscript{st} to September 20\textsuperscript{th}), and fall (September 21\textsuperscript{st} to December 20\textsuperscript{th}). However, additional efforts were made to collect samples across varying weather conditions and a range of storm sizes to produce a robust dataset. Turbidity (NTU), salinity (based upon the practical salinity scale), and temperature (\textdegree C) were measured at each site using a calibrated multi-probe instrument (YSI Inc., Yellow Springs, OH). One liter samples were collected within 3 hours of low tide in order to collect samples with minimal dilution from marine waters (NPRE is too shallow to navigate at peak low tide). The samples were collected in sterilized containers following sampling techniques outlined in standard methods (APHA 2005). After collection, samples were placed on ice and transported immediately to the University of North Carolina at Chapel Hill, Institute of Marine Sciences in Morehead City, NC for processing.

To supplement the data, historical fecal coliform concentrations were obtained from the NC Department of Environment and Natural Resources, Shellfish Sanitation Section (NCDENR-SSS; Figure 3.1a). A total of 79 samples that were collected from December 2002 to March 2004 (\(n = 79\)) during falling tide conditions were included.

\textit{Fecal indicator bacteria analyses:} All samples collected from September 2004 to August 2006 were tested for \textit{E. coli} (EC) and \textit{Enterococcus} (ENT) using the defined substrate technology test kits, Colilert\textsuperscript{®}-18 and Enterolert\textsuperscript{®} (IDEXX\textsuperscript{®} Laboratories, Westbrook, ME). The kit procedure entailed diluting the sample volume (10 ml) into 90 ml of diH\textsubscript{2}O. The QuantiTray\textsuperscript{®} then separated the volume into 49 wells of 1.86 ml and 48 wells of 0.186 ml. Conversion of positive wells from these tests to a most probable number (MPN) value was conducted following Hurley and Roscoe (1983). Although literature cites false-positives occurring in tropical and subtropical marine and estuarine waters (Pisciotta et al.
(2002), studies conducted in NC coastal estuarine waters have not demonstrated any measurable rate of false-positive results using Colilert®-18 for EC enumeration (Noble et al., unpublished).

Newport River Estuary samples collected by the NCDENR-SSS from December 2002 to March 2004 were processed by Multiple Tube Fermentation for the quantification of fecal coliform (FC) using A1-M (AOAC 1990). Previous analyses of estuarine water samples taken throughout eastern NC have shown that 93% of the FC are EC (n=3020, Kirby-Smith and Noble, unpublished data). Thus, for the purposes of this study we consider the NCDENR-SSS FC measurements to be representative of EC concentrations.

For statistical analysis, the currently used threshold for shellfish water quality management was applied for general comparison; the geometric mean threshold of 14 FC MPN per 100 ml (NSSP 2005). The NPRE is also actively utilized for boating, sailing, and other forms of recreation. Thus, the “Tier 1” single sample threshold of 104 ENT MPN per 100 ml was applied as a means to compare this estuarine waterbody with other recreational waters.

Rainfall: Daily observed measurements were reported from seven rain gauges surrounding the NPRE to obtain full coverage and to account for the heterogeneous nature of NC coastal rainfall patterns (Figure 3.1b). Five (5) of the stations data were from the State Climate Office of North Carolina, NC Climate Retrieval and Observations Network Of the Southeast (NC CRONOS) Database: Morehead City 2 WNW (ID# 315830), Beaufort Smith Field (ID# KMRH), Atlantic Beach WP (ID# 310356), and Croatan (ID# NCRN). Two stations included daily rainfall observations from volunteers for the NCDENR-SSS: Newport/Mill Creek (“A”) and Beaufort/Ware Creek (“B”).
Hydrological driven model: Fecal contamination in estuaries is influenced by several hydrologic factors. Two factors that are particularly relevant in the NPRE are rainfall, which leads to increased microbial loading, and distance to shore, which addresses microbial fate and transport into the estuary. The dependency of FIB concentration $B$ with these hydrologic factors may be expressed for sample $i$ as

$$B_i = \beta_0 + \beta_1 \text{rain}_{1i} + \beta_2 \text{rain}_{2i} + \beta_3 d_i + \epsilon_i$$  \hspace{1cm} (1)$$

where $B_i$ is the MPN estimate of FIB concentration for sample $i$, $\text{rain}_{1i}$ is the rainfall for that sample over an antecedent period of a few days, $\text{rain}_{2i}$ is the rainfall for an antecedent period preceding that of $\text{rain}_{1i}$, $d_i$ is the distance from the sample to the shore, $\epsilon_i$ is an error term, and $\beta_0$, $\beta_1$, $\beta_2$ and $\beta_3$ are linear regression coefficients. This model allows investigation of the effect of various levels of antecedent rainfall on fecal contamination. Additionally, the distance to shore allows testing whether overland flow into the estuary acts as a fecal microbial source, in which case microbial concentration are expected to decrease with increasing distance to shore. Linear regression theory can be used to obtain the regression coefficients, which can then be used to model the hydrological trend in the log-transform FIB concentration $Y=\log(B)$ as the space/time function

$$h_Y(p) = \log(\beta_0 + \beta_1 \text{rain}_1(p) + \beta_2 \text{rain}_2(p) + \beta_3 d(p))$$  \hspace{1cm} (2)$$

for any space/time point $p=(s,t)$, where $s=(s_1,s_2)$ is the spatial coordinate and $t$ is time.

For each sample $i$, the straight-line distance between the sample site and its corresponding closest point on the shore of the NPRE was calculated ($d_i$ in Eq. 1). Additionally, the daily rainfall recorded at seven rain gauges surrounding the NPRE (Fig. 1) was calculated using the space/time BMElib numerical implementation (Christakos et
al.2002) of the classical ordinary kriging method (Journel and Huijbregts 1978; Cressie 1993). This leads to estimates of daily rainfall at the location of each sample site and for the day of sampling as well as each of it preceding 14 days. The rain_{1i} and rain_{2i} variables (Eq. 1) were constructed from these kriging estimates using various non-overlapping antecedent periods of daily rainfall.

*Bayesian Maximum Entropy (BME) estimation:* The theory of space/time random field (S/TRF) was used to model the variability and uncertainty associated with the distribution of FIB concentrations across space and time. Let $B(p)$ be the S/TRF describing the distribution of a FIB, and $Y(p)=\log(B(p))$ be its log-transform. The log-transform residual S/TRF $X(p)$ is defined as

$$X(p) = Y(p) - h_Y(p)$$

This equation expresses that the S/TRF $X(p)$ models the space/time variability and uncertainty associated with the difference between the S/TRF $Y(p)$ and the hydrologic function $h_Y(p)$.

Bayesian Maximum Entropy (BME), a powerful MATLAB numerical toolbox of Modern Spatiotemporal Geostatistics implementing the BME theory (Christakos 1990, 2001; Serre and Christakos 1999), was applied to obtain space/time maps of FIB concentration across the NPRE. This framework has been successfully applied in several water quality studies (Serre et al. 2004, Akita et al. 2007). As demonstrated in these studies, BME presents the flexibility of providing the space/time simple, ordinary and universal kriging methods as its linear limiting case, while it can be expanded to a non-linear estimator if non-linear knowledge bases (e.g. soft data, non Gaussian distributions, etc.) need to be considered. Implementation of the BME method using the BMElib numerical package (Christakos et al.
2002) requires specifying the general and site specific knowledge bases characterizing the Space/Time Random Function (S/TRF) \( X(p) \), and produces BME estimates at any space/time point of interest.

The general knowledge base describes systematic space/time trends and dependencies in the S/TRF \( X(p) \). In this work, the general knowledge base consists of the space/time mean trend function \( m_X(p) = E[X(p)] \), and the covariance function \( c_X(p, p') = E[(X(p) - m_X(p))(X(p') - m_X(p'))] \) of the S/TRF \( X(p) \), where \( E[.] \) is the expectation operator. The mean trend function is a statistical moment of order 1 characterizing the systematic space/time trends in \( X(p) \), while the covariance function is a statistical moment of order 2 describing the dependency of \( X \) between points \( p \) and \( p' \). These mean trend and covariance functions are obtained from FIB data using the BMElib numerical package (Serre and Christakos 1999; Christakos et al. 2002).

The site-specific knowledge base describes information that is only relevant to specific points where measurements were performed. In this work the site specific knowledge base consists in the soft data provided by the results of the sample analysis at each of the space/time location \( p_i \) where a sample \( i \) was collected. The FIB test kits (Colilert\textsuperscript{®}-18 for EC and Enterolert\textsuperscript{®} for ENT) provide for each sample \( i \) the number of wells found to be positive for the FIB of interest (49 wells of 1.86 ml, 48 wells of 0.186 ml). From these two numbers of positive wells the Maximum Likelihood (ML) methodology provides for each sample the MPN estimate \( B_{MPN} \) and the 95% Confidence Interval (CI) lower and upper bounds \( B_L \) and \( B_U \), respectively, for the log-normally distributed concentration \( B(p_i) \) expressed in FIB/100ml. It follows directly that, given site specific knowledge, the log-transform residual FIB concentration \( X(p_i) \) is statistically characterized by a Gaussian
probability density function (PDF) with mean \( \log(B_{MPN}) - h_Y(p) \) and standard deviation \( \log(B_U) - \log(B_L) )/(2*1.96) \). Hence the site specific knowledge base \( S \) consists of Gaussian soft data for \( X(p) \) at each space/time sampling points \( p_i \).

General knowledge and site specific knowledge was processed as described above using the BMElib numerical package to obtain BME estimates of the log-transform residual S/TRF \( X(p) \) across the NPRE for each day of the period of study. The BME estimate for a given day is a function of data collected on that day, as well as data collected on days prior and following that day. The estimation error associated with a BME estimate of \( X(p) \) at an estimation point is fully characterized by the BME posterior PDF. The expected value and corresponding estimation error variance of the corresponding FIB concentration at that estimation point is then simply obtained by adding the hydrologic driven trend \( h_Y(p) \), and back log-transforming the BME posterior PDF for \( X(p) \). This results in BME maps showing the space/time distribution of FIB concentration across the NPRE.

RESULTS

*Hydrological driven model*

The kriging estimates of daily rainfall for the \( \text{rain}_{1i} \) and \( \text{rain}_{2i} \) variables were constructed using various non-overlapping antecedent periods. The most statistically significant fit hydrologic linear regression model (Eq. 1) was selected and corresponds for \( \text{rain}_{1i} \) in the 4-day antecedent rainfall (day of sampling, 3 days prior) and for \( \text{rain}_{2i} \) in the preceding non-overlapping 10-day antecedent rainfall (day 13 to 4 prior to sampling). Antecedent rainfall \( (\text{rain}_{1i}, \text{rain}_{2i}) \) and distance shore \( (d_i) \) explain 61% and 4% of variability seen in EC \( (F=134.20, p<0.00001) \) and ENT concentrations \( (F=3.85, p=0.02) \), respectively, in the NPRE.
during the study period (Table 3.1). The coefficients determined for EC showed that for every centimeter of rainfall in the 4-day antecedent period ($\beta_1$) and 10-day antecedent period ($\beta_2$), there was an increase of 48.4 and 6.5 FIB per 100 ml, respectively (Table 3.1). The coefficients determined for ENT showed that for every centimeter of rainfall in the 4-day antecedent period ($\beta_1$) and 10-day antecedent period ($\beta_2$), there was an increase of 112.6 and a decrease of 33.3 FIB per 100 ml, respectively (Table 3.1). The distance coefficient ($\beta_3$) revealed that for every kilometer away from the shore, EC decreased by 0.62 MPN/100 ml and ENT 0.11 MPN per 100 ml (Table 3.1).

**Covariance model**

Using the hydrological driven model ($h_p(p)$), the expected value $\log(B_{\text{MPN}}) - h_p(p)$ and standard deviation ($\log(B_U) - \log(B_L)/(2*1.96)$ was obtained for each sample $i$ for the soft data. From these $X$-data, the covariance experimental values shown with circles in Fig. 3.2 for EC were calculated. These experimental covariance values were fit to a covariance model shown in a plain line in Fig.2, which corresponds to the following equation:

$$c_X(r, \tau) = \sigma^2_X \exp\left(-\frac{3r}{a_r}\right) \left( \sum_{j=1}^{2} c_j \exp\left(-\frac{3\tau}{a_{ij}}\right) + c_3 e \cos\left(\frac{\pi \tau}{a_{i3}}\right) \right).$$

*Enterococcus* (ENT) resulted in a similar model (data not shown). A nugget effect was removed for covariance models to account for measurement error, which was estimated from the average of the measurement error variance for each data points to be 0.27 and 0.67 ($\log\text{FIB}/100\ ml)^2$ for EC and ENT, respectively. The population variance $\sigma^2_X$ and spatial component $a_r$ for EC were 3.30 ($\log\text{FIB}/100\ ml)^2$ and 70 km, respectively. Three nested covariance functions were used to model the temporal component of the space/time covariance model of EC, where the first structure showed a $c_1$ of 0.6 for $a_{t1}$ of 15 days, the
second structure showed a $c_2$ of 0.2 for $a_{t2}$ of 150 days, and a third structure showed a $c_3$ of 0.2 for $a_{t3}$ of 51 days. For ENT, the population variance $\sigma^2_X$ and spatial component $a_r$ were 8.68 (log FIB/100 ml)$^2$ and 70 km, respectively. Three nested covariance functions were used to model the temporal component of ENT, where the first structure showed a $c_1$ of 0.5 for $a_{t1}$ of 15 days, the second structure showed a $c_2$ of 0.3 for $a_{t2}$ of 300 days, and a third structure showed a $c_3$ of 0.2 for $a_{t3}$ of 55 days.

