FACTORS THAT INFLUENCE THE PRESENCE OF FECAL INDICATOR BACTERIA FROM THREE POTENTIAL EXPOSURE PATHWAYS

by

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This labor of love and hate is dedicated to my son, Lincoln. You were my driving force during the last few months. I wanted to be a good role model for you and knew the dissertation didn't define me, but the completion of it did. Through you I began to understand the scarcity and value of time, and above all, you made me realize the physical and mental toughness I never knew I had.

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ABSTRACT

Fecal indicator bacteria (FIB) are good predictors for the presence of pathogens associated with fecal contamination in recreational waters and criteria have been created as a benchmark to assess risk to human health. The two most traditionally used FIB for this purpose are culturable *Escherichia coli* in freshwaters and enterococci in marine settings and members of the Order *Bacteroidales* have been mentioned heavily in the literature as supplementary indicators. It has been well documented in the literature that fecal indicator bacteria can be modulated by a number of factors, such as temperature, ultraviolet light, land use, and rainfall.

This collection of studies yielded information on a how factors could modulate FIB from a variety of transport pathways to human exposure. In surface waters, concentration- and loading-based results for *E. coli* and *Bacteroidales* were highest in summer and spring, and lowest in the winter and fall, respectively. *Bacteroidales* concentrations were positively correlated with temperature and total suspended solids and negatively correlated with dissolved oxygen. In beach sand, *E. coli* concentrations were highest in the upper 0-10 cm of the foreshore samples where beachgoers typically congregate. For substrate types typically used in stormwater infrastructure, plate count concentrations increased considerably from initial spiking dose, but results were dependent on strain of bacteria and substrate type (concrete, metal, PVC).

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CHAPTER 1 INTRODUCTION

1.1 Brief background of Clean Water Act

Protection of water resources is a worldwide concern and countries implement pollution control strategies to prevent these impacts to a delicate natural resource. The federal government set forth the Federal Water Pollution Control Act in 1948 to address water pollution concerns which was amended in 1972 into what is more commonly known as the Clean Water Act. The Clean Water Act has developed water quality standards to protect human health and aquatic life by reducing toxins in surface waters. States, territories, and tribes use these water quality standards, and the accompanying narrative or numeric criteria, as guidance to protect the designated use(s) of a particular water body (e.g., fish and aquatic life, drinking water supply, recreational waters) from a specific pollutant (EPA 1986; 2012). Recreational water quality criteria were developed in 1986 and revised in 2012 to assess the level of risk associated with microbial pollutants, such as bacteria and viruses, concomitant with fecal bacteria. Each year it is estimated that 50 million respiratory and 120 million gastrointestinal illnesses occur globally from swimming in wastewater polluted water (Shuval 2003). Fecal bacteria in surface waters can originate from point sources like wastewater treatment plants, sewer overflows, stormwater pipes, and concentrated animal feeding operations (CAFOs) or from nonpoint sources, such as runoff of feces previously deposited on the landscape by livestock, manure application, wildlife, and domestic pets (EPA 2001).

1.2 Fecal indicator bacteria

Fecal indicator bacteria are a group of bacteria inhabiting the intestines of warm-blooded animals and are used in water quality assessments to indicate potential exposure

risk from pathogens associated with fecal waste (EPA 1986, 2001). The most practical way to circumvent the expense and difficulty of detecting true fecal pathogens is to use surrogates. The EPA recommends the fecal indicator bacteria (FIB) *Escherichia coli* and enterococci as two ideal proxies for fecal pathogens when monitoring or assessing water quality. *Escherichia coli* is a Gram negative, rod-shaped, motile, facultative anaerobe that is classified as a thermotolerant coliform (fecal coliform) capable of lactose fermentation and growth after 24 h @ 44.5°C for 24 h. Enterococci are Gram positive, spherical shaped (coccus), nonmotile, facultative anaerobes that can be grown at a wide range of conditions (10-45°C, and in 6.5 % NaCl). Selection of these two FIB came from early foundational studies where Cabelli et al. (1982; 1983) found enterococci correlated with gastrointestinal illnesses in freshwater and marine waters and Dufour (1984) surmised *E. coli* was a more suitable predictor for these illnesses in freshwaters. Members of the Order *Bacteroidales* have been considered as an alternative or supplementary indicator because they are more dominantly found (10^9 cells/gram human feces) in the microbial community of the gut (Sghir et al. 2000; Madigan and Martinko 2006), they can be indicative of recent fecal input due to low survival rates in the environment (Fiksdal et al. 1985; Kreader 1995), and they are useful in fecal source tracking studies (Bernard and Field, 2000, Layton et al. 2000). With the advent of quantitative polymerase chain reaction (qPCR) assays targeting 16S rRNA genes or 23SrRNA genes, results of fecal contamination testing can be delivered with faster turnaround times than culture methods.

1.3 Influence of abiotic factors on fecal indicator bacteria in multiple exposure pathways

The common theme among every chapter of the dissertation is the how the influence of a variety of factors modulate levels of FIB. The role these factors play on FIB presence is presented in three unique exposure pathways that could ultimately impact human health. The following sections give a brief background on studies highlighting the impact of specific factors that influence FIB in particular exposure pathways.

1.3.1 Surface waters

In order to effectively manage surface waters and to efficiently assign protection to human health, a clearer understanding of the influence or concurrence of environmental factors with FIB is necessary. Abiotic factors, such as elevated salinity and solar radiation have been shown to decrease fecal coliform survival (Šolić and Krstulović 1992; Muela et al. 2000). Cooler sediment temperatures in coastal waters were found to increase survival (Craig et al. 2004) whereas *E. coli* concentrations were higher in warmer months in other studies (Traister and Anisfeld 2006, Amorim et al. 2014). *Bacteroidales* 16S rRNA genetic targets tend to be detected in surface waters more frequently at lower temperatures (Okabe and Shimazu 2007; Balleste and Blanch 2010; Bell et al. 2010). The effects of other environmental factors, such as salinity, dissolved oxygen, and sunlight on survival of members of the *Bacteroidales* have also been investigated with varying results (Baughn and Malamy 2004; Okabe and Shimazu 2007; Walters and Field 2009; Bae and Wuertz 2009; Balleste and Blanch 2010).

Chapter 2 of this dissertation investigates the influence of season on *E. coli* concentration (MPN/100 mL) and flow-based loading measurements (MPN/day) for

inland waters at baseflow conditions. In Chapter 3, investigates in seasonal concentrations and loadings for *Bacteroidales* and their relationship with physicochemical water parameters (temperature, dissolved oxygen, conductivity, total suspended solids, and pH). These samples were collected from surface waters in watersheds known to exceed recreational water quality criteria.

1.3.2 Beach sand

Exposure to coastal water pollution poses both a public health and economic burden (Henrickson 2001) at a worldwide level (Pruss 1998; Shuval 2003). An overlooked contributor to public health risk in these coastal waters may be the beach sand itself (Sabino et al. 2014). Epidemiological studies have demonstrated that with beach sand can result in gastrointestinal illness (Bonilla et al. 2007; Heaney et al. 2009) and that enteric illnesses are associated with densities of fecal indicator organisms in beach sand (Heaney et al. 2012). Recent research conducted by Heaney et al. (2014) reported that factors like sand-wetting, fecal indicator concentrations in adjacent water, and wave height had an influence on numbers of fecal indicators found in sand. Most research to date has investigated the presence of fecal indicators in beach sand from coastal and Great Lakes locations (Zhang et al. 2015; Yamahara et al. 2012; Alm 2003; Eichmiller 2014; Staley et al. 2015), but other inland recreational beaches are less represented in the literature (Wilson et al. 2016; Levin-Edens et al. 2012; Marion et al. 2010).

The novel research presented in Chapter 4 was carried out at a temperate, inland manmade beach adjacent a river impoundment. The goal of the study was to compare *E. coli* concentrations by both distance from shoreline and depth in sand to determine potential for exposure to FIB or pathogens at different locations at a freshwater beach.

1.3.3 Pipe infrastructure

Negative consequences have been linked to bacterial colonization of drinking water and wastewater infrastructure (Lazarova and Manem 1995; Liu et al. 2016), while research regarding colonization of stormwater infrastructure (e.g., pipes, channels) by fecal indicators is lacking. However, McCarthy (2009) suggested that the build-up of FIB in crevices from stormwater pipes could influence concentrations in surface waters and potential human exposure. Bacteria have been shown to colonize drinking infrastructure differentially depending on the substrate type. For instance, in one study, fixed bacterial biomass on cement coupons was found to be 2.6 times higher than on PVC coupons (Niquette et al. 1999), and in another, heterotrophic plate counts of a biofilm were 100 times higher on galvanized steel coupons than PVC, copper, and stainless steel (Jang et al. 2011).

If occurring, bacterial attachment and subsequent biofilm development in stormwater drainage pipes could create an environmental reservoir of FIB and potential fecal pathogens that could contribute to impairment of nearby surface waters. The aims of Chapter 5 of the dissertation are to understand if attachment of FIBs to stormwater pipe material is possible and if there is a preference for a particular substrate type (i.e., concrete, metal, and PVC). Results from this study will give direction to water quality stakeholders and modelers who would be interested in knowing whether pipe selection could be important in initial infrastructure development or remediation of compromised infrastructure.

1.4 Summary of projects

In summary, these collective studies support the idea that levels of fecal indicator bacteria can be influenced by a variety of factors. In Chapter 2 and 3, seasonal patterns of *E. coli* and *Bacteriodales* concentrations collected from samples in surface waters at baseflow were highest in the summer and loadings were highest in the spring. Elevated concentrations of these two FIBs in the summer could be driven by warmer temperatures that were shown to have a positive correlation with FIB concentrations in Chapter 2. Increased activity/movements in summer and fecal deposition of wildlife or domestic animals near riparian zones or in-stream coupled with reduced water levels concentrating these levels could explain high FIB concentrations in the summer. Antecedent rainfall during winter and spring rainy seasons could have increased flow leading to higher overall loading even during baseflow sampling. Water quality managers responsible for improving impaired recreational surface waters should be cognizant of baseflow concentration and loading patterns and water quality parameters that could be influencing these. Patterns that are contradictory to baseline data need to be prioritized as a surface water that needs further investigation.

For Chapter 4, incremental distances from shoreline and depth of sampling in sand were important factors in *E. coli* concentrations sampled at a freshwater, inland beach. The *E. coli* concentrations were predominately detected in the upper 0-10 cm of sand in the foreshore (i.e., wave-impacted area) followed by the upper 0-10 cm of the backshore (dry area) and nearshore (water-inundated area) sampling locations. Sensitive members of the population, like children, could be at increased risk to *E. coli* exposure since they oftentimes play in the foreshore area and dig in sand. This study is impetus to

continue investigation of fecal indicator bacteria and associated pathogens especially since freshwater, beaches, exclusive of the Great Lakes, are not required by the federal government to be monitored.

To summarize Chapter 5, FIBs (two *E. coli* strains and *Enterococcus faecalis*) and types of material (concrete, metal, PVC) used to construct stormwater pipe had an effect on concentrations using culture and qPCR methods. All substrates were colonized by all strains over the course of the study. Metal was the least preferred material for colonization for the two *E. coli* strains and *E. faecalis* was the strongest colonizer overall, even on metal. There are likely structural and chemical properties of both the bacteria and pipe materials that promote this attachment. Attachment to the substrates was present in scanning electron microscopy images with evident of likely biofilm formation for *E. faecalis*. The patterns for qPCR results were not similar to patterns of culture-based methods, but using this method still allowed for detection on these substrates. This research shows FIBs could attach to stormwater infrastructure, survive and multiply between rain events, and ultimately be a fecal source to a receiving stream.

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CHAPTER 2 WATERSHED ANALYSIS OF SEASONAL CONCENTRATION- AND LOADING-BASED RESULTS FOR *ESCHERICHIA COLI* IN INLAND WATERS

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2.1 Abstract

Fecal indicator bacteria, such as *Escherichia coli*, are frequently monitored in recreational waterbodies as indicators of potential fecal pathogen presence and exposure. The present watershed analyses investigated the influence of season on *E. coli* concentration (MPN/100 mL) and loading (MPN/day) measurements for inland waters at baseflow conditions. The master dataset collected during 2007-2012 for three watersheds included 896 *E. coli* (Colilert) samples with simultaneous flow taken for a subset (39 %) of samples. The outcomes for grouped watersheds were reflected in most cases for individual watersheds. Concentration- and loading-based results were highest in summer and spring, and lowest in the winter and fall, respectively. The comparison of these two measurement techniques (concentration and loading) highlight the impact of flow data during base-flow conditions for inland waters and reveal that caution should be used when inferring one method's results from another.

2.2 Introduction

Protection and restoration of water resources is important worldwide to prevent human exposure to waterborne fecal pathogens. Singly or collectively, fecal sources such as combined sewer overflows, sanitary sewer overflows, septic tank failure, illicit sewer connection to stormwater infrastructure, bypass events from wastewater treatment plants, livestock and pastures, domestic pets, and wildlife have the potential to transfer fecal pathogens into recreational waterbodies (USEPA 2001). For example, Shuval et al. (2003) estimated that worldwide 120 million gastrointestinal illnesses and 50 million respiratory cases per year are due to recreating in waterbodies influenced by municipal wastewater; in California similar illnesses were projected to cause a public health burden and subsequent economic loss of $3.3 million per year (Dwight et al. 2005).

Waterbodies are monitored for the presence of fecal contamination and possible waterborne pathogens by enumeration of *Escherichia coli*, which is used as a fecal indicator bacteria (FIB). Based on epidemiological studies at coastal and inland beaches where positive correlations between *E. coli* densities and gastrointestinal illnesses were found (USEPA 2003; 2010), many states now include *E. coli* sampling in their water quality monitoring programs regardless of waterbody type (e.g., lakes, inland streams, rivers, estuaries, oceans) or climate (e.g., temperate, arid, tropical).

Results from *E. coli* monitoring are typically analyzed either on a concentration-based or loading-based approach. Concentration-based analysis is utilized because of the ease of sample collection and established water quality criteria that allow for the evaluation of human health risk to fecal contamination (Hörman et al. 2004; Marion et al. 2010; Amorim et al. 2014). Loading-based analysis takes into account time specific water

flow conditions allowing for the determination of relative contribution of a stream to a larger system (e.g., watershed; Gentry et al. 2006), but at a cost of increased time and expense. Both concentration- and loading-based analyses have been used to investigate fecal inputs (Stumpf et al. 2010; Gentry-Shields et al. 2012; Rowny and Stewart 2012); but simultaneous comparisons are not prevalent (Converse et al. 2011), especially for inland waters (Dorevitch et al. 2010).

Escherichia coli monitoring has been shown to be influenced by changes in season (Traister and Anisfeld 2006; Converse et al. 2011; Amorim et al. 2014; North et al. 2014), however the results from previous researchers have shown differing patterns depending on the type of analysis performed (concentration- vs loading-based analysis). For example, Traister and Anisfeld (2006), utilizing a concentration-based analysis, found that *E. coli* concentrations increased from spring to summer and decreased in the winter during a year-long study in a forested and urban watershed in the Hoosic River Basin in the northeastern U. S. This is in contrast to Converse et al. (2011) who showed the highest loading values for *E. coli* being in November during a coastal storm water study using a loading-based analysis.

Despite the wide use of *E. coli* as an FIB, very little literature exists directly comparing the results of common approaches to *E. coli* analyses. The goal of the present study was to perform a simultaneous analysis on the influence of season on *E. coli* concentrations and loading measurements for inland waters. Specifically, the objectives were to 1) compile a dataset that would allow for the direct comparison of *E. coli* concentrations and loadings; 2) determine the influence of season on *E. coli*

concentrations and loadings independent of one another; and 3) conduct a comparison of these independent analyses.

2.3 Materials and Methods

A master dataset was compiled from multiple studies by Nashville Tennessee's Metro Water Services, Stormwater Division/National Pollutant Discharge Elimination System Office. The master dataset included *E. coli* concentrations from three watersheds (Browns, Richland and Mill) in Nashville TN, USA over a period of 2007-2012 (total of 896 samples). A subset of the *E. coli* data in the master database also had corresponding flow measurements recorded (~39 %). These three watersheds (Browns, Richland, and Mill) were identified as ideal candidates for the present study because they were frequently monitored, had the most complete data and were listed on the 303(d) list as impaired due to pathogens (TDEC 2014). All data included in the master dataset was sampled following Tennessee Department of Environment and Conservation (TDEC) *Standard Operating Procedure for Chemical and Bacteriological Sampling of Surface Water* (TDEC 2009). Briefly, all samples were collected during baseflow stream conditions (<0.1" rainfall within last 72 h). Stream velocity (ft/sec) was measured using a Swoffer Model 3000 Current Velocity Meter-Flowmeter and was used along with stream cross sectional area to calculate flow (cubic feet per second, CFS). All samples were analyzed for *E. coli* within 6 hours of collection using the EPA-approved Colilert® method (TDEC 2009) (IDEXX Laboratories, Westbrook, Maine), in which a 100mL water sample is distributed into a series of aliquots. The presence or absence of metabolic activity among the aliquots is used to derive the maximum likelihood estimate of *E. coli* concentration, reported as most probable number per 100 mL (MPN/100 mL) in the

sample. Loadings were calculated by multiplying *E. coli* concentration (MPN/100 mL) by simultaneous flow measurements and were reported as MPN/day.

