

Evaluation of *Bacteroidales* 16S rRNA Genetic Markers as a Microbial
Source Tracking Tool in a Canadian Agricultural Watershed

by

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DALHOUSIE UNIVERSITY

Department of Process Engineering and Applied Science

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DEDICATION PAGE

I dedicate this thesis to Jamie Doran and Christine Baugh. Their introduction to the world of biochemistry and microbiology is the reason that I pursued higher education.

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ABSTRACT

Waterborne pathogen presence caused by fecal pollution is a leading cause of morbidity and mortality worldwide. In developed countries, this problem can result in waterborne outbreaks. Research suggests that there is a need for better fecal indicators because current methods (total coliforms and *E. coli*) are insufficient. This study investigated *Bacteroidales* 16S rRNA markers as a microbial source tracking tool in an agricultural watershed. Correlations between pathogens and markers were also investigated. Water quality monitoring was conducted following the validation of ruminant-, bovine-, human-specific, and universal *Bacteroidales* markers. Results revealed a positive relationship between *E. coli* and the universal marker. Ruminant- and bovine-specific marker detection was associated with increased runoff due to precipitation; however, the human associated marker was not detected. Furthermore, no correlations could be made between *Campylobacter*, *Salmonella*, or *E. coli* O157:H7. These techniques have potential to become a powerful tool; however, further research is needed.

LIST OF ABBREVIATIONS USED

ALOD	Assay Limit of Detection
ARA	Antibiotic Resistance Analysis
BB	Bolton Broth
BMP	Beneficial Management Practices
BPW	Buffered Peptone Water
BSA	Bovine Serum Albumin
<i>C. coli</i>	<i>Campylobacter coli</i>
<i>C. jejuni</i>	<i>Campylobacter jejuni</i>
<i>C. lari</i>	<i>Campylobacter lari</i>
<i>C. parvum</i>	<i>Cryptosporidium parvum</i>
CAFO	Concentrated Animal Feeding Operation
CCME	Canadian Council of the Ministers of the Environment
CFU	Colony Forming Units
CSO	Combine Sewer Outflow
DO	Dissolved Oxygen
dsDNA	Double Stranded DNA
<i>E. coli</i>	<i>Escherichia coli</i>
FIB	Fecal Indicator Bacteria
FIO	Fecal Indicator Organism
GFP	Green Fluorescent Protein
HUS	Hemolytic Uremic Syndrome
IAC	Internal Amplification Control
IBS	Irritable Bowel Syndrome
IMS	Immunomagnetic separation
LD-MST	Library-Dependent Microbial Source Tracking
LH-PCR	Length Heterogeneity Polymerase Chain Reaction
LI-MST	Library-Independent Microbial Source Tracking
MPC	Magnetic particle concentrator
MPN	Most Probable Number
MST	Microbial Source Tracking
mtDNA	Mitochondrial DNA
NTU	Nephelometric Turbidity Unit
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline with Tween
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
PMA	Propidium Monoazide

qPCR	Quantitative PCR
rDNA	Ribosomal DNA
REP-PCR	Repetitive Extragenic Palindromic PCR
RDP	Ribosomal Database Project
rRNA	Ribosomal RNA
RVS	Rappaport-Vassiliadis Soy Broth
SE	Sampling Event
ssDNA	Single Stranded DNA (I)
T-RFLP	Terminal-Restriction Fragment Length Polymorphism
TBW	Thomas Brook Watershed
TFTC	Too Few To Count
TNTC	Too Numerous To Count
TSB	Trypticase Soy Broth
TSS	Total Suspended Solids
TSS	Total Suspended Solids
USEPA	United States Environmental Protection Agency
VBNC	Viable But Nonculturable
WHO	World Health Organization
ρ	Spearman's Rank Coefficient

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CHAPTER 1. INTRODUCTION

Since the acceptance of the germ theory in the 19th century, the linkage between poor sanitation and degraded water quality has become increasingly evident. Conceived and strengthened over time by research and keen observation, the germ theory states that many diseases are caused by microorganisms rather than by spontaneous generation (Hardy, 1984). John Snow's discovery in 1854 that a cholera epidemic was being propagated by fecally contaminated water revolutionized the way we think about sanitation and our drinking water supplies (Lipp et al., 2002). More than 150 years later, however, the World Health Organization (WHO) reports that over a billion people around the world do not have access to clean drinking water and proper sanitation (WHO, 2011a). Second only to pneumonia, the WHO also reports that diarrheal disease is one of the leading causes of death among children under 5 years of age; transmission of these diseases are increased by unsafe water supplies and insufficient sanitation (WHO, 2011a). Although these statistics may conjure up images of third world countries, water-borne disease outbreaks can, and do, occur in developed areas. In countries such as Canada, modified farming practices, urbanization, and intense weather events are having an impact on the amount of enteric pathogens that could enter into water sources (Hrudey et al., 2006; Wilkes et al., 2011). As science and technology progresses, techniques to detect fecal pollution in drinking, irrigation, and recreational waters are evolving. It is anticipated that these new tools will allow water managers to more effectively assess, and manage, microbial water quality issues, and thus decrease the incidence of water-borne disease outbreaks.

The primary methods for detecting microbial water pollution have not changed significantly in nearly a century. Because of their high diversity and low concentrations, pathogens are often difficult to detect in water (Leclerc et al., 2001). Alternatively, microorganisms that are found in high concentrations in the gut of warm-blooded mammals are used as indicators of fecal pollution. The presence of these microorganisms, known as indicator organisms, does not necessarily mean that pathogens are also present in the water, but it suggests that recent fecal contamination has occurred (Field & Samadpour, 2007). Worldwide, water quality monitoring is typically conducted by culture-based detection of bacterial indicator organisms such as total and fecal coliforms, and *Escherichia coli* (*E. coli*) (Anderson et al., 2005). Although this technique is useful, it does have its limitations. The ability for some strains of *E. coli* to persist, or naturalize, in the environment can limit the usefulness of this organism as an indicator of recent fecal contamination (Anderson et al., 2005; Byappanahalli et al., 2003; Power et al., 2005). Furthermore, this technique is unable to differentiate between sources of fecal contamination (Bernhard & Field, 2000a). However, over the last two decades, the field of microbial water pollution research has rapidly advanced with the aid of new nucleic acid based technologies.

Microbial source tracking (MST) technologies are designed to not only determine if recent fecal contamination has occurred, but also the source of the contamination. This concept has had many variations during its rapid evolution. Initially, it was noted that the ratio of fecal coliforms to fecal streptococci might indicate whether or not fecal pollution was from a human source (Scott et al., 2002; Simpson et al., 2002). Many library-dependent techniques (assays based on databases of microbial isolate characterizations) were also developed using various biological markers such as antibiotic resistance,

immunological and chemical utilization analysis (Wiggins et al., 1999) or DNA fingerprinting (Field & Samadpour, 2007). Due to the cumbersome nature of library-dependent techniques, which requires comprehensive strain libraries from all variables of interest to be developed, recent research has focused more on library-independent methods. There are a variety of library-independent MST methods, including detection of host-specific molecular markers (Simpson et al., 2002). These techniques will be discussed in greater detail in another section. Although research into MST is quickly progressing, there are still many questions to be answered before any of these methods can be implemented for routine water quality monitoring.

The value in MST is clear; the ability to better detect recent fecal contamination and determine the source will enable better mitigation of the problem. Furthermore, the high throughput nature of some MST techniques could reduce the time between the contamination event and the corrective action, preventing outbreaks, and ultimately, saving lives.

CHAPTER 2. LITERATURE REVIEW

2.1 INFLUENCES OF WATERBORNE DISEASE ON HUMAN HEALTH

The concept that water can be a source of disease, and that pathogens can reside in feces along with normal flora, was proposed in the 1880's. However, to this day microbial water pollution is still a major issue on a global scale (Leclerc et al., 2001). The bacterium *Vibrio cholera*, the causative agent of Cholera, can be spread through untreated water contaminated with human feces. In the mid-1800s it was described and determined to be cause of many waterborne epidemics; however, is still a leading cause of disease and death in developing countries due to the limited access to clean water (Lipp et al., 2002). In developed areas where access to clean water is essentially universal, other waterborne pathogens predominate and affect the population in the form of outbreaks rather than as a ubiquitous threat (WHO, 2003). The pathogens of most concern in developed nations include pathogenic *E. coli*, *Salmonella*, *Campylobacter*, *Giardia*, *Cryptosporidium*, Rotavirus, and Norovirus (Dechesne & Soyeux, 2007; WHO, 2003). Outbreaks generally occur when there is a failure in source water protection, treatment processes, or monitoring regimes of a drinking water supply (Hrudey et al., 2006).

2.1.1 WATERBORNE PATHOGENS OF MOST RELEVANCE TO HUMAN HEALTH

E. coli and *Campylobacter* are Gram-negative bacteria and within their genus' there are some species that are pathogenic (Sherman et al., 2010; WHO, 2011b). Pathogenic *E. coli*, most notably *E. coli* O157:H7, asymptomatically colonize the gut of various ruminants (Sherman et al., 2010). The pathogen can be spread to humans through

fecally contaminated products (Sherman et al., 2010) and ingestion of fewer than 10 *E. coli* O157:H7 bacteria can cause disease (Rogers et al., 2011). Symptoms are often severe and can cause diarrhea, hemolytic uremic syndrome (HUS), blood clots, strokes and even death (Sherman et al., 2010; WHO, 2011b). The infective dose of *Campylobacter* is also low but infection is more common and less severe than *E. coli* (Brettar & Höfle, 2008; WHO, 2011b). *Campylobacter jejuni* (*C. jejuni*), *Campylobacter coli* (*C. coli*), and *Campylobacter lari* (*C. lari*) are major sources of the identified waterborne disease in humans. Birds are a known carrier of these bacteria (Sherman et al., 2010) but it is also found to occur naturally in the environment (WHO, 2011b). *Campylobacter* is the cause of approximately 14% of diarrheal infection worldwide and symptoms range from watery diarrhea to dysentery (Sherman et al., 2010). Both of these infections can cause long-term effects including chronic kidney problems and Guillain Barré syndrome; survivors of these diseases have shown increased susceptibility to irritable bowel syndrome (IBS) (Sherman et al., 2010).

Cryptosporidium and *Giardia* are protozoan pathogens that are major causes of disease. They cannot reproduce outside of the host but oocysts or cysts that are expelled into the environment can survive and remain infectious for extended periods of time (Robertson & Gjerde, 2006). Protozoa are more resistant than bacteria to chemical deactivation but their larger size makes them easier to physically remove from water (Hrudey et al., 2003; WHO, 2011b). *Cryptosporidium parvum* (*C. parvum*) has an uncommonly diverse host range and has been found in over 150 animals including humans, mice and sheep; cows, however, cows are the most important zoonotic reservoir for this parasite with as many as 10^{10} oocytes shed daily from an infected calf (Neumann et al., 2005). *Giardia* infections are more common than *Cryptosporidium* infections

(Hunter, 2003; WHO, 2011b) but it has also been suggested that human *Cryptosporidium* infection could be very underestimated (Neumann et al., 2005). As little as 30 *Giardia* cysts can cause infection in humans, which can induce mild to severe cases of diarrhea. *Giardia* is shed into the environment by infected animals, including beavers, muskrats, and humans (Wallis et al., 1996).

Viruses are the smallest pathogens, which can make it easier for them to evade detection and physical water sanitation methods, such as flocculation. They are generally very species specific, which means that they are less likely to have animal reservoirs (Neumann et al., 2005). Rotavirus is the most common cause of gastrointestinal disease in children causing hospitalization and sometimes death (Neumann et al., 2005). Norovirus causes acute gastroenteritis in all age ranges; the symptoms are less severe than Rotavirus and the infection is usually cleared within 3 days (Neumann et al., 2005). Infected individuals can excrete large numbers of virus and water contaminated with this waste can be highly infective (Neumann et al., 2005).

A pathogen that is shed into the environment in high numbers, is infectious at low doses, can survive in the environment for extended periods of time, is resistant to treatment, or is able to multiply outside the host, is of most concern to human health (Rosen, 2000). The prevalence of these organisms in water depends on a wide range of factors. Geographic location has a strong influence on which pathogens are likely to emerge. The types of animals, the proximity in which humans live with these animals, and waste management practices can all have a strong influence on which pathogens would be of greatest concern in a given area. Temperature, turbidity, surface water discharge, and general sanitation of the receiving water bodies are also all important for pathogen survival (Wilkes et al., 2011).

2.1.2 RECENT OUTBREAKS ASSOCIATED WITH MICROBIAL POLLUTION

The Walkerton Ontario outbreak of *E. coli* O157:H7 and *C. jejuni* is an example of how multiple failures in water protection and safety protocols can end in tragedy. In the spring of 2000, the rural farming community of Walkerton received a series of higher than normal rainfall events resulting in over 130 mm of rain in four days (Salvadori et al., 2009). The increased surface water runoff caused fecal material from a nearby farm that had spread manure as fertilizer on an adjacent field to enter into a shallow municipal water well (Hrudey et al., 2003; Salvadori et al., 2009). Improper monitoring and treatment prior to, and during, the outbreak led to an extended period of time in which the community consumed the contaminated water. Ultimately, in a town of 4,700 people, 2300 were affected by gastroenteritis, 65 required hospitalization, 27 of the cases resulted in HUS, and 7 died (Hrudey et al., 2003). A follow-up study conducted over 2 years after the initial incident showed that the affected residents had a 4.8% higher incidence of IBS when compared to similar populations (Sherman et al., 2010). The outbreak in Walkerton demonstrated a multi-level breakdown in source protection, treatment, distribution, monitoring and response (Hrudey et al., 2003) but other outbreaks have demonstrated that problems do not have to arise on all fronts to have adverse effects.

Increased rainfall combined with poorly treated water was the suspected cause of 1,400 causes of viral gastroenteritis in Uggeløse, Denmark (Laursen et al., 1994). In North Battleford, Saskatchewan, an outbreak of *Cryptosporidium*, which is resistant to chlorination, affected 1,900 residents because of improper water filtration (Stirling et al., 2001). In Bramham, England, malfunctions in the treatment process and subsequent monitoring resulted in 3,000 cases of gastroenteritis from an unknown source (Short,

1988). Heavy precipitation and snowmelt was thought to be the cause of a *C. parvum* outbreak in 1993, which affected over 400,000 people and caused over 50 deaths in Milwaukee, USA (Curriero et al., 2001). The source and/or cause of waterborne disease outbreaks cannot always be determined and it is not often that one single factor will contribute to an outbreak. Thus, multi-barrier approaches to protecting drinking water are typically applied in order to reduce the instances out outbreak (Hrudey et al., 2003).

A large number of waterborne outbreaks are suspected as being unreported due to multiple factors. Diseases can go unreported due to their lack of severity, short duration of the illness, self-medication, or lack of diagnostic testing (Schuster et al., 2005). One study reports that in Canada as many as 300 unreported cases of gastroenteritis might occur for every case that has been diagnostically verified (Flint et al., 2004). Variation between provinces and territories for water treatment management systems and monitoring procedures also leads to discrepancies in reporting (Schuster et al., 2005). In a study examining disease outbreaks in Canada from 1974 to 2001, the lack of consistent monitoring and reporting is evident. In only 35% of the documented outbreaks could it definitively be concluded that the cause was waterborne and over 40% of the outbreaks could not be linked back to any particular source. In 47% of the cases examined, the pathogen responsible for the outbreak was not determined. The uncertainty increases when considering rural and private water sources (Schuster et al., 2005).

2.2 THE EFFECTS OF LAND USE ON MICROBIAL WATER POLLUTION

Land use and precipitation characteristics have a major influence on the influx of microbial pollution into water systems (Ferguson et al., 2003). In urban areas the amount of impervious ground is substantially higher than in undeveloped or agricultural areas.

Pavement, storm sewers, culverts, and other hydraulic modifications in urban environments increase the amount and velocities of stormwater runoff (Arnone & Walling, 2007). Urban stormwater can contain many pollutants, including pathogens; for this reason, many stormwater outflows are considered point sources for microbial pollution (Arnone & Walling, 2007). Also, some urban centres have combined sewage systems, which carry a mixture of storm water and sewage to treatment facilities. During moderate to heavy precipitation events, combined sewer overflows (CSOs) can occur (Arnone & Walling, 2007; Dechesne & Soyeux, 2007). The US Environmental Protection Agency (USEPA) estimates that CSOs discharge over 4,500 billion liters of waste into receiving waters each year (Arnone & Walling, 2007).

The hydrology, and sources of microbial pollution, in rural and agriculturally dominated areas is very different than in urbanized areas. Within landscapes dominated by agriculture, there are many factors to consider, including terrain modifications, alterations to waterways, livestock densities, livestock access to waterways, manure management, and fertilizer use (WHO, 2011b). The residential practices in rural areas are also often different than in cities. Septic systems are used for wastewater treatment, and can influence local water quality (Peed et al., 2011). As well, individual or shared well systems are often used as potable water sources; this practice can lead to varying levels of monitoring and management of the water quality (Schuster et al., 2005).

2.2.1 AGRICULTURAL SOURCES OF MICROBIAL WATER POLLUTION

The increasing need for food has fueled the change of approximately 40% of the earth's terrain into agricultural land (Patz et al., 2008). Concentrated animal feeding operations (CAFOs) are becoming more commonplace and therefore the potential for

pathogens to enter water systems is increased due to the sheer volume of animals, and waste materials, confined in small spaces. Surface water in open lots with high animal traffic is the most susceptible to pathogenic contamination; runoff from these areas can, in turn, contaminate groundwater and nearby surface water bodies (Rosen, 2000). Although pathogen concentrations may be reduced, low-intensity livestock operations can also be a significant source of pathogen loading into adjacent water systems in the same manner (Harmel, 2010). Overgrazing should be avoided because it can not only increase the amount of feces deposited in a pastured area but it can also change the hydraulic characteristics of the pastures leading to increased surface runoff (Harmel, 2010).

Various Beneficial Management Practices (BMPs) have been proposed to reduce pollutant loading into water systems in agricultural watersheds. For example, riparian buffers act as a filter to impede the movement of surface water runoff and associated pathogens into watercourses (Mankin et al., 2006); they can also serve as a physical barrier to keep livestock away from water bodies (Rosen, 2000). Pastures without fencing or a thick riparian zone can allow livestock access to streams and rivers leading to the direct deposition of feces into this water (Sinclair et al., 2009). Providing animals with food, water, and shelter away from the water bodies can help reduce the desire for the livestock to access the streams and rivers (Rosen, 2000). Waters in which animals have direct access show a marked increase in microbial contamination levels (Fremaux et al., 2009).

Waste management and storage is an issue because of the large volumes of manure that can be produced on commercial farms. Operators can use methods such as composting, constructed wetlands, and wastewater lagoons to aid in waste management. By raising the temperature of the product, composting, if managed and mixed properly, is

an effective way to reduce and/or eliminate pathogens from solid manure (Erickson et al., 2009). Constructed wetlands are often used as a cost-effective treatment system for liquid manure or runoffs from solid manure piles. In these systems, microbial populations in liquid effluents are reduced by sedimentation, exposure to ultraviolet radiation, natural die-off, and predation (Tanner et al., 1998; Tunçsiper, 2007). Wastewater lagoons are also used for liquid manure storage and partial treatment; lagoons can be designed and operated as aerobic or anaerobic treatment system. These lagoons reduce microbial concentrations using similar mechanisms to those employed within constructed wetlands. The effectiveness of this process depends on the length of time that material remains stored in the lagoon (Rosen, 2000). After sufficient treatment of the effluent, it can be used to fertilize croplands.

The use of biological waste as fertilizer is a common practice due the increasing cost of commercial fertilizers. Liquid and solid wastes can either be surface applied or injected into the soil. With respect to minimizing pathogen persistence and transport surface application of manure is more suitable during warm periods when little precipitation is expected. The lack of moisture, exposure to ultraviolet radiation, increased temperatures, and predation can increase die-off of residual microbes (Lau & Ingham, 2001; Warnemuende & Kanwar, 2002). During the springtime, when increased precipitation and storm events are expected, injection of manure into the subsurface soil can help prevent transport with surface runoff (Rosen, 2000); however, it may increase transport through subsurface drainage water (Warnemuende & Kanwar, 2002). This method allows for reduction of microbial contamination by adsorption and filtration of the microbes, and also can expose enteric microbial populations to predation. In both cases, the manure should be applied when minimal vegetation is present to ensure that

any microbial contamination has maximum exposure to ultraviolet radiation and conditions favourable to desiccation (Rosen, 2000).

2.2.2 RESIDENTIAL SOURCES OF MICROBIAL WATER POLLUTION

Agricultural operations are not the only potential source of microbial pollution in rural areas. Unlike urban areas, household waste is generally managed in a decentralized manner using individual septic systems (Peed et al., 2011). Household wastewater is first partially treated in a septic tank where the solids, including the microorganisms, are allowed to settle. The mass of solids is reduced by anaerobic digestion and the liquid phase of the wastewater is allowed to enter into a soil absorption field where the effluent is further purified by percolation through the soil. The water is returned to the hydraulic continuum through groundwater dispersion and evapotranspiration (Brown, 1998; Peed et al., 2011). Although scheduled upkeep and maintenance of these systems may be required by law, it is often not strictly enforced and therefore at the discretion of the homeowner. Failure to properly maintain these systems could result in untreated wastewater effluent contaminating surface water and groundwater systems (Peed et al., 2001).

As with wastewater treatment, potable water supply systems can be quite different in rural communities. Many households in rural areas use on-site wells to access groundwater. The combination of faulty septic systems and potable water extracted from groundwater wells could, and has in the past, have adverse health effects. It has been shown that over 30% of private wells in Canada exceed the national guidelines for bacterial contamination (Neumann et al., 2005). These guidelines, set out by The Canadian Council of the Ministers of the Environment (CCME), state that potable water sources should not contain any fecal coliforms (< 1 coliform in 100 ml of water).

2.3 INDICATORS OF MICROBIAL WATER POLLUTION

2.3.1 FECAL INDICATOR ORGANISMS AS A TOOL TO WARN OF RECENT FECAL CONTAMINATION

It has been understood for some time that the presence of feces in water used for drinking, recreational, and agricultural purposes can cause disease (Leclerc et al., 2001). Up to 50% of an animal's wet fecal biomass is comprised of bacteria that often live symbiotically with the host by aiding in the digestion of food (McBee, 1971). Humans and animals can, however, be carriers of pathogens; these pathogens may or many not cause disease in the host. Pathogens harboured in the gut of warm-blooded animals can be deposited into the environment and spread to other animals, or humans, through ingestion (Arnone & Walling, 2007). It is not feasible to directly test for pathogen presence in water samples for many reasons. Their concentration in the environment (if present) can be low, possibly even below current limits of detection, however, they can still be hazardous due to the low infective doses of many pathogens. The copious amounts of different pathogens makes it currently impossible to test for their presence not only because of the sheer numbers, but also because of the plethora of culturing methods or detection methods required for positive identification (Field & Samadpour, 2007; Leclerc et al., 2001). The use of indicator organisms came about to circumvent this problem.

Indicator organisms are non-pathogenic microorganisms, most commonly bacteria, which can be used to indicate if feces, and therefore possibly pathogens of fecal origin, are present in a body of water. The ideal indicator organism has many characteristics. First it must be abundant in the feces of *all* warm-blooded animals; this ensures that even after dilution, detection of the fecal indicator organism (FIO) is likely to occur. For instance, *E. coli* is found in the intestinal tract of all warm-blooded animals

where it makes up 1% of the bacterial biomass (Leclerc et al., 2001). If the FIO is not ubiquitous in all mammalian intestinal tracts, false negative tests could occur that indicate a water body is safe when it may not be. Indicator organisms should also be temporally and geographically stable so that water quality testing can be standardized. Indicator organisms should mimic pathogen survival and should not have the ability to naturalize in the environment once they have been excreted from the host. Once an enteric pathogen leaves the host, it typically does not establish itself in the environment. If the organism is able to survive long periods of time or naturalize in the environment it loses its effectiveness to denote recent fecal contamination and warn of possible pathogen presence (Field & Samadpour, 2007). Testing for the ideal indicator should also be relatively straightforward and fast. The need for specialized equipment and highly trained staff would not be conducive for implementation of standardized testing. Rapid testing procedures are also essential to prevent potential exposure of pathogens to the public. Finally, the ideal indicator organism would be able to differentiate between the sources of fecal contamination (Field & Samadpour, 2007). Source identification would help to warn investigators of which pathogens may be of most concern and it would also help with mitigation of the problem. It should be noted that indicator organisms currently used for routine water monitoring do not meet all of the above criteria. Deficiencies associated with current indicator organisms will be outlined in the next section.

2.3.2 CURRENT PRACTICES OF USING FECAL COLIFORMS AND *E. COLI* AS FIOS

As our understanding of microorganisms grows and technology advances, researchers have begun to realize that the accepted FIOs may be inadequate indicators of fecal pollution. The use of fecal coliforms and *E. coli* as FIOs is an almost universal

practice (Leclerc et al., 2001). Coliforms are a broad group of Gram-negative, rod shaped and lactose fermenting bacteria that can be found in the environment or in the feces of warm-blooded mammals, including *Enterobacter* and *Klebsiella*. Coliform bacteria grow at 37°C and ferment lactose. Fecal coliforms are a subset of the coliform (or “total coliform”) group, which are able to grow and ferment lactose at 44.5°C. This ability is taken to indicate that the organisms are recently introduced into the cooler environment from their normal habitat in the gut of warm-blooded animals. *E. coli* is a member of the fecal coliform group (Leclerc et al., 2001).

Testing for coliform bacteria is simple; water is filtered through a membrane to concentrate the bacteria, the bacteria captured on the filter is then incubated on selective media, finally the number of colony forming units (CFUs) are enumerated and the number of CFUs/ 100 mL are determined. Another method to determine FIO concentrations is the most probable number technique (MPN), which statistically determines MPN/ 100 mL of coliforms by their fermentation characteristics using a dilution series. Governments and regulatory bodies worldwide have adopted maximum limits of CFUs/ 100 mL to ensure the safety of different types of water systems. If tested water samples possess levels that exceed the accepted maximums, action is taken to warn the public and prevent exposure (Arnone & Walling, 2007; Davies & Mazumder, 2003). In Canada, the CCME states that there should be no coliform bacteria in drinking water (< 1 CFU/ 100 mL). These guidelines also state that there should be less than 100 CFU/ 100 mL and 200 CFU/ 100 mL of coliforms in irrigation and recreational water, respectively.

Fecal coliforms and *E. coli* were chosen as indicator organisms because it was thought that they displayed many of the desired FIO characteristics. They are fairly

abundant and ubiquitous in mammalian feces, testing for them is simple and straightforward, assays are cost effective and results can be attained within two days (Leclerc et al., 2001). Because waterborne disease outbreaks are still a problem in the developed world, microbial water quality monitoring is still an evolving field. There are many indications that the current detection methods are insufficient. A two-day gap between water sampling and attaining results may seem quick, but in cases when the fecal pollution contains pathogenic microorganisms, this lag time can be life threatening. The ability of *E. coli* to persist in the environment is also of great concern because it may not mimic pathogen survival and could cause false positive results. Furthermore, the presence of fecal coliforms and *E. coli* in the water does not give any indication of where the contamination came from (Field & Samadpour, 2007). For these reasons, research into new and improved FIOs continues.

2.4 ADVANCES IN MICROBIAL SOURCE TRACKING

The need for more information when it comes to incidences of fecal water pollution has given rise to new technologies for microbial source tracking (MST). As the name suggests, MST aims to not only detect recent fecal contamination events but also to identify the source of the pollution. Since its inception, many different types of MST methodologies have been proposed. The two main branches of MST consist of library-dependent MST and library independent MST (Field & Samadpour, 2007; Roslev & Bukh, 2001; Simpson et al., 2002; Stoeckel et al., 2004).

2.4.1 LIBRARY-DEPENDENT MICROBIAL SOURCE TRACKING

Library dependent microbial source tracking (LD-MST) was the major focus of early MST research, and still garners some attention today. The basis behind LD-MST is

the construction of an intensive library, or database, of chosen characteristics exhibited by the selected fecal indicator organism. Before environmental samples are ready for analysis, fecal samples from all animals relevant to the watershed in question must be collected. Organisms isolated from the fecal samples must then be individually analyzed using the chosen LD-MST technique. Once a sufficient database has been constructed, water samples can be analyzed using the same method. The resulting profiles from the environmental samples can then be compared to the original library to determine the source of the microbial pollution. There are many phenotypic, biochemical, and molecular characteristics that have been considered for LD-MST; two well-known LD-MST techniques are antibiotic resistance analysis and a form of DNA fingerprinting known as ribotyping.

Antibiotic resistance analysis (ARA) uses the antibiotic resistance profile of a given FIO, such as fecal streptococci, to narrow down the source of fecal pollution (Wiggins et al., 1999). This method is based on the theory that humans are exposed to different antibiotics than livestock and the intestinal microflora of each type of host will consequently become resistant to different antibiotics. Furthermore, the assumption is made that wild animals are not exposed to any antibiotics and therefore FIOs from these animals would not be resistant to any antibiotics. Samples are taken and derived FIO are subsequently grown on an assortment of antibiotics; their resistance patterns are compared to the previously constructed library to determine the source of the pollution (Field & Samadpour, 2007; Meays et al., 2004; Stoeckel et al., 2004).

This method is problematic for a number of reasons. Consumption of heavily medicated livestock could cause human strains to be falsely identified as livestock strains if the antibiotic resistance profile of human microflora consequently begins to resemble

that of their food source (van den Bogaard, 2000). False identification of wild animals as livestock could also occur if the wild animals have regular access to the medicated feed used on some farms (Field & Samadpour, 2007; Meays et al., 2004). Identification may also become difficult if different livestock are subjected to the same types of antibiotics (Field & Samadpour, 2007) and some sources, including wild animals and unmedicated livestock, cannot be differentiated using this method (Meays et al., 2004).

