AN EVALUATION OF NEW AND TRADITIONAL APPROACHES TO MONITOR

DRINKING WATER QUALITY IN BRITISH COLUMBIA

by

Corinne Andrea Krentz

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Abstract

Microbiological monitoring of drinking water is a critical element in the source-to-tap framework. This research provides an assessment of traditional and alternative approaches to drinking water monitoring, explores new applications for existing indicators and evaluates a new tool to manage fecal contamination in water.

British Columbia (BC) Drinking Water Protection Regulation requires that drinking water samples be tested at an accredited laboratory. This creates challenges related to accessibility for some systems. The objective was to evaluate the agreement between indicator bacteria test results obtained with the current approach and an alternative approach using a presence/absence test close to the point of sample collection. Samples were collected from 83 small systems in the South Cariboo, BC. The agreement measured using Cohen's kappa was moderate to substantial for total coliforms (0.64 ± 0.11) and *E. coli* (0.73 ± 0.20).

The value of monitoring total coliforms and *E. coli* in parallel is a topic of current debate. The objective was to evaluate the potential for non-*E. coli* total coliform events to predict *E. coli* occurrence in subsequent drinking water samples. Life table analysis of microbiological testing data from small systems in BC showed that systems with a positive non-*E. coli* total coliform result were twice as likely to observe an *E. coli*-positive result in a subsequent water sample compared to systems that had not tested positive for total coliforms previously (RR=2.04).

Routine drinking water samples containing indicator bacteria are not analyzed further to investigate sources of fecal contamination. The objective was to develop a tool to identify fecal contamination sources in BC drinking water and evaluate the tool using water samples with evidence of fecal contamination. Markers associated with human, livestock and wildlife contamination were selected. Singleplex and multiplex polymerase chain reaction (PCR) was used to test contaminated raw water and drinking water samples for presence of host-associated markers. Low sensitivity of the multiplexed reaction limits its use to detect levels of markers present in contaminated drinking water samples. Singleplex PCR using host-associated markers is a promising tool to identify fecal contamination sources in small volumes of raw and drinking water.

Preface

The research presented in chapter 3 was conducted in partnership with the Interior Health Authority. It required and received a certificate of approval for a minimal risk study from the UBC Behavioral Research Ethics Board (H11-00382). Approval was also granted from the Interior Health Authority Research Ethics Board (File#2011-009).

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List of Symbols and Abbreviations

ATP	Adenosine Triphosphate
ATCC	American Type Culture Collection
BC	British Columbia
BLAST	Basic Local Alignment Search Tool
BP	Base Pairs
CFU	Colony Forming Unit
CPRG	Chlorophenol Red β-D-Galactopyranoside
DDH$_2$O	Double Deionized Water
FST	Fecal Source Tracking
G	Gram
GAL	β-D-Galactopyranoside
GLU	β-D-Glucopyranoside
GLUC	β-D-Glucuronide
HRS	Hours
IBDG	Indoxyl-β-D-Glucuronide
IHA	Interior Health Authority
IPTG	Isopropyl-β-D-galactoside
L	Litre
M	Molar
MPN	Most Probable Number
MST	Microbial Source Tracking
MUG	Methylumbelliferyl-β-D-Glucuronide
MUGAL	4-Methylumbelliferyl-β-D-Galactopyranoside
NCBI	National Center for Biotechnology Information
ONPG	Ortho-Nitrophenyl-β-D-Galactopyranoside
PCR	Polymerase Chain Reaction
PHO	Provincial Health Officer
PMA	Propidium Monoazide

PFGE	Pulsed Field Gel Electrophoresis
PPHMRL	Provincial Public Health Microbiology Reference Laboratory
QMRA	Quantitative Microbial Risk Assessment
ROS	Reactive Oxygen Species
SE	Standard Error
T_a	Annealing Temperature
TC	Total Coliforms
T_m	Melting Temperature
TMAO	Trimethylamine-N-Oxide
TTC	2, 3, 5 Triphenyltetrazolium Chloride
US EPA	United States Environmental Protection Agency
UV	Ultraviolet
VBNC	Viable But Non-Culturable
α	Confidence level for statistical analysis

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Last but certainly not least, I owe a world of thanks to my family and to Stewart. It has been a long haul and I will always be grateful to you for being there for me the entire time.

Chapter 1: Introduction

Clean water is a basic human necessity. In Canada there is an abundant supply of freshwater that is sufficient to meet the quantities demanded for agricultural, industrial and domestic purposes even though many major water bodies flow north toward sparsely populated areas. Total withdrawals represent a mere 1% of water resources in Canada (Gleick 2003). However, provision of water that meets health-based standards for drinking remains a challenge in many rural areas of the country where disinfection treatment is absent or inadequate and is of particular concern as the demand for clean drinking water continues to increase (Levin et al 2002).

Enteric diseases continue to be prevalent in developed countries despite the availability of drinking water disinfection technologies. According to Health Canada's estimates unsafe water is responsible for 90 deaths and 90,000 illnesses in Canada each year (Sierra Legal Defense Fund 2006). Safe drinking water is defined as water that meets the Guidelines for Canadian Drinking Water Quality for harmful chemicals and microbiological contaminants and poses no significant health risk to consumers (Health Canada 2010).

Drinking water systems that apply a multi-barrier approach can better ensure that drinking water is safe when it reaches the consumer, as no single barrier can adequately address the range of hazards that may be present in water. Multi-barrier or "systems-based" strategies are commonly used to reduce waterborne hazards through use of critical control points (Barron et al 2002, Jagals and Jagals 2004). These principles were derived from the hazard analysis and critical control points system, which is widely used in the food production industry. When applied to provision of safe drinking water, critical control points are identified as any potential points of contaminant entry from source to tap. Barriers or

controls placed at these points aid in risk mitigation by either reducing the probability of

contaminant entry or physically removing or inactivating waterborne hazards that are present.

Types of barriers include source water protection; adequate treatment to reduce chemical and

microbiological hazards; prevention of quality degradation during distribution; an evaluation

process to ensure that each element is performing properly; and effective response activities

to address adverse water quality events (Havelaar 1994, Krewski et al 2004). The multi-

barrier framework is illustrated in Figure 1.
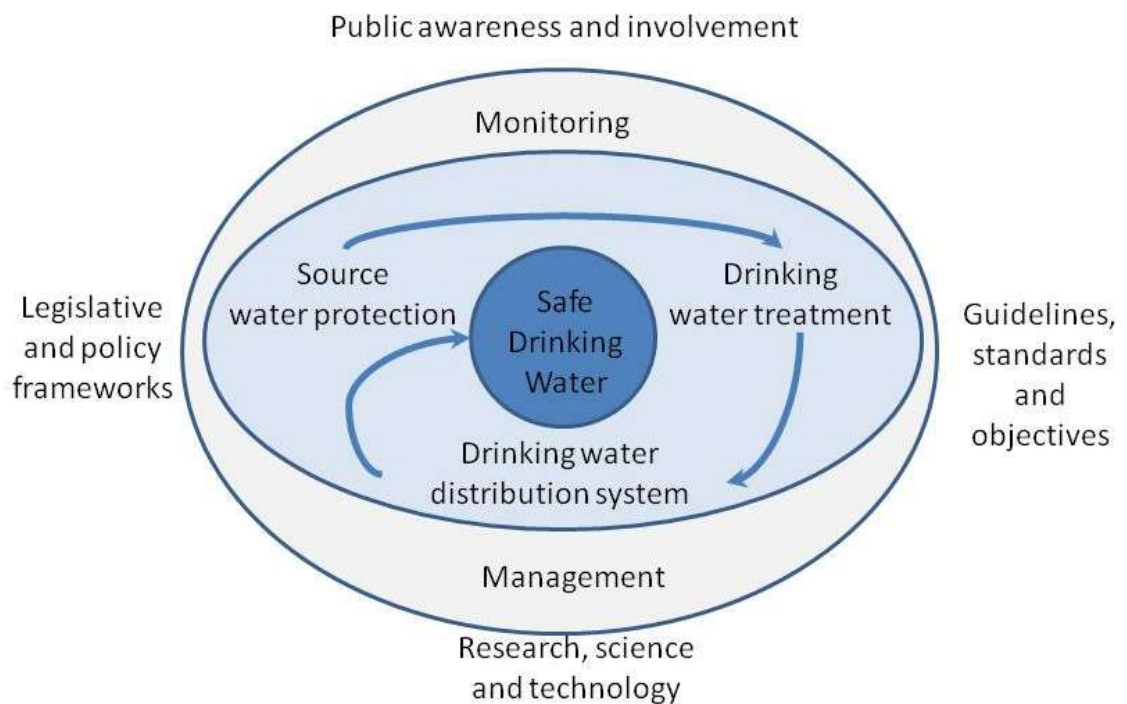
Figure 1. Multi-Barrier Framework for Safe Drinking Water. This holistic framework
involves several elements that contribute to provision of safe drinking water.
(Adapted from Kendall 2007)

Monitoring is a critical element in the safe drinking water framework. It serves as a means to determine how well source-to-tap barriers are functioning through provision of information about changes in water quality. This includes evaluating performance of treatment systems to remove and/or inactivate microorganisms, and testing water at the consumer's tap to indicate whether microorganisms of human health significance might be present. From a public health perspective, monitoring is a means to identify emerging water quality issues that can in turn be used to implement response measures to minimize adverse public health outcomes from consumption of unsafe drinking water. In support of this important role, many developed nations have implemented legislation that requires monitoring of a suite of human health relevant chemical and microbiological parameters to protect public health. While both chemical and microbiological agents in water have risks associated with their ingestion, the acute risk posed by microorganisms of health significance outweighs that posed by chemical compounds at the levels found in drinking water in most systems (Ashbolt 2004).

Ideally, drinking water would be tested in real-time for every microorganism of human health significance to identify waterborne threats. In reality, technologies to simultaneously detect all human pathogens likely to be found in water are not yet at the stage of development that would enable their widespread use for routine drinking water testing. Although important research advances in concentration and detection technologies have been made, an approach that uses surrogate indicators to evaluate microbial drinking water quality is still used internationally.

The concept of using bacterial surrogates to indicate fecal contamination was introduced in 1891 (Tallon 2005). Culture-based methods were used to selectively grow the group of

bacteria that were observed to be present in human and non-human feces. This group was collectively referred to as the total coliform group and contains many species including *Escherichia coli*. Their presence in water has been used to indicate poor water quality from a microbiological perspective. The presence of *E. coli* is a more specific indicator of recent fecal contamination.

Since the initial discovery of the association between total coliform bacteria and fecal contamination, a great deal of evidence has emerged that points out limitations with the use of total coliforms as surrogates. A desirable indicator should be present when pathogens are present and not detected when pathogens are absent. Indicator bacteria are generally more readily inactivated by chlorine-based disinfection treatments compared to protozoa and viruses (Hoff and Akin 1986, USEPA 1999), so water with no detectable viable bacteria may in fact contain other microbial pathogens. Conversely, several species of bacteria from the total coliform group have been isolated from non-fecal environments including plants. In light of these findings, many jurisdictions have removed total coliforms from the list of required testing parameters and only require testing for *E. coli*, which is considered a more suitable indicator as it is almost exclusively fecal in origin (Edberg et al 2000, Leclerc et al 2001).

While it is clear from previous studies that total coliform presence alone is not a reliable indicator of pathogen presence (Tallon et al 2005), some evidence suggests that it may be a more sensitive indicator than *E. coli*. Total coliforms are more abundant in human feces than *E. coli*, and are present at higher densities in treated and untreated water compared to fecal coliforms (Payment et al 1985). In a preliminary evaluation of microbiological monitoring data, cases were observed where total coliforms tended to occur in drinking water days or

weeks before *E. coli* were detected. These cases are discussed in Chapter 4. In this thesis research, the hypothesis that drinking water systems with prior non-*E. coli* total coliform bacteria events have a greater likelihood of detecting *E. coli* in future water samples was tested.

An additional measure to gauge the microbiological quality of drinking water in a region is the proportion of systems with boil-water notices in effect, which reflects the number of water systems with inadequate or no disinfection treatment. In 2008, 530 drinking water systems in British Columbia (BC) were on a boil-water notice (Eggerston 2008). These notices mainly affect small to medium sized systems (Figure 2), in particular small systems in the Interior region of BC (Kendall 2008). The regional boundaries of BC's five health authorities are shown in Figure 3.

Multiple lines of evidence support the claim that small systems are more susceptible to contamination than medium or large municipal systems that serve many people. Larger systems have the resources to implement source water protection plans, construct treatment facilities and conduct frequent water quality testing throughout the distribution system. Smaller systems typically face challenges related to staffing, financing, access to approved testing facilities and often have more difficulty meeting drinking water quality standards (Shanaghan 1994, Adams et al 2005, Kendall 2008). The unmet treatment needs of small systems can also be attributed in part to lack of affordability of disinfection technologies on a small scale. Cretikos et al (2010) and Rosen et al (2009) found that small-sized systems had a higher incidence of *E. coli* in drinking water samples than larger drinking water systems. Wilson et al (2009) reported more waterborne outbreaks occur in populations served by small drinking water systems, than in communities of more than 1000 people. A preliminary
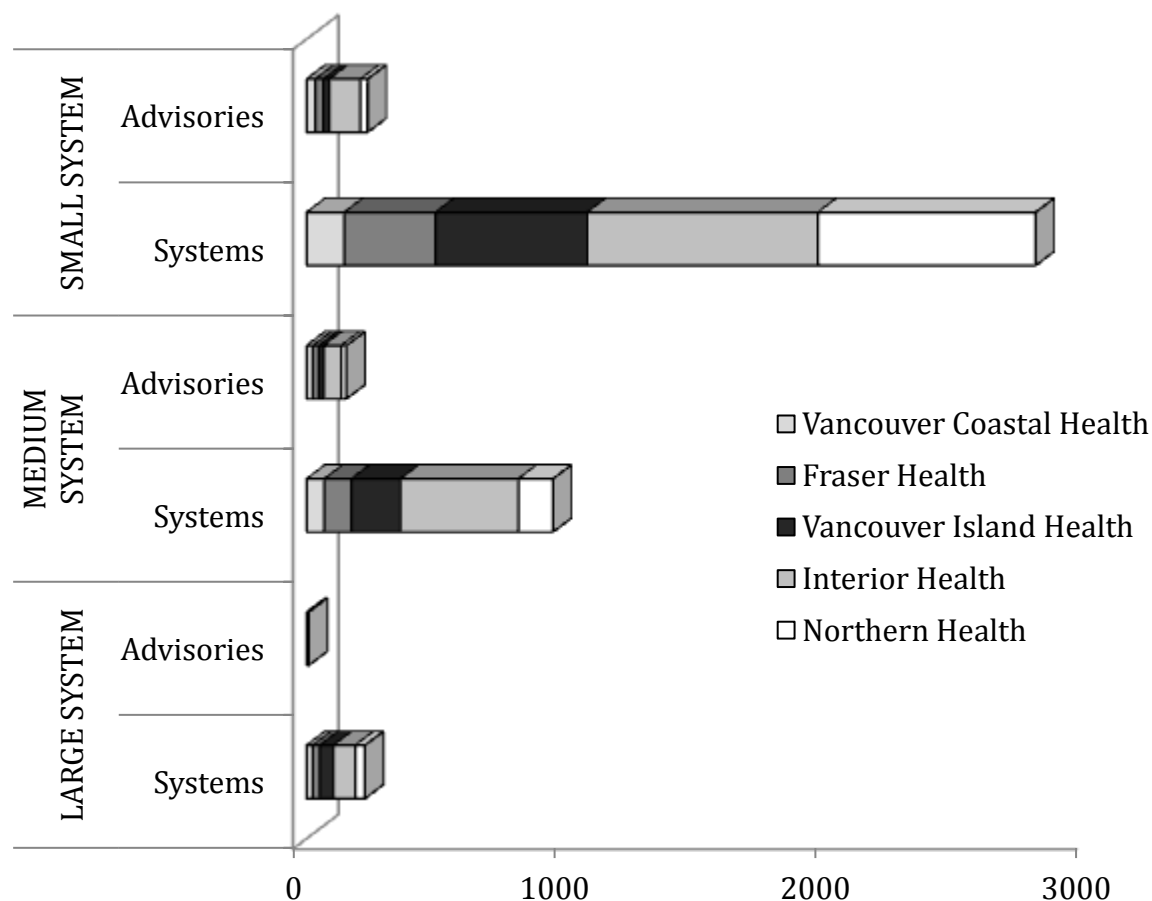
Figure 2. Boil-Water Notices in British Columbia According to System Size and Region. Small systems are defined as having fewer than 15 connections; medium systems have 15 to 300 connections and large systems have more than 300 connections.

analysis of drinking water system data in BC revealed that small systems also have higher rates of non-compliance with monitoring requirements, compared to larger-sized systems. In some regions of BC, more than half of the small systems did not meet microbiological monitoring requirements in 2009. Given that people served by small water systems are at higher risk of consuming microbiologically unsafe drinking water than those served by large municipal systems, it is particularly important that purveyors of small water systems monitor water quality regularly and receive timely test results.
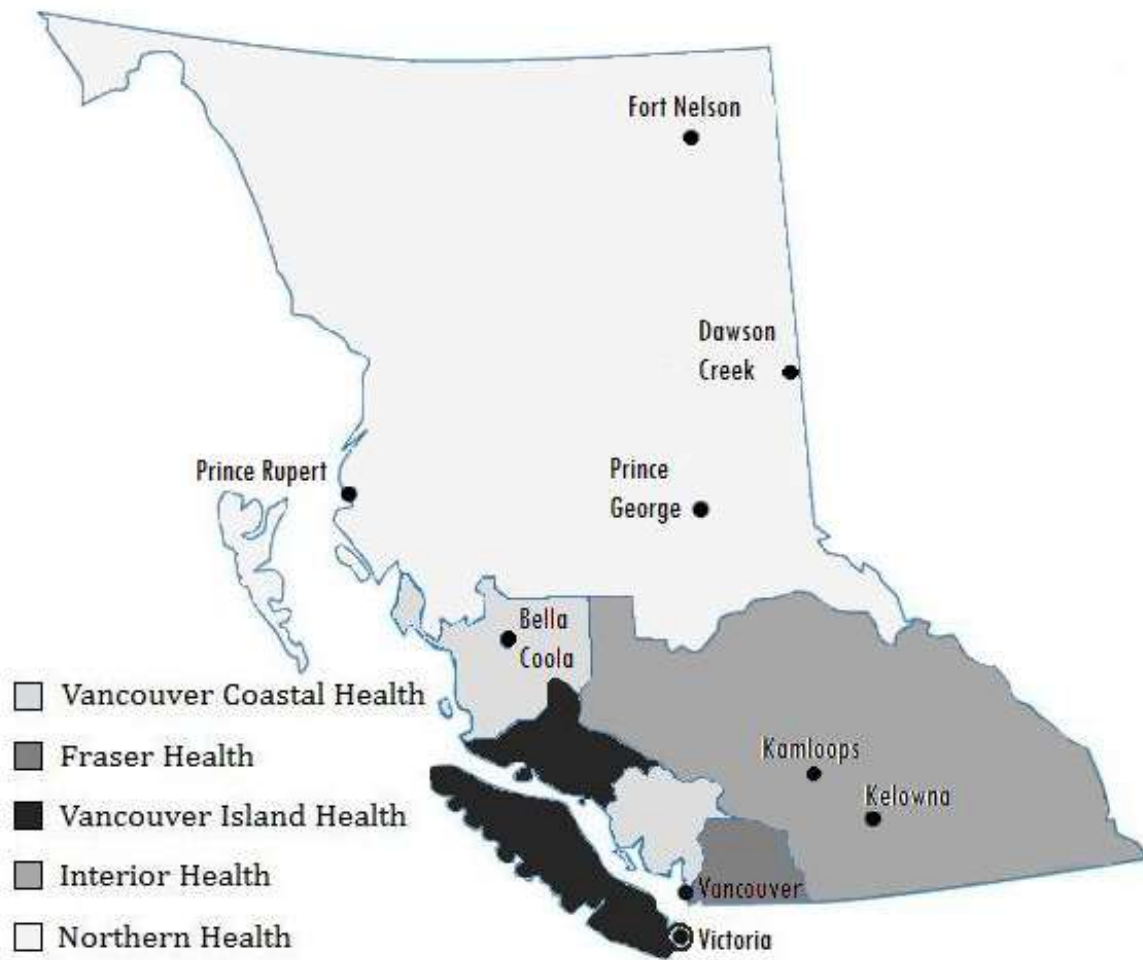
Figure 3. Boundaries of the Five Regional Health Authorities in British Columbia.
(Adapted from Kendall 2011)

The 2008 Provincial Health Officer's (PHO) Report identified that systems located in rural

areas face difficulty with regard to accessing microbiological testing services in a timely

manner. The current approach to microbiological water monitoring in BC involves

transporting samples to a PHO-approved laboratory, which may take up to 30 hours from the

time of sample collection. A qualitative method to test water samples near the point of

collection has potential to provide testing results faster than the current testing approach.

However, no studies have investigated the agreement between testing approaches in a field

setting where variables associated with transport and differences in testing method may have an effect on results. An objective of this thesis research was to evaluate the use of a qualitative method to monitor drinking water for total coliform bacteria and *E. coli* in a region of BC with a high density of small drinking water systems.

The current microbiological testing framework is heavily reliant on end-point testing for identification of water quality problems. Under most circumstances when there is a water sample positive for indicator bacteria no further testing is performed to investigate the source of contamination. This is largely due to a lack of validated tools and a paucity of data on their application to contaminated drinking water samples. Host-associated markers of fecal contamination have been identified previously for animals that are common water polluters in North America including humans, cattle, swine, poultry, deer, dogs and wild birds. An objective of this thesis research was to develop a multiplex assay to test contaminated water samples simultaneously for host-associated markers and to evaluate the assay in the field using heavily contaminated water samples from known sources, raw water samples and drinking water samples.

The overall aim of this collective work was to identify tools and evaluate approaches to better monitor and manage the risk of acquiring acute gastrointestinal illness from consumption of drinking water. This work contributes to the growing toolbox for investigating the source of fecal contamination in water. Identification of fecal pollution source(s) is important as it improves understanding of contamination sources impacting a watershed and may lead to enhanced drinking water quality, through the development of better management practices and protection policies.

1.1 Organization of Thesis

Chapter 2 provides background information on drinking water quality standards, monitoring practices and policies in Canada and BC and presents a literature review of current tools used in drinking water monitoring and management. An overview of the impacts of drinking water-related illnesses in BC is also provided.

Chapter 3 describes the field study conducted in the South Cariboo, BC to evaluate the comparability of two approaches to test drinking water for presence of indicator bacteria using different enzyme/substrate methods and sample holding conditions. This work was conducted in collaboration with Interior Health Authority Officials and was approved by both the University of British Columbia Research Ethics Board and the Interior Health Authority Ethics Board. This chapter addresses the following research questions:

1. What is the level of agreement for the detection of total coliform bacteria and *E. coli* in drinking water samples tested 23-29 hours after collection using a culture-based enzyme/substrate test compared to samples tested less than 7 hours after collection using a qualitative enzyme/substrate test?

2. Which factors (*i.e.,* testing method, holding temperature, holding time) have a significant effect on the agreement of total coliform and *E. coli* results?

Chapter 4 examines the predictive value of non- *E. coli* total coliforms in water from small distribution systems in BC. The relative risk of detecting *E. coli* in a distribution system given prior incidences of non-*E. coli* total coliforms was determined. The general value of total coliforms as a water quality indicator was discussed. The following research question was investigated:

1.   Is the probability of obtaining an *E. coli*-positive result from a drinking water system

     given that one or more prior samples have tested positive for non- *E. coli* total

     coliforms greater than the probability of obtaining an *E. coli*-positive test result from a

     system given that no prior samples have tested positive for total coliform bacteria?

Chapter 5 presents the results of a study that investigated the prevalence of host-associated

markers of fecal contamination in the feces of species that are common water polluters in BC

(*i.e.*, humans, cattle, pigs, horses, deer, chickens, seagulls, Canada geese). The utility of

polymerase chain reaction (PCR) tests to detect host-associated markers in water with

various degrees of fecal contamination was also evaluated. Research questions were as

follows:

1.   Which of the host-associated markers tested are prevalent in feces of host groups from

     the Lower Mainland, BC and not detectable by PCR in the feces of non-host species?

2.   Are detectable levels of these host-associated markers present in (i) environmental

     waters that are heavily contaminated with fecal pollution (ii) raw water and (iii)

     inadequately treated drinking water using multiplex PCR and singleplex PCR?

Chapter 6 provides a discussion of the significance of the research findings and describes

how they fit into the current water quality monitoring framework. Conclusions and

recommended areas of further research are presented.

Chapter 2: Literature Review

It has long been recognized that water plays a role in the transmission of disease (Snow 1855). Microbial pathogens that spread through human populations via the fecal-oral route can use water as a vehicle to reach susceptible hosts. Waterborne pathogens in drinking water are estimated to cause 19.5 million cases of illness in the United States each year (Reynolds et al 2008). No estimates of the disease burden due to waterborne agents implicated in acute gastrointestinal illness in Canada were available, but the incidence rate of acute gastrointestinal illness from any exposure route is 1.3 episodes per person-year in BC, Canada (MacDougall et al 2008).

The purpose of this chapter is to provide an overview of agents implicated in waterborne outbreaks and endemic acute gastrointestinal illness and to review the impacts of waterborne disease in BC. The pathways by which pathogens are transmitted including their zoonotic potential are discussed, and the tools used to identify and manage microbial risks to human health from consumption of unsafe drinking water are reviewed.

2.1 Agents Involved in Waterborne Illnesses

A total of 1,415 human pathogens have been identified (Taylor et al 2001), and those bacterial, viral and protozoan species associated with water-related disease in North America are shown in Table 1. Protozoa with plausible waterborne transmission routes that have rarely been implicated in waterborne outbreaks such as *Acanthamoeba* spp., *Balantidium coli*, *Blastocystis hominis*, Microsporidia and *Naegleria fowleri* were not included in Table 1 (Karanis et al 2007). Many human pathogens transmissible via water cause acute gastrointestinal illness, while others may target specific organs including the lungs and liver.

Table 1. Waterborne Human Pathogens and the Diseases They Cause [a]

Microorganism	Classification (Primary or Opportunistic)	Primary Route of Entry	Illness
Bacteria			
Aeromonas hydrophila	Opportunistic	Ingestion	Gastroenteritis
Arcobacter butzleri [b]	Opportunistic	Ingestion	Gastroenteritis
Campylobacter jejuni [b]	Primary	Ingestion	Campylobacteriosis (gastrointestinal)
Shiga toxigenic *Escherichia coli*	Primary	Ingestion	Gastroenteritis and colitis
Helicobacter pylori [b]	Opportunistic	Ingestion	Gastritis; infection associated with increase in risk of developing ulcer and stomach cancer
Legionella pneumophila [b]	Opportunistic	Inhalation	Legionellosis (respiratory)
Mycobacterium avium and *M. bovis*	Opportunistic	Inhalation Ingestion	Tuberculosis (respiratory)
Plesiomonas shigelloides	Primary	Ingestion	Gastroenteritis
Salmonella enterica [b]	Primary	Ingestion	Salmonellosis (gastrointestinal)
Shigella sonnei [b]	Primary	Ingestion	Shigellosis (gastrointestinal)
Vibrio cholerae	Primary	Ingestion	Cholera (gastrointestinal)
Yersinia enterocolitica	Primary	Ingestion	Yersiniosis (gastrointestinal)
Protozoa			
Cryptosporidium spp.	Primary	Ingestion	Cryptosporidiosis (gastrointestinal)
Cyclospora cayetanensis	Primary	Ingestion	Cyclosporiasis (gastrointestinal)
Entamoeba histolytica [b]	Primary	Ingestion	Amoebiasis (gastrointestinal)
Giardia spp. (Assemblage A or B)	Primary	Ingestion	Giardiasis (gastrointestinal)
Isospora belli	Opportunistic	Ingestion	Isosporiasis (gastrointestinal)
Toxoplasma gondii	Opportunistic	Ingestion	Toxoplasmosis
Viruses			
Human Adenovirus [b]	Opportunistic	Inhalation or Ingestion	Infections of upper respiratory tract; gastroenteritis
Astrovirus [b]	Primary	Ingestion	Gastroenteritis
Caliciviruses (includes Norovirus)	Primary	Ingestion	Gastroenteritis

Microorganism	Classification (Primary or Opportunistic)	Primary Route of Entry	Illness
Enterovirus [b] (includes polioviruses, coxsackieviruses and echoviruses)	Primary	Inhalation or Ingestion	Upper respiratory illness
Hepatitis A virus	Opportunistic	Ingestion	Infection of the liver
Hepatitis E virus	Opportunistic	Ingestion	Infection of the liver
Rotavirus	Primary	Ingestion	Gastroenteritis

[a] Adapted from the Contaminant Candidate List 3 (US EPA 2009)
[b] Representative of a group of closely related microorganisms

Acute gastrointestinal illness is caused by ingestion of contaminated drinking water or recreational water (*i.e.*, lakes, swimming pools), while respiratory illness is the result of infection from inhalation of water particles carrying infective microorganisms.

Most waterborne illness cases present with mild symptoms and are self-limiting in that immunocompetent individuals often clear their infection without seeking medical attention (*i.e.*, *Cryptosporidium*). Others such as *E. coli* O157:H7 infections are more severe, have a high hospitalization and case-fatality rate relative to illnesses caused by other waterborne pathogens (Mead et al 1999, Scallan et al 2011) and require medical support or treatment. Fatalities associated with consumption of unsafe water are relatively rare in developed countries and usually afflict vulnerable subpopulations. These include young infants and people with other existing medical conditions that affect the immune system (Provincial Health Services Authority 2009). Impaired innate or humoral immune defenses make individuals more susceptible to infection from opportunistic pathogens. Examples of opportunistic microorganisms commonly associated with respiratory infections include *Legionella* spp. and *Mycobacterium* spp.

2.2   Outbreaks of Waterborne Disease

Waterborne outbreaks can involve multiple types of infectious agents. The etiology of 288 waterborne disease outbreaks that occurred between 1974 and 2001 in Canada is shown in Figure 4. Many of the unidentified agents were suspected to be norovirus based on the incubation period and patient symptoms (Yoder et al 2007).

One of the most well-known waterborne outbreaks that has occurred in Canada was the Walkerton, ON outbreak which took place in 2000 (Hrudey and Hrudey 2007). The bacterial agents responsible for an estimated 2,300 illnesses and seven deaths were *E. coli* O157:H7 and *Campylobacter jejuni* (Hrudey et al 2003). This outbreak was a key driving force behind implementation of integrated water management plans to better ensure the provision of safe drinking water in the province of Ontario, Canada.
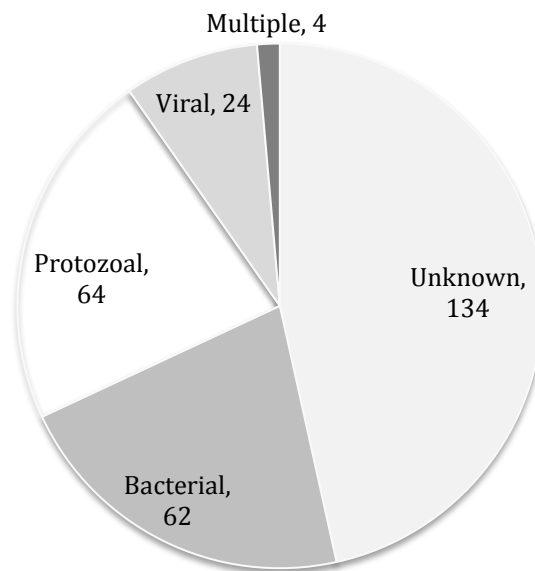
Figure 4. Etiology of Waterborne Disease Outbreaks in Canada from 1974-2001. (Adapted from Schuster et al 2005)

Documented outbreaks of waterborne disease in BC are relatively infrequent today. No

outbreaks were reported in BC between 2005 and 2007 (Kendall 2008). Certainly in the

1980's and 1990's many waterborne outbreaks occurred throughout the province (Isaac-

Renton et al 1993, Sierra Legal Defense Fund 2003, Hrudey and Hrudey 2007), but

improvements in water treatment and management have contributed to a substantial

reduction in the frequency at which community wide waterborne outbreaks occur. However,

the rate of enteric illness is still of concern with thousands of cases reported in BC each year

(Provincial Health Service Authority 2011). Rates of reportable enteric diseases in BC from

1996 to 2011 are shown in Figure 5.
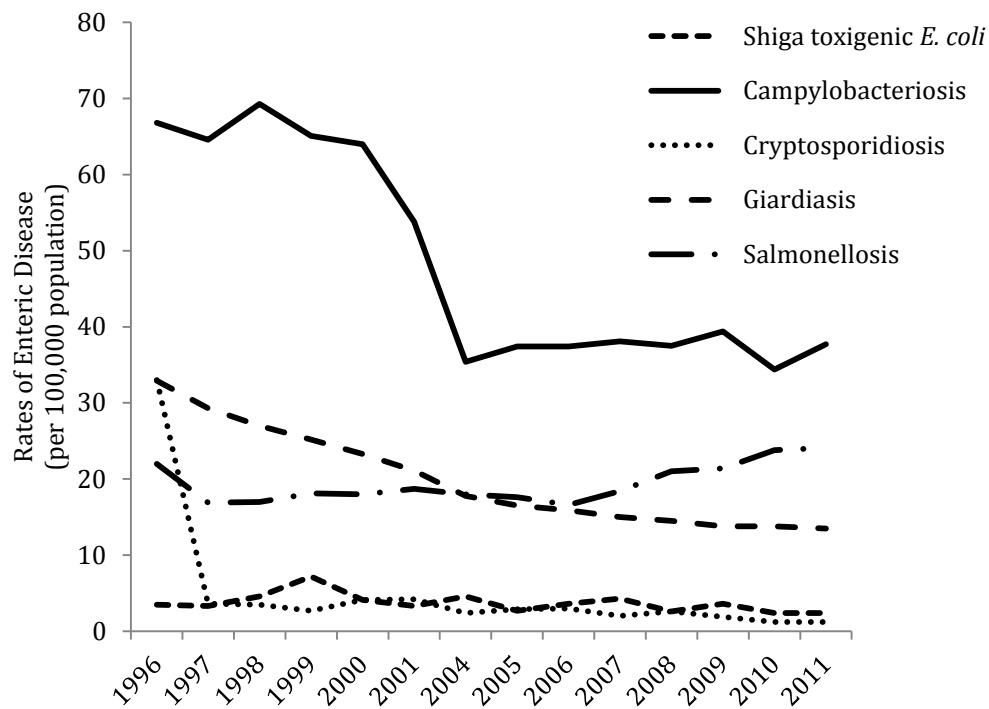
Figure 5. Rates of Reported Enteric Illnesses in British Columbia from 1996 to 2011.
(Adapted from Provincial Health Services Authority 2011)

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2.3 Estimates of Enteric Illness Associated with Water Consumption

Human pathogens that spread through the fecal-oral route can be transmitted by one or more exposure pathways including direct person-to-person contact, direct animal-to-person contact or consumption of contaminated food and water. Certain microorganisms are more commonly associated with either foodborne or waterborne transmission. For example, protozoan pathogens such as *Cryptosporidium* and *Giardia* are the causative agents of most waterborne disease outbreaks in Canada and cryptosporidiosis and giardiasis are not usually linked with consumption of contaminated food (Hall et al 2005). In comparison, bacterial pathogens such as *Campylobacter* spp., *Salmonella* spp. and Shiga toxigenic *E. coli* are more commonly associated with contaminated food (Mead et al 1999, Hall et al 2005).