Five individual sites within each watershed were utilized, except Browns, in which six sites were utilized because of ease of site access. Sampling seasons were defined as June, July, August (summer); September, October, November (fall); December, January, February (winter); and March, April, May (spring). Due to a catastrophic flood event, May 2010 was excluded from analysis. Yearly data were combined by season, across years, to incorporate a wide range of site, seasonal, and yearly variation to provide a robust estimate of FIB concentration and loadings. Sample sizes varied by year and season and can be found in Figures 2.1 and 2.2.

The three impaired watersheds lie within a temperate climate (annual average 15°C) with warm summers (July average 26°C) and mild winters (January average 2°C) (https://ag.tennessee.edu/climate/Pages/climatedatatn.aspx). The watersheds are classified as part of the Outer Nashville Basin Level IV ecoregion. No wastewater treatment plants directly impact these watersheds nor does any concentrated animal feeding operation exist in these areas. Size, land use, population, and imperviousness of the watersheds are presented in Table 2.1.

One-way Analysis of Variance (ANOVA) tests were performed on the three watersheds grouped together as well as on each watershed individually to assess seasonal differences for both concentrations and loadings. The grouped watershed allowed for an overall, robust assessment of the watersheds. If ANOVAs indicated significant seasonal differences, Tukey's post-hoc tests were performed to detect differences among seasons. Normality and equality of variances were assessed before statistical analyses were

performed and bacteria concentrations and loadings were $\log_{10}$ transformed if assumptions were not met. An alpha (α) = 0.05 was used as the significance level for all statistical analyses. IBM SPSS Ver 20 (Armonk, NY: IBM Corp) was used for all statistical analyses.

Table 2.1. General land use and watershed features for three central Tennessee impaired watersheds

	Watershed		
Land use (%)	Browns	Richland	Mill
Residential	49	56	41
Commercial	10	11	20
Industrial	9	3	9
Transportation	8	7	7
Open	24	23	23
Watershed features			
Size (hectares)	3,237	5,868	5,261
Imperviousness (%)	30	19	40
Est. 2010 population	51,370	29,995	52,571

2.4 Results and Discussion

Monitoring for fecal indicator bacteria, specifically *E. coli*, is a common approach for water quality regulators to assess human health risks from fecal contamination. The employment of *E. coli* as a monitoring tool is useful in a variety of water quality programs, such as stormwater runoff monitoring for watershed studies (Jamieson et al. 2003; Converse et al. 2011), risk assessment to beach-goers (USEPA 2012), gauging effectiveness of best management practices to minimize fecal inputs (Leisenring et al. 2012), and incorporation into total maximum daily loading calculations (USEPA 2001). The present watershed assessment was a unique opportunity to evaluate whether the effect of season influenced concentration- and loading-based analyses, both at the individual watershed level and after combining multiple watersheds together.

Significant differences using concentration-based analyses were observed by season when watersheds were analyzed collectively ($F_{3,892} = 81.169$, $p < 0.01$) and individually (Browns: $F_{3,299} = 46.785$, $p < 0.01$; Richland: $F_{3,339} = 36.506$, $p < 0.01$; Mill: $F_{3,246} = 13.764$, $p < 0.01$) and showed summer concentrations being the highest and statistically greater than winter for both grouped and individual watershed analyses (Figure 2.1a, b, c and d). Previous researchers have shown a similar seasonal trend (Traister and Anisfeld 2006; Koirala et al. 2008; Wilkes et al. 2009; North et al. 2014). For example, in a coastal urban bathing area in Portugal, mean *E. coli* concentrations were statistically highest in the summer for three of the four beaches studied (Amorim et al. 2014). Traister and Anisfield (2006) found higher *E. coli* levels in the summer in developed watershed in Upper Hoosic River watershed in Massachusetts. The high *E. coli* concentrations observed both by previous researchers and in the present study are

likely due to either lowered water levels and flow in the summer (Figure 2.1a, b, c, d) causing *in situ* and imported *E. coli* to become more concentrated (Cha et al. 2010) or increased *E. coli* replication due to increased temperature (North et al. 2014) or both. Another potential factor influencing the observed seasonal concentration results could be the integration of *E. coli* from sediment or the riparian soil matrix (i.e. naturalization) into water. Concentrations of naturalized soil *E. coli* inputs from three temperate watersheds in the US were reported to be the highest in summer and fall and lowest in winter and spring months (Ishii et al. 2006). Regardless of the cause, the pattern of higher *E. coli* concentrations in summer months appears to be rather consistent and the results of the present study support previous findings.

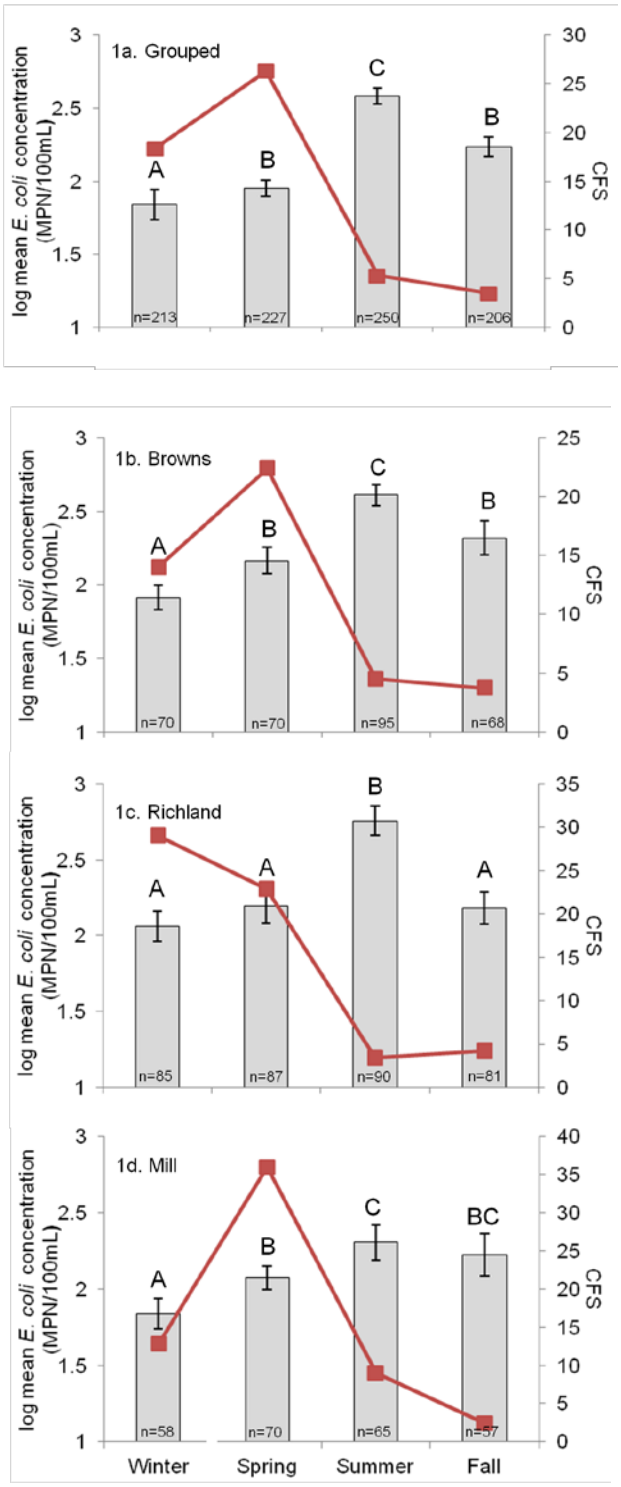

Figure 2.1. a, b, c, d. Bars are seasonal comparisons for log mean *Escherichia coli* concentrations (MPN/100 mL) and average flow in grouped (1a) and in Browns (1b),

Richland (1c), and Mill (1d) watersheds. Seasons not sharing similar letters are significantly different from each other. Data presented as mean ± 95 % confidence intervals. The red line represents average flow in cubic feet for second (CFS).

Significant differences were also observed using loading-based analyses when watersheds were analyzed collectively ($F_{3,343} = 30.635$, $p < 0.01$) and individually (Browns: $F_{3,128} = 11.055$, $p < 0.01$; Richland: $F_{3,108} = 16.018$, $p < 0.01$; Mill: $F_{3,99} = 9.726$, $p < 0.01$) and showed significantly higher loadings in the spring compared to fall in all analyses (Figure 2.2a, b, c, and d). These results (seasonal *E. coli* loadings during base flow conditions) are unique to the literature and fill a critical knowledge gap. Previous research by Converse et al. (2011) showed increased loads in fall compared to all other months tested, however this research was investigating *E. coli* loadings in storm water samples, not base flow conditions. Likewise, adequate previous research exists on base flow seasonal patterns of *E. coli* concentration (Traister and Anisfeld, 2006; Wilkes et al. 2009; Amorim et al. 2014), but few studies take into account *E. coli* loading (Jamieson et al. 2003; Gentry et al. 2006; Vidon et al. 2008). Results from the present study, showing high loadings in the spring compared to all other seasons (except in the Mill watershed) were not surprising given that spring is historically the rainiest season in central Tennessee. More frequent storm events lead to increased base flow conditions (Whittenberg 2003), and since loading is a function of flow, high loadings are plausible These results are in agreement with reports of significantly higher *E. coli* loads in the winter/spring than the summer/fall for streams in agriculturally drained watersheds (Vidon et al. 2008). Though flow appears to be the driving force for the loading increases

observed in the spring (Figure 2.2a, b, c, d), other factors such as sediment resuspension and increasing water temperatures (North et al. 2014) may also have contributed. It is also interesting to note that the high loading values observed in summer were not due to high flow, but instead were concentration-driven (Figure 2.2a, b, c, d).

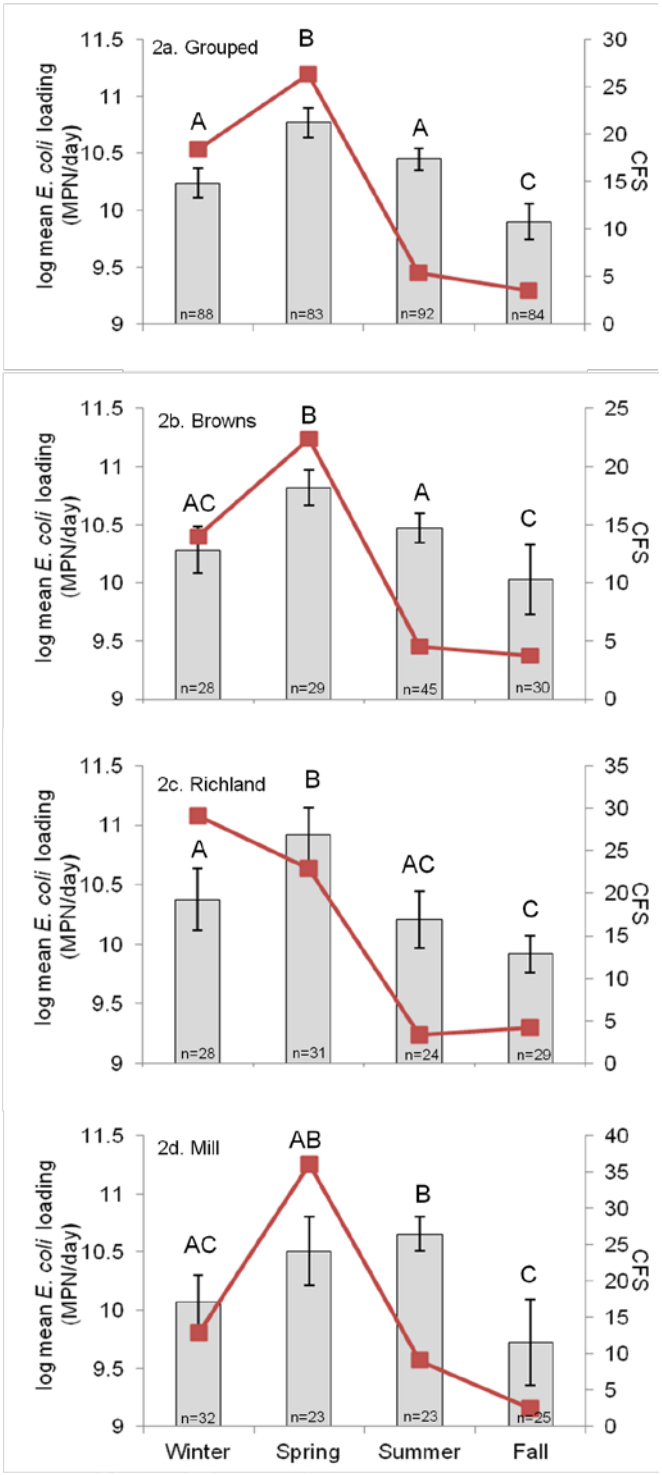

Figure 2.2. a, b, c, d. Bars are seasonal comparisons for log mean *Escherichia coli* loading (MPN/day) in grouped (2a) and in Browns (2b), Richland (2c), and Mill (2d) watersheds. Seasons not sharing similar letters are significantly different from each other.

Data presented as mean ± 95 % confidence intervals. The red line represents average flow in cubic feet for second (CFS).

Concentration analysis is typically used for comparison to pre-determined water quality criteria to assess exposure of fecal pathogens to humans, whereas loading analysis is used in the relative partitioning of fecal loads from point and nonpoint sources in a watershed for total maximum daily load (TMDL) programs (USEPA 2001). These two analysis types, although both focused on *E. coli*, have two different goals and the results of the present study show that the use of results from one analysis type should not be used as a surrogate for the other. More specifically, since loading data takes into account concentration, these results highlight the impact of flow data during base-flow conditions for inland waters and the necessity of obtaining flow data to accurately predict loading values. One technique used as a replacement for site-specific flow data has been the use of modeling programs; though concerns of inherent error have been reported (Shirmohammadi et al. 2006), such as use of limited data to model spatially and temporally variable parameters (e.g., sediment characteristics and flow patterns).

The analysis of both grouped and individual watersheds for the effect of season allowed for the incorporation of a wide variety of data across years and sites, while still allowing for watershed-specific analyses. Using these types of analyses in the future to develop background loading and/or concentration values would provide a means to better understand the impact of storm events, assess best management practice effectiveness, and elucidate long-term changes in land use or hydrological dynamics of the watershed.

Additionally, it is recommended that larger scale analyses of *E. coli* be performed that take into account other factors such as geographic region and climate.

In summary, the approach used in this study proved to be a useful tool for determining seasonal effects at both a large scale and a watershed-specific scale. Concentration- and loading-based results for *E. coli* were highest in summer and spring, and lowest in the winter and fall, respectively. Given that these two commonly used techniques showed different results, care should be taken to not infer data gathered from one analysis technique to the other.

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CHAPTER 3 SEASONAL LOADING AND CONCENTRATION PATTERNS FOR FECAL *BACTEROIDALES* QPCR MARKERS AND RELATIONSHIPS TO WATER QUALITY PARAMETERS AT BASEFLOW

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3.1 Abstract

Bacteria belonging to the Order *Bacteroidales* predominate the intestines of warm-blooded animals and monitoring of these bacteria can indicate fecal pollution impacts to a waterbody. Differences in seasonal concentrations and loadings for *Bacteroidales* and their relationship with physicochemical water parameters were investigated in temperate, inland streams. Seasonal samples (*n = 321*) were collected during baseflow in three central Tennessee, USA watersheds. To estimate total fecal bacteria in receiving streams, general *Bacteroidales* 16S rRNA gene targets were analyzed by quantitative PCR and reported as concentration and loadings for individual and combined watersheds. In most cases, *Bacteroidales* marker concentrations were highest during spring/summer and loading values were highest in the spring. *Bacteroidales* concentrations were positively correlated with temperature and total suspended solids and negatively with dissolved oxygen, while no consistent correlations were found between loadings and abiotic factors. Temperature, total suspended solids, and dissolved oxygen are likely drivers influencing seasonal patterns for *Bacteroidales* concentrations. Researchers and water quality stakeholders should carefully consider measurement type (concentration versus loading), season, and water quality parameters as elements that could impact results when developing fecal monitoring projects.

Keywords Fecal indicator bacteria, *Bacteroidales*, qPCR, season, water quality parameters

3.2 Introduction

Fecal pollution in freshwater ecosystems throughout the world is known to negatively impact both ecological functions and human health. For instance, excessive fecal inputs from concentrated agricultural feeding operations cause an increase in nutrient concentrations and subsequent eutrophication (Mallin and Cahoon 2003), while elevated fecal bacteria concentrations in recreational waters have been associated with increased swimmer illness rates (Prüss 1998; Wade et al. 2008). Sources of fecal pollution include direct inputs, such as sewer overflows, or from indirect contributions like stormwater runoff carrying domestic animal waste. In freshwater ecosystems, fecal pollution and associated pathogens responsible for causing human illness are typically monitored at stream baseflow by culturing *Escherichia coli*, fecal indicator bacteria (FIB) pervasive in warm-blooded animals' gastrointestinal tracts (USEPA 1986, 2001).

Fecal bacteria species within the Order *Bacteroidales* are recognized as alternative FIB (Fiksdal et al. 1985; Kreader 1995; USEPA 2005) that can provide complementary information to *E. coli* monitoring. *Bacteroidales* are obligate anaerobes that are specific (Dick et al. 2005) or selective (Layton et al. 2006) within the digestive system of their host making them particularly useful to determine both the sources and amount of fecal contamination. Rapid culture independent quantitative polymerase chain reaction (qPCR) assays for both general *Bacteroidales* 16S rRNA markers and those specific for human or other animal hosts have been developed (Bernhard and Field 2000a, b; Dick et al. 2005; Seurinck et al. 2005; Layton et al. 2006; Okabe et al. 2007; Shanks et al. 2009). The *Bacteroidales* are considered to be good indicators of recent fecal inputs because they have generally low survival rates in the environment (Fiksdal et

al. 1985; Dick et al. 2010), although recent research indicates long-term persistence outside of a gut is possible (Weidhaas et al. 2015).

