MICROBIAL POLLUTION SOURCE IDENTIFICATION IN RURAL / URBAN MIXED WATERSHEDS

By

Amira Oun H Oun

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Environmental Engineering —Doctor of Philosophy

2016

ABSTRACT

MICROBIAL POLLUTION SOURCE IDENTIFICATION IN RURAL/ URBAN MIXED WATERSHEDS

By

Amira Oun H Oun

The majority of the reported water impairments are related to microbial agents. Since it is not feasible to directly monitor all pathogens, the Environmental Protection Agency (EPA) has established microbial water quality criteria based on fecal indicator bacteria (FIB). Reliance on the current approach, based on FIB, for assessing the risk associated with water contamination is inadequate for protecting public health. Recently, the use of microbial indicators, as a tool for risk assessment and policy planning and implementations, has been significantly enhanced by using molecular-based methods for Microbial Source Tracking (MST). Combinations of one or more molecular based-methods are increasing in recent studies, but to date there is no single method that could be applied to all types of faecally contaminated water systems. The selection of a particular method could be affected by several factors such as the complexity of the environment under study, the number of sources suspected to be implicated in contamination events, funds available to perform studies, and the technical expertise available to produce and analyze the data. In this study multiple MST methods have been investigated in combination with land use data and hydrological conditions at a watershed scale in Michigan. Two beaches in Saginaw Bay watershed and one creek in Red Cedar River watershed have been assessed for water quality and potential sources of contamination have been identified.

Dedicated to my children who tolerated their childhood time during my journey.
Love you: Mariam, Mohamed, Ahmed, Rihanna and Aisha

ACKNOWLEDGEMENTS

The accomplishment of these milestones was only possible by the great people who surrounded me. First of all, I am delighted to convey my high appreciation to my advisor, Dr. Irene Xagoraraki, who gave me the opportunity to conduct my doctoral research in her laboratory while offering me valuable support, encouragement, and advice. Through many of our weekly meetings, she carved my path that significantly increased my knowledge in the field; especially when genuinely considering my thoughts, initiatives and thoughtfully analyze my work outcomes. A sincere thanks to my co-advisor Dr. Thomas Voice who first admitted me to the program and served as my supervisor during my Masters degree. His guidance, support, and constructive comments have made this work successful and professional. I also would like to thank my dissertation committee members Dr. Phanikumar S. Mantha, and Dr. David T. Long, thank you all for the productive input and motivation during the development of this research. I would also like to extend my thanks to my colleagues from Dr. Irene group, Dr. Mariya Munir, Dr. Ziqiang Yin, Huiyun Wu, and Evan O'Brien. A warm thanks to Lori Larner, Margaret Connor, Mary Morz and Laura Taylor for their administrative support during my studies. I am grateful to the Libyan Ministry of High Education and Scientific Research for their scholarship funding through the years of my graduate studies at Michigan State University. A warm thanks to my two loving sisters Abeer Oun (in Libya) and Dalal Oun (in Canada). I am grateful to my loving husband Haithem Aboujrad for his love and unconditional support through my endeavors. Finally, I am very grateful to my parents Nagia and Oun who have always given me the strength and wisdom to be sincere in my work; they have fostered high moral standards since my early childhood, and of course, I am grateful for being honored such love and affection.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

CWA – Clean Water Act

DNA - Deoxyribonucleic Acid

E. coli - Escherichia coli

FIB - Fecal Indicator Bacteria

GIS - Geographical Information System

MDEQ - Michigan Department of Environmental Quality

MST- Microbial Source Tracking

NCG - Next Generations Sequencing

PBC - Partial Body Contact

PCR - Polymerase Chain Reaction

qPCR- Quantitative Polymerase Chain Reaction

rRNA - Ribosomal RNA

TBC - Total Body Contact

TMDL - Total Maximum Daily Load

USEPA – United State Environmental Protection Agency

SDWA - Safe Drinking Water Act

NPDES - National Pollutant Discharge Elimination System

CHAPTER 1

INTRODUCTION

Protection of water sources from pathogenic microbial contamination is essential to human health and monitoring methodologies that detect and/or quantify fecal contamination in resource waters are well established. Policies related to water quality exist since the early 1970's. For instance, the Safe Drinking Water Act (SDWA) regulates the nation's public drinking water supply (EPA, 2004). For "fishable and swimmable" water the regulation is covered by the water quality monitoring strategies described at the Clean Water Act (CWA), Section 303. EPA recommends using the fecal indicator bacteria (FIB) *Enterococcus* and *Escherichia coli (E. coli)* as indicators of fecal contamination of fresh water (Simpson et al., 2002). A significant reduction of loads from point sources under the National Pollutant Discharge Elimination System (NPDES) helps to meet the standards in many watersheds. Recently, nonpoint sources of bacterial pollution sources including, but not limited to, storm water runoff, combined sewer flows, wastewater discharges, failing septic systems, poor livestock management, pet and wildlife waste, and litter, have surpassed point sources as the major source of fecal contamination of surface water (USEPA 2000 and 2005).

Currently, total coliforms, fecal coliforms, *Escherichia coli* and Enterococci are the most used bacterial indicators in studies related to water quality monitoring and health risk assessments worldwide (Simpson et al., 2002). The indicator bacteria themselves are expected to be non-pathogenic, but ideally they should present survival characteristics similar to the pathogens of concern (Meays et al., 2004). Although the concept of bacterial indicators is widely accepted there is an ongoing debate about their use or at least some concern regarding which organism should be used as the "ideal indicator" because the association between the pathogens and fecal indicators is not direct and not strong enough.

Escherichia coli (*E. coli*) is widely used as a bacterial indicator organism to test environmental samples for fecal contamination. This bacterium is a gram-negative, rod shaped gammaproteo bacterium that belongs to the fecal coliform group of bacteria. The primary habitat of *E. coli* is known to be the lower intestine of warm-blooded animals (Bower et al., 2005) and since it represents part of the fecal mass of most animals, its presence in the environment (second habitat) is directly related to this type of contamination and potential presence of pathogens whose pathway include fecal contamination. These characteristics, thus, make *E. coli* a good bacterial indicator (Scott et al., 2002). Recently, it was reported that *E. coli* can not only exist, but reproduce in the environment - their second habitat - and their use as an indicator could be impacted. On this note, scientists started to invest in studying other alternative indicators such as *Bacteroides* (Ahmed et al., 2009).

Identifying the origin of fecal contamination is principal in assessing associated potential health risks as well as the actions necessary to determine optimal remediation strategies. The usefulness of the microbial indicators as tools for risk assessment can be significantly enhanced by the development of testing methods and analysis techniques that can define specific sources of these organisms. The concept that tracing the source of fecal pollution using microbiological, genotypic, phenotypic, and chemical methods has been termed Microbial Source Tracking

(MST). In that context, the next natural step on dealing with water quality and microbial contamination is to invest on identifying the source of the non-point microbiological contamination. This information would allow proper regulations to be written and enforced, as well as help environmental engineers to mitigate the issue supported by the best available technologies. Currently, MST is an experimental science and there is no standard method that has been adopted mainly because improvement in precision and accuracy is needed for the development of standard operational protocols to enforce future policies development (Meays et al., 2004; EAP, 2011).

MST techniques attempt to determine the source of microbiological contamination in the environment which can be caused by humans, wildlife or domestic animals. The underlying assumption of this approach is that the host-specificity of the microorganism is influenced by a selective pressure that may occur in the host guts (Ahmed et al., 2009). MST for bacteria have been recommended as a cost-effective approach for studies related to targeted in-steam monitoring, sanitary or watershed surveys and dye septic systems failing (EAP, 2011). MST can be divided in molecular and biochemical techniques, which search for specific characteristics from the microbial indicators and allow scientists to identify the source of the contamination, and chemical methods which relay on detecting chemicals associated with human activities (Vogel et al., 2007). Determining which method or combination of methods to use for any given situation will depend on a number of factors including: specific question to be answered, detail required to answer the question (human/non-human, differentiate non-human hosts, different specific environments, etc), economics and logistics (available resources and investments, lab structure, cost of analysis, etc).

By definition an ideal indicator would be non-pathogenic, rapidly detected, easily enumerated and have survival characteristics similar to the pathogenic of concern (Scott et al., 2002; Ishii et al., 2008). For microbial source tracking, an additional characteristic needs to be added to this list: that is discriminatory power between hosts of the microbial indicator. Identifying bacterial sources is a key first step to control microbial contamination allowing a more cost- effective management of microbial risks, especially in situations where the total maximum daily loads of contaminant sources are not met and best management practices are required (Simpson et al., 2002; Staley et al., 2012).

If microbial source tracking (MST) is required, then alternative analytical methods should be used. Appropriate rapid MST methods to distinguish human and non-human contamination would be host-specific PCR tools targeting genetic markers (bacterial, or viral), these methods have long been suggested as alternative indicators to the fecal coliforms (Carrillo, M et al, 1985), and have become increasingly recognized as an identification tool for various fecal sources. The quantitative PCR method might also be used for long-term watershed studies where large numbers of samples are analyzed. qPCR is known as "real-time PCR" because results can be obtained within hours of sample collection, potentially before indicator bacteria enumeration results are obtained, as compared to months for library-dependent methods.

Recently Host-specific PCR for Bacteroidales molecular markers have been developed and usedin many MST studies. *Bacteroides* have been used to isolate a specific marker and investigate land use and water quality impairments (Peed LA, et al., 2011, Verhougstraete et al., 2014). A study conducted by Furtula et al. (2012), confirmed ruminant, pig, and dog fecal contamination in an agriculturally dominated watershed (Canada) using *Bacteroides* markers. Another study by Verhougstraete et al., 2014, provides a water quality assessment for a large number of watersheds in Michigan and found that human fecal contamination was prevalent.

In addition to Bacteroidales, human adenovirus (HAdV) have been suggested as potential MST tools (Ahmed et al., 2010, Harwood, V. J. et al, 2014). Several hexon-based real-time PCR assays have been developed to detect generic HAdVs. In addition, integrated cell culture PCR (ICC-PCR) techniques have been used to detect infectious HAdV (Lee et al., 2004). Bovine adenovirus (BAdV) were proposed as useful tools for identification of water pollution sources and appeared to be the most widely used animal viruses for MST purposes (Ahmed et al., 2010; Hundesa et al., 2006). Yet the evaluation of human and bovine adenovirus as microbial source tracking tools has not been widely examined.

More recently an emerging extension of MST concept has been used to simply characterize the entire microbial community (eg next generation sequencing) in a water sample and determine any potential similarities. The use of community analysis in MST is very recent development and remains an active research area. Next generation sequencing may potentially overcome the limitation of the single marker and improve sensitivity for host-specific molecular methods, but they also require the highest level of expertise for managing and analyzing large, complex data sets. As a result of these limitation, community analysis should be conceder method of last alternative, only to be employed when it is suspected that information that can be gained could not gathered from simpler and more cost effective approaches.

Quantification of one or more genetic markers may not provide a complete set of information to accurately identify fecal source(s) as well as to characterize fecal loadings from diffuse sources in the water bodies. As an approach to improve source-tracking capability, the outcomes of marker-based tools can be interpreted in combination with environmental reference information such as land use (Peed et al., 2011; Reischer et al., 2008) under varying hydrological conditions. This approach is expected to allow more accurate interpretation of microbiological fecal source tracking data and make the MST tools more powerful (Reischer et al., 2011, Verhougstraete, M. P., et al, 2015).

Objectives

The overall objectives of this study are:

- Investigate the use of combining MST methods with geographical information system (GIS) analysis to determine potential non-point sources of contamination in mixed watersheds.
- Characterize the load of microbial pollution in sediments
- Evaluate the use of *Bacteroides* and viruses host specific marker qPCR assays as a MST marker.
- Determine the effect of hydrological factors (precipitation and flow rate) on microbial water quality.

REFERENCES

REFERENCES

Ahmed, W., Goonetilleke, A., & Gardner, T. (2010). Human and bovine adenoviruses for the detection of source-specific fecal pollution in coastal waters in Australia. Water research, 44(16), 4662-4673.

Ahmed, W., Goonetilleke, A., Powell, D., Chauhan, K., & Gardner, T. (2009). Comparison of molecular markers to detect fresh sewage in environmental waters. water research, 43(19), 4908-4917.

Bower, P. A., Scopel, C. O., Jensen, E. T., Depas, M. M., & McLellan, S. L. (2005). Detection of genetic markers of fecal indicator bacteria in Lake Michigan and determination of their relationship to Escherichia coli densities using standard microbiological methods. Applied and environmental microbiology, 71(12), 8305-8313.

Byappanahalli, M. N., Yan, T., Hamilton, M. J., Ishii, S., Fujioka, R. S., Whitman, R. L., & Sadowsky, M. J. (2012). The population structure of Escherichia coli isolated from subtropical and temperate soils. Science of the total environment, 417, 273-279.

Harwood, V. J., Staley, C., Badgley, B. D., Borges, K., & Korajkic, A. (2014). Microbial source tracking markers for detection of fecal contamination in environmental waters: relationships between pathogens and human health outcomes. *FEMS microbiology reviews*, *38*(1), 1-40.

Hundesa, A., de Motes, C. M., Bofill-Mas, S., Albinana-Gimenez, N., & Girones, R. (2006). Identification of human and animal adenoviruses and polyomaviruses for determination of sources of fecal contamination in the environment. *Applied and environmental microbiology*, *72*(12), 7886-7893.

Ishii, S., & Sadowsky, M. J. (2008). Escherichia coli in the environment: implications for water quality and human health. Microbes and Environments, 23(2), 101-108.

Lee, C., S. H. Lee, E. Han, and S. J. Kim. 2004. Use of cell culture-PCR assay based on combination of A549 and BGMK cell lines and molecular identification as a tool to monitor infectious adenoviruses and enteroviruses in river water. Appl. Environ. Microbiol. 70:6695–6705.

Meays, C. L., Broersma, K., Nordin, R., & Mazumder, A. (2004). Source tracking fecal bacteria in water: a critical review of current methods. Journal of environmental management, 73(1), 71-79.

Scott, T. M., Rose, J. B., Jenkins, T. M., Farrah, S. R., & Lukasik, J. (2002). Microbial source tracking: current methodology and future directions. Applied and environmental microbiology, 68(12), 5796-5803.

Simpson, J. M., Santo Domingo, J. W., & Reasoner, D. J. (2002). Microbial source tracking: state of the science. Environmental science & technology, 36(24), 5279-5288.

Staley, C., Reckhow, K. H., Lukasik, J., & Harwood, V. J. (2012). Assessment of sources of human pathogens and fecal contamination in a Florida freshwater lake. Water research, 46(17), 5799-5812.

U.S Environmental Protection Agency. 2000a. Improved enumeration methods for the recreational water quality indicators: Enterococci and Escherichia coli. Office of Science and Technology, Washington, DC. EPA/821/R-97/004. 55 pp.

U.S Environmental Protection Egency.2005 Microbial Source Tracking Guide Document. Office of Science and Technology, Washington, DC. EPA/ 600-R-05-064

U.S Environmental Protection Egency.2011 Microbial Source Tracking Guide Document. Office of Science and Technology, Washington, DC. EPA/ 11-03-038

CHAPTER 2

EFFECTS OF BIOSOLIDS AND MANURE APPLICATION ON MICROBIAL WATER QUALITY IN RURAL AREAS IN THE US

Abstract

Most of the waterborne disease outbreaks observed in North America are associated with rural drinking water systems. The majority of the reported waterborne outbreaks are related to microbial agents (parasites, bacteria and viruses). Rural areas are characterized by high livestock density and lack of advanced treatment systems for animal and human waste, and wastewater. Animal waste from livestock production facilities is often applied to land without prior treatment. Biosolids (treated municipal wastewater sludge) from large wastewater facilities in urban areas are often transported and applied to land in rural areas. This situation introduces a potential for risk of human exposure to waterborne contaminants such as human and zoonotic pathogens originating from manure, biosolids, and leaking septic systems. This paper focuses on waterborne outbreaks and sources of microbial pollution in rural areas in the US, characterization of the microbial load of biosolids and manure, association of biosolid and manure application with microbial contamination of surface and groundwater, risk assessment and best management practice for biosolids and manure application to protect water quality. Gaps in knowledge are identified, and recommendations to improve the water quality in the rural areas are discussed.

Waterborne Outbreaks in Rural Areas in the U.S

Most of the waterborne disease outbreaks worldwide and in North America are associated with rural drinking water systems. According to Craun *et al.* (Nicholson, F et al.,2004), in the United States during the 12 years period of 1991–2002, 207 waterborne disease outbreaks and 433,947 illnesses were reported; 42% of these outbreaks occurred in non-community water systems, 22% occurred in individual systems such as private wells, and only 36% occurred in community systems. In most cases the drinking water supply in rural areas is provided by groundwater wells that in some cases are shallow. Between 1981 and 1998, 50% (210 of 417) of the reported waterborne disease outbreaks were linked to contaminated groundwater (Meslin, F., 1996, Slifko, T. et al., 2000). 696 outbreaks have been reported in the US between 1971 and 2000, of which 59% were linked to groundwater (Sobsey, M. et al., 2001).

Most of the reported waterborne diseases outbreaks in North America were related to microbial agents (parasites, bacteria and viruses), some to chemical agents, and some were of unknown etiology. For example, a survey by the Centers for Disease Control and Prevention (CDC) reported that one sixth (5 out of 30 cases) of drinking water-associated waterborne disease outbreaks during 2003–2004 were of unknown etiology because of a lack of available analytical methods (Hubálek, Z., 2003). The US EPA suspects that many of the outbreaks due to unidentified sources were caused by enteric viruses (Gannon, V. et al., 2004). Zoonotic and human viruses are of particular interest because viruses are the smallest of all pathogens and their small size can facilitate transport through the soil. Viruses have low die-off rates and viral infections may lead to chronic health effects.

Runoff carrying manure-borne microbial pathogens have been implicated in some of the largest water and food borne outbreaks (Cliver, D. and Moe, C., 2004, Palmer, S. et al., 2005, Pourcher, A. et al., 2005); in contrast, it is important to state that there are no scientifically documented outbreaks or excess illnesses that have occurred from exposure to pathogens associated with treated biosolids (Bofill-Mas, S. et al., 2006). An outbreak that occurred in a small farming community in Canada demonstrated that the potential of human infections caused by zoonotic pathogens (pathogens that may infect both animals and humans) has serious consequences (Cliver, D. and Moe, C., 2004, Pourcher, A. et al., 2005). More than 2300 people in the town of Walkerton, Ontario suffered gastrointestinal illness and seven died when the water supply was contaminated by manure pathogens from a nearby farm after more than five inches of rain fell over a five-day period in May 2000 (Cliver, D. and Moe, C., 2004, Pourcher, A. et al., 2005).

During March and April 1993 water contaminated with *Cryptosporidium* oocysts from Lake Michigan caused a massive waterborne outbreak of cryptosporidiosis among residents of Milwaukee, Wisconsin. The source of contaminated water was most likely runoff from rural areas during high rainfall. It was estimated that 403,000 residents living in a five-county area and numerous visitors to the city of Milwaukee were sick during this outbreak and 58 lost their lives (Pourcher, A. et al., 2007).

In recreational waters, fecal contamination from bathers has been an important source of exposure. During 1971–2000, 259 outbreaks associated with recreational activities have been reported, 144 outbreaks (56%) were associated with recreation in untreated natural water such as lakes and streams; the remaining of the recreational outbreaks occurred in treated water such as swimming pools (Monpoeho, S. et al., 2004). Fecal contamination from bathers, septic tanks, runoff from agricultural areas, and other sources was identified as the important cause of outbreaks in untreated recreational waters. Zoonotic bacterial agents such as *E. coli* O157:H7 and O121:H19 caused 38% of the outbreaks associated with untreated natural recreational water (Nicholson, F. et al., 2004, Monpoeho, S. et al., 2004).

Sources of Microbial Pollution in Rural Areas in the U.S

The nexus between water quality and public health in the rural environment is complex. Non-point sources of contaminants are common and there is little monitoring of the system or the types of pollutants and emerging contaminant risks. Human and zoonotic pathogens and other microbial pollutants are a particular concern. Water quality research tends to focus on urban areas and sophisticated treatment systems. Water and waste management in rural areas is often minimal and water quality monitoring is sporadic. Rural areas typically have high density livestock housing and lack of advanced treatment systems for animal and human waste and wastewater. Animal waste from livestock production facilities is often applied to the land without prior treatment. Domestic wastewater is often treated on-site by septic systems without regular monitoring.

There is a potential risk of human exposure to waterborne contaminants such as human and zoonotic pathogens originating from manure, biosolids, and leaking septic systems in rural areas (Monpoeho, S. et al., 2001, Kudva, I. et al., 1998). Livestock manure has been spread on the land as fertilizer and also for disposal. Manure and other wastes of various livestock often contain high concentrations of pathogens. Levels of pathogen in manure depend on the source animal, the animal's state of health, and how the manure was stored or treated before use. Non-point sources of contamination by manure include pastured animals, roaming wild animals, and leaching or runoff from agricultural areas. Point sources of manure contamination include animal feedlots, animal housing facilities, and manure storage areas, such as lagoons (Sinton, L. et al., 2007), resulting in soil and water contamination.

Biosolids (treated municipal wastewater sludge that meets standards for use as a fertilizer or soil conditioner); from large wastewater utilities in urban areas are often transported and applied to land in rural areas. Land application of biosolids provides agricultural benefits and presents a cost effective method for disposal of treated sludge following wastewater treatment; however, reuse of this product presents health concerns that must be addressed. Health concerns include pathogen transmission to food, contamination of ground water or surface water from field runoff, and build-up of heavy metals or organic contaminants (King, G. et al., 2011, Daniel, T. et al., 1998). Because biosolids may contain human pathogens (Monpoeho, S. et al., 2001, Scott, T. et al., 2002), exposure to biosolids has raised human health-related concerns. The microbial load of biosolids that are routinely applied on farm land varies depending on the type of sludge treatment and common sludge treatment processes do not completely inactivate pathogens.

Characterization of Biosolids and Manure

In 2004, a US national biosolids survey indicated that about 6.50 million tons of dry biosolids were produced in the US; and approximately 55% of the total was applied on land as soil amendment (Bofill-Mas, S. et al., 2006, Easterling, D. et al., 2000). In 2006, the US EPA (Easterling, D. et al., 2000) estimated that more than 7.10 million tons of dry biosolids per year are produced in the US; about 50% of which are land applied. Land application of biosolids has increased since restrictions were placed on ocean dumping disposal. However, due to public concern over potential hazards, in some areas of the US land application of Class B biosolids has been banned. This is particularly true in California, where in many areas Class A land application has replaced Class B land application (Atherholt, T. et al.,1998).

Biosolids contain organic matter and nutrients, and when applied to farmland can improve productivity and reduce the need for manufactured fertilizer inputs (Tate, K. et al., 2000, Kistemann, T. et al., 2002). The main benefits of biosolids are through the supply of nitrogen, phosphorus, potassium, calcium, and magnesium, biosolids also supplying the essential plant micro nutrients (e.g., sulfur, manganese, zinc, copper, iron, molybdenum, and boron) (Gary, H. et al., 1983). Biosolids are a valuable source of nutrients although it contains lower N, P, and K compared to commercial fertilizers, especially high-grade ones (Niemi, R. and Niemi, J., 1991). A study of nutrient levels in biosolids (Doran, J. and Linn, D., 1979), with more than 240 samples collected and analyzed in Pennsylvania (aerobically digested, anaerobically digested, or alkali-treated) between 1993 and 1997 showed average N, P, and K contents of 4.74%, 2.27% and 0.31%, respectively. Nutrient values of biosolids vary with sources of wastewater and wastewater treatment processes, in general compared to cattle manure, biosolids have lower P, the N:P ratio is around (3.1–3.4) (Doran, J. and Linn, D., 1979, Jawson, M. et al., 1982), and have limited amount of K (Culley, J. and Phillips, P., 1982).

Even though biosolids are a beneficial soil amendment they may also pose a potential threat to the environment and pose risks to human health. There are potential hazards with land application since several contaminants can be present in biosolids including human pathogens (Atherholt, T. et al.,1998, Kistemann, T. et al., 2002, McMurry, S. et al., 1998, Howell, J. et al., 1996). Pathogens contained in biosolids include viruses, bacteria, and animal and human parasites (protozoa and helminthes), which may cause various human diseases and illnesses (Kistemann, T. et al., 2002, Fleming, R. and Bradshaw, S., 1992). The characteristics and properties of biosolids vary depending on the quality and origin of sludge, along with the type of treatment processes (Shipitalo, M. and Gibbs, F., 2000, Shipitalo, M. and Protz, R., 1987, Drees, L. et al., 1994). Incomplete destruction, contamination from external sources, and changes in environmental factors during storage can lead to regrowth or reactivation of pathogens. Typical concentrations of some pathogens and indicators in biosolids are summarized in Table 1.

The rapid growth of concentrated animal feeding operations (CAFOS) has caused an increase in the amount of manure produced annually in US. There are approximately 450,000 CAFOs in the US including dairy, cattle, pig, and poultry farms. An estimated 450 million tons of wet weight manure or 90 million tons of dry solids manure are generated annually from about 92 million swine, 109 million cattle, 292 million turkeys, and 7.5 billion chickens in the US (Pagliai, M. et al., 1995, Geohring, L. and Van Es, H., 1994, Evans, M. and Owens, J., 1972). US EPA estimates that a single dairy cow produces approximately 55 kg/day of wet manure (Dean, D. and Foran, M., 1992). Livestock and poultry manure can provide large quantities of valuable nutrients for crop and pasture when applied to land as a fertilizer but most of this manure is applied untreated. Historically, the environmental impacts of animal manure were principally associated with nutrients (McLellan, J. et al., 1993). Manure application rates are usually based on crop N requirement which greatly increases soil P levels because, the N: P ratios of manure (2:1 to 4:1) are significantly smaller than N: P uptake ratios (6:1 to 8:1) for most crops. The excess P and N (as NO3) from manure application can be transported in runoff or leached into the groundwater. Recently, however, other constituents such as naturally excreted hormones and pathogens have been of interest (Jamieson, R. et al., 2002, Kon, T. et al., 2009, McLellan, S. and Salmore, A., 2003).

Although livestock manure is a good source of nutrients for crops and a natural way of recycling waste, raw manure is also a potential source of human pathogens if improperly stored and mishandled, or not managed correctly, fecal contamination from livestock manure handling and storage facilities is one of the most important sources of water microbiological pollution (Easterling, D. et al., 2000). Animal manure depending on its origin can be a source of zoonotic pathogens such as Hepatitis E virus, Rotavirus A (some strains), Adenovirus (some strains), *Aeromonashydrophila, Yersinia enterocolitica, Vibrio cholerae, Leptospira*, *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Salmonella* spp., *Listeria monocytogenes*, *Cryptosporidium parvum*, and *Giardia lamblia* (Rose, J. and Verhougstrate, M., 2008, Sengelov, G. et al., 2003, Pei, R. et al., 2006, Pruden, A. et al., 2007, Batt, A.L. et al., 2006, Chee-Sanford, J. et al., 2001, Sapkota, A. et al., 2007, Song, W. et al., 2007, Witte, W., 1998, Smith, K.E. et al., 1999) The concentration of some pathogens and indicators in manure are summarized in Table 2.

In the US, the quantity of manure application as a soil amendment is 30 times higher than the biosolids application (Easterling, D. et al., 2000. Tate, K. et al., 2000, Geohring, L. and Van Es, H., 1994, Evans, M. and Owens, J., 1972). This is mostly due to high manure production and public acceptance of manure application practice for thousands of years (Jahne, M. et al., 2014). In addition, most of the studies focus on the pathogens in biosolids, and there is lack of comparison studies for pathogens in manure (Easterling, D. et al., 2000, Jahne, M. et al., 2014). In comparison, the benefits of biosolids as soil amendments are similar to those provided by animal manure, both provide important plant nutrients and organic matter. Animal manure contains more P than crops require if the application rate is based on N needs (McLellan, J. et al., 1993). Most of the N in biosolids and manure is organic and becomes available to crops as it is mineralized. As a potential source of human pathogens public perception is that, biosolids pose higher risk to the public than animal manure. In fact, the risks associated with their use are not greater than the risks associated with untreated manure use. For example levels of *E.coli* indicator bacteria and *Salmonella sp* pathogens in manure are similar to biosolids (Tables 1 and 2). Overall quantitative data on total pathogen content and indicators in manure is limited comparing with biosolids.