The proportion of each reportable enteric illness that is attributable to food has been estimated for several groups of microorganisms in previous studies (Mead et al 1999, Hall et al 2005). While a similar analysis has not been conducted for water-related illnesses, the transmission routes for pathogens such as *E. coli* O157:H7, *Cryptosporidium* and *Giardia* have been evaluated in separate studies. Estimates of the proportion of *E. coli* O157:H7 (Rangel et al 2005) infections attributable to water in the United States between 1982 and 2002 was 18%. An investigative analysis of reported cases of cryptosporidiosis in Ontario, Canada found that water was the probable source of infection for 48% of cases (Majowicz et al 2001). Greig et al (2001) investigated the source of infection for 8,347 cases of giardiasis in Ontario and found that water was the most probable source in 62% of cases.

Reliable source attribution information for campylobacteriosis and salmonellosis cases was not available. Hall et al (2005) reported that 67-83% of the campylobacteriosis cases investigated in Australia were foodborne. It was assumed that a negligible number of cases

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involving *Campylobacter* spp. were attributable to person-to-person or animal-to-person contact because the infectious dose of *Campylobacter* is relatively high (Allos 2001). Hall et al (2005) estimated that 81-93% of salmonellosis cases were foodborne. It was assumed that the rate person-to-person transmission of *Salmonella enterica* was similar to that of *E. coli* (8%) estimated by Rangel et al (2005), as these pathogens share common characteristics. The proportions of campylobacteriosis and salmonellosis attributable to consumption of contaminated water were estimated by taking the remaining proportion of illness that was not accounted for by food or direct person-to-person contact.

The water-attributable proportions were used to calculate the average number of annual cases of enteric illness in BC that were due to consumption of water contaminated with *Campylobacter* spp., *Cryptosporidium*, *Giardia, Salmonella* spp. and Shiga toxigenic *E. coli* (Table 2). An estimated 21-32% of enteric illness cases in BC that were caused by these enteric pathogens were associated with contaminated water. This agrees with the results of a randomized intervention trial conducted by Payment et al (1991) who found that 35% of gastrointestinal illness cases were from consumption of water.

2.4 Impacts of Waterborne Illness in British Columbia

Waterborne illness directly impacts the well-being of affected individuals, but also presents an economic burden to the Canadian healthcare system and/or sick individuals and caregivers. Impacts of waterborne disease can be measured in a given region using incidence rates of acute gastrointestinal illness acquired through the waterborne exposure route, or economic costs associated with caring for individuals with illnesses acquired through water. It is important to quantify impacts of waterborne illness in order to evaluate the effectiveness of intervention strategies such as water treatment, source protection or new legislation.

Table 2. Estimated Annual Cases of Waterborne Illnesses in British Columbia

	Average Annual Cases Reported 2007-2011	Proportion of Cases Attributed to Water	Average Annual Cases Associated with Water	Reference
Campylobacteriosis	1664	0.17 - 0.33	283 - 549	Hall et al 2005
Cryptosporidiosis	79	0.48	38	Majowicz et al 2001
Shiga toxigenic *E.coli* Infection	136	0.18	24	Rangel et al 2005
Giardiasis	627	0.62	390	Greig et al 2001
Salmonellosis	970	0 - 0.11	0 - 107	Hall et al 2005
Total	3476		745 - 1109	

Acute gastrointestinal illness acquired through any pathway incurs costs associated with treatment and hospitalization (*i.e.*, physician visits, diagnostic laboratory tests, medication, travel) and missed employment for sick individuals and caregivers. Henson et al (2008) estimated that the economic burden of acute gastrointestinal illness in BC was CAN$514 million annually. The annual cost to treat cases of waterborne illnesses in BC is currently unknown in part due to the lack of information regarding the number of cases of acute gastrointestinal illnesses that were acquired from exposure to contaminated water. The cost to treat illnesses caused by waterborne pathogens are significant, as suggested by a study conducted in the United States; this study estimated USD$539 million is spent annually to treat diseases caused by three waterborne pathogens (Beach et al 2010).

Using the rates of reportable communicable illnesses in BC (PHSA 2011) and the estimated cost to treat a sick individual made by Henson et al (2008), an estimated CAN$1.0-1.5 million is spent annually in BC to treat the 745 to 1109 cases of acute gastrointestinal illnesses that were acquired from exposure to contaminated water (Table 2). This approximation is likely an underestimate due to underreporting, lack of inclusion of waterborne illnesses with viral etiology or illnesses caused by opportunistic pathogens that are acquired by ingestion of water or inhalation of water aerosol containing pathogens.

2.5   Waterborne Transmission of Zoonotic Pathogens

Some types of pathogens can cause infection in both non-human vertebrate animals and humans. These microorganisms are referred to as zoonotic pathogens and can be transmitted to humans via direct contact with infected animals or contaminated objects, ingestion of contaminated food or water, inhalation of aerosols containing the pathogen or via an insect vector. According to the World Health Organization expert group on waterborne zoonoses

(WHO 2004), waterborne zoonotic pathogens will continue to be a public health concern in coming years due in part to increasingly intensive cattle, swine and poultry farming operations to meet global demand for meat products (Létourneau et al 2010) and increasing domestic pet populations in developed countries. In addition, increases in international travel and global trade provide more opportunities for introduction of pathogens to non-endemic areas.

Many zoonotic pathogens are capable of surviving for extended periods in the environment and can contaminate groundwater or surface water. Thus, water used for drinking or recreational purposes that is impacted by human or animal fecal inputs poses a substantial health risk to humans. Assessment of risk associated with fecal contamination from a particular host requires an understanding of the pathogens of human health significance known to be associated with that host. The zoonotic potential of each bacterial, protozoan and viral human pathogen transmissible by ingestion of contaminated water or inhalation of contaminated water droplets was considered based on evidence presented in peer-reviewed literature. This information can be used to qualitatively evaluate the relative risk that fecal contamination from different groups of hosts poses to humans and to develop appropriate response plans. Host groups were defined as primates, livestock (*i.e.*, cattle, sheep, pigs, goats, poultry), wildlife (*i.e.*, deer, beavers, elk, bears, wild cats, raccoons), domestic pets and wild birds. Evidence of direct transmission between vertebrate animals and humans via consumption of contaminated meat was not included in Table 3.

Table 3. Waterborne Microbial Pathogens and Their Animal Hosts [a]

Microorganism [b]	Hosts					References
	Primate	Livestock [c]	Wildlife [d]	Domestic Pets [e]	Wild Birds [f]	
Bacteria						
Aeromonas hydrophila	Y	Y	N	N	Y	Gray 1984, Glünder and Siegmann 1989, Janda et al 2010
Arcobacter butzleri	Y	Y	UK	Y	UK	Van Driessche 2003, Collado and Figueras 2011
Campylobacter jejuni	Y	Y	N	Y	Y	Ogden et al 2009
Shiga toxigenic *Escherichia coli*	Y	Y	Y	Y	Y	Ferens and Hovde 2011
Helicobacter pylori	Y	N	N	N	N	Tanih et al 2010
Legionella pneumophila	Y	UK	UK	UK	Y	Clark 2003
Mycobacterium avium and *M. bovis*	Y	Y	Y	Y	Y	Biet et al 2005
Plesiomonas shigelloides	Y	N	UK	Y	UK	Arai et al 1980, González-Rey et al 2011
Salmonella enterica	Y	Y	Y	Y	Y	Quessy and Messier 1992, Renter et al 2006, Frye and Fedorka-Cray 2007, Compton et al 2008, Simpson 2008
Shigella sonnei	Y	N	N	N	N	Hale and Keusch 1996
Vibrio cholerae	Y	N	N	N	Y	Vezzulli et al 2010
Yersinia enterocolitica	Y	Y	Y	Y	Y	Fukushima et al 1984, Simpson 2008, HPA 2009, French et al 2010
Protozoa						
Cryptosporidium spp.	Y	Y	Y	Y	N	Xiao and Fayer 2008, Monis and Thompson 2003
Cyclospora cayetanensis	Y	N	N	N	N	Chacín-Bonilla 2010
Entamoeba histolytica	Y	N	N	N	N	Li and Stanley 2009

Microorganism [b]	Hosts					References
	Primate	Livestock [c]	Wildlife [d]	Domestic Pets [e]	Wild Birds [f]	
Giardia spp. (Assemblage A or B)	Y	Y	Y	Y	N	Xiao and Fayer 2008, Thompson 2004
Isospora belli	Y	N	N	N	N	Lindsay et al 1997
Toxoplasma gondii	Y	N	Y	Y	N	Aramini et al 1998, HPA 2009
Viruses [g]						
Human Adenovirus (dsDNA)	Y	UC	N	N	UC	Jogler et al 2006, Shin 2009
Astrovirus (ssRNA)	Y	N	N	N	N	Benedictis et al 2011
Caliciviruses (includes Norovirus) (ssRNA)	Y	UC	N	UC	N	Bank-Wolf et al 2010
Enterovirus (includes polioviruses, coxsackieviruses and echoviruses) (ssRNA)	Y	N	N	N	N	Shiroki et al 1997
Hepatitis A virus (ssRNA)	Y	N	N	N	N	Balayan 1992
Hepatitis E virus (ssRNA)	Y	Y	Y	N	N	Meng 2010
Rotavirus (dsRNA)	Y	Y	N	N	N	Martella et al 2010

[a] Abbreviations: UK unknown, no studies were found that investigated prevalence; UC uncertain, evidence to suggest biological plausibility is not convincing

[b] List adapted from US EPA Contaminant Candidate List 3

[c] Livestock—more than one case reported in at least one of the following: cattle, sheep, pigs, goats, poultry

[d] Wildlife— more than one case reported in at least one of the following: deer, beavers, elk, bears, wild cats, raccoons

[e] Domestic Pets— more than one case reported in dogs or cats

[f] Wild birds— more than one case reported in geese, seagulls, ducks

[g] Infection of non-human animals with virus recovered from humans, or human infection definitively linked with a non-human virus strain was taken as evidence of cross-species transmission.

2.5.1   Waterborne Bacterial Zoonoses

Many of the bacterial species listed in Table 3 have been recovered from a wide range of animal hosts. Prevalence rates varied for different hosts within a given category. In many cases there was a lack of studies regarding the prevalence of pathogens in wildlife and limited information on the prevalence of some bacterial pathogens such as *Plesiomonas shigelloides* in different host groups.

Aeromonas hydrophila is capable of causing infection in both immunocompetent and immunocompromised humans but is also known to infect fish (Janda et al 2010). *A. hydrophila* has been recovered from cattle, sheep and pig feces (Gray 1984) and from the feces of wild birds, particularly carnivorous birds with aquatic habitats (Glünder and Siegmann 1989). While *A. hydrophila* has been isolated from dogs and deer (Pal et al 1989, Andre-Fontaine et al 1995), these are not believed to be significant reservoirs. The high prevalence of *A. hydrophila* in fresh water (Janda et al 2010) suggests that water may play a significant role in spread of this bacterium to different hosts.

Arcobacter butzleri is considered a significant zoonotic pathogen (Collado and Figueras 2011) and is associated with enteritis in humans and animals. It has been isolated using culture-based methods from the colon of healthy domestic geese (Atabay et al 2008) and the feces of livestock including cattle (40%), sheep (16%) and pigs (44%) (Van Driessche 2003). Virulent strains have also been recovered from dog feces and poultry (Douidah et al 2012). The finding that *A. butzleri* is more prevalent in water with fecal contamination (Collado et al 2008) suggests that water is a plausible route of transmission for this pathogen, but to date no clinical isolates have been genetically matched to those obtained from suspected environmental sources (Collado and Figueras 2011).

Campylobacter jejuni is more commonly found in poultry than in other types of livestock (Ogden et al 2009, Soller et al 2010) and is prevalent in the feces of wild birds (Quessy and Messier 1992, Ogden et al 2009) suggesting that avian species are a significant reservoir for this pathogen. Limited information indicates that some wildlife such as deer do not harbor *C. jejuni* (Van Donkersgoed et al 1990). Although *C. jejuni* has been found in the feces of some cats and dogs, the low prevalence rate (less than 5%) suggests that domestic pets do not play a significant role in disease transmission to humans (Ogden et al 2009).

Shiga toxigenic *E. coli* is capable of causing infection in a wide variety of animals and in humans. Incidence is sporadic among populations of cattle and deer (Ferens and Hovde 2011). Occasionally Shiga toxigenic *E. coli* is isolated from domestic pets and wild birds and it is routinely observed in the feces of pigs (Ferens and Hovde 2011).

Helicobacter pylori is not considered a zoonotic pathogen as no animal reservoirs have been identified (Tanih et al 2010); however some experts speculate that it has zoonotic potential (Christou 2011). Handt et al (1994) isolated *H. pylori* from gastric tissue in cats but this does not provide sufficient evidence to implicate animals in its transmission pathway.

Few studies have investigated the prevalence of *Legionella pneumophila* in different host groups. Recovery of *L. pneumophila* from geese suggests that avian species may play a role in transmission (Clark 2003).

Both *Mycobacterium avium* and *M. bovis* cause infection in humans. Apart from cattle, *M. bovis* has been isolated from a wide range of other animals including bears, deer, pigs and raccoons (Biet et al 2005). While it is plausible for *M. bovis* to be transmitted via inhalation of contaminated water particles, there are no documented cases of human infection from an

environmental source and transmission is more likely via direct contact with infected animals or consumption of contaminated milk (Biet et al 2005). Birds are the main reservoir for *M. avium*, but livestock, wildlife and domestic pets can also host this pathogen (Biet et al 2005). The bacilli shed in the feces of carriers or infected animals can persist in the environment and contaminate drinking water supplies.

Plesiomonas shigelloides has been recovered from fish and is sometimes found in feces from cats and dogs (Arai et al 1980). A recent study demonstrated genetic similarity between *P. shigelloides* isolates from humans and cats (González-Rey et al 2011). Other studies have found substantial variability among *P. shigelloides* isolates from fresh water, humans and fish (Gu et al 2006). No livestock reservoirs have been identified (Abbey et al 1993) and the role of wildlife in the spread of the bacterium is not clear.

Salmonella enterica is a well-studied zoonotic pathogen that has been isolated from the feces of infected livestock (Frye and Fedorka-Cray 2007), raccoons (Compton et al 2008), seagulls (Simpson 2008), domestic pets and to a lesser extent wild deer (Renter et al 2006). Contaminated feces can enter water supplies via agricultural, urban and forest run-off.

Shigella sonnei has a narrow host range and is only known to cause infection in primates (Banish et al 1993, Hale and Kesuch 1996). Similarly, few reservoirs of *Vibrio cholerae* other than primates are known. Zooplankton has been identified as an environmental reservoir of *V. cholerae*, and the bacterium can persist in the guts of wild birds which may feed on the zooplankton (Vezzulli et al 2010).

Yersinia enterocolitica has been recovered from feces of hogs (Letourneau et al 2010), deer (French et al 2010), rodents (HPA 2009) and the gut of dogs (Fukushima et al 1984). Contaminated feed has been implicated in the spread of *Y. enterocolitica* to hogs (Létourneau

et al 2010). The extent to which water is involved in the transmission of *Y. enterocolitica* to humans is not known, but *Yersinia* spp. have been implicated in outbreaks of waterborne disease (Schuster et al 2005).

2.5.2   Waterborne Protozoan Zoonoses

Most protozoan pathogens listed in Table 3 are recognized as zoonotic pathogens (WHO 2004). There was limited information on the host range of some protozoan pathogens that are rarely implicated in waterborne illness in developed countries. *Cryptosporidium* and *Giardia* are the two most-well-studied protozoan pathogens and there has been much taxonomic evolution since their initial discovery to better distinguish the species and subspecies that have been recovered from various hosts from one another. With this new knowledge it has become clear that different species or genotypes preferentially infect particular hosts but may cause infection in related hosts to a lesser extent.

Some *Cryptosporidium* spp. have broad host ranges and many species and genotypes are infectious to humans; however *C. hominis* and *C. parvum* are the species responsible for most human infections (Xiao and Fayer 2008). The host range of *C. hominis* includes primates, cattle, sheep and pigs. The bovine genotype (also known as *C. parvum* genotype II) has been recovered from ruminants and humans. The cervine genotype can infect humans but is more commonly associated with cattle, sheep, deer and beavers (Xiao and Fayer 2008). *Cryptosporidium canis* and *C. felis* can infect dogs and cats respectively, but these species are rarely isolated from human feces (Xiao and Fayer 2008). Taken together, these findings suggest that livestock and wildlife play a greater role in disease transmission to humans than domestic pets.

Not all subtypes of *Giardia duodenalis* are capable of causing infection in humans and the types capable of causing infection in humans are not prevalent in some non-human animal hosts (Xiao and Fayer 2008). *G. duodenalis* Assemblage A and B can infect humans. Assemblage A has been found in cattle, horses, deer, moose and domestic pets while Assemblage B has been found in sheep, cattle, horses, beavers and dogs (Xiao and Fayer 2008). *Giardia* species that infect avian hosts such as *G. ardeae* and *G. psittaci* are not infective to humans (Xiao and Fayer 2008).

Primates are the only known reservoirs for *Cyclospora cayetanensis*, *Entamoeba histolytica* (Verweij et al 2003) and *Isospora belli* (Lindsay et al 1997). Recent studies have found *C. cayetanensis* oocysts in dog feces (Chacín-Bonilla 2010) and *Isospora belli* in dog feces in India (Traub et al 2002) suggesting potential for cross-species transmission of these parasites. A rare case of *E. histolytica* infection in a wallaby has also been reported (Stedman et al 2003).

Toxoplasma gondii utilizes feline hosts to complete the sexual stage of its life cycle; therefore domestic cats (HPA 2009) and cougars (Aramini et al 1998) are significant reservoirs for this pathogen. Oocysts shed in feline feces can enter water supplies where they can infect intermediate hosts including humans, rodents (HPA 2009), birds (Cabezón et al 2011), pigs (Hill et al 2010) and deer (Simpson 2008). Water is mainly implicated in the transmission of oocysts to intermediate hosts, whereas the spread of *T. gondii* among intermediate hosts occurs primarily via consumption of contaminated tissue.

2.5.3   Waterborne Viral Zoonoses

Few of the human pathogenic viruses that are transmissible via contaminated water have been recovered from non-human hosts. Of the viruses listed in Table 3, only hepatitis E is considered zoonotic (HPA 2009, Christou 2011) but evidence suggests that some waterborne viruses have zoonotic potential (Muller and Johne 2007, Barry et al 2008, Bank-Wolf et al 2010).

Studies investigating cross-species transmission of the viruses shown in Table 3 varied in terms of the strength of evidence provided to make a convincing case for spread of human viruses to animals or animal virus strains to humans. Demonstrating substantial sequence similarity (>95%) between virus strains isolated from human and non-human animals was taken as moderate evidence of potential to infect other species. Showing *in vitro* that a human virus was capable of infecting a non-human animal cell line was also considered moderate evidence, as was recovery of strain-specific antibodies from serum. Research that definitively linked human infections with a virus from an animal source or demonstrated that a human virus strain caused infection in an animal model was considered strong evidence of cross-species transmission.

Viruses from the *Adenoviridae* family can infect humans, cattle (Sibley et al 2011), ducks, goats, sheep, deer, reptiles and horses (Cavanagh et al 2012). The potential for human adenovirus to infect non-human cells has been studied in a porcine kidney primary cell line (Jogler et al 2006) and an avian embryonic cell line (Shin 2009). This provides preliminary evidence that human adenovirus can cross species barriers at the cellular level, but *in vivo* experiments using animal models are required to investigate the interplay between host defenses and viral attack. Human adenovirus is prevalent in sewage and can persist in treated

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wastewater (Carducci et al 2009). Release of this pathogen into the environment provides an opportunity for human adenovirus to come in contact with other potential hosts.

Currently, there appears to be low potential for astroviruses (Benedictis et al 2011), enteroviruses (Shiroki et al 1997) and hepatitis A (Balayan 1992) virus to cross species barriers from humans to animals or vice-versa.

Caliciviruses such as norovirus are frequently found in human sewage, but are inactivated by treatment with chlorine (Petrinca et al 2009). This helps prevent their spread in the environment. One study demonstrated that multiple enteric virus strains including norovirus have been found together in bivalve molluscs (Vilariño et al 2009), which provides an opportunity for genetic recombination to occur via co-infection in humans that consume contaminated molluscs. Animal noroviruses discovered in cattle, sheep and rodents have low sequence similarity with human norovirus, while high sequence similarity exists between porcine and human strains (Scipioni et al 2008). To date, animal norovirus strains have not been detected in human feces but recovery of antibodies against animal norovirus in humans and antibodies against human norovirus in pigs further supports plausibility of cross-species norovirus transmission. Recently, a novel strain of norovirus was recovered from the feces of dogs with and without diarrhea (Mesquita et al 2010, Summa et al 2012). Close interactions between dogs and humans suggest that zoonotic transmission is plausible.

Hepatitis E is a recognized zoonotic pathogen. Food and/or water may be involved in transmission of hepatitis E (Tei et al 2003, Sailaja et al 2009). Four genotypes that infect humans have been identified: Genotypes 1 and 2 are exclusive to humans and 3 and 4 are found in humans and other animals. The vast majority of hepatitis E genotypes isolated from

humans in developed countries are 3 or 4 (Lu et al 2006), indicating that animals may play a significant role in spread of disease to humans. Non-human reservoirs for hepatitis E include cattle, deer, swine, birds and rabbits (Krawczynski et al 2011, Shukla et al 2011). Examples of experimental cross-species infections include successful infection of rhesus monkeys with swine hepatitis E (Meng et al 1998), and unsuccessful infection of rhesus monkeys with an avian strain (Huang et al 2004). These studies suggest that pigs may play a more significant role in transmission to humans than birds. New sequence homology evidence indicates that transmission between swine and humans has likely occurred in China (Liu et al 2012). Lui et al (2012) observed that sequence homology between viruses isolated from swine and humans was 96-98%.

Rotaviruses are considered potential zoonotic pathogens (Martella et al 2010). Serogroups A, B and C contain strains that are of clinical significance in humans, while serogroup A is most often associated with infections in animals. Reservoirs for serogroup A, B and C rotaviruses include chickens, cattle, pigs, sheep dogs and horses (Martella et al 2010). Experimental evidence for cross-species transmission includes successful infection of rabbits inoculated with a rhesus monkey rotavirus strain (Ciarlet et al 2000), and induction of diarrhea in piglets inoculated with a human rotavirus strain (Azevedo et al 2005). A high degree of genetic similarity between rotavirus strains isolated from humans and domestic pets has also been demonstrated (Martella et al 2010). Taken together, this suggests that water supplies contaminated with animal feces may contain rotavirus but the health risk this poses to humans depends on a number of factors.

Viruses have unique abilities that increase their potential to cross inter-species barriers. Viral genomes can evolve at a rapid rate which is favorable for adaption and can affect the host

range of the virus. Genetic changes in viral genomes can occur via point mutation and reassortment events when cells are co-infected with different viral strains. Given the high degree of similarity between viral strains isolated from different hosts and the potential for viruses to evolve, it seems reasonable that some viruses have potential to cross species barriers and infect new host species. Previous studies illustrated that porcine viruses have high potential to cross species barriers and infect humans, and human viruses have high potential to infect pigs (Martella et al 2010, Meng et al 2010, Bank-Wolf et al 2011). However, it is clear that water supplies contaminated with human sewage present a much greater risk of viral infection for humans relative to water contaminated with feces from other sources.

2.6 Tools for Identification of Microbial Risks

It is not technically feasible to test every drop of water consumed for all plausible waterborne human pathogens to verify that the water is microbiologically safe for consumption. Instead, small volumes of water are collected and tested that are assumed to be representative of the water quality in a drinking water system. Some measurements for physical and chemical parameters can be taken online.

Physical, chemical and microbiological measures can be used to indicate water safety. Turbidity increases have been associated with higher risk of waterborne pathogen presence in some systems (Aramini et al 2000), but the relationship between an increase in colloidal particles in water and an increase in risk of gastrointestinal illness needs to be assessed on a system-by-system basis (Allen et al 2008). Extreme weather events, such as heavy rainfall, have been associated with increased risk of waterborne illness in some instances (Thomas et al 2006). In terms of chemical indicators, free and/or total chlorine residual in a distribution

system provides a measure of water safety for large systems that chlorinate or chloraminate drinking water. However this measure is not applicable for most small systems, which do not have chlorination treatment. Testing for viable indicator bacteria that are associated with fecal contamination is a widely-accepted approach to assess microbiological risks from consumption of water.

2.6.1   Microbial Indicators Used to Monitor Water Safety

Since the early 1900s, total coliform bacteria and *E. coli* have been used as indicators or surrogates for human pathogen risk in drinking water and thus to indicate microbiological water safety. *E. coli* is a member of the total coliform group and is generally regarded as a more reliable indicator of fecal contamination than total coliforms (Edberg et al 2000, Leclerc et al 2001). Unlike most total coliforms, *E. coli* is not prevalent in the natural environment and is almost exclusively fecal in origin (WHO 2004, Leclerc et al 2001). To illustrate the benefits and limitations of using indicator bacteria, the following section will describe the desirable traits of a good indicator and discuss how using total coliforms and *E. coli* as surrogates meets or does not meet these criteria.

An ideal indicator for water safety should be detected when pathogens are present and not detected when pathogens are absent. In order to meet this requirement, the survival and/or growth kinetics of the indicator in the environment as well as the inactivation rate of indicators during disinfection treatments should be similar to that of pathogens likely to be present in water.

Survival/growth rates are affected by several factors including temperature, salinity, dissolved oxygen levels (LeChevallier et al 1996, Roslev et al 2004), presence of other

microorganisms (Hahn and Höfle 2006), available nutrients, concentration and type of chemical disinfectants present and exposure to sunlight. Physiological responses of microorganisms will affect their ability to tolerate and survive under a given environmental condition, and the stress responses of indicator bacteria and other types of microorganisms will differ. Depending on the strain and environmental conditions *E. coli* can survive in water for days to weeks, yet the time required for a 99% decrease in the initial concentration of viable cells can be as short as one day (Nwachuku and Gerba 2008). *E. coli* may thrive in favorable environmental conditions and has been shown to proliferate in warm water reservoirs (Power et al 2005) and soil (Ishii and Sadowsky 2008). A recent study investigating the relative persistence rates of bacterial pathogens and adenovirus did not find a significant difference in decay rate constants for *Salmonella* spp. and adenovirus (Bae and Wuertz 2012). Protozoan survival in aquatic environments is more prolonged (Toze et al 2010) due to their physiological structure which makes them more robust against environmental stresses. Viral pathogens require a host to replicate and some protozoan pathogens require a host to complete their lifecycle, so these pathogens are unlikely to multiply in aquatic environments.

Indicator bacteria are inactivated at a faster rate than some viruses and protozoan pathogens upon exposure to disinfection treatments including chlorine-based chemicals (Hoff and Akin 1986). Protozoan cysts and oocysts are inherently more resistant to chemical disinfectants due to the thick protective coating of the (oo)cyst. Since this protective layer is lacking for non-spore forming bacteria, greater log reductions in viable bacterial indicators are obtained during water treatment with chlorine-based disinfectants. Upon irradiation with ultraviolet light indicator bacteria and most protozoan (oo)cysts are inactivated at a similar rate (Hijnen

et al 2006), but some viruses such as adenovirus require a much higher dose to achieve the same level of reduction (US EPA 2003).

For the surrogate to be present when human pathogens are present, it should be highly prevalent in human feces. Both *E. coli* and *Bacteroides* are recovered 100% of the time from human feces, but the vast majority of bacteria present in the human large intestine are strict anaerobes (Sekirov et al 2010). Together, bacteria from the phyla Firmicutes and Bacteroidetes account for 80% of the bacteria in the colon (Eckburg et al 2005) while the relative abundance of *E. coli* in the human colon is only 1% (Leclerc et al 2001). Presumably these anaerobic bacteria would be better candidates to use as indicators of fecal contamination than enteric bacteria. However, anaerobic bacteria are more difficult to grow on nutrient media and their survival in the environment is limited as they are not well-adapted to tolerate atmospheric levels of oxygen (Wexler 2007).

An ideal indicator for water safety should also be detected quickly using standardized methods that are suitable for routine use. Bacteria from the total coliform group can be easily detected. A variety of enzyme-substrate based methods and methods that use membrane filtration followed by plating on selective media are commercially available to simultaneously detect total coliform bacteria and *E. coli*.

2.6.2   Methods to Detect Total Coliforms and *E. coli*

Traditionally, the total coliform group was defined as facultative anaerobic Gram-negative bacteria capable of lactose fermentation and production of acid and gas after 48 hours at 35°C. These culture-based methods detected total coliforms and *E. coli* based on their ability to grow in selective nutrient media. As new methods to detect total coliforms and *E. coli*

more rapidly were developed, the definition of which species were considered to be members of the total coliform group changed. These methods detected total coliforms and *E. coli* based on presence of functional enzymes specific to total coliforms and *E. coli* that modify chromogenic and/or fluorogenic substrates. Consequently, total coliforms were defined as bacteria capable of modifying a particular substrate such as ortho-nitrophenyl-β-D-galactopyranoside (ONPG) using β-galactosidase and *E. coli* as being able to utilize substrates specific to β-galactosidase and β-glucuronidase. One of the roles of β-galactosidase in the cell is to cleave lactose into its component sugars, galactose and glucose. When grown on nutrient media in which lactose is the sole carbon source, each bacterial cell will contain thousands of copies of this large protein.

Enzyme/substrate methods can be qualitative (*i.e.*, presence/absence) or quantitative. Enumeration of total coliforms and *E. coli* may be performed using membrane filtration followed by culture of captured bacteria or using liquid culture in a most probable number (MPN) format. Enzyme/substrate assays that have been approved for drinking water testing have detection limits of one CFU of total coliforms and *E. coli* per 100 mL. While 92.4% of environmental *E. coli* isolates (Martins et al 1993) and 66% of clinical *E. coli* isolates demonstrated β-glucuronidase activity (Chang et al 1989), fecal contamination in water is likely to be from multiple hosts and would contain many strains of *E. coli*. Presumably at least one of these strains would produce functional β-glucuronidase. Studies that have investigated the potential to reduce analysis time for qualitative methods by increasing sensitivity have found that using instruments to measure assay endpoint in a shorter incubation period resulted in a substantial reduction in assay specificity (Van Poucke and Nelis 1997).

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The specificity of β-glucuronidase and β-galactosidase activity to detect total coliforms and *E. coli* was discussed in recent reviews (Rompré et al 2002, Fiksdal and Tryland 2008). While β-glucuronidase activity by methylumbelliferyl-β-D-glucuronide (MUG) hydrolysis has also been observed in some species of *Shigella, Salmonella* and *Yersinia* (Feng and Hartman 1982, Kämpfer et al 1991), these bacteria are also of fecal origin and are clinically significant. One study reported β-glucuronidase activity by *p*-nitrophenyl-β-D-glucuronide hydrolysis in *Citrobacter* and *Enterobacter* species (Pérez et al 1986), while Ralovich et al (1991) found only one false-positive isolate in 971 non-*E. coli* total coliform species tested using MUG hydrolysis. Rice et al (1990) also did not observe β-glucuronidase activity in *Enterobacter* or *Citrobacter* spp. using MUG substrate. Differences in substrate specificity may explain the discrepancy in results. β-glucuronidase activity has also been reported in anaerobic *Corynebacteria* and *Bacteroides* spp. (Dahlén and Linde 1985), but this is of little significance given that these bacteria will be selected against if cells are cultured in an aerobic environment, as would be the case if detection of total coliforms and *E. coli* were of interest. False-positive *E. coli* results due to β-galactosidase activity in some marine bacteria species have been reported (Pisciotta et al 2002) and this warrants use of alternative indicators for assessment of marine waters. However, this is not of concern from a drinking water testing perspective in Canada, where freshwater resources are plentiful and marine water is not treated for potable uses.

