

Population structure and persistence of *Escherichia coli* in ditch sediments and water in
the
Seven Mile Creek Watershed

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DEDICATION

To my family: Chandrasekaran, Padma, Anooreka and Suresh Renganathan for their love and support.

ABSTRACT

Escherichia coli is currently used as an indicator of fecal contamination in freshwater systems based on the assumption that it does not grow or survive outside of the host. However, recent studies have reported the growth and persistence of *E. coli* in various habitats such as soil, sands and sediments. While the majority of studies examining the growth and survival of *E. coli* in sediments have been performed primarily under laboratory conditions, there is, however, only limited field investigations concerning the growth and survival of this bacterium in sediments and its partitioning to overlaying water. Furthermore, the contributions of sediment adapted *E. coli* to water quality impairment in ditches or tile lines have not yet been assessed. Therefore, in this study, we examined the population structure of *E. coli* and determined whether ditch sediments can serve as reservoirs of environmental *E. coli* in the Seven Mile Creek (SMC) watershed, a minor watershed located in south central Minnesota. We also examined the potential factors that might influence the spatial and temporal distribution of *E. coli* populations present in the SMC watershed. Horizontal, fluorophore-enhanced, repetitive extragenic palindromic-PCR (rep-PCR) DNA fingerprinting technique (HFERP) was used to examine the population structure of *E. coli* in the SMC watershed, and host source-specific PCR assays targeting *Bacteroidetes* were used to identify potential host sources of fecal bacteria in the SMC watershed. Results of these studies indicated that seasonal variation in the population density of *E. coli* is likely influenced

by parameters such as temperature and flow events (rainfall and runoff events). Furthermore, we report that despite an annual shift in population structure, viable *E. coli* strains were isolated repeatedly from temperate ditch sediments and water across all three years. Therefore, sites likely contain a mixture of newly acquired and resident *E. coli* strains. HFERP fingerprint analyses indicated that the resident strains were likely growing in sediments and surviving through the winter freeze-thaw cycles. Sediment-borne *E. coli* strains had HFERP DNA fingerprints that were unique to this location and distinct from known sources of *E. coli* (from fecal origin), suggesting that these *E. coli* strains became naturalized to the SMC sediments. Furthermore, multivariate analysis of variance (MANOVA) indicated that there was mixing of bacteria between sites during flow conditions, which likely influenced the distribution and mobility of naturalized *E. coli* within the watershed. Additionally, mixing occurred between the sediment and water column, and this may have resulted in the re-suspension of sediment-borne naturalized *E. coli* into the overlaying water, leading to an apparent increase in the observed levels of *E. coli*. These results suggest that ditch sediments are temporal sinks of *E. coli* and that these indigenous populations might contribute significantly to population levels of *E. coli* measured in water quality studies. This confounds the use of *E. coli* as an indicator of fecal contamination of waterways, and has implications for future water quality monitoring and TMDL determinations.

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Chapter 1. General Introduction

1.1. Contamination of waterways

Large numbers of water bodies in the United States are considered to be impaired because of contamination by pathogenic microorganisms. According to a 2004 water quality report from the U.S. Environmental Protection Agency (U.S. EPA), pathogens were the dominant cause of river and stream impairments in the United States (132). Contamination of waterways by pathogens still continues to be one of the major public health concerns in both developed and developing countries. A recent report by the World Health Organization (WHO) revealed a strong correlation between water quality and public health (141). Several water-borne pathogens have been associated with various disease outbreaks in the past (80, 88, 108, 116, 124, 135). Among these, zoonotic pathogens that are transmitted between animals and human are of major concern. Some well known zoonotic pathogens that arise as a result of poor drinking water management practices are *Campylobacter jejuni*, *Giardia lamblia*, *Cryptosporidium parvum*, *Yersinia enterocolitica*, *Salmonella* and pathogenic *Escherichia coli* (*E. coli* O157:H7). Studies have also reported the presence of newly emerging pathogenic environmental bacteria such as *Mycobacterium* spp., *Legionella* spp., and *Pseudomonas aeruginosa*, which can grow in water distribution systems (7, 42, 85, 145). These pathogens predominantly spread through contaminated drinking-, recreational-, or shellfish waters (23, 109, 129).

More than 61% of known human pathogens and the majority of emerging human pathogens are zoonotic (129). A potential source of these pathogens in waterways is the feces of humans, populations of domestic and wildlife animals (30). Hence, fecal pollution is generally regarded as the major contributor of pathogens to waterways. Several studies have shown that exposure to waters with elevated fecal bacterial counts correlates with an increased risk of gastrointestinal disease (18, 40, 107, 116, 124, 134). In addition to causing extensive human suffering and/or death, infection by pathogens also leads to significant economic losses (129). Economic loss can be in terms of the cost associated with medicine, treatment, hospitalization, and loss of productivity. For instance, in 1993, the outbreak of cryptosporidiosis in Milwaukee resulted in the loss of \$96 million for the community (29). In addition, significant economic loss can also be incurred by the closing of beaches and fisheries, when waters do not meet U.S. EPA standards (4).

1.2. Sources of fecal contamination

Sources of fecal contamination that typically contribute to pathogens in waterways include humans, livestock, and domesticated animals, agriculture, wildlife, and urban runoff/storm water. Identifying potential source(s) of fecal contamination and their contribution is essential for the assessment of health risks associated with exposure to a contaminated water body. Contaminants can be discharged into lakes, streams, coastal and ground water from either a single, identifiable and localized source (point source pollution) or from many diffuse sources (nonpoint source pollution). It is important to

distinguish between point and nonpoint sources of fecal pollution if cost-effective and directed remediation strategies have to be implemented.

1.2.1. Human and livestock

Human fecal pollution can result from point sources such as effluent from wastewater treatment plants, untreated sewage from sewer overflows and wastewater injection wells or from non-point sources such as septic tank leakage or human recreation (31, 39, 109). Fecal pollution of human origin is of utmost concern since it is thought to more likely contain human-specific enteric pathogens. However, recent reports show that feces of domesticated animals also carry pathogens that infect humans (30, 89, 137). This has placed a lot of emphasis and attention on domestic animal management.

1.2.2. Agriculture

Some enteric pathogens present in animal feces enter waterways as a result of agricultural activities. According to the National Water Quality Inventory, agriculture was ranked as one of the major sources of impairment in streams and rivers (132). Fecal bacteria from agricultural sources can originate from point sources, such as concentrated animal feedlot operations (CAFOs), or non-point sources such as runoff from manure-amended cropland and grazing fields. Manure, which is a reservoir for enteric pathogens such as *Salmonella* and *E. coli* O157:H7, may eventually enter surface water systems through subsurface drain tiles or as a result of rainfall events and snowmelt, leading to an increase in fecal bacterial counts. Livestock grazing activities have also been shown to add significant

fecal load to runoff water (38). For example, Howell et al. reported that 80% of samples collected from the primary contact water exceeded the designated USEPA standard after cattle began grazing the adjoining pasture, but only 29% exceeded before cattle were present (67).

1.2.3. Wildlife and Urban runoff

Nonpoint source contributions from wildlife (deer, raccoons, waterfowl such as geese etc) have been shown to influence the FIB levels in watersheds and are reservoirs of various gastroenteritis agents (2, 24, 117, 123). While methods have been developed to identify fecal contributions from wildlife, remediating inputs from these sources are extremely difficult (55, 60). Restricting access of wildlife to streams and rivers is not practical in most cases and may require better management practices to keep the wildlife population in check. In addition to wildlife, nonpoint contributions from urban runoff to recreational waters is of major concern (53). Fecal indicator levels in urban stormwater often exceed the concentrations considered safe for direct human contact (98). Continued expansion of urban and sub-urban communities and differential contributions from residential, industrial and commercial areas into stormwater drain systems further complicate the task of fecal source identification.

1.3. Monitoring water quality

The presence and quantity of pathogens present in the water are indicative of its quality. Although direct monitoring of pathogens will provide a more accurate assessment of associated health risks, it is often difficult and laborious to perform (124). Moreover, standards establishing safe levels of various pathogens in the waterways are currently unavailable. These drawbacks necessitated selection of alternative indicators (of fecal origin) to determine water quality. An ideal fecal indicator should be non-pathogenic, should be strongly associated with the presence of pathogens and have survival profile similar to that of the enteric pathogens in the environment, should be unable to survive outside of the intestinal tract, and should be present in sufficient densities to permit sensitive quantification (64). Based on these characteristics, fecal indicator bacteria (FIB) such as *E. coli* and *Enterococcus* spp. have been used predominantly as indicators of fresh and marine water quality, respectively (113, 118).

1.4. *E. coli* as an indicator

E. coli is a gram negative rod-shaped bacterium that is a member of the gamma-proteobacteria. *E. coli* has been widely used for monitoring purposes as its concentration in the environment is usually much higher than that of the pathogen it predicts. Several epidemiological studies have reported a strong correlation between the densities of *E. coli* in water and the risk of gastrointestinal illness (18, 40, 93, 142). Based on such

epidemiological risk assessment studies, microbiological water quality standards have been established. According to the U.S. EPA, a maximum geometric mean of 126 colony-forming units (CFU) per 100 ml, for 5 samples over a 30 day period, has been established as the *E. coli* standard for recreational waters (131). In 2008, Minnesota Rules Chapter 7050. 0222 was revised to adopt the EPA *E. coli* standard. Waters exceeding this standard will be classified as being ‘impaired’ and may be subjected to closure.

While *E. coli* was initially thought to be a harmless and commensal microorganism inhabiting the lower gut of warm-blooded animals, more recent studies have identified several pathogenic *E. coli* strains, such as enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC), that cause a wide variety of intestinal and extraintestinal diseases (76, 96). Furthermore, studies have also shown that numbers and survival characteristics of *E. coli* do not correlate well with that of several pathogenic agents such as *Yersinia enterocolitica*, *Salmonella spp.*, *Campylobacter spp.*, *Giardia spp.* and *Cryptosporidium spp.*(9, 66, 86, 91). Due to these reasons, reliability of *E. coli* as an indicator of fecal pollution has been under serious investigation.

1.4.1. E. coli in the environment

The presence of *E. coli* in the environment is considered to be indicative of recent fecal contamination. This is based on the assumption that *E. coli* does not grow or survive outside of the host intestine. However, reports suggesting growth and persistence of *E. coli* in various natural environments have been accumulating in the recent years (13, 20,

138). Primary evidence supporting *E. coli* survival in the environment was obtained from tropical soils of Hawaii (14, 16, 56), Florida (119) and Guam (47). In the light of such studies, the potential of temperate environments to act as sinks for *E. coli* has also been examined. Byappanahalli et al. reported on the persistence and growth of *E. coli* in soils and sediments of Indiana and also in coastal forest soils from Great Lakes watershed (13, 17). Studies conducted by Ishii *et al.* in Duluth Boat Club beach and Lake Superior watersheds provided evidence supporting the long term survival of *E. coli* in the temperate climates of Minnesota (70, 71). In addition to tropical, subtropical and temperate soil environments, the filamentous macroalga *Cladophora* (72, 140) and periphyton communities (82) also harbor large concentrations of *E. coli*. Furthermore, studies have shown that the DNA fingerprints of *E. coli* strains isolated from these environmental sources were unique to specific soils and location and shared no genetic similarity with *E. coli* strains isolated from various hosts (70, 71). In some cases, the same strain was shown to be present in a given site over an extended period of time. (71). Taken together, these studies suggest that certain *E. coli* strains have become naturalized to several environments and are present as stable members of the microbial community. The presence of such naturalized *E. coli* questions its use as a reliable indicator for assessing fecal contamination.

1.4.2. Factors affecting E. coli survival in the environment

The survival rate of *E. coli* populations in the environment has been shown to depend on various factors such as pH (19), salinity (19, 125), environmental pollutants (104), temperature (126), light (105, 139), predation (8, 11), available nutrients (79), soil moisture content (15, 119) and texture of the soil (35, 99). For example, higher concentrations of *E. coli* have been observed in sediments and soils along the riverbanks (35, 119). Riverbanks are characterized by high water and organic content (resulting from dense vegetation) which are conducive for sustaining *E. coli* populations. In certain cases, even soils with lower moisture content can allow *E. coli* populations to persist (25). These environmental *E. coli* can enter the water column as a result of rainfall, tidal cycles and run-off events causing an apparent increase in the observed levels of *E. coli* (35, 119). Further, Laliberte and Grimes (1982) showed that *E. coli* has the ability to survive in aquatic sediments for many days (83). Resuspension of such viable sediment-bound *E. coli* can also cause the counts to exceed the state standard (3, 34) and lead to unnecessary impairment designation and/or closure of recreational waters. Therefore, identifying the cause(s) of microbial contamination requires a clear understanding of the sources and the various factors that influence the fate of indicator organisms once they enter the waterways.

1.5. Microbial Source Tracking

Traditional methods of monitoring fecal pollution were based on the presence or absence of the fecal indicators and their count per unit volume (121, 127). Such methods provide information as to whether the water is contaminated and if so, the degree of contamination. However, monitoring the levels of fecal indicator bacteria (FIB) in a waterway alone does not provide any indication about the sources contributing to the presence of these microbes or potential pathogens. Identifying sources of fecal contamination is essential for risk analyses (human versus non-human), supplementing sanitary surveys, and for implementing cost-effective best management practices for restoring the water quality. Furthermore, determining the various bacterial sources and estimating their corresponding contributions to the water body is an essential part of each bacterial TMDL study. Establishing TMDL was mandated in 1972 by Section 303(d) of the Clean Water Act (CWA). The CWA requires each state to provide the Environmental Protection Agency (EPA) with a comprehensive quality assessment of its waterways every two years. Once a body of water has been designated as impaired by fecal bacteria, a TMDL study must be conducted to determine the ways to remedy the impairment so the water body will meet the required state and federal water-quality standards. This has led to the development of various microbial source tracking (MST) methodologies that aim to identify the origin of fecal pollution in a waterway by matching the microbes to its source. MST methods are based on the concept that intestinal microbes found in the fecal material harbor some unique traits (developed in response to varying gut conditions,

available nutrients, etc.) that are host-specific and can be identified and used to track the source.

MST techniques can be grouped into two broad categories: (1) Non-molecular (phenotype) based methods and (2) Molecular (genotype) based methods. These methods can be further classified based on the requirement for culturing of the target organism before analysis and the need for a library to identify unknown organisms in the environment (45, 112, 113, 144). Earlier phenotypic methods employed included: fecal coliform/fecal streptococci (FC/FS) ratios (49), serotyping (101) and antibiotic resistance analysis (ARA) of indicator bacteria (21, 41, 52, 60, 61, 101, 102, 143). The latter method was based on the hypothesis that *E. coli* and *Enterococci spp.* of human origin possess antibiotic resistance characteristics that were different from those of livestock and other animals. Resistance patterns obtained by testing *E. coli* and *Enterococci spp.* isolated from a water body against a variety of antibiotics is compared to antibiotic resistance patterns in a known source library to distinguish between human versus non-human fecal pollution. However, the success of ARA in accurately identifying the host species contributing to fecal pollution has been questioned in the recent years (51, 61, 95, 111). Other phenotypic methods such as FC/FS ratios and serotyping have also been proven inefficient to accurately detect sources of FIB (63, 68, 106).

To address the drawbacks of early MST methods and improve the accuracy, molecular- based (genotypic) source tracking techniques were developed. Molecular MST methods include ribotyping (22, 37, 43, 65, 103), repetitive extragenic palindromic

polymerase chain reaction (rep-PCR) (37, 65), host-specific 16S rDNA PCR (5, 6), denaturing-gradient gel electrophoresis (DGGE) (12, 44), and pulse-field gel electrophoresis (PFGE) (77, 128). The above mentioned methods, with the exception of host-specific 16S rDNA, are based on DNA fingerprinting of bacterial isolates and are library dependent (require the use of known-source isolate libraries for identifying unknown organisms in the environment). Their efficacy depends largely on the size of the library used and method of analysis (1, 62, 69, 75, 87, 123). Smaller libraries were found not to be representative of the genetic diversity of the strains observed in the environment and are less efficient in correctly classifying nonlibrary isolates (94, 143). For instance, *E. coli* harbors an extensive genetic diversity and it has been estimated that a library size of 20,000 to 40,000 isolates might be required to encompass this diversity (50, 59, 94, 120). Such larger libraries, however, are labor intensive and expensive to construct, and often suffer from the inability to match environmental isolates to fecal isolates (62, 122). It is logical to assume that the genotypes obtained from extra-intestinal habitats will be dominated by environmental *E. coli* rather than fecal *E. coli*. Therefore, attempting to classify isolates obtained from a waterway based on similarity to known-isolates might result in incorrect classifications. In contrast, failure to classify a large proportion of challenge isolates would bias the interpretations (122). The efficacy of library dependent methods is further limited by issues such as temporal and geographic variability of bacterial genotypes, long processing time, costs, and variations in the diet of animal hosts (45, 57, 58, 113, 144).