Association of Biosolids and Manure Application with Microbial Contamination of Surface and Groundwater

Runoff from agricultural areas has been suggested as one of the major sources of nonpoint-source pollution (Low, S. et al., 2007). Rainfall events may carry human pathogens in water runoff from contaminated sites to water bodies serving as recreational, irrigation or drinking water sources. The effects of nonpoint source pollutants on specific waters vary and may not always be fully assessed because they are not easy to identify. However, we know that nonpoint sources for biosolid and manure may have harmful effects on drinking water supplies, and recreation (Eisenberg, J., 2006). Nonpoint sources are difficult to control, and they pose a great threat to the integrity of the water bodies (King, G. et al., 2011, Eisenberg, J., 2006). Microbial source tracking (MST) methods have recently been used to help identify nonpoint sources, several microbial source tracking studies have pointed to the application of biosolids and manure. Lapen *et al.* (Gobernaa, M. et al., 2011), and Gottschall *et al.* (Kudva, I. et al., 1998) monitored tile- and ground- water quality for bacteria (*Escherichia coli,* enterococci, *Clostridium perfringens*) after land application of dewatered biosolids (using both spreading and surface injection application methods), and they found that there were no significant differences between using different methods of spreading biosolids, also they measured the concentration of the *E. coli* contamination in ground water.

In May 2000 runoff from a field treated with cattle manure contaminated a groundwater supply with pathogenic bacteria (Galada, H. et al., 2012, Atwill, E. et al., 2002). In addition to human pathogenic *E. coli* and *Campylobacter*, other pathogens can also be present and survive in livestock manure. For example, human pathogenic protozoa, *Giardia lamblia* and *Cryptosporidium parvum*, can also be excreted by infected livestock and be transported in runoff events (Bradford, S. et al., 2006, Muirhead, R. et al., 2006, Chetochine, A. et al., 2006). Microbial quality of runoff following land application of manure has been reported as an important source of microbial contamination of water bodies (Daniel, T. et al., 1998). Studies involving fecal bacterial contamination in streams near dairy farms and cattle pastures (Bibby, K. and Viau, E., 2011, MDEQ, 1999), surface runoff from grazed pastures (MDEQ, 1999, Jacobs, L. and McCreary, D., 2001), and subsurface runoff from manure applied fields (Evanylo, G., 2009) demonstrated the ability of rain water runoff to transport bacteria from manure to surface water supplies. Rain events can also flush manure borne bacteria through the soil profile contaminating shallow groundwater (Eash, N. et al., 1997) and springs and wells within the hydrological catchments of pastures (Barbarick, K. and Ippolito, J., 2007).

Land-applied manure has been shown to quickly enter subsurface drains by preferential flow through macro pores (Arnold, K. et al., 1994, MDARD 2014). Macro pores are large, continuous openings in the soil formed by plant roots, soil fauna, cracks, fissures and other natural phenomena. Shipitalo and Gibbs (Shipitalo, M. and Protz, R., 1987) reported that earthworm holes within 0.5 m of subsurface drains expedited the transmission of injected liquid to these drains. Soils under no-till crop management often have more continuous flow channels (macro pores) than tilled soils (Harrigan, T. et al., 2007, Harrigan, T. et al., 2006, Harrigan, T. et al., 2005), and this may contribute to the rapid movement of injected manure to the subsurface drains.Bacterial contamination of drainage effluent was reported to be most likely in: (a) artificially drained, wet soils; (b) soils receiving high rates of liquid manure; and (c) soils that demonstrated preferential flow (Harrigan, T. et al., 2007). Several studies (Arnold, K. et al., 1994, Gagliardi, J. and Karns, J., 2002, Gagliardi, J. and Karns, J., 2002, Craun, G. et al., 2006, Craun, G. 1992) reported that the application of liquid manure to drained fields resulted in elevated levels of nutrients and bacteria in the receiving waters compared to sites where liquid manure was not applied.

MST study on recreational water contamination in southeastern Lake Huron demonstrated that the dominant source of *E. coli* in lake water samples was agriculture, which supplied about 60% of the bacteria to the lake, whereas human sources provided only about three percent (Craun, G. et al., 2003). In addition, one study carried out on the South Shore Beach in Milwaukee concluded that the high *E. coli* levels were from local sources of pollution and were rarely affected by regional contamination events such as sewage overflows (Lee, S. et al., 2002). Another study by Verhougstraete and Rose (Liang, J. et al., 2006), demonstrated that two sites in Lake Michigan have been impacted by bovine pollution, (67%) samples were positive for the bacteroides cow marker. The bacteroides results indicate the major source of fecal contamination at both sites was cow manure.

Bacteria containing antibiotic resistance gene can also be released to the environment (US EPA 2006). Although the levels of antibiotics in the environment are usually far below threshold levels to have inhibitory effects on bacterial populations, they still exert selective pressure on the development of antibiotic resistance in bacteria. The presence of antibiotic resistance genes in various environmental settings including river sediments, irrigation ditch water, dairy lagoon water, drinking water treatment plants and wastewater recycling plants has been confirmed (Hrudey, S. and Hrudey, E., 2004, Curriero, F. et al., 2001). Antibiotics and antibiotic-resistant bacteria can be released into streams and groundwater near concentrated animal feeding operation facilities (Hrudey, S. et al., 2003, NRC 2002, Hoxie, N. et al., 1997, Craun, G. et al., 2004). In a study by Chee-Sanford *et al.* (NRC 2002). The presence of tetracycline resistance genes has been confirmed in waste lagoons on two swine farms, and in the groundwater underlying the two farms. These antibiotic-resistant genes may be transferred horizontally between bacterial species, even to animals and humans (Eisenberg, J. et al., 2008, Lapen, D. et al., 2008). In addition, Munir and Xagoraraki (Gottschall, N. et al.,2009) reported that 24 manure samples from three farms and 18 biosolids samples from seven different wastewater treatment plants across Michigan were analyzed for tetracycline and sulfonamide resistance genes (Tet-W, Tet-O, and Sul-I). They reported high concentrations of antibiotic resistance genes in manure and biosolids samples. The concentrations of antibiotic resistance genes in manure was significantly greater than in biosolids and the background soil samples had significantly less contaminations than the biosolids and manure.

Risk Assessment

Land application of biosolids and manure may cause human risks of infection for residents and occupational workers. Manure and biosolids, when applied to the land, may contribute to pathogens in surface water, air, soil and groundwater depending on extent of precipitation, aerosolization, and fate and transport of pathogens on surface soil, in subsurface soil media and in air (Figure 1). Human exposure to pathogens might occur from one or more of these exposure routes. Once infected, chances of illness and mortality depend on pathogen type and human susceptibility. The risk of human exposure to biosolids-associated pathogens has been assessed in several studies (Table 3). It is important to mention here that the reviewed studies are examples and do not represent a complete list of all published studies.

Risk of infection to residents and occupational workers during land application of biosolids depends on pathogen type, pathogen concentration in biosolids, pathogen concentration in air after aerosolization, pathogen concentration in soil, biosolids application methods and location of receptor from biosolids application activities (Daniel, T. et al., 1998, Venglovsky, J. et al.,2005, Venglovsky, J. et al., 2006, Thurston-Enriquez, J. et al., 2005, Singh, R. and Agrawal, M., 2008). For example, Gerba *et al.* (Venglovsky, J. et al.,2005) estimated risk of infection from enteric viruses during mixing of biosolids with soil for an assumed hypothetical exposure scenario. They reported that risk of daily infection from rotaviruses present in biosolids ranged from 7.8 × 10−4 (when mixed with soil) to 2.11 × 10−1 (without any mixing with soil) (enteric virus concentration in biosolids = 5.13 MPN/4g biosolids; soil ingestion rate = 480 mg/d). In addition, some studies conducted sampling of microbial indicators and pathogens to determine concentration of pathogens in air and water media for estimating risk of infection for different hypothetical exposure scenarios (Daniel, T. et al., 1998, Venglovsky, J. et al., 2006, Thurston-Enriquez, J. et al., 2005, Singh, R. and Agrawal, M., 2008). They observed microbial concentration with distances and sampling heights for different weather conditions (wind velocity and direction, relative humidity and temperature) to develop empirical fate and transport models. These studies have generally focused on estimating risk of microbial infection from one medium only (Gobernaa, M. et al., 2011, Venglovsky, J. et al.,2005, Venglovsky, J. et al., 2006, Thurston-Enriquez, J. et al., 2005, Singh, R. and Agrawal, M., 2008). However, different environmental compartments (*i.e.* air, surface water, soil, groundwater, vegetables) may be contaminated during land application of biosolids depending of methods of biosolids application (*i.e.*, surface application, injection method). It is important to consider risk of infection from different environmental compartments to estimate the overall risk of infection during land application of biosolids.

To incorporate risk assessment and fate of biosolids-associated pathogens in different environmental media, Eisenberg (Lu, Q. et al., 2012) and Galada *et al.* (UNIDO 1998) developed fate and transport models of pathogens after biosolids application using different application methods and predicted risks of infection for different exposure subpopulations. For example, the model "Spreadsheet Microbial Assessment of Risk: Tool for Biosolids "SMART Biosolids" included estimation of risk of infection due to more than 20 pathogens from surface water, air, groundwater, vegetables and soil during land application of biosolids (Lu, Q. et al., 2012). In general, the following information is needed to run these comprehensive multi-compartment models: (a) weather-related information; (b) pathogenic concentration in biosolids; (c) biosolids application method; (d) decay of pathogens in different environmental media; (e) exposure-related information; and (f) dose-response information of different pathogens. During land application of manure, some studies have conducted assessment of risk of pathogenic infection. Findings presented in Table 3. For example, Brooks *et al.*, 2012 (U.S. Environmental Protection Agency 2006) compared risks of microbial infection during land application of manure and biosolids and found that risks of bacterial infection were higher during manure application activities and risks of viral infection were higher during biosolids land application activities. They further reported that risks of infection were higher due to land application of biosolids than land application of manure due to high infectivity of viruses. They also mentioned that risk estimates involved uncertainties due to lack of availability of concentration levels, field-specific inactivation rates of pathogens, and pathogens decay and regrowth rates. In another recent study by Jahne *et al.*, 2014 (Lyberatos, G. et al.,2011) on risk assessment of bioaerosols from a manure application site indicated that peak risks (95th percentile values) were found to be very high (1:250) at 100 m distance from the source. During application of dairy wastewater, Dungan, 2014 (Pepper, I. et al., 2006) estimated inhalation risks of residents due to exposures of pathogens *(Campylobacter jejuni, Escherichia coli* O157:H7, non-O157 *E.coli, Listeria monocytogenes,* and *Salmonella spp.)* in bioaerosols and found that risks were higher near the source and infection, and depended on weather conditions and setback distance. They reported the need for data on: (a) aerosolization efficiency of pathogens during spray irrigation of wastewaters; (b) inactivation and deposition rates of airborne pathogens under various environmental conditions; (c) inhalation transmission and dose–response of enteric pathogens in humans; and (d) exposure frequency and duration of affected populations.

A comparison of findings of risk assessment studies on pathogenic exposure from biosolids and manure indicated that risks of infection varied with setback distance, weather conditions (wind speed and direction, relative humidity, and temperature). Pathogen levels and extent of pathogen release from biosolids and manure during aerosolization and leaching due to rain events [68,111–114] was found to differ. Pathogen-related information needs to be obtained from fields and for conditions in which biosolids and/or manure are applied. Subsequently, pathogen-related fate and transport and risk models can be used.

Best Management Practices for Biosolids to Protect Water Quality

In 1993, the US EPA (Pepper, I. et al., 2010) established standards for land-applied biosolids under 40 CFR Part 503. Part 503 describes quantitative standards, management practices, operational standards, monitoring, recordkeeping, and reporting requirements for the use and disposal of biosolids. Individual states may develop additional guidelines so long as the state guidelines meet the minimum requirements of the federal Part 503 rule. Biosolids standards include limitations for metal and other compounds, pathogen reduction, vector requirements, best management practices, and include limitations for the land application of biosolids. Biosolids are physically, chemically and biologically treated to reduce pathogens to levels specified for Class A and B designations.

Class B biosolids (restricted use) are treated but still contain detectible levels of pathogens. Sludge to produce class B biosolids is treated using a "Processes to Significantly Reduce Pathogens" (PSRP), such as aerobic digestion, anaerobic digestion, air drying, and lime stabilization, which reduce but do not eliminate pathogens. Mesophilic anaerobic digestion (MAD) is the most prevalent treatment process for Class B biosolids in the US with a mean reduction in pathogen or indicator cultivability of 1 log (US EPA 2012). Class B biosolids must meet one of the three alternatives requirements: (1) Monitoring of indicator organisms based on fecal coliform; the geometric mean of the density of fecal coliform must be less than 2 million Colony Forming Units (CFU) or Most Probable Number (MPN) per gram of total dry solid in seven grab samples of sludge within one day, at the time of biosolids use or disposal; (2) Use of PSRP to significantly reduce pathogens; (3) Use of processes equivalent to PSRP, as determined by the permitting authority. Regulations for land application of Class B biosolids limit human exposure to pathogens by delaying harvesting post application, and prohibit the use of Class B biosolids on food crops eaten directly without processing. Class B biosolids can be applied on grain and forage crops, pastures, and grassland.

Class A biosolids (unrestricted use) contain no detectible levels of pathogens and low levels metals. Class A biosolids are treated by one of several "Processes to Further Reduce Pathogens" (PFRP), such as composting, pasteurization, drying or heat treatment, or advanced alkaline treatment, which reduce pathogens to below detectable levels. Class A classification is required for application onto public-use sites and certain food crops. This includes residential areas or home gardens, road banks, parks, golf courses, schools and other similar areas. Class A biosolids must meet one of the following bacteria limitations; fecal coliform is less than 1000 MPN per gram of total dry solid or *Salmonella sp*. bacteria density is less than 3 MPN per 4 grams of total dry solid. Class A biosolids pose minimal risk associated with use on edible food crops as a result of prior treatment that eliminates pathogens.

The Part 503 rule specifies upper limits for nine trace elements (Arsenic, Cadmium, Copper, Lead, Mercury, Molybdenum, Nickel, Selenium and Zinc) in sewage sludge to be land applied. Four options exist for meeting pollutant limits: The ceiling concentration limits (CCL), exceptional quality (EQ), cumulative pollutant loading rate (CPLR), and annual pollutant loading rate (APLR). Subpart D of the 503 rule addresses alternatives for limiting vectors such as rodents, birds and other organisms that could potentially spread disease by transporting pathogens from the application site. The goal of vector attraction reduction efforts is to either reduce the attractiveness of the biosolids to vectors by reducing organic matter (e.g., digestion, alkaline addition) or prevent vectors from coming into contact with the biosolids (e.g., subsurface injection or tillage incorporation within a short period of time after land application).

When biosolids are applied on agricultural land, they are applied at an agronomic rate to meet nitrogen requirements of the crop (CFR 40, Part 503.11) and prevent nitrogen loss to the environment. Individual states are allowed to expand the federal Part 503 rules to address state specific needs and concerns. In Michigan, Part 24 Rules of MDEQ (Viau, E. and Peccia, J., 2009) expands the Part 503 definition to include phosphorus and potassium in total nutrient management plans for crops grown at biosolids land application sites (Viau, E. and Peccia, J., 2009). Biosolids cannot be applied to agricultural land if the phosphorus Bray P1 soil test is greater than 150 ppm or 170 ppm based on the Mehlich 3 soil test.

Specified management practices for the land application of biosolids prohibit application of bulk sewage sludge if the site is likely to adversely affect a threatened of endanger species, cannot be applied to flooded, frozen or snow-covered ground or within 10 m from water bodies (CFR 40, Part 503.14). The state of Michigan expanded the general restrictions in Part 24 Rules to include a separation distance of 600 m from municipal wells, 30 m from domestic wells, homes and commercial buildings, and 15 m from surface waters. A minimum separation distance of 0.75 m between the soil surface and groundwater is required when biosolids are applied. There are many regional variations in required set-back distances and other management practices to protect the environment and public health (Wong, K. et al., 2010, Guzman, C. et al., 2007, Animal Health Institute 2002, Burkholder, J. et al., 2007, Wilson, S., 2007). Because biosolids are frequently used as a source of crop nutrients there are restrictions on the timing of biosolids application and delay time between land application and crop harvest. Michigan Part 24 Rules prohibits the harvest of food crops for periods ranging from 14 to 38 months depending upon the crop following application and the method of application. A landowner cannot harvest food crops, feed crops, and fiber crops for 30 days after biosolids are applied. Livestock are not permitted to graze on land for 30 days after a biosolids application.

In conclusion, current biosolid treatment technologies that have been required by Part 503 Rule, with existing regulations and guidelines governing the use of biosolid as a soil amendment, are sufficient to protect human health and the environment (Easterling, D. et al., 2000).To date there is no documented scientific evidence that the Part 503 Rule has failed to protect public health.

Best Management Practices for Livestock Manure to Protect Water Quality

Runoff from the farmstead, pastures and fields where manure has been stored, deposited or applied can transport pathogens, sediment, organic solids, and nutrients to surface waters. Tillage and manure management practices that quickly move manure into the soil and root zone will protect water quality by recycling valuable crop nutrients and stabilizing potential contaminants by filtration, and sorption.

Best management practices (BMPs) are proven practical and affordable soil and water conservation and management approaches. BMPs vary from region-to-region and field-to-field because of variability in site-specific conditions and for flexibility to address local concerns. BMPs are determined through a collaborative effort of local stakeholders including farmers, extension educators, agribusiness professionals, regulatory agencies and technical service providers, and include cover crops, conservation tillage, buffer strips and set-backs along with soil and manure testing to implement management practices that prevent the loss of nutrients and biological contaminants to the environment.

All states have Right-to-Farm laws. For example, in Michigan, the Farm Act (P.A. 1981, No. 93) was adopted and amended in 1987 (P.A. 1987, No. 240) to protect the environment and to protect crop and livestock producers from nuisance suits if they are in compliance with Federal, state and local laws, and follow recommended manure management practices (US EPA 2001). P.A. 1981, No. 93 authorized the development of Generally Accepted Agricultural and Management Practices (GAAMPs) for farms and farm operations. GAAMPs for Manure Management and Utilization are scientifically based, updated annually, and establish base-level management options for runoff control and wastewater management, odor management, construction design and management for manure storage and treatment facilities, and manure land application (US EPA 2001).

When manure is applied to cropland it is important to identify the fields and high-risk areas in fields where runoff is likely to occur. High risk areas include sloping ground, fields adjacent to ditches and waterways, bottom land that tends to flood in wet weather, tile drained land, and drained land with surface inlets. Vegetative filters, buffer strips and grass waterways have long been used to separate cropped or manure-applied land from nearby waterways. Widely used soil conservation practices that stabilize soil and prevent overland flow, runoff and soil erosion will help prevent manure contaminants from reaching waterways (Jongbloed, A. and Lenis, N., 1998).

Conservation tillage leaves crop residue on the soil surface and is an effective way to reduce runoff and erosion. Low-disturbance aeration tillage is compatible with no-till cropping and creates an absorptive surface in untilled ground that inhibits overland flow by fracturing the soil, increasing surface roughness, improving infiltration, and conserving crop residues (Hanselman, T. et al., 2003). Tillage is generally helpful in disrupting preferential flow paths. Tillage disrupts macropores, delays manure movement, and can greatly decrease bacteria concentration in effluent (Johnson, A. et al., 2006). Problems are likely when high rates of manure are applied and when manure is applied on wet ground or when tiles lines are flowing. Efforts to minimize problems must include low application rates (Johnson, A. et al., 2006, Gerba, C. and Smith, E., 2005). Soil conservation practices that stabilize soil will help keep manure in the proper place. High risk soils typically are fine-textured soils with night crawler burrows. Sandy loams are lower risk.

Overland flow of manure contaminants can contribute to localized ponding and preferential flow to subsurface drains. Cover crops protect the soil from wind and water erosion, recycle nutrients, and improve soil structure and fertility. Cover crops create an effective barrier to overland flow and manure contamination of waterways (Hanselman, T. et al., 2003). When manure is applied to a bare soil surface, nutrients and other contaminants accumulate at the surface and increase the chance of nutrient and bacterial transport in runoff water. When manure is applied to a vegetative surface, the plant biomass and organic matter filters bacteria and nutrients.

Cover crops inhibit overland flow, filter sediment and organic materials, assimilate nutrients, and extract water from the soil thereby increasing water holding capacity. Cover crops can influence the persistence of indicator organisms and pathogens in the root zone. Work by Gagliardi and Karns (Guan, T. and Holley, R., 2003) has shown greater persistence of *E. coli* O157:H7 on rye roots (47–96 d) and alfalfa roots (92 d) than in bare soil (25–41 d), but its persistence on crimson clover and hairy vetch roots were similar to bare soil. When manure slurry is applied to a vegetative surface the near-surface zone of high biomass and organic matter can enhance adsorption, straining and filtering of pathogens. Lim *et al.* (Hutchinson, M. et al., 2005) showed complete coliform removal of up to 2 × 107 colony forming units (cfu) 100 mL−1 in passing a 6.1 m tall fescue filter strip. Coyne *et al.* (Coyne, M.S. et al., 1995) reported 43%–

74% removal of coliforms up to 108 cfu 100−1 mL in passing a 9 m mixed Kentucky bluegrass and tall fescue filter strip. Technologies have been developed to treat manure nutrients and some of these technologies may also promote removal and degradation of pathogens such as physical treatments (separation of the solids and liquids in manure slurry by settling, filtration, screening, or drying), chemical treatments (addition of coagulants, such as lime, alum, and organic polymers to manure), and biological treatment (composting and anaerobic digestion).

Conclusions and Recommendations

A comprehensive management plan is needed to address problems of microbial water pollution in rural systems in a sustainable manner. There is a need to integrate the values and needs as well as the efforts of all stakeholders within a risk analysis framework. A key cornerstone of sustainable rural development is effective management of water and waste. Cooperation of farmers, township officials and individual homeowners is critical to achieve an effective plan. Awareness and education of responsible parties is a first step to an integrated plan and requires education of farmers, township officials, individual homeowners and water quality professionals regarding the benefits and risks associated with the use of biosolids and manure to help them make informed decisions about their choices. Interdisciplinary collaborations of scientists, engineers, and other professionals are essential.

Risk assessment frameworks for human exposure to contaminants in rural systems need to be evaluated. These comprehensive frameworks need to include identification of direct and indirect exposure pathways, pathogen data compilation, and risk assessment model development for land application of animal manure compared to biosolids. Quantitative data is a key component in the development of an effective risk assessment framework.

Pollution source tracking can help identify origins of pollution (manure or biosolids). Microbial source tracking tools can be coupled with geographical information system (GIS) data to help identify nonpoint sources in rural areas and minimize the human risks. The associations between bacterial communities and nutrient and chemical concentrations need to be assessed in order to determine if specific microbial community structure could be associated with specific types of chemical inputs and land uses.

Another important issue is the identification of transport mechanisms of contaminants from manure and biosolids to groundwater and surface water. At this point simplistic safety guidelines, such as setback distances, are used for locating wells and waste application sites. More elaborate investigation based on physicochemical processes such as sorption, speciation, and biotic and abiotic transformations of contaminants in soil and water are needed. To achieve that, advanced source tracking laboratory techniques coupled with nested field sampling and process modeling is required.

Existing techniques for managing agricultural waste, such as land application in various crop systems, need to be re-evaluated based on their potential to remove and inactivate contaminants. Over the past decades, waste management focused on the effects of nutrients, especially N and P, on water quality as the most important environmental concern. Microbes originating from biosolid and manure applications are often low on the priority for regulation and best management practices.In many cases, untreated manure may be applied in the setback areas where biosolids land application is prohibited; relatively few regulations govern land application of manure. Federal and state requirements regarding manure pathogens content are needed in order to preserve a water quality in rural areas.

The critical issues that need further research and attention include risk assessment models and determination of exposure pathways, optimization and use of quantitative analytical methods, source tracking and transport mechanisms, and optimization of waste and water treatment. Investigation of alternative techniques for treatment of animal waste, such as anaerobic membrane bioreactors, as well as investigation of the feasibility of co-treatment of human and agricultural waste is of great interest.

Acknowledgments

The authors would like to thank Michigan State University, East Lansing (U.S.A.) and Indian Institute of Technology, Delhi (India).

APPENDIX

Table 2.1 Example pathogens and indicators in class B-biosolids.

Organism	Detection method	Concentration	Reference
		Pathogens	
Salmonella sp.	culture	1.2–3.2 MPNCU/g	a
		0.487–0.954 MPN/4 g	b
		40.1MPN/4 g	c
Cryptosporidium	Laser scanning cytometry	4.3/10 g	d
Adenoviruses	qPCR	5×10^5 copies/g	e
		7.5×10^5 copies/g	b
		1.59×10^4 copies/g	f
	Cell culture	480 MPN/4g	b
Human polyomavirus	qPCR	8.05×10^2 copies/g	f
		2.5×10^5 copies/g	b
Enteroviruses	qPCR	1.9×10^4 copies/g	b
		4.8×10^3 copies/g	a
		3.3×10^4 copies/g	g
	RT-PCR	1.2×10^4 copies/g	h
		$1.0^6 \times 10^4$ copies/g	i
	Cell culture	480 MPN/4 g	b
		38.2 MPNCU/g	h
		9 MPNCU/g	i
		15–80 MPNCU/g	a
Noroviruses GI	qPCR	5×10^4 copies/g	b
	Cell culture	480 MPN/4 g	b
Noroviruses GII	qPCR	1.5×10^5 copies/g	b
	Cell culture	480 MPN/4 g	b
		Indicators	
Somatic coliphages	Cell culture	5.5×10^2 PFU/10 g	d
		2.09×10^5 PFU/4 g	c
Total coliform	Culture	7.64×10^5 MPN/4 g	c
Enterococci	Culture	7.2×10^5–2.6×10^6 MPN/g	a
		6.4×10^5 MPN/g	g
E. coli	Culture	4.4×10^5–1.1×10^6 MPN/g	a
		7.2×10^5 MPN/g	g
		10^4 MPN/g	b

Notes: qPCR-quantitative polymerase chain reaction; MPNCU-most probable number colony forming unit; RT-PCR-reverse transcription polymerase chain reaction; PFU-plaque forming unit.

a. (Munir, M. and Xagoraraki, I., 2011)
b. (Shipitalo, M. and Protz, R., 1987)
c. (Howell, J. et al., 1996)
d. (Drees, L. et al., 1994)
e. (Shipitalo, M. and Gibbs, F., 2000)
a. (Gerba, C. et al., 2002)
b. (Brooks, J. et al., 2005)
c. (Tanner, B. et al., 2008)
d. (Gerba, C. et al., 2008)

Table 2.2 Example pathogens and indicators in animal manure.