*Space/Time Bayesian Maximum Entropy Maps*

The covariance model was used as general knowledge in BMElib to obtain the BME posterior PDF of FIB at any location and time of interest. The expected value and chosen quantiles (2.5% and 97.5%) of the BME posterior PDF provide a relevant BME estimate and BME 95% confidence interval, respectively. These BME estimates and their 95% confidence intervals can be used to construct plots showing how the FIB changes as a function of time at some particular sites of interest (Fig. 3.3) or to construct maps showing the spatial distribution of the FIB across the NPRE for a particular time or specific sampling event (Figures 3.4 and 3.5).

Specific sampling events were chosen with regards to season (winter versus summer) and rainfall (0 cm versus >3.81 cm) to represent a range of non-detects ($<1.0 \times 10^0$) to 3.0 x $10^3$ MPN per 100 ml BME estimations of EC and ENT concentrations in the NPRE via space/time maps (Figure 3.4 and 3.5). The winter examples observed a temperature range of 10 to 14 (°C), salinity range of 11 to 36, and turbidity range of 1 to 11 (NTU), while the summer observations demonstrated a temperature range of 27 to 29 (°C), salinity range of 9 to 29, and turbidity range of 4 to 23 (NTU). Movies that incorporate space/time maps for the entire study period of EC and ENT distributions in the NPRE can be obtained via
The color scale to the right of S/T maps represent FIB concentrations ranging from 2.2 to 1100.0 MPN per 100 ml for EC (Figure 3.4) and 7.4 to 2980.0 MPN per 100 ml for ENT (Figure 3.5).

The distribution of EC during the dry winter example demonstrated that concentrations are near the standard for shellfish harvesting waters (14 MPN per 100 ml; Figure 3.4 a). However, the EC concentrations surpass the acceptable threshold for shellfish harvesting waters after substantial rainfall in the winter (>3.81 cm; Figure 3.4c). During warmer conditions, EC concentrations surpass the standard regardless of rainfall (Figure 3.4b and d). For ENT (Figure 3.5), the winter months illustrate that during dry conditions the estuary meets the recreational water quality water standard of 104 ENT MPN per 100 ml (panel a). The warmer dry conditions also show ENT meeting the water quality standard with the exception of a western portion (Figure 3.5 b). However, after substantial rainfall (>3.81 cm), the northwest portion of the estuary surpasses the acceptable threshold in the winter and escalates beyond the acceptable level during the summer (Figure 3.5b and d).

DISCUSSION

The FC impairment of the NPRE can be attributed to a variety of causes; however, nonpoint source pollution via stormwater runoff is a main contributor (Coulliette and Noble 2008). The occurrences of such impairments are likely to increase in NC and other coastal areas if the trend of growing developments and subsequent alterations to land-water interfaces continues, as such literature supports this current trend (Coulliette and Noble 2008; Evanson and Ambrose 2006; Shehane et al. 2005; Reeves et al. 2004; Ackerman and Weisberg 2003;
Noble et al. 2003; Kistemann et al. 2002; Mallin et al. 2000). Numerous water quality studies will be necessary to assess fecal contamination, although the limited budget and ability to assess water quality for all over a range of temporal and spatial conditions presents a serious dilemma. Therefore, with the ever changing landscape and hydrology, we present an established framework, such as S/T BME, which proves useful in integration of multiple parameters that influence impairment.

The hydrologic linear regression model showed that 61% of the variability in EC concentrations, a conservative estimate of FC, could be attributed to antecedent rainfall and distance to shore, while only 4% of the variability in ENT concentrations was accounted by the same model (Table 3.1). This finding suggests that a linear relationship between EC and rainfall is a suitable model; however, such a linear relationship may not be suitable model for ENT. Therefore, future research should be completed to find if a better suited model can be found for ENT. Coulliette and Noble (2008) reported similar findings in the NPRE with EC having a significant positive linear relationship with rainfall within the previous 48 hours (0.179, p=0.05), while ENT did not. The 4-day (day of sampling to 3 days prior) antecedent rainfall appears to contribute the greatest concentration of EC and ENT, as compared to 10-day rainfall preceding the sampling date (day 4 to day 13 prior to sampling). The study period was noted to be a “wet year” as compared to historic rainfall averages (Coulliette and Noble 2008), thus the 10-day antecedent rainfall may not have played a large of a role as would be expected in a “normal” or “dry” years (i.e. where long periods between storms might be expected to accumulate fecal contamination which can then be flushed into the estuarine system). The distance to shore illustrated a negligible decay for both FIB types. The minimum decay detected in FIB concentrations away from shore is mostly likely due to
the NPRE being relatively shallow (few meters) and being impacted by wind and tide-induced mixing. Additional sample collection and analyses along transects across the land-water interface would improve our knowledge of the distance from shore relationship.

The hydrological model acknowledged and quantified the impact of antecedent rainfall and distance to shore with respect to FIB concentrations, although more significant statistically with EC than ENT. The theory of TRF provides a useful framework to model the uncertainty and space/time variability associated with the residual log-transformed microbial concentrations (i.e. the difference between log-transformed concentrations and log transformed hydrological driven model). The BME framework provides a rigorous mathematical framework to process knowledge about the space/time variability and soft data about this residual log-transformed S/TRF, which then leads to informative maps of the distribution of FIB in the NPRE.

The population variance ($\sigma^2$, $(\log\text{FIB}/100 \text{ ml})^2$) for residual log transformed EC and ENT, which corresponds to standard deviations of 1.82 $(\log\text{FIB}/100 \text{ ml})$ and 2.95 $(\log\text{FIB}/100 \text{ ml})$ standard deviations, respectively, illustrates that residual transformed EC concentrations had a tighter range of variability than residual log transformed ENT concentrations for the study period. *E. coli* (EC) may persist or have a more steady constant source into the NPRE than ENT, as suggested in the findings of Coulliette and Noble (2008), which would lead EC having a lower residual variance. The spatial range of the covariance model (70 km) for both residual EC and ENT is quite large, indicating that FIB concentrations are being contributed from estuarine-wide sources rather than from one specific area of the NPRE. This valuable insight suggests that environmental influences may be providing uniform estuarine-wide fecal contamination (i.e. wildlife). An additional
perspective is that despite growing development and businesses, one specific area of the watershed is not yet impacting the NPRE more than the other areas.

The temporal component of the covariance model consists of three structures of 15, 150, and 51 days for EC, and 15, 300, and 55 days for ENT. The structure indicating that 60% of the EC and 50% of the ENT residual variability has an autocorrelation that disappears after 15 days, relates to the lunar cycle of spring tides (every 14.76 days; Kvale, E.P. 2006). Spring tides, when the Earth, Moon, and Sun are nearly aligned, result in pronounced high and low tides (Kvale, E.P. 2006). The extreme high tides have the potential to wash FIB from neighboring banks (Shibata et al. 2004), as well as allowing freshwater to flow into the NPRE during the ultra-low tides. Such hydrological patterns could potentially introduce higher FIB concentrations during time period. The second structure has a temporal range of 20% for 150 days for EC and 350 days for ENT, which spans the scale of seasonal variation for EC, where concentrations are significantly different in the winter versus summer (Coulliette and Noble 2008). However, ENT appears not be influenced by this seasonal scale, which was also observed in Coulliette and Noble (2008). The structure approximating 51 to 55 days for EC and ENT, respectively, corresponds to cycles of 102 to 110 days, respectively, that may be a synodically and/or tropically driven neap-spring cycles. However, a detailed tidal analysis of the NPRE would be necessary to confirm such a hypothesis.

The resulting S/T maps provide critical tools to understand the distribution of FIB in the NPRE. The examples shown signify that dry winter months, which correspond to the oyster harvesting season in NC of October 1st to March 30th, met the shellfish harvesting standard of 14 MPN per 100 ml. However, after substantial rainfall 3.81 cm (1.5 inches), the
NPRE does not appear to meet this requirement. Warmer months, regardless of rainfall, result in EC concentrations exceeding the threshold for the entire NPRE. In regards to ENT, the NPRE meets the recreational water quality threshold (104 MPN per 100 ml) except for warmer months after substantial rainfall.

Once a covariance model has been established for a waterbody, such as the impaired NPRE, S/T maps provide quantitative and reproducible evidence on which to base water quality management decisions. One main application for the S/T analyses, as was shown here, would be to evaluate the management action plan at the end of each year. As development continues to increase and land-water interfaces are altered, it is vital to examine how such changes impact FIB concentrations in the NPRE with respect to the water quality thresholds. An additional analysis would be to examine the length of time required after rainfall for the shellfish harvesting waters to return to an acceptable level. Such results would provide an economic benefit to the harvesters if the waters could be opened earlier, or an economic benefit to government to direct them when to sample to re-open the waters without needing additional sampling effort. The associated variance and confidence intervals could also be included in this analysis. For example, the model on a specific date may have more confident estimations (90%) and management would be able to trust these estimations without supportive sampling. While another scenario may involve 50% confidence in the estimates on a particular date, thus sampling would be necessary to compliment the models predictions.

Management is also interested in using such described applications for proposals to be submitted to the National Shellfish Sanitation Program to advance water quality regulation efforts for shellfish harvesting waters. However, for best use of these modeling approaches
data need to be continuously added across a range of weather conditions. Collaborations between researchers and management would be necessary, as government funding is limited to determine the safety of waters when they are designated as “opened.” It is also important to note that the models provide a more accurate prediction of concentrations seen in the estuary as compared to current approaches, thereby collaborations would improve scientific knowledge and public health. Overall, the presented research provides insight to the FC impairment and further acknowledges that rainfall is a significant contributor to NPS pollution, especially the 4-day antecedent rainfall, while the produced S/T maps provide a realistic representation of the FIB distribution across the NPRE and show promise for future management.
FIGURES

Figure 3.1 The Newport River Estuary (NPRE). The sampling stations (A) are identified by letters and numbers (sampling location identifiers), where circles relate to samples taken from August 2004 to September 2006, while squares relate to the historical samples taken by NCDENR-SSS from December 2002 to March 2004. Rain gauges (B) are illustrated in triangles for the rain data collected by NCDENR-SSS volunteers and the pentagon stations are those data reported from NCDOT.
Figure 3.2. The spatial (km) and temporal (days) components of the covariance model for *E. coli* (EC)
Figure 3.3. Temporal plot illustrating BME estimation calculations. *E. coli* monitoring data (dot centered at the MPN with error bars extending from the lower and upper bounds of the measurement error 95% CI), the hydrologic driven model (dashed line), the BME estimate (plain line) and the BME 95% CI (dotted lines) are shown below.
Figure 3.4 Space/time maps of the NPRED illustrating *E. coli* (EC) estimations. The maps show the Bayesian Maximum Entropy (BME) estimation of EC concentrations for selected events, where the color bar on the right of the maps represents EC Most Probable Number (MPN) per 100 ml. The x-axis and y-axis demonstrate the longitude and latitude coordinates.
Figure 3.5 Space/time maps of the NPRE illustrating *Enterococcus* (ENT) estimations. The maps show the Bayesian Maximum Entropy (BME) estimation of ENT concentrations for selected events, where the color bar on the right of the maps represents ENT Most Probable Number (MPN) per 100 ml. The x-axis and y-axis demonstrate the longitude and latitude coordinates.

(a) winter, 0 cm

(b) late spring, 0 cm

(c) winter, >3.81 cm

(d) summer, >3.81 cm
Table 3.1 Linear regression coefficients of the hydrological driven model. The coefficients, $\beta_1$, $\beta_2$ and $\beta_3$, correspond to $B_i=\beta_0+\beta_1 \text{rain}_1+\beta_2 \text{rain}_2+\beta_3 d_i+\epsilon_i$ (Eq.1) obtained for *E. coli* and *Enterococcus*. The 90% confidence intervals are listed in parentheses under each regression coefficient.

<table>
<thead>
<tr>
<th></th>
<th><em>E. coli</em></th>
<th><em>Enterococcus</em></th>
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<tr>
<td>$R^2$</td>
<td>0.61</td>
<td>0.04</td>
</tr>
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<td></td>
<td>(F=134.20, p&lt;0.00001)</td>
<td>(F=3.85, p=0.02)</td>
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<tr>
<td>$\beta_1$</td>
<td>48.4</td>
<td>112.6</td>
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<td>FIB MPN/ 100 ml/ cm</td>
<td>(44.40 to 52.40)</td>
<td>(36.10 to 189.20)</td>
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<td>$\beta_2$</td>
<td>6.5</td>
<td>-33.3</td>
</tr>
<tr>
<td>FIB MPN/ 100 ml/ cm</td>
<td>(1.80 to 11.30)</td>
<td>(-124.30 to 58.0)</td>
</tr>
<tr>
<td>$\beta_3$</td>
<td>-0.62</td>
<td>-0.11</td>
</tr>
<tr>
<td>FIB MPN/ 100 ml/ km</td>
<td>(-1.15 to -0.08)</td>
<td>(-11.20 to 10.97)</td>
</tr>
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REFERENCES


CHAPTER 4.1

TRANSPORT AND PARTITIONING OF FECAL CONTAMINATION INTO A HIGH PRIORITY SHELLFISH HARVESTING AREA IN EASTERN NORTH CAROLINA

INTRODUCTION

One of the requirements under section 303(d) of the Clean Water Act is to establish a Total Maximum Daily Load (TMDL) for a pollutant that causes a waterbody to exceed its specific water quality use, thereby rendering it “impaired” (NCDENR 2008). However, waterbodies are often divided into multiple sections for governmental management purposes. When impairment occurs, TMDL development is necessary for all sections encompassed in the impaired waterbody. For example, the Newport River Estuary (NPRE) in the White Oak River Basin in eastern North Carolina (NC) has eight individual fecal coliform (FC) impairments with areas ranging from 0.17 km$^2$ to 12.95 km$^2$ (NCDENR 2008). Allocated funds and personnel are not adequate to study each section with the intensity required for either TMDL development or assessment. Thus, a reasonable approach would be to focus research efforts on one impaired section within the NPRE to develop a conceptual
understanding of the fate and transport of the pollutant sources, and then apply the research findings and associated predictive information to the other areas of the impaired waterbody. The “model” could then be applied to the additional impaired sections with deviations from the original model then taken into account.