Once deposited onto a watershed and transported into a waterbody, fecal bacteria encounter fluctuations in physicochemical water quality parameters (e.g., pH, dissolved oxygen, temperature, total suspended solids) and flow that can be season dependent or not. Understanding how *Bacteroidales* genes may persist in the environment will benefit users of fecal source tracking applications, assist water quality managers in watershed management plans using alternative indicators, and raise awareness to water quality regulators considering these organisms as risk-based fecal indicator bacteria supplementary to traditional FIB, such as *E. coli*. Most research to understand how environmental abiotic factors may influence the fate of *Bacteroidales* has been conducted in laboratory microcosm experiments and to a more limited extent in field situations. Using conventional PCR in a laboratory microcosm study using river water, Kreader (1998) found that DNA of *Bacteroides distasonis* was detectable for significantly longer at 4°C compared to higher temperatures (14, 24, 30°C). Subsequent laboratory and field microcosm research using qPCR has supported the finding that *Bacteroidales* 16S rRNA genetic targets tend to be detected more frequently at lower temperatures (Okabe and Shimazu 2007; Bell et al. 2009; Balleste and Blanch 2010). The effects of other variables including salinity, dissolved oxygen, and sunlight on survival of members of the *Bacteroidales* have also been investigated (Baughn and Malamy 2004; Okabe and Shimazu 2007; Bae and Wuertz 2009; Walters and Field 2009; Balleste and Blanch 2010).

Research to investigate fecal bacteria relationships with abiotic factors in a field setting has focused primarily on stormwater monitoring approaches using conventional culturable FIB, but not *Bacteroidales*. For instance, it has been shown that FIB are often increased during storm conditions in inland watersheds (Rowny and Stewart 2012) and tidal creek headwaters (Stumpf et al. 2010), indicating that runoff is a factor for increased FIB concentrations instream. Little research in regards to relationships between genetic targets and factors has been conducted on inland streams at baseflow conditions. However, recent research conducted in northeast Georgia, USA at baseflow conditions found that the prevalence of *stx2* gene from Shiga toxin producing *E. coli* (STEC) was negatively correlated with water temperature, pH, and conductivity (Bradshaw et al. 2016).

Seasonal changes and dynamic flow and water quality parameters could modulate the presence of *Bacteroidales* markers, yet literature is sparse on this topic. Nonetheless, no seasonal fluctuations of *Bacteroidales* markers were found in a coastal study near Monterey Bay, California (Schriewer et al. 2010), while other FIB, such as *E. coli*, have been detected in higher concentrations in the spring and summer months with lower concentrations in the cooler seasons (Stallard et al. 2016; Traister and Anisfeld 2006). Flow-based *E. coli* loading measurements, which are important components of stormwater management programs (e.g., total maximum daily loads (TMDLs)), were found to be higher during the rainy, spring season at baseflow conditions in inland watersheds (Stallard et al. 2016) and during November for a coastal stormwater study (Converse et al. 2011).

The objective of the present study was to examine relationships between general *Bacteroidales* genetic markers and abiotic factors from a concentration and flow-based (loading) measurement standpoint in three central Tennessee USA watersheds at baseflow. Specific objectives were to 1) determine seasonal trends for *Bacteroidales* concentrations and loadings and 2) examine relationships between *Bacteroidales* concentrations/loadings and water quality parameters (temperature, dissolved oxygen, conductivity, total suspended solids, and pH) both in individual and grouped watersheds. These findings will fill in data gaps for understanding how environmental factors could play a role in *Bacteroidales* presence for temperate, inland waters at baseflow stream conditions. This information will be useful for water quality stakeholders considering a comprehensive approach to conducting water quality activities (e.g., fecal tracking studies, water quality monitoring, watershed management planning) instead of sampling solely for a single FIB.

3.3 Materials and Methods

3.3.1 Study design

During 2009-2012, sites within three pathogen-impaired watersheds (Browns, Richland, Mill; Tennessee Department of Environment and Conservation [TDEC], 2014) located in central Tennessee were sampled and analyzed for fecal bacteria presence during summer (June, July, August), fall (September, October, November), winter (December, January, February), and spring (March, April, May). There was a major flood event in May 2010 and these samples were omitted from analysis. The number of yearly samples, combined by season for each watershed, ranged from 12-31 with a total of 321 samples. Data was pooled seasonally across multiple years to account for a wide range of

site, seasonal, and yearly variation, thus providing a very robust estimate of FIB. Sample sizes are presented within figures and tables.

3.3.2 Study site characterization

The three watersheds under study fall within the Outer Nashville Basin Level IV ecoregion (TDEC 2000) and exhibit a mild climate with an annual mean temperature of 15°C (https://ag.tennessee.edu/climate/Pages/climatedatatn.aspx). The ecoregion consists of non-cherty limestone bedrock, deciduous forests land cover, and low to moderate gradient streams; however, the selected watersheds are heavily developed and may not conform to ecoregion characteristics. There is no direct influence of wastewater treatment plants or concentrated animal feeding operations within the watersheds. Each of the three watersheds are approximately 5,000 hectares and land uses within the watersheds are largely similar, with approximately 50 % residential, 10-20 % commercial and 25 % open area. However, the imperviousness of Mill watershed is 40 %, which is 10-20 % higher than the other two watersheds in the study (a more detailed description of the watersheds was previously given in Stallard et al. 2016

3.3.3 Sample collection

Samples were collected during baseflow stream conditions (< 0.1” rainfall within last 72 h) following TDEC standard protocols (TDEC 2009). For *Bacteroidales* analysis, approximately 110 mL of water was collected from the subsurface at the thalweg of each stream in a sterile polyethylene container, placed on ice, and transported to the laboratory within required hold time (< 6 h) for sample processing and analysis. A one liter sterile polypropylene bottle was used for the collection of samples for total suspended solids (TSS) and processed by Method 2540D in Standard Methods (APHA 2005). Concurrent

with sample collection, stream velocity (ft/sec) was taken for most samples by a Swoffer Model 3000 Current Velocity Meter-Flowmeter and used to calculate flow (cubic feet per second, CFS). Overall loadings to assess gene copies/day to a given sampling site were calculated by multiplying *Bacteroidales* concentrations (gene copies/µl) by simultaneous flow measurements. During each sample collection, pH (Hach Sension meter), dissolved oxygen (DO) (mg/L), conductivity (µS/cm), and temperature (°C) (YSI 85) were recorded.

3.3.4 Bacteroidales *analysis*

An aliquot of 1.3 mL was transferred from the 110 mL sample volume to a 1.5 mL microcentrifuge tube and stored at -80°C until analysis. Storage time before DNA analysis never exceeded six months. The number of *Bacteroidales* 16S rRNA gene copies was determined for stream samples by the AllBac real-time quantitative PCR assay without DNA extraction (Fode-Vaughan et al. 2001; Layton et al. 2006). A total volume of 25 µl per well was used for each qPCR carried out on 96-well plates. Each PCR well contained 2.5 µl of stream sample, standard, or negative control, 11 µl of Brilliant® II qPCR Master Mix (Agilent Technologies, Inc, Santa Clara, CA), 400 nM AllBac 296f forward and 412r reverse primers, 300 nM Taqman AllBac 375Bhqr fluorescent probe, and 9.5 µl of sterile HPLC water. Plasmid DNA standards containing the *Bacteroidales* 16S rRNA gene target were used to generate a standard curve with dilutions from 1×10^7 to 10 copies per µl for each run. A 2.5 ul aliquot taken from a sewage treatment plant influent sample served as the positive control, was run in separate wells, and confirmed as a positive signal with an average Ct of 27. Filter-sterilized HPLC water was used for the no template control. All standards, negative, and positive controls were performed in

triplicate. Stream samples were initially run in triplicate; however, samples in 2010 and 2011 were processed in duplicate to increase number of samples per plate. The limit of detection (LoD) was determined by running 20 replicates of diluted plasmid standard. The lowest plasmid standard concentration with detections > 95 % was 5 copies/µl. Six samples (4 from Browns and 2 from Richland) were below this value and these were assigned a value of half the detection limit (2.5 copies/µl) which allows for statistical comparisons and does not bias the result as zero in case the method limit of detection was not sensitive enough to detect this value. An additional well following every sample replicate set was spiked with 2.5 x 10^5 copies of the plasmid DNA plus sample to evaluate qPCR inhibition of each sample. Percentage of spike recovery was calculated as copy number recovered in the spiked well minus the copy number in an unspiked sample well divided by 2.5 x 10^5 then multiplied by 100. Only one sample generated a spike recovery of < 70 %, indicating qPCR inhibition and was rerun with a 10-fold dilution. Gene targets were amplified using a CFX 96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The direct qPCR amplification protocol was as follows: 55°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 60°C for 30 s. *Bacteroidales* 16S rRNA gene concentrations in a stream sample are reported as copies/µL and loadings are presented as copies/day.

3.3.5 Statistics

One-way Analysis of Variance (ANOVA) tests were performed to assess seasonal differences for concentrations and loadings of *Bacteroidales* for individual and grouped watersheds. When significant seasonal differences were found by ANOVA, Tukey's post-hoc tests were carried out to detect differences among seasons. Log_{10}

transformations were implemented on bacteria concentrations and loadings if assumptions of normality and equality of variances were not met. A non-parametric Spearman's rank order test was used for each watershed to assess correlations between *Bacteroidales* concentrations and loadings with temperature, DO, conductivity, TSS, and pH. An alpha (α) = 0.05 was used as the significance level for all statistical analyses performed with IBM SPSS Ver 20 (Armonk, NY: IBM Corp).

3.4 Results and Discussion

3.4.1 Seasonal Bacteroidales *concentrations*

Bacteroidales concentrations were highest in the warmer months of spring and/or summer in two of the three watersheds (Browns - $F_{3, 121} = 3.457$, $p < 0.05$; Richland - $F_{3, 117} = 16.518$, $p < 0.01$), as well as when the data from the three watersheds were grouped ($F_{3, 317} = 8.449$, $p < 0.01$) (Figure 3.1). Interestingly, in Mill watershed, there were no significant differences among the seasons for *Bacteroidales* concentrations ($F_{3, 71} = 1.286$, $p = 0.286$).

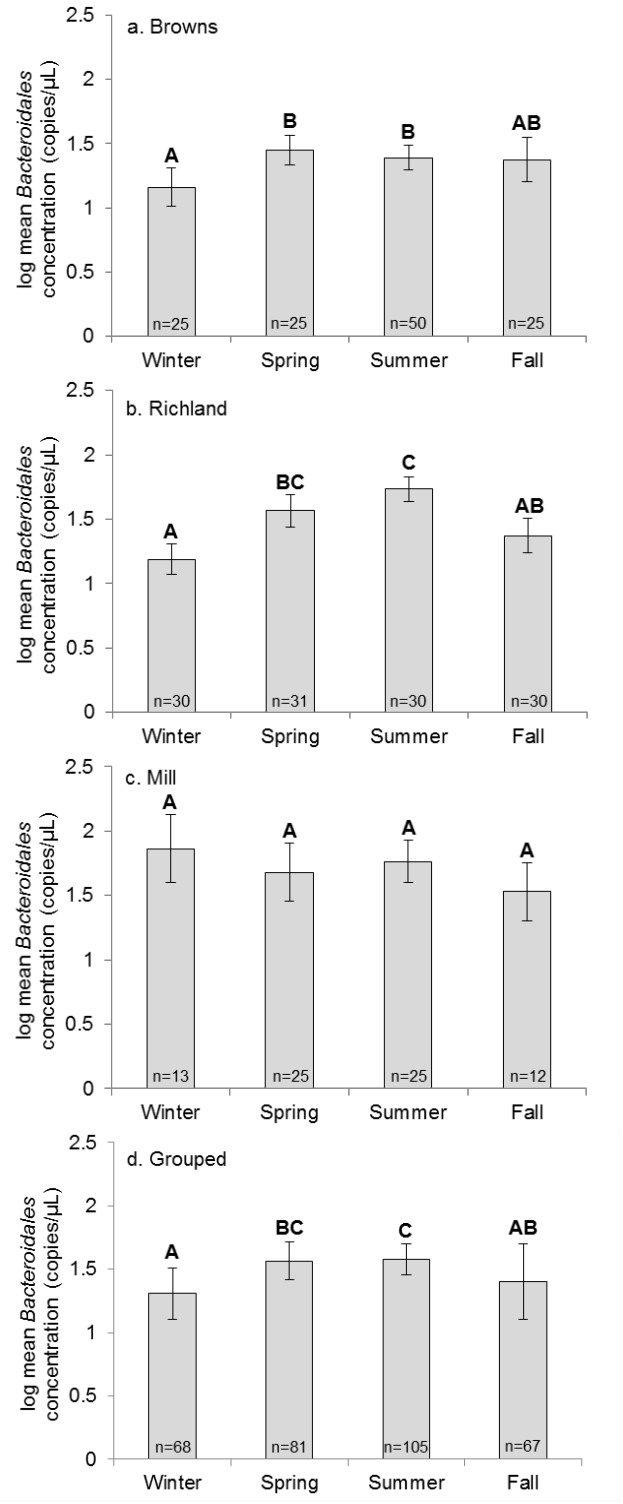

Figure 3.1. a, b, c, d Bars are seasonal comparisons for *Bacteroidales* concentrations (copies/µL) in Browns (a) Richland (b), Mill (c), and grouped (d) watersheds. Seasons

not sharing similar letters are significantly different from each other. Data presented as mean ± 95 % confidence intervals.

The pattern of higher *Bacteroidales* concentrations in the warmer seasons vs lower in the winter that was seen in the grouped watersheds and two out of three individual watersheds was similar to that seen in a prior study from our laboratory measuring *E. coli* concentrations (Stallard et al. 2016). In fact, there is a significant, but weak correlation between *E. coli* and *Bacteroidales* concentrations for grouped watersheds ($p < 0.05$; $r = 0.113$). These data imply that while *Bacteroidales* (measured as AllBac) and *E. coli* concentrations are related, there are most likely environmental factors which are impacting the two differently. *Bacteroidales* patterns in the present study do not agree with the only other report of *Bacteroidales* seasonal concentrations. Schriewer et al. (2010) reported no seasonal fluctuations of *Bacteroidales* (or fecal coliforms) around Monterey Bay, California, although no statistical comparisons were reported. Perhaps the lack of seasonal trends was due to the specific *Bacteroidales* gene marker targeted (BacUni in Schriewer *et al*. versus AllBac in the present study). Additionally, streams, rivers, and estuaries were all compiled to assess seasonal trends in the Schriewer study, whereas only freshwater streams with no marine influence were sampled in the present study.

It is plausible that increased activity/movements near riparian zones in warmer months from domestic animals or wildlife could increase fecal inputs both in riparian zones and in-stream. Increases in *Bacteroidales* concentrations during the warmest summer months may be more pronounced because stream flow and water levels are

reduced at this time. In the possibility that *Bacteroidales* is surviving in the environment, the concentration patterns seen in the present study may be due to reduced metabolic activity in cooler winter temperatures followed by growth and replication on the watershed in warmer months and transportation to streams by spring rainfall events.

3.4.2 Seasonal Bacteroidales *loadings*

For grouped watersheds, *Bacteroidales* spring loadings were higher than all other seasons ($F_{3, 270} = 16.142$, $p < 0.01$) (Figure 3.2). For individual watersheds, *Bacteroidales* loadings were found to be highest in spring for Browns ($F_{3,93} = 9.539$, $p < 0.01$), while for Richland watershed, loadings during spring were significantly higher than summer and fall, but not winter ($F_{3,107} = 6.808$, $p < 0.01$). As with concentrations, loadings in Mill were similar in all seasons ($F_{3, 62} = 2.204$, $p = 0.096$). Also, as with concentrations, there is a significant correlation between *E. coli* and *Bacteroidales* loadings for grouped watersheds ($p < 0.001$; $r = 0.542$) (for complete *E. coli* data, see Stallard et al. 2016).