Another problem with ARA is that the antibiotic resistance genes are inherently transient resulting in extreme spatial and temporal variability. The genes can be lost if the bacterium is not under selective pressure to retain them and they can be easily attained from another species carrying the resistance genes via lateral transfer (Field & Samadpour, 2007). However, once a library is developed, ARA can be beneficial because it is inexpensive and it requires little training to perform (Meays et al., 2004).

DNA fingerprinting techniques comprise the majority of the LD-MST techniques. The DNA fingerprint is formed when DNA from environmental isolates are analyzed using various molecular techniques to tease out differences in the genetic structure of FIOs from different animals. There are many manifestations of DNA fingerprinting (Field & Samadpour, 2007).

Repetitive DNA sequences are used to identify unique DNA fingerprints a commonly used technique called Rep-PCR. This technique uses primers that target conserved repetitive elements found within bacterial genomes (Simpson et al., 2002). As microflora evolve in their host, they adapt to their environment, which causes mutations in their DNA that can be used to discriminate between bacteria found in other host-species (Stoeckel et al., 2004). After DNA is extracted from the fecal or environmental samples, it is amplified by PCR. The amplicons are then resolved on agarose gel; intra-

species differences caused by various host environments can result in varying amplicon lengths. The resulting fingerprint can be compared to those previously deposited in the library; similarities can reveal the source of the fecal pollution (Parveen et al., 1999). Although this method is more specific than ARA, it also requires specialized equipment, expensive reagents, and properly trained technicians (Simpson et al., 2002).

Although LD-MST techniques have some benefits, there are also many disadvantages. The complex, costly, and time-consuming construction of a comprehensive database of isolates prior to watershed or environmental analysis is a major limiting factor. Further research is also needed on some fundamental aspects of these methods; researchers have yet to even determine the appropriate number of isolates needed in a database before the analysis is statistically significant (Simpson et al., 2002). Temporal and spatial stability can also be an issue; libraries are typically constructed only for a single watershed or geographic area and research has shown that the libraries cannot be transposed to other areas, even ones in close proximity to each other (Field & Samadpour, 2007; Simpson et al., 2002; Stoeckel et al., 2004). Finally, the construction of a library requires cultivation of bacterial isolates. Because these methods are culture dependent, the method inherently excludes many bacteria that are potentially useful but difficult to culture (Field & Samadpour, 2007).

Community finger-printing is a form of LD-MST that is not culture based. Research into this method using terminal restriction fragment length analysis (T-RFLP) is limited because proof of concept studies indicated that significant overlap between bacterial communities from various fecal sources would hinder source identification (Field & Samadpour, 2007).

2.4.2 LIBRARY-INDEPENDENT MICROBIAL SOURCE TRACKING

Research and development of library-independent microbial source tracking (LI-MST) techniques has been the focus of the majority of recent MST research. By avoiding the cumbersome task of developing a reference library, LI-MST shows greater potential for widespread application. Like DNA fingerprinting, most LI-MST methods exploit the differences in the nucleic acid sequences of FIOs. Unlike LD-MST, however, analysis is culture independent and therefore the chosen indicator organism is not restricted by its culturability (Roslev & Bukh, 2011). This characteristic has led to the employment of FIOs that were previously not considered. The variability in LI-MST methods comes not from the techniques used, but from which organisms and genes are selected as targets. Endpoint PCR and agarose gel electrophoresis were used in the early stages of LI-MST (Bernhard & Field, 2000b); however, the development of qPCR and DNA probes has revolutionized the field (Dick et al., 2005; Layton et al., 2006; Reischer et al., 2006). Initial research is needed to first identify target DNA sequences that can differentiate between hosts, but once these sequences are determined, they appear to be spatially and temporally stable (Gourmelon et al., 2010; Marti et al., 2011; Mieszkin et al., 2009). One approach gaining interest is to probe environmental samples for mitochondrial DNA (mtDNA) found in the host endothelial cells that are sloughed off, along with feces, during excretion (Caldwell & Levine, 2009; Martellini et al., 2005). Another popular assay targets the rRNA genes of the *Bacteroidales* bacteria. However, these assays are still in the primary stages of research and their benefits and limitations are not yet fully understood.

2.4.2.1 HOST-SPECIFIC MOLECULAR MARKERS AS TARGETS FOR MICROBIAL SOURCE TRACKING

Although FIOs are chosen for their ubiquity, small differences that occur due to living in different host environments can be harnessed to reveal further information about microbial pollution. Differences in the diet and anatomy of various animals can have an impact on the bacterial flora that inhabits the gut of these animals (McBee, 1971; Stoeckel et al., 2004). Prokaryotic and eukaryotic cells alike have genes that encode rRNA genes; these genes are found in all cells and are highly conserved because they are essential for metabolic function (Wittmann, 1976). Most mutations of the rRNA gene would be detrimental to the function of the cell and would likely be lethal. However, certain helices found in the structure of the rRNA do contain variable regions and minor adjustments to these areas may help the cell adapt to changes in its environment, such as temperature or pH (Woese, 1987). Unlike prokaryotic cells, eukaryotic cells contain the energy producing organelle known as the mitochondrion. Regions of mtDNA evolve at a rate greater than nuclear DNA and, like rRNA genes, they are also found in large copy numbers (Gerber et al., 2001). The variable genetic regions of both ribosomal and mitochondrial DNA are what MST can exploit. In fact, it is the variable regions of rDNA or mtDNA that allow for the development of host-specific markers and the different detection methods.

The shift from culture-dependent to culture-independent techniques has opened up a wide variety of options for FIOs. The intestinal tract is an anaerobic environment where both facultative anaerobic bacteria, such as *E. coli*, and anaerobic bacteria, such as *Bacteroidales* reside. Previously, a large number of ideal anaerobic organisms has been excluded for use as FIOs because they were undiscovered or their cultivation was too

complicated for practical use (Rajilić-Stojanović et al., 2007; Simpson, Kocherginskaya, et al., 2002). *Bacteroidales* are found in much higher concentrations than *E. coli* in animal intestinal tracts, but until recently have not been suggested as FIOs (Fiksdal et al., 1985). The *Bacteroidales* 16S rRNA gene, which has many copies per cell, has become a popular target of MST assays (Parveen et al., 1999; Simpson, Santo Domingo et al., 2002). Some host-specific markers have been detected at levels of 10^7 copies or higher in one gram of feces (Layton et al., 2006; Reischer et al., 2011). Eukaryotic cells have also been overlooked as indicators of fecal pollution but culture-independent detection methods have paved the way for many assays that target mtDNA. Within each cell there are many mitochondria and within each mitochondria there are several copies of the mtDNA. Endothelial cells that line the intestinal tract of mammals are shed along with feces; in fact, one gram of fecal material can contain up to 10^7 copies of mtDNA (Ballesté et al., 2010). Dilution of the cells within receiving watercourses is more of a concern with culture-independent methods, but cell concentration techniques can be used to help address for this shortcoming (Simpson, Santo Domingo, et al., 2002). Without the constraints of culturing, the high copy number of *Bacteroidales* 16S rDNA and mtDNA can be capitalized on by using a common molecular tool known as polymerase chain reaction (PCR).

2.4.2.2 POLYMERASE CHAIN REACTION AS A TOOL FOR MICROBIAL SOURCE TRACKING

The techniques employed by various LI-MST assays are generally similar; the differences are in the target DNA. PCR is a common technique employed by LI-MST and also by various LD-MST methods (Fig. 2.1). PCR uses cellular components and machinery (deoxyribose nucleotides and DNA polymerase) to target and amplify small

amounts of sample DNA in an extracellular reaction. Before a PCR can be executed primers that flank the desired target sequence must be created. Primers are small pieces of single stranded DNA (ssDNA) that are complementary to their target DNA sequence; they facilitate commencement of DNA replication by providing a priming point for the DNA polymerase. DNA polymerase is an enzyme found in all cells that catalyzes the replication of ssDNA into double stranded DNA (dsDNA). PCR volumes are generally less than 50 μ L and are carried out in specialized equipment called a thermocycler. There are three basic heating steps to a PCR protocol: denaturation, annealing, and elongation. Sample dsDNA is first denatured into ssDNA at high temperatures; the thermocycler raises the temperature of reaction mixtures to denature, or separate, the DNA. Once the DNA is in single strand form, the temperature is lowered to allow for the primers to bind, or anneal, to their complementary sequences. After the annealing step is complete, the temperature is raised to activate the specialized thermotolerant DNA polymerase. The enzyme binds to the primers and elongates the ssDNA using free nucleic acids present in the PCR mixture. These three steps are usually repeated 30 to 40 times, creating exponential amplification of the target DNA, known as amplicons (Peake, 1989). This is known as traditional or end-point PCR because results are obtained only at the end point of the reaction. This type of analysis is purely qualitative and results are reported as positive/negative or presence/absence.

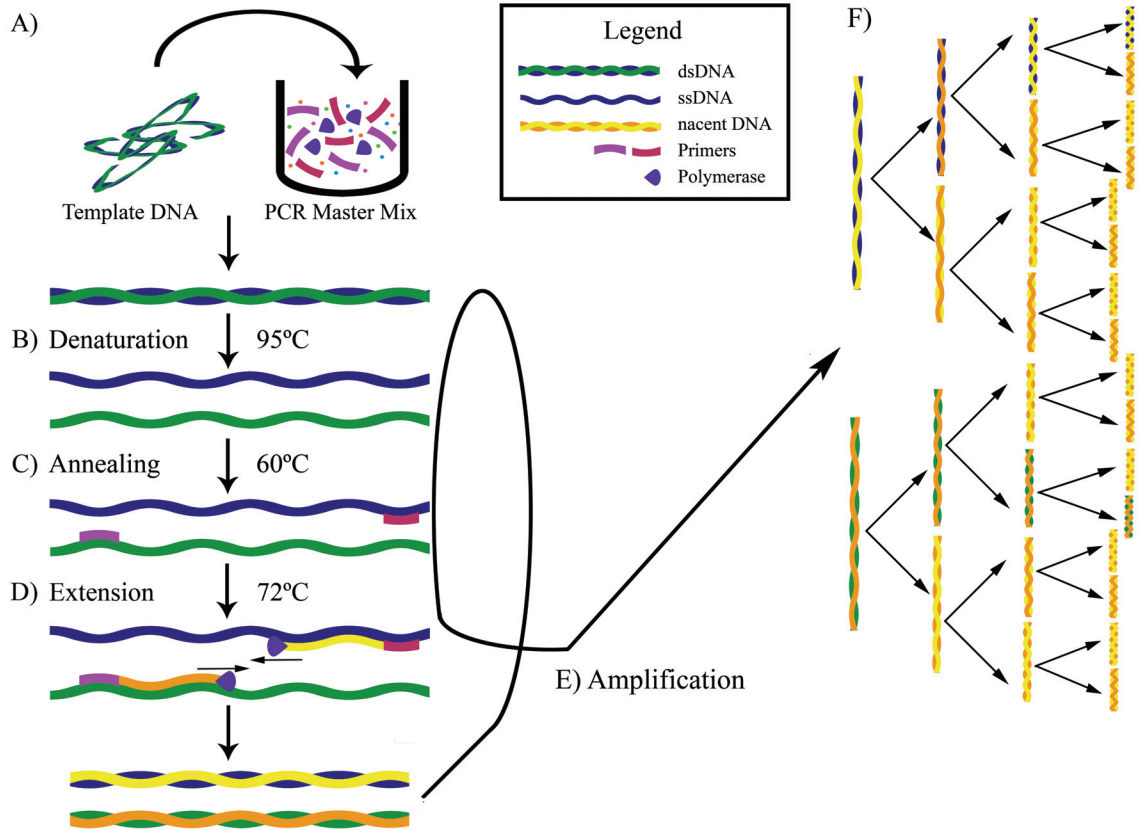


Fig. 2.1: Basic principles behind DNA amplification using polymerase chain reaction PCR amplifies small amounts of DNA to detectable levels using components and machinery derived from cells. Template DNA is added to a PCR master mix containing primers and the polymerase enzyme (A). The reaction temperature is raised to 95°C in order to denature dsDNA (B). Following denaturation, the reaction temperature is lowered (i.e.: 60°C) to enable annealing of the primers to the DNA (C). Once the primers have annealed to the ssDNA, the temperature is raised to 72°C to activate the polymerase enzyme. The polymerase enzyme uses the ssDNA to replicate the template DNA (D). This cycle is repeated approximately 30 times (E) resulting in exponential amplification of the target DNA (F).

Further progress in MST has improved upon the end-point PCR assays by using quantitative PCR (qPCR) to detect host-specific markers in a quantitative manner. qPCR employs the same principles as end-point PCR, except that it has a lower detection limit, often uses fluorescent DNA probes to continuously monitor formation of dsDNA and can determine the starting quantity of target DNA once proper standard curves have been made (Fig. 2.2). Probes are designed to work in conjunction with the primers to increase

the specificity of the target sequence quantification in a sample. Two supplementary molecules flank the probe: a fluorescent tag and a quencher molecule that prevents the tag from emitting a signal. During the annealing step of the qPCR, the probe binds a specific target sequence found within the amplifying gene. Again, from the primer, the DNA polymerase begins replicating the ssDNA. The polymerase inherently has 5' exonuclease activity, which will degrade the probe and release it from the sequence that is being replicated. Degradation of the probe causes disassociation of the fluorescent tag from the quencher, resulting in a fluorescent signal. This signal is detected and transformed into data that together with a previously established standard curve can be used to calculate the starting quantity of the marker in a given sample (Heid et al., 1996). Standard curves are constructed with known quantities of target DNA, and the time it takes to detect a threshold fluorescence signal (C_t) is plotted on the y-axis vs. the logarithm of the DNA or target organism concentration on the x-axis.

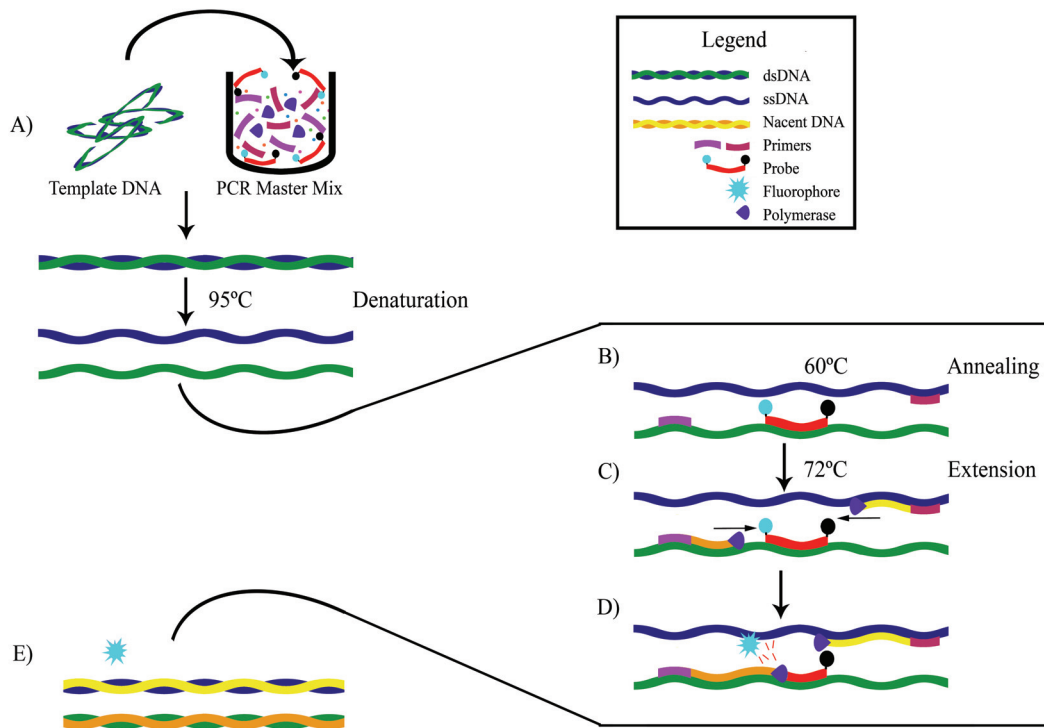


Fig. 2.2: Basic principles behind Probe-Based Quantitative PCR

Quantitative PCR is an adaptation of end-point PCR. In addition to the primers and polymerase, the master mix contains sequence specific probe that is flanked with a fluorophore and a quencher molecule (A). During the annealing step, the probe anneals to the target DNA (B). The polymerase begins replicating the ssDNA from the primers (C). The inherent exonuclease activity of the polymerase degrades the probe, thereby releasing the fluorophore (D). With each cycle the fluorescent signal increase, enabling quantification of the starting quantity of DNA (E).

Ubiquitous genes are logical targets for LI-MST and there have been many reported in the literature with varying degrees of success. Bernhard and Field first described the PCR detection of *Bacteroidales* and *Bifidobacterium* 16S rRNA genes in 2000. By isolating and amplifying the universal 16S rDNA from individual fecal samples, they were able to detect host-specific patterns using length-heterogeneity PCR (LH-PCR) and terminal restriction fragment length polymorphism (T-RFLP) analysis. Both of these methods are used to analyze the differences in gene fragment lengths that can occur when mutations cause nucleic acids to be inserted or deleted. Their research resulted in the design of primers that detected human-specific *Bacteroidales* 16S rDNA and ruminant-

specific *Bacteroidales* 16S rDNA in environmental water samples (Bernhard & Field, 2000a). This assay was the first of its kind, although many primers designed by other researchers targeting different 16S rDNA sequences and hosts were to subsequently improve upon these initial results. In short, the early assays analyzed DNA extracted from bacteria found in water samples by using end-point PCR to amplify the host-specific marker. The presence or absence of amplicons was determined by resolving the DNA by agarose gel electrophoresis. In other words, if the host-specific marker targeted by the primers was present in the sample, it would be verified by the presence of the amplicon on the agarose gel. If no DNA amplification occurs, then it was concluded that sample did not contain the host-specific markers targeted by the primers. Feces from target hosts were used to validate that these assays functioned as intended (Bernhard & Field, 2000b). However, it is important to note that end-point PCR assays are purely qualitative, giving the investigator a positive or negative result.

2.5 BACTEROIDALES 16S rRNA GENES AS MOLECULAR INDICATORS OF FECAL POLLUTION

Currently, many researchers are focused on developing MST assays to detect *Bacteroidales* 16S rDNA in environmental samples. *Bacteroidales* are an order of Gram-negative, anaerobic, bacillus bacteria. The order is composed of four families: Bacteroidaceae, Prevotellaceae, Rikenellaceae, and Porphyromonadaceae. Although most of the *Bacteroidales* indicator bacteria come from the genus *Bacteroides*, which are members of the Bacteroidaceae family, there are a small number of markers that target species from the genus *Prevotella*, members of the Prevotellaceae family. *Bacteroidales* may be an excellent group of indicator organisms for many reasons. Detection is more likely because they are the most abundant group of bacteria found in the intestinal tract of

warm-blooded mammals, comprising over 30% of the gut microbiota (Backhed et al., 2005). They are also anaerobic and therefore would not persist in the environment (Kreader, 1995). However, the most appealing characteristic for the use of *Bacteroidales* as an MST indicator is the host-specific markers harboured in their rDNA that enable differentiation between sources of fecal pollution (Bernhard & Field, 2000a).

Over the past decade, there has been a great deal of research into uncovering various *Bacteroidales* 16S rDNA host-specific markers. Because human and agricultural microbial pollution constitute the most important forms of microbial pollution, they have had the most research attention (Dechesne & Soyeux, 2007). The first publications attempting to use *Bacteroidales* as molecular markers focused on differentiating between human and bovine sources of fecal pollution (Bernhard & Field, 2000a). Several different assays targeting both human-specific and bovine-specific *Bacteroidales* have been reported in the literature with varying degrees of success. A number of markers have also been determined for other agriculturally significant animals such as pigs (Mieszkin et al., 2009; Okabe et al., 2007), poultry (Lu et al., 2007), and horses (Dick et al., 2005). Furthermore, the contribution of domestic animals and wildlife to microbial pollution has not been ignored; for example host-specific markers to detect dog (Dick, Bernhard et al., 2005; Kildare et al., 2007), elk/deer (Dick et al., 2005), Canada goose (Fremaux et al., 2010; Lu et al., 2009), and muskrat (Marti et al., 2011) feces in water have been developed.

Once a potential marker has been developed, it must first be validated before it can be used for environmental monitoring (Ahmed et al., 2009; Roslev & Bukh, 2011). Validation of the markers determines how useful they are for water quality monitoring by

testing the specificity and sensitivity of each assay against feces from target and non-target species.

The sensitivity of an assay is a reflection of its ability to detect target host feces in a sample and it is determined by testing the primer and probe set against numerous fecal samples originating from the target host species. The amount of correctly identified samples (true positives) is compared to the total number of samples. Specificity of the assay is a reflection of the ability of the assay to exclude, or not cross-react, with non-target host feces and it is determined by testing the primer and probe set against fecal samples of many different animals from a broad range of non-target species. Negative results are compared to the total number of samples, which include both the true negative and the false positive results. Many of the assays have been applied to different regions following their original publication. As will be discussed in a future section, some of these assays have shown temporal and spatial stability whereas other assays have proven to be less reliable.

2.5.1 DETECTION OF HUMAN AND RUMINANT FECAL POLLUTION USING MOLECULAR TECHNIQUES

In early 2000, Bernhard and Field published the first paper targeting *Bacteroidales* 16S rDNA as fecal indicators (Bernhard & Field, 2000a). They were able to distinguish human from bovine fecal contamination by analyzing the lengths of *Bacteroidales* 16S rRNA gene fragments using two techniques (i) LH-PCR and (ii) T-RFLP analysis. The markers were established using DNA extracted from human and bovine fecal samples. Because the markers were not tested against fecal samples from species other than human and bovine, the specificity is not reported. However, the assays proved to be highly reproducible and sensitive. The possibility of designing primers to target these host-

specific DNA fragments was discussed, as was the potential to use qPCR to quantify pollution sources (Bernhard & Field, 2000a).

Later that year, a second paper by the same group built upon the initial research by targeting the host-specific markers with novel primers. Primers targeting a human-specific gene cluster (HF assay) and a bovine-specific gene cluster (CF assay) harboured within the *Bacteroidales* 16S rDNA were developed. The specificity of the HF and CF assays was tested against feces from 10 non-target species. The human assay did not cross-react with any of the fecal samples. The bovine assay, however, cross-reacted with all ruminant fecal samples indicating that it is more appropriate for detection of microbial pollution from ruminants in general rather than bovine sources specifically (Bernhard & Field, 2000b). It was noted that greater specificity might be obtained by using a more diverse range of species for comparison of host-specific markers during assay development.

Tested against an array of fecal specimens from other species, the initial bovine and human markers yielded mixed results. Researchers who applied Bernhard and Field's techniques within their own studies had varying degrees of success. In one study, CF assay showed between 76% and 99.9% specificity. It was also highly cross-reactive with pig feces (Shanks et al., 2010). This cross-reactivity with swine samples has been demonstrated in other studies, as well (Fremaux et al., 2009). Another study testing the specificity and sensitivity of these markers found that CF assay showed only 26% sensitivity but 100% specificity, not reacting with pig or any other non-target fecal specimens. This same study demonstrated low sensitivity of the HF assay. The specificity of these assays was lower than that of the ruminant markers. Interestingly, the HF assay was highly cross-reactive to poultry feces, resulting in higher specificity for these samples

than human feces (Ballesté et al., 2010). This novel research demonstrated a proof of concept, but also demonstrated a need for further research and development of improved assays.

The need for assays of higher sensitivity and specificity was evident. As qPCR became more accessible, most of the research focused on developing TaqMan® assays rather than end-point PCR, which is only qualitative and less sensitive. Layton *et al.* (2006) developed the commonly used AllBac qPCR assay to detect non-species specific, or universal, *Bacteroidales*; however, the focus on host-specific marker development dominates the current research. Using the Taqman approach, two papers published by one group were able to positively identify both ruminant (Reischer et al., 2006) and human (Reischer et al., 2007) markers in water. The BacR assay was developed to detect ruminant fecal contamination and proved to be 100% sensitive and specific for ruminant feces. To determine specificity, 146 non-ruminant feces samples were tested, including human. The limit of detection for this assay was determined to be 100-fold more sensitive than Bernhard and Field's original qualitative ruminant assay (Reischer et al., 2006).

Development of the BacH assay sought to create a more sensitive and specific test for detecting human fecal contamination. Due to sequence variability within the human fecal samples, the investigators chose to design two probes, which when combined into one assay showed high sensitivity. This TaqMan based assay showed 98% sensitivity for human feces and 99.7% specificity. Of the 302 non-human fecal samples tested, only one false positive occurred. The feces of a domestic cat resulted in a false positive test; due to the close proximity in which domestic animals and their owners live, it is possible that fecal bacteria can be inadvertently exchanged between the two hosts through the fecal-oral route. This type of exchange may be permanent or transient, but should be taken into

consideration when trying to determine the origin of fecal contamination (Reischer et al., 2007).

The BacR and BacH assays are common assays used to detect ruminant and human fecal pollution, respectively. Literature in support of these assays has suggested that they are geographically stable. Fecal samples from Austria were used to create both assays, however, the BacH assay has been validated in different areas ranging from Canada (Tamblao et al., 2012) to Australia (Ahmed et al., 2009). Validation of the BacR assay has also been reported in Wales (Wyer et al., 2010) and France (Gourmelon et al., 2010). Although information about this assay is limited, some studies suggest that it may also be widely applicable. However, the problem still exists that in many agricultural areas more than one kind of ruminant can be a source of fecal pollution, whether domestic or wild.

There have been efforts to distinguish between different ruminant populations. Understanding the land use practices of a region can help determine the source of ruminant fecal contamination (Reischer et al., 2011), however, the ability to use MST to pinpoint the specific culprit would be extremely beneficial. The CowM2 (Shanks et al., 2008) assay was developed for the purpose of identifying bovine fecal contamination. This assay has yet to be validated in any other geographic region; however in order to develop the primer and probe set, bovine fecal samples were taken from three different farms across the United States. Sensitivity and specificity for the CowM2 assay was 100% (Shanks et al., 2008). In contrast to other *Bacteroidales* assays discussed, this assay targets the HDIG domain protein, which is believed to be directly involved in the bacteria-host interaction. Targeting these genes may enable greater specificity but the limit of detection may be affected because these genes would not be present in high copy

numbers, like the 16S rRNA genes (5 per cell in *Bacteroidetes* (Case et al., 2007)) are. Indeed, in a follow-up study to the originally published work, the assay was confirmed to be less sensitive than its 16S rRNA counterparts, in that the limit of detection of the CowM2 marker was lower than the less specific 16S rRNA assays (Shanks et al., 2010). At this point, pairing the CowM2 assay with a ruminant assay, such as BacR, is likely the most effective method to determine bovine fecal contamination.

With the progression of the MST field, the database of host-specific markers targeting a variety of different animals grows, as demonstrated in Table 2.1. As one might expect, the agriculturally significant species chicken (Lu et al., 2007), horse (Dick, Bernhard, et al., 2005), and pig (Mieszkin et al., 2009) have been targeted. Assays have also been developed to target less conventional animals, such as dog (Dick, Simonich, et al., 2005; Kildare et al., 2007), elk and deer (Dick et al., 2005), Canada goose (Fremaux et al., 2010; Lu et al., 2009), and even muskrats (Marti et al., 2011).

Table 2.1: Host-specific *Bacteroidales* Assays

Target Host	Assay	Reference
Bovine	CowM2, CowM3	Shanks et al., 2008
Canada Goose	CGOF1-Bac, CGOF2-Bac	Fremaux et al., 2010
Chicken	CP	Lu et al., 2007
Deer/ Elk	EF990	Dick, Simonich, et al., 2005
Dog	DF475	Dick, Simonich, et al., 2005
Horse	HoF597	Dick, Simonich, et al., 2005
Human	HF Cluster BacH	Bernhard & Field, 2000a, 2000b Reischer et al., 2007
Muskrat	MuBa01	Marti et al.
Ruminant	CF Cluster BacR	Bernhard & Field, 2000a, 2000b Reischer et al., 2006
Pig	Pig-1-Bac, Pig-2-Bac PF163	Mieszkin et al., 2009 Dick, Bernhard, et al., 2005
Dog	BacCan CG-Prev f5	Kildare et al., 2007 Lu et al., 2009
Universal	AllBac	Layton et al., 2006

2.5.2 ISSUES ASSOCIATED WITH MST *BACTEROIDALES* ASSAYS

Aside from the issues of sensitivity and specificity of markers, and the geographic and temporal stability, which have already been discussed, there are still significant knowledge gaps in the field of LI-MST that need to be addressed. First, more information regarding the persistence of *Bacteroidales* markers in the environment is needed. Secondly, the inability of current assays to differentiate between viable and dead *Bacteroidales* needs greater attention. Lastly, the information regarding the correlation between *Bacteroidales* markers and pathogens is weak. Although these issues are slowly being addressed, there is still limited information on the subject.

2.5.2.1 MARKER PERSISTENCE IN THE ENVIRONMENT

It is often stated that the need for improved fecal indicator organisms, such as *Bacteroidales*, is due to the fact that some enteric *E. coli* populations have been reported to persist and become naturalized in aquatic environments (Anderson et al., 2005; Byappanahalli et al., 2003; Power et al., 2005). Because *Bacteroidales* are anaerobic, it is presumed that they cannot persist in the environment for long periods of time and would therefore indicate recent fecal contamination. However, studies addressing the persistence of *Bacteroidales* markers are somewhat limited. Marker persistence (as in the stability of the DNA found in the live or dead bacteria) is dependent on many variables, including water temperature, salinity, exposure to sunlight, predation, and marker specific differences.