Organism	Concentration	Reference
Pathogens		
Salmonella sp.	2.8×10^5 CFU/25g	a
Listeria	1.7×10^4 CFU /g	a
E. coli O157:H7	2.2×10^6 CFU/g	b
Indicators		
Enterococci	1.5×10 MPN/g	c
E. coli	10^5–10^6 CFU /g	c
	5.5×10^7 MPN/g	a

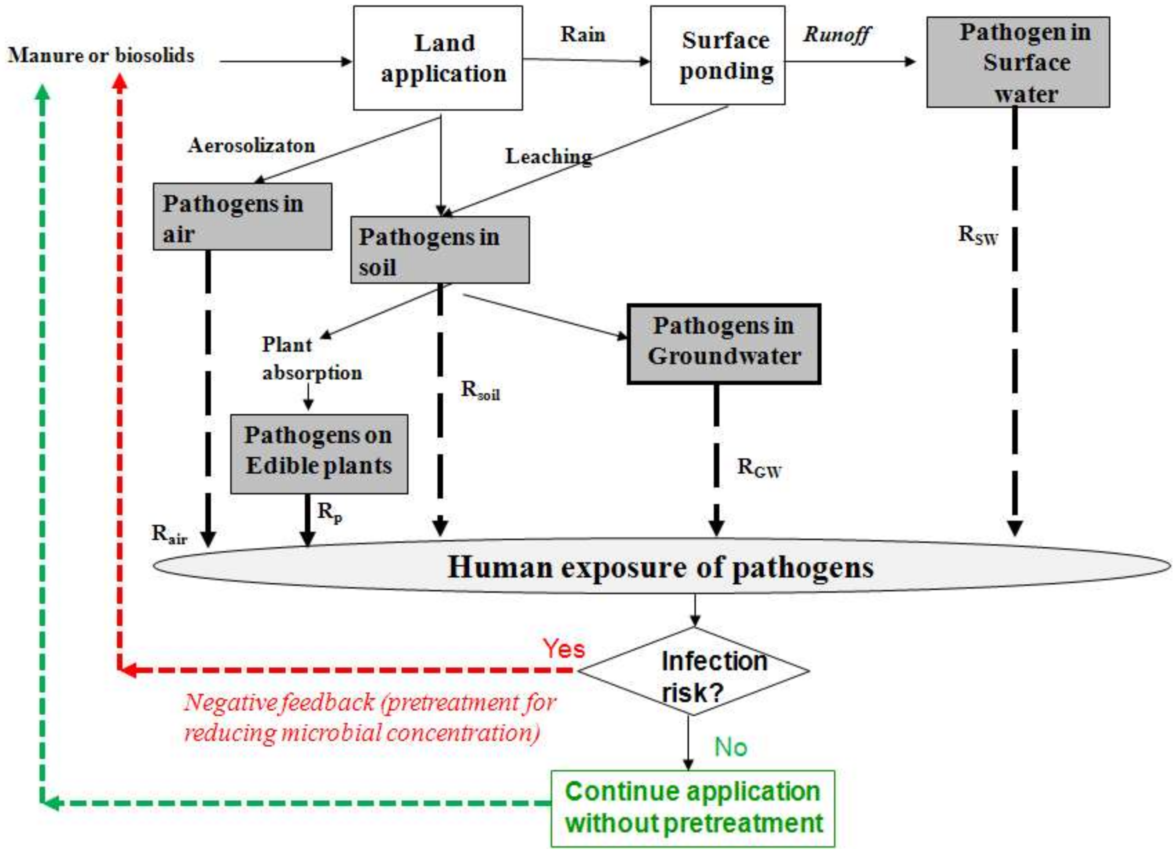
Notes: CFU-colony forming unit; MPN-most probable number.

a. (Kumar, A. et al., 2012)
b. (Brooks, J. et al., 2012)
c. (Dungan, R., 2014)

Table 2.3 Example studies focusing on risk assessment during land application activities of biosolids and/or manure.

Ref.	Exposure Scenario	Microorganisms Type	Exposed Population	Findings/Risk Estimate
Biosolids				
A	Ingestion of biosolids-contaminated soil	Rotavirus	Residential population(480 mg/d soil ingestion rate)	Risk of infection: 7.8×10^{-4} (when mixed with soil); 2.11×10^{-1} (without any mixing with soil)
B	Inhalation of indicator organisms from air during land application activity	Coliphage MS-2, *E. coli*, coxsackievirus A21	Occupational workers (for 1 to 8 h of exposure; 0.1 virus particles/g biosolids)	Risk of infection at 2 m: 1.64×10^{-7} to 1.31×10^{-6}
B	Inhalation of indicator organisms from air during land application activity	Coliphage MS-2, *E. coli* , coxsackievirus A21	Residential population (for 1 to 8 h of exposure; 0.1 virus particles/g biosolids)	Risk of infection at 30.5 m: 1.5×10^{-8} to 1.2×10^{-7}
C	Inhalation of indicator organisms from air during land application of class B biosolids	Coliform bacteria, coliphages	Occupational workers	annual risk of infection: 7.8×10^{-4} to 2.1×10^{-2}
D	Direct contact of Class B biosolids and subsequent ingestion (without incorporation in soil)	*Ssalmonella*	Residential (soil ingestion = 480 mg/d)	Risk of infection = 5.7×10^{-3}
D	Direct contact of Class B biosolids and subsequent ingestion (with 100 times dilution with soil)	*Salmonella*	Residential (soil ingestion = 480 mg/d)	Risk of infection = $5.5\ 10^{-5}$
D	Direct contact with class A residuals following regrowth of *Salmonella*	*Salmonella*	Residential(soil ingestion = 480 mg/d)	Risk of salmonella infection = 8×10^{-1}
D	Direct contact with class A residuals following regrowth of *Salmonella* (with 100 times dilution with soil)	*Salmonella*	Residential (soil ingestion = 480 mg/d)	Risk of salmonella infection = 2.64×10^{-1}
E	Direct contact of Class B biosolids	echovirus-12, enterovirus types 68–71, adenoviruses, rotaviruses, and noroviruses genotype-I	Residential population	Risk of infection: 4.45×10^{-5}
Manure				
F	Exposures from fomite, soil, crop, and aerosol exposures from manure and biosolids	Bacteria and viruses	Occupational and residential population	Greatest risk from direct consumption of contaminated soil ; Greater bacterial risks from manure and greater viral risks from biosolids
G	Exposure of dairy wastewater	*Campylobacter jejuni, E.coli*O157:H7, non-O157 *E.coli, Listeria monocytogenes,* and *Salmonella* spp.	Residential	daytime risk is less than 10^{-6} at distance > 1 km
H	Inhalation exposure during dairy manure application	*Enterococcus*spp., *E.coli, Salmonella* spp.,*Campylobacter* spp., *E. coli* O157:H7	Occupational (8h)	Median risk of infection: 1:500 (at 100 m), 1:100,000 (at 1,000 m)

Figure 2.1 A schematic linking biosolids and manure application to human exposure.



Notes: Five exposure routes: R_{air} (exposure through air route); R_p (exposure through consumption of edible plants); R_{soil} (exposure through contact and ingestion of soil); R_{GW} (exposure through ingestion of groundwater); and R_{SW} (exposure through ingestion of surface water); SW (surface water); GW (groundwater)

REFERENCES

REFERENCES

A National Biosolids Regulation, Quality, End Use, and Disposal Survey; North East Biosolids and Residuals Association (NEBRA): Tamworth, NH, USA, **2007**.

Animal Health Institute, 2002. Available online: http://www.ahi.org (accessed on 29 June 2014).

Arnold, K.; Dunn, J.; Carpenter, J.D. Best Management Practices for Biosolids Land Application; Extension Bulletin WQ426; University of Missouri Extension: Columbia, MO, USA, 1994.

Atherholt, T.B.; LeChevallier, M.W.; Norton, W.D.; Rosen, J.S. Effect of rainfall on Giardia and Crypto. J. Am. Water Works Assoc. 1998, 90, 66–80.

Atwill, E.R.; Hou, L.; Karle, B.M.; Harter, T.; Tate, K.W.; Dahlgren, R.A. Transport of cryptosporidium parvum oocysts through vegetated buffer strips and estimated filtration efficiency. Appl. Environ. Microbiol. 2002, 68, 5517–5527.

Barbarick, K.A.; Ippolito, J.A. Nutrient assessment of a dry land wheat agroecosystem after 12 years of biosolids applications. Agron. J. 2007, 99, doi: 10.2134/agronj2006.0221.

Batt, A.L.; Bruce, I.B.; Aga, D.S. Evaluating the vulnerability of surface waters to antibiotic contamination from varying wastewater treatment plant discharges. Environ. Pollut.2006, 142, 295–302.

Bibby, K.; Viau, E.; Peccia, J. Viral metagenome analysis to guide human pathogen monitoring in environmental samples. Lett. Appl. Microbiol. 2011, 52, 386–392.

Bofill-Mas, S.; Albinana-Gimenez, N.; Clemente-Casares, P.; Hundesa, A.; Rodriguez-Manzano, J.; Allard, A.; Calvo, M.; Girones, R. Quantitation and stabilityof human adenoviruses and polyomavirusJCPyV in wastewater matrices. Appl. Environ. Microbiol. 2006, 72, 7894–7896.

Bradford, S.A.; Tadassa, Y.F.; Pachepsky, Y. Transport of Giardia and manure suspensions in saturated porous media. J. Environ. Qual. 2006, 35, 749–757.

Brooks, J.P., Maxwell, S.L.; Rensing, C.; Gerba, C.P.; Pepper, I.L. Occurrence of antibiotic-resistant bacteria and endotoxin associated with the land application of biosolids. Can. J. Microbiol.2007, 53, 616–622.

Brooks, J.P.; McLaughlin, M.R.; Gerba, C.P.; Pepper, I.L. Land application of manure and class B biosolids: An occupational and public quantitative microbial risk assessment. J. Environ. Qual. 2012, 41, 2009–2023.

Brooks, J.P.; Tanner, B.D.; Gerba, C.P.; Haas, C.N.; Pepper, I.L. Estimation of bioaerosol risk of infection to residents adjacent to a land applied biosolids site using an empirically derived transport model. J. Appl. Microbiol. 2005, 98, 397–405.

Burkholder, J.; Libra, B.; Weyer, P.; Heathcote, S.; Kolpin, D.; Thorne, P.S.; Wichman, M. Impacts of waste from concentrated animal feeding operations on water quality. Environ. Health Perspect. 2007, 115, 308–312.

Chee-Sanford, J.C.; Aminov, R.I.; Krapac, I.J.; Garrigues-Jeanjean, N.; Mackie, R.I. Occurrence and Diversity of Tetracycline Resistance Genes in Lagoons and Groundwater Underlying Two Swine Production Facilities. Appl. Environ. Microbiol. 2001, 67, 1494–1502.

Chetochine, A.S.; Brusseau, M.L.; Gerba, C.P.; Pepper, I.L. Leaching of phage from class B biosolids and potential transport through soil. Appl. Environ. Microbiol. 2006,72, 665–671.

Cliver, D.O.; Moe, C.L. Prospects of Waterborne Viral Zoonoses. In Waterborne Zoonoses: Identification, Causes and Control; Cotruvo, J.A., Dufour, A., Rees, G., Bartram, J., Carr, R., Cliver, D.O., Craun, G.F., Fayer, R., Gannon, V.P.J., Eds.; WHO: London, UK, 2004.

Coyne, M.S.; Gilfillen, R.A.; Rhodes, R.W.; Blevins, R.L. Soil and fecal coliform trapping by grass filter strips during simulated rain. J. Soil Water Conserv. 1995, 50, 405–408.

Craun M.F.; Craun G.F.; Calderon R.L. Beach M.J. Waterborne Outbreaks in the United States. J. Water Health2006, 4, 19–30.

Craun, G.F.; Calderon, R.L.; Craun, M.F. Waterborne outbreaks caused by zoonotic pathogens in the USA. In Waterborne Zoonoses; IWA Publishing: London, UK, 2004; pp. 120–135.

Craun, G.F.; Calderon, R.L.; Nwachuku, N.Causes of waterborne outbreaks reported in the United States. In Drinking Water and Infectious Disease: Establishing the Links;Hunter, P.R., Waite, M., Ronchi, E., Eds.;CRC Press: London, UK, 2003; pp. 1991–1998.

Craun, G.F.Waterborne disease outbreaks in the United States of America: Causes and prevention. World Health Stat. Q.1992, 45, 192–199.

Culley, J.L.B.; Phillips, P.A. Bacteriological quality of surface and subsurface runoff from manuredsandy clay loamsoil. J. Environ. Qual. 1982, 11, 155–158.

Curriero, F.C.; Patz, J.A., Rose, J.B.; Lele, S. The association between extreme precipitation and waterborne disease outbreaks in the United States, 1948–1994. Am. J. Pub. Health 2001, 91, 1194–1201.

Daniel, T.C.; Sharpley, A.N.; Lemunyon, J.L. Agricultural phosphorus and eutrophication: A symposium overview.J. Environ. Qual. 1998, 27, 251–257.

Dean, D.M.; Foran, M.E. The effects of farm liquid waste applications on tile drainage. J. Soil Water Conser. 1992, 47, 368–369.

Doran, J.W.; Linn, D.M. Bacteriological quality of runoff water from pastureland. Appl. Environ. Microbiol. 1979, 37, 985–991.

Drees, L.R.; Karathanasis, A.D.; Wilding, L.P.; Blevins, R.L. Micromorphological characteristics of long-term no-till and conventionally tilled soils. Soil Sci. Soc. Am. J.1994, 58, 508–517.

Dungan, R.S. Estimation of Infectious Risks in Residential Populations Exposed to Airborne Pathogens During Center Pivot Irrigation of Dairy Wastewaters. Environ. Sci. Technol.2014, 48, 5033–5042.

Eash, N.S.; McClurkan, J.; Burns, R.T. Best Management Practices (BMP's) for Land Application of Biosolids; University of Tennessee Agricultural Experiment Station and Tennessee Division of Water Pollution Control, University of Tennessee: Knoxiville, TN, USA, 1997.

Easterling, D.R.; Evans, J.L.; Groisman, P.Y.; Karl, T.R.; Kunkel, K.E.; Ambenje, P. Observed variability and trends in extreme climate events: A brief review. Bull. Am. Met. Soc.2000, 81, 417–425.

Easterling, D.R.; Thomas, K.R.; Gallo, K.P. Observed climate variability and change of relevance to the biosphere. J. Geophys. Res.2000, 105, 20101–20114.

Eisenberg, J.N. Application of a Dynamic Model to Assess Microbial Health Risks Associated with Beneficial Uses of Biosolids; Water Environment Research Foundation: Alexandria, U.S.A., 2006; pp. 1–124.

Eisenberg, J.N.S.; Moore, K.; Soller, J.A.; Eisenberg, D.; Colford, J.M., Jr. Microbial risk assessment framework for exposure to amended sludge projects. Environ. Health Perspect.2008, 116, 727–733.

Evans, M.R.; Owens, J.D. Factors affecting the concentration of fecal bacteria in land drainage water. J. Gen. Micro. 1972, 71, 477–485.

Evanylo, G.K. Agricultural Land Application of Biosolids in Virginia; Publication 452–302; College of Agriculture and Life Sciences, Virginia Polytechnic Institute and State University: Blacksburg, VA, USA, 2009.

Fleming, R.J.; Bradshaw, S.H. Contamination of Subsurface Drainage Systems during Manure Spreading; ASAE Paper No. 92-2618; American Society of Agricultural Engineers. St. Joseph, MI, USA, 1992.

Gagliardi, J.V.; Karns, J.S. Persistence of Escherichia coli O157:H7 in soil and on plant roots. Environ. Microbiol.2002, 4, 89–96.

Galada, H.G.; Gurian, P.L.; Joe, A.; Kumar, A.; Olson, B.; Olson, M.; Richter, E.; Teng, J.; Zhang, H.; Xagoraraki, I.; et al. Site Specific Risk Assessment Tool for Land Applied Biosolids; Water Environment Research Foundation: Alexandria, U.S.A., 2012.

Gannon, V.P. J.; Bolin, C.; Moe, C.L. Waterborne Zoonoses: Emerging Pathogens and Emerging Patterns of Infection. In Waterborne Zoonoses: Identification, Causes and Control; Cotruvo, J.A., Dufour, A., Rees, G., Bartram, J., Carr, R., Cliver, D.O., Craun, G.F., Fayer, R., Gannon, V.P.J., Eds.; WHO: London, UK, 2004.

Gary, H.; Johnson, S.; Ponce, S. Cattle grazing impact on surface water quality in a Colorado front range stream. J. Soil Water Conserv. 1983, 38, 124–128.

Geohring, L.D.; Van Es, H.M. Soil Hydrology and Liquid Manure Applications. In Proceedings of the Liquid Manure Application Systems Conference, Rochester, NY, USA, 1994; pp. 166–174.

Gerba, C.P.; Castro-del, C.; Brooks, J.O.; Pepper, I.L. Exposure and risk assessment of salmonella in recycled residuals. Water Sci. Technol.2008, 57, 1061–1065.

Gerba, C.P.; Pepper, I.L.; Whitehead, L.F., III. A risk assessment of emerging pathogens of concern in the land application of biosolids. Water Sci. Technol. 2002, 46, 225–230.

Gerba, C.P.; Smith, E.J.Sources of pathogenic microorganisms and their fate during land application of wastes. J. Environ. Qual.2005, 34, 42–48.

Gobernaa, M.; Podmirsega, S.; Waldhubera, S.; Knappa, B.; Garcíab, C.; Insama, H. Pathogenic bacteria and mineral N in soils following the land spreading of biogas digestates and fresh manure. Appl. Soil Ecol. 2011, 49, 18–25.

Gottschall, N.; Edwards, M.; Topp, E.; Bolton, P.; Payne, M.; Curnoe, W.E.; Coelho, B.B.; Lapen, D.R. Nitrogen, phosphorous, and bacteria tile and groundwater quality following direct injection of dewatered municipal biosolids into soil. J. Environ. Qual.2009, 38, 1066–1075.

Guan, T.Y.; Holley, R.A. Pathogen survival in swine manure environments and transmission of human enteric illness: A review. J. Environ. Qual. 2003, 32, 383–392.

Guzman, C.; Jofre, J.; Montemayor, M.; Lucena, F. Occurrence and levels of indicators and selected pathogens in different sludges and biosolids. J. Appl. Microbiol.2007, 103, 2420–2429.

Hanselman, T.A.; Graetz, D.A.; Wilkie, A.C. Manure-borne estrogens as potential contaminants: A review. Environ. Sci. Techn. 2003, 37, 5471–5478.

Harrigan, T.M. Manure on Tile-Drained Cropland. Michigan Dairy Review; Michigan State University: East Lansing, MI, USA, 2005; Volume 10, pp. 10–12.

Harrigan, T.M.; Mutch, D.R.; Snapp S.S. Slurry-Enriched Seeding of Biosuppressive Covers. Appl. Eng. Agric. 2006, 22, 827–834.

Harrigan, T.M.; Northcott, W.; Rector, N.; Bolinger, D. Keeping Land-Applied Manure in the Root Zone: Part 1: Sediment and Contaminant Runoff; Extension Bulletin WO-1036; Michigan State University: East Lansing, MI, USA, 2007.

Harrigan, T.M.; Northcott, W.; Rector, N.; Bolinger, D. Keeping Land-Applied Manure in the Root Zone: Part 2: Tile-drained Land;Extension Bulletin WO-1037; Michigan State University, East Lansing, MI, USA, 2007.

He, Z.L.; Alva, A.K.; Calvert, D.V.; Li, Y.C.; Stoffella, P.J.; Banks, D.J. Nutrient availability and changes in microbial biomass of organic amendments during field incubation. Compost. Sci. Util.2000, 8, 293–302.

Howell, J.M.; Coyne, M.S.; Cornelius, P.L. Effect of sediment particle size and temperature on fecal bacteria mortality rates and the fecal coliform/fecal streptococci ratio. J. Environ. Qual. 1996, 25, 1216–1220.

Hoxie, N.J.; Davis, J.P.; Vergeront, J.M.; Nashold, R.D.; Blair, K.A. Cryptosporidiosis—Associated mortality following a massive waterborne outbreak in Milwaukee, Wisconsin. Am. J. Health 1997, 87, 2032–2035.

Hrudey, S.E.; Hrudey, E.J. Safe Drinking Water: Lessons from Recent Outbreaks in Affluent Nations; IWA-publishing: London, UK, 2004.

Hrudey, S.E.; Payment, P.; Huck, P.M.; Gillham, R.W.; Hrudey, E.J. A fatal waterborne disease epidemic in Walkerton, Ontario: Comparison with other waterborne outbreaks in the developed world. Water Sci. Technol. 2003, 47, 7–14.

Hubálek, Z. Emerging human infectious diseases: Anthroponoses, zoonoses, and sapronoses. Emerg. Infect. Dis. 2003, 9, 403–404.

Hue, N.V. Sewage sludge. In Soil Amendments and Environmental Quality;Lewis Publishers: Boca Raton, FL, USA,1995; pp. 199–247.

Hutchinson, M.L.; Walters, L.D.; Avery, S.M.; Munro, F.; Moore, A. Analyses of livestock production, waste storage, and pathogen levels and prevalences in farm manures. Appl. Environ. Microb. 2005, 71, 1231–1236.

Jacobs, L.W.; McCreary, D.S. Applying Biosolids to Land in Michigan; Extension Bulletin E-2780; Michigan State University: East Lansing, MI, USA, 2001.

Jahne, M.A.; Rogers, S.W.; Holsen, T.M.; Grimberg, S.J.Quantitative microbial risk assessment of bioaerosols from a manure application site. Aerobiologia2014,doi:10.1007/s10453-014-9348-0.

Jamieson, R.C.; Gordon, R.J.; Sharples, K.E.; Stratton, G.W.; Madani, A. Movement and Persistence of Fecal Bacteria in Agricultural Soils and Subsurface Drainage Systems: A Review. Can. Biosyst. Engin.2002, 44, 1–9.

Jawson, M.D.; Elliott, L.F.; Saxton, K.E.; Fortier, D.H. The effect of cattle grazing on indicator bacteria in runoff from a Pacific Northwest watershed. J. Environ. Qual.1982, 11, 621–627.

Johnson, A.C.; Williams, R.J.; Matthiessen, P. The total potential steroid hormone contribution of farm animals to freshwaters: The United Kingdom as a case study. Sci. Total Environ.2006, 362, 166–178.

Jongbloed, A.W.; Lenis, N.P. Environmental concerns about animal manure. J. Anim. Sci. 1998, 76, 2641–2648.

King, G.; Brooks, J.P.; Brown, S.; Gerba, C.; O'Connor, G.A.; Pepper, I.L. Land application of organic residuals: Public health threat or environmental benefit; American Society for Microbiology: Washington, DC, USA, 2011.

Kistemann, T.; Claben, T.; Koch, C.; Dangendorf, F.; Fischeder, R.; Gebel, J.; Vacata, V.; Exner, M. Microbial load of drinking water reservoir tributaries during extreme rainfall and runoff. Appl. Environ. Microbiol. 2002, 68, 2188–2197.

Kon, T.; Weir, S.C.; Howell, E.T.; Lee, H.; Trevors, J.T. Repetitive element (REP)-polymerase chain reaction (PCR) analysis of Escherichia coli isolates from recreational waters of southeastern Lake Huron.Can. J. Microbial.2009,55, 269–276.

Kudva, I.T.; Blanch, K.; Hovde, C.J. Analysis of Escherichia coli O157: H7 survival in ovine or bovine manure and manure slurry. Appl. Environ. Microbiol.1998, 64, 3166–3174.

Kumar, A.; Wong, K.; Xagoraraki, I. Effect of Detection Methods on Risk Estimates of Exposure to Biosolids-Associated Human Enteric Viruses. Risk Anal.2012, 32, 916–929.

Lapen, D.R.; Topp, E.; Edwards, M.; Sabourin, L.; Curnoe, W.; Gottschall, N.; Bolton, P.; Rahman, S.; Coelho, B.B.; Payne, M.; et al. Effect of liquid municipal biosolids application method on tile and groundwater quality. J. Environ. Qual.2008, 37, 925–936.

Lee, S.H.; Levy, D.A.; Craun, G.F.; Beach, M.J.; Calderon, R.L. Surveillance for waterborne-disease outbreaks—United States, 1999–2000. Morb. Mortal. Wkly. Rep. Surveill. Summ. 2002, 51, 1–47.

Liang, J.L.; Dziuban, E.J.; Craun, G.C.; Hill, V.; Moore, M.R.; Gelting, R.J.; Calderon, R.L.; Beach, M.J.; Roy, S.L. Surveillance for waterborne disease and outbreaks associated with drinking water and water not intended for drinking-US, 2003–2004. MMWR Surveill. Summ.2006, 55, 31–65.

Lim, T.T.; Edwards, D.R.; Workman, S.R.; Larson, B.T.; Dunn, L. Vegetated filter strip removal of cattle manure constituents in runoff. Trans. ASAE1998, 41, 1375–1381.

Low, S.Y.; Paez-Rubio, T.; Baertsch, C.; Kucharski, M.; Peccia, J. Off-site Exposure to Respirable Aerosols Produced during the Disk-incorporation of Class B Biosolids. J. Environ. Eng. 2007, 133, 987–994.

Lu, Q.; He, Z.L.; Stoffella, P.J. Land application of biosolids in the USA: A review. Appl. Environ. Soil Sci.2012, doi:10.1155/2012/201462.

Lyberatos, G.; Sklivaniotis, M.; Angelakis, A.N. Management of Biosolids in EUREAU Countries. Fresenius Environ. Bull.2011, 20, 2489–2495.

McLellan, J.E.; Fleming, R.J.; Bradshaw, S.H. Reducing Manure Output To Streams from Subsurface Drainage Systems; ASAE Paper No. 93–2010; American Society of Agricultural Engineers. St. Joseph, MI, USA, 1993.

McLellan, S.L.; Salmore, A.K. Evidence for localized bacterial loading as the cause of chronic beach closings in a freshwater marina.Water Res.2003, 37, 2700–2708.

McMurry, S.W.; Coyne, M.S.; Perfect, E. Fecal coliform transport through intact soil blocks amended with poultry manure. J. Environ. Qual. 1998, 27, 86–92.

Meslin, F.X. Global Aspects of Emerging and Potential Zoonoses: A WHO Perspective. In Proceedings of the 1st International Conference on Emerging Zoonoses, Jerusalem, Israel, 24–28 November 1996.

Michigan Commission of Agriculture and Rural Development (MDARD). Generally Accepted Agricultural and Management Practices for Manure Management and Utilization; Michigan Commission of Agriculture and Rural Development: Lansing, MI, USA, 2014.

Michigan Department of Environmental Quality, MDEQ. Administrative Rules, Part 24, Land Application of Biosolids; Michigan Department of Environmental Quality, Surface Water Quality Division: Lansing, MI, USA, 1999.

Monpoeho, S.; Maul, A.; Bonnin, C.; Patria, L.; Ranarijaona, S.; Billaudel,S.; Ferré, V. Clearance of Human-Pathogenic Viruses from Sludge: Study of Four Stabilization Processes by Real-Time Reverse Transcription-PCR and Cell Culture. Appl. Environ. Microb. 2004, 70, 5434–5440.

Monpoeho, S.; Maula, A.; Mignotte-Cadiergues, B.; Schwartzbrod, L.; Billaudel, S.; Ferre, V. Best Viral Elution Method Available for Quantification of Enteroviruses in Sludge by Both Cell Culture and Reverse Transcription-PCR. Appl. Environ. Microbiol.2001, 67, 2484–2488.

Muirhead, R.W.; Collins, R.P.; Bremer, P.J. Numbers and transported state of Escherichia coli in runoff direct from fresh cowpats under simulated rainfall. J. Appl. Microbiol. 2006, 42, 83–87.

Munir, M.; Xagoraraki, I.Levels of Antibiotic Resistance Genes in Manure, Biosolids, and Fertilized Soil.J Environ. Qual. 2011,40, 248–255.

National Research Council (NRC): Committee on Toxicants and Pathogens in Biosolids Applied to Land. Biosolids Applied to Land: Advancing Standards and Practices; The National Academies Press: Washington, DC, USA, 2002; pp. 1–12.

Nicholson, F.; Groves, S.; Chambers, B. Pathogen survival during livestock manure storage and following land application. In ADAS Gleadthorpe Research Center, Meden Vale, Mansfield, Nottinghamshire NG20 9PF, UK; National Academy Press: Washington, DC, USA, 2004.

Niemi, R.M.; Niemi, J.S. Bacterial pollution of waters in pristine and agricultural lands. J. Environ. Qual.1991, 20, 620–627.

Pagliai, M.; Raglione, M.; Panini, T.; Maletta, M.; La Marca, M. The structure of two alluvial soils in italy after 10 years of conventional and minimum tillage. Soil Tillage Res. 1995, 34, 209–223.