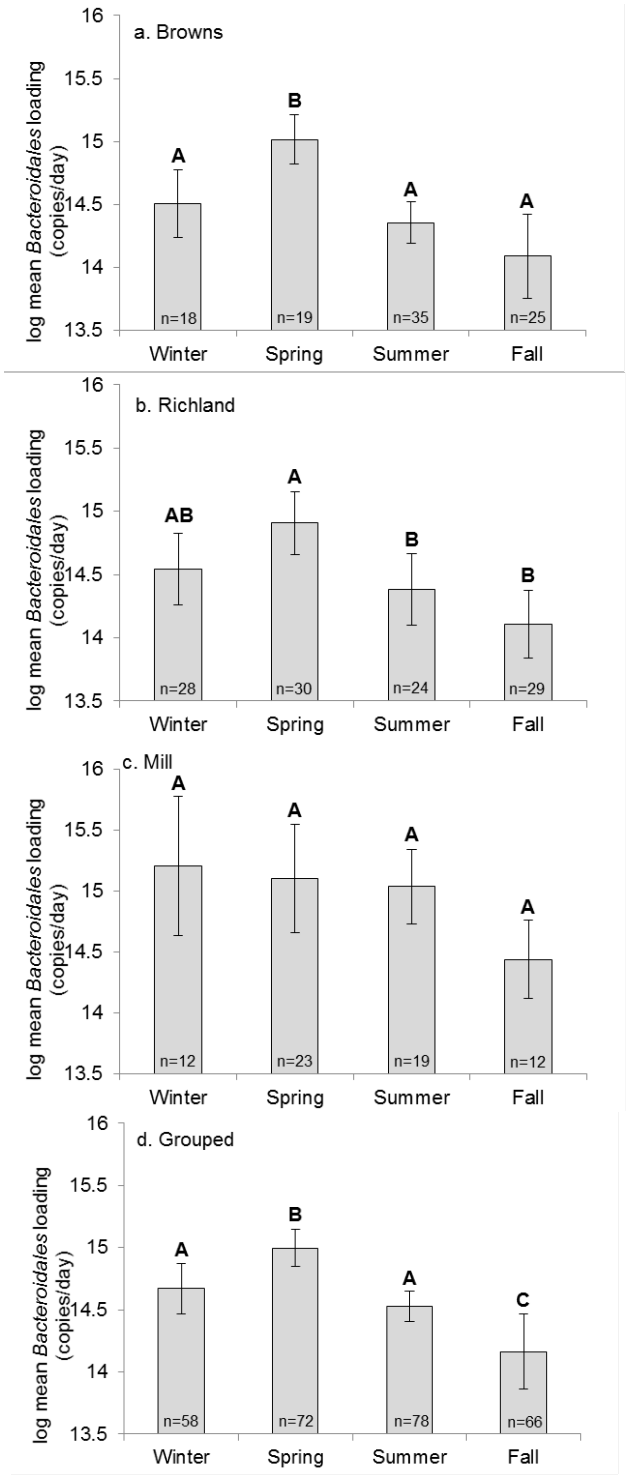

Figure 3.2. a, b, c, d Bars are seasonal comparisons for *Bacteroidales* loadings (copies/day) in Browns (a) Richland (b), Mill (c), and grouped (d) watersheds. Seasons

not sharing similar letters are significantly different from each other. Data presented as mean ± 95 % confidence intervals.

Loadings for *Bacteroidales* were highest predominantly in the spring even though the highest concentrations occurred typically in the summer. Antecedent rainfall during winter and spring rainy seasons could have increased flow leading to higher loading even during baseflow sampling. Similarly the increased flow could have agitated and resuspended historically deposited FIB in stream sediments leading to higher loadings. Another potential factor influencing *Bacteroidales* seasonal patterns for loadings in Browns, Richland, and the grouped watersheds could be the integration of FIB into the water, sediment or riparian soil matrix (i.e. naturalization). Vierheilig et al. (2012) found a high level of AllBac *Bacteroidales* marker in pristine alpine soils (10 cm depth) void of fecal pollution or animal activity. This suggests that the *Bacteroidales* may have been from feces deposited at an earlier time that became integrated into soil microbiota or possibly derived from resident soil microorganisms. Likewise, Byappanahalli et al. (2006) have suggested that naturalized *E. coli* in soil from a Great Lakes watershed could be a fecal input to Great Lakes waterbodies. Also, Ishii et al. (2006) reported the concentrations of naturalized soil *E. coli* from three temperate Lake Superior, USA watersheds were highest in summer and fall and lowest in winter and spring months. High spring loadings in central Tennessee streams in the present study could be originating from naturalized soil FIB that are being transported by runoff during the rainy season. It is probable that the majority of *Bacteroidales* genetic markers seen in the

present study are associated with fecal deposition at some point in time because *E. coli* were also detected at high levels at this time in the same watersheds (Stallard et al. 2016) and the concentration and loading values for *Bacteroidales* and *E. coli* are correlated.

3.4.3 Concentrations and loadings vs water quality parameters

Bacteroidales concentrations consistently showed significant, though weak-moderate, negative correlations with DO and positive correlations with temperature in Browns (DO $r = -0.275$, temperature $r = 0.200$), Richland (DO $r = -0.424$, temperature $r = 0.542$), and the three watersheds grouped (DO $r = -0.201$, temperature $r = 0.255$), but not Mill watershed individually (Table 3.1). When investigated individually or grouped, weak-moderate positive correlations were found for TSS and *Bacteroidales* concentrations in the three watersheds under study (r values ranged from 0.195-0.334), while concentrations were not correlated with conductivity in any of the watersheds.

Table 3.1. Spearman's rank correlation analyses for *Bacteroidales* concentrations and loadings with dissolved oxygen, temperature, total suspended solids, conductivity, and pH (values represent Spearman's *r*, with sample sizes in parentheses)

		Bacteroidales concentration	*Bacteroidales* loading
Dissolved oxygen (mg/L)	Browns	-0.275** (125)	0.036 (97)
	Richland	-0.424** (121)	-0.049 (111)
	Mill	0.096 (75)	0.111 (66)
	Grouped	-0.201** (321)	0.034 (274)
Temperature (°C)	Browns	0.200* (125)	-0.094 (97)
	Richland	0.542** (121)	0.012 (111)
	Mill	0.008 (75)	-0.025 (66)
	Grouped	0.255** (321)	-0.032 (274)
Total suspended solids (mg/L)	Browns	0.236* (75)	-0.132 (65)
	Richland	0.334** (91)	-0.025 (83)
	Mill	0.281* (70)	0.323** (65)
	Grouped	0.195** (236)	-0.009 (213)
Conductivity (µS/cm)	Browns	0.129 (125)	-0.345** (97)
	Richland	-0.060 (121)	-0.233* (111)
	Mill	-0.021 (75)	-0.173 (66)
	Grouped	0.046 (321)	-0.200** (274)
pH	Browns	-0.077 (125)	-0.277** (97)
	Richland	-0.294** (121)	-0.329** (111)
	Mill	0.378** (75)	0.446** (66)
	Grouped	0.014 (321)	-0.054 (274)
Bacteroidales concentration	Browns	1.000 (125)	0.457** (97)
	Richland	1.000 (121)	0.475** (111)
	Mill	1.000 (75)	0.810** (66)
	Grouped	1.000 (321)	0.603** (274)
Bacteroidales loading	Browns	0.457** (97)	1.000 (97)
	Richland	0.475** (111)	1.000 (111)
	Mill	0.810** (66)	1.000 (66)
	Grouped	0.603** (274)	1.000 (274)

** Correlation is significant at the 0.01 Level.

* Correlation is significant at the 0.05 level

Correlations between abiotic factors and *Bacteroidales* loadings were more inconsistent than those with concentrations. Again, Mill watershed was frequently found

to be different than the other two in relation to loading correlations. Unlike with concentrations, no significant correlations were revealed for loadings with DO or temperature in either individual or grouped watersheds. However, *Bacteroidales* loadings were correlated negatively with conductivity in Browns ($r = -0.345$), Richland ($r = -0.233$) and the grouped ($r = -0.200$) watersheds and were positively correlated with TSS in Mill ($r = 0.323$). Negative correlations between loadings and pH were seen in Browns ($r = -0.277$) and Richland ($r = -0.329$), while a positive correlation was seen in Mill ($r = 0.446$).

Not surprisingly, strong positive associations between *Bacteroidales* concentrations and loadings were exhibited by all individual and grouped watersheds. However, the very strong correlation at Mill watershed ($r = 0.810$) compared to Browns ($r = 0.457$) and Richland ($r = 0.475$) watersheds again seemed to indicate differences at Mill.

Seasonal patterns of concentrations and loadings are likely attributable to seasonal variation of associated water parameters, at least in part. Understanding what factors play a role in the presence of FIB would inform water quality regulators to allocate resources for collecting data on such. For *Bacteroidales* concentrations in the present study, two of the three individual as well as the grouped watersheds had positive and negative relationships with temperature and oxygen, respectively. These temperature and DO associations make sense in conjunction with each other since temperature and DO are negatively correlated in aquatic systems. However, these results were dissimilar to prior laboratory and microcosm studies that revealed *Bacteroidales* gene targets were more persistent in colder temperatures (Kreader 1998; Bell et al. 2009; Schulz and Childers

2011). High temperature was cited as the key factor affecting increased decay of environmental *Bacteroides* strains in both a laboratory study and an on-site river study where sewage-filled dialysis bags were exposed to stream conditions (Balleste and Blanch 2010). Differences between the present study and others may be explained by site specific differences in abiotic factors and effects related to study design. For example, in the present study, samples and measurements were taken directly from field sites and no fecal samples were manipulated or introduced into closed systems (e.g. dialysis bags) and attributes of water parameters in laboratory mesocosms may have degraded and caused more rapid decay of bacteria or DNA from dead/non-viable bacteria. It is also possible that there were continuous or intermittent inputs of fecal bacteria in the present study at some locations.

Though several studies have investigated sediment as a secondary habitat for *Bacteroidales,* there has been little exploration of TSS as an influential factor for *Bacteroidales* persistence. Turbidity, a parameter which detects the amount of suspended solids in a water column by measuring scattered light instead of a concentration like TSS, was found to have a positive correlation with *Bacteroides* loads (kg/day) and a stronger positive correlation was seen when sampling from headwaters to tailwaters in an eastern Tennessee watershed (Gentry et al. 2007). In the present study, among all watersheds there was a consistent positive correlation of TSS with *Bacteroidales* concentrations, suggesting that suspended solids may play an overlooked role in gene persistence by a variety of mechanisms. For instance, the suspended particles could serve as infrastructure for attachment of *Bacteroidales* (viable or non-viable) and increase overall densities of *Bacteroidales* gene copies. The solids could provide interstitial spaces for growth and act

as a habitat for these anaerobic bacteria during baseflow, however, additional research needs to be conducted to verify if these gene copies are from viable cells.

In the present study, conductivity displayed no relationship with *Bacteroidales* concentrations for all watersheds. This is interesting because TSS and conductivity typically have a positive relationship with each other, but did not in this study (data not shown). The suspended solids measured in the present study may contain chemical properties less likely of carrying an electrical charge and could have confounded the possibility of conductivity having a relationship with *Bacteroidales* concentrations. A negative correlation was found for *Bacteroidales* loadings and conductivity in Browns and Richland, but no correlation was found in Mill watershed, again highlighting that Mill does not follow patterns of the other watersheds. The measurement of pH did not produce a clear pattern for *Bacteroidales* concentration or loadings. These data are similar to those from a watershed study in eastern Tennessee that reported conductivity and pH to not be associated with FIB (*E. coli*) concentrations (Gentry et al. 2006). Perhaps there are other water quality analytes, like total organic carbon or nitrogen that could be underlying this lack of correlation between pH and conductivity and FIB.

3.4.4 Importance of differences in Mill watershed

In Mill watershed, the finding of no patterns for seasonal *Bacteroidales* concentration and loadings, as well as lack of correlations with temperature and DO, is quite important and could signify a watershed with a constant fecal input. In fact, there have been a number of documented intermittent sewer overflows at various places in Mill watershed (Metro Nashville Water Services, personal communication). Perhaps unique watershed characteristics at Mill, such as high imperviousness and subsequent increased

runoff, may contribute to higher concentrations or loadings of FIB, thus minimizing a seasonal pattern. A human-associated fecal *Bacteroidales* marker (HuBac) was also analyzed and was slightly above detection in Mill watershed on a few occasions (data not shown). Water quality managers responsible for improving impaired recreational waterbodies should take note of patterns contradictory with baseline data from within the watershed and from other watersheds as a reason to prioritize a watershed for further investigation.

3.5 Conclusions

The present study demonstrates seasonal patterns for *Bacteroidales* concentrations and loadings in a freshwater system at baseflow and highlights the fact that temperature, TSS, and dissolved oxygen are repeatedly significant in these patterns. Because regulatory water quality sampling or fecal tracking studies can occur at infrequent intervals, and since peaks for *Bacteroidales* concentrations and loadings can happen in different seasons, water quality managers must carefully select the type (concentration vs loading) and time (season) of sampling that best fit water quality goals (i.e., source reduction or human health protection). For example, winter sampling might be best for detection of sewer overflow or septic tank seepage because fecal indicators are generally lower in the colder months and any spike in concentrations might be indicative of a recent direct input. Also, a priority ranking for monitoring, fecal source-tracking, and remediation should be considered for sites, such as Mill watershed in the present study, which do not follow typical patterns for FIB concentrations associated with seasonal and water parameters, as this could indicate a constant fecal input.

Once baseline data have been gathered in a watershed that show the AllBac marker to be correlated with traditional fecal indicator bacteria, such as *E. coli*, it would be beneficial to utilize the AllBac qPCR assay because of its faster turnaround time compared to conventional *E. coli* methods. However, when AllBac and *E. coli* patterns do not correlate or the AllBac patterns don't match typical patterns for traditional FIB, stakeholders would need to investigate to explain this phenomenon and/or deem the utility of the marker limited. This would be warranted because of the possibility that the lack of correlation is being caused by an input of *Bacteroidales* from nonfecal sources. Continued field studies to reveal how *Bacteroidales* and *E. coli* behave under the same environmental influences will be imperative going forward when considering *Bacteroidales* markers as a surrogate or supplement to traditional indicators.

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Conflict of Interest

The authors declare no conflict of interest.

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CHAPTER 4 THE INFLUENCE OF DISTANCE FROM SHORE AND SAND DEPTH ON *ESCHERICHIA COLI* CONCENTRATIONS IN SAND FROM AN INLAND RECREATIONAL BEACH

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4.1 Introduction

Protection of swimmers from exposure to potential fecal pathogens at coastal beaches in the United States is implemented by monitoring fecal indicator bacteria (FIB, i.e., enterococci or *Escherichia coli*) (USEPA 2000). Presence of these FIB in recreational waters has been shown to have a correlative relationship to GI illness in swimmers (Cabelli 1983; Dufour et al. 1984; Wade et al. 2003). Exposure to coastal water pollution poses both a public health and economic burden at a worldwide level (Pruss 1998; Shuval 2003). Even at a local setting, illness incidences and economic loss can be substantial. For example, in a case study of two beaches in California, gastrointestinal illness rates were estimated to be 36,778 per year with an associated cost of over $1.3 million in medical care and loss of income (Dwight 2005). Despite the economic and health impact impaired beach water quality could produce, only 45 % (2708 of 6063) of sites on the National List of Beaches in the US, which are required to establish a state monitoring and public notification program, are routinely monitored. Inland beaches, other than those adjacent to the Great Lakes, are not included under the federally mandated program.

Fecal bacteria in recreational beach water can originate from multiple sources (e.g., runoff, animals, humans, and sewage overflows) and beach sand has received increasing attention as a reservoir of fecal bacteria (Alm et al. 2003; Beversdorf et al. 2007; Ishii et al. 2007; Halliday and Gast 2011), which could in turn pose an exposure threat to beach visitors through resuspension into the water column (Edge and Hill 2007; Ge et al. 2012; Phillips et al. 2014; Whitman et al. 2014) or through direct sand contact. Epidemiological studies have demonstrated contact with beach sand can result in

gastrointestinal illness (Bonilla et al. 2007; Heaney et al. 2009) with evidence of enteric illness associated with fecal indicator organism densities in beach sand (Heaney et al. 2012). Presence of antibiotic resistant strains of fecal indicator bacteria (Alm et al. 2014; de Oliveria et al. 2008) and pathogenic microorganisms, such as *Camplyobacter* spp. (Bolton et al. 1999; Yamahara et al. 2012), *Staphlyococcus aureus* and methicillin-resistant *Staplyocoocus aureus* (MRSA, Shah et al. 2011; Yamahara et al. 2012; Goodwin et al. 2012), *Vibrio vulnificus*, and *Cryptosporidium* spp. (Shah et al. 2011; Abdelzaher et al. 2010) and pathogenic yeasts (Shah et al. 2011) have been detected in beach sand. Several accounts have reported fecal bacteria in beach sand at considerably higher densities compared to surrounding waters (Alm et al. 2003; Elmanama et al. 2005; Whitman and Nevers 2003; Bonilla et al. 2007; Abdelzaher et al. 2010). Collectively, the reasons mentioned above lend cause for continued exploration of beach sand as both a source of indicator organisms and etiology of human disease.

Waterborne gastrointestinal illnesses have a greater impact on sensitive populations defined as the elderly, very young, pregnant, or immunocompromised individuals (Gerba 1996). Spatial distribution of fecal bacteria in beach sand could be an important factor in identifying locations where risk of exposure to pathogens could be of greatest concern to these populations. Researchers document foreshore sand at Great Lakes beaches as the area with highest fecal bacterial levels compared with backshore or nearshore (Whitman and Nevers 2003; Kinzelman et al. 2004). Fecal bacteria were concentrated in the intertidal zone for subtropical beaches in Florida (Shibata et al. 2004; Wright et al. 2011) or the supratidal zone in Hawaii beach sand where recreators typically congregate for non-swimming activities such sand play, sunbathing, and eating (Oshiro

and Fujioka 1995; Cui et al. 2013). Solo-Gabriele et al. (2016) recommended the consideration of spatial distribution of fecal bacteria when developing beach sand monitoring programs.

Surveillance and spatial distribution studies of fecal indicators and microbial communities in beach sand throughout the U. S. have been conducted primarily in subtropical or temperate coastal, estuarine, and Great Lakes beaches (see review in Halliday and Gast 2011). The novel research presented here was carried out on a temperate, inland manmade beach adjacent to a river impoundment. The purposes of this study were to 1) test for differences in *E. coli* at incremental distances from shoreline; 2) test for differences in *E. coli* at 3 depths in the beach sand; and 3) to determine if any interactions existed between distance and depth.