Due to the various limitations mentioned above, library-independent methods are increasingly recognized as potential alternatives for source tracking. Some library independent methods may require a culturing step as in the case of macroarray colony hybridization (55). The macroarray hybridization assay provides a quantitative measure of fecal inputs from a specific source by screening colonies isolated from environmental samples for the presence of host-specific markers. However, culturing can introduce a bias as it cannot account for bacteria that are in the viable but nonculturable (VBNC) state. This issue can be circumvented by the use of library-independent, culture-independent methods such as DNA based PCR assays. These are high throughput assays that use primers to target host specific 16S rRNA sequences. Primers that are currently available detect the presence of *Bacteroides* sp. strains - obligate anaerobes that may constitute ~ 33% of the fecal flora. They are also host specific and have limited survival in the environment making them useful for source tracking (46, 81). This method has been used effectively to identify feces from human, poultry, pig, cattle and horses (6, 36, 84, 114, 115, 136). This assay can also be performed using real time quantitative PCR technology to provide the amount of fecal inputs (28, 114, 115). In addition to eliminating culture-dependent biases, these methods reduce the processing time greatly and are therefore more suitable for TMDL studies. However, it should be noted that fecal contamination is generally determined by monitoring the counts of fecal indicator bacteria such as *E. coli* and not *Bacteroides* sp. Hence, it is difficult to correlate the results of *Bacteroides*-based PCR assays to *E. coli* count data.

The MST methods described above have shown promise; however, they still suffer from various limitations. One approach to overcoming drawbacks is to use a combination of methods for source identification (97, 133). The field of microbial source tracking is still in its early stages and effort must be directed towards validating the above methods for use by regulatory agencies and developing new methods that are rapid, robust, have high throughput, and are effective over broad geographical region. Further research in this area must also be directed towards developing and validating markers to identify animal hosts for which source tracking tools are currently unavailable.

1.6. Summary of thesis

Contamination of waterways by bacteria has placed several water bodies in the US on the Impaired Waters List. The state of Minnesota has been working towards improving water quality and the success of the programs developed lies in our understanding of the fate and ecology of indicator organisms in the environment, and the source(s) that contribute to water quality impairments. Therefore, in Chapter 2, I examined the population structure, persistence and the source(s) of *Escherichia coli* populations in the water and sediments of the Seven Mile Creek (SMC) watershed, a minor watershed located in south central Minnesota. Initial studies by Matthew J. Hamilton in the SMC watershed (from July 2008 to July 2009) provided preliminary evidence suggesting the presence of persistent *E. coli* strains in this environment (54). However, a detailed understanding of population structure and persistence of *E. coli* in ditch sediments required larger dataset. Consequently, I undertook a follow-up study at the same sites within the SMC watershed from August 2009 to October 2010. Specifically, I analyzed *E. coli* count data to determine if this bacterium is present in drainage ditches in the SMC watershed and whether they exceeded the State's regulatory standard. I used the HFERP DNA fingerprinting technique to examine the genetic structure, survival, and distribution of *E. coli* populations present at the SMC watershed. I used host-specific PCR assay to identify the potential sources of fecal bacteria present in the sediment and water samples. Lastly, I also compared the DNA fingerprints of *E. coli* obtained from the SMC

samples to those in a known source library in an attempt to determine the potential sources of *E. coli* isolates obtained from the SMC watershed. Results from our field investigations suggest that certain populations of *E. coli* have become naturalized to this environment and their dynamics are influenced by temperature, rainfall, and other abiotic factors. The presence of naturalized *E. coli* in the SMC will have implications for future water quality studies as it confounds the use of this bacterium as an indicator of fecal contamination. In Chapter 3, I summarized overall conclusions from my studies and provided some future directions for the research presented in Chapter 2.

CHAPTER 2

Genetic structure, distribution, and persistence of *E. coli* populations found in ditch sediment and water in the Seven Mile Creek Watershed

2.1. Introduction

The contamination of waterways by pathogenic microorganisms is a major cause of river and stream impairment in the U.S. (132). Due to persistent contamination by microorganisms, many waterways still do not meet the 1972 Clean Water Act's goals and water quality standards (132). One of the major routes by which these microorganisms enter waterways is through feces from humans and animals (30). Thus the fecal loading of aquatic environments is of major concern to regulatory agencies, and environmental and health professionals. Waterways contaminated with feces are reservoirs for zoonotic pathogens and therefore pose a major threat to public health (116, 141). These pathogens can be transmitted to the public through surface and groundwater, water-related recreational activities and by the consumption of shellfish (23, 109, 129).

Escherichia coli is a gram negative, rod-shaped, bacterium that inhabits the lower gut of warm-blooded animals. The presence of *E. coli* in water is considered to be indicative of recent fecal contamination, and signals the presence of potential human pathogens such as *Salmonella*, *E. coli* O157:H7, *Shigella*, *Cryptosporidium parvum*, and enteric viruses. Sources of fecal coliform bacteria in environment include runoff from feedlots and manure-amended agricultural land, septic system leakage, untreated sewage from sewer overflows, human recreation, wildlife and urban runoff (71, 75). Several epidemiological studies have reported a strong correlation between the densities of *E. coli* in water and the risk of gastrointestinal illness (18, 40, 93, 134, 142).

The use of *E. coli* as an indicator of fecal contamination in freshwater systems is based on the assumption that it does not grow or survive outside of the host intestine. However, several studies have now provided evidence suggesting that *E. coli* can grow and persist in soil, sands, sediment, and in periphyton and algal communities in temperate climates of Minnesota, Indiana and Michigan (13, 14, 16, 17, 47, 56, 71, 119).

Earlier studies examining the growth and survival of *E. coli* in environment were performed primarily under laboratory conditions (26, 32, 33, 48). This is in part due to difficulties in monitoring populations of target *E. coli* strains in the environment, and the inability to distinguish indigenous *E. coli* strains from new inputs of fecal-borne *E. coli*. Initial studies from our laboratory have shown that some *E. coli* genotypes are unique to specific soils and location, and shared no genetic similarity with *E. coli* strains isolated from known-source animals (70, 71). Furthermore, these unique *E. coli* genotypes were shown to be present in soils over extended periods of time and to persist through the temperature extremes and repetitive freeze thaw cycles (17, 70, 71). Taken together, these studies suggest that certain *E. coli* strains multiply and survive in soils and are present as stable members of the microbial community. Such strains are referred to as “naturalized *E. coli*”. The presence of naturalized *E. coli* questions the use of this bacterium as a reliable indicator for assessing fecal contamination of waterways and has obvious ramifications for TMDL determinations and fecal monitoring programs.

The survival and persistence of naturalized *E. coli* populations in the environment can be influenced by various factors such as pH, salinity, temperature, environmental pollutants, sunlight, predation, available nutrients, organic matter content, soil moisture content, and variation in soil texture (8, 11, 15, 19, 35, 79, 99, 104, 119, 126, 139). Environmental *E. coli* can enter the water column via run-off events, resuspension of sediments, and tidal cycles, resulting in an apparent increase in the observed levels of *E. coli* relative to more gentle and constant flow conditions (35, 119). Resuspension of viable soil- or sediment-borne *E. coli* can also cause counts to exceed the state standard (3, 34) and lead to unnecessary impairment designation and/or closure of recreational waters. Therefore, identifying the cause(s) of microbial contamination of waterways requires a clear understanding of the sources (fecal and non-fecal) of FIB and the various factors (hydro-climatologic conditions, physical, chemical and biological) that influence the fate of these microorganisms once they enter waterways.

Despite finding FIB in the environment, there have been only a limited number of field investigations concerning the growth and persistence of *E. coli* in sediments (70). Furthermore, no studies have been conducted to assess the contribution of these naturalized bacteria to water quality impairments in ditches or tile drainage systems. Consequently, the objectives of this study were to: 1) examine sediment and water samples from the SMC watershed for the presence and numbers of *E. coli*, 2) determine the potential sources of fecal contamination at the SMC watershed by using host-specific PCR assays, 3) use HFERP DNA fingerprint analyses to examine the growth, survival

and genetic structure of *E. coli* populations obtained from water and sediment samples, and determine if these bacteria are newly acquired or naturalized to these environments and, 4) examine the potential factors influencing the distribution of *E. coli* populations present at SMC water and sediment.

2.2. Materials and Methods

2.2.1. Site Description

The Seven Mile Creek (SMC) watershed is a part of the Middle Minnesota River Basin and is a minor watershed located in south central Minnesota, 14 miles from Mankato and south of St. Peter in Nicollet County. The Creek begins above MN Highway 99 as a series of drainage ditches and runs for about 6.1 miles southward, after which it drains into the Minnesota River. The watershed spans an area of 23,551 acres (36.8 sq miles), which amounts to nearly 3% of the Middle Minnesota major watershed and 8% of Nicollet County. The majority of the watershed (about 86%) is comprised of cultivable lands devoted primarily to corn and soybean production. The remaining area consists of deciduous forest, grassland, wetlands, and animal feedlots. There are 24 feedlots for cattle, dairy cows and hogs located within the watershed (Figure 2.2). Manure from the feedlot operation is used for fertilizing the agricultural fields and ~10-20% of the area within the watershed receives manure every year. Most rainfall to the SMC is observed from April to July and this, combined with snowmelt and run off, is

thought to cause manure from feedlots and fields to enter into the creek. In addition, the creek also receives input from three public drainage ditches, County Ditch (CD) 46, CD 24, and CD 13, which were constructed to drain the western, southern and northern portions of the watershed. After July, the open drainage ditches often dry-up completely and do not supply water to the creek. For the purpose of this study, we chose four sites within the SMC watershed, designated as SM1-SM4 (Figure 2.1), to examine in detail.

2.2.2. Sample collection and concentration

Sampling was done every two weeks from July to October in 2008, and from April to October in 2009 and 2010. Water samples were collected as previously described (10). Sediment samples were collected from the top 2 cm of the sediment layer in sterile, 207 ml capacity Whirl-Pak bags (eNasco, Fort Atkinson, WI). Samples were shipped overnight on ice and held at 4°C until processed. Bacteria in the water and sediment samples were concentrated by membrane filtration at 25°C within 24 hrs of sample collection. Membrane filtration for cell enumeration was performed once and filtration for DNA extraction was done in duplicates. Approximately 200 to 600 ml of water sample from each site was filtered through a sterile 0.45- μm (47-mm- diameter) mixed cellulose ester S-Pak membrane filter (Millipore, Billerica, MA). Some samples were filtered through multiple membranes in order to circumvent membrane clogging, and to expedite the filtration process. For sediment processing, 10 g of each sample was diluted with 95 ml of gelatin-ammonium phosphate buffer extraction solution (78) in a 160-ml Corning® milk dilution bottle. Two bottles were prepared for each sediment sample.

Sterile 3 mm glass beads (10g) were added to the bottles and the bacterial cells bound to the sediment particles were released by vigorous agitation for 30 min on a shaker.

Samples were allowed to settle for an hour and aliquots (35 and 70 ml) of the upper soil-free phase were filtered through 0.45 μm membranes separately for DNA extraction and colony isolation, respectively. Membrane filters containing cells for DNA extraction were stored frozen at -80°C until used.

Filters for cell enumeration were transferred to a sterile 50 ml conical centrifuge tube containing 10 ml of phosphate buffered saline and 5 g of sterile glass beads (3 mm – dia.). Bacterial cells were released from the filters by gentle agitation for 30 min using a wrist action shaker. Sterile 50% glycerol was then added to each centrifuge tube (final concentration of 10%) and stored at -80°C until used.

2.2.3. Isolation of environmental *E. coli*

The *E. coli* present in water and sediment samples were isolated on modified mTEC agar medium as described previously (130). Approximately 4 ml of concentrated samples from centrifuge tubes were spread-plated onto the surface of 200-250 ml mTEC agar medium in Q-tray bioassay plates (20X20 cm, Genetix, Boston, MA). Plates were incubated at 37°C for 2 hrs and then transferred to 44.5°C for 16 hrs. *E. coli* produces β -D-glucuronidase that transforms the chromogen (X-Gluc) present in the modified mTEC medium to form blue colonies. After incubation, the plates were stored at 4°C for further development of the blue pigment. Typically, 24 well-isolated blue colonies from each site

(SM1-SM4) and sample type (water and sediment) were manually picked using sterile toothpicks and transferred into 96 well microtiter plates containing 150 μ l of Hogness modified freezing medium (HMFM) (0.627 g l⁻¹ K₂HPO₄, 0.177 g l⁻¹ KH₂PO₄, 0.588 g l⁻¹ trisodium citrate, 0.247 g l⁻¹ MgSO₄.7H₂O, 44 ml l⁻¹ glycerol) and Lysogeny broth (LB) (10g l⁻¹ tryptone, 5 g l⁻¹ yeast extract and 10 g l⁻¹ NaCl at pH=7.5). Cells were grown overnight at 37° C and then stored at -80° C until used.

2.2.4. Enumeration of E. coli

E. coli counts in water and sediment samples were determined using the Colilert® Quanti-Tray®/2000, a semi-automated most probable number (MPN) quantification method, as described by the manufacturer (IDEXX Laboratories, Westbrook, ME). Count data are generally expressed as MPN/100 ml for water samples and as MPN/gram (dry weight) for sediment samples.

2.2.5. DNA extraction

Total DNA from the frozen filters was extracted by using PowerSoil DNA isolation kits (MO BIO, Carlsbad, CA). The first step in the protocol was modified to include finely chopped filters as input material for extraction. The eluted DNA was quantified using an Eppendorf BioPhotometer (Eppendorf, New York, NY) and DNA samples were stored in -80° C until used.

2.2.6. 16S rRNA-based host-specific PCR assay

Water and sediment samples for host source analysis were generally obtained twice per month from sites SM1-SM4. Samples were examined for the presence of *Bacteroides* or *Brevibacterium* sp. originating from bovine, swine, human, and poultry sources by using conventional PCR and four primer sets (Table 2.1). PCR assays were performed using conditions described previously (6, 36, 115, 136). DNA was extracted from environmental samples and diluted to 3 ng/μl in sterile, nuclease free, water for use as template in the PCR assay. All amplification reactions were performed using the MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA), and carried out in a total volume of 25 μl reaction mixture containing 5 μl of template DNA, 0.1 mmol l⁻¹ of each deoxynucleoside triphosphate (Invitrogen, Carlsbad, USA), 0.4 μmol l⁻¹ of each primer (Integrated DNA Technologies, Iowa, USA), 1X Taq reaction buffer (Denville Scientific Inc., NJ, USA), 640 μg l⁻¹ of Bovine Serum Albumine (New England Biolabs, Ipswich, MA) and 1.25 U of Choice-Taq™ DNA polymerase (Denville Scientific Inc., NJ, USA). For all PCR runs, positive control (DNA isolated from human sewage or the feces of swine, chickens, or cattle) and negative control (without template DNA) were included. In addition, a universal *Bacteroidales* 16S rRNA PCR assay (AllBac296F/Bac708R) was performed on each sample to test for the overall presence of *Bacteroides* (84), and to determine if the DNA extracts were suitable for use in host-specific PCR analysis. PCR products were separated by electrophoresis on 1.7% agarose

gel containing 0.2 µg/ml of ethidium bromide and visualized by placing on a UV transilluminator (FOTODYNE Incorporated, Hartland, WI).

