Determining Sources of *E. coli* Contamination in The Minnehaha Creek Watershed using Rep-PCR DNA Fingerprinting Technology

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Table of Contents

Acknowledgements--i

Table of Contents--ii

List of Tables --v

List of Figures--vi

Chapter 1: Introduction

Introduction of the Problem---1

Water Quality--2

Environmental Aquatic Microbiology (Biofilms)-----------------------------------3

Enteric FIB--5

E. coli and *Enterococcus*--6

Factors Influencing Growth of FIB---7

Sources of FIB---9

Microbial Source Tracking Technology--10

Determining Sources of *E. coli* with Rep-PCR---------------------------------14

Implications of Contamination and Impairment of Aquatic Resources--------

--15

Minnehaha Creek Watershed---18

Thesis Outline---20

Chapter 2: Methods, Results, and Discussion

Preface---22

Introduction--23

Materials and Methods--27

Site Description---27

Sample Collection---29

Isolation of Environmental *E. coli* ---32

Enumeration of *E. coli* ---33

DNA Extraction and PCR ---33

Gel Electrophoresis ---34

Gel Staining and Imaging ---35

Image Analysis --36

Clonality --36

MANOVA Analysis --36

Multidimensional Scaling Analysis---------------------------------------36

MCWD 2017 Sampling Results

Dry Weather Results --37

Wet Weather Results---40

Dry and Wet Combined Results--43

Clones Shared Analysis Results---46

Shannon-Weaver Diversity Index Results-------------------------------46

Hydrological Discharge Results---47

MCWD 2018 Sampling Results

Control Weather Results---49

Control vs Ultra Low Flow Combined Results---------------------------52

Clones Shared Analysis Results--55

Shannon-Weaver Diversity Index Results-------------------------------55

Total Clones Per Sample Set Results------------------------------------56

Hydrological Discharge Results---57

MCWD 2017 vs 2018 Sampling Data Results

2017 vs 2018 Sampling Results--58

2017 vs 2018 Clones Shared Analysis Results------------------------62

MCWD Sources of *E. coli* Discussion---63

Hydrological Influences on *E. coli* Discussion------------------------------------69

Chapter 3: Conclusions

Future Considerations---73

Limitations---73

Recommended Actions to Reduce FIB in the MCWD------------------------75

References---76

List of Tables

Table 2.1 - Samples Sets Analyzed in the Study --30

Table 2.2 - Clones Shared Analysis 2017 with the sample set MS-all ----------------------47

Table 2.3 - Clones Shared Analysis 2017 with the sample set Wet --------------------------47

Table 2.4 – Shannon-Weaver Diversity Index 2017 ---48

Table 2.5 - Clones Shared Analysis 2018 with the sample set MS-all ----------------------56

Table 2.6 – Shannon-Weaver Diversity Index 2018 ---57

Table 2.7 - Total percentage clones per sample set---57

Table 2.8 – 2017 vs 2018 Clones Shared Analysis--63

List of Figures

Figure 1.1 - Association of Microbes with Particles---4

Figure 1.2 - Fast moving water at the end of Minnehaha Creek-------------------------------8

Figure 1.3 - Dog Walker crossing bridge with Dog over Minnehaha Creek------------------9

Figure 1.4 - Organic Debris Stuck in Street Gutter near Minnehaha Creek----------------17

Figure 1.5 - Minnehaha Creek Watershed District Map (MCWD)----------------------------18

Figure 1.6 - Minnehaha Falls Park--19

Figure 2.0 - Outflow of the Minnehaha Creek into the Mississippi River--------------------27

Figure 2.1 - Minnehaha Creek Watershed and Study Area for the Minnehaha Creek Bacterial Source Identification Study---28

Figure 2.2 - MCWD Study Area w/ Baseline Monitoring Sites for Bacterial Source Identification Study--39

Figure 2.3 - MANOVA analysis of Dry Weather 2017 Samples------------------------------38

Figure 2.4 - Multidimensional Analysis of Dry Weather 2017 Samples----------------------39

Figure 2.5 - MANOVA Analysis of Wet Weather 2017 Samples ------------------------------41

Figure 2.6 - Multidimensional Scaling of Wet Weather 2017 Samples----------------------42

Figure 2.7 - MANOVA Analysis of Wet and Dry Weather 2017 Samples ------------------44

Figure 2.8 - Multidimensional Scaling of Wet and Dry Weather 2017 Samples-----------45

Figure 2.9 - Measurement of Discharge from Jan. 1st, 2017 until Dec. 31st, 2017------48

Figure 2.10 - Measurement of Discharge from April 1st, 2017 until October 31st, 2017--48

Figure 2.12 - MANOVA analysis of Control (Normal Weather Conditions) 2018 Samples---50

Figure 2.13 - Multidimensional Scaling of Control (Normal Weather Conditions) 2018 Samples--51

Figure 2.14 - MANOVA analysis of Control and Ultra Low Flow Conditons 2018 Samples--53

Figure 2.15 - Multidimensional scaling analysis of Control and Ultra Low Flow Conditions 2018 Samples---54

Figure 2.16 - Measurement of Discharge from Jan. 1st, 2017 until Dec. 31st, 2017-----57

Figure 2.17 - Measure of Discharge from April 1st, 2018 until October 31st, 2018-------58

Figure 2.18 - MANOVA analysis of sample sets analyzed in both 2017 and 2018-------60

Figure 2.19 - Multidimensional Scaling analysis of sample sets analyzed in both 2017 and 2018---61

Figure 2.20 - Duck in Minnehaha Creek ---65

Figure 2.21 - Tile Drainage flowing out into Minnehaha Creek-------------------------------65

Figure 2.22 - Sloped Concrete Impervious Surface leading down into Minnehaha Creek---66

Figure 2.23 - Map of Lake Minnetonka (MCWD)--67

Figure 2.24 - Lake Minnetonka Boat Party July 2019---67

Figure 2.25 - Dog Walker with Dog near Minnehaha Creek------------------------------------68

Figure 2.26 - Discarded Dog Feces near Minnehaha Creek----------------------------------68

Figure 2.27 - Trash Can near Minnehaha Creek Overflowing with Human Trash--------69

Figure 2.28 – Grit Chamber Structure Diagram---71

Chapter 1: Introduction

The Problem

Recent analysis of Minnehaha Creek Watershed by the Minnesota Pollution Control Agency or MPCA in the early 2010's revealed that the total maximum daily load (TMDL) standards for *E. coli* were not being met. Meeting TMDL standards is important in order to maintain the purity of water resources for drinking and recreational use and harvesting of aquatic-derived resources. Elevated *E. coli* counts exceeding a single sample limit of 1260 MPN/ 100ml, and a geometric mean sample limit of 126 MPN/ 100ml, were found within the watershed district. Exceeding TMDL standards resulted in the impairment listing for the watershed district. Previous studies found correlations between increased *E. coli* counts and human gastrointestinal illness. These findings prompted the Minnehaha Creek Watershed District (MCWD) to determine the cause and source(s) of elevated *E. coli* levels. Furthermore, the impact of changing water levels depending on temporal wet/dry seasons in the watershed was investigated over two seasons to understand how this factor impacted microbial communities and movement of possible human pathogens throughout the watershed. Lastly, MCWD hoped to determine what actions, if any, could be undertaken to lower *E. coli* levels to those that fall within TMDL standards in order to protect water resources for human health and recreation.

Water Quality

The maintenance of water quality is essential for human health and recreation. Within a watershed, water quality is determined by the chemistry, biology, and physical properties of the system. Changes to these properties can alter the watershed subsequently impacting water quality. It is the job of watershed managers to use a variety of tools in determining the quality of particular water resources, understand possible impacts to water quality, and provide solutions to rehabilitate altered watersheds (Elshorbagy et al. 2005). Total Max Daily Load (TMDL) designates the maximum amount of a pollutant (chemical or biological) that can enter a particular water body while still maintaining a set quality standard. TMDLs exist for the human pathogen *E. coli* and they are commonly monitored by city and state agencies to protect citizens from human illness associated with *E. coli* and other fecal pathogens.

Many water bodies throughout the US are considered to be impaired due to contamination by pathogenic organisms. Pathogens are considered the dominant cause of river and stream impairment in the US (US EPA, 2009). *E. coli* and other pathogenic microorganisms can be introduced into water bodies through mismanaged sewage systems, urban runoff due to prevalent impervious surfaces, agricultural runoff from feed lots, and vessel wastewater discharge (Fong and Lipp, 2005). Various water-borne pathogens (bacteria, viruses and protozoa) have been associated with disease outbreaks (Sharma et al, 2003; Szewzyk et al, 2000). Once in the water bodies, these microorganisms can

introduce a serious health risk to those exposed through physical contact or direct ingestion.

Environmental Aquatic Microbiology (Biofilm)

Aquatic habitats such as lakes, rivers, streams, and creeks provide many different types of microbes an ideal condition to persist and grow in the environment. However, research done by Costerton (1999) found that bacteria attached to surfaces in a matrix known as a biofilm dominate microbial life in streams and similar water bodies. In fact, evidence of bacterial biofilms have been found as early as 3.25 billion years ago and this growth pattern is an important part of the prokaryotic life cycle (Hall-Stoodley et al, 2004). A biofilm is a community of microorganisms embedded in a porous extracellular matrix containing not just bacteria but also algae, protozoa, fungi, and meiobenthos that often communicate and cooperate with each other for survival (Battin et al, 2016; Stoodley et al, 1999). Biofilms can be formed by most bacteria and are common both within the human body and in the aquatic environment.

As bacteria typically live in aquatic environments attached to surfaces in biofilms, sediment particles of varying sizes provide ideal environments for bacteria to grow on top of and within. Here, nutrients can be trapped in sediment and be plentiful (Davies et al, 1995; Marino et al, 1991). Aquatic sediments trap nutrients and can provide microorganisms shelter against predators, making it an ideal place for microbes to colonize (Howell et al, 1996). In particular, sediment

composed of fine, cohesive particles, with large surface area, are ideal for microbial growth (Gannon et al. 1983, Auer and Nichaus, 1993).

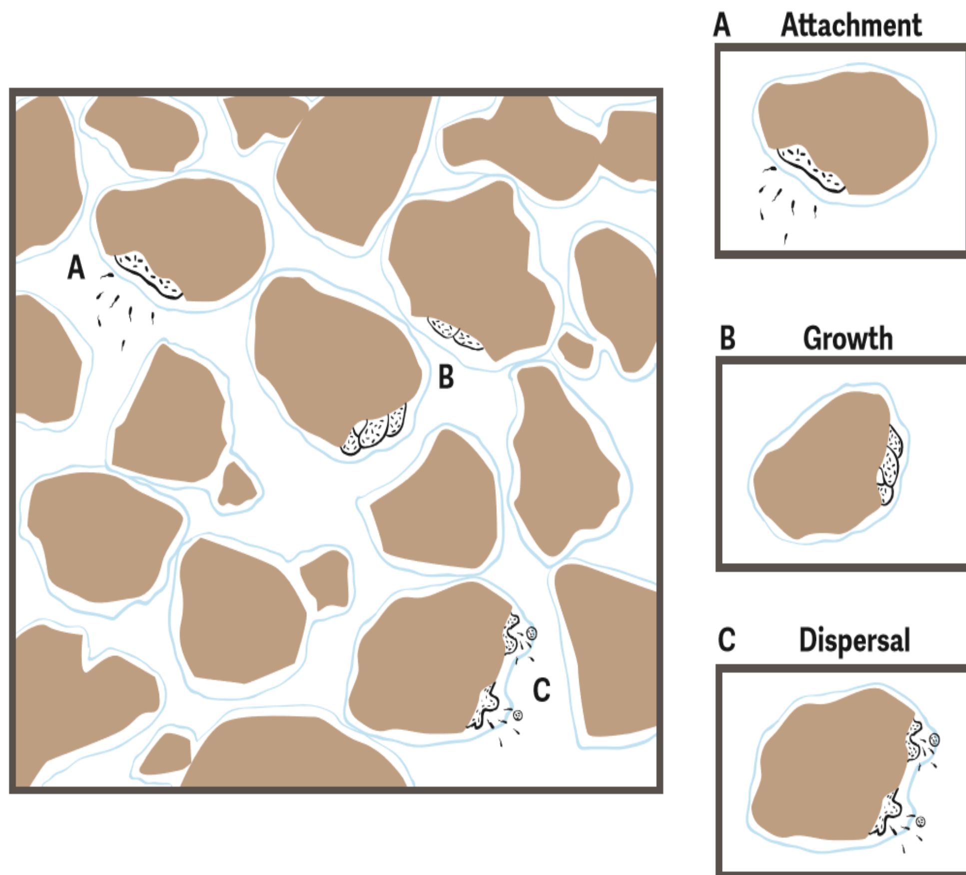


Figure 1.1 - Association of Microbes with Particles (Chen, 2018).

Once attached to sediment surfaces, bacteria become more resistant to the shear forces associated with flowing water and are considered to be part of a biofilm. (Battin et al, 2006). Creek and stream environments, making up a major portion of Minnehaha Creek, contain ideal environments for the survival and growth of microorganisms.

Enteric Fecal Indicator Bacteria

Microorganisms that can enter and thrive in the human digestive tract are known as enteric bacteria. Enteric bacteria are transmitted between humans primarily through the fecal-oral route (Fong and Lipp, 2005). The bacteria can enter the human body through the ingestion of contaminated drinking water, as well as accidentally during recreational activities such as swimming in contaminated waters. A small portion of enteric bacteria are pathogenic and are known for causing gastrointestinal illness. Furthermore, these bacteria are less known for also causing respiratory infections, conjunctivitis, hepatitis, meningitis, encephalitis, and paralysis in those with compromised immune systems (Kocwa-Haluch, 2001). While most common in drinking water supplies, enteric bacteria are also common in recreational waters and shellfish harvested from contaminated waters (Bosch et al., 2001).

Enteric bacteria are frequently used as fecal indicator bacteria (FIB) as a determinant of water quality. Their use in this manner falls on the premise that these organisms are thought to originate from an animal fecal source and can't survive out of a host organism for extended periods of time. A variety of research suggests that various enteric bacteria can, in fact, survive in both subtropical (Desmarais et al. 2001) and temperate extraintestinal environments (Ishii et al. 2006). This challenges the use of bacteria such as *E. coli* as a determinant for water quality.

Escherichia coli and *Enterococcus*

Escherichia coli (or *E. coli)* is a gram-negative bacterium within the family *Enterobacteriaceae*. *E. coli* is widely known as an important indicator bacterium to detect contamination of water bodies by microorganisms that can act as human pathogens, such as *Salmonella*, *E. coli* O157:H7, *Shigella*, *Cryptosporidium parvum* and enteric viruses (U.S. EPA, 1986). It's use as the primary indicator organism is supported by several epidemiological studies that have shown a strong correlation between *E. coli* density and risk of gastrointestinal illness (Marion et al, 2010; Wiedenmann et al, 2006; Kaper et al, 2004). Regulatory agencies, such as the United States Environmental Protection Agency, have deemed that *E. coli,* along with members of the genus *Enterococcus,* as the primary indicator organisms used to determine water quality of both freshwater and saltwater water resources (US EPA, 1997). However, recent research suggests that elevated levels of these organisms in water is not a definitive indication that human pathogens are present (Ishii et al, 2006). The presence of FIB in water resources has great impact on the decisions of watershed managers and this information is important to determine the microbiological safety of water used for human consumption and recreational activities.

Factors Influencing Growth of FIB

There are a variety of environmental factors that impact the growth of *E. coli* in the environment. These include pH, nutrient availability, oxygen concentration, salinity, hydrology, temperature, light, storm frequency, and microbial community relationships. Alone, or in combination, these factors can all impact survivability of *E. coli* and related bacteria. (Jamieson et al, 2002; Whiteman et al, 2004; Evison, 1998; Solo-Gabriele, 2000; Chandrasekaan et al, 2015). A study of river and stream fecal bacteria concentrations near Nashville, TN found that concentrations of bacteria were positively correlated with housing density, with higher concentrations of fecal bacteria being found in more densely populated areas compared to less densely populated ones, suggesting land use is important in understanding *E. coli* growth in aquatic systems (Young and Thackston 1999). Other studies have found that *E. coli* associates with filamentous macroalgae *Cladophora* and periphyton communities (Ishii et al, 2006; Whitman et al, 2003; Ksoll et al, 2007). These studies show that the growth conditions of FIB are broad, and that *E. coli* have the potential to become naturalized within environments, present as regular members of the microbial community.

