MICROBIAL SOURCE TRACKING: WATERSHED SCALE STUDY OF PATHOGEN ORIGIN, FATE, AND TRANSPORT IN THE UPPER SUGAR CREEK WATERSHED, NORTHEAST OHIO

DISSERTATION

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ABSTRACT

The provision of safe water is one of the most important challenges faced by countries due to increasing populations and industrialization. Transport of non-point source pollution, including human and animal wastes, into the environment can result in pathogenic outbreaks. However, identifying the sources of non-point source pathogenic pollution is difficult, although needed if effective remediation action is to be taken. This dissertation research project describes the application of the Microbial Source Tracking (MST) method in the Upper Sugar Creek watershed, a mixed-use watershed located in northeast Ohio. Culture independent and library independent, host-specific PCR and quantitative PCR assays (human and ruminant) of the *Bacteroidales* 16S ribosomal RNA gene were applied to identify the source of fecal contamination in the study watershed.

The effectiveness of sample processing protocols and long-term storage of environmental water samples were evaluated. Results indicated that membrane filtration for cell recovery, combined with the use of a small cell lysis container (2 mL size), produced the best efficiency and precision of DNA extraction. Long-term storage of environmental water samples before cell recovery and DNA extraction reduced DNA recovery rates.

The molecular based, quantitative PCR assay was compared with the traditional viable *E. coli* count assay and a significant ($P < 0.001$) positive correlation was measured. Thus, the molecular method for measuring *Bacteroidales* in water samples may be used to assess potential health risk due to fecal contamination. A high magnitude of general *Bacteroidales* qPCR signal was observed in samples from both a concentrated livestock operation area and a residential area. Also the ruminant- and human- host specific *Bacteroidales* PCR assays were tested for their specificity and sensitivity with local fecal samples of potential hosts, and the result validated the use of these two host specific assays at this watershed. Frequent human specific *Bacteroidales* signals were also observed in water samples from a residential area. These results were followed up by a targeted sampling method at hotspots of microbial contamination within the Upper Sugar Creek watershed. The purpose of this targeted sampling was to accurately and cost-efficiently identify the source of contamination. Spatially intensive samplings during baseflow water samples revealed that the most likely major source of fecal contamination in baseflow events was human origin from septic systems. Temporally intensive samplings conducted during both surface water baseflow and stormflow conditions from suspected agricultural fecal contamination source areas indicated sediments could be a large potential reservoir of fecal contamination during stormflow events. The fate and transport of two indicator bacteria, *Bacteroidales* and *E. coli* indicated different transport behavior.

The microbial source tracking (MST) method, when combined with targeted sampling, was able to identify sources of fecal contamination in a mixed-use watershed quickly, easily, accurately and inexpensively. This research demonstrates the power of the MST method and also makes it an attractive tool in studies of pathogen contamination in stream water samples.

Dedicated to my husband, Kenny, and to my children, Lisa and Alex

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

1.1 INTRODUCTION

1.1.1 Pathogens in environmental water

The provision of safe water is one of the most important challenges faced by countries due to increasing populations and industrialization. Even in the United States, water contaminated by microbial pathogens poses a significant threat to human health. The incidences of illness attributed to recreational water exposure appear to be increasing. The Natural Resources Defense Council (Dorfman 2005) reported that there were more beach closings and advisories in 2000 than in any previous year and 85% of these closings and advisories were due to bacterial levels that exceeded standards. Also, the Centers for Disease Control and Prevention reported 21 recreational water outbreaks in 2000, more than any single previous year since systematic surveillance began (Lee et al. 2002).

Non-point source pollution such as runoff from cropland, parking lots, lawns, mines, and septic systems accounts for more than half of the United States water quality impairments. Transport of human and animal wastes into the environment can

also result in contamination caused by pathogens such as viruses including Hepatitis A, noroviruses and rotaviruses, protozoans including *Cryptosporidium* and *Giardia*, and bacteria including *E. coli* O157:H7, *Salmonella*, and *Campylobacter*. However, there is difficulty in identifying the sources of pathogens when they are from non-point sources. This causes conflicts among agricultural, environmental and public health interests. However in many cases the blame is placed on agricultural activity.

1.1.2 Indicator bacteria

Because a wide diversity of pathogenic microorganisms exists in contaminated water, measuring all microbial pathogens in environmental water samples is difficult and costly. Instead, organisms that may indicate the presence of fecal contamination are often used as "indicator organisms" for monitoring and regulation of recreational and drinking waters. Indicator organisms are part of the normal flora and ubiquitous in the intestinal tract in warm-blooded animals. They are found at high concentrations in water samples. Therefore they are easier to measure compared to detecting the pathogen themselves. Although indicator organisms do not normally cause illness, they represent a measure of fecal contamination in water sample. For example, coliform bacteria such as *Escherichia coli* (*E. coli*) have been used as a common indicator bacterium to detect fecal contamination in water environments.

1.1.3 New technique for quantification of indicator bacteria

Viable plate counts or most probable number (MPN) techniques are frequently used for quantification of active microbial cells in environmental water samples. These techniques are time consuming because of the lengthy incubations and requirement of preparing multiple dilution samples. Techniques based on the polymerase chain reaction (PCR) to measure specific DNA sequences provide rapid and sensitive detection of bacteria. A major advantage of the PCR method is that the incubation step is not necessary. Therefore nonculturable species can also be used as indicator bacteria. However general PCR methods provide limited information on the number of microbes, and this required development of the quantitative PCR (qPCR) assay to overcome this disadvantage.

1.1.4 Microbial Source Tracking (MST)

To monitor and control microbial contamination in water environments, microorganisms relevant to public health are indirectly regulated using indicator bacteria and setting a total maximum daily loads (TMDL) criteria. These regulatory levels have been established for impaired waters by states and other municipal authorities, with oversight by the U.S. Environmental Protection Agency (U.S. EPA). To assist in the control of microbial contamination, identification of the contamination source is very useful (Simpson et al., 2002). This is because if the origin of the microbial contamination can be correctly identified, the most effective remediation action can be taken.

To identify sources of fecal contamination, various Microbial Source Tracking (MST) methods have been recently proposed and studied (Boehm et al., 2003; Dombek et al., 2000; Gilpin et al., 2003; Field and Samadpour 2007; Gourmelon et al., 2007; Jamison et al., 2004; Matellini et al., 2005; Meays et al., 2004; Schott et al., 2002; Simpson et al., 2002). Some methods do not target microorganisms directly, but instead focus on substances such as caffeine (Sankararamakrishnan and Guo 2005), fecal sterols and stanols (Nichols et al., 1996; Wu et al., 2007), or laundry brighteners (Poiger et al., 1999) in environmental water as potential indicators of human fecal contamination. The advantage of these methods is the ease of analysis due to the stability of chemical substance in water samples, compared to the rapid changes that can occur on microbial communities in water. However, because these other methods are indirect measurement of fecal contamination, their concentrations do not always correlate with the pathogens of interest.

The methods used in MST with microorganisms can be divided into two groups. Those based on culturing (phenotyping) and those based on molecular methods (genotyping). Also methods are categorized as library (collection of microorganisms from different potential sources as well as from the watershed under study) dependent and library independent (Simpson et al., 2002).

Culture-based (phenotyping) and library-dependent methods, based upon multiple antibiotic resistance, test isolates of culturable indicator bacteria against a series of antibiotics in order to discriminate human and animal sources. For example, Moore et al., (2005) applied multiple antibiotic resistant methods on a watershed scale using *Enterococcus spp.* However the disadvantage of the method is the

requirement of huge number of samples to construct the library. Also comparative studies (Moore et al., 2005) have shown that antibiotic resistant methods are less reliable compared to the ribotyping method.

Molecular (genotyping) methods are called DNA fingerprinting techniques. Examples include ribotyping (Carson et al., 2001, 2003), pulsed-field gel electrophoresis (PFGE) (Stoeckel et al., 2004) and denaturing gradient gel electrophoresis (DGGE) (Farnleitner et al., 2004). These methods still require library construction and the ability to indentify microbial sources depends on the library size constructed. Finally, molecular (genotyping) and library-independent methods, which are used in this dissertation study, use PCR to detect specific genetic markers in extracted DNA from environmental water samples. If the genetic marker is known to be specifically associated with the host pathogen, then this method may also be called host-specific PCR.

1.1.5 The indicator bacterium *Bacteroidales*

Several molecular and library independent MST methods, using order *Bacteroidales,* have been investigated (Bernhard and Field 2000a,b; Dick et al., 2005a,b; Kreader 1995). Recent developments and availability of quantitative PCR techniques have led to quantitative analysis of both general *Bacteroidales* and host-specific *Bacteroidales* as indicator bacteria (Dick and Field 2004; Okabe et al., 2007; Yanpara-Iquise et al., 2008; Kildare et al., 2007; Layton et al., 2006).

A proper functioning microbial contamination indicator organism should not reproduce outside of the animal host, and should be correlated with the presence of

pathogens (Field and Samadpour 2007). Using *Bacteroidales* as an indicator bacterium is quite advantageous because

1) It is a numerically dominant bacteria group in warm-blooded animal feces over coliforms and enterococci, which have been used as indicator bacteria (Eckburg et al., 2005). For example, very high numbers of *Bacteroidales* have been measured in human (Holdeman, et al., 1976) and cattle fecal communities (Bernard and Field 2000a). High sensitivity would thus be expected because of their abundance.

2) *Bacteroidales* are anaerobes and are assumed to have limited survival and reproduction after their release into the environment. Fiksdal et al. (1985) observed that the viable counts of *B. fragilis* declined more rapidly than those of *E. coli* in freshwater environment. Also factors influencing the persistence of *Bacteroidales* in environmental water have been studied (Bell et al., 2009; Dick et al., in press).

3) Host specificity of *Bacteroidales* has been well studied. Assays can identify *Bacteroidales* in feces from ruminants, humans, dogs, pigs, horses and elk (Bernhard and Field 2000a; Dick et al., 2005a,b; Layton et al., 2006; Okabe et al.,2007). These host-specific assays have been tested in various geologic areas include North America (Lamendella et al., 2006; Shanks et al., 2006;

Vogel et al., 2007), Europe (Seurinck et al., 2005a,b; Gawler 2007), Japan (Okabe et al., 2007), and New Zealand (Gilpin et al., 2003).

In previous studies, the terms of Genus *Bacteroides* and Order *Bacteroidales* have both been used to classify the group of microorganisms that are fecal anaerobes abundant in warm-blooded animal and used as indicators of fecal bacteria contamination. In this dissertation, we define the term "*Bacteroidales*" as our target microorganism group used for microbial source tracking and for quantification according to the term used such as "*Bacteroidales* rRNA gene fragment" in the studies of Dick et al. (2005 a, b).

1.1.6 Important considerations on *Bacteroidales* host specific PCR and qPCR

One of the concerns regarding the use of the PCR method for monitoring bacteria contamination in water is the inhibition of the PCR reaction due to humic substances originated from sediment and colloidal particle in the environmental water sample. Studies have been conducted to overcome these inhibition problems (Ijzerman et al., 1997; Jiang et al., 2005; Kreader 1996; Lakay et al., 2007; McKeown 1994; Tebbe and Vahjen 1993). In addition, consistency of DNA extraction from different environmental water samples must be monitored closely to provide proper quantification of target genes in environmental water samples. However, no study has been conducted regarding the DNA extraction process consistency.

1.2 OBJECTIVES

The primary objectives of this dissertation research project were (1) to investigate correlations between land use patterns and microbial contamination, (2) to identify the source of microbial contamination so that effective control strategies can be developed, and (3) to determine the applicability of the microbial source tracking method in the Upper Sugar Creek watershed. We used a culture independent and library independent, host-specific *Bacteroidales* 16S ribosomal RNA gene PCR assay and a quantitative PCR assay as tools of our microbial source tracking study.

In Chapter 2, we examine how chemical / physical water parameters are associated with levels of the indicator bacteria in the Upper Sugar Creek watershed. The goal here was to identify parameters that can be measured rapidly and inexpensively that could potentially point to sites that may be contaminated with fecal material. This is a preliminary study and the data are being used to guide further research on the connection between pathogen contamination and levels of various chemical / physical measurements in stream water samples.

In Chapter 3, the objective was to evaluate the effectiveness of sample processing protocols for the microbial source tracking method. We compare bacterial cell concentration methods and DNA extraction methods from environmental water sample prior to bacterial DNA analysis. The goal is to determine the best sample processing protocol that exhibits the most efficient and consistent recovery rate of our target genes of the indicator bacterium, *Bacteroidales,* and of spiked control. Additionally, the effect of sample water storage on quantity of our target gene was

investigated to determine if long-term storage of environmental water samples is possible.

In Chapter 4, the MST method was applied to our study site, the Upper Sugar Creek watershed located in northeast Ohio. The MST method involved a combination of host-specific *Bacteroidales* 16S rRNA gene PCR assays (human and ruminant) to identify the source of fecal contamination, and a general *Bacteroidales* 16S rRNA gene quantitative PCR (qPCR) assay to investigate the magnitude of fecal contamination. Before the application of the MST method to environmental water samples, host sensitivity and specificity of PCR assays were validated using local fecal samples from potential host sources. In addition, this new molecular based quantitative PCR assay is compared with the traditional indicator bacteria culture method to confirm the use of this molecular method for measuring health risk due to fecal contamination.

Finally, in Chapter 5, we conduct spatially and temporally targeted sampling along with watershed scale sampling to detect and characterize suspected fecal contamination hotspots. The goal for this study was to identify the source of fecal contamination more accurately and cost efficiently, in watershed scale studies, by using these targeted sampling methods. Also, the transport mechanism of the indicator bacteria in baseflow and stormflow conditions is investigated by temporal targeted sampling and these results were compared with the traditional indicator bacteria culture method.

1.3 STUDY AREA: UPPER SUGAR CREEK WATERSHED

Our study watershed, the Upper Sugar Creek Watershed is located in Wayne County, Ohio. In 2000, the Ohio EPA labeled the Sugar Creek Watershed as the second most impaired in Ohio. The Sugar Creek Watershed contributes to the hypoxia in the Gulf of Mexico (U.S. EPA 2000) because it is located in the headwaters of the Muskingum Watershed, Ohio's largest watershed that contributes flow to the Ohio River, and the eventually into the Mississippi River and the Gulf of Mexico. The Upper Sugar Creek watershed is located between the cities of Wooster and Orrville, with the village of Smithville at its center. Farm sizes (owned and leased land) average 287 acres (ca. 116 hectares) (Parker et al., 2007). The Upper Sugar Creek watershed contains different land uses that can serve as potential contaminant sources including residential areas, crop fields, livestock operations (dairy, sheep, horse, and swine), and natural forested areas. This mixed-use watershed is suitable for examining the source of microbial contamination from human activity, agricultural activity and/or wildlife.

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CHAPTER 2

CHEMICAL / PHYSICAL PARAMETERS AS PREDICTIVE INDICATORS OF BACTERIAL CONTAMINATION OF ENVIRONMENTAL WATER

2.1 ABSTRACT

Contamination of water samples is often associated with elevated concentrations of various physical and chemical parameters. Also, use of molecular methods for Microbial Source Tracking (MST) with order *Bacteroidales* is a powerful and innovative way to identify the source of pathogen contamination. Effective MST at the watershed scale requires selection of the appropriate sampling spots to help us diagnose areas of high levels of fecal contamination. Rapid and inexpensive methods for screening a large number of samples with various levels of fecal contamination are needed. For potential screening methods, association among chemical / physical parameters and indicator bacteria level was studied. The numbers of *E .coli*, that indicate potential fecal contamination, did not show a strong correlation with suspended solids or with turbidity. However, *E. coli* numbers were

positively correlated with conductivity in this study watershed. Conductivity information can be readily obtained in the field, and we were able to use this information to identify, in initial studies, sampling spots in watersheds that may be useful for MST studies.

2.2 INTRODUCTION

Transport of human and animal waste into aquatic systems can result in contamination with pathogenic microorganisms. This has become an increasingly serious health concern. Fecal contamination is commonly identified by using indicator bacteria such as fecal coliforms. However, the traditional culture method for fecal coliforms normally requires 24 hours of incubation time to obtain the results. Recently, molecular methods have been applied to environmental water samples for use in Microbial Source Tracking (MST) and for quantification of indicator bacteria. The advantage of molecular methods is the rapid response that can be achieved compared to traditional culture methods. However, there is also a greater cost associated with molecular methods compared to culture methods.

The aim of this dissertation study was to conduct a MST study at the watershed scale. To perform MST, we need to first select the appropriate sampling spots within the targeted watershed. It is essential to choose sampling sites that will help us diagnose areas of high levels of fecal contamination. This requires rapid and

inexpensive methods for screening a large number of samples with various levels of fecal contamination.

Some studies for estimating contamination level using other parameters have been conducted previously. Lucena et al, (1988) studied relationship among physico-chemical values and indicator bacteria values obtained from river water samples and found that the positive correlation between COD and indicator bacteria level. Charef et al., (2000) presented a smart sensor system which used neural network models to predict COD level using pH, Temperature, and conductivity values. More recently, Yazdi and Scholz (2010) assessed multiple regression analysis and neural network models for the prediction of the indicator bacteria values of storm water samples, and concluded that multiple regression analyses were not applicable for prediction of indicator bacteria levels but neural network model predicted indictor bacteria levels relatively well. For our purpose, quantitative prediction of indicator bacteria value is not necessarily. However, it is helpful if we are able to identify suspicious spots of high fecal contamination before analyzing actual indicator bacteria numbers which is costly or time consuming. In this chapter, a study was carried out to determine the correlation between fecal contamination and the readily available physico-chemical variables using parameters obtained from the study watershed.

2.3 MATERIALS AND METHODS

2.3.1 Sampling and sample processing

Environmental water samples were taken from the Upper Sugar Creek watershed located near Smithville, Ohio. Samples were taken from twelve sampling sites within the watershed every month from June 2006 to October 2006. A total of 48 samples were planned to be collected, but due to low water levels at some sampling locations, less than 48 samples were collected. At the field sampling sites, field data (i.e. temperature, pH, dissolved oxygen, turbidity, and conductivity) were collected by a YSI sonde (YSI Inc.) immediately before water sampling. Forty-two water samples (2000 mL each) were collected from the headwater streams in the Upper Sugar Creek watershed and placed in a cooler, taken to the laboratory and processed within six hours.

Viable *E. coli* counts as Most Probable Number (MPN) were obtained immediately when samples were brought to the laboratory using the Colilert ® Method with Quanti-Tray/2000™ (IDEXX, ME). These MPN values were treated as colony forming unit (CFU) for this study. Samples were processed and analyzed for suspended solids according to standard methods (APHA, 1995). DNA samples were extracted from 100 mL aliquots using the UltraClean® Soil DNA Isolation kit according to the manufacture's instruction (MO BIO Laboratories Inc., Carlsbad, CA).

DNA quality and total DNA concentrations were calculated using 260/280 and 230/280 ratios obtained by the NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

2.3.2 Statistical calculations

The data set was treated as random sampling data to evaluate the associations between various physical, chemical and biological properties of water sampled from the Upper Sugar Creek watershed. After the trimming of data sets due to missing variables, 37 complete cases remained and were used for the final statistical analysis. The Anderson-Darling Normality test was performed on all variables of the data sets, and pH and dissolved oxygen data were found not to follow a normal distribution. Because extreme values may be the most important points to explain the association between investigated properties, these data points should not be discarded. Therefore, a non-parametric statistical test was performed.

2.4 RESULTS AND DISCUSSION

We assumed the number of *E. coli* was positively related to turbidity and suspended solids values. Living microorganisms are often transported by soil particles to which they are attached (Crabill et. al., 1999). We also assumed that suspended solids measurements made in the laboratory would be positively related to

turbidity measurements made in the field, since the turbidity would be caused by suspended solids.

Table 2.1 summarizes the non-parametric Spearman rank-order correlation test performed against selected variables including field data and lab-measured data. Some significant relationships were observed between suspended solids and other variables. Some of these relationships can be explained as chemical/physical interactions. Turbidity and suspended solids are positively correlated, and show a moderate coefficient value (0.463, α=0.05). Also the 260/230 value was positively correlated with suspended solids (0.376, α=0.05). This is because suspended solids measurements are greatly influence by high amounts of soil particles, and these soil particles are enriched in humic acid contents, that causing high 260/230 value when DNA was extracted. The number of *E. coli* was moderately and positively correlated with conductivity (0.344, α=0.05). However, there was no strong correlation with other variables. Therefore, the hypothesis that there was a relationship between the number of *E. coli* in a water sample and turbidity or suspended solids values was rejected. However, it was remarkable that conductivity information that is readily available from field measurements would be a possible predictive indicator of fecal contamination.