4.2 Material and Methods

4.2.1 Field sampling

Sand core samples were collected for *E. coli* analysis during the summer of 2015 at Cedar Creek Recreational Area. Cedar Creek Recreational Area beach (36.2771N,-86.5084W) is approximately 1,200 square meters (m^2), is located on Old Hickory Reservoir, an impoundment of the Cumberland River in central Tennessee, USA, and is managed by the U.S. Army Corps of Engineers. This specific beach was chosen because no prior sampling of *E. coli* exists, it is a freshwater, inland beach in a temperate setting which is rarely assessed, and was not impacted by point sources such as wastewater treatment plants or stormwater pipes. The recreational area contains a swim beach with approximately 125’ linear meters of sandy shoreline (Figure 4.1). On the beach, the foreshore is defined as the wave impacted shoreline, the backshore as the drier sandy area

not impacted by wave action, and the nearshore as the water-inundated area to the water side of the shoreline (Whitman et al. 2014).

Sampling sites for the foreshore were chosen at 0.3m from either side of the shoreline at five 10-m increments down the beach (0, 10, 20, 30, 40 m). Other sampling sites were selected at 3, 6, 9, and 12 m away from the shoreline at the same ten meter increments in inundated nearshore waters and the dry backshore portion of the beach (Figure 4.1). Sand sampling points away from the shoreline that were consistently covered with reservoir water (nearshore) were delineated with a negative sign. From each sampling point, sand was collected from 0-10, 10-20, and 20-30 cm depths. Nearshore (*n = 59*) and inundated foreshore (*n = 15*) sand samples were collected on August 27. Backshore (*n = 50*) and upper foreshore (*n = 15*) sample were collected on September 7 (Labor Day). No precipitation, implying no runoff from surrounding areas, occurred between the two sampling events and no dam releases were indicated based on elevation changes from 8/27/15 (445.1') to 9/8/15 (444.9') (http://oldhickory.uslakes.info/Level/).

Figure 4.1. Sampling transect for sand core samples taken at Cedar Creek Recreational Area, Tennessee. Samples were taken at points where two lines intersect. Backshore (3, 6, 9, 12 m), foreshore (0.3 to -0.3 m), and nearshore (-3, -6, -9,-12 m) areas are indicated by arrows.

Each sand core sample was collected with a piece of clean, sanitized 1 1/4” inside diameter Schedule 40 PVC pipe. For each exuded 10 cm section of the core, a test tube was carefully plunged into the center of the core to collect a subsample in order to avoid

possible contamination near the edge of the core. Test tubes were immediately placed on ice for transport to the laboratory.

4.2.2 E. coli sample processing

Samples were processed following the Boehm et al. (2009) method. Briefly, 12 g of sand from each sampling point were added to 120mL of phosphate buffered saline (PBS), hand shaken by the same experimenter in a sterile glass bottle for 2 min, and then allowed to settle for 1 min. Exactly 100 mL of shaken supernatant were transferred into EPA compliant fecal coliform testing bottles and used for the determination of *E. coli* using Colilert defined substrate technology (IDEXX Laboratories, Westbrook, Maine). Autoclaved sand was spiked with IDEXX Quanti-cult® organisms (*Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *E. coli*) and processed similar to samples to evaluate sample recovery on a presence/absence basis. Sand samples were then dried in an oven at 60°C for approximately 48 h until constant weight was reached. The *E. coli* concentrations are reported as MPN/100g/dry weight of sand. For statistical purposes, samples were combined into the following three categories (distance from shoreline): foreshore (0.3 and -0.3 m), backshore (3, 6, 9, 12 m), and nearshore (-3, -6, -9, and -12 m).

4.2.3 Statistics

Due to violation of assumptions, an ANOVA could not be run and interactions between distance from shore and depth of sand were not investigated. Instead, three individual Kruskal-Wallis H non-parametric tests were performed to assess differences in *E. coli* concentrations for the independent variables 1) distance from shoreline, 2) sampling depth, and 3) sampling depth pooled by distance from shoreline. For post-hoc

analysis, pairwise comparisons for each test were run using Dunn's procedure with Bonferroni correction for multiple comparisons. Statistical analyses were performed with IBM SPSS Software Version 23.

4.3 Results

As determined by boxplot visualization, the equal distribution assumption was violated for groups within each Kruskal-Wallis test, so results for mean ranks of *E. coli* concentrations were compared instead of median values. Values in parentheses are mean ranks unless otherwise denoted. Significant difference in mean ranks were found in *E. coli* concentrations among the three categories of distance from shoreline: ($\chi^2 = 15.165$, $df = 2$, $p = 0.001$; Figure 4.2) with both foreshore (*80.15*, $p < 0.05$) and backshore (*81.52*, $p = 0.001$) having significantly higher *E. coli* concentrations than nearshore (*55.08*).

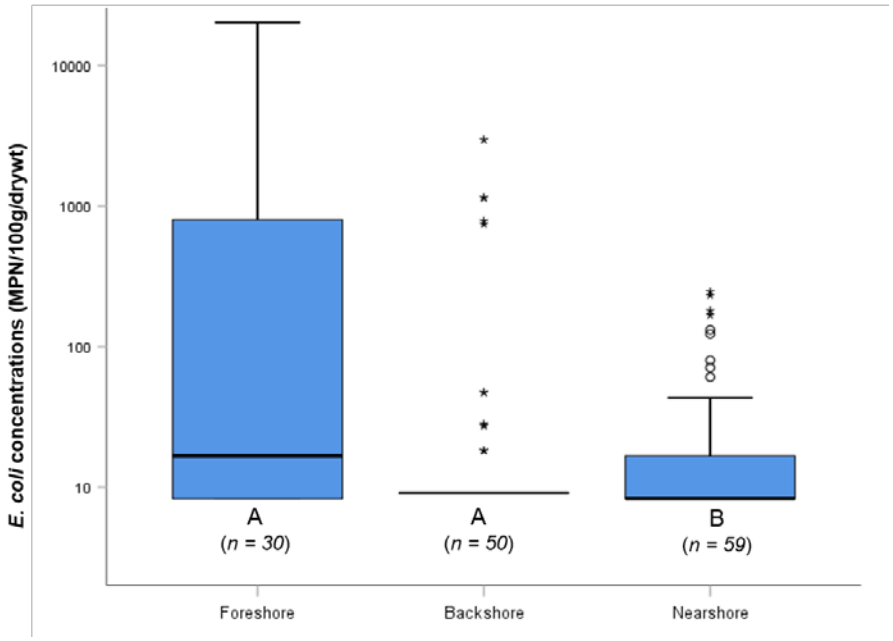

Figure 4.2 Comparison of *E. coli* concentrations (MPN/100g/dry wt of sand) for foreshore (0.3 to -0.3 m), backshore (3, 6, 9, 12m), and nearshore (-3, -6, -9,-12 m) sampling locations sampling areas at Cedar Creek Recreational Center. Boxplots not sharing the same letter have statistically different mean ranks as analyzed by a Kruskal-Wallis H non-parametric test. Sample sizes presented in parentheses. Asterisks above boxplots represent extreme outliers and circles are mild outliers.

The three depths layers were different from each other ($\chi^2 = 46.333$, $df = 2$, $p < 0.001$; Figure 4.3) with the mean rank (*101.17*) for *E. coli* concentrations in the 0-10 cm stratum being statistically higher than 10-20 (*57.09*, $p < 0.001$) or 20-30 cm (*50.93*, $p < 0.001$) layers, but these two depths were similar to each other.

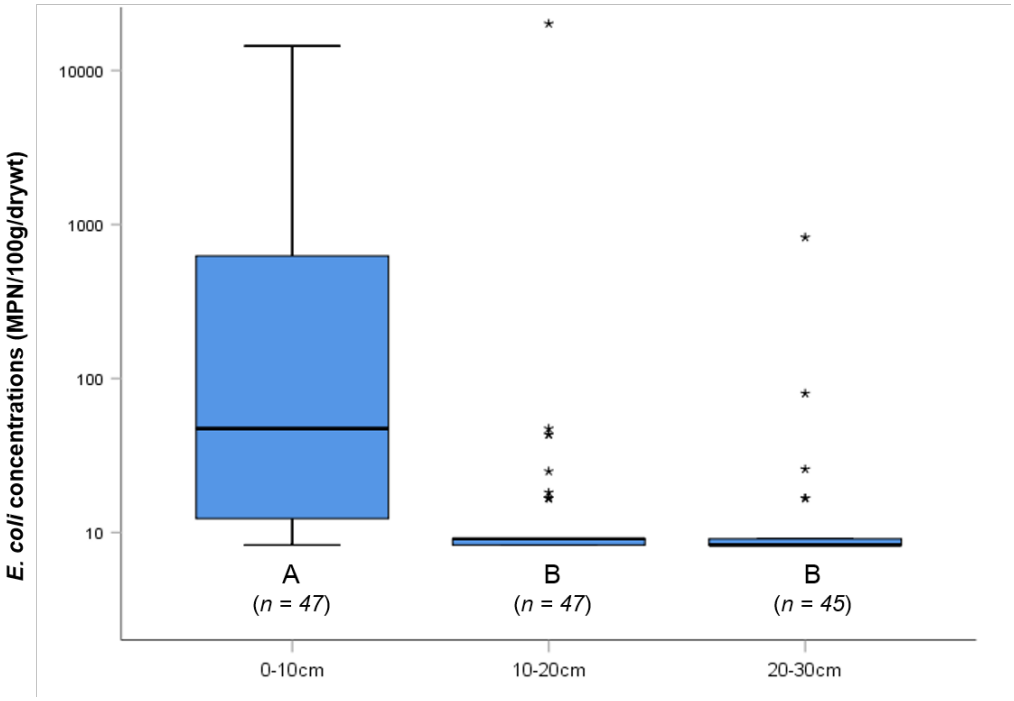

Figure 4.3 Comparison of *E. coli* concentrations (MPN/100g/dry wt of sand) for depth (0-10, 10-20, 20-30 cm) at Cedar Creek Recreational Area. Boxplots not sharing the same letter have statistically different mean ranks as analyzed by a Kruskal-Wallis H non-parametric test. Samples sizes presented in parentheses. Sample sizes presented in parentheses. Asterisks above boxplots represent extreme outliers and circles are mild outliers

The Kruskal Wallis analysis for each sampling depth pooled by distance from shoreline revealed *E. coli* concentrations were significantly different ($\chi^2 = 69.884$, $df = 8$, $p < 0.001$; Figure 4.4). The *E. coli* concentrations in foreshore 0-10 cm (*130.10*) were significantly higher ($p < 0.01$) than most distance and depth groupings except for backshore (*98.65*) and nearshore (*88.85*) 0-10 cm. Backshore 0-10 cm (*98.65*) was significantly higher than foreshore 20-30 cm (*43.25*, $p < 0.001$) and nearshore 10-20 (*37.05*, $p < 0.001$) and 20-30 cm (*38.50*, $p < 0.001$). Nearshore 0-10 cm (*88.85*) was

higher ($p < 0.01$) than the other depths at this distance. Foreshore, backshore, and nearshore at 10-20 cm and 20-30 cm layers were not significantly different from each other.

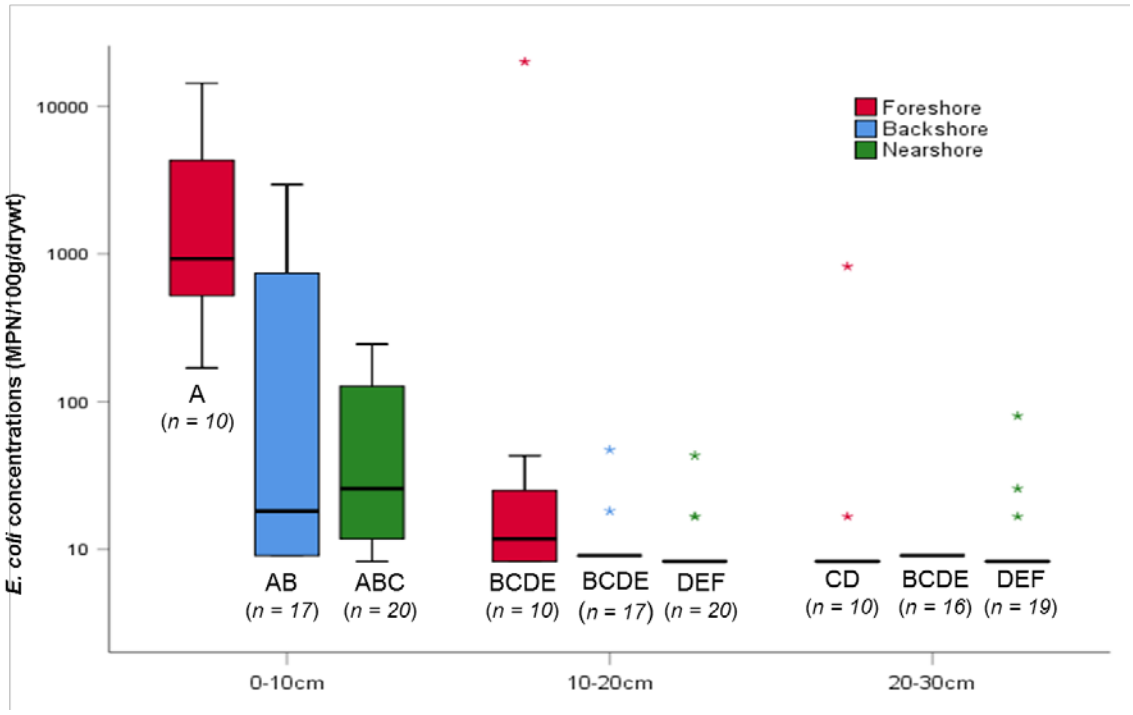

Figure 4.4. Comparison of *E. coli* concentrations (MPN/100g/dry wt of sand) for each combination of sampling depth (0-10, 10-20, 20-30 cm) and distance from foreshore (0.3 to -0.3 m), backshore (3, 6, 9, 12 m), and nearshore (-3, -6, -9,-12 m) at sampling areas at shoreline at Cedar Creek Recreational Area. Boxplots not sharing the same letter have statistically different mean ranks as analyzed by a Kruskal-Wallis H non-parametric test. Sample sizes presented in parentheses. Asterisks above boxplots represent extreme outliers and circles are mild outliers.

4.4 Discussion

Both freshwater and marine beach sands have been extensively reported to harbor fecal indicator bacteria, potential pathogens, and antibiotic resistant organisms (de Oliveria et al. 2008; Wright et al. 2011; Yamahara et al. 2012; Alm et al. 2014). The research presented here is of particular interest because most freshwater beach studies regarding the presence of these types of microorganisms have been from Great Lakes regions in the U. S. and sampling does not always include a depth profile. In several sampling points at this beach, *E. coli* concentrations exceeded water quality criteria when estimating 100g of sand being equivalent to 100 mL of water.

In conjunction with discerning the presence of fecal indicator bacteria at beaches, it is important to understand to what levels FIB occur at various locations at a beach. The three types of areas of concern include the foreshore, backshore, and nearshore with activities in each of these area potentially exposing sensitive populations to fecal pathogens. At Cedar Creek Recreational Area, the highest *E. coli* concentrations were found in foreshore, followed by backshore, then nearshore samples. In comparison, *E. coli* counts were an order of magnitude higher in foreshore sand than submerged sand samples at a Great Lakes beach (Whitman and Nevers 2003). These results also follow the general trend for freshwater beaches elaborated in Whitman et al. (2014) where wet foreshore sand contains higher FIB concentrations than inundated sand or dry backshore. However, for three beaches in Florida, *E. coli* was highest in backshore, followed by foreshore, and nearshore (Bonilla et al. 2007). Interestingly, in the Bonilla study, a dose-response was established between GI illness and time spent in wet (foreshore) sand, but this did not occur for dry (backshore) sand where *E. coli* levels were the highest.

Escherichia coli abundances were also higher in backshore than foreshore and nearshore samples in Hawaii sand (Cui et al. 2013) and it was suggested *E. coli* do not survive well in high-salinity marine conditions. However, the present study is in a freshwater environment and *E. coli* likely thrive in the foreshore area because of minimal wave action and no tidal fluctuations leading to consistently moist foreshore conditions.

In the present study, the highest levels of *E. coli* were found in the 0-10 cm upper layer and were substantially lower in the 10-20 and 20-30 cm layers. In agreement with this study, Alm et al. (2003) found *E. coli* densities from a Great Lakes beach to be highest in the uppermost 0-5 cm and 5-10 cm layer and at comparable densities to those at Cedar Creek. Though concentrations decreased in the 10-15 and 15-20 layers from the Alm study, mean *E. coli* densities were approximately 2-4 times higher than in the present study. *Escherichia coli* was not found deeper than 5cm in Florida river sediment cores (Desmarias et al. 2002), whereas *E. coli* was detectable to depths of 60 cm of stream bank sediment cores from Houston, Texas (Brinkmeyer et al. 2015). However, these were in riverine settings, not beaches.