The persistence of the AllBac marker in microcosms spiked with horse manure was investigated with respect to fecal aggregate size, fecal concentration, water temperature, and filtered versus non-filtered stream water (Bell et al., 2007). This study

demonstrated that the persistence of *Bacteroidales* markers in stream water samples was most affected by the presence of biological agents, such as protozoa. Microcosms containing unfiltered stream water spiked with manure had significantly lower AllBac marker decay rates than the microcosms containing filtered stream water spiked with manure. The starting concentration and fecal particle size did not appear to affect the decay rates of the markers. Temperature was also a factor in the persistence of the marker; the marker persisted longer in unfiltered stream water at lower temperatures. The investigators postulated that this could be due to the fact that at lower temperatures protozoa are less active and therefore predation is less of a factor. This trend indicates that there would be seasonal variability in marker persistence due to the difference in water temperature throughout the year. It would also stand to reason that the rate of marker degradation would be higher in warmer climates.

In another study, Dick *et al.* (2010) used microcosms to determine persistence of the AllBac, HF, and HumBac markers in relation to culturable *E. coli* concentrations. They suggested that the AllBac marker should not be used as an indicator of recent fecal contamination because a background level of the marker was detected. The AllBac marker concentrations did decline over time, showing a greater than 2-log removal of marker over an 11 day period; however, resuspension of the microcosm sediment at the end of the experiment caused the marker quantity to be restored to 50% of its original concentration. Conversely, this result was not seen for the other markers tested. The HF marker showed over a 3-log marker degradation in 5 days and the concentration was not increased after resuspension of microcosm sediment. These results indicate that the AllBac marker would be an inappropriate replacement for fecal coliforms due to its

apparent persistence. This study did not consider if the markers originated from viable or dead cells (Dick et al., 2010).

Recently, information regarding the AllBac assay has been revealed to help explain the high detection rate in environmental samples. The persistence of the AllBac marker in the environment could be explained by information revealed by van der Wielen *et al.* (2010). Investigation into the sequence similarity between *Bacteroidales* of fecal and non-fecal origin has revealed that the AllBac assay, intended to detect *Bacteroidales* originating from the intestinal tract of warm-blooded animals, would cross-react with *Bacteroidales* found in the environment and in the hind-gut of insects (van der Wielen & Medema, 2010). Vierheilig *et al.* (2012) further confirmed these findings. In their study, they determined that the AllBac assay detected approximately the same marker concentrations in soil samples taken from a pristine environment and from soil samples taken beside a cowpat located in a pasture, indicating the unspecific nature of the marker.

2.5.2.2 DIFFERENTIATION BETWEEN VIABLE AND NON-VIABLE CELLS

Because PCR can amplify DNA from viable, distressed or dead cells (Josephson et al., 1993), the question of cell viability still remains. One approach that has been employed to address this issue is the use of Propidium Monoazide (PMA), which is a chemical that intercalates DNA. PMA is a photo-inducible compound that crosslinks after exposure to light. DNA containing cross-linked PMA is rendered insoluble and therefore cannot be amplified by the polymerase during PCR. Furthermore, the PMA molecule is unable to penetrate the intact cellular membranes of viable cells, therefore only extracellular DNA or DNA from dead cells is affected (Nocker et al., 2006).

Bae and Wuertz (2009) investigated the use of PMA to detect viable *Bacteroidales* DNA in environmental water samples. The influence of turbidity in environmental samples was investigated because the effectiveness of PMA is dependent on exposure to light. They found that in samples without environmental solids, PMA did not affect the amplification of viable cells. At higher solids concentration (TSS = 1000 mg/L), the ability of PMA to inhibit amplification of heat-treated cells was observed. To effectively cross-link the DNA in dead/injured cells, PMA requires light activation and high TSS concentrations may interfere with light penetration into the solution. The group was able to optimize the reaction by determining the best combination of PMA concentration and radiation exposure time resulting in successful PMA-PCR at high TSS concentrations (Bae & Wuertz, 2009). Although some papers discuss the use of PMA to address the problem of determining viability (Roslev & Bukh; Walters et al., 2009), few studies have actually applied this method.

2.5.2.3 CORRELATION BETWEEN MARKER DETECTION AND PATHOGEN PRESENCE

Establishing correlations between indicator organisms and pathogen presence appears to be one of the most arduous tasks in the MST field. Information regarding these trends is limited and inconclusive. Rogers *et al.* (2011) investigated the decay rates of both pathogenic bacteria transformed with green fluorescent protein (GFP) and fecal indicators in manure amended soil. Persistence of *E. coli* O157:H7, *Salmonella enterica*, and *Campylobacter* spp. was compared with persistence of bovine- and swine-specific *Bacteroidales* markers (CowM2, CowM3, PF163, and PigBac1, respectively) and traditional fecal indicators. This study concluded that the *Bacteroidales* assay would not be an appropriate indicator for microbial pollution due to runoff from manure-amended

soils. The cultivatable FIO *Enterococci* and *E. coli* and the qPCR assays to detect pathogens exhibited similar survival rates to the GFP pathogens. However, the *Bacteroidales* markers declined at a much faster rate than the GFP pathogens, suggesting that these markers may not be appropriate for identifying risks associated with non-point source microbial pollution such as runoff from crops fertilized with manure (Rogers et al., 2011).

Another study retroactively examined water samples from an Albertan watershed that was previously determined to be contaminated with *E. coli* O157:H7, *Salmonella*, or *Campylobacter* (Walters et al., 2007). The group assayed DNA from water samples previously collected from the watershed using the universal assay, the ruminant-specific (CF128 and CF193), human-specific (HF), and swine-specific (PF163) *Bacteroidales* markers. Very few samples contained more than one pathogen. The CF128 and CF193 markers were detected in 90% and 50% of the samples positive for *E. coli* O157:H7, respectively. Conversely, only 7% of the samples positive for ruminant-specific markers were positive for *E. coli* O157:H7. Detection of *Salmonella* was not adequately predicted by any *Bacteroidales* markers. Detection was increased only 2.5 times when the ruminant-specific markers were present. The presence of human-specific markers did not increase or decrease the chances of finding *Salmonella*. In contrast, detection of the human-specific marker resulted in a 10-fold increase in the likelihood of finding *Campylobacter*. Detection of *Campylobacter* was not correlated with the presence of the ruminant marker but it was detected twice as often when the swine-specific marker was present (Walters et al., 2007). Although this study did produce some interesting correlations it is clear that there are many issues that still need to be resolved with respect

to *Bacteroidales* MST assays, including marker persistence and identifying robust correlations between pathogens and specific markers.

2.6 THESIS OBJECTIVES

The feasibility of using *Bacteroidales* MST as an alternate indicator of microbial pollution in Nova Scotia has not been explored. To that end, this project attempted to assess universal, ruminant-, bovine-, and human-specific *Bacteroidales* MST assays for use in this region. The objectives of this project were three-fold: i) to validate the chosen qPCR assays using feces collected from humans and animals around Nova Scotia ii) to investigate the occurrence of universal and host-specific *Bacteroidales* markers within the study watershed using the validated assays; and iii) to determine if any correlation between the presence of fecal indicators and the pathogens *E. coli* O157:H7, *Salmonella* sp., and *Campylobacter* spp. could be revealed.

CHAPTER 3. MATERIALS AND METHODS

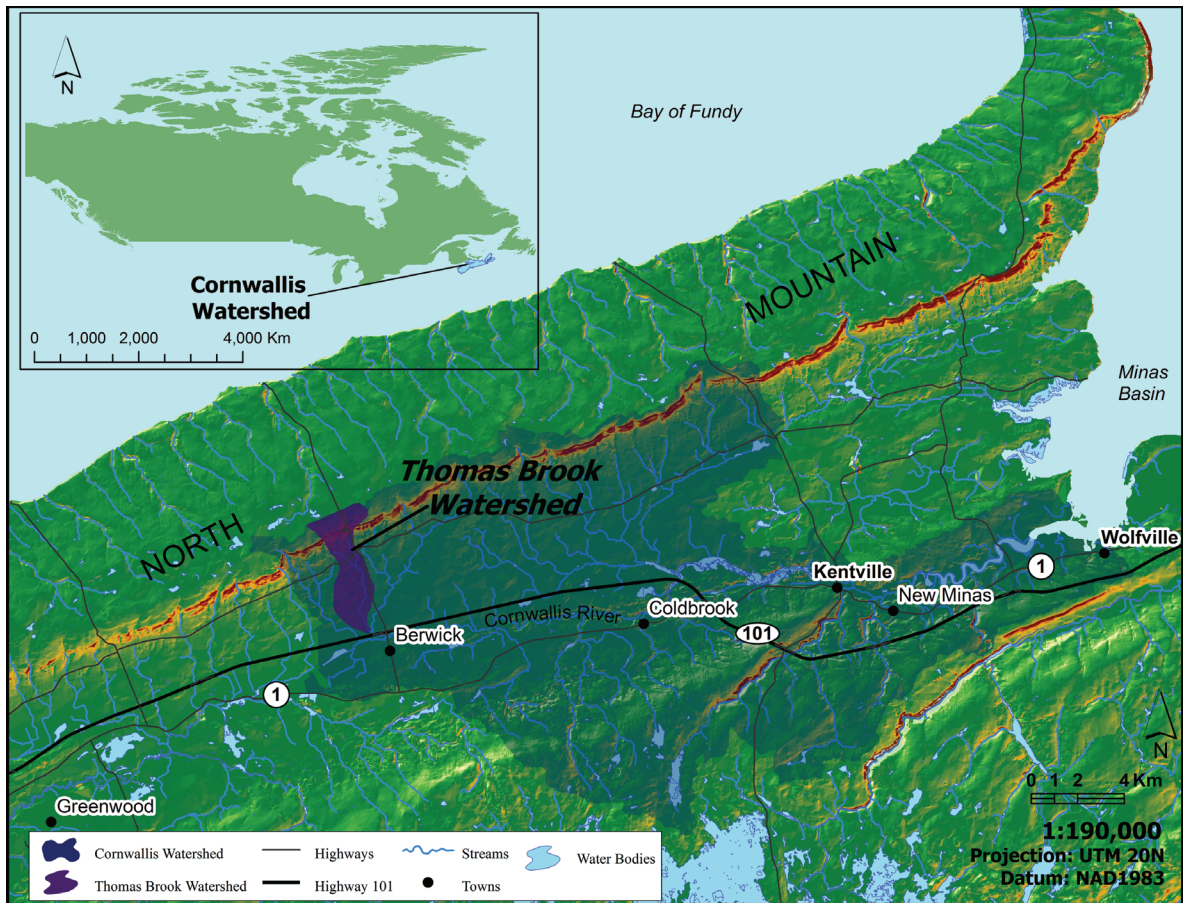
3.1 SAMPLE DESIGN

The host-specific *Bacteroidales* assays chosen for this project were developed based on populations in distant geographical areas, therefore, the feasibility of using them in Nova Scotia was assessed by first validating the chosen assays using fecal samples from humans and animals within the province. Concurrently, water samples were collected from a study watershed on a regular basis and during storm events over a 15-month period from May, 2010 to August 2011. At the time of sampling, a variety of general water quality and hydrological measurements were also taken in order to help explain variability and trends within the data. The water samples were analyzed for conventional FIOs (i.e., total coliforms and *E. coli*) and for the presence of selected pathogenic bacteria (*E. coli* O157:H7, *Salmonella* sp. and *Campylobacter* spp.) using a selective enrichment procedure. q-PCR detection using TaqMan® protocols was performed on DNA extracted from the enriched samples and directly from the water to detect pathogen and *Bacteroidales* DNA, respectively.

3.2 STUDY WATERSHED IN RURAL NOVA SCOTIA

The Thomas Brook Watershed (TBW), a sub-catchment of the Cornwallis Watershed, is located in the Annapolis Valley in Nova Scotia, Canada (Fig. 3.1). The TBW is approximately 784 hectares (Sinclair et al., 2009) and is situated on the north face of the valley, known as North Mountain. The headwaters of the TBW originate as two stream channels from the top of North Mountain. They merge in the upper third of

the watershed and drain into the Cornwallis River at the outlet on the valley floor (Jamieson et al., 2003). The slope of the stream along the upper third of the watershed, the valley wall, reaches 9% but averages a 0.5-1.3% grade for the remainder of the watershed area (Jamieson et al., 2003). Thomas Brook is a small stream of approximately 5800 m in length and seldom exceeds two meters in width.



(Sinclair et al., 2009)

Fig. 3.1: Thomas Brook Watershed, Nova Scotia, Canada, as a study watershed
The Thomas Brook subcatchment of the Cornwallis Watershed, is located on the north face of the Annopolis Valley, in Nova Scotia, Canada.

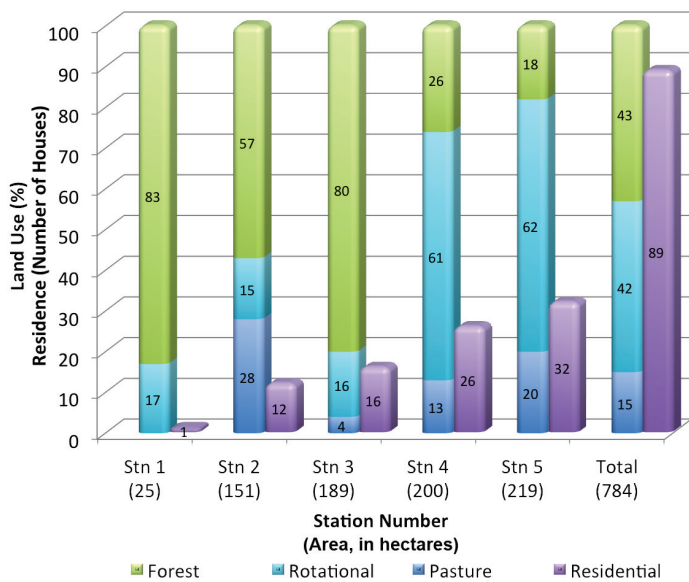
The TBW was chosen as the study watershed for numerous reasons. The Annapolis Valley is the most heavily farmed area in Nova Scotia. Within TBW, there is a mixed land use pattern consisting of forest, residential and agricultural areas. Elevated

levels of *E. coli* have been previously reported in the watershed (Jamieson et al., 2003), but previous monitoring efforts have not yielded knowledge of the primary sources of microbial contamination. Six sampling stations were chosen along the stream in an attempt to capture drainage and water quality influences from different land use designations within the watershed. Sampling stations were chosen due to their proximity to possible sources of pollution. Fig. 3.2 demonstrates the land usage breakdown of the subwatershed for each station.

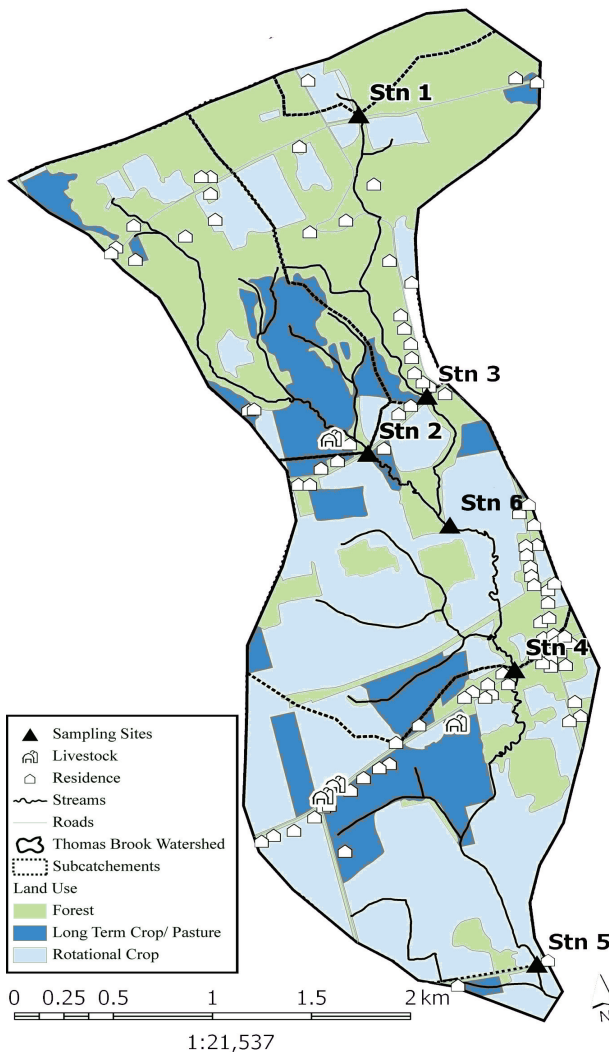
Station 1 (Stn 1) is located at the headwaters of TBW and serves as a negative control due to minimal anthropologic activity in the area. It represents 25 ha that is primarily forested (83%) with only one residential dwelling and a small amount of rotational pastured land (17%). Station 2 (Stn 2) is located downstream of a dairy farm with approximately 300 heads of cattle. In the 151 ha subwatershed area there are 12 residences as well as forested areas (57%), rotational cropland (15%) and long-term (28%) pastures. Station 3 (Stn 3) consists of 189 ha and contains a residential cluster (16 houses) that is primarily forested (80%) with little agricultural land (4% long-term pasture and 16% rotational cropland). Station 4 (Stn 4) is located downstream of where the two main branches of the watershed merge. This sampling site is also downstream of two small beef farms. There are 26 households within the Stn 4 subwatershed. The majority of the land is made up of rotational crops (62%). There is a small amount of long-term pasture (13%) and forested areas (26%). Station 5 (Stn 5) is located at the watershed outlet, prior to the stream meeting with the Cornwallis River. This is the largest subwatershed (219 ha) and is primarily made up of agricultural land (62% rotational cropland and 20% long-term pasture) with a small section of forested land (18%). There are also 32 residential dwellings in this section. Station 6 (Stn 6) is a sampling site located

downstream of Stn 2 and Stn 3. This sampling site was added due to its location at the base of a rotational crop that receives surface applied liquid dairy manure as fertilization on a yearly basis prior to the spring growing season. In total, over half of the watershed is designated as agricultural land (57%), while the rest is forested and residential (43%). The total number of residential dwellings situated within the watershed is 89.

Fig. 3.2: Mixed land use pattern of the Thomas Brook Watershed



A) Graphical breakdown of land usage for each sub-watershed in TBW and watershed totals. Land that represents forest (green), rotational (light blue) and pastured cropland (dark blue) is represented as a percent (percentage for each is stated on the bar). The number of residential dwellings is represented in purple and numerically on each bar. The area (hectares) is noted under each label.



B) Diagrammatic representation of the land usage of TBW in relation to sampling stations. The map shows the locations of livestock operations, houses, streams, roads, forest areas, and pastures. Stn 1 is at the headwater, in a location with little anthropological activity; Stn 2 and Stn 4 each are down stream of cattle operations; Stn 3 is situated in a residential cluster, and Stn 5 is at the watershed outlet where all streams culminate. Stn 6 is located downstream of Stn 2 and it is adjacent to a rotational crop that receives liquid dairy manure as fertilizer.

Adapted from Sinclair et. al, 2009

3.3 SAMPLE COLLECTION AND WATER QUALITY MONITORING

3.3.1 FECAL SAMPLE COLLECTION AND SEPTIC TANK SAMPLING FOR ASSAY VALIDATION

The chosen *Bacteroidales* assays were validated using fecal samples from humans and animals around Nova Scotia (Table 3.1). Validation of host species-specific *Bacteroidales* primers that were developed elsewhere was necessary to ensure that they were functional in Nova Scotia in terms of their specificity and sensitivity. Whenever possible, fresh individual samples were collected. Bovine samples (n=26) were collected from individual animals in and around TBW. To ensure sample diversity, feces were collected from both beef and dairy cattle of different ages ranging from calves to adults. Chicken (n=1), horse (n=2), and pig (n=3) were also collected in the Annapolis Valley, NS. To ensure that assays targeting human or ruminants did not cross-react with wildlife, fecal samples from wild animals native to Nova Scotia were collected in January 2011, at the Shubenacadie Wildlife Park (Shubenacadie, NS). Visual confirmation of individual excretion was not possible; therefore several samples from each animal pen were collected and pooled. Samples were collected from beaver, bobcat, coyote, fisher, porcupine, raccoon, red fox, river otter, snowshoe hare, and skunk. Different animal species were housed in individual pens; as a result cross-contamination with other species was unlikely.

Large-scale livestock operations typically use wastewater lagoons to treat and store animal waste. Manure and washing fluids from pens and milking room floors are regularly deposited into an open wastewater lagoon, or tank, and the resulting liquid manure is often used as fertilizer on crops. Liquid dairy manure (n=4), used to fertilize crops in TBW, was collected from a dairy farm wastewater lagoon. Liquid pig manure

(n=3) from a pig farm in the Annapolis Valley was also collected. In May 2011, septic tank samples (n=11) from households in and around TBW were sampled. The mixture of fecal material suspended in water is known as slurry. All slurry samples (livestock and septic tank) were collected using a sterile 500 mL bottle and a pole sampler. The bottle was immersed under the surface of the lagoon or tank and completely filled before being capped and placed in a cooler. All samples were transported back to the laboratory on ice and stored at 4°C until analysis.

Table 3.1: Feces collected from humans and animals within Nova Scotia for use in the *Bacteroidales* assay validation study.

Animal	Collection Area	Sample Type^a	# of Samples (N=63)
Beaver	Shubenacadie Wildlife Park, NS	Feces	1 ^b
Bobcat	Shubenacadie Wildlife Park, NS	Feces	1 ^b
Bovine	Annapolis Valley, NS	Feces	14
Bovine	Thomas Brook Watershed, NS	Slurry	4
Chicken	Annapolis Valley, NS	Feces	1 ^b
Coyote	Shubenacadie Wildlife Park, NS	Feces	1 ^b
Dog	Halifax, NS	Feces	2
Fisher	Shubenacadie Wildlife Park, NS	Feces	1 ^b
Horse	Annapolis Valley, NS	Feces	2
Human	Annapolis Valley, NS	Slurry	11
Pig	Annapolis Valley, NS	Feces	3
Pig	Annapolis Valley, NS	Slurry	3
Porcupine	Shubenacadie Wildlife Park, NS	Feces	1 ^b
Raccoon	Shubenacadie Wildlife Park, NS	Feces	1 ^b
Red Fox	Shubenacadie Wildlife Park, NS	Feces	1 ^b
River Otter	Shubenacadie Wildlife Park, NS	Feces	1 ^b
Skunk	Shubenacadie Wildlife Park, NS	Feces	1 ^b
Snowshoe Hare	Shubenacadie Wildlife Park, NS	Feces	1 ^b

^a Feces: excrement; Slurry: Combination of primarily fecal material and water

^b Pooled fecal sample consisting of 2 to 4 animals contributing

3.3.2 WATER SAMPLE COLLECTION FOR WATER QUALITY MONITORING

Investigation into the prevalence of the host-specific *Bacteroidales* markers and the traditional FIOs (total coliforms and *E. coli*) in comparison to enteric pathogens was conducted at the 6 sampling stations in TBW, described in section 3.2. Water sampling was conducted over a 15-month period beginning in May 2010 and concluding in August 2011. Sampling was conducted on a biweekly basis during the growing season (May to October) and monthly during the non-growing season. Sampling during the second growing season was truncated. During the second growing season (May to August, 2011) additional samples were collected when storm events (defined as >20 mm of precipitation in 24 hours) were predicted. Grab samples were taken at each station using a sterile 1L bottle. The bottle was rinsed multiple times with stream water before collection. The sterile bottle was placed approximately 3 to 5 mm under the stream surface to avoid collection of materials on the water surface. Bottles were capped and transported back to the laboratory on ice. Samples were kept at 4°C until processing.

3.3.3 PARAMETERS FOR ENVIRONMENTAL AND WATER QUALITY MONITORING

In order to help explain any variability that may be present in the microbiological data, numerous environmental and water quality parameters were measured (Table 3.2). Continuous monitoring of environmental and weather conditions was conducted using a HOBO Weather Station (Onset Computer Corporation, Bourne, MA). The weather station was placed adjacent to a rotational crop field near Stn 6. This site was chosen because it was situated near a sampling site and because it was in a secluded, open, area. The weather station recorded various measurements including rainfall (mm) and air temperature (°C) on 10-minute intervals.

Water quality parameters were measured at the time of sampling at the six sampling sites. Conductivity (mS/cm), pH, dissolved oxygen (DO) (mg/L) and water temperature were measured using a handheld 600R Sonde (YSI, Yellow Springs, OH). Before taking measurements, the sonde probe DO was calibrated in water saturated ambient air. Once calibrated, the probe was placed in the stream and allowed to equilibrate. Measurements were recorded after all parameters had stabilized. To ensure that the sediment in the stream was not disrupted during or before bacterial water sampling, the probe was either placed in the water just downstream of the water sampling site or placed after sampling had finished.

During the first growing season, stream flow was measured at each site using the velocity–area method (CGSB, 1991) with a pigmy current meter (Gurley Precision Instruments, Troy, NY). A FlowTracker current meter (SonTek/YSI, San Diego, CA) was used for low flow measurements, when greater sensitivity was required. During flow measurements the width of the stream channel was measured and then flow velocities, and water depths, were measured at 3 or 4 intervals across the channel. Accuracy in the stream flow measurements were ensured by choosing measurement locations that avoided curves in the stream flow path or obstructions that might cause eddies. Samples at Stn 1 were collected at a culvert outlet; flow was measured by determining the time it took to fill a bucket to 4 L. Stream flow was gauged after water samples for bacterial analysis were collected to avoid disruption of the streambed and contamination of water samples with resuspended sediment.

Water quality measurements for turbidity and total suspended solids (TSS) were completed off-site. Samples for turbidity (nephelometric turbidity units; NTU) and TSS (mg/L) were collected in a 1L bottle at the time of water collection for bacterial analysis.

Turbidity was measured using a Hach 2021AN turbidity meter (Hach, Loveland, CO). Prior to TSS analysis, 1.5 µm glass fiber filter disks (Hach) were desiccated at 103°C for 3 or more hours. The filters were then placed in a desiccator and allowed to cool. Once at room temperature, the initial weight of the filter papers (T_i) was determined and recorded. Solids from the water samples were collected on the filter papers by filtration. The filtration volume (V) was variable and depended on the turbidity of the sample water. The filter papers were returned to the oven and desiccated for another 3 or more hours. After desiccation the filters were placed in a desiccator to cool and then their final weight (T_f) was determined and recorded. TSS (mg/L) was determined using Equation 3.1.

$$\text{Equation 3.1: } TSS = \frac{T_f - T_i}{V}$$

Table 3.2: Parameters measured for environmental and water quality monitoring

	Interval	Parameter	Units
Environmental	Continuous: Daily measurements taken	Rainfall	mm
		Air Temperature	°C
Water Quality and Hydrometrics	Periodic: Measurements taken <i>in-situ</i> at time of sampling	pH	
		Dissolved Oxygen	mg/L
		Conductivity	ms/cm
		Water Temperature	°C
	Stream Flow	m ³ /s	
	Periodic: Offsite analysis	Turbidity	NTU
		TSS	mg/L

3.4 WATER SAMPLE PROCESSING FOR MICROBIOLOGICAL ANALYSES

Fig. 3.3 outlines the general procedure for microbial analysis of fecal and water samples from collection to qPCR or culture-based enumeration. The following sections will describe these steps in further detail.

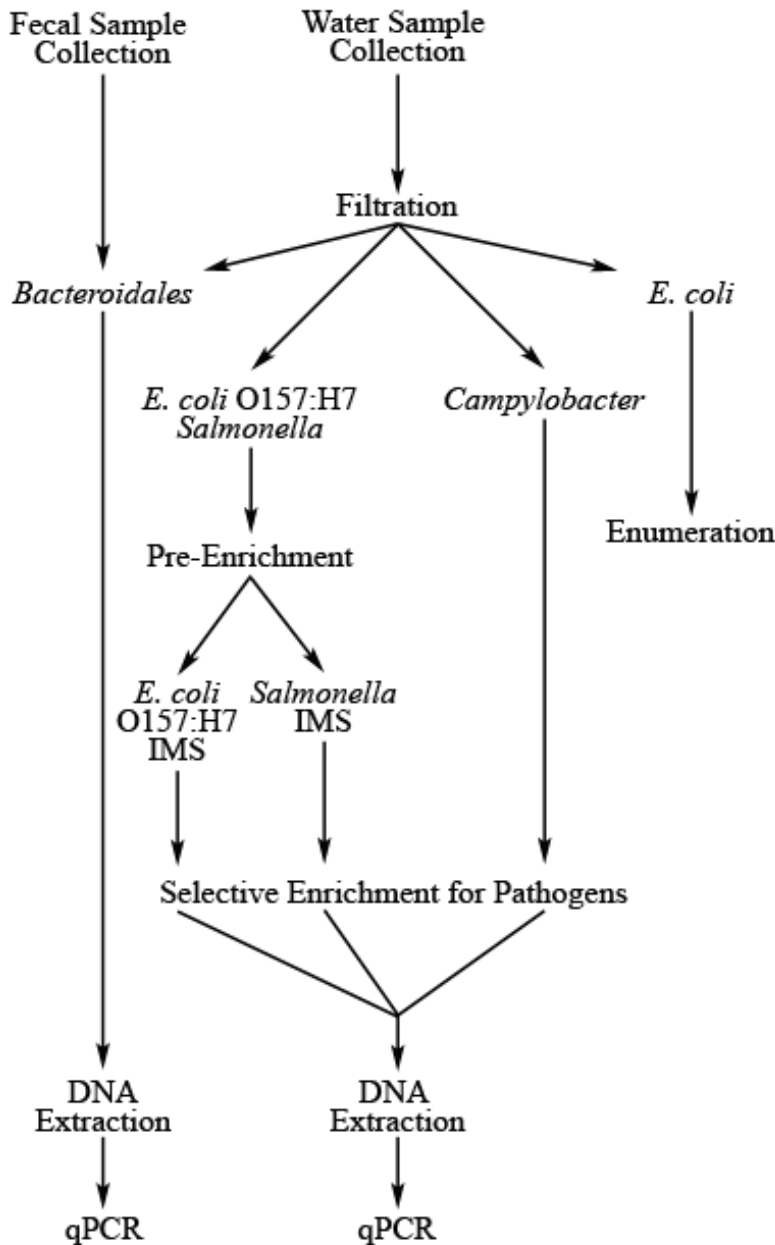


Fig. 3.3: Schematic of the microbiological methods used for water quality monitoring. DNA was extracted directly from fecal samples for *Bacteroidales* assay validation. Contents of water samples intended for *Bacteroidales*, bacterial pathogen, and traditional fecal indicator analysis were concentrated on filter membranes using vacuum filtration. DNA was extracted directly from the concentrated samples for *Bacteroidales* analysis. For detection of *E. coli* O157:H7 and *Salmonella* sp., concentrated samples were subjected to a shared pre-enrichment step followed by an individual selective enrichment, whereas *Campylobacter* spp. was enriched separately under microaerophilic conditions. All pathogen enrichments were pooled prior to DNA extraction and qPCR analysis. The membrane filtration method was used for total coliforms and *E. coli* culture based enumeration.