A further statistical analysis of the data was conducted by comparing parameters between the groups with different levels of microbiological contamination. We assumed that the values of temperature, turbidity, conductivity, suspended solids, and dissolved oxygen are significantly different between the sample groups either meet or does not meet microbiological water quality standards. The Ohio contact

recreation standard value (Table 2.2 Ohio Environmental Protection Agency, 2003) for single samples was used to divide the data set according to the *E. coli* level of each sample (Table 2.3). At first, the data was divided into two groups following grouping Method 1 which categorizes the data as MEET_STD group (Number of *E. coli* is lower than 576 CFU /100mL, n=26) and EXCEED_STD group (Number of *E. coli* is higher than 576 CFU /100mL, n=11). Kruskal-Wallis tests, the nonparametric equivalent of the analysis of variance (ANOVA), were performed for selected variables. Table 2.4 summarizes the results. They were similar with the ones obtained from the first correlation analysis. Conductivity was significantly (α=0.05) different for the two different *E. coli* plate count level groups, but no strong evidences of difference were observed for the other variables.

We also applied the Kruskal-Wallis tests on conductivity values with higher resolution of *E. coli* value groups. Samples were divided into three, instead of two groups of *E. coli* (i.e. Method 2, Table 2.3). The previous MEET_STD group was divided into two groups and then statistics applied to these three groups. The results indicated that at least one group might be different from the others (α=0.05). Finally, Pair-wise Mann-Whitney-Wilcoxon tests were performed and the low *E. coli* value group and the high *E. coli* value group were found to significantly different in conductivity at the level of α=0.05 (P=0.026) (Fig. 2.1).

The above results suggest that conductivity is related to *E. coli* counts in water samples obtained from the Upper Sugar Creek watershed. The sample size is not large, but it does indicate that conductivity could be used as a means to quickly assess

whether a stream within this watershed contains *E. coli* at levels that may need to be further investigated.

2.5 SUMMARY

Contrary to expectations, the number of *E .coli* in a water sample did not show a strong correlation with suspended solids or with turbidity at this study watershed. However, *E. coli* numbers, that indicate potential fecal contamination, was positively correlated with conductivity in this study watershed. Based on the assumption that the baseline of conductivity would not show significant differences among samples because of the relatively homogeneous geological properties in this watershed, variation of conductivity is attributed to human activity, such as effluents from wastewater treatment plants or from septic systems. This implies that fecal contamination in this study watershed was primarily due to human activity in the drainage area. Conductivity information can be obtained in the field, and we are able to use the information to identify, in initial studies, select sampling spots in watersheds that may be useful for MST study.

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	Suspended Solid	*E. coli*
Temperature	0.300*	-0.070
Dissolved Oxygen	-0.551**	-0.263
Turbidity	0.463**	-0.041
Conductivity	-0.263	0.344**
Suspended Solid	-	0.188
260/230	0.376**	0.171

**: significant at level of α=0.05, *: significant at level of α=0.10.

Table 2.1 Spearman's rank correlation coefficient matrix.

Type of standard	Type of recreational water use (Unit: CFU/100 mL)		
	Bathing waters [a]	**Primary contact** [b]	**Secondary contact** [c]
Geometric mean [d]	126	236	na
Single sample [e]	235	298	576

a. Bathing waters are suitable for swimming and other full-body-contact exposure where a lifeguard or bathhouse is present.
b. Primary-contact waters are suitable for full-body contact, such as swimming, canoeing, and scuba diving.
c. Secondary-contact waters are suitable for partial-body contact, such as wading.
d. The geometric mean is based on a minimum of five samples in a 30-day period.
e. This value cannot be exceeded in more than 10 percent of the samples collected in a 30-day period. Bathing waters are suitable for swimming and other full-body-contact exposure where a lifeguard or bathhouse is present.

Table 2.2 Ohio water-quality standards for *Escherichia coli* in recreational waters (Ohio Environmental Protection Agency, 2003) (Effective from May 1 through October 15. All values are in CFU/100 mL; na, not applicable).

		Standard values		
		Primary contact	Secondary contact	Does not meet the standard
		<298 (CFU/100mL)	<576 (CFU/100mL)	>=576 (CFU/100mL)
Sample Grouping	Method 1	**MEET_STD** (n=26)		**EXCEED_STD** (n=11)
	Method 2	**Low** (n=20)	**Medium** (n=6)	**High** (n=11)

Table 2.3 Two sample grouping methods based on number of *E. coli* defined by standard values indicated in Table 2.

Variables	P values
Temperature	**0.259**
Dissolved Oxygen	**0.435**
Turbidity	**0.842**
Conductivity	**0.043****
Suspended Solid	**0.280**
260/230	**0.251**

**: Significant at level of α=0.05

Table 2.4 P values from Kruskal-Wallis test for effect of variables on *E. coli* numbers grouped according to Method 1 (Table 2.3).

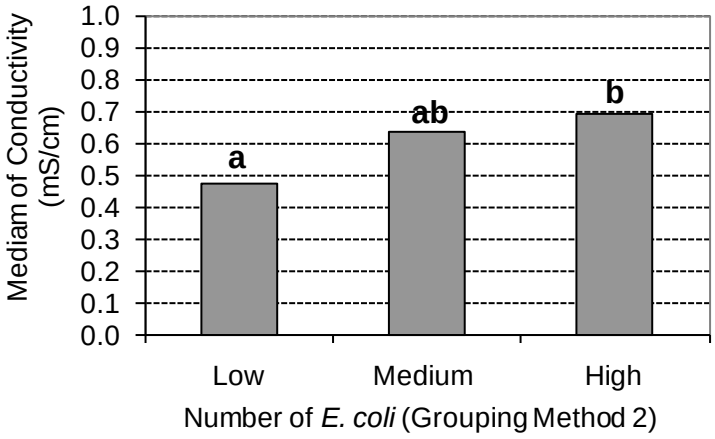

Figure 2.1 Result of conductivity value on comparison based on grouping Method 2. Bar chart shows median values for three groups of samples as segregated according to number of *E. coli*. Same subscript letter means that the conductivity values do not differ significantly based on the Mann-Whitney-Wilcoxon test at α=0.05.

CHAPTER 3

EFFECT OF EXTRACTION METHOD AND SAMPLE HOLDING TIME ON QUANTIFICATION OF BACTERIAL DNA IN ENVIRONMENTAL WATER SAMPLES

3.1 ABSTRACT

Microbial source tracking in environmental water samples using bacterial DNA requires accurate and consistent recovery rates of the source DNA. Experiments were conducted with environmental water samples using three different extraction methods in combination of different bacterial cell concentration methods (centrifugation and membrane filtration) and different physical cell lysis conditions. Some aliquots of the water samples were also stored at -20°C and -80°C for up to 48 weeks before DNA extraction. The extracted DNA samples from these treatments were analyzed for concentrations of DNA sequences of *Bacteroidales*, a fecal contamination indicator, and of *Pantoea stewartii* as a spiked positive control. The results indicated that membrane filtration is better than centrifugation for

concentrating cells in term of both extraction efficiency and consistency. The use of a small cell lysis container (2 mL size) was better than use of bigger cell lysis container (10 mL size) for extracting DNA and also yielded better extraction efficiencies and consistency. Overall, we observed good results on the use of small cell lyses container (2mL size) combined with cut membrane filter for both extraction efficiency and precision. Storage of environmental water samples for 36 weeks or 48 weeks at -20 °C or -80 °C, before cell recovery and DNA extraction, reduced DNA recovery rates significantly. Therefore long term sample water storage would not be recommended for the quantitative analysis of DNA of indicator bacteria in environmental water samples. Instead DNA should be extracted as quickly as possible from environmental samples and the DNA can then be stored.

3.2 INTRODUCTION

Excessive levels of fecal bacteria are a common cause restricting beneficial use of stream environments. Fecal indicator bacteria have been used to monitor, via culture methods, the level of fecal bacterial contamination in impaired water. Recently, molecular methods that involve the use of the quantitative Polymerase Chain Reaction (qPCR) have been applied to quantify pathogens and indicator bacteria in environmental water samples (Dick et al., 2004; Dombek et al., 2000; Kildare et al., 2007; Layton et al., 2006; Lebuhn et al., 2004; Noble et al., 2006; Okabe et al., 2007; Park et al., 2007; Seurinck et al., 2005; Shanks et al., 2008). These

methods have advantages of specificity, sensitivity, and efficiency compared to culture based methods. Ideally, the quantification of target bacterial DNA should be done with consistent recovery rates from original water samples in order to make accurate comparisons between samples. For molecular methods involving PCR, sample storage and DNA extraction method are very important factors that can cause variation in target DNA recovery rates.

Previous studies have compared (1) DNA extraction procedures for obtaining high quality DNA samples (Jara et al., 2008; Yeates et al., 1997; Yeates and Gillings, 1998) and (2) recovery consistency from solid samples such as soil samples (Wehausen et al,. 2004), However, only limited information is available concerning DNA extraction recovery consistency from environmental water samples.

For cell collection from environmental water samples, the filtration method has been used to collect microorganisms and other particulate matter from water samples (Bernhard and Field, 2000; Dick et al., 2004). The filter with collected microorganisms was either folded or rolled before being placed into the cell lysis tubes containing reagent and beads. The tubes were then mechanically agitated to facilitate cell lyses. We observed, however, that the beads could not always access all of the microorganisms on the filter paper due to the rolling or folding of the filter paper. This resulted in inconsistent recovery rates of DNA. Centrifugation has also been used to collect microbial cells from environmental water samples (Khan and Yadav, 2004). However, we observed centrifugation also did not provide consistent recovery rates of DNA since collected cells were needed to be transferred to the smaller cell lysis tubes manually. This extra cell-transferring step caused variation in

DNA recovery rates. Recently, Stoeckel et al. (2009) examined recovery of DNA from spiked water samples to make proper adjustment for target recovery in samples. Nevertheless, knowledge of efficient and consistent extraction methods are lacking. There is a need for data from well-controlled DNA extraction method comparisons using environmental water samples so that microbial source tracking can be properly conducted.

Sample holding time is also extremely important when conducting studies at a watershed scale because of the large number of samples generated. Ideally, water samples should be processed and analyzed immediately after sampling to eliminate changes in bacterial flora in sample water over time. However, processing and analysis in a timely manner is often impossible because of the time needed for sample transportation and processing. This limits the maximum number of samples from a single sampling event due to an individual laboratory's inability of sample rapid processing. However, no study has been done on storage method of water samples for genomic analysis.

The objectives of this study were to develop a DNA extraction assay suitable for quantifying bacterial DNA in environmental water samples and to investigate the effect of environmental water sample storage on DNA extraction. Stable recovery rates of DNA extraction are critical for quantification of target DNA in environmental water samples. To address this concern, we conducted a study with two hypotheses. The first was that different extraction methods would result in variation of extraction efficiency and consistency of both spiked control and a fecal contamination indicator *Bacteroidales*. A specific objective, therefore, was to compare different cell

collection / DNA extraction methods to determine the most suitable method for quantification of bacterial DNA in environmental water by the qPCR method. A second hypothesis was that environmental water sample could be stored for some time without significant loss of target DNA. The specific objective to evaluate this hypothesis was to conduct studies comparing DNA extraction after various times of storage of environmental water samples at -20 ºC or -80 ºC.

3.3 MATERIALS AND METHODS

3.3.1 Sample collection and spiking

Environmental water samples (approximately 10 liters) were collected from Upper Sugar Creek watershed in April 2008 and placed in high-density polyethylene (HDPE) containers and brought to the laboratory. Subsamples were obtained from this water sample after first inverting the container 10 times to ensure thorough mixing and then pouring out approximately 100 mL into 120 mL sized HDPE container. Subsamples for extraction method comparison were spiked with a 100 µL aliquot of *Pantoea stewartii* culture (approximately 5 x 10^6 colony forming units (CFUs) per mL). This bacterium is a pathogen affecting maize and causes the disease called Stewart's wilt. It was selected as a spike control to test for recovery rates of DNA from the water samples because we believe there would be no existence of this organism in natural water. This was indeed confirmed by negative results on qPCR of

DNA extract sample without the spike control added. Subsamples for storage method comparison were placed in a freezer at either -20 °C or -80 °C to test the effect of storage time and temperature on recovery of DNA from the water samples.

3.3.2 Comparison of DNA extraction methods

In this study, three different extraction treatments each with 10 replicates and one blank were tested for their recovery rate (Table 3.1). Two methods used filtration to collect the bacterial cell before cell lysis (Method 1 and 2). The 100 mL water subsamples were filtered using disposable vacuum filtration apparatus (Pall Corporation, NY) fitted with a 45 mm diameter filter paper (Supor 200 Membrane Disc Filters, 0.2 µm, Pall Corporation, NY). For Method 1, in order to attain maximum interaction between bacterial cells and cell lysis beads, filters that collected microorganisms and particulate matter were folded in half three times into a cone shape and then cut with a disposable razor blade three times lengthwise (Fig. 3.1) and inserted into 2 mL tubes that had been previously prepared to contain lysis buffer (approximately 600 µL) and beads (approximately 1.2 g) from UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Inc. CA). For Method 2, filters were first rolled and inserted into 10 mL tubes containing approximately 600 µL of lyses buffer and beads (approximately 1.2 g) from the same DNA extraction kit mentioned above. By using a 10 mL tube, the filter surface that contained the collected microorganism and particulate matter was exposed to lysis buffer and beads mixture. Thus none of the particles are trapped between folds of the filter paper so that maximum interaction between bacterial cells and cell lysis beads can be attained. For Method 3,

centrifugation instead of filtration was used to isolate bacterial cells out of the water samples. For this method, the 100 mL subsamples were transferred to 200 mL tubes and centrifuged for 30 minutes at 9,000 rpm (10,000 g). After centrifugation, the supernatant was discarded. To transfer the pellet to the original 2 mL lysis tube, lysis buffer from the original 2 ml tube was decanted into the tube with the pellet and the mixture was then pipetted back into the original 2 mL lysis tube containing lysis beads.

For all three methods, after cell lysis was completed, further DNA extraction processes were completed according to manufacturer's instructions. The final sample of DNA extracted from the water samples was brought to a volume of 100 μL and stored at -20°C until analysis.

3.3.3 Comparison of storage methods

Frozen 100 mL subsamples that were stored at -20°C and -80°C were thawed under running water (25°C) and the DNA extraction process was conducted as soon as the samples were completely thawed. For this study, we used Method 1 (Table 3.1) as the DNA extraction procedure for all the samples. DNA extractions from triplicate samples obtained at each storage temperature were done at 0, 16, 36 and 48 weeks of storage. The final sample of DNA extracted from the water samples was brought to a volume of 100 μL and stored at -20°C until further analysis.

3.3.4 Quantitative PCR (qPCR) analyses

Preparation of standards

For general *Bacteroidales* analyses, PCR products (694 bp) containing a target sequence (106 bp) for *Bacteroidales* 16S RNA gene was used. The initial PCR product (694 bp) was tested for product length with gel electrophoresis and DNA concentration was measured using a spectrometer (NonoDrop Technologies, Wilmington, DE). The numbers of template copies in this initial PCR product were estimated from the DNA concentration and product length as follows. Based on the assumption that the average weight of a base pair is 650 Daltons (one mole of a base pair weighs 650 g), the molecular weight of the initial PCR product (694 bp) can be estimated as 4.51×10^5 g/mole. Using Avogadro's number, 6.022×10^{23} molecules/mole and the concentration of DNA extract (ng/μL), the number of template copies per μL of DNA extract can be calculated. For the spike (*P. stewartii*) analysis, pure genomic DNA was obtained from cultured *P. stewartii* cells. The number of genomic DNA copies (i,e, template copies since one target sequence exists for each *P. stewartii* cell: Tambong et al., 2008) in extracted DNA was quantified using measured DNA concentration data using a spectrometer and the molecular weight of the total genome length of *P. stewartii* of 5.19×10^6 bp.

qPCR assays

qPCR assays were performed according to modified published protocols for general *Bacteroidales* (Layton et al., 2006) and for *P. stewartii* (Tambong et al., 2008). In our assays, we did not use Taq-Man probes, but instead used SYBR® Green Dye (BioRad Laboratories, Inc., Hercules, CA). All qPCR assays were

checked by melting curve analysis. SYBR® Green Dye is a highly specific double-stranded DNA binding dye that can detect PCR product as it accumulates during PCR cycles. However, it might also detect non-specific reaction products. Sequences of primers for the qPCR assays are listed in Table 3.2. All reactions were carried out with a BioRad iQ5 instrument (BioRad Laboratories, Inc., Hercules, CA) using 25 μL of reaction volume that included 1 μL of DNA extract as template or 1 μL of DNA free water for no-template controls. All qPCR reactions were conducted with 7-point standard curves using serial 10-fold dilutions from the master standard solution (10^8 copies / reaction). All samples and standards were analyzed in triplicate in the same run.

3.3.5 Statistical calculations

For the extraction method study, the geometric mean of qPCR triplicates was used to represent one extraction from each 100 mL of subsample. The geometric means of 10 extractions for *Bacteroidales* qPCR and of 9 extractions for spiked control (*P. stewartii*) qPCR were then used to determine extraction efficiency of the three different extraction methods and standard deviations were used to estimate extraction consistency. The results from the three extraction methods were compared using ANOVA. For the storage study, the geometric means of qPCR triplicates were also used to represent one extraction from each 100 mL of sub sample, and the geometric means of three extractions from storage triplicates of subsamples were used to represent each storage condition. Concentration data were standardized using

the non-stored water subsamples as the baseline for comparison and assigning an extraction efficiency of 100% to the non-stored samples. All statistical analyses were conducted using Minitab® v.15 (Minitab Inc, 2007).

3.4 RESULTS AND DISCUSSION

3.4.1 Extraction method comparison

We compared three extraction methods (Table 3.1) for DNA extraction efficiency and precision by analyzing spiked control of *P. stewartii* and also analyzing naturally existing *Bacteroidales.* Theoretically, each sample has approximately the same number of target microorganisms. Any variation in recovery of target DNA is thus assumed to be due to the DNA extraction process. Overall recovery rate for the spiked control ranged from 1.4% to 7.1% (n=9) for Method 3. The range of recovery rate agrees with those obtained from a similar extraction method used in a previous study (2.5% to 11%, n=15, Stoeckel et al., 2009). Average spiked control recovery rates using the other extraction methods were 17.5% and 9.6% for Method 1 and Method 3, respectively.

DNA extraction efficiency from both spiked control (Fig.3.2) and *Bacteroidales* (Fig.3.3) showed similar trends. On average, Method 1 indicated the highest average target DNA copy number and Method 3 indicated the lowest average

target DNA copy number. The coefficient of variation (CV), that is the standard deviation divided by the mean and then multiplied by 100, was also used to compare extraction consistency. Method 1 was the most precise for both spiked control and *Bacteroidales* extractions (CV, 16.3% for spiked control, and 21.5% for *Bacteroidales*). The least precise method for spiked control was Method 3 with a CV value of 60.3% and for *Bacteroidales* it was Method 2 with a CV value of 36.6%.

Overall, Method 1 that uses cut filter paper containing bacterial cells inserted in a 2 mL tube along with beads was the best method for DNA extraction for quantitative analysis in terms of both extraction efficiency and precision. Method 2 was expected to be an improvement over Method 1 because the filter surface with attached bacterial cells was more exposed to lyses buffer and beads. However, mechanical shaking seems to work better with the smaller 2 mL tubes used in Method 1, compared to the larger 10 mL tubes used in Method 2. For Method 3, the pellets created by centrifugation needed to be resuspended with buffer and then transferred to the cell lysis tube (2 mL) manually, so that subsequent cell lysis could occur. This extra step caused variability of recovery rates even though this centrifuge method collects bacterial cells without use of filter paper so that the next step involving mechanical breakage of the cells could be optimized.

We also tested the validity of using a spiked control for quantitative analysis of *Bacteroidales* in environmental water samples. The indicator bacterial cells that naturally exist in sample water are potentially attached to suspended sediment whereas the spiked control cells are freely dispersed in sample water. This could lead to different extraction mechanisms and efficiencies. If these two extraction

efficiencies are well correlated, copy number information from the spiked control qPCR could be used to monitor DNA extraction consistency from the environmental water sample. Fig. 3.4 shows scatter plots and regression analyses of spiked control (*P. stewartii*) qPCR copy number plotted against *Bacteroidales* qPCR copy number from the same extraction. All extraction methods showed positive relationships between the copy numbers of the spiked control and *Bacteroidales*. This implies that DNA extraction processes worked similarly for both species. Therefore, spiking environmental water samples with *P. stewartii* cell and analyzing the copy number in DNA extract could be a reliable way to establish quality control of the qPCR method used to estimate unknown numbers of *Bacteroidales* cells in environmental water sample.

3.4.2 Sample storage method comparison

Fig. 3.5 shows relative quantity of *Bacteroidales* 16sRNA copy numbers extracted from water samples after storage at -20°C and -80°C for periods up to 48 weeks. These data points are geometric means of triplicate of water samples with the same storage treatment. The qPCR measurements were made on extracted DNA samples after the last stored water sample was extracted. This was done by storing earlier extracted DNA samples at -20°C for 0, 12 or 32 weeks before the qPCR analysis. The *Bacteroidales* 16sRNA copy number was also determined immediately after extraction so that the data could be used to check for the rate of DNA degradation during extracted DNA sample storage. We did not observe major DNA degradation.