2.2.7. *HFERP DNA fingerprinting*

Horizontal, fluorophore-enhanced, repetitive extragenic palindromic-PCR(rep-PCR) DNA fingerprinting (HFERP) was performed using unlabeled and 6-FAM fluorescently-labeled BOXA1R primers as described previously (75). HFERP gel images were captured using a Typhoon 8610 Variable Mode Imager (Amersham Biosciences, Sunnyvale, CA). Images were processed using ImageQuant software (Amersham Biosciences) and analyzed using Bionumerics v2.1 software (Applied Maths, Sint-Martens-Latem, Belgium) as described previously (37, 75).

2.2.8. *Statistical Analysis*

Dendrograms were generated from the HFERP fingerprint data using the curve-based, Pearson's product-moment correlation coefficient and the unweighted pair group method with arithmetic means (UPGMA). Each DNA fingerprint represents a single isolate and isolates with $\geq 92\%$ similarity were considered to be clones (74). Spatial and temporal dynamics of *E. coli* populations were analyzed using the multivariate analysis of variance (MANOVA), a form of discriminant analysis, by accounting for the covariance structure (17, 37, 71). The significance of the discriminant analysis was determined from the P value associated with each MANOVA analysis, which represents the probability of obtaining same level of separation by randomly grouping the isolates. Jackknife analysis,

with maximum-similarity, was performed on the same set of isolates to determine the rates of correct assignment (94). ID bootstrap analysis (at $P = 0.9$), done using a Bionumerics script (<http://www.applied-maths.com/bn/scripts/bnscripts.htm>), was performed to identify the potential sources of *E. coli* isolates in SMC (37, 71).

Additionally, chi-square test for independence and Fisher's exact test were done to assess the statistical significance of some of the results obtained in this study.

2.3. Results

Samples for this project were collected from July - October in 2008 and from April - October in 2009 and 2010 [raw data from July 2008 to July 2009 were obtained by Matthew J. Hamilton (54)]. In this report, HFERP DNA fingerprints of *E. coli* strains obtained from 2008-2010 were included in the overall analyses (section 2.3.3) as it provided a better understanding of the changes in population structure of *E. coli* over several years and the factors influencing their structure. However, 2008 data were excluded from *E. coli* count and host source-specific PCR analyses (sections 2.3.1 and 2.3.2, respectively), as non-availability of samples from April-June 2008 might introduce a bias in the comparisons presented in these sections.

2.3.1. *E. coli* count data

Water and sediment samples were collected on a weekly basis from all four sites (SM1-SM4) and examined for the presence and numbers of *E. coli*. Individual water and sediment *E. coli* counts varied considerably over the sampling period (Figures 2.3, 2.4, 2.5 and 2.6). Some sites experienced a period of dryness during the 2009 sampling year (site SM2 was dry during mid-August; SM3 was dry most of August and late September; and SM4 was dry during late September). Therefore, only 116 of 124 water samples that were expected between April and October 2009, were collected for monitoring purposes, and of these, 42 (36.2%) contained *E. coli* counts greater than those permissible by state statute (126 CFU per 100 ml). In 2010, 35 out of 116 (30.2%) samples exceeded the state standard and all the samples that exceeded were collected from June or later. Overall, the decrease in water quality exceedances over the two year period was not significant. However, examination of *E. coli* counts from water at individual sites showed that there was a significant reduction in the number of water samples exceeding the state standard at SM3 (from 53.8% in 2009 to 24.1% in 2010). The percentage of water samples exceeding the permissible state standard across sites is shown in Table 2.2.A.

In general, rainfall did not correlate with sample collection as the dates and times of sampling were fixed. However, some sites were dry in 2009 and water samples for count analysis were obtained from these sites once rainfall occurred. For instance, water samples obtained from SM1 and SM4 during mid-August contained extremely low concentrations of *E. coli* (6.3 and 38.4 CFU/ 100 ml, respectively), and sites SM2 and

SM3 were dry. Precipitation data obtained from SMC indicated that two significant rainfall events occurred between August 18 and 27, 2009 (Figure 2.7. B). Since run-off events associated with rainfall often lead to elevated fecal counts in waterways, all water samples collected in late August exceeded the state standard (Figure 2.3).

Further analysis of the count data revealed a strong correlation between *E. coli* concentrations and temperature profile at the SMC. The average counts of *E. coli* in water and sediments across months were calculated and plotted as shown in Figure 2.8. Average monthly temperatures are shown in Table 2.3. As expected based on temperature, water samples obtained in the spring months (April and May) generally had lower concentrations of *E. coli* than did those obtained from later periods. *E. coli* concentrations in water samples began to rise in June and were predominantly elevated during summer (June to August) and early fall (September), after which time the counts started to decline. Consequently, the percentage of water samples exceeding the *E. coli* standard was higher during summer months than the rest of the sampling period (Table 2.2.B). Sediment-borne *E. coli* concentrations followed a similar trend where the majority of samples from April and May did not contain detectable levels of *E. coli* with highest concentrations observed in August or September.

2.3.2. Host source-specific PCR assay

A total of 242 samples (122 samples from April to October, 2009 and 120 samples from April to October, 2010) were analyzed to determine the potential sources of

fecal contamination and their prevalence in the SMC watershed. Among these 242 samples, 120 were obtained from water (31, 31, 28 and 30 from sites SM1, SM2, SM3 and SM4, respectively) and 122 were obtained from sediment (31, 31, 29 and 31 from sites SM1, SM2, SM3 and SM4, respectively).

The AllBac PCR assay detected *Bacteroides sp.* in all samples tested, indicating that DNA extracts were free of inhibitors and suitable for use in host-specific PCR analysis. PCR analyses indicated that 106 of 122 samples (87%) from 2009 samples were positive for bovine, 14 (11.5%) were positive for swine and 2 (1.6%) were positive for poultry-specific fecal marker. In 2010, of the 120 samples tested, 72 (60%) contained the bovine fecal marker and 22 (18.3%) contained swine-specific fecal marker. The poultry-specific LA35 marker gene was not detected in 2010 samples and the human-specific HF183 marker gene was not detected in any environmental sample obtained during this study. Overall, the results from 2009-2010 indicated that among the potential host sources of fecal contamination examined, bovine and swine were prevalent, with bovine being the most common source (Figure 2.9).

Results were further analyzed for significant differences in fecal contamination across years, sampling months, sites, and sample types. The chi-square test for independence showed that there was a significant association between year and bovine fecal contamination, $\chi^2(1, N= 242) = 22.48$, p-value = 0.000. The bovine fecal marker was detected more frequently in samples from 2009 than those from 2010 (Figure 2.10). In addition, significant differences were observed in bovine fecal contamination across

months (fisher's exact test p -value = 0.000). The bovine fecal marker was detected in all samples collected from April to July and less frequently in samples from August to October, in both 2009 and 2010 (Figure 2.10). In contrast, analysis of the PCR data showed that swine fecal contamination across years and sampling months were not significantly different (p -value > 0.05). Furthermore, no particular site or sample type contributed significantly to bovine or swine fecal loading at the SMC watershed (p -value > 0.05).

2.3.3. Genetic structure and dynamics of *E. coli* populations at the SMC watershed

The genetic and population structure of 2,425 *E. coli* strains was determined by HFERP DNA fingerprint analysis. Of these, 1364 and 1061 were from water and sediment, respectively. Analyses of HFERP DNA fingerprints revealed that *E. coli* population at the SMC watershed was genetically diverse, with relative similarity values ranging from 2.0 to 100%. A dendrogram of all isolates is not shown due to space constraints. Based on the criteria proposed by Johnson et al. (75), 434 and 293 unique strains were recovered from water and sediment samples, respectively. Furthermore, the Shannon diversity index for water and sediment isolates were 5.36 and 4.85, respectively, suggesting that *E. coli* isolates from water were more diverse than those from sediment.

MANOVA analysis of *E. coli* fingerprints grouped by year revealed an annual shift in population structure at the SMC (Figure 2.11). The first and second discriminants accounted for 100% of the variation (p -value = 0.001), indicating that the strains were

clustered tightly by year. The distribution of *E. coli* isolates was more coherent when grouped by year than by site (Figure 2.12). Furthermore, grouping of isolates by sample type within each year revealed a strong overlap between *E. coli* populations from water and sediment obtained the same year (Figure 2.13). Additionally, *E. coli* strains of same genotype were isolated from both sediment and water samples from each site on several occasions (Figure 2.14). This suggests that some *E. coli* populations that were bound to the surface of sediment particles were detached and resuspended into overlaying water as a result of mixing during flow events.

Distribution of water *E. coli* isolates across sites was explored by using Jackknife analysis (using maximum similarity values). Water isolates obtained when the sites were physically connected by water (June) were compared to those from a no flow period (September) in 2010. Results of this analysis showed that there was a greater overlap of water isolates between sites when there was flow, as indicated by their lower rates of correct classification (Table 2.4. A). During no flow conditions, water became stagnant at the sampling sites and sites became geographically isolated, allowing for differentiation of *E. coli* populations at the respective sites leading to their higher rates of correct classification (Table 2.4. B). Similar results was observed at the SMC watershed previously (54).

Additionally, the genetic relatedness of the *E. coli* isolates from the SMC to those of known source *E. coli* isolates obtained from the Minnesota fingerprint library (75) was determined by using ID bootstrap analysis at P value ≥ 0.90 . The library consisted of *E.*

E. coli fingerprints obtained from cattle and dairy cows, chicken, turkey, goose, ducks, pig, horse, human cats and dogs (75). Results of this analysis indicated that up to 1.9, 0.7 and 0.2% of the *E. coli* isolates from the SMC may have originated from human, poultry, and swine, sources respectively. However, no source could be assigned to the remaining 97.2% of the SMC *E. coli* isolates. Moreover, despite the detection of bovine-specific *Bacteroides* marker in majority of samples obtained from SMC, none of the 2,425 isolates matched the fingerprints of bovine *E. coli*. This indicated that the library used is likely not representative of *E. coli* strains observed at these sites.

2.3.4. Naturalized *E. coli* populations at the SMC

Analysis of 2,425 HFERP DNA fingerprints also revealed that some of the same strains (with $\geq 92\%$ similarity values) appeared in sediments and water over an extended period of time (Figure 2.15). This result, combined with my observation that same strain could be isolated in October of one year and again in April of the next sampling year, supports the idea that *E. coli* can persist in this environment (Figure 2.16). These persistent strains represented 10.9%, 28.9%, 14.7% and 24.5% of all *E. coli* isolated from SM1, SM2, SM3 and SM4 sites, respectively. Moreover, none of these strains matched with known source *E. coli* from Minnesota fingerprint library using ID bootstrap analysis (P-value ≥ 0.9). Therefore, based on the criteria proposed by Ishii et al. (71), the above strains were considered to be naturalized to this environment.

The genetic relatedness of these persistent SMC strains to known source *E. coli* strains from the fingerprint library was further explored by using MANOVA analysis (Figure 2.17). The *E. coli* strains naturalized to the SMC clustered into a separate group, and were distinct from *E. coli* isolated from human and animal feces (which exhibited a strong overlap). A more detailed comparison of SMC *E. coli* to known sources of *E. coli* is shown in Figure 2.18. The non-naturalized water and sediment *E. coli* strains were more closely related to *E. coli* from fecal sources (as indicated by the overlap) than were the naturalized *E. coli* population. Furthermore, MANOVA analysis explained 100% of the variation in first and second discriminants (p-value: 0.001), further supporting the hypothesis that the indigenous SMC strains could be readily distinguished from known source *E. coli* strains. In water, the highest proportion of naturalized *E. coli* strains was usually observed during the summer months when the *E. coli* counts were extremely high, suggesting that these strains may contribute to exceedances of water quality standard (Table 2.5).

Lastly, MANOVA analyses were used to compare the naturalized *E. coli* populations obtained from the SMC sediments to those obtained by Ishii et al. (70, 71) in Duluth Boat Club (DBC) beach sediments, and in temperate soils of Lake Superior watersheds. Results of this study indicated that naturalized *E. coli* populations from each location were distinct and unique to the specific environment from which they were isolated (Figure 2.19). Furthermore, naturalized *E. coli* populations from sediments were

more closely related to each other than to those from soils, suggesting that properties of the habitat likely influence the adaptation of these stains.

2.4. Discussion

In this study I aimed to determine the extent at which fecal *E. coli* bacteria persist and grow in the Seven Mile Creek watershed sediment and water samples and to also assess the potential sources of fecal contamination at SMC and the factors that were likely contributing to the elevated levels of *E. coli* observed.

2.4.1. Seasonal change in E. coli population densities

E. coli densities in SMC water samples exceeded the permissible Minnesota standard (126 CFU/100 ml) predominantly during summer and fall seasons. Several water and sediment samples from spring 2010 did not contain detectable levels of *E. coli* presumably due to low temperatures observed in Minnesota in spring (20, 35, 56, 119). Overall, changes in the densities of *E. coli* in water and sediments appeared to correlate well with the temperature profile observed during the sampling period. For example, in 2009 the average air temperatures for the month of April and May near SMC were 10° C and 15° C , respectively, and the corresponding water *E. coli* count averages were around 12 CFU/100 ml and 71 CFU/100 ml, respectively. In June, average counts increased to 197 CFU/100 ml and remained elevated throughout summer months and into early fall. Monthly averages for temperature during this period were 17 - 20° C. After this period,

however, temperatures dropped below 10° C and counts began to decline as well, and at the end of sampling in October, counts averaged at ~ 93 CFU/100 ml. These results are similar to those previously reported in soils and water of northern Minnesota, where variations in *E. coli* counts were shown to be temperature related with highest counts observed during summer (70, 71). It should be noted that sediment temperature generally increased once the overlaying water has warmed-up, and this may explain the lagging trend observed in monthly sediment count averages as compared to water counts.

In addition to temperature, rainfall also drastically influenced the dynamics and distribution of *E. coli* populations at the SMC. This observation is based on count data of samples collected following rainfall events, the desiccation of some sampling areas during times of drought, and the geographical isolation of some sample sites that were previously connected by water. For instance, several sites were dry during certain periods of August and September 2009 and rainfall, in addition to carrying high densities of *E. coli* from feedlots and farm lands into the creek, likely resulted in the displacement of sediment-bound *E. coli* into water column and their transport downstream leading to mixing within and between sites. Consequently, water samples obtained from all four sites after rainfall events (08/27/2009 and 10/07/2009) contained large numbers of *E. coli*. Rainfall has previously been shown to increase *E. coli* concentrations in freshwater and is frequently, thought to contribute to non-point source pollution of waterways (3, 34, 35, 119). Taken together my results suggest that the seasonal variation in *E. coli* counts

observed in water and sediments are most likely related to temperature, rainfall, and the patchy distribution of *E. coli* within sampling locations (17, 71).

While many of the tested samples contained *E. coli*, it is also important to note that the *E. coli* enumeration method used in this study is culture-dependent and, therefore, cannot account for *E. coli* in a viable but not culturable (VBNC) state. Several studies have shown that VNBC state confers an adaptive advantage to bacteria and is often employed for long-term survival in extreme environments (27, 110). The ability of indicator microorganisms such as *E. coli* and *Enterococci* sp., to enter the VNBC state and resuscitate from this state upon returning to favorable conditions has been previously reported (90, 100). In temperate climates of Minnesota, this strategy would allow *E. coli* to survive the repeated freeze-thaw cycles, and may possibly explain the ability of sediment-adapted *E. coli* to persist over extended periods of time in extreme environmental conditions like those observed in the SMC watershed.

2.4.2. Potential sources of fecal contamination

Water quality exceedances found in this study indicated that the watershed was impaired by fecal bacteria. In order to better understand fecal contamination at the SMC watershed, and to ensure that water quality meets the established standard in future, a pollutant source characterization was necessary. Results of host source-specific PCR assays indicated that some of the fecal bacteria at the site are likely contributed by cattle and swine. These results correlate with the trend observed in 2008 (54). This result is not

surprising as there are several feedlots for cattle, dairy cows and hogs within the watershed. Furthermore, majority of the watershed (about 86%) is comprised of cultivable lands, 20% of which receive manure every year. Runoffs from feedlots and manure amended agricultural land can serve as primary input of fecal bacteria into the creek (38, 67). In addition to rainfall and snowmelt, runoff from public drainage ditches and public drain tile systems also move water from fields into open drainage ditches. The open drainage ditches, however, do not supply water to the creek after July.