Seasonality impacts microbial communities, particularly those in temperate regions where changing seasons are accompanied by fluctuations in temperature, nutrients, light, and storm frequency (Hullar et al, 2005). Precipitation events, and the resulting storm water run-off events, are associated

with varying hydrological flows (Figure 1.2) and these can alter sediment beds in water bodies (Solo-Gabriele et al, 2000; Desmarais et al, 2002). This can

Figure 1.2 - Fast Moving Water at the End of Minnehaha Creek.

expose and redistribute microbes attached to biofilms, releasing them into the water column. (Desmarais et al, 2002; Jamieson et al, 2005; Piorkowski et al, 2014; Solo-Gabriele et al, 2000; Selvakumar and Borst, 2006; Krometis et al, 2009;). A South African study looking at *E. coli* concentrations during dry and wet seasons found that the concentration increased during the wet season (Abia et al 2015). Previous studies have shown that resuspension phenomena can cause increased fecal bacteria counts, exceeding TMDL standards (An et al 2002; Davies et al 1995; Crabill et al, 1999). Ultimately, these events trigger “false alarms” that do not likely prevent threat to human health, but regardless, result in the unnecessary closure of waters important as sources of drinking water and human recreation. Seasonality can both directly and indirectly affect microbial populations and the movement of *E. coli* throughout aquatic systems.

Sources of FIB

Point source contamination refers to that coming from a direct source that can be identified. Major sources of fecal coliforms include contamination from humans, pets, wildlife, and agricultural livestock (Byappanahalli et al, 2003, Ishii et al, 2006; Ishii et al, 2007; Jameson et al, 2002). Human fecal pollution is an important point source that can come from wastewater treatment plants, the release of sewage through poor management practices, septic tank leakage, and from human recreation activities such as swimming (Rose et al, 2001; Dufour ,1984). Pet fecal pollution (Figure 1.3) is also a possible source that is the result of neglect of pet owners in picking up their pets' fecal matter which can be washed directly into nearby water bodies and storm sewers (Cox et al, 2005). The care of farm animals and related agricultural activities can be a point source for fecal contamination. Manure from livestock can both directly enter and indirectly enter water sources. Furthermore, subsurface drains (tiles), can act as transport mechanisms to move and release fecal bacteria into surface waters (Doran et al, 1979; Howell, 1995). Wildlife are another possible point source of fecal contamination (Simmons, 2000; Sturdee et al, 1999).

Figure 1.3 - Dog Walker Crossing Bridge with Dog over Minnehaha Creek.

Microbial Source Tracking Technology

Microbial Source Tracking (MST) technology has emerged as an effective method to determine the sources of fecal pollution, along with indicator organisms often present in fecal matter, based on the assumption that certain fecal microbiota associate with a host organism (Harwood et al. 2013). Prior to widespread use of MST, fecal pollution was monitored by determining the count of FIB per unit volume, which provided a basic understanding of it a water body was contaminated, and to what degree. However, this analysis did not reveal the source of contamination, a necessary piece of information to know how to control levels of fecal indicator bacteria in water resources.

MST methods uses library- or non-library-based methods to group various indicator bacteria from different fecal sources (hosts) through isolation and then grouping them by either genotype or phenotype. The methods are based on the idea that bacteria found in the gastrointestinal tract carry unique traits that bacteria outside of a host do not have. This technique relies on analyzing signature phenotypes, DNA markers, or fingerprints unique to a specific microorganism. Adding to the library classifies the bacteria into various groups or source categories that show where the indicator microorganism is likely to have been derived from. This method of classifying indicator organisms assumes that the distribution of indicator organism phylotypes in environmental samples is similar proportionally to distribution of phylotypes in fecal samples (Anderson et al., 2005). This technology is continuing to evolve with the widespread adoption

of more quantitative methods for identifying bacteria, such as qPCR (Harwood et al. 2013) and DNA sequencing technologies.

Microbial source tracking laboratory techniques fall into two main categories. The first group are those utilizing phenotypic based methods and the second are those using genotypic based methods. There is further classification of methods if there is an additional requirement that the organism of interest be cultured before or analysis or to have a library used to identify unknown organisms (Field et al, 2007; Santo Diomingo et al, 2007; Scott et al, 2007; Yan et al 2007). Prior to the widespread adoption of genotypic based methods, phenotypic type methods for determining water quality were used and included fecal coliform/fecal streptococci ratios, serotyping, and antibiotic resistance analysis (ARA) of indicator bacteria (Parveen et al, 2001; Carroll et al, 2005; Harwood et al, 2000; Carroll et al, 2005; Edge and Hill, 2005; Harwood et al, 2003; Wiggins et al, 2003). Patterns of resistance of *E. coli* and *Enterococci spp.* found against various antibiotics are compared to a known library to distinguish human sources of fecal pollution from non-human sources. However, ARA has been found to provide inconsistent results and its reliable use as a microbial source tracking technology has been questioned (Harwood et al 2003; Griffith et al, 2003; Moore et al, 2005, Samadpour et al, 2005). Furthermore, other phenotypic methods such as serotyping and FC/FS ratios have been questioned for their inconsistency and inefficiency (Havelaar et al, 1990; Howell et al, 1996; Pourcher et al, 1991).

The reliability issues of phenotypic MST methods encouraged researchers to develop more accurate molecular-based genotypic source tracking methods. Some of these various methods developed for use in MST include ribotyping (Carson et al, 2001; Parveen et al, 1999; Dombek et al, 2000, Holloway, 2001) repetitive extragenic palindromic polymerase chain reaction (rep-PCR) (Dombek et al, 2000; Holloway, 2001), host-specific 16s rDNA PCR (Bernard et al, 2000; Bernard et al, 2000), denaturing-gradient gel electrophoresis (DGGE) (Buchan et al, 2001; Farnleitner et al, 2000), and pulse-field gel electrophoresis (PFGE) (King, 1976; Tynkkynen, 1999). These methods, apart from host-specific 16S rDNA, utilize DNA fingerprints (unique genetic sequences) of bacterial isolates, and are dependent on having a library to identify unknown organisms. The effectiveness of these methods relies on library size (Sturdee et al, 1999; Albert et al, 2003; Hassan et al, 2005; Indest et al, 2005; Johnson et al, 2004; Leung et al, 2004). This is because smaller libraries are not sufficient to fully characterize the diversity of bacteria strains (Wiggins et al, 2003; McLellan et al, 2003), especially of species such as *E. coli* which has been estimated to require 20,000 to 40,000 isolates to fully encompass its genetic diversity (Gordon, 2001; Hartl and Dykhuizen, 1984; Rocha et al, 1999). Without a library of this size, the diversity of a bacteria such as *E. coli* cannot effectively be characterized. Unfortunately, larger size libraries are very labor intensive and expensive to create, while also being unreliable in accurately matching environmental isolates to fecal isolates (Hassan et al, 2005; Stoeckel et al, 2004).

Due to these limitations, library-independent methods have become increasingly popular for microbial source tracking studies. Some of these methods, such as macroarray colony hybridization, can be used to screen large numbers of isolates in the aquatic environment to determine associated source hosts. These technologies are easily adapted to perform quick simultaneous analysis of many bacterial isolates which saves on time and costs of purchasing new materials. However, this technique requires culturing which introduces a bias towards bacteria of interest that can't be cultured (Hamilton et al, 2006). Despite this, the method is useful to provide a quantitative measure of fecal inputs from a specific environmental source organism. The use of library- and culture-independent methods provides a solution to the "viable but nonculturable" problems by using high throughput assays and primers that target host specific 16S rRNA (DNA) sequences. For instance, primers exist for *Bacteroides sp.* strains which are common members of the gut microbiome. Furthermore, they are useful for source tracking as studies have shown that they are host specific, and the targeted DNA is unlikely to survive in the environment (Bernhard and Field, 2000; Fiksdal et al, 1985; Kreader, 1995; Dick et al, 2005; Layton et al, 2006; Seurinck et al, 2005; Shanks et al, 2008; Weidhaas et al, 2010). Along with using real time qPCR technology, these methods can provide the quantity of fecal input coming from a source. Due to not having to culture the environmental isolate and being more efficient, these methods are suitable for TMDL studies, provided the funds are available to run these types of research projects.

Ultimately, if cost is an issue, cheaper, older, library-based, culture-based methods may have to be used. No one single method is limitation free, and a combination of multiple methods is the strongest and most accurate approach for truly robust microbial source tracking analysis (Noble et al, 2006; Vogel et al, 2007). Additional restraints on library independent methods include the temporal and geographic variability among different bacterial genotypes, labor and cost intensive, and variations in the diet of animal hosts (Field and Samadpour, 2007; Scott et al, 2002; Yan et al, 2007). Further research should be done on developing new markers for identifying animal sources of fecal contamination.

Determining Sources of *E. coli* with Rep-PCR

Determining the source of elevated *E. coli* counts is important to fully understanding how to protect water resources, human health, and recreation. Rep-PCR DNA fingerprinting technology is one modern genotypic method that can be used to characterize the level of bacterial diversity of various samples (Dombek et al, 2000). With this technology, specific gene sequences located between adjacent highly conserved, repetitive gene elements can be amplified using polymerase chain reaction (PCR) procedure followed by gel electrophoresis and staining to produce an image of specific DNA fingerprints. These DNA fingerprint images are then analyzed and compared to each other to determine the genetic similarity within bacterial populations. Genetic similarity is determined by comparing the presence and spatial location of densitometric

curves between fingerprints. By comparing the spatial profile of curves between two fingerprints, one can determine the genetic similarity between fingerprints, and, ultimately, the level of genetic diversity in a sample set.

Implications of Contamination and Impairment of Aquatic Resources

There are multiple implications for the impairment of aquatic environments. The impairment has both immediate and extended impacts on quality of life and human health. The detection of indicator organisms in environmental waters can result in the contamination of drinking water supplies and closure of recreational surface waters (Jamieson et al, 2005). In fact, the issue of contamination of water resources has been deemed so important that the United Nations identified improving water quality as one of the eight "Millennium Development Goals", with its goal as being a reduction in the number of people without access to safe clean water by 50% by 2015 (WHO, 2011).

While developed countries around the world process and treat surface water in treatment facilities, as well as subject these water supplies to regulation and testing before it becomes part of the public drinking water supply, developing countries might not possess or use these technologies to ensure quality drinking water. Here, people may have to obtain drinking water directly from nearby lakes, rivers, and streams. Contaminated drinking water supplies have the greatest potential to impact these kinds of communities. A study done on water supplies in a south Indian town found that of 37 water samples retrieved from a surface lake

and pumped out of a well underneath a dry river bed, 67% of samples were found to be contaminated with *E. coli* despite being considered “treated” municipal water (Brick et al. 2004). A study done in Africa found that waterborne diseases infect millions and diarrhea associated with contaminated drinking water is responsible for 2 to 2.5 million deaths every year (Fenwick 2006). Clearly, there are serious public health impacts globally for communities without access to clean water.

In the US, the EPA estimates that pathogens impair more than 480 km of rivers and shorelines and 2 million ha of lakes in the US (US EPA 2010a) and there are economic impacts associated with compromised water resources. A study of costs associated with pathogen contamination in Massachusetts estimated a price of $75 million yearly (Weiskel et al. 1996). A study on the impacts of a 1993 outbreak of cryptosporidiosis in Milwaukee found that the impact to water quality resulted in a 96-million-dollar loss for the city (Corso, 2003). It is clear, there are major financial implications, locally, nationally, and globally for contamination of water resources.

A previous study utilizing metagenomic DNA sequencing and microbial source tracking technologies was conducted on the watershed district from years 2016 to 2018 to determine the sources of elevated *E. coli* levels. The results from the analysis indicated that major sources of *E. coli* included residential lawns, in-stream sediment, soil in streambanks and riparian areas, soil from road construction, and organic debris trapped in street gutters (Figure 1.4).

Figure 1.4 - Organic Debris Stuck in Street Gutter near Minnehaha Creek.

E. coli originating from human sources was minimal. *E. coli* were found to be present, persistent, and increasing in storm drain channels during the warmer months with lower temperatures eventually decreasing populations. Adaptive management practices that were suggested included periodically cleaning grit chambers of organic debris, high in carbon compounds and other important biomolecules that provide nutrients for *E. coli* to grow. Furthermore, it was suggested that soil debris generated from construction activity be collected and be prevented from entering the watershed. Lastly, improving “Best Management Practices” or BMPs for temporary restrooms on construction sites was suggested as a solution to further reduce human contamination of watershed (Gruber, 2018).

Minnehaha Creek Watershed

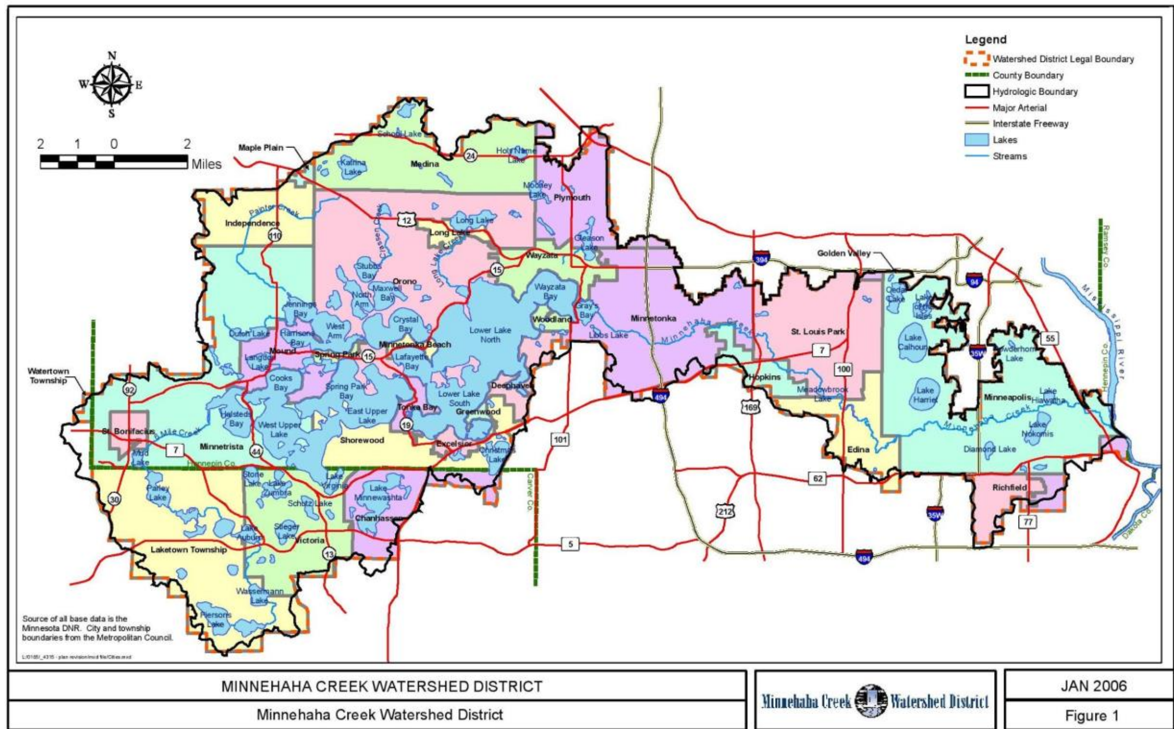


Figure 1.5 - Minnehaha Creek Watershed District Map (MCWD, 2006).

The MCWD is a governmental agency that oversees the protection of various water bodies located within a 178 square mile area primarily within the southern and western Twin Cities metropolitan area within the state of Minnesota. Figure 1.5 shows the area managed by the MCWD. These include notable areas such as Minnehaha Creek, Lake Minnetonka, Minneapolis Chain of Lakes, and Minnehaha Falls Park. These areas are commonly used as places of human recreation. Water flowing through the watershed enters the Mississippi River and travels down the river before eventually ending up in the Gulf of Mexico. Thus, the impacts to the Minnehaha Creek Watershed are not contained

Figure 1.6 - Minnehaha Falls Park - The End of Minnehaha Creek Right Before Flowing Into the Mississippi River.

within the district and there are greater implications for *E. coli* contamination. The MCWD setting is primarily urban and is particularly impacted by human activity, compared to more rural located water bodies throughout the rest of the state.