Palmer, S.; Brown, D. Morgan, D. Early Qualitative Risk Assessment of the Emerging Zoonotic Potential of Animal Diseases. Br. Med. J.2005, 331, 1256–1260.

Pei, R.; Kim, S.-C.; Carlson, K.; Pruden, A. Effect of River Landscape of the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). Water Res. 2006, 40, 2427–2435.

Pepper, I.L.; Brooks, J.P.; Gerba, C.P. Pathogens in biosolids. Adv. Agron. 2006, 90, 1–41.

Pepper, I.L.;Brooks, J.P.; Sinclair, R.G.; Gurian, P.L.; Gerba, C.P. Pathogens and indicators in United States Class B biosolids: National and historic distributions. J. Environ. Qual.2010, 39, 2185–2190.

Pourcher, A.M.; Françoise, P.B.; Virginie, F.; Agnieszka, G.; Vasilica, S.; Gérard, M. Survival of fecal indicators and enteroviruses in soil after land-spreading of municipal sewage sludge. Appl. Soil Ecol. 2007,35, 473–479.

Pourcher, A.M.; Morand, P.; Picard-Bonnaud, F.; Billaudel, S.; Monpoeho, S.; Federighi, M.; Moguedet, G. Decrease of enteric micro-organisms from rural sewage sludge during their composting in straw mixture. J. Appl. Microbiol. 2005,99, 528–539.

Pruden, A.; Pei, R.; Storteboom, H.; Carlson, K. Antibiotic Resistance Genes as Emerging Contaminants: Studies in the Northern Colorado. Environ. Sci. Tech.2006, 40, 7445–7450.

Rose, J.B.; Verhougstrate, M. Investigation of Water Quality and Sources Associated with Buck Creek Watershed; Michigan State University: East Lansing, MI, USA, 18 September 2008.

Sapkota, A.R.; Curriero, F.C.; Gibson, K.E.; Schwab, K.J. Antibiotic-resistant enterococci and fecal indicators in surface water and groundwater impacted by a concentration swine feeding operation. Environ. Health Perspect.2007, 115, 1040–1045.

Scott, T.M.; Rose, J.B.; Jenkins, T.M.; Farrah, S.R.; Lukasik, J. Microbial source tracking: Current methodology and future directions. Appl. Environ. Microbiol.2002, 68, 5796–5803.

Sengelov, G.; Agerso, Y.; Halling-Sorensen, B.; Baloda, S.B.; Andersen, J.S.; Jensen, L.B. Bacterial antibiotic resistance levels in Danish farmland as a result of treatment with pig manure slurry. Environ. Intern.2003, 28, 587–595.

Shepherd, M.A.; Withers, P.J. Phosphorus leaching from liquid digested sewage sludge applied to sandy soils. J. Agric. Sci. 2001, 136, 433–441.

Shipitalo, M.J.; Gibbs, F. Potential of Earthworm Burrows to Transmit Injected Animal Waste to Tile Drains. Soil Sci. Soc. Am. J.2000, 64, 2103–2109.

Shipitalo, M.J.; Protz, R. Comparison of morphology and porosity of a soil under conventional and zero tillage. Can. J. Soil Sci.1987, 67, 445–456.

Singh, R.P.; Agrawal, M. Potential benefits and risks of land application of sewage sludge. Waste Manag.2008, 28, 347–358.

Sinton, L.W.; Braithwaite, R.R.; Hall, C.H.; Mackenzie, M.L. Survival of indicator and pathogenic bacteria in bovine feces on pasture. Appl. Environ. Microbiol.2007, 73, 7917–7925.

Slifko, T.R.; Smith, H.V.; Rose, J.B. Emerging parasite zoonoses associated with water and food. Int. J. Parasitol. 2000, 30, 1379–1393.

Smith, K.E.; Besser, J.M.; Hedberg, C.W.; Leano, F.T.; Bender, J.B.; Wicklund, J.H.; Johnson, B.P.; Moore, K.A.; Osterholm, M.T. Quinolone-resistant campylobacter jejuni Infections in Minnesota, 1992–1998. New Eng. J. Med.1999, 340, 1525–1532.

Sobsey, M.D.; Khatib, L.A.; Hill, V.R.; Alocilja, E.; Pillai, S. Pathogens in Animal Wastes and the Impacts of Waste Management Practices on Their Survival, Transport and Fate. In White Paper, Midwest Plan Service; Iowa State University: Ames, IA, USA, 2001.

Song, W.; Huang, M.; Rumbeiha, W.; Li, H. Determination of amprolium, carbadox, monensin, and tylosin in surface water by liquid chromatography/tandem mass spectrometry. Rapid Commun. Mass. Spectrom.2007, 21, 1944–1950.

Stehouwer, R.C.; Wolf, A.M.; Doty, W.T. Chemical monitoring of sewage sludge in Pennsylvania: Variability and application uncertainty. J. Environ. *Qual.***2000**, *29*, 1686–1695.

Tanner, B.D.; Brooks, J.P.; Gerba, C.P.; Haas, C.N.; Josephson, K.L.; Pepper, I.L. Estimated occupational risk from bioaerosols generated during land application of class B biosolids. J. Environ. Qual. 2008, 37, 2311–2321.

Tate, K.W.; Atwill, E.R.; George, M.R.; McDougald, N.K.; Larsen, R.E.Crytposporidium parvum transport from cattle fecal deposits on California rangelands. J. Range Manag. 2000, 53, 295–299.

Thurston-Enriquez, J.; Gilley, J.; Eghball, B. Microbial quality of runoff following land application of cattle manure and swine slurry. J. Water Health2005, 3, 157–171.

U.S. Environmental Protection Agency. Emerging Technologies for Biosolid Management; EPA 832-R-06–005; U.S. Environmental Protection Agency: Washington, DC, USA. 2006.

United Nations Industrial Development Organization (UNIDO) and International Fertilizer Development Center (IFDC). Fertilizer Manual, 3rd ed.; Kluwer Academic Press: Norwell, MA, USA, 1998.

US EPA. Cyanobacteria and Cyanotoxins: Information for Drinking Water Systems; Fact Sheet EPA-810F11001; US EPA: Washington, DC, USA, 2012.

US EPA. Prepublication of the Ground Water Rule Federal Register Notice; EPA-HQ-OW-2002-0061; FRL-RIN 2040-AA97; US EPA: Washington, DC, USA, 2006.

US EPA. Protocol for Developing Pathogen TMDLs; EPA 841-R-00–002; United States Environmental Protection Agency: Washington, DC, USA, 2001.

US EPA. The Standards for the Use or Disposal of Sewage Sludge; Final 40 CFR Part 503 Rules; EPA 822/Z-93/001; US EPA: Washington, DC, USA,1993.

Venglovsky, J.; Martinez, J.; Placha, I. Hygienic and ecological risks connected with utilization of animal manures and biosolids in agriculture. Livest. Sci. 2006, 102, 197–203.

Venglovsky, J.; Sasakova, N.; Vargova, M.; Pacajova, Z.; Placha, I.; Petrovsky, M.; Harichova, D. Evolution of temperature and chemical parameters during composting of the pig slurry solid fraction amended with natural zeolite. Bioresour. Tech.2005, 96, 181–189.

Viau, E.; Peccia, J. Survey of Wastewater Indicators and Human Pathogen Genomes in Biosolids Produced by Class A and Class B Stabilization Treatments. Appl. Environ. Microbiol.2009, 75, 164–174.

Wilson, S.C. Hogwash! Why Industrial Animal Agriculture Is Not beyond the Scope of Clean Air Act Regulation. Pace Environ. L. Rev.2007, 24, 439.

Witte, W. Medical consequences of antibiotic use in agriculture. Science1998, 279, 996–997.

Wong, K.; Onan, B.; Xagoraraki, I. Quantification of enteric viruses, indicators and salmonella in Class B anaerobic digested biosolids by culture and molecular methods. Appl. Environ. Microb. 2010, 76, 6441–6448.

CHAPTER 3

MICROBIAL POLLUTION CHARACTERIZATION OF WATER AND SEDIMENT AT TWO BEACHES IN SAGINAW BAY, MICHIGAN

Abstract

Signing Bridge beach and Whites beach in Saginaw Bay have been designated as impaired waterbodies by Michigan Department of Environmental Quality with alarming *E. coli* levels. To quantify microbial load and determine probable sources of elevated *E. coli* levels, water and sediment samples were collected from the two beaches during swimming season of 2011. Sources of fecal pollution were assessed in the two beaches using multiple host specific molecular markers: human-associated *Bacteroides* B.thetaiotaomicron α 1-6 mannanase B.theta), bovine-associated *Bacteroides* (B. theta), human adenoviruses (HAdV), and bovine adenoviruses (BAdVs) in conjunction with land use information. Fecal indicator bacteria (*E. coli*, and enterococci) were also monitored, and they were routinely detected in the two beaches. In Singing Bridge beach 50% of water samples (n= 78) exceed the recreational water quality guidelines for *E coli*, while in Whites beach all water samples met the *E. coli* water quality standard. Human and bovine-associated *Bacteroides* and human and bovine adenoviruses were detected in both beaches indicating influence of multiple sources of contamination. Cell culture assay indicated 6 water samples in singing bridge beach, and 7 water samples in Whites beach contained infectious viruses. Results show both frequency and average concentration of B.theta and BoBac were higher in sediment samples than water samples. Elevated BoBac levels compared with B.theta levels in water and sediment samples in the two beaches, both of which

are characterized as intensely farmed areas suggest that runoff from agricultural areas was the probable dominant pollution source. The approach described in this study may help to characterize impacted beaches and design management plans.

Introduction

Water quality monitoring is essential to protect public health, and improve natural resource management and sustainability of the ecosystems (MDEQ, 1997). EPA recommends water quality criteria to reduce risks to human health caused by exposure to pathogens such as bacteria and viruses in water bodies, and each individual state authorities can use the EPA criteria as guidance when setting their own water quality standards (United States Environmental Protection Agency (USEPA), 2012). Culture methods for fecal bacterial indicators (FBI) such as *E. coli* and enterococci are generally used for routine beach monitoring, and water quality advisories or closures occur when levels of fecal indicator organisms exceed standards set by individual state authorities.

Epidemiological studies have found a correlation between indicator organisms and gastrointestinal illnesses (Cabelli et al., 1982; Pruss A., 1998; Wade et al., 2003, 2006, and 2010). However, fecal indicators are thought to have a limited value in assessing the presence and the level of human pathogens because of their poor correlation with some pathogens (Harwood, V. J., 2005, Noble R. T et al., 2001, Pusch, D et al.,2005, Lemarchand, K et al.,2003). Reliance on the current approaches (FIB) for assessing the risk associated with recreational water contact are inadequate for protecting public health, and might not be entirely protective as pathogens have been detected when culturable indicators do not exceed recommended standards (Jiang, S.et al ,2001, Lipp, E. K.et al.,2001, Noble, R. T et al, 2003). In addition, present culture methods for detecting fecal indicators take 18 to 24 hours to obtain results (Wade et al., 2008). This delay allows at-risk beaches to stay open until indicator results are available and in some cases closures occur unnecessarily when indicator bacteria levels have already fell back within acceptable and safe levels.

It is well established that the majority of these indicators are not limited to human fecal pollution but are also associated with other warm-blooded animals, indicating that the presence of these bacteria does not provide any information to the originating host source (Malakoff D et al., 2002), Which makes new approaches for rapidly and practically assessing water quality are needed. If microbial source tracking (MST) is required, then alternative analytical methods should be used. PCR-based fecal source tracking tools targeting Bacteroidales genetic markers have long been suggested as alternative indicators to the fecal coliforms (Carrillo, M et al, 1985), and have become increasingly recognized as an identification tool for various fecal sources, including humans (Bernhard and Field, 2000), cows (Bernhard and Field, 2000, Layton et al., 2006), dogs (Dick et al., 2005a), pigs (Dick et al., 2005b), horses (Dick et al., 2005b), and geese (Fremaux et al., 2010). In addition to human sources, recent studies have applied Bacteroidales genetic markers for fecal source identification of ruminants and cattle (Jent et al., 2013; Marti et al., 2013), however, these studies remain limited. Human populations may be exposed to cattle-derived fecal pathogens via a number of routes (Fayer and Lewis, 1999) including swimming or bathing in recreational waters (Cabelli et al., 1982; Keene et al., 1994). Therefore, it is important to assess fecal loading from these two primary sources (human and cattle) for proper management and remediation of fecal water pollution in mixed uses watersheds.

Quantification of one or more Bacteroidales markers may not provide a complete set of information to accurately identify fecal source(s) as well as to characterize fecal loadings from diffuse sources in the water bodies. As an approach to improve source-tracking capability, the outcomes of marker-based tools can be interpreted in combination with environmental reference information such as land use (Peed et al., 2011; Reischer et al., 2008). This approach is expected to allow more accurate interpretation of microbiological fecal source tracking data and make the MST tools more powerful (Reischer et al., 2011, Verhougstraete, M. P., et al, 2015).

In addition to Bacteroidales, human adenovirus (HAdV) have been suggested as potential MST tools (Noble et al., 2003; Ahmed et al., 2010, Harwood, V. J. et al, 2014, jiang et al., 2005). Several hexon-based real-time PCR assays have been developed to detect generic HAdVs. In addition, integrated cell culture PCR (ICC-PCR) techniques have been used to detect infectious HAdV (Chapron, C. D. et al.,2000, Choo, Y. J. et al, 2006, Lee et al., 2004, Rigotto et al, 2005). Bovine adenovirus (BAdV) were proposed as useful tools for identification of water pollution sources and appeared to be the most widely used animal viruses for MST purposes (Ahmed et al., 2010; Hundesa et al., 2006). Yet the evaluation of human and bovine adenovirus as microbial source tracking tools have not been widely examined.

Recently, there is increasing concern about sediment as a source of beach water quality impairments. Several studies have confirmed the occurrences of *E. coli* and enterococci are times higher in sediment than the overlying water, as well as the potential of sediment to act as a reservoir for the fecal organism (Verhougstraete, M. P., et al, 2015, Ishii et al. 2007, Boehm et al., 2009, Phillips et al, 2011, Alm and Burke, 2006). In order to implement successful remediation strategies in a mixed watershed a better understanding of the sediment microbial load has to be investigated, detailed pollution source tracking has to be undertaken, and the relative contributions of different sources have to be quantified

IN this study we are combining species-specific quantitative qPCR tests for human and bovine species, with the land use information derived from GIS maps. In particularly this study aims to: 1) determine the microbial pollution level of two recreational beaches at Saginaw bay using fecal indicators and virus infectivity measurements; 2) identify the probable source of contamination in the two recreational beaches using host specific human and bovine markers; 3) determine the relationship of fecal indicators (*E. coli* and enterococci) with *Bacteroides* markers; 4) characterize the microbial pollution load of sediments, 5) evaluate the use of viruses as microbial source tracking tools. Application of this approach will help to improve the pollution source assessment in Great Lakes beaches.

Material and Methods

Site Description

The present study was conducted in Saginaw Bay watershed in Michigan, US. Saginaw Bay was officially designated as one of the original 43 Great Lakes Areas of Concern (AOCs) due to stressors including elevated bacteria levels. The land-use in the watershed includes: agriculture 56%, forest 24.8%, open lands 8.9%, urban 6.1%, wetlands 3.3%, water 0.9% (National Land cover map, 2006) (fig.1a&b). The land coverage of the sub-watersheds of interest is summarized in Table1. Two sites were selected and tested weekly throughout the swimming season from June to September, 2011: Singing Bridge beach and Whitney drain (manmade drain outlet to Lake Huron), and Whites beach (Fig.1a). The beaches were selected in this study based on their historically poor beach water quality with undefined sources of pollution (Michigan Department of Environmental Quality, http://www.deq.state.mi.us/beach/, November 22, 2013). Singing Bridge beach is located in the East Branch of the Au Gres River-Rifle sub watershed where the Whitney Drain meets Lake Huron, with public access allowed. Whites beach is located in Kawkawlin-Pine sub watershed at the end of the Whites Beach road, with no public facilities, but a large open grass between the road and the water edge. Number of the closure or advisories for the two studied beaches during swimming seasons from 2003 to 2011 are shown in Table 2.

Sample collection and processing

Each beach was sampled 13 weeks throughout the swimming season from June to September, 2011. Water and sediment samples were collected from four points parallel to the shoreline in each beach. In addition, two points in upstream Whitney drain were monitored at Singing Bridge beach. Using sterile one liter bottles, water grab samples were collected at knee depths. Two water samples were collected at each sampling point, one for *E. coli* and enterococci enumeration, and the other one for nucleic acid extraction followed by *Bacteroides* qPCR assays.

The enteric viruses were collected via filtration through Nano Ceram (Argonite) filter that attached to custom designed filtration system unit (filter house and pump) based on EPA's virus adsorption-elution method (USEPA, 2001a). Sampling equipment was previously disinfected by chlorine and neutralized with sodium thiosulfate. The volume of beach water pumped through the filter ranged from 150 to 300 liters. Adjustment of pH was not needed since pH in all beaches was below 9.5 during sampling events. Sediment samples for bacterial and viral analysis were collected in a Whirl-Pak sample bags. All water, viral filtered, and sediment samples were placed on ice (4°C), stored in a cooler, transported to the Water Quality Laboratory at Michigan State University, and processed or eluted within 24 h.

E. coli and enterococci enumeration

E. coli, and enterococci in water samples were measured in duplicate using defined substrate method Colilert-18^{TM} and Enterolert^{TM}, Quanti-Tray 2000 (IDEXX Laboratories, Inc.). Undiluted samples and three serial dilutions (10 mL, 1 mL, and 0.1 mL per 100 mL made with deionized water) each mixed with reagent, had shaken 10 times and poured into the Quanti-Tray/2000 tray. Incubation and microbial enumeration were conducted following the manufacturer's protocol. Fluorescent wells were reported positive for *E. coli* and enterococci, and expressed as most probable number (MPN) of cells per 100 ml water sample (MPN/100 mL) using the manufacturer's MPN tables.

Water sample preparation

For *Bacteroides* molecular analysis, undiluted water was filtered through 0.45 µm hydrophilic mixed cellulose esters filter (Pall Corporation 66278) under partial vacuum. The filter was placed into a 50 mL sterile disposable centrifuge tube containing 45 ml of sterile phosphate buffered saline PBW, vortexed on high for 10 min, and then centrifuged (20 min; 4500 ×g) to pellet the cells. forty-three milliliters (2mL of pellet left) were decanted from the tube and the remaining pellet were stored at -80 °C until DNA could be extracted. The water sample volume that were filtered for DNA extraction was taken into account when calculating the final concentration. Overall, water volumes were ranged from 500 to 1000 ml.

Virus elution

Water samples were analyzed for enteric viruses according to the EPA's Manual of Methods for Virology (U.S. EPA 2001a). To elute absorbed viral particles, the Nano Ceram filters were backwashed with one liter of 1.5% [wt/vol] beef extract with 0.05M glycine (pH 9 to 9.5, 25°C). Then, the elution was concentrated using organic flocculation by lowering the solution pH to 3.5. The samples were centrifuged at 2,500 × g for 15 min for further concentration and the floc was resuspended in 30 ml of 0.15 M sodium phosphate (pH 9.0). Dissolved precipitates containing the viruses were centrifuged at 10,000× g for 10 min. The supernatants (approximately 30 ml) were collected (pellet was discarded), pH were neutralized (7.0 to 7.5) with 1 M HCl, and supplemented with 100 units of penicillin, 100µg of streptomycin and 0.25µg of fungizone. The eluent was filter through 0.22 µm filter and stored in aliquots at -80°C until DNA extraction were conducted.

Sediment samples for viral analysis were eluted by following the ASTM Method D4994-89: 25 g of sediment sample was eluted by adding 100 ml 10% beef extract (pH 9.0 to 9.5), stirring for 30 minutes, and centrifuged at 10,000 ×g for 30 min. The supernatant was collected in a sterile one-liter beaker. Organic flocculation was preceded by adjusting pH to 3.5 with 1N HCl and stirring for 30 minutes with low speed. The samples were centrifuged at 2500×g for 15 min to collect the pellets, the pellets resuspended in 0.01M phosphate buffered saline (PBS), pH was neutralized to (7.0-7.5) supplemented with 1 ml of Kanamycin, and 1ml of Gentamicin, the eluent was filtered through 0.22 um filter and then stored at -80°C.

Nucleic acid extraction

For water samples, both *Bacteroides* and virus DNA extraction were performed by using the MagNa Pure Compact System automatic machine (Roche Applied Sciences, Indianapolis, IN) with the corresponding kit (MagNA Pure Compact Nucleic Acid Isolation Kit I). Approximately 100 µL of DNA was extracted from 400 µL of the water sample pellet.

For sediment samples, *Bacteroides* DNA extractions were performed using UltraClean® Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Solana Beach, CA) following the manufacture's instruction manual. Nucleic acid extraction for virus was carried out in sediment samples by using the MagNa Pure Compact System automatic machine (Roche Applied Sciences, Indianapolis, IN). All extracted DNA was stored at -20°C until qPCR analysis.

qPCR amplification

Extracted DNA from water and sediment samples were analyzed for the presence of human-associated *Bacteroides* (B.theta) (Yampara et al., 2008), and bovine-associated *Bacteroides* (BoBac) (Layton et al., 2006) using TaqMan real-time qPCR assays. All qPCR assays were performed as described in the citations. For HAdV, the TaqMan probe, and forward and reverse primer (AP/AQ1/AQ2/AP) of published primers/ probe set was used for analysis of all water and sediment samples (Heim et al., 2003). BAdV analysis was conducted by using published primers/ probe set (BAV4-8F/ BAV4-8R/ BAV4-8P) (Wong and Xagoraraki, 2010). Primer sequences and product size for each assay are described in (Table 3). All qPCR quantification analysis was carried with LightCycler® 1.5 Instrument (Roche Applied Sciences, Indianapolis, IN) and LightCycler 480 Probes Master kit in sealed glass capillaries with a total reaction volume of 20 ml. The crossing point (Cp) value for each qPCR reaction automatically determined by the LightCycler® Software 4.0. Positive control was included with each qPCR run. Plasmid standard for B.theta and BoBac were generated with the Bac32F and Bac708R primers, DNA extracts from sewage (collected from wastewater treatment plant, East Lansing, MI), and cow manure (collected from Michigan State University dairy farm) were cloned into pCR 4-TOPO using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA, USA). Plasmid DNA was prepared using a Bio-Rad miniplasmid prep kit (Hercules, CA), and sequencing was carried out from vector primer sites M13 Forward and M13 Reverse following the manufacturer's instructions. Virus nucleic acid were extracted from HAdV types 2 and BAdV type 4 (obtained from American Type Culture Collection (ATCC)). The amplicons were subsequently cloned into plasmid vector based on the one-shot chemical trans- formation described in the manufacturer's instructions (TOPO TA Cloning Kit for Sequencing; Invitrogen, Carlsbad, CA, USA). Plasmid DNA carrying the cloned hexon gene was purified using Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA) and quantified by Nanodrop to serve as stock genomic equivalent copies (GEC). Stock GEC were diluted to a desired range and used for creating standard curves. Deionized molecular grade water was used as a negative control during each qPCR run. All qPCR analysis was run in triplicates. Standard curves amplification efficiencies of >95% and R^2 values of >0.98 were documented. For quality assurance steps were taken included lab blanks, field blanks, calibration checks, and duplicate samplings.

Cell culture ICC-PCR assays for virus

Cell culture experiments were conducted on samples that were positive with HAdV using qPCR in order to determine the infectivity levels in environmental samples. Viruses were cultured on the A549 human cell line (obtained from ATCC, cell passage 90 - 110) followed the total culturable virus quantal assay (EPA, 2001b), with minor modification. The cells were incubated in flasks at 37°C with growth medium (minimum essential medium with 10% fetal bovine serum, L-glutamine, Earle's salts) until at least 70 to 90% confluence was obtained. Virus eluent of environmental samples were added into healthy cells and incubated at 37°C for 1 hour with occasional rocking to ensure complete contact between the cells and viral particles. After the growth medium was decanted and discarded, the cells were washed with Dulbecco's phosphate buffered saline. Cells were maintained with minimum essential medium supplemented with L-glutamine, Earle's salts, and 2% fetal bovine serum. The cells were observed for cytopathic effect (CPE) as an indication of the presence of viable, infectious enteric viruses for 14 days. Flasks displayed CPE were taken out from the incubator, and a confirmation procedure was applied by inoculating aliquots of 1 ml of the supernatant into healthy cells, and then monitored for 7 days. All positive results were confirmed with a second passage. Viral DNA was extracted from the infected cells and integrated cell culture polymerase chain reaction ICC-PCR assays have been performed with primers/probe of total HAdV described in Table 3.

Statistical analysis

An assessment of the normality of microbial concentrations data was performed graphically by using Q-Q plot and numerically by using Shapiro-Wilk's W test. The results indicate that the FIB, *Bacteroides*, and virus data are not normally distributed (skewed). Therefore, all data were $\log_{10}$ transformed prior to all statistical analyses to reduce skewness. The parametric Pearson correlation coefficients (r) were used to measure the correlation among microbial assay data and the degree of the relationship between indicators (*E. coli* and enterococci) in both beaches. In order to examined significant differences among FIB, *Bacteroides* and virus an independent sample *t*-test (student test) was conducted. The assumptions of normality and homogeneity of variance have been assessed. Normality has been assessed as describe above and homogeneity of variance (assumes that both groups have equal error variances) assessed using Levene's Test for the equality of error variances. The *t*-test was two- tailed, with alpha levels, or the probability of rejecting the null hypothesis when it is true, set at $p < 0.05$. These tests were performed using SPSS Statistic 17.0 software (SPSS Inc., Chicago, IL) with significance (α) set at 0.05.

Results

Presences of bacterial indicators

A summary of the results for Singing Bridge beach and Whitney drain, and for Whites beach are presented in Fig.2, all values have been $\log_{10}$ transformed and are reported as the geometric mean concentrations. In total, 156 grab water samples were processed from Singing Bridge beach (n = 52) and from Whitney drain (n=26), and Whites beach (n=78). FIB were detected in both beaches in all 156 water samples. The concentration of the *E. coli* exceeded daily geometric mean of *E. coli* standard for recreational water of the state of Michigan (The daily geometric mean of 3 samples must be < 300 *E. coli* / 100ml) in 50% of samples in Singing Bridge beach often by an order of magnitude or more, and no exceedance was reported in Whites beach. Concentration of *E. coli* based on the geometric means reached the highest of 3.18 $\log_{10}$, and 1.88 $\log_{10}$ MPN/100ml for Singing Bridge beach/Whitney drain and Whites beach, respectively. For enterococci the highest concentration was 3.28 $\log_{10}$ for Singing Bridge beach/ Whitney drain, and 1.39 $\log_{10}$ MPN/100ml for Whites beach.

Pearson Correlation coefficients between fecal indicator organisms were calculated among each other in each beach. The relationship between *E. coli* and enterococci shows they are highly correlated with a statistically significant correlation at the 5% significance level at Singing Bridge beach (r=0.75 Pearson correlation coefficients, $p < 0.001$) and Whitney drain (r=0.81 Pearson correlation coefficients, $p < 0.001$). The least correlated relationship was seen when comparing between the two fecal indicators in Whites beach (r=0.13 Pearson correlation coefficients, $p < 0.26$). When comparing the concentrations of *E. coli* and enterococci across the two beaches, concentrations tended to be significantly higher in Singing Bridge beach than Whites beach for both indicators (*E. coli* and enterococci). The *E. coli* concentrations in Singing Bridge and Whites beach are significantly different ($p < 0.019$), and similarly for enterococci ($P< 0.026$).

The correlation between *E. coli* and enterococci with *Bacteroides* marker were calculated for the two beaches. In Singing Bridge, *E. coli* had a week correlation coefficients with B.theta (r= 0.166) and BoBac (r=0.23). For enterococci, the coefficients were 0.12 and 0.08, with B.theta and BoBac, respectively. In Whites beach, *E. coli* still poorly correlated with *Bacteroides* (r=0.043 for B.theta and r=0.075 for BoBac), while enterococci had a moderate correlation coefficient with B.theta (0.45), but low with BoBac (0.05).