Freezing water samples could enhance subsequent cell lysis. At the same time, storing frozen samples could also lead to loss of target DNA sequence due to degradation. Our target bacterium (*Bacteroidales*) had not previously been studied concerning whether it is possible to store either the original water sample or extracted DNA sample before further processing. Fig. 3.5 shows *Bacteroidales* target DNA sequence (106bp) quantity changes over time of sample water storage up to 48 weeks. After 16 weeks of water sample storage under -20°C, the quantity of target DNA sequence decreased to 54%, on average, of the original quantity. However, the same storage duration at -80°C did not significantly lower the quantity of target DNA sequence (ANOVA, P=0.130). It was clearly evident that if water samples were stored more than 36 weeks, even at -80°C, a significant loss of the target DNA sequence occurred. After 48 weeks of sample storage, the target gene concentrations were reduced to less than 20 % of the original amount for both -20°C and -80°C storage temperatures. Over all, long term storage of water samples under freezing conditions of -20°C would not be recommended for the quantitative analysis of *Bacteroidales* in water. However, storage of water samples at-80 °C for up to 16 weeks is possible without causing major changes in amount of DNA extracted.

3.5 SUMMARY

The results from this study suggest that DNA extraction protocols greatly affect DNA extraction efficiency and precision from bacteria. Overall, the data suggest that the combination of filtration for cell concentration and the small 2 mL tube for cell lysis provides best results. In terms of efficiency and precision of DNA extraction, copy numbers of spiked cell DNA and indicator bacteria DNA were positively correlated. Therefore, concentration information from the spiked control sample can be used to monitor extraction consistency and provide quality control of the overall quantification process.

The results also suggest that long-term storage of environmental water samples at -20°C is not suitable for DNA quantification. However -80 °C storage condition did not show significant quantity change in the number of *Bacteroidales* target DNA sequence up to 16 weeks. It is thus possible to store environmental water sample if large number of samples have to be collected at one time during a watershed scale study that involved molecular methods for microbial source tracking.

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Methods	Cell extraction method	Cell lysis tube size
Method 1	Filter extraction	2mL
Method 2	Filter extraction	10mL
Method 3	Centrifugation	2mL

Table 3.1 Tested DNA extraction methods. Each method has 9 replicates with spike, one non-spiked, and one extraction blank. See Materials and Methods section for detailed description of each method used to extract DNA from environmental water samples.

Primer	Target gene	Sequences (5' to 3')	Reference
AllBacF	*Bacteroidales* (16sRNA)	GAGAGGAAGGTCCCCCAC	Layton et al. (2006)
AllBacR		CGCTACTTGGCTGGTTCA	
RT74F	*P. stewartii* (cpsD)	TGCTGATTTTAAGTTTTGCTA	Tambong et al. (2008)
RT177R		AAGATGAGCGAGGTCAAGGATA	

Table 3.2 Primers for two qPCR assays used in this study.

	Method 1		Method 2		Method 3	
Extraction replicates	Copy number /100mL	Recovery rate	Copy number /100mL	Recovery rate	Copy number /100mL	Recovery rate
Non-spiked	ND		ND		ND	
Extraction blank	ND		ND		ND	
1	7.76 $X10^3$	15.5%	2.55 $X10^3$	5.1%	1.34 $X10^3$	2.7%
2	7.45 $X10^3$	14.9%	3.00 $X10^3$	6.0%	7.44 $X10^2$	1.5%
3	8.19 $X10^3$	16.4%	5.82 $X10^3$	11.6%	1.51 $X10^3$	3.0%
4	1.06 $X10^4$	21.3%	5.80 $X10^3$	11.6%	2.59 $X10^3$	5.2%
5	9.50 $X10^3$	19.0%	6.65 $X10^3$	13.3%	8.56 $X10^2$	1.7%
6	8.88 $X10^3$	17.8%	5.83 $X10^3$	11.7%	2.08 $X10^3$	4.2%
7	1.14 $X10^4$	22.9%	6.35 $X10^3$	12.7%	3.56 $X10^3$	7.1%
8	7.92 $X10^3$	15.8%	4.21 $X10^3$	8.4%	3.67 $X10^3$	7.3%
9	7.89 $X10^3$	15.8%	5.01 $X10^3$	10.0%	7.06 $X10^2$	1.4%
Mean	8.76 $X10^3$	17.5%	4.80 $X10^3$	9.6%	1.59 $X10^3$	3.2%

*ND: Not Detected

Table 3.3 Spiked control (*P. stewartii*) qPCR result and recovery rates (added appx. 5 $x10^4$ cells per extraction).

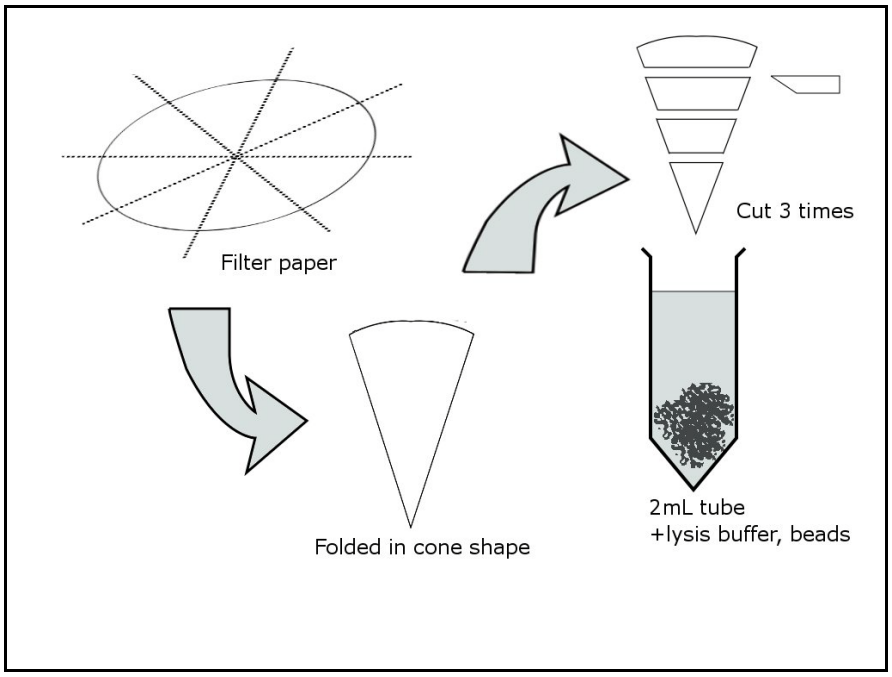
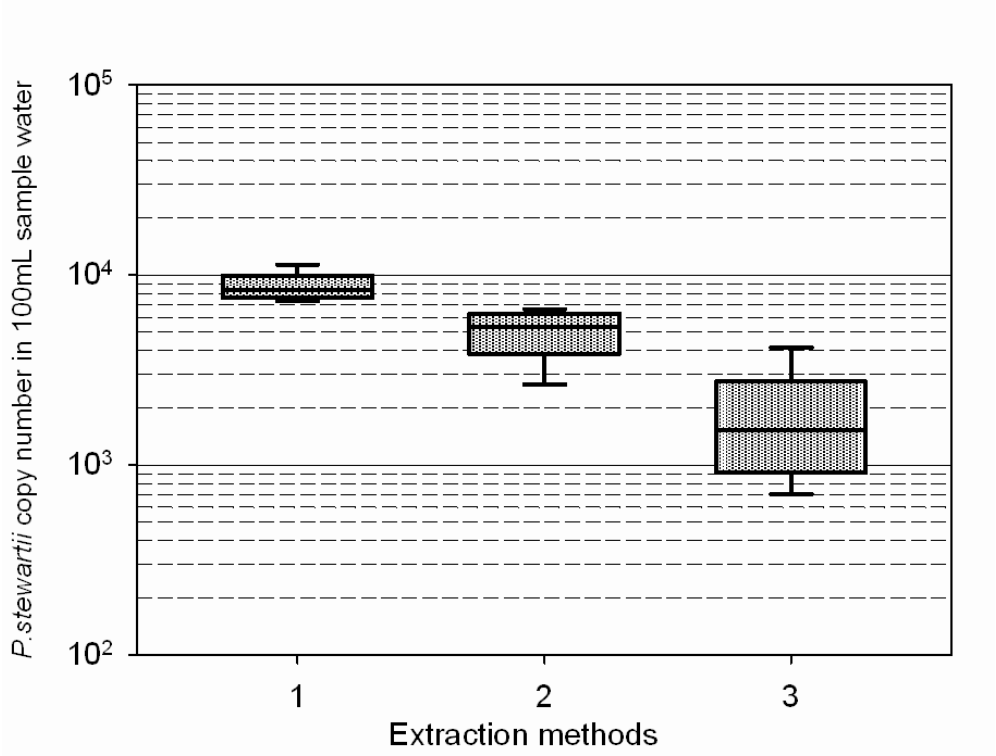
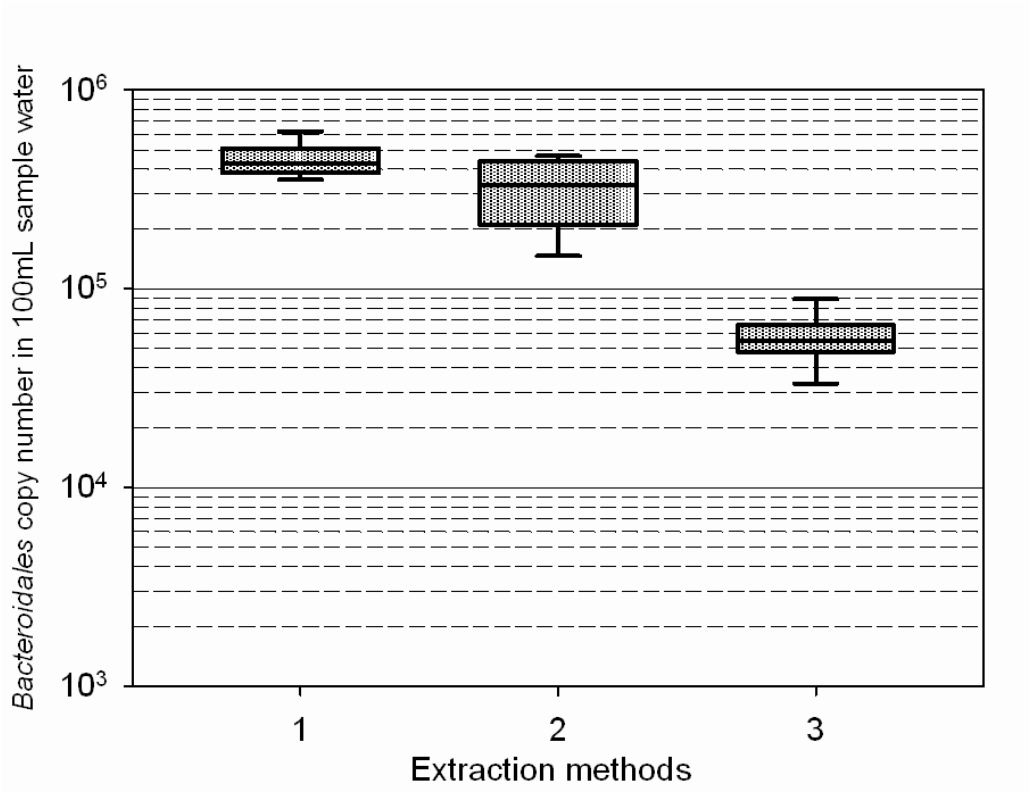


Figure 3.1 Filter cutting diagram for Method 1. Filters with collected microorganisms and particulate matter were folded in half three times, then cut with a disposable razor blade three times to be inserted in a lysis tube.



Method	Sample size	Mean	SD	CV
Method 1	9	8.76 $X10^3$	1.40 $X10^3$	16.0%
Method 2	9	4.80 $X10^3$	1.47 $X10^3$	30.6%
Method 3	9	1.59 $X10^3$	1.16 $X10^3$	72.9%

Figure 3.2 Extraction method comparison result using *P. stewartii* qPCR assay. *P. stewartii* cells (appx. 5 x 10^4 cells) added to 100 mL of sub samples before DNA extraction processing.



Method	Sample size	Mean	SD	CV
Method 1	10	4.46 X10^5	9.72 X10^4	21.8%
Method 2	10	2.90 X10^5	1.20 X10^5	41.6%
Method 3	10	5.44 X10^4	1.66 X10^4	30.5%

Figure 3.3 Extraction method comparison result using *Bacteroidales* qPCR assay.

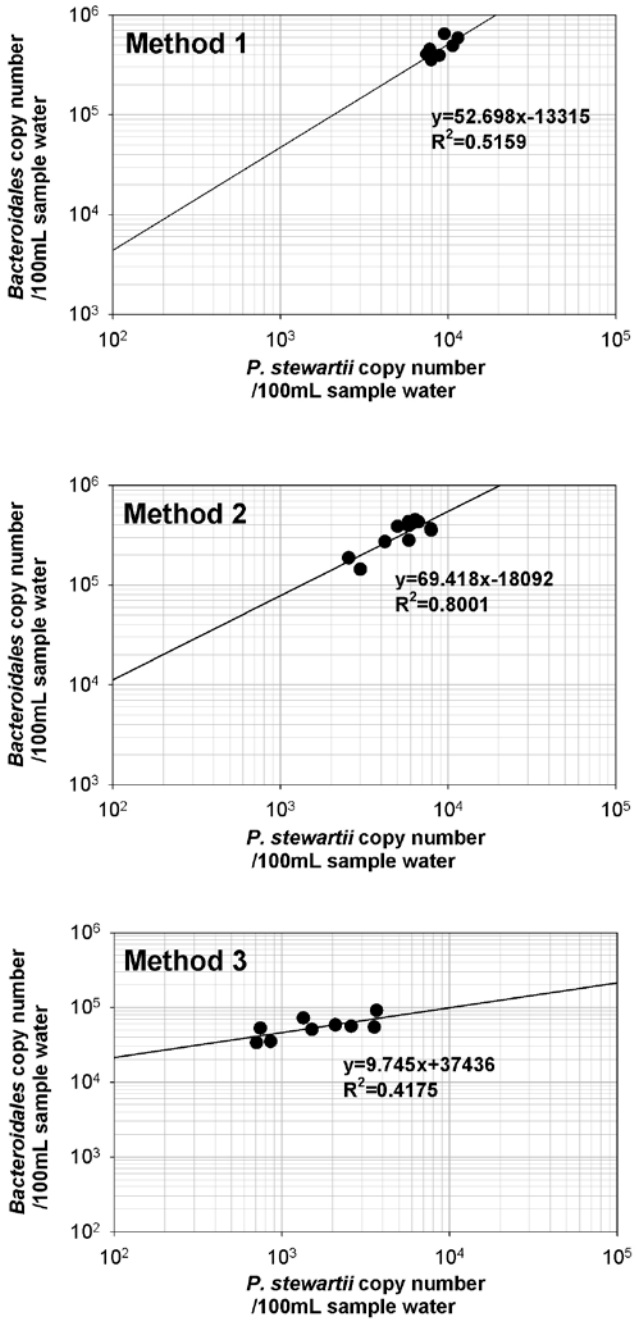


Figure 3.4 Scatter plots and regression analyses of *P. stewartii* spike copy number plotted against *Bacteroidales* copy number for three extraction methods.

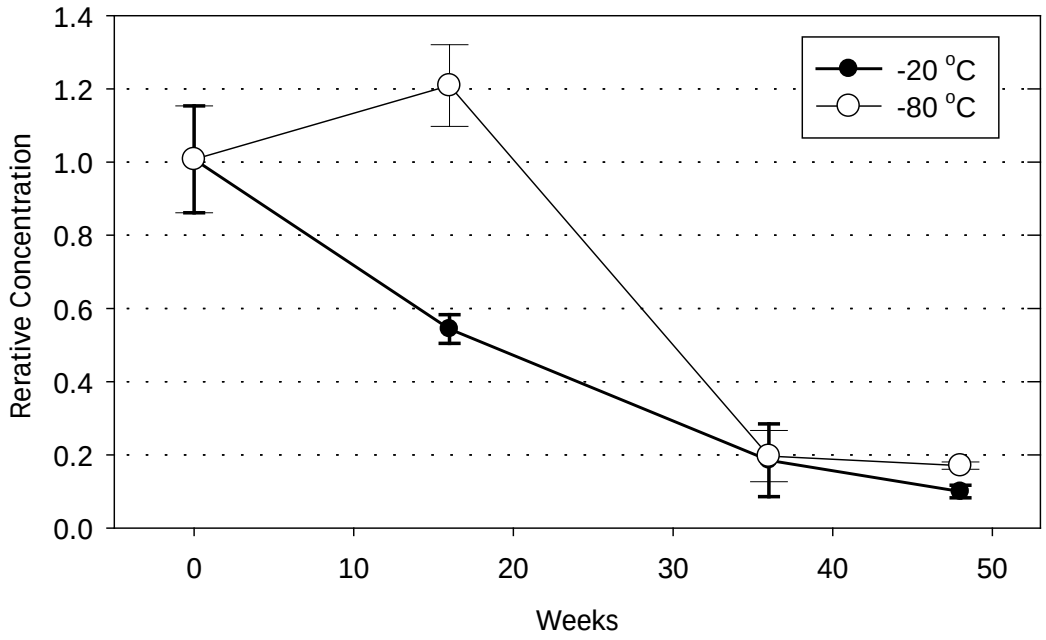


Figure 3.5 Sample storage method (0 days to 48 weeks, -20°C and -80°C) comparison results using *Bacteroidales* qPCR assay. Results were normalized by assigning the no-storage samples or time zero samples as 100%. Error bar indicates standard deviation of triplicates.

CHAPTER 4

MICROBIAL SOURCE TRACKING IN A MIXED USE WATERSHED: LIBRARY INDEPENDENT AND HOST-SPECIFIC PCR AND QUANTITATIVE PCR ASSAYS

4.1 ABSTRACT

Transport of human and animal waste into aquatic systems can result in contamination by pathogenic microorganisms. This has become an increasingly serious health concern. In this study, we determined applicability of the Microbial Source Tracking (MST) method to the Upper Sugar Creek watershed, northeast Ohio. This mixed-use watershed was suitable for examining the source of microbial contamination from human activity, agricultural activity and/or wildlife. We tested the MST method involving a combination of host-specific *Bacteroidales* 16S rRNA gene PCR assays (human and ruminant) to identify the fecal contamination source and a general *Bacteroidales* 16S rRNA gene quantitative PCR (qPCR) assay to

investigate the magnitude of fecal contamination. Viable counts of *E. coli* were also determined for statistical comparison with the general *Bacteroidales* PCR assay. Before the application of the MST method to environmental water samples, host sensitivity and specificity of PCR assays were validated using local fecal samples from potential host sources. Surface water samples were collected from 21 sites throughout the Upper Sugar Creek watershed beginning July 2008 and terminating March 2009. There was a significantly ($P < 0.001$) positive correlation between *E. coli* and *Bacteroidales* concentrations in water samples. We observed frequent human specific positive *Bacteroidales* signals in water samples from a residential area and also a high magnitude of general *Bacteroidales* qPCR signal in samples from both a concentrated livestock operation area and a residential area. These results indicate the potential application of the MST method to identify potential sources of microbial contamination and to guide land management decisions at the watershed scale.

4.2 INTRODUCTION

Understanding the source of potential pathogens in natural waterways is important for prediction of human health risk. It is also necessary for developing land management strategies for protecting water resources. However, the source of microbial contamination for most waterborne disease outbreaks cannot always be

identified. Source identification is required to determine responsibility of the contamination and to avoid conflict among public health agencies, farmers, industries, and local residents.

The goal of the Microbial Source Tracking (MST) method is to identify the source of microbial contamination in natural waters (Domingo et al., 2007; Field and Samadpour 2007; and Simpson et al., 2002). The MST has been studied by using indicator bacteria and either culture-based methods or molecular methods. The development of molecular methods provides several advantages because of its ease of use and time efficiency compared to culture-based methods that require time for cell growth and the development of a library. There is no need to create a library of cultured reference isolates due to the specificity and sensitivity of PCR. Therefore, rapid diagnosis and identification of microbial contamination in a watershed is possible.

We used microorganisms in the order *Bacteroidales* as our target organism for identifying microbial contamination since this species is reported to have host specific distributions (Dick et al., 2005a) and low survival rates in an aerobic freshwater environment (Fiksdal et al., 1985; Kreader 1998). This allows for the detection of recently, and not priorly introduced fecal contamination. Bernhard and Field (2000a, b) used length heterogeneity PCR to identify human and ruminant specific 16S rRNA genetic markers for this species. Dick et al. (2005a) studied fecal samples of eight different hosts (human, bovine, pig, house, dog, cat, gull, and elk) and then developed pig, horse and dog specific 16S rRNA genetic markers (Dick et al., 2005a, b). Among these developed host specific *Bacteroidales* markers, human

and ruminant specific *Bacteroidales* markers developed by Bernhard and Field (2000a, b) have been evaluated in the United States (Bernhard et al., 2003), France (Gourmelon et al., 2007), and Belgium (Seurinck et al., 2007). However, Gawler et al. (2007) found that sensitivity and specificity of these markers varied depending on the region of study across the world. Therefore, local validation is needed before these markers can be employed for MST in a specific watershed. In addition, several quantitative PCR (qPCR) methods for *Bacteroidales* have also been developed. These methods can quantify both total *Bacteroidales* and host specific *Bacteroidales* (Layton et al., 2006; Dick and Field, 2004; Okabe et al., 2007; Okabe and Shimazu 2007).