Of the eight impaired sections of the NPRE is Ware Creek. This creek was the focus area for research efforts in order to create such a conceptual model. Ware Creek, in eastern NC, was once considered prime habitat for shellfish harvests, thriving environmental sanctuaries, and serene waterfront homes. It epitomized the ideal NC estuarine environment which is so important for the local economy (shellfish and tourism industry), as well as for the natural flora (e.g. thriving seagrass beds and oyster reefs) and wildlife (e.g. larval and juvenile fisheries, bluefish, flounder, blue crabs, shrimp). However, the recent closure in 2006 of Ware Creek for commercial oyster harvesting drew attention to the precarious balance between estuarine ecosystem health and anthropogenic influence from growing coastal development. Overall, since 1986 there has been a 9% in increase NPRE shellfish harvesting closures (NCDENR-SSS, Patti Fowler pers. communication) illustrating the growing need to understand the fate and transport of nonpoint source pollution in effort to prevent continuing degradation of these vital estuarine waters.

Ware Creek provided an ideal tributary for an intensive microbiological water quality study. A series of ditches and culverts deposits stormwater runoff into a single narrow channel that empties directly into a previously open shellfish harvesting area if the NPRE. Other than the tributary itself and the potential for failing septic systems, there are minimal outside influences that would otherwise impact a fecal pollution study in this area. Surface water samples were collected from November 2006 to November 2007 during a range of
storm and dry weather conditions with the overall goals to intensely examine the fate and transport of fecal contamination introduced into the NPRE via Ware Creek. The objectives of the research were to (1) quantify the transport and dilution of fecal contamination input through culture and molecular-based methods, (2) partition the fecal pollution signal via two lines of evidence (molecular FIB *B. thetaiotamicron* (*B. theta*) and a separate analysis of sediment cores), and (3) identify environmental dynamics of fecal contamination in Ware Creek, specifically as related to storms.

MATERIALS AND METHODS

*Study site*

Ware Creek is located in the Northeast portion of the NPRE in the town of Beaufort and is in an area classified as Area E-4 by NC Department of Environment and Natural Resources-Shellfish Sanitation Section (NCDENR-SSS 2005; Fig. 4.1.1). Ware Creek was a previously approved area for shellfish harvesting until 2006 when the status was changed to “prohibited” due to levels of FC exceeding the water quality standards (NCDENR-SSS, Patricia Fowler pers. communication). Although land-use alterations occurred during the study (i.e. clearing of land, residential development), the area is categorized as low density residential surrounded by forested and row-crop agricultural land. The creek is tidally influenced and likely fecal contamination sources stem from wildlife (deer, peacocks), agricultural drainage (soybean), domestic animals (dogs, cats), and human sewage (failing septic systems). Previous surveys confirmed Ware Creek as a main contributor of non-point source pollution, especially during times of heavy rainfall (Kirby-Smith unpublished).
Sample locations

Sampling locations created a transect from where stormwater runoff entered Ware Creek and flushed into the closed shellfish harvesting area (Figure 4.1.1). The sites are directed downstream, where Ware Creek 1 (WC1) empties stormwater runoff from the neighboring watershed into the beginning of the transect. Ware Creek 2 (WC2) and Ware Creek 3 (WC3) continues the transect to Ware Creek 4 (WC4), which is the previously approved shellfish harvesting area. Relatively low density residential development flanked the transect. The transect is 700 m with the specific distances between sites and source shown in Figure 4.1.1.

Sample collection

Between November 2006 and November 2007, a total of 153 surface water samples were collected along the transect (upstream to downstream) of WC1, WC2, WC3, and WC4 (Figure 4.1.1). For every sampling event, three one liter surface water samples were collected within 3 hours of low tide in order to obtain samples that are impacted minimally by dilution from marine waters. In order to characterize the temporal variability associated with the FIB results, three time series (t1, t2, and t3) along the transect going downstream from WC1 to WC4 (total of three samples per site).

Sediment analyses

Following standard methods (APHA 2005), sediment cores were collected under 30 cm of water from the bank of the transect at the same sites where the surface water samples were taken. Care was taken to not disturb the bottom material. A sterile modified 60 ml syringe (tip portion removed for a flat opening) was inserted to a minimum depth of 3 cm, the syringe was slowly removed, and capped with a rubber stopper. Cores were taken in
duplicate, placed on ice, and transported immediately to the University of North Carolina at Chapel Hill’s Institute of Marine Sciences in Morehead City, NC for processing.

_Fecal indicator bacteria sample processing_

All samples were tested for *E. coli* (EC) and *Enterococcus* (ENT) using the defined substrate technology test kits, Colilert®-18 and Enterolert® (IDEXX® Laboratories, Westbrook, ME). Conversion of positive wells from these tests to a MPN value was conducted following Hurley and Roscoe (1983). Although literature cites false-positives occurring in tropical and subtropical marine and estuarine waters (Pisciotta et al. 2002), studies conducted in NC coastal estuarine waters have not demonstrated any measurable rate of false-positive results using Colilert®-18 for EC enumeration (Noble et al., unpublished). In addition, previous analyses of estuarine water samples taken throughout eastern NC have shown that 93% of the FC are EC (n=3020, Kirby-Smith and Noble, unpublished data), thus for the purposes of this study we consider our EC measurements to be conservative representations of FC concentrations.

Water quality standards were used as reference points for comparisons: the geometric mean for shellfish harvesting waters (14 FC MPN per 100 ml; NSSP 2005) and the “Tier 1” single sample threshold for recreational waters (104 ENT MPN per 100 ml).

_Sediment core processing_

The tope few centimeters of the sediment cores were selected and carefully sectioned onto Parafilm M®. The sections were split, in which one half was designated for FIB processing and the other for dry weight. For FIB processing, one set of duplicate halves for each site was placed into 100 ml of sterile phosphate buffered saline, shaken continuously for five
minutes, and pipeted (10 ml) immediately for FIB processing. For dry weight, the other
duplicate halves were combined into a pre-weighed foil dish, dried for 48 hours at 55°C,
cooled for at least 5 minutes, and weighed (grams).

*Alternative fecal indicator bacteria*

**Sample processing:** For each sample, a known volume (100-500 ml) of sample was vacuum
filtered through replicate 47mm, 0.4 µm porosity polycarbonate filters (Millipore Bedford,
MA) using a filtration manifold.

**Extraction:** The filters were frozen at -80°C until further processing. Filters were
extracted for DNA using the DNA-EZ Extraction kit (GeneRite, LLC; North Brunswick,
New Jersey). Briefly, the filter was placed in 500µl Buffer AE (QIAGEN Valencia, CA)
containing 1mm zirconium/silica beads (Biospec Corp. Bartlesville, OK), the filter was
homogenized for 2 minutes in a Bead Beater (Biospec), and the filtrate centrifuged for 1
minute at 14, 000 x g. The filtrate was removed without disturbing pelleted beads,
centrifuged as above, and the filtrate combined with an equal volume of binding buffer and
placed in a DNAsure column. The column was spun for 1 min at 12,000 x g, the filtrate
discarded, and 500µl of Wash Buffer added to the column. The column was centrifuged and
the filtrate discarded. Fifty µl of Elution Buffer was added directly to the center of the
column, incubated at room temperature for 1 min, and then spun for 1 minute at 12,000 x g.
The eluted DNA was collected and stored at -20°C until further processing.

**Analyses:** DNA extracts were analyzed by quantitative PCR (QPCR) for *B.
thetaioatomicron* (*B. theta*). Primers (BthetaFor and BthetaRev) and probe (BthetaFAM)
were constructed to correspond to a 110 bp fragment the 16S rRNA gene of *B. theta*
(Blackwood and Noble *In Prep*). Quantitative PCR (QPCR) was performed on DNA extracts
using *Bacteroides theta* specific primers and under the following conditions: TaKaRa Pre Mix Ex Taq (Mirus Bio Madison, WI), 1 µ mol l⁻¹ each primer, 0.1 µ mol l⁻¹ probe, and 5 µl DNA template for a total reaction volume of 25 µl. All reactions were performed on a SmartCycler® II (Cepheid, Sunnyvale, CA) and reaction conditions were as follows: hot start at 95°C for 10 s, followed by 45 cycles of 95°C for 15 s and 60°C for 45 s.

Quantification of the standards was conducted using *B. theta* cells enumerated via epifluorescence microscopy following the previously outlined approach (Noble and Furhman 1998) and were used to establish a 4-log standard curve, with reactions run in duplicate. QPCR amplification efficiencies of >95% and R² values of >0.99 were documented for each standard curve.

**Controls:** Appropriate controls were included for all analyses: These were as follows 1) negative extraction control, which consisted of DNA extraction containing a blank filter to ensure that contamination did not occur during sample processing, 2) Specimen processing control (SPC), where a known amount of salmon sperm DNA was added to each extraction tube prior to extraction to account for sample loss and inhibition during sample processing, 3) a no template PCR control (NTC), which consisted of PCR reaction containing no DNA template, and 4) a calibrator, the gene of interest, which was processed in the same manner as the samples. The calibrator was *B. theta* cells processed as described above. A sample was said to be inhibited or sample loss to have occurred if the C_t value of the SPC differed by more than 3 C_t’s less than that of the SPC only control. All samples were within the acceptable range for the SPC. The proposed thresholds of >5,000 *B. theta* cells per 100 ml and <5,000 *B. theta* cells per 100 ml were used as reference points to determine the likely sources of fecal pollution.
Environmental parameters

Turbidity (NTU), salinity (based upon the practical salinity scale), dissolved oxygen (mg/l), and temperature (°C) were measured at each site using a calibrated multi-probe instrument (YSI Inc., Yellow Springs, OH).

Rainfall data were obtained using the National Ocean and Atmospheric Association (NOAA) National Climatic Data Center (NCDC) station at the Michael J. Smith Field in Beaufort, NC and are available online (http://www.ncdc.noaa.gov/oac/ncdc.html). The starting time for each sampling event was reported and hourly rainfall prior to each sampling event was calculated for cumulative rainfall 12 hours prior, 24 hours prior, and 48 hours prior to the sampling event (Table 4.1.1).

The following rainfall categories were used for statistical comparisons (cumulative 48 hours), where No Rain (NR) conditions are defined as 0 cm (n=5), Moderate Rain (MR) conditions are defined as 0 cm to < 2.54 cm (n=7), and Heavy Rainfall (HR) are defined as having >2.54 cm (n=4; Table 4.1.1). The 2.54 cm (1 inch) gradations and the 48 hour cumulative rainfall window were used, respectively, due to previous research illustrating significant correlations by these groupings (Coulliette and Noble 2008).

Statistical analyses

Microsoft Office Excel 2003 was used to manage data and conduct basic calculations, while SPSS version 11.0 was used for statistical analyses. All datasets were assessed for normality and proper correlation or significance tests were applied (Howell 2002, Salkind 2004). All fecal indicator bacteria concentrations (EC, ENT, B. theta) were log\(_{10}\) transformed before analyses. For the remainder of this Chapter, \(n_E\) refers to the number of events for a specific
grouping (i.e. NR, MR, and HR), while \( n_S \) refers to the total number of samples collected and processed within the specific sampling event.

**Temporal analysis:** Events for which a complete time series (i.e. samples were collected with 20 minute intervals at the same site consecutively during a single storm event, denoted \( t_1, t_2, \) and \( t_3 \)) were grouped according to site (WC1, WC2, WC3, and WC4; \( n_E=10 \)). Events were then categorized by rainfall conditions, where for EC and ENT the following events were used for NR category: 1, 3, 4; MR category: 6 - 8, 10, 11; and HR category: 14, 15. For *B. theta*, the following events were used for the NR category: 1, 3, 4; MR category: 7, 8, 10, 11 and HR category: 14, 15. Lastly, the data were grouped by individual time points within each rain category (\( n=3 \)). Normality was assessed and traditional FIB (EC, ENT) had normal distributions for all sites, while *B. theta* had normal distributions for WC1, WC2, and WC3. Statistical analysis for the normal distributions included ANOVA one-way (two-tailed, \( \alpha=0.05 \)) with post-hoc Bonferroni for multiple comparisons. Ware Creek 4 (WC4) *B. theta* concentrations did not have a normal distribution and thus, Kruskal-Wallis was used to assess significant differences between time points.

For all of the time series data collected, we observed no statistically significant difference among FIB results, regardless of rainfall condition or site. Therefore, for the remainder of the analyses, these data were pooled and, for each site sampled (e.g. WC1) during each event, FIB results are a mean value for the three time series samples collected. Ware Creek 1 (WC1) and WC4 EC concentrations were used as examples to illustrate the low temporal variability that we observed for all FIB concentrations during the study (Figure 4.1.2).
Significant differences were not seen among the three sampling time points with respect to the rainfall categories (NR: n_E=3, n_S=9; MR: n_E=4, n_S=12, and HR: n_E=2, n_S=3). Ware Creek 1 did not show significant differences among time points (t1, t2, and t3) for NR events (F_{(2,6)}=0.857), MR events (F_{(2,9)}=0.146), or HR events (F_{(2,6)}=0.027). The overall average EC concentrations for the NR, MR, and HR rain categories were 8.82 x 10^2 (±0.08), 3.30 x 10^3 (±0.09), and 2.76 x 10^3 (±0.13), respectively. Ware Creek 4 did not show significance differences between time points (t1, t2, and t3) for NR events (F_{(2,6)}=0.116), MR events (F_{(2,9)}=0.034), or HR events (F_{(2,6)}=0.100). The overall average EC concentrations for the NR, MR, and HR rain categories were 7.02 x 10^1 (±0.15), 4.21 x 10^2 (±0.18), and 5.78 x 10^2 (±0.28), respectively. No significant differences among time points were seen for any of the Ware Creek sites for EC, ENT and B. theta (data not shown). Thus, all the data presented are mean values of the three time points for each site.