In recent years, the market has become flooded with many commercial enzyme/substrate tests used to simultaneously detect total coliforms and *E. coli* in drinking water (Table 4). These tests take 18 to 24 hours to complete, which is a substantial reduction in analysis time compared to traditional lactose fermentation methods. These enzyme/substrate tests use

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Table 4. Commercially Available Enzyme/Substrate Tests to Detect Total Coliforms and *E. coli* in Water [a]

Test Name	B-Galactosidase Substrate to Detect Total Coliforms	B-Glucuronidase Substrate to Detect *E. coli*	Additives to Aid in Total Coliform Detection	Agent to Inhibit Non-Total Coliforms	Manufacturer
Liquid Media—Presence / Absence or Most Probable Number Format					
Colilert®	ONPG	MUG	Not disclosed	Not disclosed	IDEXX Laboratories Inc. (USA)
Colisure®	CPRG	MUG	Not disclosed	Not disclosed	IDEXX Laboratories Inc. (USA)
Colitag	ONPG	MUG	TMAO for pH control	Undisclosed growth inhibitor for *Aeromonas* and *Pseudomonas*	CPI International (USA)
E. colite	X-Gal	MUG	Not disclosed	Not disclosed	Charm Sciences Inc. (USA)
m-Coli Blue 24	TTC	X-Gluc	Not disclosed	Not disclosed	Hach/Millipore (USA)
Readycult Coliforms 100	X-Gal	MUG	IPTG	Not disclosed	EMD Chemicals Inc. (USA)
Solid Media—Membrane Filtration, Selective Plating					
Brilliance *E. coli*/ Coliform Agar	Salmon-Gal	X-Gluc	None	None	Oxoid (UK)
C-EC-MF-Agar	X-Gal	MUG	IPTG	None	Biolife (Italy)

Test Name	B-Galactosidase Substrate to Detect Total Coliforms	B-Glucuronidase Substrate to Detect *E. coli*	Additives to Aid in Total Coliform Detection	Agent to Inhibit Non-Total Coliforms	Manufacturer
ChromAgar ECC	Salmon-Gal	X-Gluc	None	None	Chromagar (France)
ChromoCult®	Salmon-Gal	X-Gluc	Peptone, pyruvate, sorbit, phosphate buffer	Tergitol® 7 inhibits gram positive bacteria	EMD/Merck Laboratories (USA)
Coli ID	X-Gal	Salmon-Glu	Not disclosed	Not disclosed	bioMerieux (France)
Coliscan	Red-Gal	X-Gluc	Not disclosed	Not disclosed	Microbiology Laboratories (USA)
HiCrome ECC	Salmon-Gal	X-Gluc	None	None	Sigma-Aldrich (USA)
BBL MI Agar	MUGal	IBDG	None	Cefsulodin antibiotic	BD (USA)
Rapid *E. coli* 2	X-Gal	Salmon-Glu	Not disclosed	Not disclosed	Bio-Rad (USA)

(Adapted from Manafi 2000)

[a] Abbreviations: ONPG, Ortho-nitrophenyl-β-D-galactopyranoside; MUG, Methylumbelliferyl-β-D-glucuronide; TMAO, Trimethylamine-N-oxide; CPRG, Chlorophenol red β-D-galactopyranoside; TTC, 2, 3, 5 triphenyltetrazolium chloride; X-Gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; IPTG, Isopropyl-β-D-galactoside; Salmon-Gal, 6-chloro-3-indolyl-β-D-galactopyranoside; Red-Gal, 6-chloro-3-indolyl-β-D-galactoside; Salmon-Glu, 6-chloro-3-indolyl-β-D-glucopyranoside; MUGal, 4-methylumbelliferyl-β-D-galactopyranoside; IBDG, Indoxyl-β-D-glucuronide.

different substrate compounds, some media contain agents to inhibit growth of non-total coliforms and additives to enhance the growth and/or enzyme activity of total coliforms. Differences in media composition affect the comparability of results obtained using different methods and these variations in performance have been widely documented (Schets et al 1993, Manafi 2000, Olstadt et al 2007, Maheux et al 2008). While the identities of the chromogenic and fluorogenic substrates used in the various reagents are disclosed, the other components of the commercial reagents including additives that may be responsible for suppression of growth of non-total coliforms are proprietary. This further contributes to difficulty assessing the reasons behind observed differences in performance between reagent systems.

Additives to enhance enzyme activity include inducers isopropyl-β-D-galactoside (IPTG) and MetGlu. IPTG is a compound that increases transcriptional activity of the *lac* operon via a de-repression mechanism thereby increasing the amount of β-galactosidase produced (Tryland and Fiksdal 1998). MetGlu has been shown to increase β-glucuronidase activity in *E. coli* (Tryland and Fiksdal 1998). Transport of substrates into the cell can be improved by addition of sodium dodecyl sulfate (Manafi 2000). Enzymes such as catalases to neutralize reactive oxygen species have also been reported to enhance recovery of chlorine-stressed cells (Calabrese and Bissonnette 1990). Compounds such as trimethylamine-N-oxide (TMAO) may also be added to control pH. TMAO decomposes to generate alkaline products which counter the decrease in pH caused by the production of acid during lactose fermentation. Maintaining a slightly alkaline pH is important not only for cellular respiration, but also for optimal visualization of some fluorogenic enzyme products such as

4-MU that fluoresce in a pH-dependent manner (Chilvers et al 2001). Activity of β-galactosidase can also be enhanced by sodium lauryl sulfate (Berg and Fiksdal 1988).

Antibiotics such as cefsulodin can be added to agar to inhibit growth of some bacteria including *Pseudomonas* (Hussain et al 1991) and *Aeromonas* (Alonso et al 1996). Most *Pseudomonas* species are ONPG negative but growth can suppress detection of total coliforms. *Aeromonas* species are ONPG positive but until recently were not regarded as true members of the total coliform group (Edberg and Allen 1991a), as they are ubiquitous in the environment. Their role as human enteric pathogens is controversial as few outbreaks of diarrheal disease have been linked with *Aeromonas* (Abeyta et al 1986, Krovacek et al 1995). They have been isolated from stools of patients with gastroenteritis (Hofer et al 2006, Janda and Abbott 2010) and *A. hydrophila* is included in the United States Environmental Protection Agency (US EPA) Contaminant Candidate List (US EPA 2009).

Many of the bacteria from the Contaminant Candidate List shown in Table 1 are known to enter a viable but nonculturable (VBNC) state under stress conditions such as nutrient starvation, unfavorable growth temperature or oxidative stress (Oliver 2009). One limitation of culture based methods is their inability to detect viable but nonculturable bacteria. Cells that exhibit low levels of metabolic activity and are in a latent state will not proliferate and form colonies on standard culture media. Enzyme/substrate methods, on the other hand, are able to detect viable *E. coli* even when culturability is lost (Petit et al 2000). Other methods that are DNA-based or antigen/antibody based (Jurkevica et al 2010) have been developed to address the issue of detecting VBNC cells and further reduce analysis times.

Polymerase chain reaction (PCR) is a versatile approach to amplify nucleic acids from target microorganisms and can be designed to detect the genes that encode β-galactosidase and β-glucuronidase in total coliforms and *E. coli* respectively (Bej et al 1991, Fricker and Fricker 1994). However, traditional PCR methods are limited because they do not discriminate between live and dead cells. Reverse-transcriptase PCR which detects mRNA is one alternative technique that may be used to specifically target metabolically active cells. Some chemical agents such as propidium monoazide (PMA) have been proposed to selectively detect live cells using PCR (Nocker et al 2006, Gedalanga and Olson 2009). PMA molecules bind to extracellular double-stranded DNA and DNA of cells with breaches in membrane integrity. This modification renders the DNA unamplifiable by PCR; thus, only the DNA within viable cells is detected. While PCR methods offer an advantage in terms of analysis time, such methods are currently incapable of attaining a detection limit equal to that of culture-based methods (1 cell per 100 mL) for total coliform bacteria and *E. coli* (Juck et al 1996, Tantawiwat et al 2005, Kuo et al 2009). The specificity of primer sets used for total coliform and *E. coli* detection also requires improvement (Fricker and Fricker 1994, Tantawiwat et al 2005). For these reasons, PCR methods have not been approved by regulatory agencies to test drinking water for total coliforms and *E. coli* for the purposes of complying with drinking water regulations.

Methods that use partial or whole-cell recognition including immunoassay approaches are discussed in a review by Noble and Weisberg (2005). Equivalency of these methods with standard methods or a direct relationship with health risk has not been demonstrated, and they are prohibitively expensive and currently insufficiently sensitive to be feasible for routine monitoring of drinking water systems.

There are several practical challenges associated with developing better methods to detect pathogens in water. Detection of low concentrations of pathogens in drinking water requires a method that is highly sensitive, while use of the results to make public health decisions requires that it is highly specific. There are challenges associated with the recovery efficiency of protocols to concentrate large-volume water samples to the volume typically used for molecular analysis (*i.e.*, less than 1 mL). An additional challenge is to concentrate the microorganisms of interest without also concentrating compounds that interfere with the analytical measurement of the target agent.

2.7   Tools for Management

Human pathogens, including zoonotic microbes, can enter waterways from point sources such as sewage outfalls and non-point sources including agricultural, forest and urban run-off. Characteristics of the source water will affect the degree to which microbial contaminants present in run-off water can gain entry into the source water. For example, shallow groundwater wells and wells that are under direct influence of surface water are inherently more vulnerable to contamination from run-off than deep wells, where the aquifer is protected because it is located between two layers of impermeable material. Surface water including lakes and flowing supplies are generally considered to pose a high human health risk if consumed without disinfection treatment.

Source water protection is an important component of a holistic source-to-tap approach to reduce the risk that pathogens will be present in drinking water at the consumer's tap. Adoption of an integrated approach to risk assessment and risk management is increasing in the water sector (van den Hoven and Kazner 2009), and this necessitates the development of tools to identify sources of contamination that impact watersheds. Addressing contamination

problems at their source when possible, as opposed to relying on other barriers such as treatment to manage risks, will better ensure provision of safe drinking water at the consumer's tap. This relates to the critical control point concept described in Section 1, which aims to prevent entry of contaminants into a system.

In the event that a fecal contamination problem is detected using indicator bacteria, there are a number of tools available that researchers can use to further investigate the source of the fecal pollution. From a risk perspective, it may be sufficient to simply identify the source as human or non-human; but depending on the objective, in other cases such as to plan remedial actions it may be necessary to identify the specific sources of fecal contamination that impact a watershed.

2.7.1   Fecal Source Tracking

Fecal source tracking (FST) has become a well-established field in water management. It is concerned with identifying the particular host group responsible for fecal contamination in water by associating markers found at the site of contamination with those recovered from a suspected source. Contamination from human sources is generally considered to pose the greatest risk to human health, due to the wide array of pathogens that can be transmitted via human fecal waste. Thus, efforts to identify contamination source have largely focused on developing indicators of pollution from human sewage. Many chemical and biological indicators have been proposed to detect human-associated contamination. Presence of caffeine (Buerge et al 2003) and optical brighteners used in laundry detergents (Cao et al 2009) are some examples of chemical indicators used to detect human contamination in waterways.

Biological indicators of fecal contamination may be viral, prokaryotic or eukaryotic in nature. Microbial source tracking (MST) is the specific application of markers in microbes (*i.e.*, viruses, bacteria) to track a contamination source. Markers for human and non-human animal-associated contamination were summarized by Roslev and Bukh (2011).

The host-specific property of viruses makes them attractive candidates for source-association marker development studies. Markers using adenovirus and polyomavirus (Hundesa et al 2006, McQuaig et al 2006), enterovirus (Noble et al 2003) and norovirus (Wolf et al 2010) have been developed for human-specific strains and some non-human animal strains. To date, these markers have only been used to detect source-associated viruses in heavily contaminated liquids such as sewage and river water with fecal contamination. Improving method sensitivity is a challenge since the nucleic acids of human viral pathogens constitute a mere 0.1% of total nucleic acid material in biosolids (Bibby et al 2011). This is largely due to relative differences in genome sizes of viruses, and prokaryotic and eukaryotic cells.

The potential for commensal gut bacteria to be used as source-associated markers has been assessed in previous studies where candidate species included *Bifidobacterium* (Bonjoch et al 2004), *Enterococcus* (Ahmed et al 2008a), *E. coli* (Clermont et al 2008) and *Bacteroidales*. Obligate anaerobic bacteria belonging to the order *Bacteroidales* are promising targets as these cells are highly abundant in feces (Eckburg et al 2005), have demonstrated host-associated properties (Bernhard and Field 2000a) and are commensal bacteria in a wide range of animals. Genetic differences in the variable region of the 16S rRNA gene in *Bacteroides* species isolated from different hosts have been used in many studies to develop host-associated markers (Bernhard and Field 2000b, Dick et al 2005, Kildare et al 2007, Okabe et al 2007, Lu et al 2009, Fremaux et al 2010). The sensitivities and specificities of these

markers vary widely and no single marker has been demonstrated to be entirely host specific. Thus, the term "host-associated" is used here to describe the relationship between markers and their presumed hosts.

Other source-associated biological markers that are not part of host microbiota are the genetic material found in host epithelial cells. These exfoliated eukaryotic cells originate from the inner linings of the gut and are highly abundant (10^7 cells per gram) in human and cattle feces (Schill and Mathes 2008). Differences in mitochondrial DNA sequences from different hosts have been exploited to design host-associated markers that have been used to distinguish between fecal contamination from nine hosts (Schill and Mathes 2008).

2.7.1.1   Methods to Detect Source-Associated Markers

Methods to detect markers of source-associated fecal contamination typically involve several steps including capture of cells, culture or enrichment if necessary, purification of nucleic acid material and application of a test to determine the presence or quantity of source-associated marker. Approaches that do not require a cultivation step are advantageous, as they are more rapid and are not subject to selection bias. Many molecular methods are based on detection of one or more specific nucleic acid sequences and are culture-independent.

Capture of cells is commonly accomplished using filtration, as it allows for large volumes of water to be processed. Microbes may then be cultured on appropriate nutrient media, or nucleic acid from collected cells may be purified directly using a commercially available kit or a crude extraction method (*i.e.*, phenol/chloroform). The target marker sequence may be amplified using PCR and detected using gel electrophoresis or a fluorescent labeling system (*i.e.*, FAM, SYBR green). Simultaneous detection of multiple markers is possible using

multiplex PCR. Multiplex PCR has been used to detect host-associated viral markers (Wolf et al 2010), mitochondrial DNA markers (Schill and Mathes 2008) and human-associated 16S markers in *Bifidobacterium* (Bonjoch et al 2004). Marker approaches are considered library-independent because they do not rely on information in a database to identify the source of the contamination.

Library-dependent approaches require *a priori* characterization of microbes from all possible sources and involve matching the profile of the microbe from the contamination site to the profile of a microbe from the source. Methods that are used to generate microbial profiles include antibiotic resistance analysis (Ahmed et al 2008b), ribotyping (Carson et al 2003), pulsed field gel electrophoresis (Johnson et al 1994, Mullane et al 2007), denaturing gradient gel electrophoresis (Buchan et al 2001) and terminal restriction fragment length polymorphisms (Liu et al 1997). The relative advantages and disadvantages of these methods for FST in water are described in a review by Meays et al (2004).

2.7.2   Quantitative Microbial Risk Assessment

Quantitative microbial risk assessment (QMRA) is a tool that has emerged recently and is a component of water safety plans (Smeets et al 2010). The objective of QMRA is to estimate the human health risk posed by exposure to microbial pathogens through consumption of water from a given watershed at a given time. For example, Ahmed et al (2010) demonstrated how QMRA could be used to estimate the health risk from consumption of roof-harvested rainwater.

QMRA models take into account the types of pathogens present and their seasonal fluctuations in concentration and therefore require extensive characterization of the

watershed *a priori*. While this may be a fruitful exercise for large water systems that serve many thousands of people daily, for small water systems this is simply not feasible.

2.7.3   Other Management Tools

A number of water management tools have been developed for different purposes. These include guidance documents such as the *Comprehensive Source-to-Tap Assessment Guideline* to identify hazards and evaluate risks, water safety plans which are advocated by the World Health Organization, and best practice documents such as *Monitoring Water Quality in the Distribution System* to guide development of effective objective-oriented drinking water monitoring programs. Examples of programs that focus specifically on reducing microbial contamination of source water from livestock include the Environmental Farm Plan that promotes water quality protection using tools such as manure loading advisories to better protect water bodies surrounding agricultural areas from farmland run-off. In addition, the National Water Supply Expansion Program promotes off-site livestock watering to reduce fecal contamination in streams. Other programs that aim to improve water safety through capacity-building include Circuit Rider Programs to train and educate water purveyors in rural areas.

2.8   Drinking Water Systems in British Columbia

The province of British Columbia, Canada has approximately 4,900 regulated drinking water systems and the vast majority of these are small systems that serve less than 500 people per day (Kendall 2011). Ninety percent of British Columbians obtain their drinking water from large municipal systems. The remainder of the population is served by many small or medium-sized public systems or by private water supplies.

The British Columbia Ministry of Health is the ministry responsible for public drinking water systems in BC, and works with regional health authorities on drinking water related issues. There are five regional health authorities in BC: Fraser Health, Interior Health, Northern Health, Vancouver Coastal Health and Vancouver Island Health. Each health authority is responsible for regulatory oversight of drinking water systems within its regional boundaries through the application of the Drinking Water Protection Act.

2.8.1 Drinking Water Quality Standards

In Canada, a set of drinking water quality guidelines have been developed by Health Canada based on scientific health data (Health Canada 2010) that specify the maximum acceptable concentrations of various chemicals and microbiological indicators. Each province can adopt all or a subset of the Canadian Water Quality Guidelines, and include them in provincial drinking water legislation. The Drinking Water Protection Act applies to all drinking water systems located in BC that have more than one service connection. The Act requires drinking water purveyors to test their potable water for indicator bacteria at a prescribed frequency.

The microbiological monitoring schedules recommended in the Drinking Water Protection Regulation are based on the number of people served by the system (B.C. Reg. 200/2003). Systems that serve larger populations are monitored more frequently for indicator bacteria than those that serve fewer people. The recommended number of monthly samples for systems serving less than 5,000 people per day is four. In practice many operators of small systems serving less than 500 people submit samples for microbiological testing only once per month.

All drinking water samples submitted for compliance with the total coliform and *E. coli* requirements outlined in the BC Drinking Water Protection Regulation must be tested at a laboratory that has been approved by the Provincial Health Officer (PHO). There are thirteen PHO-approved laboratories in BC that accept external water samples for testing (EWQA 2011). Some large municipal systems such as Metro Vancouver and Capital Regional District operate their own PHO-approved laboratories for water testing.

The maximum acceptable concentration of total coliform bacteria specified in the Regulation depends on system size. Systems that submit more than one sample per month must have no samples with more than 10 CFU per 100 mL and at least 90% of samples must contain no detectable levels of total coliform bacteria (B.C. Reg. 200/2003). Small systems that submit one sample per month must have zero detectable total coliforms per 100 mL. The maximum acceptable concentration of *E. coli* is none detectable per 100 mL for all drinking water systems. Presence of total coliform bacteria or *E. coli* indicates that water may contain pathogenic microorganisms.

In the event that microbiological water quality standards are not met, a boil-water notice may be issued which means that water should be boiled prior to consumption. Depending on the circumstances, a notice may be permanent or temporary in nature. An untreated surface water supply or an untreated groundwater supply that is under the influence of surface water poses an unacceptably high risk to consumers; thus, the system will be placed on a permanent boil-water notice until such time as adequate treatment is implemented. Malfunction of equipment, indication of an adverse change in water quality (*i.e.*, an increase in turbidity or detection of *E. coli* in the distribution system), or lack of compliance with monitoring requirements outlined in the operating permit may result in the issue of a temporary boil-

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water notice or a water quality advisory until such time as the water in the distribution system

is deemed safe for human consumption.

Chapter 3: Evaluation of a Local-Testing Strategy to Monitor Microbiological Quality of Water for Small Drinking Water Systems

The objective of this study was to evaluate the agreement between two approaches to test drinking water for indicator bacteria. Duplicate drinking water samples were collected from eighty-three small water systems in the South Cariboo, British Columbia (n=271). One sample was sent to a laboratory (23-29 hours holding time) where it was tested for total coliforms and *E. coli* using ChromoCult® (Merck, Darmstadt Germany).[1] The other sample was tested at a local health unit (less than 7 hours holding time) using Colilert® presence/absence (IDEXX Laboratories Inc, Westbrook ME).[2] Cohen's kappa was used to measure the agreement for detection of total coliform bacteria and *E. coli* using the two approaches. Cohen's kappa for total coliforms and *E. coli* were 0.64 ± 0.11 and 0.73 ± 0.20 respectively. The approach using ChromoCult® along with sending the sample to a distant laboratory detected total coliforms in more samples than the approach using Colilert® and testing the sample near the point of collection. This is likely due to differences in method sensitivity rather than growth during the sample holding period. There was no apparent bias regarding the ability of Colilert® and ChromoCult® to detect *E. coli,* and the observed discordant pairs may have been due in part to unequal distribution of cells between samples. The findings of this study demonstrate that testing samples using the Colilert® presence/absence test provides results that compare well with those obtained using ChromoCult® for detection of *E. coli.* The results may be used to support a local-testing

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[1] ChromoCult® is a registered trademark of Merck KGaA, Darmstadt, Germany.
[2] Colilert® is a registered trademark of IDEXX Laboratories, Inc. Westbrook, ME, USA

approach for analysis of *E. coli* in drinking water that is timely and well-suited to systems located in remote areas.

3.1   Introduction

The Drinking Water Protection Regulation in British Columbia (BC) requires that drinking water purveyors have their finished water tested for indicator bacteria to ensure that it meets microbiological standards (BC Reg 200/2003). Under the Regulation, testing must occur at a laboratory that has been approved by the Provincial Health Officer (PHO) and samples must be tested using an internationally accepted methodology. Samples are required to reach the testing facility by 30 hours from the time of collection and sites must be re-sampled if this limit is exceeded. The delay in sample testing due to transport hinders a timely public health response in the event of an adverse water quality result that indicates fecal contamination. Therefore testing samples close to the point of collection is a desirable alternative, particularly when immediate intervention is needed. Since *E. coli* is a more reliable indicator of fecal pollution than total coliforms, detection of viable *E. coli* in drinking water necessitates immediate action whereas finding viable non-*E. coli* total coliforms in drinking water does not necessarily require an urgent response.

Both qualitative and quantitative methods can be used to determine whether or not the drinking water meets microbiological criteria. According to the Regulation (BC Reg 200/2003), presence of any detectable total coliform bacteria is an unacceptable result for small systems that are tested once per month because the standard for potable drinking water is less than one colony forming unit (CFU) of total coliform bacteria per 100 mL. Most laboratory testing facilities use quantitative membrane filtration methods rather than

qualitative enzyme/substrate methods. Most quantitative methods require additional equipment and are not suitable to implement on a small scale. There are a variety of commercially available tests on the market and these have been evaluated extensively elsewhere (Hörman and Hänninen 2006, Olstadt et al 2007, Maheux et al 2008).

Colilert[®] presence/absence testing has been used in a variety of settings to analyze water and has a detection limit of one CFU per 100 mL for total coliforms and *E. coli* (Edberg et al 1988). The test uses patented defined substrate technology to simultaneously detect total coliform bacteria and *E. coli*. In Canada, Colilert[®] has been used in First Nations communities to facilitate communities having greater control over the operations and testing of their drinking water systems. However, when some First Nations communities switched from membrane filtration to Colilert[®] presence/absence testing, the number of boil-water notices in these communities decreased (Penn 2006). While it is possible that actual changes in water quality following the change in testing approach were responsible for this phenomenon, it is also possible that the observed difference was due to a difference in method performance or a difference in microbial population dynamics as a result of different sample holding conditions. This observation emphasizes the need to better understand which variables (*i.e.*, analytical test, holding time and temperature) have a significant impact on water quality results. It also raises the question as to whether qualitative defined substrate methods are sufficiently sensitive to replace membrane filtration methods.

While some laboratory method comparison studies have found the performance of membrane filtration methods to be superior to that of defined substrate methods for the detection of total coliforms in drinking water (Olson et al 1991, Maheux et al 2008), others have found that Colilert[®] recovered higher numbers of *E. coli* or total coliforms than other approved methods

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(Niemela et al 2003, Hörman and Hänninen 2006). Still others suggest that the performance of Colilert® is comparable to other methods for the detection of total coliforms and *E. coli* in water (Edberg et al 1989, Cowburn et al 1994, Buckalew et al 2006, ISHA 2010). These conflicting conclusions suggest that method performance is, in part, dependent upon the characteristics of the water matrix analyzed, and findings from studies using one or several different water sources may not be extrapolated to water with different chemical and physical properties.

In addition to performance variability associated with the testing method, sample holding time and temperature can significantly affect the survival of total coliform bacteria in drinking water samples (McDaniels et al 1985, Pope et al 2003). Previous studies have observed significant reductions in total coliform bacteria after 24 hours at 4-5 °C (McDaniels et al 1985, Pope et al 2003), suggesting that it is desirable to analyze water samples as soon after collection as possible to obtain the most representative estimate of the total coliform population. The decline in bacterial density accelerates as holding temperature increases. Maintaining optimal cooler temperatures during sample transport is not always achieved in the summer months in BC when most of the seasonal small systems (*i.e.*, resorts, parks) are operational. It is unclear whether or not the effects of holding temperature and time have a greater impact on coliform detection than the use of a different testing method.

Given the limitations of the current testing strategy in BC and other regions with large numbers of small water systems located in rural areas, it is desirable to explore an alternative strategy that can quickly and accurately detect presence of viable total coliform bacteria and *E. coli* in drinking water. This a particular need in BC where there are a large number of small drinking water systems that sample infrequently and are generally at higher risk of

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microbiological contamination (Davies and Mazumder 2003, Cretikos et al 2010). The objective of this study was to compare the current approach to drinking water monitoring with an alternative testing approach in a project carried out in a geographical area of BC with a high density of small water systems. The alternative approach involved testing samples immediately after collection using a qualitative defined substrate method. The agreement between total coliform and *E. coli* results when the testing method and the sample holding time were changed was evaluated. The effect of sample holding temperature was also examined. This study provides an evaluation of water monitoring strategies needed for evidence-based decision making about drinking water monitoring policy and practices.

3.2   Methods

3.2.1   Sample Size

The sample size required to measure an acceptable level of agreement was estimated using three methods described by Donner (1992), Sim and Wright (2005), and Cantor (1996) for analysis involving Cohen's kappa. Sample size was calculated using a 2-tailed test, a minimum acceptable kappa value of 0.70, a kappa null value of 0.40, 90% statistical power and assuming a low (0.10) proportion of positive results. The proportion of positive results was estimated using *E. coli* testing data from previous studies that analyzed treated drinking water (Clark et al 1991, Olson et al 1994, Cowburn et al 1994). The largest of the three sample size estimates (n=241) was obtained using the method described by Sim and Wright (2005).

3.2.2   Study Area

The Interior Healthy Authority (IHA) region of BC has approximately 1,300 small water systems and as of August 2011 approximately 440 of these were on a boil-water notice

(http://www.interiorhealth.ca/YourEnvironment/InspectionReports/Pages/WaterNotifications
.aspx). Over 150 of these small drinking water systems are located in the South Cariboo, and
this area was selected as the study region (Figure 6).

3.2.3   Participation, Recruitment and Consent

Drinking water systems in the South Cariboo that met selection criteria were invited to
participate in the study. Inclusion criteria for drinking water systems included that systems
had less than 300 connections (*i.e.*, small and medium-sized systems), were registered with
the Interior Health Authority as a drinking water system serving the public, were operational
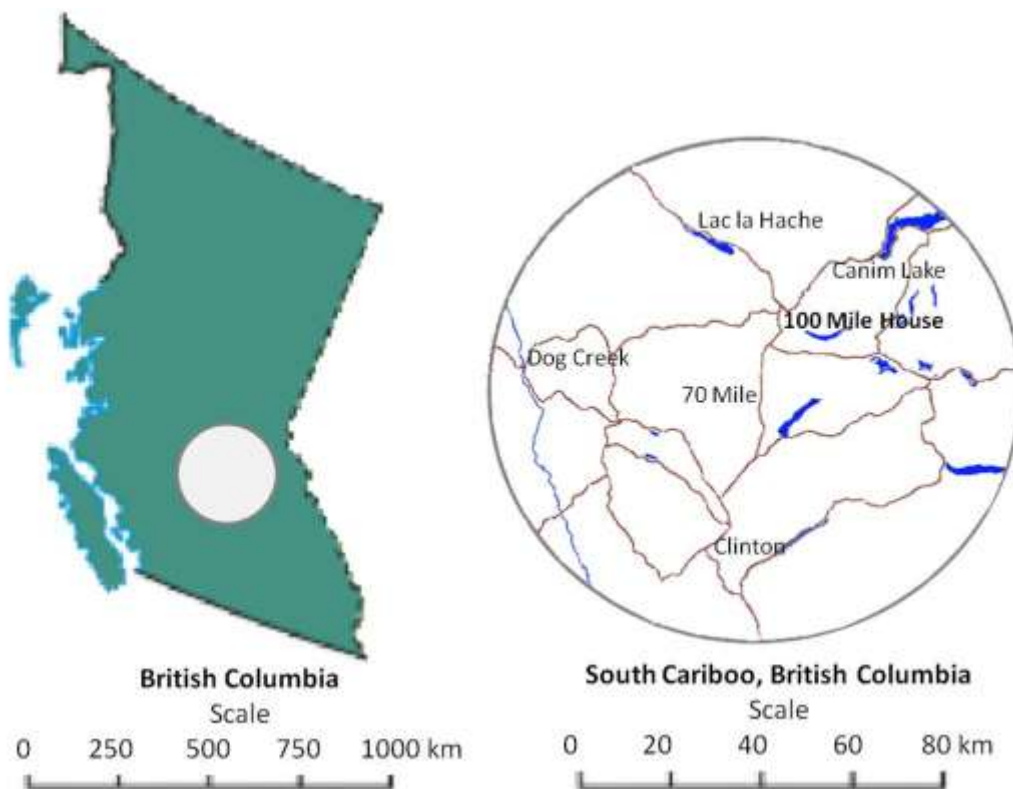
Figure 6. Map of the Province of British Columbia, Canada and the South Cariboo Region.

from at least May to September and were easily accessible by car. Purveyors of eighty-five water systems that met selection criteria were invited to participate in the study. Following ethics approval from both the University of British Columbia Research Ethics Board and Interior Health Ethics Board, drinking water purveyors were mailed a study information package. Consent to participate was obtained from the purveyors of eighty-three drinking water systems. Each participating system was assigned a unique alphanumeric code to ensure anonymity. Each purveyor was given a copy of the signed consent form, and agreed to either drop-off duplicate samples at the health unit in 100 Mile House, BC once per month or facilitate water sample collection and/or pick-up from the designated sample sites once per month.

3.2.4 Sampling Plan

Duplicate drinking water samples were collected from participating drinking water systems by the researcher or the operator. Both sampling containers were filled at the same time from the same sample site. One of the duplicate samples was sent to a PHO-approved laboratory in BC and the other sample was tested by the researcher at the 100 Mile House Health Unit. Drinking water samples were submitted monthly from June to August 2011. Samples were collected from designated sites, which are the specific locations within the distribution system that samples are taken each sampling period. There were one to three sample sites per system depending on the size and layout of the distribution system. Typically, sample sites were located immediately downstream of the treatment system and at the end of pipelines.

3.2.5   Sample Collection and Transport

Drinking water samples were collected from participating drinking water systems following standard procedures (APHA 1998). Briefly, the water was turned on and left running for 2 to 5 minutes prior to aseptic sample collection. Any aerators or screens were removed from the faucet prior to sample collection. Approximately 200 mL of water was collected in a sterile plastic bottle containing sodium thiosulphate, and a second bottle was filled immediately after the first. Both bottles were labeled with the time, date and sample site. Time and date of sample collection were used to calculate turnaround time for each sample.   Turnaround time was defined as the time elapsed from sample collection to result documentation. Holding time was the time from sample collection to commencement of sample testing.

Samples collected by the researcher were placed in a cooler with icepacks and transported to 100 Mile House Health Unit. Some water samples that were brought to the health unit by drinking water operators were transported in coolers with ice packs. Temperature of samples at the time of arrival at the health unit was not recorded. All samples were refrigerated immediately upon arrival at the health unit.

Water samples were shipped in coolers with ice packs to a PHO-approved testing facility in BC. Cooler temperature was recorded upon arrival. Drinking water testing standards in BC (Kendall 2007) require that samples be tested within 30 hours of collection. As per standard protocol, samples that were collected more than 30 hours prior to their arrival at the laboratory were discarded and the site was resampled.

3.2.6   Drinking Water Testing

One of the duplicate samples was tested at a PHO-approved testing facility for total coliforms and *E. coli* using membrane filtration followed by plating on ChromoCult® agar (Merck, Darmstadt Germany). All PHO-approved laboratories in BC scored 89% or higher on proficiency testing samples analyzed using membrane filtration methods, which indicates that the quality of the testing data obtained from the PHO-approved laboratory was acceptable (CMPT 2011). ChromoCult® agar plates were incubated for 24 hours at 35 °C and the number of *E. coli* and total coliform colonies were enumerated. If total coliforms or *E. coli* could not be quantified due to overgrowth of background bacteria, an inconclusive result was recorded. Sample data with inconclusive ChromoCult® results were discarded, as paired data was required for each sample.