Bacteroides Markers

Human-associated *Bacteroides* and bovine-associated *Bacteroides* data was plotted to provide an overview of the distribution of the mean and median concentration in both water and sediment samples for each sampling site (Fig. 2).

In Singing Bridge beach/ Whitney drain, 78 water samples were tested with qPCR for the presence and the level of the human and bovine-associated *Bacteroides*. Human-associated *Bacteroides* (B.theta) were detected in 34 samples with highest level of 2.48 $\log_{10}$ copies/100ml and bovine-associated *Bacteroides* (BoBac) were detected in 36 samples, with levels up to 3.68 $\log_{10}$ copies/100ml. In Whites beach *Bacteroides* were also tested in 78 water samples, B.theta were detected in 54 samples with highest level of 3.23 $\log_{10}$ copies/100ml and BoBac were detected in 41 samples, with levels up to 4.08 $\log_{10}$ copies/100ml. A significant trend emerged when comparing human and bovine associated *Bacteroides* in water samples among each beach. Bovine *Bacteroides* had higher concentration in both beaches and t-test shows significant statistical difference between the two markers (Singing Bridge beach, $p < 0.01$; Whitney drain $p < 0.001$; Whites beach $p < 0.001$).

In sediment samples, human-associated *Bacteroides* were detected in 46 and 40 samples in Singing Bridge beach and Whites beach, respectively. The highest concentration of B.theta in sediment reached 4.27 $\log_{10}$ copies/100gm of sediment at Singing Bridge, and 3.81 $\log_{10}$ copies/100gm of sediment in Whites beach. Bovine-associated *Bacteroides* were detected in 49 samples in Singing Bridge with highest level of 7.05 $\log_{10}$ copies/100ml, and 47 samples in Whites beach with highest level of 6.84 $\log_{10}$ copies/100ml. Occurrence and concentration of bovine associated *Bacteroides* are higher than human-associated *Bacteroides* in both beaches with pairwise comparisons, and they considered statistically significant different ($p < 0.05$, n=78).

Sediment and water pairwise comparisons indicated that 85% of the sediment samples had higher B.theta levels compared to the water, assuming 100 mL of water was equal to 100 g of sediment (Verhougstraete & Rose, 2014, Zehms et al., 2008), and 65% of the sediment samples had a higher BoBac than water samples in Singing Bridge beach. Similarly, in Whites beach B.theta and BoBac were higher in the sediment than in the water in 70% and 85% of the samples, respectively.

Presence of Enteric Viruses

Virus analysis was carried on 78 water samples, 39 for Singing Bridge beach and 39 for Whites beach. Frequencies of viruses were significantly lower than *Bacteroides* in both beaches. In Singing Bridge 6 samples were tested positive for HAdV, with concentration range from 4.7×10^0 to 1.45×10^2 copies per 100ml, and 13 samples were tested positive for BAdV with concentration range from 4×10^0 to 1.00×10^1 copies /100ml. In Whites beach, HAdV and BAdV were detected in 8 and 2 samples, respectively, of which the concentration ranged from 0.5×10^0 to 1.47×10^2 copies/100ml. In sediment sample, HAdV were detected in 3 samples in Singing Bridge beach and 6 samples in Whites beach, with concentration below 10 copies /100 g in both beaches. BAdV were not detected in any sediment samples. The occurrence and low concentration of viruses produced less meaningful results for all pairwise comparisons and were not considered statistically significant.

Cell culture analysis was only applied on 23 samples (14 water and 9 sediment) that were positive with HAdV by qPCR analysis. A total of 13 water samples (6 from singing bridge and 7 from Whites beach) and 6 sediment samples (3 from singing Bridge beach and 2 from Whites beach) showed CPE in the cell culture assay; while in the confirmation assay only 7 water samples showed CPE from Singing Bridge Beach (4 from Singing Bridge and 3 from Whites beach).

Discussion

The qPCR assays targeting Bacteroidales 16S rRNA genes are increasingly being used as molecular tools to identify fecal sources in a quantitative manner. However, it is difficult to identify unknown fecal source(s) based on the qPCR assay results only due to lack of information on routes of marker transport in the environment (Reischer et al., 2011). In the present study, we used qPCR assays to compare the fecal loading in well characterized watersheds impacted by mixed land uses, with a particular focus on identification of probable fecal pollution sources. The assay results were interpreted in relation to land use data that is expected to influence fecal marker loading to beaches.

Trend of B.theta and BoBac marker in water was similar to that of sediment (Fig. 2), and the two markers exhibited insignificant correlations at the two sampling sites. BoBac was higher than B.theta in water and in sediment samples among the two beaches. Our assays demonstrated that the two beaches were largely influenced by the cattle-originated fecal pollutants and identified runoff from agriculture areas as a predominant non-point source of fecal pollution, confirming our assumptions based on land use information (Table1). Occurrence of B.theta marker in water and sediment samples in each beach, indicate that both beaches were impacted by human fecal pollution. In Singing Bridge beach, the B.theta marker could be the result of in-stream transportation from human wastewater upstream Whitney drain. While in Whites beach the human faces impact could be the leaking from the septic system from the surrounding houses located nearby the beach shoreline. This outcome may indicate that *Bacteroides* assays were able to determine the predominant fecal pollution source at each beach during the sampling period.

Although levels of *Bacteroides* may significantly correlate with conventional indicators in wastewater (Srinivasan et al., 2011), the correlations in natural waters are usually very low.

Flood et al. (2011) observed no correlation between enterococcal counts and presence and absence of *Bacteroides* markers. Bonkosky et al. (2009) and McQuaig et al. (2009) reported that *Bacteroides* concentration was poorly correlated with fecal coliforms and enterococci. No significant correlation between *Bacteroides* and *E. coli* was found in surface water and storm water (Merrick et al., 2009; Sauer et al., 2011). Results of statistical analysis based on our data showed correlations (r) among different species of microorganisms were generally poor. A significantly weak correlation was observed between the concentration of indicators and the *Bacteroides* markers in grab water samples, the correlations coefficients range of ($0 > r < 0.4$). Our results above, strongly suggest that traditional indicators fail to represent other microbial species in natural water bodies, and therefore current criteria may not sufficiently prevent people from waterborne pathogens.

Possible reasons for the lack of correlations could be the result of overlapping patterns of inputs from other wildlife animals in the mixed watershed (dogs, deer, gulls, etc.) that are confounding any single marker correlations. Another confounding factor may be different environmental decay rates for FIB and markers (Eichmiller et al., 2014; Jeanneau et al., 2012).

One of the aims of this study is to determine the occurrence and relationships of B.theta, BoBac, HAdV, and BAdV in sediment. Our data shows the occurrence of both B.theta and BoBac was more frequent in sediment than in water. These results support the hypothesis that sediment under natural water bodies can act as a reservoir for bacteria (Verhougstraete, M. P., et al, 2015, Ishii et al. 2007, Whitman et al. 2011, Boehm, 2009), and could act as nonpoint source of bacterial pollution in Saginaw bay area. On the other hand, viruses seemed more likely suspended in water, due to relatively higher frequencies of water samples positive with viruses than in sediment.

In order to examine the use of adenovirus as MST tool, the relationship between HAdV and B.theta, and between BAdV and BoBac have been studied. Our data showed that correlation coefficients for B.theta and HAdV in Singing Bridge and Whites Beach were very week, 0.0038 and 0.002, and for BoBac and BAdV were 0.0205 and 0.0074, respectively. In our study, viruses generally had poor correlations with other microorganisms, and one of the possible reasons is that virus levels in majority of the samples were below detection limit. However, since the frequencies and concentrations of viruses are very low, it is difficult to compare virus prevalence and levels in water and sediment samples. Collectively, employed HAdV and BAdV as a microbial source tracking methods in the studied beaches were failed to identify the source of fecal contamination.

A strength of this study is the high number of samples analyzed (n=312) for multiple markers over a long time period. Future work should include a more in-depth look at transport mechanisms and other environmental parameters such as precipitation, wave, weather temperature, and the effect of environmental characteristics on FIB and *Bacteroides* marker decay.

Conclusions

The major conclusion of this work are as following: 1) Singing Bridge beach and Whites beach are impacted and occurrence of human and bovine markers indicated agriculture and human microbial source of contamination, 2) *Bacteroides* marker assays are useful for microbial source tracking studies when combined with land use information, this approach will allows for a better understanding of the sources of fecal pollution in mixed watersheds, 4) the significant different between the concentration of human and bovine-associated *Bacteroides*, can be interpreted in combination with land use data and indicated that runoff from agriculture areas are the probable source of contamination, human sewage is partially contributing to the fecal contamination in the two Saginaw Bay beaches. 3) the use of viruses as a MST tool failed to identify the source of contamination due to its low abundance in the environment, 4) sediments contain high levels of fecal organism as compared to the water samples, and sediments can act as non-point source that degrade beach water quality, 5) infectious HAdVs were detected in some water samples which did not violate *E. coli* concentration according to Michigan standard, this finding indicates the need of use of the alternative indicators along with pathogens measurement to protect public health.

Acknowledgments

This study was funded by EPA GLRI. We thank Central Michigan District Health Department for their help during sampling.

APPENDIX

Table 3.1 Watersheds and sites description

Site name	**Watershed**	**Watershed land use percentage***			
		Agriculture (%)	Forest (%)	Urban (%)	Water and wetland (%)
Singing Bridge beach and Whitney drain	Au Gres-Rifle	34.4	46.7	1.6	14.7
Whites beach	Kawkawlin–Pine	63.7	18.9	2.1	12.4

*Land-use data obtained with GIS from national land cover map, 2001

Table 3.2 Water quality exceedances from 2003 to 2011

Site name	**Coordinates**	**Description**	**Number of Closures or Advisories per Beach 2003- 2011***
Singing Bridge beach and Whitney drain	44.14334 / -83.56661	Located in Arenac County where the Whitney Drain meets Lake Huron. The beach is about 179.5 feet wide, with public access allowed.	422
Whites beach	43.92861/ -83.89051	Located in Arenac County at the end of Whites Beach Rd Surrounded by residential homes relying on septic systems for wastewater management.	144

*Data obtained from beach monitoring system (BeachGuard), Michigan Department of Environmental Quality, http://www. deq.state.mi.us/beach/

Table 3.3 Primer/ Probe set for qPCR assays tested in water and sediment sample

Assay	Primer Name	Sequence (5'-3')	Amplicon Size	Reference
B.theta (Alpha Mann.)	BtH-F	CATCGTTCGTCAGCAGTAACA	62	Yampara - Iquise et al., 2008
	BtH-R	CCAAGAAAAAGGGACAGTGG		
	BtH-P	ACCTGCTG		
BoBac	BoBac367f	GAAG(G/A)CTGAACCAGCCAAGTA	100	Layton et al., 2006
	BoBac467r	GCTTATTCATACGGTACATACAAG		
	BoBac402Bhqf	TGAAGGATGAAGGTTCTATGGATTGTAAACTT		
HAdV (Heim)	AQ1	GCCACGGTGGGGTTTCTAAACTT	129	Heim et al., 2003
	AQ2	GCCCCAGTGGTCTTACATGCACATC		
	AP	TGCACCAGACCCGGGCTCAGGTACTCCGA		
BAdV	BAV4-8F	CRAGGGAATAYYTGTCTGAAAATC	85	Wong and Xagoraraki, 2010
	BAV4-8R	AAGGATCTCTAAATTTYTCTCCAAGA		
	BAV4-8P	FAM-TTCATCWCTGCCACWCAAAGCTTTTTT-BBQ1		

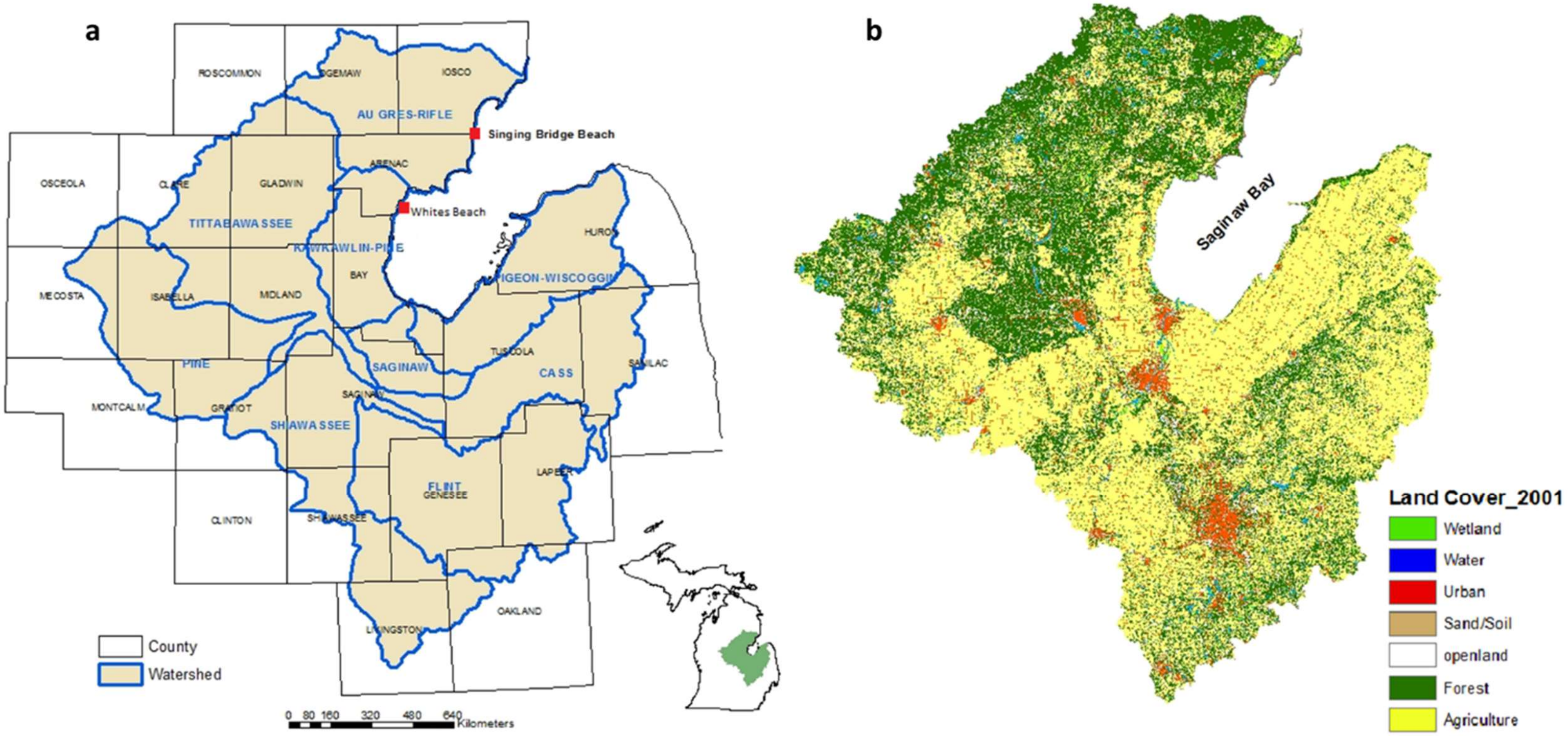


Figure 3.1 Location of watersheds of interest and sampling locations on two public beaches in Saginaw Bay. Map comprised of various spatial datasets: a) Watershed boundaries and county boundaries (U.S. Environmental Protection Agency and U.S. Geological Survey, 2005), and b) Land cover (National land cover map, NLCD 2001)

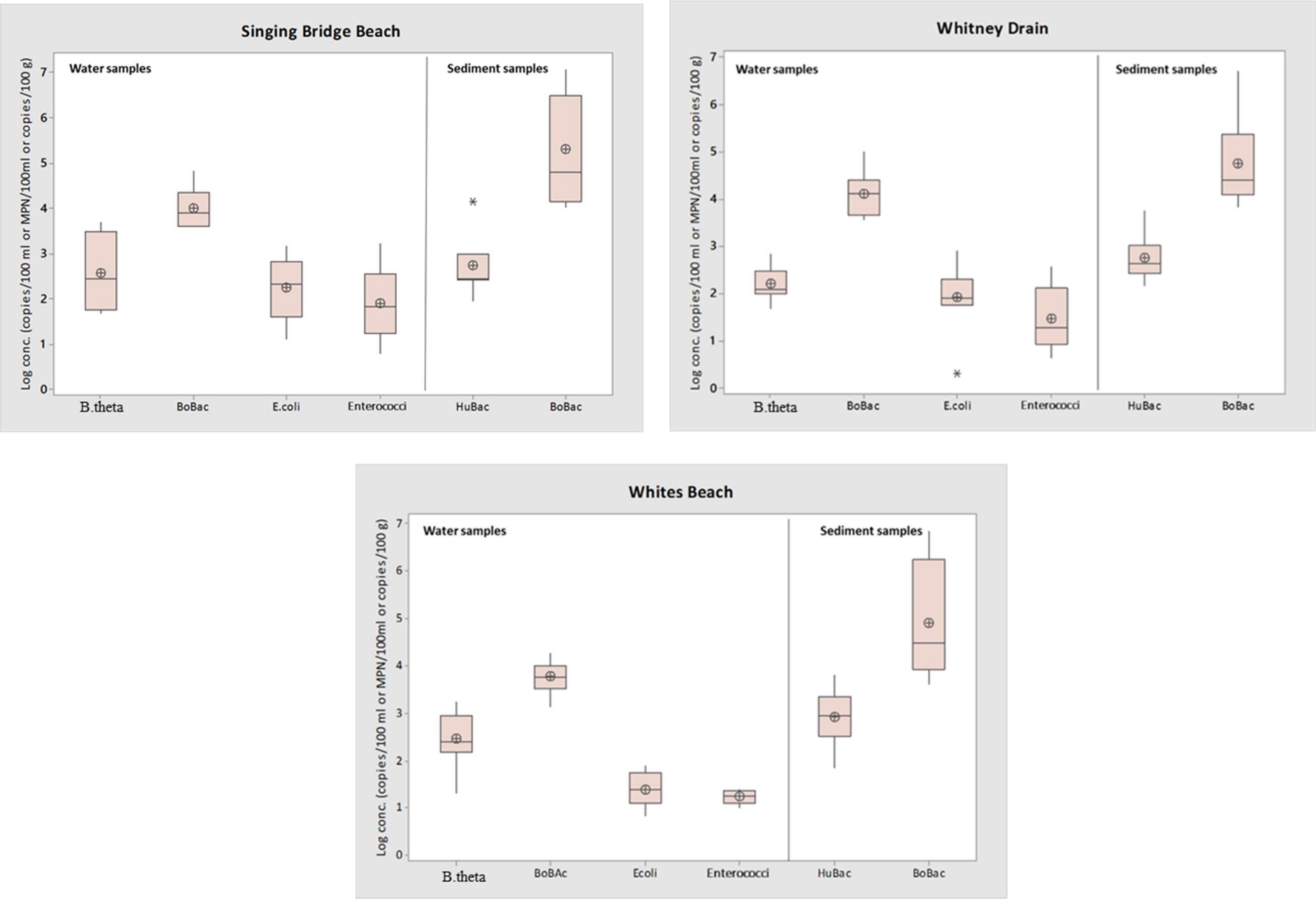


Figure 3.2 summary data from 13 weeks of sampling. Each week 2 samples were collected from Whitney Drain,4 samples from Singing Bridge beach and 6 samples from Whites beach. Figures present FIB (*E.coli* and enterococci), *Bacteroides* markers (B.theta and BoBac) in water and sediment samples for the 3 sampling locations Each box plot represents the median (line within the box), mean (circle in the box), and quartiles of all data, and whisker represent the maximum and minimum level of the markers and outliers, respectively.

REFERENCES

REFERENCES

Ahmed, W., Goonetilleke, A., & Gardner, T. (2010). Human and bovine adenoviruses for the detection of source-specific fecal pollution in coastal waters in Australia. Water research, 44(16), 4662-4673.

Alm, E.W., Burke, J., Hagan, E., 2006. Persistence and potential growth of the fecal indicator bacteria, Escherichia coli, in shoreline sand at Lake Huron. J. Great Lakes Res. 32, 401–405.

Bernhard, A. E., & Field, K. G. (2000). A PCR assay to discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA. Applied and environmental microbiology, 66(10), 4571-4574.

Boehm, A.B., Griffith, J., McGee, C., Edge, T.A., Solo-Gabriele, H.M., Whitman, R., Cao, Y., Getrich, M., Jay, J.A., Ferguson, D., Goodwin, K.D., Lee, C., Madison, M., Weisberg, S.B., 2009. Faecal indicator bacteria enumeration in beach sand: a comparison study of extraction methods inmediumto coarse sands. J. Appl.Microbiol. 107, 1740–1750.

Boehm, A.B., (2007). Enterococci concentrations in diverse coastal environments exhibit extreme variability. Environ. Sci. Technol. 41, 8227–8232.

Bonkosky, M., Hernandez-Delgado, E. A., Sandoz, B., Robledo, I. E., Norat-Ramirez, J., & Mattei, H. (2009). Detection of spatial fluctuations of non-point source fecal pollution in coral reef surrounding waters in southwestern Puerto Rico using PCR-based assays. *Marine Pollution Bulletin*, *58*(1), 45-54.

Burton, G. A., Gunnison, D., & Lanza, G. R. (1987). Survival of pathogenic bacteria in various freshwater sediments. Applied and Environmental Microbiology, 53(4), 633-638.

Cabelli, V.J., Dufour, A.P., McCabe, L.J., Levin, M.A., (1982). Swimming-associated gastroenteritis and water quality. Am. J. Epidemiol. 115, 606–616.

Carrillo, M., E. Estrada, and T. C. Hazen. 1985. Survival and enumeration of the fecal indicators Bifidobacterium adolescentis and Escherichia coli in a tropical rain forest watershed. Appl. Environ. Microbiol. 50:468–476.

Chapron, C. D., N. A. Ballester, J. H. Fontaine, C. N. Frades, and A. B. Margolin (2000). Detection of astroviruses, enteroviruses, and adenovirus types 40 and 41 in surface waters collected and evaluated by the information collection rule and an integrated cell culture-nested PCR procedure. Appl. Environ. Microbiol. 66:2520–2525.

Choo, Y.-J., and S.-J. Kim. (2006). Detection of human adenoviruses and enteroviruses in Korean oysters using cell culture, integrated cell culture- PCR, and direct PCR. J. Microbiol. 44:162–170.

Davies, C. M., Long, J. A., Donald, M., & Ashbolt, N. J. (1995). Survival of fecal microorganisms in marine and freshwater sediments. Applied and Environmental Microbiology, 61(5), 1888-1896.

Dick, L. K., Bernhard, A. E., Brodeur, T. J., Santo Domingo, J. W., Simpson, J. M., Walters, S. P., & Field, K. G. (2005b). Host distributions of uncultivated fecal Bacteroidales bacteria reveal genetic markers for fecal source identification. Applied and Environmental Microbiology, 71(6), 3184-3191.

Dick, L. K., Simonich, M. T., & Field, K. G. (2005a). Microplate subtractive hybridization to enrich for Bacteroidales genetic markers for fecal source identification. *Applied and environmental microbiology*, *71*(6), 3179-3183.

Eichmiller, J. J., Borchert, A. J., Sadowsky, M. J., & Hicks, R. E. (2014). Decay of genetic markers for fecal bacterial indicators and pathogens in sand from Lake Superior. *Water research*, *59*, 99-111.

Fayer, R., Lewis, E. J., Trout, J. M., Graczyk, T. K., Jenkins, M. C., Higgins, J., ... & Lal, A. A. (1999). Cryptosporidium parvum in oysters from commercial harvesting sites in the Chesapeake Bay. Emerging infectious diseases, 5(5), 706.

Flood, C., Ufnar, J., Wang, S., Johnson, J., Carr, M., & Ellender, R. (2011). Lack of correlation between enterococcal counts and the presence of human specific fecal markers in Mississippi creek and coastal waters. *Water research*, *45*(2), 872-878.

Fremaux, B., Boa, T., & Yost, C. K. (2010). Quantitative real-time PCR assays for sensitive detection of Canada goose-specific fecal pollution in water sources. *Applied and environmental microbiology*, *76*(14), 4886-4889.

Gunnison, D. (1999). Evaluating Microbial Pathogens in Reservoirs. Water Quality Technical Notes Collection (WQTN PD-03)', US Army Engineer Research and Development 24.

Harwood, V. J., A. D. Levine, T. M. Scott, V. Chivukula, J. Lukasik, S. R. Farrah, and J. B. Rose. (2005). Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. Applied and Environmental Microbiology 71:3163-3170Center, Vicksburg.

Harwood, V. J., Staley, C., Badgley, B. D., Borges, K., & Korajkic, A. (2014). Microbial source tracking markers for detection of fecal contamination in environmental waters: relationships between pathogens and human health outcomes. *FEMS microbiology reviews*, *38*(1), 1-40.

He, J. W., and S. Jiang. (2005). Quantification of enterococci and human adenoviruses in environmental samples by real-time PCR. Appl. Environ. Microbiol. 71:2250–2255.

Heim, A., Ebnet, C., Harste, G., & Pring Åkerblom, P. (2003). Rapid and quantitative detection of human adenovirus DNA by real time PCR. Journal of medical virology, 70(2), 228-239.

Hundesa, A., de Motes, C. M., Bofill-Mas, S., Albinana-Gimenez, N., & Girones, R. (2006). Identification of human and animal adenoviruses and polyomaviruses for determination of sources of fecal contamination in the environment. *Applied and environmental microbiology*, *72*(12), 7886-7893.

Ishii, S., Hansen, D.L., Hicks, R.E., Sadowsky, M.J., (2007). Beach sand and sediments are temporal sinks and sources of Escherichia coli in Lake Superior. Environ. Sci. Technol. 41, 2203–2209.

Jeanneau, L., Solecki, O., Wéry, N., Jardé, E., Gourmelon, M., Communal, P. Y., ... & Pourcher, A. M. (2012). Relative decay of fecal indicator bacteria and human-associated markers: a microcosm study simulating wastewater input into seawater and freshwater. *Environmental science & technology*, *46*(4), 2375-2382.

Jent, Justin R., et al. "Determining hot spots of fecal contamination in a tropical watershed by combining land-use information and meteorological data with source-specific assays." *Environmental science & technology* 47.11 (2013): 5794-5802.

Jiang, S. C., Chu, W., Olson, B. H., He, J. W., Choi, S., Zhang, J., ... & Gedalanga, P. B. (2007). Microbial source tracking in a small southern California urban watershed indicates wild animals and growth as the source of fecal bacteria. Applied Microbiology and Biotechnology, 76(4), 927-934.

Jiang, S., H. Dezfulian, and W. Chu. (2005). Real-time quantitative PCR for enteric adenovirus serotype 40 in environmental waters. Can. J. Microbiol. 51:393–398.

Jiang, S., R. Noble, and W. Chu. (2001). Human adenoviruses and coliphages in urban runoff-impacted coastal waters of Southern California. Appl. Environ. Microbiol. 67:179–184.

Keene, W. E., McAnulty, J. M., Hoesly, F. C., Williams Jr, L. P., Hedberg, K., Oxman, G. L., & Fleming, D. W. (1994). A swimming-associated outbreak of hemorrhagic colitis caused by Escherichia coli O157: H7 and Shigella sonnei. *New England Journal of Medicine*, *331*(9), 579-584.

Layton, A., McKay, L., Williams, D., Garrett, V., Gentry, R., & Sayler, G. (2006). Development of Bacteroides 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. Applied and Environmental Microbiology, 72(6), 4214-4224.

Lee, C., Lee, S. H., Han, E., & Kim, S. J. (2004). Use of cell culture-PCR assay based on combination of A549 and BGMK cell lines and molecular identification as a tool to monitor infectious adenoviruses and enteroviruses in river water. Applied and environmental microbiology, 70(11), 6695-6705.