Elevated levels of *E. coli* in the upper layer at Cedar Creek Recreational Area could be due to deposition from hosts followed by optimal conditions for growth and persistence. While sampling, a toddler wearing a diaper was observed sitting in the foreshore area playing in the sand signifying a possible route of shedding of fecal bacteria from human hosts. A more likely source of *E. coli* levels in the upper layer was Canada geese that were feeding and drinking in the foreshore nearby, but outside of the sampling area. Since the foreshore is not dynamic in the way of wave action, once fecal bacteria are deposited, it is quite possible there is minimal or slower transport to nearshore sands

and resuspension into the water column, except in the immediate vicinity. A reason for low level detections at increased depth could be due to FIB being buried but still able to survive after sand trucks periodically spread fresh sand over the entire beach to maintain esthetics of the beach. Other plausible reasons for detections beneath the 0-10 cm layer could be the protection deeper layers offer from environmental stresses (sunlight, shearing from water movement), reduced protozoan grazing, and disturbance from human activities (playing in sand).

Both distance from shoreline and depth appear to influence the levels of *E. coli* concentrations when these variables are considered separately. Because the highest levels were found in the foreshore in the 0-10 cm depth where beachgoers typically congregate and play in the wet sand, thus increasing their chances of exposure, future field studies at freshwater, inland should be conducted. Ideally, next studies need to focus monitoring of *E. coli* on the upper 0-10 cm encompassing the backshore, foreshore, and nearshore areas because exposure chance is not exclusive to the foreshore. Water samples should also be taken to determine if the sand is contributing to FIBs in surface waters and vice versa. Sanitary surveys that are used to better describe characteristics like general beach conditions (temperature, wind speed and direction, rainfall, bather load) at Great Lakes beaches should be incorporated into a more intensive monitoring approach. Likewise, more abiotic measurements, like moisture content and temperature, should be paired with the sand samples to understand if these are promoting presence and growth in the sand. In conjunction with *E. coli* as an FIB, adding on enterococci as an FIB would be interesting since this is the primary indicator used at marine beaches. Assessment of pathogens and their relationship to culturable FIB is imperative to understand if *E. coli* (or enterococci)

is a good surrogate for the presence of pathogens. If pathogens are found, an epidemiological study should be conducted to establish a risk to beachgoers at inland, freshwater beaches.

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CHAPTER 5 ATTACHMENT TO STORMWATER INFRASTRUCTURE MATERIALS DEPENDS ON SUBSTRATE TYPE AND FECAL BACTERIAL STRAIN

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5.1 Introduction

Negative consequences can be linked to bacterial colonization and biofilm formation on substrates associated with the food industry (Palmer et al. 2015; Bridier et al. 2015; Galie et al. 2018), healthcare and medicine (Costerton et al. 1999; Donlan and Costerton 2002; Gupta et al. 2016), as well as drinking water and wastewater infrastructure (Lazarova and Manem 1995; Liu et al. 2016). For example, in the drinking water industry, it is well documented that heterotrophic bacteria colonize pipes which is a cause for concern, both from an economic and health perspective (Chowdhury 2012; Lu et al. 2013). Total coliforms, fecal coliforms, and *E. coli* are regulatory indicators sometimes monitored by municipalities as surrogates to assess the potential for more harmful fecal bacteria in drinking water distribution systems.

In contrast to studies of bacterial colonization of drinking water distribution infrastructure, research regarding colonization of stormwater infrastructure (e.g., pipes, channels) by fecal indicators present in stormwater is lacking. Research efforts have instead primarily been focused on modeling the fate and transport of fecal indicator bacteria (FIB, i.e., *E. coli* and enterococci) and pathogens in stormwater runoff and surface waters because this is where recreational activity occurs and likelihood of exposure to humans is greatest. In runoff studies, FIB are shown to attach to particulates, such as soil, fecal particles, total suspended solids, and sediment (Characklis et al. 2005; Soupir et al. 2010). However, Muirhead et al. (2005) found the majority of cells (91-92 %) in a runoff study remained in an unattached state. When these unattached bacteria are carried through the network of stormwater infrastructure pipes, there is an opportunity to make contact with the pipe material itself. McCarthy (2009) suggested the build-up of

FIB in crevices from stormwater pipes could influence peak concentrations in surface waters, at least for some sites, within the first flush of a wet weather event.

The steps from initial adhesion of planktonic bacterial cells on substrates to formation of a biofilm is a multistep process organized by a combination of genetic and environmental factors (Stoodley et al. 2002; Marićc and Vraneš 2007; Gupta et al. 2016) with the first stage in biofilm development being bacterial attachment (Marshall et al. 1971; Costerton 1999). Some bacteria may have an advantage during the beginning phases of biofilm development due to structural features of the cell that promote adhesion. Specialized structures on *E. coli*, like flagella and fimbriae, assist in initial attachment to abiotic surfaces (Cookson et al. 2002; Van Houdt and Michiels 2005), and the enterococcal surface protein encoding gene (*esp*) promotes primary attachment and biofilm formation on polystyrene surfaces for *Enterococcus faecalis* (Toledo-Arana et al. 2001), but is not required for all enterococci isolates (Mohamad et al. 2004). In addition, surface pili of *E. faecalis* have been implicated in attachment in a clinical setting (Nallapareddy et al. 2006). To note, *E. coli* and enterococci are the two primary FIBs used in water quality standards to indicate impairment from fecal-derived sources in surface waters, including those receiving input from permitted stormwater conveyances.

In a review by Liao et al. (2015) on bacterial attachment to particles, properties, such as size, surface charge, organic matter concentration, surface area, and hydrophobicity were suggested as important variables in bacterial attachment. Also drinking water pipe composition has been reported to influence general bacterial concentrations as well as the presence of opportunistic pathogens (Pedersen 1990; Kerr et al. 1998; Chang et al. 2004; Wang et al. 2012), including those in a viable but

nonculturable (VBNC) state (Lee et al. 2007). The inherent physical and chemical properties of pipe material used in stormwater infrastructure may influence the suspected growth of bacteria between rainfall events. Stormwater pipes may consist of an assortment of materials, such as corrugated metal, concrete, and PVC. These same materials are used in drinking water distribution systems and research for attachment of and/or biofilm development of bacteria commonly found in drinking water on these surfaces, though not for FIB, is plentiful. For instance, fixed bacterial biomass on cement coupons were found to be 2.6 times higher than on PVC coupons placed in a Brussels drinking water distribution system (Niquette et al. 1999), and heterotrophic plate counts of a biofilm were 100 times higher on galvanized steel coupons than PVC, copper, and stainless steel under drinking water simulated hydraulic conditions (Jang et al. 2011). However, there were no differences in total cell count for heterotrophic bacteria among PVC, polyethylene, and stainless steel substrates exposed to control and ozonated water in a study by Zacheus et al (2000).

Because of the considerable evidence that different pipe materials are an important factor when discussing bacterial attachment and/or biofilm formation in drinking water infrastructure, it was hypothesized that FIB colonization of stormwater structures may occur in a similar fashion. If occurring, bacterial attachment and subsequent biofilm development in stormwater drainage pipes could create an environmental reservoir of FIB and potential fecal pathogens that could contribute to impairment of nearby surface waters during storm event flushing of the system. The aims of this study are to understand if attachment of FIBs to stormwater pipe material is possible and if there is a preference for a particular substrate type. Specific objectives

were to (1) determine the effects of FIB strains and substrate type on bacterial concentrations using culture methods, (2) to determine the effects of FIB strains and substrate type on bacterial concentrations using qPCR methods and (3) to visualize adherence to substrates for each of the species using scanning electron microscopy.

Results from this study will give direction to water quality stakeholders and modelers who would be interested in knowing whether pipe selection could be important in initial infrastructure development or remediation of compromised infrastructure. If FIB strains colonize substrates differentially, stakeholders might be more selective in choosing the appropriate strain to investigate fecal inputs from stormwater infrastructure. This study will also give guidance as to whether qPCR methods give similar results to culture methods and the limitations or modifications to consider before using this method.

5.2 Materials and Methods

5.2.1 Laboratory design

A laboratory strain of *Escherichia coli* (ATCC 25922, referred to as *E. coli*_25922) and *Enterococcus faecalis* (ATCC 29212) were tested separately for their adherence and growth on individual concrete, PVC, and a galvanized steel metal substrate in a time series experiment on days 1, 4, and 7. ATCC strains, *E. coli* (Naves et al. 2008) and *E. faecalis* (Mohamed and Huang 2007) are both reported to be biofilm producers. An additional field isolated strain (referred to as *E. coli*_field) confirmed as *E. coli* by culture (IDEXX), biochemical testing, and sequencing followed by a search in the BLAST database, was also tested.

For each of the sampling day, and for each of substrate types, seven replicate sterile conical tubes (50 mL) were prefilled with 40 mL of sterile 1 % tryptic soy broth (TSB) in water and the selected substrate added for a sample size of 21 per day. The substrate pieces had been previously washed and autoclaved. Approximate surface area for the substrates are as follows: concrete 25.42; PVC 27.58; and metal 10.37 cm^2. Preliminary growth curve studies for each strain were conducted to determine transmittance (~50 %) and dilutions for the desired initial dosing concentration. A polypropylene conical tube containing 200 µL of log phase of the selected FIB strain into 20 mL of sterile PBS was used as the dosing tube. Using a repeat pipette, an aliquot of 100 µL containing 10^5 colony forming units (CFU) was spiked into each conical tube at Time 0. The concentration of the initial dosing aliquot was confirmed by plate counts on tryptic soy agar plates. All samples and controls were placed into a preheated shaking incubator (New Brunswick Scientific, Model C24) at 30°C and allowed to shake at 150 rpm for the duration of the experiment, except for the brief period of removing samples.

On each sampling day, all seven replicates for each of the three substrates, along with controls were immediately placed on ice and processed for culture or qPCR analysis. Triplicate conical tubes with 1 % TSB and substrate, but no dosed bacteria, were carried throughout the experiment and tested for contamination on Day 7. Both TSB and phosphate buffered saline (PBS) rinsing solution were tested for contamination prior to the study.

5.2.2 Substrate processing

Each substrate was removed from its conical tube with flame-sterilized stainless steel (19 cm) forceps and rinsed for 30 s with PBS over a rinseate beaker to remove non-

adherent bacteria. Each substrate was then transferred to an individual 100 mL sterile polypropylene collection bottle prefilled with 30 mL of sterile PBS. The substrate was picked up by flame-sterilized forceps and all sides scrubbed by the same experimenter with a dedicated dental pick for a total of 1.5 min. The scrubbed substrate and dental pick were briefly agitated in the PBS contained in the collection bottle and then given a final PBS rinse for 5 s over the collection bottle while being held with sterile forceps. The final volume in the collection bottle ranged from 35-40 mL and was measured for each sample.

5.2.3 Culture methods

Each sample collection bottle containing the processed substrate was vortexed on maximum speed for 10 s before serial dilutions commenced. A preliminary experiment was conducted in advance to determine optimal dilutions needed for each specific culture method (i.e, Colilert®,Enterolert®, and plate counts). Serial dilutions (1:10) were performed by adding 100 µL of sample volume to 900 µL of PBS prefilled into sterile microcentrifuge tubes to reach the predetermined optimum dilution for each sample and counting method. For the standard plate count method on both *E. coli* strains and *E. faecalis*, 100 µL from the predetermined dilution were transferred to the center of a TSA plate and spread evenly over the surface of the agar using a borosilicate glass spreader ("hockey stick") and aseptic techniques. Plates were inverted and incubated at 35°C for 24 h and viable colonies reported as colony forming unit (CFU) per 100 mL volume or surface area (cm^2).

The Colilert® method for *E. coli* or Enterolert® for *E. faecalis* (IDEXX Laboratories, Westbrook, Maine) was performed on selected dilutions from the same

serial dilutions as above. The Colilert® and Enterolert® methods use a patented Defined Substrate Technology® (DST) nutrient indicator with a fluorescently tagged substrate to detect 4-methyl-umbelliferone-B-D-glucuronide for *E. coli* or 4-methyl-umbelliferone-B-D-glucoside for *E. faecalis*. Briefly, 100 µL from selected dilutions for the individual strain tested were placed in 100 mL of PBS prefilled in a sterile polypropylene collection bottle. Premeasured proprietary DST reagent was added to the collection bottle and swirled to dissolve media. The entire 100 mL was poured into a Quanti-Tray® 2000 with 49 large and 48 small wells, sealed, and incubated for 24 h at 35°C. Wells with actively metabolizing *E. coli* or *E. faecalis* were illuminated with a 365 nm, long-wave UV lamp attached to a viewing cabinet. Fluorescent wells were counted and results for both *E. coli* strains and *E. faecalis* were reported as MPN/cm^2. Quanti-Cult QC kit containing *E. coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* or IDEXX-QC Enterococci consisting of *E. faecalis*, *E. coli*, and *Streptococcus bovis* were analyzed similarly to samples for each Colilert or Enterolert reagent lot to determine the ability to accurately confirm the presence/absence of *E. coli* or enterococci.

5.2.4 DNA extraction

Each sample bottle containing substrate was vortexed for 5 sand 20 mL was centrifuged at 8000 x G for 10 min and supernatant decanted. The pellet was resuspended in 1.8 mL of sterile PBS and genomic DNA was extracted with the DNeasy® UltraClean Microbial Kit (Qiagen, Germantown, MD) according to manufacturer's instructions and stored at -80°C until used in qPCR assays.

5.2.5 qPCR assays

Gene copies for 23S rRNA were determined for substrate samples by the EPA-EC (*E. coli*) 23S and Entero1 qPCR assays outlined in Oladiende et al. (2014). Genomic DNA was extracted from ATCC pure cultures of *E. coli* and *E faecalis* to prepare qPCR standard curves using DNeasy® UltraClean Microbial Kits and the pure culture of *E.coli*_25922 was used as a surrogate for *E.coli*_field. Forward and reverse primers (500 µM) and probes (100 µM) (Eurofins Genomic, Louisville, KY) were rehydrated in 10 mM Tris. A total reaction volume (20 µl) on a 96-well plate consisted of 2X GoTaqProbe qPCR MasterMix (Promega, Madison, WI), 0.2 mg/mL bovine serum albumin (BSA) (Sigma Aldrich, St. Louis, MO), 1 µM of each primer, 80 nM probe and 4 µL of standard, sample, or controls in triplicate. The cycle amplification protocol using a CFX 96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) was as follows: 95°C for 20 s, then 40 cycles of 95°C for 3 s and 60°C for 30 s. Triplicate no template controls were included on every plate run.

Salmon DNA extract served as in inhibition check to determine if sample extracts contained substances that interfered with qPCR amplification. On 96-well plates separate from sample runs, a 20 µL reaction volume contained 2X GoTaqProbe qPCR MasterMix, salmon primers (1 µM) and probes (80 nM), BSA (0.2 mg/mL), salmon DNA (2 ng/mL), and 4 µL of sample extract. In place of a sample extract (regardless of strain being assessed/quantified), 4 µL of DNA extracted from *E. faecalis* was used as a no inhibition control in a set of separate triplicate wells on every inhibition check 96-well plate to signify a control where no inhibition was known to occur. Cycle conditions were as above. Assay inhibition occurred if average cycle threshold (CT) values for samples

containing salmon DNA were 1 CT greater than average CT values for the *E. faecalis* no inhibition control containing salmon.

5.2.6 Scanning electron microscopy

Bacteria, present on the different substrates, were vapor fixed for 24 h with 2 % osmium, then air dried. Each substrate was attached to a 26 mm aluminum specimen mount and coated with gold palladium. Bacteria located on representative substrates were photographed by the Middle Tennessee State University (MTSU) Interdisciplinary Microanalysis and Imaging Center (MIMIC) core using a Hitachi S-3400N Scanning Electron Microscope/Oxford Aztec INCA X-Act with an accelerating voltage of 15 kV.

5.2.7 Statistics

A two-way ANOVA was conducted to assess the effects of strain and substrate type on plate count (CFU/cm^2), IDEXX (MPN/cm^2), qPCR copies (copies/cm^2). The assumptions of normality tested by Shapiro-Wilk's test and homogeneity of variances examined by Levene's test were violated, therefore all three types of dependent variables were log-transformed to meet the assumptions. Pairwise comparisons among the levels for each independent variable were made when a main effect was found to be statistically significant ($p < 0.05$). Otherwise, when an interaction was detected from the two-way ANOVA, pairwise comparisons for each simple main effect of an independent variable within one level of the other independent variable was made. Results are reported as mean log per each concentration type with 95 % confidence intervals and sample sizes also presented in the figures.

5.3 Results and Discussion

5.3.1 Culture methods data

The data for MPN/cm^2 from IDEXX (Colilert and Enterolert) methods and CFU/cm^2 from plate counts for each individual strain were found to show very similar patterns based on visual inspection of bar graphs and by correlations between MPN/cm^2 and CFU/cm^2 (Pearson's r: *E. coli_25922 = 0.945, E. coli_field = 0.963, E. faecalis = 0.741)*. Because of this, for brevity, only plate count results are presented below. However, the fact that the two methods give similar patterns is worth notice in itself, because the IDEXX methods are often used for regulatory monitoring with the assumption that they give similar results to culture methods.

5.3.2 Two-way ANOVA

A significant interaction ($p < 0.001$) was found between strain and substrate type for plate counts (CFU/cm^2) on day 1 ($F_{4, 50} = 6.766$), day 4 ($F_{4, 50} = 20.247$), and day 7 ($F_{4, 50} = 4.373$). Day 1 qPCR results showed differences among copies/cm^2 for the main effects of species ($F_{2, 51} = 7.238$, $p < 0.05$) and substrate type ($F_{2, 51} = 55.963$, $p < 0.001$), but significant interaction between the two variables occurred on day 4 ($F_{4, 50} = 9.283$, $p < 0.001$) and day 7 ($F_{4, 46} = 3.672$, $p < 0.05$). These interactions indicate that concentrations results could not be explained without considering the independent variables with repect to one another. For instance, which substrate is most colonized depends on the strain of bacteria and alternatively, the bacteria with the highest counts depend on the substrate type. Because significant interactions were seen in most analyses (except Day1 qPCR data), separate sections are dedicated below to describe the simple effects on plate counts and copy number of each independent variable on different levels

of the interacting independent variable. Two figures for each analysis, either CFU/cm^2 or copies/cm^2, were provided to easily guide the reader through the results although data could be presented in one figure per analysis.