The other duplicate sample was tested at the environmental testing unit in the 100 Mile House Health Unit using Colilert® presence/absence (IDEXX Laboratories Inc.,Westbrook, ME). Colilert® presence/absence testing was selected for this study from the pool of commercially available tests because it has been approved by accreditation bodies such as the US EPA and the Association of Analytical Communities, and it has advantages over membrane filtration methods in terms of its simplicity of use and interpretation of results. Colilert® presence/absence testing was conducted according to the manufacturer's protocol (IDEXX Laboratories Inc. 2011). Briefly, one snap-pack containing Colilert® reagent was added to 100 mL of water and the powder was dissolved using mild agitation. Samples were incubated at 35 °C and the result was read at 24 and/or 28 hours. If the colour of the sample was at least as yellow as the colour of the Colilert® comparator, then the sample was considered positive for total coliform bacteria. Similarly, if the intensity of the fluorescence

of the sample was greater than or equal to that of the Colilert® comparator, then the sample was considered positive for *E. coli*.

Quality control testing of each lot of Colilert® reagent was conducted using *Klebsiella pneumonia* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922) and sterile water.

Confirmation testing was not performed as previous studies have indicated that Colilert® has a false-positive rate of less than 1% (Olson et al 1991, Fricker and Fricker 1996, Maheux et al 2008). Some plant and algal extracts have been shown to possess β-galactosidase and β-glucuronidase activity, thus causing interference in the Colilert® test (Davies et al 1994). This was assumed to have a negligible impact on results because most of the drinking water systems sampled in this study used groundwater supplies, which normally do not contain substantial amounts of plant material or algae.

3.2.6.1 Additional Drinking Water Testing

A subset of samples was analyzed immediately and after 24 hours using Colilert® (n=33). The initial sample was analyzed using the Colilert® presence/absence test immediately after collection as previously described, and the remaining volume (100 mL) was stored at 4 °C for 24 hours. After the 24-hour incubation period, the sample was analyzed using the Colilert® presence/absence test. These results were used to compare the analytical tests (Colilert® versus ChromoCult®) when the same sample holding period was used.

3.2.7   Statistical Analysis

3.2.7.1   Agreement between Testing Approaches

The agreement between the outcomes generated using the two testing approaches was evaluated using Cohen's kappa. Kappa is a chance-corrected measure of agreement between two categorical variables generated by two different observers or using two different approaches (Hanley 1987, Sim and Wright 2005).

The testing results from the PHO-approved laboratory were reported as quantitative values, and these were converted to dichotomous outcomes. The result was assigned a '0' if absent by Colilert® or less than one CFU per 100 mL by ChromoCult®, and a '1' if present by Colilert® or at least one CFU per 100 mL by ChromoCult®. The agreement for total coliform bacteria and *E. coli* were evaluated separately. Each pair of results was scored according to the agreement of the two approaches. Duplicates that tested positive by both methods were (1, 1); duplicates that scored negative by both methods were (0, 0); and duplicates that scored positive by one method and negative by the other were (0, 1) or (1, 0). The results were summarized in a two-by-two attribute table and these values were used to calculate Cohen's kappa (95% confidence interval) and the standard error of kappa (Fleiss 1971). AgreeStat software was used to perform the calculations (http://agreestat.com/agreestat.html). The positive and negative predictive value, specificity and sensitivity were calculated in a Microsoft Excel spreadsheet.

Samples collected from the same drinking water system on different days were assumed to be independent, and samples collected from different sites on the same day were assumed to be independent. To test this assumption, the kappa analysis was repeated using a subset of data with dependent sample data removed. When multiple samples were collected from the same

system at different sites on the same day, one or more of the records were randomly selected for elimination leaving one record per system per day.

Binomial probability was used to evaluate discordant pairs to determine the likelihood that the observed difference was due to chance (Bej et al 1991). The McNemar test was used to determine whether there was a difference between the ability of the approaches to detect total coliforms. The McNemar test statistic was calculated using a correction factor of 1.0 (Fleiss 1981, Ilstrup 1990), and the test statistic was assumed to follow a chi-square distribution as the number of discordant total coliform results was greater than 25 (Mahdi et al 2011) with one degree of freedom.

3.2.7.2   Testing Method

Results of samples tested at 24 hours post-collection using Colilert® and ChromoCult® were compared using Cohen's kappa to evaluate whether there was a difference between the abilities of the testing methods to detect total coliforms and *E. coli* when the same holding period was used. This analysis assumes that small differences in holding temperature had a negligible effect on total coliform survival.

3.2.7.3   Holding Temperature

Samples were placed into one of two groups based on the arrival temperature of the cooler. If the cooler arrival temperature was less than 8 °C, samples were placed in one group (n=125); and samples that arrived in coolers where the temperature was greater than or equal to 8 °C were placed in the other group (n=145). The proportion of samples with discordant results was calculated for each group and compared using z-test to determine whether there was a greater proportion of samples with discordant results when transport temperature

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exceeded 8 °C. A discordant result meant that a sample tested positive using the proposed approach (Colilert® with less than 7 hour holding time) and negative using ChromoCult® with 24 hour holding time, or vice-versa. The 95% confidence interval was calculated using the Wilson estimate (Brown et al 2001).

3.2.7.4   Holding Time

The results of samples tested using Colilert® at two different holding times were compared to determine whether the number of viable total coliform or *E. coli* cells decreased during the holding period. Cohen's kappa was used to analyze samples tested less than 7 hours post-collection and those tested after being held at 4 °C for 24 hours.

3.2.7.5   Cell Concentration and Distribution between Samples

The chi-squared test was used to evaluate the effect of cell concentration on agreement between Colilert® and ChromoCult® test results for total coliform bacteria. Data that contained ChromoCult® results categorized as "overgrown" or "inconclusive" were not included. Samples were placed in one of three categories based on the concentration of total coliform bacteria in the sample as determined by ChromoCult®. Poisson probabilities were used to evaluate the probability that total coliforms were present in samples that tested positive for total coliforms using ChromoCult® and negative for total coliforms using Colilert®. Bacterial counts for duplicate samples were assumed to follow a Poisson distribution, as previously demonstrated (El-Shaarawi et al 1981, Gale et al 2002).

3.2.7.6   Turnaround Time

Sample turnaround times for samples tested at the regional PHO-approved laboratory and those tested at the 100 Mile House Health Unit were compared using a two-tailed paired *t*-test (99% confidence).

3.3   Results

3.3.1   Drinking Water System Characteristics

The eighty-three drinking water systems sampled in this study supplied water to seasonal resorts and campgrounds, motels, provincial parks, restaurants and community centers. Of these, 63 used groundwater (*i.e.,* dug well, drilled well, spring), 17 used surface water (*i.e.*, lake, river) as the water source and 1 used a combination of surface and groundwater. Two systems were water dispensers in commercial establishments and the source of the water was unknown. The majority of drinking water systems sampled in this study were groundwater systems with no disinfection treatment. Among those with disinfection treatment (n=21), methods included standalone ultraviolet (UV) light treatment or UV in combination with filtration; standalone chlorination or chlorination in combination with filtration.

During the study period, 272 drinking water sample pairs were collected and tested using two different testing approaches. Over 90% of samples tested at the local health unit using Colilert® had holding times of 6 hours or less. Holding times of samples tested at the PHO-approved laboratory using ChromoCult® were 26 ± 3 hours. No inconclusive testing results were obtained using Colilert®, while 10 of the 272 samples tested were inconclusive for *E. coli* and one sample was inconclusive for total coliforms using ChromoCult®. This data was not used in calculations of agreement measures.

3.3.2   Discrepancy in Total Coliform Results

An interim analysis conducted using sample data collected in the first month of the study

identified a significant discrepancy between the two approaches for detection of total

coliforms. A disproportionately high number of samples tested positive for total coliforms

using ChromoCult® and negative using Colilert® compared to samples that tested negative

using ChromoCult® and positive using Colilert® (Table 5). While some disagreement would

be expected due to unequal distribution of bacteria in the two samples tested, the binomial

probability that all discordant pairs were total coliform-positive using ChromoCult® and total

coliform-negative by Colilert® due to chance alone was less than 0.001. Therefore,

additional hypotheses were posed and testing plan was modified, as described in section

3.2.6.1, to investigate whether the disagreement was partly due to unequal distribution of

bacteria among duplicate samples tested, changes in microbial population during sample

transport, or difference in sensitivity between ChromoCult® and Colilert® tests.

Table 5. Initial Analysis of Total Coliform Bacteria Results Using ChromoCult® and
Colilert® Tests and Different Holding Times

		Tested using ChromoCult® (holding time 26 ± 3 hrs)		
		Present	Absent	Total
Tested using Colilert® (holding time less than 7 hrs)	Present	10	0	10
	Absent	13	73	86
	Total	23	73	96

3.3.3   Overall Agreement between the Two Testing Strategies

Cohen's kappa was used to evaluate the agreement between testing results obtained using the two testing approaches. The results for total coliforms and *E. coli* are summarized in Table 6. The Cohen's kappa for total coliforms and *E. coli* were 0.64 ± 0.11 and 0.73 ± 0.20 respectively (95% confidence intervals), which indicate that agreement for both groups was substantial.

Both binomial probability and McNemar test indicated that there was a difference between the abilities of the two approaches to detect total coliform bacteria in drinking water samples. The binomial probability that at least 85% of the discordant pairs were positive for total coliforms using ChromoCult® and negative using Colilert® due to chance alone was less than 0.0001. The McNemar test statistic obtained was highly significant (*p*<0.001).

Table 6. Total Coliform Bacteria and *E. coli*[a] using ChromoCult® and Colilert® Tests and Different Holding Times

		Tested using ChromoCult® (holding time 26 ± 3 hrs)		
		Present	Absent	Total
Tested using Colilert® (holding time less than 7 hrs)	Present	42 (10)	5 (4)	47 (14)
	Absent	28 (3)	196 (245)	224 (248)
	Total	70 (13)	201 (249)	271 (262)

[a] *E. coli* data is shown in parentheses

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Using the ChromoCult® test as the reference, the negative predictive values of the Colilert®

test with a short (less than 7 hour) holding time for total coliforms and *E. coli* were 88% and

99% respectively, while the positive predictive values for total coliforms and *E. coli* were

89% and 71%, respectively. Both methods detected *E. coli* in about 5% of the samples.

Measures of agreement for the two approaches are shown in Table 7. Virtually identical

values were obtained when calculations were repeated using a subset of the data with

dependent sample results removed. This suggests that the assumption that test results from

samples collected from different sites of a system on the same day were independent did not

considerably affect the mean kappa for total coliforms or *E. coli*.

Table 7. Effect of Sample Independence for Comparison of ChromoCult® with 26 ± 3 hour
Holding Time and Colilert® with less than 7 hour Holding Time

	Total Coliforms All	Total Coliforms Independent Samples Only	*E. coli* All	*E. coli* Independent Samples Only
n	271	208	262	203
95% CI Kappa	0.64 ± 0.11	0.65 ± 0.12	0.73 ± 0.20	0.68 ± 0.22
Overall Agreement	88 %	88 %	97 %	97 %
Negative Predictive Value	88 %	87 %	99 %	98 %
Positive Predictive Value	89 %	90 %	71 %	67 %
Specificity	98 %	97 %	98 %	98 %
Sensitivity	60 %	61 %	77 %	73 %

3.3.4 Effect of Testing Method

More samples tested positive using ChromoCult® than Colilert® when sample holding times were equal (Table 8). The binomial probability of 4 or 5 of the discordant pairs for total coliforms being due to chance if there was an equal chance of observing either outcome was 0.19, which is not statistically significant.

3.3.5 Effect of Sample Holding Temperature

Sample transport temperatures ranged from 2.8 to 14.7 °C. The proportion of samples with discordant total coliform results was no different when holding temperature was greater than 8 °C (0.097) compared to when holding temperature was less than 8 °C (0.152) when 270 samples were analyzed. The 95% confidence interval of the difference in proportions was 0.06 ± 0.08.

Table 8. Total Coliform Bacteria and *E. coli*[a] Using ChromoCult® and Colilert® Tests and the Same Holding Times

		Tested using ChromoCult® (holding time 26 ± 3 hrs)		
		Present	Absent	Total
Tested using Colilert® (holding time 24 hrs)	Present	19 (4)	1 (0)	20 (4)
	Absent	4 (1)	6 (18)	10 (19)
	Total	23 (5)	7 (18)	30 (23)

[a] *E. coli* data is shown in parentheses

3.3.6   Effect of Sample Holding Time

Additional testing was conducted to investigate whether the detectable level of viable total coliform bacteria and *E. coli* in drinking water was lower when samples were held at 4 °C for 24 hours, compared to samples that were tested immediately after collection using the Colilert® presence/absence method. Initially, 8 out of 33 samples tested positive for *E. coli* using Colilert®. When a second Colilert® test was conducted after holding the sample for 24 hours at 4 °C, only five of the eight samples that initially tested positive for *E. coli* were *E. coli*-positive in the second test (Table 9). The binomial probability of all three discordant *E. coli* results being positive initially and negative after a second test, due to chance alone, was 0.125. As there were only three discordant observations, there is insufficient evidence to disprove the hypothesis that holding samples for 24 hours at 4 °C has no effect on cell viability when samples are tested using Colilert®.

Table 9. Total Coliform Bacteria and *E. coli*[a] Using Colilert® with Different Holding Times

		Tested using Colilert® (holding time 24 hrs)		
		Present	Absent	Total
Tested using Colilert® (holding time less than 7 hrs)	Present	19 (5)	2 (3)	21 (8)
	Absent	1 (0)	11 (25)	12 (25)
	Total	20 (5)	13 (28)	33 (33)

[a] *E. coli* data is shown in parentheses

3.3.7   Cell Concentration and Distribution between Samples

The relationship was examined between cell concentration and agreement between results obtained using ChromoCult® and a holding time of 24 hours and using Colilert® and a holding time of less than 7 hours. Seventy-three percent of the samples positive for total coliforms by ChromoCult® with low cell concentrations between 1 and 10 CFU per 100 mL (n=26) gave results that did not agree (Table 10). This indicates that replicate samples containing low concentrations of total coliforms are more likely to give discordant results. However, 40% (n=28) of samples with cell concentrations greater than 10 CFU per 100 mL tested positive with ChromoCult® and negative with Colilert®, indicating that there are other factors in addition to low cell concentration that are responsible for the observed bias in total coliform results.

A highly significant chi-squared value supported that there is an association between cell concentration and agreement between ChromoCult® and Colilert® tests ($\chi^2(255)= 112$, $p<0.001$). When the chi-squared result was decomposed to determine which groups were significant, samples with greater than 10 CFU total coliforms per 100 mL and samples with 1

Table 10. Observed Agreement between Colilert® and ChromoCult® Total Coliform Results and Cell Concentration

	Greater than 10 CFU/100 mL Total Coliforms by ChromoCult®	1 – 10 CFU/100 mL Total Coliforms by ChromoCult®	Less than 1 CFU/100 mL by ChromoCult®
Disagree (+,- or -,+)	8	19	5
Agree (+,+ or -, -)	20	7	196
Total	28	26	201

to 10 CFU total coliforms per 100 mL appeared to have different propensity to produce results that agree, using both ChromoCult® and Colilert® tests ($\chi^2(54)= 10.68$, $p<0.005$). The effect of cell concentration on agreement of *E. coli* test results was not investigated because all samples, with the exception of one, that were *E. coli*-positive contained less than 10 CFU per 100 mL.

The possibility was investigated that unequal distribution of cells between samples was responsible for lack of detection of total coliforms using Colilert® when total coliforms were detected using ChromoCult®. As the total coliform concentration in water increased, the Poisson probability indicated that it was increasingly unlikely that cells would be distributed between the two sample vials such that one would contain no total coliform bacteria (Table 11). Even though the proportion of discordant results decreased when cell concentration increased, the probability that nearly one third of duplicate samples contained greater than 10 CFU total coliforms per 100 mL in the sample tested using ChromoCult®, but did not contain any total coliform bacteria in the sample tested using Colilert®, was extremely low.

Table 11. Probability of Observing a Negative Total Coliform Result Using Colilert® when Different Concentrations of Total Coliforms are Present

Cell Concentration Total Coliforms by ChromoCult® (CFU/100 mL)		Number of Samples	Number of Samples Negative by Colilert®	Poisson Probability of 0 CFU/100mL Total Coliforms in a Sample	Colilert®- Negative Samples	Probability of Observed Result
Range	Mean					
1	1	8	7	3.68×10^{-1}	88%	9.12×10^{-4}
2 - 10	4.28	18	12	1.39×10^{-2}	67%	5.08×10^{-23}
>10	67.6	28	8	4.51×10^{-30}	29%	1.71×10^{-235}

3.3.8   Sample Turnaround Time

Sample turnaround times were calculated for 269 duplicate samples. Test results for drinking water samples analyzed locally using Colilert® were obtained 28 ± 7 hours (95% confidence) from the time of collection, whereas the average turnaround time for drinking water samples tested at a PHO-approved laboratory using ChromoCult® was 50 ± 7 hours (95% confidence). Eliminating the step where drinking water samples are transported to a PHO-approved laboratory resulted in a statistically significant reduction in sample turnaround time ($t(269)=143$, $p<0.01$).

3.4   Discussion

Two drinking water monitoring strategies were evaluated that differed in terms of the testing method used and the sample holding time and temperature. Water from eighty-three small and medium-sized systems in the South Cariboo region of BC was tested using Colilert® and ChromoCult®. The agreement between the two approaches was evaluated using Cohen's kappa. Previous studies have reported that agreement between results obtained using Colilert® and ChromoCult® tests varied, despite the fact that both tests use the same principle to detect total coliforms and *E. coli*. Detection of total coliforms is based on presence of a functional β-galactosidase enzyme and detection of *E. coli* is dependent upon presence of both β-galactosidase and β-glucuronidase enzyme. A negligible number of false negative test results was expected, as other studies have found that ChromoCult® and Colilert® generated less than 0.5 % false negatives (Rice et al 1990, Fricker et al 2008).

The kappa statistic is a chance-corrected measure that has been used to quantify the agreement between two independent observers for dichotomous outcomes. Guidelines to

interpret kappa values have been proposed by Landis and Koch (1977) and Muñoz and Bangdiwala (1997). By these definitions, kappa values 0.41 to 0.60 were considered moderate agreement, 0.61 to 0.80 were considered substantial agreement and greater than 0.80 were considered almost perfect agreement.

The overall agreement of the two approaches as measured by Cohen's kappa was 0.64 ± 0.11 and 0.73 ± 0.20 for total coliform bacteria and *E. coli*, respectively. Given that kappa values are generally low when the outcome measured is rare (Viera and Garrett 2005), as was the case in this study, the moderate-to-substantial agreement rating is likely an underestimate. A similar kappa value for *E. coli* was reported by Eckner et al (1998) who compared Colilert[®] and mEndo media, but they observed a greater degree of agreement for total coliform results with kappa 0.94 ± 0.06.

Others have found that Colilert[®] and ChromoCult[®] methods compare poorly for the detection of non-*E. coli* total coliforms. The tests did not always detect the same species even though it contained the *lacZ* gene that encodes β-galactosidase. In a validation study, Maheux et al (2008) tested 33 non-*E. coli* total coliform species using ChromoCult[®] and Colilert[®] and the agreement was kappa 0.31 ± 0.33. Given that the total coliform group includes many species of bacteria, not all species in the total coliform group will favor the same growth conditions. Differences in nutrient media composition of Colilert[®] and ChromoCult[®] may be partly responsible for the differences in recovery observed. In addition, Colilert[®] reagent and ChromoCult[®] agar contain different substrates for detection of total coliforms (*i.e*., ONPG in Colilert[®] and Salmon-Gal in ChromoCult[®]) and *E. coli* (*i.e*., MUG in Colilert[®] and X-Gluc in ChromoCult[®]). Variation in test performance may be due in part to differences in enzyme/substrate specificity.

The kappa values reported are conservative because the majority of water samples tested were negative for total coliform bacteria. Datasets with high proportions of either positive or negative results have been shown to yield a lower kappa value (Sim and Wright, 2005) compared to a dataset with equal proportions of positive and negative results. Lower than expected kappa values, due to high proportions of negative drinking water test results, have been reported elsewhere (Lewis and Mak 1989).

Not only was the agreement for total coliform results lower than expected, but there was also a bias in the discordant pairs. The McNemar test and binomial probability confirmed that more of the discordant pairs than would be expected by chance tested positive for total coliforms when sample holding times were 26 hours and ChromoCult® was used, and negative when sample holding times were less than 7 hours and Colilert® was used.

It appears unlikely that the bias was due to bacterial growth during the holding period, based on the results of the 33 samples tested using Colilert® immediately after collection and after 24 hours at 4 °C. On the contrary, previous studies (McDaniels et al 1985) have observed a decline in the total coliform population in the first 24 hours after sample collection when samples were held at 5 °C. The findings of Dutka and El-Shaarawi (1980) also support that the total coliform population can change during the sample holding period. They tested triplicate samples held at 1.5 °C at different time periods for total and fecal coliforms to assess their stability, defined as no difference between sample results at 1% significance. They observed that all samples tested for total and fecal coliforms were stable at 2 hours, while only 59% and 68% of samples were stable for total and fecal coliforms, respectively, at 30 hours.

The discordant *E. coli* results observed when samples were tested using Colilert[®] immediately and after 24 hours at 4 °C may be due to die-off, or these observations may have been due to chance from uneven distribution of bacteria between samples. Pope et al (2003) found that 4 out of 11 surface water sites showed significant decreases in viable *E. coli* after samples were stored at 4 °C for 30 hours (95% CI 0.10 to 0.36 log reduction). It is difficult to make direct comparisons between studies because *E. coli* survival kinetics are expected to differ according to a multitude of factors. These include the strain of *E. coli* used and degree of cell damage sustained from exposure to disinfectant; characteristics of the water matrix including disinfectant and nutrient concentration, presence of other microorganisms; and sample holding temperature.

The effect of sample holding temperature was of insufficient magnitude in this study to explain the bias in discordant total coliform results when samples were held at less than 14°C. No difference between the proportions of samples with discordant total coliform results was found when sample holding temperature was less than 8 °C versus 8 to 14 °C.

While the results of samples tested at 24 hours using Colilert[®] and ChromoCult[®] show the same trend with a bias towards detection of total coliforms using ChromoCult[®] but not Colilert[®], the small sample size (n=30) limits the weight of this observation. The likelihood that the observed results were due to chance was 19%. No such bias was observed in other validation studies comparing Colilert[®] and ChromoCult[®] (Maheux et al 2008).

Viable cell concentration affected agreement between tests. Samples containing 1 to 10 CFU total coliforms per 100 mL were less likely to produce results that agreed than samples with greater than 10 CFU per 100 mL. Similar findings have been reported by Bej et al (1991),

who observed that in all instances where PCR and Colilert® test results differed, the concentration of total coliforms was less than 5 CFU per 100 mL. Thus, some disagreement in test results can be attributed to sample-to-sample variability when viable cell concentrations are low. This may explain the observed low positive predictive value for *E. coli*, as all samples containing viable *E. coli* had less than 10 CFU per 100 mL. Other studies have also found that false-negative results were frequently observed in water samples with low total coliform concentrations (Schets et al 1993).

From a regulatory perspective, both qualitative presence/absence Colilert® testing and quantitative ChromoCult® testing are acceptable methods to analyze drinking water as both tests are capable of determining whether drinking water quality meets the criteria of less than one CFU of total coliforms per 100 mL. Over three quarters of drinking water samples tested negative for total coliform bacteria and over 95% tested negative for *E. coli*, so one could make the argument that it is unnecessary to test all samples using a quantitative method when the majority do not contain any viable total coliforms. In some cases, quantitative testing may be warranted for systems that have a history of total coliform occurrence. A hybrid testing approach, in which all samples are tested locally using a qualitative test and some samples are sent to PHO-approved laboratories for quantitative testing, may provide additional benefits, relative to the current testing approach.

One general limitation of plating media is that it may be difficult to discern whether or not total coliform colonies are present on a plate that is overgrown with background microorganisms (Pitkänen et al 2007). Although ChromoCult® contains Tergitol®7 to inhibit growth of most Gram-positive bacteria (Merck, Darmstadt Germany), detection of indicator bacteria in samples that contain high levels of Gram-negative non-total coliform bacteria may

be impaired. This is less of a problem with the Colilert® presence/absence test as it is a liquid medium, and is not affected by levels of background microflora typically found in drinking water as long as the recommended sample incubation times are followed (Edberg et al 1988) and the reagent is not used after its shelf-life expiry date (Landre et al 1998). Perhaps the greatest advantage of the Colilert® presence/absence test is that it is easy to use and does not require any specialized equipment such as a membrane filtration apparatus.

Testing samples as soon as possible after collection would avoid potential changes in sample matrices and give the most accurate indication of the microbiological quality of the drinking water being consumed. Not only is timeliness important from the perspective of preserving the integrity of the sample matrix, but it is also critical from a public health intervention and community risk standpoint. It was demonstrated that using a local testing approach provided total coliform and *E. coli* results approximately 22 hours faster than the current approach, which can take at least 48 hours from the time of sample collection to obtain microbiological water quality results. Sample transport may take up to 30 hours, and sample analysis in the laboratory is typically at least 24 hours. Given that many small drinking water system purveyors only have their water tested once per month, the probability of detecting a transient contamination event is low; but, it is nonetheless important to check for any operational malfunctions that may have occurred during treatment or distribution as pathogen intrusion can have serious health consequences.

There are several limitations of this study. Secondary hypotheses that were posed midway through the study to evaluate the effect of testing method, sample holding time and temperature on agreement of test results had a limited sample size. While it was sufficient to illustrate general trends, the statistical significance of the low frequency observations was

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limited. With regard to sampling strategy, while efforts were made to ensure that duplicate samples were homogeneous, unequal dispersion of bacterial cells between samples was possible as some microorganisms in water bind together and aggregate (LeChevallier et al 1988). In terms of controlling sample temperature during transport, sample holding temperature from the sample site to the health unit was variable, depending on whether the researcher or the purveyor transported the samples to the health unit. Not all samples brought in by purveyors were contained in coolers with icepacks. However, duplicate samples were exposed to the same temperature conditions. Thus, the lack of refrigeration of some duplicate samples during transport to the health unit was not expected to affect the agreement between results of duplicate samples. Measures of timeliness presented here may not reflect the holding or turnaround times of samples taken from other regions of BC. There are fifteen PHO-approved laboratories in BC that test drinking water for indicator bacteria for the purpose of compliance with the Drinking Water Protection Regulation. Only one of these laboratories was included in this study and, therefore, measures of timeliness will vary according to proximity of the sample site and testing facility.

Current legislation in BC requires that all drinking water samples be tested at a PHO-approved laboratory, and proposed changes are only acceptable if the performance of the alternative is equivalent or superior to that which is used currently.   While a local-testing approach using a presence/absence method would enable timelier detection of adverse changes in microbiological water quality, there appears to be a trade-off with regard to sensitivity for total coliform bacteria that warrants consideration. No significant discrepancy in testing method was evident for *E. coli*, which has greater acceptably in the regulatory,

water policy and public health communities as a water safety indicator than the use of total coliforms.

One considerable advantage of allowing drinking water testing at local health units in BC is the potential to improve surveillance of private water systems that are not registered with the local health authority. Owners of private wells are recommended to submit water samples two or three times per year (Health Canada 2008), but private well sampling rates in Canada are low (Hexemer et al 2008), in part due to inconvenience, time and cost. These barriers would be reduced if samples could be dropped off by system owners and tested locally.

3.5   Conclusions

The two monitoring approaches generated comparable total coliform (kappa 0.64 ± 0.11) and *E. coli* (kappa 0.73 ± 0.20) results overall. A disproportionately high number of total coliform-positive samples were observed using the current approach. While the sample size for additional testing was limited, the results suggest that the observed discrepancy was more likely due to differences in the commercial tests, rather than changes in the bacterial population during sample transport. Holding temperatures less than 14 °C or holding times less than 24 hours were not found to influence overall agreement of test results.

It is unclear whether the low positive predictive value is due to the low concentration of culturable *E. coli* in samples, as there were insufficient numbers of *E. coli*-positive samples to conduct further analyses. This may be an area for future research. Discordant total coliform test results were more likely to occur when samples contained 1 to 10 CFU of total coliforms per 100 mL, compared to samples that contained greater than 10 CFU per 100 mL.

Use of a qualitative defined substrate test would allow drinking water samples to be tested near the point of collection and would significantly improve turnaround times, but this comes at the expense of reducing sensitivity for detection of total coliform bacteria. The importance of this limitation depends on the purpose for microbiological testing (*i.e.*, to make decisions concerning public safety, or to evaluate operational performance) and the perceived value of total coliform bacteria as an indicator of water quality. Additional analysis including a cost-comparison of both testing approaches would further inform discussions regarding the benefits and limitations of testing approaches.

Chapter 4: The Predictive Value of Total Coliforms in Drinking Water using Life Table Analysis

With the exception of *E. coli*, bacteria from the total coliform group are not reliable indicators of fecal pollution in drinking water. Thus, the value of reporting total coliform results in drinking water has been scrutinized by many experts. Total coliform monitoring in distribution systems is no longer a required parameter in many countries but is still mandated in Canada and the United States. Although total coliform bacteria alone may not provide sufficient cause to place a drinking water system on a boil-water notice, it is often used to indicate system vulnerability to contamination. Total coliform and *E. coli* results from small drinking water systems tested over a three-year period in British Columbia were analyzed using life table analysis. Small drinking water systems that have a non-*E. coli* total coliform-positive result were found to have a slightly higher probability that a subsequent sample will contain *E. coli*, compared to small drinking water systems with no prior total coliforms detected in the distribution system (RR=2.04). One month after a non-*E. coli* total coliform-positive test, the probability of the system having an *E. coli*-positive test was nearly four times that of systems with no prior total coliforms. However, this is of minor practical significance due, in part, to the low rate of *E. coli*-positive drinking water samples, reflected in the low absolute risk increase at one month after a non-*E. coli* total coliform test (1.6%).

4.1 Introduction

Ensuring that drinking water meets Canadian health-based standards (Health Canada 2010) and is safe for human consumption is dependent upon application of a multi-barrier approach. From source-to-tap this involves adequate protection and management of

watersheds, reduction of hazards posed by harmful compounds and microorganisms using appropriate water treatment practices, and delivery of treated water via a well-maintained distribution system. Drinking water quality is monitored for indicator bacteria within the distribution system because it gives the best representation of the safety of the drinking water being consumed.

Routine monitoring for all possible waterborne pathogens is not feasible for drinking water systems. Therefore, surrogate parameters, namely total coliforms and *E. coli*, are used to measure microbiological drinking water quality. Total coliforms and *E. coli* can be detected simultaneously in a drinking water sample using a variety of chromogenic and fluorogenic tests (Edberg et al 1991b, Wang and Fiessel 2008). Total coliform bacteria inhabit the gastrointestinal tracts of animals and are present at up to thirteen million viable cells per gram of animal waste (Geldreich et al 1962). As such, their presence can be associated with fecal contamination. However, several species of bacteria belonging to the total coliform group are of non-fecal origin (Leclerc et al 2001). *E. coli* is a member of the total coliform group that is almost exclusively of gut or fecal origin. Therefore, it is generally recognized as a stronger indicator of fecal contamination than total coliforms (Edberg et al 2000).

One consequence of the fact that total coliforms are not exclusively present in the gut and feces is that a positive total coliform test result may falsely suggest that fecal contamination is present. Total coliform bacteria and *E. coli* can also be absent when waterborne pathogens are present (Keswick et al 1984, Rose et al 1991). Non-bacterial pathogens including viruses and protozoa can be more resistant to disinfection treatments (Hoff and Akin 1986) than bacterial indicators and persist for longer periods. Thus, pathogens may go undetected in water that is presumed safe for human consumption.

The reliability of total coliforms as an indicator of water safety has been questioned since the 1970s (Barrell et al 2000, Leclerc et al 2001, Reynolds 2003). In response to the mounting evidence that highlights the limitations of total coliforms as a water safety indicator, governing bodies in some jurisdictions including Australia and the European Union no longer require distribution system monitoring for total coliform bacteria (Stevens et al 2003, Standridge 2008, Figueras and Borrego 2010). Instead total coliforms are used more often with the intent of the test being a process indicator to evaluate performance of treatment operations. In Canada and the United States, total coliform testing is required for all drinking water systems, although the presence of *E. coli* remains the actionable standard measure for indicating presence of fecal contamination in drinking water. Measurement of *E. coli* in water is part of an evidence-based approach to make important decisions concerning public safety related to drinking water consumption. Currently, the presence of total coliforms in drinking water is taken as evidence that a system is vulnerable to contamination, but there is no immediate health risk as few of the members of the total coliform group are harmful to humans (Health Canada 2011). This is reflected in the current Canadian guidelines where the maximum allowable concentration of total coliform bacteria in finished water immediately after treatment or drawn from an untreated well is none detectable per 100 mL, but water within the distribution system may contain up to 10 CFU per 100 mL under some circumstances (BC Reg 200/2003).

There is a need for more sensitive measures indicative of elevated risk of illness from drinking water consumption. Fecal coliforms and *E. coli* are subgroups of the total coliform group and may thus be less sensitive to small changes in microbial water quality. A previous study showed that measured concentrations of total coliforms were consistently higher than

fecal coliform concentrations in raw and treated drinking water samples (Payment et al 1985).   Further, in a preliminary analysis of a microbiological water testing dataset, it appeared that some drinking water systems had detected non-*E. coli* total coliform bacteria days or weeks prior to an *E. coli*-positive test result.

The objective of this study was to investigate the value of non-*E. coli* total coliform bacteria as predictive indicators and determine how often non-*E. coli* total coliform events were followed by more health relevant changes in water quality. The hypothesis tested was that the probability of obtaining an *E. coli*-positive drinking water test result from a system, given that prior samples have tested positive for non- *E. coli* total coliform bacteria, is greater than the probability of obtaining an *E. coli*-positive drinking water test result from a system given that prior samples have not tested positive for total coliforms.