3.4.1 WATER SAMPLE FILTRATION FOR MICROBIAL ANALYSIS

In preparation for microbial analysis, the contents of the water samples were concentrated on a filter membrane using vacuum filtration. Samples from each station were filtered onto individual membranes in replicates of four to test for the presence of i) total coliforms and *E. coli*, ii) *E. coli* O157:H7, and *Salmonella*, iii) *Campylobacter*, and iv) *Bacteroidales*. Filtration of the water samples was done on a sterilized Microfil Filtration System (Millipore Corporation, Billerica, MA). Appropriate volumes or dilutions of water samples were filtered through a 0.45 μ M filter membrane (Pall Corporation, Ann Arbor, MA). Samples were inverted several times before filtration to ensure uniformity of sample contents.

The volume of water used for the enumeration of total coliform bacteria and *E. coli* was dependent on the clarity of the sample. Analysis of *E. coli* O157:H7 and *Salmonella* sp., *Campylobacter* spp., and *Bacteroidales* required filtration of 500 mL of sample water. After filtration of each volume, the membrane and its contents were aseptically transferred to the appropriate plating or enrichment media. Negative filtration controls for each filtration replicate were conducted by subjecting sterile H₂O to the same filtration procedures as the sample water. Membranes designated for *Bacteroidales* analysis were transferred to a 15 mL tube containing 10 mL of sample water and then stored at 4°C until further processing.

3.4.2 TOTAL COLIFORM AND *E. COLI* ENUMERATION OF WATER SAMPLES

Enumeration of total coliform and *E. coli* was accomplished using the mColiBlue24 (Hach) method, according to manufacturer's instructions. Prior to filtration, 2 mL of mColiBlue24 broth was aliquoted into the appropriate number of petri dishes (47

mm, Pall) containing absorbent pads and left to solidify. Appropriate dilutions were created by serially diluting water samples in peptone saline (PS, 0.1% peptone (Oxoid, Basingstoke, England), 0.85% NaCl (EMD, Chemicals, Darmstadt, Germany)). After sample filtration, the funnel was rinsed with a known volume of PS to ensure all cells were collected on the filter paper. The filter paper was then aseptically transferred to the petri dish. All plates were inverted and incubated at 37.5°C for 24 hours. After incubation, total coliforms and *E. coli* were enumerated by counting the number of colony forming units (CFUs) and converting these to CFU/100 mL. Enumeration of red and blue colonies represented total coliforms. Enumeration of blue colonies represented *E. coli*.

3.4.3 BACTERIAL PATHOGEN ENRICHMENT FROM WATER SAMPLES

Due to the potentially low occurrence of enteric pathogens in environmental waters, samples used for bacterial pathogen analysis required enrichment prior to qPCR analysis. Samples designated for *E. coli* O157:H7 and *Salmonella* sp. detection were subjected to a shared pre-enrichment step in 100 mL of buffered peptone water (BPW, BD, Sparks, MD). Sample filter membranes were aseptically deposited into Erlenmeyer flasks (250 mL) containing BPW, vortexed for five seconds and incubated at 37°C for 24 hours.

After 24 hours of incubation in BPW, aliquots of the enrichment samples were subjected to individual selective enrichment targeting *E. coli* O157:H7 and *Salmonella* sp. using Dynabeads anti-*E. coli* O157 (Invitrogen Dynal, Oslo, Norway) and Dynabeads anti-*Salmonella* (Invitrogen Dynal), respectively. The Dynabeads use immunomagnetic separation (IMS) to selectively concentrate the target bacteria from the pre-enriched bacterial cultures. Antibodies that target the pathogen of interest are coated onto magnetic

beads. When present, epitopes on the surface of the bacteria will bind to the bead-antibody complex. The bacteria-bead complex can then be separated from the non-target bacteria by applying a magnetic force. Several washing steps are required to ensure that all non-target bacteria are removed from the sample.

The IMS protocol was performed individually for *E. coli* O157:H7 and *Salmonella* sp. as per the manufacturer's instructions, with some modifications. BPW enrichments were vortexed prior to aliquoting 10 mL into 15 mL tubes. A negative IMS control sample was included by subjecting sterile BPW to the IMS protocol. Samples were subjected to centrifugation for 10 min at 3200 x g. The supernatant was discarded and the pellet was resuspended in 500 µL of phosphate buffered saline with Tween (PBS-T) (PBS (EMD Chemicals): 0.15 M NaCl, 0.01M phosphate, pH 7.4; with 0.05% Tween-80 (Fisher Scientific, Fair Lawn, NJ)). The suspension was transferred to a 1.5 mL Eppendorf tube. The Dynabead stock solutions were vortexed before pipetting 20 µL into the Eppendorf tubes. The bacteria-bead mixture was mechanically agitated for 25 minutes to allow sufficient binding of antibody and epitope. The sample tubes were then placed in the magnetic particle concentrator (MPC), which is a microcentrifuge tube rack with a magnet. Placement of the Eppendorf tubes in the rack creates contact between the magnet and one side of the tube, allowing for concentration of the bead-bacteria complex. The samples were rotated in the MPC for 3 minutes to allow for maximum separation and concentration of the bead-bacteria complex. The liquid from each tube was carefully aspirated while maintaining contact between the tube and the MPC. The cells were washed 3 times by removing the tubes from the MPC, adding 1 mL of PBS-T, resuspending the pellet, then performing the rotation and aspiration steps. Following the final aspiration, the microcentrifuge tubes were removed from the MPC and 1 mL of the

appropriate selective media was added to the tubes. The beads were resuspended and transferred into 9 mL of the appropriate selective media (total volume 10 mL).

Tryptic Soy Broth (TSB) (BD) was supplemented with novobiocin (MP Biomedicals, Solon, OH) to a final concentration of 20 µg/mL and used to enrich *E. coli* O157:H7. Rappaport-Vassiliadis Soya Peptone (RVS) broth (Oxoid) was used to selectively enrich for *Salmonella* sp. For this secondary enrichment step, samples were incubated at 42°C for 24 hours. Uninoculated TSB-novobiocin and RVS (controls) were incubated along with the samples.

Enrichment of *Campylobacter* spp. was performed under microaerophilic conditions using Bolton Broth (BB, Oxoid) supplemented with BB selective supplement (Oxoid SR0183E) and 5% (v/v) laked horse blood (Oxoid SR0048C). After filtration, filters were aseptically transferred to 50 mL tubes containing 40 mL of supplemented BB and then vortexed. The Campy GasPak system (BD) was used to create the microaerophilic conditions by placing the tubes with punctured caps into the GasPak EZ Incubation Container (BD) together with activated GasPak EZ Sachets (BD). The *Campylobacter* spp. enrichment was incubated at 42°C for 48 hours. A negative media control was also conducted for the *Campylobacter* protocol.

3.5 QPCR ANALYSES

3.5.1 DNA EXTRACTION FOR USE IN *BACTEROIDALES* AND PATHOGEN MARKER DETECTION

All DNA extractions were performed using the PowerSoil DNA Extraction Kit (Mo Bio Laboratories, Inc., Carlsbad, CA). DNA required for use in the *Bacteroidales* validation study was extracted directly from fecal samples. Briefly, approximately 250 mg of feces was placed in the PowerBead tubes provided. In the case of slurry samples

(liquid manure or septic samples) the PowerBead tube contents (a combination of a proprietary buffer and beads) were removed and 250 μ L of the slurry was aliquoted into the empty tube. The samples were centrifuged at 10,000 x g for 2 min. The supernatant was removed and the contents of the PowerBead tube were returned. DNA extraction was subsequently performed following the manufacturer's instructions.

Prior to the DNA extraction, the water samples designated for *Bacteroidales* analysis were vortexed for 3 minutes to dislodge bacteria from the surface of the filter membrane followed by the removal of the membrane from the 15 mL tube. Samples intended for bacterial pathogen analysis were pooled prior to DNA extraction. For each sample 2 mL of TSB-novobiocin, RVS, and BB were combined into one 15 mL tube. Tubes containing water samples and pathogen enrichments were then centrifuged at 3200 x g for 10 min. The supernatant was removed from the tubes and the pellet was resuspended using the residual liquid (< 250 μ L). The suspension was transferred to the PowerBead tubes and DNA extraction was performed as per the manufacturer's instructions. Negative DNA extraction controls were conducted by subjecting the extraction protocol to sterile water. DNA samples were stored at -20°C until the time of analysis.

3.5.2 DETECTION OF HOST-SPECIFIC *BACTEROIDALES* MARKERS AND FECAL BACTERIAL PATHOGENS BY TAQMAN® qPCR

Detection of host-specific *Bacteroidales* DNA sequences (hence forth termed “markers”) in fecal and water samples were carried out using previously published TaqMan® qPCR based assays. Assays were selected according to their relevance to the watershed and included the host unspecific universal *Bacteroidales* marker (Layton et al.,

2006), as well as the ruminant (Reischer et al, 2006), human (Reischer et al, 2007), bovine (Shanks et al., 2008), and swine (Mieszkin et al, 2009) specific markers (Table 3.3). Detection of the bacterial pathogens *E. coli* O157:H7, *Salmonella* sp., and *Campylobacter* spp. were also detected using TaqMan® qPCR based assays developed by Ibekwe et al. (2002), Cheng et al. (2008), and Lund et al. (2004), respectively (Table 3.3).

The CowM2 assay contained an internal amplification control (IAC) template DNA and a probe specific for the IAC. The IAC for the CowM2 assay was constructed as previously described by Shanks et al. (2010) and generously supplied by the Yost Lab at the University of Regina (Regina, SK). All probes, except the IAC probe, were flanked on the 5' end with the fluorophore known as FAM (6-carboxyfluorescein) and the quencher molecule BHQ1 (Black Hole Quencher 1) on the 3' end. The IAC construct was flanked by the CowM2 primer binding sites and had an internal binding site for the IAC probe. The fluorophore known as TET (a tetrachloro derivative of carboxyfluorescein) was bound to the 5' end of the IAC probe (Shanks et al., 2008); this enabled distinction between the fluorescent signals emitted by itself and the CowM2 probe. Signal generation due to the degradation of the IAC probe signified that amplification was occurring, and therefore no inhibitory compounds were present in the reaction.

Table 3.3: Oligonucleotide primers and probes used in assays for water quality monitoring

Target Organism	Primer/ Probe Name	Sequence	Reference
Universal <i>Bacteroidales</i>	AllBac295f	GAGAGGAAGGTCCCCAC	Layton et al., 2006
	AllBac412r	CGTACTTGGCTGGTTCAG	
	AllBac375Bhqr	FAM-CCATTGACCAATATTCTCACTGCT GCCT-BHQ1	
Ruminant <i>Bacteroidales</i> Species	BacR-F	GCGTATCCAACCTTCCCG	Reischer et al., 2006
	BacR-R	CATCCCCATCCGTTACCG	
	BacR-P	FAM-CTTCCGAAAGGGAGATT-BHQ1	
Human <i>Bacteroidales</i> Species	BacH-F	CTTGGCCAGCCTTCTGAAAG	Reischer et al., 2007
	BacH-R	CCCCATCGTCTACCGAAAATAC	
	BacH-Pc	FAM-TCATGATCC- CATCTG-BHQ1	
	BacH-Pt	FAM- TCATGATGCCATCTTG-BHQ1	
Bovine <i>Bacteroidales</i> Species	CowM2F	CGGCCAAATACTCTGATCGT	Shanks et al., 2008
	CowM2R	GCTTGTTGCGTTCCTGAGATAAT	
	CowM2P	FAM-AGGCACCTATGTCCTTTACCTCATC AACTACAGACA-BHQ1	
	Cow IAC-P	TET-TAGGAACAGGCGGCGACGA-BHQ1	
<i>E. coli</i> O157:H7	EaeF	GTAAGTTACTATAAAAAGCACCGTCG	Ibekwe et al., 2002
	EaeR	TCTGTGTGGATGGTAATAAATTTTTG	
	EaeP	FAM-TGGAAGCGCTCGCATTGTGG-BHQ1	
<i>Salmonella</i> sp.	InvA3F	AACGTGTTCCCGTGCCTAAT	Cheng et al., 2008
	InvA3R	TCCATCAAATTAGCGGAGGC	
	InvA3Probe1	FAM-TGGAAGCGCTCGCATTGTGG-BHQ1	
<i>Campylobacter</i> spp.	CampF2	CACGTGCTACAATGGCATAT	Lund et al., 2004
	CampR2	GGCTTCATGCTCTCGAGTT	
	CampP2	FAM-CAGAGAACAATCCGAAGTGGGACA- BHQ1	

The universal *Bacteroidales* marker (AllBac) was designed to detect all members of *Bacteroidales* and served to detect if any *Bacteroidales* were present in the samples. All assays were conducted on the iQ Real Time System (Bio-Rad Laboratories, Hercules, CA) in a reaction volume totaling 25 μ L. Each reaction contained 4 μ L of template DNA, 1X iQ Supermix (Bio-Rad; 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.2 mM of each dNTP, 25 U/mL iTaq DNA polymerase, 3 mM MgCl₂), and 0.4 mg/mL bovine serum albumin (BSA) (Promega Corporation, Madison, WI). Each reaction mixture also

contained primers and probes of varying concentrations (see Table 3.4 for details). Table 3.4 also states the thermocycling programs used for each assay.

Table 3.4: Oligonucleotide concentrations and thermocycling programs used for microbial water quality assays

Assay	Component	Final Concentration	Program
AllBac	F-Primer (6 μ M)	0.6 μ M	95°C for 10 min; 40 cycles of 95°C for 30 sec, 60°C for 45 sec
	R-Primer (6 μ M)	0.6 μ M	
	Probe (2 μ M)	0.2 μ M	
BacR	F-Primer (1 μ M)	0.1 μ M	95°C for 6 min; 50 cycles of 95°C for 15 sec, 60°C for 15 sec, 72°C for 45 sec
	R-Primer (5 μ M)	0.5 μ M	
	Probe (1 μ M)	0.1 μ M	
BacH	F-Primer (5 μ M)	0.2 μ M	95°C for 6 min; 40 cycles of 95°C for 15 sec, 61°C for 15 sec, 72°C for 45 sec
	R-Primer (5 μ M)	0.2 μ M	
	Probe C (1 μ M)	0.1 μ M	
	Probe T (1 μ M)	0.1 μ M	
CowM2	F-Primer (25 μ M)	1 μ M	95°C for 6 min; 50 cycles of 95°C for 15 sec, 60°C for 1 min
	R-Primer (25 μ M)	1 μ M	
	Probe (2 μ M)	0.08 μ M	
	Probe IAC (2 μ M)	0.08 μ M	
<i>E. coli</i> O157:H7	F-Primer (3 μ M)	0.3 μ M	95°C for 6 min; 40 cycles of 95°C for 20 sec, 55°C for 30 sec, 72°C for 40 sec
	R-Primer (3 μ M)	0.3 μ M	
	Probe (1 μ M)	0.1 μ M	
<i>Salmonella</i> spp.	F-Primer (6 μ M)	0.6 μ M	95°C for 6 min; 40 cycles of 95°C for 15 sec, 60°C for 30 sec
	R-Primer (6 μ M)	0.6 μ M	
	Probe (2 μ M)	0.2 μ M	
<i>Campylobacter</i> spp.	F-Primer (6 μ M)	0.6 μ M	95°C for 6 min; 40 cycles of 95°C for 15 sec, 60°C for 1 min
	R-Primer (6 μ M)	0.6 μ M	
	Probe (2 μ M)	0.2 μ M	

Included in every assay were two or more qPCR negative controls that were subjected to the same assay protocols but in place of template DNA, sterile H₂O was added. Each assay also included five standard samples of known target DNA concentration (10² to 10⁵ ng/ μ L) to enable creation of a standard curve and subsequent quantification of the copy numbers in the sample DNA. The standards consisted of

extracted and standardized suspensions of plasmid DNA harbouring the target sequences (Fremaux et al., 2010), which were generously provided by the Yost Lab.

3.6 DATA ANALYSIS

3.6.1 SENSITIVITY AND SPECIFICITY OF *BACTEROIDALES* ASSAYS

The sensitivity and specificity of the *Bacteroidales* assays were determined to validate use of the markers in Nova Scotia. The sensitivity (Equation 3.2) of a marker is a measurement of the ability of the assay to detect target feces. The specificity (Equation 3.3) of a marker is a measurement of the ability of the assay to exclude non-target feces. A true positive result (a) is defined as positive detection by the marker when tested against target feces. A false positive result (b) occurs when positive detection of the marker occurs when tested against non-target feces. False negative results (c) are defined as negative detection of the marker when tested against target feces. Finally, a true negative result occurs with negative detection of the marker when tested against non-target feces (Fremaux et al., 2009; Gourmelon et al., 2007).

$$\text{Equation 3.2: } \textit{Sensitivity} = \frac{a}{(a+c)}$$

$$\text{Equation 3.3: } \textit{Specificity} = \frac{d}{(b+d)}$$

3.6.2 STATISTICAL ANALYSIS OF DATA

The relationship between *E. coli* and the universal *Bacteroidales* markers were not normally distributed, therefore the relationship between these markers was determined using the Spearman's rank coefficient (Fremaux et al., 2010). The Spearman's coefficient

(rho/ ρ) was determined for the TBW by pooling all data. The ρ coefficient was also determined for each station.

The conditional probability of *Bacteroidales* marker detection was determined using the Bayes' Theorem (Kildare et al., 2007). Equation 3.4 was used to calculate the conditional probability $P\left(\frac{H}{T}\right)$ of the relevant *Bacteroidales* markers where $P\left(\frac{T}{H}\right)$ represents sensitivity of the marker, $P(H)$ represents background detection of the marker in environmental samples, $P\left(\frac{T}{H'}$) represents the percentage of false positives in the specificity study, and $P(H')$ represents the percentage of environmental samples that did not test positive for the marker.

$$\text{Equation 3.4: } P\left(\frac{H}{T}\right) = \frac{P\left(\frac{T}{H}\right)P(H)}{P\left(\frac{T}{H}\right)P(H) + P\left(\frac{T}{H'}\right)P(H')}$$

Due to the enrichment step, quantification of *Campylobacter* spp. in environmental samples was not possible; therefore, the relationship between indicator concentrations and *Campylobacter* spp. presence was investigated using logistic regression. Samples positive for *Campylobacter* spp. were given a value of '1' and samples negative for the pathogen were designated as '0'. Both the *E. coli* and universal *Bacteroidales* s were regressed against the categorical pathogen value to determine if a significant correlation existed.

CHAPTER 4. RESULTS

Over the 15-month sampling period, 178 water samples were collected from six stations in TBW. A total of 26 discrete sampling events were conducted. Of those, 3 sampling events were conducted for the purpose of storm event monitoring and resulted in multiple samples from each station taken over a span of two or more days.

Results for all qPCR assays are reported in marker copies/ 100 mL for water samples and copies/g for solid samples such as fecal material. The detection limit of the qPCR assays were determined as 1.0×10^2 copies/ 100 mL by the Yost Lab, University of Regina in a procedure outlined in Fremaux et al. (2010). Positive results that fell below the assay limit of detection (ALOD) were considered non-detects. However, for statistical purposes, positive results that fell below the ALOD were recorded as half the value of the detection limit (50 copies/ 100 mL); negative results were recorded as 0. Results for all *E. coli* enumeration were reported as CFU/ 100 mL.

4.1 MARKER VALIDATION STUDY

4.1.1 SENSITIVITY AND SPECIFICITY OF *BACTEROIDALES* ASSAYS

Markers were validated with fecal waste materials from warm-blooded animals and humans from Nova Scotia. The sensitivity of a marker represents the frequency that the assay correctly identifies the host-specific marker when tested against target feces. The sensitivity of a marker is lowered each time the assay does not detect target feces. The specificity measures how precise the assay is at detecting target host feces. The cross-reaction of a marker with non-target feces lowers the specificity of an assay.

The results of the marker validation are summarized in Table 4.1. The AllBac assay, targeting the 16S rRNA genes of all *Bacteroidales*, was 97.3% sensitive and 100% specific. Two false negatives occurred, which lowered the sensitivity of the assay.

Table 4.1: Sensitivity and specificity of the assays used in this study

Marker/ Assay	Target	Sensitivity (Frequency)	Specificity
AllBac	Universal <i>Bacteroidales</i>	97.3% (61/63)	100%
BacR	Ruminant-specific <i>Bacteroidales</i>	94.4% (17/18)	93.9%
CowM2	Bovine-specific <i>Bacteroidales</i>	88.9% (16/18)	100%
BacH	Human-specific <i>Bacteroidales</i>	64.3% (7/11)	91.9%

The BacR assay targets the 16S rRNA genes of *Bacteroidales* found in the gut of ruminants. One false negative occurred resulting in a 94.4% assay sensitivity. The sample that was a false negative for BacR marker was also a false negative for the AllBac marker but positive for the CowM2 marker. Two non-ruminant fecal samples tested positive for the BacR marker, resulting in a specificity of 93.9%. The fecal samples that cross-reacted with the BacR marker were from a septic tank (1.96×10^4 copies/ g) and a chicken fecal sample (8.37×10^3 copies/g).

The CowM2 assay, which targets the HDIG gene in *Bacteroidales* specifically residing in the gut of bovines, had 88.9% sensitivity. Two false negatives occurred to cause a lower sensitivity, however, the false negatives were from different fecal samples than the AllBac and BacR false negatives. The specificity of the CowM2 marker was 100%. The marker did not cross-react with any feces from non-targets hosts.

Finally, the BacH marker is used to detect the 16S rDNA of *Bacteroidales* living in the gut of humans. The sensitivity of this assay was lower than the other assays. Four false negative results occurred, resulting in an assay sensitivity of 64.3%. The specificity of the assay was also slightly lower than the others at 91.9%. Coyote (9.19×10^4 copies/g), domestic dog (1.67×10^4 copies/g), and fisher (3.40×10^4 copies/g) cross-reacted with the BacH marker, resulting in false positives.

4.1.2 MARKER DETECTION RATIOS

The concentration of each marker was compared to determine the differences in detection between each marker (Table 4.2). To compare the concentration of AllBac to a host-specific marker, only the AllBac concentrations from fecal samples where the host-specific marker was correctly detected were used.

Table 4.2: Comparison of marker concentrations from assay validation results

	AllBac ^a	BacR ^a	CowM2 ^a	BacH ^b
Total Average^c	2.24×10^9	1.94×10^8	1.44×10^6	1.89×10^6
Host-Specific AllBac Average^d	N/A	2.65×10^9	2.81×10^9	2.12×10^9

^a Marker concentration in copies/ g feces

^b Marker concentration in copies/ 100 mL

^c Average marker concentration of true-positive samples

^d Average concentration of the AllBac marker for fecal samples that were true positive for the respective host-specific marker

The average concentration of the AllBac marker in all fecal samples was 2.24×10^9 copies/g. The average concentration of the BacR marker was 1.94×10^8 copies/g. The concentration of the AllBac marker for the samples where BacR was detected was 10-fold higher at 2.81×10^9 copies/g. The concentration of the CowM2 and BacH markers were 1.44×10^6 and 1.89×10^6 copies/g, respectively. Both assays were 1000-fold lower than the respective AllBac concentrations in the same fecal samples.

4.2 PRECIPITATION IN TBW DURING THE STUDY PERIOD

Precipitation in the TBW was recorded daily (Fig. 4.1). During the first growing season (May 1 to September 31, 2010) precipitation was recorded on 42.8% of the days. On 11 days at least 10 mm of precipitation was recorded and of those days 10 possessed rainfall totals exceeding 20 mm. The non-growing season lasted for 210 day between October 1 2010 and April 30, 2011. Precipitation in the watershed was recorded on 55.7% of the days in this season, of which 21 days exceeded 10 mm and 10 of those days exceeded 20 mm of precipitation. The second growing season lasted for 116 days and precipitation was recorded on 43.9% of days during this period. There were 12 days that exceeded 10 mm of precipitation, however, only 5 of those days exceeded 20 mm. The influence of precipitation on flow at each of the stations, and subsequently microbial parameters, in the watershed will be discussed in the next section.

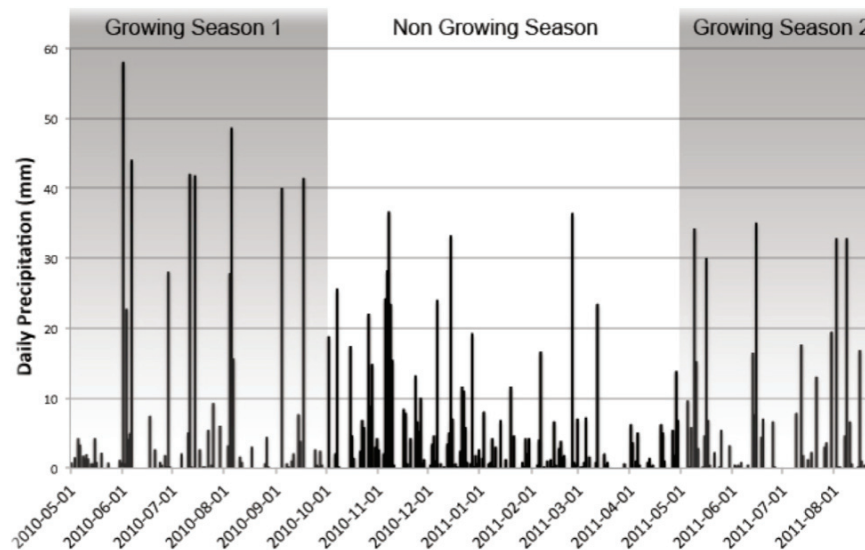


Fig. 4.1: TBW daily precipitation (mm) over the 15-month sampling period. The sampling period is broken up into 3 stages: Growing Season 1 (May 1 to September 30, 2010), Non-growing Season (October 1, 2010 to April 30, 2011), and Growing Season 2 (May 1 to August 24, 2011)

4.3 UNIVERSAL *BACTEROIDALES* MARKER (ALLBAC) CONCENTRATIONS IN THE TBW

As expected, the detection rate of the universal *Bacteroidales* marker (AllBac) was extremely high. The AllBac marker was detected in 97.2% (173/178) of the water samples tested and concentrations ranged from 1.0×10^2 to 2.0×10^8 copies/ 100 mL with an average concentration of 1.04×10^5 copies/ 100 mL. All samples were positive, however the marker concentrations of 5 non-detects were below the ALOD.

4.3.1 RELATIONSHIP BETWEEN ALLBAC AND *E. COLI* CONCENTRATIONS IN THE TBW

The relationship between the AllBac marker and *E. coli* concentrations was examined to determine if there is a significant relationship between the indicator organisms. Fig. 4.2 illustrates the general association between the indicator concentrations at each station. In every case, an increase in AllBac concentrations coincides with an increase in *E. coli* concentrations.