5.3.3 Effect of strain on plate counts for each substrate type on individual days

Mean log plate counts were different ($p < 0.001$; Figure 5.1) among the strains for metal substrate on all days (day 1 – $F_{2, 50} = 15.022$, day 4 – $F_{2, 50} = 77.103$, day 7 – $F_{2, 50} = 34.771$) but on day 7 only for concrete ($F_{2, 50} = 44.210$, $p < 0.001$) and PVC ($F_{2, 50} = 3.949$, $p < 0.05$).

On day 1 (Figure 5.1) for metal substrate, mean log *E. coli*_25922 ($p < 0.05$) and *E. faecalis* ($p < 0.001$) plate count concentrations were higher than for the *E. coli*_field strain. On days 4 and 7, *E. faecalis* concentrations were higher ($p < 0.001$) than both *E. coli*_25922 and the *E. coli*_field strain.

The pattern among the strains on day 1 and day 4 for concrete and PVC substrates indicate that initially all strains colonized these two substrates similarly and elevated concentrations (~10^8 CFU/cm^2) existed beyond the first day. By day 7, however, the concentrations for the two *E. coli* strains on concrete and PVC had fallen at least a half to one log from previous days. Though, as with the metal substrate, *E. faecalis* concentrations were sustained for longer and mean log plate counts on day 7 were higher on concrete ($p < 0.001$) and PVC ($p < 0.05$) than one or both of the *E. coli* strains.

The trend seen for these bacteria for attachment onto the substrates tested may be explained by bacterial cell surface properties. Gutman et al. (2013) suggested flagella used by *E. coli* for motility may be able to overcome repulsive forces at a substrate's interface. Fimbriae, small thin flagella-like appendages covering bacterial cells, area

required feature for initial attachment of uropathogenic *E. coli* to host tissues (Axner et al. 2011) and they cannot attach to abiotic surfaces, like polystyrene, without these structures (Prigent-Combaret et al. 2000; Cookson et al. 2002). Because *E. faecalis* concentrations are higher than the two *E. coli* strains on concrete and PVC on day 7, perhaps the coccoid shape and similar, small size promote tighter packing of *E. faecalis* onto these substrates. Likewise, the enterococcal surface protein (Esp) is one structure found to be necessary for attachment and biofilm development. In fact Toledo-Arana et al. (2001) showed that attachment and biofilm development on polystyrene and PVC plastic surfaces only occurred when Esp protein expression was restored in *esp*-deficient strains.

E. faecalis demonstrated the ability to colonize metal substrate well throughout the study and *E. faecalis* concentrations were significantly elevated over the two *E. coli* strains on days 4 and 7. If *E. faecalis* is indeed forming a biofilm as it appears in SEM pictures discussed later (see Figures 5.5 and 5.6), then features of the biofilm itself could confer the greater ability to colonize metal. A study by Barnes et al. (2012) reported the presence of extracellular DNA localized to long, filamentous strands and within the extracellular matrix of an *E. faecalis* biofilm at 4 hours post-inoculation. The presence of negatively charged eDNA could enhance the attachment to a metal coated with a positively zinc ion as tested in this study.

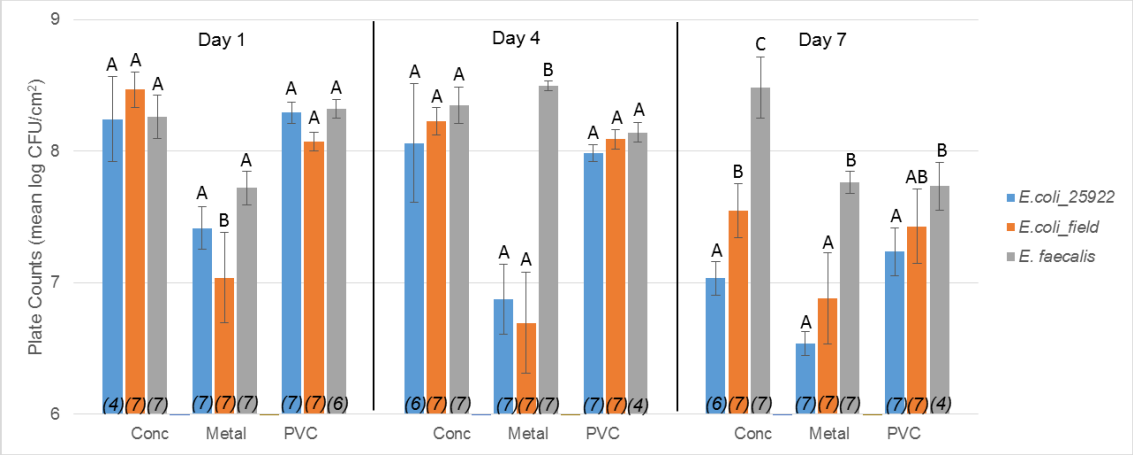

Figure 5.1. Comparisons of plate count concentrations (mean log CFU/cm^2 substrate) among strains within a substrate type on each individual day. Strains within a substrate type on each individual day not sharing the same letters are significantly different from each other. Data presented as mean log ± 95 % confidence intervals. Sample sizes are in parentheses.

5.3.4 Effect of substrate type on plate counts for each strain on individual days

There were differences in plate counts among the three substrate types on all days ($p < 0.001$, Figure 5.2) for *E. coli*_25922 (day 1 – $F_{2, 50} = 29.116$, day 4 – $F_{2, 50} = 33.456$, day 7 – $F_{2, 50} = 11.089$), *E. coli*_field (day 1 – $F_{2, 50} = 70.081$, day 4 – $F_{2, 50} = 56.461$, day 7 – $F_{2, 50} = 10.937$), and *E. faecalis* (day 1 – $F_{2, 50} = 13.631$, day 7 – $F_{2, 50} = 14.153$), except for *E. faecalis* on day 4 ($p = 0.176$).

For *E. coli*_25922 and *E. coli*_field strains, the metal substrate was the least colonized throughout the study, with concentrations on days 1, 4, and 7 being consistently lower on metal substrate than concrete and PVC. All significance values for the aforementioned tests were reported at $p < 0.001$ except for concrete vs metal for *E. coli*_25922 on day 7 ($p < 0.05$). By day 7, numbers had begun to decline and the drop off appeared to be greater for concrete and PVC than metal in the two *E. coli* strains.

E. faecalis patterns were more variable with concentrations for metal being lower ($p < 0.001$) than both concrete and PVC only on day 1. For day 4, no differences for substrate concentrations (concrete:metal $p = 1.000$; PVC:metal $p = 0.192$; concrete:PVC $p = 0.826$) were seen and day 7 concrete concentrations were higher than metal or PVC ($p < 0.001$). Interestingly, concrete concentrations of *E. faecalis* were sustained through day 7 of the study with no apparent decline.

Concrete and PVC were colonized similarly by both of the *E. coli* strains, and *E. faecalis* on day 1, with concentrations higher than metal. This is in contrast to Niquette et al. (2000) who reported that PVC coupons developed less biomass than cement-based substrates and Chang et al. (2003) where concentrations (CFU/cm^2) of heterotrophic bacteria produced a pattern of cement-lined cast iron (6.76×10^5) > galvanized steel (2.30×10^5) > PVC (2.93×10^4). However, these studies did not distinguish among the different species found in the results and the coupons were exposed to a drinking water distribution system which generally do not contain strains used in the present study. However, attachment of FIB to concrete is known to occur, at least in the case of *E. coli* which has been genetically-engineered to form a biofilm to protect concrete from deterioration by other microorganisms (Soleimani et al. 2013).

Along with bacterial properties, surface and chemical properties of different substrates could influence the attraction of the bacteria. For example, streptococci bacteria were found to be attracted more to stainless steel and zinc coupons than other metals (Flint et al. 1999). Because the galvanized metal substrates in this study were made of an iron alloy coated in zinc, it is plausible that the chemical and physical properties of the zinc coating could be playing a role in the patterns of bacterial

colonization. In addition, the high concentration maintained on concrete relative to metal and PVC for *E. faecalis* on day 7 may be explained by the alkaline conditions found on fresh concrete (Roberts et al. 2002). For instance, *E. faecalis* cells can grow at a pH of 9.6 (Franz et al. 2003), and Ran et al. (2015) found *E. faecalis* can form a dense biofilm at pH 10 within 24 hours.

Although the bacteria in this study are confined to a closed system, it is interesting to note substrate attachment was observed on all substrates and the survival lasted for 7 days without providing additional food sources. If bacteria can proliferate on substrates in the environment, perhaps one determining factor in survivability is the retention of moisture in stormwater conveyances after rain events which allows for the maintenance of hydrated bacterial cells.

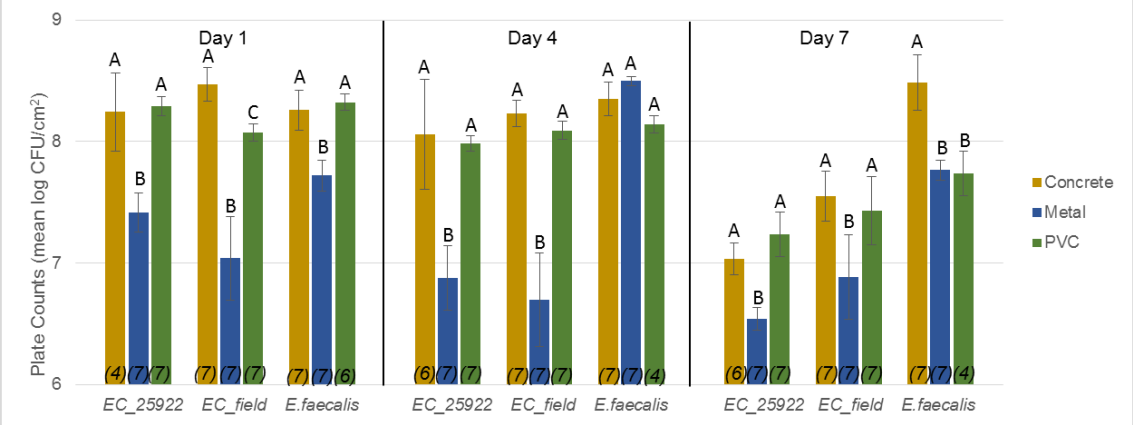

Figure 5.2. Comparisons of plate count concentrations (mean log CFU/cm^2 substrate) among substrates within a strain on each individual day. Substrate type within a strain on each individual day not sharing similar letters are significantly different from each other. Data presented as mean log ± 95 % confidence intervals. Sample sizes are in parentheses.

5.3.5 Main effects of substrate type and strain on qPCR copies on day 1

For the main effect of substrate (i.e., no interactions occurred), mean log qPCR copies (copies/cm^2) for concrete were significantly higher ($p < 0.001$) than metal and PVC substrates (Figure 5.3). For concrete and metal on day 1, *E. coli*_field and *E. faecalis* behaved more similarly and were higher than *E. coli*_25922. For differences in qPCR copy concentration among strains, mean log copies/cm^2 were higher ($p < 0.05$) in *E. coli*_field and *E. faecalis*, than for *E. coli*_25922 (Figure 5.4). It is interesting to point out that concrete had higher copy concentrations ($p < 0.001$) than metal and PVC for all strains.

5.3.6 Effect of strain on qPCR copies for each substrate type on days 4 and 7

On days 4 and 7, there were significant differences ($p < 0.001$; Figure 5.3) in mean log qPCR copies (copies/cm^2) among the strains for the concrete (day 4 – $F_{2, 50} = 19.016$, day 7 – $F_{2, 46} = 9.459$) and metal (day 4 – $F_{2, 50} = 32.668$, day 7 – $F_{2, 46} = 13.854$). There were differences in mean log copies/cm^2 among strains for PVC on day 4 ($F_{2, 50} = 5.796$, $p < 0.05$), but not on day 7 ($p = 0.815$).

Mean log qPCR copies for concrete were higher in *E. faecalis* ($p < 0.001$) and *E. coli*_field ($p < 0.05$) than for *E. coli*_25922 on days 1 and 4. Likewise for metal, mean log qPCR copies were higher ($p < 0.001$) in *E. faecalis* and *E. coli*_field than for *E. coli*_25922. However, *E. faecalis* concentrations on concrete and metal substrates were only higher than *E. coli*_field on day 4. For PVC, mean log qPCR copies for *E. coli*_field were higher ($p < 0.05$) than *E. coli*_25922 and *E. faecalis* on day 4.

The general pattern seen for concentration of qPCR copies on concrete and metal substrates was that *E. faecalis* and *E. coli*_field were consistently higher than *E.*

*coli*_25922. Since this pattern does not match that with what was seen in the plate counts, it could mean some characteristic of *E. coli*_25922 makes DNA extraction less efficient. Perhaps, *E. coli*_25922 has become accustomed to a living in laboratory setting with ideal nutritional and environmental conditions and has lost a 23r RNA gene copy which explains slightly lower copy numbers than its field counterpart, *E. coli*_field. The lack of copies found on PVC could arise from toxic byproducts being produced by PVC and promoting genotoxicity, but not at lethal levels since culture counts were high. Vinyl chloride is proven to form DNA adducts (Brandt-Rauf et al. 2012) and release of this chemical during DNA could interfere with reagents in the extraction process. On the other hand, these possible vinyl-chloride-DNA adducts could be formed at priming sites for qPCR which would reduce amplification. However, it is difficult to provide a plausible explanation for this phenomenon since the pattern among the strains for metal and concrete was not also seen for PVC.

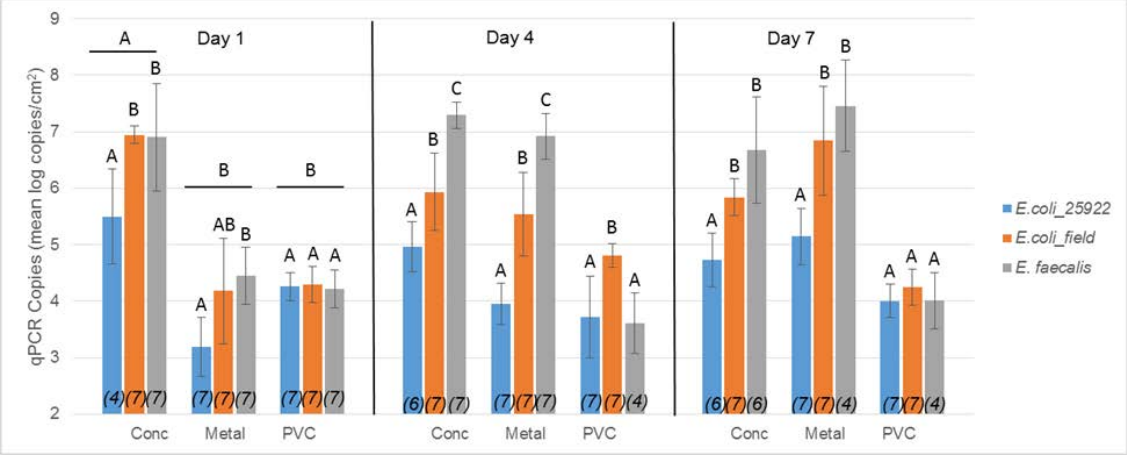

Figure 5.3. Comparisons of qPCR concentrations (mean log copies/cm^2 substrate) among strains within a substrate type on each individual day. Strains within a substrate type on each individual day not sharing the same letters are significantly different from each

other. On day 1, substrates not sharing the same letter over the bar, are different from each other. Data presented as mean log ± 95 % confidence intervals with sample sizes in parentheses

5.3.7 Effect of substrate type on qPCR copies for each strain on days 4 and 7

On days 4 and 7 there were statistically significant differences (Figure 5.4) in mean log qPCR copies (copies/cm^2) among the substrate types for *E. coli*_25922 (day 4 – $F_{2, 50} = 5.797$, day 7 – $F_{2, 46} = 3.890$, $p < 0.05$), *E. coli*_field (day 4 – $F_{2, 50} = 4.861$; $p < 0.05$, day 7 – $F_{2, 46} = 19.889$; $p < 0.001$), and *E. faecalis* (day = – $F_{2, 50} = 40.813$, day 7 – $F_{2, 46} = 22.270$, $p < 0.001$).

Mean log qPCR copies on concrete were higher than PVC for all strains (*E. coli*_25922, *E. coli*_field $p < 0.05$; *E. faecalis* $p < 0.001$) on day 4 and higher ($p < 0.001$) than PVC for *E. coli*_field and *E. faecalis* on day 7. Metal qPCR copy concentrations were significantly higher ($p < 0.001$) than PVC for *E.faecalis* on day 4 and were higher than PVC for all three strains on day 7 (*E. coli*_25922, $p < 0.05$; *E. coli*_field:*E. faecalis*, $p < 0.001$). There were no significant differences between concrete and metal concentrations for *E. coli*_field (day 4 – $p = 0.881$, day 7 – $p = 0.061$) or *E. faecalis* (day 4 – $p = 0.954$, day 7 – $p = 0.365$) on either day, as well as no differences for these two substrates on day 7 for *E. coli*_25922 ($p = 1.000$).

