UTILIZATION OF MICROBIAL SOURCE-TRACKING MARKERS TO INFORM TARGETED REMEDIATION AND PREDICT POTENTIAL PATHOGENS IN HIGH PRIORITY SURFACE WATERS

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ABSTRACT

JENNIFER GENTRY SHIELDS: Utilization of microbial source-tracking markers to inform targeted remediation and predict potential pathogens in high priority surface waters
(Under the direction of Jill Stewart)

Although *Escherichia coli* and enterococci will be recommended for use as recreational water quality standards (RWQS) for all surface waters by the U.S. EPA, measuring their levels contributes little to our knowledge of the source of contamination in nonpoint source (NPS) impacted waters. Yet understanding the sources of fecal pollution is critical for developing management plans to protect recreational waters and for assessing the associated health risks. Testing for these fecal indicator bacteria (FIB) augmented with microbial source-tracking (MST) assays may improve our ability to identify and prioritize sources that have a high likelihood of contributing human pathogens to surface waters. Yet more research is required to understand how MST methods relate to measurements of FIB in inland waters, which are predominantly impacted by NPS containing both human and animal source fecal contamination. To understand whether MST assays can aid in better targeting of remediation efforts, novel, promising MST markers were evaluated for (1) their relationship to land use, (2) their ability to predict microorganisms of public health concern, and (3) their association with FIB within two areas of the Cape Fear watershed. The results of this research suggest that MST markers are necessary for identifying and prioritizing areas with a high likelihood

of contributing human pathogens to surface waters, but that they cannot be easily utilized in a tiered approach with FIB.

To Chris

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LIST OF ABBREVIATIONS

AWQC: ambient water quality criteria

CWA: Clean Water Act

DI: deionized

DOC: dissolved organic carbon

DOM: dissolved organic matter

EEM: Emission-excitation matrix

EPA: Environmental Protection Agency

EV: enterovirus

FIB: fecal indicator bacteria

GI: gastrointestinal

MST: microbial source tracking

NPDES: National Pollutant Discharge Elimination System

NPS: nonpoint source

PARAFAC: parallel factor analysis

PCR: polymerase chain reaction

PS: point source

qPCR: quantitative polymerase chain reaction / real-time polymerase chain reaction

RT-PCR: reverse transcription polymerase chain reaction

RT-qPCR: reverse transcription quantitative polymerase chain reaction

RWQC: recreational water quality criteria

TCV: turnip crinkle virus

TMDL: total maximum daily load

Chapter 1: Introduction and Literature Review

1. Introduction

To protect the public from recreational waterborne illness, the U.S. Environmental Protection Agency (EPA) has established recommended water quality criteria, including the Ambient Water Quality Criteria (AWQC) in 1986 (1986) and the new Recreational Water Quality Criteria (RWQC; USEPA, 2012) to be adopted October 2012. If adopted by states as a water quality standard, the RWQC can be applied to other Clean Water Act (CWA) programs, including National Pollutant Discharge Elimination System (NPDES) permits, water body assessments to determine use attainment, and development of Total Maximum Daily Loads (TMDLs), in addition to beach monitoring and notification programs.

Both the AWQC (1986) and the RWQC (2012) utilize fecal indicator bacteria (FIB), specifically enterococci and *E. coli*, to signify when the risk of acute gastrointestinal (GI) illness exceeds an acceptable level. FIB are utilized because they are thought to indicate the presence of fecal matter and, potentially, pathogens in surface waters. FIB are easier and cheaper to detect than pathogens, and epidemiologic studies (e.g., Cabelli, et al., 1982; Dufour, 1984; Haile, et al., 1999; Kay, et al., 1994; Wade, et al., 2008; Wade, et al., 2006) support the use of FIB as predictors of GI rates among swimmers.

However, the correlation between FIB and GI illness is derived from studies conducted primarily at coastal waters impacted by point source (PS) pollution. In fact,

there are only a limited number of studies relating human health to contact with water contaminated by non-point source (NPS) runoff (Calderon, et al., 1991; Cheung, et al., 1990; Colford, et al., 2007; Haile, et al., 1999; McBride, et al., 1998; Wiedenmann, et al., 2006). Yet, generating this type of data is imperative because NPS runoff is the major cause of impairment for most US waters (USEPA, 2002). NPS pollution differs fundamentally from PS pollution in that, unlike PS pollution, for which rates of input and composition are typically known, NPS pollution is often diffuse, intermittent, and heterogeneous with agricultural, commercial, residential, and wildlife sources all as potential contributors of FIB and pathogens (Schwab, 2007). As a result, NPS pollution frequently carries a complex mixture of animal and/or human fecal material.

This mixture of contamination sources can be challenging for source allocation because enterococci and *E. coli* are often found in high concentrations in animal feces (see Section 3.1). Inland waters are generally dominated by more rural and agricultural land use and are thought to be primarily impacted by NPS pollution from wildlife and livestock (Dorevitch, et al., 2010). Additionally, FIB have been documented to survive and grow in sediments (Wheeler Alm, et al., 2003; Yamahara, et al., 2009). Consequently, measuring FIB levels contributes little to our knowledge of the source of contamination (Boehm, et al., 2009). Understanding the sources of fecal contamination is critical for the development of management plans to protect recreational waters. The need to identify the source of fecal pollution in affected watersheds has led to the development of a wide range of microbial source-tracking (MST) methods (Section 3.2).

Augmenting traditional FIB testing with MST assays in natural waters may improve our ability to identify and prioritize areas that have a high likelihood of

contributing human pathogens to surface waters. However, it is not yet clear how MST assays relate to measurements of FIB in natural waters impacted primarily by NPS pollution. The objective of this study was to determine whether MST markers can provide information for source allocation and human pathogen presence in the Cape Fear watershed in North Carolina, a diverse watershed with both highly urbanized areas as well large regions of industrialized agriculture, and whether the MST markers can be utilized in a tiered assessment with FIB.

Insights obtained from the inclusion of MST marker data in watershed-wide sampling studies is hypothesized to identify factors that influence fecal pollution trends in two areas of the Cape Fear basin. Furthermore, the results of this study will illustrate that molecular approaches designed to test for MST markers can be used to improve and prioritize remediation projects and provide a higher level of information toward decision making processes aimed at protecting human health.

While there are many potential MST indicators discussed in the literature, this study utilized assays for *Bacteroides* sp., *Methanobrevibacter smithii,* enteric viruses, and coliphages, all of which are promising targets for MST studies. These assays have been used to identify human fecal contamination and discriminate between animal and human fecal sources (F+ coliphages) (Section 3.2). These assays, utilizing PCR and qPCR, are potentially more sensitive, quantitative, and amenable to automation than culture-based methods (Santo Domingo, et al., 2007). However, in practice, the concentration and extraction steps necessary for molecular methods can have low and variable recoveries, and the nucleic acid extractions can contain inhibitors that increase sample-specific assay detection limits or result in false negatives. During the course of this study, the need to

predict the presence of inhibitors in environmental samples as well as to provide a quantification control for viruses during concentration and extraction processes was identified. This project improves these methodological limitations with two additional studies evaluating novel methods for measuring PCR inhibitors and developing a novel virus surrogate for improved virus detection and quantification in environmental samples.

2. Research Objectives

1. Elucidate the spatial and temporal trends of source-specific MST markers in order to prioritize areas for targeted remediation in a mixed-use watershed.
2. Identify whether sites with intense exposure to NPS from agricultural sources may pose human health risks due to presence of microorganisms of public health concern in order to inform targeted best management practices.
3. Compare MST markers to FIB indicators to determine whether MST markers can be used in a tiered assessment.
4. Determine whether the level of inhibition detected in select surface water samples is related to the presence of detectable dissolved organic matter components in order to determine what types and levels of organics cause PCR inhibition in environmental water samples.
5. Develop and demonstrate proof-of-concept for a novel process control for recovery of enteric viruses from water.

3. Literature Review

This literature review will summarize the FIB and MST literature in order to discern the utility of proposed MST markers and to identify approaches for uniting FIB and MST assays. Section 3.1 will briefly review the traditional FIB, focusing on their ability to indicate GI illness risk, fecal source, and pathogen presence. Section 3.2 will then review the current MST technologies, beginning with a synopsis of MST methods followed by a review of the MST markers in current use, focusing on their sensitivity, specificity, and correlation to pathogens. This literature review will then try to unite the FIB and MST paradigms in Section 3.3 by comparing two approaches for utilizing MST technologies with FIB and summarizing four potential applications for MST in the water quality monitoring process.

3.1 Fecal indicator bacteria: fecal coliforms, *Escherichia coli*, and enterococci

Various organisms and groups of organisms have been suggested and used as FIB. This review will only address those recommended by the US EPA, *E. coli* and enterococci, for use in all surface waters as well as fecal coliforms, which are currently utilized by many states as a water quality standard.

Fecal coliforms were recommended as a recreational water quality standard in 1972 by the US Environmental Protection Agency (US EPA). The recommended level was 200 fecal coliforms per 100 ml of water, based on the National Technical Advisory Committee's report to the Federal Water Pollution Control Administration (1968). This report was based on prospective epidemiological studies conducted by the U.S. Public Heath Service between 1948 and 1950, which indicated that gastrointestinal (GI) illness

in swimmers was significantly higher than non-swimmers when coliform densities averaged 2,300-2,400 per 100 ml (Stevenson, 1953). The recommended level was based on a statistical relationship to *Salmonella*, which was detected in 60-100% of samples when the fecal coliform density was greater than 200 colony-forming units (CFU) per 100 ml. A later study (Cabelli, 1983) determined that fecal coliforms were actually a relatively poor indicator of GI illness risk and the US EPA stopped recommending fecal coliforms as a recreational indicator following the study. Nevertheless, many states continue to use fecal coliforms as a water quality indicator.

Besides a poor correlation to GI illness, fecal coliforms have other limitations as fecal indicators. Fecal coliforms cannot distinguish between human and animal contamination, as they are found in the intestines of all warm-blooded animals, and some fecal coliforms, such as *Klebsiella pneumoniae,* do not have a fecal source (Knittel, 1975). Fecal coliforms have also been detected and found to survive for extended periods in unpolluted tropical waters, which suggests that fecal coliforms may occur naturally in tropical waters (Roll and Fujioka, 1997).

Fecal coliforms have also shown poor correlation to pathogens, especially enteric viruses (Berg and Metcalf, 1978; Noble and Fuhrman, 2001) and protozoans (Bonadonna, et al., 2002), although they have been shown to be a fairly reliable indicator of bacterial pathogens in select studies (e.g., Krometis, et al., 2010; Schriewer, et al., 2010). The correlation between fecal coliforms and pathogens was evaluated by Wu et al. (2011), who assessed 126 cases of fecal coliform and pathogen pairings in their meta-analysis. The study found that fecal coliforms were not positively associated with pathogens (OR = 0.84; 95% CI, 0.56-1.27).

Fecal coliforms were replaced as a recommended water quality standard in 1986, when the US EPA published its recommended water quality criteria for recreational waters. The new recommendations included the use of **Escherichia coli** (***E. coli)*** as the preferred FIB for use in fresh water, with levels of 126 CFU per 100 ml (based on the geometric means of at least five samples over a 30-day period) (USEPA, 1986). This standard was based on epidemiological studies that used a prospective epidemiological-microbiological design scheme to find a direct, linear relationship between GI illness and *E. coli* density (Dufour, 1984). The use of this standard was strengthened by a meta-analysis of 27 studies, which determined that *E. coli* was a consistent predictor of GI illness in freshwater: a log (base 10) increase was associated with a 2.12 (95% CI, 0.925–4.85) increase in relative risk (Wade, et al., 2003).

However, as a coliform bacteria, *E. coli* has the same limitations as the fecal coliforms, including presence in animals, replication in the environment (Desmarais, et al., 2002; Solo-Gabriele, et al., 2000), and a poor correlation to pathogens, especially enteric viruses (Nasser and Oman, 1999). Wu et al. (2011) found an overall lack of correlation between *E. coli* and pathogens (OR = 0.52; 95% CI, 0.25-1.06).

The US EPA's AWQC for recreational waters (1986) also proposed the use of **enterococci** as an FIB in marine water and fresh water. The 1986 recommendation was based on geometric means of at least five samples over a 30-day period of 35 CFU/100 ml in marine water and 33 CFU/100 ml in fresh water (USEPA, 1986) based on studies by Cabelli (1983) and Dufour (1984). A meta-analysis of 27 studies in fresh and marine waters found that a log (base 10) unit increase in enterococci was associated with a 1.34 (95% CI, 1.00–1.75) increase in relative risk for GI illness in marine waters (Wade, et al.,

2003). More recently, studies have found that enterococci measured using quantitative PCR (qPCR) were positively correlated with swimming-associated GI illness (Wade, et al., 2008; Wade, et al., 2006; Wade, et al., 2010).

Although enterococci may serve as a good indicator of GI illness at sewage-impacted beaches, the bacterium cannot be used to distinguish between human and animal fecal sources. For example, a study in Homosassa, FL found higher numbers of enterococci in a wildlife park than in canals in a residential area utilizing septic systems (Griffin, et al., 2000). Another study in Florida found that one bird fecal event contributed approximately the same enterococci load as one adult human swimmer (Wright, et al., 2009).

Studies have also found a lack of correlation between enterococci and pathogens, such as enteric viruses, *Giardia,* and *Cryptosporidium* (e.g., Bonadonna, et al., 2002; Harwood, et al., 2005; Jiang, et al., 2001). Wu et al. (2011) found no correlation between pathogens and enterococci (OR = 0.47; 95% CI, 0.24-0.94).

In summary, the FIB recommended by the EPA as water quality standards can tell us much about the human health risk, particularly at PS-impacted beaches. In fact, their use in the US has significantly contributed to an overall decrease in recreational waterborne illness (Tallon, et al., 2005). However, as a group, traditional FIB (which includes fecal coliforms, *E. coli*, and enterococci) lack correlation to many fecal pathogens (Bonadonna, et al., 2002; Geldenhuys and Pretorius, 1989; Harwood, et al., 2005; Horman, et al., 2004; Jiang, et al., 2001; Lemarchand and Lebaron, 2003; Lund, 1996; Noble and Fuhrman, 2001; Pusch, et al., 2005). Furthermore, measuring FIB levels contributes little to our knowledge of the source of contamination (Boehm, et al., 2009)

due to the possibility of a non-fecal source (Scott, et al., 2002; Simpson, et al., 2000) and growth in environments such as soil, beach sand, sediments, and bodies of water (Anderson, et al., 2005; Byappanahalli, et al., 2003a; Byappanahalli, et al., 2003b; Fujioka, et al., 1999; Hardina and Fujioka, 1991; Wheeler Alm, et al., 2003; Whitman, et al., 2003).

Understanding the sources of fecal contamination is critical for effective management plans to protect recreational waters. The inability to determine the source of fecal pollution has led to a push to develop methods that allow for the reliable discrimination between human and nonhuman microbial contamination. A wide variety of microbial, chemical, and eukaryotic source-tracking markers have been proposed, although this review will focus solely on microbial source-tracking (MST) markers.

3.2 Microbial Source-Tracking Markers

MST methods include detection of several types of microbes, including bacteria, viruses, and protozoa, which are associated with various fecal sources, including humans, animals, groups of animals, or even specific species.

3.2.1 Overview of MST methods

Methods for MST can be divided into culture-based and culture-independent methods, and can be further divided into library-dependent and library-independent methods. Library-dependent methods require a large assemblage of typed organisms from various host sources, to which new isolates are matched to their corresponding source categories by direct subtype matching or by statistical means. Conversely, library-independent methods rely on sample-level detection of a particular host-specific

organism or gene. A schematic representing the various types of MST methods is shown in Figure 1 (Santo Domingo, et al., 2007).

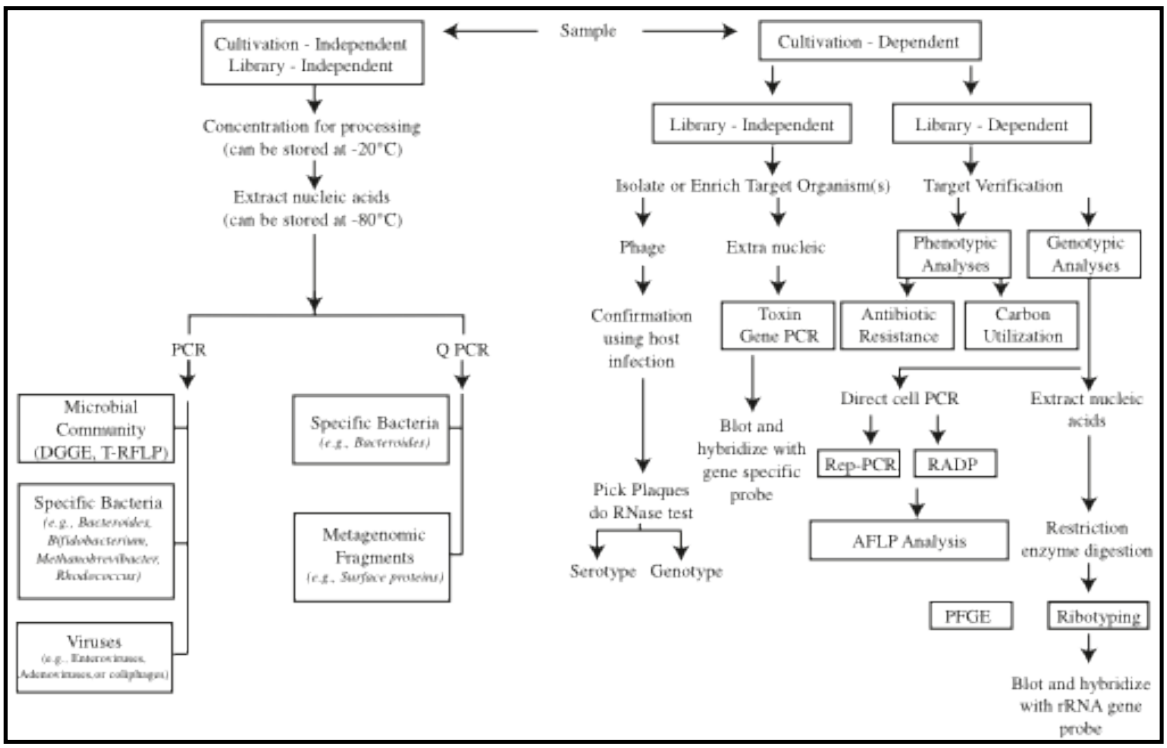


Figure 1.1. Schematic representation of common methods for tracking sources of fecal pollution using microbial analyses (from Santo Domingo, et al., 2007; permission obtained from Elsevier, 2012).

Library-dependent methods are based on the hypotheses that (1) the phenotypic or genotypic attributes of target strains (such as *E. coli* or enterococci) are host-specific and (2) the phenotypic and genotypic attributes of target strains isolated from environmental samples are similar to those found in host groups (Ahmed, 2007). Library-dependent methods are generally culture-based, including antibiotic-resistance tests and DNA fingerprinting, although they need not be (e.g., community fingerprinting).

In brief, the major advantage of library-dependent assays are that many of the techniques use FIB, which can be an advantage when comparing MST results to FIB

concentrations. Another advantage is that library-dependent techniques can be used to classify isolates from multiple fecal sources because they are based on subspecies or strains of bacteria that are associated with specific animal species (Sargeant, et al., 2011).

However, disadvantages to library-dependent methods are that subspecies likely change with respect to geography, time, rainfall, and habitat (Kuntz, et al., 2003). These changes require effective libraries to contain a large number of isolates, anywhere from 900-2,000 fecal isolates according to Jenkins et al. (2003). A large library size not only increases the time and skill required to distinguish species and subspecies, but it also increases the likelihood of including isolates that occur in the gastrointestinal tract of multiple host species (Stoeckel and Harwood, 2007; USEPA, 2005), which increases the complexity of the statistical analyses necessary and decreases the method specificity. In general, library-dependent methods have performed poorly in comparative studies (Field, et al., 2003; Griffith, et al., 2003; Moore, et al., 2005; Samadpour, et al., 2005; Stoeckel, et al., 2004), and they have been largely supplanted by library-independent methods.

Library-independent methods include both culture-based and non-culture-based assays.

Culture-based, library-independent methods include bacterial and bacteriophage methods. Bacterial culture-based methods include those that target sorbitol-fermenting bifidobacteria (Bonjoch, et al., 2005; Lynch, et al., 2002; Mara and Oragui, 1983; 1985; Resnick and Levin, 1981). Blanch et al. (2006) found culturing of sorbitol-fermenting bifidobacteria to be effective in distinguishing human from animal feces in a methods comparison study. However, due to rapid degradation of anaerobic bacteria (including bifidobacteria) in the environment (Bonjoch, et al., 2005; Carrillo, et al., 1985; Resnick

and Levin, 1981; Rhodes and Kator, 1999), bacterial culture-based methods have been largely replaced with molecular methods. Bacterial methods also include an assay that targets the *esp* gene, a virulence factor in *Enterococcus faecium* (Scott, et al., 2005). Although final detection involves PCR, a cultivation step is needed to enrich for the low densities of *Ent. faecium* harboring the *esp* gene.

Culture-based, library-independent methods also include culture of bacteriophages, mainly those that infect certain strains of *Bacteroides fragilis* and F+RNA coliphages. Culturing bacteriophages is relatively inexpensive and does not require advanced expertise, making them broadly available, and methods are available that can be used to enrich for bacteriophages, increasing the number of target microorganisms. However, an enrichment step only allows for semi-quantitative results and can create a "culture bias" due to differential selection (Stewart-Pullaro, et al., 2006). Other disadvantages to culturing phages for MST include: (1) they can only discriminate between human and non-human sources; (2) they may exhibit differential survival (Brion, et al., 2002; Long and Sobsey, 2004; Schaper, et al., 2002a); and (3) they are irregularly distributed geographically (Payan, et al., 2005).

Non-culture-based, library-independent methods have largely dominated the MST field in recent years. These methods usually involve target concentration by filtration followed by extraction of nucleic acids and amplification of target genes by PCR or qPCR (i.e., molecular methods). There are multiple benefits to using molecular methods over culture-based methods. Because the genetic marker is assayed directly from a water sample, without the need for an intervening culture step, molecular methods are not limited to the few cultivable microbes. Molecular methods are often faster and more

sensitive than culture-based methods. Additionally, multiple assays targeting various targets can be performed using the same nucleic acid extractions (Santo Domingo, et al., 2007). These extracts can also be preserved for future analyses should more sensitive assays, or assays for additional hosts, become available. However, there are also a number of drawbacks to molecular methods. Detection of specific markers may require concentrating large volumes of water (for viruses) or performing specificity tests (for bacteria such as *Bacteroides)* (Field and Samadpour, 2007).

While library-independent methods have generally performed better than library-dependent methods in validation studies, they have a number of disadvantages as well. Most library-independent methods work better for wastewater than for individual fecal samples, as host-specific markers may not be present in every individual. Additionally, there are very few library-independent methods with target species beyond humans and a few domestic animal species. Lastly, while some MST markers have been rigorously investigated, others require further validation before they can be used dependably.

3.2.2 Library-independent MST markers: Performance characteristics

The MST field is an emerging science, and much of the field has focused on the development of new MST markers and the improvement of detection and quantification methods. Due to an abundance of proposed MST markers, several review articles have been published (e.g., Field and Samadpour, 2007; Santo Domingo, et al., 2007; Stoeckel and Harwood, 2007) that describe the ideal characteristics of an MST marker. Reviewing these characteristics in detail is beyond the scope of this report; however, some of the characteristics of an ideal and useful MST marker are summarized in Figure 1.2 (excerpted from Harwood and Stoeckel, 2011). When considering which MST markers to

utilize, these marker characteristics can be a helpful guideline. Moreover, MST marker performance for each of these characteristics will determine what information individual markers can provide when utilized in monitoring studies.

Characteristic	Ideal marker	Useful marker
Specificity	Marker found only in target host species	Marker is differentially distributed among host species
Distribution in host population	Found in all members of all populations of target host species; contributes to sensitivity of method	Consistently found in host species whose feces could impact the target sites
Evenness	Quantity in the feces of individuals is similar	Quantity in aggregate sources, e.g., sewage influent; animal populations, is similar
Temporal stability in host	Frequency and concentration in host individuals and populations does not change over time	Despite variation in marker frequency and concentration in individuals, these characteristics are stable at the population level
Geographic range/ stability	The frequency and concentration in geographically separated host populations are similar	The marker can consistently be detected and quantified across the geographic area to be studied
Environmental persistence	Consistent decay rate in various matrices and habitats; no increase under any conditions; response to treatment processes and environmental insults is similar to that of pathogens	Predictable decay rate in various matrices and habitats; no increase under ambient conditions; response to treatment processes and environmental insults is characterized
Quantitative assessment	Can be accurately quantified	Accurately indicates presence/ absence of contamination source
Relevance to regulatory parameters	The marker is derived from an organism that is a regulatory tool	The marker is correlated with an organism that is a regulatory tool
Relevance to health risk	The marker is strongly correlated with risk of all types of waterborne disease, e.g., gastroenteritis, dermatitis, upper respiratory infections	The marker constitutes a health risk or is otherwise correlated with a subset of waterborne disease, e.g., viral gastroenteritis

Figure 1.2. Characteristics of an ideal vs. a useful MST marker (Harwood and Stoeckel, 2011; permission obtained with kind permission of Springer Science and Business Media).

As evaluating each MST marker for all of these characteristics is impractical, this literature review will focus on three characteristics of an MST marker that will be critical for evaluating the MST marker results for Objectives 1, 2, and 3 of this report: (1) sensitivity (how frequently a method detects a source when it is present), (2) specificity (the ability to rule out a source when it is absent), and (3) correlation to pathogens.

Multiple cross-validation studies have compared the sensitivities and specificities of select MST markers (e.g., Field, et al., 2003; Griffith, et al., 2009; Griffith, et al., 2003; Harwood, et al., 2009), with varying results (Table 1.1). Some studies have also determined correlation to pathogens (e.g., Aw and Gin, 2010; Savichtcheva, et al., 2007; Schriewer, et al., 2010; Wu, et al., 2011). This literature review summarizes the data on these two characteristics for the most popular MST methods to help discern what information MST markers can provide in monitoring and assessment.

While MST markers exist that target a number of different sources, this literature review will focus only on those MST markers with a human target, as all of the MST markers in this report are either specific to a human-source or discriminate between human-source and animal-source fecal contamination (data on the sensitivity and specificity of popular animal-specific MST markers are included in Table 1.1 for reference). Current human-source MST markers can be divided into those that target anaerobic bacterial (or archaeal) genes, bacterial toxin/virulence genes, or viruses.

Obligate Anaerobes

Anaerobic organisms have been proposed as fecal indicators because they comprise a significant portion of fecal bacteria (Madigan, et al., 2003; Matsuki, et al., 2004; Wood, et al., 1998), and because they are limited to warm-blooded animals. Due to their low oxygen tolerance, obligate anaerobes tend to have short survival periods outside of the host; thus, detection of these microbes generally indicates recent (or extensive) fecal contamination (Savichtcheva and Okabe, 2006). The most common obligate anaerobes used in current MST studies are *Bifidobacterium, Bacteroidales*, and *Methanobrevibacter*.

Bifidobacterium species are anaerobic Gram-positive bacteria belonging to the *Actinobacteria* group. *Bifidobacterium* are the first to colonize the infant digestive tract and provide multiple benefits to the host, including regulation of the gut microflora, enhancement of the immune system, and production of antimicrobial substances and vitamins (Bivati and Mattarelli, 2006).

Ten species of *Bifidobacteria* have been identified in human fecal material, and four of these, *B. adolescentis*, *B. dentium*, *B. longum*, and *B. catenulatum* were considered primarily of human origin (Bonjoch, et al., 2004; Doraj-Raj, et al., 2009; Lynch, et al., 2002; Nebra, et al., 2003). The sensitivity and specificity of several assays targeting *B. adolescentis* range from 67-100% and 84-100%, respectively (Table 1.1). A newer assay targeting *B. catenulatum* was the most promising PCR assay in an Irish evaluation study, exhibiting 100% sensitivity with human sewage samples and a specificity of 87% (Doraj-Raj, et al., 2009).

Comprehensive studies on the correlation of *Bifidobacterium* spp. presence with that of pathogens have yet to be performed.

Likely due to this assay's questionable specificity (Lamendella, et al., 2008) and rapid degradation in the environment, this organism is rarely utilized in more recent source-tracking studies.

By far, the most popular MST methods are those that target the members of the order ***Bacteroidales***, family *Bacteroidetes*, using the 16S rRNA gene. The genus *Bacteroides*, the family, and the order comprise anaerobic fecal bacteria that are abundant in the intestines of mammals. *Bacteroides* spp. may make especially good indicators of human fecal pollution as the species makes up approximately one-third of the human

fecal microflora (Madigan, et al., 2003), outnumbering fecal coliforms, *E. coli*, and *Enterococcus* spp. *Bacteroides* spp. are obligate anaerobes, so their ability to persist or grow in the environment is limited (Fiksdal, et al., 1985; Kreader, 1998). Persistence of *Bacteroidales* bacteria and genetic markers in the environment have been shown to range from 1 to 24 days, depending on temperature, the presence of predators, salinity, and marker used (Bernhard, et al., 2003; Kreader, 1998; Seurinck, et al., 2005; Walters and Field, 2009).

Multiple human-specific *Bacteroidales* assays have been developed (Bernhard and Field, 2000a; b; Converse, et al., 2009; Dick and Field, 2004), as well as *Bacteroidales* assays that can distinguish between cattle, canine, swine, horse, and elk feces (Bernhard and Field, 2000b; Dick, et al., 2005a; Dick, et al., 2005b; Layton, et al., 2006; Okabe, et al., 2007). The sensitivity and specificity for many of the human-specific *Bacteroidales* assays have been examined (Ahmed, et al., 2009; Doraj-Raj, et al., 2009; Griffith, et al., 2009; Kirs, et al., 2011; Stoeckel and Harwood, 2007). Results indicate that sensitivity and specificity vary widely depending on the assay, method, and location: between 12-100% sensitivity and 0-100% specificity (Table 1.1).

The correlation between *Bacteroides* sp. and several pathogens has been examined. A study by Schriewer et al. (2010) evaluated the ability of several *Bacteroidales* assays (universal, human, dog, and cow) to predict pathogen occurrence. The study found the universal *Bacteroidales* genetic marker to have a comparable or higher mean predictive potential for protozoan and bacterial pathogens than traditional FIB. A study by Savichtcheva et al. (2007) found that both total and human-specific

Bacteroidales 16S rRNA genetic markers were predictive for the occurrence of *E. coli* O-157, *Salmonella*, and the LT and STh genes of *E. coli* ETEC.

Assays targeting *Bacteroidales* are currently the most popular source-tracking markers, likely due to the wide range of assays and targets available.

The genus ***Methanobrevibacter***, in the order Methanobacteriales, includes the human-specific *M. smithii* and the ruminant-specific *M. ruminantium*. *M. smithii* may be useful as an indicator of sewage and human fecal pollution in the environment because of its host specificity and high abundance in the human intestine. *M. smithii* is the dominant archaean in the human gut, occurring in concentrations of 10^7–10^{10} per gram of dry weight (Bond, et al., 1971; Lin and Miller, 1998).

An assay developed by Ufnar et al. (2006) that detects the *nifH* gene of *M. smithii* was able to detect human fecal contamination in individual fecal samples, sewage, and sewage-contaminated marine water and was negative for uncontaminated marine waters, other fecal samples (cow, sheep, swine, horse, deer, goat, turkey, goose, chicken, and dog), and environmental waters contaminated with bovine waste. This assay was utilized in four other studies and found to be 98-100% sensitive in wastewaters, 9% sensitive in individual fecal samples, 20% sensitive in spiked environmental samples, and 96-100% specific using individual and composite animal fecal samples and water samples (Griffith, et al., 2009; Harwood, et al., 2009; Kirs, et al., 2011; McQuaig, et al., 2009). A quantitative PCR (qPCR) assay has also been developed that was 60-100% sensitive to all samples with known or spiked sewage inputs and 83% specific to a seawater sample spiked with gull guano (Griffith, et al., 2009; Johnston, et al., 2010).