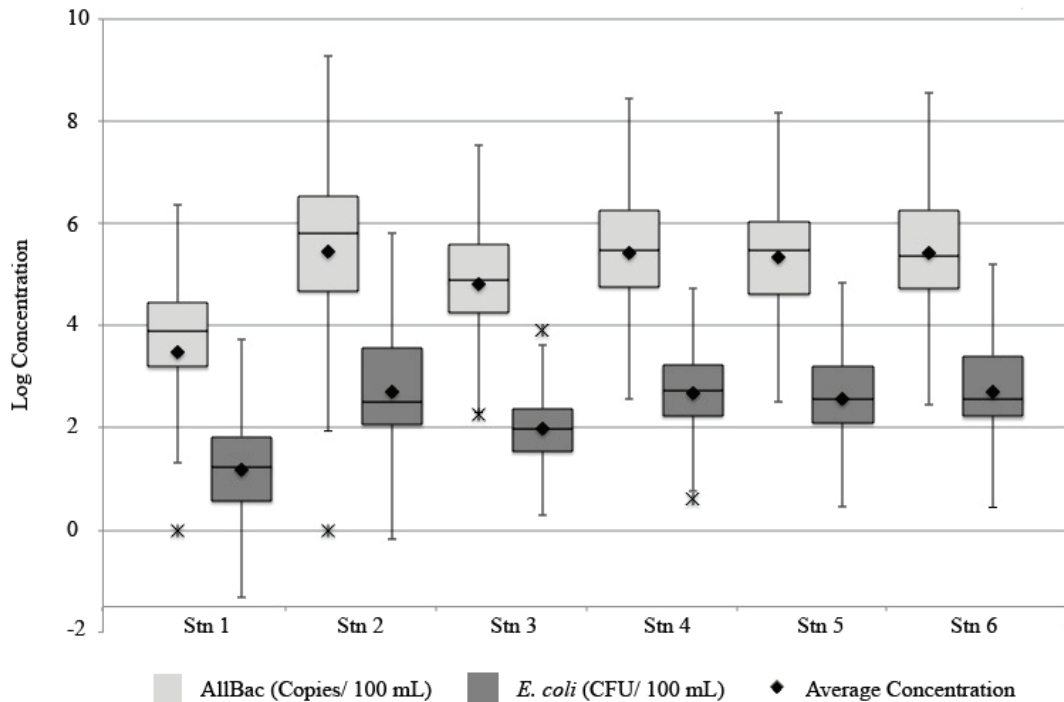


Fig. 4.2: Box plots representing AllBac markers and *E. coli* concentrations at each sampling station during the 15-month sampling period. The lower and upper bounds of the box represent the 25th and 75th percentiles, respectively. The line within the box demonstrates the median of the concentrations and the averages concentrations are indicated as diamonds. The upper and lower whiskers represent the higher and lower limits, with outliers represented as stars. The concentration of AllBac marker (light grey) and *E. coli* (dark grey) show a positive association.

As previously reported by Sinclair *et al.* (2009), the *E. coli* concentrations in the TBW routinely exceed the national guidelines set by the CCME for irrigation (100 CFU/ 100 mL) and recreational (200 CFU/ 100 mL) water use. The average concentration of *E. coli* over the sampling period was 2.10×10^3 CFU/ 100 mL. Individual station averages at all sampling stations, except Stn 1, exceeded both the 100 CFU/ 100 mL 200 CFU/ 100 mL guidelines.

As the AllBac marker and *E. coli* concentrations were not normally distributed, their association was statistically analyzed using the Spearman's rank correlation. The Spearman's rank coefficient (ρ) ranges between -1 and 1. A negative or positive ρ -value is indicative of the nature of the relationship. Values of -1 or 1 indicate perfect

negative or positive correlation, respectively; a value of 0 indicates no correlation. To determine the ρ -value for the entire watershed, results from the six stations were pooled before analyzing the data. The result ($\rho = 0.629$) showed that there was a statistically significant relationship between AllBac and *E. coli* concentrations ($\alpha = 0.01$, $p < 0.01$).

4.3.2 STN 1: ALLBAC CONCENTRATIONS IN RELATION TO *E. COLI*, FLOW, AND PRECIPITATION

Stn 1 was considered a negative control for this study because it is located at the headwater of TBW and it has little anthropologic influence. The only major inputs to this section of the stream was a spring, located just upstream of Stn 1, and precipitation. The average flow rate at Stn 1 ($4.45 \times 10^{-3} \text{ m}^3/\text{s}$) was the lowest of all the stations. The flow rate at Stn 1 had little variation and did not exceed $5.0 \times 10^{-3} \text{ m}^3/\text{s}$ (Fig. 4.3, panel A). In most cases, increased precipitation was only reflected mildly in the flow rates.

The results supported the use of Stn 1 as a negative control site. The mean of the *E. coli* (81 CFU/ 100 mL) concentrations and the AllBac marker (4.17×10^4 copies/ 100 mL) were low compared to the other stations. Stn 1 also had 80% (4/5) of the AllBac non-detects. Only 6.67% of the *E. coli* samples tested exceeded CCME standards for recreational water use and only 13.3% exceeded the guidelines for irrigation water. *E. coli* concentrations ranged from non-detect to 1.15×10^4 CFU/ 100 mL and appeared to follow a seasonal trend with lower concentrations during the colder months. AllBac marker concentrations ranged from 1.0×10^2 to 1.35×10^4 copies/ 100 mL but did not seem to be influenced by the season (Fig. 4.3, panel B).

When comparing precipitation to *E. coli* concentrations, a trend between increased precipitation and *E. coli* can be seen. There does not appear to be a trend between *E. coli*

concentration and AllBac markers. In fact, several instances occur when one indicator is detected and the other is not.

To test the association between *E. coli* and AllBac specifically at Stn 1, the Spearman's rank correlation was applied to the Stn 1 data subset. The results ($\rho= 0.167$) indicate that there is not a significant relationship ($p > 0.01$) between the indicator concentrations at this station. This anomaly is likely due to the low detection rates of both the *E. coli* and *Bacteroidales* markers.

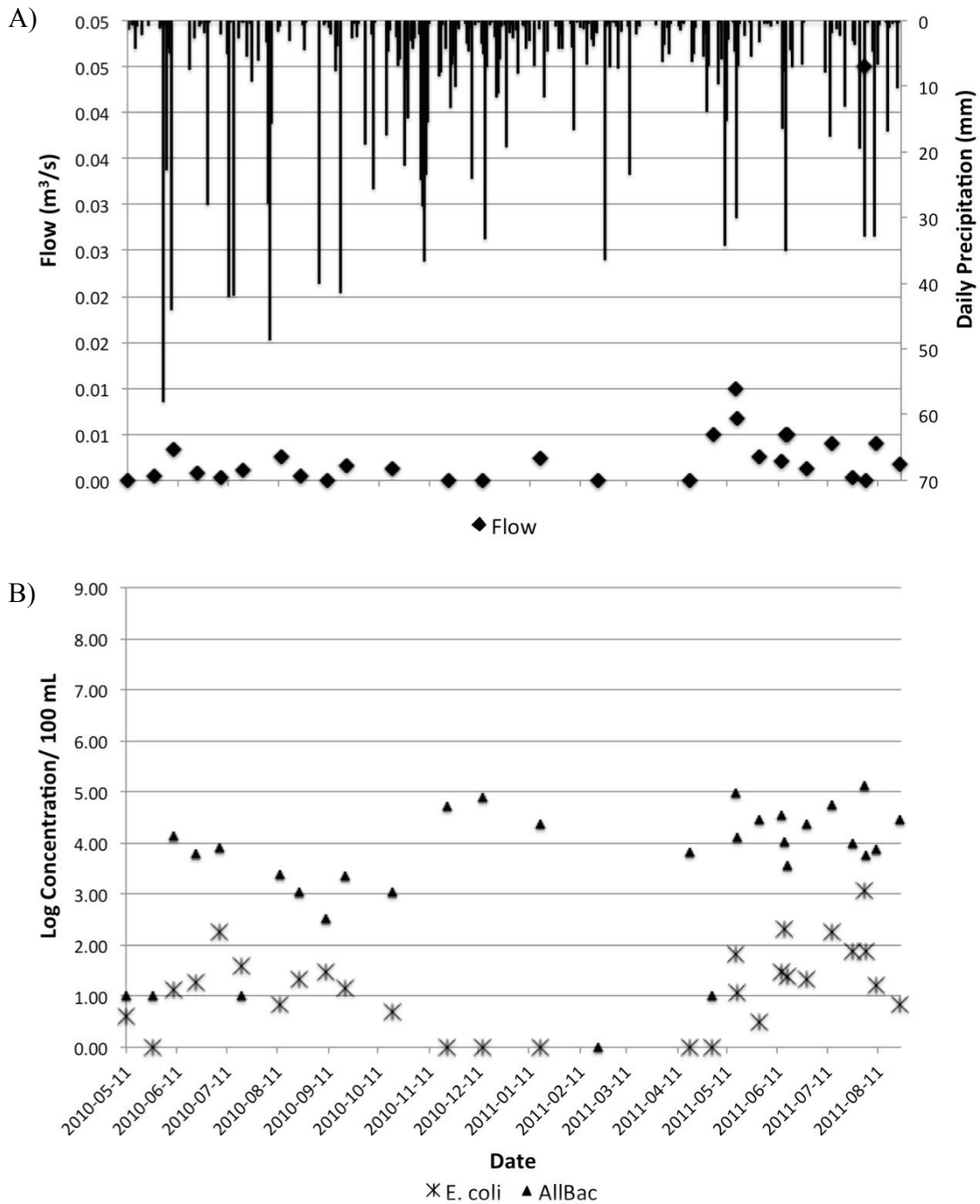


Fig. 4.3: Station 1 time series plots of flow and precipitation, and *E. coli* and universal *Bacteroidales* (AllBac) concentrations
 The flow rate (m³/s) at Stn 1 is not significantly affected by the precipitation in the TBW (A). The *E. coli* (Log CFU/ 100 mL) and AllBac concentrations (copies/ 100 mL) do not show a strong correlation (B) and do not seem to reflect increased precipitation events.

4.3.3 STN 2: ALLBAC CONCENTRATIONS IN RELATION TO *E. COLI*, FLOW, AND PRECIPITATION

There are a variety of anthropogenic activities that could influence the water system near Stn 2; most notably, a dairy farm was situated upstream of the sampling station. Stn 2 is located on one of the two main branches in TBW. The average flow rate was $8.06 \times 10^{-2} \text{ m}^3/\text{s}$ and ranged from an undetectable flow rate to $6.88 \times 10^{-1} \text{ m}^3/\text{s}$ (Fig. 4.4, panel A). The flow rate appeared to be more responsive to precipitation events during the second growing season.

The concentration of indicators reflected the location of the sampling station. *E. coli* concentrations exceeded CCME standards for recreational water use and irrigation water use in 64.5% and 80.6% of the samples tested, respectively (Fig. 4.4, panel B). The concentration of *E. coli* at Stn 2 ranged from 9.0 to 3.67×10^4 CFU/ 100 mL and the mean concentration was 4.20×10^3 CFU/ 100 mL, which was the highest out of all 6 stations in the watershed. During the non-growing season, *E. coli* concentrations were generally lower than during the first and second growing seasons, however, there did not appear to be any seasonal trends with the AllBac marker. An increase in flow rate appeared to cause an increase in both the *E. coli* and AllBac marker concentrations.

AllBac marker concentrations in the samples gathered from Stn 2 ranged from 1.0×10^2 to 2.04×10^7 copies/ 100 mL (Fig. 4.4, panel B) and the mean concentration was the highest in the watershed at 3.07×10^5 copies/ 100 mL. Only 1 sample fell below the ALOD. Increases in AllBac concentrations appeared to coincide with increases in *E. coli* concentrations. Upon further investigation, the association between the two indicators at Stn 2 was statistically significant ($p < 0.01$) and positively correlated ($\rho = 0.606$).

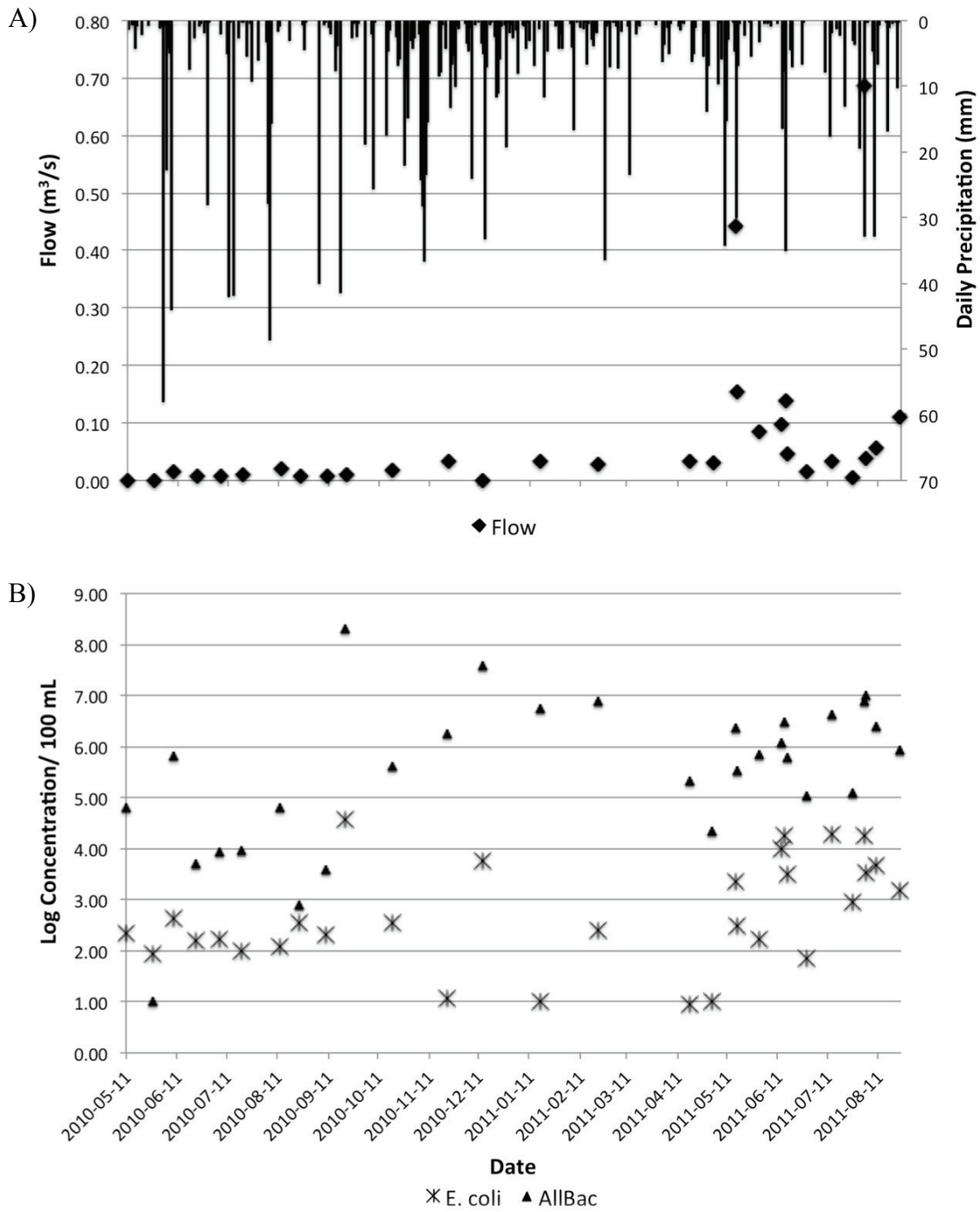


Fig. 4.4: Station 2 time series plots of flow and precipitation, and *E. coli* and universal *Bacteroidales* (AllBac) concentrations
 The flow rate (m^3/s) at Stn 2 is affected most by precipitation during the second growing season (A). The *E. coli* (Log CFU/ 100 mL) and AllBac concentrations (copies/ 100 mL) show a positive correlation (B) and appear responsive to precipitation events

4.3.4 STN 3: ALLBAC CONCENTRATIONS IN RELATION TO *E. COLI*, FLOW, AND PRECIPITATION

Stn 3 is located on the second of the two main branches in TBW. The sampling station is located in a residential cluster with sections of forested area. The average flow rate at Stn 3 was the second lowest in the watershed ($6.35 \times 10^{-2} \text{ m}^3/\text{s}$) and ranged from an undetectable flow rate to $4.07 \times 10^{-1} \text{ m}^3/\text{s}$ (Fig. 4.5, panel A).

The average *E. coli* concentration at Stn 3 was $5.45 \times 10^2 \text{ CFU}/100 \text{ mL}$, which exceeds both the irrigation and recreational CCME guidelines for water quality. However, half of the samples fell below the guidelines for irrigational water use and 73.3% were below the guidelines for recreational water use. The concentration range during the sampling period was 2.0 to $8.10 \times 10^4 \text{ CFU}/100 \text{ mL}$ with the lowest concentrations recorded during the non-growing season. Higher flow rates coincide with increases in both the *E. coli* and AllBac marker concentrations (Fig. 4.5, panel B).

The *E. coli* and AllBac marker concentrations were the second lowest of all the stations in the TBW. The average concentration of the AllBac marker was $6.32 \times 10^4 \text{ copies}/100 \text{ mL}$ and the concentration range was 1.81×10^2 to $1.36 \times 10^6 \text{ copies}/100 \text{ mL}$, with no samples falling below the ALOD. The AllBac marker did not appear to follow any seasonal trend. Using the spearman rank coefficient, it was determined that the relationship between *E. coli* and AllBac marker concentrations at Stn 3 was significant ($\rho = 0.496$, $p < 0.01$).

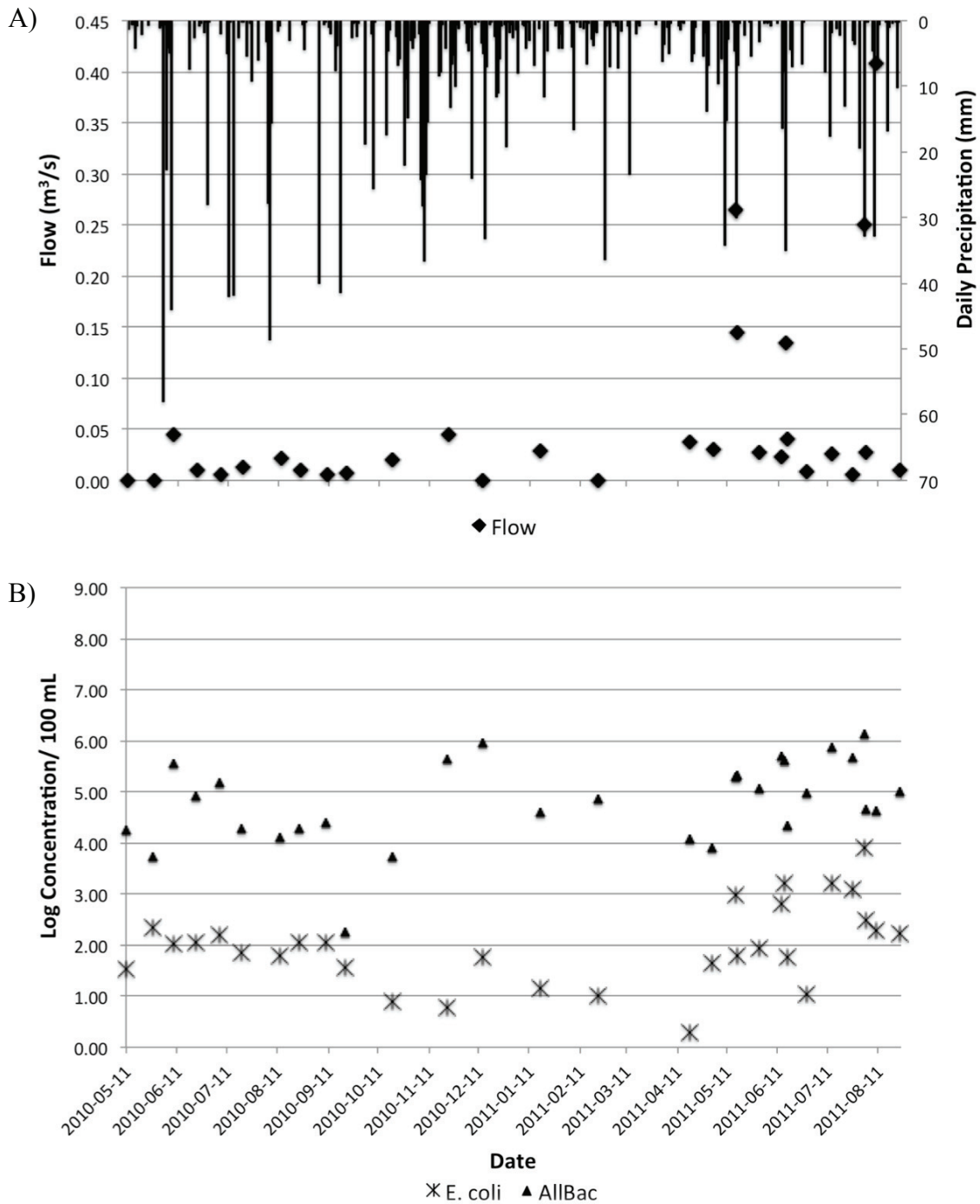


Fig. 4.5: Station 3 time series plots of flow and precipitation, and *E. coli* and universal *Bacteroidales* (AllBac) concentrations
 The flow rate (m^3/s) at Stn 3 is not significantly affected by the precipitation in the TBW (A). The *E. coli* (Log CFU/ 100 mL) and AllBac concentrations (copies/ 100 mL) showed a positive correlation (B) and appeared responsive to increased flow rates.

4.3.5 STN 4: ALLBAC CONCENTRATIONS IN RELATION TO *E. COLI*, FLOW, AND PRECIPITATION

Stn 4 is located approximately two thirds down the watershed and downstream of where the two upper branches of the watershed join. Two nearby beef farms were predicted to have the most influence on the water quality at this site. The average flow rate was the highest at Stn 4 ($2.24 \times 10^{-1} \text{ m}^3/\text{s}$) and ranged from 2.06×10^{-2} to $2.57 \text{ m}^3/\text{s}$. The measured flow rates were higher in the second growing season and corresponded to storm events (Fig. 4.6, panel A).

The average *E. coli* concentration at Stn 4 was above the CCME guidelines at $2.19 \times 10^3 \text{ CFU}/100 \text{ mL}$. *E. coli* concentrations ranged between 4 and $1.34 \times 10^4 \text{ CFU}/100 \text{ mL}$, with 20.0% and 30.0% of the samples registering below the irrigational and recreational water use guidelines, respectively. During the winter the *E. coli* concentrations decreased. Increased concentrations of both the *E. coli* and AllBac markers were consistent with increased flow and precipitation values.

The AllBac marker concentration ranged from 3.41×10^3 to $7.07 \times 10^6 \text{ copies}/100 \text{ mL}$ and the average concentration was $2.60 \times 10^5 \text{ copies}/100 \text{ mL}$. The season did not have an apparent effect on the concentration of the AllBac marker. Again, the *E. coli* and AllBac marker concentrations showed a positive and significant correlation ($\rho = 0.559$, $p < 0.01$) (Fig. 4.6, panel B).

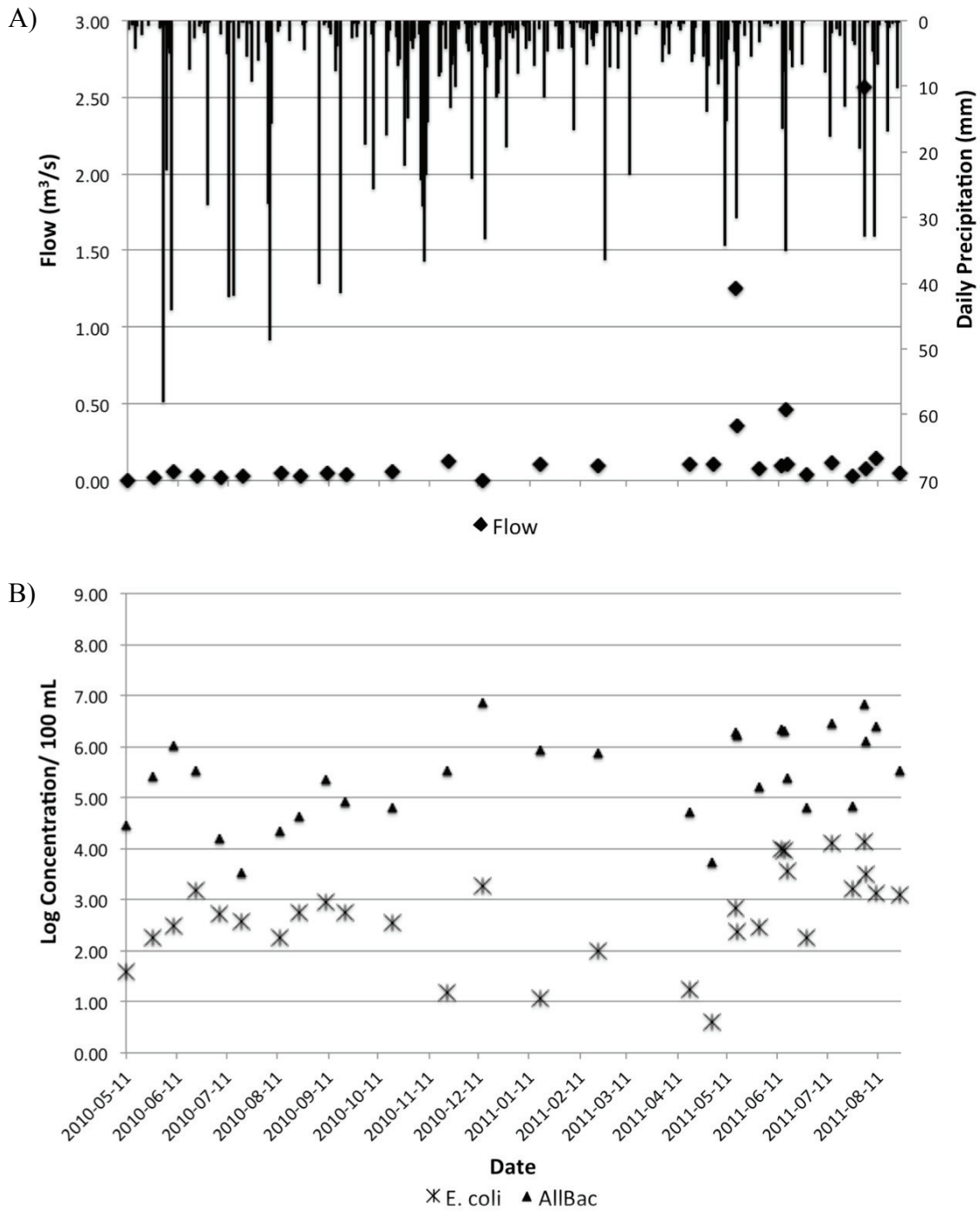


Fig. 4.6: Station 4 time series plots of flow and precipitation, and *E. coli* and universal *Bacteroidales* (AllBac) concentrations
 The flow rate (m^3/s) at Stn 4 is not significantly affected by the precipitation in the TBW (A). The *E. coli* (Log CFU/ 100 mL) and AllBac concentrations (copies/ 100 mL) showed a strong correlation (B) and reflected increased precipitation events.

4.3.6 STN 5: ALLBAC CONCENTRATIONS IN RELATION TO *E. COLI*, FLOW, AND PRECIPITATION

Because Stn 5 was located at the outlet of the watershed, the potential influences at this site were a culmination of the entire watershed. The average flow at Stn 5 was $1.17 \times 10^{-1} \text{ m}^3/\text{s}$. The lowest recorded flow velocity was $2.62 \times 10^{-2} \text{ m}^3/\text{s}$ and the highest was recorded as $3.64 \times 10^{-1} \text{ m}^3/\text{s}$ (Fig. 4.7, panel A). The influence of precipitation on flow velocities is demonstrated on Fig. 4.7 (A); increased precipitation is often followed by increased flow.

E. coli concentrations at Stn 5 rarely fell below CCME guidelines for irrigational water use (16.7%) and two thirds of the samples exceeded the guidelines for recreational water use. The average *E. coli* concentration at the watershed outlet was $1.90 \times 10^3 \text{ CFU}/100 \text{ mL}$ and ranged between 5 to $1.44 \times 10^4 \text{ CFU}/100 \text{ mL}$. The *E. coli* and AllBac marker concentrations at this station generally followed changes in precipitation and flow rate.

The AllBac marker was detected at concentrations ranging from 5.27×10^3 to $3.94 \times 10^6 \text{ copies}/100 \text{ mL}$ and the average marker concentration at Stn 5 was $2.17 \times 10^5 \text{ copies}/100 \text{ mL}$. Increased *E. coli* concentrations appeared to coincide with increased AllBac marker concentrations (Fig. 4.7, panel B). The Spearman's rank correlation confirmed that the relationship was positive and significant ($\rho = 0.459$, $p < 0.01$).