4.2   Methods

4.2.1   Drinking Water System Metadata

A database of all drinking water systems in British Columbia that were registered with regional health authorities was developed in Microsoft Excel using publicly available data (http://www.healthspace.com/clients.html). It contained the facility name, size and regional health authority.

4.2.2   Drinking Water Sample Test Data

Total coliform (TC) and *E. coli* water sample test result data were obtained from the Laboratory Information System archive at the British Columbia Public Health Microbiology Reference Laboratory in Vancouver, BC. The test data were from samples received between January 2007 and December 2009 and did not include samples tested at other PHO-approved

laboratories. Each sample record contained the collection date, client name, facility name, total coliform result (CFU per 100mL) and *E. coli* result (CFU per100mL). The sample dataset was trimmed using inclusion and exclusion criteria. Inclusion criteria included the following: Samples were collected from a registered drinking water system in BC with less than 15 connections, and the client name on the sample requisition was one of the regional health authorities in BC (*i.e.*, Fraser Health Authority, Interior Health Authority, Northern Health Authority, Vancouver Coastal Health Authority and Vancouver Island Health Authority).

The following exclusion criteria were applied. Data from systems that had less than two samples tested from 2007 to 2009 were excluded. All microbiological test results from samples of beach water, ice, sewage, swimming pool water, hot tub water and raw or pre-treatment water were excluded. Sample data from water systems with 15 or more connections were omitted, as these systems contain many sample sites representing distinct areas of the distribution system pipe network; thus, results at one location in the system may not reflect water quality at another location in the system. In comparison, all sample sites from a given small drinking water system were assumed to be interchangeable.

Quantitative total coliform and *E. coli* test results were transformed into binary variables. A zero was assigned if no total coliform bacteria were detected, and a '1' was assigned if there were at least 1 CFU per 100mL present. Sample data was grouped according to drinking water facility name (n=1519) and arranged in chronological order. If a system submitted more than one sample per month, a single sample was randomly selected to be used for each month in the analysis.

4.2.3 Life Table Analysis

A life table was calculated using the actuarial approach. All drinking water systems that had at least one non-*E. coli* total coliform-positive sample were placed in the 'Prior TC' group. Any system whose first recorded sample was positive for *E. coli* was not included in the analysis (n=25), as no subject can fail at the initial time point (Jiang and Fine 2007). The starting point for the Prior TC group was defined as the date that the result of the first total coliform-positive sample from a drinking water system was obtained. All other systems were placed in the 'No Prior TC' group. This included systems that did not have any total coliform-positive samples, and those that had an *E. coli*-positive sample that was not preceded by a non-*E. coli* total coliform result. The starting point for each system in the No Prior TC group was the earliest sample collection date in the dataset. The interval period was defined as the length of time, in months, between sample data points.

The end point was defined as the date that the first *E. coli*-positive sample result from the drinking water system was obtained. All observed end points fell into one of the interval periods. Systems were categorized as lost-to-follow-up if data was missing (*i.e.*, a sample was not submitted during the interval period) or if data collection ended before the endpoint was reached.

Life table analysis was repeated for two groups of water systems defined using different non-*E. coli* total coliform threshold criteria. Groups were defined based on occurrence or lack of occurrence of a non-*E. coli* total coliform event with a concentration of at least 10 CFU per 100 mL. The starting point for No Prior TC $\geq$ 10 CFU group was the first sample submitted after January 2007, while the starting point for the Prior TC $\geq$ 10 CFU group was the date of the first sample containing at least 10 CFU of non-*E. coli* total coliforms per 100 mL.

4.2.4   Life Table Function

The time elapsed between the starting point and each sample submitted for testing was calculated using built-in time formulas in Microsoft Excel.

The data was analyzed using the assumption that any missing data for an interval was negative for *E. coli* and a system was considered 'lost-to-follow-up' the interval after the final sample was submitted. That is, the system remained "event-free" until it either stopped submitting samples, or had an *E.* coli-positive sample. The data was also analyzed without the event-free assumption; if no sample was submitted during an interval period, the system was counted as 'lost-to-follow-up' and any subsequent data for that system was discarded.

The analysis was repeated using several interval widths. These were defined as one month, two months, three months, four months and six months. For each interval the number of systems 'at risk' of an *E. coli*-positive result, the number of systems that reached the endpoint, and the number of systems 'lost-to-follow-up' (also known as right-censored) was recorded (Breslow 1975). These values were used to calculate the probability of reaching the endpoint, or hazard, for each interval.

The probability of reaching the end of the interval without an *E. coli*-positive sample result for each interval and the cumulative probability were calculated. The cumulative probability was used to construct life table curves for the Prior TC and the No Prior TC group. Standard error for each interval was calculated using the method proposed by Peto et al (1977) shown in Equation 1 where P_i is the cumulative probability of not observing an *E. coli*-positive result, and R_i is the number of systems at risk at the beginning of interval *i*.

1 $\text{SE}(P_i) = P_i \left[(1 - P_i) / R_i \right]^{1/2}$

4.2.5   Comparison of Groups

The Mantel-Cox log-rank test was used to compare the life table functions of the Prior TC and the No Prior TC groups (Mantel 1966). This involved comparing the number of observed events to the number of expected events under the assumption that the null hypothesis was true (*i.e.*, that there was no difference between the two groups). The expected frequency of observing an *E. coli*-positive sample was calculated for each group.

The total expected frequency of observing an *E. coli*-positive sample was determined for the Prior TC and the No Prior TC group, and similarly the total observed frequency for each group was determined. A Mantel-Cox chi-squared test was used to determine whether the expected rate differed from the observed rate. The overall relative risk (RR) was calculated using Equation 2, where *O* is the number of observed *E. coli* events and *E* is the number of expected *E. coli* events.

$$2 \qquad RR = (O_{\text{Prior TC}} / E_{\text{Prior TC}}) / (O_{\text{No Prior TC}} / E_{\text{No Prior TC}})$$

The Mantel-Cox chi-squared value was compared to the critical value for the chi-squared test with 1 degree of freedom to evaluate the significance of the result. A relative risk of 1 meant that there was no difference between the groups, whereas a relative risk greater than 1 indicated that the outcome was more likely to occur in the Prior TC group compared to the No Prior TC group.

4.2.6   Descriptive Statistics

Relative risk increase and absolute risk increase were calculated as described by Barratt et al (2004). For the Prior TC group, the positive predictive value was calculated to determine the probability of an *E. coli*-positive result after one month and thirty-six months. For the No

Prior TC group, the negative predictive value was calculated to determine the probability of no *E. coli* being detected after one month and thirty-six months. Positive and negative likelihood ratios were also calculated at one and thirty-six months as an additional measure to evaluate whether total coliform presence was predictive of *E. coli* in the short and long term (McGee 2002, Deeks and Altman 2004). A positive likelihood ratio is the ratio of the probability that the system had a prior non-*E. coli* total coliform event and detected *E. coli* in a subsequent sample to the probability that the system had a prior non-*E. coli* total coliform event but did not detect *E. coli* in future samples. Similarly, a negative likelihood ratio is the ratio of the probability of *E. coli* detection in samples that were not preceded by a total coliform-positive result to the probability of no prior total coliform-positive results and no *E. coli* in subsequent samples.

4.3   Results

Of the 1544 drinking water systems included in this analysis, 815 had one or more tests that were positive for non-*E. coli* total coliform bacteria and were placed in the Prior TC group, 704 had no total coliform-positive tests or had an *E. coli* positive result that was not preceded by a non-*E. coli* total coliform-positive test and were placed in the No Prior TC group and 25 drinking water systems were removed from the dataset because the first sample in the dataset was *E. coli*-positive.   Of the total coliform-positive samples used to define the starting point for the Prior TC group, 8 were overgrown with total coliform bacteria present, 213 contained at least ten CFU of total coliforms per 100mL and 594 contained less than ten CFU of total coliforms per 100 mL.

A total of 139 and 78 outcomes were observed in the Prior TC group and No Prior TC group, respectively, by the end of the data collection period. The proportion of positive tests that

occurred in three months or less was 0.35 for the Prior TC group and 0.23 for the No Prior TC group. There was little difference between the two groups in terms of the average time to reach the outcome. The mean time to reach the outcome for systems in the Prior TC group was 10 months and 12 months for the No Prior TC group. Raw life table data is shown in Appendix A.

Five interval periods were analyzed: one month, two months, three months, four months and six months to evaluate the effect that data assumptions had on the measures of risk. The number of data points used in the analysis decreased and more information was lost as the interval width increased.

Interval width (*i.e.*, the number of months selected for the interval period) had a minor effect on the results, when the data was analyzed under the assumption that a missing sample during an interval was equivalent to a sample testing negative for *E. coli*. The same trend was observed for all interval widths when data was analyzed using the event-free assumption (Figure 7). The probability of remaining *E. coli*-free was significantly lower for systems in the Prior TC group, compared to the No Prior TC group.

The Mantel-Cox log-rank test was used to test the null hypothesis that there was no difference between the life table functions of systems with prior total coliforms and systems with no previous record of total coliforms in the distribution system. This difference was statistically significant ($p<0.01$) for all interval widths when data was analyzed using the event-free assumption. However, the difference between groups was not significant when the event-free assumption was not used and interval widths were less than 3 months (Figure 8).
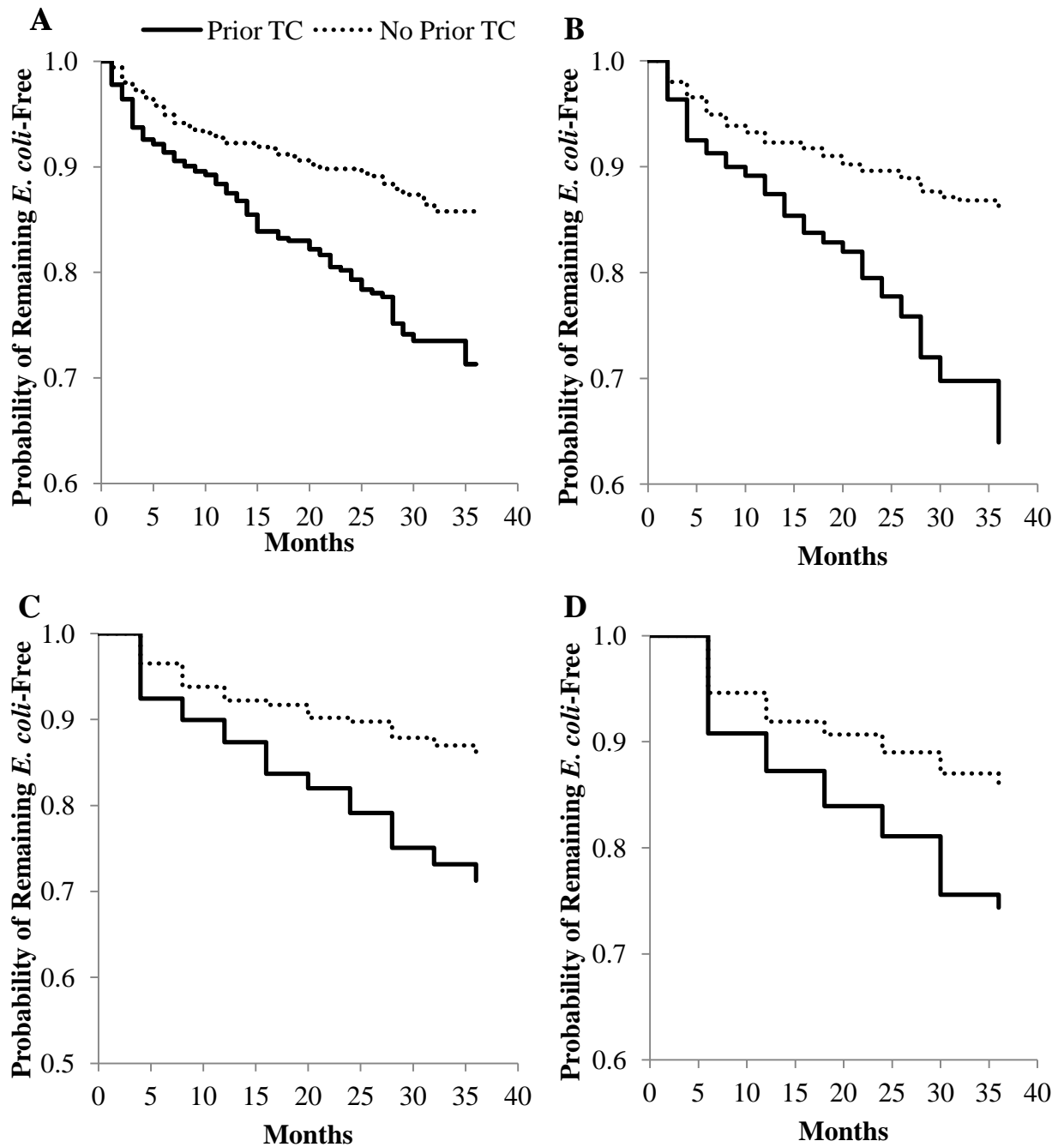
Figure 7. Life Table Functions of Drinking Water Systems with Prior Total Coliform Events and No Prior Total Coliform Events Using the Event-Free Assumption. Interval periods of one (A), two (B), four (C) and six (D) months are shown.

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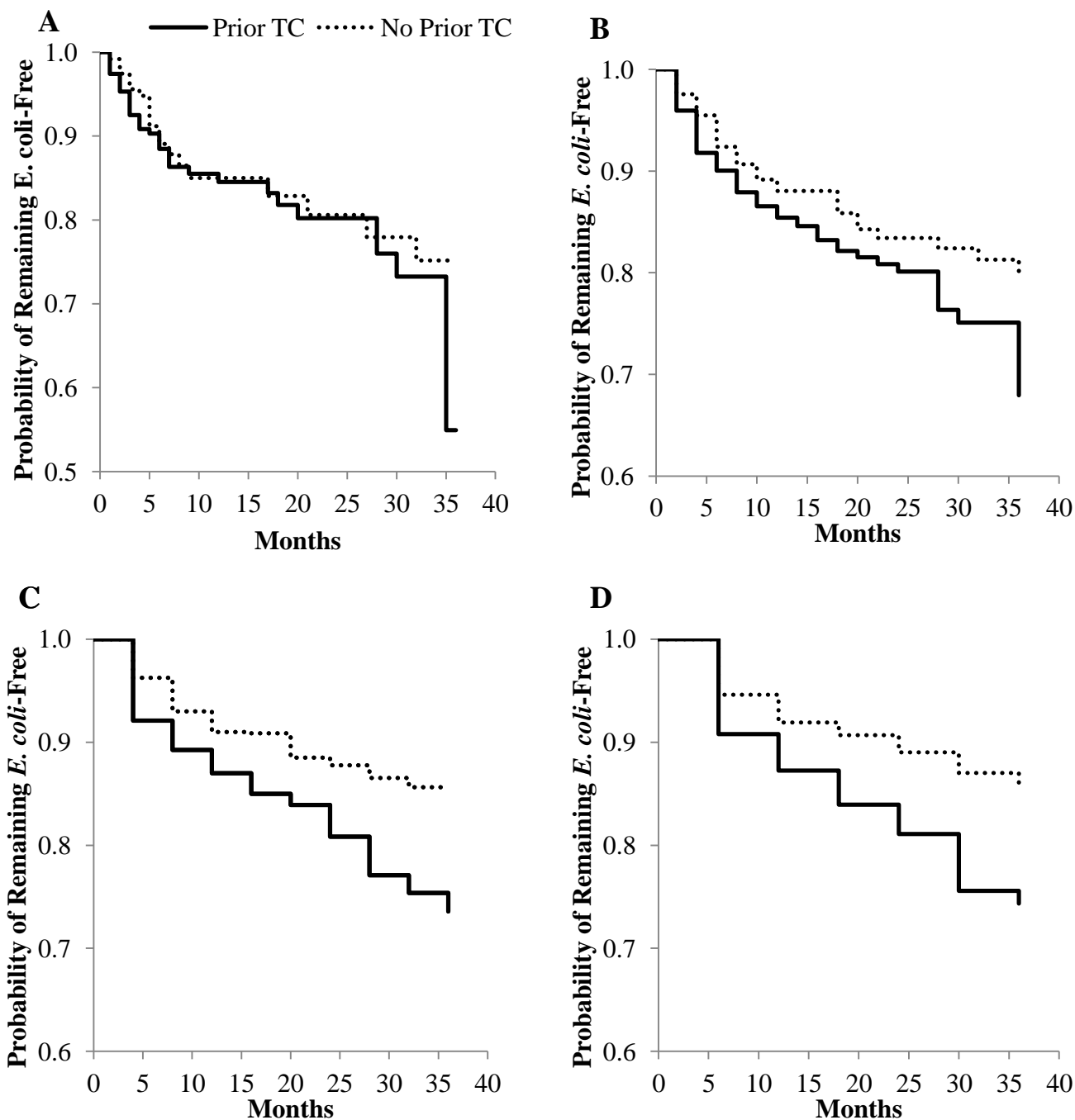
Figure 8. Life Table Functions of Drinking Water Systems with Prior Total Coliform Events and No Prior Total Coliform Events without the Event-Free Assumption. Interval periods of one (A), two (B), four (C) and six (D) months are shown.

The difference between the probability of observing an *E. coli* in the No Prior TC group and the Prior TC group was usually the most substantial in the first interval relative to the other intervals. The hazard ratios for the first interval in some scenarios were greater than 2 (Table 12). This meant that one to four months after a non-*E. coli* total coliform-positive result, these drinking water systems were twice as likely to have *E. coli* detected in a subsequent drinking water sample. Up to one month after non-*E. coli* total coliform bacteria were detected in a water sample, the water system was almost four times more likely to have *E. coli* in a water sample, compared to a system that did not previously detect non-*E. coli* total coliform bacteria.

Life table data used to calculate descriptive statistics is shown in Table 13. It is noteworthy that nearly two-thirds of the systems that detected *E. coli* had prior non-*E. coli* total coliform events and this is not a trivial number. The low event rates are reflected in the low absolute

Table 12. Relative Risk of *E. coli* Presence in Drinking Water

Interval Width	Mantel-Cox Chi-Squared	Relative Risk	Hazard Ratio in Interval 1
With Event-Free Assumption			
One Month	26.5 **	2.04	3.88
Two Months	29.7 **	2.12	1.84
Three Months	24.2 **	1.98	2.33
Four Months	23.4 **	1.96	2.19
Six Months	21.4 **	1.90	1.75
Without Event-Free Assumption			
One Month	2.80	1.49	3.17
Two Months	3.81	1.41	1.63
Three Months	9.78 *	1.66	2.19
Four Months	11.0 **	1.69	2.10
Six Months	12.4 **	1.70	1.71

$* p < 0.01; ** p < 0.001$

Table 13. Life Table for Prior TC and No Prior TC Groups at 1 and 36 Months [a]

	1 Month		36 Months	
	Prior TC	**No Prior TC**	**Prior TC**	**No Prior TC**
Events	18	4	139	78
Systems Lost	0	0	665	537
Non-Events	797	700	11	89
Total Systems	815	704	815	704
Event Rate	0.0221	0.00568	0.171	0.111

[a] Assumption that systems that did not submit a sample during the interval period were event-free

risk increase at one and thirty-six months. These values are 6.0 % at thirty-six months and 1.6 % at one month, which illustrates that there is little difference in the absolute rates at which *E. coli*-positive samples were observed between the two groups. The large relative risk increase at one month indicates that there is a large difference in the event rates between the two groups in relative terms. However, this can be misleading. Even though the event rate for the Prior TC is higher than that of the No Prior TC group, the event rates for both groups are low. The odds ratio at one month shows that a system with a prior non-*E. coli* total coliform result has 4 times the odds (*i.e.*, the ratio of the probability of having an *E. coli* positive sample and the probability of not having an *E. coli* in water) of a system with no prior total coliforms of having an *E. coli*-positive sample. Given that the odds are extremely low in the first place (1:175 for the No Prior TC group), 4 times those odds is still a small number (1:44) but is substantial, considering that British Columbia has hundreds of small water systems.

The positive and negative predictive values for non-*E. coli* total coliform-positive results are summarized in Table 14. The positive predictive values were low due in part to the low

Table 14. Descriptive Statistics of Prior TC and No Prior TC Groups at 1 and 36 Months [a]

	1 Month	36 Months
Absolute Risk Increase	0.0164	0.0598
Odds Ratio	3.95	1.65
Positive Predictive Value of TC Test	0.0311	0.171
Negative Predictive Value of TC Test	0.986	0.889
Positive Likelihood Ratio	1.22	1.23
Negative Likelihood Ratio	0.553	0.748

[a] Assumption that systems that did not submit a sample during the interval period were event-free

outcome frequency and the large number of systems. The slightly higher positive predictive value at thirty-six months compared to one month after a non-*E. coli* positive result suggests that the total coliforms are better predictors of future contamination in the long term. However, even the long-term predictive value appears too low to be of practical significance.

The positive likelihood ratios are greater than one, which indicates an association between having a positive non-*E. coli* total coliform result and having an *E. coli*-positive result in the future. The negative likelihood ratios are less than one, which indicates that having no prior total coliform-positive results is associated with not having an *E. coli*-positive result in the future. The strength of these associations is weak to moderate as both the positive and negative likelihood ratios are close to one.

Similar results were obtained when different criteria were used to sort water systems into one of two groups. Water systems that had at least one non-*E. coli* total coliform event, in which the concentration was at least 10 CFU per 100 mL, were approximately twice as likely to have viable *E. coli* in a future water sample, compared to water systems with no total coliform events or events with less than 10 CFU of total coliforms per 100 mL (RR=1.93).

The Prior TC ≥ 10 CFU group had 392 systems while the No Prior TC ≥10 CFU group had

1,111 systems. The life table function is shown in Figure 9.

The threshold total coliform concentration (*i.e.*, 1 or 10 CFU per 100 mL) used to sort water

systems into groups had a minor effect on the results. At the end of the data collection

period, the cumulative probability of remaining *E. coli*-free was 0.67 for water systems with

at least one TC event where the concentration of total coliforms was at least 10 CFU per 100

mL. By comparison, the cumulative probably of remaining *E. coli*-free was 0.71 for water

systems with a TC event where the concentration was at least 1 CFU per 100 mL.
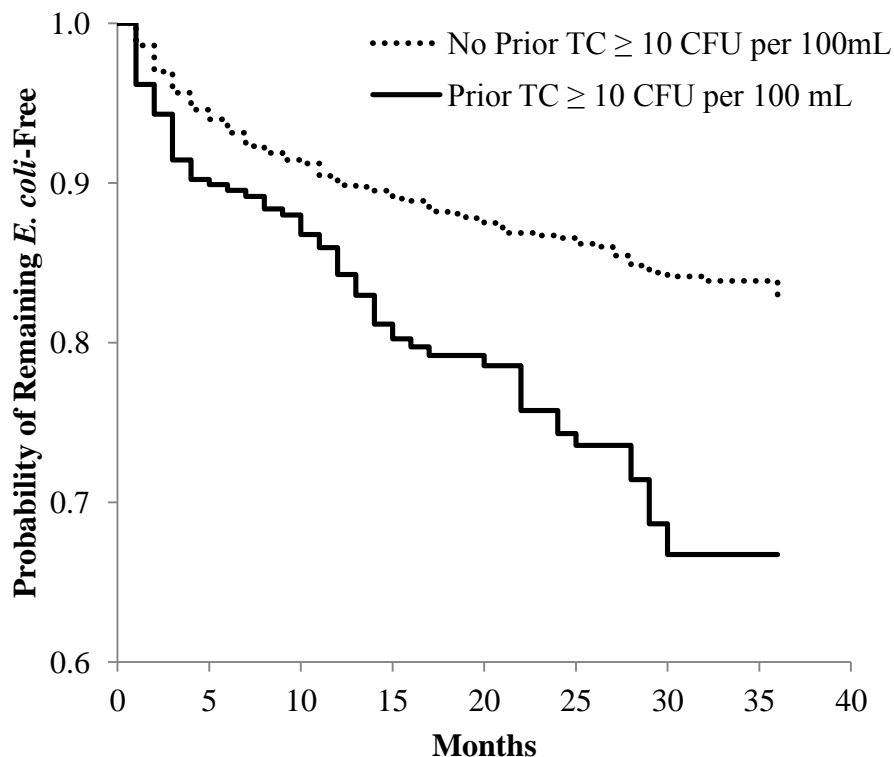
Figure 9. Life Table Functions of Water Systems with No Prior TC ≥10 CFU per 100 mL
and Systems with Prior TC ≥10 CFU per 100 mL. Interval width was one month and the
event-free assumption was used.

4.4   Discussion

First the role of total coliforms as indicators of fecal contamination will be presented, followed by a discussion of the potential for non-*E. coli* total coliforms to act as an early indicator of a vulnerability in one or more of the barriers in place to reduce microbial hazards. The likelihood that these system failures indicated by non-*E. coli* total coliforms will precede human health-relevant changes in microbial water quality has not been investigated previously.

The public health relevance of total coliform bacteria in water has been scrutinized for decades. Criticisms of total coliforms as public health indicators point to a lack of evidence that demonstrates a strong association between total coliforms and outbreak occurrence (MacKenzie et al 1994, Craun et al 1997), endemic gastrointestinal illnesses (Payment et al 1993), or presence of protozoan or viral pathogens (Keswick et al 1984, Rose et al 1991). While some studies have found a weak relationship between total coliform presence and presence of pathogens (Wilkes et al 2009), the relationship appears to be site-dependent.

Since system failures that lead to microbiological contamination of treated drinking water are infrequent, most total coliform-positive results will be false positives (Hrudey and Leiss 2003). False positive results are those that indicate that fecal contamination is present when it is not. They can be due to presence of interferents that have enzyme activity, water sample contamination during collection or presence of total coliforms in water that are of non-fecal origin. Natural reservoirs including soils and plants (Grimont et al 1981, Brady et al 2008) have been documented for many species belonging to the total coliform group. Some types of total coliform bacteria are recognized plant pathogens (Chung et al 1993, Topley 1997, Leclerc et al 2001).

By comparison, *E. coli* would presumably falsely indicate fecal contamination less frequently than non-*E. coli* total coliforms because *E. coli* is almost exclusively of fecal origin. Thus, non-*E. coli* total coliforms in drinking water are less likely to indicate a true danger to human health than *E. coli*. Further support that non-*E. coli* total coliforms are less likely than *E. coli* to indicate a true hazard is the finding of Strauss et al (2001), that the odds ratio for acquiring acute gastrointestinal illness from consumption of water that tested positive for *E. coli* was 1.52, while that for total coliforms was 0.39.

It is clear that the value of non-*E. coli* total coliform bacteria in estimating the immediate risk to human health is limited. High levels of fecal contamination in water should be indicated by presence of *E. coli* along with other species from the total coliform group, since *E. coli* is present at concentrations that range from 10 to 10,000 CFU per gram of feces (Leclerc et al 2001). However, non-*E. coli* total coliforms may have predictive value as non-*E. coli* total coliforms in water indicate an increase in microbial loading rate, which is the result of a failure of system barriers to effectively reduce microbiological hazards. If such weaknesses in system barriers are not addressed, the system may be vulnerable to future contamination events, which could have human health consequences.

It is possible that non-*E. coli* total coliforms may be able to outcompete *E. coli* for finite resources under culture conditions because they are likely to be more abundant initially in a water sample than *E. coli*, giving them a substantial growth advantage. As such, non-*E. coli* total coliforms may be detected in some instances where small amounts of contamination are present but *E. coli* is not detected. In a previous study total coliform bacteria were detected at higher frequency and in higher concentrations than fecal coliform bacteria in filtered, ozonated and finished water (Payment et al 1985). This suggests that total coliforms may be

a more sensitive indictor than fecal coliforms, including *E. coli*, for small increases in microbial loading rates.

It appears that the frequency at which small increases in microbial loading rates, indicated by non-*E. coli* total coliforms, is followed by detection of *E. coli* in drinking water is low. The fact that one month after a non-*E. coli* total coliform-positive test, only 3% of systems had further evidence of fecal contamination supports the argument that most non-*E. coli* total coliform-positive results do not predict human-health-significant contamination events in the short term. Other groups have also shown that positive predictive values for rare hazards tend to be low (Hrudey and Leiss 2003).

Drinking water samples infrequently tested positive for *E. coli* so the event rate was low for both groups of small water systems analyzed. The difference in absolute risk between the group of systems with prior non-*E. coli* total coliforms and the group with no prior total coliforms indicates that systems with prior total coliforms are only slightly more likely to have *E. coli* in a future water sample because *E. coli*-positive events are extremely rare for both groups at one month. Consequently, the absolute risk increase for systems that had previously detected non-*E. coli* total coliforms was only 1.6% at one month, and only slightly higher in the long term with an absolute risk increase of 6.0% up to three years after the initial non-*E. coli* total coliform event.

Although the absolute risk increase was low, the relative risk value showed that drinking water systems that had non-*E. coli* total coliforms in the distribution system were twice as likely to have an *E. coli*-positive test in the months following the initial total coliform event, compared to systems with no prior total coliforms (RR=2.04). The hazard ratio suggested that systems in the Prior TC group were nearly four times more likely to detect *E. coli* one

month after a non-*E. coli* total coliform event, compared to systems in the No Prior TC group. The likelihood ratios suggest that an association between detection of non-*E. coli* total coliforms and detection of *E. coli* in a subsequent water sample exists, but both the positive and negative likelihood ratios fall short of those considered significant for practice. Thus, it is a challenge to interpret non-*E. coli*-total-coliform-positive test results when there is no evidence of an operational failure in the water system.

In order to interpret the meaning of microbiological testing results, it is necessary to understand the multitude of factors that govern microbial loading rates and survival in different environments. Microbial water quality of surface water may be affected by factors that can vary widely temporally and spatially such as weather patterns (*i.e.*, heavy rainfall, long periods of drought), seasonal variations in temperature and watershed land uses (*i.e.*, agricultural, urban, industrial). The complex interactions that occur between each of these factors and their impacts on water quality accounts for some of the challenges faced in developing predictive approaches to evaluating human health-relevant changes in water quality. Some predictive water quality models have been proposed (Ailamaki et al 2003, Wu et al 2009) but are not widely used in practice.

Limitations of this study include that there were few systems for which monthly sampling data was available for the entire three-year period. Many systems submitted samples on an irregular basis, or for a limited period of time. This resulted in a high number of systems classified as 'lost' and a large discrepancy in sample size from the beginning to the end of the study period. The data points in the final intervals of the life table analysis were based on a small number of systems, which means that a single *E. coli* event had a greater impact on the cumulative probability of having an *E. coli*-positive sample in the final intervals. Information

regarding type of water treatment was not available so it was not possible to control for total coliform events that may have occurred due to treatment malfunctions or *E. coli* events that may have been prevented due to installation of new treatment systems. It was assumed that all water samples were submitted for routine purposes, but collection may have been related to a specific event (*i.e.*, turbidity spike, water-main break) in which case a sample bias towards total coliform and *E. coli*-positive samples may exist. Water system metadata that may be used to indicate a change in microbiological water quality including turbidity measurements, chlorine residual and documented water-main breaks were not available. Analysis of the relationship between total coliform events in small water systems and these variables may be an area of future study.

These results show that drinking water systems with one or more non-*E. coli* total coliform-positive test results had a greater chance of obtaining an *E. coli*-positive result in subsequent months, relative to systems with no prior total coliforms detected in the distribution system. The absolute risk increase, however, is too low to justify executing immediate hazard-reduction measures based on detection of non-*E. coli* total coliforms alone, but evidence of non-*E. coli* total coliforms warrants an appropriate precautionary response. When using microbiological data to make risk management decisions, application of the appropriate degree of precaution is critical and should consider the likelihood of obtaining false-positive and false-negative errors (Hrudey and Leiss 2003). For rare events, the probability of obtaining a false negative is low but the false positive rate is high. On one hand, erring on the side of caution that total coliforms may be an early indicator of poor water quality, and hence a possible threat to human health, may be reasonable. It is clear however that issuing a boil-water notification immediately based on total coliform results alone may not be

defensible before the possibility of a false-positive error has been ruled out. This latter type

of response could have the downside of decreasing public confidence in drinking water

providers in the event of a type I error. On the other hand, if after careful examination of

other risk factors the decision-maker determines there is an unacceptably high risk from

consumption of the water, acting quickly to issue a public notification (in the event of a true

contamination incident) could prevent numerous illnesses and potentially hospitalizations.

A stepwise response to non-*E. coli* total coliform-positive drinking water test results would

be appropriate to verify that the result was not due to sampling error, determine the likelihood

of fecal contamination through further testing and characterize the inputs to the system. The

majority of samples containing non-*E. coli* total coliforms are not indicative of fecal

contamination but may represent a failure in the multi-barrier system to reduce microbial

hazards, so the first follow-up response should be to rule out the possibility of sample

contamination during collection by re-sampling the site. This is the first course of action

recommended by Health Canada after a total coliform-positive result is obtained (Health

Canada 2011). Other responses may include increased diligence in system operations and

characterization of the total coliform bacteria detected to gather further evidence as to

whether the system is impacted by fecal contamination. Upon collection of additional

evidence that fecal contamination exists, performing a sanitary survey and applying microbial

source tracking molecular tools would be appropriate.

4.5   Conclusions

This research demonstrated using life table analysis that presence of non-*E. coli* total

coliform bacteria indicates a slightly higher likelihood that a small drinking water system will

test positive for *E. coli* in the following months compared to a small system that had no prior

total coliforms detected (RR=2.04). But, given the rarity of viable *E. coli* detection in

drinking water, the absolute risk increase is low. Although the presence of non-*E. coli* total

coliform bacteria does not in itself indicate a health risk, they have value in the context of

drinking water distribution system monitoring on the basis that they indicate a need to

perform further investigations to confirm or dismiss the potential that the system is impacted

by fecal pollution.