M. smithii presence has not been evaluated for correlation to enteric pathogens. Results from studies evaluating this marker are promising, but more research is required to further confirm the marker's sensitivity, specificity, and correlation to pathogens.

Bacterial toxin/virulence genes

Toxin genes of pathogenic FIB strains (e.g., *E. coli* and enterococci) may also serve as host-specific indicators of fecal pollution. The **enterococcal surface protein**, ***esp***, of *E. faecium,* has been proposed as a human-specific indicator of fecal pollution. This marker has been evaluated in studies with mixed results. The first PCR assay targeting this gene was positive for 97% of sewage and septic samples and negative for livestock and bird waste (Scott, et al., 2005), but required a membrane filtration step 48 h before PCR detection (Scott, et al., 2005). Without the initial enrichment step, the assay's sensitivity and percentage of correct classification declined to 4.4 and 5.9%, respectively (Balleste, et al., 2010). Other studies have found the *esp* PCR assay to be 90-100% sensitive to sewage, septic system samples, and sewage-spiked samples and 68-100% specific to individual fecal and spiked water samples (Ahmed, et al., 2008; Griffith, et al., 2009; Kirs, et al., 2011). Several recent studies have suggested that the *esp* gene may not be exclusive to human fecal pollution (Ballesté, et al., 2010; Byappanahalli, et al., 2008; Layton, et al., 2009). Due to its required enrichment step and possible lack of specificity, the *esp* assay may not serve as an optimal MST marker in environmental monitoring programs.

Viruses: Bacteriophages and human enteric viruses

Due to their constant presence in sewage and polluted waters, bacteriophages have been proposed as indicators of fecal pollution. Bacteriophages are also possible

indicators of viral pollution because their size, morphology, structure, and behavior in the environment closely resemble that of human enteric viruses (Gerba, 2000). The most popular phages used in current MST studies are the F+RNA coliphages and phages that infect specific strains of *B. fragilis*.

F+RNA coliphages have been used to predict the source of fecal pollution because animal and human feces contain different serotypes (Scott et al., 2002). Groups I and IV are generally associated with nonhuman animals, while group II isolates are generally associated with human and pig feces, and group III isolates are generally associated with human feces (Griffin, et al., 2001), although some studies have reported that Group II and III genotypes have been identified from a small number of animal-source samples (Schaper, et al., 2002b; Stewart, et al., 2006) and that genotype I has been isolated from municipal sewage (Griffin, et al., 2000; Stewart-Pullaro, et al., 2006). Until recently, relatively few strains were completely characterized, limiting the ability of molecular assays to be sensitive to all members within each genotype. However, Friedman et al. recently characterized 19 F+RNA strains (Friedman, et al., 2009b) and developed new genotype-specific primer sets accordingly (Friedman, et al., 2009a). These new F+RNA assays have yet to be tested for sensitivity and specificity with reference samples.

Older F+ coliphage assays, as well as somatic assays, have been examined in three separate multi-laboratory studies (Field, et al., 2003; Griffith, et al., 2009; Noble, et al., 2003). Recorded sensitivities of F-RNA coliphage assays for human wastewater and fecal samples have ranged from 0-99% sensitivity in blind and wastewater samples, and 11-100% specificity (Table 1.1). Sensitivities for somatic and F+ coliphage assays were

found to be 100% and 30-100%, respectively, with specificities of 67-100% and 100%, respectively (Table 1.1).

Correlations between coliphages and pathogens have been examined, and positive correlations have been found between somatic coliphages and adenoviruses (Aw and Gin, 2010), enteroviruses (Mocé-Llivina, et al., 2005), and culturable enteric viruses (Payment and Franco, 1993), and between F+ coliphages and noroviruses (Aw and Gin, 2010; Dore, et al., 2000) and adenoviruses (Wu, et al., 2011). A promising coliphage for use as a water quality standard is the F+ coliphage (Havelaar and Pot-Hogeboom, 1988) due to its similarity in size, shape, structure, and genetic makeup to many human enteric viruses. F+ coliphages have been found to be more stable than human enteroviruses in environmental waters (Sinton, et al., 2002), exhibit high resistance to water purification processes (Kott, et al., 1974), and are resistant to inactivation in natural environments and sewage treatment processes, likely making them conservative indicators for enteric viruses. In their meta-analysis, Wu et al (2011) found that coliphages had a positive correlation to multiple pathogens, with an odds ratio for total coliphages and F+ coliphages of 1.29 (95% CI = 0.82-2.05) and 1.27 (95% CI = 0.48-3.35), respectively.

The **bacteriophages that infect *Bacteroides* spp.** have also been proposed as MST markers. Several bacteriophages that infect *Bacteroides* spp. have been found to be fairly human-specific, including those that infect *B. fragilis* HSP40 (Grabow, et al., 1995), *B. thetaiotamicron* GA17 (Blanch, et al., 2006; Payan, et al., 2005), and *B. fragilis* GB124 (Ebdon, et al., 2007; Payan, et al., 2005). The use of bacteriophages that infect *Bacteroides* to indicate animal waste has also been described (Payan, 2006). MST studies that included bacteriophages infecting *Bacteroides* spp. found the indicators to be highly

predictive of human fecal contamination. One study found that the sensitivity and specificity of bacteriophages infecting *B. thetaiotamicron* were among the most predictive of human wastewater, and were included in a model that was 100% predictive of the fecal source in wastewaters (Blanch, et al., 2006). A second study, which analyzed river water, municipal wastewater treatment effluent, and animal fecal samples for multiple indicators, found that bacteriophages infecting strain GB124 were present in all municipal wastewaters and were not detected in fecal samples from animals (Table 1.1) (Ebdon, et al., 2007).

Correlation have been demonstrated between the presence of *B. fragilis* bacteriophage and enteric viruses and to detectable enteric virus genomes (Armon and Kott, 1996; Gantzer, et al., 1998). *B. fragilis* phage also persist comparable to or longer than enteric viruses, coliphages, and polioviruses in surface waters (Chung and Sobsey, 1993).

Viral pathogens themselves are also a promising tool for assessing water quality. Over 100 types of pathogenic viruses are excreted in human and animal wastes (Melnick, 1984), and they are shed in extremely high numbers: typically between 10^5 and 10^{11} virus particles per gram of stool from infected hosts (Farthing, 1989). Enteric viruses are known to cause a significant portion of waterborne disease (Fogarty, et al., 1995), but viruses are also believed to be responsible for a significant percentage of waterborne outbreaks in which the etiological agent is unknown. Commonly studied groups of enteric viruses include Picornaviridae (polioviruses, enteroviruses, coxsackieviruses, hepatitis A virus, and echoviruses), Adenoviridae (adenoviruses), Caliciviridae (noroviruses, caliciviruses, and astroviruses), and Reoviridae (reoviruses and rotaviruses).

While the use of animal-specific viruses as MST markers has been proposed, the majority of research has focused on the use of human-specific viruses. These include human polyomaviruses, adenoviruses, enteroviruses, and noroviruses.

Human polyomaviruses (HPyVs) are nonpathogenic to individuals with a healthy immune system and infect a large portion of the human population (Behzad-Behbahani, et al., 2004; Knowles, et al., 2003). Nested PCR (McQuaig, et al., 2006) and qPCR assays (McQuaig, et al., 2009) that detect both JCV and BKV species have been designed and tested to have 50-100% sensitivity in fecal, wastewater, and blind samples, and 100% specificity in non-human fecal samples (Table 1.1).

Human adenoviruses are frequently found in urban rivers associated with human fecal contamination (Castingnolles, et al., 1998; Chapron, et al., 2000; Tani, et al., 1995). The sensitivities of these assays range from 40-92%, while the specificities of some have been calculated between 67-100% (Table 1.1).

Human **enteroviruses**, including poliovirus, echovirus, and Coxsackie A and B viruses, have been found in activated sludge, sewage outfalls, and fresh and marine waters associated with human fecal contamination (Griffin, et al., 1999; Jiang, et al., 2001; Kopecka, et al., 1993; Noble and Fuhrman, 2001; Reynolds, et al., 1998). A microbial source-tracking comparison study (Noble, et al., 2003) found that one enterovirus RT-PCR assay (Tsai, et al., 1993, with minor modifications) had a sensitivity of 38% and a specificity of 100% (Table 1.1). Similarly, a quantitative reverse-transcriptase PCR (RT-qPCR) assay (e.g., Gregory, et al., 2006) had a 20-25% sensitivity to spiked water samples and 100% specificity to gull feces-spiked seawater (Griffith, et al., 2009).

Noroviruses may also serve as possible MST markers, due to the fact that several norovirus genogroups are specific to humans (only genogroups I, II, and IV have been detected in humans, though genogroups I and II predominantly cause gastrointestinal disease) (Koopmans, 2002), and because norovirus genogroups I and II are thought to be the leading cause of viral gastroenteritis worldwide (Atmar, 2006). Noroviruses have been detected in treated wastewaters and surface waters (Astrom, et al., 2009; da Silva, 2007; Lodder and de Roda Husman, 2005; Ueki, et al., 2005). A methods comparison study (Griffith, et al., 2009) found norovirus assays to have a 0-80% sensitivity in sewage-spiked samples and 100% specificity to gull-feces spiked seawater (Table 1.1).

The advantages of utilizing pathogenic viruses for water quality management are significant. Viruses are very host specific; thus, detection of various viral targets can solidify the determination of the source of contamination. Quantification in stormwater and contaminated surface waters may be an important tool for quantifying contaminant load and potential human health risk, respectively. On the other hand, pathogenic viruses generally infect only a small percentage of any given population, making them relatively rare targets. Additionally, before consistent use in field studies, viral detection assays will require reliable process controls, as the required concentration and extraction steps generally have low and variable recoveries.

Summary

Currently, there is no consensus among researchers or regulatory agencies regarding the best MST markers. No single technique is capable of determining all possible fecal sources accurately. While results from MST techniques can provide important insight into the sources of fecal contamination and, possibly, the presence of

pathogens, the choice of marker will provide different types of information. For example, viral markers, such as enteroviruses and adenoviruses, tend to have relatively high false-negative rates in individual fecal samples, leading to a low sensitivity, but their narrow host range allows them to be exceedingly specific. Thus, they can more definitively indicate the source of fecal pollution, but they cannot reliably rule out the presence of human sewage. Conversely, markers such as human-associated *Bacteroidales* have a lower average specificity than viruses, but their overwhelming numbers in human hosts allow for increased sensitivity. Moreover, each of the markers mentioned above have varying levels of precision. For examples, coliphages can distinguish between human and non-human fecal contamination, whereas different *Bacteroides* markers may be specific to animal species (e.g., elk, dog, human). Lastly, only some MST markers have been evaluated for correlation to pathogens. It is critical that water quality managers using these techniques have a good understanding of the abilities and limitations of MST markers.

3.3 FIB and MST utilization

Federal recommendations for and state agency approaches to microbial water quality testing will continue to primarily utilize FIB standards (USEPA, 2012). The results of these standards will likely be used as the sole or principal tool for mitigation of water pollution, even though research indicates that the presence, source, and public health significance may not be reliably assigned using fecal indicator organism assessments alone (Section 3.1). While testing water quality for these approved standards may provide a framework for gauging water-body health from a human health perspective, it only provides a fraction of the information necessary to initiate

remediation when those same standards are not met (Kinzelman, et al., 2011). For example, failure to meet FIB-based water quality standards results in impairments of water bodies based on their designated use, but it does not provide adequate data for developing a pollution reduction strategy, implementing BMPs, or effectively allocating NPS contributions in the development of TMDLs. Thus, according to Gawler et al. (2007), monitoring programs would benefit by combining MST assays with FIB standards. Incorporation of MST data with FIB standards would enable inclusion of historical data relating FIB to human health risk. On the other hand, many researchers believe that problems with the traditional FIB markers outweigh any benefits in their continued use as water quality indicators. It may be more practical to completely replace FIBs with the newer MST methods, as some of these markers have shown correlations to health. Nevertheless, because state regulatory agencies will have the option to continue utilizing culture-based FIB methods, many will continue to utilize these traditional methods exclusively. Thus, we propose that regulatory agencies would do well to incorporate MST assays with the traditional FIB assays rather than utilize FIB markers alone. Incorporation of MST markers with FIB may enhance monitoring data, assist in the development of TMDLs and BMPs by elucidating pollutant sources, and may be utilized to gauge water-quality improvements, and possibly gauge the risk of fecal pollution (Gawler, et al., 2007).

This section will describe two common approaches for MST incorporation with FIB, outlining their advantages and disadvantages. This section will then summarize four potential applications of MST markers, outlining what type of approach would be useful for each application.

3.3.1 MST utilization: Toolbox and tiered approaches

The two most popular methodologies for combining FIB and MST indicators are the "toolbox" and "tiered assessment" approaches.

The toolbox approach utilizes multiple assays to allow for a more accurate interpretation of the data. Groups of MST markers that are optimized for different project goals, locations, etc., are on hand and ready for use as specific situations demand (Harwood and Stoeckel, 2011). An example of utilization of the toolbox approach is the study by Vogel et al. (2007), which used multiple MST assays, including *E. coli* rep-PCR, coliphage typing, and *Bacteroidales*16S rDNA to determine the source of fecal contamination in a Nebraska watershed. The use of multiple assays allowed for increased confidence in the interpretation of assay results and prevented erroneous results when one of the assays was not detected.

Thus, an advantage to this approach is an increased correct classification of sources over using only one MST assay. The use of multiple methods for detection of contamination from one source can be used to support one another, alleviating the uncertainty of results from imperfections in all current MST methods (Harwood and Stoeckel, 2011). Another advantage of the toolbox approach is that as MST markers would be utilized throughout a study, data on the spatial, climate, and seasonal trends of MST markers would be available, allowing for better interpretation of changes in marker concentrations following remediation projects (Kinzelman, et al., 2011).

However, the toolbox approach has several disadvantages. A major disadvantage is the increased expense of using multiple assays. Many MST assays require expensive equipment and reagents and/or technical expertise (Hartel, 2011). As EPA's RWQC

recommendations will become national recommendations for all surface waters designated for primary contact recreation (swimming, bathing, surfing) (USEPA, 2012), the limited resources, especially manpower and financial resources, of regulatory agencies are likely to be spread thin across water bodies with varying actual use levels. For example, monitoring schemes that aim to fully characterize a water body may require temporally and spatially exhaustive sampling plans. These resource-intensive requirements may preclude a monitoring agency from utilizing expensive assays as continual monitoring tools.

Given these advantages and disadvantages, the use of a toolbox approach may be best suited to remediation projects, as the comprehensive data would allow for better interpretation of remediation projects post-implementation.

In contrast to the toolbox approach, a tiered approach utilizes multiple levels of monitoring, generally employing lower cost methods that broadly measure contamination, such as FIB, as continual monitoring tools followed by more expensive, technically demanding tools, such as MST markers, in targeted areas where higher levels of FIBs are detected (Harwood and Stoeckel, 2011). A tiered approach was utilized by Noble et al. (2006) to assess and quantify fecal contamination in Santa Monica Bay, California. The study employed *E. coli*, *Enterococcus* sp., *Bacteroides* sp., and enteroviruses to identify sources of FIB in an urban watershed.

An advantage to the tiered approach is the decreased expense compared to the toolbox approach due to a selective use of MST assays. In contrast to the toolbox approach, more expensive analyses such as MST assays could be used only for targeted sites, or "hot spots." Given per sample costs of ~$30 for qPCR assays versus <$10 for

culture-based FIB methods (Griffith and Weisberg, 2011) and limited state and local budgets, the cost savings in using MST assays only sparingly could be a pivotal factor for monitoring agencies deciding between tiered and toolbox approaches. It is important to note, however, that the proposed RWQC allow for use of qPCR-based assays for *Enterococcus* as a regulatory standard (USEPA, 2012). If monitoring agencies choose to adopt molecular methods for water quality standards, initial monetary investment costs for assays targeting additional markers, such as those for MST markers, would be minimal. However, the initial monetary jump from culture-based detection of FIB to molecular-based detection may be beyond the reach of many monitoring agencies: one recent estimate for initiating qPCR in a laboratory, including capital costs associated with buying all necessary equipment and revamping old laboratory space to new molecular sample requirements (e.g., qPCR hoods), was approximately $100K (Griffith and Weisberg, 2011).

A major disadvantage for the tiered approach is that a lack of association between first-tier and second-tier markers (e.g., FIB and MST markers) could drastically reduce the approach's ability to identify and reduce fecal pollution sources. Another disadvantage of this approach is that use of the marker just before and after remediation may not reveal much about an increase or decrease in a particular source, as the seasonal, climate, and spatial variability of an MST marker will not be defined (Benham, et al., 2011).

Given these advantages and disadvantages, a tiered approach may be best suited to identifying and prioritizing impaired water bodies, as opposed to use in remediation projects.

3.3.2 Potential applications of MST

The use of a tiered or toolbox approach will likely be dependent on how an MST method is to be utilized for water quality monitoring. Harwood and Stoeckel (2011) have proposed that there are four potential applications for MST in water quality monitoring:

(1) Assessing the source(s) of fecal contamination in recreational or drinking waters;
(2) Prioritizing impaired water bodies for total maximum daily load (TMDL) implementation (or other interventions);
(3) Apportioning sources for TMDL plans; and
(4) Assigning or relieving responsibility for fecal pollution.

One application for MST markers in a monitoring program is **(1) assessing sources of fecal contamination in recreational water.** At present, water quality monitoring is done with the use of FIB, which cannot indicate the source of fecal contamination. Few monitoring agencies currently integrate MST assays with FIB as a first step in water quality monitoring. However, this may change as MST methods develop and become standardized.

The second application for which MST markers can be utilized is **(2) prioritization of impaired water bodies for total maximum daily load (TMDL) implementation or other interventions.** Once water bodies are listed as impaired (as determined by FIB concentrations above regulatory standards), states must develop a TMDL to guide remediation efforts. As approximately 40% of assessed surface waters in the US are impaired (USEPA, 2009), prioritizing waterways for TMDL interventions is critical. A practical approach for ranking water bodies was outlined by Wapnick et al. (2009). This "weight of evidence approach," based on the World Health Organization's (WHO) Annapolis Protocol (WHO, 1999), ranks water bodies according to probable

pollution sources and human use. It facilitates decision-making about which areas will be investigated by MST methods and which will receive more limited attention and resources.

The third application for which MST markers can be utilized is **(3) source apportionment for TMDL plans.** This application is primarily concerned with producing pollutant load reductions in order to achieve water quality monitoring standards. As these standards are generally based upon the 1986 USEPA recommendations for recreational water quality utilizing FIB (USEPA, 1986; 2003), this application is primarily concerned with reducing these FIB to acceptable levels. Given that the project will need to apportion contributions from various fecal sources, quantitative markers will be necessary to identify the sources of FIB loading (Harwood and Stoeckel, 2011). These quantitative measurements will need to be connected to assessments of flow, fate and transport, and seasonal changes, as well as assessments of antecedent rainfall, groundwater levels, etc. for sufficient power of the MST markers to allocate sources of FIB loading.

The fourth application for which MST markers can be utilized is **(4) forensic applications, (i.e., assigning (or relieving) responsibility for pollution.** It is very likely that some of the source allocation assessments and implementation plans for remediation that are developed under TMDL plans will enter the legal system, as strategies such as altering animal waste disposal practices may not be undertaken willingly (Teaf, et al., 2011). This last application would require technically and legally defensible MST methods for assigning responsibility for fecal pollution. As this application is attempting to apportion contributions from specific fecal sources, quantitative markers will be

necessary to determine the relative sources of fecal loading, as will highly precise markers that can identify the species of domesticated animal or even a specific herd or flock that is the major contributor of fecal pollution (USEPA, 2005).

For applications 3 and 4, correlation of the MST markers with FIB standards is preferred in order to allocate the sources of FIB to different sources, especially during certain times (e.g., particular storm events) or within certain areas (e.g., tributaries as opposed to receiving waters). This was emphasized by Kinzelman et al. (2011), who stated that for MST markers to be of relevance to the recreational water quality community, which includes the regulators charged with achieving water-quality objectives based on FIB levels, MST must closely correlate with the FIB regulatory parameters. Thus, a toolbox approach may be the best approach for these applications because there is a better likelihood of one or several MST markers correlating with FIB levels. Moreover, a toolbox approach would be appropriate for these applications, especially application 4, because the use of multiple methods for detection of one source can be used to support one another, alleviating the uncertainty of results from imperfections individual MST methods (Harwood and Stoeckel, 2011).

For applications 1 and 2, a toolbox approach could also be utilized, but, as mentioned earlier, a tiered approach may be preferred for identifying and prioritizing sources of fecal contamination due to the reduced costs of this approach compared to the toolbox approach. However, the process for determining whether or not a tiered approach is appropriate for these applications has not been established. Several studies have determined the applicability of a tiered approach in select basins based on correlations between FIB standards and MST markers (e.g., Converse, et al., 2009; Sauer, et al.,

2011). Correlation between MST markers and FIB standards would be beneficial, as the first tier in many tiered approaches is likely to be FIB assays. We propose here that there are also two other possibilities for evaluating the use of a tiered approach.

First, if a correlation is not found between MST and FIB organisms, a tiered approach could be evaluated based on the percentage of MST-positive samples that would be missed if only FIB standards were used. For example, if prioritization of water bodies for TMDL plans is to be based on those areas predominantly impacted by human-source fecal pollution, the FIB assays in the first tier should indicate, or "flag," at least a majority of the sites positive for human-source MST markers. This criterion was utilized by Sauer et al. (2011), who recommended against a tiered approach because high FIB levels would have failed to flag 35% of the samples with high concentrations of a human-specific *Bacteroidales* marker and would have flagged 33% of samples as priority that had low or no evidence of the marker. Obviously, several components of this criterion are subjective and may vary between studies, including what % of MST-positive samples should be indicated by high FIB levels and also what constitutes "high" levels of FIB indicators. We suggest that at least 50% of samples positive for an MST marker of interest (e.g., a human-source marker) be indicated by high FIB levels. We also suggest that "high" FIB levels be defined as levels above regulatory thresholds, as these thresholds are used to categorize water bodies as in compliance or impaired. This criterion would likely only be necessary if MST and FIB are not correlated.

Second, a tiered approach could be evaluated based on whether or not the MST markers provide sufficiently different information from the FIB standards. For example, if an MST marker is not largely specific to a particular source, it may not provide the

level of information necessary to identify the source of fecal contamination in individual samples. For example, if an MST marker is only 50% specific to a particular source, it may not provide enough additional information from FIB to necessitate its use in a tiered approach, although an MST marker may still provide useful information for a study, for example if the marker has shown a relationship to human health. The level of MST marker specificity required for usefulness in a tiered approach has not yet been established and is likely to vary depending on the goals of individual projects.

Thus, determining whether or not a tiered approach is applicable for identifying and prioritizing sources of fecal contamination is not straightforward. One or all of the above criteria could be utilized to determine whether this approach can be used with individual MST markers.

3.4 Conclusions

Recommendations for water quality monitoring for primary contact recreation are currently based on traditional FIB standards (Section 2). The results of these monitoring assessments are frequently used as the sole or primary factor for mitigating contamination, even though much of the literature indicates that FIB assessments alone do not reliably indicate the presence, source, and possibly public health significance of fecal contamination. While testing for FIB may provide a framework for gauging water quality from a human health standpoint, particularly in PS-impacted waters, it only provides a fraction of the information necessary to initiate remediation in most waters when those same standards are not met (Kinzelman, et al., 2011). Thus, while routine monitoring via FIB will likely continue to utilized by regulatory agencies for assessing water quality (USEPA, 2012), these techniques are limited in their utility. MST

technologies, when applied throughout water quality assessment, prioritization, and remediation stages, can improve the identification and remediation of fecal contamination.

A tiered approach, if applicable, could prove most beneficial for regulatory agencies when attempting to identify and prioritize NPS contributions of fecal contamination. Monitoring programs that include MST technologies have the potential to discriminate between human versus nonhuman sources and better indicate the presence of pathogens. It would be desirable for MST markers to correlate with FIB standards for ease of incorporation into existing water quality monitoring regulations. However, it is not yet clear how MST assays relate to measurements of FIB in natural waters impacted primarily by NPS pollution. The objective of this study was to determine whether MST markers can provide information for source allocation and human pathogen presence in the Cape Fear watershed in North Carolina, a diverse watershed with both highly urbanized areas as well large regions of industrialized agriculture, and whether the MST markers can be utilized in a tiered assessment with FIB.

3.5 Approach

With a focus on the Cape Fear watershed, the objectives for this research were met using field-based and lab-based studies. The approach to address each objective is outlined below.

Objective 1. Elucidate the spatial and temporal trends of human-specific MST markers in order to prioritize areas for targeted remediation in a mixed-use watershed.

This study focused on a mixed-use sub-watershed in the Research Triangle, North Carolina. This area is experiencing rapid growth leading to increased NPS pollution and water body impairment of freshwater supplies. Impacted waters include Jordan Lake, which is located within an urbanizing watershed and serves as a source of drinking water for surrounding municipalities. Monthly raw water samples from 15 stream locations within the Jordan Lake watershed were analyzed for human-specific source tracking markers. The sites were chosen to represent varying land uses. This research helped to identify hot spots for targeted restoration plans. For this goal, we utilized the anaerobic human-source markers *Bacteroides* sp. and *M. smithii*, and the enteric viruses, enterovirus and norovirus.

Objective 2. Identify whether sites with intense exposure to NPS from agricultural runoff may pose human health risks due to presence of microorganisms of public health concern in order to inform best management practices.

Industrial hog production has grown rapidly in North Carolina in the last few decades, and the state is now ranked second in hog production within the country. The rapid growth of the hog CAFO industry has raised concern about proper waste disposal, as chronicled in reports from the National Pew Commission on Industrial Food Animal Production (2008) and the US Government Accountability Office (2008a). Wastes from CAFOs are land-applied and can contribute to NPS run-off. This project evaluated coliphages as indicators of microbial water quality in surface waters adjacent to hog farm waste application sites. Results were compared to measures of traditional FIB and to counts of presumptive antibiotic-resistant *Staphylococcus* and hepatitis E virus. For this goal, we chose coliphages due to the study's location in a densely agricultural area, the

ability of F-RNA coliphages to distinguish between human and animal sources, and the correlation of both somatic and F+ coliphages to pathogens.

Objective 3. Compare MST markers to FIB indicators to determine whether MST markers can be used in a tiered assessment.

This comparison was conducted utilizing data generated from evaluating Objectives 1 and 2. Comparisons between MST markers and FIB counts were based on (1) correlations between MST and FIB or (2) the % of MST-positive samples "missed" by FIB levels below regulatory thresholds, and (3) the specificity of MST markers.

4. Methods Development

In addressing Objectives 1-3, this study utilized several molecular assays, including assays for *Bacteroides* sp., *Methanobrevibacter smithii,* and enteric viruses. These assays, utilizing qPCR, are potentially more sensitive, quantitative, and amenable to automation than culture-based methods (Santo Domingo, et al., 2007). However, in practice, the concentration and extraction steps necessary for molecular methods can have low and variable recoveries, and the nucleic acid extractions can contain inhibitors that increase a sample's limit of detection or result in false negatives. Utilization of quantitative markers is a high priority goal for much of the MST field (Santo Domingo, et al., 2007). However, before incorporation of quantitative data, multiple performance characteristics will have to be defined for MST markers and their methods to ensure accurate quantification and interpretation (see review in Harwood and Stoeckel, 2011). In terms of accurate quantification, Wuertz et al. (2011) established that there are two major challenges that face accurate quantification of molecular MST markers from natural water samples: (1) assessing the recovery efficiency of sample processing steps, including concentration of targets and extraction and purification of nucleic acids, and (2) assessing the impact of PCR inhibitors. During the course of this study, both of these issues impacted detection and quantification of MST markers, especially the viral targets. Thus, this project addressed these methodological limitations with two additional objectives. One objective evaluated novel methods for measuring PCR inhibitors and another developed a novel virus surrogate for improved virus detection and quantification in environmental samples.

Objective 4. Determine whether the level of inhibition detected in select surface water samples is related to the presence of detectable dissolved organic matter components in order to determine what types and levels of organics cause PCR inhibition in environmental water samples.

When concentrating large volumes of water for molecular methods, it is possible, and sometimes unavoidable, to co-concentrate PCR inhibitors, such as humic substances and metals, with the target of interest (Abbaszadegan, et al., 1993). Major inhibitors of PCR common in the water environment are humic substances, including humic and fulvic acids, which are the major constituents of organic material in the aquatic environment (Steinberg and Muenster, 1985). A number of studies have examined the removal of humic substances from samples, but few have examined the type and levels of humic substances that correlate to levels of PCR inhibition. It was expected that levels of certain humic substances would correlate to increased levels of PCR inhibition in samples. Understanding this correlation will help researchers to predict what types of samples may have increased levels of inhibition, and suggest appropriate measures for reducing or removing inhibitory substances. The level of inhibition was evaluated in conjunction with abiotic parameters, land use, and the level of antecedent rainfall.

Objective 5. Develop and demonstrate proof-of-concept for a novel process control for recovery of enteric viruses from water.

Modern molecular techniques such as PCR offer powerful tools for rapid detection of an almost infinite variety of microbial contaminants in water. Processing samples in preparation for PCR typically involves concentration and extraction steps with low and variable recovery efficiencies. Controls for bacterial targets have been developed and are

widely available (Siefring, et al., 2008). However, widely adaptable controls that can be used to estimate recovery efficiencies of viruses during sample processing are lacking. These controls are necessary to make the best use of the new detection technologies and to improve method sensitivity for detection of viral pathogens in water. It was expected that a plant virus, turnip crinkle virus, would serve as an adequate viral extraction control due to its similar morphology, size, and genetic material to human enteric viruses. This control will aid researchers in effectively quantifying levels of viral pathogens in environmental samples.