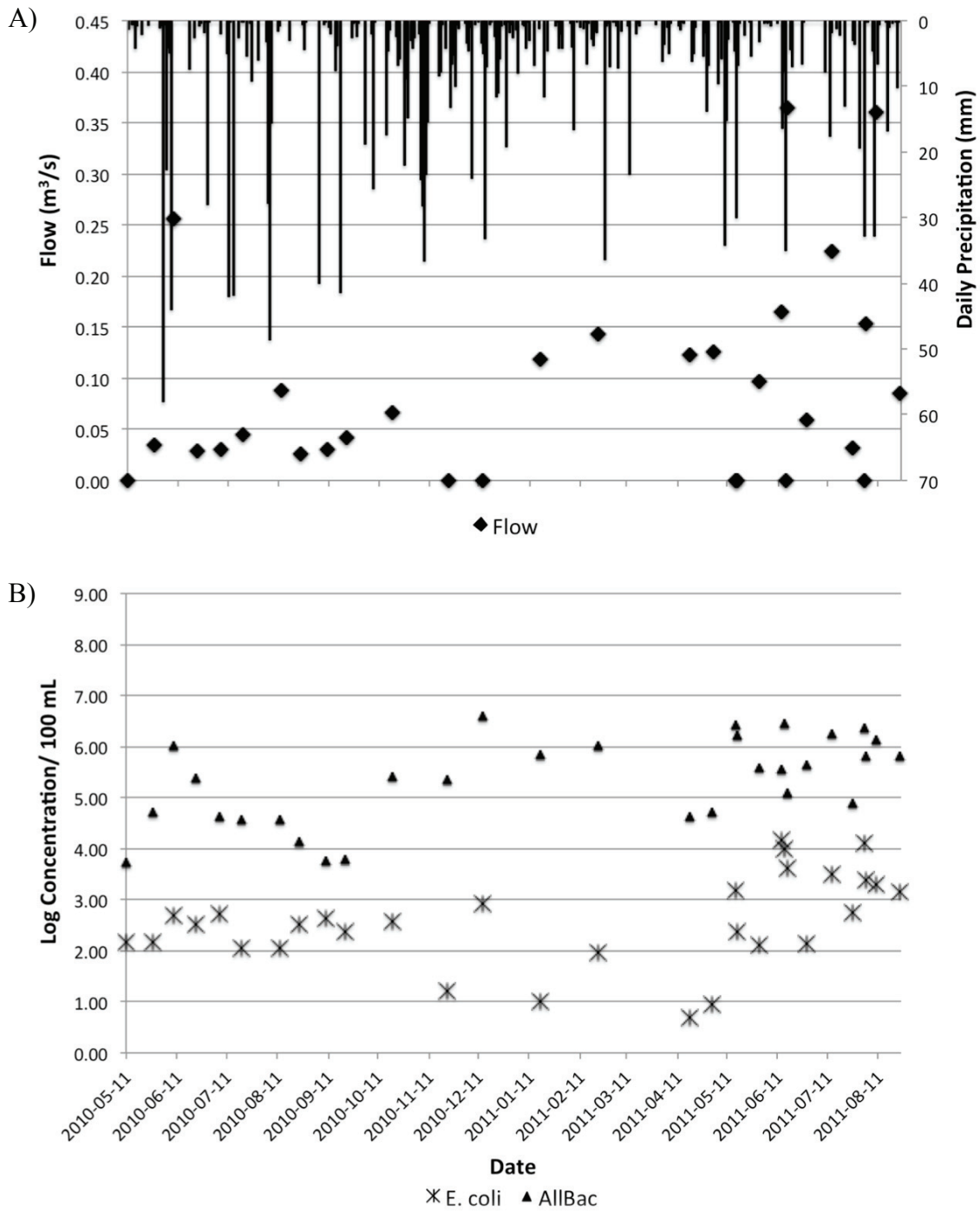


Fig. 4.7: Station 5 time series plots of flow and precipitation, and *E. coli* and universal *Bacteroidales* (AllBac) concentrations
 The flow rate (m^3/s) at Stn 5 is affected by the precipitation in the TBW (A). The *E. coli* (Log CFU/ 100 mL) and AllBac concentrations (copies/ 100 mL) showed a positive correlation (B) and reflected increased precipitation events.

4.3.7 STN 6: ALLBAC CONCENTRATIONS IN RELATION TO *E. COLI*, FLOW, AND PRECIPITATION

Stn 6 was also found in the lower third of the watershed and bordered a rotational crop that received liquid dairy manure as fertilizer; therefore the main influence at this site was thought to be agricultural. The average flow at Stn 6 was the second highest in the watershed ($1.35 \times 10^{-1} \text{ m}^3/\text{s}$) and ranged from 6.96×10^{-3} to $1.05 \text{ m}^3/\text{s}$ (Fig. 4.8, panel A). Increased precipitation appeared to have a positive influence on the flow rates measured at the site.

The average *E. coli* concentration was 3.69×10^3 CFU/ 100 mL, which exceeds the CCME guidelines for both the irrigation and recreational water uses. *E. coli* concentrations ranged from 9 to 2.71×10^4 CFU/ 100 mL. Only 17.2% of the samples were below the irrigational water use guideline and 31.0% were under the recreational use guideline. *E. coli* concentrations were lower during the winter, however there does not appear to be any seasonality to the AllBac marker concentrations (Fig. 4.8, panel B). Both *E. coli* and AllBac marker concentrations show trends with the flow rates measured at the site.

The average AllBac marker concentration was 2.61×10^5 copies/ 100 mL, which was higher than the watershed average. The AllBac marker concentrations ranged from 8.55×10^3 to 1.16×10^7 copies/ 100 mL. The Spearman's rank coefficient ($\rho = 0.664$) confirmed that there was a positive relationship ($p < 0.01$) between *E. coli* and AllBac marker concentrations at Stn 6.

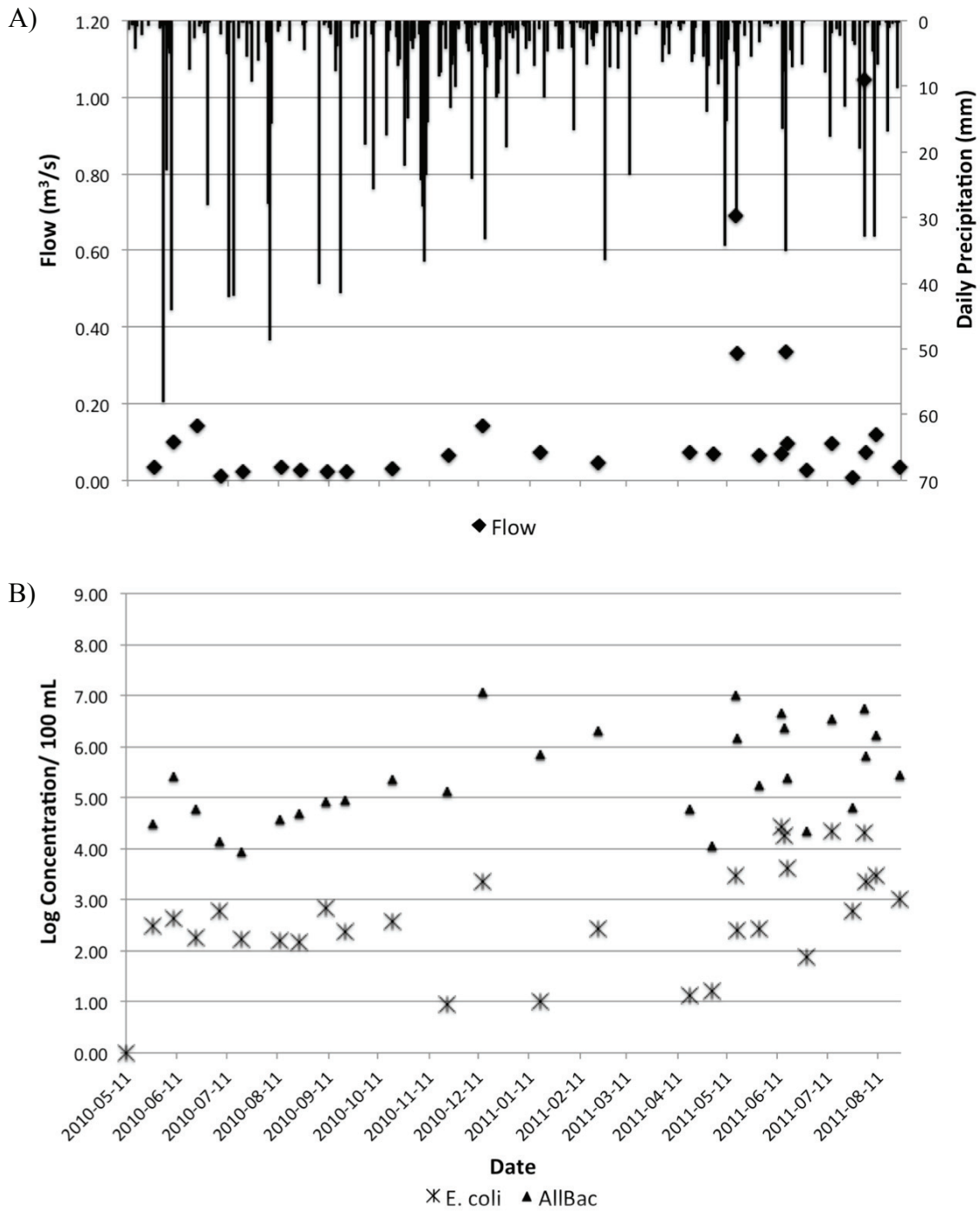


Fig. 4.8: Station 6 time series plots of flow and precipitation, and *E. coli* and universal *Bacteroidales* (AllBac) concentrations
 The flow rate (m^3/s) at Stn 6 was somewhat affected by the precipitation in the TBW (A).
 The *E. coli* (Log CFU/ 100 mL) and AllBac concentrations (copies/ 100 mL) showed a strong positive correlation (B) and reflected increased precipitation events.

4.4 RUMINANT- AND BOVINE-SPECIFIC *BACTEROIDALES* MARKERS (BACR)

4.4.1 CONDITIONAL PROBABILITY OF BACR AND COWM2 DETECTION IN THE ENVIRONMENT

The ruminant-specific BacR assay was used to detect microbial pollution resulting from contamination of the water column with feces from ruminants and the bovine-specific CowM2 marker was used to further determine if the contamination could be traced back to cattle. To determine the probability that detection of the BacR or CowM2 markers in water samples was actually caused by the presence of ruminant or bovine feces, respectively, the Bayes' Theorem was applied to the results obtained in the validation and water quality monitoring studies. The sensitivity and specificity of the assays, which were determined in the assay validation study, had a major influence on the probability that detection of the marker signifies the actual presence of target feces.

Equation 3.4 (Section 3.6.2) was used to calculate the conditional probability of the BacR and CowM2 markers. Table 4.3 outlines the variables used to calculate the conditional probability of each marker.

Table 4.3: Variables and results of the BacR and CowM2 Bayes' Theorem calculation

Marker	$P\left(\frac{T}{H}\right)$	$P(H)$	$P\left(\frac{T}{H'}\right)$	$P(H')$	$P\left(\frac{H}{T}\right)$
BacR	94.4%	14.1%	6.10%	85.9%	71.8%
CowM2	88.9%	3.37%	0%	96.3%	100%

The two cross-reactions of the BacR marker with a septic tank sample and chicken feces lowered the conditional probability to a 71.8% chance that when the BacR marker is detected, the water has been contaminated with ruminant feces. The fact that the CowM2 marker had 100% specificity resulted in a conditional probability of 100%, therefore, if

the CowM2 marker has been detected in the water, there is a 100% chance that bovine fecal pollution is present.

4.4.2 ROUTINE MONITORING OF BACR AND COWM2 IN ENVIRONMENTAL WATER SAMPLES

Of the 178 samples collected during the sampling period, 136 samples were collected during routine monitoring. The purpose of routine monitoring is to establish average values for all the relevant variables during a variety of environmental conditions. As expected, the detection frequency of the BacR and CowM2 markers was low. Only 5.88% (8/136) of the samples tested positive for the BacR marker during routine monitoring; these were observed over four sampling events (SE04, SE10, SE13, and SE22), which will be explained in the following sections. The CowM2 marker was detected in only two samples (1.47%), which were obtained during one sampling event (SE25).

SE04 occurred on June 22, 2010. This was during a dry period with only 10 mm of rain falling in the week prior to sample collection. The BacR marker was detected at Stn 4, downstream of the two beef farms. The concentration of the marker was 9.10×10^3 copies/ 100 mL. The flow rate at Stn 4 on that day was one of the lowest recorded at $2.89 \times 10^{-2} \text{ m}^3/\text{s}$, however, the *E. coli* count (1.54×10^3 CFU/ 100 mL) was in the upper third of the values recorded at that site. No other sites had BacR marker detections for this sampling event.

SE10 was conducted on September 21, 2010. In the three days prior to the sampling event, no precipitation occurred, however 41.4 mm of rain fell on the fourth day prior to the sample collection. In total, 52.8 mm of rain fell in the week before the samples were taken. Stn 2, downstream of the dairy farm, was the only site where the

BacR marker was detected during SE10. The concentration of the BacR marker was 1.12×10^4 copies/ 100 mL. The flow rate at that time was recorded as 9.77×10^{-3} m³/s, which was in the lower quarter of the recorded flow rates at that site. The *E. coli* count for SE10, however, was the highest recorded value at that site (3.67×10^4 CFU/ 100 mL).

Two sites, Stn 2 and Stn 6, tested positive for the BacR marker during SE13. SE13 was conducted during the non-growing season on December 13, 2010. That day 5 mm of precipitation was recorded. The week prior to sampling, a total of 4.2 mm of precipitation fell. On the 8th day before sampling, 24 mm of rain occurred. On the date of SE13, the average daily temperature was 11.8°C, which was approximately 13 to 16°C warmer than any other day that week. Any precipitation that fell before the sampling date likely fell as snow and remained as such until the temperature warmed. This may have caused increased runoff into the stream system, however, the flow rate measured only at Stn 6 during this sampling event due to logistical issues.

The concentration of the BacR marker at Stn 2 was 1.03×10^5 copies/ 100 mL. The *E. coli* concentration was 5.76×10^3 CFU/ 100 mL, which was in the top 20% of concentrations at that station. Stn 6, which was below a rotational crop that received springtime manure fertilization, was the second site at which BacR was detected during SE13. The BacR concentration at Stn 6 was 5.65×10^4 copies/ 100 mL. The flow rate was recorded as 1.44×10^{-1} m³/s, which was the highest recorded flow value during routine monitoring. The *E. coli* concentration (2.29×10^3 CFU/ 100 mL) was in the top third of values recorded at the Stn 6.

SE22 was the last routine-sampling event that resulted in BacR marker detection. The marker was detected at four out of the six sites (Stn 2, 4, 5, and 6). SE22 occurred on July 13, 2011. The total precipitation for the week prior to sampling was 25.8 mm, 17.6

mm of which fell the day before sampling. The BacR concentration at Stn 2 was recorded as 6.33×10^3 copies/ 100 mL. The flow rate was 3.43×10^{-2} m³/s, which was above the median at that station. The *E. coli* concentration (1.90×10^4 CFU/ 100 mL) was the second highest, behind the SE10 already discussed. At Stn 4 the BacR concentration was recorded as 4.43×10^4 copies/ 100 mL. The flow rate registered as 1.18×10^{-1} m³/s and was in the top quarter of recorded values at that station. The *E. coli* concentration (1.30×10^4 CFU/ 100 mL) was the second highest recorded at that site. SE22 was the only routine sampling event in which the BacR marker was detected at Stn 5, the watershed outlet. The concentration of the marker was 3.27×10^3 copies/ 100 mL. The flow rate (2.24×10^{-1} m³/s) and *E. coli* concentrations (3.13×10^3 CFU/ 100 mL) were in the top 20% of recorded values at Stn 5. Finally, the BacR marker was detected at 4.02×10^4 copies/ 100 mL at Stn 6. The flow at this site was 9.52×10^{-2} m³/s, which was in the middle third of the recorded values. Conversely, the *E. coli* concentration was 2.12×10^4 CFU/ 100 mL, which was the second highest recorded concentration at that site.

Interestingly, the CowM2 marker was not co-detected in any routine monitoring samples that contained the BacR marker. The CowM2 marker was detected twice during routine sampling, both during SE25, at Stn 2 and Stn 5. SE25 was conducted on August 8, 2011; on the day before the sampling event, 32.8 mm of precipitation fell. The CowM2 marker concentration at Stn 2 was 2.04×10^4 copies/ 100 mL. Both the flow (5.66×10^{-2} m³/s) and *E. coli* concentration (4.79×10^3 CFU/ 100 mL) at that site were in the top third of the recorded values. At Stn 5, the CowM2 marker concentration was 1.07×10^5 copies/ 100 mL. The flow rate for SE25 at that site was 3.60×10^{-1} m³/s, which was the second highest recorded value. The *E. coli* concentration (1.92×10^3 CFU/ 100 mL) was in the top quarter of the values for that site.

Not surprisingly, the BacR and CowM2 markers were not detected at Stn 1, the watershed headwater, or Stn 3, the residential site.

4.4.3 STORM EVENT DETECTION OF BACR AND COWM2 IN ENVIRONMENTAL WATER SAMPLES

Storm event sampling was conducted during the second growing season. Attempts were made to collect samples throughout the storm event when greater than 20 mm of precipitation were forecast. The increased precipitation causes surface water runoff that would carry microbial contamination into the stream system. Increased surface runoff can be reflected in increased flow rates. Because of their proximity to ruminant and bovine contamination sources, four of the six sampling sites were hypothesized to detect either, or both, of the ruminant or bovine marker during storm events. Samples were also collected as the storm subsided, to determine how quickly the markers disappeared from the stream environment. Three storm events were monitored in this study and corresponded to sampling events SE18, SE20, and SE24.

4.4.3.1 SE18: DETECTION OF HOST-SPECIFIC MARKERS DURING STORM EVENT MONITORING

SE18 occurred on May 15th and 16th, 2011. On May 15th (SE18-A), 30 mm of precipitation fell, followed by 6.8 mm the following day (SE18-B). As expected, no BacR or CowM2 markers were detected at the watershed headwater (Stn 1) or at the residential site (Stn 3). Similarly, neither marker was detected at Stn 2. All other stations had varying levels of detection for both BacR and CowM2.

The flow at Stn 1 decreased from $1.0 \times 10^{-2} \text{ m}^3/\text{s}$ during SE18-A to $6.67 \times 10^{-3} \text{ m}^3/\text{s}$ during SE18-B. Both the *E. coli* and AllBac concentrations reflected that trend. The *E. coli* concentration was recorded as 65 CFU/ 100 mL for SE18-A, and then the concentration dropped to 12 CFU/ 100 mL on the second day. In a similar fashion, the

AllBac concentration decreased from 9.79×10^4 copies/ 100 mL on SE18-A to 1.28×10^4 copies/ 100 mL on SE18-B.

Flow rates at Stn 3 were some of the highest during SE18. During SE18-A, the flow was $2.64 \times 10^{-1} \text{ m}^3/\text{s}$ and decreased to $1.45 \times 10^{-1} \text{ m}^3/\text{s}$ during SE18-B. The *E. coli* count for SE18-A was 9.75×10^2 CFU/ 100 mL, which exceeded the station average (5.45×10^2 CFU/ 100 mL). There was a substantial decrease in the concentration (63 CFU/ 100 mL) after the rainfall (SE18-B). In contrast, the AllBac concentrations were slightly higher for SE18-B than for SE18-A, at 2.16×10^5 and 1.95×10^5 copies/ 100 mL, respectively.

Three out of the four hypothesized sites had detectable ruminant or bovine markers (Fig. 4.9). It was somewhat surprising that the BacR or CowM2 marker was not detected at Stn 2, which is downstream of the dairy farm. The BacR marker was detected, however, at Stn 4, 5 and 6. The only site during SE18 at which the CowM2 marker was detected was Stn 6.

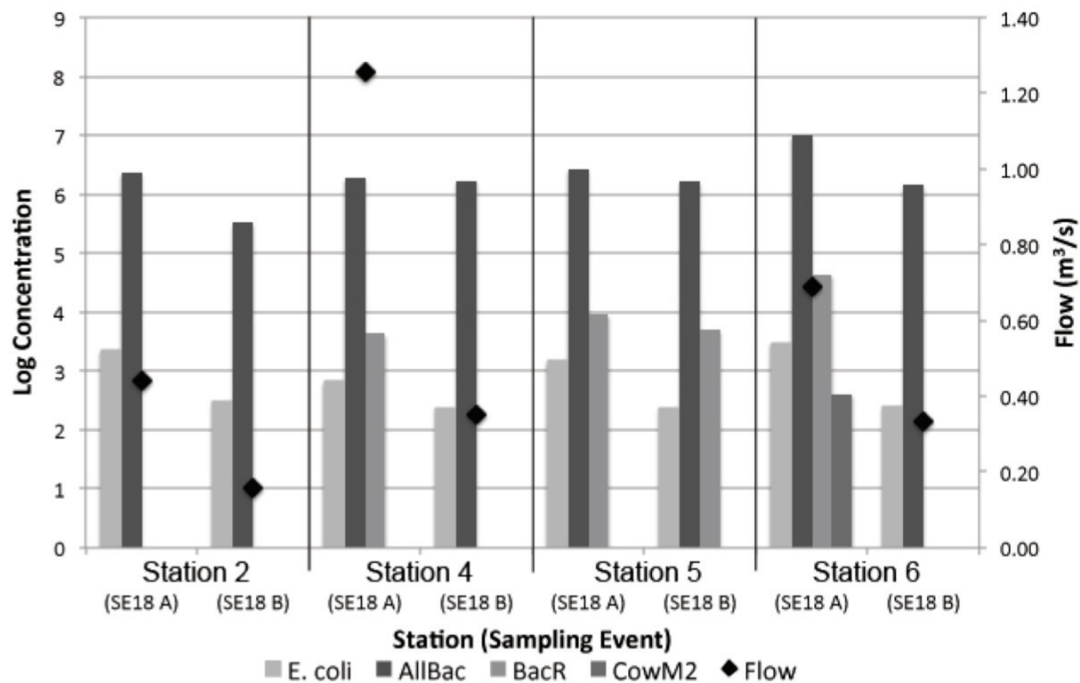


Fig. 4.9: *E. coli*, AllBac, BacR, and CowM2 concentrations and flow during SE18 Values for *E. coli* (Log CFU/ 100 mL), AllBac, BacR, and CowM2 (Log copies/ 100 mL) concentrations in relation to flow (m³/s) at Stn 2, Stn 4, Stn 5, and Stn 6 during a storm event (SE18) in May, 2011. Due to logistical reasons, the flow was not gauged at Stn 5.

Both the flow rate, and AllBac concentration at Stn 2 exceeded the station average during SE18-A at $4.43 \times 10^{-1} \text{ m}^3/\text{s}$ and 2.30×10^6 copies/ 100 mL, respectively. The *E. coli* concentration for SE18-A was 2.28×10^3 CFU/ 100 mL, which was above the station median. During SE18-B, the flow rate and AllBac concentrations decreased but still remained above the station averages at $1.55 \times 10^{-1} \text{ m}^3/\text{s}$ and 3.30×10^5 copies/ 100 mL, respectively. The *E. coli* concentration dropped to 3.12×10^2 CFU/ 100 mL, which was close to the station median.

The first detection of the BacR marker during SE18 was at Stn 4. The flow rate during SE18-A ($1.25 \text{ m}^3/\text{s}$) was the second highest recorded flow at that station. The flow remained higher than baseline flows during SE18-B at $3.53 \times 10^{-1} \text{ m}^3/\text{s}$. The *E. coli*

concentration (6.73×10^2 CFU/ 100 mL) was only slightly above the station median and fell within the bottom third of station concentrations during SE18-B at 2.37×10^2 CFU/ 100 mL. The AllBac concentrations remained fairly consistent over the two sampling times, ranging from 1.87×10^6 copies/ 100 mL for SE18-A to 1.72×10^6 copies/ 100 mL for SE18-B. The BacR marker was detected at Stn 4 during SE18-A, when the flow and *E. coli* concentrations were the highest. The concentration of the BacR marker was 4.28×10^3 copies/ 100 mL. The ruminant marker was not detected during the second sampling event, when the flow had decreased.

Due to inaccessibility to the stream at Stn 5, the flow was not measured during SE18. The *E. coli* concentration during SE18-A was in the top third of station concentrations (1.50×10^3 CFU/ 100 mL), but was lower than the station median during SE18-B (2.42×10^2 CFU/ 100 mL). The AllBac concentration for SE18-A was 2.70×10^6 copies/ 100 mL, which was the third highest recorded value at that station. The marker concentration decreased to 1.67×10^6 copies/ 100 mL during SE18-B, however, this value was still in the top quarter of the concentrations at Stn 5. The BacR marker was detected during both sampling events but the concentration decreased from 9.05×10^3 copies/ 100 mL for SE18-A to 4.95×10^3 copies/ 100 mL for SE18-B.

Both the BacR and CowM2 markers were detected at Stn 6 during SE18. The flow rate measured during SE18-A (6.89×10^{-1} m³/s) was the second highest recorded at that station. The flow remained high during SE18-B at 3.33×10^{-1} m³/s, which was the fourth highest at that station. The high flow rates were not necessarily reflected in the *E. coli* concentrations. SE18-A had the fourth highest concentration at 1.81×10^4 CFU/ 100 mL, however, the concentration fell below the station median during SE18-B to 2.48×10^2 CFU/ 100 mL. Like the flow, the AllBac concentration during SE18-A was the second

highest (9.81×10^6 copies/ 100 mL) at Stn 6 and decreased to 1.45×10^6 copies/ 100 mL during SE18-B, which remained in the top third of station values. The BacR and CowM2 markers were only detected during SE18-A. The concentrations of the BacR and CowM2 markers were 4.38×10^4 and 4.12×10^2 copies/ 100 mL, respectively.

4.4.3.2 SE20: DETECTION OF HOST-SPECIFIC MARKERS DURING STORM EVENT MONITORING

The sampling event SE20 took place over a four-day period from June 13th to 16th, 2011. Sample collection started on June 13th (SE20-A) when 16.4 mm of precipitation fell. No collection was conducted on June 14th, when only 7.5 mm of rain occurred; however, on June 15th more samples were collected when 35 mm of rain fell (SE20-B). The following day, when SE20-C was conducted, there was no precipitation.

Neither the BacR nor CowM2 were detected at Stn 1 or Stn 3. The flow at Stn 1 ranged from 2.00×10^{-2} m³/s on SE20-A to 5.00×10^{-2} m³/s on SE20-B and SE20-C. The *E. coli* counts were below the station median for SE20-A and SE20-C (24 and 29 CFU/ 100 mL, respectively), however, the *E. coli* concentration for SE10-B was the second highest recorded value at that station (2.07×10^2 CFU/ 100 mL). The trend was not reflected in the AllBac marker count, where the concentration during SE20-A was in the top quarter of the recorded values at Stn 1 (3.57×10^4 copies/ 100 mL), the SE20-B samples were close to the station median (1.07×10^4 copies/ 100 mL), and the sample from SE20-C (3.68×10^3 copies/ 100 mL) was in the lower third of the concentrations recorded at Stn 1.

The flow rate at Stn 3 during SE20-A (2.32×10^{-2} m³/s) was lower than the station median but increased to the top quarter of flow velocities (1.35×10^{-1} m³/s) during SE20-B before decreasing to 3.98×10^{-2} m³/s for SE20-C. The *E. coli* concentration for SE20-A

was 6.50×10^2 CFU/ 100 mL, which was in the top quarter of Stn 3 values. The concentration increased to 1.61×10^3 CFU/ 100 mL for SE20-B and then fell below the station median for SE20-C (57 CFU/ 100 mL). The AllBac concentrations followed a decreasing trend of 4.87×10^5 copies/ 100 mL for SE20-A, 4.13×10^5 copies/ 100 mL for SE20-B, and 2.16×10^4 copies/ 100 mL for SE10-C. Both SE20-A and -B were in the top quarter of AllBac concentrations for Stn 3, however, SE20-C was in the lower third.

The BacR marker was detected at all of the predicted sites (Stn 2, Stn 4, Stn 5, and Stn 6) during SE20. However, the only station at which the CowM2 marker was detected was Stn 6 (Fig. 4.10).

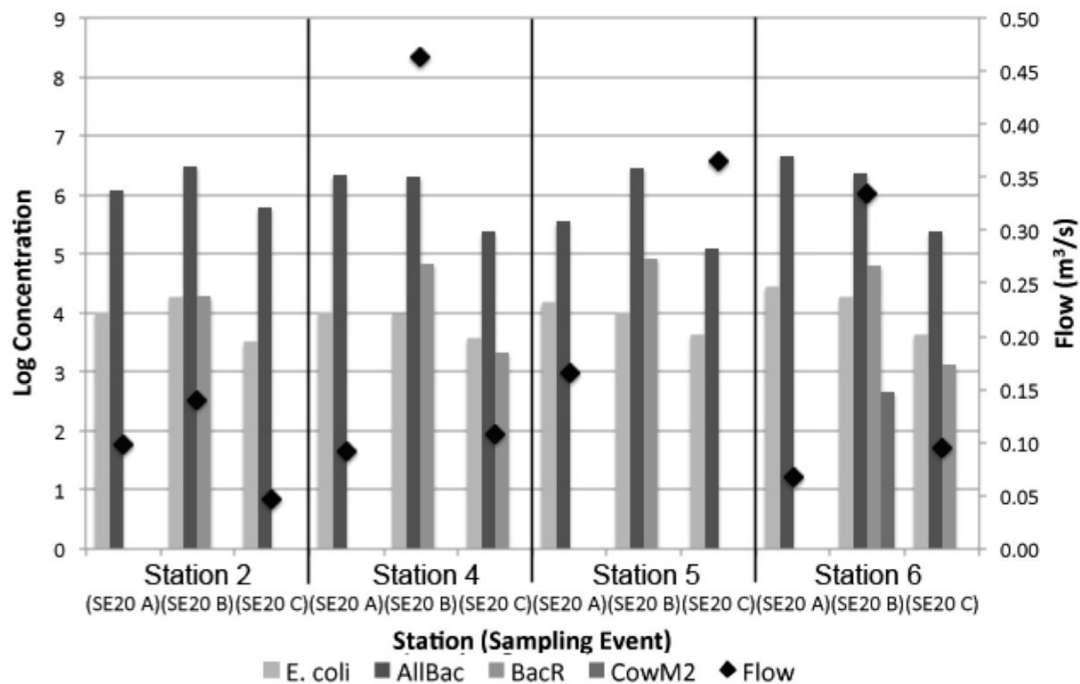


Fig. 4.10: *E. coli*, AllBac, BacR, and CowM2 concentrations and flow during SE20 Comparison of *E. coli* concentrations (CFU/ 100 mL) and AllBac, BacR and CowM2 marker concentrations (copies/ 100 mL) in relation to flow velocity (m³/s) recorded at Stn 2, Stn 4, Stn 5, and Stn 6 during a storm event in June 2011.