**Spatial analysis:** Fecal indicator bacteria (FIB) concentrations from each event (n_E=16) during the study were grouped according to their sampling locations, WC1, WC2, WC3, and WC4. The data for each site was separated according to rainfall categories (NR, MR, and HR). Selected events having three time points for each site were used for significances testing between sites and rain categories. For EC and ENT, the following events were used for NR category: 1, 3, 4; MR category: 6 - 8, 10, 11; and HR category: 14, 15. For B. theta, the following events were used for the NR category: 1, 3, 4; MR category: 7, 8, 10, 11; and HR category: 14, 15. All datasets were normally distributed, this significance testing used ANOVA (two-tailed, α=0.05) with post-hoc Bonferroni for multiple comparisons.
**Rainfall and FIB:** The overall mean FIB concentrations (EC, ENT, and *B. theta*) from each event were correlated to the 12, 24, and 48 hour cumulative rainfall prior to sampling using Spearman Rank statistical computation (rainfall data was not normally distributed; two-tailed, $\alpha=0.05$). Further statistical comparisons were conducted for rainfall categories and used sampling events that had three time points for each sites. For NR conditions, events 1, 3, and 4 were used for statistical significance. For MR conditions, events 6, 7, 8, 10 were used for statistical significance. For HR conditions, events 14 and 15 were used for statistical significance.

**Environmental parameter correlations:** All parameters were examined for normality prior to correlation analysis. Temperature, EC, and ENT showed normal distributions and thus, Pearson product correlation analysis was used for the comparisons within the three observations (two-tailed, $\alpha=0.05$). All remaining parameters did not have normal distributions and Spearman Rank correlation was used (two-tailed, $\alpha=0.05$).

**Resuspension analysis:** Data was separated into “windy” and “calm” storms, where “windy” storms were defined as having greater than or equal to 23 km/hr winds and less than 0.99 cm of rainfall within 48 hours. “Calm” storms ($n_S=30$) were defined as having less than 23 km/hr winds and greater than or equal to 0.99 cm of rainfall. Sampling events that met the “windy” storm ($n_S=48$) requirements were 1, 3, 4, and 6, while events categorized as “calm” storms were 7, 8, 13, and 16 (Table 4.1.1). Fecal indicator bacteria (EC, ENT, and *B. theta*), turbidity, and salinity were tested for significant differences between the groups using independent t-tests for normal distributed data (EC, ENT, salinity) comparisons and Mann-Whitney was used for data not normally distributed (turbidity), where significance was set at ($\alpha=0.05$)
RESULTS

Transport, loss, and loading of fecal indicator bacteria

Ware Creek is dramatically impacted by fecal contamination during rainfall events. As mentioned in the statistical temporal analysis (methods section), the temporal signal was homogeneous for FIB (EC, ENT, and \textit{B. theta}) concentrations according to rain groupings (NR, MR, and HR) and thereby, allowed for further investigation into transport in relation to loss, loading, and spatial distributions. The dilution effect across the transect was consistent for all FIB and across all rain categories, although the percent lost is variable depending on the indicator. Spatially, the shellfish harvesting site (WC4) resulted in lower FIB concentrations when less than 2.54 cm of rainfall occurred due to dilution effects. However, once rainfall exceeded 2.54 cm, runoff inundates the whole system and the traditional FIB (EC and ENT) concentrations are similar across the entire transect. The molecular FIB, \textit{B. theta}, was found in similar concentrations across all sampling locations and rain categories.

\textbf{Transport:} The dilution of FIB concentrations were approximately 100 MPN or cells per 100 ml for every 100 meters for EC, ENT, and \textit{B. theta} regardless of rain category (Figure 4.1.3; ENT data not shown). Average concentrations of FIB for rain categories (NR, MR, and HR) at each site are listed in Table 4.1.2. Statistical analysis showed that during NR (0 cm) conditions WC4 was significantly different from all other sites for EC (F_{(3,32)}=22.95, p<0.001) and ENT (F_{(3,32)}=6.35, p<0.005). Ware Creek 4 (WC4) resulted in significant differences as compared to WC1 and WC2 under MR (>0 to <2.54 cm) conditions for EC (F_{(3,56)}=5.09, p<0.005) and ENT (F_{(3,56)}=5.49, p<0.005). However, during HR (>2.54 cm) sites were not significantly different for EC (F_{(3,20)}=2.34) or ENT (F_{(3,20)}=0.26), concentrations. \textit{B. theta} resulted in similar differences for NR conditions, where WC4 was
significantly different than WC2 and WC3 ($F_{(3,31)}=6.84, p=0.001$). However, $B. \theta$eta concentrations between sites were not significantly different in MR ($F_{(3,44)}=1.77$) and HR ($F_{(3,19)}=1.16$) conditions.

Loss and loading: The percent losses from WC1 to WC4 (shellfish harvesting area) for EC concentrations were 93%, 86%, and 80% for NR, MR, and HR, respectively. The percent losses for ENT concentrations were 91%, 91%, and 50% for NR, MR, and HR, respectively. $B. \theta$eta experienced losses of 69%, 58%, and 81% for NR, MR, and HR, respectively. The average loading rates for WC1, where the stormwater runoff emptied into Ware Creek, was $2.14 \times 10^9$, $1.96 \times 10^9$ and $5.32 \times 10^8$ for EC (MPN per L per 10 min), ENT (MPN per L per 10 min), and $B. \theta$eta (cells per L per 10 min), respectively. When the dilution effect described in the transport section was applied (loss of 100 MPN or cells per 100 ml for every 100 meters) to the loading rates for EC, $1.50 \times 10^5$ MPN per 100 ml per 10 minutes will reach WC4 (shellfish harvesting area). When similar assumptions were met for ENT and $B. \theta$eta, $1.35 \times 10^5$ MPN and $3.76 \times 10^4$ cells per 100 ml per 10 minutes will reach WC4 (shellfish harvesting area).

Partitioning of the fecal signal

Regardless of site and rain category, all $B. \theta$eta concentrations were below 5,000 cells per 100 ml with one exception for WC2 during HR conditions (Figure 4.1.4). Even though current research is still ongoing regarding $B. \theta$eta as an alternate indicator, we speculate that concentrations of <5000 cells per 100 ml are indicative of waters that are not impacted significantly by human fecal contamination.

Sediment cores showed EC MPN per grams of $7.84 \times 10^1$, $7.71 \times 10^1$, $4.94 \times 10^1$, and $1.38 \times 10^0$ for WC1, WC2, WC3, and WC4, respectively. Enterococcus (ENT) sediment
cores resulted in $2.33 \times 10^3$, $2.77 \times 10^3$, $1.24 \times 10^3$, and $2.03 \times 10^1$ MPN per grams for WC1, WC2, WC3, and WC4, respectively. Using Total Suspended Solids (TSS) data to convert MPN per grams to MPN per 100 ml, EC sediment core data revealed that 0.01% to 0.03% of water column concentrations were due to bacteria attached to suspended solids. 

*Enterococcus* (ENT) sediment core data revealed that 0.08% to 0.53% of water column concentrations were due bacteria attached to suspended solids.

**Relationship of environmental parameters and resuspension with fecal indicator bacteria**

Observations from all sites represented a wide range of observations of turbidity (0 to 1321 NTU, n=146), salinity (0.05 to 32, n=148), temperature (11.04 to 35.08 °C, n=148), and DO (0 to 25.7 mg/L, n=148). Salinity had a significantly positive correlation with turbidity and temperature, while having a significantly negatively correlation with DO (Table 4.1.3). Turbidity was significantly correlated with DO and temperature, while DO showed a significant negative relationship with temperature (Table 4.1.3).

The FIB had significant positive relationships with one another (EC with ENT, n=151; EC with *B. theta*, n=140; ENT with *B. theta*, n=140; Table 4.1.3). Both EC and ENT showed significant negative relationships with salinity and DO, while EC was significantly positively correlated with temperature. *B. theta* had a negative relationship with DO, while resulting in a significant positive relationship with temperature.

Rainfall for 12, 24, and 48 hours prior to sampling were significantly correlated with EC (0.573, 0.543, 0.591; p<0.05) and ENT (0.705, 0.708, 0.698; p<0.01) for all data during the study period. *B. theta* did not have a significant relationship with rainfall (12 hr: 0.029, 24 hr: 0.021, 48 hr: 0.021).
*E. coli* (EC) and ENT revealed significantly different concentrations in “windy” versus “calm” storms (EC: \(t_{(75.25)} = 8.40, p<0.001\); ENT: \(t_{(76)} = 3.45, p=0.001\)). The mean concentrations for EC in “windy” and “calm” storms were \(3.33 \times 10^2 \pm 0.10\) and \(4.31 \times 10^3 \pm 0.09\) MPN per 100 ml, respectively. The mean concentrations for ENT in “windy” and “calm” storms were \(3.31 \times 10^2 \pm 0.12\) and \(1.53 \times 10^3 \pm 0.14\) MPN per 100 ml, respectively. Salinity \((t_{(76)}=-0.323)\) and turbidity \((z=-0.216)\) were not significantly different.

**DISCUSSION**

The recent closure of Ware Creek during the research study period provided a rare opportunity to examine the potential application of the tributary as a baseline model for the additional TMDL developments within the NPRE. Ware Creek is close in proximity and illustrated similar characteristics to the NPRE, such as stormwater runoff being a major contributor of fecal pollution and persistence of traditional fecal indicator bacteria FIB (Coulliette and Noble 2008). Thus, Ware Creek is a valid baseline tributary in which to model additional NPRE TMDLs. It should be noted that the study period (November 2006 to November 2007) was experiencing exceptionally dry conditions with 26.7 cm (10.5 inches) below the historic average for the estuary (1948 to 2006 averages from the State Climate Office of North Carolina, NC Climate Retrieval and Observations Network Of the Southeast (NC CRONOS) Database; Morehead City 2 WNW, ID# 315830). It is suggested that adjustments be made in the model parameters or future research should be conducted during “normal” rainfall conditions. With that being acknowledged, the research findings demonstrated Ware Creeks dynamics when non-point source (NPS) pollution enters the system (i.e. falling tide), revealed the amplitude of dilution and loss of FIB along the
transect, and partitioned the FIB signal using *B. theta* and sediment data, which are all vital components for future model development.

This research clearly shows that rainfall transported pollution from the neighboring land into Ware Creek tributary. The results also showed a 1:1:1 dilution effect (MPN or cells per ml per meter) for all rain categories (NR, MR, HR) and FIB (EC, ENT, *B. theta*; Figure 4.1.3). Due to ratio’s uniformity, dilution is believed to be the main function of the decrease seen versus settling or decay. This ratio is valuable for the baseline model, although the ratio could change for a larger transect than the one used for this study (700 m). Loading estimates for moderate rain conditions (>0 cm to <2.54 cm), when assuming the 1:1:1 dilution effect and constant flow, show that approximately 10,000 to 100,000 MPN or cells per 100 ml of FIB will reach the shellfish harvesting area (WC4) every 10 minutes. Although volume estimates would improve this analysis, it is reasonable to envision how quickly FIB concentrations would escalate and surpass water quality requirements.

Ware Creek showed no temporal signal (Figure 4.1.2), while spatially, the upstream location concentrations (WC1, WC2, WC3) were consistent within individual rain categories (NR, MR, HR; Table 4.1.2). The focus of FC TMDL development is to understand the “allowable” input into the waterbody, so conducting research during falling tide (~3 hours) incorporated the majority of FIB entering Ware Creek as nonpoint source runoff. Therefore, knowing that the concentrations of FIB do not vary significantly during this window (falling tide), allows the model to adjust one parameter of the individual area’s input concentration versus having to calculate a hydrological fluctuation function if a temporal signal was present. In addition, the quantification of FIB along the upstream portions of the transect illustrated consistent concentrations within similar rain categories (NR, MR, HR) and sites,
as well as between sites (WC1, WC2, WC3) for EC and ENT (Table 4.1.2). This provides a spatial baseline for the rain categories, in which additional parameters can be added, such as the significant dilution effect seen once the transect opens up at the shellfish harvesting area (WC4). For example, EC has an overall average of 86% loss in concentration from the source (WC1) to the shellfish harvesting area (WC4). The common factor accounting for significant differences was rainfall, as it influences the entering FIB concentrations and mutes any spatial signal after >2.54 cm of rainfall.

The important aspect of FIB water quality studies is whether the contamination is from a human source, thus containing potential human pathogens that may pose a public health risk. The incorporation of \textit{B. theta} as a molecular FIB provided insight of likely sources of fecal pollution in Ware Creek. \textit{B. theta} was significantly correlated with \textit{E. coli} (EC) and \textit{Enterococcus} (ENT) (Table 4.1.3), showed a similar temporal trend, demonstrated a significantly negative relationship with DO (mg/l), as well as a significantly positive relationship with temperature (Table 4.1.3.). In addition to the advantage of real-time processing for \textit{B. theta} (<3 hours) as compared to traditional FIB (18 – 24 hours), these findings support \textit{B. theta} as a reliable and comparative FIB to EC and ENT, as well as its’ anaerobic nature leading to detection of recent fecal pollution. The “partitioning” threshold of concentrations above 5,000 cells per 100 ml has been proposed as an indication of potential human sources. \textit{B. theta} is present at 100X more in the human gut than EC or ENT, found in much lower concentrations in warm-blooded animals, hence the reasoning for the proposed threshold for 5,000 cells for 100ml. This threshold was never exceeded during the study.
In addition to partitioning the likely sources entering from the neighboring land, it was important to assess the possibility of persistent and reservoir populations. Previous research has shown sediments as a source of such populations (Fries et al. 2008; Byappanahalli and Fujioka 2004; Shibata et al. 2004; and Desmarais et al. 2002). The concentrations found in sediment ranged from approximately 1.3 to 78.4 MPN per gram and 20.3 to 277.0 MPN per gram for EC and ENT (n=12; dry weight), respectively. These findings are within the ranges found in NPRE sediments, where approximately 2.5 to 762.7 MPN per gram and 2.5 to 1072.7 MPN per gram (n=4; dry weight) were found for EC and ENT, respectively. Similar results were observed in the nearby Neuse River Estuary with particle attached ENT (Fries et al. 2007). Although, reservoir populations of traditional FIB are found, the FIB concentrations estimated in the water column due to suspended solids in NR conditions (0 cm rain) were determined to be negligible (<1%). Further work should be conducted to investigate how MR and HR conditions would impact these results, as resuspension of sediments are also a vital factor to include in a model.