Table 1.1. Sensitivities and specificities for Microbial Source Tracking Assays

Test	Target	Host category	Sample type	Sensitivity (n)	Specificity (n)	Reference
Anaerobic Bacterial and Archaeal Genes						
Bifidobacterium adolescentis PCR	Bi-ADO 1/2	Human	Wastewater	1.00 (22)	0.84 (60)	Bonjoch et al. (2004)
Bifidobacterium adolescentis colony hybridization	S-S-B.ado-0182-a-S-27	Human	Individual Feces	0.92 (12)	1.00 (85)	Lynch et al. (2000)
Bifidobacterium adolescentis colony hybridization	S-S-B.ado-0182-a-S-28	Human	Wastewater	0.67 (3)	1.00 (3)	Lynch et al. (2000)
Bifidobacterium adolescentis PCR	BiADO 1/2	Human	Individual feces/wastewater	0.85 (26), 1.00 (33)	0.84 (119)	Doraj-Raj et al. (2009)
Bifidobacterium catenulatum PCR	BiCAT 1/2	Human	Individual feces/wastewater	0.46 (26), 1.00 (33)	0.87 (119)	Doraj-Raj et al. (2009)
Bacteroidales PCR (two trials)	HF183F, HF134F/Bac708R	Human	Blind samples	0.70, 1.00 (10, 14)	1.00, 1.00 (6, 7)	Field et al. (2003)
Bacteroidales PCR	HF183F/Bac708R	Human	Individual feces	0.20–0.85 (7–25)	0.85–1.00 (46–73)	Bernhard et al. (2000)
Bacteroidales PCR	HF183F/Bac708R	Human	Wastewater	1.00 (41)	1.00 (75)	Bernhard et al. (2000)
Bacteroidales PCR	HF183F/Bac708R	Human	Blind samples	0.80 (5), 1.00 (2)	1.00 (5)	Griffith et al. (2009)
Bacteroidales PCR	HF183/Bac708R	Human	Individual fecal samples and Wastewater	1.00 (22)	0.87 (67)	Kirs et al. (2011)
Bacteroidales PCR	HF183/Bac708R	Human	Wastewater, Septic Systems	0.96 (55)	0.87(117)	McQuiag et al. (2009)
Bacteroidales PCR	HF183/Bac708R	Human	Individual feces/wastewater	0.12 (26), 0.70 (33)	1.00 (119)	Doraj-Raj et al. (2009)
Bacteroidales PCR	Bac32F/Bac708R	Human	Wastewater, Non-human feces	1.00 (53)	0.96 (316)	Harwood et al. (2009)
Bacteroidales PCR	HF134	Human	Wastewater	0.3 (40)	0.81 (72)	Balleste et al. (2010)
Bacteroidales PCR	HF183	Human	Wastewater	0.5 (40)	0.71 (73)	Balleste et al. (2010)

Bacteroides PCR	HF183	Human	Wastewater, Non-human feces	1.00 (50)	0.99 (136)	Ahmed et al. (2009)
Bacteroides PCR	BacHum	Human	Wastewater, Non-human feces	1.00 (50)	0.94 (136)	Ahmed et al. (2009)
Bacteroides PCR	HuBac	Human	Wastewater, Non-human feces	1.00 (50)	0.63 (136)	Ahmed et al. (2009)
Bacteroides PCR	BacH	Human	Wastewater, Non-human feces	1.00 (50)	0.94 (136)	Ahmed et al. (2009)
Bacteroides PCR	Human-Bac	Human	Wastewater, Non-human feces	1.00 (50)	0.79 (136)	Ahmed et al. (2009)
Bacteroidales, qPCR	BacHum	Human	Individual Feces and Combined Fecal Samples, Wastewater	0.67, 1.00 (18,14)	0.98 (41)	Kildare et al. (2007)
Bacteroidales, qPCR	HuBac	Human	Individual Feces and Combined Fecal Samples, Wastewater	0.89, 1.00 (18,14)	0.61 (41)	Kildare et al. (2007)
Bacteroidales, qPCR	HF183	Human	Individual Feces and Combined Fecal Samples, Wastewater	0.61, 1.00 (18,14)	0.73 (41)	Kildare et al. (2007)
Bacteroidales, qPCR	HF183	Human	Blind samples	0.80 (5), 0.50 (2)	1.00 (5)	Griffith et al. (2009)
Bacteroidales, qPCR	HF183	Human	Blind samples	1.00 (5), 1.00 (2)	0.00 (5)	Griffith et al. (2009)
Bacteroidales qPCR	HF183F/reverse primer described	Human	Individual feces	0.86 (7)	1.00 (19)	Seurinck et al (2005)
Bacteroidales qPCR	HF183F/reverse primer described	Human	Wastewater	1.00 (4)	NR (NR)	Seurinck et al (2005)
Bacteroides thetaiotamicron PCR	BT 1/2	Human	Individual feces/wastewater	0.65 (26), 0.39 (33)	NR (NR)	Doraj-Raj et al. (2009)
Bacteroides thetaiotamicron PCR	BFD	Human	Blind samples	1.00 (5), 0.25 (2)	0.50 (5)	Griffith et al. (2009)
Bacteroides thetaiotamicron qPCR	BFD	Human	Blind samples	1.00 (5), 0.75 (2)	1.00 (5)	Griffith et al. (2009)
Bacteroides vulgatus PCR	BV 1/2	Human	Individual feces/wastewater	0.88 (26), 1.00 (33)	0.86 (119)	Doraj-Raj et al. (2009)
Bacteroidales PCR (two trials)	CF128F, CF193F/Bac708R	Ruminants and pseudoruminants	Blind samples	1.00 (7, 9)	0.89, 0.92 (9, 12)	Field et al. (2003)
Bacteroidales PCR	CF128F/Bac708R	Ruminants and pseudoruminants	Individual feces	0.97, 1.00 (31, 20)	1.00, 1.00 (20, 28)	Bernhard et al. (2000), Fogarty et al.

						(2005)
Bacteroidales PCR	CF128F/Bac708R	Ruminants and pseudoruminants	Wastewater	1.00 (75)	0.93 (14)	Bower et al. (2005)
Bacteroidales PCR	CF193F/Bac708R	Ruminants and pseudoruminants	Individual feces	1.00 (31)	1.00 (28)	Bernhard et al. (2000)
Bacteroidetes PCR	CF128	Ruminant	Wastewater	0.26 (19)	1.00 (95)	Balleste et al. (2010)
Bacteroidetes PCR	CF193	Ruminant	Wastewater	0 (19)	0.99 (94)	Balleste et al. (2010)
Bacteroidales PCR	CF128/Bac708	Ruminant	Individual fecal samples and Wastewater	0.85 (27)	0.65 (51)	Kirs et al. (2011)
Bacteroidales PCR	CF128F/Bac708R	Cattle	Individual feces	1.00 (19)	0.73 (40)	Bernhard et al. (2000)
Bacteroidales PCR	CF193F/Bac708R	Cattle	Individual feces	1.00 (19)	0.70 (40)	Bernhard et al. (2000)
Bacteroidales PCR	RumB1F/BacPreR	Ruminant	Individual fecal samples and Wastewater	0.97 (74)	0.97 (103)	Doraj-Raj et al. (2009)
Bacteroidales PCR	Bac32F/RumD1R	Ruminant	Individual fecal samples and Wastewater	0.91 (74)	1.00 (103)	Doraj-Raj et al. (2009)
Bacteroidales PCR	Bac32F/RumD2R	Ruminant	Individual fecal samples and Wastewater	1.00 (74)	0.95 (103)	Doraj-Raj et al. (2009)
Bacteroidales PCR	CF128F/Bac708R	Ruminant	Individual fecal samples and Wastewater	0.95 (74)	0.94 (103)	Doraj-Raj et al. (2009)
Bacteroidales, qPCR	BacCow	Cattle	Individual Feces, Combined Fecal Samples	1.00 (8)	0.94 (51)	Kildare et al. (2007)
Bacteroidales, qPCR	BoBac	Cattle	Individual Feces, Combined Fecal Samples	1.00 (8)	0.96 (51)	Kildare et al. (2007)
Bacteroidales PCR	DF475F/Bac708R	Dog	Blind samples	0.40 (15)	0.86 (7)	Field et al. (2003)
Bacteroidales, qPCR	BacCan	Dog	Individual Feces, Combined Fecal Samples	0.63 (8)	0.90 (51)	Kildare et al. (2007)

Methanobrevibacter smithii PCR	Mnif-342f/Mnif-363r	Human	Wastewater, Septic Systems	0.98(55)	0.99(117)	McQuiag et al. (2009)
Methanobrevibacter smithii PCR	Mnif342f/Mnif3363r	Human	Wastewater, Non-human feces	1.00 (44)	0.98 (343)	Harwood et al. (2009)
Methanobrevibacter smithii PCR	Mnif142f/Mnif363r	Human	Individual Fecal Samples, Wastewater samples	0.09 (11), 1.00 (11)	0.96 (67)	Kirs et al. (2011)
Methanobrevibacter smithii PCR	nifH	Human	Blind samples	0.20 (5), 0.00 (2)	1.00 (5)	Griffith et al. (2009)
Methanobrevibacter smithii qPCR	nifH	Human	Blind samples	0.60 (5), 1.00 (2)	0.83 (5)	Griffith et al. (2009)
Methanobrevibacter smithii Luminex	nifH	Human	Blind samples	0.90 (5), 0.75 (2)	0.83 (5)	Griffith et al. (2009)
Methanobrevibacter ruminatium PCR	Mrnif	Ruminants	Individual Fecal Samples	0.84 (98)	1.00 (187)	Ufnar et al. (2007)
Methanobrevibacter ruminatium PCR	Mrnif	Ruminants	Wastewater	1.00 (4)	1.00 (644)	Ufnar et al. (2007)
Ruminant Methanogen PCR	Mrnif	Ruminants	Blind samples	0.00 (5), 0.00 (2)	1.00 (5)	Griffith et al. (2009)
Swine Methanogen PCR	P23-2	Swine	Individual Fecal Samples	0.84 (25)	0.99 (260)	Ufnar et al. (2007)
Swine Methanogen PCR	P23-3	Swine	Wastewater	0.63 (8)	1.00 (641)	Ufnar et al. (2007)
Swine Methanogen PCR	P23	Swine	Blind samples	0.00 (5), 0.00 (2)	1.00 (5)	Griffith et al. (2009)
Bacterial Toxin/Virulence Genes						
Enterococcus faecium esp gene PCR	Primers described	Human	Wastewater, Non-human feces	0.97 (65)	1.00 (102)	Scott et al. (2002)
Enterococcus faecium esp gene PCR, no enrichment	Primers described	Human	Wastewater	0.04 (23)	0.98 (54)	Balleste et al. (2010)
Enterococcus faecium esp gene PCR, using enrichment	Primers described	Human	Wastewater	0.77 (13)	0.68 (22)	Balleste et al. (2010)
Enterococcus faecium esp gene PCR	Primers described	Human	Wastewater, Non-human feces	0.90 (42)	1.00 (155)	Ahmed et al. (2008)
Enterococcus faecium esp gene PCR	Primers described	Human	Blind samples	1.00 (5), 1.00 (2)	1.00 (5)	Griffith et al. (2009)
Enterococcus faecium	ESPF/ESPR	Human	Individual fecal	NT, 1.00	0.86 (33)	Kirs et al.

esp gene PCR			samples, wastewater	(3)		(2011)
Viruses						
Bacteroides fragilis phage	Host strain HSP40	Human/Nonhuman	Wastewater	1.00 (36)	0.90 (20)	Tartera et al. (1989)
Bacteroides fragilis phage	Host strain HSP40	Human/Nonhuman	Fecal Samples	0.13 (90)	1.00 (145)	Grabow et al. (1995)
Bacteroides fragilis phage	Host strain RYC2056	Human	Wastewater	0.99 (108)	0.22 (110)	Blanch et al. (2006)
Bacteroides thetaiotamicron phage	Host strain GA17	Human	Wastewater	0.99 (73)	0.93 (71)	Blanch et al. (2006)
Bacteroides fragilis phage	Host strain GB-124	Human	Wastewater/ Pooled Non-human feces	1.00 (110)	1.00 (30)	Ebdon et al. (2007)
Coliphage, somatic	EPA 1601	Human	Blind samples	1.00 (5), 1.00 (2)	1.00 (5)	Griffith et al. (2009)
Coliphage, somatic	2-step enrichment	Human	Blind samples	1.00 (5), 1.00 (2)	0.67 (5)	Griffith et al. (2009)
Coliphage, F+	2-step enrichment	Human	Blind samples	0.30 (5), 1.00 (2)	1.00 (5)	Griffith et al. (2009)
Coliphage, F+	EPA 1601	Human	Blind samples	0.75 (5), 1.00 (2)	1.00 (5)	Griffith et al. (2009)
Coliphage, F+DNA	CLAT	Human	Blind samples	0.20 (5), 0.50 (2)	1.00 (5)	Griffith et al. (2009)
Coliphage, F+RNA	CLAT	Human	Blind samples	0.30 (5), 0.50 (2)	1.00 (5)	Griffith et al. (2009)
Coliphage, F+RNA, genotyping or sequencing	Primers and probes described	Human	Blind samples	0.33, 0.67 (3)	0.75, 1.00 (4)	Field et al. (2003)
Coliphage, F+RNA, hybridization, GII	Primers and probes described	Human	Wastewater	0.99 (103)	0.34 (82)	Blanch et al. (2006)
Coliphage, F+RNA, hybridization, GIII	Primers and probes described	Human	Wastewater	0.96 (103)	0.12 (82)	Blanch et al. (2006)
Coliphage, F+RNA, hybridization, GIV	Primers and probes described	Nonhuman	Wastewater	0.91 (82)	0.35 (103)	Blanch et al. (2006)
Coliphage, F+RNA, hybridization, GI	Primers and probes described	Nonhuman	Wastewater	0.95 (82)	0.11 (103)	Blanch et al. (2006)
Coliphage, F+RNA, genotyping or sequencing	Primers and probes described	Nonhuman	Blind samples	0.00, 0.00 (4)	0.33, 0.33 (3)	Field et al. (2003)

Polyomavirus, nested PCR	Primers and Probes Described	Human	Wastewater, Septic Systems	1.00(50)	1.00(25)	McQuaig et al. (2006)
Polyomavirus, nested PCR	Primers and Probes Described	Human	Blind samples	0.80 (5), 0.50 (2)	1.00 (5)	Griffith et al. (2009)
Polyomavirus qPCR	SM2/P6/KGJ3	Human	Wastewater, Septic Systems	1.00(55)	1.00(117)	McQuiag et al. (2009)
Polyomavirus PCR	SM2/P6	Human	Wastewater, Non-human feces	1.00 (41)	1.00 (332)	Harwood et al. (2009)
Polyomavirus PCR	HPyVsF/HPyVsR	Human	Individual fecal samples and wastewater	0.59 (22)	1.00 (67)	Kirs et al. (2011)
Adenovirus, nested PCR	Primers described	Human	Wastewater	0.92 (12)	1.00 (31)	Malquer de Motes et al. (2004)
Adenovirus, nested PCR	Primers described	Human	Blind Samples	0.50 (8)	1.00 (3)	Noble et al. (2003)
Adenovirus, nested PCR	hexAA1885/hexAA1913, hexAA1893/hexAA1905	Human	Wastewater, Septic Systems	0.87(55)	NR	McQuiag et al. (2009)
Adenovirus, nested PCR	Primers described	Human	Blind samples	0.40 (5), 0.50 (2)	0.67 (5)	Griffith et al. (2009)
Adenovirus, nested PCR	Primers described	Cattle	Pooled Fecal Samples	0.75 (8)	1.00 (35)	Malquer de Motes et al. (2004)
Adenovirus, nested PCR	Primers described	Swine	Pooled Fecal Samples	0.74 (23)	1.00 (20)	Malquer de Motes et al. (2004)
Adenovirus, nested PCR	Primers described	Swine	Pooled Fecal Samples	0.84 (38)	NR	Hundesa et al. (2009)
Adenovirus, nested PCR	Primers described	Swine	Wastewater	1.00 (8)	NR	Hundesa et al. (2009)
Adenovirus qPCR	Q-PAdV-F/Q-PAdV-P/Q-PAdV-R	Swine	Pooled Fecal Samples	0.89 (38)	1.00 (unknown)	Hundesa et al. (2009)
Adenovirus qPCR	Q-PAdV-F/Q-PAdV-P/Q-PAdV-R	Swine	Wastewater	1.00 (8)	NR	Hundesa et al. (2009)
Enterovirus RT-PCR	Primers described	Human	Blind Samples	0.38 (8)	1.00 (4)	Noble et al. (2003)
Enterovirus RT-qPCR	Primers described	Human	Blind samples	0.20 (5),	1.00 (5)	Griffith et al.

				0.25 (2)		(2009)
Enterovirus RT-PCR	Primers described	Cattle	Individual feces	0.76 (95)	0.63 (54)	Ley et al. (2002)
Enterovirus RT-PCR	Primers described	Cattle, deer	Individual feces	0.63 (145)	0.75 (4)	Ley et al. (2002)
Enterovirus qRT-PCR	Primers and probe described	Cattle	Individual feces	0.78 (193)	0.42 (100)	Jimenez-Clavero et al. (2005)
Norovirus qRT-PCR	JJV2F/COG2R/RING2-TP	Human	Sewage	0.79 (14)	1.00 (4)	Gregory et al. (2010)
Norovirus RT-PCR	Primers described	Human	Blind samples	0.80 (5), 0.00 (2)	1.00 (5)	Griffith et al. (2009)
Norovirus RT-PCR	Primers described	Human	Blind samples	0.00 (5), 0.00 (2)	1.00 (5)	Griffith et al. (2009)

Chapter 2: Microbial Source-Tracking Markers Display a Relationship to Land Use but not Rainfall

1. Introduction

Nonpoint source (NPS) pollution is the leading contributor to water quality problems in the United States. Unlike point source (PS) pollution, for which the composition and rates of input are generally known, NPS pollution comes from many diffuse sources, including agricultural, commercial, residential, and wildlife (Schwab, 2007). As a result, NPS pollution frequently contains a complex mixture of animal and/or human fecal contamination. The need to identify the source of fecal pollution is becoming a priority for states and territories in the U.S. in order to meet water quality standards and to develop and implement total maximum daily loads (TMDLs). A TMDL determines the maximum amount of a pollutant that an impaired waterbody can receive to still meet its water quality objectives, and allocates this amount to waste loads from PS and NPS, natural background, and a margin of safety (USEPA, 2001c).

The ability to identify fecal pollution sources is also increasingly useful in compliance with microbial water quality standards for recreational waters, where fecal pollution is currently monitored using fecal indicator bacteria (FIB). The number of beach closures and advisories in the U.S. increased to 24,091 in 2010, the second highest number since the National Resources Defense Council began recording these events 21 years ago (Dorfman and Rosselot, 2011). These closures and advisories have a major economic impact on coastal communities that depend on recreational tourism for revenue (Rabinovici, et al., 2004). Conversely, the public health costs associated with exposure to

fecal pathogens likely has economic impacts as well. While public advisories and closures associated with recreational waters are implemented regardless of the type of contamination, most postings are the result of unknown sources (Dorfman and Rosselot, 2011). Understanding the source of fecal contamination impacting recreational areas can be used to protect public health, minimize economic impacts, and determine effective remedial actions.

Traditional FIB, including fecal coliforms, enterococci, and *E. coli*, cannot indicate the source of the fecal contamination due to the presence of these bacteria in both animal and human sources. Consequently, alternative indicators have been proposed for identifying the sources of fecal pollution (Simpson, et al., 2002; USEPA, 2005). Identifying human-source contamination is thought to be especially important, as human fecal waste contains human-specific pathogens that typically pose a greater health risk (Schoen and Ashbolt, 2010).

Microbial source-tracking (MST) assays are in various stages of development, and several reviews have been published summarizing the status of source tracking and outlining the benefits and limitations of some of these assays (e.g., Hagedorn, et al., 2011; Scott, et al., 2002; Stoeckel and Harwood, 2007). Some of the most promising human-source-tracking methods in current use rely on quantitative PCR (qPCR), which can make results available within hours of sample collection. Methods for cultivation of FIB traditionally require 18-24 hours. The *nif*H qPCR assay (Johnston, et al., 2010; Ufnar, et al., 2006) for *Methanobrevibacter smithii* is a relatively new, promising assay for human-source fecal pollution that has not been tested in mixed-use watersheds. Assays for *Bacteroides* sp. are widely used for MST, as the bacteria are frequently

detected in feces and fecally contaminated waters. Among potential genetic targets for *Bacteroides* sp., the HuBac qPCR assay has the highest recorded sensitivity in individual fecal samples and wastewater (Kildare, et al., 2007; Seurinck, et al., 2005). Assays for enterovirus and norovirus are also utilized in MST studies (Noble, et al., 2003), as viruses are highly host specific and detection of source-specific viral targets can solidify the determination of the source of contamination.

The goal of this research was to utilize each of these MST assays in a field study examining the effects of land use and levels of impervious surfaces in order to gauge how increasing development is associated with levels of human fecal contamination in inland watersheds. Association of land use changes with human MST markers and enteric pathogens would suggest areas with greater health risks and could indicate effective remediation strategies to mitigate NPS pollution (e.g., expanding sewer lines, targeting stormwater best management practices). The MST markers were also tested for correlations to antecedent rainfall and other abiotic factors, as well as to concentrations of traditional FIB to determine whether regulatory thresholds were protective against waters testing positive for human-source contamination.

2. Materials and methods

2.1 Study sites and sample collection

Water samples were collected from 15 sites on Morgan Creek, New Hope Creek and Northeast Creek tributaries within the B. Everett Jordan Lake catchment, as described in Rowny and Stewart (2012). Jordan Lake is the largest reservoir within the Cape Fear River Basin in North Carolina, covering approximately 13,940 acres. Sites

were selected to represent a range of land use, impervious surface cover, and watershed area (Figure 1).

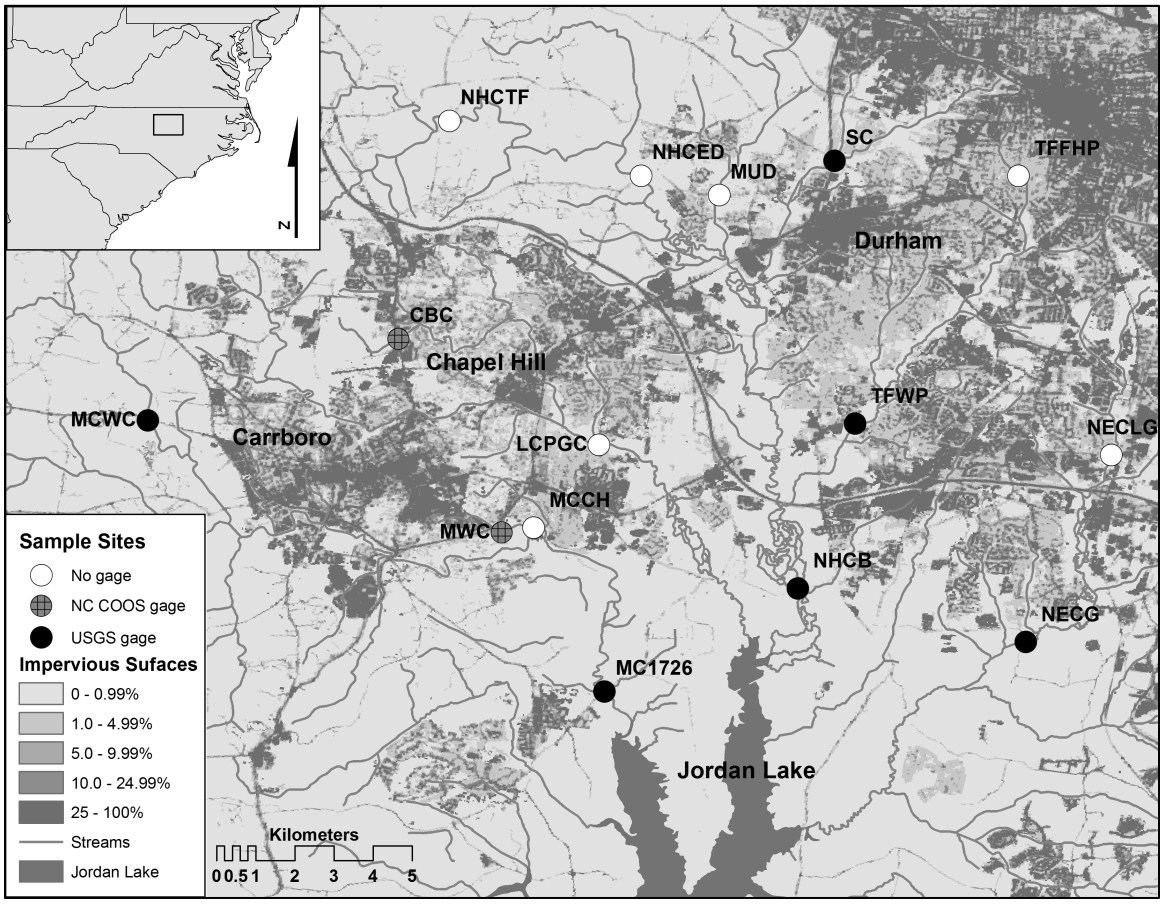


Figure 2.1. Study area showing sampling points along tributaries of Jordan Lake, N.C. (USA). Sample sites are labeled as follows: 1) Morgan Creek at NC-54 West (White Cross); 2) Meeting of the Waters Creek at Laurel Hill Rd.; 3) Morgan Creek near Chapel Hill; 4) Little Creek at Pinehurst Golf Course; 5) Morgan Creek at NC-1726; 6) New Hope Creek at NC-1107 (Blands); 7) Northeast Creek at SR1100 (Genlee); 8) Northeast Creek at SR1182 (Lowes Grove); 9) Third Fork NHC at Woodcroft Parkway; 10) Third Fork Creek NHC at Forest Hills Park; 11) Sandy Creek At Cornwallis Rd; 12) Mud Creek at NC 751; 13) New Hope Creek at Erwin Road; 14) New Hope Creek at Turkey Farm Road; 15) Crow Branch Creek at MLK.

Land use characteristics and level of impervious surfaces within each site were determined as described in Rowny and Stewart (2012) using the 2005 Multi-Resolution Land Characterization Consortium National Land Cover Data. The percent of land covered by impervious surfaces (%IS) was used to bin sites into three levels of

development intensity: low, medium and high. The low intensity class included the five sites with the lowest % IS (0.5 to 4.0%); the intermediate intensity class included the five sites with intermediate % IS (4.1 to 14.4 %); and the high intensity class included the five sites with the largest % IS (15.0 to 34.0%).

Samples were collected from each site once a month at approximately the same time of day during background dry weather (DW) for one year between April 2010 and March 2011. Sampling was delayed if precipitation in excess of 2.5 cm occurred in the 72-hr period proceeding planned collection. Samples were also collected once during three rain events occurring in September 2010, November 2010, and January 2011. Rain events were defined as at least three days without appreciable rainfall followed by a rainfall event that was anticipated to increase stream flow at least four times (4X) over pre-storm rates. The rain event sample was collected between 2-4 hours after the onset of precipitation, when the soil became saturated and water began to run off the surface. This was designed to capture the "first flush" of runoff. Hourly precipitation data was obtained from the National Oceanic and Atmospheric Administration U.S. Climate Reference Network (USCRN, 2011) Durham station. Antecedent precipitation was calculated for each sampling time-point by independently summing the preceding 2, 24, 48 and 168 (1 week) hourly observations. Stream flow for 6 of the 15 sites was collected by USGS gauges that collect flow rate data four times an hour. Stream flow rate was determined by matching sample collection time with the most proximate USGS gauge observation time. Two sampling sites were located at NC Coastal Ocean Observing System (NC COOS) gauges that collect stream flow rate data four times an hour. However, the gauge at Crow Branch Creek was prone to malfunction, and these stream flow data were not included in

the analysis. Stream flow rates at the remaining NC COOS site (Meeting of the Waters Creek) were determined in the same manner as at the USGS sites.

During dry weather sampling events, two 1-L grab samples were collected in sterilized polypropylene bottles from each of the sites. Samples were collected in an upstream fashion to minimize the collection of disturbed sediments. At the time of sampling, a YSI Professional Pro was used to record water temperature, dissolved oxygen, conductivity, total dissolved solids, and pH. Water samples were immediately placed on ice and transported to the laboratory for processing within 6 hrs of collection. A bench-top Hach 2100N nephelometer was used to determine turbidity once the samples had been returned to the lab.

During rain events, water samples were collected in the same manner as during the dry-weather events, with the exception that physical parameters were determined at the laboratory rather than in the field. An additional 500 ml water sample was collected in a sterile polypropylene bottle for recording the water temperature on-site using a digital thermometer. The sample was sealed, placed on ice, and transported to the laboratory to be tested with a YSI Professional Plus to determine the other physical parameters.

In total, 204 samples were collected during dry weather and rain events, including 159 dry weather samples and 45 rain event samples. Samples could not be collected for 21 dry weather samples because the tributaries were either dry or frozen at the time of collection.

2.2 Detection of MST Species

Two-hundred ml of water from each site was filtered onto 47-mm diameter, 0.45-μm pore-size polycarbonate filters (Millipore, Bedford, MA) using a four-place filtration

manifold and vacuum pump assembly with filter funnels (Millipore, Bedford, MA), as suggested by Haugland et al. (2005). Filters were transferred into sterile 2-ml screw-cap tubes and stored at -80°C until DNA extraction. Bacterial DNA was extracted from filters within 2 weeks of concentration using the MoBio Powersoil kit (MoBio, Carlsbad, CA) to elute a final concentrated DNA sample in 100-µl nuclease-free sterile water. The DNA samples were stored at -80°C until further processing.

TaqMan primers and probes were used to assay for *M. smithii* (Johnston, et al., 2010) and *Bacteroides* sp. (Layton, et al., 2006). The PCR reaction mixture for both primer sets contained 2 µl of sample, each primer at a concentration of 500 nM, the probe at a concentration of 120 nM, 12.5 µl of 2X RT-PCR buffer, and nuclease-free water for a total reaction mixture of 25 µL (Quantitect Probe PCR kit). The reaction mixture was assayed on a Cepheid SmartCycler (Sunnyvale, CA) using the following conditions: (i) 2 min at 50°C, (ii) 15 min at 95°C, (iii) 45 cycles of 1 s at 95°C and 1 min at 50°C. All amplification reactions were carried out in duplicate. Standard curves of each microorganism were used for quantification and results were reported as genome copies per 100 ml. The qPCR limit of detection for the *M. smithii* assay (*nif*H) was <10 genome copies per reaction (<167 copies per 100 ml) using purified genomic DNA (courtesy of C. Johnston); and the limit of detection for the *Bacteroides* sp. assay (HuBac) was <18 genome copies per reaction (<300 copies per 100 ml) using purified genomic DNA (ATCC, Manassas, VA). Samples that yielded a "non-detect" qPCR result were assigned a concentration of <167 genome copies per 100 ml for *nif*H and a concentration of <300 genome copies per 100 ml for HuBac. For statistical analyses, the qualified values of <167 and <300 were converted to 16 and 30, respectively.

2.3 Inhibition Control

A specimen processing control (SPC) was utilized to measure the amount of matrix inhibition by adding a known amount of DNA to each bacterial concentrate and a blank at the end of the extraction step. Salmon Sperm Testes DNA (SKETA) (Sigma, St. Louis, MO) was added at a final concentration of 20 ng per 100 µl extracted DNA. TaqMan primer and probe (Applied Biosystems, Inc., Foster City, CA) sequences for the SPC were as described by Haugland et al. (2005). The primers and probe target a segment of the ribosomal RNA gene operon, internal transcribed spacer region 2 of chum salmon, *Oncorhynchus keta*. The primers and probe were synthesized by MWG Biotech (High Point, NC). All qPCR sample reactions were considered to be inhibited with a Ct value 1.8 cycles or higher than that of the blank and were recalibrated using the difference between the average Ct in the control samples and in the environmental samples. Specifically, a ΔCt value was calculated by subtracting the mean SKETA Ct value for the uninhibited control reactions from the sample SKETA Ct. The ΔCt value was applied in the equation $(E + 1)^{(\Delta C_T)}$, where E is the amplification efficiency calculated from the control standard curve using the equation: E = [10^(-1/slope)] - 1. This calculation provides an estimate for each qPCR of the constant by which the corresponding sample estimate must be multiplied in order to reflect the actual number of DNA copies that would have been obtained if no inhibition had occurred. This approach is similar to that described by Pfaffl et al. (2001).

Samples in which SKETA failed to amplify or amplified later than 3.3 cycles (equal to a 1-log decrease in the qPCR amplification relative to the control) greater than the average Ct of the blank control were considered too inhibited for reliable

quantification of DNA concentration. These samples were diluted 1:10 with molecular-grade DNase-free water and reanalyzed. Diluted samples with a SKETA Ct delay of 3.3 cycles more than the control and that still showed no sample DNA amplification were scored as having a DNA concentration below the limit of detection (i.e., negative). Diluted samples with a SKETA delay of 3.3 cycles and for which a sample DNA concentration could then be estimated were corrected as described above using the SKETA Ct method with the inclusion of the appropriate dilution factor.

Twenty-four samples (12%) were inhibited past a threshold of 3.3 cycles for the SPC. Fourteen of these were dry weather samples (9% of all dry weather samples) and 10 of these were rain event samples (22% of all rain event samples). Half of the 24 inhibited samples were collected in November 2010 (dry weather and rain event samples). Each inhibited sample was diluted and re-tested, with one additional sample proving to be positive for the HuBac marker and no additional samples proving to be positive for the *nif*H marker.