All flow rates at Stn 2 were in the upper half of the recorded velocities. Flow rates during sampling events SE20-A, SE20-B and SE20-C were 9.85×10^{-2} , 1.39×10^{-1} and $4.59 \times 10^{-2} \text{ m}^3/\text{s}$, respectively. The *E. coli* and AllBac markers followed the same trend as the flow data. The *E. coli* concentrations were $9.76 \times 10^3 \text{ CFU}/100 \text{ mL}$ for SE20-A, $1.77 \times 10^4 \text{ CFU}/100 \text{ mL}$ for SE20-B, and $3.11 \times 10^3 \text{ CFU}/100 \text{ mL}$ for SE20-C. The AllBac concentrations ranged from $1.22 \times 10^6 \text{ copies}/100 \text{ mL}$ for SE20-A, $3.04 \times 10^6 \text{ copies}/100 \text{ mL}$ for SE20-B, and $6.04 \times 10^5 \text{ copies}/100 \text{ mL}$ for SE20-C. The BacR marker was only detected in sample SE20-B ($1.88 \times 10^4 \text{ copies}/100 \text{ mL}$), which was during the greatest flow and most precipitation.

The changes in flow rate between sampling events were the most extreme at Stn 4. During SE20-A, the flow was measured at $9.09 \times 10^{-2} \text{ m}^3/\text{s}$, it increased to $4.64 \times 10^{-1} \text{ m}^3/\text{s}$ during SE20-B, and then decreased to $1.08 \times 10^{-1} \text{ m}^3/\text{s}$ during SE20-C. The *E. coli* concentrations did not reflect this trend, and instead decreased for each sampling event. The *E. coli* concentrations were $1.02 \times 10^4 \text{ CFU}/100 \text{ mL}$ for SE20-A, $9.25 \times 10^3 \text{ CFU}/100 \text{ mL}$ for SE20-B, and $3.50 \times 10^3 \text{ CFU}/100 \text{ mL}$ for SE20-C. The AllBac markers also decreased during the successive sampling events. SE20-A and SE20-B concentrations were similar at 2.13×10^6 and $2.04 \times 10^6 \text{ copies}/100 \text{ mL}$, respectively. The AllBac marker concentration decreased an order of magnitude ($2.44 \times 10^5 \text{ copies}/100 \text{ mL}$) for SE20-C. The BacR marker was detected at Stn 4 during SE20-B and SE20-C at concentrations of 6.89×10^4 and $2.95 \times 10^3 \text{ copies}/100 \text{ mL}$, respectively.

The flow during SE20-C was the highest recorded value at Stn 5 ($3.64 \times 10^{-1} \text{ m}^3/\text{s}$), however the flow during SE20-B was not recorded due to logistical issues. The flow during SE20-C was $1.54 \times 10^{-1} \text{ m}^3/\text{s}$. The *E. coli* concentration ($1.44 \times 10^4 \text{ CFU}/100 \text{ mL}$) during SE20-A was also the highest recorded value at Stn 5. The concentrations

during SE20-B (1.00×10^4 CFU/ 100 mL) and SE20-C (4.00×10^3 CFU/ 100 mL) were the third and fourth highest, respectively. The AllBac marker concentration started at 3.56×10^5 copies/ 100 mL for SE20-A, and then increased to 2.91×10^6 copies/ 100 mL for SE20-B, and fell to 1.21×10^5 copies/ 100 mL for SE20-C. The BacR marker, registering a concentration of 8.53×10^4 copies/ 100 mL, was detected only during SE20-B when the AllBac marker was at its highest concentration.

The flow rates at Stn 6 followed the same pattern as the other sites. The flow during SE20-A (6.81×10^{-2} m³/s) was the lowest for this storm-sampling event and was close to the station median. It then increased to 3.34×10^{-1} m³/s during SE20-B, which was the third highest recorded value at Stn 6. The flow during SE20-C decreased to 9.43×10^{-2} m³/s, which was still in the top third of the station flow velocities. Again, the *E. coli* concentrations had a decreasing trend. SE20-A had the highest recorded *E. coli* concentration (2.71×10^4 CFU/ 100 mL) at Stn 6. SE20-B and SE20-C had the fourth and fifth highest concentrations at the site, which were recorded as 1.81×10^4 and 4.09×10^3 CFU/ 100 mL, respectively. The AllBac markers followed the same decreasing trend as *E. coli*. For SE20-A the AllBac marker concentration was 4.62×10^6 copies/ 100 mL, the concentration for SE20-B was 2.27×10^6 copies/ 100 mL, and for SE20-C it was 2.40×10^5 copies/ 100 mL. The BacR marker was detected in both the SE20-B and SE20-C samples. The marker concentration of SE20-B was 6.47×10^4 copies/ 100 mL and it decreased to 1.30×10^3 copies/ 100 mL for SE20-C. The CowM2 marker was also detected in the SE20-B sample at 4.56×10^2 copies/ 100 mL.

4.4.3.3 SE24: DETECTION OF HOST-SPECIFIC MARKERS DURING STORM EVENT MONITORING

The last storm event sampling was conducted on August 2nd and 3rd, 2010. The first samples (SE24-A) were collected on August 2nd, when 38.8 mm of precipitation fell. The second set of samples (SE24-B) occurred on the following day when only 0.2 mm of rain fell.

Again, the ruminant and bovine markers were not detected at Stn 1 and Stn 3. At Stn 1 during SE24-A the highest recorded flow rates ($4.51 \times 10^{-2} \text{ m}^3/\text{s}$), *E. coli* concentration ($1.15 \times 10^3 \text{ CFU}/100 \text{ mL}$), and AllBac marker concentration ($1.33 \times 10^5 \text{ copies}/100 \text{ mL}$) for the site occurred. For SE24-B, the flow was not recorded but the *E. coli* concentration remained in the upper quarter of the recorded data at 76 CFU/ 100 mL. The AllBac marker concentration, however, dropped below the station median to $5.63 \times 10^3 \text{ copies}/100 \text{ mL}$.

The flow rates at Stn 3 were the third highest recorded at the site during SE24-A ($2.64 \times 10^{-1} \text{ m}^3/\text{s}$) but the flow rate was under the station median for SE24-B ($2.74 \times 10^{-2} \text{ m}^3/\text{s}$). The *E. coli* concentration for SE24-A was $8.10 \times 10^3 \text{ CFU}/100 \text{ mL}$, which was the highest recorded for the site. The concentration decreased to 3.01×10^2 for SE24-B, but still remained in the upper third of the recorded values. Again, the AllBac marker concentration was the highest recorded value at Stn 3 during SE24-A ($1.36 \times 10^6 \text{ copies}/100 \text{ mL}$) but dropped below the station median for SE24-B ($4.62 \times 10^4 \text{ copies}/100 \text{ mL}$).

The BacR marker was detected in the greatest number of samples during SE24. The marker was detected at all of the predicted sites, and detected on both days of the sampling event at all but one of predicted sites (Stn's 2,4,5, and 6). The CowM2 was detected twice, both during SE24-A, but this time at Stn 2 and Stn 4 (Fig. 4.11). At every

site, the *E. coli* and BacR marker concentrations decreased from SE24-A to SE24-B. The decreasing trend also presented itself in the AllBac marker concentrations at all sites except Stn 2 and in the all flow velocities (Stn 5 flow during SE24-A was not recorded).

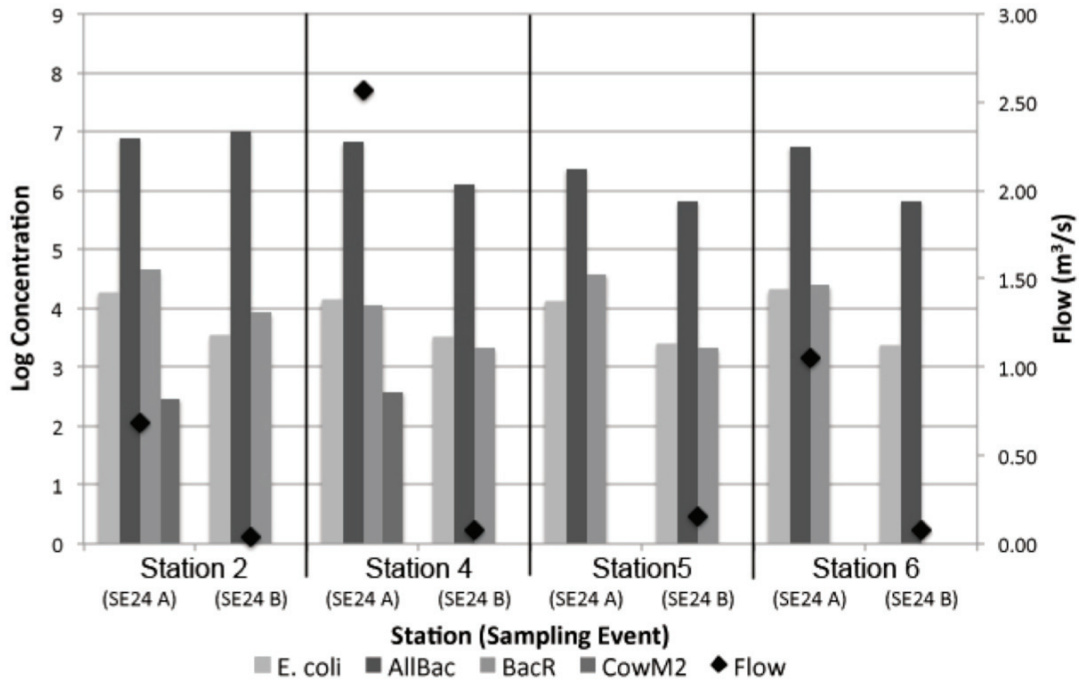


Fig. 4.11: *E. coli*, AllBac, BacR, and CowM2 concentrations and flow during SE24. Comparison of *E. coli* concentrations (CFU/ 100 mL) and AllBac, BacR and CowM2 marker concentrations (copies/ 100 mL) in relation to flow velocity (m³/s) recorded at Stn 2, Stn 4, Stn 5, and Stn 6 during a storm event in August 2011.

The flow rate at Stn 2 was the highest recorded value during SE24-A (6.88×10^{-1} m³/s) but decreased to 3.89×10^{-2} m³/s during SE24-B, which was still above the station median. The *E. coli* concentration for SE24-A was 1.83×10^4 CFU/ 100 mL, which was the third highest at that site and then decreased to 3.35×10^3 CFU/ 100 mL for SE24-B. The AllBac concentrations at Stn 2 increased slightly from 7.91×10^6 to 1.04×10^7 copies/ 100 mL for SE24-A and SE24-B, respectively. BacR was detected in both the SE24-A (4.41×10^4 copies/ 100 mL) and SE24-B (8.39×10^3 copies/ 100 mL) samples

and followed a decreasing trend. The CowM2 marker was also detected in the SE24-A samples at a concentration of 2.95×10^2 copies/ 100 mL.

All measured parameters at Stn 4 followed a decreasing trend from SE24-A to SE24-B. The flow decreased from $2.57 \text{ m}^3/\text{s}$, the highest recorded flow in the entire watershed, to $8.06 \times 10^{-2} \text{ m}^3/\text{s}$, just above the station median. The *E. coli* concentration decreased from 1.34×10^4 CFU/ 100 mL, the highest recorded value at the site, to 3.23×10^3 CFU/ 100 mL, which was still in the upper quarter of the recorded values. The AllBac concentration was the second highest value recorded at the site (6.70×10^6 copies/ 100 mL) and then decreased to 1.27×10^6 CFU/ 100 mL, which was in the upper third of the values. The BacR marker was detected during both sampling events at Stn 4. The concentration of SE24-A was 1.40×10^4 copies/ 100 mL and then decreased to 2.13×10^3 copies/ 100 mL for SE24-B. The CowM2 marker was also detected during SE24-A, at a concentration of 3.85×10^2 copies/ 100 mL.

The flow during SE24-A was not recorded at Stn 5 but registered at $1.54 \times 10^{-1} \text{ m}^3/\text{s}$ during SE24-B. The *E. coli* concentration was the second highest recorded at the site (1.30×10^4 CFU/ 100 mL) for SE24-A but decreased to 2.44×10^3 CFU/ 100 mL for SE24-B, which was in the upper quarter of recorded values at the site. The AllBac marker concentration decreased from 2.34×10^6 copies/ 100 mL to 6.61×10^5 CFU/ 100 mL, both above the station median. The BacR marker was also detected at Stn 5 and decreased from 3.83×10^4 copies/ 100 mL during SE24-A to 2.09×10^3 copies/ 100 mL during SE24-B. The CowM2 marker, however, was not detected.

The flow during SE24-A at Stn 6 was the highest recorded value at that site ($1.05 \text{ m}^3/\text{s}$) and then decreased $7.28 \times 10^{-2} \text{ m}^3/\text{s}$, which was close to the station median. The *E. coli* concentration decreased from 2.02×10^4 CFU/ 100 mL during SE24-A to 2.28×10^4

CFU/ 100 mL during SE24-B; both these concentrations were in the upper third of the recorded values at this site. Similarly, the AllBac concentration decreased from 5.67×10^6 copies/ 100 mL during SE24-A to 6.70×10^5 copies/ 100 mL during SE24-B. The BacR marker was only detected during SE24-A (2.45×10^4 copies/ 100 mL) and the CowM2 marker was not detected.

4.5 HUMAN-SPECIFIC *BACTEROIDALES* MARKER (BACH)

4.5.1 CONDITIONAL PROBABILITY OF BACH DETECTION IN THE ENVIRONMENT

The conditional probability was calculated to determine if detection of BacH in water samples represents a strong probability of the existence of human fecal contamination. The conditional probability was calculated using the same equation and variables as were used to calculate the conditional probability of BacR and CowM2; the result was a conditional probability of 4.29% (Table 4.4), meaning that detection of the BacH marker may not accurately predict the presence of human fecal contamination.

Table 4.4: Variables and results of the BacH Bayes' Theorem calculation

Marker	$P\left(\frac{T}{H}\right)$	$P(H)$	$P\left(\frac{T}{H'}\right)$	$P(H')$	$P\left(\frac{H}{T}\right)$
BacH	64.3%	5.62%	8.11%	99.4%	4.29%

4.5.2 DETECTION OF BACH IN ENVIRONMENTAL WATER SAMPLES

The BacH marker was detected in only one sample (0.562%) throughout the entire 15-month sampling period. The BacH marker was detected during the SE12 sampling event at the watershed outlet, Stn 5. SE12 occurred on November 22, 2010. In the week prior to the sampling period, 21 mm of precipitation was recorded. Between November

5th and November 10th, 2010, 127.8 mm of rain was recorded, however, this event was 17 days before sample collection.

4.5.3 SUMMARY OF *BACTEROIDALES* WATER QUALITY MONITORING

Although the sample size during increased precipitation events is small, an increasing trend between precipitation volume and the BacR and CowM2 marker detection can be seen (Fig. 4.12). Precipitation volumes between 30 and 40 mm reflected the highest percent detection for both markers. Volumes over 50 mm showed a very high frequency of detection for the BacR marker (66.7%, n = 6). Detection of *E. coli* and AllBac markers appear independent of precipitation volumes. At their lowest detection rate, *E. coli* was detected above CCME guidelines for irrigational water quality (>100 CFU/ 100 mL) in 53% of the samples and the AllBac marker was detected above 1×10^4 copies/ 100 mL in 73% of the samples.

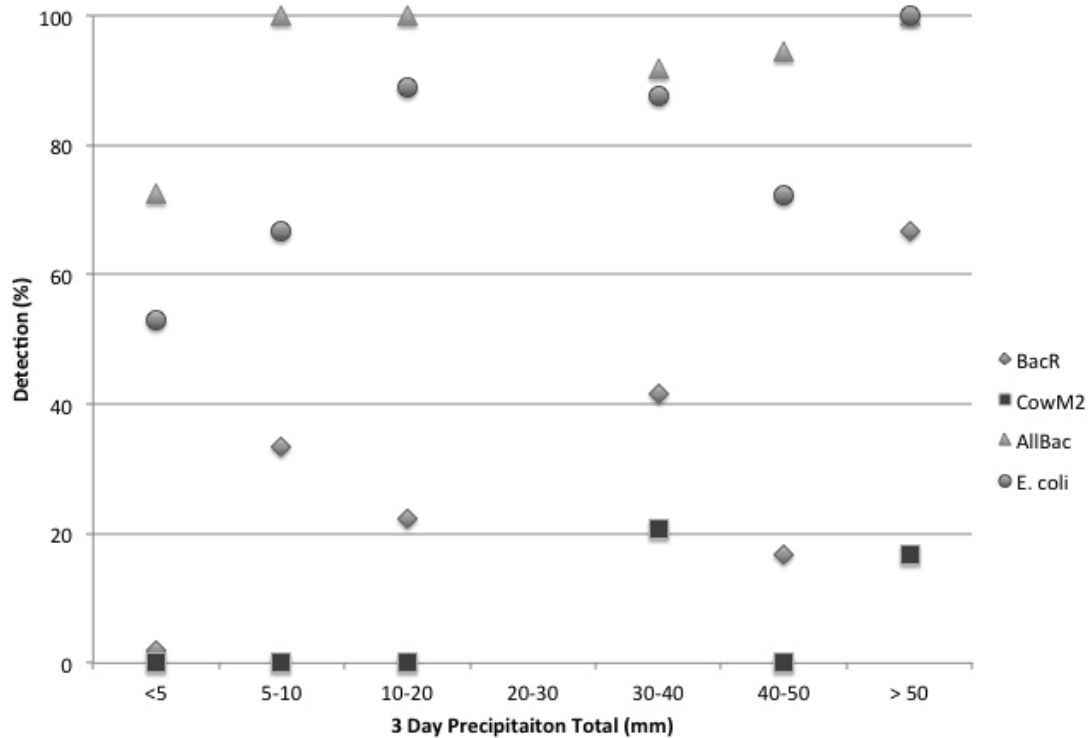


Fig. 4.12: Influence of Increased Precipitation on Host Specific BacR and CowM2 Marker Detection in Comparison with *E. coli* and AllBac Detection Rates
 The frequency of host-specific marker detection increases as precipitation volume increases. Precipitation volume is measured as the total volume of precipitation (mm) on the two days prior to and the day of sampling. No precipitation volumes between 20 and 30 mm were recorded during this monitoring program. Detection rate of *E. coli* is recorded as the percentage of total samples that exceeded the CCME guidelines for irrigational water quality. Detection rate of the AllBac marker is recorded as the percentage of samples exceeded 1×10^4 copies/ 100 mL

Fig. 4.13 summarizes the water quality monitoring data collected over the sampling period. Station 1 and station 3 are the least impacted sampling sites in the TBW. Stations 2, 4, 5, and 6 are highly impacted; the CCME guidelines are routinely exceeded and the ruminant and bovine host-specific markers were detected.

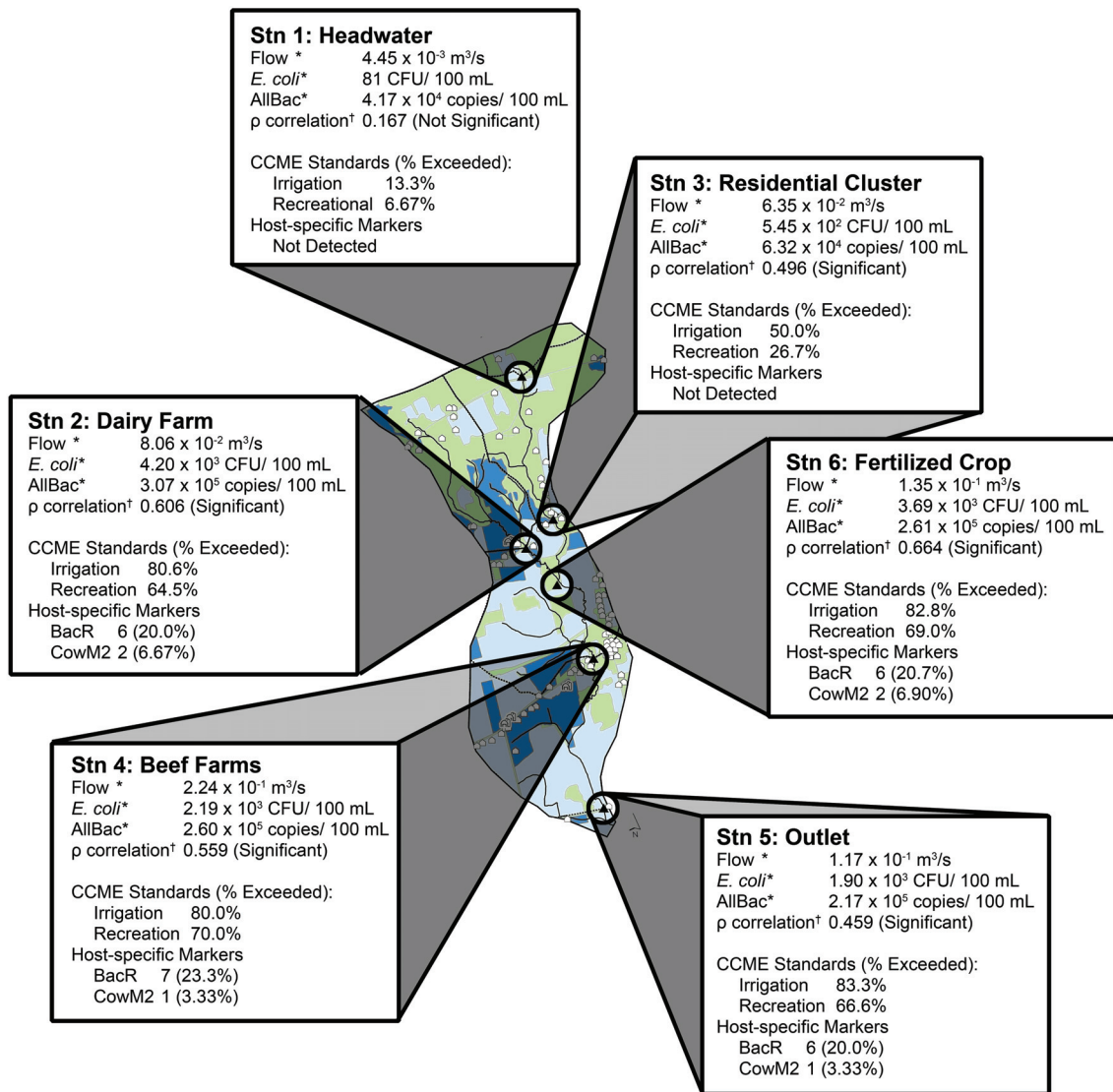


Fig. 4.13: Water Quality Monitoring Data for the 15-Month Sampling Period
 Flow rates, *E. coli* concentrations, and AllBac marker concentrations for each station stated as averages. ρ correlation value demonstrates the relationship between *E. coli* and AllBac concentrations. Percent of samples that exceed the CCME guidelines for irrigational and recreational water use are also indicated. Frequency and percentage of host-specific BacR and CowM2 markers detected at each station includes routine monitoring and storm events.

4.6 PATHOGEN DETECTION

4.6.1 SALMONELLA SP. AND E. COLI O157:H7 IN ENVIRONMENTAL WATER SAMPLES

Using selective enrichment and IMS, environmental water samples were tested for *Salmonella* and *E. coli* O157:H7. Over the 15-month sampling period, neither pathogen was detected in any of the water samples. Positive controls were periodically used to ensure that culture conditions and qPCR methods were optimal.

4.6.2 CAMPYLOBACTER SPP. IN ENVIRONMENTAL WATER SAMPLES

Because of the enrichment process, quantifying *Campylobacter* populations in the water samples was not possible. Therefore the results of the *Campylobacter* qPCR assays were classified as either positive or negative. Out of the 149 samples that were tested for *Campylobacter*, 114 (76.5%) were positive. Using logistic regression to assess the relationship between *E. coli* concentrations and the presence of *Campylobacter*, it was determined that there was no significant relationship. The same statistical analysis procedure revealed that there was also no statistically significant relationship between the AllBac marker and the presence of *Campylobacter*.

Further analysis was conducted to compare the percentage of positive *Campylobacter* samples within two modalities representing *E. coli* levels above and below the CCME irrigation guidelines (> 100 CFU/ 100 mL and < 100 CFU/ 100 mL, respectively; Fig. 4.14). Of the 94 samples that were above the guidelines, 79 (84.0%) were positive for *Campylobacter*. A high incidence of *Campylobacter* (64.6%) was also seen in the 55 samples that that fell below the guidelines.

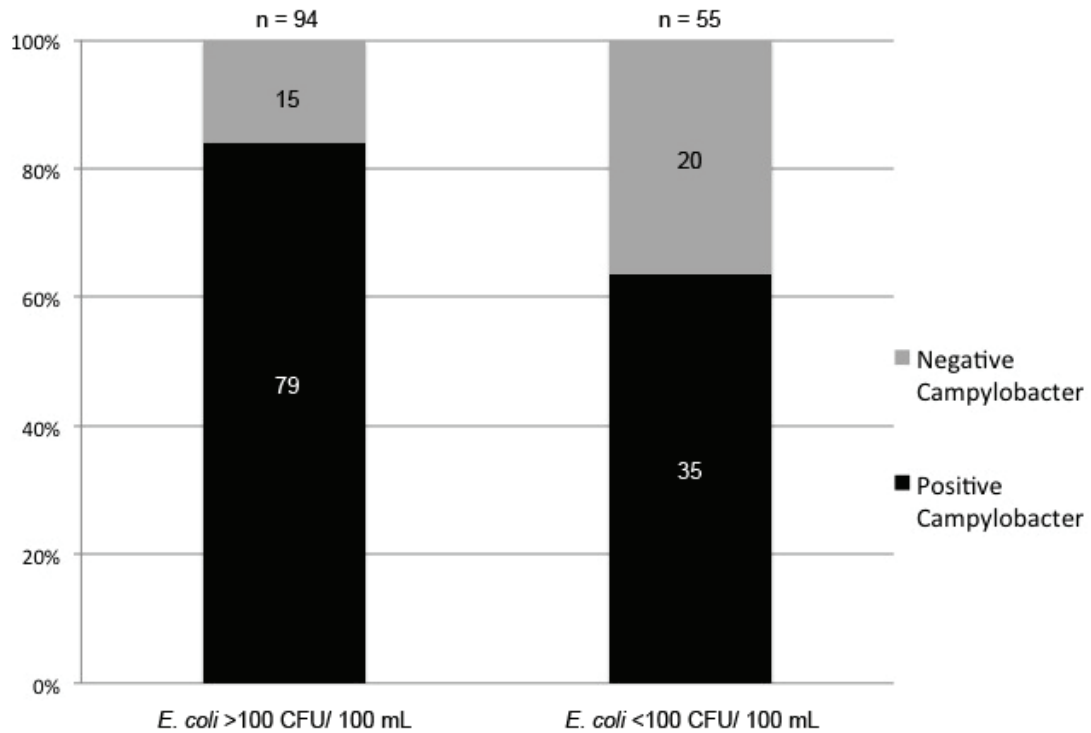


Fig. 4.14: Relationship between *Campylobacter* and the CCME *E. coli* guidelines
 The presence of *Campylobacter* was high in samples that fell both above and below the CCME irrigational guidelines for *E. coli*, however a slightly higher incidence of *Campylobacter* is seen in samples exceeding the guidelines (84.0%) compared to samples that fell below the acceptable guidelines (63.6%).

4.7 WATER QUALITY PARAMETERS

Numerous water quality measurements were taken during each sampling event, such as water temperature, pH, dissolved oxygen, conductivity, turbidity, and TSS. Table 4.5 summarizes the average values and standard deviations of each water quality parameter at station one through six, and also provides the averages for the entire watershed.

Table 4.5: Averages and standard deviations of water quality parameters at each station and for TBW.

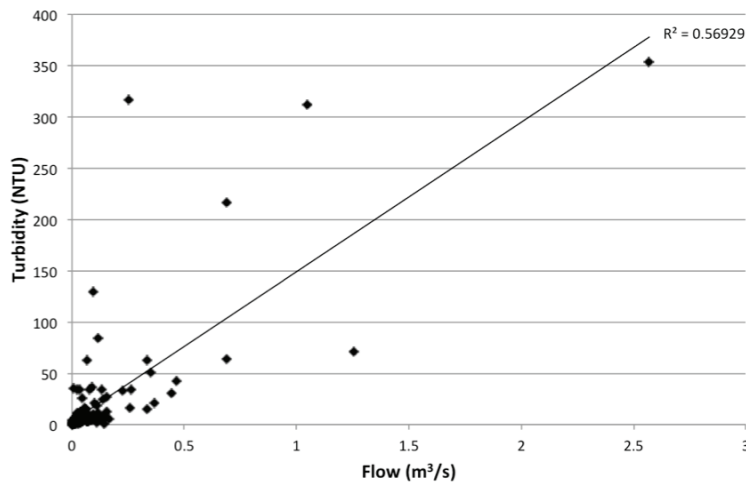
	Temp (°C)	Conductivity (mS/cm)	DO (mg/L)	pH	Turbidity (NTU)	TSS (mg/L)
Stn 1^a	12.7 (5.40)	0.121 (0.098)	9.90 (2.20)	7.17 (0.50)	1.43 (1.55)	13.42 (28.70)
Stn 2^a	13.7 (5.90)	0.321 (0.086)	9.91 (2.05)	7.39 (0.37)	17.16 (42.61)	6.65 (9.24)
Stn 3^a	12.2 (5.30)	0.339 (0.159)	10.7 (1.80)	7.18 (0.37)	21.41 (62.55)	5.73 (9.32)
Stn 4^a	12.7 (4.30)	0.287 (0.126)	10.3 (1.90)	7.31 (0.44)	31.68 (70.80)	12.39 (13.43)
Stn 5^a	12.7 (4.50)	0.121 (0.020)	9.90 (1.58)	7.17 (0.47)	18.58 (26.90)	25.11 (55.62)
Stn 6^a	12.6 (5.30)	0.285 (0.106)	10.5 (1.70)	7.39 (0.45)	29.13 (66.27)	36.69 (76.46)
Total^b	12.1 (5.10)	0.328 (0.094)	10.8 (1.90)	7.29 (0.42)	20.02 (51.64)	16.82 (42.25)

^a Average (Standard deviation) of parameter at the given TBW station

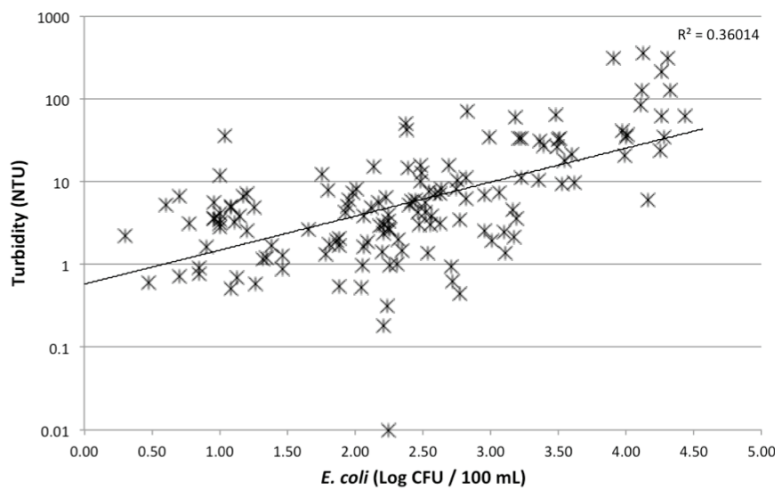
^b Average (Standard deviation) of parameter for the TBW watershed

The average water temperature, conductivity, DO, and pH do not vary significantly between each station. The turbidity and TSS values vary widely throughout the sampling events, as demonstrated by the standard deviation. Positive correlations can be seen between flow, *E. coli*, and AllBac markers and turbidity (Fig. 4.15).