Results of these studies also indicated that the fecal loading of the SMC was less frequent during the fall months. This observation may be related to lack of run-off and sporadic rainfall events observed during this time. Interestingly, all water and sediment samples obtained during Fall 2010 did not contain the bovine marker gene. Although rainfall events occurred during this period, they were infrequent and did not track well with our sampling dates.

The samples were also analyzed for fecal bacteria of human and poultry origin. With the exception of two sediment samples from April 2009 that tested positive for poultry marker, poultry fecal contamination was not observed throughout the study (54). In addition, there were no positives for the human *Bacteroides* marker gene during the study. Interestingly, however, DNA fingerprints of SMC *E. coli*, when tested against a Minnesota known source library, suggested that 1.9% of *E. coli* isolates at the SMC may be of human origin. Although, only 0.7% of the SMC isolates matched with poultry-specific *E. coli*, these isolates were obtained in July, August, and September 2009.

In contrast, the poultry-specific PCR assay did not detect any positives in samples during this period. Despite the prevalence of bovine and swine-specific *Bacteroides* marker gene, none of the SMC *E. coli* isolates matched with bovine *E. coli*, and only 0.2% matched with *E. coli* of swine origin. It must be noted that host source-specific PCR assays were based on using marker genes originating from *Bacteroides* and *Brevibacterium sp.* strains, whereas the DNA fingerprint analyses were done using *E. coli*. These differences may explain the discrepancies observed in results. Furthermore, 24% of total samples (water and sediment) were negative for the presence of all of the host source marker genes tested using the PCR assay. Since the watershed consists of forests, grasslands, and undeveloped areas, it is likely that other sources such as wildlife also impact the fecal loading of SMC. However, due to a lack of validated probes, we were unable to test for fecal bacteria from these other potential sources.

Taken together, these results suggest that the Minnesota DNA fingerprint library that was used is likely not representative of the genetic diversity of the strains observed at the SMC watershed. DNA fingerprinting is a library-dependent MST method and its efficacy, therefore, is largely dependent on the size of reference library (1, 59, 62, 69, 75, 94, 120, 123, 143). In addition, failure to assign a source for majority (97.2%) of the SMC *E. coli* may also be due to temporal or geographic limitations of the library which primarily consists of *E. coli* strains collected from across Minnesota between 2000 and 2005 (45, 57, 58, 113, 144). Comparisons done using a more comprehensive reference library containing *E. coli* isolates obtained from livestock, wildlife and manure within the

SMC watershed would serve to overcome the above limitations. In addition, employing a combination of MST methods will increase the efficiency of source identification. Such an approach has gained widespread use in the recent decade (97, 133).

2.4.3. Population structure of *E. coli* in the SMC watershed

HFERP DNA fingerprint analyses revealed that the population structure of *E. coli* at the SMC watershed was extremely diverse. In particular, *E. coli* isolates from water were more diverse than those from sediment, suggesting that water is greatly impacted by continuous inputs of *E. coli* from varied sources. The genetic diversity amongst *E. coli* isolates from the SMC was further explored by using MANOVA analysis – the isolates grouped by year. While there was an annual change in the population of *E. coli* at the SMC, a certain fraction of that population was the same during all three years of study (as seen from the overlapping cluster at the center of Fig. 2.11). Dendrogram consisting of all the SMC *E. coli* fingerprints revealed that some of the same *E. coli* strains were repeatedly isolated from the SMC sediments from 2008 to 2010 (Figure 2.15) and some *E. coli* strains of same genotype appeared at the end of one sampling year and then again in the beginning of the next sampling year (Figure 2.16). These results support our contention that some sediment-borne *E. coli* strains persisted over winter months, through freeze-thaw cycles, and became stable members of this sediment microbial community. Similar findings have been reported in temperate soils by Ishii et al. (71) and Byappanahalli et al. (17). Further support for the autochthonous nature of these *E. coli* strains was provided by MANOVA analysis showing that DNA fingerprints of

naturalized *E. coli* strains in the SMC were distinct and readily distinguished from those of *E. coli* isolated from human and animal feces. The existence of naturalized *E. coli* in sediments was previously suggested by Ishii and co-workers in Duluth Boat Club beach sediments. However, their sampling period was much shorter than ours (70). MANOVA analysis also indicated that there was mixing of bacteria between the water column and sediment within sites. Consequently, naturalized *E. coli* from sediment can be resuspended into the water column leading to an apparent increase in the observed levels of *E. coli* (73). Results from our study indicated that this is likely causing some water samples to exceed the state standard (Table 2.5). Furthermore, my analysis showed that mixing of *E. coli* strains between the sites and their transport downstream during flow conditions (rainfall and runoffs) likely influence the distribution and mobility of naturalized *E. coli* within the watershed. Our result is consistent with those reported by Ishii et al. (71) and Solo-Gabriele et al.(119) who showed that sediment- and soil-borne *E. coli* can move due to flow and runoff events. Mixing within and between sites may explain the lack of coherency observed in MANOVA analysis when DNA fingerprints of *E. coli* isolates were grouped by site (Figure 2.12).

Results from our study also supported growth of *E. coli* in temperate sediment and or water. Analysis of the dendrogram containing DNA fingerprints revealed that clonal isolates (DNA fingerprints with $\geq 92\%$ similarity) from a single strain were isolated many times in the same sample (Figure 2.15) and in some sediment samples, all the isolates obtained appeared to be from a single strain. It is extremely likely that *E. coli*

were growing in the sediments and water at SMC as the isolation technique used in our study did not include an enrichment step that would allow for single isolates to multiply. Growth and survival of *E. coli* strains in temperate sediments and water compromises the use of this bacterium as an indicator of fecal contamination and may misdirect abatement strategies developed for TMDLs.

Finally, a comparison of naturalized *E. coli* populations from SMC sediments, Duluth Boat Club beach sediments, and temperate soils of Lake Superior watersheds indicated that these strains were distinct and unique to the sediments and soils in which they were isolated. This is likely due to differences in the properties of sediments and soils studied and to the primary origins of *E. coli* in these soils. Chemical and physical properties such as available nutrients (organic carbon content, total nitrogen and phosphorus), moisture content, texture, composition, and particle size have been shown to play an important role in determining the persistence and adaptation of *E. coli* in the environment (15, 32, 33, 35, 48, 71, 119). This might also explain why naturalized *E. coli* populations from sediment were more closely related to each other than to those from soils. However, a detailed examination of sediment composition and characteristics at the SMC watershed is required to shed more light on this matter and to better understand how sediments act as reservoirs of *E. coli*.

2.5. Acknowledgements

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Table 2.1. Conventional PCR primer sets used in this study.

Primer	Sequence (5'→ 3')	Fecal source	Target organism	Size (bp)	Ref.
HF183F Bac708R	ATCATGAGTTCACATGTCCG CAATCGGAGTTCTTCGTG	Human	<i>Bacteroides-Prevotella</i>	525	(6)
PF163F Bac708R	GCGGATTAATACCGTATGA CAATCGGAGTTCTTCGTG	Swine	<i>Bacteroidales</i>	563	(36)
LA35F LA35R	ACCGGATACGACCATCTGC TCCCCAGTGTCAGTCACAGC	Poultry	<i>Brevibacterium</i> sp.	571	(136)
CowM3F CowM3R	CCTCTAATGGAAAATGGATGGTATCT CCATACTTCGCCTGCTAATACCTT	Bovine	<i>Bacteroides</i>	122	(115)

Table 2.2. Exceedance of water quality standard across sites and months.

A.

	2009	2010
	% of samples exceeding State standard	% of samples exceeding State standard
SM1	32.3	27.6
SM2	40.0	34.5
SM3	53.8	24.1
SM4	20.7	34.5

B.

	2009	2010
	% exceedance	% exceedance
April	0	0
May	12.5	0
June	56.25	37.5
July	60	15
August	58.33	75
September	37.5	60
October	37.5	16.67

Table 2.3. Average monthly air temperatures (° C) during the sampling period.

Average monthly temperatures (° C)		
	2009	2010
April	9.56	10.89
May	15.6	14.69
June	19.13	21.83
July	19.94	23.92
August	19.61	23.69
September	17.73	16.95
October	8.3	10.81

Table 2.4. Jackknife analysis of DNA fingerprints from water isolates obtained during flow and no flow conditions at the SMC.

A.

Jackknife analysis of 2010 water <i>E. coli</i> isolates obtained during flow condition				
	Maximum similarities (%)			
	SM1	SM2	SM3	SM4
SM1	62.50	12.50	4.17	16.67
SM2	20.83	58.33	4.17	12.50
SM3	12.50	8.33	83.33	20.83
SM4	4.17	20.88	8.33	50.00

B.

Jackknife analysis of 2010 water <i>E. coli</i> isolates obtained during no flow condition				
	Maximum similarities (%)			
	SM1	SM2	SM3	SM4
SM1	75.00	0.00	8.33	0.00
SM2	8.33	100.00	8.33	0.00
SM3	4.17	0.00	79.17	8.33
SM4	12.50	0.00	4.17	91.67

Table 2.5. Potential effect of naturalized strains on exceedance of water quality standards.

Date	Site	Corresponding water <i>E. coli</i> counts (CFU/100ml)	Percentage of naturalized <i>E. coli</i>
08/26/2008	W2	2400	29 (696) ^a
08/26/2008	W3	1500	42 (630)
09/22/2008	W4	1400	21 (294)
06/23/2009	W4	600	67 (402)
07/21/2009	W3	1500	21 (315)
10/29/2009	W3	650	50 (325)
08/25/2010	W2	2400	12 (288)
08/25/2010	W4	1900	8 (152)

^aValue in parenthesis denotes the potential contribution of naturalized strains to the *E. coli* counts observed in CFU/100 ml.

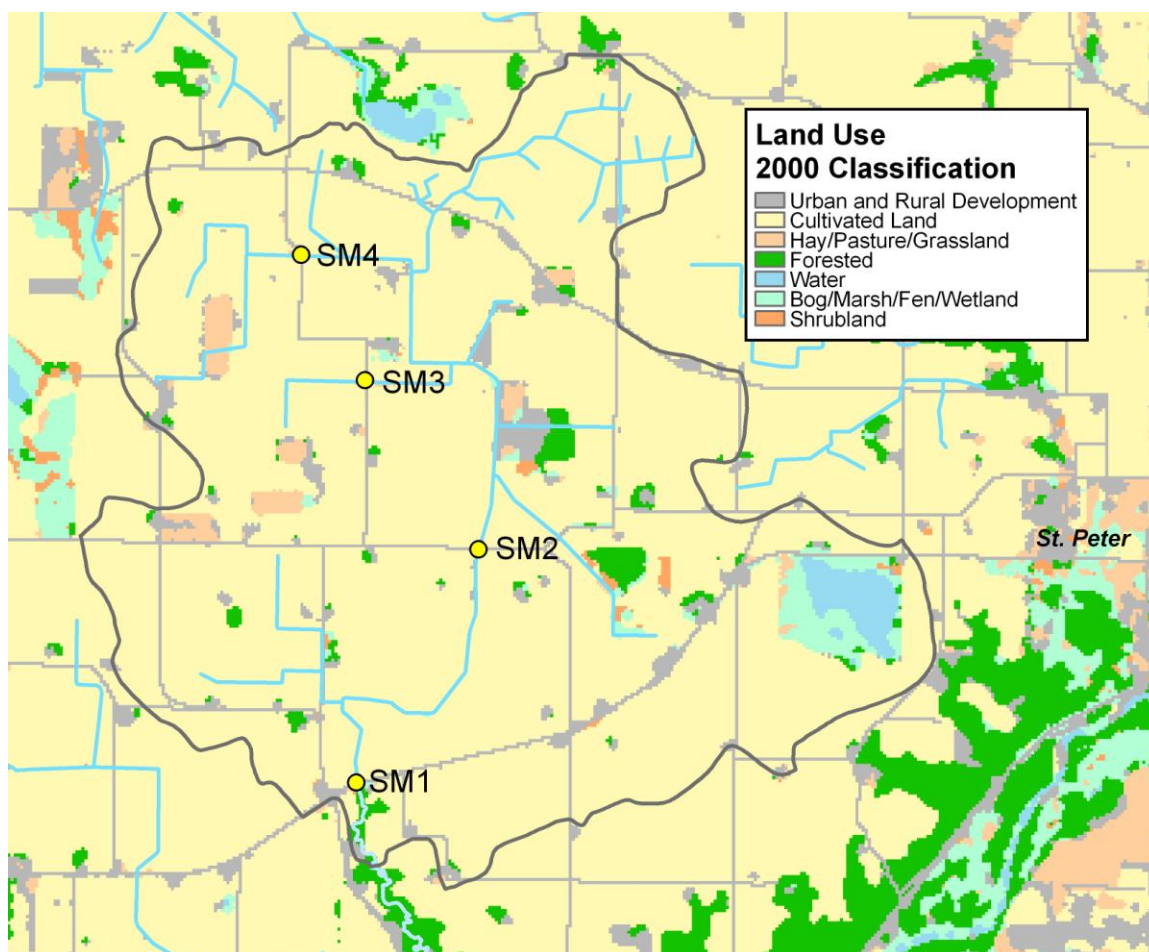


Figure 2.1. GIS land use map of the Seven Mile Creek watershed. Samples sites, SM1- SM4 are denoted by yellow circles. The creek flows from SM4 towards SM1.

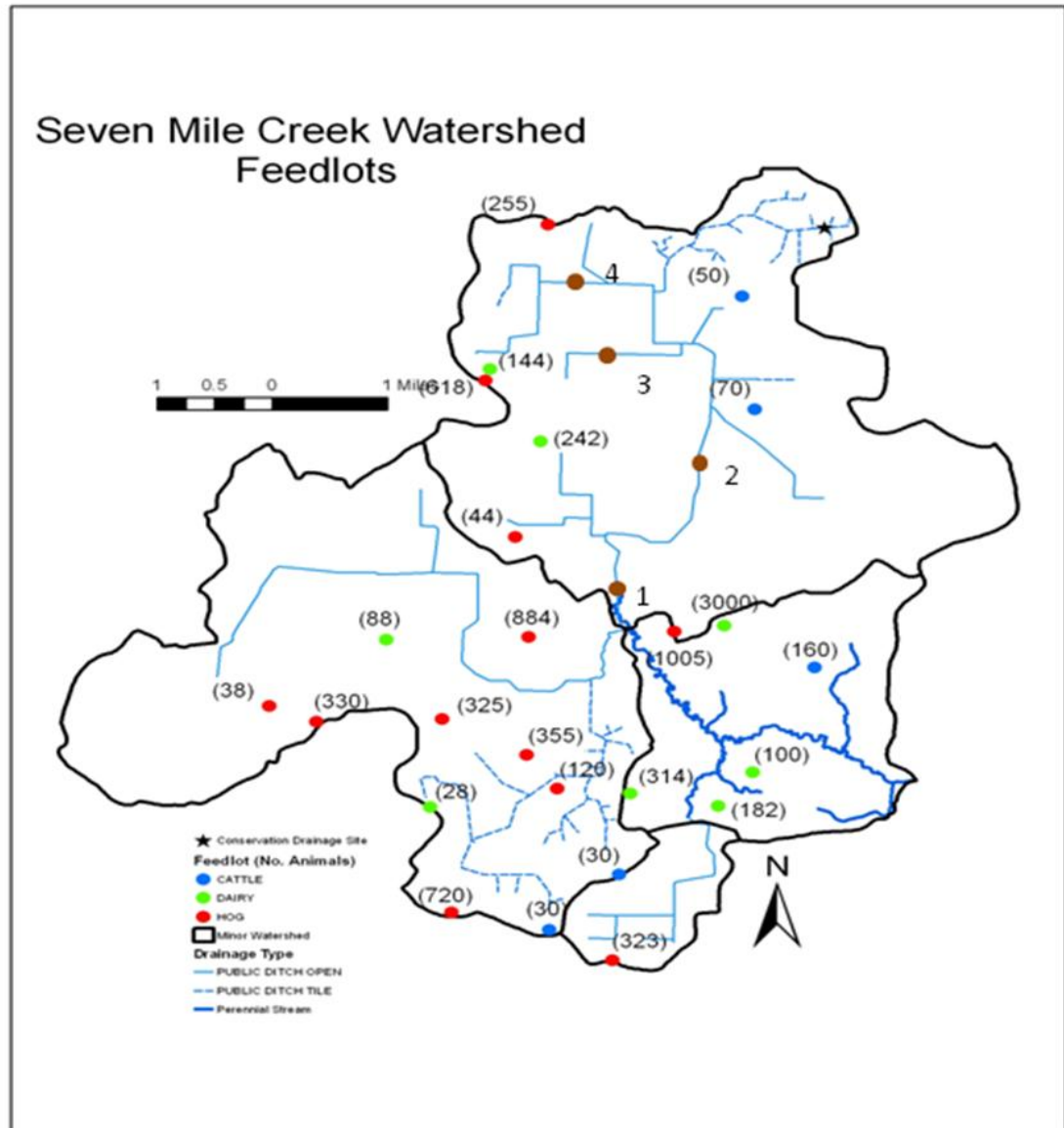


Figure 2.2. Distribution of feedlots across the watershed. Feedlots for cattle, dairy and hog are marked by (●), (●) and (●) circles, respectively. The sampling sites SM1-4 are denoted (●), numbered 1-4, respectively.