2.4 Viral Analyses

Somatic and F+ coliphage were enumerated using the Single Agar Layer (SAL) method (USEPA, 2001b). Samples in which no coliphages were detected were assigned a concentration of <2 PFU per 100 ml. For statistical analyses, the qualified value of <2 was converted to 1.

The human enteric viruses, enterovirus (EV), norovirus genogroup I (NoV GI), and norovirus genogroup II (NoV GII), were enumerated using qPCR. The viruses were concentrated using the adsorption-elution method described by Katayama et al. (2002) and modified by Fong et al. (2005b). Briefly, 500 ml of water was adjusted to pH ~4.0

using a 1 N solution of acetic acid. This was passed through a 47-mm diameter, 0.45-μm pore-size HA (mixed cellulose ester) membrane filter (Millipore, Billerica, MA) using a sterile glass filter housing. The filter was rinsed with 100 ml 0.5 M sulfuric acid (pH 3.0). Viruses were eluted from the membrane with 10 ml 1-mM sodium hydroxide (pH 10.5-10.8). The eluent was added to 0.1 ml of 50 mM sulfuric acid (pH 3.0) and 0.1 ml of 100X Tris EDTA (pH 8.0) in a sterile 15 ml polypropylene tube. The eluent was further purified and concentrated using Centriprep YM-50 concentrator columns (Millipore, Billerica, MA) to a final volume of 2 ml. Concentrates were stored at -80°C. RNA was extracted from 200 μl of viral concentrate within one week of concentration using the Qiagen RNeasy Mini kit to elute a final concentrated RNA sample in 50-μl nuclease-free sterile water (Qiagen, Valencia, CA).

TaqMan primers and probes were used to assay for NoV GI, NoV GII (Jothikumar, et al., 2005), and EV (Donaldson, et al., 2002). The RT-PCR reaction mixture for all primer sets contained 2 μl of sample, each primer at a concentration of 500 nM, each probe mixture at a concentration of 120 nM, 12.5 μl of 2X RT-PCR buffer, 0.3 μl of 25X RT-PCR enzyme mix, and nuclease-free water for a total reaction mixture of 25 μL (Quantitect Probe RT-PCR kit). The reaction mixture was subjected to a one-step assay on a Cepheid SmartCycler (Sunnyvale, CA) using the following conditions: (i) RT for 30 min at 50°C, (ii) 15 min at 95°C, (iii) 45 cycles of 15 s at 94°C and 1 min at 60°C. All amplification reactions were carried out in duplicate. Samples that gave a positive result in either or both of the duplicate reactions were amplified by RT-PCR again. Only after a sample gave a second positive result was it counted as an overall positive. Standard curves (of NoV GI, NoV GII, or EV) were used for quantification and

results were reported as genome copies per 100 ml. The qRT-PCR limit of detection for EV was 300 genome copies per reaction (1.5×10^3 genome copies per 100 ml) using EV genomic RNA; for NoV GI was 200 genome copies per reaction (1.0×10^3 genome copies per 100 ml) using a NoV GI.4 RNA transcript (courtesy J. Vinjé, CDC); and for NoV GII was 10 genome copies per reaction (3.0×10^0 genome copies per 100 ml) using a NoV GII.1 RNA transcript (courtesy J. Vinjé, CDC).

2.5 Statistical Analysis

All statistical analyses were performed using SAS statistical software (Cary, NC). Microbial concentrations were log10 transformed prior to statistical analyses. Spearman rank analyses were used to assess the relationship between alternative indicator concentrations (HuBac, *nif*H, somatic and F+ coliphages) and antecedent rainfall, land use, level of impervious surface, log10 transformed concentrations of fecal indicator bacteria, and physical parameters. Dry weather and rain event microbial data were compared using a Mann-Whitney U test. All tests were considered significant at $p < 0.05$.

3. Results

3.1 MST marker detection

Study results demonstrate environmental detection of tested MST markers in a mixed-use watershed. The *Bacteroides* sp. marker (HuBac) was detected in 98% (199 of 204) of samples analyzed, including 97% (155 of 159) of dry weather samples and 98% (44 of 45) of rain event samples (Table 2.1). This suggests the presence of the HuBac marker at all sites examined, regardless of level of development, land use type, season, physical parameters, or antecedent rainfall.

The *M. smithii* marker (*nif*H) was detected in 30% (59 of 196) of samples analyzed (8 samples were not analyzed due to sample degradation): 30% (46 of 151) of dry weather samples and 29% (13 of 45) of rain event samples (Table 2.1). The *nif*H marker was never detected at sites 10, 13, and 15, suggesting the absence of *M. smithii* at these sites. Sites 13 and 15 are largely forested (>75%) and have low levels of impervious surfaces (<4%). Conversely, site 10 is largely developed (99.3%) and has a high level of impervious surfaces (17.1%). The *nif*H marker was detected at least once at the other 12 sites and was detected most frequently (>70%) at sites 2, 6, and 7. Site 2 is a highly developed site (85.7%) with a high percentage of impervious surfaces (34%). Sites 6 and 7 have an intermediate level of development, with 40.4% and 57.1% of land developed and 8.8% and 14.4% covered with impervious surfaces, respectively.

3.2 MST marker concentration

The concentrations of the two markers detected at each site were correlated to land use and level of impervious surface using the Spearman rank order method. Both markers were positively correlated to the percent of land developed and the percent of impervious surface. Both markers were negatively correlated to the percent of land used for agriculture and forest (Table 2.2).

Using a Mann-Whitney U test, the concentrations of neither MST marker was significantly different between dry weather and rain events. Additionally, neither marker concentration was significantly correlated to the level of rainfall 2, 24, 48, or 168 hours preceding the sampling event. Geometric mean concentrations for HuBac at each of the 15 sites ranged between $4.3x10^3$ and $9.6x10^4$ genome copies per 100 ml during dry weather and between $6.7x10^3$ and $7.8x10^5$ genome copies per 100 ml during rain events

(Table 2.3). Geometric mean concentrations for *nif*H at each of the 15 sites ranged between $<1.7x10^0$ and $3.8x10^2$ genome copies per 100 ml during dry weather and between $<1.7x10^0$ and $1.3x10^3$ genome copies per 100 ml during rain events (Table 2.3).

3.3 Comparison to fecal indicator bacteria

The concentrations of both markers were compared to concentrations of fecal coliforms and *E. coli* as detected using standard membrane filtration (Rowny and Stewart, 2012). The HuBac marker was correlated to both fecal coliforms ($r = 0.233$, $p < 0.001$) and *E. coli* ($r = 0.266$, $p < 0.001$). The *nif*H marker was not correlated to either fecal indicator bacteria (FIB). The number of samples in which the *nif*H marker was detected was then compared to the number times in which fecal coliforms and *E. coli* concentrations exceeded recommended regulatory thresholds for recreational waters (200 and 126 CFU per 100 ml, respectively) (USEPA, 1976; 1986) (Table 2.4). In the 59 samples positive for the *nif*H marker, *E. coli* levels exceeded 126 CFU per 100 ml in only 27 samples (46%) and fecal coliform levels exceeded 200 CFU per 100 ml in only 26 samples (44%).

3.4 Virus detection

Somatic coliphages were detected in 79% (161 of 204) of samples analyzed, including 84% (134 of 159) of dry weather samples and 60% (27 of 45) of rain event samples. Geometric mean concentrations for somatic coliphages at each of the 15 sites ranged between $1.7x10^1$ and $7.6x10^1$ PFU per 100 ml. F+ coliphages were detected in 50% (102 of 204) of samples analyzed, including 53% (85 of 159) of dry weather samples and 38% (17 of 45) of rain event samples. Geometric mean concentrations at each of the 15 sites ranged between $8.4x10^0$ and $6.0x10^1$ PFU per 100 ml.

Coliphages did not show a seasonal trend. However, somatic and F+ coliphage concentrations were positively correlated with temperature ($p < 0.001$) and precipitation 24 h ($p < 0.001$), 48 h ($p < 0.001$), and one week ($p < 0.001$) prior. Coliphages were also positively correlated with both fecal coliform concentrations ($p < 0.001$) and *E. coli* concentrations ($p < 0.001$). Additionally, coliphage concentrations were correlated with dissolved oxygen content ($p < 0.001$), conductivity ($p < 0.001$), pH ($P < 0.001$), turbidity ($p < 0.001$), and total dissolved solids ($p < 0.001$).

Enteric viruses were rarely detected in the 204 samples. Enterovirus and norovirus genogroup I were never detected. Norovirus genogroup II (NoV GII) was detected twice, both times at a concentration of 3.0 genome copies per 100 ml. NoV GII was detected once in a dry weather sample (at site 5 in October 2010) and once in a rain event sample (at site 7 in November 2010).

4. Discussion

Utilizing 15 sites representing various land uses within the Jordan Lake watershed in North Carolina, this study utilized two MST markers, *nif*H and HuBac, and two enteric virus assays, EV and NoV, to establish an association between increasing urbanization and water quality in the study area.

Associations were found between land use and the concentrations of both *M. smithii* (*nif*H) and *Bacteroides* sp. (HuBac). The concentrations of the HuBac and *nif*H markers were both associated with increasing development and impervious surfaces. The higher concentrations of human-source fecal indicator microorganisms in more urbanized watersheds suggest that land use changes associated with development, as well as

increases in watershed impervious cover, affect water quality. These results are consistent with a study examining fecal indicator bacteria (FIB) in this watershed (Rowny and Stewart, 2012) as well as a study examining coliphage concentrations along tidal creeks (DiDonato, et al., 2009). The tributaries examined in this study are representative of central North Carolina, where relatively rapid urbanization has resulted in heavy stormwater inputs of fecal contamination to receiving waters and resultant water quality impairments (Characklis and Krometis, 2009). The MST results indicate that not only does increasing anthropogenic land use result in impaired water quality, but it may also result in an increasing human health risk. Water quality in these tributaries is of particular concern as they feed into B. Everett Jordan Lake, a popular recreation area and a drinking water source for parts of the Research Triangle area of North Carolina.

The MST markers *nif*H and HuBac were also evaluated for their relationship to precipitation levels, and an increase in the concentrations of both markers was observed during rain events. This was expected, as stormwater routinely contains high levels of fecal indicator bacteria and is a major contributor to water quality degradation in urban beaches, lakes, and rivers (Marsalek and Rochfort, 2004). Nevertheless, this increase was not statistically significant, and there was no correlation between antecedent rainfall and either marker. This is in contrast to the fecal indicators *E. coli* and enterococci, which displayed strong, positive correlations to antecedent rainfall levels in the same samples (Rowny and Stewart, 2012). Although these results may be a product of the sampling scheme, which utilized only one grab sample collected during the "first flush" of rain events, these results are consistent with a previous study examining *Bacteroides* levels in relation to amount of rainfall (Sauer, et al., 2011). Previous research has demonstrated

environmental reservoirs for FIB (i.e., sediments) that can be mobilized during rainfall (Krometis, et al., 2007). Lack of correlation of either MST marker to rainfall suggests that the anaerobic MST markers utilized in this study are more specific for recent, land-based contamination events as opposed to resuspension in the water column. Additionally, a lack of correlation to rainfall further supports that site-specific characteristics are important for the detection of MST markers and characterization of NPS pollution in a watershed.

Both somatic and F+ coliphages were correlated to the amount of antecedent rainfall 24, 48, and 168 (1 week) hours prior to sampling. This is consistent with two previous reports that found coliphage to be seasonal, with correlations to rainfall and temperature (Jiang, et al., 2007; Reyes and Jiang, 2010). An increase in coliphages during rain events may signal an increased public health risk, as correlations between coliphages and human illness have been identified in previous studies (Colford, et al., 2007; Wiedenmann, et al., 2006). One of these studies (Wiedenmann, et al., 2006), determined a no observable adverse effect level (NOAEL) of 10 somatic coliphages for freshwater. Our study found somatic coliphage above this level in 53% (107) of samples.

Studies have also found correlations between coliphages and viral pathogens (Aw and Gin, 2010; Mocé-Llivina, et al., 2005; Wu, et al., 2011). In our study, neither coliphage was correlated with viral pathogens due to the low number of samples positive for enteric viruses. Enterovirus and norovirus genogroup I (GI) were never detected in our samples. This is surprising, given that human enteroviruses have been found in waters associated with human fecal contamination (Griffin, et al., 1999; Jiang, et al., 2001; Noble and Fuhrman, 2001), and noroviruses have been detected in treated

wastewaters and surface waters (Astrom, et al., 2009; Lodder and de Roda Husman, 2005). It is possible that the assays used in this study were not sensitive enough to detect viruses present in our samples. The qPCR assay for EVs used herein (Donaldson, et al., 2002) was found to have a detection limit of 9.3 viral particles per ml in seawater, but had a detection limit of 300 genome copies per reaction in our laboratory. The NoV GI assay has been reported to have a detection limit of 10 viral genomes per reaction (Jothikumar, et al., 2005), but the detection limit for this study was 200 genome copies per reaction. Due to the high detection limits for these two assays, it is possible that EV and NoV GI were present in our samples, but were not detected. More work is needed to improve methods for concentration of enteric viruses from complex, environmental samples and to increase the sensitivity with which pathogens can be detected from water.

Both samples positive for norovirus GII were detected at 3.0 copies per 100 ml (30 copies per L) and were detected at sites immediately downstream from wastewater treatment plants (sites 5 and 7). The concentrations detected are approximately equivalent to the estimated values for chlorinated secondary effluent, based on reported raw sewage norovirus densities of $10^3 – 10^6$ genome copies per L (Haramoto, et al., 2006; Katayama, et al., 2008) and an estimated removal of 2.2 - 3.0 logs (Haramoto, et al., 2006). Our results indicate a risk for norovirus illness at these two tributaries using a report by Schoen and Ashbolt (2010), which estimated that approximately 9 norovirus genomes per L in a waterbody would yield a risk of 0.03 GI illnesses per swim event. Further, as these tributaries feed into B. Everett Jordan Lake, a waterbody used as a drinking water source, inadequate or failing treatment processes could lead to insufficient removal of the viral pathogens from source waters.

There was a strong disagreement between the two MST markers regarding the percent of samples containing human-source pollution: *Bacteroides* sp. (HuBac) was detected in 98% of samples while *M. smithii* (*nif*H) was detected in only 30% of samples. Because the HuBac marker was so ubiquitous in our samples, despite the sites in our study representing various land use types and intensities and, likely, sources of contamination, it is likely that this marker was amplifying non-human targets. This conclusion is supported by recent studies, which found this assay to cross-react with animal fecal samples, including cattle, swine, sheep, horse, dog, duck, and kangaroo (Ahmed, et al., 2009; Layton, et al., 2006). Conversely, previous studies have found the *nif*H marker (using qPCR) to be 86% specific to human feces (Griffith, et al., 2009).

Previous studies have proposed using a tiered approach in monitoring schemes (Boehm, et al., 2009; Stewart, et al., 2008). For one tiered approach, samples are tested with a variety of methods, beginning with culture-based methods for traditional FIB (e.g., *E. coli* and enterococcus). Samples exceeding water quality standards or with high levels of FIB are subjected to further analyses including PCR, qPCR, or virus analysis. In this study, the HuBac marker was detected in 98% of samples at concentrations that correlated with both *E. coli* and fecal coliforms. These results suggest that the HuBac marker does not provide sufficiently different or additional information than traditional FIB to be included in a tiered approach.

However, the *nif*H marker was not correlated with either FIB in this study. Less than 50% of the samples positive for human source pollution (using the *M. smithii nif*H marker) would have been indicated by high levels of fecal coliforms or *E. coli*. In fact, 76 samples had *E. coli* levels above regulatory thresholds with no *M. smithii* present (Table

2.4). This suggests that other sources (besides human inputs) are contributing to the presence of *E. coli*, which is consistent with previous reports that found natural contributions of FIB in waters with minimal or no known human influence were sufficient to exceed water quality thresholds (Griffith, et al., 2010). In our study, if FIBs were used as a metric for identifying and prioritizing tributaries in this watershed, over half of the tributaries with evidence of human sewage contamination would not have been given a high priority. These findings demonstrate the extent to which *E. coli* levels may not represent sewage contamination in the environment. Thus, environmental monitoring in areas with multiple sources of pollution may require qPCR for MST markers as part of a first step in a tiered assessment. In our study, we found that qPCR for *M. smithii* using the *nif*H marker may be a good candidate for a human-source contamination indicator in environmental monitoring schemes.

The *nif*H marker was consistently detected at sites 2, 6, and 7. Sites 6 and 7 both have an intermediate level of development but are located directly downstream from a water reclamation facility and a wastewater treatment plant, respectively. Site 2 is a highly developed site receiving much of the UNC-Chapel Hill stormwater runoff, which may be contaminated by sewage from leaking sewer drains as the University works to test and update its infrastructure. There is growing evidence that stormwater systems can be contaminated with (human) sewage from aging, failing infrastructures and illicit cross-connections between the stormwater and sewage systems (Gaffield, et al., 2003; Rajal, et al., 2007; Salmore, et al., 2006). This study suggests that the *nif*H qPCR assay can be used to detect human sewage inputs and can indicate areas where appropriate remediation strategies could be implemented.

Conclusions

This study evaluated several MST assays in a field study against land use and levels of impervious surfaces in order to gauge how increasing development is associated with levels of human fecal contamination in inland watersheds. The concentrations of two MST markers, HuBac and *nif*H, were positively associated with increasing development and impervious surfaces. Higher concentrations of the MST markers in more urbanized watersheds suggest that land use changes associated with development, as well as increases in watershed impervious cover, affect water quality and may also result in an increasing human health risk. These results suggest that environmental monitoring schemes utilizing the *nif*H qPCR assay can detect human sewage inputs and could indicate areas for targeting appropriate remediation strategies. However, these monitoring programs should utilize qPCR for MST markers such as *nif*H as a first step in a tiered assessment, as less than 50% of the samples positive for human source pollution using the *nif*H assay would have been indicated by high levels of fecal coliforms or *E. coli*. In contrast to these FIB, the MST markers evaluated were not correlated to antecedent rainfall levels, possibly indicating that the anaerobic MST markers utilized in this study are more specific for recent, land-based contamination events as opposed to resuspension of particle-associated organisms in waterways. Enteric viruses evaluated in this study were rarely detected, indicating a need to improve methods for concentration of viruses from complex, environmental samples and to increase the sensitivity with which pathogens can be detected from water. Nevertheless, norovirus genogroup II was detected twice during the study, and its presence, together with the MST markers, suggest areas in

our study with potentially greater health risks. These markers also indicate areas where resources and remediation strategies might best be directed.

Table 2.1. Percent of samples positive for *Bacteroides* sp. (HuBac) or *M. smithii* (*nif*H) at each site.

		Site														
		Low Level of Development					Intermediate Level of Development					High Level of Development				
Marker	Sample Type	1	3	13	14	15	4	5	6	7	12	2	8	9	10	11
HuBac	Dry Weather	100 (11/11)	100 (12/12)	91 (10/11)	89 (8/9)	100 (6/6)	100 (11/11)	91 (10/11)	92 (11/12)	100 (12/12)	100 (11/11)	100 (12/12)	100 (10/10)	100 (11/11)	89 (8/9)	100 (12/12)
	Rain Event	100 (3/3)	100 (3/3)	100 (3/3)	100 (3/3)	100 (3/3)	100 (3/3)	100 (3/3)	100 (3/3)	100 (3/3)	100 (3/3)	100 (3/3)	100 (3/3)	100 (3/3)	100 (3/3)	100 (3/3)
*nif*H	Dry Weather	9 (1/11)	0 (0/12)	0 (0/10)	13 (1/8)	0 (0/5)	27 (3/11)	36 (4/11)	83 (10/12)	75 (9/12)	10 (1/10)	75 (9/12)	22 (2/9)	20 (2/10)	0 (0/8)	36 (4/11)
	Rain Event	33 (1/3)	67 (2/3)	0 (0/3)	33 (1/3)	0 (0/3)	33 (1/3)	67 (2/3)	33 (1/3)	100 (3/3)	33 (1/3)	67 (2/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)

Table 2.2. Significant correlations between MST markers and land use using the nonparametric Spearman Rank method. Statistically significant correlations were characterized by P-values < 0.05.

Land Use	HuBac	*nif*H
% Developed	0.209**	0.19818**
% Agriculture	-0.20046**	-0.1592*
% Forest	-0.21503**	-0.18217**
% Impervious surface	0.21921**	0.23498**

*p < 0.05
**p < 0.01

Table 2.3. Geometric mean concentrations of *Bacteroides sp*. (HuBac) and *M. smithii* (*nif*H) markers at each site during dry weather and rain sampling events.

		Sites with Low Level of Development				
Marker	Sample Type	1	3	13	14	15
HuBac	Dry Weather	$2.4x10^4$	$4.7x10^4$	$1.5x10^4$	$4.3x10^3$	$2.7x10^4$
	(Min, Max)	($4.2x10^2$, $2.0x10^6$)	($6.4x10^3$, $4.0x10^5$)	($3.0x10^2$,$1.0x10^6$)	($3.0x10^2$, $3.0x10^4$)	($2.1x10^3$,$4.0x10^5$)
	Rain Events	$2.3x10^5$	$1.9x10^5$	$4.9x10^4$	$9.5x10^3$	$1.7x10^4$
	(Min, Max)	($2.5x10^3$, $4.5x10^7$)	($4.5x10^4$, $1.3x10^6$)	($1.2x10^4$, $1.4x10^5$)	($8.2x10^2$, $1.2x10^6$)	($3.0x10^2$, $3.1x10^5$)
*nif*H	Dry Weather	$1.6x10^2$	$<1.7x10^2$	$<1.7x10^2$	$1.6x10^2$	$<1.7x10^2$
	(Min, Max)	($9.1x10^1$, $1.7x10^2$)	(NA)	(NA)	($1.3x10^2$, $1.7x10^2$)	(NA)
	Rain Events	$1.3x10^3$	$2.7x10^2$	$<1.7x10^2$	$2.6x10^2$	$<1.7x10^2$
	(Min, Max)	($1.7x10^2$, $7.7x10^4$)	($8.2x10^1$, $1.5x10^3$)	(NA)	($1.7x10^2$, $5.8x10^2$)	(NA)
		Sites with Intermediate Level of Development				
Marker	Sample Type	4	5	6	7	12
HuBac	Dry Weather	$4.7x10^4$	$6.1x10^4$	$2.0x10^4$	$9.6x10^4$	$8.1x10^4$
	(Min, Max)	($5.7x10^2$, $2.1x10^6$)	($3.0x10^2$, $7.6x10^5$)	($3.0x10^2$, $2.2x10^5$)	($1.2x10^4$, $3.1x10^6$)	($1.6x10^3$, $1.2x10^7$)
	Rain Events	$1.4x10^5$	$2.5x10^4$	$9.2x10^3$	$6.2x10^4$	$6.7x10^3$
	(Min, Max)	($8.2x10^4$, $2.9x10^5$)	($5.9x10^3$, $1.6x10^5$)	($7.5x10^2$, $4.5x10^4$)	($1.6x10^4$, $1.7x10^5$)	($6.0x10^2$, $7.7x10^4$)
*nif*H	Dry Weather	$1.6x10^2$	$2.9x10^2$	$3.8x10^2$	$3.8x10^2$	$2.3x10^2$
	(Min, Max)	($5.8x10^1$, $2.2x10^2$)	($1.7x10^2$, $1.5x10^3$)	($1.7x10^2$, $2.0x10^3$)	($1.3x10^2$, $4.0x10^3$)	($1.7x10^2$, $3.1x10^3$)
	Rain Events	$2.1x10^2$	$3.4x10^2$	$5.0x10^2$	$2.2x10^2$	$<1.7x10^2$
	(Min, Max)	($1.7x10^2$, $3.0x10^2$)	($5.4x10^1$, $4.1x10^3$)	($1.7x10^2$, $4.4x10^3$)	($1.0x10^2$, $4.7x10^2$)	(NA)
		Sites with High Level of Development				
Marker	Sample Type	2	8	9	10	11
HuBac	Dry Weather	$8.5x10^4$	$6.7x10^4$	$6.4x10^4$	$1.7x10^4$	$6.0x10^4$
	(Min, Max)	($1.0x10^4$, $9.3x10^5$)	($2.0x10^3$, $8.5x10^5$)	($6.6x10^3$, $4.7x10^5$)	($3.0x10^2$, $1.4x10^5$)	($9.5x10^3$,$8.4x10^5$)
	Rain Events	$3.7x10^5$	$7.8x10^5$	$1.4x10^5$	$8.4x10^4$	$3.4x10^4$
	(Min, Max)	($1.9x10^5$, $1.2x10^6$)	($4.1x10^4$, $8.7x10^6$)	($8.1x10^3$, $1.9x10^6$)	($3.9x10^3$, $1.1x10^6$)	($2.4x10^4$, $5.6x10^4$)
*nif*H	Dry Weather	$2.9x10^2$	$2.3x10^2$	$1.6x10^2$	$<1.7x10^2$	$2.0x10^2$
	(Min, Max)	($5.4x10^1$, $2.9x10^4$)	($1.5x10^2$, $2.6x10^3$)	($3.9x10^1$, $4.4x10^2$)	(NA)	($4.5x10^1$, $3.4x10^3$)
	Rain Events	$5.0x10^2$	$<1.7x10^2$	$<1.7x10^2$	$<1.7x10^2$	$<1.7x10^2$
	(Min, Max)	($1.7x10^2$, $2.7x10^3$)	(NA)	(NA)	(NA)	(NA)

NA = not applicable

Table 2.4. Agreement between regulatory thresholds for the traditional fecal indicator bacteria (FIB), *E. coli* and fecal coliforms, and presence of the *M. smithii nif*H sewage markers.

Traditional FIB	Percent Correlation (n)	
(CFU/100ml)	*nif*H detected	*nif*H not detected
E. coli >126	13 (27)	38 (76)
E. coli <126	16 (32)	33 (67)
Fecal coliforms >200	13 (26)	32 (66)
Fecal coliforms <200	16 (33)	39 (79)

Chapter 3: Presumptive Antibiotic-Resistant *Staphylococcus* and Hepatitis E Virus in Waters Proximal to Swine Concentrated Animal Feeding Operations

1. Introduction

Agriculture in the United States has shifted from numerous small operations to fewer, larger industrial operations. The number of Concentrated Animal Feeding Operations (CAFOs) in the U.S. has increased, from 3,600 in 1982 to almost 12,000 in 2002 (USGAO, 2008b). In North Carolina, farms with 5,000 or more hogs account for 75% of all swine operations (USDA, 2007). Consequently, the number of swine in North Carolina has also increased dramatically, from 3.7 million in 1991 to over 10 million in 1998, making North Carolina the second leading state in national pork production (Edwards and Ladd, 2000). In fact, five adjacent counties in eastern North Carolina (Bladen, Duplin, Greene, Sampson, and Wayne) were estimated to have a population of over 7.5 million swine in 2002 (USGAO, 2008b). This number of swine can produce up to 15.5 million tons of manure annually (USGAO, 2008b). Swine manure in North Carolina is typically collected and stored in open-pit lagoons before the liquid waste is sprayed onto agricultural fields for disposal. As a result of runoff and percolation events, components of manure, including zoonotic and human pathogens, may impact surface water quality proximal to swine CAFOs (Anderson and Sobsey, 2006; Campagnolo, et al., 2002; Jongbloed and Lenis, 1998; Krapac, et al., 2001; Sayah, et al., 2005; Thurston-Enriquez, et al., 2005). Pathogens potentially present include *Salmonella, Campylobacter, Yersenia*, *Listeria*, enteropathogenic *E. coli*, *Ascaris suum*,

Cryptosporidium, Giardia intestinalis, and viruses such as hepatitis E virus, enteric calicivirus, and rotavirus.

Specific swine production practices, including the dosing of swine with sub-therapeutic levels of antimicrobials for growth promotion, can promote known and emerging bacterial pathogens. An estimated 10.3 million pounds of antimicrobials are used annually in US swine production for growth promotion and to improve feed efficiency (Mellon, et al., 2001). These antimicrobials include some of the same drugs used in human clinical medicine, including tetracycline, erythromycin, lincomycin, virginiamycin, and ampicillin (USFDA, 2004). The use of antimicrobials in swine feed and water selects for antibiotic-resistance among commensal and pathogenic bacteria in swine (Aarestrup, et al., 2000; Bager, et al., 1997; Wegener, 2003) and may result in high levels of drug-resistant bacteria and resistance genes in swine waste (Chee-Sanford, et al., 2001; Haack and Andrews, 2000; Parveen, et al., 2006). Previous studies have identified antibacterial-resistant enterococci (Haack and Andrews, 2000; Sapkota, et al., 2007) and *E. coli* (Anderson and Sobsey, 2006; Parveen, et al., 2006; Sayah, et al., 2005) in waters impacted by swine waste. However, to our knowledge there are no data in the published literature regarding the presence of antibiotic-resistant *Staphylococcus* in surface waters proximal to swine CAFO spray fields.

The rapid growth of the hog CAFO industry has raised concern about proper waste disposal, as chronicled in reports from the National Pew Commission on Industrial Food Animal Production (2008) and the US Government Accountability Office (2008b). The goal of this study was to determine whether two microorganisms of public health concern, antibiotic-resistant *Staphylococcus* and hepatitis E virus, and potential

indicators for human enteric viral pathogens, coliphages, were present in creeks adjacent to swine CAFO spray fields, and were correlated to traditional fecal indicator bacteria used to monitor recreational water quality.

2. Materials and Methods

2.1 Study sites and sample collection

The study area is in the coastal plain region of North Carolina, US, where there is a high density of swine CAFOS. To determine the impact of swine lagoon spray fields on adjacent water quality, we looked for sampling locations located in public access waters upstream and downstream of three spray fields (Sites 1-3). We also looked for sampling sites that were at headwater locations that would not be downstream from other sources of contamination; however, no suitable and accessible sites were identified. The diversity and density of livestock operations and homes in the study area meant that "upstream" sampling locations are potentially downstream from many other sources of fecal waste. Nevertheless, we use the letters A and B, respectively, to denote the sampling locations upstream and downstream of each site.

From mid-February to mid-August 2010, surface water samples were collected weekly from sampling sites and were processed for all targets: antibiotic-resistant *Staphylococcus*, hepatitis E virus (HEV), and coliphages. Samples were also collected monthly from mid-September 2010 to mid-January 2011 and were processed for antibiotic-resistant *Staphylococcus* in order to capture seasonal trends. Sterile 4-L polycarbonate bottles (Nalgene) were used for sample collection. Water samples were taken in the late morning or early afternoon and transported on ice to the laboratory.

Rainfall data was obtained through the State Climate Office of North Carolina from the Williamsdale field lab in Duplin County. Hourly increments of rainfall were combined to compare the amount of precipitation 24 and 48 hours before sampling to microbial concentrations.

2.2 Enumeration and isolation of antibiotic-resistant *Staphylococcus*

All surface water samples, except samples collected on February 15, 2010, were analyzed for antibiotic-resistant *Staphylococcus* following the experimental procedure documented by Goodwin and Pobuda (2009). Duplicate water samples were filtered using a 47-mm, 0.45 μm HA membrane filter (Millipore, Bedford, MA), placed onto CHROMagar™ MRSA (BD BBL™) plates, and incubated at 37°C overnight. Due to a high amount of growth on CHROMagar™ MRSA plates, samples were filtered in multiple dilutions. Colonies with the morphological characteristics of *Staphylococcus aureus* (i.e., mauve with a matte halo) were counted after 18-24 hours of incubation. Up to ten of these colonies were selected from each sample site and streaked onto CHROMagar™ Staph aureus (BD BBL™) plates for isolation. After incubation at 37°C for 18-24 hours, all colonies with a morphology characteristic of methicillin-resistant *Staph. aureus* (MRSA) (mauve with a matte halo) were inoculated in 0.75 ml Brain Heart Infusion broth with 15% glycerol, and stored at -80°C until further characterization. Samples in which no *Staphylococcus* isolates were detected were assigned a concentration value of <1 CFU per volume filtered. For statistical analyses, this concentration was converted to one-half the lower detection limit value (i.e., 0.5 CFU).