Rainfall demonstrated significant relationships with *E. coli* (EC) and *Enterococcus* (ENT) for 12, 24, and 48 hour groups. The significant inverse relationship of salinity with EC and ENT also illustrated that as freshwater via stormwater runoff decreased salinity, the NPS pollution was transporting fecal contamination into the tributary. The additional comparison of “calm” versus “windy,” an analysis to determine whether runoff (“calm” storm) or resuspension (“windy” storm) were accountable for increased FIB concentrations, showed that “calm” storms produced significantly higher EC and ENT concentrations. A related study in the NPRE using Bayesian Maximum Entropy (BME) developed a covariance model that accounted 50 to 60% of the variability in FIB concentrations was due to the 4-day
antecedent (day of sampling and three days prior; Coulliette et al., In Prep.). These lines of evidence emphasize the importance of rainfall and the subsequent inclusion into a model, as well as the need to understand the transport of contamination via this NPS stormwater runoff.

This detailed study quantified several parameters for a baseline FC TMDL (temporal signal, spatial signal, dilution effect, driving factors, etc.). During the study, a majority of the events exceeded the FC shellfish harvesting allowable threshold of 14 MPN per 100 ml. Meanwhile, during times of heavy rainfall Enterococcus exceeded the threshold of 104 MPN per 100 ml for recreational waters. However, acknowledging that this fecal contamination may not be from a human source based upon B. theta results, this research emphasizes the need to further investigate sources and reasoning for closures of valuable shellfish harvesting waters. Although, regardless of source, it is clear that NPS stormwater runoff needs to be controlled. Best management practices are necessary and vital for remediation and long-term protection of this tributary.
FIGURES

Figure 4.1.1 Ware Creek tributary. The sampling locations are within the Newport River Estuary in eastern North Carolina.

**APPROXIMATE DISTANCE**

- WC1:WC2 = 106 m
- WC2:WC3 = 124 m
- WC3:WC4 = 538 m

From source (WC1):
- WC2 = 105 m
- WC3 = 220 m
- WC4 = 700 m

**DOWNSTREAM**
Figure 4.1.2 Temporal signal of *E. coli* (EC). The figures below represent Ware Creek 1 and Ware Creek 4 (NR: n_E=3, n_S=9; MR: n_E=4, n_S=12, and HR: n_E=2, n_S=3). Error bars are ±1 standard error.

(a) Ware Creek 1

(b) Ware Creek 4
Figure 4.1.3 Transport and dilution effect on *E. coli* (EC) and *B. theta*. Error bars are +1 standard error.

(a) *E. coli*

(b) *B. theta*
Figure 4.1.4 Concentrations of *B. theta* in Ware Creek. The error bars do not appear at this scale.
TABLES

Table 4.1.1. Ware Creek sampling events and associated rainfall parameters.

<table>
<thead>
<tr>
<th>Event ID</th>
<th>Rainfall (cm) 12/24/48 prior to sampling</th>
<th>Rain Category</th>
<th>Wind (km/hr)</th>
<th>Storm Category</th>
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<tr>
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<td>0/0/0</td>
<td>NR</td>
<td>23</td>
<td>Windy</td>
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<tr>
<td>2</td>
<td>0/0/0</td>
<td>NR</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0/0/0</td>
<td>NR</td>
<td>23</td>
<td>Windy</td>
</tr>
<tr>
<td>4</td>
<td>0/0/0</td>
<td>NR</td>
<td>27</td>
<td>Windy</td>
</tr>
<tr>
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<td>0/0/0</td>
<td>NR</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.74/0.74/0.74</td>
<td>MR</td>
<td>23</td>
<td>Windy</td>
</tr>
<tr>
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<td>MR</td>
<td>10</td>
<td>Calm</td>
</tr>
<tr>
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</tr>
<tr>
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<td></td>
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<tr>
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<td>MR</td>
<td>35</td>
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<td>10</td>
<td>Calm</td>
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<td>1.68/4.55/4.55</td>
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<td>0/18.29/18.83</td>
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<td>21</td>
<td>Calm</td>
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Table 4.1.2. Fecal indicator bacteria (FIB) and rainfall. The following are shown below: (a) *E. coli* MPN per 100 ml (EC), (b) *Enterococcus* MPN per 100 ml (ENT), and (c) *B. theta* cells per 100 ml (± 1 standard error). Statistically differences are noted by \(^A\) for being significantly different from WC1, WC2, and WC3, \(^B\) for being significantly different from WC1 and WC2; and \(^C\) for being statistically different from WC2 and WC3 using ANOVA (two-tailed, \(\alpha=0.05\)) with post-hoc Bonferroni for multiple comparisons.

(a) EC

<table>
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<td>(n_r=5, n_s=48)</td>
<td>(n_r=7, n_s=74)</td>
<td>(n_r=4, n_s=30)</td>
</tr>
</tbody>
</table>
| WC1    | \(8.82 \times 10^2 \, (\pm 0.08)\)  \\
|        | \(n=12\) | \(3.30 \times 10^3 \, (\pm 0.09)\)  \\
|        |       | \(n=19\) | \(2.76 \times 10^3 \, (\pm 0.13)\)
|        |       |       | \(n=9\)
| WC2    | \(9.17 \times 10^2 \, (\pm 0.09)\)  \\
|        | \(n=12\) | \(2.94 \times 10^3 \, (\pm 0.16)\)  \\
|        |       | \(n=19\) | \(2.50 \times 10^3 \, (\pm 0.18)\)
|        |       |       | \(n=7\)
| WC3    | \(1.01 \times 10^3 \, (\pm 0.13)\)  \\
|        | \(n=12\) | \(1.81 \times 10^3 \, (\pm 0.16)\)  \\
|        |       | \(n=19\) | \(2.35 \times 10^3 \, (\pm 0.16)\)
|        |       |       | \(n=7\)
| WC4\(^A\) | \(7.02 \times 10^1 \, (\pm 0.15)\)  \\
|        | \(n=12\) | \(4.21 \times 10^2 \, (\pm 0.18)\)  \\
|        |       | \(n=17\) | \(5.78 \times 10^2 \, (\pm 0.28)\)
|        |       |       | \(n=7\)
| AVERAGE | \(5.03 \times 10^2 \, (\pm 0.09)\) | \(1.70 \times 10^3 \, (\pm 0.08)\)  \\
|        |       |       | \(1.87 \times 10^3 \, (\pm 0.10)\)

(b) ENT

<table>
<thead>
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<th>MR</th>
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</thead>
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<td>(n_r=7, n_s=74)</td>
<td>(n_r=4, n_s=30)</td>
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</tbody>
</table>
| WC1    | \(7.86 \times 10^2 \, (\pm 0.18)\)  \\
|        | \(n=12\) | \(3.35 \times 10^3 \, (\pm 0.16)\)  \\
|        |       | \(n=19\) | \(6.58 \times 10^3 \, (\pm 0.26)\)
|        |       |       | \(n=9\)
| WC2    | \(4.94 \times 10^2 \, (\pm 0.17)\)  \\
|        | \(n=12\) | \(2.32 \times 10^3 \, (\pm 0.15)\)  \\
|        |       | \(n=19\) | \(4.37 \times 10^3 \, (\pm 0.27)\)
|        |       |       | \(n=7\)
| WC3    | \(5.04 \times 10^2 \, (\pm 0.15)\)  \\
|        | \(n=12\) | \(1.36 \times 10^3 \, (\pm 0.18)\)  \\
|        |       | \(n=19\) | \(4.78 \times 10^3 \, (\pm 0.25)\)
|        |       |       | \(n=7\)
| WC4\(^A\) | \(4.73 \times 10^1 \, (\pm 0.19)\)  \\
|        | \(n=12\) | \(1.74 \times 10^2 \, (\pm 0.27)\)  \\
|        |       | \(n=17\) | \(2.35 \times 10^2 \, (\pm 0.31)\)
|        |       |       | \(n=7\)
| AVERAGE | \(2.53 \times 10^2 \, (\pm 0.13)\) | \(1.29 \times 10^3 \, (\pm 0.11)\)  \\
|        |       |       | \(4.35 \times 10^3 \, (\pm 0.13)\)
(c) *B. theta*

<table>
<thead>
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<th>NR(^A)</th>
<th>MR(^B)</th>
<th>HR</th>
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<td>(n_E=7, n_S=72)</td>
<td>(n_E=4, n_S=28)</td>
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<td>(2.67 \times 10^3 (\pm 0.04)) (n=19)</td>
<td>(4.52 \times 10^3 (\pm 0.18)) (n=8)</td>
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<tr>
<td>WC2</td>
<td>(4.15 \times 10^3 (\pm 0.15)) (n=10)</td>
<td>(2.33 \times 10^3 (\pm 0.20)) (n=19)</td>
<td>(7.27 \times 10^3 (\pm 0.09)) (n=7)</td>
</tr>
<tr>
<td>WC3</td>
<td>(3.26 \times 10^3 (\pm 0.13)) (n=10)</td>
<td>(2.67 \times 10^3 (\pm 0.10)) (n=19)</td>
<td>(5.36 \times 10^3 (\pm 0.09)) (n=7)</td>
</tr>
<tr>
<td>WC4</td>
<td>(\text{C}4.65 \times 10^2 (\pm 0.12)) (n=9)</td>
<td>(1.98 \times 10^3 (\pm 0.12)) (n=15)</td>
<td>(3.31 \times 10^3 (\pm 0.14)) (n=6)</td>
</tr>
<tr>
<td><strong>AVERAGE</strong></td>
<td>(1.91 \times 10^3 (\pm 0.09))</td>
<td>(2.18 \times 10^3 (\pm 0.08))</td>
<td>(3.71 \times 10^3 (\pm 0.14))</td>
</tr>
</tbody>
</table>
Table 4.1.3. Correlations of environmental parameters and FIB in Ware Creek. Statistically significant values were determined for all correlations ($\alpha = 0.05$, two-tailed) using the Spearman rank correlation coefficient (SR) analysis, except for EC:ENT and FIB:Temperature when Pearson product moment was used ($\alpha = 0.05$, two-tailed).

<table>
<thead>
<tr>
<th></th>
<th>Salinity (NTU)</th>
<th>Turbidity (mg/l)</th>
<th>DO (mg/l)</th>
<th>Temperature ($^\circ$C)</th>
<th>EC MPN/100 ml</th>
<th>ENT MPN/100 ml</th>
<th>B. theta cells/ 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salinity</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
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<td>1.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DO (mg/l)</td>
<td>-0.430</td>
<td>0.163</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Temperature ($^\circ$C)</td>
<td>0.435</td>
<td>0.237</td>
<td>-0.534</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EC MPN/100 ml</td>
<td>-0.251</td>
<td>0.109</td>
<td>-0.477</td>
<td>0.264</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ENT MPN/100 ml</td>
<td>-0.340</td>
<td>0.156</td>
<td>-0.354</td>
<td>-0.005</td>
<td>0.807</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>B. theta cells/ 100 ml</td>
<td>-0.072</td>
<td>0.084</td>
<td>-0.189</td>
<td>0.275</td>
<td>0.424</td>
<td>0.318</td>
<td>1.0</td>
</tr>
</tbody>
</table>
REFERENCES


Blackwood, A.D. and R.T. Noble (In Prep). Development of a rapid Quantitative PCR method for the quantification of *Bacteroides thetaiotaomicron* an alternative indicator of fecal pollution. To be submitted to *Journal of Applied Microbiology*.


CHAPTER 4.2

COMPARISON OF FECAL COLIFORM AND ESHERICHIA COLI WATER QUALITY DETECTION METHODS

INTRODUCTION

North Carolina Department of Environment and Natural Resources, Shellfish Sanitation Section (NCDENR-SSS) follows the National Shellfish Sanitation Program (NSSP) recommendations of using fecal coliforms (FC) to determine the open or closed status of waters for shellfish harvesting. For a shellfish harvesting area to be classified as “open,” the FC median or geometric mean can not exceed 14 most probable number (MPN) or colony forming unit (CFU) per 100 ml (NSSP 2005). In addition, depending on the method used, no more than 10% of the samples can exceed 28 to 43 MPN per 100 ml (NSSP 2005). There are five NSSP approved methods for the detection of FC in shellfish growing and surveys and classification: A-1M decimal dilution MPN, A-1M 12-tube single dilution MPN, American Public Health Association (APHA) decimal dilution MPN, APHA 12-tube single dilution MPN, and mTEC (NSSP 2005). The NCDENR-SSS currently uses the A-1
dilution procedure, which is a multiple tube fermentation (MTF) procedure that generates data in MPN per 100 ml (A-1M 1990).