Within MCWD there are some potentially unique sources for microbes to inhabit. Grit chambers are large, man-made concrete structures that contain baffles. These baffles slow down water, allowing sediment and heavier materials such as trash and vegetation to settle to the bottom of the structure where it is trapped. The purpose of these grit chambers is to keep these types of materials out of surface waters. However, if not cleaned, materials can build up in grit chambers, reducing their effectiveness. Grit chambers are often attached to street gutters and materials flowing through gutters may enter grit chambers before emptying out into the Minnehaha Creek.

Coupons are small pieces of brick, concrete, tile or metal placed into the creek. The bricks are exposed to flowing water within the water column and are also exposed to sunlight entering the water surface. They act as additional

surfaces that can be colonized by biofilm bacteria. These coupons can be removed from the stream and can be processed to analyze bacteria growing in the surface biofilms.

Lastly, street gutter drains exist throughout suburban neighborhoods within the MCWD study area. These gutter drains are used to remove storm water and loose vegetation from streets. Large amounts of water carrying organic matter enter these street gutter drains during storm events. Drain tiles are often attached to street gutters and transport storm water and snow melt into the Minnehaha Creek.

Thesis Outline

Elevated levels of fecal indicator bacteria in water bodies are a concern to watershed managers as well as the public that use these resources for recreation and source of drinking water. Water bodies that do not fall within TMDL standards become impaired resulting in their closure. It is necessary to understand the sources of elevated levels of FIB to determine the proper course of action to lower their levels to fall within TMDL limits. Furthermore, factors such as variable hydrology must be investigated to determine how these factors impact the movement of fecal indicator bacteria within a particular aquatic system. In order to accomplish these tasks, it is essential to fully understand the origin, fate, and ecology of fecal indicator organisms, Therefore, in Chapter 2, I deduce sources of *E. coli* and hydrological impacts to their community and

movement within the Minnehaha Creek Watershed District, located within the greater Twin Cities Metropolitan area in east central Minnesota. Specifically, I analyzed genetic diversity of a variety of sample sets obtained from specific locations along the Minnehaha Creek to compare levels of diversity and understand how various populations are related to one another. I also compared the impact of variable hydrological conditions on population and how these changing flow levels impact the movement of FIB in the watershed system. I used Rep- PCR DNA fingerprinting technology to examine the level of genetic diversity within *E. coli* populations present in the Minnehaha Creek. The results of the investigation suggest that Lake Minnetonka and Minnehaha Creek sediment are strong sources of *E. coli* within the watershed district. It is likely that certain populations of *E. coli* have become naturalized, attaching themselves via biofilms to surfaces in these locations. Following precipitation events, these bacteria are periodically pulled back up into the water column, contributing to increased *E. coli* counts and subsequent impairment listings following sampling. Street gutter drains and grit chambers are also a potential source of *E. coli* within the MCWD but are likely dependent on changes in hydrological flows that physically disrupt *E. coli* biofilms within these structures, releasing *E. coli* into the water column and out into the creek. The presence of naturalized *E. coli* has implications for future studies on water quality as their existence conflicts with their use as a reliable indicator of fecal contamination. In Chapter 3, I have summarized findings, described limitations of the study, and provided recommended actions and future directions for research presented in Chapter 2.

Chapter 2. Determining Sources of Environmental *E. coli* populations in the Minnehaha Creek Watershed

Preface

In 2013, total maximum daily load values for *E. coli* were not being met within the Minnehaha Creek Watershed District (MCWD) in Minnesota. Elevated levels of *E. coli* are of public health concern as they are used to indicate the possible presence of pathogenic bacteria that can cause illness in humans. The Minnehaha Creek, which occupies acres urban and suburban Minneapolis, empties into the Mississippi River - which ultimately flows out into the Gulf of Mexico. Poor water quality in the Minnehaha Creek, due in large part to microbial contamination, has the potential to impact larger regions and water bodies in the US and North American. Despite their importance, however, the sources of *E. coli* are not known. In this study I used rep-PCR DNA fingerprinting technology to determine the source(s) of elevated *E. coli* within the Minnehaha Creek during the years 2017 and 2018. Additionally, hydrological data was recorded to determine effects of altered hydrological flow regimes on change in diversity of *E. coli*. MANOVA and multidimensional scaling analysis suggested that Lake Minnetonka and sediment within the Minnehaha Creek are important sources of *E. coli* that are not greatly impacted by changing hydrological conditions. An analysis of 2017 vs 2018 data sets also showed that street gutters and grit chambers may also be an important source of *E. coli* contamination - but could be variable and dependent on higher hydrological flows to flush *E. coli* into the

Creek. Results of this study showed that once *E. coli* and other fecal indicator bacteria enter water bodies, through a variety of sources including human, wildlife, pet, sewage, agricultural, etc., that these populations can grow and persist within both lake and sediment environments. This, together with new bacterial inputs each year, contribute to elevated *E. coli* counts leading to the impairment of water bodies. Variable hydrological flows have the possibility to move *E. coli* from surface biofilms into the water column leading to increased *E. coli* counts, particularly in structures such as street gutters and grit chambers.

Introduction

Maintaining water quality of aquatic resources is necessary to ensure public health and safety. Water quality can come under attack from biological contaminants and can put people at risk for human illnesses through drinking or coming into physical contact with contaminated waters (Bens et al, 1998; Cox et al, 2005; Roach et al, 1993; Sharma et al, 2003). One of the major ways through which biological contaminants enter the waterways is through the feces of humans and other animals (Cox et al, 2005). TMDLs exist for bacterial pathogens such as *E. coli* to ensure water quality (Said et al, 2004). The exceedance of TMDLs is a major issue for watershed managers and those involved in environmental and public health. This often results in the closure of lakes, creeks, rivers and other places of human recreation. *E. coli* and other fecal bacteria can come from many possible sources and their distribution and

movement within an aquatic system can be impacted by changes in hydrology. It is important to investigate the source of these bacteria and how the environment impacts their growth and movement so that actions can be taken to reduce their levels to fall within TMDL standards.

E. coli and *Enterococcus* are both commonly found in the human gastrointestinal tract and are used as fecal indicator organisms, one determinant of water quality (Klein, 2003; Fong and Lipp, 2005). *E. coli* is a gram-negative bacterium common in the gastrointestinal tract of warm-blooded animals. *Enterococcus* is a gram-positive bacterium that is also common in the gastrointestinal tract of humans with many. Some members of the genera *Escherichia* and *Enterococcus* are known to cause human illness. The presence of these bacteria is considered indicative of recent fecal contamination and their presence signals the possible presence of other pathogens such as *Salmonella*, *E. coli* O157:H7, *Shigella,* or *Cryptosporidium parvum*. They are used as a determinant of water quality as epidemiological studies have shown a relationship between FIB (such as *E. coli)* and human illness (Marion et al 2010; Wiedenmann et al, 2006; Cabelli, 1982, Pruss, 1998). There are multiple important sources of fecal coliform bacteria and FIB in the environment, including agricultural activities, septic leakage, human recreation, pets, and wildlife (Ishii et al, 2006; Indest et al, 2005).

The use of *E. coli* as a FIB assumes that it is a common member of the fecal gut microbiota and relies on ideal conditions of the host to survive, therefore, it is incapable of surviving and establishing itself outside of the host.

Current research suggests that *E. coli* can become naturalized in the environment within sand, soil, and sediment of tropical and temperate climates and this compromises its use as an indicator of recent fecal contamination (Byappanahalli et al, 2003; Byappanahalli et al, 2006; Ishii et al, 2007; Ishii et al, 2007; Ishii et al, 2006). Studies on the ideal growth conditions and survival of *E. coli* in the environment have primarily been conducted in the laboratory setting rather than out in the environment. This is because of the lack of proper technologies available to monitor populations of *E. coli* and distinguish naturalized microbial communities from recent fecal contaminants. Studies have shown that some *E. coli* genotypes are unique to location and shared no genetic similarity with *E. coli* from known source hosts (Ishii et al. 2006, Kaper et al, 2004). These studies show that *E. coli* are more than capable of surviving outside of the host and becoming regular members of the microbial community within the outside environment.

There are many factors that influence the survival and growth of naturalized *E. coli* within the environment. Some of these factors include pH, nutrient availability, insolation, salinity, hydrology, temperature, light, storm frequency, and microbial community relationships. (Jamieson et al, 2002; Whiteman et al, 2004; Evison, 1998; Solo-Gabriele, 2000; Chandrasekaan et al, 2015). Environmental populations of *E. coli* can start when fecal *E. coli* enters the water column. Once in the water column, bacteria survive and grow by establishing biofilms, a community of microorganisms held together by an extracellular matrix (Costerton, 1999). Bacteria growing in biofilms receive

additional nutrients and protection from predators. Precipitation events affect existing populations of *E. coli* already in the water, pulling them apart from biofilms and back into the water column, contributing to a temporary increase in observed levels of *E. coli* (Solo-Gabriele et al, 2000; Desmarais et al, 2002). *E. coli* have been found to survive through temperature extremes and freeze thaw cycles meaning they can persist for extended periods of time in the environment (Byappanahalli et al, 2006; Ishii et al, 2006; LaLiberte et al, 1982). Resuspension of environmental *E. coli* into the water column often results in exceedances in TMDLs resulting in the closure of waterways for human recreation (YJ et al, 2002; Davies et al, 1995).

The objectives of this present study were to: 1) Examine sediment, coupon, and water samples from the Minnehaha Creek Watershed for populations of *E. coli ;* 2) Determine the potential sources of fecal contamination in the Watershed by using Rep-PCR technology; 3) Determine impacts of variable hydrology on the persistence and movement of *E. coli* within the watershed and changes in genetic diversity; and 4) Determine actions to be taken to reduce levels of *E. coli* contamination within the Minnehaha Creek Watershed District.

Materials and Methods

Site Description

Minnehaha Creek Watershed District is a watershed district located within a 178 square mile area primarily within the southern and western Twin Cities metropolitan area of the state of Minnesota. The watershed is composed primarily of cultivated lands devoted to agriculture (30%) with parks and open space consisting of 22% and water consisting of 15%, respectively. Residential housing accounts for 7%. Remaining land is primarily composed of vacant land; wetlands, forest, or woodland. 29 communities fall within the boundaries of the watershed. Notable areas within the watershed include Minnehaha Creek, Lake Minnetonka, Minneapolis Chain of Lakes, and Minnehaha Falls Park (Figure 2.0). These areas are commonly used as areas of human recreation. Water flowing through the watershed enter the Mississippi River and travel down the river before eventually ending up in the Gulf of Mexico.

Figure 2.0 - Outflow of the Minnehaha Creek into the Mississippi River at Minnehaha Falls Park.

The Minnehaha Creek is a 22-mile-long tributary that runs east from Gray's Bay Dam, an outlet on Lake Minnetonka, through various suburban cities located within the southern Twin Cities metro, before exiting Minnehaha Falls at Minnehaha park and flowing into the Mississippi River. Given Minnehaha Creek's proximity to a major metropolitan area, it is particularly impacted by human activity compared to more rural areas located throughout the rest of the state.

Water temperature and hydrological flow data were obtained from the United States Geological Survey through the Minnehaha Creek Watershed District Website. For this study, a variety of sample sites within the study area (Figure 2.1) were chosen by a separate environmental engineering firm, Burns and McDonnell, to sample for microbial diversity. The sites were chosen based on ease of sampling access (Figure 2.2).

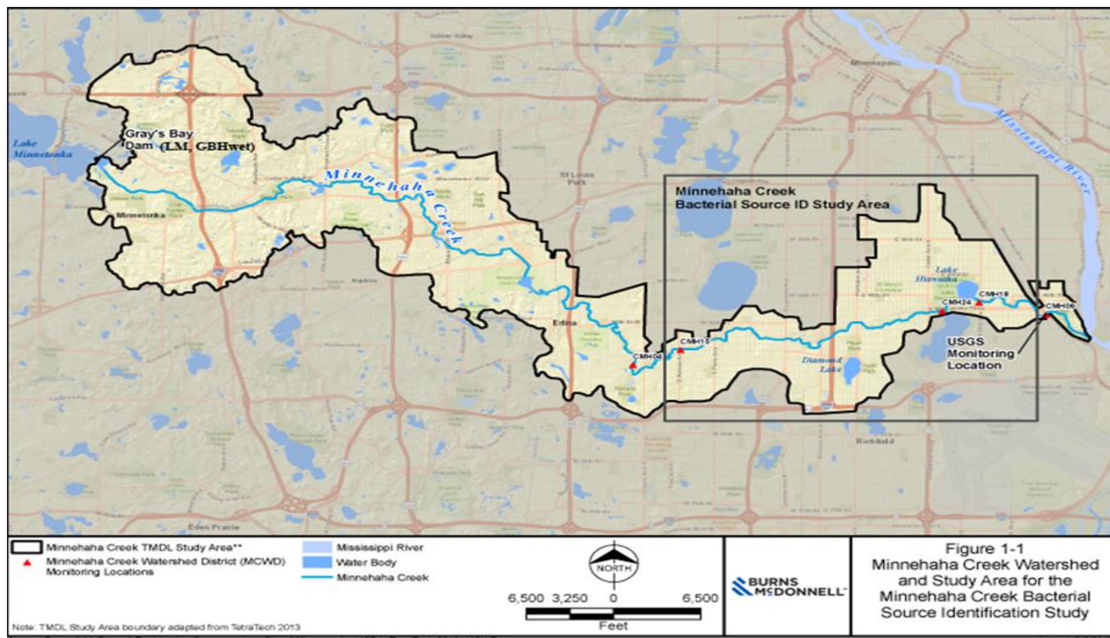


Figure 2.1 - Study Area for the Minnehaha Creek Bacterial Source Identification Study. All samples were taken from various locations within the study area (District, 2013).

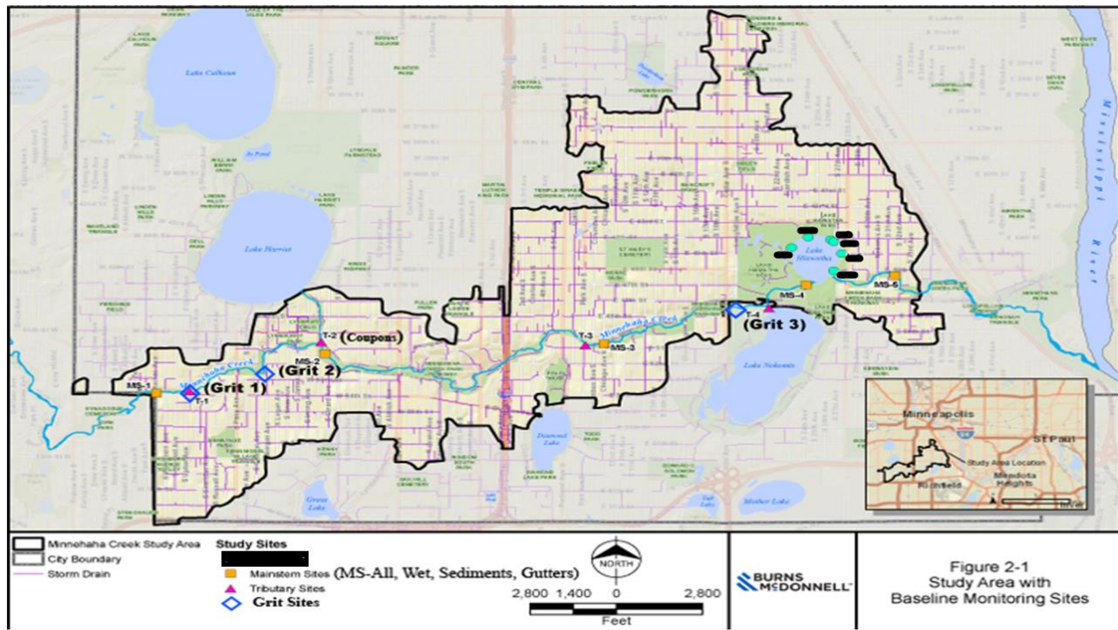


Figure 2.2 - MCWD Study Area with Baseline Monitoring Sites for Bacterial Source Identification Study (Burns and McDonnell, 2017). Mainstream sites (MS-all, Wet, Sediments, and Gutter) were considered "study area sites".