The host specific *Bacteroidales* PCR assay and the quantitative *Bacteroidales* assay are very important tools for the identification of fecal contamination in impacted watersheds. Some studies of natural stream water samples have characterized the association between *E. coli* concentration and host specific *Bacteroidales* PCR results (Lamendella et al., 2007; Shanks et al., 2006) and between pathogen concentration and the general *Bacteroidales* PCR results (Walters et al., 2007). However, the *Bacteroidales* qPCR assay has not been strictly validated as a quantitative method in the same way that the commonly used *E. coli* indicator bacteria have been validated as an indicator of pathogen contamination.

The objectives of this study were, therefore, to (1) evaluate the specificity and sensitivity of host-specific *Bacteroidales* markers as indicators of fecal contamination, (2) apply these host-specific markers to environmental samples taken from the Upper Sugar Creek watershed to assess the origin of bacterial contamination, (3) quantify

total *Bacteroidales* in the same environmental samples to assess the magnitude of fecal contamination, and (4) compare results of general *Bacteroidales* concentrations using the qPCR method and quantities of *E. coli* using a general culture method.

4.3 MATERIALS AND METHODS

4.3.1 Study site

We collected water samples from the Upper Sugar Creek Watershed (Fig. 4.1) located in Wayne County, Ohio. In 2000, the Ohio Environmental Protection Agency (EPA) labeled the Sugar Creek Watershed as the second most impaired in Ohio. The Sugar Creek Watershed contributes to the hypoxia in the Gulf of Mexico (US EPA 2000) because it is located in the headwaters of the Muskingum Watershed, Ohio's largest watershed that contributes flow to the Ohio River. The Upper Sugar Creek, as a headwater of the Sugar Creek Watershed, is located between the cities of Wooster and Orrville, with the village of Smithville at its center. Farm sizes (owned and leased land) average 287 acres (ca. 116 hectares) (Parker et al., 2007). This watershed contains different land uses that can serve as potential contaminant sources. These land uses include residential areas, crop fields, livestock operations (dairy, sheep, horse, and swine), and natural forested areas. This mixed-use watershed is suitable for examining the source of microbial contamination from human activity, agricultural

activity and/or wildlife. Also, Demouchelle (2006) conducted a general PCR assay for *Bacteroidales* in well water collected from Wayne County, Ohio. The results of this study clearly showed fecal contamination in this area.

4.3.2 Fecal sampling

Fecal samples were collected for use as positive controls in tests of host specificity of the *Bacteroidales* markers and to help establish the bacterial source tracking method in the Upper Sugar Creek Watershed. Wild animal and domestic animal fecal samples were collected from within the watershed and livestock feces were collected from the Ohio Agricultural Research Center (OARDC) farm facility located just 6 km outside of the watershed. Individual human fecal samples were voluntary collected from individuals within the state of Ohio. A total of 20 individual cow, pig, dog, wild geese, chicken, sheep, and deer fecal samples and seven individual human fecal samples were collected. All samples were individually stored in sterile 50 mL conical centrifuge tubes, transported to the laboratory and stored at -20°C prior to DNA extraction and analyses. Also, two wastewater-treatment plant (WWTP) and two septic influent samples were also collected from within Wayne County for the purpose of serving as positive controls of human contamination. These influent samples were stored in sterile 200mL containers and transported to the laboratory and processed for DNA extraction within six hours.

4.3.3 Water sampling

Stream water samples were taken from 21 sampling sites within the Upper Sugar Creek watershed (Fig. 4.1). This watershed has different land uses with potential contaminant sources such as residential areas with and without a municipal sewage system, crop fields, livestock operations (daily, sheep, house, and swine), and natural forested areas. The selected sampling sites have been well characterized in previous studies (Parker et al., 2007) and long-term water quality studies in the Upper Sugar Creek Watershed are on-going. Five sets of samples were collected beginning July 2008 and terminating March 2009. We focused on capturing the microbial community in water column at base flow condition to provide better comparison of results from the different samplings. Water samples (approximately 100 mL) containing suspended sediment were taken from each sampling sites. The water samples were placed in a cooler and taken to the laboratory and processed within six hours. Viable *E. coli* counts were obtained immediately when samples were brought to the laboratory using the Colilert ® Method with Quanti-Tray/2000™ (IDEXX, ME).

4.3.4 DNA extraction

Fecal DNA was extracted from approximately 0.5 g of fecal sample using the PowerSoil™ DNA Isolation kit according to the manufacture's instruction (MO BIO Laboratories Inc., Carlsbad, CA). To achieve consistent extraction efficiency for quantitative analysis, DNA extraction from water samples were conducted using Method 1 as described in the previous chapter of this dissertation. 100 mL of

environmental water samples and 10 mL of sewage and septic samples diluted to 100 mL with DNA free water were filtered through a membrane filter with 0.2 µm openings. The bacterial cells collected on the filters were processed using the PowerSoil™ DNA isolation kit according to the manufacture's instruction except using membrane filter with bacterial cells instead of using soil as DNA source. All extracted DNA samples were tested for presence of *Bacteroidales* DNA using the general *Bacteroidales* primer set (Table 4.1). The concentrations of DNA in fecal DNA extract samples are usually greater than in water DNA extract samples. The DNA samples from the fecal samples were, therefore, diluted to 4 ng/µL after measurement of DNA concentration with the NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE). This dilution step also prevented the inhibition of the PCR reaction by humic materials also extracted from the fecal samples. All extracted DNA samples were stored at -20 °C until further processing.

4.3.5 General and host specific PCR

Fecal DNA general and host specific PCR

General *Bacteroidales* PCR analysis using the primer set described in Table 4.1 was conducted on all samples to check for the presence of *Bacteroidales* signal in the extracted DNA. Each 25 µL final PCR mixture contained 1× Taq polymerase buffer (20mM Mg^{2+}), 1.25 U of Takara Ex Taq™ polymerase (Takara Bio Inc. Japan), each primer at a concentration of 0.2 µM, dNTPs mixture at a concentration of 200 µM each, and non-acetylated bovine serum albumin at 0.04% in concentration. A

thermal cycler (MJ Research, Watertown, MA) was used for all reactions with the following conditions: an initial heating at 94°C for 3 min for predenaturation, 30 cycles consisting of 94°C for 1 min, 53 °C for 45 sec, and 72°C for 2 min were conducted. A final 7 min extension period was conducted at 72°C.

Ruminant- and human-specific *Bacteroidales* markers (Table 4.1) were tested for host sensitivity and specificity using DNA extracted from fecal samples. All forward primers specific for each marker (CF128f and CF183f for ruminant specificity and HF134f and HF183f for human specificity) were paired with the general reverse primer (Bac708r). The PCR running conditions were the same as described for the general *Bacteroidales* PCR assay except the annealing temperatures were 62 °C for both ruminant marker sets and 63 °C for both human marker sets. After the fecal sample DNA concentrations were adjusted, approximately 4 ng of fecal DNAs were amplified by PCR. Ten individual cow fecal samples randomly selected from 20 fecal samples and seven individual human feces were analyzed. In addition, two WWTP influent samples and two septic influent samples were pooled and both pooled DNA samples were used to test primer sensitivity. Primer specificity was further investigated using pooled fecal DNA samples that included from pig, deer, sheep, dog, goose, and horse.

All PCR products were visualized by electrophoresis using 1% agarose gel stained with ethidium bromide stain and compared using a 100 bp DNA ladder (Promega, Madison, WI). Sizes of amplicons for each primer pair are described in Table 4.1.

Water sample host specific PCR

Before the host specific analyses, general *Bacteroidales* PCR analyses were conducted using the primers set described in Table 4.1. These were conducted on DNA obtained from all water samples collected from the five samplings between August 2008 and March 2009 to check for the existence of *Bacteroidales*. Human and ruminant specific PCR assays described above were also conducted on the DNA obtained from the water samples. The PCR conditions used for DNA from water samples were the same as described above with exception that 35 cycles, instead of 30 cycles, were used due to the possibility of low template concentration. All PCR products were visualized using electrophoresis as described above.

4.3.6 Quantitative PCR

A set of primers that targeted the 16S rRNA gene of general *Bacteroidales* (AllBacf and AllBacr, Table 4.1) was used for qPCR to assess the magnitude of fecal contamination in the samples. All qPCR assays were performed using the iQ™ SYBR® Green Supermix and iQ5 real-time PCR detection system (Bio-Rad, Hercules,CA). Each 25 µL PCR mixture contained 1X of iQ Supermix, each primer at a concentration of 0.3 µM, non-acetylated bovine serum albumin at 0.06% concentration, and 1 µL of DNA extraction sample. The 1 µL of DNA theoretically corresponds to 1 mL of water sample. A real-time thermal cycler was used for all reactions with the following conditions: an initial heating at 95°C for 3 min for predenaturation followed by 40 cycles consisting of 95°C for 15 sec and 60°C for 1

min. After completion of the reaction, the melt curve was analyzed to check for accuracy of data. For each qPCR run, all samples were analyzed in triplicate. PCR inhibitors in the samples were determined to be negligible based upon results obtained after 10-fold and 100-fold dilutions.

4.4 RESULTS AND DISCUSSION

4.4.1 Host specific PCR

Fecal samples host specific PCR

Ruminant and human specific *Bacteroidales* 16S rRNA gene markers were tested for sensitivity and specificity. Sensitivity of markers was determined as follows:

$$\text{Sensitivity} = a / (a+b) * 100$$

Where a is the number of true positives (i.e. fecal samples that were positive for the PCR marker of its own species) and b is the number of false negatives (fecal samples that were negative for the PCR marker of its own species) (Gawler et al., 2007).

Figure 4.2 shows the electrophoresis image of PCR products using primers for two different ruminant specific markers. These markers displayed 100% sensitivity for individual cow feces DNA. However these markers did not show perfect

specificity of detection of cow feces. They also tested positive for other ruminant species (deer and sheep). This result was expected because ruminant specific markers cannot differentiate between different ruminant species as these animals share the same type of *Bacteroidales* due to similar gastrointestinal systems. In addition, one of the markers (CF128f) was positive for pig feces DNA. A false positive response with pig feces DNA when using a ruminant specific marker has been observed in a previous study (Gawler et al., 2007). Overall, it seems as if host specific PCR analyses can be combined with land use data analyses to assess potential sources of fecal contamination. This information can then be used in making land management decisions at the watershed scale.

The results obtained when using human specific markers to test fecal DNA samples showed variability in intensity of the target band (Figure 4.3). One of seven individual human fecal samples was negative for both human specific markers. Therefore specificity of both markers is calculated as 86%. This result indicates that human gastrointestinal flora has variability, and *Bacteroidales* species that carry these two markers might not always exist in humans. This would cause a false negative PCR result. However, positive signals were observed with both human specific marker sets when DNA from wastewater influent and septic influent samples were tested.

No false positives were observed with other host DNA pools or individual cow fecal DNA samples. These results were similar to data published by others (Bernhard and Field, 2000 a, b; Ahmed et al., 2008). We concluded these primers sets

are applicable for use in microbial source tracking of human fecal contamination in the Upper Sugar Creek Watershed.

Water samples host specific PCR

General *Bacteroidales* PCR markers were detected in all water samples except the samples from site 8. This site is a community-maintained natural spring used as a drinking water supply. Ruminant and human specific markers, previously tested with fecal samples, were used to investigate the existence of ruminant fecal contamination and human fecal contamination in the five sets of environmental water samples collected between July 2008 and March 2009. Over all, fewer samples were positive for any of the host specific markers compared to the general *Bacteroidales* marker (Table 4.2). This is understandable in that the host specific markers are a subset of the general *Bacteroidales* markers, and are thus found at lower concentrations (Bernhard and Field 2000 a, b).

We did not observe consistency in the ruminant signals at any of the sampling sites. In July of 2008, both ruminant markers showed strong positive signals in samples from site 21. Site 21 had a relatively small sub watershed that had a pasture where dairy cows were allowed to graze. The tile lines from this pasture contribute to the surface water collected at this sample site. The CF128f ruminant marker was only weakly positive in samples from sites 5 and 6 taken in August 2008. The ruminant marker, CF193f, was weakly positive in samples taken from site 18 taken in August 2008. Sites 5 and 6 and Site 18 have large areas that contribute to water flow and simple contamination source estimations were not possible. There were no ruminant

positives found in samples collected during the months of September and October in 2008 and March in 2009.

Both human markers generated a greater number of positive signals across the study watershed than did the ruminant markers. We especially observed constant strong positives for human markers at Sites 14 and 21. According to field observation, the sub-watershed of sampling Site 14 contained a residential area that had a septic system located near the stream. Site 21, in addition to including a pasture sub watershed, was also suspected to have septic outlet pipes contributing to the water at the sampling site.

The recurrent human specific positives throughout the watershed were rather unexpected, because water quality issues in this rural watershed often are assumed to be associated with agricultural activity. We could not define the contribution of human activity compared to agricultural activity toward the fecal contamination in this study watershed because no quantitative analyses were conducted for host specific markers. However, our findings do suggest detectable amounts of human fecal contamination at the base flow condition in this watershed and further studies are warranted.

4.4.2 Quantitative PCR

All samples except Sample Site 8 contained a detectable amount of the general *Bacteroidales* marker with concentrations ranging from 10^5 to 10^9 DNA target segments per 100 mL sample (Fig. 4.4). There was variation in marker concentrations among the five sampling events. However, recurrent high quantities

were observed at sampling Sites 14 and 21. This consistent high marker concentration indicates that continuous inputs of fecal contamination seem to be occurring at these sites. Combining results from host specific PCR assay and our quantitative general *Bacteroidales* results leads us to the conclusion that there are, hotspots of fecal contamination located closely upstream of these two sampling sites. Human feces are the likely source of this contamination.

A possible association between fecal contamination and land use could also be made by observation of the area. As described above, the sub-watershed at sampling Site 14 had a residential area with a septic system located along the stream. Sampling Site 21 had a pasture for dairy cows and tile lines from this pasture, along with suspected septic outlet pipes, contributed to the water flow and sample obtained from this site. A more intensive sampling and analysis could be conducted to better assess contamination at hotspots identified by our microbial source tracking method. Identifying the source of contamination can be useful information in designing successful water quality management at the point source level and at the watershed scale.

4.4.3 Correlation between *Bacteroidales* qPCR method and Viable *E. coli* count

Viable counts of *E. coli* were determined for statistical comparison with the *Bacteroidales* qPCR assay (Fig.4.5). Non Parametric Spearman's rank correlation coefficient was calculated based on the collected data pairs (n=59). There was a statistically moderate positive correlation (Spearman's rank correlation coefficient: 0.545, $P<0.001$) observed between *E. coli* and *Bacteroidales* quantity in water

samples. This result agrees with the results from a previous study (Gentry et al., 2007; Okabe et al., 2007).

E. coli is a culturable aerobic bacterium and is widely used as a fecal indicator to regulate water quality. *Bacteroidales*, the subject of this study, is an anaerobic bacterium and is expected to have limited survival after release into the environment. The positive correlation between *E. coli* and *Bacteroidales* numbers in our base flow environmental water samples imply both could be used as indicator bacteria for use in measuring health risk due to pathogenic bacteria in the watershed. However, *Bacteroidales* was found at levels about 1000 times greater than *E. coli* and it is thus a more sensitive indicator of fecal contamination. In addition, because of its limited survival in the environment and the availability of specific host markers, *Bacteroidales* may also be a more specific indicator of fecal contamination, especially recent contamination.

4.5 SUMMARY

The results of this study indicate that a combination of host specific *Bacteroidales* 16s rRNA gene PCR assays and general *Bacteroidales* quantitative16s rRNA gene PCR assay can identify potential fecal contamination hotspots in a watershed. Host specific human *Bacteroidales* markers clearly distinguished human fecal contamination from agricultural / wildlife fecal contamination. Ruminant

specific *Bacteroidales* markers were not able to differentiate between agricultural sources such as cows and wildlife source such as deer. However, a combination of ruminant specific PCR analyses and land use data analyses could be used to make the microbial source tracking method more accurate and a powerful tool. In addition, we observed a positive correlation between general *Bacteroidales* concentration from qPCR assay and *E. coli* concentration enumerated by general culture method. This result suggests that both could be used as indicator bacteria for measuring health risk due to pathogenic bacteria in the watershed.

Overall, this study confirm the potential application of the microbial source tracking method for use in aiding land management decisions to control microbial contamination at the watershed scale.

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Purpose	Name	Sequence (5'-3')	Annealing Temp.	Target Amplicon	Reference
General *Bacteroidales* PCR primer sets	Bac32f	AAC GCT AGC TAC AGG CTT	53ºC	676bp	Bernhard and Field 2000a
	Bac708r	CAA TCG GAG TTC TTC GTG			
Ruminant specific *Bacteroidales* forward primers	CF193f	TAT GAA AGC TCC GGC G	62ºC	471bp	Bernhard and Field 2000b
	CF128f	CCA ACY TTC CCG WT ACT C	62ºC	580bp	
Human specific *Bacteroidales* forward primers	HF134f	GCC GTC TAC TCT TGG CC	63ºC	574bp	
	HF183f	ATC ATG AGT TCA CAT GTC CG	63ºC	525bp	
General *Bacteroidales* quantitative PCR primer sets	AlllBacf	GAG AGG AAG GTC CCC CAC	60ºC	109bp	Layton et al., 2006
	AlllBacr	CGC TAC TTG GCT GGT TCA G			

*1 Bac708r was used as a reverse primer for all the host specific forward primers.

Table 4.1 Primers used in this study.

	Human specific *Blacteroidales*										Ruminant specific *Biacteroidales*									
	HF134f					HF183f					CF128f					CF193f				
Site	July 08	Aug 08	Sep 08	Oct 08	Mar 09	July 08	Aug 08	Sep 08	Oct 08	Mar 09	July 08	Aug 08	Sep 08	Oct 08	Mar 09	July 08	Aug 08	Sep 08	Oct 08	Mar 09
1		+	+	+	+				+	++										
2	+																			
3	+	+	+	+	+	+		+		+										
4																				
5	+				+	+						+								
6		+					+					+								
7																				
8																				
9	+				+	+				+										
10																				
11	+					+														
12			+																	
13																				
14	++	++	++	++	++	++	++	++	++	++										
15	+					+														
16																				
17			+			+														
18	++	+	++			++	++	+									+			
19	++				++					++										
20	++																			
21	++	++	++	++	++	++	++		++	++	++					++				

Table 4.2 Host specific PCR result for all samples. No host-specific positives were found at sites 4, 7, 8, 10, 13, and 16. +: Weak positive ++: Strong positive. The intensity of these positive signals was judged by comparison to reference positive controls on an electrophoresis gel image.

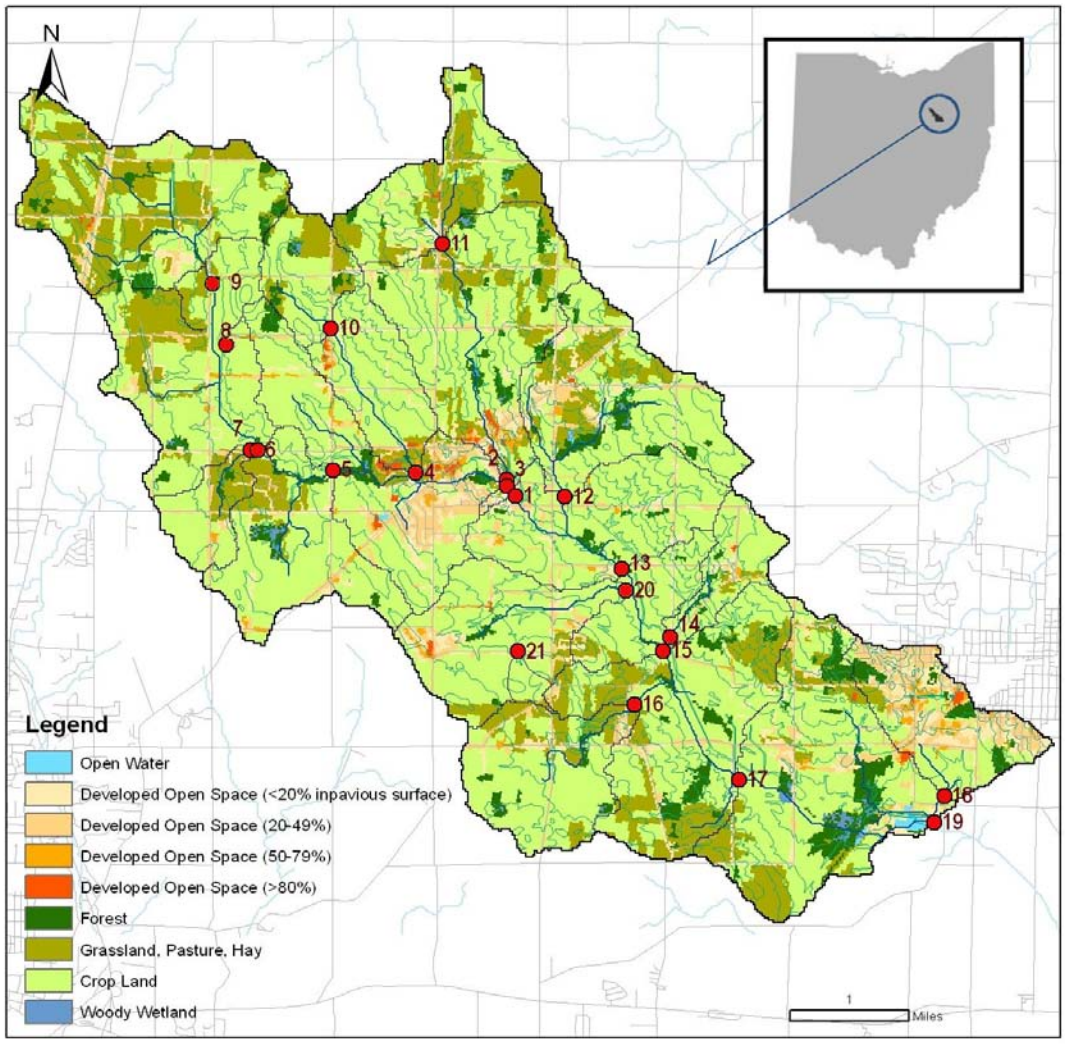

Figure 4.1 The Upper Sugar Creek Watershed mapped with land use data (NLCD 2001, USDA). The red dots show the location of 21 sampling sites.