Lee, C., S. H. Lee, E. Han, and S. J. Kim. (2004). Use of cell culture-PCR assay based on combination of A549 and BGMK cell lines and molecular identification as a tool to monitor infectious adenoviruses and enteroviruses in river water. Appl. Environ. Microbiol. 70:6695–6705.

Lemarchand, K., and P. Lebaron. 2003. Occurrence of *Salmonella* spp. and *Cryptosporidium* spp. in a French coastal watershed: relationship with fecal indicators. FEMS Microbiol Letts 218:203-209.

Lipp, E. K., S. A. Farrah, and J. B. Rose. 2001. Assessment and impact of microbial fecal pollution and human enteric pathogens in a coastal community.Mar. Pollut. Bull. 42:286–293.

Malakoff, D. (2002). Microbiologists on the trail of polluting bacteria. Science, 295(5564), California shoreline. J. Water Health 1:23–31.

Marino, R. P., & Gannon, J. J. (1991). Survival of fecal coliforms and fecal streptococci in storm drain sediment. Water Research, 25(9), 1089-1098.

Marti, R., Gannon, V. P., Jokinen, C., Lanthier, M., Lapen, D. R., Neumann, N. F., ... & Topp, E. (2013). Quantitative multi-year elucidation of fecal sources of waterborne pathogen contamination in the South Nation River basin using Bacteroidales microbial source tracking markers. *Water research*, *47*(7), 2315-2324.

McQuaig, S. M., Scott, T. M., Lukasik, J. O., Paul, J. H., & Harwood, V. J. (2009). Quantification of human polyomaviruses JC virus and BK virus by TaqMan quantitative PCR and comparison to other water quality indicators in water and fecal samples. *Applied and environmental microbiology*, *75*(11), 3379-3388.

Merrick, N. N. (2009). Microbial Source Tracking; Library Independent Host-Specific Bacteroidales 16S rRNA Gene PCR Assay in a Mixed Use Watershed.

Michigan Department of Environmental Quality, MDEQ (1997), A Strategic Environmental Quality Monitoring Program for Michigan's Surface Waters: MI/DEQ/SWQ-96/152, p. 7-39.

Michigan Department of Environmental Quality, MDEQ (2008) Guidance for Delisting Michigan's Great Lakes Areas of Concern, revised. MI/DEQ/WB-06-001.

Michigan Department of Environmental Quality, MDEQ (2012). Water Quality and Pollution Control in Michigan 2010 Sections 303(d), 305(b), and 314 Integrated Report. MI/DEQ/WB-10/001.

Michigan Department of Environmental Quality, MDEQ (2013). Michigan beach monitoring year 2013 annual report, MI/DEQ/WRD-14/025.

Noble, R. T., and J. A. Fuhrman. 2001. Enteroviruses detected by reverse transcriptase polymerase chain reaction from the coastal waters of Santa Monica Bay, California: low correlation to bacterial indicator levels. Hydrobiologia 460:175-184.

Noble, R. T., Weisberg, S. B., Leecaster, M. K., McGee, C. D., Dorsey, J. H., Vainik, P., & Orozco-Borbon, V. (2003). Storm effects on regional beach water quality along the southern California shoreline. Journal of Water and Health, 1(1), 23-31.

Peed, L. A., Nietch, C. T., Kelty, C. A., Meckes, M., Mooney, T., Sivaganesan, M., & Shanks, O. C. (2011). Combining land use information and small stream sampling with PCR-based methods for better characterization of diffuse sources of human fecal pollution. *Environmental science & technology*, *45*(13), 5652-5659.

Phillips, M.C., Solo-Gabriele, H.M., Piggot, A.M., Klaus, J.S., Zhang, Y., 2011. Relationships between sand and water quality at recreational beaches. Water Res. 45, 6763–6769.

Pruss, A. 1998. Review of epidemiological studies on health effects from exposure to recreational water. Int. J. Epidemiol. 27:1–9.

Public Sector Consultants Inc, PSC (2002). Targeting environmental restoration in the Saginaw area of concern (AOC), 2001 Remedial Action Plan Update, prepared for: The Partnership for the Saginaw Bay Watershed. Lansing, Michigan.

Public Sector Consultants Inc, PSC (2011). An Assessment and Status Report of Beach Closings in the Saginaw River/Bay Area of Concern, prepared for: The Partnership for the Saginaw Bay Watershed, Bay City, Michigan.

Pusch, D., D. Y. Oh, S. Wolf, R. Dumke, U. Schroter-Bobsin, M. Hohne, I. Roske, and E. Schreier. 2005. Detection of enteric viruses and bacterial indicators in German environmental waters. Arch Virol 150:929-47.

Reischer, G. H., Haider, J. M., Sommer, R., Stadler, H., Keiblinger, K. M., Hornek, R., ... & Farnleitner, A. H. (2008). Quantitative microbial faecal source tracking with sampling guided by hydrological catchment dynamics. *Environmental microbiology*, *10*(10), 2598-2608.

Reischer, G. H., Kollanur, D., Vierheilig, J., Wehrspaun, C., Mach, R. L., Sommer, R., ... & Farnleitner, A. H. (2011). Hypothesis-driven approach for the identification of fecal pollution sources in water resources. *Environmental science & technology*, *45*(9), 4038-4045.

Rigotto, C., T. C. Sincero, C. M. Simoes, and C. R. Barardi. 2005. Detection of adenoviruses in shellfish by means of conventional-PCR, nested-PCR, and integrated cell culture PCR (ICC/PCR). Water Res. 39:297–304.

Sauer, E. P., VandeWalle, J. L., Bootsma, M. J., & McLellan, S. L. (2011). Detection of the human specific Bacteroides genetic marker provides evidence of widespread sewage contamination of stormwater in the urban environment. *Water research*, *45*(14), 4081-4091.

Srinivasan, S., Aslan, A., Xagoraraki, I., Alocilja, E., & Rose, J. B. (2011). Escherichia coli, enterococci, and Bacteroides thetaiotaomicron qPCR signals through wastewater and septage treatment. *Water Research*,*45*(8), 2561-2572.

Surface Water Quality Division (SWQD). 1994.Saginaw River/Bay Remedial Action Plan, Draft 1995 Biennial Report, Volume I. Lansing, Mich.: MDNR.

U.S.EPA, 2001a. Manual of methods for virology, Chapter 14. EPA 600/4-84/013 (N14).

U.S.EPA, 2001b. Manual of Methods for Virology, Chapter 15: Total Cultural Virus Quantal Assay. EPA 600/4-84/013 (N15).

U.S.EPA, 2012. Guidelines for Water Reuse, Chapter 1: EPA/600/R-12/618.

Van Donsel, D. J., & Geldreich, E. E. (1971). Relationships of salmonellae to fecal coliforms in bottom sediments. WATER RESEARCH, VOL 5, NO 11, P 1079-1087, NOVEMBER 1971. 3 FIG, 3 TAB, 21 REF.

Van Loosdrecht, M. C., Lyklema, J., Norde, W., & Zehnder, A. J. (1989). Bacterial adhesion: a physicochemical approach. Microbial Ecology, 17(1), 1-15.

Verhougstraete, M. P., & Rose, J. B. (2014). Microbial investigations of water, sediment, and algal mats in the mixed use watershed of Saginaw Bay, Michigan. Journal of Great Lakes Research, 40, 75-82.

Verhougstraete, M. P., Martin, S. L., Kendall, A. D., Hyndman, D. W., & Rose, J. B. (2015). Linking fecal bacteria in rivers to landscape, geochemical, and hydrologic factors and sources at the basin scale. *Proceedings of the National Academy of Sciences*, *112*(33), 10419-10424.

Wade, T. J., N. Pai, J. N. Eisenberg, and J. M. Colford, Jr. 2003. Do U.S. Environmental Protection Agency water quality guidelines for recreational waters prevent astrointestinal illness? A systematic review and meta-analysis. Environ. Health Perspect. 111:1102–1109.

Wade, T.J., Calderon, R.L., Sams, E., Beach, M., Brenner, K.P., Williams, A.H., Dufour, A.P., 2006. Rapidly measured indicators of recreational water quality are predictive of swimming-associated gastrointestinal illness. Environ. Health Perspect.114, 24–28.

Wade, T.J., R.L. Calderon, KP Brenner, E. Sams, M. Beach, R. Haugland, L. Wymer, and A. P. Dufour. 2008. High Sensitivity of Children to Swimming-Associated Gastrointestinal Illness Results Using a Rapid Assay of Recreational Water Quality. *Epidemiology*, 19: 375-383.

Wade, T.J., Sams, E., Brenner, K.P., Haugland, R., Chern, E., Beach,M.,Wymer, L., Rankin, C., Love, D., Li, Q., Noble, R., Dufour, A., 2010. Rapidlymeasured indicators of recreational water quality and swimming-associated illness at marine beaches: A prospective cohort study. Environ. Heal. 9, 66.

Whitman, R.L., Nevers, M.B., Przybyla-Kelly, K., Byappanahalli, M.N. (2011). Physical and biological factors influencing environmental sources of fecal indicator bacteria in surface water. In: Sadowsky, M.J., Whitman, R.L. (Eds.), The Fecal Bacteria. American Society for Microbiology, Washington, DC, pp. 111–134.

Witt, D. J., & Bousquet, E. B. (1988). Comparison of enteric adenovirus infection in various human cell lines. Journal of virological methods, 20(4), 295-308.

Wong, K., & Xagoraraki, I. (2010). Quantitative PCR assays to survey the bovine adenovirus levels in environmental samples. Journal of applied microbiology, 109(2), 605-612.

Yampara-Iquise, H., Zheng, G., Jones, J. E., & Carson, C. A. (2008). Use of a Bacteroides thetaiotaomicron-specific α-1-6, mannanase quantitative PCR to detect human faecal pollution in water. *Journal of applied microbiology*, *105*(5), 1686-1693.

Zehms, T. T., McDermott, C. M., & Kleinheinz, G. T. (2008). Microbial concentrations in sand and their effect on beach water in Door County, Wisconsin. Journal of Great Lakes Research, 34(3), 524-534.

CHAPTER 4

MICROBIAL POLLUTION SOURCE IDENTIFICATION AT A TMDL SUB-WATERSHED IN MICHIGAN UNDER VARYING HYDROLOGICAL CONDITIONS

Abstract

A significant portion of the Red Cedar River Watershed, located in Ingham and Livingston Counties, Michigan, is impaired due to elevated *E. coli* levels. This study was conducted in Sloan Creek, a sub-watershed of the Red Cedar River watershed. The sub-watershed area encompasses intensive agriculture, dairy and beef farming, homes with on-site septic systems, and one stream segment, Sloan Creek, listed as impaired due to high *E. coli* concentrations that exceed water quality standard. Bacterial Total Maximum Daily Load (TMDL) for the sub-watershed has been approved by USEPA in 2014. The current work aims to determine whether microbial water quality standards are being met, and to identify the probable source of microbial contamination in Sloan Creek sub-watershed by using: a fecal indicator (*E.coli*), host-specific human and bovine Bacteroidales genetic markers, and microbial diversity identification using Illumina sequencing and metagenomics analysis. These methods were used in combination with environmental information include land use, precipitation, and stream flowrate. Water samples were collected from three sites in the sub-watershed twice a week and following rain events during spring and summer of 2015. *E. coli* was routinely detected in the three sampled sites. 68% of samples (n= 192) exceed the recreational water quality guidelines for *E. coli* by several orders of magnitude. High concentrations of human and bovine-associated *Bacteroides* were detected in the three sites indicating influence of multiple sources of fecal contamination. Metagenomics analysis showed

presence of bacterial sequences of human and animal origin. Rainfall, and flowrate were significantly correlated to *E. coli* concentrations at the creek. Results suggest that the probable sources of contamination are leakage from septic systems and runoff from a concentrated animal feeding operation nearby Sloan Creek.

Introduction

Michigan water quality is assessed by using *E. coli* concentration to determine partial body contact (PBC) the entire year, and total body contact (TBC) during May1 to October 31. Under the Clean Water Act Section 303(d) It is required that the Michigan Department of Environmental Quality (MDEQ) determines if the water quality standards are being met. If the *E. coli* levels in a water body exceed the standard, then MDEQ must develop a total maximum daily load TMDL for a bacteria-impaired water body (USEPA 1997). Once a TMDL is established, a watershed protection plan must be developed and adopted to reduce contaminant sources in order to attain TMDL goals (USEPA 2008). This plan must include source identification and development of strategies for mitigation of these sources (USEPA 2001).

MDEQ estimates that about half of Michigan's river miles are impaired according to *E. coli* concentrations in 2014 (MDEQ, 2014). This makes identification of sources of fecal contaminants in small creeks and sub-watershed of significant importance for calculating the pollution load that rivers can receive from the sum of point and non-point sources in the sub-watersheds and remain in compliance with water quality standards (USEPA 1991). Microbial contamination in beaches and drinking water intake were given more attention than streams and creeks in sub-watershed because it paus a great risk to human health due to direct human exposure (Kistemann, et al., 2002, Wong, et al., 2009, Almeida and Soares, 2012). However, investigating and identifying the microbial contamination in water streams is a first tire for protecting human health, given the fact that creeks are considered the main responsible source for contaminates load that entering the lakes through rivers.

Identifying the origin of pollution in complex watersheds requires continuous monitoring and costly comprehensive investigation via long term field sampling at several locations under various hydrological conditions (wet and dry seasons) or (base flow and stormflow) (Soranno PA, et al., 2011). A synoptic sampling scheme have been suggested to characterize water quality under a single flow condition at a single point in time across wide-ranging areas (Verhougstraete et al., 2014, Grayson et al., 1997). Compared with long-term comprehensive investigations, the synoptic approach reduces the number of samples, cost, and time to examine pollution sources, On the other hand, synoptic sampling may miss discharges from human sources, and information about the effect of hydrological condition on microbial load can be missing too.

Indicator organisms are monitored when determining pathogen contamination (Simpson et al. 2002; Stoeckel and Harwood 2007). *E. coli* are typically sampled to indicate the presence or absence of contamination from fecal matter. Using an indicator organism to solve fecal contamination problems in surface waters presents several challenges: first, fecal coliform is an indicator of fecal pollution and not a direct measure of fecal contamination because of the poor correlation with pathogens (Harwood, V. J., 2005, Noble R. T et al., 2001, Pusch, D et al.,2005, Lemarchand, K et al.,2003). Second, fecal coliform does not identify sources of fecal pollution. Therefore, it is imperative that prior to any remediation strategy, microbial source tracking study (MST) is designed to identify the potential sources of pollution.

Appropriate rapid MST methods to distinguish human and non-human contamination would be host-specific PCR Bacteroidales molecular markers. Recently *Bacteroides* have been used to isolate a specific marker and investigate land use and water quality impairments (Peed LA, et al., 2011, Verhougstraete et al., 2014). A study conducted by Furtula et al. (2012), confirmed ruminant, pig, and dog fecal contamination in an agriculturally dominated watershed (Canada) using *Bacteroides* markers. Another study by Verhougstraete et al., 2014, provides a water quality assessment for a large number of watersheds in Michigan found that human fecal contamination was prevalent using a synoptic sampling approach, the base flow in the studied watersheds was generally dominated by groundwater and not by wastewater treatment effluent.

More recently an emerging extension of MST concept has been used to simply characterize the entire microbial community (eg next generation sequencing (NGS)) in a water sample and determine any potential similarities. the use of community analysis in MST is very recent development and remains an active research area. Next generation sequencing may potentially overcome the limitation of the single marker and improve sensitivity for host-specific molecular methods, but they also require the highest level of expertise for managing and analyzing large, complex data sets. As a result of these limitation, community analysis should be conceder method of last alternative, only to be employed when it is suspected that information that can be gained could not gathered from simpler and more cost effective approaches. It is likely best suited to large TMDL projects or water with high economic value.

To date, most published studies of microbial contaminants are limited by reliance on quantification of FIB and MST markers that target single source only and by low numbers of samples. And some other studies investigated large watershed using synoptic sampling approach. To the best of our knowledge this is the first study conducted in Sloan creek sub-watershed implemented a continues sampling scheme and host specific markers along with NGS analysis as a confirmation step to assess microbial contaminant concentrations and defined the *E. coli* source(s) within the watershed during both base flow and storm events runoff. In response of TMDL, this paper aim to: 1) quantify levels of *E.coli* in Sloan creek under base flow and storm water conditions, 2) Identify the source of fecal contamination in Sloan creek using human and bovine specific *Bacteroidis* markers and NGS, 3) determine the effect of hydrological factors (precipitation and flowrate) and land use on microbial water quality at small sub-watershed.

Material and Methods

Site description and data source

The Red Cedar River flows about 50 miles through rural and agriculturally dominant land in the south-central lower peninsula of Michigan USA. The Red Cedar drains into the Grand River and, eventually, Lake Michigan. For this study, Sloan Creek sub-watershed of Red Cedar River watershed in Ingham county is selected for investigation due to elevated *E. coli* that exceed the Michigan WQS for total and partial body contact (ICD Red Cedar Monitoring Project 2013, MDEQ, 2014). The MDEQ ranked this sub-watershed as a top priority subgroup in the TMDL area based on their stressor analysis. They are two main water streams within the sub-watershed Sloan Creek and Button drain, the two streams drain agriculture and residential areas into Red Cedar River. Figure 1&2 shows the Red Cedar River watershed and Sloan Creek sub-watershed.

According to Red Cedar river watershed management plan 2015, Sloan creek sub-watershed contains a human population of 2,127, living at a density of 112 people per square mile. About 393 homes are estimated to be serviced by septic systems. This sub-watershed has an estimated 3,080 large animals, including 3,000 cows, 40 horses and 40 pigs, sheep, goats and alpacas. Most of the cows are housed at a Concentrated Animal Feeding Operation Mar-Jo-Lo Farms (CAFO), although smaller farms are also present. Large animal density is estimated to be

174 animals per square mile, the highest of any of the Red Cedar River sub-watersheds. Excluding the CAFO, there are an average of 10 animals per farm, and 12 animals per square mile. Suspected sources of bacteria in the sub-watershed include human, agricultural and wildlife inputs. There are no known point-source sewage inputs to Sloan creek or Button drain, but both streams apparently have animal and human nonpoint sources (Red Cedar River Management Plan, 2015).

According to TMDL for *E. coli* in Portions of the Red Cedar River and Grand River Watersheds 2012; Mar-Jo-Lo Farms CAFO manifested about 5.3 million gallons of liquid waste in 2009. A total of 1.8 million gallons of liquid waste, and 5,500 tons of solid waste were not manifested, and were spread by Mar-Jo-Lo Farms CAFO. The Comprehensive Nutrient Management Plan (CNMP) 2009 Annual Report has identified 613 acres of land as available for the spreading of their non-manifested waste. All of these identified available acres are within the TMDL source area. In May-June and August-November of 2009, manure was land applied to nearly all of the available acres, and had the potential to impact *E. coli* concentrations in Sloan Creek as well as downstream areas.

Red Cedar river watershed and Sloan creek sub-watershed are defined and characterized with Esri ArcMap GIS software using various data source. The National Hydrography dataset (NHD) from USGS was used for channel and stream network (Figure 1). The National Elevation dataset (NED), 30 m resolution, from the USGS was used for the Digital Elevation Model (DEM) for slope and surface runoff direction estimation (Figure 2).

Land use data was based on 30-meter resolution National Land Cover Database (NLCD 2011; http://www.mrlc.gov/nlcd11_data.php). Land Cover NLCD Classification System include 16 thematic classes were reclassified using the Anderson Land Use/Land Cover Classification system, into 8 land cover categories (Figure 3&Table1). Sloan Creek sub-watershed is classified as a rural and agriculturally dominant area.

Precipitation data, were obtained from Michigan Automated Weather network (MAWN) (Enviro-weather, 2015), East Lansing MSUHort, Michigan station, (42.6734, -84.4870), (www.agweather.geo.msu.edu/mawn/). Discharge in Sloan Creek was collected from United States Geological Survey gauging station (USGS 04112000) located at Sloan creek near Williamston city, Michigan (42.6758, -84.3638).

Water sample collection and processing

Two tributaries, Sloan creek and Button drain, within the sub-watershed were selected for sampling. Samples were collected in sterile one liter bottles, the bottles were autoclaved at the lab and rinsed three times with source water before use. Three grab water samples were collected in duplicate, one from each tributary and the last sample has been taken from the mouth when Sloan creek meet Red cedar river (Figure 2b). Two water samples were collected at each location: one for *E. coli* analysis and one for *Bacteroides* analysis.

A comprehensive long term sampling scheme was designed to collect samples at least twice per week and following each rain event during spring and summer 2015, from March 22 to August 26. A total of 192 samples (64 from each sampling location) were collected. The base flow conditions for *E. coli* and *Bacteroides* and water discharge were recorded by samples collected in March, three weeks before the study was started. Sampling sites were established down streams considering watershed elevation slop, watershed plans and accessibility. All sampling sites were located at bridge crossings. The three sampling location are named to correspond with the road intersections as following: Location 1 (Sloan), at the mouth of Sloan

Creek when its drains into Red Cedar River, Location 2, Sloan creek tributary (Meridian), and Location 3 Button drain (Every). Samples were stored on ice and analyzed in the laboratory within 2 to 4 hrs. of collection.

Water analysis

Water samples were analyzed for *E.coli* Concentration using defined substrate method Colilert-18TM Quanti-Tray 2000 (IDEXX Laboratories, Inc.). *E. coli* were measured in duplicate directly or diluted with phosphate-buffered saline solution (PBS) (pH = 7.2) to three serial dilutions 100, 10^{-1}, 10^{-2}, each sample mixed with reagent, had shaken 10 times, and poured into the Quanti-Tray/2000 tray. Samples incubated at 35 °C (±0.5 °C) for 24 h (±2 hr). Microbial enumeration was conducted following the manufacturer's protocol and *E. coli* reported as MPN/100ml.

Molecular analysis

All water samples were tested for human and bovine-associated *Bacteroides* molecular markers quantitatively using qPCR. 500 mL of water sample were filtered through 0.45 µm hydrophilic mixed cellulose esters filter (Pall Corporation 66278) under partial vacuum. The filter was placed into a 50 mL sterile disposable centrifuge tube containing 45 ml of sterile phosphate buffered saline PBW, vortexed on high for 10 min, and then centrifuged (30 min; 4500 ×g; 20°C) to pellet the cells. Sample were concentrated down to 2 mL by decanted forty-three milliliters from the tube and the remaining pellet were stored at -80 °C until DNA could be extracted. After thawing samples, 100 µL DNA was extracted from 400 µL pellet using MagNa Pure Compact System automatic machine (Roche Applied Sciences, Indianapolis, IN) with the corresponding kit (MagNA Pure Compact Nucleic Acid Isolation Kit I). Two host-associated quantitative polymerase chain reaction (qPCR) methods were utilized to identify and quantify sources of fecal pollution within the sub-watershed. Human-associated *Bacteroides* B. thetaiotaomicron α-1–6 mannanase TaqMan assay according to Yampara-Iquise et al., 2008 were used with the published primers / probe set: BtH-F forward primer TCGTTCGTCAGCAGTAACA and BtH-R Reverse primer AAGAAAAAGGACAGTGG; and BtH-P probe 6FAM-ACCTGCTG-NFQ. Bovine-associated *Bacteroides* 16srRNA (BoBac) TaqMan assay (Layton et al., 2006) were used to detect the presence and magnitude of bovine fecal contamination, with the primers / probe set as described in citation: forward primer BoBac367f (GAAG(G/A)CTGAACCAGCCAAGTA), Reverse primer BoBac467r (GCTTATTCATACGGTACATACAAG), and BoBac402Bhqf probe (TGAAGGATGAAGGTTCTATGGATTGTAAACTT). All qPCR quantification analysis was carried with LightCycler® 1.5 Instrument (Roche Applied Sciences, Indianapolis, IN) and LightCycler 480 Probes Master kit with a total reaction volume of 20 ml. DNA extracted from samples was analyzed in triplicate with 5 ml of extract used for template. Corresponding qPCR programs are described in citations. The crossing point (Cp) value for each qPCR reaction automatically determined by the LightCycler® Software 4.0. One copy of the targeted gene is assumed present per cell, and thus one gene copy number corresponded to one equivalent cell, then gene copies were converted to and reported as copies/100mL.

In order to prepare the standards, the DNA was extracted from ATCC (number 29148D-5) genomic DNA, for B. thetataiomicron, and from bovine feces obtained from Michigan State University dairy farm for BoBac. The amplified PCR products for the target genes were cloned into one shot chemically competent *E.coli* using TOPO TA Cloning kit for Sequencing

(Invitrogen Inc., Carlsbad, CA, USA), according to the protocol provided by the manufacturer. Plasmids were extracted with QIAprep Spin MiniPrep kit (Valencia, CA, USA) and were sequenced at the Research Technology Support Facility (RTSF) at Michigan State University that confirmed the insertion of the target inside the vector. The plasmids were quantified using Qubit Fluorometric Quantitation (Thermo Fisher Scientific) and then serially diluted ten-fold to construct qPCR standard curves. Triplicates of dilutions ranging from 10^8 to 10^0 were used for the standard curve. plasmid standard was included during each qPCR run as a positive control and molecular-grade water was used in place of DNA template for negative controls.

Microbial community analysis

Bacterial DNA extracts for the samples from August 16th, 17th, and 18th of 2015 were sequenced on an Illumina platform (Illumina HiSeq, Roche Technologies) at the Research Technology Support Facility (RTSF) at Michigan State University. DNA-Seq libraries were prepared using the Rubicon Genomics ThruPLEX DNA-seq Kit. After preparation, libraries underwent quality control and were quantified using Qubit dsDNA, Caliper LabChipGX and Kapa Biosystems Library Quantification qPCR kit. The libraries were pooled together and this pool was loaded on an Illumina MiSeq v2 standard flow cell. Sequencing was done in a 2x250bp format with a v2 500 cycle reagent cartridge. Base calling was performed by Illumina Real Time Analysis (RTA) v1.18.54 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v1.8.4. The flexible read trimming tool for Illumina NGS data called Trimmomatic was used for trimming the raw reads from the Illumina sequencer and removing adapters (Bolger et al. 2014). The trimmed reads were assembled into contig files so as to reduce the chances of false positive detection using an iterative de Bruijn graph de novo assembler for short reads sequencing data with highly uneven sequencing depth called IDBA-UD (Peng et al. 2012).

The assembled contig files were uploaded to the MG-RAST web server for analysis. MG-RAST is an online database for the annotation and analysis of metagenomes (Meyer et al. 2008). MG-RAST searches the non-redundant M5NR and M5RNA databases in which each sequence is unique using the best hit classification option which reports the functional and taxonomic annotation of the best hit in the M5NR for each feature. Data was analyzed based on organism abundance and on the functional distribution at the subsystem hierarchy with maximum E-value cutoff of 1E-5, minimum percent identity cutoff of 60% and minimum alignment length cutoff of 15 bps. The number of hits is defined as the number of occurrences of the input sequence in the database. Analysis includes the phylogenetic comparisons and functional annotations against the database and the results are expressed in the form of abundance profiles. The abundance listed by MG-RAST presents the estimate of the number of sequences that contain a given annotation, found by multiplying each selected database match (hit) by the number of representatives in each cluster (Wilke et al. 2013).

Wet weather hydrology

Out of 192 total sampling events 147 occurred during wet weather, defined for this study as measurable rainfall on the same morning as the sampling event; among them four events are conceder as a first flush. 3/31/2015, 4/23/2015, 5/4/2015, and 5/13/2015, defined as the first rainfall after three dry days (John F. Griffith, 2009). The hydrograph and the rain events are presented in figure 4, both the flowrate and rainfall fluctuated during the sampling period. In general, two main storm are observed during the sampling period, which caused a huge increase to the flowrate in the creek. The first storm was on 6/14/2015 with precipitation of 1.67 in and the second on 6/22/2015 with perception of 1.76 in. Besides rainfall, groundwater can be a big source of the flowrate, which provided the base-flow during the sampling time. Flowrate reached the highest value of 158 cfs on June 23, one day after the largest rain event. However, after June 28 to the end of the sampling, there were multiple rainfall events with several peaks of the flowrate, but not much increase. It shows the rainwater was captured by the soil and vegetation on the ground and did not contribute to the flowrate in the river.