Sample Collection

Samples containing water, sediment, and coupons were obtained by Burns and McDonnell field technicians for the years 2017 and 2018 and dropped off at the Sadowsky laboratory using insulated coolers. "Wet Flow" samples were obtained on September 19th, 2017. "Wet Flow" conditions were characterized by presence of a significant precipitation event(s) within the past 72 hours prior to sampling. "Dry Flow" samples were obtained on October 20th, 2017. "Dry Flow" Conditions were characterized by lack of precipitation within the past 72 hours prior to sampling. "Control (Normal) Flow" samples were obtained on August 1st, 2018. "Control (Normal) Flow" conditions were

characterized by a normal level of precipitation for the area within the past 72 hours prior to sampling. "Ultra-Low Flow" samples were obtained on August 23rd, 2018. "Ultra-Low Flow" conditions were characterized by presence of an extended drought within the past 72 hours prior to sampling. Samples from specific locations within the study area were obtained for years 2017 and 2018, respectively (Table 2.1).

Table 2.1 - Samples Sets Analyzed in the Study

Sample Set	Sample Type and Description for 2017	Sample Set	Sample Type and Description for 2018
Gutter	Dry Weather – spatial composite from street gutter drains in the study area	Gutter	Dry Weather – spatial composite from street gutter drains in the study area
MS-All	Dry Weather – spatial/temporal composite at all study area sites	MS-All	Dry Weather – spatial/temporal composite at all study area sites
Grit Chamber	Dry Weather – spatial composite from 5 grit chamber structures in study area	Grit Chamber	Dry Weather – spatial composite from 5 grit chamber structures in study area
Coupons	Dry Weather – composite of three coupon structures deployed in study area	Coupons	Dry Weather – composite of three coupon structures deployed in study area
LM	Dry Weather – spatial composite from Lake Minnetonka	LM	Dry Weather – spatial composite from Lake Minnetonka
Sediments	Dry Weather – spatial composite from study area sites	Sediments	Dry Weather – spatial composite from study area sites
Wet	Wet Weather – spatial/temporal composite from study area sites	T-2 UL	Extremely Dry Weather - from Tributary Site 2
GBH Wet	Wet Weather – spatial composite from Lake Minnetonka at Gray's Bay Dam	MS-All UL	Extremely Dry Weather - spatial/temporal composite at all study area sites

Water Samples

Water samples supplied as a composite mixture were directly filtered through a microbiological using a Büchner flask. Water samples supplied in individual containers were shaken vigorously for approximately twenty seconds and then equal volumes of each individual container were combined to make a composite sample. The newly made composite samples were then filtered. A minimum of 1-liter volume of composite sample water was vacuum filtered through 0.22 um nitrocellulose filters to collect and concentrate bacteria.

Sediment samples

Sediment samples were supplied as 100-gram composites containing equal masses of individual samples from each of five study sites. Sediment samples were mixed with 1 liter of sterile ammonium phosphate gelatin buffer (Kingsley 1981) and then shaken for 15 minutes using the low setting of an Eberbach horizontal shaker.

The silt from the sediment was allowed to settle for an additional 10 minutes following shaking. Following the rest period, a minimum of 1-liter volume of supernatant from each sample was vacuum filtered through 0.22um nitrocellulose membrane filters.

Coupon samples

Individual coupons were placed in 1-liter jars with 200 ml of ammonium phosphate buffer and 10 ml of 3mm sterile glass beads. The jars were shaken for 15 minutes using the low setting of an Eberbach horizontal shaker in order to separate biofilm bacterial from the coupon into the supernatant.
The silt from the sediment was allowed to settle for an additional 10 minutes following shaking. Following the rest period, a minimum of 1-liter volume of supernatant from each sample was vacuum filtered through 0.22um nitrocellulose membrane filters.

Isolation of Environmental *E. coli*

Following filtration, dilution plating was performed in replicate using Membrane Thermotolerant *E. Coli* (MTEC) agar plates for each of the filtrates. Excess filtrate that was not plated onto MTEC agar plates was immediately frozen at -70C in sterile 50% glycerol solution. Isolation of 75 *E. coli* occurred for each sample set with eight sample sets for each of the sampling years. This totaled 600 colony isolates for each year, which was the number of isolates agreed upon for the study. In the event that less than 75 *E. coli* colonies could be isolated on MTEC agar plates, additional dilution plating could be done utilizing excess filtrate to achieve at least 75 *E. coli* colonies.

Enumeration of *E. coli*

Following dilution plating, MTEC agar plates were incubated at 37C for 2 hours, followed by 44.5C for an additional 12 hours. Following incubation, MTEC agar plates were analyzed. *E. coli* colonies expressed an opaque, deep blue color and were targeted for further isolation. The positive blue colony isolates were picked from MTEC agar plates into HMFM medium in 96-well microtiter plates, and wrapped in plastic wrap/aluminum foil for immediate freezing at -70C as part of future PCR reaction.

DNA Extraction and PCR

HMFM medium 96 well plates were removed from freezer and set out in a sterile hood vent to thaw. Once thawed, bacteria within wells of the well plate were stamped onto plate count agar plates using sterile pronged stamper. Plate count agar plates were incubated overnight at 37C.

The following day, plate count agar plates were removed from the incubator and brought to a sterile hood vent.

1ul of wet cells were picked from the plate count agar plate colonies into 100 ul of sterile 0.05M NaOH solution within each well of a 96 well plastic DNA extraction plate using a 1ul plastic inoculation loop. Wet cells were scraped off into NaOH solution and friction was generated by spinning the inoculation loop to ensure that cells had been released from the loop and entered the NaOH solution. Three positive control PIG 294 controls were also picked and added to three separate remaining wells of the DNA extraction plate. The DNA extraction

plate was covered with Microseal A film and firmly sealed. The sealed DNA extraction plate was heated to 95 C for 10 minutes in a PTC-100 thermal cycler. Following cool down period to approximately 35C, the plate was transferred to an Eppendorf plate centrifuge and was centrifuged for 10 minutes at 640 RPM. Following centrifugation, 2ul of the supernatant was used as the DNA template for PCR with the below conditions using the BOX A1R primer sequence in a new sterile 96 well PCR plate (per reaction): Mastermix Ingredient and Volumes was adapted from (Johnson et. al., 2004). The Thermal Cycler Cycle was the same as referenced in (Johnson et. al., 2004). Following PCR, the plate was frozen at -20C until ready for gel electrophoresis.

Gel Electrophoresis

Gel electrophoresis was performed by adding 5ul of 6x Blue Dextran Ficoil 500 loading dye to each 25ul of PCR Product using a distriman repetitive pipette. 13ul of this dyed PCR product was loaded into a 250ml 1.5% agarose gel using the Horizon 20-25 gel system with 30-prong gel comb. A separate 1kb PLUS DNA ladder was loaded at the left end, middle, and right end of each gel. 23 *E. coli* samples were included on each gel, along with the ladder, and a positive control. Gels were run using the Horizon 20-25 gel system at 70V for 16 hours with 0.5X TAE buffer on very slow recirculation using peristaltic pumps in a 4C cold room.

Gel Staining & Imaging

Gels were stained in 0.5ug/ml ethidium bromide and 0.5X TAE for 30 minutes. These were then imaged and saved using a FotoDyne Foto/UV 26 imager.

Image Analysis

Gel images were converted from BMP to 8-bit TIFF format using Adobe Photoshop CS2. These TIFF images were then imported and analyzed with Bionumerics version 3.5 using Pearson's Curve-Based coefficient with background subtraction. Cluster analysis was exported and supplied to Burns & McDonnell engineering firm for dissemination.

Clonality

Clones, for the purpose of this experiment, were recognized as *E. coli* fingerprints sharing a genetic similarity of greater than 88%. This was determined through the use of the Pearson's curved-based coefficient which uses densitometric curve data to indicate the presence of repetitive gene sequences using computer software. The curve data describes the position of horizontal bands in addition to their relative density. The more similar the curve profile of two fingerprints, the more genetically similar they can be considered. Clonality is a state in which two fingerprints share a genetic similarity of greater than 88% based on comparison of curve profiles. These two fingerprints can be considered to share "clonality".

MANOVA Analysis

A Multivariate analysis of variance or MANOVA analysis was performed to determine statistical differences between *E. coli* fingerprints and compare genetic similarity of samples to one another. Sample sets each consisted of 75 analyzed fingerprints for a total of 600 fingerprints per year; 1200 fingerprints were analyzed for the entire study. Samples sets that had fingerprints that "overlapped" were considered to be more genetically similar than fingerprints that didn't "overlap". Sample sets found nearby one another on the MANOVA were considered to share an "relationship".

Multidimensional Scaling Analysis

The data was reanalyzed used a multidimensional scaling approach in order to better visualize genetic diversity relationships between samples. Multidimensional scaling approach produces a 3D image that can provide additional information on genetic diversity similarities.

RESULTS

MCWD 2017 Sampling Data Results

Dry Weather Results

Results of 2017 dry weather data are shown in Figures 2.3 and 2.4. An overlap between sediment and coupon DNA fingerprints was found (Figure 2.4). Moreover, fingerprint data overlapped between *E. coli* from MS-all and the Gutter fingerprints. A low level of overlap was found between LM and Sediment fingerprints as was an relationship between gutter and MS-all sample sets, supported by analysis of dendrogram data. LM appeared to be the least distinct and overlapped with most other sample sets. The sample set with the greatest level of diversity was LM as shown by the spread of LM fingerprints in Figure 2.4.

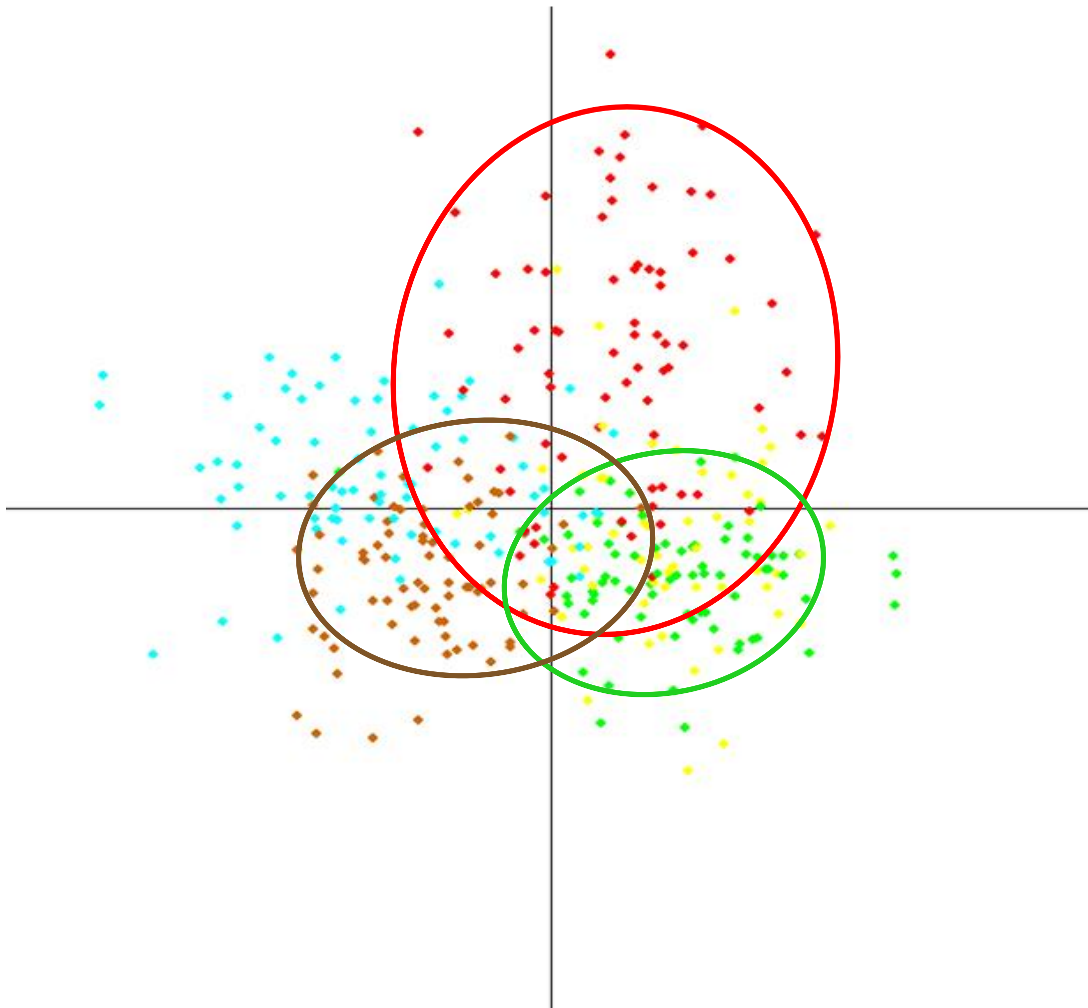


Figure 2.3 - MANOVA Analysis of Dry Weather 2017 Samples. Each dot represented a unique DNA isolate fingerprint from a particular sample set. Each sample set contained 75 isolates.

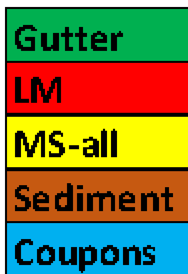
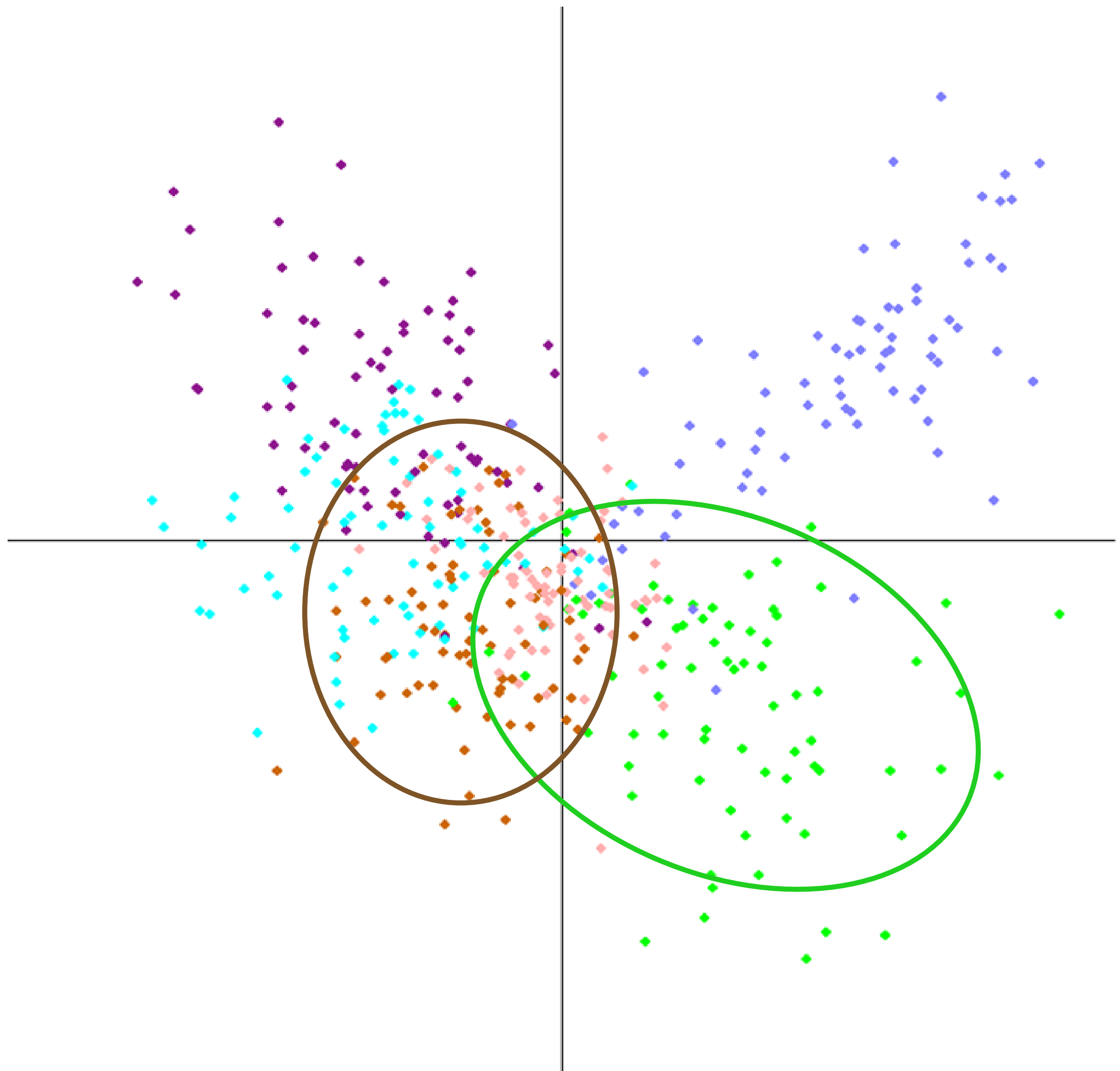


Figure 2.4 - Multidimensional Analysis of Dry Weather 2017 Samples. Each dot represented a unique DNA isolate fingerprint from a particular sample set. Each sample set contained 75 isolates.