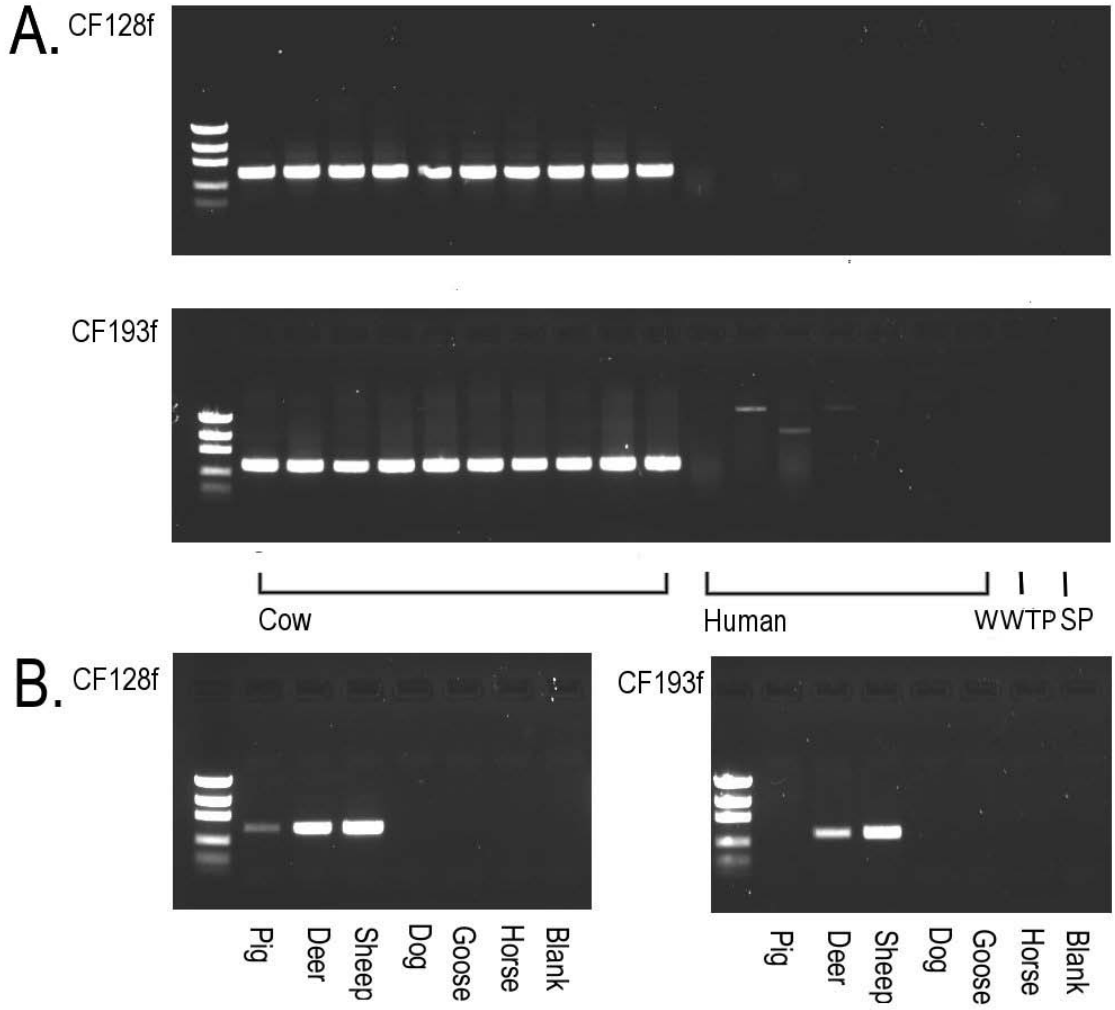

Figure 4.2 Host specificity PCR results with two ruminant specific primers (CF128f, CF183f). (A) Ruminant specific primer distinguished cow fecal DNA from human fecal DNA, WWTP influent DNA (WWTP), and septic influent DNA (SP). (B) Deer and sheep fecal DNAs were also positive for both markers and pig fecal DNA was positive for CF128f.

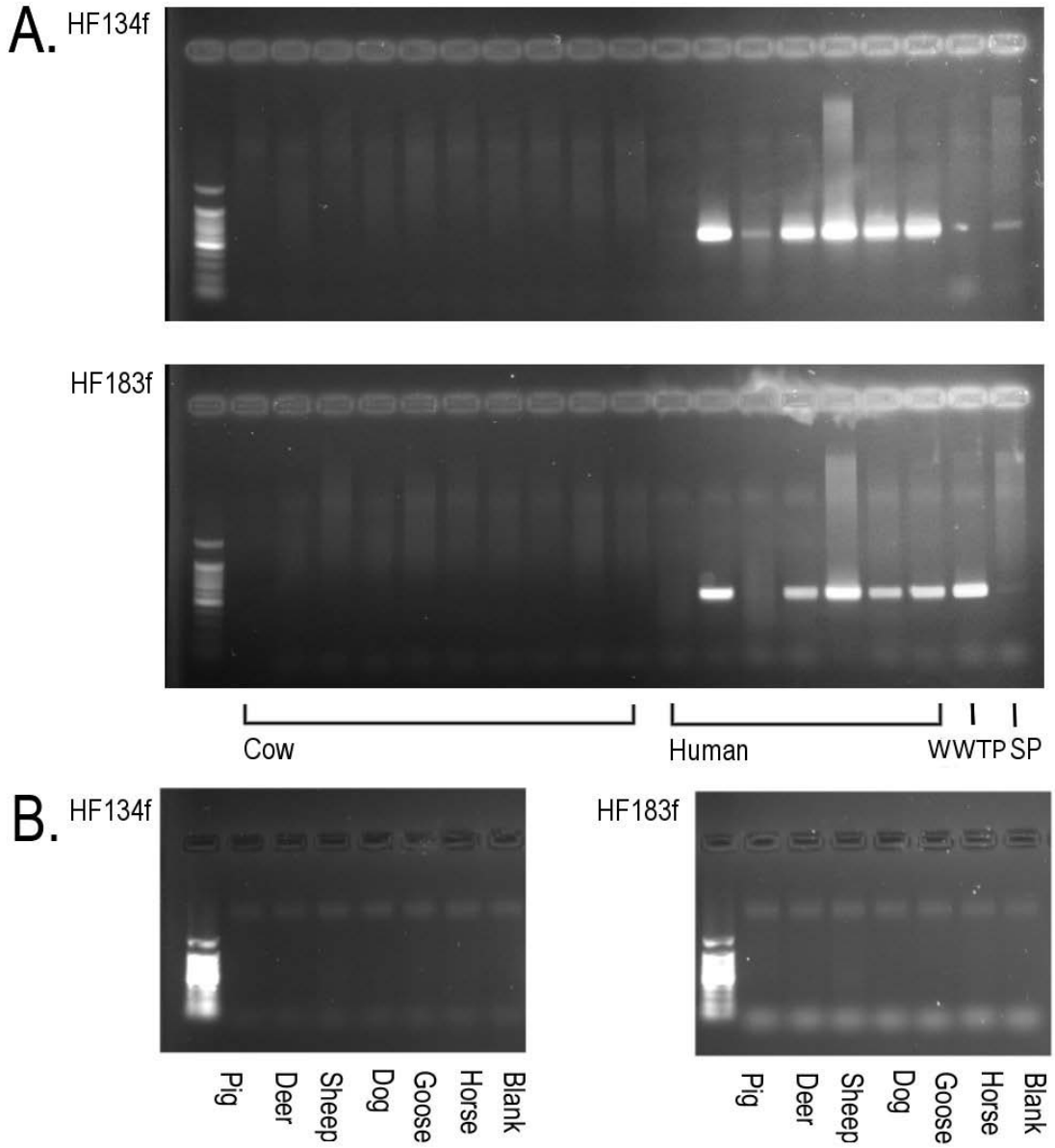

Figure 4.3 Host specificity PCR results with two human specific primers (HF134f and HF183f). (A) False positive on cow fecal DNA was not observed. One out of 7 individual human fecal samples was false negative for both primers. WWTP influent DNA (WWTP), and septic influent DNA (SP) both tested positively. (B) No false positives were observed on other host DNA samples.

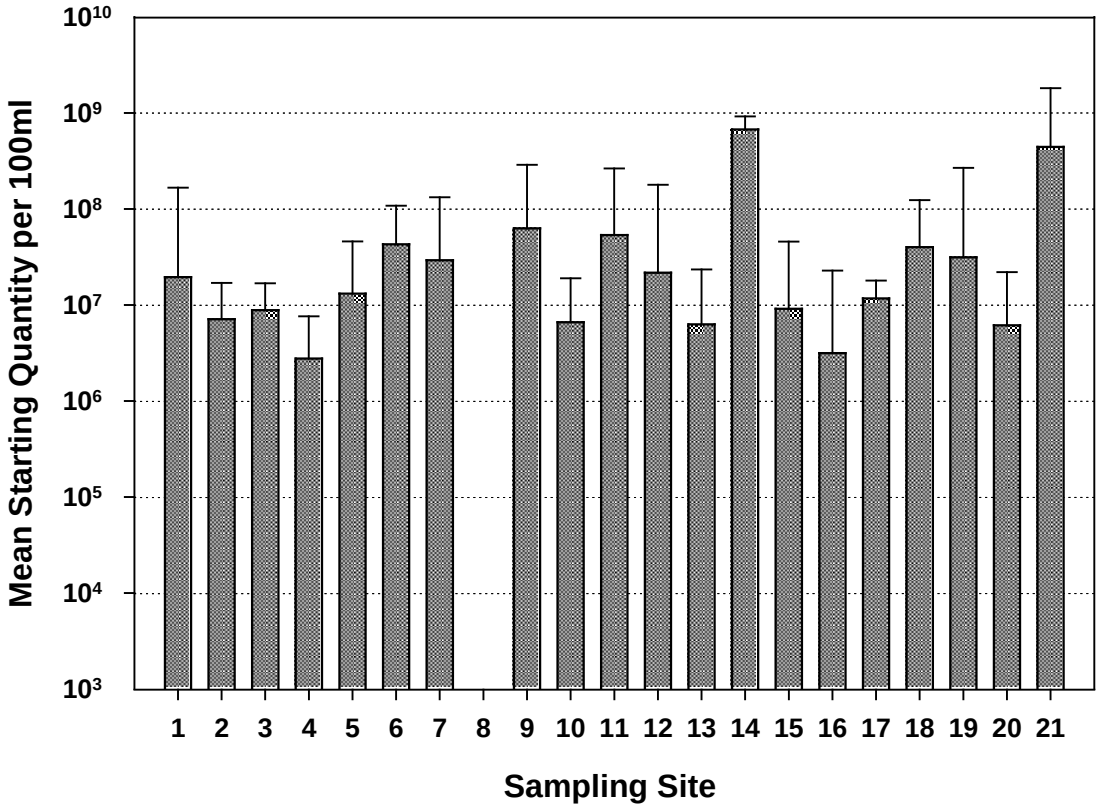

Figure 4.4 qPCR results with general *Bacteroidales* 16S rRNA gene markers. Values are the geometric mean of five samplings and the error bar indicates one standard error.

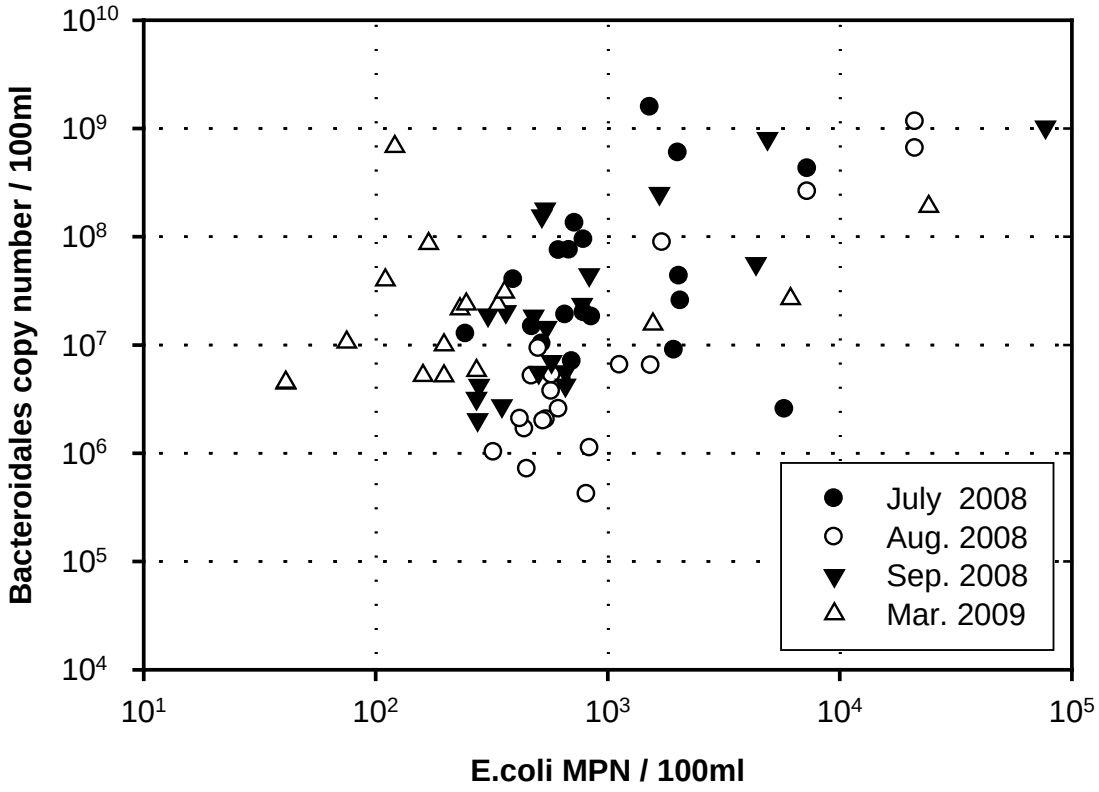

Figure 4.5 Scatter plot showing the relationship between *E. coli* most probable number and *Bacteroidales* qPCR results. Spearman's rank correlation coefficient was 0.545 (n=59, P<0.001). This is a non-parametric test and, therefore, a correlation equation cannot be generated.

CHAPTER 5

IDENTIFYING MICROBIAL CONTAMINATION HOTSPOT WITH TARGETED SAMPLING AND QUANTITATIVE PCR

5.1 ABSTRACT

A major limitation with the microbial source tracking method to identify the source of a microbial contamination is cost. A targeted sampling method was conducted to find hotspots of microbial contamination and identify the source of contamination accurately and cost efficiently. The first samplings were done at base flow conditions with spatially intensive samplings from suspected residential and agricultural fecal contamination source areas. The second samplings were done as temporally intensive samplings conducted during both baseflow condition and stormflow conditions from suspected agricultural fecal contamination source areas. Finally, because *Bacteroidales* is a relatively new indicator bacterium, we combined the *Bacteroidales* 16S rRNA gene assay with the *E. coli* viable count assay to study the differences and similarities of fate and transport of these two indicator bacteria.

When targeted sampling was combined with the Microbial Source Tracking (MST) method and the quantitative indicator bacteria assay, the combination was able to identify sources of fecal contamination quickly, easily, and inexpensively. These lowered costs should make MST method more attractive as a way of identifying the source of fecal contamination. Once fecal contamination hotspots are detected, action can then be taken to mitigate or eliminate the contamination.

5.2 INTRODUCTION

The goal of Microbial Source Tracking (MST) is to identify the source of microbial contamination in natural waters. Microbial source tracking has been applied using phenotypic-, genotypic-, and chemical-methods (Scott et al., 2002; Simpson et al., 2002; Field and Samadpour 2007). Mostly, the target species when MST is applied are either pathogenic or indicator bacteria which originate from the intestinal systems of warm-blooded animals.

Recently order *Bacteroidales* has been studied as an indicator bacterium for widely diverse environments (Ahmed et al., 2008; Bernhard and Field 2000a,b; Bernhard et al., 2003; Dick et al., 2004, 2005; Fogarty and Voytek 2005; Kreader 1995, 1998; Okabe et al., 2007a,b; Shanks et al., 2006; Yampara-Iquise et al., 2008). Order *Bacteroidales* is reported to have host specific distributions (Dick et al., 2005) and low survival rate in aerobic freshwater environments (Fiksdal et al., 1985; Kreader 1998). Therefore, it is only expected to be detected when there is recently introduced fecal contamination. In the previous chapter, a watershed scale MST study using *Bacteroidales* host specific 16S rRNA gene PCR assay (Bernhard and Field 2000a) and *Bacteroidales* 16S rRNA gene quantitative PCR assay (Layton et al., 2006) was described. It was concluded that the sampling and analysis method used could detect suspected areas with fecal contamination sources at the watershed scale level, and that there were hotspots of contamination in the studied watershed. Also,

the previous study included only a limited number of baseflow water samples and could not detect any fecal contamination in storm runoff.

A major limitation with the MST method to identify the source of a microbial pathogen pollutant is cost. In order to obtain accurate contamination source information, a large number of samplings and sample analyses have to be done, and the genotypic analyses can be quite costly. Thus there is a need to develop more cost effective, targeted sampling methods that can be coupled with MST to provide practical use for regulatory agencies and researchers.

Indicator bacteria exist in environmental waters both during base flow periods and during storm flow events. Previous studies have shown that microbial contamination input and stream stage height were strongly correlated (Hunter et al., 1992). Enteric bacteria are also known to be mostly associated with fine sediment particles in aquatic systems (Gannon et al., 1983; Auer and Niehaus, 1993). Therefore, sediment particles and both indicator and pathogenic bacteria are probably moved into stream waters during more intense storm events when there are high flows and movement of sediment. However, the influence of sediment transport on microbial transport is not fully understood and survival of the microbial community in sediment environments is quite complex. There are several studies on the relationship between sediment concentrations and presence of bacteria in water bodies. For example, Howell et al. (1996) found a high survival rate of coliform bacteria in settled sediments. In contrast, Maki and Hicks (2002) reported that the *Salmonella typhimurium* survival rate in the water column containing suspended sediment did not enhance the survival of the organism. Anderson et al. (2005) studied the survival rate

of different phylotypes of *E. coli* and found that different phylotypes of *E. coli* exhibited different survival rates in the environment. Their results suggested that when specific phylotypes are able to survive in settled sediment, they become dominant in the sediment environment. The consequence is this phylotype will be the dominant species in the water column when storm events occur. The fate and transport of our target indicator bacteria, *Bacteroidales* has not been studied and is unknown.

The objective of the study reported in this chapter was to find hotspots of fecal contamination and identify the source of fecal contamination accurately and cost efficiently by using a targeted sampling method. To fulfill this objective, targeted samplings were conducted in two different ways. The first samplings were done at base flow conditions with spatially intensive samplings from suspected residential and agricultural fecal contamination source areas. Our first hypothesis is that the spatial intensive sampling method during baseflow would pinpoint the exact location of contamination at hotspots. The second samplings were done as temporally intensive samplings conducted during both baseflow condition and stormflow conditions. Our second hypothesis is that the base and storm samplings would reveal transport mechanisms of the indicator bacteria as being either associated with sediment or freshly introduced as land surface flow during the stormflow events. Finally, because *Bacteroidales* is relatively new as a pathogen indicator bacterium, we combined the *Bacteroidales* 16S rRNA gene assay with the *E. coli* counting assay to study the differences and similarities of fate and transport of these two indicator bacteria.