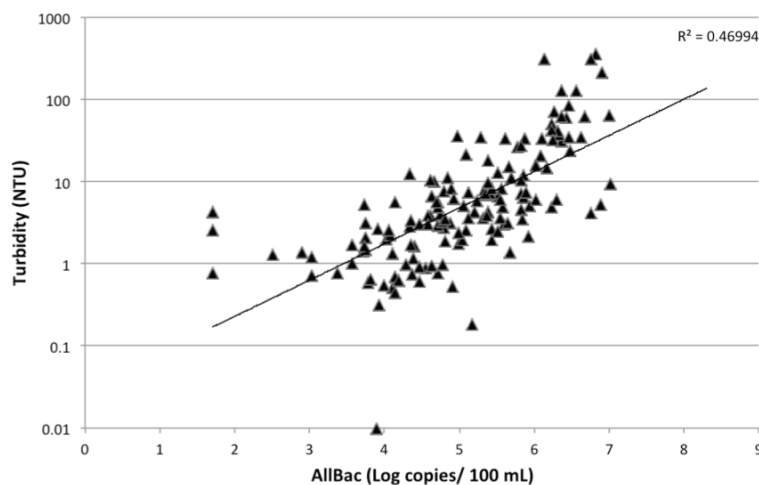
Fig. 4.15: Relationship of flow rates, *E. coli* and *Bacteroidales* with turbidity in TBW



A) The flow rates (m^3/s) plotted against the turbidity (NTU) at each station show a positive linear relationship between the two variables ($R^2 = 0.569$)



B) A weakly positive correlation ($R^2 = 0.360$) can be seen between *E. coli* concentrations (CFU/100 mL) and turbidity of the samples collected from TBW.



C) The correlation between universal *Bacteroidales* AllBac marker concentrations (copies/100 mL) and turbidity (NTU) was also positive ($R^2 = 0.470$)

CHAPTER 5. DISCUSSION

5.1 MARKER VALIDATION STUDY

The need for more informative indicators of fecal water pollution is clear. With mounting evidence of *E. coli* naturalization in aquatic environments (Anderson et al., 2005; Byappanahalli et al., 2003; Power et al., 2005) and the inability of traditional FIOs to distinguish between fecal pollution sources (Field & Samadpour, 2007), an alternate strategy to warn of microbial pollution is essential. Advances in molecular techniques, such as qPCR, have the potential to significantly advance the field of water quality monitoring. The evolution from using unspecific and culture-dependent FIOs to employing host-specific and culture-independent FIOs is occurring rapidly.

The use of the *Bacteroidales* 16S rRNA gene as a tool for MST had not been previously investigated in Nova Scotia. In a watershed such as TBW, where microbial pollution levels are frequently above CCME water quality standards (Jamieson et al., 2003; Sinclair et al., 2009), determining fecal pollution sources would be extremely beneficial. Since this form of MST is still in its infancy, the chosen qPCR assays required validation before being implemented as tools for water quality monitoring in this region.

The assay validation study was successful, in that the chosen assays were successfully validated for use in this study, and for other studies to be conducted in other Nova Scotia water systems with similar pollution sources. This is an important step as even though the *Bacteroidales* assays appear to be temporally and geographically stable (Ahmed et al., 2009; Fremaux et al., 2010; Gourmelon et al., 2010; Shanks et al., 2008;

Wyer et al., 2010), validation in a large number of diverse environments is required before this tool can be universally applied. .

The sensitivity and specificity of the AllBac assay was 97.3 and 100%, respectively, and the average concentration of the AllBac marker was 2.24×10^9 copies/ g feces. These results are consistent with the original study published by Layton et al. (2006), which revealed a high degree of sensitivity and specificity of the AllBac primers and probe by testing it against feces and by searching for sequence homology of *Bacteroides* within the Ribosomal Database Project (RDP). Furthermore, the RDP search revealed an extremely low chance of cross-reaction (< 0.1%) with bacteria outside the *Bacteroidetes* phylum (Layton et al., 2006).

The results of the BacR validation were similar to the original paper by Reischer et al. (2006). The sensitivity and specificity of the BacR marker in the original paper (Reischer et al., 2006) were both reported as 100%. The sensitivity and specificity were slightly lower in this study, and were recorded as 94.4 and 93.9%, respectively. One bovine fecal sample resulted in a false negative BacR detection; however, the reasons for this are unclear. The fecal sample tested positive for the CowM2 marker and other fecal samples from cows on the same farm were collected and tested positive for the BacR marker. A study conducted in France further confirmed these results and reported 89% sensitivity and 100% specificity (Mieszkin et al., 2009). The CowM2 showed 100% specificity in both the original literature (Shanks et al., 2008) and this validation study. The lack of cross-reactivity is not surprising since the target gene is not the 16S rRNA gene but is involved in bacterial-host interaction (Shanks et al., 2008).

Differences in the concentration of host-specific markers detected in fecal samples indicate that the detection rates will vary between environmental assays. The heterogenic

nature of the 16S rRNA gene (Case et al., 2007) may affect the concentrations. Not all copies of the 16S rRNA gene within a *Bacteroidales* cell must contain the host-specific DNA sequence; in fact, there may be a subpopulation of *Bacteroidales* within the host that does not contain any copies of the host-specific marker.

The average concentration of the AllBac marker in the ruminant feces, 2.65×10^9 copies/ g, was an order of magnitude higher than the average BacR marker (1.94×10^8 copies/ g). The average concentration of the CowM2 marker (1.44×10^6 copies/ g) was 1000 fold lower than detection of the AllBac markers in the same feces (2.81×10^9 copies/ g). In bovine fecal samples that possessed both the BacR and CowM2 marker, the latter was detected 100 fold lower. The differences in detection rates of the marker in environmental water samples could be partially explained by these differences.

Conversely, the low sensitivity (64.3%) of the BacH assay was surprising. Reischer et. al (2007) originally reported higher sensitivity and specificity for the BacH assay. The marker was detected in 100% of the samples originating from combined sewage sources (municipal wastewater and cesspit samples). The sensitivity decreased to 95% when testing individual human fecal samples. Another study conducted in Australia reported similar sensitivity for the BacH assay (100%) when testing influent from a sewage treatment plant (Ahmed et al., 2009). Furthermore, Tambelo et al. (2012) reported constant low-level detection of the BacH marker in a Saskatchewan watershed that is influenced by an upstream wastewater treatment plant. The contrast between results reported in the literature and the results obtained in this study could be due to the nature of the samples.

The samples used in this study were collected from septic tanks, which may have contributed to the lower sensitivity. Fecal bacteria found in municipal wastewater,

cesspits, and individual fecal samples would be more concentrated than bacteria found in septic systems. The individual characteristics of the septic systems could have adversely affected the detection rate. High water usage, such as running showers, dishwashers, or washing machines, prior to tank sampling may have diluted the marker. Predation of *Bacteroidales* is suspected of having the most significant affect on marker persistence and decay rates (Bell et al., 2007). Since septic systems are biologically active systems that are used to breakdown organic material (Peed et al., 2011), predation could play a role in lowering the detection rates of the BacH within the septic system.

With similar detection rates as the CowM2 marker, the BacH marker may be more difficult to detect. The concentration of the BacH marker was 1000 fold lower than the concentration of the AllBac marker in the same septic tank samples. The watercourse would further dilute seepage of untreated septic contents making detection very difficult in lower concentrations.

The specificity of the BacH assay as originally reported by Reischer et. al. (2007) was 99.6%. Ahmed et al. (2009) found the BacH assay to be 94% specific. The specificity as determined in this validation study was 91.7%. The low level of cross reactivity of this marker in geographically diverse areas indicates that it is geographically stable.

5.2 MARKER DETECTION IN ENVIRONMENTAL WATER SAMPLES

Variations in marker stability and persistence in the environment also likely play a role in the different detection rates of different markers. In environmental water samples, the difference in marker concentrations increased to 2 orders of magnitude between samples that concurrently detected the AllBac and BacR markers. However, the difference between concentrations of the AllBac marker and the CowM2 marker in

environmental samples remained consistent at 3 orders of magnitude lower for CowM2, as did the 100 fold difference in the detection rate of BacR and CowM2 markers.

Little information is available on the persistence of the BacR and CowM2 markers. One study indicates that the decay rate of both the BacR and BacH markers are comparable to decay rates of *E. coli*, however the water body in which the study took place did not appear to have naturally high levels of *E. coli* that may have naturalized in the environment (Sokolova et al., 2012). In another recent study, the persistence of the host-specific BacR, CowM2, and BacH markers in an aquatic environment were investigated and contrasted to the persistence of *E. coli* and the AllBac marker. The host-specific markers did not persist in the environment for more than 12 days; however, *E. coli* and the AllBac marker were still detectable on the last day of the study (Tambalo et al., 2012). The persistence of the CowM2 marker has also been investigated in manure-amended soils (Rogers et al., 2011). The results indicated that the CowM2 marker decays at a rate much quicker than traditional FIOs and *Bacteroidales* 16S rDNA markers, which lead to the suggestion that this marker would be more appropriate for point-source or recent non-point source fecal pollution events rather than detection of fecal material originating from manured cropland (Rogers et al., 2011).

A combination of markers relevant to the watershed in question has been suggested when trying to determine the sources of microbial pollution within an impacted watershed (Reischer et al., 2011). When considering the differences between the BacR and CowM2 markers, it may be difficult to establish if bovine feces are absent in the absence of the CowM2 marker if the BacR marker is detected in low concentrations. Tambalo et al. (2012) reported that the BacR marker persisted in the environment longer than the CowM2 marker (7 days versus 3 days). In instances of high BacR concentrations

and no CowM2 detection, the possibility of other ruminant influences should be considered, however bovine fecal contamination cannot be disregarded. The samples can be tested for other ruminant-specific markers if they have been developed, such as the deer-specific assay (Dick, Simonich, et al., 2005).

Positive detection can be more informative than non-detection of markers. Probability calculations of both the BacR and CowM2 markers determined that if these markers are detected in the samples, there is a high probability (71.8% and 100%, respectively) that host feces are present in the water.

Persistence of the AllBac marker in environmental samples has been previously reported, which suggests that it is not suitable for use as an indicator of recent fecal pollution events (Dick et al., 2010). In fact, recent research into the AllBac marker suggests that this assay detects environmental members of *Bacteroidales* (Vierheilig et al., 2012) as well as *Bacteroidales* found in the hind gut of insects (van der Wielen & Medema, 2010).

In TBW, where consistently high levels of *E. coli* have been recorded, the AllBac was detected in virtually all samples (97.2%). This level of detection is consistent with other findings and support the fact that the AllBac marker may not reveal enough information about the state of water quality to be useful as an indicator. Using Spearman's rank correlation, an association between *E. coli* concentrations and the AllBac was evident ($\rho= 0.629$). This is not surprising since the persistence of *E. coli* in environmental samples has also been reported. This data may have a greater reflection on the natural ecology of the TBW stream environment than on fecal water pollution.

During baseline monitoring in the TBW, the BacR was detected in only 5.88% of the samples. Even though the sampling took place during baseline monitoring, further

investigation into precipitation prior to these sampling events revealed that most of the marker detection could be explained by rainfall occurring in the days preceding the sampling event. Only one of these sampling events was during a dry period with low flow. Activity at nearby agricultural operations or deposition of fecal material from wild ruminants into the watercourse may have occurred and caused the detection of the BacR marker during the dry period. The rest of the positive BacR sampling events were after moderate precipitation and periods of increased flow rates. *E. coli* levels were consistently higher than average when the BacR marker was detected. Interestingly, the CowM2 marker was not co-detected in any of these samples. However, some marker concentrations were low enough that if present, the CowM2 marker may have fallen below the ALOD.

A large amount of rain fell the day before the only sampling event during baseline monitoring in which the CowM2 marker was detected. Curiously, the BacR marker was not detected in these samples. Differences in marker persistence between BacR and CowM2 may explain detection of only the CowM2 marker at Stn 5, the watershed outlet. The marker would have likely travelled through the watershed from Stn 2 or Stn 4, resulting in greater exposure to degradative factors. Lack of co-detection of the BacR marker at Stn 2 is inconsistent with other findings. Even though detection of these markers were classified as baseline sampling events, in all but one case, their presence can be explained by increased precipitation.

The sole detection of the BacH marker was during baseline monitoring and cannot be explained by conventional factors such as high flow or large precipitation events. The marker was detected at Stn 5, in which there are 32 residential dwellings located in this subcatchment. An intense storm event that occurred 17 days prior to the sampling event is

unlikely to have caused the detection of the marker at the watershed outlet. Again, information on the persistence of the BacH marker is limited; however, the decreased detection rates found in the BacH validation study suggest that this is not a persistent marker.

Research and application, thus far, regarding the BacH marker has not included septic tank samples. Validation of the marker has been done using human feces and mixed fecal samples from municipal sewage systems or cesspits (Ahmed et al., 2009; Reischer et al., 2007). Application of the marker for use in environmental water samples has been applied to systems that are impacted by this same type of concentrated pollution (Reischer et al., 2007; Sokolova et al., 2012).

If the flow rate at Stn 3 (station located downstream of the residential cluster) during storm events was assumed to be $1.24 \times 10^{-1} \text{ m}^3/\text{s}$, the BacH marker would have to be deposited in the stream system at a rate of 1.07×10^{10} copies/ day before it could be detected above the ALOD. A typical average household water usage is approximately 1000 L/ day and the validation study determined that the BacH marker was present in septic tanks at an average concentration of 1.21×10^7 copies/ L; therefore, a faulty septic system would disperse on the order of 1.21×10^{10} copies per day into the environment. This number is approximately equal to the marker concentration required for detection during a storm event, when the markers are more likely to reach the stream system. In other words, for the BacH marker to be detected, the entire daily output of a faulty septic system would have to reach the stream in order to have concentrations above the limit of detection. This situation is unlikely because some of the bacteria would die off for various reasons or be dispersed throughout the surrounding soil rather than being deposited into the stream. If multiple septic systems were failing, the likelihood of detecting the BacH

marker would increase. These results indicate that the BacH marker may not be suitable for detecting microbial pollution due to individual faulty septic systems and that a marker with lower detection limits would be required for this situation.

5.3 MARKER DETECTION DURING STORM EVENTS

It is known that storm events can cause an influx of microbial pollutants into watercourses due to surface runoff from contaminated areas (Ackerman & Weisberg, 2003; Curriero et al., 2001). Such trends were seen in this study. Increase precipitation during storm event monitoring generally resulted in an increase in flow rates, *E. coli* counts, and AllBac marker concentrations. Directly after a storm event, the BacR marker and sometimes the CowM2 marker, could also be detected.

The purpose of the storm event monitoring was to determine if the host-specific marker would be detected, and to also determine how long they persisted in the stream system after the event. When BacR and CowM2 markers were detected during the storm, their concentrations often returned to undetectable levels in the samples collected the day after the storm. In the cases where the host-specific markers were still detectible during the post-storm sample collection, their concentrations were approximately an order of magnitude lower than the previous day.

The storm event monitoring also verified the hypothesis that the watershed headwater catchment, Stn 1, and the residential cluster catchment, Stn 3, would not be influenced by bovine fecal contamination. Neither the ruminant nor the bovine marker was detected at these stations. The detection of bovine fecal contamination would have been challenging to explain, however, both these stations contain forested areas that may be inhabited by other wild ruminants, such as deer. Lack of the BacR marker during

storm events at either station suggests that wild ruminants are not likely to be a significant source of fecal contamination in this watershed.

These storm-monitoring results support the BacR and CowM2 assays for use as indicator organisms. These markers are detected during storm events and rapidly decrease as the precipitation subsides and surface runoff decreases. As precipitation volumes increased over a 3-day period, the percentage of BacR and CowM2 marker detection also increased. A 42% BacR detection rate was seen during periods associated with 30-40 mm of precipitation over 3 days. This detection rate increased to 67% when the precipitation volume exceeded 50 mm. Although the sample size for storm periods is small, this is an interesting trend that supports further investigation. Conversely, the *E. coli* and AllBac marker did not always show a decreasing trend with precipitation and their concentrations consistently remained high. Independent of the precipitation trends, *E. coli* and AllBac concentrations remained high. *E. coli* was detected above the CCME guidelines for irrigational water 53% of the time. Similarly, the AllBac marker was detected above 1×10^4 copies/ 100 mL in at least 73% of the samples assayed.

5.4 PATHOGEN DETECTION IN ENVIRONMENTAL WATER SAMPLES

As previously discussed, the presence of indicator organisms does not necessarily reflect the presence of pathogens in the water column. There is a need for indicators that better reflect the survival of enteric pathogens. This claim is supported by the data collected in this study.

Consistently high levels of *E. coli* did not correspond to the detection of *Salmonella* or *E. coli* O157:H7. In the 15-month sampling period, neither pathogen was detected despite the fact that *E. coli* levels frequently exceeded CCME guidelines for

irrigation and recreational water quality.. The *Salmonella* assay detects a gene found in serotypes of *Salmonella enterica*, including *Salmonella* Typhi (Cheng et al., 2008). The pathogenic *E. coli* assay is designed to specifically detect the *E. coli* serotype O157:H7, which causes HUS (Ibekwe et al., 2002). An assay this specific is required when determining if pathogenic *E. coli* is present because the *E. coli* species is so ubiquitous and the pathogenic strains need to be separated from the non-pathogenic strains, such as those that are used as indicator organisms.

The presence of FIOs does not necessarily denote the presence of pathogenic bacteria. To determine if there is any possible association between either the BacR or the CowM2 marker, more sampling would have to be conducted and the pathogens would have to be detected in the environmental samples. The fact that *Salmonella* and *E. coli* O157:H7 were not detected in the stream system could mean that they were not present in the animal population or, if present, their concentration was so low that it was not detected.

High instances of enteric disease in an impacted agricultural watershed in southern Alberta, Canada, prompted a study to determine the prevalence of *Salmonella* and *E. coli* O157:H7 in the water. Over a two-year study period, *Salmonella* and *E. coli* O157:H7 were only detected in 6.2 and 0.9% of the water samples, respectively (Johnson et al., 2003). This study demonstrates that even in watersheds with high instances of enteric disease, the pathogen detection rates can be very low. Another study, conducted in an impacted mixed land use watershed in California, USA, investigated pathogen presence in relation to FIOs (Schriewer et al., 2010). *Salmonella* was detected in only 7% of the samples, whereas *E. coli* O157:H7 was not detected in any samples. *Campylobacter* was also detected in 5% of the water samples. Schriewer et al. were not able to identify any

associations between *Bacteroidales* s and *Salmonella*, *E. coli* O157:H7, or *Campylobacter*.

In contrast to the *Salmonella* and *E. coli* O157:H7 results, thermophilic *Campylobacter spp.* was detected in over 75% of the samples. The primer and probes that were used in this study were not specific to a certain pathogenic species. Rather, they detected a variety of thermophilic strains, such as *C. jejuni*, *C. coli*, *C. upsaliensis*, and *C. lari*, originating primarily from chickens (Lund et al., 2004). Although not tested, Lund et al. (2004) also noted that that *C. lanienae*, which is found in the gut of bovine species, should be detected by the assay because it also harbours binding sites for the primers and probes. *C. jejuni* is most often associated with birds, but it has also been associated with bovine fecal contamination (Hrudey et al., 2003). Detection of *Campylobacter* originating from bovine species would be more likely in TBW because there are no poultry farms in the area.

The results of the *Campylobacter* study are in contrast with the results of the *Salmonella* and *E. coli* study. Due to the enrichment step, quantification of the original *Campylobacter* concentration was not possible; samples were designated either positive or negative for bacteria presence. As there was such a high frequency of detection, no significant associations between *E. coli* or AllBac marker concentrations could be determined using logistic regression.

Although the frequency of *Campylobacter* (84.0%) is higher in samples that exceed the CCME guidelines for irrigational water use, a large number of samples that fall under the guidelines are were also positive for *Campylobacter* (64.6%). These results call into question the ability of the CCME guidelines to predict or warn of the risk to *Campylobacter* exposure.

The survival mechanisms of *Campylobacter* spp. are not well understood, however it has been suggested that the bacteria can persist in the environment for up to 4 months after being released from the host animal (Rollins & Colwell, 1986; Thomas et al., 1999). Deposition into the environment can cause morphological changes due to poorly understood stress responses, rendering the cells viable but nonculturable (VBNC); these responses appear to enable the cells to survive cold, UV, and aerobic stresses for extended periods of time (Murphy et al., 2006). The high frequency of *Campylobacter* detection could be caused by the ability of the bacteria to persist. If this is the case, host-specific *Bacteroidales* markers that are indicative to recent fecal contamination would not be a useful indicator to warn against *Campylobacter* presence.

5.5 WATER QUALITY PARAMETERS

Measurements of the water quality parameters were conducted to gain an overall understanding of the stream environment. Parameters such as temperature, conductivity, DO and pH remained consistent throughout the study period and did not appear to have an affect on the FIOs or marker concentrations. The turbidity of the water is often affected by increased precipitation (Curriero et al, 2001). In fact, turbidity did appear to have a positive relationship with the flow rate of the stream. Not surprisingly, as precipitation and flow rates increased, the turbidity of the water also increased as streambed sediment was disrupted and particulate from the surface water runoff was deposited into the stream. Although not as strong as the relationship with flow, both the *E. coli* and AllBac marker concentrations showed a positive relationship with turbidity. The concentrations were elevated when turbidity was high. This fits with the persistence hypothesis of both *E. coli* and AllBac because as the sediment is resuspended in the water

column the indicators also become resuspended and therefore are detected at higher concentrations. Along with sediment, the surface runoff would also contain bacteria and other organic matter, which would also increase the indicator concentrations. The ability of turbid water to protect organism from UV radiation could also be a factor in elevated concentration levels (Wyer et al., 2010).

5.6 USE OF *BACTEROIDALES* AS A TOOL IN WATER QUALITY MANAGEMENT

The potential for the host-specific *Bacteroidales* assays to be used as a tool for water quality management has been demonstrated in this study. With strategic planning of sampling sites and a basic understanding of the potential sources of pollution within the watershed, it would be possible to implement these assays to aid in assessing sources of microbial pollution in a rural setting, especially with respect to bovine and ruminant fecal pollution. In an impacted watershed, such as TBW, these assays could be useful in first determining what kind of BMPs would be beneficial to the watershed in question by providing information on the source of the microbial water pollution. After implementation of the BMPs, these assays would continue to be useful in monitoring the watershed to determine if the BMPs are having a positive impact on water quality.

Furthermore, the assays could be used in instances of isolated fecal contamination to aid in source identification and mitigation of the problem; however, currently there are too many unknown variables for this method to be used as an alternate indicator for fecal water pollution. Knowledge gaps are exposed as research into the MST field continues but addressing these issues could lead to more powerful assays that may one day be used as stand-alone indicators of microbial water pollution, or in conjunction with other new or traditional indicators of fecal water pollution.

CHAPTER 6. CONCLUSIONS

6.1 PROJECT SUMMARY

The Thomas Brook Watershed in Nova Scotia presented a unique opportunity to conduct a MST study. The watershed is very small and has well defined pollution sources, which made it an ideal location to conduct a preliminary MST study to determine if this method is applicable for use in rural agricultural watersheds. Assays targeting the *Bacteroidales* 16S rRNA genes were chosen after careful consideration of the land use information in the watershed. The universal AllBac marker was used to monitor the non host-specific *Bacteroidales* in the watershed. The ruminant-specific BacR and bovine-specific CowM2 assays were chosen to detect any microbial pollution originating from the dairy farm operation located upstream of Stn 2, the two beef farms located upstream of Stn 4, and the manure amended crop at Stn 6. The human-specific BacH marker was chosen to detect microbial pollution due to the residential cluster upstream of Stn 3.

Using target and non-target host feces collected from in and around the TBW, the AllBac, BacR and CowM2 markers were validated. The sensitivity and specificity of the AllBac (97.3% and 100%, respectively), BacR (94.4% and 93.9%), and CowM2 (88.9% and 100%) markers were high and confirmed that they are valid for use in this study. Samples were collected from local septic systems in order to validate the BacH marker. Although the specificity (91.9%) was comparable to the other assays, the sensitivity (64.3%) was lower than expected. It is likely that the temporal nature of individual septic systems influenced these results. Dilution of the marker by the high volume of water used by a household could negatively impact the value of this marker for use as an indicator of

microbial water pollution originating from faulty septic systems. It is also possible that the BacH marker degrades within the septic tank.

Baseline water quality monitoring of the watershed confirmed that the *E. coli* concentrations at every station, except the headwater, exceeded the CCME guideline of 100 CFU/ 100 mL for irrigational water. This routine monitoring also revealed that the AllBac marker was essentially ubiquitous and, therefore, would not be an appropriate indicator for fecal water pollution. Detection of the BacR and CowM2 markers during baseline monitoring revealed that in the rare instances where a host-specific marker was detected, it was primarily during times of increased precipitation and flow rates.

Storm event monitoring provided insight into the effects of heavy rainfall (> 20 mm/ day) on flow rates and marker concentrations in the TBW. The BacR and CowM2 markers were detected only at sites hypothesized to be affected by bovine fecal contamination. Increased surface water runoff during heavy rainfall presumably carried the microbial contamination into the stream system, which allowed for detection of the ruminant and bovine markers. Followup monitoring revealed a sharp decline or disappearance of the host-specific markers on the recession limb of storm hydrographs. These results support the use of the host-specific markers as indicators of recent fecal contamination.

Detection of the BacR and CowM2 markers at Stn 2, Stn 4, and Stn 6 supported the hypothesis that the dairy farm at Stn 2, the beef farms at Stn 4, the manure amended crop at Stn 6 would have an impact on water quality. Marker detection at Stn 5 was likely due to a combination of the pollution sources at the watershed outlet. Neither marker was detected at Stn 3, for which there were no identified bovine fecal contamination sources.

The BacH marker was detected only once during the study period. The low sensitivity of the marker could have an affect on the ability of the assay to detect fecal water pollution originating from faulty septic systems. It is also possible that the majority of the septic systems in the watershed were functioning properly during this study and, therefore, there was very little fecal contamination to be detected.

Associations between host-specific markers and pathogen presence could not be identified. Neither *Salmonella* nor *E. coli* O157:H7 were detected during the study. Conversely, the *Campylobacter* marker was detected so often that it was not possible to discern a statistical relationship between the presence of this pathogen and indicator organisms (*E. coli* or *Bacteroidales* markers)

6.2 FUTURE DIRECTIONS

The use of *Bacteroidales* 16S rRNA genes as a tool for MST has great potential. As with any emerging field, there are knowledge gaps that need to be addressed in order to further assess these assays for use in watershed scale studies.

Although there are studies that have investigated the persistence of some host-specific markers, more information regarding the survival of these markers in the environment under various circumstances still needs to be addressed. Information on the persistence of pathogens or pathogenic markers should also be investigated and compared to the persistence of the host-specific *Bacteroidales* markers. Furthermore, an assay that has the ability to distinguish between viable and non-viable bacteria would be beneficial when seeking information on recent fecal contamination events. Finally, this information could be combined to strengthen existing assays or create new assays that can be used in microbial source tracking. Information on the persistence of markers originating from

both viable and non-viable *Bacteroidales* would be useful to determine which assay would better reflect the survival rates of enteric pathogens in the environment.

Further investigation into the cross-reactivity of the AllBac marker with *Bacteroidales* of environmental and insect origin is important in understanding the data already collected, and may help explain the persistent nature of the marker. Development of a universal *Bacteroidales* marker that exclusively detects bacteria originating from the gut of warm-blooded animals could provide more informative results for MST studies.

Once the foundations of the MST field have been strengthened, many more host-specific markers can be developed that target many different potential microbial pollution sources. Assessment of mixed land-use areas that are home to various wild, domestic, and agricultural animals would benefit from a large database of host-specific markers.

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