Wet Weather Results

Results of 2017 wet weather analyses are shown in Figure 2.5 and 2.6. An overlap existed between DNA fingerprints from *E. coli* isolated from coupons and sediment, which indicated a relationship between these two sample sets (Figure 2.5). The Grit sample set fingerprints were found mostly in middle of MANOVA axis, which indicated lack of distinctness, and overlap with other sample sets. A relationship existed between sediment and coupon sample sets with fingerprints. Grit also appeared to have a similar level of relation with these two sample sets, as shown by the cluster of fingerprints of these three sample sets in Figure 2.6. The Gutter sample set was fairly indistinct, and fingerprints were found throughout the multidimensional sphere. An analysis of dendrogram data showed that the GBH Wet and Wet sample sets both contained a high percentage of clones. Clone clusters of GBH Wet were seen on the left-hand side of sphere, and clone clusters of Wet sample set were seen on the top right side of the multidimensional scaling diagram, respectively (Figure 2.6). The gutter set was found to be the most diverse, as shown by the spread of the fingerprints in Figure 2.6.



Gutter
Wet
Sediment
Coupons
GBHwet
Grit

Figure 2.5 - MANOVA Analysis of Wet Weather 2017 Samples. Each dot represented a unique DNA isolate fingerprint from a particular sample set. Each sample set contained 75 isolates.

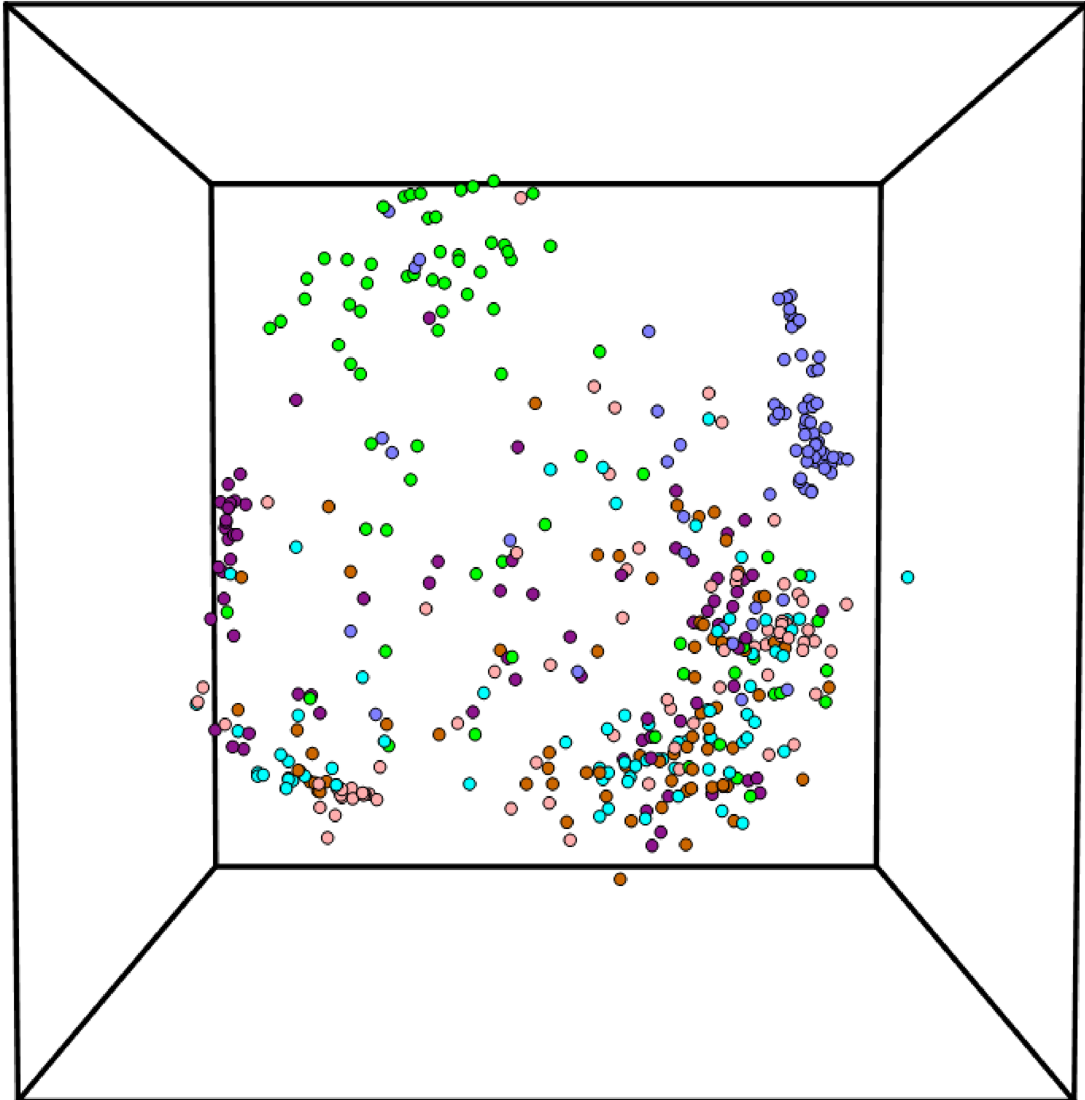
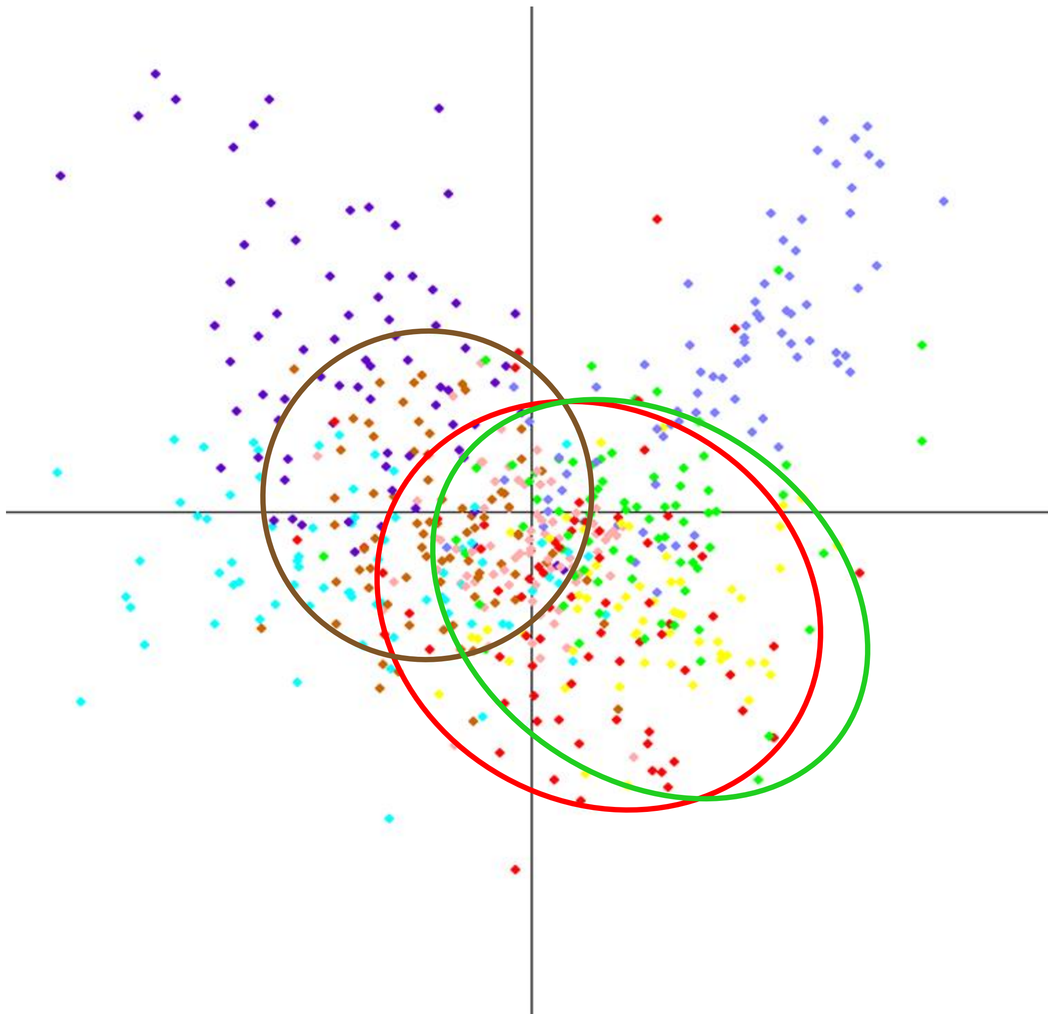


Figure 2.6 - Multidimensional Scaling of Wet Weather 2017 Samples. Each dot represented a unique DNA isolate fingerprint from a particular sample set. Each sample set contained 75 isolates.

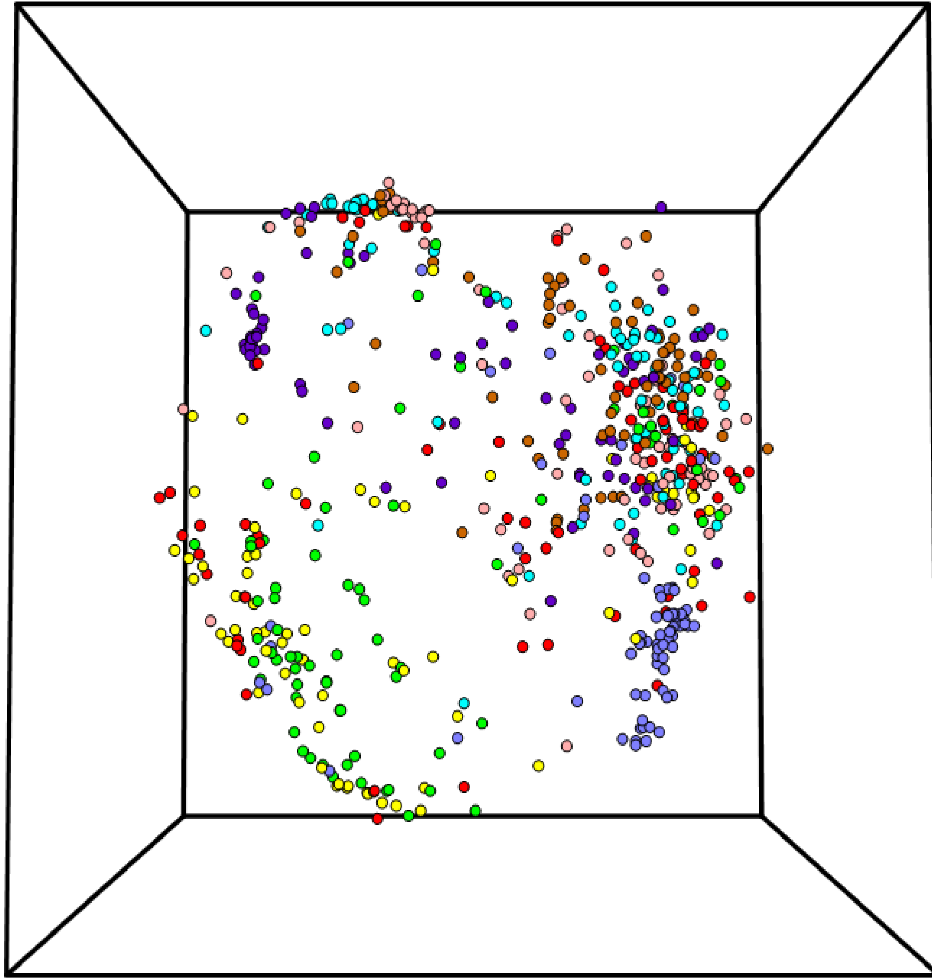
Dry and Wet Combined Results

Results of the combined analysis of 2017 wet and dry weather data are shown in Figures 2.7 and 2.8. DNA fingerprints from GBH wet, Coupons, and Sediment sample sets were generally located on the left side of the MANOVA axis (Figure 2.7). Fingerprints from Wet, gutter, and MS-all sample sets were generally focused on the right side of axis. There was some overlap between Gutter and MS-all fingerprints, which indicated a relationship between these two sample sets. GBHwet and Wet sample sets were fairly independent from all other sample sets and were reasonably distinct from other sample sets. LM was fairly indistinct with moderate levels of fingerprints present in all MANOVA quadrants. Grit was found in the middle of the axis and was the least distinct of all sample sets and shared some overlap with all other sample sets. A relationship existed between sediment, grit, and coupon sample sets. LM and Gutter sample sets all showed high levels of diversity as shown in Figure 2.8. Sediment sample set also was fairly diverse with fingerprints stretching across both left hand MANOVA quadrants shown in Figure 2.7.



Gutter
LM
MS-all
Sediment
Wet
GBHwet
Coupons
Grit

Figure 2.7 - MANOVA Analysis of Wet and Dry Weather 2017 Samples. Each dot represented a unique DNA isolate fingerprint from a particular sample set. Each sample set contained 75 isolates.



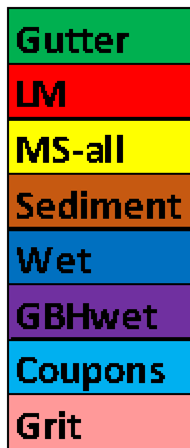


Figure 2.8 - Multidimensional Scaling of Wet and Dry Weather Combined 2017 Samples. Each dot represented a unique DNA isolate fingerprint from a particular sample set. Each sample set contained 75 isolates.

Clones Shared Analysis Results

The coupons sample set was found to share the most number clones with MS-all with 7 (Table 2.2). Sediment sample set was found to share the least clones with MS-all with 1. Gutter sample set was found to share the most clones with Wet with 3 (Table 2.3). Sediment, coupons, and Grit were found to share the least clones with Wet with 0.

Table 2.2 - The table shows the number of clones shared with the sample set MS-All. Any two fingerprints with greater than 88% similarity were deemed clones.

Group	**Clones shared with MS-all**
LM	4
Gutter	6
Coupons	7
Sediment	1

Table 2.3 - The table shows the number of clones shared with Wet sample set. Any two fingerprints with greater than 88% similarity were deemed clones.

Group	**Clones Shared with Wet**
Gutter	3
Sediment	0
Coupons	0
GBHwet	1
Grit	0

Shannon- Weaver Diversity Index Results

The diversity among 2017 Sample Sets was analyzed using Shannon-Weaver Diversity

Index (Table 2.4). The Gutter sample set was found to have highest diversity, while the Wet sample set was found to have the lowest diversity.

Table 2.4 - The diversity index was a measure of the level of genetic diversity between 75 *E. coli* colonies picked for each sample set. Higher numbers indicated higher diversity.

Group	Shannon-Weaver Diversity Index
Wet	2.420
MS-all	4.133
Gutter	4.170
LM	4.008
GBHwet	3.666
Coupons	3.806
Sediment:	3.599
Grit	3.693

Hydrological Discharge Results

In 2017, the discharge rate increased in late March following snowmelt rising above 100 ft^3/sec (Figure 2.9 and 2.10). Apart from a small dip in July/August, discharge remained above 100 cubic feet per second until the beginning of November. Peak discharge occurred in mid-May and beginning in October with recorded discharges of nearly 500 ft^3/sec. Overall daily discharges for 2017 were higher than median daily statistic for nearly the entire year.