Water quality managers with the NCDENR-SSS are in the process of converting completely over from the use of the MTF method to a membrane filtration (MF) method, mTEC. Currently, water samples that are being collected for FC enumeration are being analyzed using both the MTF A-1 and MF mTEC methods during specified testing periods (NCDENR-SSS, Patricia Fowler pers. communication). Previous research has shown mTEC to yield comparable data, as well as the method being less expensive and time consuming (e.g. Rippey et al. 1987). This methodology (mTEC) can also be used to enumerate *E. coli* (EC) through a confirmation step (Rippey et al. 1987), thus providing additional information for assessing water quality in NC waters.

Although the NSSP will most likely continue to approve FC as the indicator of choice for managing shellfish harvesting waters, numerous water quality laboratories have identified the possibility of EC specific tests, such as or Colilert®-18 (defined substrate technology test kits from IDEXX® Laboratories, Inc.) as a suitable replacement for the measurement of the entire FC group. Thus, understanding the relationship between EC specific tests, such as Colilert®-18, and traditionally used methods for enumeration of FC, such as mTEC, is important.

Colilert®-18 can be used to enumerate both total coliforms and EC, has a wider effective quantification range than traditional MTF tests due to the increased number of wells considered in the MPN calculation, and requires only minutes of setup and processing time. Although Colilert®-18 specifically detects EC and not all additional members of the FC group (such as *Citrobacter*, *Serratia*, and *Klebsiella*), previous analyses of estuarine water
samples taken throughout eastern NC have shown that 93% of the FC are EC (n=3020, Kirby-Smith and Noble, unpublished data). Those results indicate that Colilert®-18 would yield a conservative estimate of the FC concentration, but results would not be statistically different from FC results.

In an effort to aid the NCDENR in the advancement of shellfish harvesting water quality methods, water samples taken in Ware Creek were processed using mTEC and Colilert®-18. Results demonstrate (1) the relationship between FC and EC enumeration determined by mTEC and (2) the comparison of EC enumeration using Colilert®-18 versus EC enumeration using mTEC.

MATERIALS AND METHODS

Study site

Ware Creek is located in the Northeast portion of the NPRE in the town of Beaufort and is in an area classified as Area E-4 by NC Department of Environment and Natural Resources-Shellfish Sanitation Section (NCDENR-SSS 2005; Fig. 4.1.1). Ware Creek was a previously approved area for shellfish harvesting until 2006 when the status was changed to “prohibited” due to levels of FC exceeding the water quality standards (NCDENR-SSS, Patricia Fowler pers. communication). Although land-use alterations occurred during the study (i.e. clearing of land, residential development), the area is categorized as low density residential surrounded by forested and row-crop agricultural land. The creek is tidally influenced and likely fecal contamination sources stem from wildlife (deer, peacocks), agricultural drainage (soybean), domestic animals (dogs, cats), and human sewage (failing septic systems). Previous surveys confirmed Ware Creek as a main contributor of non-point source pollution, especially during times of heavy rainfall (Kirby-Smith unpublished).
Sample locations

Sampling locations created a transect from where stormwater runoff entered Ware Creek and flushed into the closed shellfish harvesting area (Figure 4.1.1). The sites are directed downstream, where Ware Creek 1 (WC1) empties stormwater runoff from the neighboring watershed into the beginning of the transect. Ware Creek 2 (WC2) and Ware Creek 3 (WC3) continues the transect to Ware Creek 4 (WC4), which is the previously approved shellfish harvesting area. Relatively low density residential development flanked the transect. The transect is 700 m with the specific distances between sites and source shown in Figure 4.1.1.

Sample collection

Between November 2006 and November 2007, a total of 153 surface water samples were collected along the transect (upstream to downstream) of WC1, WC2, WC3, and WC4 (Figure 4.1.1). For every sampling event, three one liter surface water samples were collected within 3 hours of low tide in order to obtain samples that are impacted minimally by dilution from marine waters. In order to characterize the temporal variability associated with the FIB results, three time series (t1, t2, and t3) along the transect going downstream from WC1 to WC4 (total of three samples per site).

mTEC assay

All samples were tested in duplicate for FC and EC following the methods as described in Rippey et al. (1987). The mTEC procedure included serial dilution of the water samples, membrane filtration of the serial dilutions, and a series of incubations (35°C for 2 hours, 44.5°C for 20 to 22 hours). After incubation, yellow colonies were counted as FC and those remaining yellow after exposure to the urease agent for 10 minutes were counted as EC.
Results were reported as CFU per 100 ml. The dilution with a countable range of colonies (20 to 200) was used for determining final concentrations.

*Colilert®*-18 assay

All samples were tested for EC using the defined substrate technology test kit, *Colilert®*-18 (IDEXX® Laboratories, Westbrook, ME). Conversion of positive wells from these tests to a MPN value was conducted following Hurley and Roscoe (1983). Although literature cites false-positives occurring in tropical and subtropical marine and estuarine waters (Pisciotta et al. 2002), studies conducted in NC coastal estuarine waters have not demonstrated any measurable rate of false-positive results using *Colilert®*-18 for EC enumeration (Noble et al., unpublished).

*Statistical analyses*

Microsoft Office Excel 2003 was used to manage data and conduct basic calculations (i.e. averages, linear equation), while SPSS version 11.0 was used for statistical analyses (i.e. normality, correlations). All datasets were assessed for normality and the proper correlation analysis was applied (Howell 2002, Salkind 2004). All concentrations were $\log_{10}$ transformed before analyses. All datasets were normal and the Pearson product moment correlation (PP) was used to assess significant correlations. A significant relationship was defined by an alpha ($\alpha$) of 0.05 (two-tailed).

**RESULTS AND DISCUSSION**

The detection of FC and EC using mTEC were significantly correlated ($r^2 = 1.0$, $p<0.001$).

The slope of the linear regression shows that 96% of EC detected were also FC (Figure 4.2.1) with a range of 76% to 100% (Table 4.2.1). Previous research in NC estuarine waters has also reported that 93% of the FC are EC (n=3020, Kirby-Smith and Noble, unpublished)
data). These results indicate that EC is the predominant member of the FC population in NC estuarine waters, and that using EC as a proxy for FC would be a viable alternative.

The enumeration of EC via Colilert®-18 and mTEC was also significantly correlated ($r^2 = 0.866$, $p<0.001$). The slope illustrates that Colilert®-18 enumeration was generally higher than mTEC (for every 10 MPN per 100 ml of EC measured via Colilert®-18, there were 8.6 MPN per 100 ml EC measured via mTEC). Researchers have noted that MF methods can provide lower numbers than other methods, specifically in more turbid waters, due to interference of total suspended solids for the formation of colonies on membrane filters. Given the relatively high concentration of total suspended solids that can be found in Ware Creek waters (0 to 1322 NTU), we hypothesize that Colilert®-18 might be a superior means for enumerating EC in these waters. The statistically sound performance of IDEXX technology, specifically Colilert®-18, makes it an attractive water quality method.
Figure 4.2.1 Comparison of mTEC detection of fecal coliforms (FC) and *E. coli* (EC; n = 144). The error bars represent ±1 standard error.
Figure 4.2.2 Comparison of Colilert®-18 and mTEC methods for measuring EC (n = 144). The error bars represents ±1 standard error.

$y = 0.79x + 0.40$

$r^2 = 0.866$ (p<0.001)
Table 4.2.1 Sampling events, sample sizes, and the EC:FC ratio (mTEC).

<table>
<thead>
<tr>
<th>Event</th>
<th>Sample Sizes (time points, total event samples)</th>
<th>EC/ FC ratio</th>
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<tbody>
<tr>
<td>3</td>
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</tr>
<tr>
<td>4</td>
<td>4, 12</td>
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</tr>
<tr>
<td>5</td>
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</tr>
<tr>
<td>6</td>
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</tr>
<tr>
<td>7</td>
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</tr>
<tr>
<td>8</td>
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</tr>
<tr>
<td>16</td>
<td>4, 4</td>
<td>0.97</td>
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</table>
REFERENCES


INTRODUCTION

The genus *Campylobacter* contains four thermotolerant species (*Campylobacter jejuni, Campylobacter coli, Campylobacter lari,* and *Campylobacter upsaliensis*) that are bacterial human pathogens. These pathogens cause gastrointestinal illness (Thomas et al. 1999) at an approximate infectious dose of 500 organisms (Robinson 1981). Specifically, *C. jejuni* is listed as the leading cause of bacterial illness in the United States (U.S.) with an estimated two to four million cases per year (Center for Disease Control and Prevention). In general, thermotolerant *Campylobacter* spp. were to blame for 36 outbreaks due to contaminated water supplies and raw milk from 1978 to 1987, as well as 38 outbreaks from 1988 to 1996 due to contaminated “other” foods (i.e. undercooked poultry; Nachamkin and Blaser 2000). Improvements in home kitchen practices, pasteurization processes, and chlorination practices have reduced foodborne and waterborne (drinking water supplies) outbreaks (Nachamkin and Blaser 2000). However, multiple European studies show the

Limited research has been conducted regarding thermotolerant Campylobacter spp. in U.S. environmental waters.

The Newport River Estuary (NPRE) is a North Carolina (NC) coastal estuarine system (453.25 km²) within the White Oak River Basin (Figure 2.1, NCDENR-SSS 2005). The NPRE is environmentally (i.e. seagrass beds, endangered/threatened aquatic species), economically (i.e. shellfish industry), and recreationally (i.e. tourism, boating) an important coastal waterbody in NC. Between 1990 and 2000, the towns neighboring the NPRE (Morehead City and Newport, NC) have shown population increases of 27.2% and 33.1%, respectively (NCSD 2000). The NPRE is also experiencing water quality degradation, exceeding the (14 MPN or CFU per 100 ml) fecal coliform threshold for shellfish harvesting waters (NSSP 2005), and is currently on the 319(d) list for impairment. Ware Creek (WC) is a tributary of the NPRE and was once a popular area for shellfish harvesting (Figure 4.1.1). WC was closed to harvesting in 2006 due to fecal coliform exceedances.

Previous water quality studies in the NPRE and WC examined the distribution and dynamics of fecal indicator bacteria (FIB), E. coli (EC) and Enterococcus (ENT). This study was designed to investigate whether human pathogens were a part of this fecal contamination. Campylobacter spp. were chosen due to their association with avian reservoirs (Waldenstrom et al. 2002; Engvall et al. 2002; Koenradd et al. 1997), which are highly prevalent in eastern NC and have the potential for escalated prevalence of
*Campylobacter* spp. during the shellfish harvesting season (October to March; Wilson and Moore 1996; Jones et al. 1990; Bolton et al. 1987). Estuarine water samples collected in the NPRE and WC from February 2006 to July 2007 were analyzed for the presence of *Campylobacter* spp. by traditional culture and molecular methods, and their relationships with FIB and environmental conditions were examined.

**MATERIALS AND METHODS**

**Study sites and sampling locations**

**Newport River Estuary**: The NPRE (Figure 2.1) is located north of Morehead City and Beaufort, NC and is in an area classified as Area E-4 by the NC Department of Environment and Natural Resources-Shellfish Sanitation Section (NCDENR-SSS 2005). This estuary has an average depth of 1 m and is a well-mixed system with an average residence time of 6 days or 12 tidal cycles. Flushing stems from the Atlantic Ocean and is controlled through the Beaufort Inlet (Kirby-Smith and Costlow 1989). The surrounding land-uses consist of approximately 45% forestland, 38% wetlands, 9% residential, 5% bays/estuaries, and 3% cropland (NCDENR-SSS 2005). There are also two point-source discharges (wastewater treatment plants). Associated with varied land-uses are sources of fecal contamination including wildlife (deer, raccoon, bear, or waterfowl), small farm operations (horse, cow, hog), and agricultural drainage (animal biosolids application). The most likely sources of human contamination are subdivision stormwater runoff, septic tank failure, and treated wastewater from the Morehead City and Newport Wastewater Treatment Plants (NCDENR-SSS 2005, NCDENR-SSS 2000).

**Ware Creek**: Ware Creek is located in the Northeast portion of the NPRE in the town of Beaufort, NC and is in an area classified as Area E-4 by the NCDENR-SSS (NCDENR-
Ware Creek was previously an approved area for shellfish harvesting until 2006 when the status was changed to “prohibited” due to levels of fecal coliforms (FC) exceeding water quality standards (Patti Fowler, personal comm.). Although land-use alterations occurred during the study (i.e. clearing of land, residential development), the area is categorized as low density residential with surrounding forested and agricultural land. The creek is tidally influenced and possible fecal contamination sources are wildlife (deer, peacocks), agricultural drainage (soy bean), domestic animals (dogs, cats), and human sewage (failing septic systems). Previous surveys have confirmed Ware Creek as a main contributor of non-point source pollution, especially during times of heavy rainfall (Kirby-Smith unpublished).