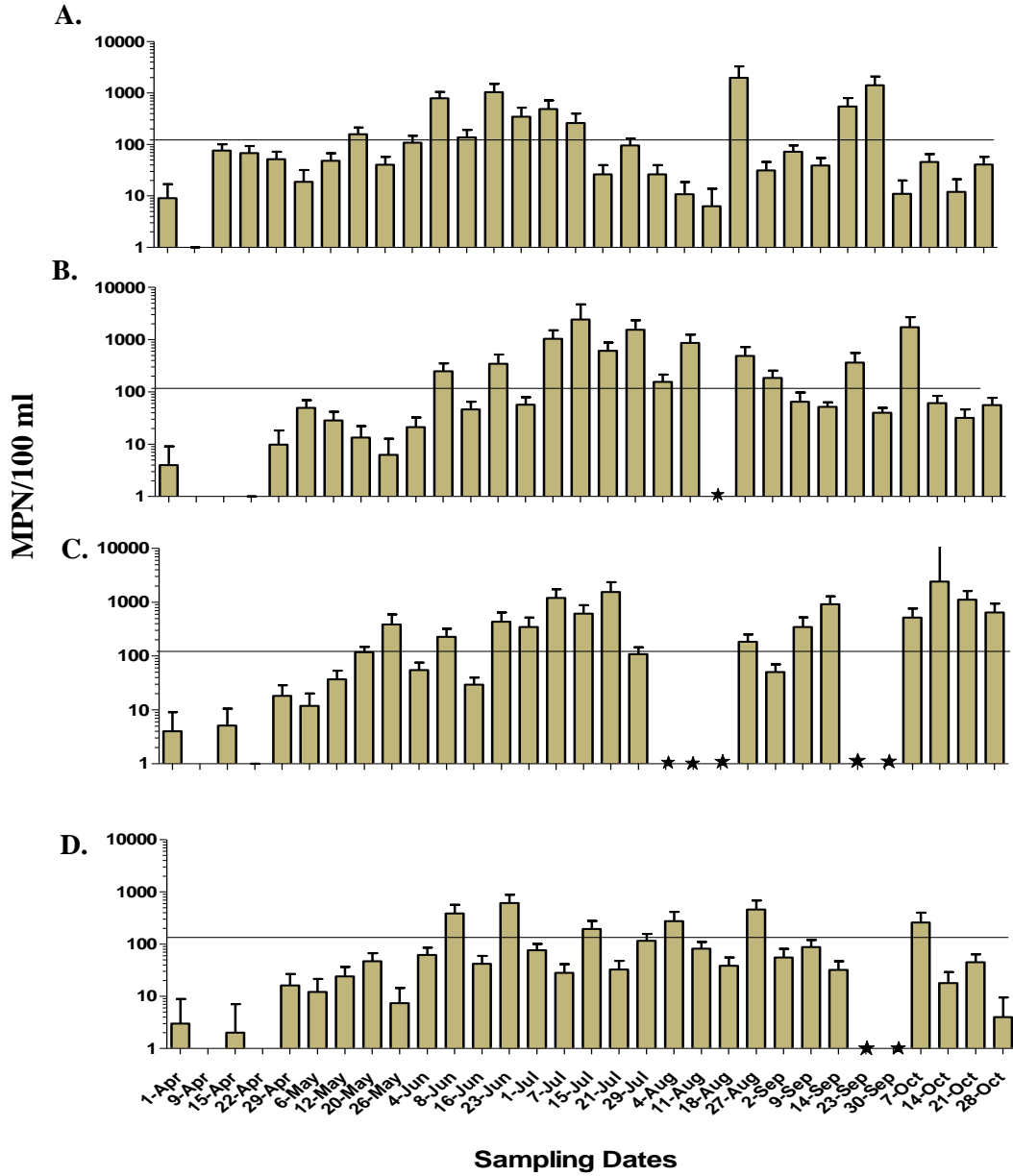


Figure 2.3. Individual water *E. coli* counts from April to October 2009. Counts from SM1-SM4 are shown separately in panels A, B, C and D, respectively. State standard for water is marked by the horizontal black line at 126 CFU/100 ml. ★ Sites were dry during these dates and samples could not be obtained.

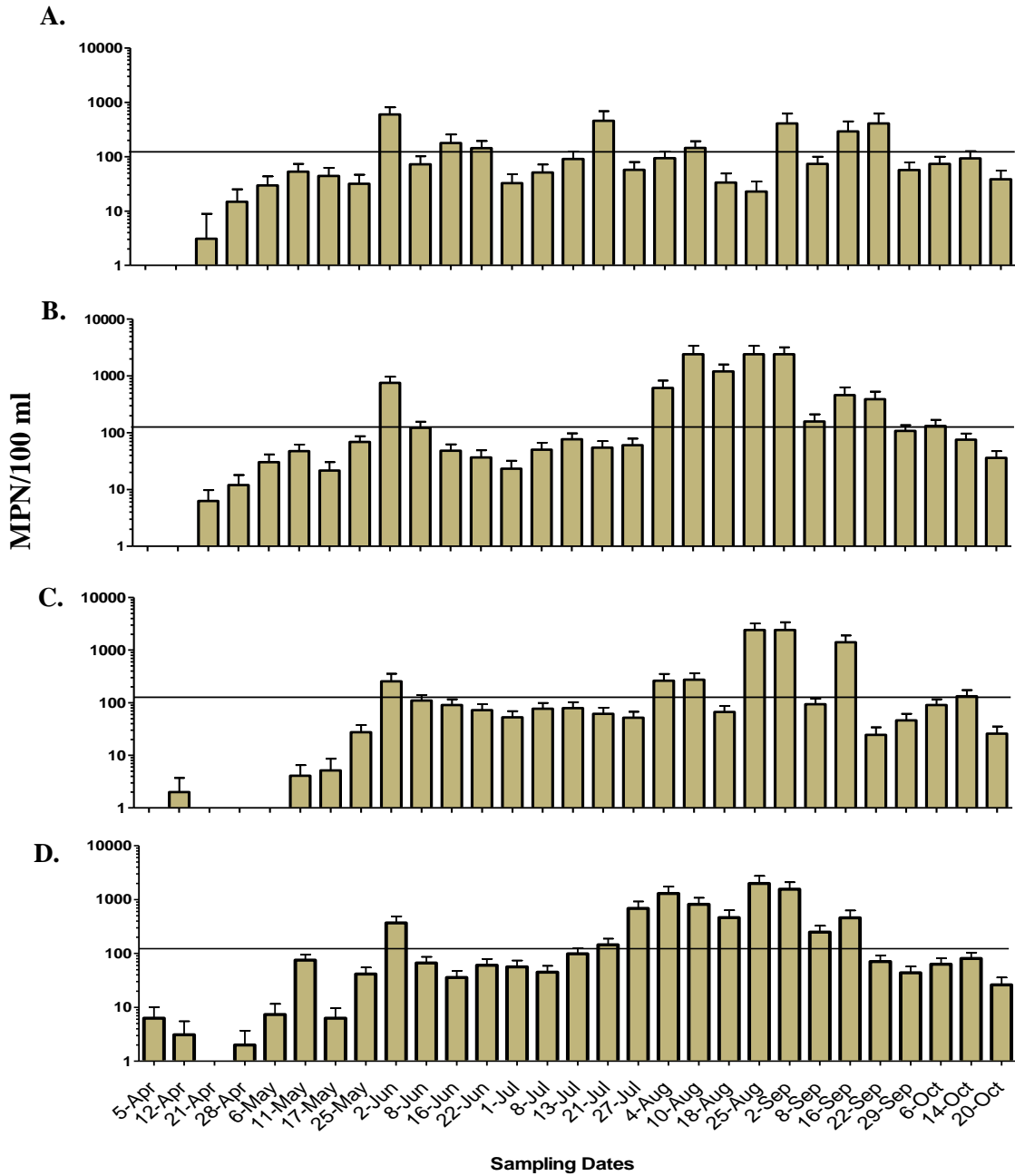


Figure 2.4. Individual water *E. coli* counts from April to October 2010. Counts from SM1-SM4 are shown separately in panels A, B, C and D, respectively. State standard for water is marked by the horizontal black line at 126 CFU/100 ml.

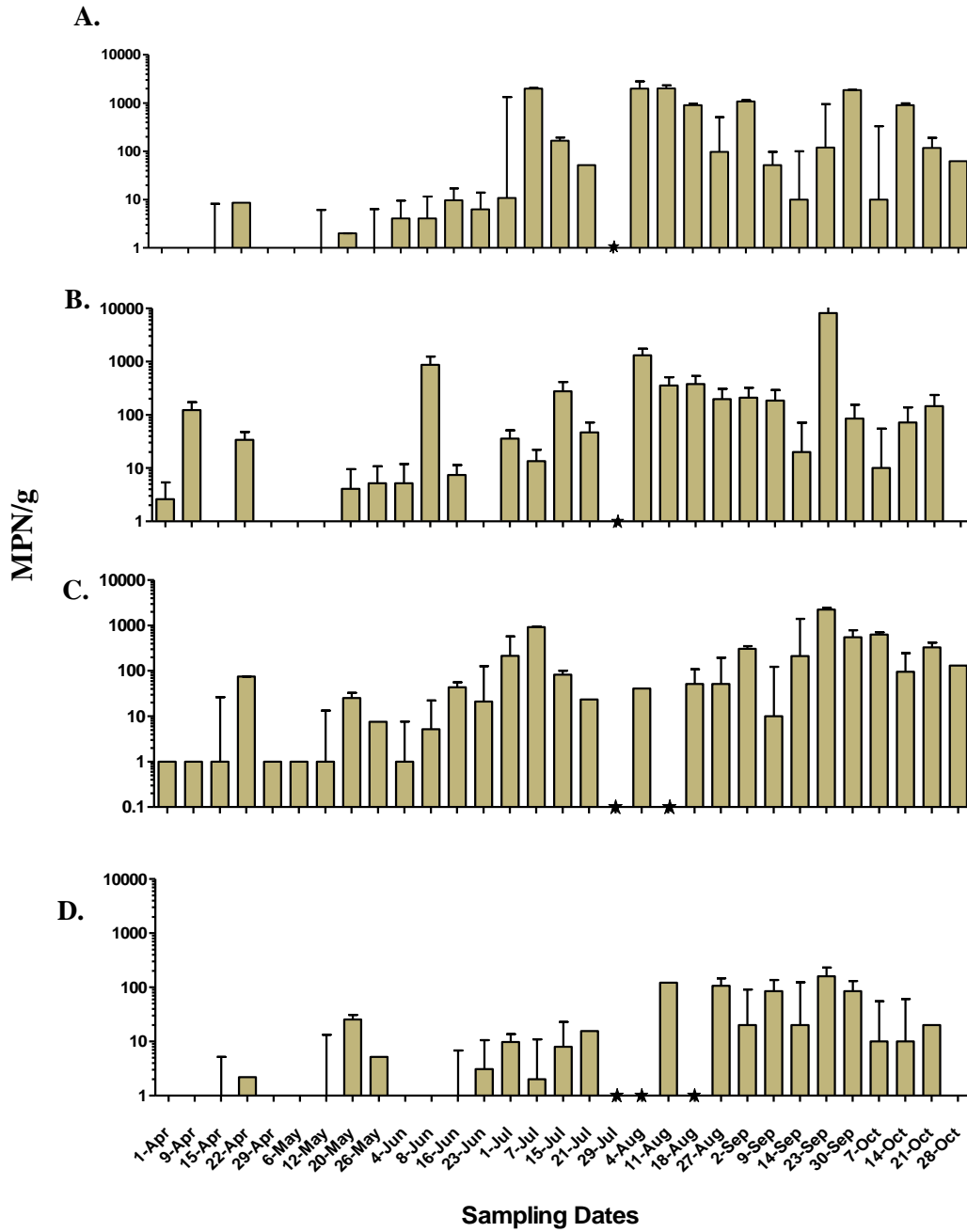


Figure 2.5. Individual sediment *E. coli* counts from April to October 2009. Counts from SM1-SM4 are shown separately in panels A, B, C and D, respectively. ★ Sites were dry during these dates and samples could not be obtained.

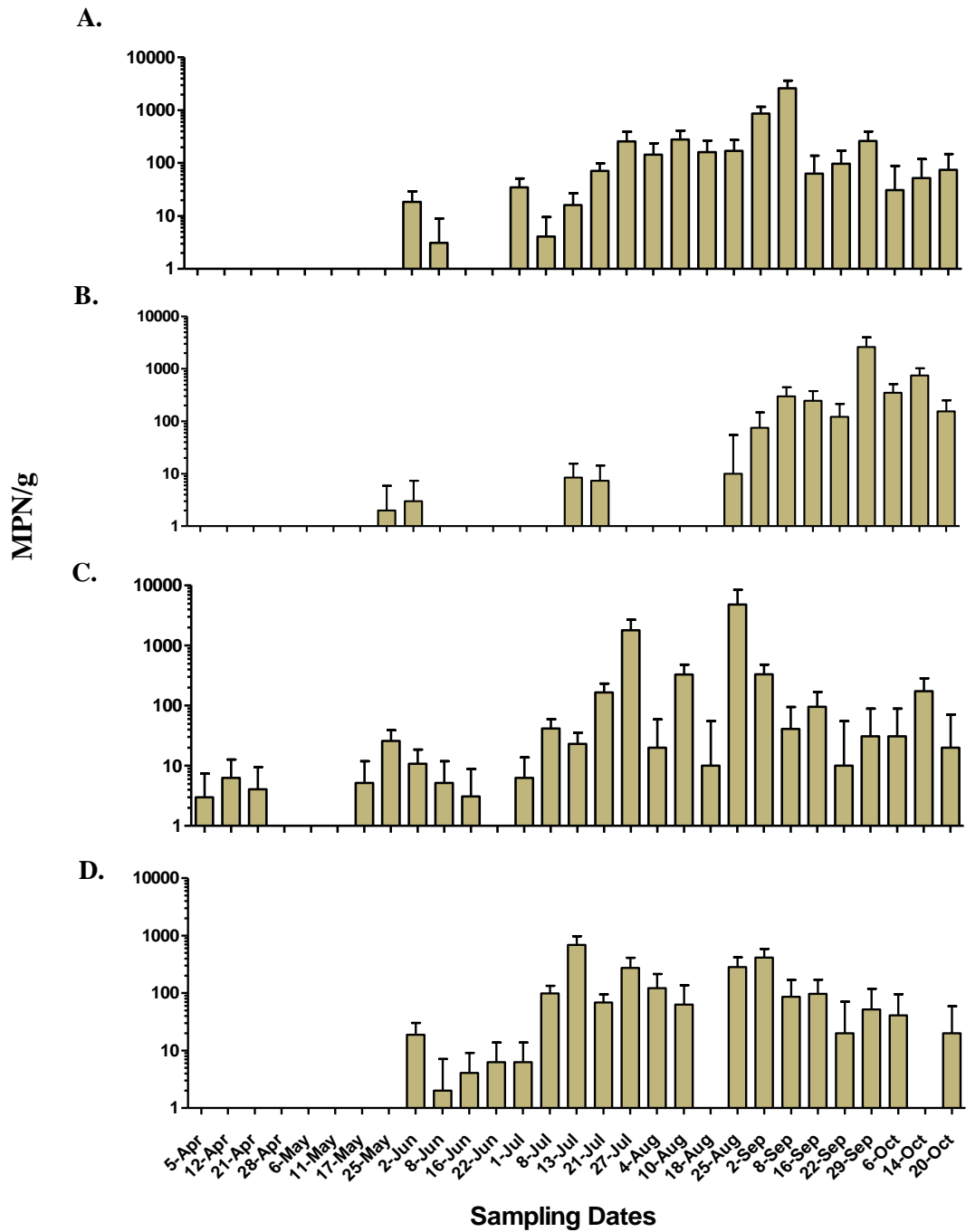


Figure 2.6. Individual sediment *E. coli* counts from April to October 2010. Counts from SM1-SM4 are shown separately in panels A, B, C and D, respectively.