5.3 MATERIALS AND METHODS

5.3.1 Study watershed, sampling locations, and schedule

We collected water samples from the Upper Sugar Creek Watershed located in Wayne County, Ohio. The study watershed characteristics were described in detail in the previous chapter (Chapter 4). Land uses include residential areas, crop fields, livestock operations (dairy, sheep, horse, and swine), and natural forested areas. This mixed-use watershed is suitable for examining the source of microbial contamination from human activity, agricultural activity, and/or wildlife.

5.3.2 Spatial intensive sampling

Two spatial intensive sampling sites were identified based on results obtained from the measurements made of concentrations of general *Bacteroidales* in five sets of baseflow water samples collected between July, 2008 and March, 2009. Recurrent high general *Bacteroidales* quantities were observed at Site 14 and Site 21 (Fig. 4.1). This implies that continuous input of fecal contamination was occurring at these two sites. By the observations of land use around these two sampling sites, a possible association between fecal contamination and land use could be made.

The sub-watershed of Site 14 (Fig. 4.1) had a residential area with septic systems located along the stream and no apparent agricultural source. First, six sampling spots (No.1 to 6 on Fig. 5.1) were selected upstream of the Site 14 and three sets of samples were collected from these sampling spots. Next, additional four

sampling spots (No.7 to 10 on Fig. 5.1) were added at the down stream of the Site 14 and another three sets of samples were collected from total of 10 sampling spots to pinpoint the source of the fecal contamination at the hotspots.

For Site 21, the historical information collected from local residents indicated this sub-watershed had a natural spring fed farm pond (Fig. 5.2). The pond area is backfilled and used as pasture currently. Sampling Site 21 was located 20 feet downstream of three pipe outlets, and these pipe outlets would be the only sources of water feeding to Site 21. These pipe outlets were likely either tile line outlets of spring water or septic outlet of residential buildings located nearby. Three sampling points (Fig.5.2) including original Site 21 and two of upstream pipe outlets from which continuous flow observed were selected and three sets of samples were collected to pinpoint the source of the fecal contamination at these hotspots.

These water samples (approximately 100 mL) were collected from each sampling points between October, 2008 and September, 2009. The water samples were placed in a cooler and taken to the laboratory and processed within six hours. Viable *E. coli* counts were obtained immediately when samples were brought to the laboratory using the Colilert ® Method with Quanti-Tray/2000™ (IDEXX, ME).

5.3.3 Storm flow sampling

One storm flow sampling site was selected based on the land use management and previous watershed scale baseflow sampling results described in Chapter 4. The selected Site 10 (Fig 5.3) and its sub-watershed has a dairy pasture upstream. Also tile line outlets are located just above the sampling site. However, we did not observe

high fecal contamination level with general *Bacteroidales* qPCR in previous baseflow samples and also did not observe ruminant fecal contamination with ruminant specific *Bacteroidales* PCR assay (see Chapter 4). We hypothesized that if pathogens do show up at this sampling site, it would be due to a different transport mechanism. Instead of being introduced during baseflow, stormflow would be much more important.

At this sampling Site 10, four sampling points were selected upstream of the original sampling site. There is no major agricultural influence were expected at two of upstream sampling points 10-1 and 10-2, and sampling point 10-3 would not get runoff from the daily pasture and tile line outlets. In order to study the effect of stormflow to fecal contamination runoff, total of three sets of baseflow samples and four sets of storm flow samples were taken between August 2009 and September 2009. These water samples (approximately 100 mL) were placed in a cooler and taken to the laboratory and processed within six hours. Viable *E. coli* counts were obtained immediately when samples were brought to the laboratory using the Colilert ® Method with Quanti-Tray/2000™ (IDEXX, ME).

5.3.4 DNA extraction

DNA extraction from the water samples were conducted using Method 1 as described in Chapter 3 of this dissertation. Briefly, 100 mL of the baseflow water samples were filtrated through a membrane filter with 0.2 μm openings. Stormwater samples were expected to contain more suspended solid and therefore 20 mL of sampled water were diluted to 100 mL with DNA free water. They were then also filtered through a membrane filter with 0.2 μm openings. This dilution step prevented

incomplete filtering due to clogging of the membrane and also prevented the inhibition of the PCR reaction by humic materials which are often abundant in stormwater samples. The bacterial cells collected on the filters were processed using the PowerSoil™ DNA isolation kit according to the manufacture's instruction except using the membrane filters containing the bacterial cells instead of using soil as the DNA source. All extracted DNA samples were stored at -20°C until further processing.

5.3.5 General and human host specific *Bacteroidales* quantitative PCR

A set of general primers that targeted the 16S rRNA gene of *Bacteroidales* (AllBacf: GAG AGG AAG GTC CCC CAC, Allbacr: CGC TAC TTG GCT GGT TCA G, Layton et al., 2006) was used for qPCR to assess the magnitude of fecal contamination in the samples. Also, selected samples were analyzed with a set of human specific primers that also targeted the 16S rRNA gene of *Bacteroidales* (HF183f: ATC ATG AGT TCA CAT GTC CG, HF183r: TAC CCC GCC TAC TAT CTA ATG, Seurinck et al., 2004). All qPCR assays were performed using the iQ™SYBR® Green Supermix and the iQ5 real-time PCR detection system (Bio-Rad. Hercules, CA). Each 25 µL PCR mixture contained 1X of the iQ Supermix, each primer at a concentration of 0.3 µM, non-acetylated bovine serum albumin at 0.06% concentration, and 1 µL of DNA extraction sample. The 1 µL of DNA theoretically corresponds to 1 mL of water sample. A real-time thermal cycler was used for all reactions with the following conditions: an initial heating at 95°C for 3 min for predenaturation followed by 40 cycles consisting of 95°C for 15 sec and 60°C for 1

min. After completion of the reaction, the melt curve was analyzed to check for accuracy of data. For each qPCR run, all samples were analyzed in triplicate. PCR inhibitors in the samples were determined to be negligible based upon results obtained after 10-fold and 100-fold dilutions.

5.3.6 Human and ruminant host-specific PCR

In addition to quantitative PCR assays, human- and ruminant-specific *Bacteroidales* PCR analysis using the primer set described in Table 4.1 (Chapter 4) were conducted on storm and base flow samples. Each 25 µL final PCR mixture contained 1× Taq polymerase buffer (20mM Mg^{2+}), 1.25 U of Takara Ex Taq^{TM} polymerase (Takara Bio Inc. Japan), each primer at a concentration of 0.2 µM, dNTPs mixture at a concentration of 200 µM each, and non-acetylated bovine serum albumin at 0.04% in concentration. A thermal cycler (MJ Research, Watertown, MA) was used for all reactions with the following conditions: an initial heating at 94°C for 3 min for predenaturation, 35 cycles consisting of 94°C for 1 min, 62 °C for both ruminant marker sets or 63 °C for both human marker sets for annealing, 53 °C for 45 sec, and 72°C for 2 min were conducted. A final 7 min extension period was conducted at 72°C. All PCR products were visualized by electrophoresis using 1% agarose gel stained with ethidium bromide stain and compared using a 100 bp DNA ladder (Promega, Madison, WI). Sizes of amplicons for each primer pair are described in Table 4.1 (Chapter 4).

5.4 RESULTS AND DISCUSSION

5.4.1 Spatial intensive sampling

Suspected human hot spot

At the Site 14 sub-watershed, where recurrent high general *Bacteroidales* quantities were observed during previous watershed scale sampling, a total of 48 samples were quantitatively analyzed for the general *Bacteroidales* marker and the human specific *Bacteroidales* marker (Fig. 5.4). Each sampling point from 1 to 6 represents the geometric mean of six samplings collected from October 2008 to September 2009. Sampling points 7 to 10 represent means of three samplings collected from August 2009 to September 2009 for general *Bacteroidales* maker and for human specific *Bacteroidales* maker.

In these samples, the general *Bacteroidales* marker was detected in all 48 samples at concentrations ranging from 3.1×10^4 to 4.4×10^9 copies per 100 mL sample water. The human specific *Bacteroidales* marker was detected in all samples. Four samples from sampling points 2 and 3 in October 2008 and sampling points 1 and 2 in June 2009 show levels below the PCR quantification limit which was 10^2 copies per reaction. One reaction required 1µL of template DNA, which theoretically originated from 1mL of sample water, Therefore quantities of these samples were recorded as 10^4 per 100 mL of sample water. The highest concentration of human specific *Bacteroidales* marker observed was 5.8×10^6 copies per 100 mL sample water.

Samples were taken throughout the year except during the winter. The highest general *Bacteroidales* and human specific *Bacteroidales* numbers were consistently observed at the sampling points 5, 6, or 7, which were close to the suspected septic outlets. As shown in Fig. 5.4, the differences in the log levels of the indicator bacteria concentrations among sampling points were evident. The identification of human marker at sampling points 5, 6, or 7, where septic outlets were found, suggest the contamination was likely of human origin. However, human specific marker was also observed upstream of the visible septic outlets so that it is possible that introduction of the marker could have also been some other source upstream. Since the flow rates were not logged in this study, it was impossible to calculate the load of human specific *Bacteroidale*s marker at each sampling point, but these contaminated hotspots were simply judged by the indicator concentrations.

Figure 5.5 shows the result for the *E. coli* Most Probable Number (MPN) assays along with the results from general *Bacteroidales* marker assay as a comparison. For the *E. coli* MPN assay, each sampling point from 1 to 6 represents the mean of five samplings collected in April 2009 to September 2009. The sampling points 7 to 10 represent the means of three samplings collected in July 2009 to September 2009. As expected, the general *Bacteroidales* copy numbers were always higher than the *E. coli* counts in the same sample. This is because *Bacteroidales* are a more dominant species than *E. coli* in fecal matter. Both indicator bacteria, however, showed a similar trend in concentration change along the stretch of the stream sampled. This supports the idea that *Bacteroidales* can be used as a sensitive fecal contamination indicator bacterium.

Suspected Agricultural hot spot

At the Site 21 sub-watershed, a total of three sampling points were quantitatively analyzed for general *Bacteroidales* marker and human specific *Bacteroidales* marker (Fig. 5.6). The results from three different baseflow sampling events in March, June, and July 2009 are shown separately. In these samples, the general *Bacteroidales* marker was detected in all 9 samples at concentrations ranging from 1.9×10^7 to 1.7×10^9 copies per 100 mL sample water. This sub-watershed was dominated by dairy activities and it was assumed that a human specific marker would be not detected. However, the human specific *Bacteroidales* marker was detected in all samples, even from the outlet pipe assumed to be a spring tile line (sampling point 2 in Fig. 5.6). The March and June samples show lower concentration below the PCR quantification limit which was 10^2 copies per reaction. One reaction required 1µL of template DNA, which theoretically originated from 1mL of sample water, Therefore quantities of these samples were recorded as 10^4 per 100 mL of sample water. The magnitude of human specific marker at sampling point 2 was lower than at sampling point 1 for all three sampling events. This suggests there might be a septic system connected to this outlet (sampling point 2) that was thought to only contain spring water samples or that the spring was contaminated by some other means. However, based on the difference in human specific marker concentration, the outlet pipe 1 (sampling point 1) contributed more fecal contamination to the down stream water than outlet pipe 2 (sampling point 2).

Fig. 5.7 shows the result for the *E. coli* Most Probable Number (MPN) assays along with the results from general *Bacteroidales* marker qPCR assay as a

comparison. The results from three different baseflow sampling events are shown separately. As expected, the general *Bacteroidales* copy numbers were always higher than *E. coli* counts in the same sample. The *E. coli* MPN number for sampling point 1, collected in March 2009, resulted in maximum detection limit (i.e. 2.4×10^4 MPN /100mL sample water). Therefore it is not indicated at a bar chart. The fact that this sample had the highest MPN value among the three sampling spots agreed with the trends observed for the other two sampling events that occurred in June and July. Similar to Site 14, both *Bacteroidales* and *E. coli* indicator bacteria showed similar trends in concentration differences among the three sampling points.

5.4.2 Storm flow study

Baseflow samples

Two sets of base flow samples from the four sampling points were also analyzed for quantities of general *Bacteroidales* marker (Fig. 5.8). The purpose of analyzing base flow samples was to investigate change in concentration of the fecal contamination marker along the flow path and to compare the concentration of the fecal contamination markers in base flow with storm flow samples from the same sampling points. Both sets of base flow samples indicated an almost 2-log higher concentration of general *Bacteroidales* marker at upstream sampling point 10-4 compared to the most down stream sampling point 10-1. This suggests there was a fecal contamination source upstream of this sampling site and this contamination was diluted when it reached sampling point 10-1.

In the previous Chapter 4, suspected fecal contamination hotspots were identified if average general *Bacteroidales* copy number was more than 10^8 copies/100mL sample water. Two samples from sampling point 10-4 indicated 7.9 x 10^7 and 6.2 x 10^8 copies/100mL sample water. Therefore, sampling point 10-4 would be defined as a potential fecal contamination hotspot. More intensive sampling would be needed to confirm this.

Stormflow samples

Fig. 5.8 also shows the results from the general *Bacteroidales* marker quantitative assays with three sets of storm flow samples. There was no statistical difference in marker concentrations among the sampling points due to the small number of samples collected. However, the trend was that concentrations of the indicator bacteria went up between sampling points 10-2 and 10-1 at all three sampling events. At this site, it was thought that agricultural sources were the most important influence on stormflow properties. Host specific (human and ruminant) *Bacteroidales* PCR were conducted both on baseflow and storm flow samples in order to investigate the source of *Bacteroidales* detected by the quantitative PCR (Table 5.1).

No ruminant-associated signal was observed in the two sets of base flow samples. However, all three storm sampling events resulted in ruminant-associated positive signals at sampling point 10-1 where pasture water runoff would be expected. Also, some ruminant signals were observed at the sampling point 10-2. This implies that the agricultural farm facility located between 10-2 and 10-3 could also be a source of agricultural fecal contamination during stormflow events. In addition,

human-associated signals were observed, even in baseflow at sampling points 10-4, 10-3 and 10-1. This finding is consistent with the general *Bacteroidales* qPCR results. Thus the contamination source upstream of these sampling points can be attributed to human sources. Also, unexpectedly, quite frequent human-associated signals were observed in stormflow samples.

Sediments are expected to be a large potential reservoir of indicator bacteria and pathogenic bacteria. Even though *Bacteroidales* would not survive in sediments, the target genes from non-viable cells potentially exist in sediments and may be detected with the molecular assays. Since the molecular assays used in this study do not differentiate between viable and non-viable cells, general and host specific *Bacteroidales* would be expected to be detected more often in stormflow than baseflow samples due to sediment disturbance. The association between viable pathogenic bacteria existence and "detectable" indicator bacteria *Bacteroidales* target gene existence in stormwater needs to be further investigated.

Two indicator bacteria fate and transport effects from storm study

In Chapter 4, *E. coli* viable counts and general *Bacteroidales* marker qPCR results in baseflow samples were moderately, but statistically positively correlated. *E. coli* MPN numbers were analyzed with four set of stormflow samples from four sampling spots at Site 10 and with three baseflow samples from same sampling spots. The results are shown in Fig. 5.9. Compared to similar analysis using general *Bacteroidales* maker (Fig. 5.8), *E. coli* numbers in stormflow clearly showed higher numbers at all sampling points in baseflow samples. In contrast, *Bacteroidales*

marker did not show any differences between the numbers taken from stormflow and baseflow samples. Thus the fate and transport of the two indicator microorganisms are different. Possible reasons are that live *E. coli* cells survived in the environment within sediments, and possibly reproduced. They were then flushed out into stream waters during storm events. *Bacteroidales* does not survive well in the environment and so only more recently introduced cells and nonviable cells would be detected. Therefore *Bacteroidales* number did not show significant difference between stormflow and baseflow samples.

With respect to MST methods, different behavior of these two indicators in stormflow would be a key factor in the future use of these two indicator bacteria. Further studies on the association of *Bacteroidales* and *E. coli* fate and transport in watersheds are needed to ensure the successful use of the MST method.

5.5 SUMMARY

This study was the first to combine targeted sampling and microbial source tracking (MST) using *Bacteroidales* as an indicator bacterium. When targeted sampling was combined with the MST method and the quantitative indicator bacteria assay, the combination was able to identify sources of fecal contamination quickly, easily, and inexpensively. These lowered costs should make MST method more attractive as a way of identifying the source of fecal contamination. In the case of the spatial targeted sampling study, the most likely major source of fecal contamination

in baseflow events was found to be of human origin from septic systems. In the case of storm flow events, sediments seemed to be large potential reservoirs of fecal contamination. Once fecal contamination hotspots are detected, action can then be taken to mitigate or eliminate the contamination.

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Human Markers

		HF134F					HF183F				
		Baseflow		Storm flow			Baseflow		Storm flow		
	Sampling date	8/27/09	9/15/09	8/20/09	8/28/09	9/23/09	8/27/09	9/15/09	8/20/09	8/28/09	9/23/09
Sampling site Downstream------Upstream	10-4	+	++	++	+	+		+	++	+	+
	10-3	+		++	++	+			++	++	+
	10-2			++	++				++	+	
	10-1		+	+		++		+	+		+

Ruminant Markers

		CF128F					CF193F				
		Baseflow		Storm flow			Baseflow		Storm flow		
	Sampling date	8/27/09	9/15/09	8/20/09	8/28/09	9/23/09	8/27/09	9/15/09	8/20/09	8/28/09	9/23/09
Sampling site Downstream------Upstream	10-4										
	10-3										
	10-2			++	+				+		
	10-1			+	++	+			+	+	+

Table 5.1 Site 10 host specific *Bacteroidales* PCR results from storm and base flow samples. +: Weak positive ++: Strong positive. The intensity of these positive signals was judged by comparison to reference positive controls on an electrophoresis gel image.

Figure 5.1 Sampling spots at sampling site 14.Ten sampling spots were located up and down stream of original sampling spot for site 14 (number 5). Flow runs from north east to south west, and runs into main sugar creek (Adapted and modified from: Imagery: ©2010 DigitalGlobe, GeoEye, State of Ohio/OSIP, USDA Farm Service Agency, Map data: ©2010 Google).

Figure 5.2 Left: sampling spots at sampling site 21.Three sampling spots are indicated as 1: flow from pipe located on the right, 2: flow from pipe located on the left, and 3: original sampling spot for site 21, which is located on the other side of the culvert pipe. Right: overview of sub-watershed of sampling site 21. No surface flow was observed, this pasture was backfilled spring pond area.

Figure 5.3 Sampling spots at site 10. This tributary runs from North to South. Original site 10 samples were taken at the sampling point 1 on this aerial photo (Adapted and modified from: Imagery: ©2010 DigitalGlobe, GeoEye, State of Ohio/OSIP, USDA Farm Service Agency, Map data: ©2010 Google).

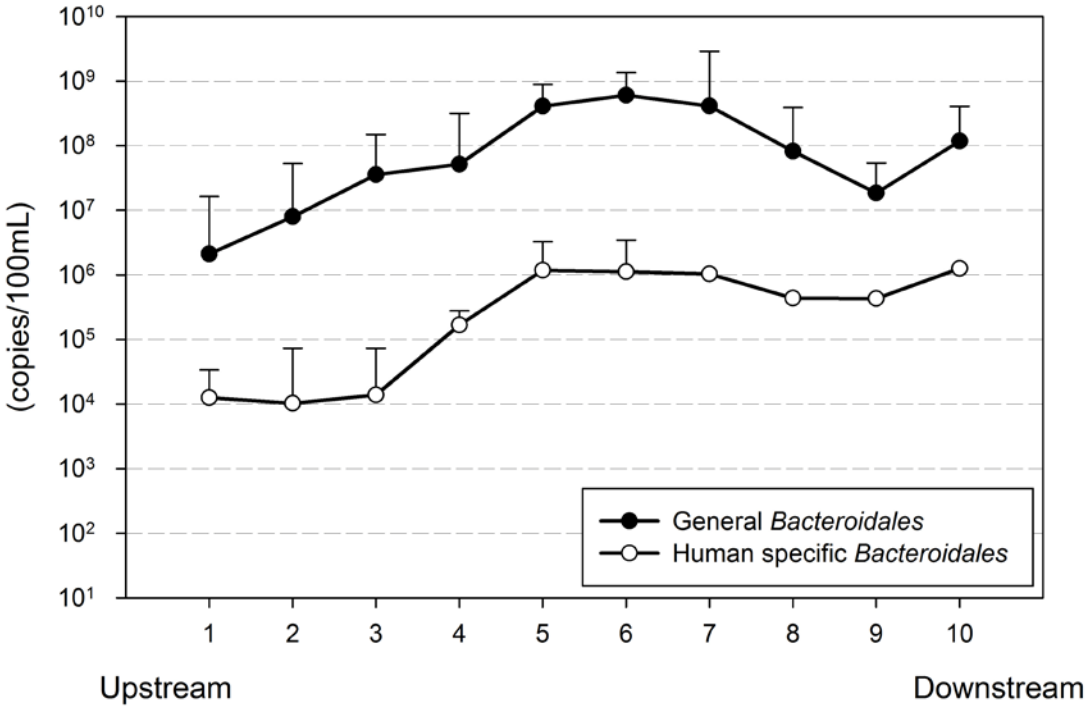

Figure 5.4 Site 14 general *Bacteroidales* and human specific *Bacteroidales* qPCR results. Data points indicate geometric means of multiple samplings (July 2008 to March 2009). Error bar indicates standard deviation from these multiple samplings, No error bars generated for sample point 7 to 10 with Human specific *Bacteroidales*, these data points represent one set of samples.