Sample collection: Between February 2006 and July 2007, a total of 16 surface water samples were collected from four different sites in the NPRE (n=11) and two sites in WC (n=5). Sampling sites for the NPRE (Recreational Site (RS), Calico Creek (CC), Closure Line (CL), and Harlowe Creek (HC)) were chosen based on existing NCDENR-SSS stations and on-going sanitary surveys (2000). Chosen sites were located in areas near land and had avian influences. The sampling sites for WC (Yarborough Dock (YD) and shellfish sanitation section Ware Creek sampling site (SSS-WC)) were surrounded by avian feeding areas. In order to collect samples with minimal dilution from marine waters, one liter samples were collected within 3 hours of low tide. Water samples were collected in sterilized containers following sampling techniques outlined in standard methods (APHA 2005), placed on ice, and transported immediately to the University of North Carolina at Chapel Hill, Institute of Marine Sciences in Morehead City, NC for processing.
Campylobacter spp. analysis

Isolation and enrichment: For each surface water sample, 500 to 1000 ml were vacuum filtered through replicate 47 mm, 0.45 um Zetaphor® filters (Mathewson et al. 1983; CUNO, Inc., Meriden, CT) using a filter funnel and receiver (Nalgene, Inc). The filters were placed in a total volume of 10 ml of Prestons broth (Bolton and Robertson 1982a) containing 2 ml of Campylobacter Growth Supplement (Oxoid, Hampshire, England), 4 ml of an antibiotic solution (0.000119 mg of polymyxin B sulfate, 5 mg of rifampicin, 5 mg of trimethoprim lactate, 5 mg of amphotericin in dionized H₂O), and 50 ml of laked horse blood (Hemostat Laboratories, Dixon, CA). The samples were incubated under microaerophilic conditions at 37°C for 4 hours (for resuscitation of injured cells) followed by 44 hours at 42°C. One milliliter of the primary enrichment was subjected to a secondary enrichment in a total volume of 9 ml of fresh Preston broth containing the mentioned additives, and was incubated microaerophilically at 42°C for 24 hours to reduce the likelihood of molecular detection of dead cells. Fifty microliters of the secondary enrichment was subcultured onto Columbia blood agar (CBA with five antimicrobics and 10% Sheep Blood; Becton, Dickinson and Company, BBL™, Sparks, MD) and incubated microaerophilically at 42°C. To ensure pure cultures, isolated colonies were subcultured onto CBA one to two additional times and incubated microaerophilically at 42°C for 24 hours. Purified colonies were subjected to biochemical tests and secondary enrichments were subjected to molecular analysis. Controls were run in parallel with the isolation and enrichment samples and included positive controls (Source: Collette Fitzgerald of the Center for Disease Control and Prevention; C. jejuni (D5014), C. coli (D5083), or C. lari (D0558)), as well as a sterility negative control.
**Biochemical analyses:** Isolated colonies were tested for oxidase and catalase activity (BBL™ Reagent Droppers, Sparks, MD). Colonies that were oxidase and catalase positive were identified as presumptive positive *Campylobacter* spp.

**Molecular analyses:** One milliliter aliquots of secondary enrichments were washed using a series of centrifugation steps at 7000 rpm for 20 minutes with 1X Phosphate Buffered Saline (PBS) (Savill et al. 2001). To release DNA, the washed cells were heat shocked at 100°C for 12 minutes (Savill et al. 2001). Centrifugation followed to pellet cell debris, leaving sample DNA suspended in the supernatant (Savill et al. 2001). The lysed cells were then stored at -80°C until PCR processing. A multiplex set of primers were used to detect portions of *Campylobacter* 23S rRNA enabling both genus and species identification (Wong et al. 2004). *Therm* primers identified the presence of thermophilic *Campylobacter* (246 bp band), while *lpxA* primers targeted *C. jejuni* (99 bp band) and *ceuE* primers target *C. coli* (695 bp band; Wong et al. 2004). PCR products were visualized via gel electrophoresis using ethidium bromide. The detection limit for the multiplex PCR is <6 MPN per poultry pack (rinse of poultry packaging; Wong et al. 2004). Samples positive for the *Therm* gene but not *C. jejuni* or *C. coli* were subjected to PCR using *C. lari* specific primers. These primers target the 23 rRNA and positive samples should show the thermotolerant band (222 bp) and *C. lari* band (177 bp; modified Eyers et al. 1993; Eyers et al. 1994). The detection limit for the *C. lari* PCR corresponds to twelve bacteria and as little as 0.062 pg of DNA can be visualized on the agarose gel (Eyers et al. 1993).

**Fecal indicator bacteria analyses**

All samples were tested for *E. coli* (EC) and *Enterococcus* (ENT) using the defined substrate technology test kits, Colilert®-18 and Enterolert® (IDEXX® Laboratories, Westbrook, ME). Conversion of positive wells from these tests to a MPN value was conducted following
Hurley and Roscoe (1983). Although false-positives have been documented in tropical and subtropical marine and estuarine waters (Pisciotta et al. 2002), studies conducted in NC coastal estuarine waters have not demonstrated any false-positive results using Colilert®-18 for EC enumeration (Noble et al., unpublished). In addition, previous analyses of estuarine water samples taken throughout eastern NC have shown that 93% of the FC are EC (n=3020, Kirby-Smith and Noble, unpublished data). Thus, for the purposes of this study we consider our EC measurements to be conservative representations of FC concentrations.

Environmental parameter measurements: Turbidity (NTU), salinity (based upon the practical salinity scale), dissolved oxygen (mg/l), and temperature (°C) were measured at each site using a calibrated multi-probe instrument (YSI Inc., Yellow Springs, OH). Due to the heterogeneous nature of rainfall in coastal NC, daily rainfall data were collected from three rain gauges situated for full coverage of the NPRE (Figure 2.1a). Rain gauge “A” is located at the Michael J. Smith Field Airport in Beaufort NC, is maintained by the National Ocean and Atmospheric Association (NOAA) National Climatic Data Center (NCDC) and data are available online (http://www.ncdc.noaa.gov/oa/ncdc.html). Rain gauge “B”, located in Mill Creek in Newport, NC, is maintained by volunteers for NCDENR-SSS. Rainfall gauge “C”, located in Ware Creek in Beaufort, NC, is maintained by a volunteer from Duke University Marine Laboratory. For comparison of FIB to rainfall levels, data from the closest rain gauge was used for each site, and due to sampling constraints (boating during foul weather) a 48 hour rainfall total was used for analyses.

RESULTS

Campylobacter spp. detection

A total of 75% (12 out of 16) samples from WC and NPRE had colony growth similar to the positive control Campylobacter spp. grown for side-by-side comparisons (Table 5.1).
A total of 33% (4 out of 12) and 92% (11 out of 12) of samples with colony growth were presumptive positives and catalase positives, respectively. All samples, regardless of colony growth were tested for thermotolerant *Campylobacter* spp. using multiplex PCR. The thermotolerant band was present in 44% (7 out of 16) of samples. Of the samples positive for colony growth, 58% (7 out of 12) were confirmed to be thermotolerant. All samples confirmed to be a thermotolerant species were determined to be *C. lari* by the species specific PCR. No samples presented the *C. coli* or *C. jejuni* bands.

*Relationship of thermotolerant Campylobacter spp. with environmental parameters*

Environmental parameters were measured during the winter, spring, and summer (Table 5.2). The samples were further grouped as positive for *C. lari* (n=7) and those negative for thermotolerant *Campylobacter* spp. (n=9). The samples positive for *C. lari* had an average temperature of 12.4°C (10.1 to 13.8°C), salinity of 29.5 (11.8 to 35.4), turbidity of 4.8 NTU (1.2 to 10.6 NTU), DO of 8.6 mg/l (7.8 to 9.8 mg/l), and 0.3 cm (0 to 1.3 cm) for 48 hour rainfall. The samples negative for thermotolerant *Campylobacter* spp. had an average temperature of 19.5°C (10.0 to 32.5°C), salinity of 26.7 (18.2 to 32.8), turbidity of 22.3 NTU (1.8 to 99.2 NTU), DO of 7.5 mg/l (4.2 to 10.0 mg/l), and 1.9 cm (0.2 to 6.9 cm) for 48 hour rainfall.

*Relationship of thermotolerant Campylobacter spp. with FIB*

The samples were grouped according to the presence or absence of thermotolerant *Campylobacter* spp. and were compared to the respective EC and ENT concentrations (MPN/100 ml; Figure 5.1). One sample positive for *C. lari* had an exceptionally high average concentration of FIB (ClsLine: 2.8 x 10^2 MPN per 100 ml versus (n=6), 4.0 x 10^0
MPN per 100 ml), and as a result averages were calculated for the confirmed positives with and without the outlier (Figure 5.2). The samples confirmed as *C. lari*, including the outlier, had average EC and ENT concentrations of $4.8 \times 10^1$ ($2.5 \times 10^0$ to $2.8 \times 10^2$) MPN per 100 ml and $1.1 \times 10^2$ ($2.5 \times 10^0$ to $7.4 \times 10^2$) MPN per 100 ml, respectively. With exclusion of the outlier, EC and ENT concentrations were $9.0 \times 10^0$ ($2.5 \times 10^0$ to $3.6 \times 10^1$) MPN per 100 ml and $8.8 \times 10^0$ ($2.5 \times 10^0$ to $1.5 \times 10^1$) MPN per 100 ml, respectively. Samples negative for thermotolerant *Campylobacter* spp. showed EC and ENT concentrations of $1.8 \times 10^2$ ($2.5 \times 10^0$ to $6.3 \times 10^2$) MPN per 100 ml and $2.0 \times 10^2$ ($2.5 \times 10^0$ to $7.8 \times 10^2$) MPN per 100 ml, respectively. Samples positive for *C. lari* were below the FC shellfish harvesting single-sample threshold of 14 MPN per 100 ml, while samples negative for *C. lari* had FC concentrations that exceeded this water quality threshold. All samples were taken when conditions met the rainfall cutoff (below 3.81 cm or 1.5 in) and shellfish harvesting waters would be open.

DISCUSSION

Thermotolerant *Campylobacter* spp. were detected in WC and NPRE estuarine waters during the study time period (February 2006 to July 2007). Although 75% of the samples grew morphologically similar colonies to the positive controls, only 33% of these colonies were confirmed as presumptive positives (i.e. catalase and oxidase positive) due to the low numbers of confirmed oxidase reactions. Biotyping is known to be inconsistent and thus, the lack of oxidase positives may have been due to media components interfering with the reaction. In contrast to the low oxidase positives from the colonies, 92% were catalase positive. When presumptive positives and catalase positives were compared with PCR
analyses, the presumptive positive test presented false-negatives and catalase positive samples presented false-positives.

All samples positive for thermotolerant *Campylobacter* spp. were confirmed to be *C. lari* (Table 5.1). *C. jejuni* or *C. coli* were not detected during the study. *C. lari* has been reported as the most prevalent thermotolerant *Campylobacter* spp. in coastal recreational waters (Obiri-Danso and Jones 1999) and shellfish (Endtz et al. 1997; Wilson and Moore 1996). In contrast, *C. jejuni* is mainly associated with foodborne outbreaks (i.e. poultry, beef) (Engvall et al. 2002; Labarca et al. 2002), *C. coli* is mainly associated with swine (Engvall et al. 2002), and *C. upsaliensis* is mostly associated with household pets (i.e. canine) (Engvall et al. 2002; Labarca et al. 2002). Avian species are also important reservoirs for thermotolerant *Campylobacter* spp. (Waldenstrom et al. 2002; Engvall et al. 2002; Koenradd et al. 1997), and waterfowl could be the source of *C. lari* in the WC and NPRE samples. Waldenstrom et al. (2002) showed that shoreline-foraging birds were the main carriers of this human pathogen (compared to ground-foraging or plant-eating species), while another study documented gulls as the only source of *C. lari* (compared to a variety of other warm-blooded animals showing the presence of *C. coli* and/or *C. jejuni* in their feces) (Engvall et al. 2002). In addition, *C. lari* survive longer than *C. coli* or *C. jejuni* in increasing temperatures and sunlight (Obiri-Danso and Jones 2001; Thomas et al. 1999).

The *C. lari* positive samples in this study were detected in the winter (February 2006) and early spring (March 2006; Table 5.1 and 5.2). These results agree with studies indicating that seasons with lower temperatures (Wilson and Moore 1996; Jones et al. 1990; Bolton et al. 1987) and less UV radiation (Obiri-Danso and Jones 2001; Jones et al. 1990; Butler et al. 1987) have a higher prevalence of thermotolerant *Campylobacter* spp. One U.S. study
conducted in Georgia coastal waters, however, reported higher concentrations of *Campylobacter* spp. in the warmer months (Vereen Jr. et al. 2007). The *C. lari* positive samples in this study were also associated with lower levels of rainfall (0.3 cm) as compared to the negative samples (1.9 cm). This contradicts Bolton et al. (1987) and Vereen Jr. et al. (2007) where heavy rainfall correlated to higher loads of *Campylobacter* spp. However, heavy rainfall in that study caused wastewater treatment failures. Other measured environmental parameter observations, salinity, turbidity, and DO, were consistent during the study period. Despite the associations found with seasons and rainfall, the sample size of this study is too small to make conclusive statements and further research is necessary.

There was no apparent relationship with FIB and further samples are needed to establish any correlations (Figure 5.21). Numerous studies have shown a lack of correlation with fecal water quality indicators (Savill et al. 2001; Carter et al. 1987; Obiri-Danso and Jones 1999; Arvanitidou et al. 1995; Bolton 1987; Rosef et al. 2001; Alonso and Alonso 1993), while one U.S. contradicts these findings and found significant correlations with fecal coliforms, temperature, nutrients (Vereen Jr. et al. 2007). It is important to note that for the presented research, FIB concentrations met the shellfish harvesting water quality limit (14 MPN or CFU per 100 ml) when *C. lari* was detected (Figure 5.1, dotted line). This finding demonstrates a public health risk when shellfish harvesting waters are open and thermotolerant *Campylobacter* spp. could be present. In addition, the shellfish harvesting season (October to March) is when the largest numbers of thermotolerant *Campylobacter* spp. are found in environmental waters (Wilson and Moore 1996; Jones et al. 1990; Bolton et al. 1987). As mentioned, avian species are major reservoirs for this pathogen and specifically waterfowl native to the NPARE are speculated to be the source of the *C. lari*
detected. Tauxe et al (1987) documented a summer peak in human cases of Campylobacteriosis due to the increased barbequing of meats (Tauxe et al. 1987) and increased exposure to contaminated water through summer recreational activities. However, results presented here suggest the largest threat of Campylobacteriosis is through foodborne exposure to raw or undercooked shellfish harvested from WC and NPRE, or through ingestion of contaminated water during the cooler months (below 15°C). The sites used for this study included one location open to shellfish harvesting (HC), one location that was open and then closed during the study period to shellfish harvesting (WC), and three locations currently closed to shellfish harvesting (RS, CC, CL). Results showed that all sites, regardless of status, were contaminated with \( C. lari \) in March 2006 during shellfish harvesting season. The presence of a human bacterial pathogen in waters used for shellfish harvesting and recreational activities raises concerns. Future work is needed, especially in U.S. coastal waters, to examine the depth of this threat and potential sources of thermotolerant \( Campylobacter \) spp. as well as to provide remediation suggestions for management practices that would reduce this threat.
FIGURES

Figure 5.1. Relationship between *Campylobacter* spp. and FIB. The dotted line is to relate the FIB concentrations to the water quality threshold for shellfish harvesting areas. The asterisk notes the data not including the outlier and the error bars represent ±1 standard error.
Table 5.1 *Campylobacter* spp. detection in the Newport River Estuary and Ware Creek. The following notations are used: “n/a” refers to non-applicable, “Therm(+)” refers to the sample being positive for the thermotolerant band (246 bp) from multiplex PCR (Wong et. al. 2004), and “*C. lari* (+)” refers to the presence of the *C. lari* band (177 bp) and the thermotolerant band (222 bp) using the species specific PCR (Eyers et al. 1993, Eyers et al. 1994).