Chapter 5: Identification of Fecal Contamination Sources in Water Using Host-Associated Markers

In British Columbia, drinking water is tested for total coliforms and *Escherichia coli* but there is currently no routine follow-up testing to investigate fecal contamination sources in samples that test positive for indicator bacteria. Reliable microbial source tracking (MST) tools to rapidly test water samples for multiple fecal contamination markers simultaneously are currently lacking. The objectives of this study were (1) to develop a qualitative MST tool to identify fecal contamination from different host groups, and (2) to evaluate the MST tool using fecal samples from host groups, heavily contaminated water samples, raw water and drinking water samples with evidence of fecal contamination. Host-associated markers were selected for humans, livestock (cattle, chickens, pigs, horses), wildlife (deer, Canada geese, seagulls) and domestic pets (dogs). Singleplex and multiplex PCR were used to test water from heavily polluted sites (n=7), and raw (n=14) and drinking water samples (n=18) with evidence of fecal contamination for presence of fecal markers associated with humans, cattle, seagulls, pigs, chickens and Canada geese. The multiplex MST assay correctly identified the suspected contamination source in 5 out of 7 contaminated waterways, demonstrating that it may have utility for heavily contaminated sites. The detection limit of singleplex PCR was generally 100 times lower than that of multiplex PCR. More than half of the raw and drinking water samples analyzed using singleplex PCR contained at least one host-associated marker. Singleplex PCR was capable of detecting host-associated markers in small sample volumes and is, therefore, a promising tool to further analyze water samples submitted for routine testing.

5.1   Introduction

Consumption of microbiologically contaminated drinking water poses a substantial human health risk. In Canada, the estimated rate of acute gastrointestinal illness attributable to water is 0.11 cases per person per year (Messner et al 2006), and Health Canada estimates that consumption of unsafe drinking water is responsible for 90 deaths each year (Sierra Legal Defense Fund 2006). Illnesses acquired through consumption of contaminated water also pose a substantial economic burden. The estimated annual economic burden of acute gastrointestinal illness reflecting food, water and person-to-person exposure in British Columbia (BC) is CAN$514 million (Henson et al 2008). This includes costs associated with diagnosis, treatment and missed employment. Waterborne diseases are largely preventable through application of multi-barrier approaches that consider potential points of pathogen entry into a water supply system from source to tap.

In BC, drinking water is routinely tested for *E. coli* to ensure the absence of fecal contamination. Approximately 1% of drinking water samples tested annually from across the province contained viable *E. coli* (Kendall 2008). Often, the source of contamination is unknown. Identification of probable contamination sources may be used to manage the issue and may have importance in estimating risk as different fecal sources pose variable human health risks (Soller et al 2010). Health officials responsible for conducting investigations of adverse water quality events have limited tools available to provide additional information about the fecal contamination source.

A suite of microbial source tracking (MST) tools have emerged to address the need to identify fecal contamination sources, and many microbial source tracking markers targeting host-associated bacteria and viruses have been identified (Roslev and Bukh 2011). These

markers have been used in the development of various MST tests, but no single test has been identified as superior or entirely reliable (Field and Samadpour 2007). Most researchers recommend a 'toolbox' approach using multiple targets, in order to increase confidence that the fecal contamination source is accurately identified. The vast majority of these tests use PCR to detect and/or quantify host-associated markers. PCR offers the advantages of rapid analysis time, high sensitivity and versatility to detect targets from all types of microorganisms.

Bacteria belonging to the order Bacteroidales have emerged as the frontrunners of host-associated microbial targets, due to their high prevalence rate across members of the same host group (Shanks et al 2010a, Shanks et al 2010b), high relative abundance in feces of many hosts (Wang et al 1996, Harmsen et al 1999), and relatively high degree of host association (Bernhard and Field 2000a, Dick et al 2005, Shanks et al 2007, Haugland et al 2010). Studies investigating the persistence and decay rate of *Bacteroides* markers have largely focused on isolating specific factors that have the greatest impact on decay rate such as sunlight, presence of predatory microorganisms, temperature and salinity (Ballesté et al 2010, Dick et al 2010, Green et al 2011, Schulz and Childers 2011, Solecki et al 2011). These studies have taken place under controlled conditions using microcosms and little is known about persistence of these markers in complex natural environments. Also, studies have largely focused on impaired surface waters and little is known about marker prevalence in groundwater systems or treated drinking water. This is significant as close to 40% of small drinking water systems in BC rely on groundwater as a drinking water source (Drinking Water Review Panel 2002), and vulnerability to contamination is often poorly characterized. Contaminated groundwater has also been implicated in approximately three quarters of

waterborne outbreaks in the United States (Reynolds et al 2008).

When used in combination with fecal indicator testing in cases where the fecal contamination source is not obvious or to confirm findings from a visual site inspection, a qualitative tool capable of detecting multiple sources of fecal contamination could be used to further analyze routine water samples that test positive for fecal indicator bacteria such as *E. coli*.

The objective of this study was firstly to develop a qualitative test to detect multiple markers of fecal contamination from hosts that are common polluters of waterways in BC. Singleplex and multiplex PCR was applied to design an assay capable of discriminating between different host-associated markers based on amplicon size. Markers for fecal contamination from the following host groups were included: Humans, cattle, pigs, seagulls, dogs, deer, chickens, and Canada geese. The second aim was to determine whether the assay could detect environmentally relevant concentrations of host-associated fecal markers in water with heavy microbial pollution loads, raw water and inadequately treated drinking water.

5.2 Methods

5.2.1 Host-Associated Primer Identification and Modification

A review of MST literature identified validated host-associated PCR primers for wildlife (*i.e.*, deer, gulls, geese); livestock (*i.e.*, cattle, horses, pigs, chickens); dogs and humans. Primers were chosen based on sensitivity and selectivity described in peer-reviewed studies. Selectivity is defined as the extent that a method can detect the marker of interest in a complex mixture without being affected by other components in the matrix (Vessman et al 2001). In environmental science, 'selectivity' is often used interchangeably with 'specificity' (Hrudey and Leiss 2003). An additional consideration was the ability of primer pairs to

generate amplicons of a unique size that could be resolved on an agarose gel. Primer

sequences, target gene and/or microorganism, melting temperature (T_m), amplicon size are

listed in Table 15. Primers targeting DNA in the 16S rRNA gene are shown in Figure 10.

If two different primer pairs generated PCR products of similar size, one of the primer sets

was modified to increase or decrease the amplicon size. Sequences for target amplification

regions were obtained from GenBank and multiple sequence alignments were performed

using BioEdit software (Ibis Biosciences, Carlsbad, CA). Unique segments were identified

and used to design new primers. The primers to detect the horse-associated marker were

modified to reduce amplicon size using an uncultured equine intestinal bacterium sequence

(GenBank Accession number AB056669.1). The National Center for Biotechnology

Information (NCBI) Primer Basic Local Alignment Search Tool (BLAST) was also used to

confirm that no close matches to uncultured bacteria isolated from non-hosts existed in the

environmental sequence database (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

5.2.2   Fecal Sample Collection

Composite fecal samples were collected aseptically from the following hosts: human

(slurries from two household septic systems), cattle (Charlois and Holstein), chicken (two

populations), deer (European fallow and black-tailed), western seagull, Canada goose (two

populations with different diets), potbelly pig, dog (various breeds) and horse (various

breeds). Fecal samples were collected using sterile utensils and transferred into sterile plastic

containers. Samples were stored at -20 °C until analysis. Each composite sample was made

up of feces from four to nine hosts. The freshness of the fecal samples at the time of

collection varied. Although freshness can affect the types and relative amounts of

Table 15. Host-Associated PCR Primers

Host	Target	Primer Name	Primer Sequence (5' to 3')	References	T_m (°C)	Amplicon Length (bp)
Human	Human-associated *Bacteroides 16S*	HF183 F HF 183 R	ATC ATG AGT TCA CAT GTC CG TAC CCC GCC TAC TAT CTA ATG	Bernhard and Field 2000a, Seurinck et al 2005	57 56	83
Cow	Bovine-associated *Bacteroides 16S*	BacBov1 F BacBov1 R	AAG GAT GAA GGT TCT ATG GAT TGT AAA GAG TTA GCC GAT GCT TAT TCA TAC G	Lee et al 2010	61 62	101
		BacBov2 F BacBov2 R	GGA TTA CAG CCC TAC GGG TTT TA GGA GTT AGC CGA TGC TTA TTC ATA TAA		62 62	101
Pig	Swine-associated *Bacteroides 16S*	Pig1-Bac F Pig1-Bac R	AAC GCT AGC TAC AGG CTT AAC CGG GCT ATT CCT GAC TAT GGG	Mieszkin et al 2009	56 63	129
		Pig2-Bac F Pig2-Bac R	GCA TGA ATT TAG CTT GCT AAA TTT GAT ACC TCA TAC GGT ATT AAT CCG C	Okabe et al 2007	62 59	116
Horse	Bacteroidales *16S*	HoF597 F Bac708 R	CCA GCC GTA AAA TAG TCG G CAC ATG TTC CTC CGC TCG TA [*]	Dick et al 2005	58 62	354
		Ho2 F Ho2 R	CCA GCC GTA AAA T(A/C)G TCG G TTC GCT TGG CGT CTC AGC CA	This study	62 70	265
Dog	Canine-associated *Bacteroides 16S*	BacCan-545 F BacUni690 R	GGA GCG CAG ACG GGT TTT AAT CGG AGT TCC TCG TGA TAT CTA	Kildare et al 2007	64 60	145

Host	Target	Primer Name	Primer Sequence (5' to 3')	References	T$_m$ (°C)	Amplicon Length (bp)
Deer	*Enterococcus Mut2*	D40 F D40 R	TCA TGA TCT GTG CAA TAT TCG AC AGC AAT TAC TGA AGA AGA AGT TTT G	Soule et al 2006	60 58	326
	cytB	cytB F cytB R	AGG AGT ACT ACT TCT AGT CCT [*] GAT TAA GAT AGA TGA GAC TAG GGC [*]	Schill and Mathes 2008	46 55	210
Goose	Prevotella *16S*	CGPrevf5 F CGPrevf5 R	CCC ACC AAG CCG TCG AT GCT TAA CCT GCG GCC TG	Lu et al 2009	61 59	327
		CGOF1 F CGOF1 R	GTA GGC CGT GTT TTA AGT CAG C GTG TCT CAG TA(T/C) AAC GGC ACG GTT [*]	Fremaux et al 2009	57 65	70
Gull	*Catellicoccus marimammalium 16S*	Gull-2 F Gull-2 R	TGC ATC GAC CTA AAG TTT TGA G GTC AAA GAG CGA GCA GTT ACT A	Lu et al 2008	59 57	412
Chicken	*Bacteroides*	CP2-9 F CP2-9 R	GTA AGA CAG CAA CCC CAT GTA ACC TAT GGT TCA ACA CGC TTTA	Lu et al 2007	58 59	251
	Brevibacterium *16S*	LA35 F LA35 R	ACC GGA TAC GAC CAT CTG C TCC CCA GTG TCA GTC ACA GC	Weidhaas et al 2010	61 62	571

[*] Primer modified for this study

1   aaattgaagagtttgatcatggctcagattgaacgctggcggcaggcctaacacatgcaagtcgaacg|gtaacaggaagaagcttgcttctttgctgac|gagtggcggacggg
                                                                    1 gcatgaatttagcttgctaaatttgat
                                                                    2 tgcatcgacctaaagtttttgag

114   tgagtaatgtctgggaaactgcc|tgatggagggggataactactggaaacggtagctaataccgcataacgtcgcaagaccaaagagggggtaccttcgggcctcttgccatcg|
                              1 gcggattaataccgtatgaggt
                              3 atcatgagttcacatgtccg

227 |gatgtgcccagatggc|attagctagtaggtgggtaacggctcacctaggcgacgatccctagctggtctgagaggatgaccagccacactggaactgagacacggtccagac
                  3 cattagatagtaggcggggta

340   tcctacgggaggcagcagtggggaatattgcacaatgggcgcaagcctgatgcagccatgccgcgtgtatgaagaaggccttcgggttgtaaa|gtactttcagcgggggaggaa
                                                                                4 ggattacagccctacgggtttta

453 |gggagtaaagttaatacctttgctcattgacgttaccogcagaag|aagcaccggctaactccgtgccagcagccgcggtaatacggagggtgcaagcgttaatcggaattact
                  2 tagtaactgctcgctctttgac
              4 ttatatgaataagcatcggctaactcc

566   g|cgcacgcaggcggtttgttaagtcagatgtgaaatccccgggctcaacctgggaactgcatctgatactggcaagcttgagtctcgtagagggggggtagaattccag|gtgta
           5 gtaggccgtgtttttaagtcagc   5 aaccgtgccgttatactgagacac

679   gcggtgaaatgcgtagagatctggaggaataccggtggcgaaggcggccccctggacgaagactgacgctcaggtgcgaaagcgtggggagcaaacaggattagataccctgg

792   tagtccacgccgtaaacgatg|ccgacttggaggttgtgcccttgaggcgtggcttccggagctaacgcgttaagtcgac|cgcctggggagtacggccgcaaggttaaaactca

1018 aatgaattgacggggggcccgcacaagcggtggagcatgtggtttaattcgatgcaacgcgaagaacccttacc|tggtcttgacatccacggaagttttcagagatgagaatgtg

1131 |ccttcgggaaccgtgag|acaggtgctgcatggctgtcgtcagctcgtgttgtgaaatgttgggttaagtcccgcaacgagcgcaaccctt|atcctttgttgccagcggtccgg

1244 |ccgggaactcaaaggagactgccagtgataaact|ggaggaaggtgggggatgacgtcaagtcatcatggcccttacgaccagggctacacacgtgctacaatgg|cgcatacaaa

1357 |gagaagcgacctcgcgagagcaagcggacctcataaagtgcg|tcgtagtccggattggagtctgcaactcgactccatgaagtcggaatcgctagtaatcgtggatcagaatg

1470 ccacggtgaatacgttccecgggccttgtacacaccgcccgtcacaccatgggagtgggttgcaaaagaa|gtaggtagcttaaccttcgggagggcgctta|ccactttgtgatt

1583 catgactggggtgaagtcgtaacaaggtaaccgtaggggaacctgcggttggatcacctcctta

1 Swine
2 Gull
3 Human
4 Bovine
5 Canada goose

Figure 10. *E. coli* 16S rRNA Gene. (numbering from Brosius et al 1978) Primer sets for the 16S rRNA gene of bacteria specific to swine, gull, human, bovine, and Canada goose feces are underlined and numbered. The nine hypervariable regions are illustrated in boxes.

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microorganisms present, it was less critical in this study where only DNA persistence, and not cell viability, was being examined.

5.2.3   Genomic DNA Extraction and Quantification

Genomic DNA was extracted from approximately 100 mg of fecal sample using a MoBio Soil DNA Isolation Kit according to the manufacturer's protocol (MoBio Laboratories Inc, Carlsbad, CA). The extracted DNA was stored at -20 °C in 10 mM Tris buffer. Fecal DNA extracts were quantified using the Qubit system (Invitrogen, Carlsbad, CA). This fluorescence technology provides more accurate DNA quantification data than UV spectrometry based methods when the sample matrix contains interferents that absorb light at 260nm (O'Neill et al 2011).

5.2.4   MST Assay Development

Each host-associated primer pair was evaluated using singleplex PCR. This involved determining (1) whether the target sequence was present in the feces of presumed hosts and non-presumed hosts; (2) whether the target sequence would be amplified from fecal DNA extracts from presumed hosts in the presence of genomic bacterial DNA from the feces of non-hosts; and (3) the detection limit of the assay. Primer pairs that were found to be strongly host-associated through empirical and *in silico* analysis were pooled and evaluated in a multiplex format.

5.2.4.1   Marker Prevalence in Hosts

Singleplex PCR was used to determine if the target sequence was present in a host fecal DNA sample. Each reaction contained one illustra PureTaq Ready-to-go PCR bead (GE Healthcare, Waukesha, WI), 20.5 μL double-deionized water (ddH$_2$O), 2.5 μL of template

DNA, 1 μL of 6.25 μM forward primer and 1 μL of 6.25 μM reverse primer (Invitrogen, Carlsbad, CA).

A positive control using broad spectrum 16S rRNA gene primers was included to verify that the sample did not contain significant levels of PCR-inhibitory compounds and that bacterial DNA was present (*i.e.*, the DNA extraction procedure was successful). These broad spectrum 16S rRNA primers 8f 5'-AGAGTTTGATCATGGCTCAG and 519r 5'-GWATTACCGCGGCKGCTG have been validated by Eden et al (1991) and Lane et al (1985) respectively. Each reaction contained 12.5 μL Qiagen Hot StarTaq Master Mix, 9.4 μL ddH$_2$O, 0.3 μL of 20 μM forward primer 0.3 μL of 20 μM reverse primer, and 2.5 μL of template DNA. The following cycling conditions were used for all reactions using 16S rRNA primers: One 15 min incubation step at 95 °C to activate the HotStarTaq; thirty-five cycles of 1 min at 94 °C, 1min at 56 °C and 1 min at 72 °C.

5.2.4.2   Evaluation of Selectivity

In silico analysis of selected primers was conducted using NCBI BLAST. Each primer pair was searched against the GenBank non-redundant sequence database. BLAST was used to identify most probable matches between the primer query sequence and those contained in the database, including uncultured and environmental sample sequences. Significant matches had an expected value of less than 0.002, at least 95% identity with the primer sequence and at least 90% coverage. The description of the sequence source was recorded and classified as host or non-host.

The selectivity of each primer set was evaluated to determine the ability of the test to differentiate the target of interest in the presence of other components. Genomic DNA from each non-host fecal sample was pooled to make "selectivity mixes" for each host. Each

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selectivity mix contained 2 µL of extracted fecal DNA from each non-host species to obtain similar relative amounts of microbial DNA from each. The selectivity mixes contained 1 µL of extracted fecal DNA from dairy cattle and 1 µL from beef cattle to ensure that the mix was representative of the microorganisms found in the feces of both varieties of cattle, as these have been shown to be different (Durso et al 2010). The selectivity mix containing DNA from all non-hosts was used as the template DNA for the PCR with host-associated primers. Each reaction contained 2.25 µL of selectivity mix, one illustra PureTaq Ready-to-go PCR bead (GE Healthcare, Waukesha, WI), 1 µL of 6.25 µM host-associated forward primer and 1 µL of 6.25 µM host-associated reverse primer and 20.75 µL ddH$_2$O. The following cycling conditions were used: 50 °C for 2 min, 95 °C for 5 min, and 35 cycles of 94 °C for 30 sec, 61 °C for 30 sec and 72 °C for 40 sec. The absence of PCR products verified that the concentration of the target sequence was below the detection limit of the assay in fecal matter from the non-host groups tested. These experiments were repeated to verify the results.

The ability of host-associated primers to amplify the target sequence in the presence of non-target sequences from non-hosts was evaluated by adding host fecal DNA to the selectivity mix and amplifying the target region using host-associated primers. Each reaction contained 2.25 µL of selectivity mix and 0.25 µL of host fecal DNA, one illustra PureTaq Ready-to-go PCR bead (GE Healthcare, Waukesha, WI), 1 µL of 6.25 µM host-associated forward primer and 1 µL of 6.25 µM host-associated reverse primer 20.5 µL ddH$_2$O and used the same cycling conditions described above. Presence of an amplicon of the correct size indicated that the test was selective for the target evaluated. Non-selective primers were not evaluated further for sensitivity and were not included in the multiplex PCR.

5.2.4.3 Evaluation of Sensitivity

The sensitivity of the test was evaluated for human, cattle, pig, chicken, seagull and Canada goose feces in ddH_2O. Approximately 150 mg of feces from each host group was weighed and suspended in 1 mL of ddH_2O. This solution was used to prepare 50 mL solutions containing 100 mg of feces, 1 mg of feces and 100 mL serial dilutions containing 0.01 mg and 0.0001 mg of feces. Samples (50 mL) were passed through a 0.45 μm GN-6 Metricel filter (Pall Corporation, Port Washington, NY) and the filter was cut into small pieces. The DNA from the cells captured on the filter was extracted using a MoBio Soil DNA Isolation Kit (MoBio Laboratories Inc, Carlsbad, CA). The DNA from each fecal sample was analyzed using singleplex PCR with host-associated primers in triplicate. If 2 out of 3 or more of replicates in a particular dilution were positive, the assay was considered capable of detecting the marker at that concentration. A separate reaction using broad spectrum 16S primers was included to verify that PCR inhibition did not occur.

5.2.4.4 Multiplex PCR

Host-associated primers for human, cattle, pig, chicken, seagull and Canada goose fecal contamination were evaluated in a multiplex PCR. A positive control containing fecal DNA from all hosts and a negative control to assess primer-primer interactions (*i.e.*, formation of dimers) and contamination was included.

Gradient multiplex PCR was used to empirically determine the optimal annealing temperature and number of cycles to minimize non-specific PCR product amplification. Each reaction contained one illustra PureTaq Ready-to-go PCR bead (GE Healthcare, Waukesha, WI); 0.5 μL of fecal DNA from each host (chicken, human, pig, cow, gull); 10 μL of primer mixture; and 12.5 μL ddH_2O. The negative control contained a PCR bead, 10 μL of

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primer mixture; and 15 μL ddH$_2$O. The primer mixture contained each of the following

primers: CP2-9F, CP2-9R, HF183F, HF183R, Pig2-BacF, Pig2-BacR, BacBov2F,

BacBov2R, Gull2F and Gull2R. The final concentration for chicken (CP2-9) and Canada

goose (CGOF1) primers was adjusted to 500 nM while the final concentration for human

(HF183), pig (Pig2-Bac), cow (BacBov2) and seagull (Gull2) primers was 300 nM.

Annealing temperatures tested were 55.1 °C, 57.1 °C, 59.5 °C and 61.2°C.

The optimal theoretical annealing temperature was determined as follows. The melting

temperature (T_m) for each primer and product were calculated using a modified nearest

neighbor method described by Von Ahsen et al (2001) shown in Equation 3. Na^+_{eq} is sodium

ion equivalents; GC is the GC content of the primer or product and bp is the nucleotide

length of the primer or PCR product.

3 T_m (°C) = 77.1 °C + 11.7 log [Na^+_{eq}] + 0.41 GC – 528/ bp

The average optimal annealing temperature (T_a^{OPT}) was calculated using the method

described by Rychlik et al (1990) shown in Equation 4.

4 T_a^{OPT} (°C) = 0.3 T_m^{primer} + 0.7 $T_m^{product}$ – 14.9

The calculated average optimal annealing temperature (61 °C) was tested experimentally

using gradient PCR (55.1 °C, 57.1 °C, 59.5 °C and 61.2 °C) and 34 cycles to confirm that

these cycling conditions amplified targets successfully while minimizing non-specific

products.

5.2.5   Separation and Visualization of Amplicons

PCR-amplified samples were loaded onto a 2.0 % agarose gel stained with GelRed Nucleic

Acid Stain (Biotium Inc, Hayward, CA). Lanes were loaded with a mixture that consisted of

4 μL of PCR product mixed with 3 μL of gel loading buffer. One lane of each gel contained 4 μL of Bio-Rad EZ load 100 bp molecular ruler (Bio-Rad Laboratories Inc, Hercules, CA). The gel was run at 100 V for 90 minutes, visualized under UV light and photographed using a Bio-Rad Gel Doc imaging system (Bio-Rad Laboratories Inc, Hercules, CA). The size of each amplicon was determined visually.

5.2.6   Amplicon Sequencing

Amplicons were sent to the Centre for Molecular Medicine and Therapeutics (Vancouver, BC) for sequencing using an Applied Biosystems 3130xl 16-capillary genetic analyzer (Life Technologies, Carlsbad, CA).

5.2.7   Microbial Source Tracking Assay Applications

5.2.7.1   Sample Sites

5.2.7.1.1   Heavily Contaminated Environmental Water

Seven heavily contaminated sites that were known to contain fecal inputs from one or more host species were identified. Site 1 was located two kilometres downstream from a sewage treatment plant (secondary) outfall that discharged about 500,000 m^3/day of effluent. Site 2 was a stream that was suspected to contain sewage from failing municipal infrastructure. Site 3 was a roadside ditch located in an agricultural area adjacent to a hog farm and a dairy farm. Site 4 was a lagoon within an urban park inhabited by Canada geese and other waterfowl. Site 5 was a ditch impacted by run-off from a nearby chicken farm. Site 6 was a coastal area with some nearby industrial activities, and the beach was inhabited by seagulls. Site 7 was a slough located in an agriculturally intensive area. Samples collected from the Metro Vancouver drinking water distribution system were used as negative control samples (site 8). The Seymour and Capilano watersheds are protected and do not have significant

inputs from urban or agricultural sources. However, it is possible for wildlife including deer, birds and rodents to access the watershed. Metro Vancouver drinking water is treated using filtration and chlorine disinfection (Seymour) and chlorine only (Capilano).

5.2.7.1.2   Raw and Drinking Water

E. coli-positive raw water (n=14) and drinking water (n=7) samples submitted to the Provincial Public Health Microbiology Reference Laboratory (PPHMRL) in Vancouver, BC for routine microbiological testing were obtained over a one-month period. Water samples sites were located across the province and the source of contamination was unknown. Up to 200 mL of water is submitted, of which 100 mL is used for indicator bacteria testing.

Large-volume drinking water samples (n=11) were collected from small drinking water systems in the Interior Health Authority region of BC. Samples were collected from systems that had recently submitted a drinking water sample that tested positive for total coliform bacteria. This was undertaken to determine if larger volumes are needed to provide adequate detection of host-associated fecal markers in raw and treated drinking water. Information about probable contamination sources was obtained through communications with health officials, water system operators and visual inspections of the area.

5.2.7.2   Sample Collection and Indicator Bacteria Analysis

Samples from heavily contaminated sites were collected in sterile 10-L containers, transported to the PPHMRL and processed within 6 hours. Samples from each site were tested using Colilert® to confirm that total coliforms and/or *E. coli* bacteria were present (IDEXX, Carlsbad, CA). Samples were analyzed as per the manufacturer's instructions.

Raw and drinking water samples were collected in 200 mL sterile bottles and transported to the PPHMRL in coolers with icepacks. All samples were tested for total coliforms and *E.*

coli within 30 hours of sample collection. Raw water samples were tested for total coliforms and *E. coli* using Colilert® Quanti-trays (IDEXX, Carlsbad CA). Drinking water samples were tested for total coliforms and *E. coli* using ChromoCult®. The sample was passed through a 0.45 μm membrane filter, the filter was placed on ChromoCult® media and incubated at 35 °C for 24 hours. The remaining volume of each raw and drinking water sample was stored at 4 °C. All raw and drinking water samples that tested positive for *E. coli* were used for further analysis, and all water samples that tested negative for *E. coli* were discarded by PPHMRL staff.

Ten litre samples were collected from eleven drinking water systems that had recently submitted a drinking water sample that contained viable total coliform bacteria. These included samples from untreated surface water and groundwater and inadequately treated surface water. Water samples were transported to the laboratory in coolers with ice packs, refrigerated immediately, and processed within 24 hours. Sampling containers were cleaned with 10% bleach after each use and were rinsed with water from the sample site before collecting the water sample.

5.2.7.3   Water Sample Processing

Water samples were passed through a 0.45 μm GN-6 Metricel mixed cellulose esters filter (Pall Corporation, Port Washington, NY). The filter funnel was treated in between samples to minimize the likelihood of cross-contamination. The filter funnel was rinsed thoroughly with deionized water and placed in a 10% bleach solution for five minutes followed by thorough rinsing with deionized water. A negative process control using deionized water was prepared as the final sample filtered to evaluate cross-contamination in the filtration process.

In cases where the filter clogged with sediment, the entire 10-L volume of sample was not processed and the volume of water filtered was recorded. The membrane filter was transfered onto a sterile surface where it was sliced into small pieces using a sterile scapel. The pieces of the filter were transferred into a Bead Solution tube from a MoBio Soil DNA Isolation Kit and the DNA extraction was performed as per the manufacturer's instructions (MoBio Laboratories Inc., Carlsbad, CA). All small volume raw and drinking water samples were processed immediately after the indicator bacteria result was obtained.

5.2.7.4 Microbial Source Tracking using Singleplex and Multiplex PCR

DNA from each water sample was screened for presence of multiple host-associated markers using the multiplex PCR assay described here. A 16S control was included for each heavily contaminated known-source sample and for a subset of the raw and drinking water samples. Raw and drinking water samples were also tested for presence of host-associated markers using singleplex PCR because the singleplex PCR had a lower detection limit for some markers than the multiplex PCR. Singleplex reactions contained one illustra PureTaq Ready-to-go PCR bead (GE Healthcare, Waukesha, WI); 2.5 μL of DNA from the water sample; 1 μL of 6.25 μM host-associated forward primer and 1 μL of 6.25 μM host-associated reverse primer; and 18 μL ddH$_2$O. All water samples were tested for human (Bernard and Field 2000a), bovine (Lee et al 2010) and swine-associated marker (Okabe et al 2007) using the singleplex cycling conditions described previously. Raw water samples and drinking water samples with surface water sources were tested for presence of gull-associated marker (Lu 2008) and Canada goose-associated marker (Fremaux et al 2009) in singleplex format, using the cycling conditions previously described.

5.2.7.5   Interpretation of Gel Images

All gel images were captured using a 2.0 second exposure time. A visible band in at least 2 out of 3 replicates was considered a positive result for a particular marker. If a faint band was visible on the agarose gel in a location that corresponded to the size of any of the host-associated marker amplicons, the presence of that host-associated marker was inconclusive.

5.3   Results

5.3.1   *In Silico* Analysis

Each primer sequence was queried against an environmental database to determine whether the top matches were bacteria that were isolated from the presumed host. Database hits are summarized in Table 16. Hits for the presumed human marker HF183 were mostly from human sources, although the reverse primer sequence had high similarity (*i.e.,* 100% identity, 100% query coverage) with an uncultured Bacteroidetes bacterium isolated from pig intestine (HQ701473.1).

Of the two cattle-associated primer pairs BLASTed, primers for BacBov2 had fewer non-host matches than BacBov1. Both markers have been found in bacteria that were isolated from human sources. Similarly, the top matches for Pig2-Bac primer pairs were pig-specific, while Pig1-Bac primer pairs had matches from cow, human, chicken and various other sources. The primer pair for the CGOF1 Canada goose marker had top matches that were bacteria from Canada geese feces while the CGPrev primers did not. The primer pairs for seagull-associated marker had no non-host hits and several hits from bacteria isolated from seagull feces. All hits for chicken-associated markers CP2-9 and LA35 had E-values greater than 0.002 and searches revealed few significant matches for both host and non-host sources.

Table 16. Primer BLAST Results

Primer	Presumed Host	Non-host hits [a]	Host hits [a]	Source descriptions considered "Host"	Source descriptions considered "Non-host"
HF183 F	Humans	No	Yes [b]	Human feces, human skin	None
HF 183 R	Humans	Yes [b]	Yes [b]	Human feces, human skin	Pig intestine
BacBov1 F	Cattle	Yes	Yes	Cow feces, farm milk	Human feces, rat feces, human skin, seal colon
BacBov1 R	Cattle	Yes	No	None	Human skin, human feces, human sputum, chicken litter
BacBov2 F	Cattle	No	Yes	Cow gut, cow feces	None
BacBov2 R	Cattle	Yes	Yes	Cow gut, cow feces/manure	Human sewage
Pig1-Bac F	Pigs	Yes [b]	Yes [b]	Pig feces	Human sputum/mouth, human feces, chicken cecum
Pig1-Bac R	Pigs	Yes [b]	Yes [b]	Pig feces/manure, Pig intestine	Cow feces, rock hyrax feces, gorilla feces, lemur feces, chimpanzee feces, flying fox feces, white-faced saki feces
Pig2-Bac F	Pigs	No	Yes	Pig feces/manure/ slurry, Pig intestine	None
Pig2-Bac R	Pigs	No	Yes	Pig feces	None
CGPrevf5 F	Canada geese	Yes [b]	Yes [b]	Canada goose feces	Cow rumen, sheep rumen, rat feces, pig feces, camel rumen, yak rumen, human intestine, human feces, human sputum, horse feces
CGPrevf5 R	Canada geese	Yes [b]	Yes [b]	Canada goose feces	Wallaby foregut, human feces, pig tonsil, pig feces, cattle feces, lemur feces, gorilla feces, chimp feces, baboon feces, bonobo feces

Primer	Presumed Host	Non-host hits [a]	Host hits [a]	Source descriptions considered "Host"	Source descriptions considered "Non-host"
CGOF1 F	Canada geese	No	Yes	Canada goose feces, geese cecum	None
CGOF1 R	Canada geese	No	Yes	Canada goose feces	None
Gull-2 F	Gulls	No	Yes	Seagull feces	None
Gull-2 R	Gulls	No	Yes [b]	Seagull feces	None
CP2-9 F	Chickens	Yes [b]	No	None	Human chromosomal DNA, zebra finch chromosomal DNA
CP2-9 R	Chickens	No	No	None	None
LA35 F	Chickens	Yes [b]	Yes [b]	Poultry litter	Corn
LA35 R	Chickens	No	Yes [b]	Poultry litter	None

[a] Criteria for a significant hit was at least 95% identity, at least 90% coverage and expected value less than 0.002

[b] All hits had expected values greater than 0.002; those with at least 95% identity and 90% coverage are described

5.3.2   Verification of Marker Presence in Host Feces

Fecal samples from nine host groups were used to demonstrate proof of concept and verify that each host-associated marker was present in feces from the presumed host. Hosts included two types of cows (beef and dairy), two flocks of Canada geese, two types of deer (European fallow and black-tailed), horses, humans, two chicken populations, dogs, seagulls and a pig.