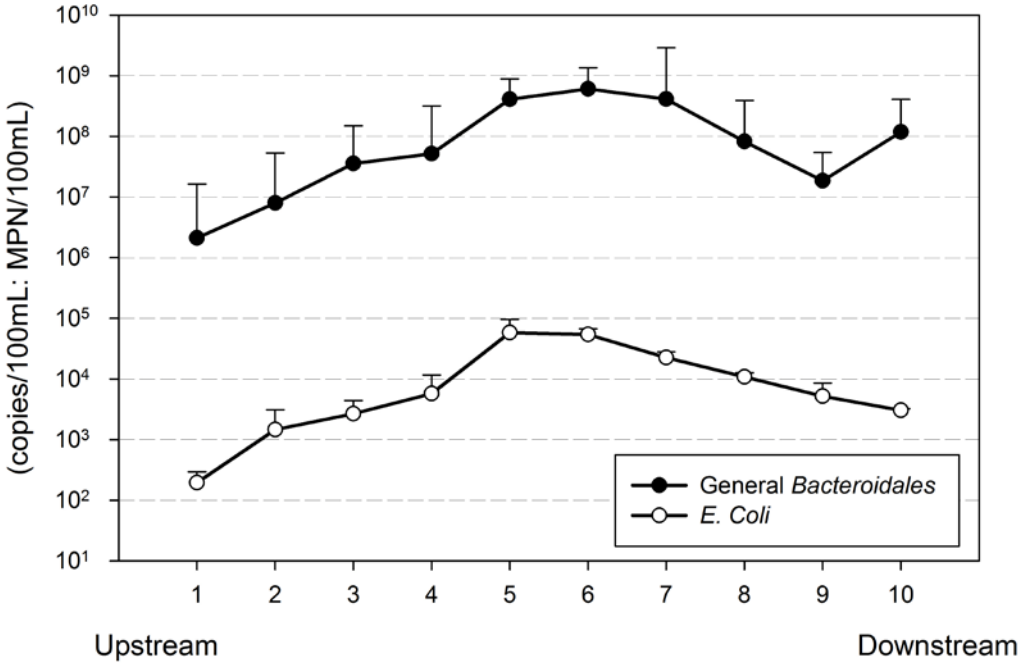

Figure 5.5 Site 14 General *Bacteroidales* qPCR result and *E. coli* Most probable number. Error bar indicates standard deviation of multiple samplings.

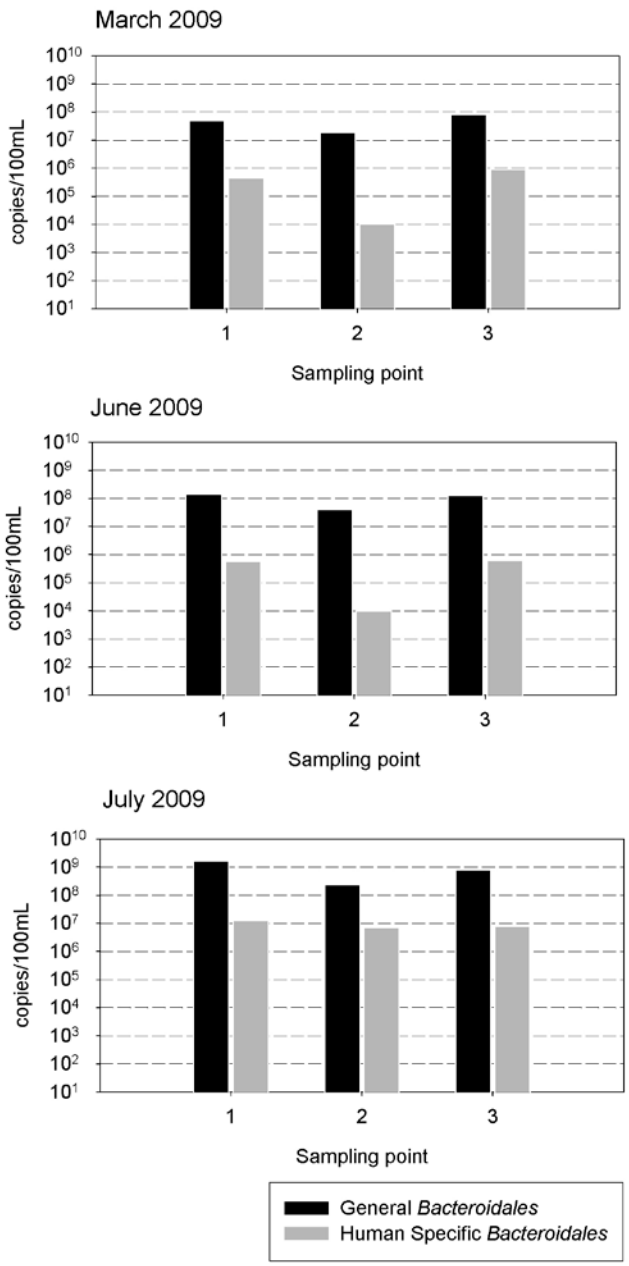

Figure 5.6 Site 21 general and human specific *Bacteroidales* qPCR results. Samples were taken three different sampling events.

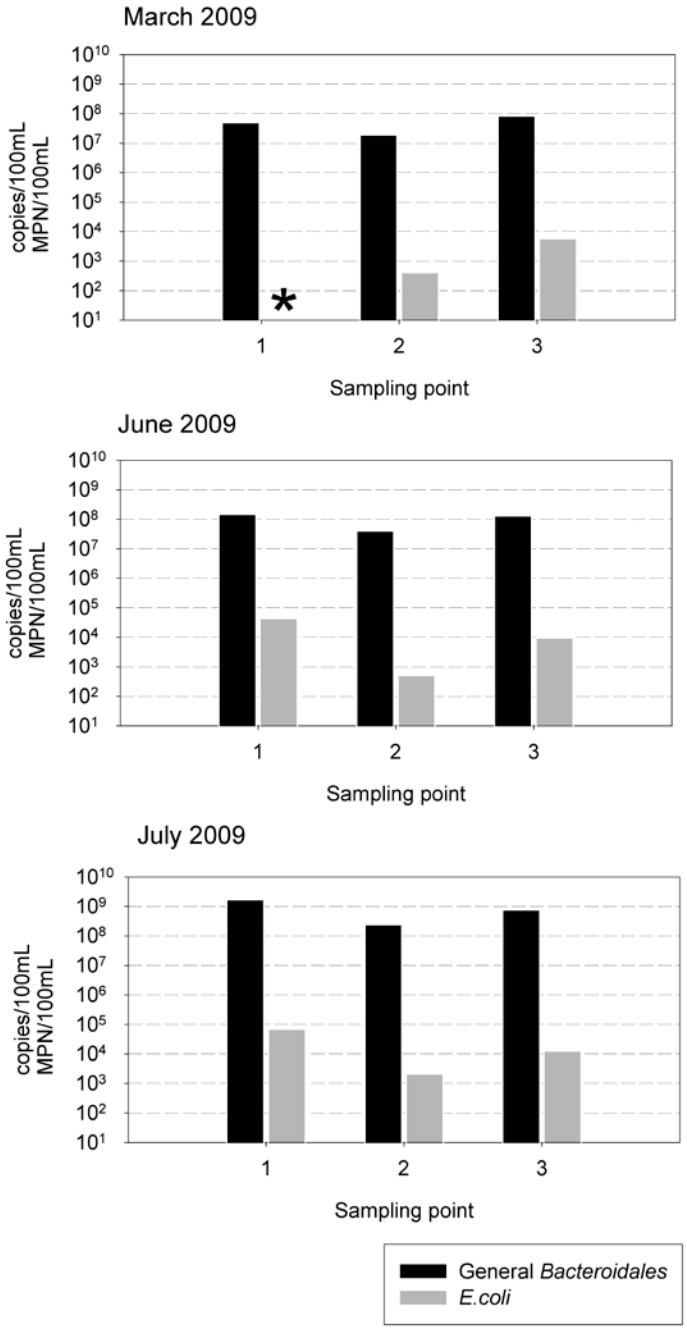

Figure 5.7 Site 21 general *Bacteroidales* qPCR result and *E. coli* most probable number result. Samples were taken three different sampling events.
*sample from the sampling point 1 on March 2009, *E. coli* analysis resulted in maximum detection limit (2.4 x 10^4 MPN/100mL sample water).

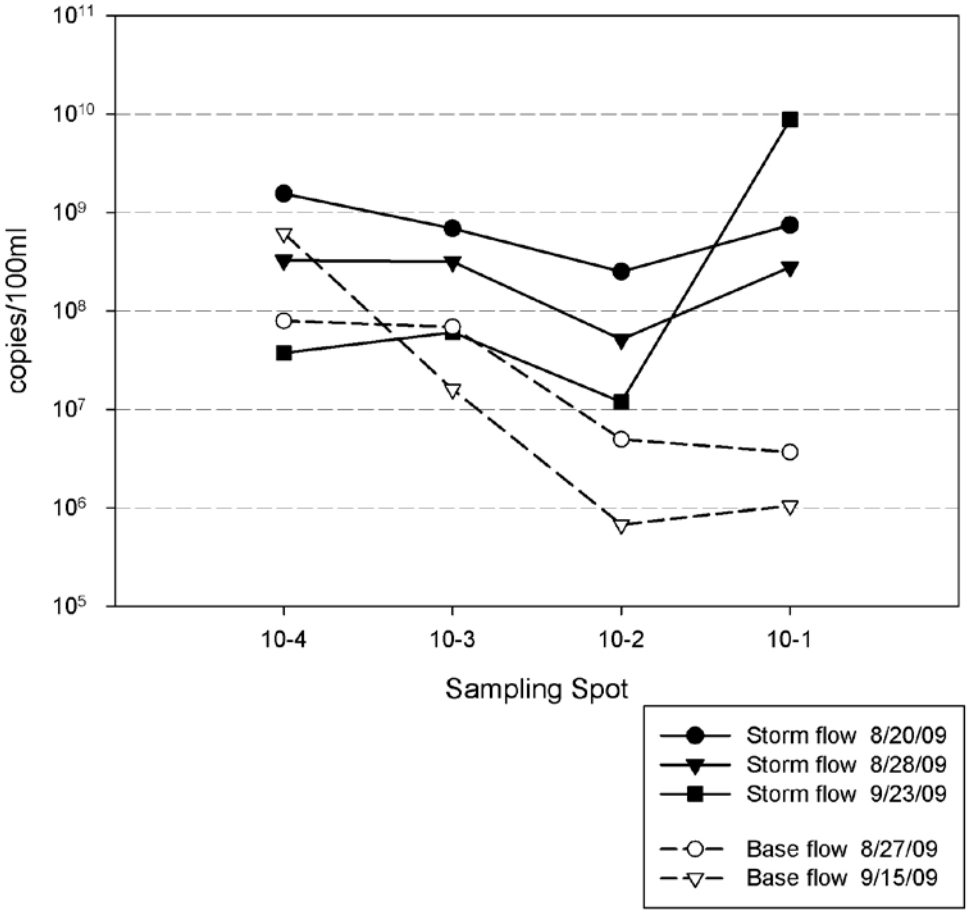

Figure 5.8 Site 10 general *Bacteroidales* qPCR result from storm and baseflow samples.

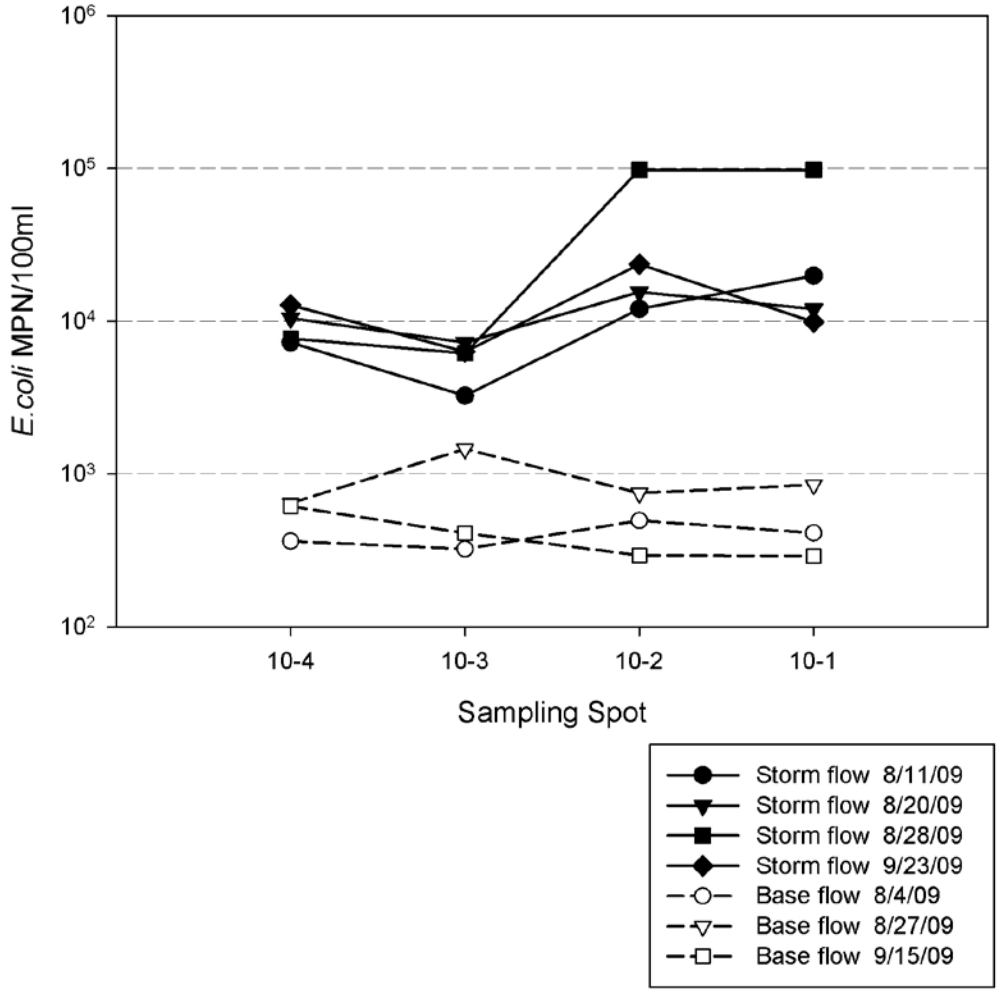

Figure 5.9 Site 10 *E. coli* Most Probable Number from storm and base flow samples.

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APPENDIX

Date: 07/19/2006

Siite Number		Description	Temperature ˚C	pH	DO mg/L	Turbidity NTU	Conductivity mS/cm	E.coli MPN/100ml	SS mg/L	DNA conc, ng/ul	260/280	260/230
1	1-1	Forested area up										
2	1-2	Forested area down (stream #20)	24.5	7.3	1.5	25.7	0.05	310	13.00	244.36	1.45	0.93
3	2-1	Barn with horse up/septic tank up (stream #20)	23.8	7.4	2.3	23.4	0.00	281	164.00	338.42	1.41	0.93
4	2-2	Barn with horse down (stream #20)	24.0	7.6	3.6	16.0	0.00	262	3.00	192.78	1.44	0.94
5	4-1	Pond with water fowl down (main stream)	27.9	8.6	4.0	21.3	0.34	74	-	18.75	1.98	0.50
6	5-1	Dairy pasture down (stream #6 and #7)						4480	-	8.78	1.63	0.23
7	6-1	Town up (main stream)	22.3	8.3	6.0	3.3	0.56	265	-	4.67	2.35	0.42
8	6-2	Town down (main stream)	21.3	8.3	5.4	2.6	0.55	288	-	4.52	3.21	0.41
9	7-1	WWTP up (main stream)	22.1	8.3	6.0	2.0	0.55	382	-	9.02	2.22	0.72
10	7-2	WWTP down (main stream)	21.9	8.3	5.2	1.8	0.56	265	11.00	4.41	1.91	0.43
11	8-1	Pig operation up (stream #23)	27.8	7.6	4.5	12.0	0.41	146	11.00	39.53	2.06	1.45
12	8-2	Pig operation down (stream #23)	27.3	8.2	4.5	5.6	0.69	2460	12.00	3.71	3.28	0.25
-	blank	Field blank	-	-	-	-	-	<1	0	-	-	-

Date: 09/11/2006

Siite Number		Description	Temperature ˚C	pH	DO mg/L	Turbidity NTU	Conductivity mS/cm	E.coli MPN/100ml	SS mg/L	DNA conc, ng/ul	260/280	260/230
13	1-1	Forested area up										
14	1-2	Forested area down (stream #20)	16.3	7.9	3.1	16.5	6.73	1353	55.20	210.70	1.58	1.05
15	2-1	Barn with horse up/septic tank up (stream #20)										
16	2-2	Barn with horse down (stream #20)	15.6	8.2	6.9	10.5	5.16	7270	29.80	18.66	1.57	1.10
17	4-1	Pond with water fowl down (main stream)	19.7	0.3	6.1	24.9	0.45	231	11.70	25.60	1.96	0.56
18	5-1	Dairy pasture down (stream #6 and #7)	16.9	7.9	5.4	44.1	0.57	487	10.60	11.52	1.50	0.47
19	6-1	Town up (main stream)	16.9	8.5	8.5	0.0	0.74	269	2.60	9.22	2.25	0.19
20	6-2	Town down (main stream)	16.5	8.3	7.3	0.0	0.67	305	3.90	5.89	2.98	0.71
21	7-1	WWTP up (main stream)	17.5	8.3	7.8	1.5	0.76	143	1.30	4.63	2.08	0.69
22	7-2	WWTP down (main stream)	18.0	8.6	9.1	1.1	0.75	331	6.60	8.85	1.54	0.73
23	8-1	Pig operation up (stream #23)	-	-	-	-	-	-	-			
24	8-2	Pig operation down (stream #23)	18.7	0.6	3.9	115.0	1.78	>2419	39.40	103.03	2.10	1.75
-	blank	Field blank	-	-	-	-	-	<1	0	-	-	-

continued

Table A.1 Water quality data used for the analysis in Chapter 2.