Statistical analysis

All statistical analyses were performed using SPSS Statistics software (Version 19.0) with a significance $\alpha = 0.05$. *E. coli* and *Bacteroides* concentrations were log-transformed to achieve normality and meet the assumptions of a parametric test. Simple t-tests were used to determine the differences in mean concentrations of target organisms among each other and with the perception and the flowrate. The t-test was two- tailed, with alpha levels, or the probability of rejecting the null hypothesis when it is true, set at $p < 0.05$. Pearson's correlation coefficient was used to test the relationship between *E.coli* and *Bacteroides* markers. A linear regression analysis was performed using scatter plots of $\log_{10}$ cells/100 mL of *Bacteroides* and $\log_{10}$ MPN/100mL of *E. coli* and to determine the potential source of increased *E. coli* concentrations relative to discharge.

Results

Seasonal effects on Microbiological water quality

Three sites within Sloan creek sub-watershed were monitored for the presence of microbial fecal indicator (*E. coli*) to assess microbial water quality at Sloan creek sub-watershed. Monitoring results for *E. coli* concentrations across the three sites are presented with the rain events and the water hydrograph in Figure 5. The highest concentrations of *E. coli* were found in Sloan site (at the mouth of Sloan creek). The concentration of *E. coli* reached 7270 MPN/100mL on June 18th. *E. coli* concentrations also peaked on this day at Meridian site (6131 MPN/100 mL) and at Every site (2500 MPN/100 mL). High concentrations of *E. coli* were found within 24 to 72 hrs following each rain event. If Michigan's single sample limit of 130 MPN/100 mL (Michigan Department of Environmental Quality 2002) is used, then of our192 samples: 73% (49 of 64) of Sloan samples, 78% (50 of 64) of Meridian samples, and 50% (32 of 64) of Every samples exceeded the water quality guidelines for the state of Michigan.

E. coli concentration during spring under base flow conditions in the absence of recent storm runoff were assumed as a reference condition, 3 weeks before the sampling event was started, the Sloan site was sampled 3 days / week for *E. coli* to help figure out the *E. coli* reference condition, then reference condition were calculated as geometric mean of all 9 samples (21 MPN/100mL). *E. coli* levels for Sloan, Meridian, and Every sites were compared for samples collected in dry weather (base flow) versus wet weather (rainfall events). There was often a corresponding increase in *E coli* concentrations flowing each rain event, but not all high *E coli* concentrations were associated with rain events. Of the 49 occasions when *E coli* concentration exceeded (130 MPN/100 mL), there were rain data available for 40.

For all three sites all *E. coli* concentrations were significantly elevated and has strong correlated with the water discharge (Sloan $p < 0.001$, $r=0.65$; Meridian $P< 0.004$, $r= 0.5$ Every $P<0.001$, $r= 0.68$). Over all There was no significant difference in levels of *E. coli* among the three sites. *E. coli* concentrations for Sloan site was significantly correlated with Meridian and Every ($r^2 = 0.85$ for Meridian, $r^2 = 0.9$ for Every) (Figure 6). *E.coli* has significant relation with BoBac and B.theta in Sloan and Meridian sites ($P< 0.05$) and r ranged from 0.31 to 0.5. For Every site BoBac has insignificant relation with *E.coli* level but B.theta has significant relation with *E. coli* ($r=0.618$, $P<0.01$). All statistical analysis for *E.coli* are shown in Table 4.1.

Quantitative microbial source tracking

Sloan creek receives drainage from 19 square miles of Sloan creek sub-watershed (agricultural, rural, and suburban land use). The detection of *Bacteroides* specific markers compared to culturable *E. coli* levels in Sloan site, which represent the total load that Sloan creek carry to Red Cedar river, over the 64-days sample period are shown in Figure 7a&b. Our result showed a significant correlation between the two markers (B.theta and BoBac) and *E.coli* ($P<0.05$). On average, the levels of the human (B.theta) and bovine (BoBac) *Bacteroides* genetic makers showed large differences among sites, however, these differences were not statistically significant ($P>0.05$). A total of 64 samples in each sampling sites were tested for human and bovine *Bacteroides* markers of which 49 were collected during rainfall runoff periods and 14 during base flow. Human *Bacteroides* (B. theta) were present in 25% of Sloan, 27% of Meridian, and 14% of Every samples (Figure7a). Overall average concentrations of B.theta human marker at Sloan site were the highest among the three sites with average of 1.1×10^7 genomic copies/100mL. At Meridian site the average of the B.theta human marker concentration was 1.87×10^5 genomic copies/100mL, while Every site was the lowest among the three sites with average concentration of 2.5×10^{1} genomic copies/100mL. Occurrence of BoBac in the three site was 28% at Sloan, 22% at Meridian, 11% at Every samples. BoBac concentrations ranged from not detected to 7.6×10^{9} genomic copies/100mL in runoff event samples at Sloan creek, and 7.52×10^{7} genomic copies/100 mL at Meridian, and 5.3×10^{3} at Every. At the mouth of Sloan creek, the difference in runoff event compared to base flow occurrence was dominated by bovine *Bacteroides*. The seasonal differences in *Bacteroides* markers over the watershed were compared for the three sites (Figure 7a&b). Wet weather had a significant effect on *Bacteroides* marker levels, when compared the concentration of the two markers with the flow rate, the relation of B.theta and BoBac markers occurrence and mean concentrations in samples from all three sites were greater in runoff samples than in samples collected during base flow periods. There were conclusive seasonal trends in the *Bacteroides* markers for the three sites. Human and bovine markers were elevated in the wet season at Sloan site ($p < 0.04$, $r=0.58$ for B. theta, $P < 0.027$, $r=0.76$ for BoBac). The same trend was observed in Meridian site, both human and bovine *Bacteroides* markers were significantly higher in wet season when the flowrate was high ($p < 0.032$, $r=0.46$ for B. theta, $P < 0.045$, $r=0.69$ for BoBac). There was no significant different for both B.theta and BoBac during base flow and storm flow in Every site.

Metagenomics analysis

Results from MG-RAST analysis are shown in Figures 8a &b. Bacterial sequences comprised over 80% of the total sequences in the three samples analyzed. The genus *Bacteroides* accounted for 3.89% on the 16th, 0.37% on the 17th, and 10.89% on the 18th of all bacterial sequences. Numerous *Bacteroides* species were detected by MG-RAST (Figure8 b), with one of the most common being human *Bacteroides* fragilis, which accounted for 33% of all *Bacteroides* sequences on the 16th. And Bovine *Bacteroides* (Mycobacterium bovis) with 117 hits in the same sample. Several *E.coli* wild-type strains were detected in the three analyzed samples such as *E.coli* B, *E.coli* W, *E.coli* K12. Table 4.2 listed some of the bacteria and human and bovine-associated *Bacteroides* strains that detected in the three samples.

Discussion

Michigan climate is characterized by hot humid summers, cold winters with snowfall, and wet springs. The state receives a good amount of perception throughout the year, averaging 30-40 inches annually. This climate creates a long period of pollutant build-up deposited on surfaces during dry weather (October -March) and then washed away in spring when the snow starts to melt into rivers and lakes. The initial storms of the spring season usually thought to have higher pollutants concentrations, which create a first flush phenomenon. But this is not always true in mid-Michigan climate because the first flush runoff can be started in early March when the snow starts to melt witch cannot carry pollutants that stick to the soil surface before the soil temperature start to increase during the summer (wet season).

The sampling scheme and study design should be given a special attention when conducting microbial source tracking study, with taking in consideration all the regional hydrological conditions and pollution dynamics because these are crucial factors for the significance of the results (Kay et al., 2007, Reischer et al., 2008).

This study was established and evaluated a comprehensive sampling concept for Sloan Creek sub-watershed with consideration for the whole seasonal hydrological conditions. The sampling scheme design as a continued sampling to characterize and compare between the spring first flush phenomenon, which depend on the snow melt as a driving force, and the summer first flush which depend on the rainfalls as driving force. And to study the impact of creeks on the river water quality. With the overall goal of providing a practical and theoretical monitoring and source tracking template that could be rolled out to other Red Cedar River sub-watersheds of concern within Ingham County and be adopted by other counties in the same region.

The impact of river outfalls on lake water quality depends on numerous interacting factors such as the pollutants loads that the river carries to the lake. The delivery of contaminants by multiple creeks greatly complicates understanding of the source contributions. During the study period Sloan creek continuously delivered water with high concentrations of *E. coli* to Red Cedar river. Time series graph shows that rainfall, and flowrate were significantly related to *E. coli* concentration at the creek (see result section, Figure5). The *E. coli* concentration have a wide range between the base flow and the storm flow, with a sharp increase following each rain event. Similar to our finding, studies have been conducted to describe the variability of microbial indicator concentrations or sources in watershed scale found a strong relationship between fecal indicators and wet weather. Reeves et al. (2004) carried out a series of field studies to identify the spatial distribution of fecal indicator bacteria in dry and wet weather run-off. In the Hoosic River watershed, bacterial levels were found to be higher in summer than in winter, and higher during storms than during base flow conditions (Traister & Anisfeld 2006). By studying the density and sources of *E. coli* in multiple watersheds, Meays et al. (2006) found that the fecal coliforms (FC) counts varied by year, month and site, for each of the watersheds.

Another finding of this study is that, late season storms had a greater frequency of water quality exceedances compared to early season storm events, possibly because in early spring the soil temperature is still low which make the deposited pollution hard to release in to the stream.

Another reason could be the effect of cold temperature on *E. coli*.

The importance of precipitation and streamflow in the transport of protozoan and bacterial pathogens and fecal indicator bacteria in lotic systems has been frequently reported (Ferguson et al., 2003; Dorner et al., 2006; Wu et al., 2011), but only a handful of studies have related these environmental factors to MST markers transport. Similar to previous studies that have shown that bacterial loading rates in rivers increase during hydrologic events (Kistemann et al., 2002; Krometis et al., 2007), MST marker loading rates for the present study also increase with rain events, in the three of the study sites. Interestingly, the highest concentration of *E. coli* was measured on June 18 four days after the first large rain event in the season June 14th (1.67 in), a sharp increase was observed in June 18th (7270 MPN/100mL) comparing with June 17 (272.3 MPN/100mL), then *E.coli* continue to decrease gradually until the end of the season and falling back within the base flow condition. It is worth to mention that in August 10th the largest rain event in the season was recorded (2.27 in) but *E. coli* did not raise much.

Bacteroides markers went through similar trend as *E. coli* trend with some delay on the time series, the highest concentration of the two *Bacteroides* markers BoBac and B.theta are reached their highest concentration during the sampling season on June 27, which had rain event of (0.91 in). Even though *Bacteroides* markers highest hits were not tied with *E. coli* but both *Bacteroides* markers were have a sharp increase on June 15 within 24 hr from the first large rain event, B.theta was raised from not detected on June 14 to $1.35x10^6$ on June 15, and reached the highest on June 27 ($6.99x10^8$). BoBac act similar as B.theta, its raised from not detected on June 14 to $4.3x10^4$ on June 15, and the highest concentration was on June 27 ($7x10^9$). Late on the season on August 10 the largest rain event was recorded (2.27 in), *E. coli* had a sharp increase from 357.5 MPN/100 mL on August 9 to 1046.2 MPN/100mL on August 10, similarly the BoBac have been raised from not detected to 6.97×10^4. No human B.theta marker was detected. This can be an evident that source of *E. coli* in August is bovine feces since no human *Bacteroides* were detected.

Knowledge of the influences of land use on source water quality, especially on sources of fecal contaminants, is also critical for efforts towards proper management within a watershed. The management based on land use can be effective for mitigating rainfall impacts to the water body of watersheds with appropriate water qualify data (Long &Plummer 2004). The degree of microbial contamination may be predictable according to the types of land use of a watershed. A study revealed that bacteria in storm water run-off varied from higher to lower concentrations by the following order of land use types: recreational, agricultural, urban and open space (Tiefenthaler et al. 2008). Land use information is also useful for determining sources of fecal contamination. This study demonstrated that natural contributions of fecal indicator bacteria at watershed with minimal human influence (urban land use = 9%) are sufficient to generate exceedances and degrade the water quality during the storm event.

This paper was able to defined the probable source of contamination in Sloan creek sub-watershed by using fecal indicator (*E.coli*) and host specific marker with NGS as a confirmation step combined with land use and hydrological information. According to the Total Maximum Daily Load for *E. coli* in Portions of the Red Cedar River and Grand River Watersheds 2012; Mar-Jo-Lo Farms CAFO spread a total of 1.8 million gallons of liquid waste, and 5,500 tons of solid waste in May-June and August-November, this manure applied time helped us to interpret our data as following, in June when the heavy rain events started runoff carry out the manure from the surface to the creek, and similarly on August we observed a high *E.coli* and high BoBac with no human marker detected. The human marker was detected and had the potential to impact

E. coli concentrations mainly in June within the heavy rain season, which indicate a leakage from septic system. The land use information helped us to confirm our assumption because human B.theta were detected more frequently in Meridian site which located within the urban area and surrounded with homes. Another approach that give our work confident to identify the *E. coli* source is the use of MST tool along with the microbial community analysis (NGS), since host specific markers have some limitation such as specificity of the qPCR assays, we applied the NGS as robust tool for MST, on the three samples those of which had the highest *E. coli* concentrations following largest rain events. The result of June 16 shows occurrence of bovine Bacteroides (Mycobacterium bovis), and human *Bacteroides* fragilis which belong to the same genus of B. thetaiotaomicron.

Conclusion

The study examined the influences of hydrological conditions and land use on *E. coli* concentration and sources in the Sloan creek sub-watershed in Michigan. *E. coli* concentration was strongly influenced by precipitation and stream flowrate. this study analyzed a high number of samples for multiple markers over a long time period allowing for the observation of long term patterns. These patterns would have been difficult to understand using short-term monitoring synoptic sampling scheme. Monitoring the Sloan Creek during the dry and wet season with a set of molecular markers revealed a complicated pattern of human and animal fecal inputs. This MST study in mixed use watershed was successfully able to provide a Multiple lines of evidence to identify the sources of fecal pollution in Sloan Creek using host specific markers with microbial community analysis (NGS). This study provides a sampling scheme and MST path to assess and improve water quality at Red Cedar River sub-watersheds scales.

APPENDIX

Table 4.1 Statistical value for Ecoli concentration in the three sampling location in correlation with BoBac and B.theta obtained from t-test and Pearson's correlation at level of significant <0.05

Location	Flowrate	BoBac	B.theta
Location 1	r=0.54, P<0.0001	r= 0.316, P<0.001	r=0.317, P<0.011
Location2	r=0.5, P<0.024	r=0.5, P<0.001	r=0.45, P<0.024
Location3	r=0.68, P<0.001	r= 0.013, P<0.918	r=0.618, P<0.01

Table 4.2 Bacterial stains detected by using NGS in the 16, 17, and 18 August samples

Species	Host
Butyrivibrio fibrisolvens	Ruminant (cows and deer)
Mycobacterium bovis	Cattle
BacteroidesPrevotella rumincola	Ruminant
Ruminoccus albus	Cattle, sheep, and goats
Ruminoccus flavefacients	Ruminant and wildlife
Bacteroides thetaiotaomicron	Human
Bacteroides fragilis	Human
Bacteroides vulgatus	Human
Bacteroides helcogenes	Swine

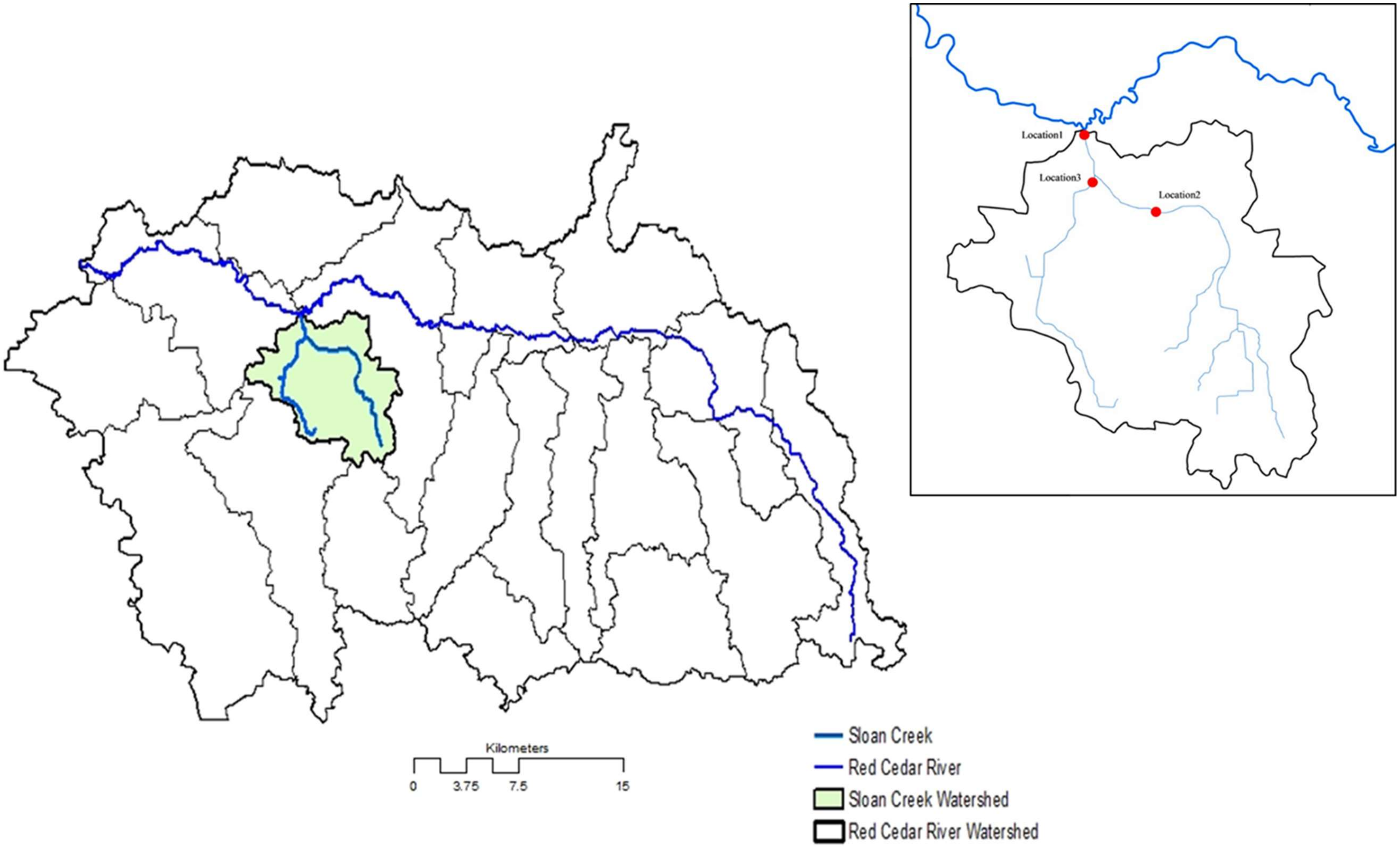


Figure 4.1 Red Cedar River watershed and the sub-watersheds boundaries. Sloan Creek sub-watershed is shown in green

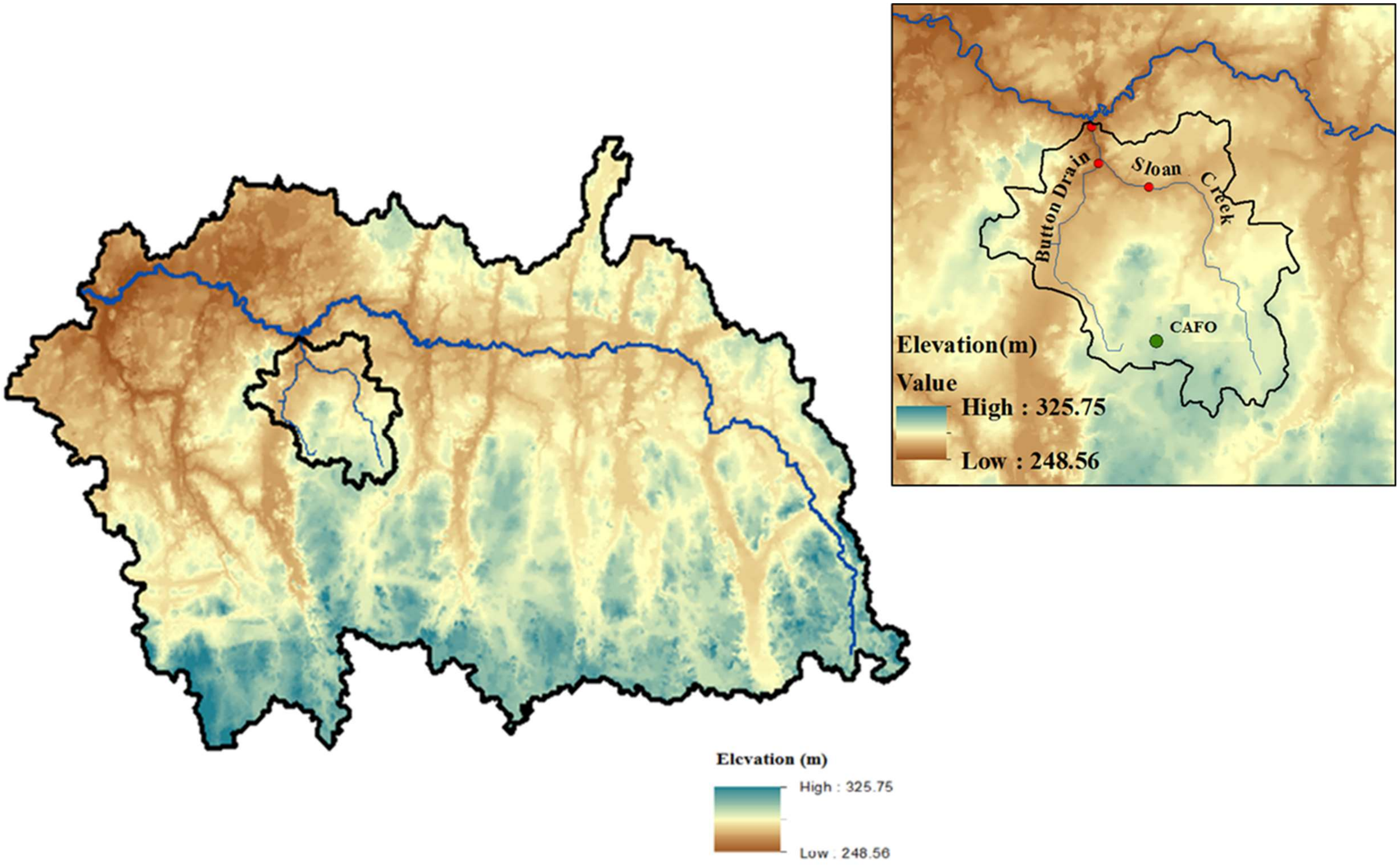


Figure 4.2 Red Cedar River watershed and Sloan Creek sub-watershed Digital Elevation Model (DEM). Sloan Creek sub-watershed has a relatively low relief with the maximum elevation recorded as 324 m and a minimum of 249 m.

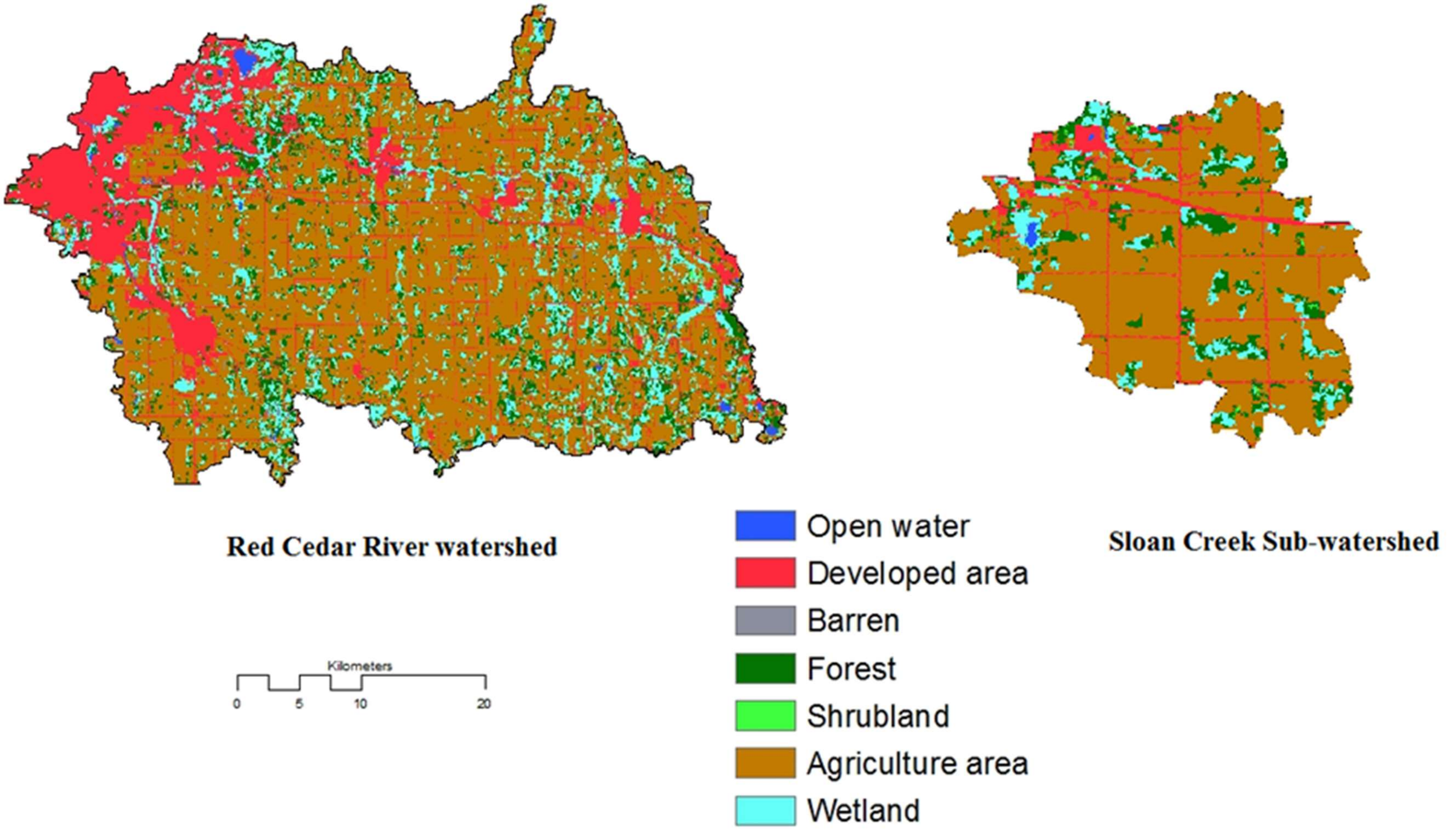


Figure 4.3. Red Cedar River watershed and Sloan creek sub-watersheds Land-use, data source: National land Cover database (NLCD) 2011.