### WARE CREEK
(Feb 2006 - April 2006: SSS-WC; June and July 2007: YD)

<table>
<thead>
<tr>
<th>Date</th>
<th>Colony Growth</th>
<th>Presumptive Positive</th>
<th>Catalase Positive</th>
<th>Multiplex PCR</th>
<th><em>C. lari</em> PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feb 2006</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>n/a</td>
</tr>
<tr>
<td>March 2006</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Therm (+)</td>
<td><em>C. lari</em> (+)</td>
</tr>
<tr>
<td>April 2006</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
<td>-</td>
<td>n/a</td>
</tr>
<tr>
<td>June 2007</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>n/a</td>
</tr>
<tr>
<td>July 2007</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>n/a</td>
</tr>
</tbody>
</table>

(n = 5)

### NEWPORT RIVER ESTUARY

<table>
<thead>
<tr>
<th>Date (Site ID)</th>
<th>Colony Growth</th>
<th>Presumptive Positive</th>
<th>Catalase Positive</th>
<th>Multiplex PCR</th>
<th><em>C. lari</em> PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feb 2006</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RS</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Therm (+)</td>
<td><em>C. lari</em> (+)</td>
</tr>
<tr>
<td>CC</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
<td>-</td>
<td>n/a</td>
</tr>
<tr>
<td>CL</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Therm (+)</td>
<td><em>C. lari</em> (+)</td>
</tr>
<tr>
<td>HC</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>n/a</td>
</tr>
<tr>
<td>March 2006</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RS</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Therm (+)</td>
<td><em>C. lari</em> (+)</td>
</tr>
<tr>
<td>CC</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Therm (+)</td>
<td><em>C. lari</em> (+)</td>
</tr>
<tr>
<td>CL</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Therm (+)</td>
<td><em>C. lari</em> (+)</td>
</tr>
<tr>
<td>HC</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Therm (+)</td>
<td><em>C. lari</em> (+)</td>
</tr>
<tr>
<td>April 2006</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RS</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
<td>-</td>
<td>n/a</td>
</tr>
<tr>
<td>CL</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>n/a</td>
</tr>
<tr>
<td>HC</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
<td>-</td>
<td>n/a</td>
</tr>
</tbody>
</table>

(n = 11)

Total: 12/16 (75%) 7/16 (44%)

Total positive: 4/12 (33%) 11/12 (92%) 7/12 (58%) 7/12 (58%)
Table 5.2 Environmental parameter observations for *Campylobacter* spp. samples.

<table>
<thead>
<tr>
<th>Date</th>
<th>Temperature (°C)</th>
<th>Salinity</th>
<th>Turbidity (NTU)</th>
<th>DO (mg/l)</th>
<th>Rainfall (48 hrs, cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ALL SAMPLES</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>February 2006 (n=5)</td>
<td>10.3 (10.0-10.6)</td>
<td>23.2 (11.8-31.3)</td>
<td>4.1 (1.2-10.6)</td>
<td>9.6 (9.2-10.0)</td>
<td>0.99 (0.6-1.3)</td>
</tr>
<tr>
<td>March 2006 (n=5)</td>
<td>13.2 (12.7-13.8)</td>
<td>32.8 (29.6-35.4)</td>
<td>4.4 (2.7-8.3)</td>
<td>8.2 (7.8-8.6)</td>
<td>0</td>
</tr>
<tr>
<td>April 2006 (n=4)</td>
<td>21.7 (21.0-22.2)</td>
<td>28.0 (24.0-32.8)</td>
<td>18.0 (6.9-29.0)</td>
<td>7.3 (6.8-7.6)</td>
<td>0.6 (0.2-1.0)</td>
</tr>
<tr>
<td>June 2007 (n=1)</td>
<td>24.7</td>
<td>27.5</td>
<td>21.2</td>
<td>5.9</td>
<td>6.91</td>
</tr>
<tr>
<td>July 2007 (n=1)</td>
<td>32.5</td>
<td>26.6</td>
<td>99.2</td>
<td>4.2</td>
<td>4.6</td>
</tr>
<tr>
<td><strong>POSITIVE SAMPLES (C. lari)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=7</td>
<td>12.4 (10.1-13.8)</td>
<td>29.5 (11.8-35.4)</td>
<td>4.8 (1.2-10.6)</td>
<td>8.6 (7.8-9.8)</td>
<td>0.3 (0-1.3)</td>
</tr>
<tr>
<td><strong>NEGATIVE SAMPLES</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=9</td>
<td>19.5 (10.0-32.5)</td>
<td>26.7 (18.2-32.8)</td>
<td>22.3 (1.8-99.2)</td>
<td>7.5 (4.2-10.0)</td>
<td>1.9 (0.2-6.9)</td>
</tr>
</tbody>
</table>
REFERENCES


CHAPTER 6
SYNTHESIS

North Carolinas (NC) coastal communities, similar to coastal communities worldwide, depend on the local estuaries and beaches for personal enjoyment, economic livelihood, and as thriving environmental sanctuaries. This southeastern state is ranked 8th in the nation for tourism, which brings in billions of dollars each year (NCDC 2004). The Newport River Estuary (NPRE) was the focus estuary for the presented dissertation research, which is reported to be the most productive shellfish harvesting area in the White Oak River Basin. This estuary was responsible for 3.63% of NC’s total commercial shellfish profit from 1996 to 2006, thereby contributing to the economy (White Oak River Basinwide Water Quality Plan 2007; NCDMF landing data). In addition, the White Oak River Basin is listed as containing “some of the most biologically significant habitats along the Atlantic Coast” (White Oak River Basinwide Water Quality Plan 2007). All of these ecosystem services are intimately connected, and when the water quality is poor, a cascade affect causes recreational and shellfish harvesting activities to be suspended, as well as disrupting the delicate balance of the estuarine ecosystem. For the protection of our estuarine ecosystems and the eastern NC way of life, it is vital to be proactive in water quality research. Specifically, water
quality research that assists in building tools useful to water quality managers, identifies methodologies to partition sources of pollution, and quantifies contaminant concentrations and loading with an eye towards remediation, are such necessary actions before the damage is irreversible.

The overall goal of this dissertation was to conduct research on the microbiological contamination found in the NPRE, and to assess the patterns, dynamics, and relationships of this contamination as related to stormwater. The 319(d) listing for fecal coliform impairment in the NPRE immediately raised a red flag for the estuarine area, as it was one of the few remaining productive and open harvesting areas. The research presented in this dissertation incorporated advanced techniques and modeling approaches that should provide an example for future study of similar estuarine environments. Hopefully, the work presented in this dissertation can be applied to a range of estuarine environments worldwide.

A major finding of this dissertation is that stormwater runoff is the predominant vector of fecal pollution to the NPRE. Water quality managers in eastern NC, in North Carolina Department of Environment and Natural Resources (NCDENR), have long understood the adverse impact of rainfall on microbiological water quality and have been using rainfall as a measure to close shellfish harvesting waters. The historic management threshold was 5.08 cm (2.0 inches) from 1990 until 1994 (NCDENR Shellfish Sanitation Section - Patricia Fowler, person. comm.). The currently imposed management strategy for the NPRE, implemented since 1994, is to close shellfish harvesting areas after 3.81 cm (1.5 inches) within 24 hours (NCDENR Shellfish Sanitation Section - Patricia Fowler, person. comm.). However, this research illustrated that just 2.54 cm (1.0 inch) of rainfall leads to significant increases of fecal contamination as compared to lesser rainfall levels. Hence, one
major finding of this dissertation is that the currently used rainfall standard may need to be reevaluated in the face of growing coastal development, and that a suggested recommendation for a lower management rainfall threshold to close shellfish harvesting waters could be 2.54 cm in 24 hours.

A second major accomplishment of this dissertation is the integration of water quality data from the entire NPRE into a framework of Space/Time Bayesian Maximum Entropy (S/T BME) modeling. The application of this modeling approach for microbial water quality determination is novel, cutting edge research and should provide a platform for the use of this type of modeling for a range of new water quality applications. The ability to incorporate error, uncertainty, and variability that is natural to water quality sampling with actual fecal indicator bacteria (FIB) measurements to create visual aids of fecal pollution is invaluable for management agencies. The models and maps developed using S/T BME from the NPRE data supported previous findings and demonstrated significant relationships of FIB with rainfall, antecedent rainfall and distance to shore, while also providing visual maps illustrating the distribution of fecal pollution in the estuary. This research provides a solid foundation for future work in which continuous or real-time FIB data could be incorporated into a S/T BME model database and act to supplement management decision-making by providing maps to show where problem areas are based upon rainfall, thus saving resources.

A major area for advancement of water quality research is the use of rapid, molecular techniques for managing waters. It has become well known that *E. coli* (EC) and *Enterococcus* (ENT) can persist in tropical and subtropical sands, sediments and waters (Fries et al. 2008; Byappanahalli and Fujioka 2004; Shibata et al. 2004; and Desmarais et al. 2002). There has been a goal in recent years to discover alternate indicators of fecal
contamination that are more accurate in identifying recent contamination and specific to human fecal pollution. In this dissertation, \textit{Bacteroides thetaiotaomicron (B. theta)}, was proposed as an alternate fecal indicator bacteria capable of indicating fresh fecal contamination. \textit{B. theta} is found in very high concentrations in human fecal matter (100X more than EC or ENT) and is a facultative anaerobe, thus is a more specific indicator of human and recent fecal contamination (Wang et al. 1996). \textit{B. theta} concentrations were quantified via quantitative polymerase chain reaction (QPCR) techniques as a “second tier” within the water quality studies presented in Chapter 4. Over the course of the work conducted in Ware Creek in Chapter 4 the following was demonstrated: 1) stormwater runoff dramatically influenced the high priority shellfish harvesting waters of Ware Creek with FIB loading on the order 10,000 to 100,000 MPN or cells per 100 ml reaching shellfish harvesting area every 10 minutes, 2) that rainfall amounts above 2.54 cm (1 inch) inundates the system with fecal contamination and concentrations plateau to 1000’s FIB per 100 ml, and 3) evidence from using \textit{B. theta} to partition probable fecal sources supported that high concentrations of indicator bacteria found during heavy rainfall were not likely to be of human origin.

Although the mentioned findings above illustrate that it is highly likely that majority of the fecal pollution in the NPRE being of environmental and wildlife sources, the presence of the zoonotic human bacterial pathogen, \textit{Campylobacter lari}, still raises concerns. A major problem with the currently used FIB based management system for recreational and shellfish harvesting water quality assessments is that zoonotic pathogens have been assumed by many to be a minor concern. \textit{C. lari}, most likely stemming from the large avian coastal populations in eastern NC, is a potential zoonotic pathogen that could pose a public health
risk. Traditional FIB (EC, ENT) were not correlated with *C. lari* in this research project (although the data set is small), and this finding is similar to a majority of other water quality studies. The pathogen was actually found in higher concentrations when waters were open for shellfish harvesting season (no rain, winter temperatures), which poses potential foodborne transmission. This work was conducted on a small-scale, however, and more intensive sampling is needed to make conclusive statements about the need for a *Campylobacter* specific water quality assessment in eastern NC.

In this dissertation I have demonstrated a wide range of approaches used to characterize fecal pollution in the NPRE. The aim was to conduct work in the field during times (storm events) when previous sampling efforts had not been adequately conducted, and to place the resulting wealth of data into several modeling (S/T BME, Bayesian) modeling frameworks for improved management of these estuarine waters. Additionally, a major goal was to identify the potential sources, variability, and loading of microbial contaminants, in an effort to begin to understand the possible BMP scenarios that might be appropriate for implementation in the NPRE. Finally, in addition to serving the purpose for my dissertation, the presented data is part of a larger ongoing 319 project with the North Carolina Division of Water Quality regarding a Total Maximum Daily Load (TMDL) model to determine the maximum allowable limit of fecal coliforms that can be released into the NPRE without violating water quality standards. Thus, my large dissertation datasets are being integrated into a probabilistic or Bayesian network model to support this TMDL development, which is part of Duke Ph.D. dissertation project (Andrew D. Gronewold). The overall model will be used to assist in management decisions, estuary remediation, and future research.
The primary goal for future work in the NPRE should address remediation efforts and stormwater control. Implementation of best management practices may help re-open the closed areas and keep the currently opened areas, opened. Future work should include identifying sources of environmental and wildlife sources of fecal contamination, as well as further investigation of molecular technology for human-specific tracers of human pollution. This work should also include further examination of *Campylobacter* spp. as a public health risk, perhaps in combination with viral pathogens in waters overlying high priority shellfish harvesting beds, high use beach recreational waters, and shellfish meats.
REFERENCES