Table A.1 continued

Date: 10/03/2006

	Siite Number	Description	Temperature ˚C	pH	DO mg/L	Turbidity NTU	Conductivity mS/cm	E.coli MPN/100ml	SS mg/L	DNA conc, ng/ul	260/280	260/230
25	1-1	Forested area up										
26	1-1	Forested area down (stream #20) big Pipe	16.84	7.54	5.70	33.8	1.100	496	3.70	30.01	1.63	0.69
27	1-2	Forested area down (stream #20) small pipe	16.11	6.67	5.28	22.0	0.460	959	6.40	24.4	1.74	0.68
28	2-2	Barn with horse down (stream #20)	15.60	7.80	6.76	18.3	1.650	2489	23.00	26.75	1.71	0.48
29	4-1	Pond with water fowl down (main stream)	18.78	8.07	6.83	900.0	0.369	156	194.30	20.21	2.84	0.19
30	5-1	Dairy pasture down (stream #6 and #7)	17.05	7.94	6.04	23.2	0.530	288	32.60	9.82	1.71	0.32
31	6-1	Town up (main stream)	16.87	8.57	9.64	39.0	0.705	110	1.90	4.74	1.32	0.3
32	6-2	Town down (main stream)	15.83	8.47	9.16	26.6	0.653	41	0.30	5.89	3.01	0.29
33	7-1	WWTP up (main stream)	11.40	8.40	8.86	14.0	0.722	216	0.70	9.14	2.2	0.45
34	7-2	WWTP down (main stream)	16.80	8.32	8.73	8.9	0.719	462	0.30	13.4	1.99	0.35
35	8-1	Pig operation up (stream #23)	18.16	8.22	8.01	49.5	0.224	95	14.10	101.83	2.07	1.28
36	8-2	Pig operation down (stream #23)	17.39	7.91	6.18	9.9	1.640	631	1.40	11.92	1.84	0.35
-	blank	Field blank	-	-	-	-	-	<1	0	-	-	-

Date: 10/25/2006

	Siite Number	Description	Temperature ˚C	pH	DO mg/L	Turbidity NTU	Conductivity mS/cm	E.coli MPN/100ml	SS mg/L	DNA conc, ng/ul	260/280	260/230
37	1-1	Forested area up										
38	1-1	Forested area down (stream #20) big Pipe	7.77	7.26	6.44	105.0	0.479	>2419.6	14.14	116.31	1.35	0.78
39	1-2	Forested area down (stream #20) small pipe	8.10	7.47	5.00	132.0	0.237	228	18.14	56.98	1.38	0.68
40	2-2	Barn with horse down (stream #20)	6.84	7.62	9.50	71.1	0.310	2667	9.86	61.83	1.26	0.64
41	4-1	Pond with water fowl down (main stream)	7.74	8.07	11.33	10.8	0.381	97	11.00	73.99	1.64	0.63
42	5-1	Dairy pasture down (stream #6 and #7)	8.43	8.03	9.35	10.9	0.504	96	12.00	13.76	1.46	0.36
43	6-1	Town up (main stream)	7.51	8.33	118.00	12.1	0.602	368	2.14	13.23	1.84	0.44
44	6-2	Town down (main stream)	7.44	8.36	11.22	3.6	0.575	529	2.14	9.15	1.69	0.39
45	7-1	WWTP up (main stream)	7.55	8.43	12.01	3.7	0.587	839	1.14	11.15	1.61	0.5
46	7-2	WWTP down (main stream)	7.83	8.03	9.50	3.5	0.611	645	2.57	39	1.48	0.72
47	8-1	Pig operation up (stream #23)	6.11	8.14	14.00	73.0	0.332	41	10.71	30.64	2.03	0.91
48	8-2	Pig operation down (stream #23)	7.40	8.11	11.30	2.0	0.901	85	1.43	4.7	2.42	0.3
-	blank	Field blank	-	-	-	-	-	<1	0	-	-	-

Bacteroidales qPCR 8/13/08

	Extraction Process (unit: copies/ mL of sample water)					
Replicates	15ml Tube extraction	Geomean	2ml Tube extraction	Geomean	Centrifuge	Geomean
blank	0.00E+00	-	1.25E+03	1.06E+03	0.00E+00	-
blank	1.06E+02		8.95E+02		0.00E+00	
1	1.58E+05	1.58E+05	4.48E+05	4.69E+05	7.23E+04	6.23E+04
1	-		4.91E+05		5.37E+04	
2	2.09E+05	1.87E+05	4.70E+05	4.53E+05	6.72E+04	7.20E+04
2	1.67E+05		4.37E+05		7.72E+04	
3	1.46E+05	1.43E+05	3.85E+05	4.03E+05	5.56E+04	5.28E+04
3	1.41E+05		4.22E+05		5.01E+04	
4	4.74E+05	4.33E+05	3.72E+05	3.85E+05	4.78E+04	5.06E+04
4	3.95E+05		3.99E+05		5.36E+04	
5	3.33E+05	3.94E+05	5.15E+05	4.90E+05	5.38E+04	5.58E+04
5	4.65E+05		4.66E+05		5.79E+04	
6	4.40E+05	4.30E+05	6.75E+05	6.46E+05	3.27E+04	3.49E+04
6	4.21E+05		6.19E+05		3.73E+04	
7	2.69E+05	2.81E+05	3.81E+05	3.94E+05	5.65E+04	5.82E+04
7	2.93E+05		4.07E+05		6.00E+04	
8	4.48E+05	4.51E+05	6.08E+05	5.88E+05	6.00E+04	5.45E+04
8	4.54E+05		5.69E+05		4.95E+04	
9	2.69E+05	2.72E+05	4.19E+05	3.55E+05	9.00E+04	9.05E+04
9	2.75E+05		3.01E+05		9.11E+04	
10	4.09E+05	3.87E+05	3.51E+05	3.66E+05	3.32E+04	3.37E+04
10	3.66E+05		3.81E+05		3.43E+04	

continued

Table A.2 qPCR results for comparison of sample processing methods. Used for the analysis in Chapter 3.

Table A.2 continued

P.stewartii qPCR 8/04/08

	Extraction Process (unit: copies/ mL of sample water)					
Replicates	15ml Tube extraction	Geomean	2ml Tube extraction	Geomean	Centrifuge	Geomean
No PS	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
No PS	0.00E+00		0.00E+00		0.00E+00	
blank	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
blank	0.00E+00		0.00E+00		0.00E+00	
1	2.43E+03	2.55E+03	7.46E+03	7.76E+03	1.44E+03	1.34E+03
1	2.68E+03		8.08E+03		1.25E+03	
2	3.25E+03	3.00E+03	6.65E+03	7.45E+03	8.12E+02	7.44E+02
2	2.77E+03		8.35E+03		6.81E+02	
3	5.14E+03	5.82E+03	7.52E+03	8.19E+03	1.49E+03	1.51E+03
3	6.60E+03		8.93E+03		1.53E+03	
4	5.56E+03	5.80E+03	1.08E+04	1.06E+04	2.76E+03	2.59E+03
4	6.06E+03		1.05E+04		2.43E+03	
5	6.51E+03	6.65E+03	9.70E+03	9.50E+03	1.01E+03	8.56E+02
5	6.80E+03		9.31E+03		7.25E+02	
6	5.78E+03	5.83E+03	9.49E+03	8.88E+03	2.40E+03	2.08E+03
6	5.89E+03		8.31E+03		1.81E+03	
7	6.52E+03	6.35E+03	1.16E+04	1.14E+04	4.64E+03	3.56E+03
7	6.19E+03		1.13E+04		2.73E+03	
8	4.39E+03	4.21E+03	7.60E+03	7.92E+03	4.02E+03	3.67E+03
8	4.03E+03		8.26E+03		3.35E+03	
9	4.96E+03	5.01E+03	7.39E+03	7.89E+03	7.06E+02	7.06E+02
9	5.06E+03		8.43E+03		7.06E+02	

.

Environmental Water Sample Storage

(Unit: copies/ mL of sample water)

Sample #	DAYS	-20C		Sample #	DAYS	-80C	
121	0days	**1.09E+06**	100%	121	0days	**1.09E+06**	100%
122	0days	**1.13E+06**	104%	122	0days	**1.13E+06**	104%
123	0days	**8.55E+05**	78%	123	0days	**8.55E+05**	78%
226	4m	**5.44E+05**	50%	229	4m	**1.28E+06**	117%
227	4m	**5.97E+05**	55%	230	4m	**1.31E+06**	120%
228	4m	**5.19E+05**	48%	231	4m	**1.10E+06**	101%
257	9m	**3.05E+05**	28%	260	9m	**1.84E+05**	17%
258	9m	**1.32E+05**	12%	261	9m	**1.38E+05**	13%
259	9m	**1.28E+05**	12%	262	9m	**2.78E+05**	26%
263	12m	**1.20E+05**	11%	266	12m	**1.78E+05**	16%
264	12m	**8.54E+04**	8%	267	12m	**1.81E+05**	17%
265	12m	**9.90E+04**	9%	268	12m	**1.62E+05**	15%

Table A.3 qPCR results for comparison of sample water storage methods. Used for the analysis in Chapter 3.

July 2008 sampling data

Site Number	Temperature ˚C	pH	DO mg/L	Turbidity NTU	Conductivity mS/cm	*E.coli* MPN/100ml	*Bacteroidales* copy/100ml
1	22.70	8.48	7.44	3.40	0.59	2014	5.93E+08
2	22.10	8.30	6.69	7.90	0.58	245	1.26E+07
3	22.90	8.49	7.32	3.10	0.58	789	1.98E+07
4	21.00	8.21	8.06	2.00	0.51	521	1.02E+07
5	20.90	8.38	7.53	6.10	0.65	616	7.44E+07
6	19.40	7.74	6.25	7.00	0.36	1935	8.95E+06
7	20.20	8.38	6.85	3.80	0.64	393	4.00E+07
8	11.86	7.63	3.50	2.20	0.11	0	0.00E+00
9	19.50	7.94	6.08	9.90	0.94	722	1.33E+08
10	18.70	7.91	4.93	4.90	0.97	2064	2.55E+07
11	21.30	8.08	4.79	18.60	0.87	657	1.89E+07
12	23.50	8.90	-	3.80	0.30	473	1.47E+07
13	22.80	8.41	7.50	4.70	0.55	426	0.00E+00
14	21.90	8.05	6.73	16.80	0.45	7270	4.25E+08
15	23.00	8.45	7.87	5.20	0.08	789	9.37E+07
16	21.80	8.22	7.36	3.70	0.49	5794	2.54E+06
17	22.80	8.39	7.41	5.60	0.52	703	7.03E+06
18	22.60	8.35	7.55	13.50	0.05	855	1.81E+07
19	23.00	8.30	7.03	8.10	0.48	683	7.46E+07
20	20.30	8.21	7.68	3.60	0.56	2035	4.31E+07
21	16.90	7.04	6.38	8.90	0.56	1523	1.57E+09
Blank						0	-

continued

Table A.4 Water quality data of field samples. Used for the analysis in Chapter 4.

Table A.4 continued

August 2008 sampling data

Site Number	Temperature ˚C	pH	DO mg/L	Turbidity NTU	Conductivity mS/cm	*E.coli* MPN/100ml	*Bacteroidales* copy/100ml
1	21.07	9.16	6.97	36.20	0.654	545	2.04E+06
2	20.91	9.13	6.59	38.80	0.626	616	2.55E+06
3	21.28	9.24	7.00	36.10	0.508	637	4.72E+06
4	20.05	8.97	5.85	34.70	0.541	450	7.12E+05
5	20.64	9.19	7.20	41.20	0.724	1130	6.49E+06
6	19.81	8.94	5.36	60.50	0.371	1720	8.81E+07
7	20.22	9.28	6.81	42.80	0.831	573	3.72E+06
8	12.54	8.57	2.97	37.90	0.657	0	0.00E+00
9	17.24	8.67	5.67	38.30	0.950	1539	6.45E+06
10	18.12	8.93	5.60	64.40	1.030	439	1.67E+06
11	20.32	9.09	5.53	44.50	0.924	576	5.27E+06
12	20.26	9.01	6.76	40.90	0.536	471	5.14E+06
13	21.21	9.14	6.25	38.30	0.652	323	1.02E+06
14	20.10	9.12	7.00	36.70	0.547	21160	6.53E+08
15	21.38	9.32	6.66	40.80	0.628	839	1.11E+06
16	20.11	9.06	7.22	35.20	0.537	813	4.17E+05
17	21.42	9.25	6.96	39.50	0.612	504	9.26E+06
18	18.55	9.28	7.90	51.00	0.865	7270	2.60E+08
19	21.42	9.29	6.63	48.30	0.347	420	2.07E+06
20	18.82	8.71	7.19	33.40	0.515	529	1.97E+06
21	18.79	8.25	7.07	34.00	0.866	21160	1.15E+09
Blank						0	-

continued

Table A.4 continued

September 2008 sampling data

Site Number	Temperature ˚C	pH	DO mg/L	Turbidity NTU	Conductivity mS/cm	*E.coli* MPN/100ml	*Bacteroidales* copy/100ml
1	16.20	9.36	7.96	40.10	0.685	504	5.59E+06
2	16.21	9.24	7.65	43.10	0.106	573	7.02E+06
3	16.11	9.29	7.79	41.80	0.649	657	5.65E+06
4	14.58	9.16	8.87	38.70	0.293	272	3.22E+06
5	16.07	9.45	8.78	44.50	0.434	350	2.75E+06
6	13.77	8.98	7.26	53.30	0.005	4352	5.65E+07
7	14.31	9.80	8.41	48.20	0.931	776	2.37E+07
8	11.90	10.20	4.34	38.80	0.011	0	0.00E+00
9	13.86	9.22	7.44	41.50	0.001	538	1.80E+08
10	13.13	9.15	7.89	41.00	0.339	657	4.26E+06
11	14.11	9.38	8.56	44.40	0.001	520	1.56E+08
12	13.73	9.21	8.36	46.00	0.583	4884	8.06E+08
13	16.35	9.87	9.40	51.60	0.018	364	2.03E+07
14	14.40	9.96	8.73	40.60	0.272	77010	1.03E+09
15	15.92	9.45	8.19	41.80	0.369	545	1.45E+07
16	14.86	9.19	8.87	36.10	0.106	275	2.04E+06
17	16.24	9.47	9.07	48.90	0.002	480	1.85E+07
18	13.66	9.37	9.34	54.60	0.008	833	4.45E+07
19	16.57	9.49	7.87	47.10	0.675	305	1.89E+07
20	14.69	8.97	8.31	39.00	0.455	279	4.24E+06
21	19.14	9.06	7.46	42.50	0.001	1670	2.51E+08
Blank						0	-

continued

Table A.4 continued

October 2008 sampling data

Site Number	Temperature ˚C	pH	DO mg/L	Turbidity NTU	Conductivity mS/cm	*E.coli* MPN/100ml	*Bacteroidales* copy/100ml
1	5.62	8.65					1.56E+07
2	4.01	8.54					3.73E+06
3	4.56	8.56					6.53E+06
4	4.82	8.80					1.55E+06
5	4.07	8.68					1.21E+07
6	3.82	8.20					8.00E+07
7	2.95	8.62					2.55E+08
8							0.00E+00
9	4.61	8.24					2.34E+08
10	5.16	8.38					1.33E+07
11	3.55	8.55					1.47E+08
12	3.96	8.64					1.72E+07
13	4.49	8.69					1.29E+07
14	6.50	8.53					7.10E+08
15	5.17	8.89					7.94E+06
16	5.31	8.33					1.63E+06
17	4.40	8.79					1.79E+07
18	6.53	8.69					1.57E+07
19	4.80	8.70					1.50E+07
20	7.60	8.49					2.24E+06
21	12.50	8.42					7.54E+08
Blank							-

continued

Table A.4 continued

March 2009 sampling data

Site Number	Temperature ˚C	pH	DO mg/L	Turbidity NTU	Conductivity mS/cm	*E.coli* MPN/100ml	*Bacteroidales* copy/100ml
1						242	2.63E+07
2						231	2.15E+07
3						1565	1.55E+07
4						41	4.42E+06
5						246	2.38E+07
6						110	3.98E+07
7						336	2.36E+07
8						0	0.00E+00
9						6131	2.67E+07
10						197	5.17E+06
11						24196	1.90E+08
12						41	4.52E+06
13						272	5.79E+06
14						>24196	6.73E+08
15						160	5.20E+06
16						169	8.62E+07
17						197	9.98E+06
18						359	3.06E+07
19						121	6.80E+08
20						75	1.06E+07
21						>24196	4.92E+07
Blank						0	-

Site 14 hotspots General *Bacteroidales* (copies/ 100ml)

Sampling Date	30-Oct	31-Mar	16-Jun	14-Jul	17-Aug	15-Sep	Geomean	SD
Site	1	2	3	4	5	6		
1 upstream	2.10E+04	3.33E+05	3.09E+04	2.01E+03	1.63E+04	1.22E+04	2.10E+04	1.42E+05
2	5.11E+04	1.14E+06	6.22E+04	9.63E+03	4.78E+05	1.51E+04	7.94E+04	4.51E+05
3	6.97E+04	3.04E+06	1.81E+05	6.91E+05	9.80E+05	7.84E+04	3.56E+05	1.14E+06
4	7.01E+04	2.47E+06	6.78E+06	2.81E+05	5.39E+05	1.10E+05	5.19E+05	2.64E+06
5	1.95E+06	6.17E+06	1.23E+07	6.85E+06	1.11E+07	4.14E+05	4.09E+06	4.75E+06
6	7.17E+06	8.99E+06	1.75E+07	2.53E+06	1.90E+07	8.96E+05	6.04E+06	7.51E+06
7				1.20E+06	4.41E+07	1.31E+06	4.11E+06	2.47E+07
8				5.42E+05	5.69E+06	1.82E+05	8.25E+05	3.08E+06
9				7.31E+05	2.90E+05	3.02E+04	1.86E+05	3.54E+05
10 downstream				5.65E+06	1.28E+06	2.25E+05	1.18E+06	2.88E+06

Site 14 hotspots Human specific *Bacteroidales* (copies/ 100ml)

Site	30-Oct	31-Mar	16-Jun	14-Jul				
Sampling Date	1	2	3	4			Geomean	SD
Site	1.25E+02	4.36E+02	1.00E+02	4.52E+01			1.25E+02	1.76E+02
2	1.00E+02	1.32E+03	1.00E+02	8.20E+00			1.03E+02	6.28E+02
3	1.00E+02	1.25E+03	3.52E+01	6.03E+01			1.38E+02	5.92E+02
4	2.41E+02	2.63E+03	2.06E+03	8.71E+02			1.68E+03	1.09E+03
5	1.57E+04	4.32E+03	7.53E+03	5.00E+04			1.18E+04	2.10E+04
6	5.83E+04	9.08E+03	9.78E+03	1.59E+04			1.12E+04	2.35E+04
7				1.03E+04			1.03E+04	
8				4.36E+03			4.36E+03	
9				4.30E+03			4.30E+03	
10 downstream				1.26E+04			1.26E+04	

continued

Table A.5 Site 14 hotspot *Bacteroidales* and *E.coli* data. Used for the analysis in Chapter 5.

Table A.5 continued

Site 14 hotspots *E.coli* (MPN/100mL)

Site	30-Oct	31-Mar	16-Jun	14-Jul	17-Aug	15-Sep	Geomean	SD
	1	2	3	4	5	6		
1 upstream		2.33E+02	3.10E+02	3.10E+02	1.22E+02	1.03E+02	1.95E+02	9.94E+01
2		>24186	9.80E+02	4.10E+02	3.87E+03	2.93E+03	1.46E+03	1.63E+03
3		>24186	7.30E+02	4.08E+03	4.61E+03	3.67E+03	2.66E+03	1.74E+03
4		1.73E+04	6.22E+03	1.09E+03	7.70E+03	6.92E+03	5.74E+03	5.90E+03
5		1.99E+04	3.87E+04	8.66E+04	1.12E+05	8.81E+04	5.80E+04	3.83E+04
6		>24186	4.61E+04	7.27E+04	5.79E+04	4.36E+04	5.39E+04	1.33E+04
7				2.76E+04	2.42E+04	1.70E+04	2.25E+04	5.40E+03
8				9.59E+03	1.30E+04	1.01E+04	1.08E+04	1.84E+03
9				9.59E+03	4.11E+03	3.57E+03	5.20E+03	3.33E+03
10 downstream				2.99E+03	3.26E+03	2.87E+03	3.03E+03	1.98E+02

Site 21 hotspots General *Bacteroidales* (copies/ 100ml)

Date	29-Oct	30-Mar	15-Jun	13-Jul	Geomean	SD
Sampling point						
1	7.54E+06	4.92E+05	1.47E+06	1.69E+07	2.30E+06	9.20E+06
2		1.87E+05	4.03E+05	2.41E+06	5.66E+05	1.23E+06
3		8.36E+05	1.31E+06	7.68E+06	2.03E+06	3.82E+06

Site 21 hotspots Human specific *Bacteroidales(copies/ 100ml)*

Date	29-Oct	30-Mar	15-Jun	13-Jul	Geomean	SD
Sampling point						
1	1.26E+04	4.55E+03	5.65E+03	1.25E+05	1.48E+04	6.91E+04
2		1.00E+02	1.00E+02	7.22E+04	8.97E+02	4.16E+04
3		9.08E+03	6.21E+03	8.06E+04	1.66E+04	4.22E+04

Site 21 hotspots *E.coli* (MPN/100mL)

Date	29-Oct	30-Mar	15-Jun	13-Jul	Geomean	SD
Sampling point						
1		>24186	4.35E+04	6.87E+04	5.47E+04	1.78E+04
2		4.10E+02	5.10E+02	2.11E+03	7.61E+02	9.54E+02
3		5.81E+03	9.60E+03	1.27E+04	8.91E+03	3.44E+03

Table A.6 Site21 hotspot *Bacteroidales* and *E. coli* data. Used for the analysis in Chapter 5.

Site 10 hotspots general *Bacteroidales* and *E.coli*

sampling point		AllBac Geomean copy/100mL	E. coli MPN/100mL
Base set1	4-Aug		
Upstream	10-4	-	364
	10-3	-	323
	10-2	-	496
original 10	10-1	-	411
Base set2	27-Aug		
upstream	10-4	7.90E+07	645
	10-3	6.86E+07	1459
	10-2	4.94E+06	750
original 10	10-1	3.67E+06	850
Base set3	15-Sep		
upstream	10-4	6.15E+08	613
	10-3	1.60E+07	410
	10-2	6.75E+05	294
original 10	10-1	1.05E+06	291
Storm set0	11-Aug		
upstream	10-4	-	7270
	10-3	-	3255
	10-2	-	12033
original 10	10-1	-	19863
Storm set1	20-Aug		
upstream	10-4	1.55E+09	10462
	10-3	6.89E+08	7270
	10-2	2.50E+08	15531
original 10	10-1	7.44E+08	12033
Storm set2	28-Aug		
upstream	10-4	3.26E+08	7710
	10-3	3.16E+08	6170
	10-2	5.15E+07	98040
original 10	10-1	2.78E+08	98040
Storm set3	23-Sep		
upstream	10-4	3.73E+07	12740
	10-3	6.08E+07	6310
	10-2	1.19E+07	23590
original 10	10-1	8.77E+09	9870

Table A.7 Site10 hotspot *Bacteroidales* and *E. coli* data. Used for the analysis in Chapter 5.