2.3 Detection of hepatitis E virus

Water samples collected from mid-February 2010 to mid-August 2010 were analyzed for HEV using reverse-transcription real-time PCR (RT-qPCR). Viruses were concentrated using the adsorption-elution method described by Katayama et al. (2002) and modified by Fong et al. (2005a). Briefly, 1 L of water was adjusted to pH ~4.0 using a 1 N solution of acetic acid. This sample was passed through a 47-mm, 0.45 μm pore size HA membrane filter (Millipore) using a sterile glass filter housing. The filter was rinsed with 100 ml 0.5 M sulfuric acid (pH 3.0). Viruses were eluted from the membrane with 10-ml 1 mM sodium hydroxide (pH 10.5-10.8). The eluent was added to 0.1 ml of 50 mM sulfuric acid (pH 3.0) and 0.1 ml of 100X Tris EDTA (pH 8.0) in a sterile 15 ml polypropylene tube. The eluent was further purified and concentrated using Centriprep YM-50 concentrator columns (Millipore) to a final volume of 2 ml. Concentrates were stored at -80°C.

RNA was extracted from 200 μl of viral concentrate using the QIAamp One-For-All Nucleic Acid kit (Qiagen, Valencia, CA), following the protocol for liquid transport media, to extract nucleic acids into 100-μl buffer AVE using a BioRobot Universal System (Qiagen). Nucleic acid samples were stored at -80°C overnight. RNA was reverse-transcribed using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA). The cDNA synthesis mixture contained 5 μl of nucleic acid, 1 mM of a specific reverse primer (JVHEVR), 10 mM of a dNTP mix, 2 μl of 10X RT buffer, 1 μl of the MultiScribe reverse transcriptase, and nuclease-free water for a total reaction mixture of 20 μl. The reaction mixture was subjected to reverse transcription on an Applied Biosystems 7900 (Life Technologies)

using the following conditions: (i) 10 min at 25°C, (ii) 120 min at 37°C, and (iii) 5 min at 85°C. The cDNA product was stored at -80°C.

TaqMan primers and probes were used to assay for HEV cDNA (Jothikumar, et al., 2006). The qPCR reaction mixture contained 2 µl of cDNA, 400 nM of each primer, 100 nM of probe, 10 µl of 2X Probe PCR Mix, 400 ng/µl of BSA, 150 ng/µl T4 gene 32 protein, and nuclease-free water for a total reaction mixture of 20 µL (Quantitect Probe PCR kit). The reaction mixture was subjected to qPCR on an Applied Biosystems 7900 using the following conditions: (i) 1 min at 60°C, (ii) 15 min at 95°C, (iii) 45 cycles of 15 s at 94°C and 1 min at 60°C. All amplification reactions were carried out in duplicate. Samples that gave a positive result in either or both of the duplicate reactions were amplified by qPCR again. Only after a sample gave a second positive result was it counted as an overall positive.

2.4 Coliphage detection and isolation

Water samples collected from mid-February 2010 to mid-August 2010 were analyzed for somatic and F+ coliphage using the Single Agar Layer (SAL) Method (USEPA, 2001b). Up to 8 F+ coliphage plaques from each sample were isolated for characterization in 2 ml TSB and stored at -80°C. Samples in which no coliphages were detected were assigned a concentration of <1 plaque forming unit (PFU) per 100 ml. For regression analyses, the qualified value of <1 was converted to 0.5 PFU per 100 ml.

For samples in which no F+ coliphages were detected, an enrichment technique was used to detect F+ coliphages following US EPA Method 1601 (USEPA, 2001a). One plaque from each dilution (300 ml, 30 ml, and 3 ml) was isolated for further characterization in 2 ml TSB and stored at -80°C.

To distinguish F-RNA and F-DNA coliphages, 5 µl of all F+ isolates were spotted on two plates, one containing *E. coli* Famp and a one containing *E. coli* Famp plus 10 mg/ml RNase A. Plaque formation on the *E. coli* Famp and the *E. coli* Famp plus RNase plates indicated an F-DNA coliphage. Plaque formation on the *E. coli* Famp plate but not the *E. coli* Famp plus RNase plate indicated an F-RNA coliphage. A positive control F-RNA strain (MS2) was spotted on all plates.

2.5 Typing of F-RNA coliphage isolates

Coliphage isolates that did not propagate on the RNase plates (positive for RNA genome) were genotyped using reverse-transcription PCR (RT-PCR). RNA was extracted from 200 µl of viral isolation or enrichment using the RNeasy Mini kit (Qiagen, Valencia, CA) to elute the RNA sample in 50-µl nuclease-free sterile water. The RNA was immediately subjected to RT-PCR according to Friedman et al. (2009a). The 50-µl reaction volume contained 5 µl of viral RNA, 200 µM forward and reverse primer, 0.5 µl RNase Inhibitor, 10 µl 5X buffer, 400 µM dNTPs, and 2 µl of RT enzyme (Qiagen One-Step RT-PCR kit). The RT-PCR reaction was performed using a MasterCycler gradient (Eppendorf, Hamburg, Germany). Thermal cycle conditions were as follows: 50°C for 30 min, 95°C for 15 min, followed by 45 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, with a final extension of 72°C for 10 min. Isolates were first classified into their respective genera, *Levivirus* or *Allolevivirus,* using the MJV82 forward and either the *Levivirus* JV41 reverse or the *Allolevivirus* JV81 reverse primer (Vinje, et al., 2004). Each isolate was then assayed for each genogroup using genogroup specific primers (Friedman, et al., 2009a). The prototype strains MS2 (GI), GA (GII), QB (GIII), and SP

(GIV) were used as positive controls. Amplicons were separated by gel electrophoresis in 1.5% agarose, stained with ethidium bromide, and visualized under UV light.

2.6 Statistical Analyses

All statistical analyses were conducted in SAS 9.1 (SAS Inc., Cary, NC) statistical software with a level of statistical significance set at $p < 0.05$. Microbial concentrations were log-transformed prior to analysis. The Mann-Whitney U test was performed to test the statistical significance of differences between microbial concentrations in the A and B sampling locations at each site. Kruskal-Wallis one-way ANOVAs were used to test the statistical significance of differences in microbial concentrations between seasons. The relationship of microbial concentrations to antecedent rainfall and fecal indicator bacteria was determined using Spearman Rank correlations (for *Staphylococcus* and coliphage concentrations) and generalized logistic regressions (for F-RNA coliphage presence).

3. Results

Samples were collected from site 1 fifteen times at both A and B locations, and from sites 2 and 3 thirty-one times at both A and B locations. Site 1 became too dry to obtain samples after early June 2010. Sites 4 and 5 were added June 8, 2010 as B locations only and were sampled 14 times each. An additional site 6 was added mid-October as a B location only and was sampled 5 times. A total of 186 samples were collected for this study.

Of 183 water samples analyzed for antibiotic-resistant *Staphylococcus*, 92% had at least one colony with a characteristic morphology (mauve with matte halo) of methicillin-resistant *Staphylococcus aureus* (MRSA) with growth in the presence of salt

(25g/L) and cefoxitin (Table 3.1). In order to evaluate whether the isolates were MRSA, we characterized a subset (45) of our isolates using PCR of the *clf*A, 16S, and *mec*A genes (data not presented). Most (96%) of this subset was positive for the Staphylococcal 16S rRNA gene but negative for the *clf*A and *mec*A genes, suggesting that our isolates are a species other than *S. aureus* and are not carrying the *mec*A variant represented by the primer set. Two isolates (4%) were negative for all three PCR assays. Pending further characterization of all isolates, these bacteria are referred to only as presumptive antibiotic-resistant *Staphylococcus* (PARS) in this report.

A total of 98% of samples analyzed were positive for somatic coliphages, and 85% were positive for F+ coliphages (Table 3.1). Of the 660 F+ coliphages isolated and subjected to RNase testing, 21 isolates (3%) were RNA phage. Genotyping revealed that all of the F-RNA coliphages belonged to genogroup I.

Hepatitis E virus (HEV) was detected once during the study period at site 3A in March, 2010.

3.1 Comparison by sample location

All samples collected from sites 1A, 1B, 5B, and 6B had at least one PARS colony. The site with the lowest percentage (79%) of water samples positive for PARS was Site 4B (Table 1). The geometric mean PARS concentrations for each site ranged between 12.0 CFU per 100 ml (1.7, 35.7) at site 1B and 50.2 CFU per 100 ml (8.3, 308.0) at site 2A (Table 2). Mann-Whitney U tests revealed that the concentrations of PARS at sites 1A and 1B and at sites 3A and 3B were not different. However, the mean concentration of PARS at site 2B was lower than at site 2A ($p < 0.003$) (Table 3.3). Stratifying by rainfall did not affect these results.

All samples from sites 2A, 2B, 4B, and 5B were positive for at least 1 somatic coliphage (1 PFU per 100 ml), and all samples from sites 4B and 5B were positive for at least 1 F+ coliphage (1 PFU per 100 ml) (Table 3.1). The geometric mean somatic coliphage concentrations ranged between 5.5 PFU per 100 ml (0.5, 1000.0) at site 1A and 96.2 PFU per 100 ml (8.0, 766.0) at site 5B (Table 3.2). The geometric mean F+ coliphage concentrations ranged between 2.5 PFU per 100 ml (0.5, 18.0) at site 1A and 20.0 PFU per 100 ml (3.0, 84.0) at site 4B (Table 3.2). Mann-Whitney U tests revealed that coliphage concentrations at A and B sites were not generally different (Table 3). Stratifying by rainfall did not affect these results.

The 21 F-RNA coliphage isolates were detected at least once at all sites examined except 4B (Table 3.3). The largest percentage of F-RNA coliphages were isolated in samples from site 3A (24%).

3.2 Temporal and seasonal distribution

The geometric mean concentrations of PARS, somatic coliphages, and F+ coliphages detected in each season during the study period is presented in Table 3.4. Concentrations of PARS were highest in the fall (October-December) ($p < 0.04$). Somatic coliphage geometric mean concentrations were similar for all seasons examined: winter, spring and summer. F+ coliphage concentrations were higher in the summer (July-September) than in the winter or spring ($p < 0.0001$).

All F-RNA coliphages were isolated from sampling time-points in February, April, and August 2010, and 65% were isolated from coliphage enrichments in February 2010.

3.3 Correlation to rainfall

Peak rainfall was recorded in the spring. Higher somatic coliphages coincided with peak rainfall, but higher PARS and F+ coliphage concentrations did not. Spearman Rank analyses revealed that PARS was not generally correlated to rainfall, although concentrations were negatively correlated with 24h antecedent rainfall levels across all downstream sites ($p < 0.03$). While coliphages were not correlated to rainfall across all A or B sites, somatic coliphage concentrations were correlated with 48h antecedent rainfall at sites 2B ($p < 0.01$) and 5B ($p < 0.03$), and F+ coliphage concentrations were correlated with 24h antecedent rainfall at site 1A ($p < 0.04$) (Table 3.5).

Generalized logistic regressions revealed that the presence of F-RNA coliphages was not correlated to antecedent rainfall. For the one sample positive for HEV, no precipitation was recorded during the 48 hours preceding sample collection.

3.4 Correlation to fecal indicator bacteria

PARS concentrations were also compared to the concentrations of the traditional fecal indicator bacteria (FIB) fecal coliforms, enterococci, and *E. coli* (as recorded in Myers, 2011). Spearman Rank correlations revealed that PARS concentrations were positively correlated with fecal coliforms ($p < 0.0003$) and *E. coli* ($p < 0.003$) across all A sampling sites combined (Table 3.6).

The concentrations of somatic and F+ coliphages were also compared to the concentrations of the FIB (as recorded in Myers, 2011). Spearman rank analyses revealed that somatic coliphage concentrations were correlated with fecal coliform concentrations ($p < 0.02$), *E. coli* ($p < 0.009$), and *Enterococcus* sp. ($p < 0.04$) across all A sites, and *E.*

coli across all B sites ($p < 0.001$). F+ coliphage concentrations were correlated with fecal coliforms across all A ($p < 0.01$) and B ($p < 0.0004$) sites (Table 3.6).

Generalized logistic regressions revealed that the presence of F-RNA coliphages was not correlated to FIB concentrations.

For the sample positive for HEV, F+ coliphages were detected in the water sample at 5 PFU per 100 ml, but F-RNA coliphages were not detected. Conversely, the water sample contained levels of somatic coliphages, fecal coliforms, *E. coli*, and enterococci that were too numerous to count (TNTC). The water sample also contained a high concentration of PARS, 290 CFU per 100 ml.

4. Discussion

In this study we investigated surface waters proximal to swine lagoon waste spray fields for the presence of microorganisms of public health concern, including presumptive antibiotic-resistant *Staphylococcus* (PARS) and hepatitis E virus, and for potential enteric virus indicators, coliphages.

This is the first study to examine CAFO-impacted waters for the presence of antibiotic-resistant *Staphylococcus*. Antibiotic-resistant *Staphylococcus*, specifically methicillin-resistant *S. aureus* (MRSA), has been cultured from swine nasal swabs in the United States (Smith, et al., 2009) and abroad (Cui, et al., 2009; de Neeling, et al., 2007; Huijsdens, et al., 2006; Khanna, et al., 2008; Lewis, et al., 2008; Pomba, et al., 2009), but it has not been evaluated in swine waste. Given the high percentage (92%) of water samples positive for PARS, our research suggests that there is a possibility of transfer of antibiotic-resistant *Staphylococcus* from farms to surrounding waters. This is supported

by previous data that found that the movement of resistant bacteria and resistance determinants from swine CAFOs into the environment can be extensive (Chee-Sanford, et al., 2001; Sapkota, et al., 2007). Movement from swine lagoon spray fields to the environment would represent a potential additional transmission route of antibiotic-resistant *Staphylococcus*, in addition to that between agricultural animals and associated workers (Cui, et al., 2009; Khanna, et al., 2008; Smith, et al., 2009; Van Cleef, et al., 2010) and to food products (Pesavento, et al., 2007; Pu, et al., 2009; van Loo, et al., 2007). Movement of antibiotic-resistant *Staphylococcus* to the environment may also promote the horizontal transfer of resistance genes within the environment. It is important to note, however, that *S. aureus* and MRSA have been isolated from marine water, stream water, and sand samples from areas not impacted by swine CAFOS in California (Goodwin and Pobuda, 2009), Florida (Abdelzaher, et al., 2010), Hawaii (Tice, et al., 2010; Viau, et al., 2011a), and the Pacific Northwest (Soge, et al., 2009). Thus, this study attempted to incorporate quantitative results using culture-based methods.

Our study is the first attempt, to our knowledge, to quantify levels of antibiotic-resistant *Staphylococcus* in waters adjacent swine CAFO spray fields using culture-based methods. We detected a geometric mean concentration of 18.0 CFU per 100 ml (1.7, 308.0) across all sites. Initial genetic testing on a small number of isolates suggests that these bacteria are not MRSA, but that they are positive for the 16S rRNA gene of *Staphylococcus* spp. only. These results were surprising as this method, originally designed for use with clinical isolates, was reported to have been successfully adapted for use with environmental seawater and beach sand samples at recreational beaches (Goodwin and Pobuda, 2009). The CHROMagar™ MRSA culture-based method – using

the appearance of a mauve colony on the membrane filter in conjunction with a mauve with matte halo isolate appearance – had a 92% positive predictive accuracy when compared with PCR (*clf*A, 16S, and *mec*A genes) (Goodwin and Pobuda, 2009). Reasons for why this culture method was less specific for MRSA in our study are unclear, though it may be related to our use of CHROMagar™ Staph aureus plates for secondary isolation of colonies. Goodwin and Pobuda (2009) speculated that accurate enumeration of *S. aureus* and MRSA from environmental samples depends on a combination of technician experience, control of filter volumes, control of incubation times, and proper isolation of colonies needing further identification.

The sites in this study were located in an area with a high density of swine CAFOS; nevertheless, we attempted to determine the impact of individual swine lagoon spray fields on water quality in adjacent streams. PARS concentrations were compared at the upstream and downstream locations of three sites (1A, 1B, 2A, 2B, 3A, 3B). Our results indicated that, on average, PARS concentrations were not different between A and B sites. Similarly, somatic and F+ coliphage concentrations were not different at A and B sites. We suspect that these results are due to diffuse contamination of surface waters with swine waste in addition to low levels of rainfall during the study period. The geographic area examined in this study is located in a county with an estimated swine population of 2,285,224 in 2007 (USDA, 2007). Furthermore, North Carolina has been reported to utilize more antibiotics for growth-promotion than the total amount used clinically in all of the United States (Florini, et al., 2005). Similar concentrations of PARS and coliphages at A and B sites may also be due to low levels of rainfall during the study period. Precipitation levels exceeded 1 inch only three times during the study. In

fact, this study found that, in general, microbial concentrations were not positively correlated to rainfall levels. The levels of rainfall that occurred during the study period would not likely result in a "flush" of waste downstream of the spray fields examined.

It is also possible that other fecal sources could be responsible for the diffuse concentrations of PARS and coliphages. There are numerous poultry CAFOs, septic systems, and cattle grazing in open fields in this area, in addition to the ubiquitous swine CAFOs. Moreover, many rural homes in the area use septic systems for sewage disposal. Nevertheless, swine and poultry CAFOs are estimated to be the largest contributors to fecal waste in this area (Wing, 2012), combined with the fact that all of the F-RNA coliphage isolates belonged to genogroup I (GI), indicating an animal-source (e.g., pigs, cattle, sheep) of fecal pollution in the surface waters (Osawa, et al., 1981). Furthermore, much of the liquid fecal waste in this area is likely from swine, as swine CAFOs use liquid waste management systems (lagoons and spray fields) to dispose of fecal waste, whereas almost all poultry CAFOs in the area use dry litter waste management systems (Wing, 2012).

Given the ubiquity of swine CAFOs in this area, we could not categorize our A sites as un-impacted and we could not assess whether the concentrations of PARS detected at these sites are different from those in un-impacted surface waters. Only a few reports have published quantitative results of *Staphylococcus* in environmental samples, and these only include the concentrations of *S. aureus* and MRSA (Fowler, 2005; Tice, et al., 2010). Nevertheless, previous studies have found that the concentrations of antibiotic-resistant microorganisms, including *Enterococcus* sp. and *E. coli*, tend to be higher in swine CAFO-impacted waters than in un-impacted waters (Anderson and Sobsey, 2006;

Sapkota, et al., 2007).

In contrast to PARS and coliphage concentrations, fecal indicator bacteria (FIB) concentrations were consistently higher at B sites than A sites (Myers, 2011). These bacteria are used in the US to indicate the presence of fecal contamination surface waters. The reason for the increase in FIB from A to B sites and no change in the microorganisms examined are unclear, although it may be related to the presence of naturalized FIB resulting from nutrient inputs from agricultural runoff (Ferguson and Signoretto, 2011). This hypothesis is supported by the fact that swine-specific microbial source-tracking markers were detected in similar concentrations at both A and B sites (Myers, 2011).

PARS concentrations were not correlated to FIB across all A and B sites. However, somatic coliphages were positively correlated with *E. coli* across A and B sites, and F+ coliphages were positively correlated with fecal coliforms across A and B sites. None of the microorganisms examined were correlated with enterococci. These results indicate that FIB cannot predict the presence of increased PARS. Conversely, these results indicate that coliphages may be able to be used in a tiered assessment with *E. coli* and fecal coliforms (the FIB used for water quality monitoring in NC) in this area.

HEV was detected once during the study period, at site 3A. In industrialized countries, little is known about possible sources and transmission routes for endemic human HEV infections. Research is often impeded by the rare detection of outbreaks, occurrence of asymptomatic infections, and a long and variable incubation period for disease (Lewis, et al., 2010). However, previous research has suggested the possibility of zoonotic transmission routes for HEV (Meng, 2009; Pavio, et al., 2008), and a systematic

review found that in industrialized countries, specifically Europe, zoonotic transmission seemed likely (Lewis, et al., 2010). Another recent meta-analysis found a significant association between occupational exposure to swine and human HEV IgG seropositivity in 10 of 13 cross-sectional studies (Wilhelm, et al., 2011). Hepatitis E infection in humans is very similar to hepatitis A; symptoms include jaundice, anorexia, abdominal pain, and hepatomegaly with fever, nausea and vomiting (Emerson and Purcell, 2003). Hepatitis E is generally self-limiting, but fatal hepatitis E caused by genotype 3 (which includes swine HEV) has been reported in Japan (Mizuo, et al., 2005) and in Europe (Dalton, et al., 2008; Kraan, et al., 2004; Mennecier, et al., 2000; Péron, et al., 2007), often in older males with underlying chronic liver disease.

To our knowledge, this is the first study to examine the presence of HEV in creeks impacted by swine CAFO waste over time. Previous studies have detected HEV in swine (Choi, et al., 2003; Dell'Amico, et al., 2011; Huang, et al., 2002; Kase, et al., 2008; Takahashi, et al., 2003) and swine lagoons (Kasorndorkbua, et al., 2005; McCreary, et al., 2008; Pina, et al., 2000). Only two other studies have examined impacted surface waters for HEV (Karetnyi, et al., 1999; Kasorndorkbua, et al., 2005), but neither examined water samples during multiple seasons. One of these studies (Karetnyi, et al., 1999) detected HEV in a tile outlet draining a field to which manure had been applied. Because HEV in developed countries is thought to be predominantly from zoonotic origins (when travel to developing countries can be ruled out) (Nelson, et al., 2011), HEV in this sample most likely originated from swine. This hypothesis is further supported by the presence of three swine-specific microbial source-tracking markers in this sample (Myers, 2011).

Due to challenges in detecting HEV in environmental samples containing high

concentrations of organics (and likely PCR inhibitors), this study also examined water samples for the presence of F-RNA coliphages, potential indicators for human enteric viruses. Of 660 F+ isolates, only 21 (3%) were F-RNA coliphage. This was surprising, given that two previous studies utilizing similar methods found F-RNA coliphages to represent 19-50% of all F+ isolates in swine wastewaters (Cole, et al., 2003; Lee, et al., 2009). One study that examined surface waters impacted by swine feces found that 18% of F+ coliphage isolates were F-RNA (Cole, et al., 2003). The reason for the lower percentage of F-RNA coliphages in environmental waters than in swine wastewaters may be due to dissimilar inactivation rates of F-DNA and F-RNA phages after being sprayed onto fields.

Results indicated that all of the F-RNA isolates belonged to genogroup I (GI), indicating an animal source of fecal pollution (Osawa, et al., 1981). This was surprising, as previous studies have found GI F-RNA coliphages to represent 19-60% of all F-RNA isolates in swine wastewaters (Cole, et al., 2003; Lee, et al., 2009) and 0% (0 of 3) of all F-RNA isolates in surface waters impacted by swine feces (Cole, et al., 2003). The higher percentage of GI in our swine-impacted water samples could be a product of the enhanced persistence of GI over other genogroups in environmental waters (Brion, et al., 2002; Cole, et al., 2003; Long and Sobsey, 2004; Schaper, et al., 2002a). It could also be a factor of selective enrichment in some of our samples (Sobsey, et al., 2006), as 65% of the F-RNA coliphages were detected in coliphage enrichments from February 2010. However, the predominance of GI F-RNA phages could also be a product of the improved primer set utilized in this study. This was the first study to utilize novel primers for F-RNA coliphages (Friedman, et al., 2009a) in environmental samples. These primers

were constructed from at least five strains per genogroup and greatly increased the diversity of detectable strains.

Nevertheless, the presence of F-RNA coliphages did not correspond to the presence of HEV in our samples, although the largest percentage of F-RNA coliphages were isolated in samples from site 3A, where HEV was detected. Additionally, F+ coliphage concentrations were not increased (5 PFU per 100 ml) in the HEV-positive water sample. This was surprising, given that previous studies have shown F+ coliphages to correspond to enteric viruses, including noroviruses (Aw and Gin, 2010; Dore, et al., 2000) and adenoviruses (Wu, et al., 2011). Conversely, somatic coliphage concentrations were higher in this sample than all other samples. Previous studies have found correlations between somatic coliphages and adenoviruses (Aw and Gin, 2010), enteroviruses (Mocé-Llivina, et al., 2005), and culturable enteric viruses (Payment and Franco, 1993). Additionally, concentrations of all three FIB (Myers, 2011) and PARS were extremely high in this sample, indicating that this particular sample was highly fecally contaminated.

Conclusions

The majority of samples, 92%, were positive for PARS, with a geometric mean concentration of 18.0 CFU per 100 ml (1.7, 308.0). The ubiquity of swine CAFOs prevented us from being able to compare concentrations with those from un-impacted sites in this area. Hepatitis E virus (HEV) was detected in one surface water sample, which also contained high concentrations of fecal indicator bacteria, *Staphylococcus*, and somatic coliphages, indicating deteriorated water quality. F-RNA coliphages were

detected in 7% of all samples examined. All belonged to genogroup I (GI), indicating an animal-source of fecal pollution. The ubiquity of presumptive antibiotic-resistant *Staphylococcus*, as well as the presence of HEV and F-RNA coliphages, in the samples examined in this study indicate a possible human health risk in waters proximal to swine CAFO spray fields which should be explored in future studies.

Table 3.1. Percentage of surface water samples positive for presumptive antibiotic-resistant *Staphylococcus* (PARS), somatic coliphages, and F+ coliphages by site.

Site	% Positive (n)		
	Staphylococcus	Somatic coliphages	F+ coliphages
All A samples	97 (73)	97 (65)	83 (65)
All B samples	92 (106)	99 (89)	87 (89)
Site 1A	100 (13)	85 (13)	85 (13)
Site 1B	100 (13)	100 (13)	77 (13)
Site 2A	97 (30)	100 (26)	77 (26)
Site 2B	93 (30)	100 (26)	88 (26)
Site 3A	97 (30)	100 (26)	88 (26)
Site 3B	90 (30)	96 (26)	77 (26)
Site 4B	79 (14)	100 (12)	100 (12)
Site 5B	100 (14)	100 (12)	100 (12)
Site 6B	100 (5)	NA	NA

NA= not applicable

Table 3.2. Geometric mean concentrations of presumptive antibiotic-resistant *Staphylococcus* (PARS; CFU per 100 ml) and coliphages (PFU per 100 ml) at each site.

Site	*Staphylococcus* Geo. Mean (CFU/100 ml)	Min, Max	Somatic coliphages Geo. Mean (PFU/100 ml)	Min, Max	F+ coliphages Geo. Mean (PFU/100 ml)	Min, Max
All A samples	22.5	1.7, 308	20.4	0.5, 1000	6.3	0.5, 82
All B Samples	15.5	1.7, 260	27.5	0.5, 1000	7.2	0.5, 99
1A	12.4	1.7, 120	5.5	0.5, 187	2.5	0.5, 18
1B	12.0	1.7, 35.7	17.8	3.0, 104	3.0	0.5, 69
2A	50.2	8.3, 308	17.9	2.0, 67.0	9.2	0.5, 82
2B	21.0	2.9, 260	22.1	1.0, 172	8.6	0.5, 83
3A	13.0	1.7, 290	45.1	4.0, 1000	6.7	0.5, 63
3B	12.8	1.7, 160	35.4	0.5, 1000	4.5	0.5, 99
4B	14.3	3.3, 60.0	11.6	2.0, 30.0	20.0	3.0, 84
5B	20.1	6.7, 38.3	96.2	8.0, 766	13.1	4.0, 74
6B	13.1	8.6, 26.7	NA	NA	NA	NA

NA= not applicable

Table 3.3. Water samples positive for F-RNA coliphages.

Date	Site	No. RNA Phages	Culture Method	Genogroup
4/1/10	1A	1	SAL[a]	I
2/16/10	1B	1	enrichment	I
2/24/10	1B	1	enrichment	I
4/1/10	1B	1	SAL	I
2/16/10	2A	1	enrichment	I
2/24/10	2A	2	enrichment	I
4/1/10	2A	1	SAL	I
2/24/10	2B	2	enrichment	I
8/10/10	2B	1	SAL	I
2/16/10	3A	1	enrichment	I
2/24/10	3A	3	enrichment	I
8/10/10	3A	1	SAL	I
2/16/10	3B	1	enrichment	I
2/25/10	3B	3	enrichment	I
8/10/10	5B	1	SAL	I

[a]SAL= single agar layer method

Table 3.4. Geometric mean concentrations of presumptive antibiotic-resistant *Staphylococcus* (PARS; CFU per 100 ml) and somatic and F+ coliphages (PFU per 100 ml) by season.

Season	n	*Staphylococcus*		Somatic Coliphages		F+ Coliphages	
		Geo. Mean (PFU/100 ml)	Min, Max	Geo. Mean (PFU/100 ml)	Min, Max	Geo. Mean (PFU/100 ml)	Min, Max
Winter	26	17.1	1.7, 290.0	21.7	0.5, 1000.0	3.5	0.5, 72.0
Spring	76	14.4	1.7, 308.0	26.1	0.5, 722.0	3.5	0.5, 92.0
Summer	62	22.0	1.7, 300.0	23.1	0.5, 766.0	21.8	2.0, 99.0
Fall	17	25.5	6.7, 140.0	NA	NA	NA	NA

NA= not applicable

Table 3.5. Significant Spearman Rank correlation coefficients of presumptive *Staphylococcus* (PARS) and somatic and F+ coliphage concentrations with 24- and 48-hour antecedent rainfall amounts.

		Correlation with rainfall		
Site	Rainfall amt (in.)	Staphylococcus	Somatic	F+
All Upstream	24h	NS	NS	NS
	48h	NS	NS	NS
All Downstream	24h	-0.2134*	NS	NS
	48h	NS	NS	NS
1A	24h	NS	NS	0.5707*
	48h	NS	NS	NS
1B	24h	NS	NS	NS
	48h	NS	NS	NS
2A	24h	NS	NS	NS
	48h	NS	NS	NS
2B	24h	NS	NS	NS
	48h	NS	0.4776*	NS
3A	24h	NS	NS	NS
	48h	NS	NS	NS
3B	24h	NS	NS	NS
	48h	NS	NS	NS
4B	24h	NS	NS	NS
	48h	NS	NS	NS
5B	24h	NS	NS	NS
	48h	NS	0.6320*	NS
6B	24h	NS	NA	NA
	48h	NS	NA	NA

[a]Correlations based on Spearman Rank analyses.
*$p < 0.05$
NA = not applicable
NS = not significant

Table 3.6. Significant Spearman Rank correlation coefficients between presumptive antibiotic-resistant *Staphylococcus* (PARS) or somatic and F+ coliphage concentrations and fecal indicator bacteria.