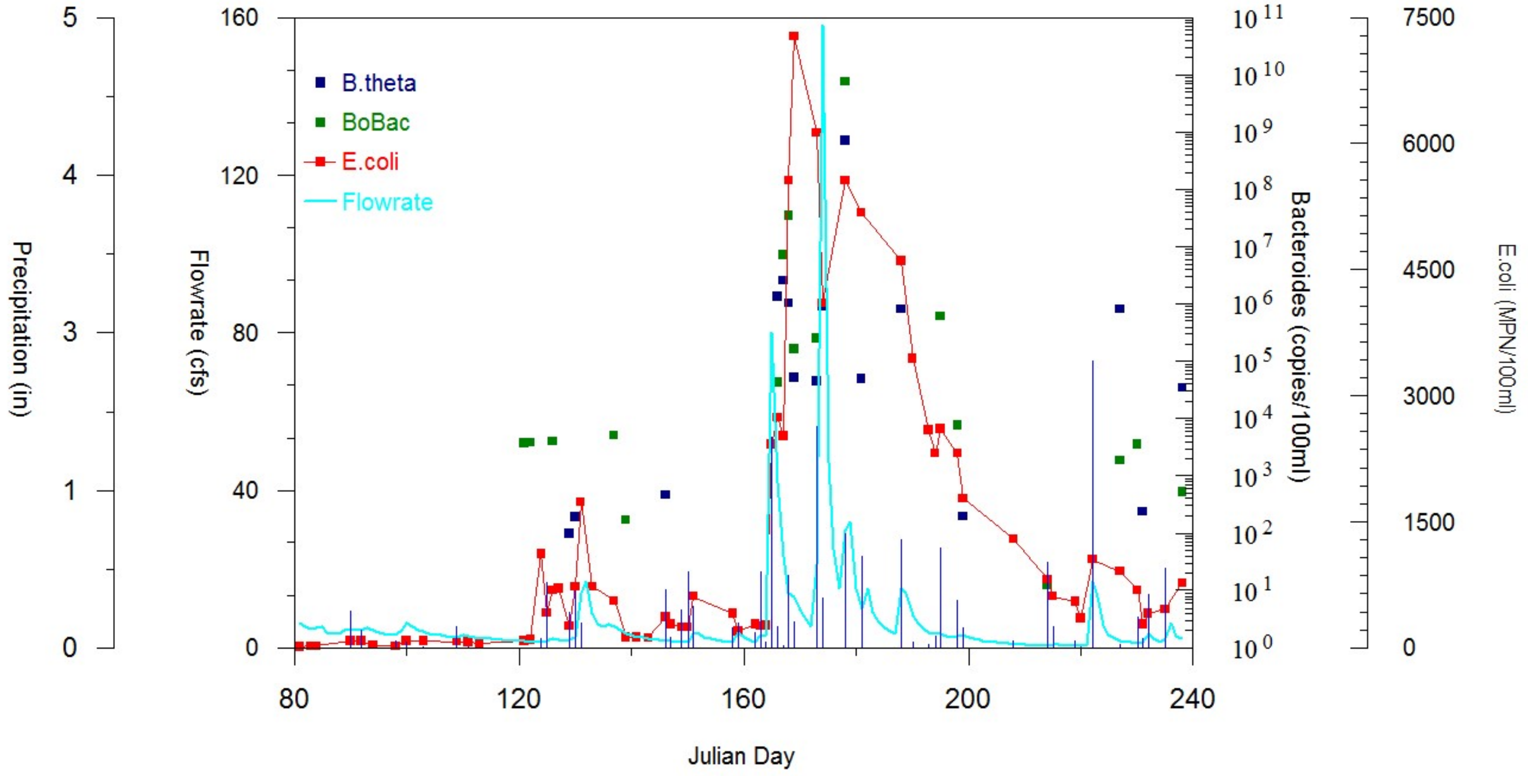
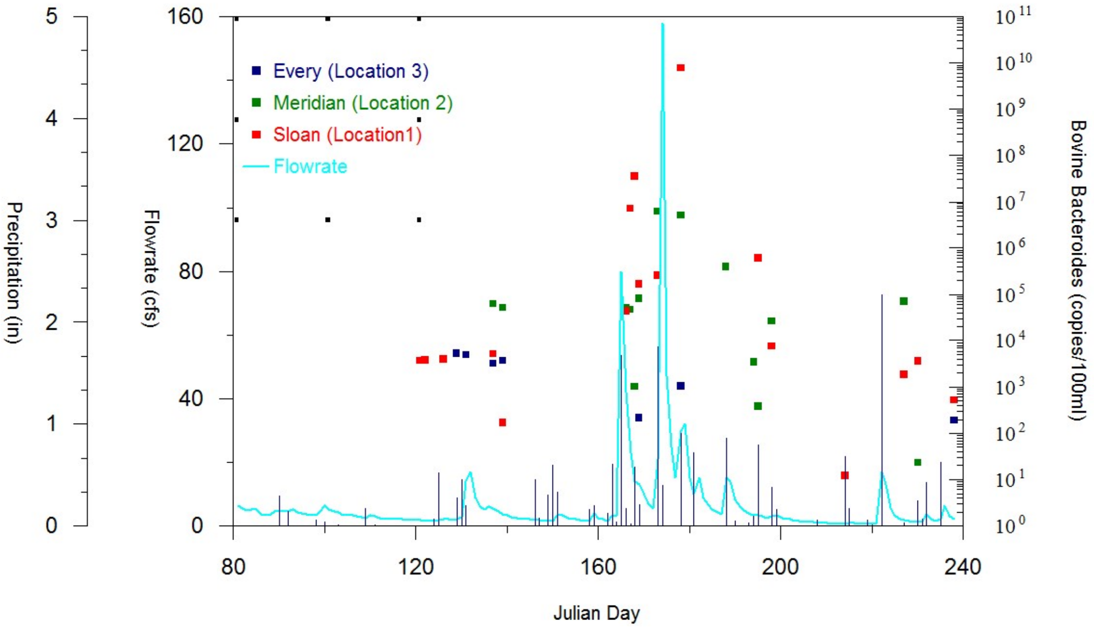


Figure 4.4 Hydrograph of Sloan Creek discharge during the study period. Discharge levels are daily mean values. Small squares mark the 3 measured organisms, *E. coli* in red, Bovine *Bacteroides* in green, human *Bacteroides* in bleu. Drop bleu lines represent precipitations. Time line is shown in Julian day calendar (80 = 3/21/2015, 240= 8/28/2015)



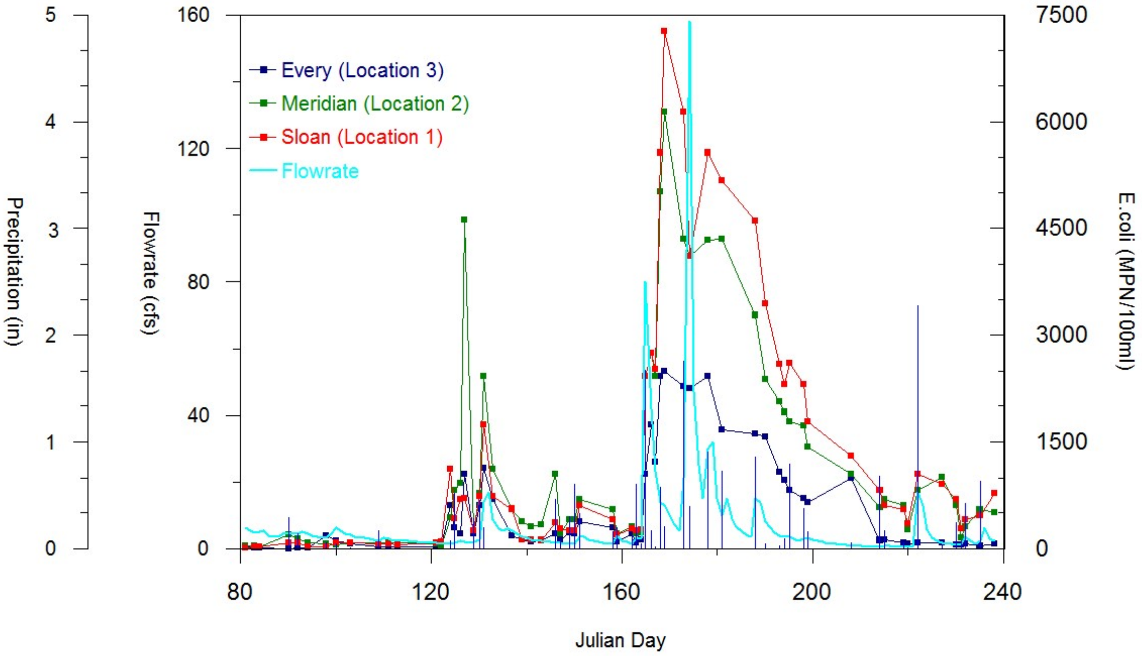
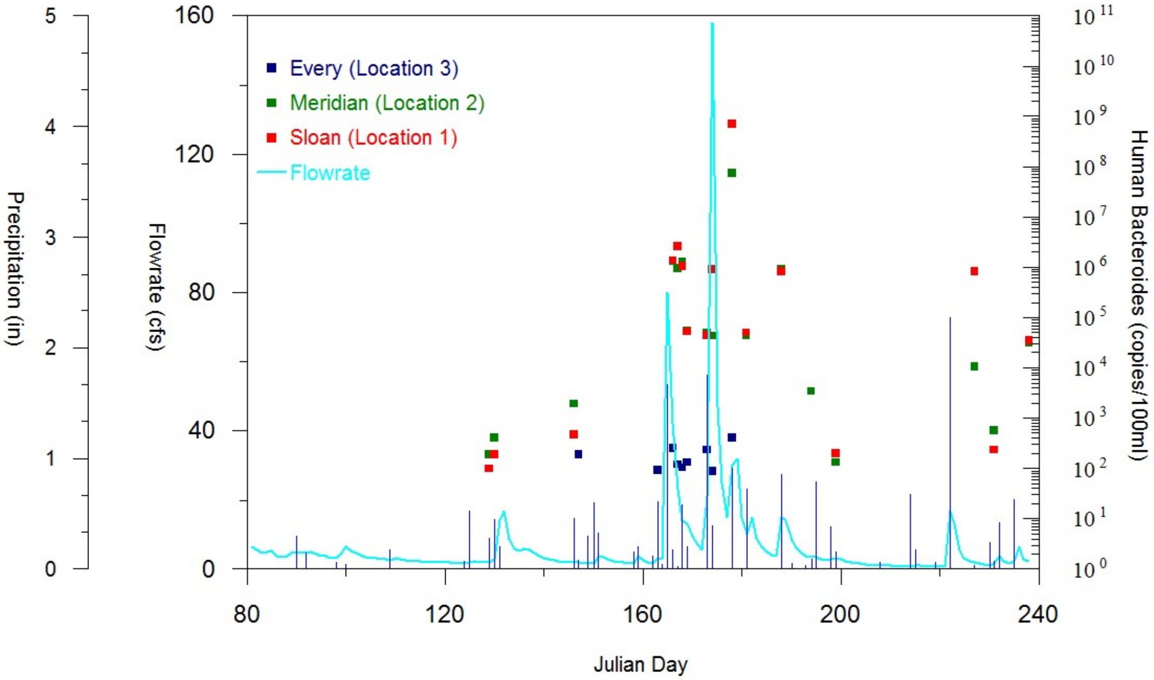


Figure 4.5 a,b,&c Hydrograph of Sloan Creek discharge during the study period. Discharge levels are daily mean values. The figures summery of the monitoring organism in the 3 sampling loctions a) *E.coli* , b) bovine *Bacteroides,* c) human *Bacteroides*.

Figure 4.5 (cont'd)



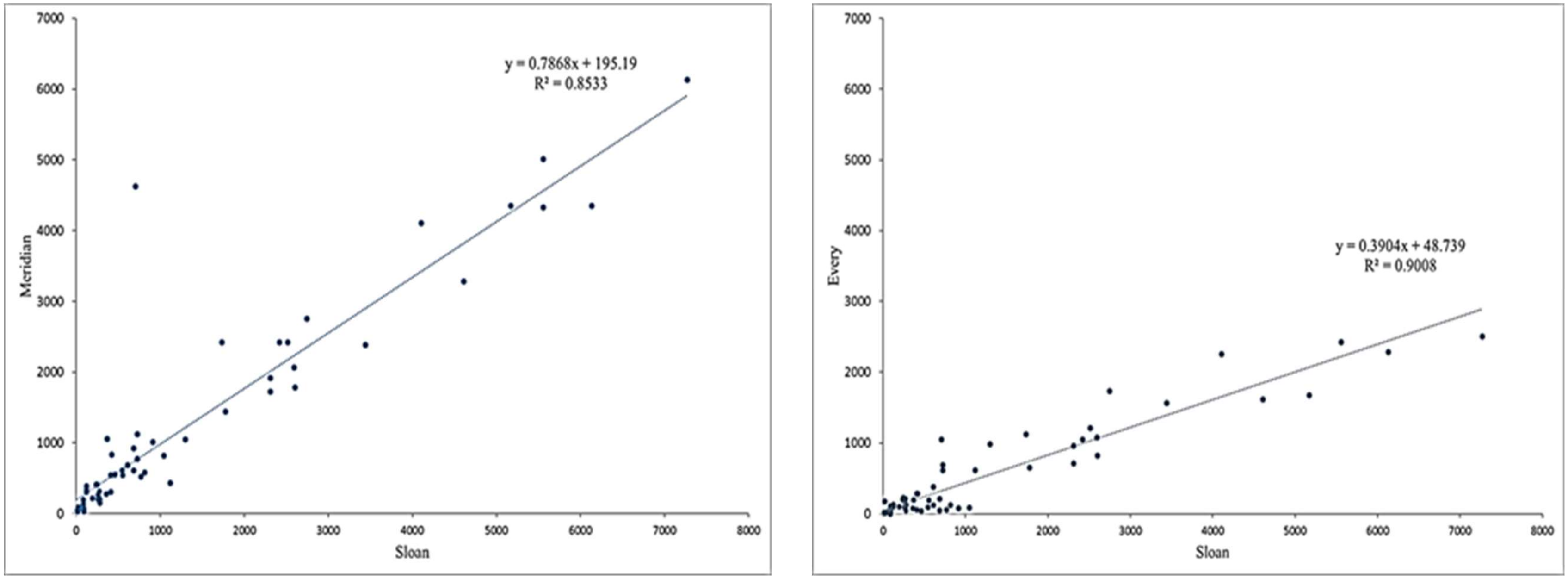


Figure 4.6. corrolation between E.coli concentrations between Sloan (location1) and the other two locations Meridian (location2), and Every (location3).

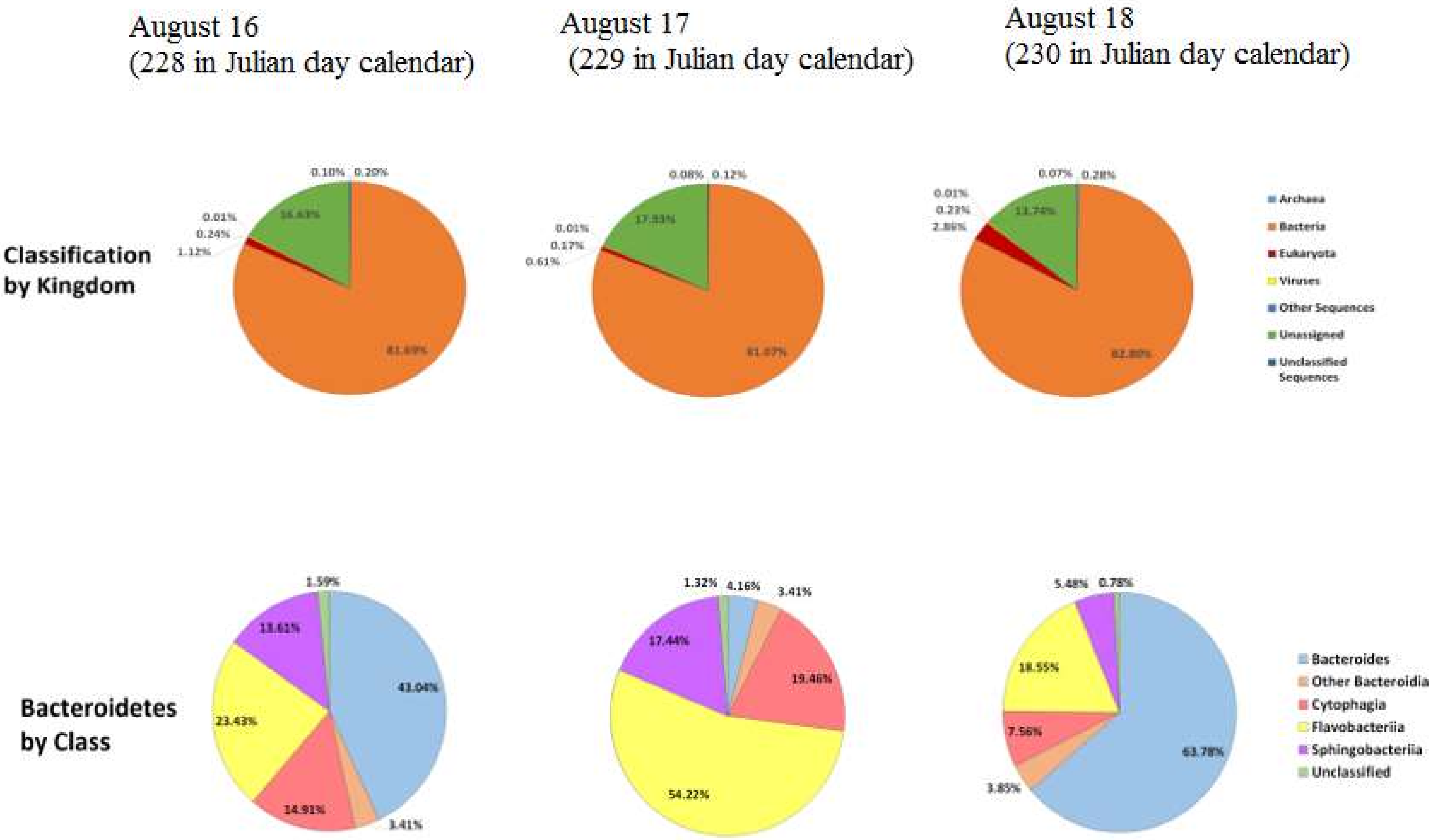


Figure 4.7: (a) Metagenome summary for three samples with the highest *E. coli* concentration (b) Breakdown of affiliated bacterial sequences shows *Bacteroides* are the dominant species among Proteobacteria

REFERENCES

REFERENCES

Almeida, C., & Soares, F. (2012). Microbiological monitoring of bivalves from the Ria Formosa Lagoon (south coast of Portugal): A 20 years of sanitary survey. *Marine pollution bulletin*, *64*(2), 252-262.

Dorner, S. M., Anderson, W. B., Slawson, R. M., Kouwen, N., & Huck, P. M. (2006). Hydrologic modeling of pathogen fate and transport. Environmental science & technology, 40(15), 4746-4753.

Furtula, V., Osachoff, H., Derksen, G., Juahir, H., Colodey, A., & Chambers, P. (2012). Inorganic nitrogen, sterols and bacterial source tracking as tools to characterize water quality and possible contamination sources in surface water. Water research, 46(4), 1079-1092.

Grayson, R. B., Gippel, C. J., Finlayson, B. L., & Hart, B. T. (1997). Catchment-wide impacts on water quality: the use of 'snapshot'sampling during stable flow. Journal of Hydrology, 199(1), 121-134.

Harwood, V. J., Levine, A. D., Scott, T. M., Chivukula, V., Lukasik, J., Farrah, S. R., & Rose, J. B. (2005). Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. Applied and Environmental Microbiology, 71(6), 3163-3170.

Kay, Euan R., David A. Leigh, and Francesco Zerbetto. "Synthetic molecular motors and mechanical machines." Angewandte Chemie International Edition 46, no. 1-2 (2007): 72-191.

Kistemann, T., Claßen, T., Koch, C., Dangendorf, F., Fischeder, R., Gebel, J., ... & Exner, M. (2002). Microbial load of drinking water reservoir tributaries during extreme rainfall and runoff. Applied and environmental microbiology, 68(5), 2188-2197.

Krometis, L. A. H., Characklis, G. W., Simmons, O. D., Dilts, M. J., Likirdopulos, C. A., & Sobsey, M. D. (2007). Intra-storm variability in microbial partitioning and microbial loading rates. Water Research, 41(2), 506-516.

Layton, A., McKay, L., Williams, D., Garrett, V., Gentry, R., & Sayler, G. (2006). Development of Bacteroides 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. Applied and Environmental Microbiology, 72(6), 4214-4224.

Lemarchand, K., and P. Lebaron. 2003. Occurrence of Salmonella spp. and Cryptosporidium spp. in a French coastal watershed: relationship with fecal indicators. FEMS Microbiol Letts 218:203-209

Long, S. C., & Plummer, J. D. (2004). Assessing land use impacts on water quality using microbial source tracking.

Noble, R. T., & Fuhrman, J. A. (2001). Enteroviruses detected by reverse transcriptase polymerase chain reaction from the coastal waters of Santa Monica Bay, California: low correlation to bacterial indicator levels. Hydrobiologia, 460(1-3), 175-184.

Peed, L. A., Nietch, C. T., Kelty, C. A., Meckes, M., Mooney, T., Sivaganesan, M., & Shanks, O. C. (2011). Combining land use information and small stream sampling with PCR-based methods for better characterization of diffuse sources of human fecal pollution. Environmental science & technology, 45(13), 5652-5659.

Pusch, D., Oh, D. Y., Wolf, S., Dumke, R., Schröter-Bobsin, U., Höhne, M., ... & Schreier, E. (2005). Detection of enteric viruses and bacterial indicators in German environmental waters. Archives of virology, 150(5), 929-947.

Reischer, G. H., Haider, J. M., Sommer, R., Stadler, H., Keiblinger, K. M., Hornek, R., & Farnleitner, A. H. (2008). Quantitative microbial faecal source tracking with sampling guided by hydrological catchment dynamics. Environmental microbiology, 10(10), 2598-2608.

Santhi, C., Arnold, J. G., Williams, J. R., Hauck, L. M., & Dugas, W. A. (2001). Application of a watershed model to evaluate management effects on point and nonpoint source pollution. Transactions of the ASAE, 44(6), 1559.

Simpson, J. M., Santo Domingo, J. W., & Reasoner, D. J. (2002). Microbial source tracking: state of the science. Environmental science & technology, 36(24), 5279-5288.

Soranno, P. A., Wagner, T., Martin, S. L., McLean, C., Novitski, L. N., Provence, C. D., & Rober, A. R. (2011). Quantifying regional reference conditions for freshwater ecosystem management: a comparison of approaches and future research needs. Lake and Reservoir Management, 27(2), 138-148.

Stoeckel, D. M., & Harwood, V. J. (2007). Performance, design, and analysis in microbial source tracking studies. Applied and Environmental Microbiology, 73(8), 2405-2415.

Tiefenthaler, L. L., Stein, E. D., & Schiff, K. C. (2008). Watershed and land use–based sources of trace metals in urban storm water. Environmental Toxicology and Chemistry, 27(2), 277-287.

Traister, Elena, and Shimon C. Anisfeld.(2006) "Variability of indicator bacteria at different time scales in the upper Hoosic River watershed." Environmental science & technology 40, no. 16: 4990-4995.

USEP 1991. United States Environmental Protection Agency (USEPA). 1991. Guidance for Water Quality

Verhougstraete, M. P., Martin, S. L., Kendall, A. D., Hyndman, D. W., & Rose, J. B. (2015). Linking fecal bacteria in rivers to landscape, geochemical, and hydrologic factors and sources at the basin scale. Proceedings of the National Academy of Sciences, 112(33), 10419-10424.water. Water Res 46(4):1079–1092.

WMDEQ, 2014. Water quality and pollution control in Michigan, Integrated report. MI/DEQ/WRD-14/001

Wong, M., Kumar, L., Jenkins, T. M., Xagoraraki, I., Phanikumar, M. S., & Rose, J. B. (2009). Evaluation of public health risks at recreational beaches in Lake Michigan via detection of enteric viruses and a human-specific bacteriological marker. water research, 43(4), 1137-1149.

Wu, J., Rees, P., & Dorner, S. (2011). Variability of E. coli density and sources in an urban watershed. Journal of water and health, 9(1), 94-106.

Yampara-Iquise, H., Zheng, G., Jones, J. E., & Carson, C. A. (2008). Use of a Bacteroides thetaiotaomicron-specific α-1-6, mannanase quantitative PCR to detect human faecal pollution in water. Journal of applied microbiology, 105(5), 1686-1693.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

The present work was able to identify potential non-point sources of fecal contamination in the selected watersheds in Michigan. MST approaches and sampling concepts were established and evaluated with consideration of seasonal hydrological variability and coupled with geographical information system (GIS) data. The suggested approach was tested in two selected watersheds. A large scale watershed (Saginaw Bay) that drains into large waterbody (Lake Huron) and a small scale sub-watershed (Sloan Creek) that drains into a creek (Sloan creek). Since the effectiveness of the MST method depends on the size of the watershed and the number of potential pollution sources, we applied different sampling schemes in each study. For Saginaw Bay, mixed-use large watershed, a synoptic sampling scheme has been designed to characterize water quality during swimming season under a base flow condition at a wide-ranging area. For Sloan creek, mixed- use sub-watershed, a continuous monitoring and comprehensive investigation approach via long-term sampling under various hydrological conditions has been used (wet and dry seasons, base flow and storm flow). The specific conclusions of each study have been written at the end of chapters 3 and 4. From these two microbial source tracking investigations we are able to derive the following general conclusions:

- In mixed-use watersheds, the dominant land use in the watershed is the probable dominant source of pollution, and the percentage of the land use in theparticular watershed indicates relative contribution of pollutants sources

- The significance of MST results are influenced by study design, selection of MST markers, sampling scheme, and watershed data collection (land use, surface elevation, hydrological factors). An MST study should include multiple sampling locations, collection of multiple samples during a sampling event, and multiple sampling events during the period of interest (e.g., summer or winter) or variable hydrologic conditions (e.g., base or storm flow).
- *Bacteroides* marker assays are useful for microbial source tracking studies when combined with land use information. This approach will allow for a better understanding of the sources of fecal pollution in mixed watersheds. In this study, *Bovine Bacteroides* 16srRNA (BoBac) has performed high specificity and sensitivity to identify the source of bovine fecal pollutions in agriculture-dominant watershed. *Bacteroides* thetaiotaomicron α-1–6 mannanases (B. theta) gene, has a high human specificity. To the best of our knowledge, this was the first study that used the B.theta gene in river/creeks water and linked its presence to land use patterns.
- Sediments contain high levels of fecal organisms as compared to water samples, and can act as a non-point sources that degrade beach water quality.
- The use of viruses as an MST tool failed to identify the source of contamination due to their low abundance in the environment. Meanwhile, infectious human adenoviruses (HAdVs) were detected in some water samples which did not violate *E. coli* concentrations according to Michigan standards. This finding indicates the need for using of alternative indicators along with pathogens measurement to protect public health.

- The use of community analysis (Next Generation Sequencing) provides multiple lines of evidence for identifying fecal source and improve the usefulness of MST results. The use of NGS in MST studies is very recent development and remains an active research area.
- Finally, in both studies the correlation between the molecular marker (human and bovine-associated *Bacteroides*, and human and bovine adenoviruses and fecal indicators is week. This makes MST analysis result likely be the most useful to confirm the presence or absence of a particular source, or to gain a qualitative understanding of the types and relative abundance of different sources. MST methods should be used to supplement rather than replace current methods.

Recommendations

To continue to improve our understanding of the MST and source identification in mixed used watershed the following are suggested:

- Quantify the relative contributions of human and animal sources to the total microbial pollution load, by using multiple quantitative qPCR *Bacteroides* tests and determine the ratios indicating the relative contribution of pollutant sources. The proposed ratios are presented below.

Ratio 1: Human Bacteroides / All Bacteroides

Ratio 1 will determine the relative contribution of human sources (for example, failing septic systems).

Ratio 2: Bovine Bacteroides / All Bacteroides

Ratio 2 will determine the relative contribution of bovine sources (farming).

Ratio 3: (Total Ruminant – Bovine Bacteroides) / All Bacteroides

Ratio 3 will determine the approximate relative contribution of deer (wildlife).

Ratio 4: Porcine Bacteroides / All Bacteroides

Ratio 4 will determine the relative contribution of porcine sources (farming).

Ratio 5: Chicken Bacteroides / All Bacteroides

Ratio 5 will determine the relative contribution of chicken (farming).

Ratio 6: Geese Bacteroides / All Bacteroides

Ratio 6 will determine the relative contribution of wildlife Geese (wildlife).

- Next generation sequencing can provide new insights of MST study.
- Microbial methods in combination with modeling will result in better understanding of diffused non-point sources.