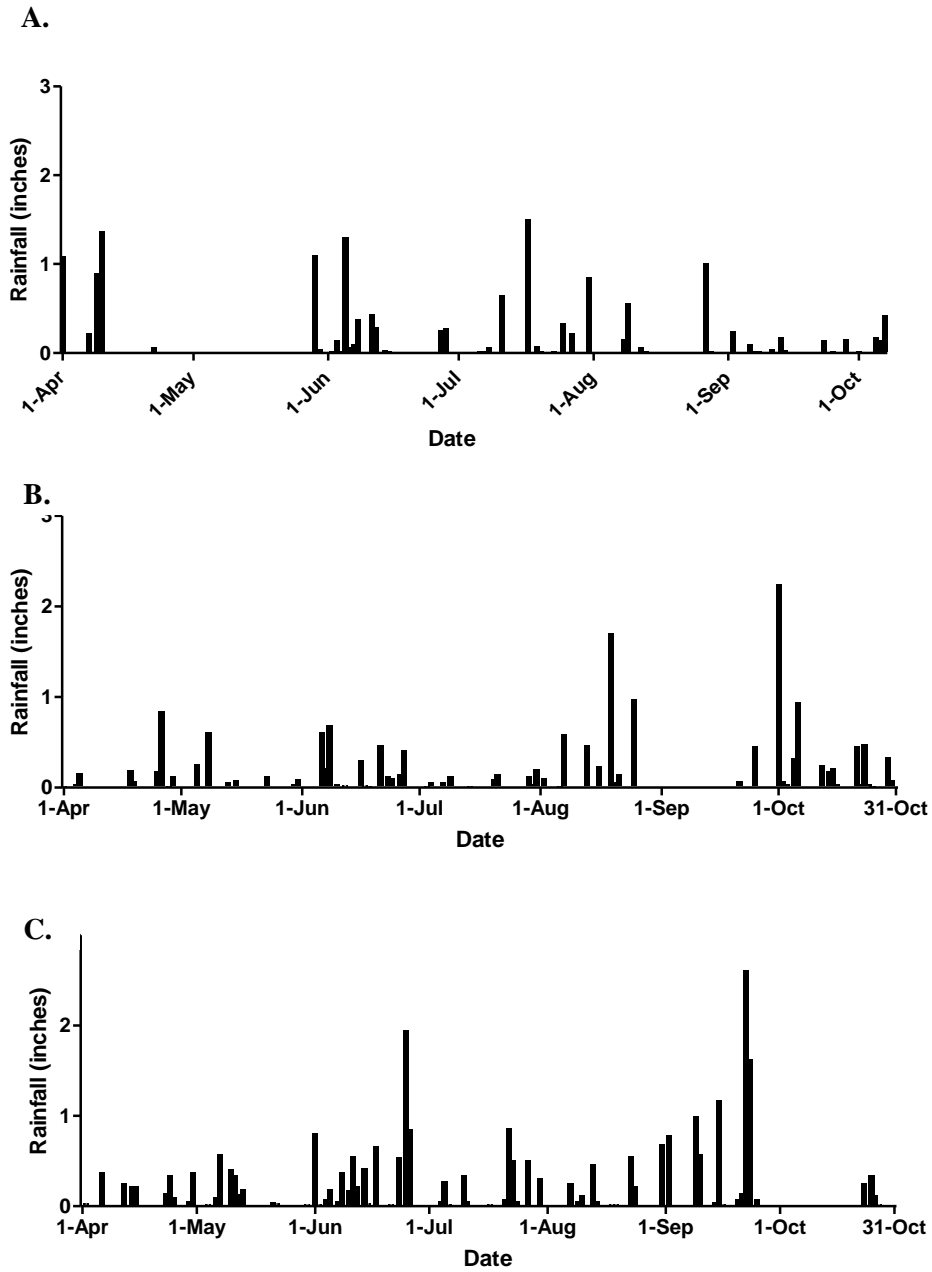


Figure 2.7. Precipitation data by month for the 2008, 2009 and 2010 sampling periods are shown in panels A, B and C, respectively.

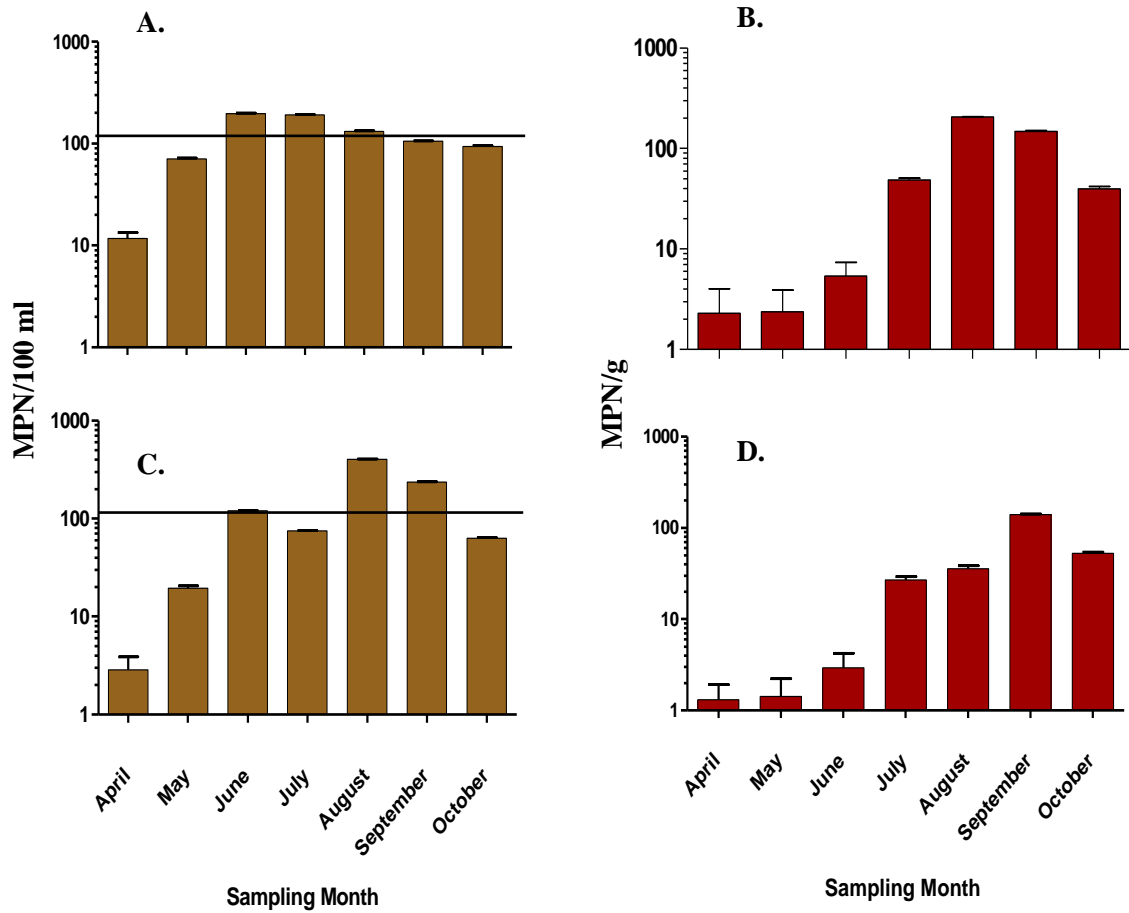


Figure 2.8. The average water and sediment *E. coli* counts across months. Average water counts (in MPN/ 100ml) from 2009 and 2010 are shown in panels A and C, respectively. Average sediment counts (in MPN/ g dry weight) from 2009 and 2010 are shown in panels B and D, respectively. Water *E. coli* standard is denoted by the horizontal line at 126 CFU/100 ml.

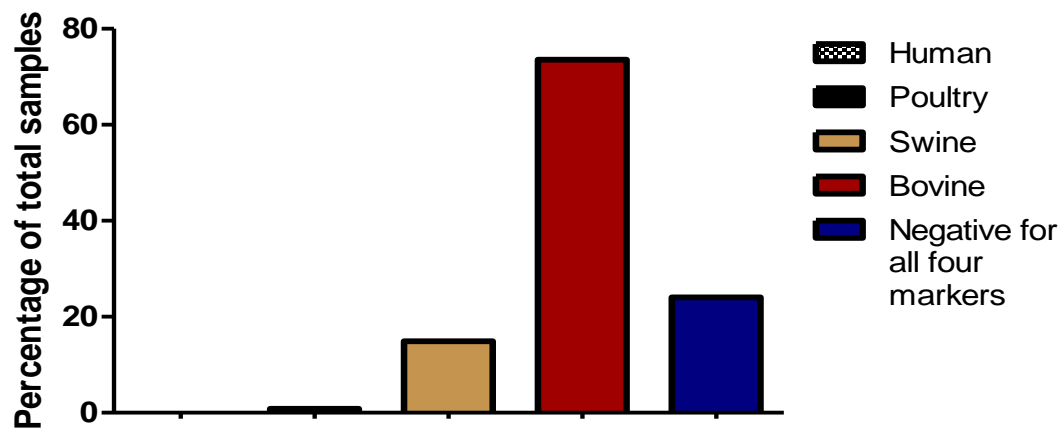


Figure 2.9. Summary of host species specific PCR assays for 2009-2010. Fecal bacterial markers tested include those originating from bovine, human, poultry and swine sources. Note: The percentages do not add up to a 100% as several samples were positive for more than one host source tested.

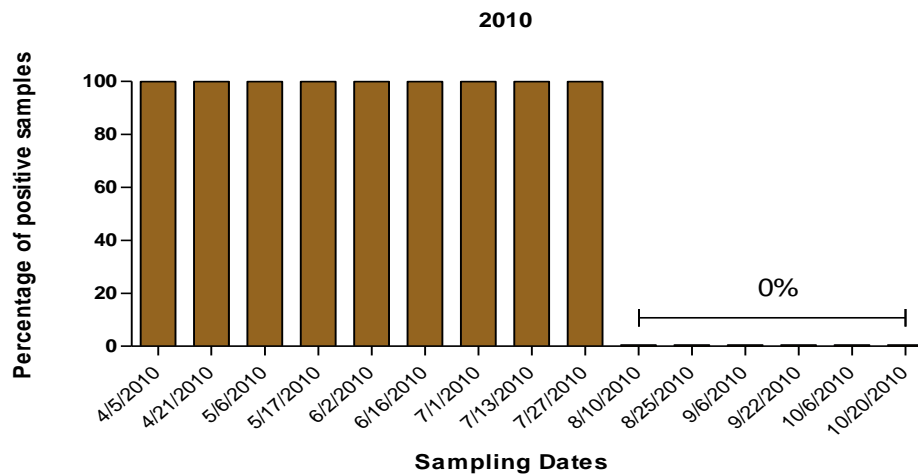
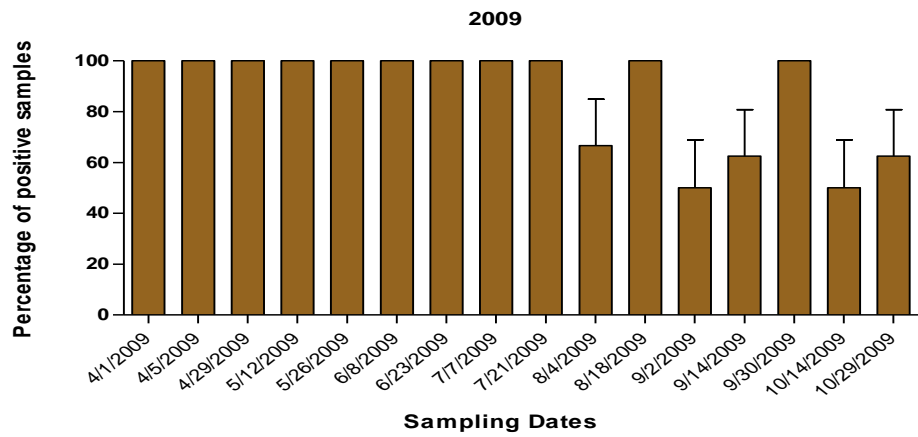


Figure 2.10. Comparison of samples positive for bovine fecal marker by sampling year and dates. Percentages for sampling dates were obtained by averaging the site contributions.

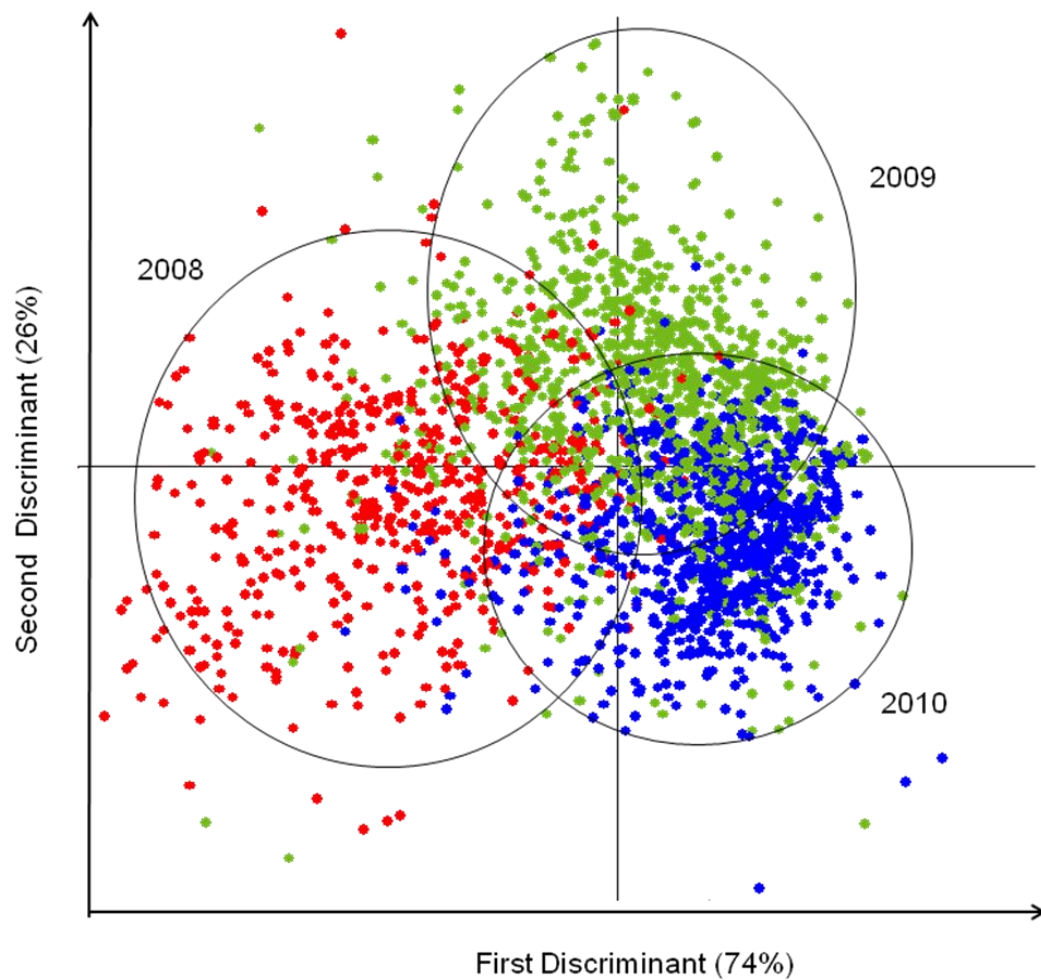


Figure 2.11. MANOVA analysis of all SMC *E. coli* isolates grouped by year. Isolates from 2008, 2009 and 2010 are represented by (●), (●) and (●), respectively.