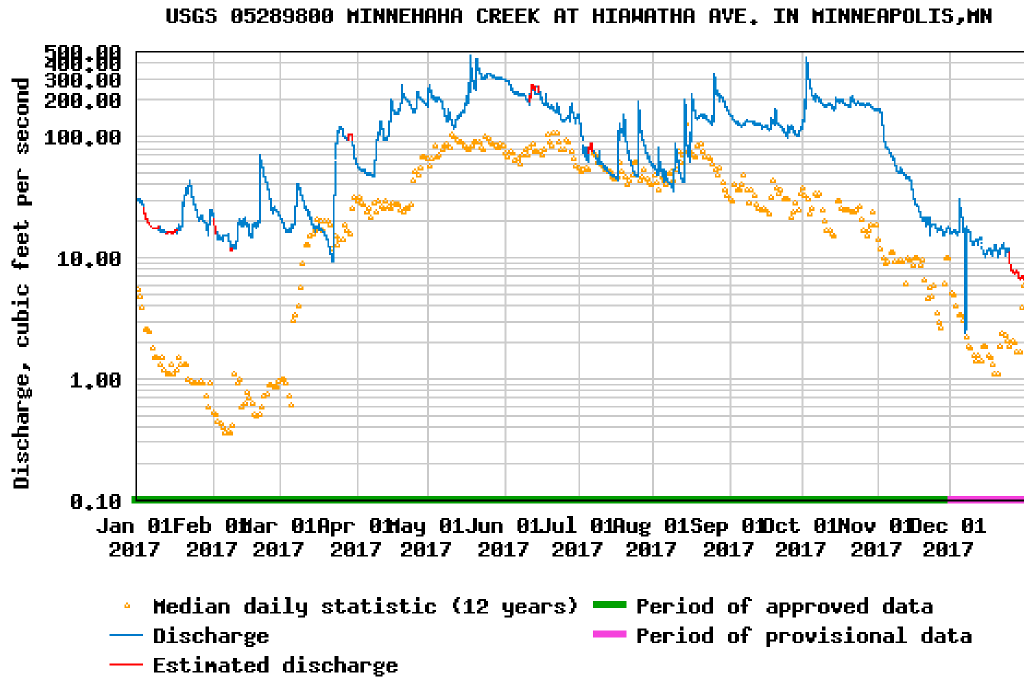


Figure 2.9 - Measurement of Discharge from Jan. 1st, 2017 until Dec. 31st, 2017.

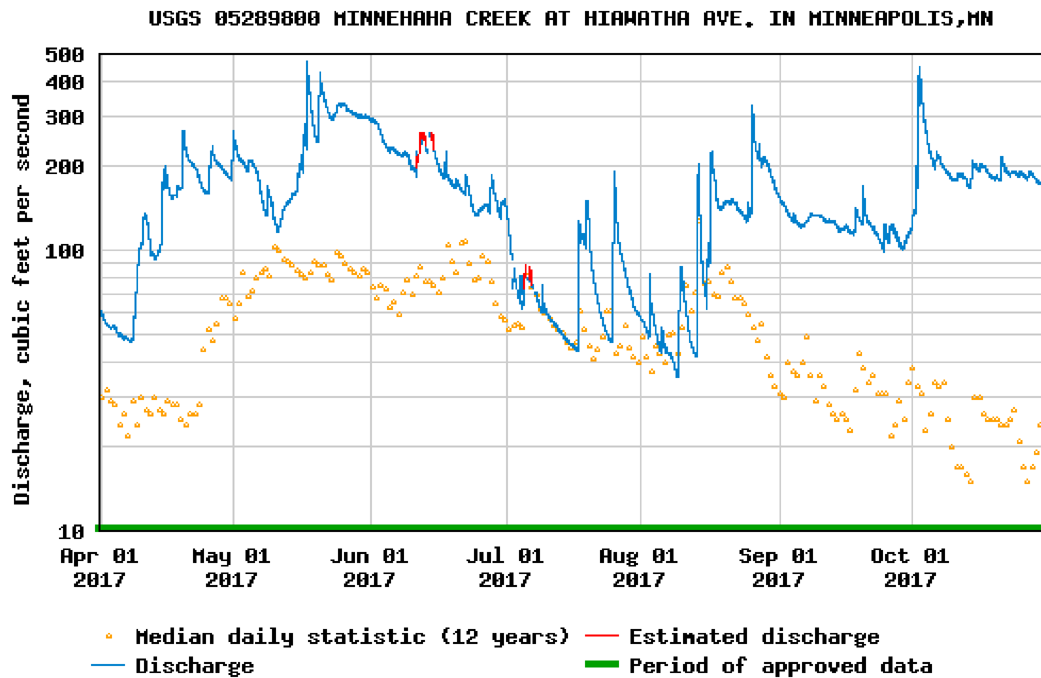
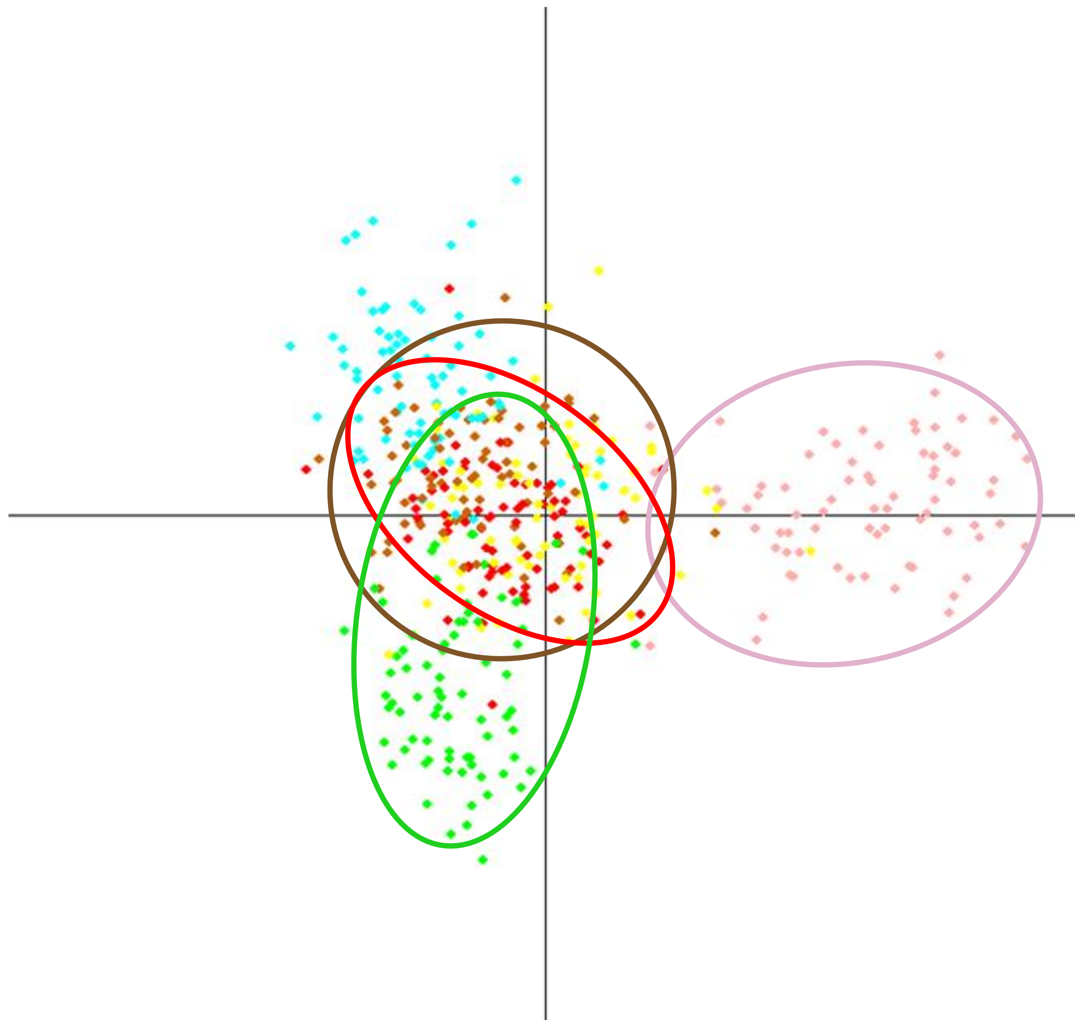


Figure 2.10 - Measure of Discharge from April 1st, 2017 until October 31st, 2017.

MCWD 2018 Sampling Data Results

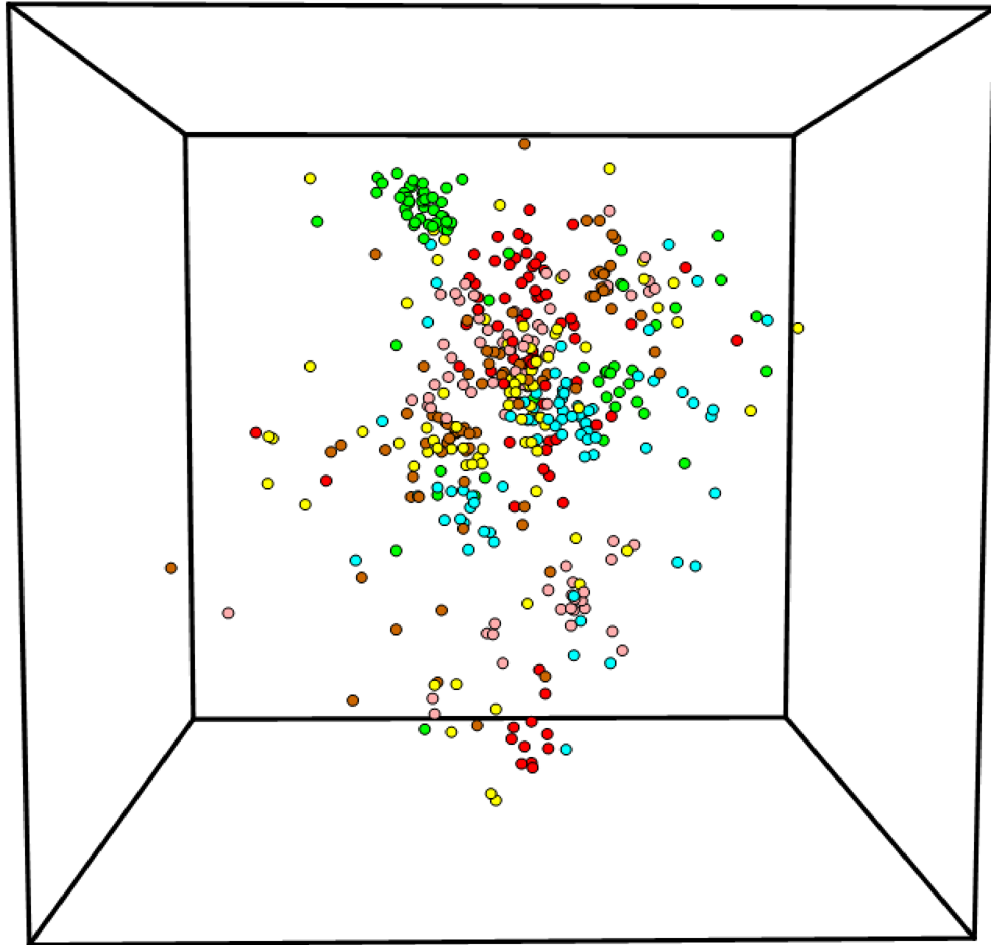
Control Weather Results

DNA fingerprint data from 2018 samples was analyzed by MANOVA (Figures 2.12 and 2.13). Fingerprints from coupon, sediment, and gutter sample sets were generally found on the left side of axis. In contrast, fingerprints from Grit sample set were found on the right side of axis. MS-all fingerprints were generally found in the center of axis (Figure 2.12). LM sample set was fairly indistinct and shared overlap with all other sample sets. LM sample set was indistinct with fingerprints spread throughout the sphere. MS-all sample set was also fairly indistinct and spread out. Through analysis of dendrograms, Gutter sample set contained a fairly large clone cluster which showed up in the top left of sphere (Figure 2.13) A relationship existed between sediment and grit as shown by close proximity of these sample sets' fingerprints (Figure 2.13). LM was the sample set with the greatest level of diversity and shared overlap with other sample sets as shown in Figure 2.12.



Gutter
LM
MS-all
Sediment
Coupons
Grit

Figure 2.12 - MANOVA Analysis of Control (Normal Weather Conditions) 2018 Samples. Each dot represented a unique DNA isolate fingerprint from a particular sample set. Each sample set contained 75 isolates.



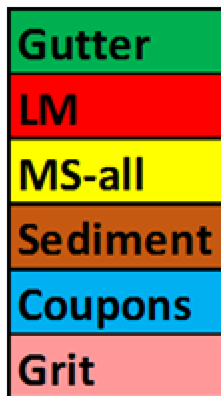


Figure 2.13 - Multidimensional Scaling of Control (Normal Weather Conditions) 2018 Samples. Each dot represented a unique DNA isolate fingerprint from a particular sample set. Each sample set contained 75 isolates.

Control vs Ultra Low Combined Results

Results of DNA fingerprint analyses for 2018 control and ultra-low flow samples are shown in Figures 2.14 and 2.15. Fingerprints from Coupons, Sediment, MS-all UL, and Gutter sample sets were generally found on the left side of the MANOVA axis. Grit fingerprints were most distinct and found on the right side of the MANOVA axis (Figure 2.14). Fingerprints from T2 UL, MS-all and LM sample sets were mostly in the center of the axis. LM and Sediment sample sets were both indistinct and shared similarities with almost all sample sets. MS-all, sediment, and LM sample set was fairly indistinct with fingerprints spread throughout the sphere (Figure 2.15). Analysis of dendrograms showed that Gutter sample set contained a large cluster of clones which could be seen in bottom left corner of sphere. There was a fair amount of overlap between Wet and GBHWet sample sets. LM and sediment were fairly diverse and shared overlap with every other sample set. Grit was also diverse despite also being quite distinct from most other sample sets (Figure 2.14).