		Correlation with fecal indicator bacteria (FIB)		
Site	FIB	Staphylococcus	Somatic	F+
All upstream	Fecal coliforms	0.4108***	0.2865*	0.3143*
	E. coli	0.3404**	0.3204**	NS
	Enterococcus	NS	0.2610*	NS
All downstream	Fecal coliforms	NS	NS	0.3679***
	E. coli	NS	0.3441**	NS
	Enterococcus	NS	NS	NS
Site 1A	Fecal coliforms	NS	NS	0.5964*
	E. coli	NS	NS	NS
	Enterococcus	NS	NS	NS
Site 1B	Fecal coliforms	NS	NS	NS
	E. coli	NS	NS	NS
	Enterococcus	NS	NS	NS
Site 2A	Fecal coliforms	0.4215*	NS	NS
	E. coli	0.4614*	0.2297	NS
	Enterococcus	NS	0.3958	NS
Site 2B	Fecal coliforms	NS	0.2832	0.4672*
	E. coli	NS	0.3998*	NS
	Enterococcus	NS	NS	NS
Site 3A	Fecal coliforms	0.5444**	NS	NS
	E. coli	0.5657**	NS	NS
	Enterococcus	NS	NS	NS
Site 3B	Fecal coliforms	NS	NS	NS
	E. coli	NS	NS	NS
	Enterococcus	NS	NS	NS
Site 4B	Fecal coliforms	NS	-0.6025*	NS
	E. coli	NS	NS	NS
	Enterococcus	NS	NS	NS
Site 5B	Fecal coliforms	NS	NS	0.7417**
	E. coli	NS	NS	NS
	Enterococcus	NS	NS	NS
Site 6B	Fecal coliforms	NS	NA	NA
	E. coli	NS	NA	NA
	Enterococcus	-0.6156	NA	NA

NS = not significant
*$p < 0.05$
**$p < 0.01$
***$p < 0.001$
NA= not applicable

Chapter 4: QPCR Inhibition is Related to Fluorescent Dissolved Organic Matter Components Using Excitation-Emission Matrix Spectroscopy and PARAFAC

1. Introduction

Methods for water quality testing are transitioning from traditionally culture-based to molecular assays using host-specific qPCR. The reasons for this transition include avoiding cultivation, which can save both time and expenses, and the potential for molecular methods to be sensitive, quantitative, and amenable to automation (Santo Domingo, et al., 2007). Additionally, multiple assays aimed at different targets can be performed using the same nucleic acid extractions, and the extracts can be preserved for future analyses should more sensitive assays, or assays for additional hosts, become available (Santo Domingo, et al., 2007). Quantitative PCR (qPCR) assays are especially promising, as they would allow for increased sensitivity, quantification, speed, and automation. However, before a qPCR method can be used routinely as part of environmental monitoring programs, the accuracy and sample limit of detection (SLOD) of a method must be defined. Both of these are influenced by inhibitors present in nucleic acid extractions that can increase the SLOD much higher than a laboratory-obtained method detection limit, skew calculated marker concentrations, or even result in false-negatives.

Common inhibitors of PCR in natural waters include humic substances, e.g. humic and fulvic acids, which are the major constituents of aquatic dissolved organic matter (DOM) (McKnight, et al., 2003). Humic substances are able to cause inhibition

because they are comprised of large, complex structures similar to DNA; these structures within humic substances can compete with the target DNA for polymerases, ions, or nucleotides. Inhibition due to humic substances has been recorded previously (Abbaszadegan, et al., 1993; Kreader, 1996).

Because inhibitors can be co-concentrated with molecular targets when concentrating large volumes of water for molecular methods (Abbaszadegan, et al., 1993), several methods have been evaluated to remove or reduce inhibition. Adjuvants such as T34 gene protein, polyvinylpyrrolidone, and bovine serum albumin can be added directly to the PCR reaction to reduce inhibition (Fuhrman, et al., 2005; Kreader, 1996; Monpoeho, et al., 2000). Removal of inhibitors has been reported with the use of density gradient centrifugation using cesium chloride (Leff, et al., 1995; Ogram, et al., 1987), hexadecyltrimethylammonium bromide (Cho, et al., 1996), polyvinylpolypyrrolidone (Frostega˚rd, et al., 1999; Zhou, et al., 1996), gel electrophoresis (Zhou, et al., 1996), and Sephadex G-100 and G-200 columns (Abbaszadegan, et al., 1993; Miller, et al., 1999). Variable inhibitor removal efficiencies have also been reported for different DNA extraction/purification methods (Martin-Laurent, et al., 2001; Widjojoatmodjo, et al., 1992). Many of these methods are costly, labor-intensive, lengthy, or result in significant, or even complete loss of DNA during recovery procedures (Ijzerman, et al., 1997; Kuske, et al., 1998; Moré, et al., 1994; Zhou, et al., 1996).

Because none of these methods has proven fully effective at removing inhibitors for qPCR, amplification efficiency controls for PCR have been developed to detect levels of inhibition present in samples (Wilson, 1997). One such control, Salmon sperm DNA, is added directly to samples pre- or post-extraction to provide a convenient and sensitive

test for inhibition; this control also allows for a correction in measurement variability (Haugland, et al., 2005). It would be advantageous to be able to predict which types of samples may contain various types and levels of PCR inhibitors, especially the humic and fulvic acids present in environmental water samples. A broadly applicable method to assess the type and level of these inhibitors in a sample would aid in minimizing the number of false-negative results and increase the degree of accuracy of a molecular method.

A common method for estimating gross DOM concentration utilizes estimates of dissolved organic carbon, or DOC, as all organic compounds are on average 50% carbon by mass. However, while DOC is an important parameter in estimating the total concentration of all organic molecules comprising DOM, it cannot provide information about DOM chemical composition, including the structures and the abundance of functional groups. Yet it is likely that only a subset of organic molecules within the DOM pool are involved in PCR inhibition. Thus, DOC may not be useful for predicting PCR inhibition.

Several analytical approaches have been used to study the chemical and isotopic composition of DOM. The application of recent advances in optical methods to understand the light-absorbing fraction of DOM has significantly advanced knowledge of DOM source and reactivity. Emission-excitation matrix (EEM) has been used to characterize the source of DOM in natural waters (e.g., DOM derived from leaf litter and crop residues) (Coble, et al., 1990) and in municipal wastewater treatment sludge (Chen, et al., 2003). EEM spectroscopy measures emission spectra across a range of excitation wavelengths, resulting in a landscape surface defined by the fluorescence intensity at

pairs of excitation and emission wavelengths (Chen, et al., 2003). Locations on the EEM corresponding to one or more peaks of maximum fluorescence intensities are then noted (defined as "peak picking"). The EEM signals of DOM samples have previously been delineated into regions, corresponding to humic acid, fulvic acid, aromatic proteins, and microbial by-products (Hudson, et al., 2007).

Because EEM spectroscopy can detect differences in DOM source, it has been investigated as a tool to predict the level of PCR inhibition in sample concentrates from biosolids (Rock, et al., 2010). Rock et al. (2010) investigated whether EEM profiling (quantification of the area under peaks in different EEM regions) of biosolids samples was to linked to levels of PCR inhibition. The authors found that EEM profiling could be used to predict the level of inhibition in biosolid samples, although these findings were not justified with statistical correlations.

Recent studies have determined that the "peak picking" method (including in Rock, et al., 2010) can obscure patterns within a dataset because the EEM spectra of DOM contain many broad and overlapping emission curves (Murphy, et al., 2011). Understanding the underlying variability captured in an EEM has been significantly advanced through parallel factor analysis (PARAFAC), a statistical modeling approach that separates a dataset of EEMs into mathematically and chemically independent components (each representing a single fluorophore or a group of strongly co-varying fluorophores) (Bro, 1997), providing a "fingerprint" of DOM and its humic-like and protein-like fluorescing components.

The objective of this study was to investigate relationships between PARAFAC analysis of EEMs and levels of qPCR inhibition present in environmental water samples.

The level of qPCR inhibition detected in samples was compared to the total concentration of DOC detected in samples (a measure of DOM), three measures of DOM source in samples (specific ultraviolet absorbance, slope ratio, and fluorescence index), as well as the concentration of total fluorescent DOM and the concentrations of fluorescent DOM components detected by PARAFAC analysis of EEMs. Because the chemical composition of DOM (and thus the likely moieties contributing to inhibition) vary with physical and watershed parameters (Wang, 2011), the level of inhibition present in samples was also compared to land use, level of impervious surfaces, rainfall, and physical parameters (temperature, dissolved oxygen, conductivity, total dissolved solids, pH, and turbidity) to determine if these are factors would also impact qPCR inhibition.

2. Materials and Methods

2.1 Study sites and sample collection

Environmental water samples were collected from 15 sites on Morgan Creek, New Hope Creek and Northeast Creek tributaries, within the B. Everett Jordan Lake catchment in North Carolina, US. Sites were selected to represent a range of land use, levels of impervious surface, and watershed area (as described in Rowny, 2011).

Land use characteristics and level of impervious surfaces within each site were determined as described previously (Rowny, 2011) using the 2005 Multi-Resolution Land Characterization Consortium's National Land Cover Data. Sites were divided into three levels of land development, as measured by percent of land covered by impervious surfaces (% IS) in the watershed upstream of each sampling point. The five sites with the lowest % IS were assigned to the 'Low intensity' development class (0.5 to 4.0%); the

five sites with intermediate % IS were assigned to the 'Intermediate intensity' class (4.1 to 14.4 %); and the five sites with the largest % IS were assigned to the 'High intensity' class (15.0 to 34.0%).

Samples were collected from each site bimonthly at approximately the same time of day during background dry weather (DW) for one year between April 2010 and March 2011. Samples were not collected if precipitation in excess of 2.5 cm occurred in the 72-hr period proceeding planned collection; sampling was delayed until these conditions were met. Samples were also collected at four time points during three rain events occurring in September 2010, November 2010, and January 2011. Rain events were defined as of at least three days without appreciable rainfall followed by a rainfall event more than 2 hours in duration with at least 1.0 inch of precipitation. The first sample was taken immediately before the rain started and was used as a baseline sample. The second sample was taken within an hour after the first sample to capture the initial surface runoff. The third sample was collected 2-6 hours after the second, depending on the severity of the rain event, to collect during peak discharge. The fourth sample was collected 2-12 hours after the third sample during the end of rain event. The four samples were taken to coordinate with the baseline, rising limb, peak, and falling limb of the hydrograph. Hourly precipitation data was obtained from the National Oceanic and Atmospheric Administration's U.S. Climate Reference Network (USCRN, 2011) Durham station. Antecedent precipitation was calculated for each sampling time-point by independently summing the preceding 2, 24, and 48 hourly observations. Stream flow for six of the 15 sites was collected by USGS gauges that collect flow rate data four times an hour. Stream flow rate was determined by matching sample collection time with the most

proximate USGS gauge observation time. Two sampling sites were located at NC Coastal Ocean Observing System (NC COOS) gauges that collect stream flow rate data four times an hour. However, the gauge at Crow Branch Creek was prone to malfunction, and these stream flow data were not included in our analysis. Stream flow rates at the remaining NC COOS site (Meeting of the Waters Creek) were determined in the same manner as at the USGS sites.

During dry weather sampling events, two 1-L grab samples were collected in sterilized polypropylene bottles for qPCR inhibition characterization and one 40-ml sample was collected in a sterile amber glass vial for DOM characterization from each of the sites. At the time of sampling, a YSI Professional Pro was used to record water temperature, dissolved oxygen, conductivity, total dissolved solids, and pH. Water samples were immediately placed on ice and transported to the laboratory for processing within 6 hrs of collection. A bench-top Hach 2100N nephelometer was used to determine turbidity once the samples had been returned to the lab.

During rain events, water samples were collected in the same manner as during the dry-weather events, with the exception that physical parameters were determined at the laboratory rather than in the field. An additional 500 ml water sample was collected in a sterile polypropylene bottle for recording the water temperature on-site using a digital thermometer. This sample was sealed, placed on ice, and transported to the laboratory to be tested with a YSI Professional Plus to determine the other physical parameters.

2.2 Determination of qPCR inhibition level

Half of the dry weather samples (the first time-point each month) and one-quarter of the rain event samples (the second time-point of each rain event) were measured for

inhibition (198 samples). Two-hundred ml water from each site was filtered onto 47-mm diameter, 0.45-μm pore-size polycarbonate filters (Millipore, Bedford, MA) using a four-place filtration manifold and vacuum pump assembly with filter funnels (Millipore, Bedford, MA), as suggested by Haugland et al. (2005). Filters were transferred into sterile 2-ml screw-cap tubes and stored at -80°C until DNA extraction. Bacterial DNA was extracted from filters within 2 weeks of concentration using the MoBio Powersoil kit (MoBio, Carlsbad, CA) to elute a final concentrated DNA sample in 100-μl nuclease-free sterile water. The DNA samples were stored at -80°C until further processing.

A specimen processing control (SPC) was utilized to measure the amount of matrix inhibition by adding a known amount of DNA to each bacterial concentrate and a blank at the end of the extraction step. Salmon sperm testes DNA (SKETA) (Sigma, St. Louis, MO) was added at a final concentration of 20 ng per 100 μl of extracted DNA. TaqMan primer and probe (Applied Biosystems, Inc., Foster City, CA) sequences for the SPC were as described by Haugland et al. (2005) and were synthesized by MWG Biotech (High Point, NC). The primers and probe target a segment of the ribosomal RNA gene operon, internal transcribed spacer region 2 of chum salmon, *Oncorhynchus keta*. The qPCR reaction mixture contained 2 μl of sample, each primer at a concentration of 500 nM, the probe at a concentration of 120 nM, 12.5 μl of 2X PCR buffer, and nuclease-free water for a total reaction mixture of 25 μL (Quantitect Probe PCR kit). The reaction mixture was assayed on a Cepheid SmartCycler (Sunnyvale, CA) using the following conditions: (i) 2 min at 50°C, (ii) 15 min at 95°C, (iii) 45 cycles of 1 s at 95°C and 1 min at 50°C. All amplification reactions were carried out in duplicate. A standard curve of salmon sperm DNA was used for quantification of the control and samples. The level of

inhibition in each sample was calculated as the difference between the control and sample concentrations divided by the control concentration, and the qPCR inhibition was reported as percent inhibited. As this research is novel, there is no standard method for reporting raw inhibition levels within samples. This results of the research is reported as differences in ng of DNA rather than Ct values in order to (1) present linear data, as opposed to the logarithmic data given by Ct values, and (2) account for differences in the amount of SKETA added to samples due to PCR runs occurring weeks apart.

2.3 EEM and PARAFAC

All of the dry weather samples (except for those from February and March 2011) and all of the rain event samples were analyzed for DOM (401 samples). Water samples were collected in clean, fluorescent DOM-free, 40-ml amber glass vials. After sample collection, water samples were filtered through a pre-rinsed Millipore Millex 0.2 μm or Millex 0.22 μm syringe filter (Billerica, MA) within 2 hours or were refrigerated until analysis. The syringe was rinsed thoroughly with laboratory-grade water between samples. Following filtration, samples were stored in a clean, fluorescent DOM-free, amber 40-ml vials. Once filtered, absorbance and fluorescence spectra were determined. After absorbance and fluorescence determined were measured, samples were transferred to 24-ml clear glass vials, acidified using hydrochloric acid to bring samples to a pH of approximately 2-3 (to remove inorganic carbon), and refrigerated until dissolved organic carbon (DOC) content was measured.

DOC was measured using a Shimadzu TOC 5000 Analyzer (Columbia, MD), which analyzes organic carbon using the High Temperature Combustion Method (APHA, 1998). Organic carbon calibration standards were prepared fresh from a working solution

of 21.25 mg potassium hydrogen phthalate in 100-ml laboratory-grade water. Standards and blanks were placed between every 6-7 samples to check the reliability of the instrument.

The UV-Visible absorbance spectrum of each sample was measured between 200-700 nm in increments of 1 nm using a Hewlett Packard 8452A Diode Array Spectrophotometer. Sample absorbance spectra were made in reference to DI water. For this study, decadic absorbance values were reported as a function of absorbance wavelength. The spectral slope ratio (SR) was calculated for each sample based on the absorbance spectrum following the procedures of Helms et al. (2008). The specific ultraviolet absorbance (SUVA) was reported as the decadic absorbance at 254 nm in a 1-cm cuvette divided by the DOC concentration (mg C/L). SUVA is strongly, positively correlated to the average aromatic carbon content of the DOM measured by ^{13}C NMR (Cory, et al., 2007; Weishaar, et al., 2003), and thus SUVA is a proxy for the aromaticity and source (i.e., terrestrial vs. microbial) of DOM (Weishaar, et al., 2003). High SUVA values indicate more terrestrial DOM and low SUVA values indicate more microbial DOM.

Excitation-emission matrices (EEMs) were collected using a Fluorolog-321 Spectrofluorometer (HORIBA Jobin Yvon Inc, Edison, NJ) equipped with a new charge-coupled device (CCD) detector. Daily instrument checks (lamp scan, Raman water peak scan, and a clean cuvette check scan) were performed to ensure the instrument was calibrated correctly and to minimize instrument specific responses. Samples were placed in the same 1-cm quartz cuvette used to measure absorbance intensities. The EEMs were collected by measuring fluorescence intensity over excitation wavelengths of 240-450 nm

in increments of 5 nm using the CCD to capture an emission spectrum over the range of 320-550 in increments using a range of integration times and wavelength bins optimized for maximum dynamic range for each sample. The optimum dynamic range for each sample was determined from the UV-Vis absorbance of the sample, which was measured prior to the EEM. Dynamic range was chosen to maximize detection of emission at low excitation wavelength without saturating the CCD detector at high excitation wavelength. The EEMs were corrected for inner-filter effects and for instrument-specific excitation and emission corrections in Matlab version 7.7 (MathWorks, Natick, MA) (Cory, et al., 2010).

The fluorescence index (FI) was calculated as the ratio of emission intensity at 470 nm over the emission intensity at 520 nm at an excitation wavelength of 370 nm (Cory, et al., 2010; McKnight, et al., 2001) from the corrected EEM. FI is a qualitative measure of the relative proportion of the terrigenous and microbial organic matter, and is less resolved than a full EEM (Cory, et al., 2010; McKnight, et al., 2001).

PARAFAC was used to decompose fluorescence signals in the dataset of EEMs into unique components that can be used to characterize and quantify changes in DOM fluorescence. Due to the inherent heterogeneity of DOM, each component likely represents spectra of groups of similarly fluorescing constituents (Stedmon, et al., 2003). PARAFAC uses an alternating least squares algorithm to minimize the sum of squared residuals in a three way model (Stedmon and Bro, 2008; Stedmon, et al., 2003). The data is decomposed into a set of trilinear terms and a residual array (Equation 1).

$$x_{ijk} = \sum_{f=1}^{F} a_{if} b_{jf} c_{kf} + \varepsilon_{ijk}, i = 1,.\,,I; j = 1,.\,,k = 1,.\,,K;$$

Equation 1. PARAFAC Equation

In the equation, x_{ijk} is the fluorescence intensity of emission of sample *i* at the *j*th emission wavelength and the *k*th excitation wavelength for a PARAFAC model with *F* number of components. The terms a, b, and c represent the concentration, emission spectra, and excitation spectra, respectively, of the different fluorophores. The a_{if} term is directly proportional to the concentration of the *f*th analyte of the *i*th sample, while b_{jf} and c_{kf} are scaled estimates of the emission and excitation spectra at wavelengths j and k respectively for the fth analyte. The e_{ijk} term represents any unexplained signal that can be from residual noise and un-modeled variability.

In order to interpret the PARAFAC scores as actual concentrations of the components in a sample, the identity of the responsible fluorophore and subsequent second order calibration would be needed (Stedmon and Bro, 2008). However, this specific identification of fluorophores is not possible with complex, heterogeneous mixtures such as DOM because components likely represent a group of co-varying fluorophores and the relationships between component spectra, absorptivity, and fluorescence quantum yield are unknown for each group. Therefore, Fmax values (the fluorescence of each component at the respective excitation and emission maximum of each component in each sample) were used to quantify and compare changes in DOM components. While Fmax values provide estimates of the relative concentration of each component in a sample, it is important to note that comparison of the relative concentrations between components depends on their quantum efficiency and their

response to changes in the local molecular environment. Estimates for the precision of the Fmax values of each component were obtained from the triplicate analyses of each sample.

The PARAFAC model was run and validated following Stedmon and Bro (2008) using the DOMFluor Toolbox in Matlab version 7.7. The PARAFAC model was developed using a dataset of 401 samples, with 206 samples collected during dry samples and 195 samples collected during rain events. A total of 43 samples (26 dry samples and 17 rain event samples) were identified as outliers during the PARAFAC modeling procedure and were removed from the model, leaving 358 total samples with 180 dry samples and 178 rain event samples.

Using PARAFAC, six unique components were identified in this dataset, summarized in Table 4.1. Components 1, 2, 3, and 4 (C1, C2, C3, C4) are all associated with precursor organic matter derived from terrestrial sources, with C2 being the most strongly associated with terrestrial organic matter of the components. C3 is more blue-shifted in emission peak maximum which implies a stronger association with microbial precursor organic matter of lower aromatic carbon content. C1 and C4 are a mix of terrestrial and microbial. C5 is amino acid-like or tryptophan-like. C6 was determined to be identical to a component identified in an independent study of the fluorescent signature of wastewater effluent DOM collected from the Orange Water and Sewer Authority (OWASA) located nearby (Polera, 2010).

The six component model was validated following the four-way split-half analysis procedure of Stedmon and Bro (2008). The model explained 99.8% of the variation

within the dataset, with variation explained by each component decreasing in order from C1 to C6.

2.4 Statistical Analyses

All statistical operations were conducted in JMP 9.0 or SAS 9.1 (SAS Inc., Cary, NC) statistical software with a level of significance set at $p < 0.05$. The Mann-Whitney U test was performed to test the significance of difference between inhibition in dry weather and rain event samples. Kruskal-Wallis one-way ANOVAs were used to test the significance of difference in inhibition between seasons and land development categories. The relationship of inhibition to level of antecedent rainfall, discharge, level of development, level of impervious surface, physical parameters, dissolved organic carbon (DOC), fluorescence index (FI), specific ultraviolet absorbance (SUVA), slope ratio, and dissolved organic matter component concentrations was determined using Spearman Rank analyses.

The data was evaluated in two sets - one with all inhibition data and one with only samples in which inhibition was present (non-zero) to better determine the factors responsible for increased qPCR inhibition. Within each of these sets, the data was grouped by sample type (dry weather and rain event) and by development intensity because there was no reason to expect that the factors that explain differences in dry weather vs. rain event samples were the same as the factors that explain differences between land use categories.

3. Results

3.1 Complete Data Set

One-hundred and eight samples (55%) exhibited some amount of inhibition. Thirty-two samples (17%) were inhibited to such an extent that there was >50% difference between the control and sample concentrations, and 8 samples (4%) were inhibited to such an extent that there was >90% difference (1-log) in the control and sample concentrations.

3.1.1 Correlation of Inhibition to Season and Rainfall Levels

QPCR inhibition was similar across all seasons, although inhibition tended to be higher in the fall and spring. A Kruskal-Wallis one-way analysis of variance (ANOVA) found no difference between seasons for any land use category.

The level of qPCR inhibition detected in samples collected during dry weather and rain events samples were similar, and a Mann-Whitney U test found no difference between the two types of samples. However, a Spearman Rank analysis found inhibition to be negatively correlated to antecedent rainfall 2-hr ($X = -0.566$; $p = 0.049$), 24-hr ($X = -0.589$; $p = 0.021$), and 48-hr ($X = -0.566$; $p = 0.028$) previous in rain event samples from areas with high development intensity.

3.1.2 Correlation of Inhibition to Land Use and Physical Parameters

QPCR inhibition was similar across sites with different levels of development, and a Kruskal-Wallis ANOVA found no difference between high, intermediate, and low levels of development. Using Spearman Rank analyses, inhibition was not correlated to land use, level of impervious surface, or watershed size.

Inhibition was not correlated to most physical parameters, including temperature, pH, turbidity, total dissolved solids, or dissolved oxygen, although inhibition was negatively correlated to conductivity ($X = -0.271$; $p = 0.046$) in dry weather samples from areas with an intermediate level of development.

3.1.3 Correlation of Inhibition Level to DOM quantity and quality

Using Spearman Rank analyses, inhibition was not correlated to DOC, SUVA, or SR. However, inhibition was correlated to FI for rain event samples from areas with intermediate levels of development (Table 4.2). Inhibition was also correlated to the level of total fluorescent DOM (FDOM) and to several of the component concentrations (using Fmax values and percent values) identified in some samples (Table 4.2).

3.2 Partial Data Set Using Only Inhibited Samples

3.2.1 Correlation of Inhibition to Season and Rainfall Levels

QPCR inhibition was similar across all seasons for all land use categories, although inhibition tended to be higher in the fall and spring. A Kruskal-Wallis one-way analysis of variance (ANOVA) found no difference between seasons except at low land use categories ($p < 0.0006$), where inhibition was significantly higher in the spring.

The level of qPCR inhibition detected in samples collected during dry weather and rain events were similar, and a Mann-Whitney U test found no difference between the two types of samples. However, Spearman Rank analyses found inhibition to be positively correlated to antecedent rainfall 2-hr ($X = 0.661$; $p = 0.035$), 24-hr ($X = 0.680$; $p = 0.031$), and 48-hr ($X = 0.667$; $p = 0.035$) previous for rain event samples from areas with low levels of development.

3.2.2 Correlation of Inhibition to Land Use and Physical Parameters

QPCR inhibition detected in samples at sites with different levels of development were similar, and a Kruskal-Wallis ANOVA found no difference between inhibition in samples from areas with high, intermediate, and low levels of development. Using Spearman Rank analyses, inhibition was not correlated to land use, level of impervious surface, or watershed size, although inhibition was positively correlated to the level of forest cover ($X = 0.363$; $p = 0.049$) in dry weather samples from areas of high development.

The relationship between inhibition and physical parameters (temperature, dissolved oxygen, pH, conductivity, turbidity, total dissolved solids) was assessed using Spearman Rank analyses. In the dry weather samples from areas of high development, inhibition was negatively correlated to total dissolved solids ($X = -0.373$; $p = 0.043$) and positively correlated to turbidity ($X = 0.577$; $p = 0.003$). In dry weather samples from areas of low development, inhibition was negatively correlated to pH ($X = -0.622$; $p = 0.001$). In the rain event samples from areas of low development, inhibition was negatively correlated to total dissolved solids ($X = -0.669$; $p = 0.034$) and conductivity ($X = -0.758$; $p = 0.011$).

3.2.3 Correlation of Inhibition to DOM quantity and quality

Spearman Rank analyses determined that inhibition was not correlated to SR but was correlated to DOC, FI, and SUVA in select sample groups (Table 4.3). QPCR inhibition was also correlated to the concentration of component 2 (using Fmax value) and to the percent of several components in select samples (Table 4.3).

4. Discussion

This study found an overall positive correlation of qPCR inhibition to several humic-acid DOM components, including C1, C2, and C4. There were also positive correlations between qPCR inhibition and %C1 and %C2. C1, C2, and C4 are humic substance-like, which have been shown to cause PCR inhibition (Abbaszadegan, et al., 1993; Kreader, 1996). Of these fractions of fluorescent DOM, C2 is most strongly associated with terrestrially derived humic acids. In contrast to C2, C1 and C4 are more strongly associated with the fulvic acid fraction of DOM, and are likely derived from multiple sources of organic matter. There was also one positive correlation between qPCR inhibition and a protein-like component C5. These protein-like components (breakdown products of proteins) have not yet been evaluated for their contribution to PCR inhibition in natural waters.

Interestingly, there was a general trend of negative correlation between qPCR inhibition and C3 (and %C3). C3 is primarily microbially-derived DOM components, comprised of biomolecules low in aromatic carbon (e.g. lipids, protein, carbohydrates and chitin). The inverse correlation between qPCR inhibition and %C3 may be due to the importance of terrestrially derived carbon. For example, as the "less terrestrial" component (e.g., C3) increases relative to the total fluorescence, the "more terrestrial" components (e.g., C2) decrease (as a % of total fluorescence). The importance of terrestrially derived DOM components on qPCR inhibition is supported by correlations detected between qPCR inhibition and FI and SUVA, both strong markers of terrestrial sources of DOM. For example, both FI and SUVA are strongly correlated with the aromatic carbon content of DOM (Weishaar, et al., 2003). High SUVA values indicate

more terrestrially derived DOM and low SUVA values indicate more microbially derived DOM. A positive correlation was detected between qPCR inhibition and SUVA at areas with low development intensity, indicating a positive correlation between qPCR inhibition and terrestrially derived DOM. For FI, lower values correspond to more terrestrially derived carbon and higher values correspond to microbially derived precursor organic matter. Two negative correlations were detected between qPCR inhibition and FI at a low and intermediate development intensity sites, indicating a negative correlation between microbially derived DOM. All of these correlations support a relationship between increasing terrestrially-derived DOM and qPCR inhibition in areas where terrestrially derived DOM would be assumed to be dominant (e.g., rural areas).

Alternatively, the reason for the negative correlation between qPCR inhibition and C3 may be related to the nucleic acid extraction method. It is possible that extraction methods disproportionately remove various types of humic substance complexes from environmental samples over others. The extraction method used (MoBio PowerSoil kit) may have preferentially removed fulvic acid complexes in C3 over types of humic substance complexes (such as those in C1 or C2). If this is the case, the extraction method would have removed the majority of qPCR inhibitors from samples in which most of the qPCR inhibition was due to C3. Consequently, the level of qPCR inhibition would be expected to decrease as the fraction of C3 (%C3) increases. This hypothesis is supported by the results of Rock et al (2010), who found that both fulvic acid, microbial by-product-like (e.g., C3) and humic acid-like compounds (e.g., C2) corresponded to increases in qPCR inhibition better than other FDOM components examined.

These data suggest that not only do different extraction methods remove varying levels of humic substances, but they may also remove varying types of these qPCR inhibitors. Rock et al. (2010) came to a similar conclusion: an extraction method they examined was found to be less efficient at removing aquatic/marine humic acids than other types of humic material. Fractionation of humic substances during extractions may have important implications for environmental sample processing. It is well known that PARAFAC modeling of EEMs provides information on the humic substance present in environmental samples, and the results of this study suggest that tracing the different types of humic substances through the extraction process using PARAFAC could yield new insight into which extraction method maybe best suited to removing those components. Future studies should aim to explore the relationship between DOM components and qPCR inhibition levels in a controlled setting in order to test this theory.

This study found no direct relationship between qPCR inhibition and land use. This was surprising, given that it was assumed that streams in more rural areas would have higher DOM, including humic substances, and thus, higher levels of qPCR inhibition. However, qPCR inhibition was rarely related to total DOM. Rather, it was more often associated with the individual components of DOM that predominate in various watershed compartments. This is supported by the fact that the correlations to DOM components can be grouped by land use category. For example, the majority of positive correlations to C2 (and %C2) were detected in areas of low development intensity. This agrees with the results of Wang (2011), who determined that C2 was the most terrestrial of all the components, more likely to be associated with soil and plant matter of terrestrial origin (and thus not derived from autochthonous processes).

Conversely, all of the negative correlations to C3 (and %C3) were detected in areas of high development intensity. Likewise, Wang (2011) determined that C3 was the most "blue shifted," which implies a more microbial source, and was highest in areas of high development intensity.

The correlation between qPCR inhibition and DOM components would also explain this study's seemingly contradictory Spearman Rank correlations between qPCR inhibition and rainfall. In the full dataset, inhibition was negatively correlated with rainfall in areas of high development intensity. In the partial dataset of only inhibited samples, inhibition was positively correlated to rainfall in areas of low development intensity. The correlations between qPCR inhibition and DOM components together with the rainfall results would suggest that rainfall in areas of low development increases the percentage of terrestrially-derived DOM components, such as those complexes making up C2, in streams, while rainfall in areas of high development results in increases in the percentage of microbially-derived DOM components, such as those complexes making up C3. This is supported by Wang (2011), who found that C3 only increased significantly during rain events in areas of high development intensity. Thus, while qPCR inhibition showed no direct relationships to land use or rainfall, both of these categories are likely related to the correlation between qPCR inhibition and DOM components.

The correlation between qPCR inhibition and DOM components was less clear when examining qPCR inhibition during each season. Inhibition was higher in the fall and spring for most land use categories, but this difference was rarely significant. Increased inhibition in the fall and spring is likely due to increased overall DOM (including individual components) in these two seasons.