Initially for qPCR copies it appears that concrete is better colonized relative to metal and PVC on day 1. By days 4 and 7, however, concentrations on the metal substrate increased and were very similar to concrete, while the PVC concentrations remain lower throughout. It seems unlikely that this is due to lack of failure to effectively

scrape cells from PVC relative to concrete and metal, since the corresponding plate counts were not low for PVC. One potential explanation is that there are more VBNC bacteria that remain on the metal and concrete substrates relative to PVC, possibly due to greater biofilm formation and retention of these cells on concrete and metal. Because qPCR detects for target sequences in both viable, but nonculturable and live culturable cells, the higher level of copies on the metal and concrete substrate could be from capture and amplification of both of these.

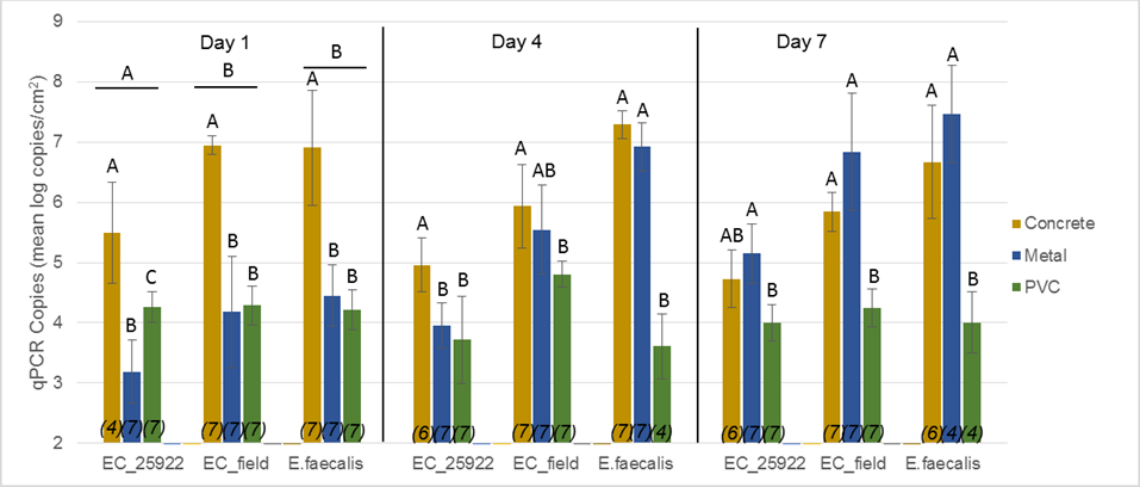

Figure 5.4. Comparisons of qPCR concentrations (mean log copies/cm2 substrate) among substrates within a strain on each individual day. Substrate type within a strain on each individual day not sharing similar letters are significantly different from each other. On day 1, strains not sharing the same letter over the bar, are different from each other. Data presented as mean log ± 95 confidence intervals with sample sizes in parentheses

5.3.8 Scanning Electron Microscopy

The presence of *E. coli*_25922 and *E. faecalis* were visually detected on all three substrates by scanning electron microscopy (Figures 5.5-5.7). Concrete surfaces contained crevices which could provide the strains a place for shelter (Figure 5.5a,b,c). Based on the plate count concentrations on day 7 (Figure 5.1), *E. faecalis* had higher levels on concrete than *E. coli*_25922 which could be explained by the spherical shape of this strain making it easier to enter crevices and attach to globular structures of the concrete (Figure 5.5c). There is a possibility that *E. faecalis* may be producing extra polymeric substances as evidenced by the string-like structure or pili found suggesting biofilm production (Figure 5.5c).

For the metal substrates (Figure 5.6b,c), strains appear to be congregating more so around surface features that are rough and irregular in shape that could indicate corrosion instead of smoother surface. The increased positively charged sites in corroded areas associated with iron and zinc oxides can allow for more bacterial attachment (Ams et al 2004). Some of the *E. coli*_25922 cells in Figure 5.6b tend to be hollow and possibly dead as compared to the more intact *E. faecalis* cells which would support the higher levels of viable cells as seen in plate count concentrations (Figure 5.1). Some of the *E. faecalis* cells appear to be embedded in an extracellular polymeric substance present in a biofilm (Figures 5.5c and 5.6c). However, no analysis was completed to confirm biofilm production.

Both *E. coli*_25922 and *E. faecalis* are prolific on the PVC substrate (Figure 5.7b,c). The *E. coli*_25922 strain has the appearance of adhering in a single layer,

whereas *E. faecalis* cells tend to be clumpy and congregate. A few cells of *E. faecalis* can even be found situated within the holes of the PVC.

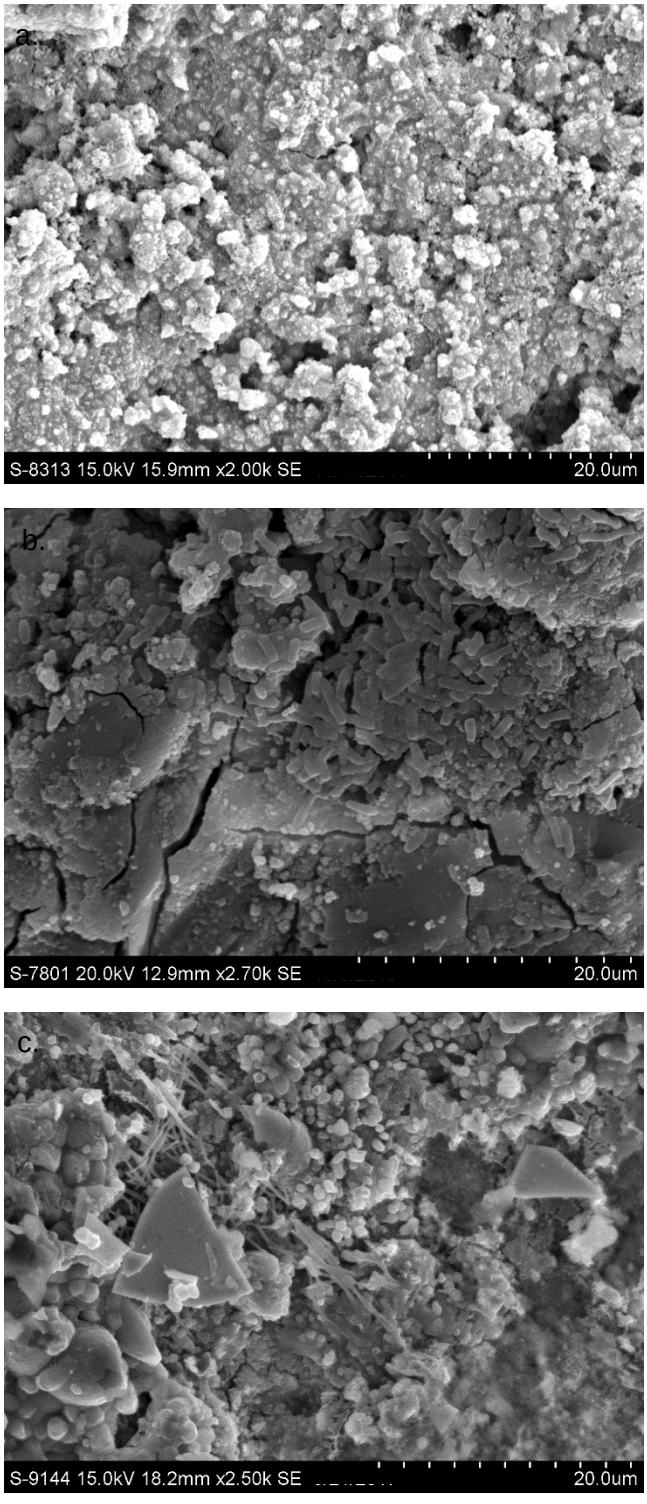

Figure 5.5. a, b, c. Scanning electron microscopy images of background (5.5a), *E. coli*_25922 (5.5b), and *E. faecalis* (5.5c) detected on concrete substrates.

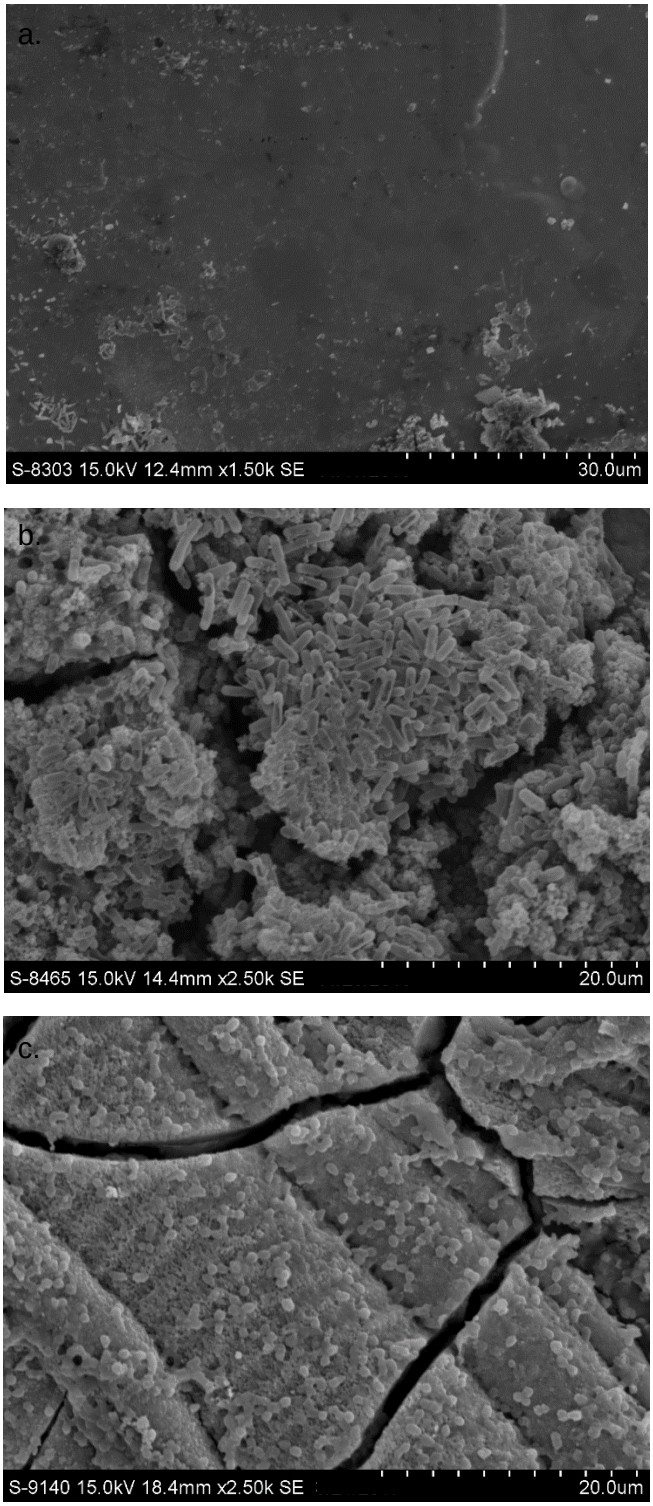

Figure 5.6. a, b, c. Scanning electron microscopy images of background (5.6a), *E. coli*_25922 (5.6b), and *E. faecalis* (5.6c) detected on metal substrates.

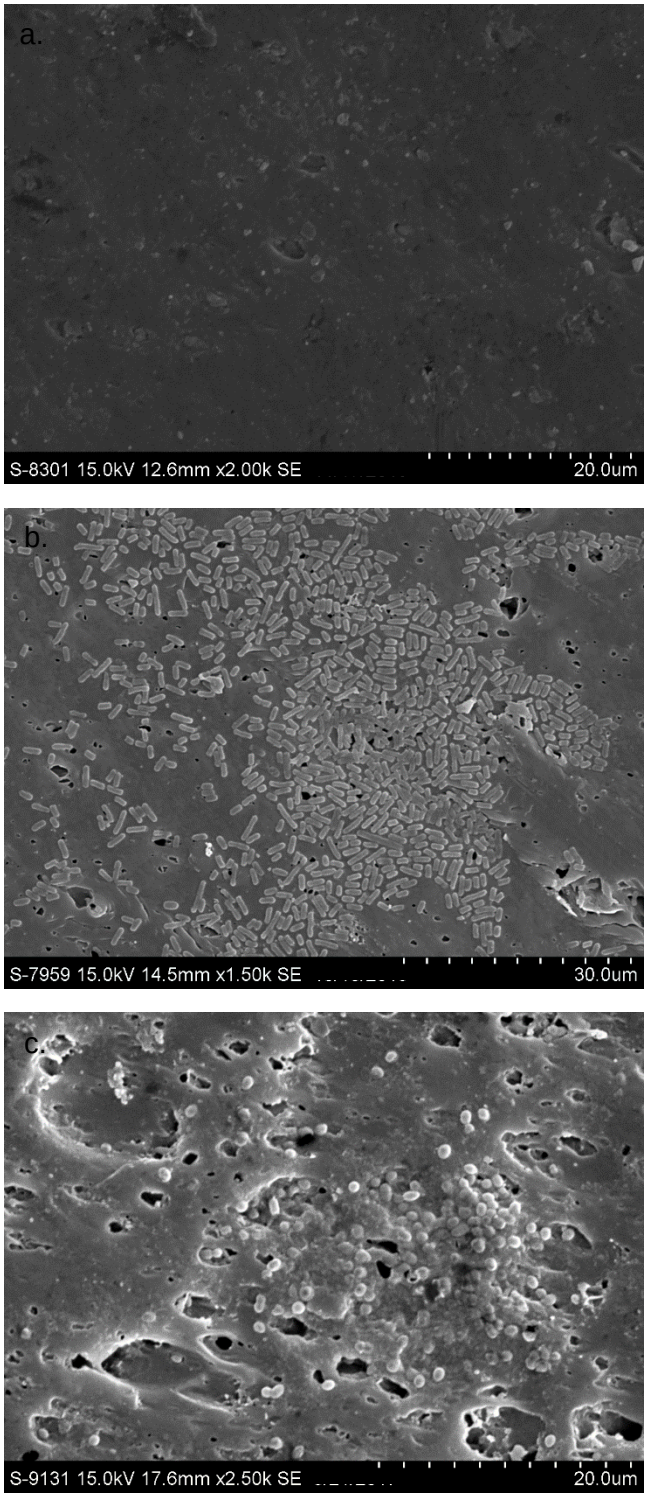

Figure 5.7. a, b, c. Scanning electron microscopy images of background (5.7a), *E. coli*_25922 (5.7b), and *E. faecalis* (5.7c) detected on PVC substrates

5.4 Conclusions

The present study demonstrated that the FIB tested were are able to attach, survive, and potentially replicate on concrete, metal, and PVC which are construction components of stormwater infrastructure. This was shown by plate count methods, IDEXX tests, qPCR, and scanning electron microscopy. Samples were spiked at initial dose of 10^5 CFU (equating to 2.5 x 10^3 CFU/mL) and plate count concentrations increased considerably to between 10^6 and 10^9 CFU/cm^2 by day 7, depending on strain of bacteria and substrate type. Regardless of substrate type, *E. faecalis* maintained the highest counts/cm^2 over seven days. Metal was the least colonized by the two *E. coli* strains and *E. faecalis* initially, but thereafter *E. faecalis* had little trouble attaching to the metal. Future studies will be necessary to investigate the physical or chemical properties of the bacterial cell and/or the substrate likely responsible for these interactions. Though the qPCR results sometimes exhibited different patterns than plate counts, this method would be an appropriate screening tool for the rapid detection of FIB. The enterococci group of FIB has an EPA-approved qPCR method for quickly estimating risk to fecal pathogens and this tool could be used at stormwater outfalls to assess fecal contamination from different pipe materials. Although some might view the detection of both DNA from dead and live cells a limitation from the qPCR method, this information could still be useful in microbial source tracking studies to detect the source of FIB in stormwater infrastructure. Overall, this research shows FIB could attach to stormwater infrastructure, survive and multiply between rain events, and ultimately serve as a fecal source to a receiving stream during a subsequent storm.

Briefly, I will provide a set of limitations that have occurred throughout this study. First, a field strain of an *Enterococcus spp*. should have been isolated and tested in parallel to *E. faecalis* similar to *E. coli*_25922/*E. coli*_field. The initial dosing concentrations should have been more closely verified to concentrations that were typical of levels found in the environment. In the least, pH should have monitored in conjunction with daily sample processing, instead of at the end of the study. Better images for SEM for all days and including field strains should have been attempted. A preliminary study using a biofilm detection method should have been performed on a dummy samples in advance to work out kinks in the methodology instead of obtaining unusable information with actual samples. A shaking method was used in order to simulate some type of agitation and suspension of bacteria that may be seen in stormwater pipes during a rain event. Perhaps a more realistic rpm could have been selected. Also, for a publication, there will have to be an explanation of why sample sizes were not all 7 by the end of the study. I did not address the results for controls from conicals containing bacteria in TSB media, but with no substrate. Nor did I discuss densities of bacteria in overlying media in conicals containing substrate. There should have been some type of delineation between dead and live cells with either propidium monoazide qPCR or BacLight viability stains. The same amount of time was spent on scrubbing the individual substrates although these were quite different in surface areas and characteristics. Lastly, there should have been some type of normalization of scrub time based on a feature of the substrate like surface area.

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