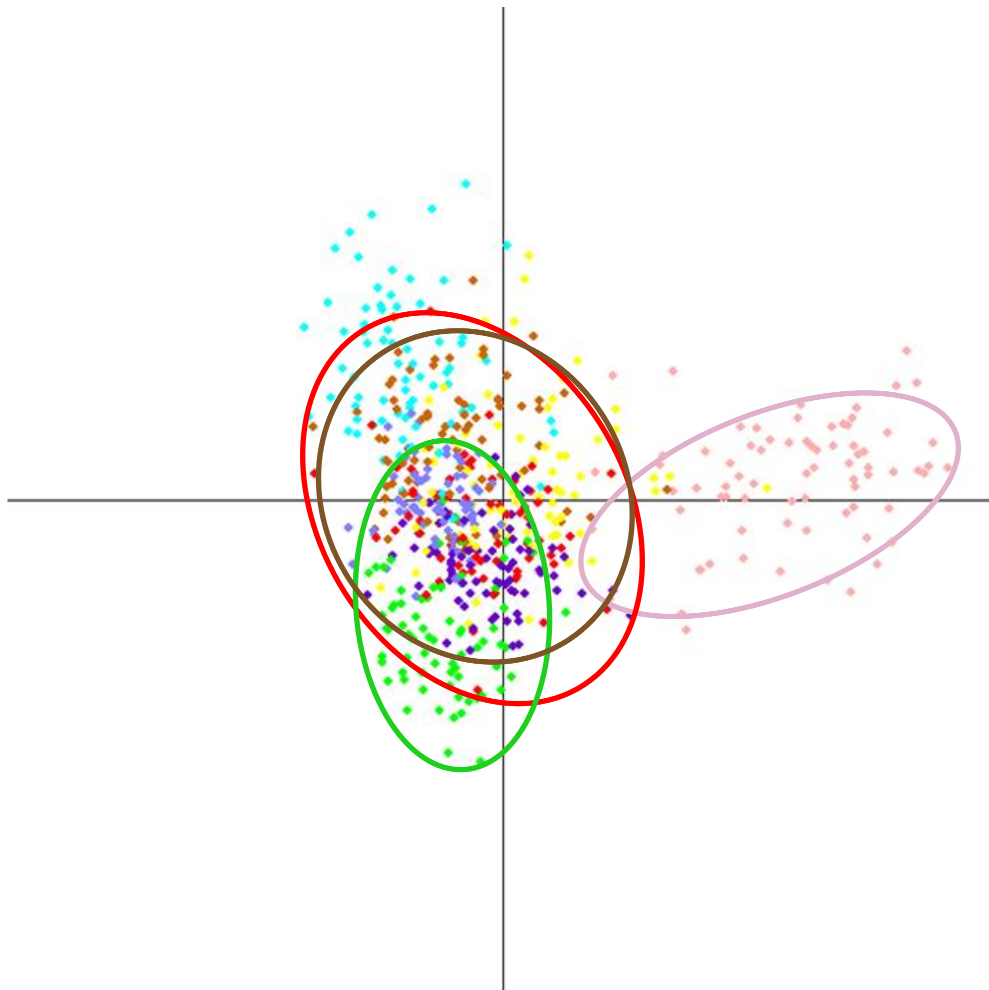
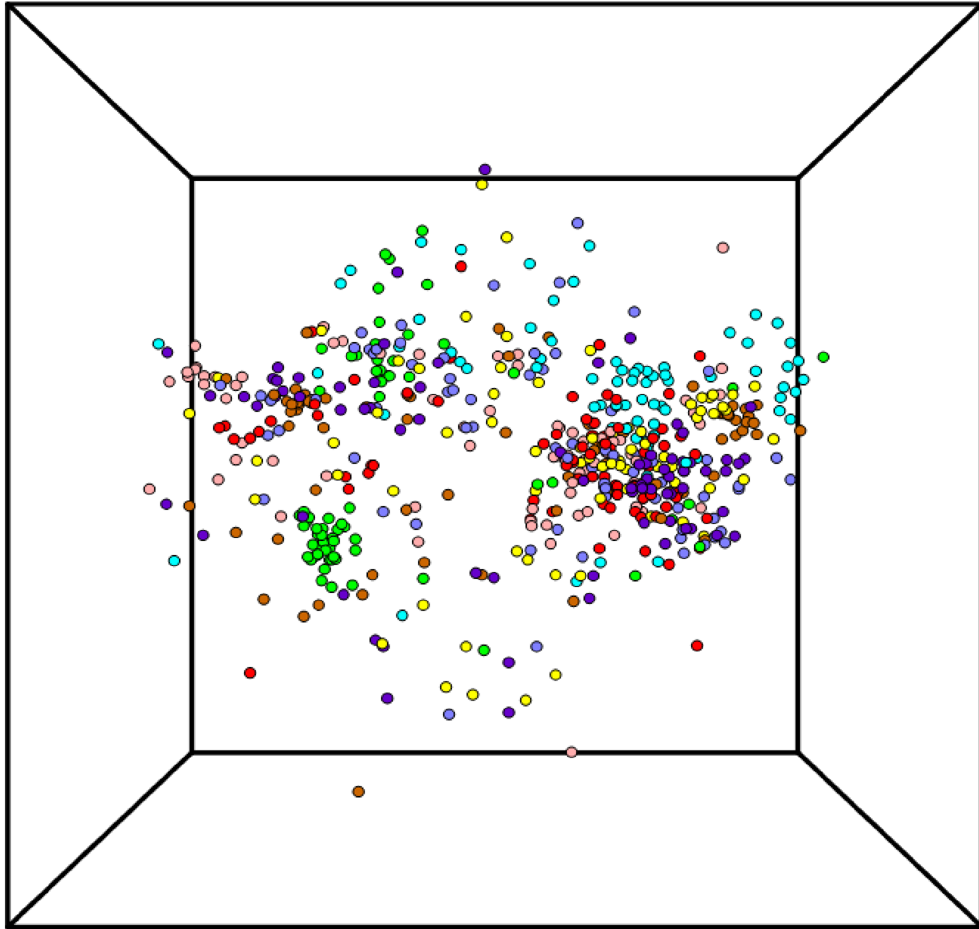


Figure 2.14 - MANOVA Analysis of Control and Ultra Low Flow 2018 Samples. Each dot represented a unique DNA isolate fingerprint from a particular sample set. Each sample set contained 75 isolates.



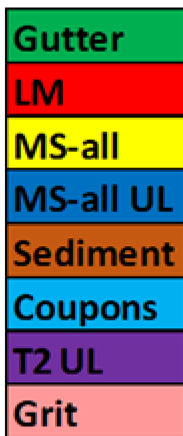


Figure 2.15 - Multidimensional Scaling of Control and Ultra Low Flow 2018 Samples. Each dot represented a unique DNA isolate fingerprint from a particular sample set. Each sample set contained 75 isolates.

Clones Shared Analysis Results

An analysis of clones in the shared 2018 data set is shown in Tables 2.5 and 2.6. The Grit sample set shared 5 clones with MS-all sample set . LM, MS-all UL, Sediment, and T2 UL sample sets shared the least clones with 2.

Table 2.5 – Shows the number of clones other sample sets shared with MS-all. Any two fingerprints with greater than 88% similarity were deemed clones.

Group	Clones shared with MS-all
Gutter	3
LM	2
MS-all	2
MS-all UL	2
Sediment	2
Coupons	3
T2 UL	2
Grit	5

Shannon Weaver Diversity Index Results

Analyses of clonal diversity (Table 2.6) of 2018 sample sets showed that MS-all UL sample set was found to have highest genetic diversity. In contrast, the Gutter sample set was found to have the least genetic diversity.

Table 2.6 - Indicates level of genetic diversity in each sample set. Higher number indicates higher diversity.

Group	Shannon-Weaver Diversity Index
Gutter	1.98
LM	3.37
MS-all	3.76
MS-all UL	4.07
Sediment	3.11
Coupons	3.12
T2 UL	3.45
Grit	3.41

Total Clones Per Sample Set Results

The total number of clones in each sample set is shown in Table 2.7. The Gutter sample set had the highest number of clones (55) and subsequently low genetic diversity. In contrast, the MS-all UL had the lowest number of clones.

Table 2.7 - Shows the number and total percentage of clones in each sample set. Each sample set contained 75 *E. coli* colonies.

Sample Set	Clones	% Clones
Gutter	55	73.30%
LM	53	70.70%
MS-all	33	44.00%
MS-all UL	25	33.30%
Sediment	48	64.00%
Coupons	51	68%
T2 UL	46	61.30%
Grit	51	68%

Hydrological Discharge Results

Discharge data for 2018 (Figures 2.16) showed there were increases in discharge in mid-April following snowmelt rising above 100 cubic ft^3/sec. Apart from a small dip in August/September, discharge remained above 100 ft^3/sec. until mid-November. Peak Discharge occurred in mid-September, reaching recorded discharge of nearly 700 ft^3/sec. Overall daily discharges for 2018 were often higher than median daily statistic for the year.

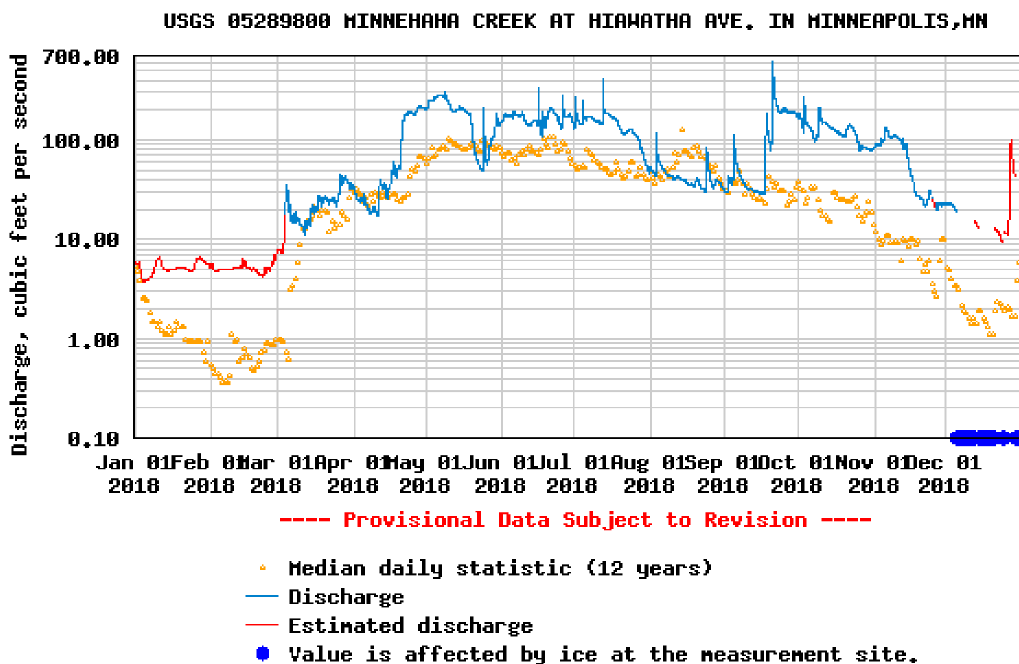


Figure 2.16 - Measurement of Discharge from Jan. 1st, 2018 until Dec. 31st, 2018.

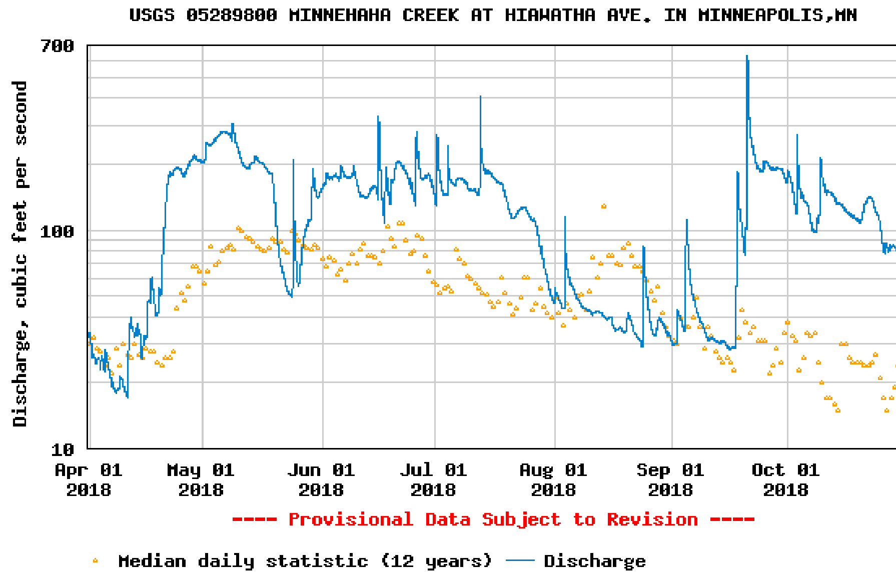


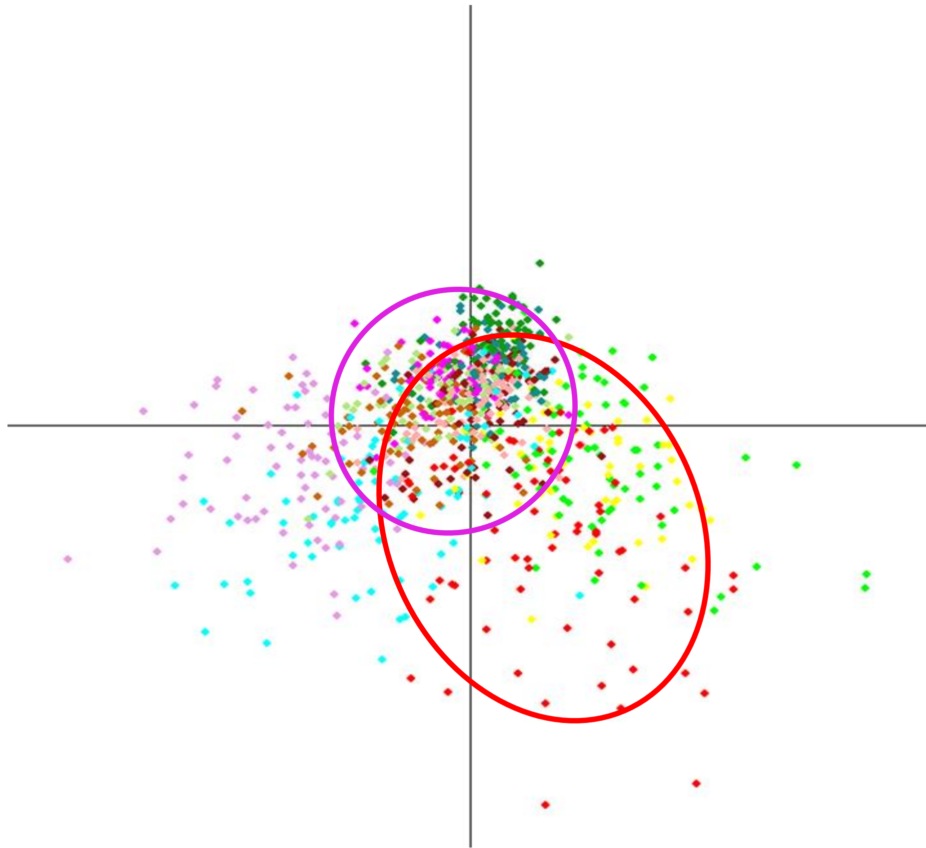
Figure 2.17 - Measure of Discharge from April 1st, 2018 until October 31st, 2018.

MCWD 2017 vs 2018 Sampling Results

2017 vs 2018 Sampling Results

Multidimensional scaling was used to analyze the relationship between 2017 and 2018 results (Figures 2.18 and 2.19). The 2018 gutter sample set was significantly less distinct than the Gutter 2017 sample set. Similarly, the LM 2018 sample set was less distinct than LM 2018 sample set. MS-all 2018 sample set less distinct than MS-all 2017 sample set. Sediment 2017 sample set and Sediment 2018 sample set were both non-distinct and fingerprints were found near the middle of the MANOVA axis. Coupon 2018 sample set was less distinct than Coupon 2017 sample set. Grit 2018 sample set was less distinct than Grit 2017 sample set. Gutter 2018 sample set was less distinct than Gutter 2017. LM 2018 sample set was less distinct than LM 2017 sample set. MS-all 2018 and

MS-all 2017 sample sets were similar with MS-all 2018 more strongly associated with many other samples sets from both years. Sediment 2017 and 2018 sample sets were quite similar with a fair amount of overlap. Coupon 2017 and Coupon 2018 had a strong level of overlap. Grit 2017 and Grit 2018 were also fairly similar and shared overlap.



Gutter2017
Gutter2018
LM2017
LM2018
MS-all2017
MS-all2018
Sed2017
Sed2018
Coup2017
Coup2018
Grit2017
Grit2018

Figure 2.18 - MANOVA Analysis of sample sets analyzed in both 2017 and 2018. Each dot represented a unique DNA isolate fingerprint from a particular sample set. Each sample set contained 75 isolates.