An interesting comparison between this study and that of Wang (2011) is that the specific DOM components C2 and C3 were correlated to both the level of qPCR inhibition and levels of culturable fecal indicator bacteria (FIB). The highest correlation between C2 and FIB occurred in areas of low development intensity and the highest correlation between C3 and FIB occurred in areas of high development intensity (Wang, 2011). Wang (2011) proposed that DOM, specifically C2 and C3, may serve as ecological controls on FIB, whereas this study proposes that DOM, especially C2 and C3, also serve as inhibitors of qPCR. These results imply that specific complexes of humic substances may, in opposing ways, impact measures of FIB used for monitoring recreational water quality and qPCR assays, such as those used to track the sources of fecal contamination. These results would suggest that DOM may be partly responsible for the discrepancy between FIB and pathogens or MST markers (as quantified by qPCR) in this watershed (Shields et al., 2012).

Conclusions

Specific fluorescent dissolved organic matter (DOM) components as measured by PARAFAC modeling of EEMs are correlated to qPCR inhibition. These components are predominantly terrestrially-derived, humic acid-like DOM components and microbially-derived, fulvic acid-like DOM components. These components are also correlated to increases in fecal indicator bacteria as measured by culture methods, suggesting that these DOM components may simultaneously influence both FIB measurements used for water quality monitoring and qPCR measurements, such as those used for fecal contamination source tracking. This study demonstrated that PARAFAC modeling of

EEMs can determine if the relative concentrations DOM components present in a sample can impact qPCR success. This method may also be able to determine if a chosen extraction method would be able to address these inhibitors.

Table 4.1. Fluorescence characteristics of component EEMs from PARAFAC model.

Component	Excitation/Emission (nm/nm)	EEM Region (Coble et al., 1996)	Description	Origin and Description
C1	(250)325/425	A&M	Fulvic acid-like	Mixed: humic DOM associated with biological production of autochthonous sources
C2	250/500	A&C	Humic acid-like	Terrestrial: ubiquitously observed
C3	250/410	A	Fulvic acid-like	Mixed: more likely to be associated with microbially-derived precursor material
C4	260(370)/445	A&C	Fulvic acid-like	Mixed: more likely to be associated with terrestrial DOM
C5	280/350	B&T	Protein-like	Amino acid (tryptophan-like)
C6	(280)350/380		Wastewater	Wastewater DOM

Table excerpted from Wang et al., 2011

Table 4.2. Significant Spearman correlation coefficients ($p<0.05$) between qPCR inhibition and dissolved organic matter (DOM) for full dataset.

	Sample Type					
DOM Component	DW[a] High	DW Interm.	DW Low	Rain Event High	Rain Event Interm.	Rain Event Low
DOC	NS[b]	NS	NS	NS	NS	NS
FI	NS	NS	NS	NS	-0.6279	NS
SUVA	NS	NS	NS	NS	NS	NS
SR	NS	NS	NS	NS	NS	NS
C1	NS	NS	0.419	NS	NS	NS
C2	NS	NS	0.378	NS	NS	NS
C3	NS	NS	NS	-0.613	NS	NS
C4	NS	NS	0.467	NS	NS	NS
C5	NS	NS	0.492	NS	NS	NS
C6	NS	NS	NS	NS	NS	NS
Total FDOM	NS	NS	0.370	NS	NS	NS
%C1	NS	NS	NS	NS	-0.536	0.651
%C2	NS	NS	NS	NS	NS	NS
%C3	-0.477	NS	NS	-0.768	NS	NS
%C4	NS	NS	NS	NS	NS	NS
%C5	NS	NS	NS	NS	NS	NS
%C6	NS	NS	NS	NS	NS	NS

[a]DW = dry weather samples
[b]NS = not significant

Table 4.3. Significant Spearman correlation coefficients between qPCR inhibition and dissolved organic matter (DOM) in inhibited sample dataset.

	Sample Type					
DOM	DW[a] High	DW Inter.	DW Low	Rain Event High	Rain Event Inter.	Rain Event Low
DOC	NS	NS	0.538	NS	NS	NS
FI	NS	NS	-0.527	NS	NS	NS
SUVA	NS	NS	NS	NS	NS	0.770
SR	NS	NS	NS	NS	NS	NS
C1	NS	NS	NS	NS	NS	NS
C2	NS	NS	0.521	NS	NS	NS
C3	NS	NS	NS	NS	NS	NS
C4	NS	NS	NS	NS	NS	NS
C5	NS	NS	NS	NS	NS	NS
C6	NS	NS	NS	NS	NS	NS
Total FDOM	NS	NS	NS	NS	NS	NS
%C1	NS	NS	NS	NS	NS	0.782
%C2	0.497	NS	0.544	NS	NS	NS
%C3	-0.811	NS	NS	NS	NS	NS
%C4	NS	NS	NS	NS	NS	NS
%C5	NS	NS	NS	NS	NS	NS
%C6	NS	NS	NS	NS	NS	NS

[a]DW = dry weather samples
NS = not significant

Chapter 5: Similar Concentration and Extraction Recoveries Allow for Use of Turnip Crinkle Virus as a Spike and Recovery Control for Enteroviruses in Water

1. Introduction

Enteric viruses, including norovirus and rotavirus, are widely believed to be the main etiological agents of waterborne disease (Cabelli, 1983; Fogarty, et al., 1995; Soller, et al., 2010). They are also believed to be responsible for a significant percentage of waterborne outbreaks in which the etiological agent is unknown. Historically, viral outbreaks have been difficult to study, in part due to the relatively low densities of viruses in the environment and the lack of cell culture systems to grow most viruses. Modern molecular techniques such as polymerase chain reaction (PCR) now offer powerful tools for rapid detection of many enteric viruses in water. PCR avoids the need for cultivation, which can save both time and expenses, and has the potential to be sensitive, inexpensive, quantitative, and amenable to automation (Santo Domingo, et al., 2007). Additionally, multiple assays targeting multiple targets can be performed using the same nucleic acid extractions. These extracts can also be preserved for future analyses should more sensitive assays become available. Conventional and quantitative PCR assays now exist for several viral pathogens and indicators. However, loss during concentration and extraction procedures and/or inefficient reverse transcription and PCR can result in an underestimation of virus load or even result in false negatives.

Many techniques have been proposed to address the effects of PCR inhibitors present in samples. Adjuvants such as T34 gene protein, polyvinylpyrrolidone, and

bovine serum albumin can be added directly to the PCR reaction to reduce inhibition (Fuhrman, et al., 2005; Kreader, 1996; Monpoeho, et al., 2000). Removal of inhibitors has been reported with the use of density gradient centrifugation using cesium chloride (Leff, et al., 1995; Ogram, et al., 1987), hexadecyltrimethylammonium bromide (Cho, et al., 1996), polyvinylpolypyrrolidone (Frostega˚rd, et al., 1999; Zhou, et al., 1996), gel electrophoresis (Zhou, et al., 1996), Sephadex G-100 and G-200 columns (Abbaszadegan, et al., 1993; Miller, et al., 1999), antigen-antibody reaction (Schwab, et al., 1996), cation exchange resin (Abbaszadegan, et al., 1993), and gel chromatography (Abbaszadegan, et al., 1993; Borchardt, et al., 2003). Variable inhibitor removal efficiencies have also been reported for different DNA and RNA extraction/purification methods (Aw, et al., 2009; Martin-Laurent, et al., 2001; Rock, et al., 2010; Widjojoatmodjo, et al., 1992). Many of these methods are costly, labor-intensive, lengthy, or result in significant, or even complete, loss of nucleic acids during recovery procedures (Abbaszadegan, et al., 1993; Ijzerman, et al., 1997; Kreader, 1996; Kuske, et al., 1998; Monpoeho, et al., 2000; Moré, et al., 1994; Schwab, et al., 1996; Zhou, et al., 1996).

Since none of these methods has proven fully effective at removing inhibitors, nucleic acid amplification efficiency controls have been developed to quantify PCR inhibition for viral assays (e.g., Gregory, et al., 2006; Gregory, et al., 2010; Hata, et al., 2011), as well as to address the recovery efficiency during the nucleic acid extraction procedure (Hata, et al., 2011). Previous studies have also utilized β-actin RNA (Conn, et al., 2011) and MS2 RNA (Dreier, et al., 2005; Mormann, et al., 2010) to measure the efficiency of viral RNA extraction procedures.

Few studies have attempted to quantify the recovery efficiency of concentration procedures, although some studies have utilized process controls. Mormann et al. (2010) utilized an MS2 virus as a process control for norovirus in experimentally contaminated foods. However, this study did not compare the recovery efficiency of MS2 during the concentration or extraction procedures with the target virus (norovirus) of interest. Moreover, MS2 is not an adequate process control for many environmental samples, including wastewater and waters impacted by sewage, as the virus occurs naturally in these samples. Engineered alternatives, including armored RNA, have also been utilized as process controls in clinical and environmental samples (Beld, et al., 2004; Bressler and Nolte, 2004; Drosten, et al., 2001; Eisler, et al., 2004). The benefits of armored RNA controls include safety, stability, and physical characteristics that mimic natural viruses; however, only a limited number of armored RNA controls are commercially available, and the procedure for making these controls is expensive and labor-intensive. Natural viruses may be a better alternative to armored RNA controls for use as viral surrogates because they would better mimic natural viruses during extraction and concentration procedures. Some natural viruses, namely plant viruses, are easier and less expensive to proliferate and quantify than armored RNA. A plant virus surrogate would also avoid the safety issues of laboratory technicians working with pathogenic viruses.

To be a potential viral surrogate, a plant virus would need to have a similar size, morphology, and genetic material to human enteric viruses. The potential viral surrogate should also be absent from environmental samples of interest. One option is Tombusviruses, which are non-enveloped virions with a 28-35 nm diameter and an isometric nucleocapsid (icosahedral; T=3) similar to many human enteric viruses. Turnip

crinkle virus (TCV) is a Tombusvirus consisting of a positive sense, single-stranded RNA genome of ~ 4 kb with reported spreads only in the UK and former Yugoslavia (Hollings and Stone, 2005).

The goal of this study was to evaluate TCV for use as a viral surrogate for human enteroviruses during concentration and extraction procedures. TCV was evaluated for its ability to (1) concentrate with similar efficiency to a target enterovirus and (2) contain RNA that is extracted and recovered with similar efficiency to that of echovirus 12, a commonly utilized enterovirus representative (e.g., Gregory, et al., 2006; Viau, et al., 2011b). TCV was also evaluated to determine whether it was (3) present in native samples.

2. Materials and methods

2.1 Standards

2.1.1 Echovirus 12 and turnip crinkle virus

A stock of a model enterovirus, echovirus 12 (EV), was obtained from the laboratory of Mark Sobsey at the University of North Carolina at Chapel Hill. The stock was quantified using a plaque assay with FRhK-4 cells and had a titer of $1.23x10^9$ PFU/ml.

A stock of turnip crinkle virus (TCV) was obtained from the laboratory of Steve Lommel at North Carolina State University. The plant virus was propagated on *Nicotiana benthamiana* plants under greenhouse conditions for 7 days and was purified from leaf tissue as described for CarMV (Lommel, et al., 1982) with a few modifications. Briefly, 100 g of infected leaves were homogenized in a blender with 400 ml of extraction buffer

containing 0.2 M sodium acetate buffer, pH 5.2, 0.1% β-mercaptoethanol, and water. The extract was expressed through four layers of cheesecloth and centrifuged at 8,200 rpm for 15 min in a Sorvall GSA rotor (Thermo Scientific, Asheville, NC) then decanted through Miracloth (Thermo Fisher Scientific Remel Products, Lenexa, KS). Four volumes of supernatant were mixed with one volume of 40% polyethylene glycol (PEG 6000) in 1 M NaCl, stirred for 1 hr at 0°C, and was then centrifuged at 8,200 rpm for 15 min. The pellet was re-suspended in 100 ml of 20 mM sodium phosphate buffer, pH 7.4-7.6, and centrifuged at 8,500 rpm for 15 min. The supernatant was further purified by two cycles of differential centrifugation and fractionation on linear log sucrose gradients in 20 mM sodium phosphate, pH 7.4-7.6, for 2 hr in a 70 Ti rotor (Beckman Coulter, Inc., Indianapolis, IN) at 5°C at 47K and 52K, respectively. After each centrifugation, the pellet was re-suspended in 1 ml 20 mM sodium phosphate buffer and stored at 4°C overnight. The sample was then vortexed and centrifuged at 13,200 rpm for 15 min. The supernatant was collected and stored at 4°C overnight or until use. An absorbance at 250 nm of a 10^{-2} dilution of TCV was measured using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific Remel Products), and the viral concentration was determined using the formula: Virus concentration (mg/ml) = (A_{260} x 100)/(virus extinction coefficient). This value was converted to viruses/ml for a final concentration of 4.84 x 10^{15} viruses/ml.

A serial dilution was created of both EV and TCV using PCR-grade nuclease-free water and was stored at 4°C.

2.1.2 Viral RNA standards

Viral RNA was extracted from the EV stock using the RNeasy Mini Kit (Qiagen, Valencia, CA). Purified RNA was eluted into 30 μl of nuclease-free water and was reverse-transcribed using the RNA PCR Core Kit (Applied Biosystems, Foster City, CA) with random hexamers according to the manufacturer's directions. The cDNA was PCR amplified with primers EchoFwd and EchoRev, targeting the 5' non-translated region of the enterovirus genome (Table 5.1). The PCR reaction mixture contained 20 μl of the reverse transcription reaction, each primer at a concentration of 150 nM, 2 mM of $MgCl_2$, 8 μl of 10X PCR Buffer II, 0.5 μl of AmpliTaq DNA Polymerase, and nuclease-free water for a total reaction mixture of 100 μL (RNA PCR Core kit). The reaction mixture was assayed on an Eppendorf MasterCycler gradient (Eppendorf, Hamburg, Germany) using the following conditions: (i) 2 min at 95°C, (iii) 35 cycles of 1 min at 95°C and 1 min at 60°C, and (iii) a final extension of 7 min at 72°C. The double-stranded DNA product was quantified using a PicoGreen Kit (Life Technologies, Grand Island, NY) and a Nanodrop ND-3300 Fluorescent Spectrophotometer (Thermo Fisher Scientific Remel Products). The quantified DNA was converted to copies/ml, assuming one DNA copy per genome. A 10-fold serial dilution was created and subjected to RT-qPCR using enterovirus-specific primers and probes (Table 5.1).

A solution of purified DNA plasmid containing the entire TCV genome was obtained from the laboratory of Steve Lommel at North Carolina State University. The plasmid was quantified using a PicoGreen Kit and Nanodrop ND-3300 Fluorescent Spectrophotometer. The quantified DNA was converted to copies/ml, assuming one DNA

copy per genome. A 10-fold serial dilution was created and subjected to RT-qPCR using TCV-specific primers (Table 5.1).

The RT-PCR reaction mixture for both viruses contained 2 μl of sample, each primer at a concentration of 500 nM, each probe mixture at a concentration of 120 nM, 12.5 μl of 2X RT-PCR buffer, 0.3 μl of 25X RT-PCR enzyme mix, and nuclease-free water for a total reaction mixture of 25 μl (Quantitect Probe RT-PCR kit). The reaction mixture was subjected to a one-step assay on a Cepheid SmartCycler (Sunnyvale, CA) using the following conditions: (i) RT for 30 min at 50°C, (ii) 15 min at 95°C, (iii) 45 cycles of 15 s at 94°C and 1 min at 60°C. All concentration standards were amplified in duplicate.

2.1.3 Salmon sperm DNA standard

Salmon sperm DNA (SKETA) was obtained from the laboratory of Rachel Noble at the University of North Carolina at Chapel Hill. The DNA was previously quantified at a concentration of 10 μg/ml. A serial dilution of SKETA was created with nuclease-free water and was subjected to qPCR with TaqMan primer and probe sequences as described by Haugland et al. (2005). Primers and probe were synthesized by MWG Biotech (High Point, NC). A standard curve was created using a 10-fold dilution series of SKETA DNA.

2.2 Viral RNA extraction experiments

2.2.1 Spiking of samples with preliminary viral concentration and RNA extraction

Sterile 47-mm, 0.45-μm cellulose acetate/nitrate (Type HAWP, denoted as HA; Millipore Corp., Bedford, MA) filters were folded into quarters and aseptically placed into four 2-ml screw-cap tubes containing an equal mix (0.15 g/0.15 g) of 0.5 mm and 0.1

mm zirconia/silica beads (BioSpec Products, Inc., Bartlesville, OK). One ml of Buffer RLT (Qiagen RNeasy Plant Mini Kit) modified to contain 2% polyvinylpyrrolidone-40 (Calbiochem, Bloomington, IN) and 10 ml of β-mercaptoethanol (Fisher Scientific, Pittsburgh, PA) was added to each tube. EV was added to three of the tubes at a final concentration of $1.23x10^5$ PFU/ml, and TCV was added to the same three tubes at a final concentration of $1.0x10^8$ viruses/ml. The last tube was set aside as a negative extraction control sample. Salmon sperm DNA (SKETA) was added to all four tubes at a concentration of 100 ng DNA/ml. Nucleic acid was extracted from all samples using a Qiagen RNeasy Plant Mini Kit (Valencia, CA) following a modified plant and fungi isolation protocol as previously described (Conn, et al., 2011). Nucleic acid was eluted into 100 µl nuclease-free water and stored at -80°C before RT-qPCR analysis for TCV and EV as described in Section 2.1.2. The samples were also analyzed for SKETA with qPCR as described in Section 2.1.3.

2.2.2 Spiking of extraction samples with secondary viral concentration

In order to estimate the stock viral concentrations of TCV and EV in terms of genome copies, a relative concentration was determined by spiking a second set of triplicate extraction tubes with the EV and TCV concentrations (in genome copies/ml) estimated using RT-qPCR in Section 2.2.1. Three 2-ml screw-cap tubes containing an HA filter and zirconia/silica beads were spiked with $1.23x10^8$ PFU/ml of EV and $1.2x10^7$ viruses/ml of TCV (Table 5.2). A negative extraction control (containing no TCV or EV viruses) was processed alongside the three samples.

2.2.3 Determination of virus stock concentrations

Following the second extraction experiment, the concentration of each virus stock was converted to genome copies/ml using the formulas:

$$X_0 * D_1 = X_a$$

$$X_a * D_2 = X_b$$

Where X_0 = the initial spiking concentration (copies/ml); X_a = the concentration (genome copies/ml) detected by RT-qPCR from the first spiking experiment; X_b = the concentration (genome copies/ml) detected by RT-qPCR in the second spiking experiment; D_1 = difference between the first and second spiking viral concentrations; D_2 = difference between concentrations of viruses detected in the first and second spiking experiments. Using these formulas, the initial spiking concentrations of each virus could be converted to genome copies/ml using the formula: $X_0 = (X_b / D_2) / D_1$.

In the first spiking experiment, EV was spiked at a concentration of $1.23x10^5$ PFU/ml, and TCV was spiked at a concentration of $1.0x10^8$ viruses/ml. The average recovered concentration for EV was $4.2x10^6$ genome copies/ml, and the average recovered concentration for TCV was $1.2x10^7$ genome copies/ml (Table 5.2). A second set of triplicate extraction tubes was then spiked with $4.2x10^6$ PFU/ml of EV and $1.2x10^7$ viruses/ml of TCV. The average recovered viral concentrations were $1.3x10^8$ genome copies/ml of EV and $1.0x10^6$ genome copies/ml of TCV (Table 5.2). Using the formulas above, the initial spiking concentration of each virus (in genome copies/ml) was determined to be $1.0x10^8$ genome copies/ml for TCV and $1.3x10^8$ genome copies/ml for EV. For TCV, the virus concentration in genome copies/ml was equivalent to the concentration in viruses/ml (i.e., $1.0x10^8$ viruses/ml = $1.0x10^8$ genome copies/ml). For

EV, the concentration in genome copies/ml was approximately 3-logs higher than the concentration in PFU/ml (i.e., $1.23x10^5$ PFU/ml = $1.3x10^8$ genome copies/ml).

2.3 Viral concentration experiments

2.3.1 Virus concentration in deionized water

To determine the concentration and extraction efficiencies of each virus without PCR inhibitors, both viruses were spiked into a set of triplicate 250-ml deionized water samples at a concentration of $1.9x10^7$ genome copies/100 ml, using the stock concentrations estimated in section 2.2. A fourth water sample was used as a negative control. All four water samples were filtered onto 47-mm diameter, 0.45-μm pore-size HA filters using a four-place filtration manifold and vacuum pump assembly with filter funnels (Millipore). Filters were aseptically transferred into sterile 2-ml screw-cap tubes and the RNA was immediately extracted as described in Section 2.2.1 and analyzed by RT-qPCR as described in Section 2.1.2.

2.3.2 Virus concentration in an environmental water sample

To determine the concentration and extraction efficiencies of each virus in water likely containing PCR inhibitors, each virus was spiked into water obtained from Morgan Creek in Chapel Hill, North Carolina, USA. The water was first tested for the presence of the two viruses by concentrating 250 ml onto an HA filter, extracting the RNA, and analyzing for the presence of target viruses using RT-qPCR as described in Section 2.3.1. The water sample was negative for both EV and TCV.

Both viruses were spiked into triplicate 250-ml environmental water samples at a concentration of $9.6x10^7$ genome copies/100 ml, using the stock volumes estimated in section 2.2. A fourth water sample was used as a negative control. All four water samples

were concentrated, and the RNA was extracted and analyzed with RT-qPCR as in Section 2.3.1.

2.3.3 TCV detection in sewage

The ability to detect TCV in wastewater was determined by spiking TCV into a wastewater sample obtained from a utility in Chapel Hill, North Carolina, USA. The influent sample was first tested for the presence of TCV by concentrating 100 ml onto an HA filter, extracting the RNA, and analyzing with RT-qPCR as in Section 2.3.1. The sewage sample was negative for TCV.

TCV was then spiked into 100 ml sewage at a concentration of $2.4x10^9$ genome copies/100 ml, using the stock volume estimated in section 2.2. The sample was concentrated, and the RNA was immediately extracted and analyzed using RT-qPCR as described in Section 2.3.1.

2.4 TCV detection in native samples

To determine whether TCV was present in surface waters from disperse geographic locations in the US, various samples were examined for the presence of TCV. Samples of 500 ml were shipped overnight on ice from Doheny Beach in Dana Point, CA; Lake Carroll in Tampa, FL; and South Shore Beach in Milwaukee, WI. Immediately upon arrival, 250 ml was concentrated, and the RNA was extracted and analyzed for TCV as described in Section 2.3.2.

To determine whether TCV was present in sewage influent from disperse geographic locations in the US, various samples were examined for the presence of TCV. Samples of 500 ml were shipped overnight on ice from one wastewater treatment utility near Oakland, CA; Tampa, FL; Milwaukee, WI; Chapel Hill, NC; Raleigh, NC; Tucson,

AZ; Columbus, OH; and from two facilities near Santa Ana, CA. Immediately upon arrival, 250 ml was concentrated, and the RNA was extracted and analyzed for TCV using RT-qPCR as described in Section 2.3.3.

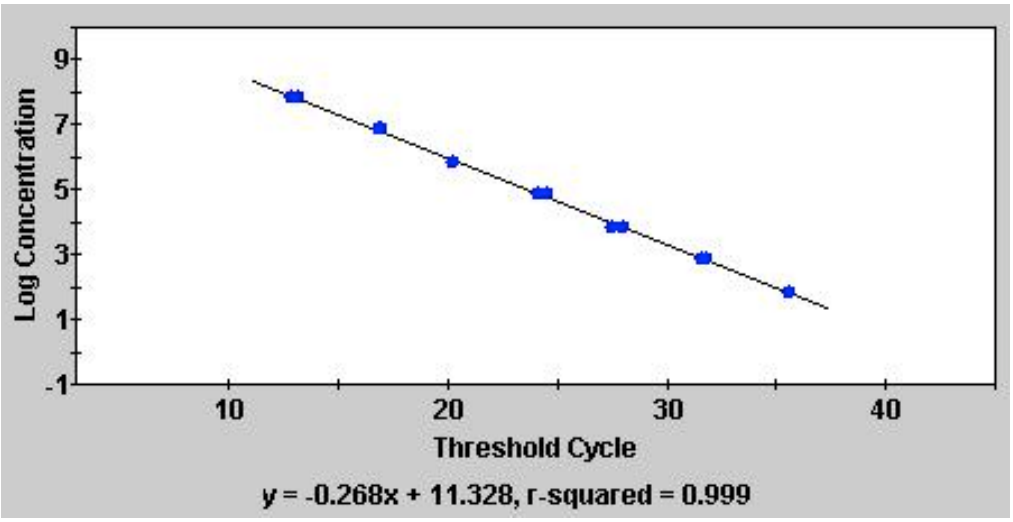
2.5 Statistics

Percent recovery (% recovery) was calculated by dividing the average final concentration (in genome copies/ml) in each sample by the initial concentration. An overall % recovery for each virus was calculated by averaging the % recovered in each sample. Percent loss (% loss) was calculated by subtracting the % recovery from 100%.

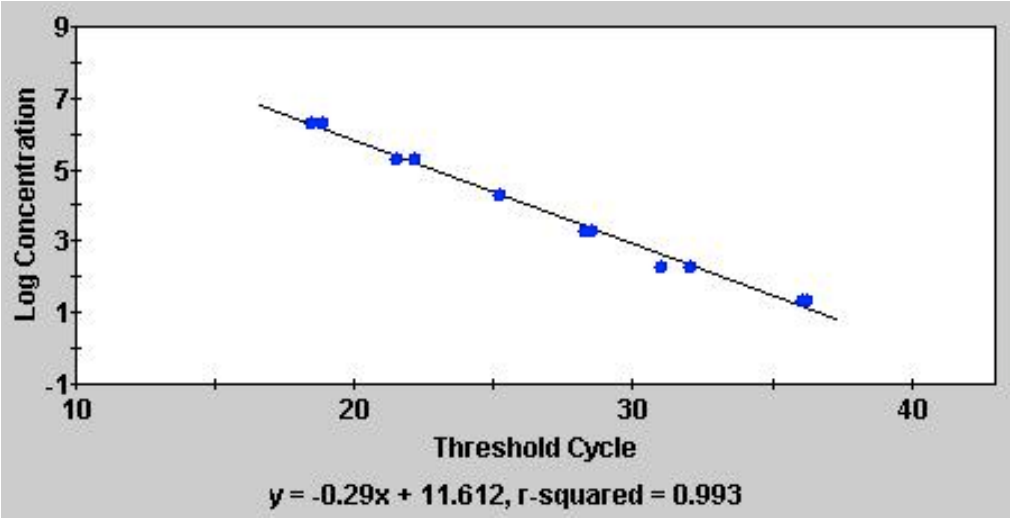
3. Results

3.1 RT-qPCR efficiency

The amplification efficiency and slopes of the echovirus 12 (EV) and turnip crinkle virus (TCV) standard curves were similar (Figs. 5.1a and 1b). Both standard curves showed a high linearity, with a coefficient of determination (R^2) of 0.999 for EV (Fig. 5.1a) and 0.993 for TCV (Fig. 5.1b) and amplification efficiencies of 92.6% and 97.5%, respectively. The dynamic range of the RT-qPCR assays was wide, ranging from 72 to $7.2x10^8$ genome copies (8 logs) for the EV standard and from 20 to $2.0x10^6$ genome copies (6 logs) for the TCV standard.



1a.



1b.

Figure 5.1. RT-QPCR Standard curves for (a) enterovirus using an Echovirus 12 DNA transcript and (b) turnip crinkle virus using a turnip crinkle virus DNA plasmid.

3.2 Determination of viral extraction efficiencies

Two sets of extraction experiments were performed. In the first set, EV was spiked at a concentration of $1.3x10^8$ genome copies/ml, and TCV was spiked at a concentration of $1.0x10^8$ genome copies/ml. The average recovered concentration for EV was $4.2x10^6$ genome copies/ml and the average recovered concentration for TCV was $1.2x10^7$ genome copies/ml (Table 5.2). A second set was then spiked with $5.5x10^9$

genome copies/ml of EV and $1.2x10^7$ genome copies/ml of TCV. The average recovered viral concentrations were $1.3x10^8$ genome copies/ml of EV and $1.0x10^6$ genome copies/ml of TCV (Table 5.2). The average % loss for EV was 97.2% and for TCV was 89.6% during extraction (Table 5.2). Negative extraction controls for both experiments were negative for EV and TCV.

Salmon sperm DNA (SKETA) was spiked into the first set of extraction samples at a concentration of 100 ng DNA/ml lysis buffer. The average recovered concentration was 15 ng DNA per ml, giving an average % loss for SKETA of 85% (Table 5.2).

3.3 Concentration and extraction efficiencies of viruses in water and wastewater samples

To determine the concentration and extraction efficiencies of each virus without inhibitors, each virus was spiked into deionized (DI) water samples at a concentration of $1.9x10^7$ genome copies/100 ml, using the stock volumes estimated in section 3.2. The average loss for EV was 99.96% and for TCV was 99.98% (Table 5.3). The lowest detectable spiking concentration for TCV in deionized water was $4.0x10^4$ genome copies/100 ml.

To determine the concentration and extraction efficiencies of each virus with inhibitors present, both viruses were spiked into environmental water samples at a concentration of $9.7x10^7$ genome copies/100 ml, using the volumes estimated in section 3.2. RNA was extracted from the filters and recovered concentrations for each virus were determined using RT-qPCR. The average loss for EV was 99.996% and for TCV was 99.991% in the environmental water sample (Table 5.4). The lowest detectable level of TCV in the environmental sample was $1.0x10^6$ genome copies/100 ml.

The ability to detect TCV in wastewater was determined by spiking TCV at a concentration of $2.4x10^9$ genome copies per 100 ml of wastewater influent obtained from a wastewater treatment facility in Chapel Hill, North Carolina. The average loss for TCV in sewage influent was 99.997%. The lowest detectable level of TCV in the sewage influent sample was $1.0x10^7$ genome copies/100 ml.

3.4 Presence of TCV in native water and wastewater samples

For TCV to be a useful surrogate control virus, it should not be present in environmental waters where it may be utilized. Surface water samples from several locations around the US, including Doheny Beach in Dana Point, CA; Morgan Creek in Chapel Hill, NC; Lake Carroll in Tampa, FL; and South Shore Beach in Milwaukee, WI were analyzed for the presence of TCV. All surface water samples were negative for TCV.

For TCV to be a useful surrogate control virus, it should not be present in wastewaters where it may be utilized. Wastewater influent samples from several locations around the US were examined for the presence of TCV. Wastewater influent samples from facilities in or near Oakland, CA; Santa Ana, CA; Tampa, FL; Milwaukee, WI; Chapel Hill, NC; Raleigh, NC; Tucson, AZ; and Columbus, OH were analyzed for the presence of TCV. All wastewater influent samples were negative for TCV.

4. Discussion

This study examined the ability of turnip crinkle virus (TCV), a plant Tombusvirus, to be utilized as a viral surrogate for human enteroviruses in concentration and extraction procedures from environmental water samples. TCV was examined for its

ability to (1) concentrate with a similar recovery to an enterovirus and (2) contain RNA that is extracted with a similar recovery to an enterovirus. TCV was also evaluated for its (3) absence from native water and wastewater samples. TCV was found to have a similar recovery to echovirus 12 (EV) when concentrated from sterile and environmental waters and was absent from all native waters and wastewaters examined, making it an ideal spike-and-recovery viral surrogate for human enteroviruses.

TCV was determined to have a similar recovery to EV when concentrated from a sample with no PCR inhibitors (DI water). Both viruses were estimated to have approximately 3-log losses, suggesting that adsorption of the virus to a cellulose acetate/nitrate (HA) filter during concentration and lysis of the virus capsid during extraction are similar for the two viruses. These results indicate that TCV is a suitable virus surrogate for EV, and likely a suitable virus surrogate for other human enteroviruses as they have similar sizes, morphologies, and genomes to echovirus 12. Thus, TCV could be used to quantitatively estimate enterovirus loss incurred during concentration and extraction steps when using the methods described herein. TCV may also be able to serve as a viral surrogate for other enteric viruses, including norovirus or hepatitis A or E, as these viruses have similar morphology and genome structure to enteroviruses. Future studies can utilize comparison studies similar to those undertaken in this report to determine whether TCV can serve as a viral surrogate to other enteric viruses of interest.