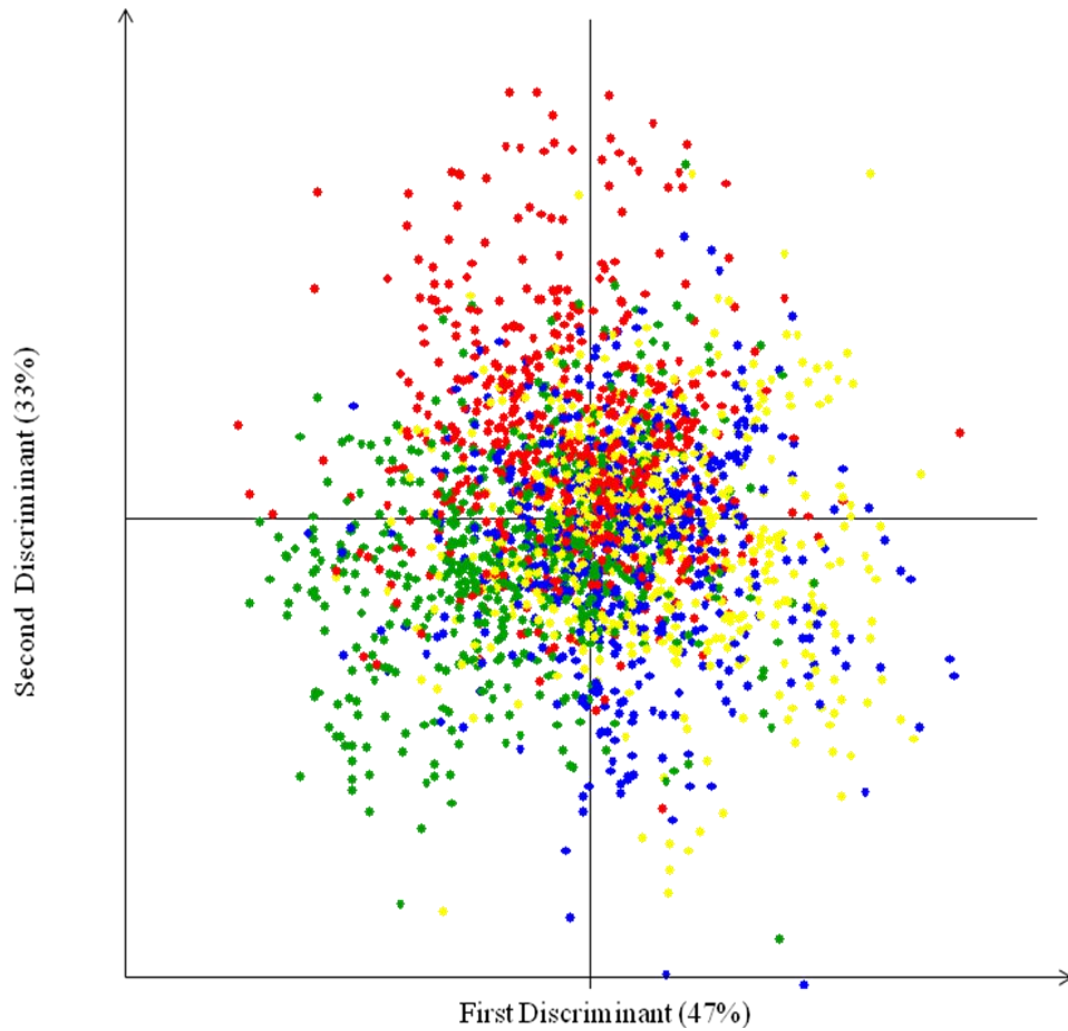


Figure 2.12. MANOVA analysis of SMC *E. coli* isolates grouped by site (2008-2010). *E. coli* isolates from SM1, SM2, SM3 and SM4 are denoted by (●), (●), (●) and (●), respectively.

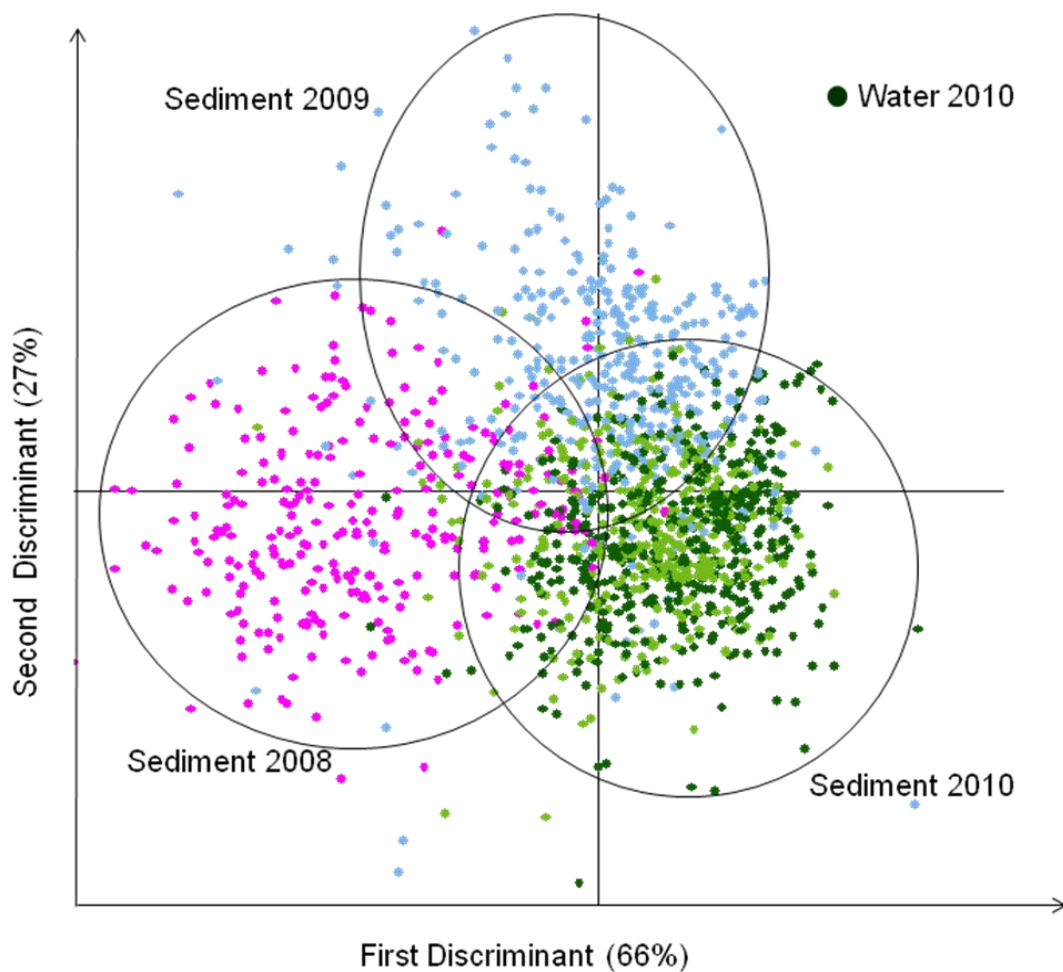


Figure 2.13. MANOVA analysis of SMC *E. coli* isolates grouped by sample type. Sediment isolates from 2008, 2009 and 2010 are denoted by (●), (●) and (●), respectively. Water *E. coli* isolates from 2010 is shown by (●). Similar trend was observed with water isolates from 2008 and 2009.

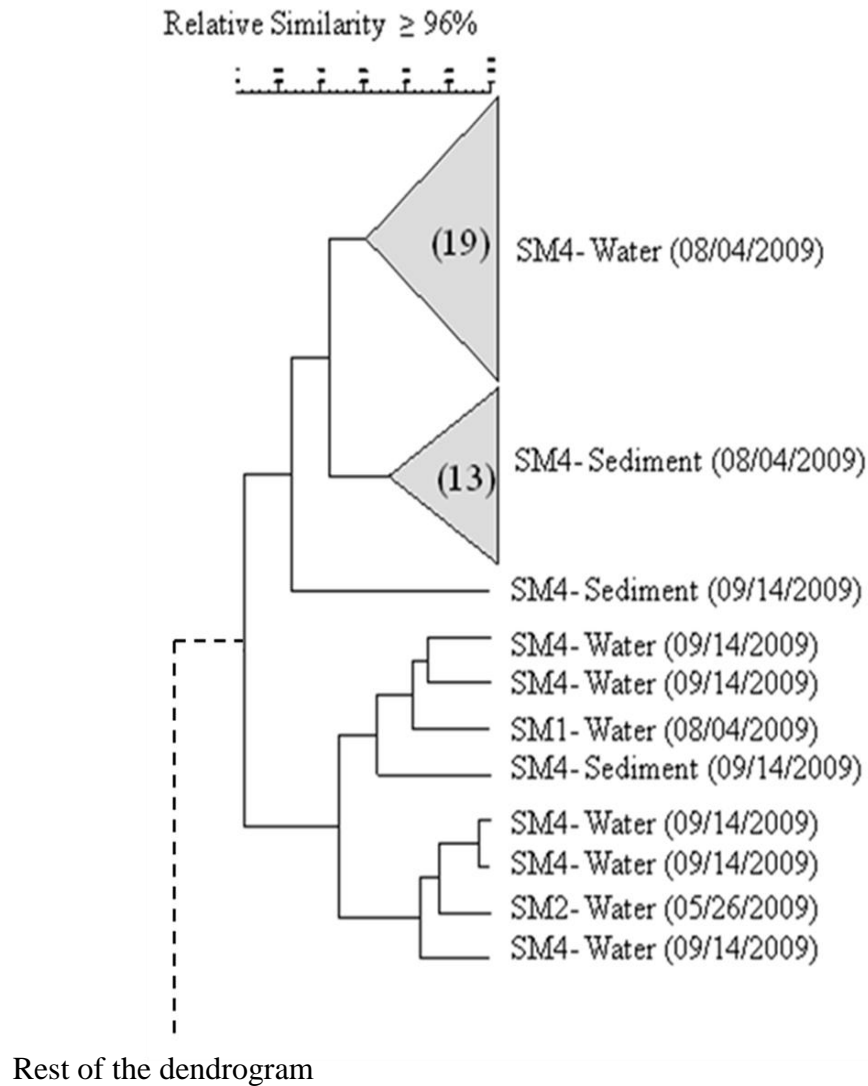


Figure 2.14. Partial dendrogram generated using HFERP DNA fingerprint data from water and sediment isolates (of same genotype) obtained between July and September 2009. Value in parenthesis denotes the number of isolates in each group.

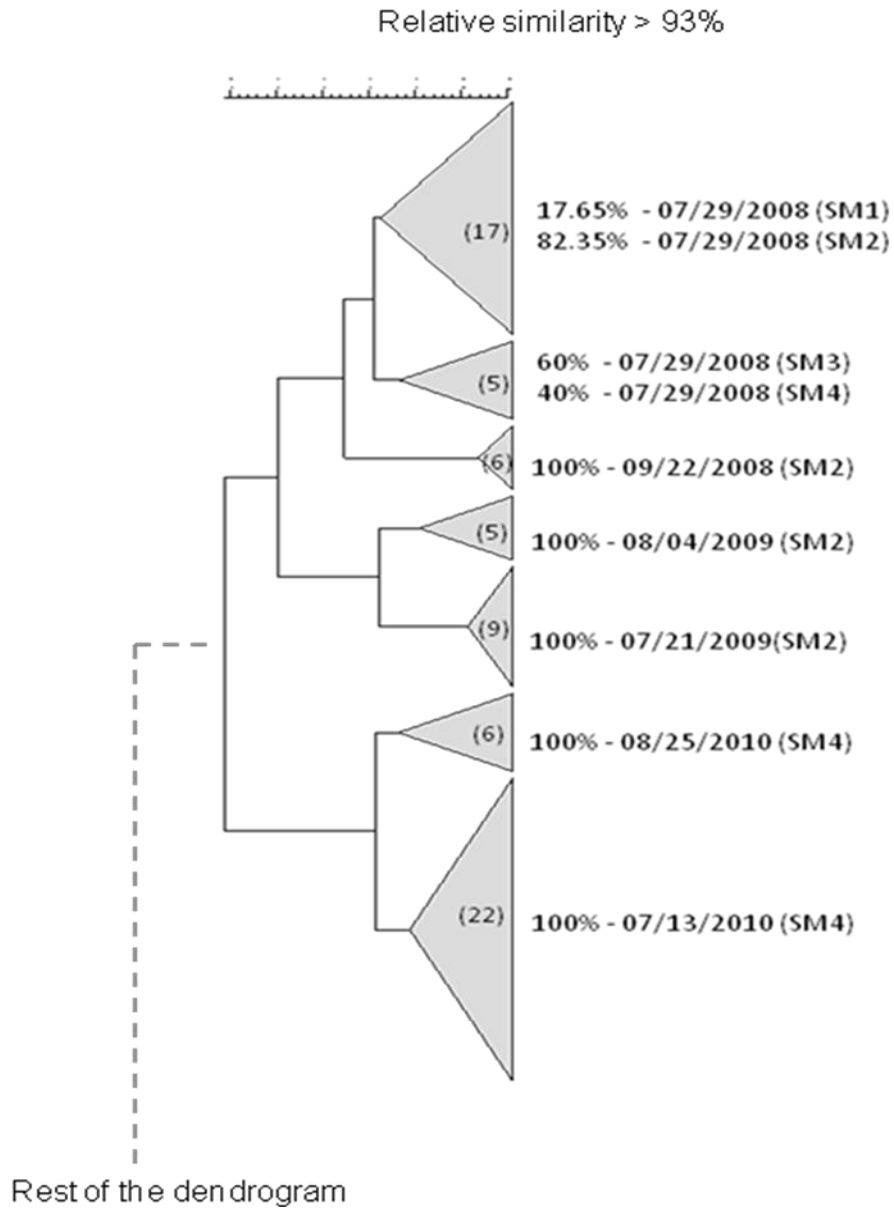


Figure 2.15. Partial dendrogram of SMC naturalized *E. coli* strains (of same genotype) obtained between July 2008 and October 2010. Isolates were collapsed in the dendrogram for purpose of representation. The number of *E. coli* isolates in each group and percentages of the samples from which these *E. coli* isolates were obtained are shown.