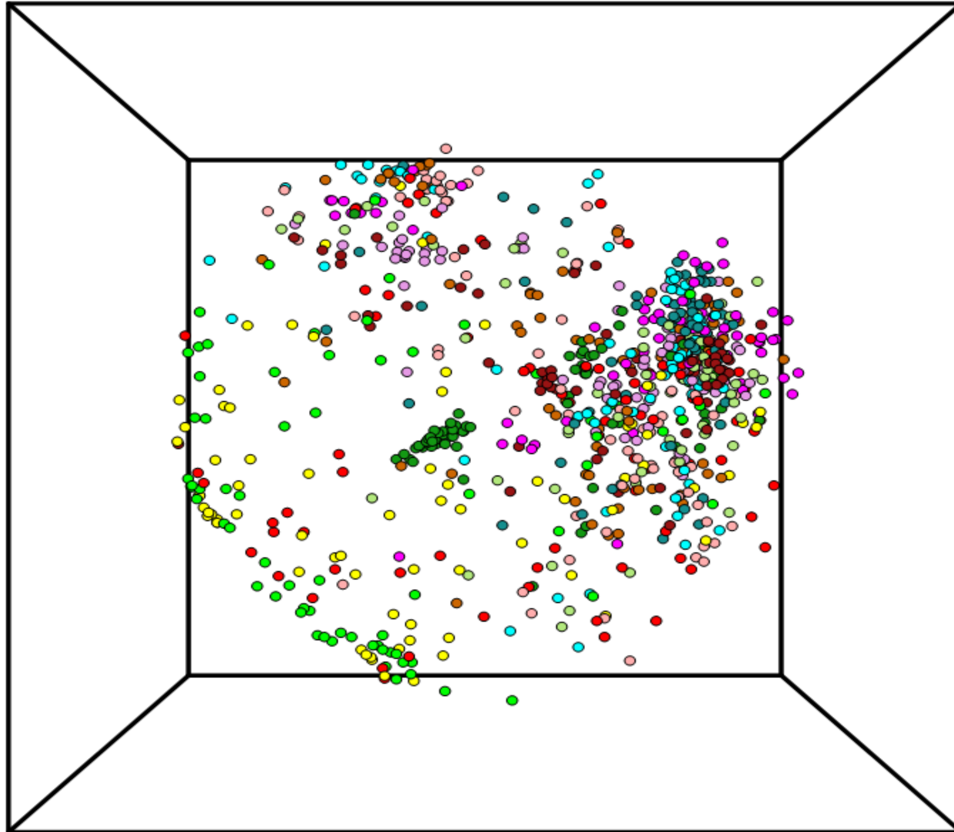


Figure 2.19 - Multidimensional Scaling of sample sets analyzed in both 2017 and 2018. Each dot represented a unique DNA isolate fingerprint from a particular sample set. Each sample set contained 75 isolates.

2017 vs 2018 Clones Shared Analysis Results

The number of *E. coli* fingerprint clones shared between the 2018 and 2017 is shown in Table 2.8. The Grit was the sample set that shared the most clones over both years, with five. Sediment shared one and Gutter, LM, MS-all, Coupons, shared none.

Table 2.8 - Table shows the clones shared between sample sets of consecutive years (2017 and 2018).

Sample Set	Clones shared both years
Gutter	0
LM	0
MS-all	0
Sediment	1
Coupons	0
Grit	5

Discussion

MCWD Sources of *E. coli* Discussion

In this study, the DNA fingerprints of *E. coli* were isolated from several sample locations in the MCWD and were obtained to characterize microbial diversity and determine possible sources of elevated *E. coli* levels within the Minnehaha Creek. Additionally, impacts of variable hydrology on microbial diversity were analyzed over the two-year study period in order to understand how variable flow due to presence or absence of precipitation events subsequently impact the change in the sources of *E. coli* in the watershed and how *E. coli* populations move throughout the watershed.

Through an analysis of various sample sets of years 2017 and 2018 in the Minnehaha Creek, my analysis suggested that the Lake Minnetonka sample set and Sediment sample set were the strongest sources of elevated *E. coli* levels in the MCWD. These sample sets were most consistently non-distinct and shared overlap with most other sample sets for sampling events in 2017 and 2018.

Lake Minnetonka sample set seemed to be one of the least distinct, and it's fingerprints shared overlap with many other sample sets' fingerprints. In dry weather, there was less flow through the Minnehaha creek and thus less bacteria from the lake ended up washing into Minnehaha creek. Still, past research has shown despite low flow conditions, *E. coli* are still able to move through soil, sands, and sediment (Ishii et al, 2007), particularly when storm or wave action

impacts the biofilms. Furthermore, LM had a variety of potential inputs of *E. coli* due to the large amounts of recreational areas in and around Lake Minnetonka.

Sediment was found to be fairly indistinct and shared overlap with most other sample sets for both years. Previous research found that sediment can be a sink for *E. coli* (Hood and Ness, 1982; LaLiberte et al, 1982). This is supported by the findings that aquatic bacteria are known to associate with sediment particles in order to form biofilms which provide bacteria with protection and nutrient acquisition (Davies et al, 1995; Marino et al, 1991). A similar study done (Chandrasekaran 2011) found that ditch sediments were temporal sinks of *E. coli* composed of indigenous populations that contribute to elevated *E. coli* counts during sampling. It is likely that sediment present throughout Minnehaha Creek was a strong source of elevated *E. coli*. It is likely that throughout years, E. coli are becoming naturalized within Lake Minnetonka water/sediment and sediment within Minnehaha Creek. Supported by findings from past studies, these non-point source bacteria are likely to be the main contributor to the continued findings of increased numbers of *E. coli* in the MCWD.

While non-point sources were likely to be the primary source of *E. coli* contamination, new point source inputs each year further exacerbate the water quality issue. There were a variety of inputs that could have deposited *E. coli* into Lake Minnetonka or sediment within the Minnehaha Creek. One of these inputs included wildlife. Wildlife was prevalent in and around Lake Minnetonka. Despite Minnehaha Creek running through an urban metropolitan area, ample parks, preserves, and wooded areas existed along Lake Minnetonka which

allowed refuge and habitat to wildlife such as deer, turkeys, ducks, geese, birds, squirrels, rabbits, rodents, etc. (Figure 2.20). It is likely that wildlife were an important source of fecal *E. coli* contamination.

Figure 2.20 - Duck in the Minnehaha Creek.

Urban influences were a large and important impact Lake Minnetonka and the Minnehaha Creek. Drain tiles, which were common along streets surrounding the Minnehaha Creek, removed water and debris from surrounding neighborhoods, and could have been a particular source of fecal contamination of creek sediment. Fecal contamination from wildlife or pets could have been drained into tiles and quickly transported straight into the lake (Figure 2.21). These tiles also provided microbes with protection from solar radiation, temperature fluctuations, and desiccation making them a possible source of *E. coli.*

Figure 2.21 - Tile Drainage Flowing Out into Minnehaha Creek.

Concrete surfaces found throughout areas surrounding the creek could have provided a “highway” of sorts for *E. coli* and other fecal bacteria to quickly enter waterways, drop out of the water column, and form biofilms on sediments and other surfaces. Concrete structures, such as the one below (Figure 2.22), would have quickly moved organic debris and water containing *E. coli* directly in the Creek.

Figure 2.22 - Sloped Concrete Impervious Surface Leading Down into Minnehaha Creek.

A map of Lake Minnetonka (Figure 2.23) shows a variety of small cities and towns that surrounded and were enveloped by the lake. There were a variety of parks in and around the lake for pet owners to bring their pets as well as partake in recreational activities such as swimming. These were all potential sources of *E. coli* in Lake Minnetonka. Events such as the Lake Minnetonka Boat Party (Figure 2.25) attracted large numbers of people in a small area and these types of events had the potential to contaminate the lake with fecal bacteria. Recreational areas such as picnic areas could attract wildlife due to leftover food and trash and public beaches could be a recreational area for young children and babies which could be potential point sources of contamination.

Figure 2.23 - Map of Lake Minnetonka (MCWD, 2006). The boundaries of Lake Minnetonka fall within the Minnehaha Creek Watershed District shown in red.

Figure 2.24 - Lake Minnetonka Boat Party July 2019 (Wagner, 2019).

Pets were a likely point source of possible *E. coli* contamination in Lake Minnetonka and the Minnehaha Creek. Public sidewalks ran through a multitude of residential neighborhoods around Lake Minnetonka and along streets nearby the Minnehaha Creek where it was common to regularly see residents walking dogs (Figure 2.25). While it is unlawful to leave dog feces on the ground, it was common to see dog feces left in grassy areas on the side of sidewalks or on walking trails (Figure 2.26). If left, the pet feces had the potential to be washed away directly into the creek or indirectly through being washed into street gutters ending up in the Lake or in creek sediment.

Figure 2.25 - Dog Walker with Dog near Minnehaha Creek.

Figure 2.26 - Discarded Dog Feces near Minnehaha Creek. A strong storm event could have the potential to wash this into the creek.

It was common to see trash cans available for neighborhood residents to use on street corners. However, trash cans could have been source of *E. coli* if unattended trash was left to accumulate in an overflowing trash can, particularly in the summer when it was warm, and wildlife was active. Furthermore, dog walkers could've have thrown doggie bags into the trash can. This particular trash can (Figure 2.27) was within 10 meters of the Minnehaha Creek and was overflowing with discarded trash. During a storm event, this trash could have tipped over, releasing trash that into the nearby creek and into creek sediment.

Figure 2.27 - Trash Can near Minnehaha Creek Overflowing with Human Trash.

Hydrological Influences on *E. coli*

A comparison of data for the year 2017 revealed that, based on MANOVA and Multidimensional Scaling analysis, there was a general increase in genetic distinctness among sample sets going from dry to wet data (Figure 2.3, Figure 2.5). In particular, the Gutter sample set for wet weather was found to be more

genetically distinct than in dry weather as supported by dendrogram data. This is evident by the greater spread of Gutter fingerprints across the MANOVA (Figure 2.5) compared to Gutter fingerprints for Dry samples (Figure 2.3). Samples unique to Wet sampling date, Wet sample set and GBHwet samples, were both found to be fairly distinct and diverse suggesting that the precipitation event prior to wet sampling date could have increased the flow necessary to move existing biofilm *E. coli* or new point source inputs of *E. coli* into these sampling areas.

2017 grit was the most indistinct and shared some genetic similarity with all other samples (Figure 2.7). The fingerprints were located near the MANOVA center axis. The grit chamber tank was exposed to stormwater containing organic debris hosting different *E. coli* populations from different areas. Past research has found that FIB populations could colonize and survive within grit chambers and similar structures (Zhang and Llulla, 2006). A comparison of 2017 to 2018 Grit suggests that precipitation events have strong impacts on *E. coli* diversity and distinctness. In 2018, Grit sample set was found to be extremely distinct and more diverse. With lower overall discharges recorded for 2018, it is possible that the lower flows resulted in fewer new inputs into grit chambers as well as a general isolation of current populations the grit chamber, promoting growth of genetically identical *E. coli*. The isolation and lowering of new inputs would explain the increase in distinctness of *E. coli* but not necessarily explain the increase in diversity. In fact, this lower flow phenomena would be more likely to decrease the diversity of *E. coli* in grit chambers based on the reasoning above.

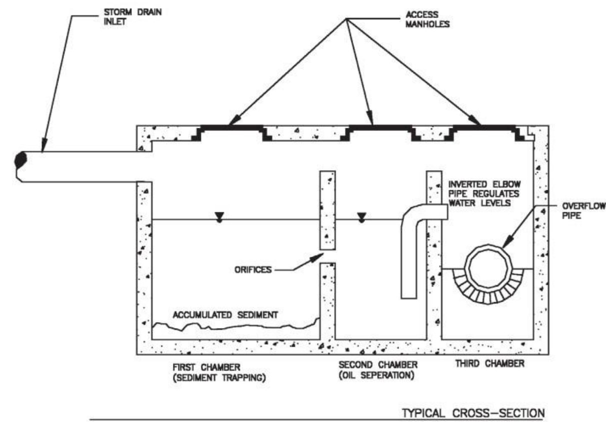


Figure 2.28 – Grit Chamber Structure Diagram (Massachusetts Stormwater Handbook, 2007).

Furthermore, Grit chambers were similar to street gutters in that they could have both protected microorganisms from harmful radiation, temperature fluctuations, and trap organic debris that microbes can grow on (Figure 2.28). Another possible explanation for the large change in diversity and distinctness between years is if grit chambers were altered or cleaned between the sampling dates of 2017 and 2018 that could have removed established *E. coli* biofilms within grit chambers. It was likely that grit chambers were an important source of *E. coli* in the Minnehaha Creek following precipitation events, when flows were heavier and could have more effectively removed *E. coli* from grit chamber biofilms and pulled them into the water column.

Gutter 2018 sample set seemed to be less distinct and less diverse compared to Gutter 2017 sample set. In Figure 2.18, Gutter 2017 fingerprints were spread throughout both the top and bottom right side of the MANOVA,

whereas Gutter 2018 fingerprints were clustered closer to the center MANOVA axis and almost entirely in the top right quadrant. The position of the 2018 fingerprints indicated that they were less genetically unique and overall there was less genetic diversity in this sample set. Lower flow due to fewer storm events would have reduced the amount of storm water harboring new *E. coli* reaching street gutters resulting in reduced diversity. Also reduced storm water flows could have meant less fecal matter being released into street gutters, which would otherwise be a strong input of clones into the gutter sample set. Alternatively, there could have been more localized growth within gutters in 2018 than in 2017, resulting in greater distinctness and diversity. Gutter was a likely source of *E. coli* for periods following recent precipitation events, but its importance was likely dependent on altered flow conditions, due to these precipitation events, which acted to release biofilm bacteria from surfaces into the creek water column.

The discussion of hydrological activity was particularly important for Gutter and Grit Chamber sample sets. These two sample sets seemed to be strongly impacted by fluctuations in water discharge due to precipitation, compared to other sample sets. Changes in these conditions could have had a fairly significant effect in the amount of organic debris that entered into street gutters and grit chambers, respectively. Both of these structures were designed to control the buildup of various materials in the street and in the stream/river, respectively, following storm events. However, if excess material was trapped in street gutters and grit chambers due to extremely turbulent weather events or lack of cleaning of structures, this could have inhibited normal functioning of

these structures and also could have promoted bacteria growth on excess organic matter trapped in structures, leading to larger amounts of *E. coli* growth.

Chapter 3. Conclusions

Future Considerations

While this experiment was performed using Rep PCR fingerprinting technology, other technologies such as qPCR could have been used to provide a more in-depth picture of *E. coli* sources. However, costs of qPCR are still significant and without proper funding for this type of experiment, more affordable alternatives such as Rep-PCR must be used. In addition, rep-PCR fingerprinting allowed us to determine clonality and qPCR does now. Currently, adequate funding is a major hurdle in the field of environmental sciences. Without very large budgets for labs and public agencies to purchase newer equipment, these technologies will not be implemented until costs of technology come down drastically.

Limitations

Rep PCR fingerprinting technology was useful to give a broad picture of relationships among sample sets but lacks the power to provide a more in-depth analysis. This is partly due to the fact that Rep PCR only looks at a small portion (repetitive sequences) of the microbial genome. Furthermore, Rep PCR analysis only provides us with dendrograms which allow us to understand relationships

between a very limited number of just 75 fingerprints per sample set. This is significant as previous research has found that *E. coli* can require as many as 20,000 to 40,000 isolates to fully understand the diversity of *E. coli* present in a sample. (Gordon, 2001; Hartl and Dykhuizen, 1984; Rocha et al, 1999). As an alternative to this, with a whole genome sequence, one would be able to target specific genes specific to pathogenic strains of *E. coli*. Assuming we knew how many of these genes are typical in any one *E. coli* genome, we would be able to quantify exactly how many pathogenic bacteria are in a single sample. By using this information along with existing methods that link specific *E. coli* to source hosts (ex. Dog, cow, etc.), we would be able to quantify which source inputs are contributing the most pathogenic *E. coli*.

In regard to quality of sampling data, diversity of a particular sample set can be skewed if the sampling technique is poor, resulting in an unrepresentative sample. This can vastly overestimate the clonality present in a sample and can occur if fecal matter from human, livestock, wildlife, or pet is accidentally introduced into a sample. This would greatly increase the clonality and greatly decrease the overall diversity present in the sample, giving an altered diversity profile of the particular sample location.

Recommended Actions to Reduce FIB in the MCWD

Results of this study indicated that Lake Minnetonka water and Sediment from the creek were likely the greatest sources of elevated *E. coli* levels in the MCWD. These sample sets were most consistently non-distinct and shared overlap with most other sample sets for sampling events in 2017 and 2018. Moreover, during the wet season with high flow conditions, the street gutters and Grit chambers were also likely sources of FIB.

One recommended action to reduce fecal bacterial loading would be to periodically clean gutters and grit chambers of debris to remove organic material that bacteria could use to grow. Pet owners should have greater penalties placed upon them for neglecting their pet waste. Alternatively, cities could place dog waste bags in neighborhoods to make it more convenient for pet owners to deal with their pet waste. Some greater restrictions could be placed on activities such as the Minnetonka boat party which is likely to be a large point source of fecal contamination into Lake Minnetonka.

Lastly, watershed managers could more strongly control discharge into the creek at Lake Minnetonka through by opening the dam at Gray's Bay Dam in a manner that would create a more gradual increase of water into the creek which would be less likely to disrupt sediment biofilms and stir up sediment *E. coli* into the water column.

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