In addition to similar recoveries in deionized water, TCV and EV had similar recoveries in a water sample likely containing PCR inhibitors. Both viruses had losses of approximately 4 logs, suggesting that inhibitors present in the environmental sample acted similarly on both viruses. Because the cause of inhibition in this sample is unknown

(e.g., humic substances vs. proteins or tannic acids), it would be difficult to determine whether these inhibitors would be similar to those present in other environmental samples or whether they would affect correlations between TCV and enteric viruses. Comparison studies should be undertaken to determine if TCV will be applicable in target waters, and these studies can also determine what concentration of TCV will be applicable for spike-and-recovery in target waters. This study determined that a minimum concentration of $1.0x10^6$ genome copies/100 ml was necessary for use in environmental waters, given a 99.991% loss and the sample limit of detection determined for the environmental sample. However, a lower quantity may be more applicable with different concentration and extraction methods.

A surprising result of this study was the low recovery efficiency of the concentration and extraction methods: the general recovery (including concentration, extraction, and reverse transcription steps) was 0.03% for EV and 0.02% for TCV. These methods have been applied elsewhere to successfully detect enteric viruses (Conn, et al., 2011; Gregory, et al., 2006; Noble, et al., 2006). In fact, the recoveries found in this study are much lower than the concentration recovery published by Fuhrman et al. (2005), who estimated a general recovery of 51% ($r^2 = 0.99$) from 50-ml freshwater samples using similar methods. Differences in recovery efficiencies between the two studies are likely due to variations in the elution step. In this study, we quartered the HA filter and placed it directly into a 2-ml screw-cap tube for extraction according to Conn et al. (2011), whereas Fuhrman et al. (2005) placed the filter into a Whirl-Pak bag for the addition of lysis buffer and removed the liquid for RNA extraction. In preliminary tests, Fuhrman et al. (2005) found that folding the filter into quarters and placing into a microcentrifuge

tube for extraction yielded much lower recovery. The dramatic difference in recovery efficiencies due to small changes in detection procedures reaffirm the necessity of utilizing controls during all parts of the concentration and extraction process.

As opposed to the similarities in the general recovery efficiencies of the two viruses, their extraction efficiencies were less similar (although they were within 1 log of each other). Due to the differences in extraction efficiency, TCV should be utilized in conjunction with an RNA–based extraction control, as opposed to using TCV alone or with a DNA-based control. Utilization of both a spike-and-recovery surrogate and a nucleic acid extraction control are recommended by Santo Domingo et al. (2007) for achieving accurate quantification of microbial targets. RNA extraction controls such as β-actin RNA have been reported in the literature (Conn, et al., 2011), although this control was unavailable in the US at the time of this study.

This study used a relative quantification method to estimate the virus stock concentrations and initial spiked virus concentrations in terms of genome copies/ml. For EV, the concentration was 3 logs higher using the molecular-based quantification method than the culture-based method. These results are not surprising, as previous studies have demonstrated that viral quantification using different methods can result in drastically different estimated concentrations due to the presence of non-infectious viruses. This phenomenon is generally referred to as the particle-to-PFU ratio, and for different viruses this ratio can be anywhere from 1:1 to 10,000:1 (Racaniello, 2007). Previous studies have demonstrated that one enterovirus, poliovirus, can have a particle-to-PFU ratio of anywhere from 1:1 to 1,000:1 (Racaniello, 2007). Because different quantification

methods can give drastically different measures of virus quantity, similar quantification techniques should be utilized for both spiking and recovery concentrations.

The discrepancy between the two EV quantification methods may also be due to the presence of naked RNA present in the virus stock. This seems to be supported by the fact that while the general (including concentration) recoveries between TCV and EV were similar, the extraction recoveries were more different (although they were still within 1 log of each other). However, the extraction experiments were used to estimate the quantity of viruses used for spiking the concentration experiments. If naked RNA had been included in the estimated viral quantifications, this value would have resulted in a significantly lower concentration efficiency for EV than for TCV, as naked RNA would not have adsorbed to the filter during concentration experiments. Because the two concentration efficiencies were similar, this is not a likely explanation for the discrepancy between the two quantification methods. Regardless, studies could include RNase, which eliminates naked RNA, followed by an RNase inhibitor, before concentration and extraction experiments. This method was utilized successfully by Mormann et al. (2010) as a pretreatment step to allow for detection of intact virus particles.

As opposed to the discrepancy between the EV quantification methods, the method used to quantify TCV using a spectrophotometer produced a quantity that was equivalent to the quantity estimated by molecular methods (RT-qPCR). This is surprising, as this method is not commonly utilized for quantification of enteric viruses, most likely due to a lower density of enteric viruses in samples (a concentration of at least $1x10^{13}$ viruses/ml was required for this method) than densities possible with plant viruses. Because similar quantities of TCV are achieved when using both quantification

methods, this virus is an ideal spike-and-recovery virus surrogate. Furthermore, TCV can be easily grown in typical greenhouse conditions and isolated with well-documented methods. This ease of production and quantification make TCV a better candidate than armored RNA, which can be expensive to manufacture. Moreover, because TCV is a plant virus, it may be safer than animal viruses such as murine or porcine norovirus for consistent use in the laboratory.

We demonstrate here that TCV will serve as an excellent control for the detection of enteroviruses from US waters. All of the environmental water and wastewater samples investigated in this report were negative for the virus. No samples from outside the US were investigated for the presence of TCV, although it is likely that TCV can serve as a positive control for a broader geographic area, as reported spreads include only the UK and the former Yugoslavia. Nevertheless, environmental samples from different geographic areas should be tested for the virus before its use as a quantitative control.

The development of recovery controls has been recognized as a need for providing reliable detection and quantification of microbes when processing environmental samples for water quality monitoring (Santo Domingo, et al., 2007; USEPA, 2007). Most quality control efforts, however, have been directed toward the construction of amplification efficiency controls for PCR (e.g., Gregory, et al., 2006; Gregory, et al., 2010; Hata, et al., 2011). The incorporation of the virus surrogate presented here is recommended in conjunction with amplification efficiency controls for standardizing the detection and quantification of enteric viruses from environmental samples.

Conclusions

The development of recovery controls for processing environmental samples has been recognized as a need for providing reliable and quantitative data for water quality monitoring, yet most quality control efforts have focused on amplification efficiency controls for PCR. This study determined that a plant virus, turnip crinkle virus (TCV) can serve as a spike-and-recovery virus surrogate during concentration and extraction procedures. Because these steps can have low recovery efficiencies, as demonstrated in this study, a recovery control is absolutely necessary for confidence in detection and quantification of viruses from environmental samples. TCV, being absent from US waters and wastewaters and demonstrating similar recovery efficiencies to human enteroviruses, is recommended for this purpose.

Table 5.1. Primer and probe identities, target genes, sequences, and amplicon lengths for qPCR assays used in this study.

Primer/Probe	Target	Sequence (5'-3')	Size (nt)	Source
EV1F	Enteroviruses	CCCTGAATGCGGCTAAT	143	Gregory et al. (2006)
EV1R		TGTCACCATAAGCAGCCA		
EV probe		[FAM]ACGGACACCCAAAGTAGTCGGTTC[BHQ-1]		
EchoFwd	Echovirus 12	GCGTTTCGCTCCGCACAACC	509	This study
EchoRev		CAGGCCAGTCTCGTGTGCCC		
TCV-F	Turnip Crinkle Virus	CCTCTGACTTCTCGGTCCTG	109	This study
TCV-R		CTGCTCCTCAGTTGTGACCA		
TCV-Probe		[FAM]AGGTAGTGTCCAATGGGCTG[BHQ-1]		

Table 5.2. Viral concentrations used for spiking, recovered concentrations post-spiking, percent lost (% loss), and percent recovered (% recovered) for extraction and RT-qPCR of echovirus 12, turnip crinkle virus, and salmon sperm DNA.

Organism	Spiked Concentration[a]	Recovered Concentration[a]	% Loss	% Recovered
EV	$5.5x10^{9}$	$1.3x10^{8}$	97.6	2.4
Std Dev		$9.7x10^{7}$		
EV	$1.3x10^{8}$	$4.2x10^{6}$	96.8	3.2
Std Dev		$1.8x10^{6}$		
TCV	$1.0x10^{8}$	$1.2x10^{7}$	87.6	12.4
Std Dev		$2.1x10^{6}$		
TCV	$1.2x10^{7}$	$1.0x10^{6}$	91.5	8.5
Std Dev		$1.4x10^{5}$		
SKETA	$1.0x10^{2}$	$1.5x10^{1}$	85.3	14.7
Std Dev		$9.6x10^{0}$		

[a] In genome copies/ml for EV and TCV or ng DNA/ml for SKETA
EV = echovirus, TCV = turnip crinkle virus

Table 5.3. Viral concentrations used for spiking, recovered concentrations post-spiking, percent lost (% loss), and percent recovered (% recovered) for concentration in deionized water, extraction, and RT-qPCR of echovirus 12 and turnip crinkle virus.

Virus	Spiked Concentration[a]	Recovered Concentration[a]	% Loss	% Recovered
EV	$1.9x10^7$	$6.5x10^3$	99.97	0.03
Std Dev		$2.8x10^3$		
TCV	$1.9x10^7$	$3.8x10^3$	99.98	0.02
Std Dev		$1.5x10^3$		

[a] In genome copies/ml

EV = echovirus, TCV = turnip crinkle virus

Table 5.4. Viral concentrations used for spiking, recovered concentrations post-spiking, percent lost (% loss), and percent recovered (% recovered) for concentration in environmental water, extraction, and RT-qPCR of echovirus 12 and turnip crinkle virus.

Virus	Spiked Concentration[a]	Recovered Concentration[a]	% Loss	% Recovered
EV	$9.7x10^7$	$3.6x10^3$	99.996	0.004
Std Dev		$4.6x10^2$		
TCV	$9.7x10^7$	$8.8x10^3$	99.991	0.009
Std Dev		$1.4x10^3$		

[a] In genome copies/ml

EV = echovirus, TCV = turnip crinkle virus

Table 5.5. Viral concentration used for spiking, recovered concentration post-spiking, percent lost (% loss), and percent recovered (% recovered) for concentration in sewage influent, extraction, and RT-qPCR of turnip crinkle virus.

Virus	Spiked Concentration[a]	Recovered Concentration[a]	% Loss	% Recovered
TCV	2.42×10^{9}	1.67×10^{5}	99.997	0.003
Std Dev		9.35×10^{4}		

[a] In genome copies/ml
TCV = turnip crinkle virus

Chapter 6: Conclusions

Given the need to identify the source of fecal pollution in surface waters impacted by nonpoint source (NPS) pollution, as well as the inability of fecal indicator bacteria (FIB) to indicate the source, a wide range of microbial source-tracking (MST) methods have been developed. However, water quality monitoring has traditionally been based on FIB, and the US EPA will likely continue its recommendation of FIB, specifically enterococci and *E. coli*, as recreational water quality standards in October of 2012 (USEPA, 2012). Thus, much of the recent research in this field has focused on supplementing the traditional FIB with the new MST assays. However, it is not yet clear how MST assays relate to measurements of FIB in natural waters impacted primarily by NPS pollution. The overall objective of this study was to determine whether MST markers can provide information for source allocation and human pathogen presence in the Cape Fear watershed in North Carolina, a diverse watershed with both highly urbanized areas as well as large regions of industrialized agriculture, and whether the MST markers can be utilized in a tiered assessment with FIB. This overall objective was divided into 3 individual objectives:

1. Elucidate the spatial and temporal trends of source-specific MST markers in order to prioritize areas for targeted remediation in a mixed-use watershed.
2. Identify whether sites with intense exposure to NPS from agricultural sources may pose human health risks due to the presence of

microorganisms of public health concern in order to inform targeted best management practices.

3. Compare MST markers to FIB indicators to determine whether MST markers can be used in a tiered assessment.

The first objective of this research was to elucidate the spatial and temporal trends of source-specific MST markers in order to prioritize areas for targeted remediation in a mixed-use watershed. The results of chapter 2 confirmed that two human-source markers, *Methanobrevibacter smithii* and norovirus genogroup II, could help prioritize specific areas for remediation by elucidating the spatial and temporal trends of human-source fecal pollution.

The *nif*H marker was positively associated with increasing development and impervious surfaces. Higher concentrations of the human-specific MST marker in more urbanized watersheds suggest that land use changes associated with development, as well as increases in watershed impervious cover, affect water quality and may also result in an increasing human health risk. Water quality has been shown to be impacted by increasing development in studies examining traditional FIB in this watershed (Rowny, 2011) and examining FIB and coliphage concentrations along tidal creeks (DiDonato, et al., 2009). The results from this study suggest that environmental monitoring schemes utilizing the *nif*H qPCR assay could indicate areas for targeting appropriate remediation strategies for human fecal contamination.

Norovirus genogroup II (NoV GII) was detected twice during the study, and its presence, together with the *nif*H MST marker, suggests specific sites in our study with potentially greater health risks that were not indicated by FIB analysis. Both NoV GII-

positive samples were detected at 3.0 copies per 100 ml (30 copies per L) and were detected at sites immediately downstream from wastewater treatment plants (sites 5 and 7). Our results indicate a risk for NoV illness for these two sites using a report by Schoen and Ashbolt (2010), which estimated that approximately 9 NoV genomes per L would yield a risk of 0.03 GI illnesses per swim event. Further, as these tributaries feed into B. Everett Jordan Lake, a waterbody used for drinking water purposes, inadequate or failing treatment processes could lead to insufficient removal of the viral pathogens from source waters.

The second objective of this study was to identify whether sites with intense exposure to NPS from agricultural sources may pose human health risks due to the presence of microorganisms of public health concern in order to inform targeted best management practices. The results of chapter 3 confirmed that sites with intense exposure to NPS from specific sources of agricultural contamination may pose human health risks due to the presence of presumptive antibiotic-resistant *Staphylococcus* (PARS) and hepatitis E virus (HEV). Moreover, the study demonstrated that coliphages are useful for indicating the source of fecal pollution and for indicating the presence of potential viral pathogens, information required for informing best management practices.

A high percentage (92%) of water samples in Chapter 3 were positive for PARS, at a geometric mean concentration 18.0 CFU per 100 ml (1.7, 308.0) across all sites. Antibiotic-resistant *Staphylococcus*, specifically methicillin-resistant *S. aureus* (MRSA), has been cultured from swine nasal swabs in the United States (Smith, et al., 2009) and abroad (Cui, et al., 2009; de Neeling, et al., 2007; Huijsdens, et al., 2006; Khanna, et al., 2008; Lewis, et al., 2008; Pomba, et al., 2009), but it has not been evaluated in swine

waste. Our research suggests that there is a possibility of transfer of PARS from farms to surrounding waters. Movement from swine lagoon spray fields to the environment would represent an additional transmission route of antibiotic-resistant *Staphylococcus*, in addition to that between agricultural animals and associated workers (Cui, et al., 2009; Khanna, et al., 2008; Smith, et al., 2009; Van Cleef, et al., 2010) and to food products (Pesavento, et al., 2007; Pu, et al., 2009; van Loo, et al., 2007). Movement of antibiotic-resistant *Staphylococcus* to the environment may also promote the horizontal transfer of resistance genes within the environment.

In contrast to PARS, HEV was detected only once during the study. To our knowledge, this is the first study to examine the presence of HEV in creeks impacted by swine CAFO waste over time. Previous studies have detected HEV in swine (Choi, et al., 2003; Dell'Amico, et al., 2011; Huang, et al., 2002; Kase, et al., 2008; Takahashi, et al., 2003) and swine lagoons (Kasorndorkbua, et al., 2005; McCreary, et al., 2008; Pina, et al., 2000). Only two other studies have examined impacted surface waters for HEV (Karetnyi, et al., 1999; Kasorndorkbua, et al., 2005), but neither examined water samples during multiple seasons. One of these studies (Karetnyi, et al., 1999) detected HEV in a tile outlet draining a field to which manure had been applied. Because HEV in developed countries is thought to be predominantly from zoonotic origins (when travel to developing countries can be ruled out) (Nelson, et al., 2011), HEV in this sample most likely originated from swine. This hypothesis is further supported by the presence of three swine-specific microbial source-tracking markers in this sample (Myers, 2011). The human health risk of swine HEV in surface waters is as yet unknown, as zoonotic transmission of HEV has not yet been demonstrated. Nevertheless, previous research has

suggested the possibility of zoonotic transmission routes for HEV (Meng, 2009; Pavio, et al., 2008), and a systematic literature review found that in industrialized countries zoonotic transmission seemed likely (Lewis, et al., 2010).

While the presence of HEV did not correspond to the presence of F-RNA coliphages in our samples, somatic coliphage concentrations were higher in this sample than all other samples. Previous studies have found correlations between somatic coliphages and adenoviruses (Aw and Gin, 2010), enteroviruses (Mocé-Llivina, et al., 2005), and culturable enteric viruses (Payment and Franco, 1993). These results reaffirm the need for improved detection and quantification methods for viruses in environmental media, but also suggest that elevated somatic coliphage concentrations may be able to indicate the presence of HEV in environmental waters. F-RNA genotyping also aided in determining the presence of animal fecal contamination, likely swine, in the surface waters adjacent to swine lagoon spray fields. Monitoring of FIB alone would not have been sufficient to predict the presence of HEV and PARS or the source of fecal contamination.

In summary, the results of Chapters 2 and 3 indicate that MST markers provide valuable information on the spatial and temporal trends of specific sources of fecal contamination and can indicate specific areas that have a high likelihood of contributing human pathogens to surface waters. Specifically, in Jordan Lake watershed, MST markers indicated that increasingly urbanized areas were greater contributors of human fecal contamination than the more agricultural or forested areas. Moreover, MST markers indicated specific sites, downstream of wastewater treatment plants and downstream of the University of North Carolina campus, that may be impacted by human-source fecal

contamination. Detection of MST markers, including enteric viruses, suggests that these sites be more closely monitored for human pathogens. Conversely, in the eastern part of the Cape Fear Basin, which is impacted by a dense concentration of swine CAFOs, the presence of PARS and HEV, as well as the ubiquity of coliphages, suggests that current waste management practices may be associated with the dissemination of microorganisms of public health concern in waters proximal to CAFO spray fields. Inclusion of coliphages in this study aided in determining a major source of fecal pollution, using F-RNA coliphage genotyping, and indicated the presence of HEV, information required for informing best management practices. The results of these chapters illustrate that MST markers can be used to improve and prioritize remediation projects and provide a higher level of information toward decision making processes aimed at protecting human health.

The third objective of this research was to determine if the FIB and MST markers could be utilized in a tiered assessment by comparing the presence and concentrations of each using data from Objectives 1 and 2.

Coliphages were utilized for both Objectives 1 and 2 (Chapters 2 and 3, respectively), and data from both suggests that somatic and F+ coliphages were correlated to FIB. In chapter 2, somatic and F+ coliphages were positively correlated with both fecal coliforms and *E. coli*. In chapter 3, somatic coliphages were positively correlated to *E. coli*, and F+ coliphages were positively correlated to fecal coliforms. These results suggest that somatic and F+ coliphages can be utilized in combination with FIB in a tiered assessment. Inclusion of these coliphages in a monitoring program may better indicate the presence of enteric viruses, such as norovirus or adenovirus, than FIB

alone (Chapter 1, Section 3.2). Conversely, the F-RNA used for identifying sources of fecal pollution in this study were not correlated to the FIB. Results indicated that using FIB indicators, 73%, 40%, and 7% of the F-RNA-positive samples would not have been flagged by concentrations of fecal coliforms, *E. coli*, and enterococci, respectively, above regulatory thresholds. This data indicates that F-RNA genotyping could possibly be utilized in a tiered assessment with *E. coli* and enterococci, as the % of F-RNA positive samples that was flagged by these two FIB was above 50%. The addition of F-RNA to a tiered assessment would be useful for determining the sources of fecal pollution present and may aid in discriminating between animal and human sources of fecal contamination (Chapter 1, Section 3.2). Nevertheless, the specificity of the F-RNA genotyping assay utilized in this research has not yet been evaluated. The ability of this assay to correctly discriminate between sources will provide an important piece of information for deciding whether F-RNA genotyping can be utilized in a tiered assessment with FIB.

In addition to coliphages, several human-specific MST markers, including two anaerobic microbes, *Bacteroides* sp. and *M. smithii*, and two enteric viruses, enterovirus and norovirus, were utilized for Objective 1.

The *Bacteroides* sp. marker (HuBac) was correlated to both fecal coliforms and *E. coli*. This was expected as *Bacteroidales* markers are frequently used for apportioning the sources of FIB in water bodies. For example, Reischer et al. (2008) utilized a ruminant-specific *Bacteriodales* marker (BacR) to explain the variance in *E. coli* during high-flow events in a karstic spring. A strong correlation between the two markers allowed the authors to explain 72-80% of the *E. coli* variance. The idea of utilizing MST markers to predict, or index, the concentration of FIBs in water bodies is attractive. In fact, this

objective has been the main driving force for the development of MST technologies, as it would allow water managers to better implement TMDLs, which are based upon reducing FIB concentrations. For this use, nonparametric correlations between MST markers and FIBs, such as those utilized in this research, are not sufficient because, while nonparametric correlations can suggest an association, they cannot describe the proportion of the variance in the regulatory parameters of interest (i.e., FIB). Utilization of MST markers for TMDL purposes instead requires parametric correlations/regressions to quantify the variance in FIB concentrations explained by MST results (Kinzelman, et al., 2011). Currently, only a few studies have reported a parametric relationship between MST and FIB at the catchment scale, and in several instances *Bacteroidales* markers and FIB were not correlated (e.g., Sauer, et al., 2011; Stapleton, et al., 2009).

The positive correlation between FIB and HuBac indicates that the HuBac marker could be utilized in combination with FIB in a tiered assessment to identify and prioritize areas for remediation. This was expected, as *Bacteroidales* markers are the most frequently used markers for identifying fecal pollution sources, and markers are available that can detect a wide array of sources, including human, dog, ruminant, bovine, equine, elk, and gull fecal pollution. Moreover, statistical approaches have been designed to better determine the relative contribution of each source and to account for individual marker inaccuracies (Kildare, et al., 2007; Wang, et al., 2010).

However, the HuBac marker did not appear to be specific to human sewage in our study. This observation is supported by reports that found the HuBac marker is not specific to human waste; one study found the HuBac marker to cross-react with multiple animal fecal samples, including cattle, swine, sheep, horse, dog, duck, and kangaroo

samples (Ahmed, et al., 2009). Thus, the HuBac marker did not provide sufficiently different or additional information from the FIB in our study and would not be useful in a tiered assessment for identifying human fecal pollution. Other *Bacteroidales* markers that may be useful in a tiered assessment are listed in Table 1.1.

As opposed to the HuBac marker, the human-specific *M. smithii* marker (*nif*H) was not correlated to either FIB evaluated. In fact, less than 50% of the samples positive for the *nif*H marker would have been indicated by levels of fecal coliforms or *E. coli* above regulatory thresholds. This data suggests that the *nif*H marker cannot be used in a tiered assessment with FIB.

A lack of correlation between the *nif*H marker and FIB may be due to the contribution of non-human and natural sources of FIB in the watershed. This hypothesis is consistent with previous reports that found levels of FIB in waters with minimal or no known human influence were sufficient to exceed water quality thresholds (Griffith, et al., 2010). Conversely, previous studies have found the *nif*H marker (using conventional PCR) to be 96-99% specific to human feces (Harwood, et al., 2009; Kirs, et al., 2011; McQuaig, et al., 2009).

Lack of correlation between FIB and *nif*H in Chapter 2 was also surmised to be partly due to the influence of inhibition caused by dissolved organic matter (DOM) in the water samples. Results from evaluating Objective 4 indicated that particular DOM components may simultaneously influence both culture-based FIB measurements used for water quality monitoring and qPCR measurements, such as those used for MST.

As a reminder, Objective 4 evaluated whether the level of inhibition detected in select surface water samples was related to the presence of detectable dissolved organic

matter components. Results indicated that QPCR inhibition was correlated to several DOM components, predominantly terrestrially derived, humic acid-like DOM components and microbially derived, fulvic acid-like DOM components. Specifically, qPCR inhibition was correlated to several humic-substance DOM components, including C1, C2, C3, and C4. The importance of these DOM components on qPCR inhibition is supported by the results of Rock et al (2010), who found that both fulvic acid, microbial by-product-like (e.g., C3) and humic acid-like compounds (e.g., C2) corresponded to increases in qPCR inhibition better than other DOM components examined.

Interestingly, Wang (2011) determined that two of these components, C2 and C3, were also positively correlated to FIB concentrations and were proposed to serve as ecological controls for FIB. These results imply that specific complexes of humic substances may affect FIB levels and MST qPCR assays in opposing ways, suggesting that DOM is partly responsible for the discrepancy between (culture-based) FIB and (qPCR-based) MST markers in this watershed.

Promisingly, Objective 4 also demonstrated that PARAFAC modeling of EEMs may be able to determine whether a chosen nucleic acid extraction method could address these inhibitors. Results of Objective 4 suggested that the extraction method used in Chapter 2 (MoBio PowerSoil kit) preferentially removed the fulvic acid complexes of C3 over humic acid complexes, such as those in C1 or C2. The hypothesis that various nucleic acid extraction methods disproportionately remove particular types of humic substances over others is supported by Rock et al. (2010), who found that an extraction method they examined was less efficient at removing aquatic/marine humic acids than other types of humic material. These results could have important implications for

environmental sample processing. It is well accepted that PARAFAC modeling of EEMs provides information on the humic substance present in environmental samples, and the results of this study suggest that tracing the different types of humic substances through the extraction process using PARAFAC could yield new insight into which extraction method maybe best suited to removing those components.

Moreover, these results have implications on the utilization of both FIB and MST measures in water quality monitoring. These results suggest that utilizing similar methods for both measures (e.g., both culture-based or non-culture-based) could aid in improving the correlation between the two types of markers. This is in line with previous research that demonstrated an improved correlation between *E. coli* and *Bacteroides* sp. when similar methods (qPCR) were utilized (versus culture and qPCR) (Sauer, et al., 2011). The disparity between culture-based on non-culture based methods has been thought to be due to differential attenuation of whole microorganisms (enumerated by culture-based methods) versus nucleic acids (enumerated by molecular methods), such as during wastewater treatment (Stapleton, et al., 2009). However, this research suggests that DOM inhibitors will also impact this correlation and provides further support for the use of similar methods when utilizing multiple microbial methods for water quality monitoring.

The last human-source MST markers utilized for Objective 1 were enteric viruses, including enterovirus and norovirus. Low levels of detected viruses prevented comparisons with FIB. In Chapter 2, norovirus genogroup II (NoV GII) was detected only twice, and enterovirus and norovirus genogroup I (NoV GI) were never detected. This was surprising, given that human enteroviruses have been found in waters associated with human fecal contamination (Griffin, et al., 1999; Jiang, et al., 2001; Noble and

Fuhrman, 2001), and noroviruses have been detected in treated wastewaters and surface waters (Astrom, et al., 2009; Lodder and de Roda Husman, 2005). The reason for the non-detects may have been due to the high detection limits for both viruses (a detection limit of >300 viral genomes per reaction for enterovirus and >200 viral genomes per reaction for NoV GI). Low detection rates of these viruses indicate that more work is needed to improve methods for concentration of enteric viruses from complex, environmental samples and to increase the sensitivity with which pathogens can be detected from water.

Thus, the goal of Objective 5 was to develop and demonstrate proof-of-concept for a novel process control for recovery of enteric viruses from water. The development of recovery controls has been recognized as a need for providing reliable detection and quantification of microbes when processing environmental samples for water quality monitoring (Santo Domingo, et al., 2007; USEPA, 2007). Most quality control efforts, however, have been directed toward the construction of amplification efficiency controls for PCR (e.g., Gregory, et al., 2006; Gregory, et al., 2010; Hata, et al., 2011). This study aimed to provide a proof-of-concept for the use of turnip crinkle virus (TCV), a plant Tombusvirus, to be utilized as a viral surrogate for human enteroviruses in concentration and extraction procedures from environmental water samples.

Results from Objective 5 indicated that TCV has a similar recovery to echovirus 12 (EV), a representative human enterovirus, when concentrated from samples with no PCR inhibitors (DI water) and from environmental water samples likely containing PCR inhibitors. These results suggest that adsorption of the virus to an HA filter during concentration and lysis of the virus capsid during extraction are similar for the two

viruses. Moreover, inhibitors present in the environmental sample likely act similarly on both viruses. These results indicate that TCV is a suitable virus surrogate for EV, and likely a suitable virus surrogate for all human enteroviruses as they have similar sizes, morphologies, and genomes to echovirus 12. Thus, TCV can be used to quantitatively estimate enterovirus loss incurred during concentration and extraction steps when using the methods described. TCV may also be able to serve as a viral surrogate for other enteric viruses, including norovirus or hepatitis A or E, as these viruses have similar morphology and genome structure to enteroviruses.

Results from Objective 5 also indicated that TCV can serve as a control for the detection of enteroviruses from US waters, as all of the environmental water and wastewater samples investigated in this report were negative for the virus. No samples from outside the US were investigated for the presence of TCV, although it is likely that TCV can serve as a positive control for a broader geographic area. Reported spreads of the virus include only the UK and the former Yugoslavia.

At present, reliable quantification of viral targets is hampered by losses occurring during the concentration and extraction stages. Yet accurate quantification of viruses would be useful for making informed decisions on the sources of contamination and for prioritizing impaired systems. Use of TCV as a viral processing control will improve the accuracy of viral detection methods and with the use of improved detection and quantification methods enteric viruses can be reliably used as MST markers.

In conclusion, the results of this research suggest that MST markers are necessary for identifying and prioritizing areas with a high likelihood of contributing human pathogens to surface waters, but that they cannot be easily utilized in a tiered approach

with culture-based FIB. As it is unlikely that these two types of indicators will be coupled in any natural system, a tiered approach is not appropriate for regulatory monitoring. These two types of markers provide very different types of information, and MST markers provide valuable information for determining the source of fecal contamination. Thus, incorporation of MST markers with traditional FIB in a toolbox approach may be more suitable.

Alternatively, with these markers in a management toolbox, it may be more practical to work towards a complete paradigm shift in monitoring, wherein MST markers serve as a principal measure of water quality. The MST markers could in turn be followed by direct pathogen analyses, which is increasingly feasible through the development of molecular methods and the improvement of processing techniques. However, this paradigm shift will not be possible until several advancements have been made in the field of MST. First, the precision and reproducibility of MST methods will need to be validated across multiple laboratories, across wide geographical areas, and the MST markers will need to eventually be validated by international standards organizations in the EU and USA (Kinzelman, et al., 2011). Second, before implementation as a baseline-monitoring tool, epidemiological studies that correlate MST markers to health outcomes will be required. Lastly, efforts to improve our confidence in the quantification abilities of MST markers will be necessary. These efforts will include development of algorithms for calculating source-specific loads (utilizing host distribution and relative abundance of markers in hosts and non-hosts) and improvement of processing and detection methods to quantify losses due to filtration and extraction as well as PCR inhibition (Santo Domingo, et al., 2007). Much of this work is currently

being done, but it will likely take a large evidence base before the regulatory community will consider replacing FIBs as the recommended standards for environmental waters. Nevertheless, this should not detract from the significant potential utility of MST markers as tools for water quality management. This report presents only a small sample of the peer-reviewed literature evidence base that MST markers offer useful insight into fecal contamination source.

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