Relative similarity $\geq 92\%$

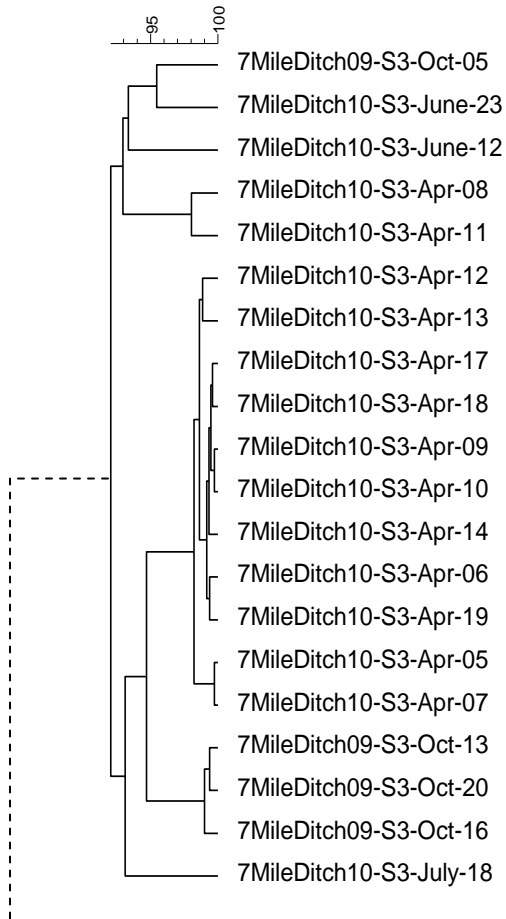


Figure 2.16. Partial dendrogram of HFERP DNA fingerprint data consisting of SM3 sediment *E. coli* isolates (of same genotype) obtained between October 2009 and July 2010

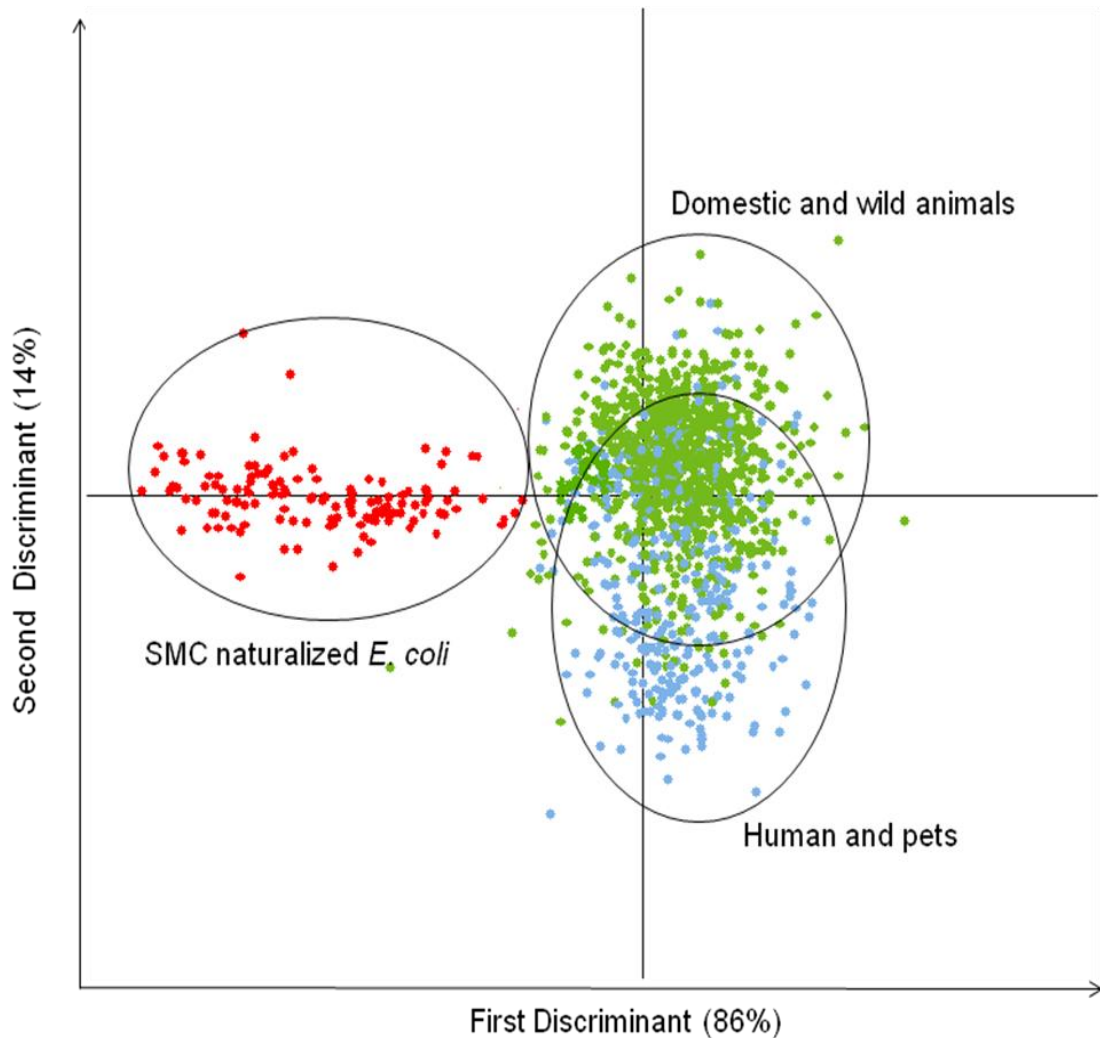


Figure 2.17. MANOVA of HFERP DNA fingerprints from *E. coli* strains. (A) MANOVA of HFERP DNA fingerprints obtained from SMC naturalized *E. coli* strains (●) and *E. coli* isolated from feces of human (●) and animal sources (●).

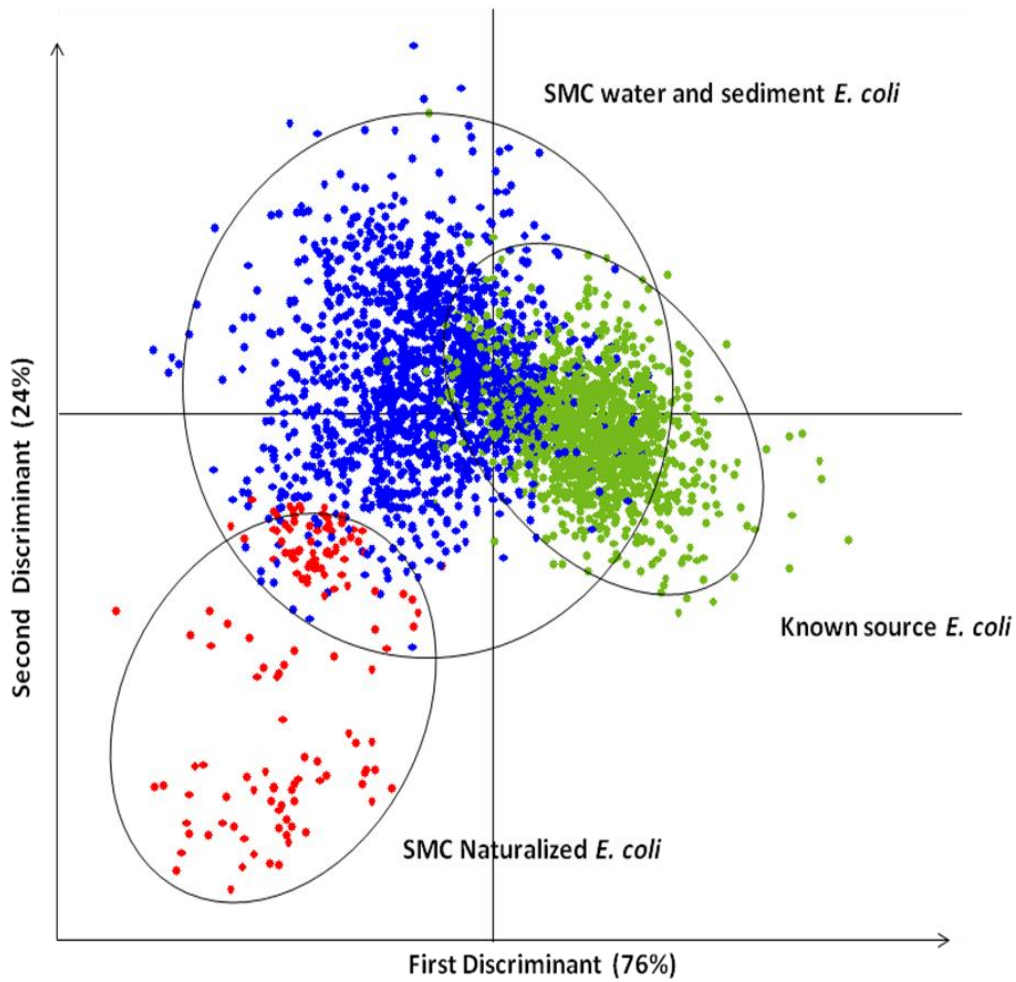


Figure 2.18. MANOVA of HFERP DNA fingerprints of SMC naturalized *E. coli* (●), non-indigenous SMC water and sediment *E. coli* (●) and *E. coli* isolated from known sources (●).

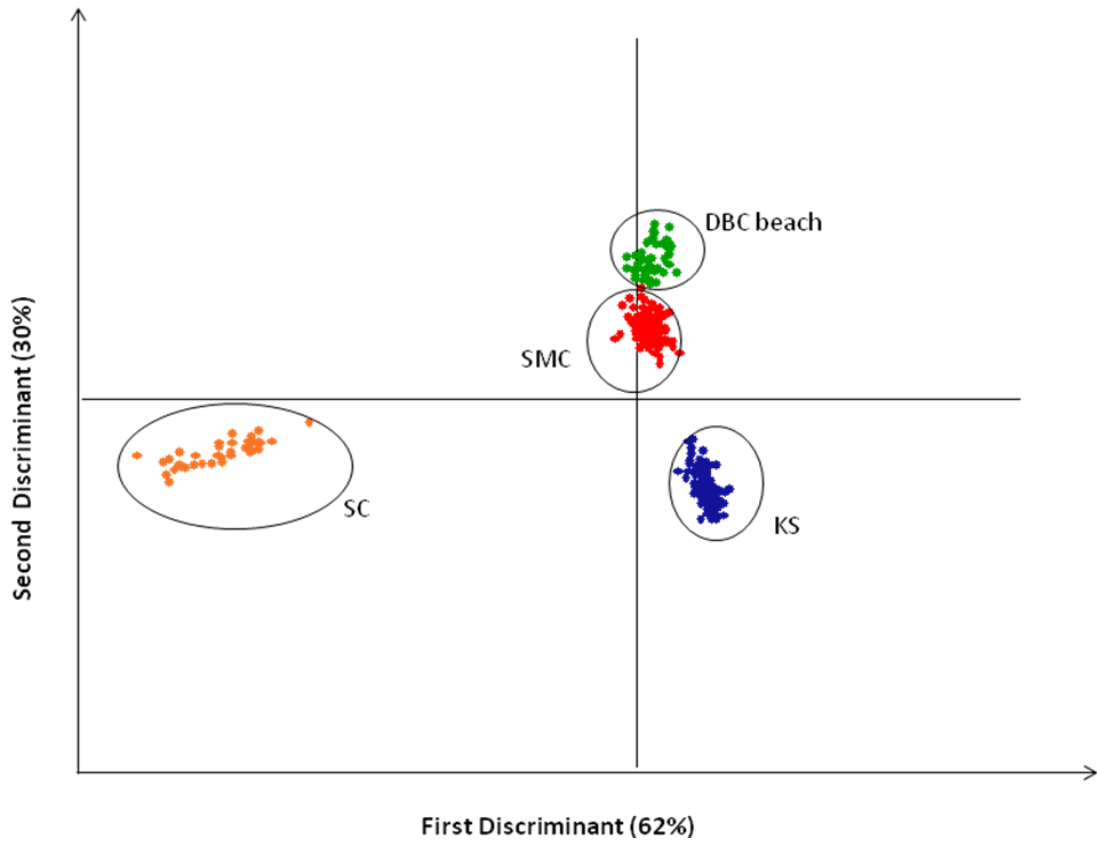


Figure 2.19. MANOVA of HFERP DNA fingerprints of naturalized *E. coli* strains from the Seven Mile Creek sediments (●), Duluth Boat Club beach sediments (●), Kingsbury Stark site soil, Lake Superior watershed, Duluth (●), and St. Louis Clyde site soil, Lake Superior watershed, Duluth (●).

Chapter 3. Conclusions and Future Directions

Understanding the fate and ecology of *E. coli* in the environment is essential for determining its reliability as an indicator of water quality and for assessing public health risks. With recent reports suggesting the persistence of *E. coli* in soils, water and beach sands, the likelihood of temperate sediment to act as an alternate source and sink of *E. coli* in environment was evaluated. The present study allowed for collection of a large dataset, and lead to a detailed understanding of population structure, distribution and sources of *E. coli* in temperate ditch sediments than was possible previously.

In conclusion, in this study I report that the SMC watershed was heavily contaminated with *E. coli* and seasonal variations in the concentrations of *E. coli* observed in the SMC were likely influenced by temperature profiles, rainfall and runoffs. The *E. coli* levels in the SMC watershed were above the permissible 126 CFU/ 100 ml limit at least 30% of the tested times indicating impairment by fecal bacteria. Microbial source tracking using HFERP DNA fingerprinting and host source-specific PCR assays identified fecal bacteria in the SMC that may be of bovine, swine, poultry and human origin.

Examination of genotypes of *E. coli* strains obtained from the SMC revealed that the population structure of *E. coli* was extremely diverse with shifts occurring on an annual scale. Despite this shift, some *E. coli* strains remained constant over time and were repeatedly isolated from sediments and water across all three years. Therefore, *E.*

coli populations present in SMC can be viewed as a mixture of new acquired (due to runoffs are fecal inputs) and persistent strains. Persistent strains are likely naturalized to the sites, capable of growing in sediments, and surviving through the repeated freeze-thaw cycles.

Distribution of *E. coli* population in the SMC was greatly influenced by flow events. Mixing of water occurred between sites during flow and likely affected the mobility of naturalized *E. coli* among the sites located within the watershed. Additionally, mixing occurred between sediment and water column, and this may result in re-suspension of sediment-borne naturalized *E. coli* into overlaying water leading to an apparent increase in the observed levels of *E. coli*. Overall, these results suggest that ditch sediments are temporal sinks and sources of *E. coli* in environment, which confounds the use of *E. coli* as an indicator and has implications for future water quality monitoring and TMDL determinations.

As mentioned in the discussion section, there are several avenues for future research in this area. Results from our study suggested that use of conventional indicators (such as *E. coli*) in combination with alternative indicators (genera *Bacteroides* and *Bifidobacterium*, spore-forming *Clostridium perfringens* and bacteriophage and coliphages) can serve to overcome some of the limitations associated with current monitoring procedures. However, for such an approach to gain widespread use, adequate epidemiological studies supporting the use of these alternative indicators needs be conducted.

Results obtained in this study also emphasized the need for a reference library containing *E. coli* isolates obtained from livestock, wildlife and manure within the SMC watershed. Comparison of *E. coli* strains from this reference library to those isolated from SMC might help to explain why the ID bootstrap analysis failed to identify majority of the SMC isolates and also aid in validating the naturalized *E. coli* strains obtained from the SMC.

Additionally, indigenous *E. coli* strains obtained in this study can be incubated in natural, non-sterile, non-amended sediments from the watershed as a confirmatory experiment to show that these strains have the ability to grow and persist in sediments. Such incubation studies have been previously reported for soil naturalized *E. coli* strains (71).

Furthermore, the ability of *E. coli* to grow and persist in the environment likely emphasizes a genetic adaptation which can be explored by using omics-based approaches. Genome sequencing of naturalized *E. coli* strains and comparisons with those of fecal-borne *E. coli* (present in host-intestine) will provide insight into the genetic differences that confer adaptive advantages to the habitat (host intestine or environment) from which these strains are isolated. A recent study by Luo et al.(92) has used such an approach to identify some genes that are of ecological and functional significance to each of these group. The environment-specific gene set that they indentified included several genes of unknown function, a complete pathway for diol utilization (energy substrate), and the gene for lysozyme production (hydrolysis of bacterial cell walls). The latter two

are important for resource acquisition and survival in the environment. In contrast, the gastrointestinal *E. coli* included several genes involved in the transport and use of nutrients thought to be abundant in the gut. Moreover, their genome data also suggested that environmental bacteria are highly unlikely to represent a risk to public health. Taken together, results from our study and that of Luo et al.(92) underscore the need to reevaluate the use of *E. coli* as an indicator of fecal pollution in natural ecosystems. In future, molecular assays that utilize genes unique to fecal-borne *E. coli* over environmental *E. coli* as biomarkers must be developed as they will provide a more accurate assessment of fecal contamination in environment than is possible by current methods.

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