DNA from fecal matter was extracted and tested for presence of host-associated markers using PCR primers described in peer-reviewed papers (Table 17). Both cattle markers (BacBov1 and BacBov2) were detected in beef and dairy cow fecal samples, and the PCR primers did not amplify any non-specific products from cattle fecal DNA. Thus, these markers were considered candidates for further selectivity testing. Fecal DNA from one of the flocks of Canada geese contained both Canada goose-associated markers (CGPrevf5 and CGOF1), but only CGPrevf5 was detected in both flocks of Canada geese. CGPrevf5 PCR primers amplified non-specific products from geese fecal DNA; thus CGOF1 was selected for further selectivity testing.

Neither the D40 marker, nor the *cytB* mitochondrial DNA gene specific to deer were detected in a composite fecal sample from European fallow deer. A fecal sample from a black-tailed deer also did not contain the D40 marker. Horse-associated markers were not detected in the composite samples collected from various breeds of horses. The successful amplification of 16S controls indicated that lack of marker detection was not due to PCR inhibition or problems with the DNA extraction protocol. The lack of amplification of markers in horse and deer fecal DNA may be attributed to low abundance of these markers in host feces,

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Table 17. Summary of Marker Prevalence, Selectivity and Sensitivity

Marker (Host)	Abundance in Feces (copies per g)	Host Distribution	Selectivity		Sensitivity		References
			Specificity	Tested Against	Limit of Detection (mg feces per L)	DNA Copies per Reaction	
HF183 (Humans)	10^5 to 10^9	87.5% (n=16)	100% (n= 27)	Cat, deer, dog, duck, elk, goat, llama, pig, seagull, sheep	1.4×10^{-3} [b]	10	Bernhard and Field 2000a, Seurinck et al 2005
BacBov1 and BacBov2 [a] (Cattle)	Not determined	100% (n=18)	90.4% (n=52)	Cat, deer[c], dog[c], goose, gull, horse, raccoon, pig, human	Not determined	10	Lee et al 2010
Pig1Bac and Pig2Bac (Pigs)	$10^{8.6}$ and $10^{8.5}$	100% (n=25)	100% (n=54)	Human, bovine, horse, sheep	Not determined	1.6	Mieszkin et al 2009
Ho2 (Horses)	Not determined	90% (n=10)	Not determined	None	Not determined	100 (theoretical)	Dick et al 2005
BacCan (Dogs)	Not determined	63% (n=8)	90.2% (n=51)	Bovine, cat[c], horse, human[c], gull	Not determined	1	Kildare et al 2007
D40 (Deer)	Not determined	19.2% [d] (n=52)	Not determined	Cow[c], dog, human[c], waterfowl	Not determined	Not determined	Soule et al 2006

Marker (Host)	Abundance in Feces (copies per g)	Host Distribution	Selectivity		Sensitivity		References
			Specificity	Tested Against	Limit of Detection (mg feces per L)	DNA Copies per Reaction	
CGOF1 (Canada Geese)	10^3 to 10^9	57% (n=101)	99.7% (n=291)	Human, cow, pig, chicken, gull, pigeon[c], duck, swan, moose, deer, caribou, bison, goat, horse	Not determined	Not determined	Fremaux et al 2010
Gull2 (Gulls)	10^{10} to 10^{14}	70.7% (n=58)	100% (n=299)	Pig, cow, human, goat, sheep, horse, cat, dog, squirrel, deer, possum, vulture, raccoon, hedgehog, bobcat, ape, elephant, sea lion, seal, whale, porpoise, Canada goose, turkey, pigeon, duck, chicken, penguin, parrot, dove, pelican, ibis	2.4×10^{-4}	Not determined	Lu et al 2008
LA35 (Chickens)	10^7 to 10^9	76.1% (n=57)	93% (n=116)	Cow, pig, ducks[c], goose[c], human[c]	0.1	30	Weidhaas et al 2010
CP2-9 (Chickens)	Not determined	40% (n=70)	100% (n=36)	Cow, pig, human, Canada goose	1	Not determined	Lu et al 2007

[a] Evaluated together in multiplex

[b] Dry weight

[c] Found to have the marker

[d] Percent of isolates with marker, samples were pooled from 4 hosts

genetic variation in the marker sequence or differential marker prevalence in populations from different geographic regions.

The HF183 marker for human fecal contamination was detected in samples collected from two different household septic tanks; the BacCan marker was detected in a composite dog fecal sample; and the Gull2 marker was detected in a composite seagull fecal sample. Both pig markers (Pig1-Bac and Pig2-Bac) were detected in the pig fecal sample. No non-specific PCR products were amplified by any of these primer pairs when tested with host fecal DNA. Thus, these markers for human, dog, seagull and pig were further evaluated to determine primer selectivity.

Two chicken fecal markers (CP2-9 and LA35) were tested in two different populations of chickens. LA35 was not detected in either sample of DNA extracted from chicken feces. CP2-9 was present in one of the composite samples of chicken feces, but absent in the composite sample from a different chicken population. Therefore, CP2-9 was selected for further selectivity testing.

Sequenced amplicons from cow and seagull fecal samples had close matches in the NCBI database, confirming that the expected products were obtained. Sequenced products from human, chicken and pig did not have significant matches in the databases searched using BLAST.

5.3.3   Evaluation of PCR Primer Selectivity Using Fecal DNA

Once the presence of the marker in host feces was confirmed, the degree of host-association for each marker was evaluated using pooled samples containing fecal DNA from multiple potential hosts and the results are shown in Figure 11. A marker that was present in multiple
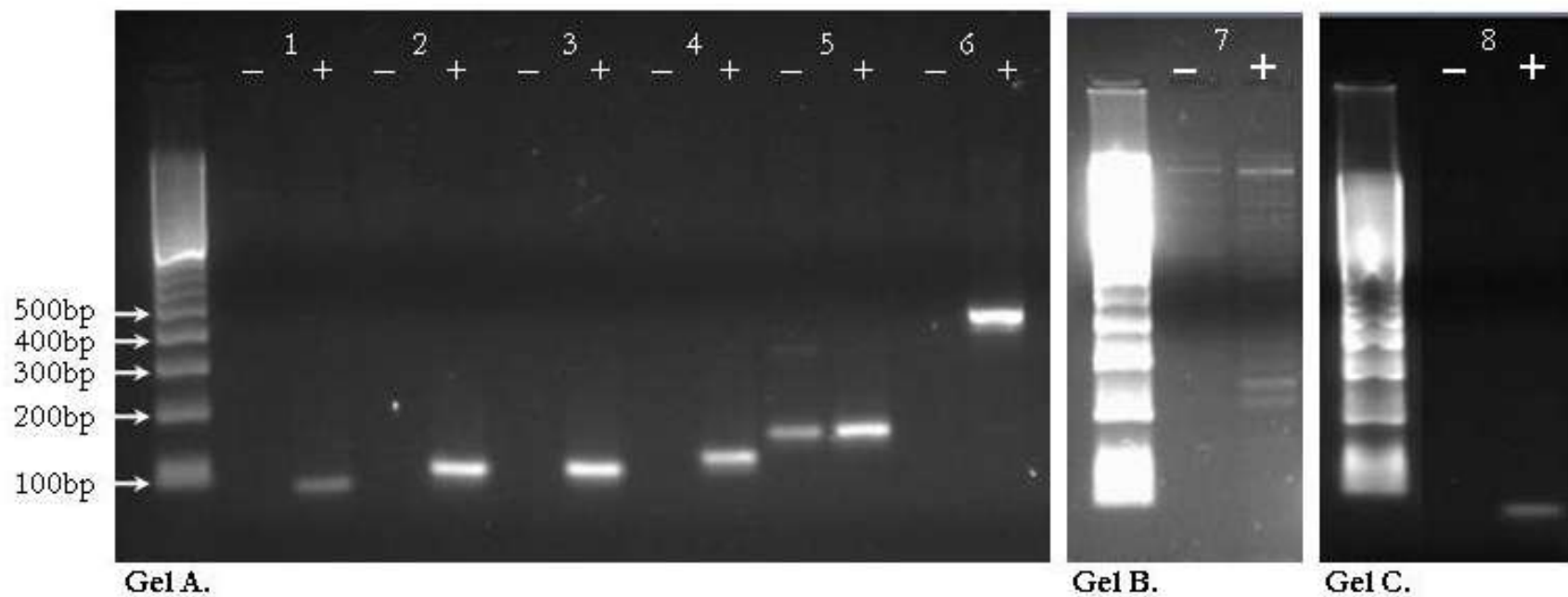
Figure 11. Primer Selectivity Evaluation Using Singleplex PCR. The 100 bp molecular ruler is shown in the leftmost lane of each agarose gel. Lanes labeled with a '-' contain DNA from all other host groups except the host group for which the primer is intended, and lanes labeled with a '+' contain fecal DNA from all host groups. Numbers indicate the host group for which the primer is intended to amplify the host-associated marker from. **Gel A**. (1) human primer HF183, (2) beef cattle primer BacBov-2, (3) dairy cattle primer BacBov-2, (4) pig primer Pig2-Bac, (5) dog primer BacCan, (6) seagull primer Gull-2. **Gel B**. (7) chicken primer CP2-9. **Gel C**. (8) Canada goose primer CGOF1.

hosts was not considered strongly associated with a particular host. Presence of an amplicon

of the correct size in samples containing fecal DNA from the presumed host, and absence of

the amplicon in the pooled sample with fecal DNA from other animals that were not

presumed host groups indicated that the marker was strongly associated with the presumed

host group. Findings are summarized in Table 18.

5.3.4   Detection Limit for Singleplex and Multiplex Assays

The detection limit for each host-associated marker (HF183, Gull-2, BacBov2, Pig2-Bac,

CGOF1, and CP2-9) was evaluated using water samples spiked with feces. In general, the

singleplex PCR outperformed the multiplex PCR, detecting markers at concentrations up to

100 times lower than the lowest concentration that could be detected using the multiplex

assay (Table 18). The CP2-9 marker was detected in DNA extracted from 150 mg of chicken

feces, but it was not detected in water spiked with 100 mg of feces, using either singleplex or

multiplex PCR. Likewise, the CGOF1 marker was not detected in water spiked with 100 mg

of feces using multiplex PCR, but was detected in fecal DNA extracts from 150 mg of feces.

The numbers of marker copies detectable per reaction for various host-associated targets

have been described elsewhere and are summarized in Table 17. The detectable amount of

marker in relation to the contamination level of the original sample was determined, as this

estimate takes into account DNA losses that occur during sample processing. The human-

associated marker (HF183) was detected using singleplex PCR in samples that originally

contained as little as 1 mg of human sewage, and in samples that originally contained at least

100 mg of human sewage using multiplex PCR. The amount of total fecal DNA contained in

each reaction that corresponds to the lowest amount of fecal contamination detectable by

multiplex PCR is shown in Table 18. Total fecal DNA concentrations for samples that

Table 18. Experimental Evaluation of Marker Selectivity and Sensitivity in Feces

| Host | Marker | Singleplex | Multiplex | | |
		Detection Limit (mg Host Feces in Sample)	Detected in Non-Host	Detection Limit (mg Host Feces in Sample)	Detection Limit (ng Total Fecal DNA)
Cow	BacBov1	ND [a]	Yes	ND	ND
	BacBov2	0.01	No	1	0.190
Human	HF183	1	No	100	0.279
Swine	Pig1-Bac	ND	Yes	ND	ND
	Pig2-Bac	1	No	1	0.235
Seagull	Gull2	0.01	No	1	0.185
Chicken	CP2-9	>100	Yes	>100	>2.12
	LA35	ND	No	ND	ND
Canada	CGPrev	ND	Yes	ND	ND
Goose	CGOF1	1	No	>100	>0.328
Dog	BacCan	ND	Yes	ND	ND

[a] Not Determined

corresponded to the lowest fecal contamination amount detectable using singleplex PCR were below the quantification limit (0.5 ng/mL) of the Qubit system (Invitrogen, Carlsbad, CA).

The 16S PCR results indicated that amplification was not affected by inhibitory compounds (*i.e.,* humic acids) at any of the dilutions used. The presence of a weak 16S band in the lane containing the negative process control showed that some bacterial carryover occurred during sample processing; however, absence of bands that corresponded to the sizes of host-associated markers in the PCR negative process control indicated that there was no carryover

of marker DNA. One possible explanation is the presence of bacterial DNA in commercially available *Taq* DNA polymerase, which is well established in the literature (Hughes et al 1994, Maiwald et al 1994).

5.3.5 MST Assay Optimization

DNA template volumes were increased from 2.5 to 5.0 µL to improve the detection capability of the PCRs; no inhibitory effects were evident as a result of this change. The effect of increasing the template volume on the detection of human-associated marker was a 100-fold decrease in detection limit.

Optimal annealing temperatures for each primer are shown in Table 19. The average of these (61 °C) was used as the optimal annealing temperature for the multiplex PCR.

Table 19. Annealing Temperature Optimization

	Human	**Cow**	**Pig**	**Chicken**	**Goose**	**Seagull**
Forward Primer						
T_m (°C)	59.9	64.3	60.4	62.2	64.3	60.6
%GC	45.0	50.0	29.6	47.6	50.0	40.9
Length (bp)	20	22	27	21	22	22
Reverse Primer						
T_m (°C)	62.2	63.4	62.5	60.6	66.3	62.5
%GC	47.6	37.0	45.5	40.9	50.0	45.5
Length (bp)	21	27	22	22	24	22
Tm primer avg	61.0	63.9	61.4	61.4	65.3	61.5
Amplicon						
T_m (°C)	80.2	77.6	85.0	81.3	80.2	88.3
%GC	45.8	36.6	53.0	38.0	48.6	53.2
Length (bp)	83	101	116	251	70	412
T_a^{OPT} (°C)	59.6	58.6	63.0	60.4	60.8	65.4

5.3.6   MST Assay Applications

The MST assay was used to analyze three types of water samples for host-associated markers. These included (1) water from sites with heavy fecal contamination loads from known or suspected sources to demonstrate biological likelihood, (2) drinking water from systems with suspected contamination sources and (3) samples of raw and drinking water from across the province of British Columbia submitted to PPHMRL for routine total coliform and *E. coli* testing to determine whether detectable levels of host-associated markers were present.

5.3.6.1   Sites with Known Fecal Contamination Sources

Water samples collected from heavily contaminated sites, where the origin of the fecal pollution was known, were used to demonstrate feasibility and biological likelihood. Assay performance was evaluated in a complex water matrix and verified that host-associated marker DNA persisted in the environment at sufficient levels to be detected by PCR. Results showed that the multiplex PCR was capable of identifying the likely contamination source in 5 out of 7 sites (Table 20), with few or no non-specific amplicons generated (Figure 12). No markers were detected at site 5 using multiplex PCR nor was the chicken fecal marker detected in the multiplex positive control that contained chicken fecal DNA, but the chicken-associated fecal marker was detected from water collected at site 5 using singleplex PCR.

Table 20. Host-Associated Marker Presence in Heavily Polluted Sites

Site	Expected Inputs	Assay Format	Human HF183	Cow BacBov2	Pig PigBac2	Chicken CP2-9	Gull Gull2	Canada Goose CGOF1
1	Human and birds	Singleplex	+	+	-	-	+	+
		Multiplex	+	-	-	-	+	-
2	Human only	Singleplex	+	+	-	-	-	-
		Multiplex	+	-	-	-	-	-
3	Cow and Pig	Singleplex	+	+	+	-	-	-
		Multiplex	-	+	+	-	-	-
4	Waterfowl	Singleplex	+	+	-	-	+	+
		Multiplex	-	-	-	-	-	+
5	Chicken	Singleplex	+	+	-	+	-	INC [a]
		Multiplex	-	-	-	-	-	-
6	Gulls	Singleplex	+	+	-	-	+	-
		Multiplex	+	-	-	-	+	-
7	Cow	Singleplex	INC	+	-	-	-	-
		Multiplex	-	-	-	-	-	-
8	None	Singleplex	INC	-	-	-	-	-
		Multiplex	-	-	-	-	-	-

[a] INC Inconclusive
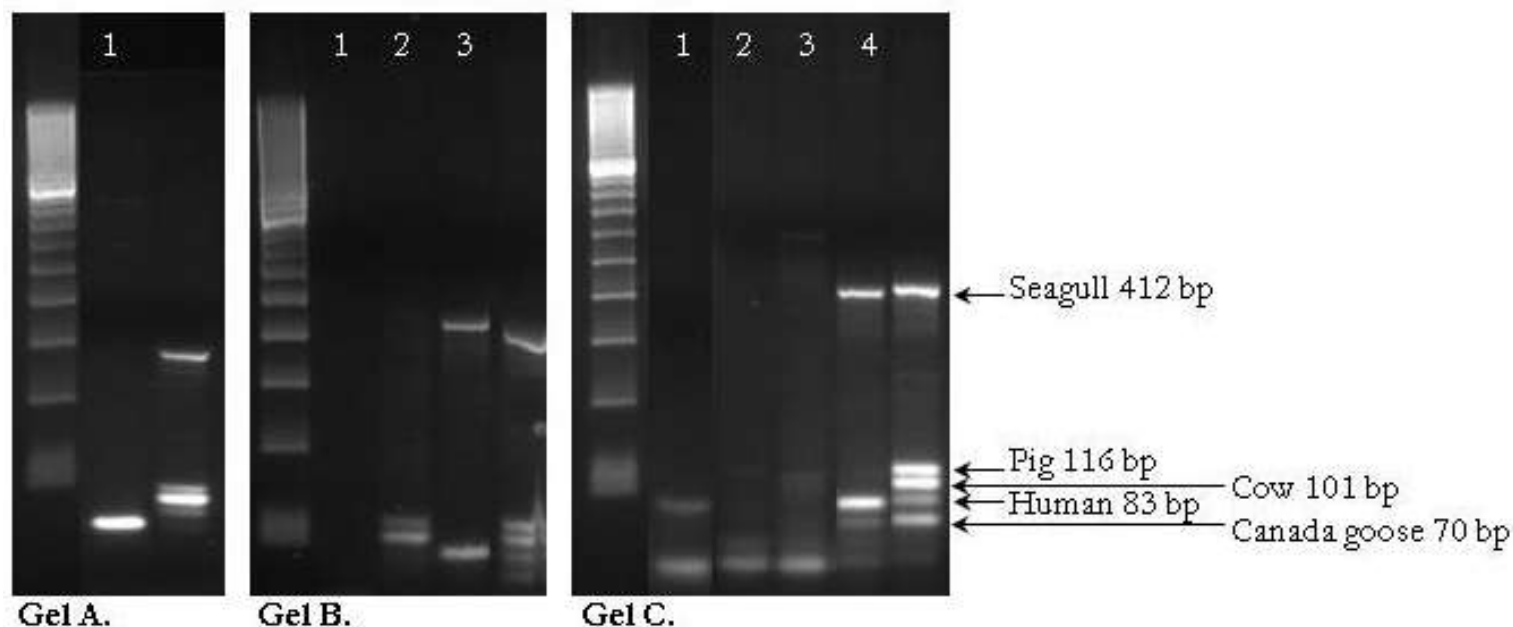
Figure 12. DNA Extracted from Water Impacted by Known Sources of Fecal Contamination Analyzed Using Multiplex PCR. The leftmost lane of each gel contains a 100 bp molecular ruler and the rightmost lane is the positive control which contains all six host-associated primers used in the multiplex and fecal DNA from the corresponding six host groups. **Gel A**. (1) Site 4 which was expected to contain fecal markers from waterfowl. **Gel B**. (1) Site 8 did not contain any host-associated markers as expected for treated municipal water, (2) Site 3 contained both cow and pig fecal markers as expected, (3) Site 6 contained the expected seagull marker and an unexpected human-associated fecal marker. **Gel C**. (1) Site 2 as expected contained the marker for human fecal contamination, (2) Site 5 was expected to contain the marker for chicken fecal contamination but this marker was not amplified, (3) Site 7 was expected to contain the bovine-associated marker but this target was not detected, (4) Site 1 contained the expected markers for human contamination and seagulls.

In addition to detecting the marker(s) associated with the suspected contamination source, the singleplex PCR often detected other host-associated markers. For site 1, it was possible that the river also contained run-off from nearby farms and fecal contamination from birds. However, it was unexpected that site 2 was impacted by bovine contamination. This site is located in an urban area and no known livestock operations are nearby. Unexpected human-associated fecal markers were detected in samples collected from sites 3-5, but the possibility that these may be due to leaking septic fields from nearby homes cannot be ruled out. Detectable levels of bovine-associated fecal markers were not expected in samples from sites 4-6. The observed bovine markers at these sites may be due in part to the widespread nature of livestock farming in the Fraser Valley and long persistence of *Bacteroides* in the environment. An additional possibility is that there are low concentrations of bovine-associated *Bacteroides* from sources other than cattle.

5.3.6.2 Drinking Water Samples with Suspected Contamination Source

Seven of the 11 large-volume drinking water samples collected from drinking water systems that tested positive for total coliform bacteria contained detectable concentrations of host-associated markers using singleplex PCR. These are summarized in Table 21. Only one of the large-volume samples that tested positive for human-associated marker using singleplex PCR also gave a positive result using multiplex PCR, which was not surprising given that the detection limits for host-associated markers using singleplex PCR were substantially lower than those using multiplex PCR.

The HF183-positive site was an untreated groundwater source and the most likely source of contamination was a septic tank. Two of the water systems were suspected to be impacted by fecal contamination from cattle. One was a shallow well near a cattle ranch and the other

Table 21. Characteristics of Water Samples Analyzed Using Singleplex and Multiplex PCR [a]

Sample Type (Raw, Drinking)	Source Water	Total Coliforms (CFU/100mL)	*E. coli* (CFU/100mL)	Volume Filtered (L)	Host-Associated Marker(s) Detected using Singleplex PCR	Host-Associated Marker(s) Detected using Multiplex PCR
Small Volume Water Samples						
Drinking	Flowing Supply	8.7	3.1	0.15	Human, Bovine	None
Drinking	Unknown	OG, +	OG, +	0.11	Human	None
Drinking	Unknown	170	1.0	0.15	Human, Bovine	None
Drinking	Groundwater	51	1.0	0.16	None	None
Raw	Lake/Reservoir	120	3.1	0.18	Bovine, Gull	Gull
Raw	Lake/Reservoir	150	1.0	0.13	None	None
Raw	Flowing Supply	37	15	0.11	None	None
Raw	Flowing Supply	16	1.0	0.14	Human	None
Raw	Flowing Supply	80	1.0	0.17	Human	None
Raw	Lake/Reservoir	35	1.0	0.15	Human	None
Raw	Flowing Supply	79	1.0	0.15	Human, Bovine	None
Raw	Combined	12	1.0	0.10	Human, Bovine	None
Raw	Flowing Supply	9.8	1.0	0.12	None	None
Raw	Unknown	140	21	0.17	Bovine, Gull	Gull
Drinking	Flowing Supply	1.0	1.0	0.15	None	None
Raw	Combined	9.8	1.0	0.10	Bovine	None
Drinking	Flowing Supply	1.0	1.0	0.16	None	None
Raw	Flowing Supply	9.8	1.0	0.12	None	None
Drinking	Surface	2.0	1.0	0.15	None	None
Raw	Flowing Supply	12	1.0	0.14	None	None
Raw	Flowing Supply	1400	1400	0.12	None	None
Large Volume Water Samples						
Drinking	Groundwater	2.0	1.0	10	Human	None
Drinking	Flowing Supply	18	<1.0	10	Human, Bovine	None
Drinking	Groundwater	OG, +	OG, +	10	Human	Human
Drinking	Groundwater	OG, +	OG, +	10	Human	None

Sample Type (Raw, Drinking)	Source Water	Total Coliforms (CFU/100mL)	*E. coli* (CFU/100mL)	Volume Filtered (L)	Host-Associated Marker(s) Detected using Singleplex PCR	Host-Associated Marker(s) Detected using Multiplex PCR
Drinking	Groundwater	9.0	1.0	10	None	None
Drinking	Lake	150	<1.0	7.0	Human, Bovine, Canada goose	None
Drinking	Lake	4.0	<1.0	2.5	None	None
Drinking	Combined	8.0	<1.0	10	Human	None
Drinking	Groundwater	4.0	<1.0	10	None	None
Drinking	Groundwater	OG, +	OG, -	8.5	Bovine	None
Drinking	Groundwater	62	<1.0	10	None	None

[a] 'OG, +' Plate was overgrown; at least one total coliform or *E. coli* colony was visible; 'OG, -' Plate was overgrown; no total coliform colonies were visible

was a natural spring in an area with many free roaming cattle. Samples from these two systems tested positive for both bovine and human markers using singleplex PCR. These findings suggest that either human fecal contamination may be more widespread than suspected, or the strength of host-association for the bovine fecal marker may be weaker than these empirical and *in silico* selectivity evaluations suggested.

5.3.6.3   Raw and Drinking Water with Unknown Fecal Contamination Sources

DNA extracted from fourteen raw and seven drinking water small-volume samples that tested positive for *E. coli* were analyzed for presence of host-associated fecal markers using singleplex and multiplex PCR (Table 22). The source water types for these samples are described in Table 23. Avian markers were only detected in surface waters but no other trend between source water type and host-associated markers present was apparent.

Table 22. Proportion of Raw and Drinking Water Samples Containing Host-Associated Markers Using Singleplex and Multiplex PCR [a]

	Assay Format	Human HF183	Cow BacBov2	Pig PigBac2	Gull Gull2	Canada Goose CGOF1
Small Volume Raw Water	Singleplex	5/14	5/14	0/14	2/14	1/14
	Multiplex	0/14	0/14	0/14	2/14	0/14
Small Volume Drinking Water	Singleplex	3/7	2/7	0/7	0/7	0/6
	Multiplex	0/7	0/7	0/7	0/7	0/7
Large Volume Drinking Water	Singleplex	6/11	3/11	0/11	0/3	1/3
	Multiplex	1/11	0/11	0/11	0/11	0/11

[a] Some samples contained multiple markers; distribution of host-associated markers detected in each sample is described in Table 23.

Table 23. Distribution of Host-Associated Markers in Raw and Drinking Water Samples by Source Water Type

	Human Only	Bovine Only	Human and Bovine	Bovine and Avian	Human, Bovine, Avian	No Markers Detected
Small Volume Raw Water						
Lake/Reservoir	1			1		1
Flowing Supply	2		1			5
Combined		1	1			
Groundwater						
Not Provided				1		
Small Volume Drinking Water						
Lake/Reservoir						1
Flowing Supply			1			2
Combined						
Groundwater						1
Not Provided	1		1			
Large Volume Drinking Water						
Lake/Reservoir					1	1
Flowing Supply			1			
Combined	1					
Groundwater	3	1				3
Total	8	2	5	2	1	14

Eight out of fourteen raw water samples contained detectable concentrations of host-associated fecal markers using singleplex PCR; and markers were detected in two out of fourteen samples using multiplex PCR. Of the eight samples that tested positive using singleplex PCR, three contained the human-associated marker exclusively, one was positive for bovine-associated marker exclusively, two samples contained both bovine and human markers, and two contained both bovine and avian markers. Both samples that tested positive for host-associated markers using multiplex PCR detected the gull-associated marker, which agreed with the results obtained using singleplex PCR. Three out of seven drinking water samples contained detectable levels of host-associated fecal markers using

singleplex PCR, but no host-associated markers were detected using multiplex PCR for small-volume drinking water samples. Of the three small-volume drinking water samples that tested positive for host-associated markers using singleplex PCR, one sample contained the human-associated marker exclusively and the other two samples contained both human and bovine-associated markers.

5.4   Discussion

Holistic approaches to water management require an understanding of factors that have potential to influence water quality at every point between the water source and the consumer's tap, and the barriers in place to mitigate risks. Monitoring is a critical element in the multi-barrier approach to ensure provision of potable water, as it enables us to identify and investigate changes in water quality that represent weaknesses in the system.

Drinking water samples are collected on a routine basis for microbiological monitoring purposes and are tested for viable total coliforms and *E. coli*. In most events where viable total coliforms or *E. coli* are detected in a routine water sample, no further testing is conducted to investigate where the contamination might be coming from. When an initial investigation has been done and the result is determined not to be a false positive due to sampling or analytical error, there is a need for microbial source tracking tools that could be used in combination with indicator bacteria testing to provide information about the fecal contamination origin.

In this study, microbial source tracking primer sets were used for bacteria associated with the feces of humans, cattle, pigs, horses, dogs, geese, gulls, chickens and deer in a singleplex and multiplex PCR format to analyze contaminated water intended for human consumption in BC. The prevalence of these host-associated bacteria in groundwater and surface water

used for human consumption, and inadequately treated drinking water has not previously been evaluated in previous studies.

Fecal samples from each of the nine hosts were tested to confirm host-associated marker presence and found that the host-associated markers for human (HF183), pig (Pig2-Bac), cow (BacBov2), seagull (Gull2), Canada goose (CGOF1) and chicken (CP2-9) were present at detectable levels in feces using multiplex and singleplex PCR. Target DNA sequences for HF183, Pig2-Bac, BacBov2, Gull2 and CGOF1 are located in the 16S rRNA operon and each bacterial genome can contain between 1 and 15 copies depending on the species. Bacterial genomes on average possess 5.5 copies of the 16S rRNA gene and *Bacteroides* spp. have 7 copies per genome (Klappenbach et al 2000). The poultry marker CP2-9 was not amplified consistently from host fecal samples and this low sensitivity may be due to the fact that the marker is present in only a single copy per genome. Differential amplification of chicken fecal DNA from different populations of chickens also suggests that the marker may not be conserved across poultry populations or that it has low relative abundance in poultry feces. Low relative abundance of *Bacteroides* in avian feces has been reported by others (Fogarty and Voytek 2005, Liu et al 2010).

Markers for deer and horse were not detected using singleplex PCR in composite fecal samples. The lack of marker detection in horse feces was unexpected, as a similar marker was present in 90% of horse fecal samples (Dick et al 2005). This may be due to a lack of conservation of the sequence targeted by the modified reverse primer used in this study among the equine-associated subgroup of *Bacteroides*. Lack of detection of deer-associated *cytB* marker in deer feces may be due to the low concentration of mitochondrial DNA relative to bacterial and other DNA present in fecal DNA extracts. The distribution of the

D40 marker in different populations of deer is not well characterized and previous studies have used fecal samples from four deer (Soule et al 2006). Also, the fact that target sequences for both deer and horse are present in one copy per genome results in a lower detection limit relative to markers that target the 16S rRNA gene. These findings indicate that the equine and deer-associated markers used here would not be suitable to detect environmentally relevant concentrations of fecal contamination from these hosts in waterways.

There is limited information regarding the geographic distribution of host-associated markers for equine species, domestic pets and wildlife including deer. Geographic differences in human marker prevalence has been demonstrated by Jenkins et al (2009) who did not detect human fecal-associated marker in any of the five sewage samples analyzed in Kenya. Some factors suspected to contribute directly to variations in gut microbiota that are associated with geography such as diet, have not been adequately supported by previous findings (Simpson et al 2002, Sadet et al 2007). Other factors including animal age and breed have been demonstrated to contribute in part to gut microbiota variation in canines (Simpson et al 2002), but evidence to date supports that the composition of microbiota in the gut is influenced by a number of host factors (Sadet et al 2007, Durso et al 2010, Benson et al 2010).

The degree of host-association of each marker was evaluated both experimentally and *in silico*. No cross-reactivity with non-host fecal DNA for the human (HF183), pig (Pig2-Bac), cow (BacBov2), seagull (Gull2), Canada goose (CGOF1) or chicken (CP2-9) markers using PCR was observed. A search of an environmental database revealed that bacteria isolated from non-presumed hosts contained sequences that closely matched those targeted by reverse

primers for BacBov2 and HF183. It is not surprising that the HF183 reverse primer sequence

is highly similar to others found in the sequence database because it lies just outside of the

V2 region in the 16S rRNA gene. The HF183 forward primer is located within the V2 region

and had no matches with bacteria from non-hosts in the database. Therefore, based on *in

silico* and experimental evidence from this study and others (Bernhard and Field 2000a,

Dorai-Raj et al 2009), amplification of an 83 bp product is a good indicator of human

contamination. The reverse primer for BacBov2 is located within the V3 region of the 16S

rRNA gene, but bacteria of human fecal origin also a contained highly similar (*i.e.,* 100%

identity, 100% query coverage) sequence (DQ886177.1). The forward primer for BacBov2

is located in a conserved region. Together, this indicates that presence of BacBov2 marker

could be from human sources of contamination.

In cases where *in silico* evidence indicates that host-associated bacteria have been isolated

from multiple hosts and the non-hosts are not native to the area of intended use for the tool,

the relevance of the bacterium being found in these hosts does not in itself pose a specificity

issue. However, finding that the bacterium is not found exclusively in a single host raises the

concern that the bacterium may be capable of inhabiting the gut of other hosts including

those that may be found in the region where the tool is intended to be used and these hosts

may not be represented in the environmental database queried.

A multiplex approach was developed to detect host-associated markers simultaneously in

water containing feces. When the assay was applied to heavily polluted water samples, the

suspected major contamination source was correctly identified in 5 out of 7 sites, without

amplifying markers associated with host groups that were not expected to impact the sample

site. Other studies have also found that PCR amplification of host-associated *Bacteroides*

markers was able to correctly identify contamination sources from humans and ruminant species (Bernhard and Field 2000b), and generated fewer false positives compared to library-based methods. While the singleplex PCR was able to detect the most probable contamination source(s) from all 7 of the heavily polluted sites including the poultry marker in run-off water from a poultry farm and the cattle marker from the agricultural site that were not detected using multiplex PCR, singleplex PCR also detected host-associated markers from unexpected contamination sources. Bovine-associated marker was detected from five sites that were not known to be impacted by bovine contamination. Some cross-reactivity of BacBov1 and/or BacBov2 with bacteria in dog feces has been reported elsewhere (Lee et al 2010), which may explain why the BacBov2 marker was detected at site 2 within an urban area. Human marker was detected from three sites that were not expected, but the possibility that septic tanks were responsible for the human contamination cannot be ruled out.

More than half of the raw and drinking water samples analyzed contained at least one of the five host-associated markers using singleplex PCR, suggesting that this method may be a useful addition to current water quality testing to provide information about risk and appropriateness of existing barriers, including treatment regime. The information could also be used to address microbial contamination issues at the source. Other groups have demonstrated that presence of a human-associated marker is not correlated with presence of viable fecal indicator bacteria (van der Wielen and Medema 2010), and human-associated markers can be detected when no indicators of infective microorganisms exist. Thus, the use of the MST assay would be best applied as a secondary test if and only if viable indicator bacteria are detected. Here, host-associated fecal markers were detected in water samples where only total coliforms and not *E. coli* were present, suggesting that presence of any

bacteria from the total coliform group should be investigated further to rule in or rule out fecal contamination. The controversial role of total coliform bacteria as an indicator of fecal contamination is discussed further in Chapter 4.

Six of the thirty-two raw and drinking water samples tested contained both human and bovine-associated markers. HF183 has not been previously reported in bovine feces (Bernhard and Field 2000a, Mieszkin et al 2009), but some bovine-associated *Bacteroides* species have been recovered from human feces (Mieszkin et al 2009). The BacBov2 marker specifically was not detected in human sewage here or in previous studies (Lee et al 2010). *In silico* analysis showed that sequences similar to BacBov2R have been found in human sewage, and BacBov2F is located outside of a hypervariable region, which suggests that amplification of a 101 bp product from human sewage using these primers is possible. Based on these lines of evidence, it is possible that samples containing both HF183 and BacBov2 were exclusively contaminated by human feces. Alternatively, the site may have been impacted by both human and bovine inputs. Interpretation of MST results can be complicated in cases where the markers exhibit some cross-reactivity with non-hosts and MST molecular test results should be compared with findings from a visual site inspection.

The six host-associated markers were able to persist in water samples at levels detectable by singleplex PCR from the time of initial contamination to analysis. Other studies investigating marker persistence highlight that testing for host-associated markers needs to take place as soon as possible after sample collection, as DNA degrades in the environment in response to sunlight, high temperature, DNases and predatory microorganisms (Ballesté and Blanch 2010, Green et al 2011). Previous studies estimating the decay of HF183 marker using microcosms showed that the time required for a 99% reduction in the initial

concentration of marker was 2 to 3 days (Dick et al 2010, Liang et al 2012). The decay kinetics for cow-associated *Bacteroides* markers were similar to those of human markers (Liang et al 2012), and marker DNA was detected up to 14 days after microcosms were spiked with 0.7 g/L of cow feces.

In this study, the number of fecal samples used to verify marker presence for some host groups was small; however, the sensitivity and degree of host-association for the markers used here have been tested to varying extents in previous studies (Bernhard and Field 2000a, Lu et al 2007, Lu et al 2008, Mieszkin et al 2009, Fremaux et al 2010, Lee et al 2010). Although efforts were made to minimize the potential for sample contamination and cross-contamination, some water samples that were not expected to be impacted by humans gave an inconclusive result for the human-associated marker when tested using singleplex PCR. There is a possibility that sample contamination occurred, as the human-associated bacterial marker has been recovered from human skin (Kong et al 2011). However, all negative process controls and PCR controls were negative for HF183.

The information provided by this MST assay is valuable in evaluating the risk posed by consumption of contaminated drinking water. Studies have demonstrated that water contaminated with human sewage poses a greater risk of acute gastrointestinal illness than exposure to contamination from other sources, with the possible exception of cattle feces depending on the degree of contamination (Soller et al 2010). Human feces contains more pathogens that are capable of infecting humans compared to feces from other species, largely due to the high degree of host-specificity of viruses. Inadequately treated water is an effective vehicle for the transmission of viruses that cause respiratory and gastrointestinal illness such as adenovirus, calicivirus and enterovirus. Unlike viruses, zoonotic protozoan

parasites such as *Cryptosporidium* and *Giardia* spp. have a broad host range (Thompson 2004, Xiao and Fayer 2008). However, studies have shown that neither of the two *Giardia* genotypes that infect humans (Assemblage A and B) were carried by birds and less than 20% of the *Giardia* spp. isolated from cattle were infective to humans (O'Handley et al 2000). Transmission of Assemblage A *Giardia* can also occur between wildlife (*i.e.*, beavers, deer) and humans, and between dogs and humans via ingestion of cysts (Thompson 2004). Common waterborne bacterial pathogens such as *Campylobacter jejuni* and enterohemorrhagic *Escherichia coli* can be found in livestock and humans (Soller et al 2010), and to a lesser extent domestic pets and wildlife (Ferens and Hovde 2011). *Campylobacter* is highly prevalent in poultry (Ogden et al 2009), whereas *E. coli* O157:H7 is more commonly found in cattle and pigs than other livestock (Chapman et al 1997, Soller et al 2010). Thus, identification of contamination source can provide insight into the types of pathogens that may be present.

For a more in-depth investigation of risk, it is possible to quantify host-associated markers using real-time PCR. Interpretation of quantitative host-associated marker data for risk assessment purposes should consider factors that affect the concentration of host-associated marker detected. These include variability of host-associated marker concentration in feces from animal to animal; in water due to environmental conditions that affect marker degradation rates (*i.e.*, predation, temperature, sunlight), differential marker loading rates in receiving waters due to weather conditions; and in the final DNA extract due to differences in extraction efficiency, both from variations in sample matrix and the protocol used (Silkie and Nelson 2009). If quantification of host-associated markers is desired, an additional

consideration would be to measure and account for PCR inhibition for each sample matrix, as this has been shown to vary depending on source water type (Gibson et al 2012).

While the multiplex format may have uses as a preliminary screening tool when high concentrations of fecal contamination are expected, the higher sensitivity of the singleplex PCR is advantageous to detect contamination in drinking water samples where the concentration of host-associated markers is expected to be low.

Future MST work should focus on developing standardized procedures for water sample processing including concentration of target cells and DNA extraction to better enable comparisons of findings between studies. Additional studies to improve understanding of the factors that contribute to animal-to-animal variation in microbial gut populations would fill a significant knowledge gap. In terms of field applications, more research that compares the performance of different MST methods on samples containing biologically relevant concentrations of fecal contamination is needed. The potential for these host-associated markers to be used in outbreak investigations could be explored in a future study. Measurement of the tangible benefits of MST work is also lacking, and more studies reporting statistics on improvements (or lack thereof) in water quality as a result of MST tool application may encourage stakeholders to support the further research needed.

5.5   Conclusions

Detection of host-associated markers in samples containing indicator bacteria can provide valuable information about potential sources of fecal pollution impacting source or drinking water. Multiplex PCR may not be sensitive enough to detect the low levels of host-associated markers that would be expected in contaminated drinking water samples, but may be used to identify the main contamination source in applications where fecal pollution loads

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are high such as raw water. The higher sensitivity of the singleplex PCR suggests that it would be a more suitable approach to detect environmentally relevant concentrations of host-associated markers in water impacted by fecal contamination from humans, cattle, pigs, chickens, seagulls or Canada geese. Interpretation of MST data should consider both up-to-date information available in environmental databases and experimental data that includes likely sources of contamination in order to evaluate potential cross-reactivity. The MST assay is a promising approach that may be a useful addition to the water management toolbox. This test that can be applied to the existing water monitoring framework to provide additional evidence that could be used in conjunction with sanitary surveys and local knowledge, to rule in or rule out potential contamination sources.

Chapter 6: Conclusions

The Plan-Do-Check-Act cycle provides a useful model for continuous assessment and improvement of water monitoring and management programs (Deming 1950, Singh and Singh 2012). Evaluations of current practices and proposed alternatives are necessary in order to improve drinking water monitoring programs and the tools used to make decisions about water safety. This work contributes to the body of knowledge on water monitoring and management tools. It provides an assessment of location-appropriate methods to detect microbiological indicators in drinking water, an evaluation of the potential for non-*E. coli* total coliforms to be used as a means to identify drinking water systems that have weaknesses in their microbial contamination barriers, and describes the development of a new test to characterise microbiological contamination in raw and drinking water.

6.1 Implications for Routine Testing of Drinking Water from Small Systems in Remote Areas

Given that microbiological monitoring is a critical component of a holistic framework to provide safe drinking water and should be conducted regularly as required by the British Columbia Drinking Water Protection Regulation, a lack of compliance with monitoring requirements is consistent with a vulnerability in a drinking water system's multi-barrier framework. More than half of the sampling sites of small drinking water systems in Northern Health Authority and Interior Health Authority regions in BC were overdue for sampling and testing for total coliforms and *E. coli*. Both Northern Health Authority and Interior Health Authority regions have a large number of small water systems and some of these systems are located in areas that are difficult to access and are far from a PHO-approved testing facility.

The study conducted in the South Cariboo, which is located within the Interior Health Authority region, provides comparison data for two testing approaches that may be used to support the use of a simple qualitative test for routine drinking water monitoring that can be performed close to the point of sample collection.

The overall agreement of the current approach used to test drinking water for indicator bacteria compared to the proposed local-testing approach as measured by Cohen's kappa was 0.64 ± 11 for total coliforms and 0.73 ± 0.20 for *E. coli*. Agreement was lower than expected, given that both methods use a similar detection principle and have been approved by the US EPA for testing of drinking water for indicator bacteria. When water sample holding conditions are within the acceptable limits and two different testing methods that have both been shown to compare favourably to an accepted standard method are used, there is a difference in the total coliform results that cannot be explained by unequal distribution of bacteria in duplicate samples tested. This discrepancy may be due to differences in the formulations of the testing reagents which affect the recovery of stressed bacterial cells. There was no bias in terms of the abilities of either approach to detect *E. coli*. However, due to the low frequency of *E. coli* in drinking water, the comparisons for detection of *E. coli* are based on a small number of observations. In addition, agreement between the two testing approaches was affected by cell concentration and because most *E. coli*-positive samples contained less than 10 CFU per 100mL the probability for disagreement was higher. While the qualitative test that can be performed close to the point of collection offers advantages in terms of result turnaround time, the trade-off for sensitivity of total coliform detection as well as data quality issues should be considered before transitioning to a local-testing approach.

First Nations communities in Canada currently test their own drinking water samples for total coliforms and *E. coli* using the Colilert® enzyme/substrate test at community laboratories. One third of the drinking water samples collected from water systems on First Nation lands in 2006/2007 were sent to a PHO-laboratory for testing as a quality assurance measure (Kendall 2008). This program may serve as a template for local health units such as 100 Mile House Health Unit that may benefit from adopting a similar testing approach.

The local-testing approach addresses the issue of timeliness of testing results as there is no need to transport samples great distances to PHO-approved laboratories. Testing results obtained using the local-testing approach were typically 24 hours faster than those reported by the PHO-approved laboratory. The potential for cost savings to be realized, by no longer requiring courier services, is also a possibility.

This study does not address the criticism that total coliform and *E. coli* testing may be of limited value from a statistical perspective, since some small water systems are only tested once per month. As such, the likelihood of detecting a sporadic change in water quality triggered by a weather event for example would be low. This sampling frequency is a function of the limited resources available for sample testing and the participation level of purveyors of small systems in the water monitoring program.

Limitations inherent in the use of indicator bacteria, including the lack of association in some cases between presence of total coliforms and *E. coli* and the presence of non-bacterial pathogens, and the inability of total coliform bacteria to indicate presence of some opportunistic bacterial pathogens, would not be affected by a change to a local-testing approach like the one described here.

6.2   Implications for Interpretation of Total Coliform Bacteria in Drinking Water

Although testing finished water in distribution systems for total coliforms is mandated in British Columbia (BC) and other provinces in Canada, the relevance of total coliforms to water safety is controversial. Given the number of limitations associated with their use as water safety indicators described in Chapter 4, the justification for the testing and reporting of this group of bacteria under the British Columbia Drinking Water Protection Regulation deserves to be challenged. The presence of total coliform bacteria indicates vulnerability of a drinking water system's barriers to penetration from waterborne pathogens; however, the likelihood that these non-*E. coli* total coliform events would lead to a more severe contamination event in the future had not been investigated previously.

Life table analysis was used to evaluate the potential for non-*E. coli* total coliforms to be used as a tool to identify drinking water systems that have a high likelihood of observing *E. coli* in treated drinking water. Evidence indicated that non-*E. coli* total coliforms have some utility in terms of their ability to predict occurrence of *E. coli* in subsequent drinking water samples. A retrospective analysis of microbiological testing data from drinking water systems showed that drinking water systems with a positive non-*E. coli* total coliform result are more likely to observe an *E. coli*-positive result in a subsequent water sample, compared to water systems that have not tested positive for total coliforms previously (RR=2.04). Given the low frequency of *E. coli* occurrence in drinking water, the difference in absolute risk one month after the non-*E. coli* total coliform result (1.6%) and up to three years after the non-*E. coli* total coliform result (6.0%), may appear small but is significant.

These findings support focusing initiatives to improve water management on small systems with one or more prior non-*E. coli* total coliform events and other attributes currently used to

assess the degree to which water systems are susceptible to microbiological contamination. Non-*E. coli* total coliform occurrence in drinking water indicates a change in water quality that warrants follow-up actions, including re-sampling to verify that the result was a true positive; if so, there is a need to conduct further investigations to determine the cause (*i.e.*, treatment system malfunction).

6.3 Implications for Incorporating Fecal Source Tracking Tools into Routine Water Testing

Source water protection through management of fecal contamination inputs is an important element of the multi-barrier approach to reduce the risk of acute gastrointestinal illness from consumption of water. Few tools are currently available to provide reliable information about microbiological contamination sources, especially when the fecal pollution is from non-point sources. A two-tiered testing approach, to further analyze routine water samples that test positive for total coliform bacteria and/or *E. coli* for presence of host-associated fecal markers, would provide useful information for health officials to evaluate risk and for stakeholders to guide remedial plans.

A PCR assay was developed to identify fecal contamination sources in water using a combination of host-associated markers previously identified in peer-reviewed journals for host groups that were considered common polluters of water in BC. These included humans, livestock and hobby farm animals (cattle, chickens, pigs, horses), wildlife (deer, Canada geese, seagulls) and domestic pets (dogs). The performance of the assay was assessed using fecal samples from different hosts, heavily contaminated water samples and raw and drinking water samples with evidence of fecal contamination. Host-associated markers for human, bovine, pig, chicken, dog, seagull and Canada goose feces were detected using multiplex

PCR in fecal DNA from host populations in the Lower Mainland, BC. Human, bovine, pig, seagull and Canada goose markers demonstrated sufficient selectivity to be combined in a multiplex PCR format to simultaneously detect multiple targets. However, the detection limit of the multiplexed PCR assay was up to two orders of magnitude higher than the limit of detection of host-associated markers using singleplex PCR, and this affected the ability of the multiplex PCR to detect low concentrations of target DNA present in contaminated drinking water samples.

Host-associated markers were detected in small volumes of raw water and drinking water that had evidence of fecal contamination using singleplex PCR. Multiplex and singleplex PCR can provide useful information when they are applied together using a qualitative approach; multiplex PCR can be used as a preliminary screening tool to identify major sources of contamination and singleplex PCR can be used to identify or rule out other contributing sources that are present in lower amounts. Singleplex PCR may be more appropriate for analysis of small volumes of samples that are left-over after routine testing for indicator bacteria, since low levels of fecal contamination would be expected.

This assay is a promising test that can be easily adapted to the current work-flow to analyze routine water samples in PHO-approved laboratories. The discrimination of host-associated markers is based on differences in the sizes of the amplicons generated, so no specialized fluorescence detectors or software systems are required to analyze PCR results. The test uses a standard thermocycler for PCR amplification of targets and agarose gel electrophoresis for detection, which are common pieces of equipment in a laboratory that conducts molecular testing.

A limitation of the size-based approach to distinguish between amplicons is the potential for amplification of one or more non-specific products of the same apparent size, as one of the expected products associated with a particular host group. Therefore, test results should be considered together with field data and other evidence to identify the likely source(s) of fecal contamination.

6.4   Areas for Further Study

The merits and drawbacks of bacteriological drinking water quality monitoring in the context of water-related public health challenges in developed countries today has been a topic of much discussion recently (McGuinness 2012). This research explored questions about the suitability of the current testing framework to meet unique challenges faced by small water systems in BC, the value of information provided by microbiological tests to better understand public health risk in systems with non-*E. coli* total coliform events, and the potential for value-added testing to identify sources of fecal contamination in water.

In light of the finding that the agreement of total coliform and *E. coli* results obtained using a presence/absence approach was moderate to substantial compared to those obtained using a membrane filtration test at an accredited laboratory, the next step may be to design a proficiency testing strategy to evaluate the quality of the testing data produced at basic laboratories using a presence/absence methodology. A cost comparison analysis of current and alternative testing approaches should be undertaken to determine whether cost savings would be realized testing water samples near the point of collection using a presence/absence methodology. Cost comparisons may include other promising emerging technologies such as loop-mediated isothermal amplification (Plutzer and Karanis 2009) that are robust, cost-effective and not reliant on a single marker. Future research efforts may be directed toward

evaluating emerging technologies, such as loop-mediated isothermal amplification in a field setting for routine bacteriological testing.

The findings of this research support that testing water for total coliforms and *E. coli* in parallel has relevance in the water industry. Water systems that reported non-*E. coli* total coliforms in drinking water had a significantly higher probability of having an *E. coli*-positive result. Future research may investigate the relationship between turbidity spikes, low chlorine residual, extreme weather events, main-breaks and operational failures and detection of *E. coli* in distribution system samples from systems across BC over the long-term. This could potentially be used to construct a model to predict the likelihood of an *E. coli* occurrence.

Research to evaluate improvements in water quality or reduction in illness from intervention approaches or implementation of a water management program is needed. For example, Payment et al (1991) demonstrated that installation of point-of-use water treatment devices in homes resulted in a 35% reduction in the number of the cases of gastrointestinal illness reported compared to the control group. Recently, a facility was constructed to treat the drinking water supplied to residents of Metro Vancouver, BC. It would be interesting to evaluate the change in reported acute gastrointestinal illness rates before and after the treatment plant went online.

This research demonstrated that routine raw and drinking water samples that contain *E. coli* also frequently contain detectable levels of host-associated markers for humans, cattle, pigs, seagulls or Canada geese using singleplex PCR. This test should be added to the toolbox available to Drinking Water Officers in BC for investigation of drinking water quality issues. Next steps may include implementing the method at selected PHO-approved laboratories that

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have the capacity to perform the test and conducting internal method validation studies. Future MST research may focus on improving the sensitivity of the test, potentially by using conformationally locked nucleic acids in PCR primers (Alonso et al 2010). Application of the MST test to *E. coli*-positive recreational water samples submitted for routine testing purposes could also be explored in a future study. Further research on the relationship between the presence of host-associated markers and the presence of human pathogens is also needed.

Despite its limitations, indicator bacteria testing continues to be the primary means to evaluate water safety in Canada. As we continue to evaluate and improve upon the tools we use to identify and investigate water quality issues, it is important that these tools are relevant to the needs of water purveyors, health officials and policy-makers. Much work remains to be done as we continue to make progress towards provision of safe drinking water in BC; researchers should continue to work closely with stakeholders to ensure that future research efforts advance knowledge in a mutualistic manner.

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Appendices

Appendix A   Life Table Analysis Data

Table A1. Life Table Analysis of the Prior TC and No Prior TC Group Using the Event-Free Assumption

	Prior TC Group						No Prior TC Group						
	At	Reached		Pr	Cumulative Pr		At	Reached		Pr	Cumulative Pr		Hazard
Int	risk	Outcome	Lost	(no *E.coli*)	(no *E.coli*)	SE	risk	Outcome	Lost	(no *E.coli*)	(no *E.coli*)	SE	Ratio [a]
One Month Interval Width													
1	815	18	0	0.98	0.98	0.01	704	4	0	0.99	0.99	0.00	3.88
2	797	11	46	0.99	0.96	0.01	700	10	2	0.99	0.98	0.01	0.99
3	740	20	35	0.97	0.94	0.01	688	5	8	0.99	0.97	0.01	3.78
4	685	8	44	0.99	0.93	0.01	675	5	11	0.99	0.97	0.01	1.62
5	633	3	33	1.00	0.92	0.01	659	5	11	0.99	0.96	0.01	0.64
6	597	5	14	0.99	0.91	0.01	643	6	5	0.99	0.95	0.01	0.90
7	578	5	14	0.99	0.91	0.01	632	5	16	0.99	0.94	0.01	1.10
8	559	3	13	0.99	0.90	0.01	611	2	8	1.00	0.94	0.01	1.65
9	543	3	4	0.99	0.90	0.01	601	3	4	0.99	0.93	0.01	1.11
10	536	2	7	1.00	0.89	0.01	594	1	4	1.00	0.93	0.01	2.22
11	527	5	14	0.99	0.88	0.01	589	3	6	0.99	0.93	0.01	1.89
12	508	5	14	0.99	0.88	0.01	580	3	9	0.99	0.92	0.01	1.91
13	489	4	15	0.99	0.87	0.01	568	0	5	1.00	0.92	0.01	UD
14	470	7	20	0.98	0.85	0.02	563	0	19	1.00	0.92	0.01	UD
15	443	8	17	0.98	0.84	0.02	544	2	19	1.00	0.92	0.01	4.92
16	418	0	26	1.00	0.84	0.02	523	1	15	1.00	0.92	0.01	<0.01
17	392	3	20	0.99	0.83	0.02	507	3	21	0.99	0.91	0.01	1.30
18	369	1	20	1.00	0.83	0.02	483	1	9	1.00	0.91	0.01	1.33
19	348	0	23	1.00	0.83	0.02	473	2	4	1.00	0.91	0.01	<0.01
20	325	3	21	0.99	0.82	0.02	467	2	17	1.00	0.90	0.01	2.19
21	301	2	11	0.99	0.82	0.02	448	2	7	1.00	0.90	0.01	1.50

	Prior TC Group						No Prior TC Group						
					Cumulative							Cumulative		
Int	At risk	Reached Outcome	Lost	Pr (no *E.coli*)	Pr (no *E.coli*)	SE	At risk	Reached Outcome	Lost	Pr (no *E.coli*)	Pr (no *E.coli*)	SE	Hazard Ratio [a]
22	288	4	8	0.99	0.80	0.02	439	0	11	1.00	0.90	0.01	UD
23	276	1	3	1.00	0.80	0.02	428	0	7	1.00	0.90	0.01	UD
24	272	3	12	0.99	0.79	0.02	421	0	11	1.00	0.90	0.01	UD
25	257	3	5	0.99	0.78	0.02	410	2	13	1.00	0.89	0.01	2.38
26	249	1	18	1.00	0.78	0.02	395	1	15	1.00	0.89	0.01	1.61
27	230	1	30	1.00	0.78	0.02	379	3	19	0.99	0.88	0.02	0.57
28	199	6	29	0.97	0.75	0.03	357	2	20	0.99	0.88	0.02	5.64
29	164	2	31	0.99	0.74	0.03	335	2	18	0.99	0.87	0.02	2.20
30	131	1	27	0.99	0.74	0.03	315	0	23	1.00	0.87	0.02	UD
31	103	0	17	1.00	0.74	0.04	292	0	18	1.00	0.87	0.02	<0.01
32	86	0	21	1.00	0.74	0.04	274	1	15	1.00	0.87	0.02	<0.01
33	65	0	18	1.00	0.74	0.05	258	0	20	1.00	0.87	0.02	UD
34	47	0	10	1.00	0.74	0.06	238	0	30	1.00	0.87	0.02	UD
35	37	1	7	0.97	0.71	0.06	208	0	58	1.00	0.87	0.02	UD
36	29	0	18	1.00	0.71	0.07	150	2	59	0.98	0.86	0.03	<0.01
Total		139	665					78	537				

Table A2. Life Table Analysis of the Prior TC and No Prior TC Group Using Different Interval Widths, without the Event-Free Assumption

		Prior TC Group			Cumulative			No Prior TC Group			Cumulative		
Int	At risk	Reached Outcome	Lost	Pr (no *E.coli*)	Pr (no *E.coli*)	SE	At risk	Reached Outcome	Lost	Pr (no *E.coli*)	Pr (no *E.coli*)	SE	Hazard Ratio [a]
Six Month Interval Width													
1	815	65	218	0.91	0.91	0.01	704	35	111	0.95	0.95	0.01	1.71
2	532	19	92	0.96	0.87	0.01	558	14	136	0.97	0.92	0.01	1.37
3	421	14	104	0.96	0.84	0.02	408	5	56	0.99	0.91	0.01	2.88
4	303	9	76	0.97	0.81	0.02	347	6	49	0.98	0.89	0.02	1.83
5	218	11	112	0.93	0.76	0.03	292	6	49	0.98	0.87	0.02	3.03
6	95	1	66	0.98	0.74	0.04	237	3	96	0.98	0.86	0.02	1.02
Total		119	669					69	497				
Four Month Interval Width													
1	815	57	188	0.92	0.92	0.01	704	24	133	0.96	0.96	0.01	2.10
2	570	15	168	0.97	0.89	0.01	547	16	143	0.97	0.93	0.01	0.92
3	387	8	49	0.98	0.87	0.02	388	7	69	0.98	0.91	0.01	1.11
4	330	8	53	0.97	0.85	0.02	312	1	27	1.00	0.91	0.02	7.87
5	269	3	63	0.99	0.84	0.02	284	7	25	0.97	0.89	0.02	0.49
6	203	7	24	0.96	0.81	0.02	252	2	26	0.99	0.88	0.02	4.38
7	172	7	42	0.95	0.77	0.03	224	3	19	0.99	0.87	0.02	3.31
8	123	2	65	0.98	0.75	0.03	202	2	23	0.99	0.86	0.02	2.10
9	56	1	29	0.98	0.74	0.05	177	0	55	1.00	0.86	0.02	UD
Total		108	681					62	520				

	Prior TC Group						No Prior TC Group						
Int	At risk	Reached Outcome	Lost	Pr (no *E.coli*)	Cumulative Pr (no *E.coli*)	SE	At risk	Reached Outcome	Lost	Pr (no *E.coli*)	Cumulative Pr (no *E.coli*)	SE	Hazard Ratio [a]
Three Month Interval Width													
1	815	49	175	0.93	0.93	0.01	704	19	170	0.97	0.97	0.01	2.19
2	591	13	185	0.97	0.91	0.01	515	15	118	0.97	0.94	0.01	0.79
3	393	10	60	0.97	0.88	0.02	382	7	101	0.98	0.92	0.01	1.30
4	323	5	37	0.98	0.87	0.02	274	4	33	0.98	0.90	0.02	1.06
5	281	8	34	0.97	0.84	0.02	237	0	18	1.00	0.90	0.02	UD
6	239	2	39	0.99	0.83	0.02	219	3	18	0.99	0.89	0.02	0.64
7	198	2	40	0.99	0.83	0.02	198	4	21	0.98	0.87	0.02	0.53
8	156	4	13	0.97	0.80	0.03	173	1	19	0.99	0.87	0.02	4.37
9	139	3	22	0.98	0.78	0.03	153	1	9	0.99	0.86	0.03	3.48
10	114	6	35	0.94	0.74	0.04	143	1	5	0.99	0.85	0.03	8.74
11	73	0	44	1.00	0.74	0.04	137	1	10	0.99	0.85	0.03	<0.01
12	29	1	0	0.97	0.71	0.07	126	2	53	0.98	0.83	0.03	1.72
Total		103	684					58	575				
Two Month Interval Width													
1	815	29	166	0.96	0.96	0.01	704	14	254	0.98	0.98	0.01	1.63
2	620	22	159	0.96	0.92	0.01	436	8	105	0.98	0.96	0.01	1.95
3	439	7	103	0.98	0.90	0.01	323	9	73	0.97	0.93	0.01	0.58
4	329	7	51	0.98	0.88	0.02	241	4	49	0.98	0.91	0.02	1.25
5	271	4	23	0.98	0.87	0.02	188	3	13	0.98	0.89	0.02	0.93
6	244	3	19	0.99	0.86	0.02	172	2	21	0.99	0.88	0.02	1.03
7	222	2	17	0.99	0.85	0.02	149	0	9	1.00	0.88	0.02	UD
8	203	3	23	0.98	0.84	0.02	140	0	14	1.00	0.88	0.03	UD
9	177	2	18	0.99	0.83	0.03	126	3	7	0.98	0.86	0.03	0.49
10	157	1	30	0.99	0.82	0.03	116	2	14	0.98	0.84	0.03	0.38
11	126	1	8	0.99	0.82	0.03	100	1	5	0.99	0.84	0.03	0.80
12	117	1	9	0.99	0.81	0.03	94	0	6	1.00	0.84	0.03	UD
13	107	0	8	1.00	0.81	0.03	88	0	4	1.00	0.84	0.04	UD

	Prior TC Group						No Prior TC Group						
Int	At risk	Reached Outcome	Lost	Pr (no *E.coli*)	Cumulative Pr (no *E.coli*)	SE	At risk	Reached Outcome	Lost	Pr (no *E.coli*)	Cumulative Pr (no *E.coli*)	SE	Hazard Ratio [a]
14	99	4	15	0.96	0.77	0.04	84	1	1	0.99	0.83	0.04	3.65
15	80	1	19	0.99	0.76	0.04	82	0	6	1.00	0.83	0.04	UD
16	60	0	24	1.00	0.76	0.05	76	1	4	0.99	0.81	0.04	<0.01
17	36	0	19	1.00	0.76	0.06	71	0	5	1.00	0.81	0.04	UD
18	17	1	13	0.90	0.69	0.09	66	1	15	0.98	0.80	0.04	5.57
Total		88	724					49	605				
One Month Interval Width													
1	815	18	237	0.97	0.97	0.01	4	426	0.01	0.99	0.99	0.00	3.17
2	560	10	197	0.98	0.95	0.01	4	91	0.02	0.98	0.97	0.01	1.24
3	353	9	97	0.97	0.92	0.01	3	44	0.02	0.98	0.96	0.02	1.55
4	247	4	50	0.98	0.91	0.02	1	18	0.01	0.99	0.95	0.02	2.22
5	193	1	34	0.99	0.90	0.02	4	17	0.04	0.96	0.91	0.03	0.15
6	158	3	24	0.98	0.88	0.02	2	12	0.02	0.98	0.89	0.03	0.88
7	131	3	12	0.98	0.86	0.03	1	10	0.01	0.99	0.88	0.03	1.75
8	116	0	7	1.00	0.86	0.03	1	6	0.02	0.98	0.86	0.04	<0.01
9	109	1	8	0.99	0.86	0.03	1	3	0.02	0.98	0.85	0.04	0.56
10	100	0	7	1.00	0.86	0.03	0	3	0.00	1.00	0.85	0.04	UN
11	93	0	3	1.00	0.86	0.03	0	3	0.00	1.00	0.85	0.05	UN
12	90	1	7	0.99	0.85	0.04	0	3	0.00	1.00	0.85	0.05	UN
13	82	0	3	1.00	0.85	0.04	0	0	0.00	1.00	0.85	0.05	UN
14	79	0	1	1.00	0.85	0.04	0	3	0.00	1.00	0.85	0.05	UN
15	78	0	8	1.00	0.85	0.04	0	3	0.00	1.00	0.85	0.05	UN
16	70	0	4	1.00	0.85	0.04	0	0	0.00	1.00	0.85	0.05	UN
17	66	1	4	0.98	0.83	0.04	1	2	0.03	0.98	0.83	0.05	0.63
18	61	1	3	0.98	0.82	0.04	0	0	0.00	1.00	0.83	0.06	UN
19	57	0	4	1.00	0.82	0.05	0	0	0.00	1.00	0.83	0.06	UN
20	53	1	2	0.98	0.80	0.05	0	1	0.00	1.00	0.83	0.06	UN
21	50	0	4	1.00	0.80	0.05	1	1	0.03	0.97	0.81	0.06	<0.01

	Prior TC Group						No Prior TC Group						
	At	Reached		Pr	Cumulative Pr		At	Reached		Pr	Cumulative Pr		Hazard
Int	risk	Outcome	Lost	(no *E.coli*)	(no *E.coli*)	SE	risk	Outcome	Lost	(no *E.coli*)	(no *E.coli*)	SE	Ratio [a]
22	46	0	2	1.00	0.80	0.05	35	0	1	1.00	0.81	0.06	UN
23	44	0	2	1.00	0.80	0.05	34	0	0	1.00	0.81	0.06	UN
24	42	0	1	1.00	0.80	0.06	34	0	2	1.00	0.81	0.06	UN
25	41	0	0	1.00	0.80	0.06	32	0	1	1.00	0.81	0.06	UN
26	41	0	0	1.00	0.80	0.06	31	0	0	1.00	0.81	0.06	UN
27	41	0	1	1.00	0.80	0.06	31	1	1	0.97	0.78	0.07	<0.01
28	40	2	4	0.95	0.76	0.06	29	0	0	1.00	0.78	0.07	UN
29	34	0	4	1.00	0.76	0.06	29	0	0	1.00	0.78	0.07	UN
30	30	1	5	0.96	0.73	0.07	29	0	1	1.00	0.78	0.07	UN
31	24	0	5	1.00	0.73	0.08	28	0	0	1.00	0.78	0.07	UN
32	19	0	6	1.00	0.73	0.09	28	1	0	0.96	0.75	0.07	<0.01
33	13	0	6	1.00	0.73	0.11	27	0	0	1.00	0.75	0.07	UN
34	7	0	3	1.00	0.73	0.14	27	0	1	1.00	0.75	0.07	UN
35	4	1	0	0.75	0.55	0.18	26	0	0	1.00	0.75	0.07	UN
36	3	0	2	1.00	0.55	0.21	26	0	2	1.00	0.75	0.07	UN
Total		57	757					25	655				

[a] Hazard Ratio is undefined because the denominator was zero

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