OCCURRENCE AND POTENTIAL PREDICTORS OF VEROTOXIGENIC *ESCHERICHIA COLI* (VTEC), *SALMONELLA*, AND *LISTERIA MONOCYTOGENES* IN SURFACE WATER USED FOR PRODUCE IRRIGATION IN THE LOWER MAINLAND OF BRITISH COLUMBIA, CANADA

by

JUSTIN DESMOND FALARDEAU

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Abstract

Foodborne pathogens such as verotoxigenic *Escherichia coli* (VTEC), *Salmonella*, and *Listeria monocytogenes* may be present in surface waters, thus having the potential to contaminate fresh produce during crop irrigation. The objectives of this study were to determine the occurrence of VTEC, *Salmonella*, and *L. monocytogenes* in surface waters used for produce irrigation in the Lower Mainland of British Columbia, and to investigate potential predictors of their presence.

Water samples (n = 223) were collected from three and four irrigation ditches in both the Serpentine and Sumas watersheds, respectively, between February 2015 and August 2016. VTEC colonies on water filters were detected using a verotoxin colony immunoblot developed for the detection of all VTEC serotypes, and isolates were confirmed via multiplex PCR for virulence genes *vt1* and *vt2*. Detection of *Salmonella* and *L. monocytogenes* was completed using Health Canada Methods MFHPB-20 and MFHPB-30, respectively. Generic *E. coli* (EC) and fecal coliforms (FC) were enumerated using 3M™ Petrifilm™ Count Plates, and by membrane filtration with growth on m-FC agar with 0.01% rosalic acid; this was followed by transfer to nutrient agar containing 4-methylumbelliferyl-β-D-glucuronide. Meteorological data were collected from Environment Canada records, and agricultural data were collected from the British Columbia Agricultural Land Use Inventories.

The most commonly occurring pathogen was *L. monocytogenes* (11.2%), followed by VTEC (4.93%), and *Salmonella* (2.68%). Pathogen recovery was more common in the Serpentine watershed ($p < 0.05$), especially during the winter and fall seasons ($p < 0.05$). Pathogen occurrence in these locations was correlated with FC ($r = 0.431$) and EC ($r = 0.408$), but only by

using the membrane filtration method. Pathogens were still recovered when indicator concentrations were low. Pathogen occurrence was also correlated with the proximity to upstream livestock (r_s = -0.886) and the level of precipitation the day before sample collection (r = 0.203).

In conclusion, VTEC, *Salmonella*, and *L. monocytogenes* are present in surface waters used for irrigation in the Lower Mainland of British Columbia. There is potential to predict their presence, but further research is required to confirm factors affecting pathogen occurrence, which entail longer sampling times and increased sampling locations.

Preface

Subsection 2.2.3.1.1, Detection and isolation of *Listeria monocytogenes*, and subsection 2.2.3.2.1, detection and isolation of *Salmonella* were conducted by Silliker JR Laboratories in Burnaby, British Columbia. The author, Justin Falardeau, submitted water samples to be tested and collected any isolates recovered from positive samples.

Subsection 2.2.3.1.2, Serotyping and pulsed-field gel electrophoresis of *Listeria monocytogenes* was conducted by Dr. Franco Pagotto, Ms. Karine Hébert, and Mr. Kevin Tyler at the Listeriosis Reference Service, Health Canada, Ottawa, Ontario. The author, Justin Falardeau, submitted bacterial isolates to be tested and performed the analysis and interpretation of results.

Subsection 2.2.3.2.2, Serotyping of *Salmonella* was conducted by Dr. Gitanjali Arya and Dr. Cornelis Poppe at the OIE Reference Centre for Salmonellosis, National Microbiology Laboratory, Public Health Agency of Canada, Guelph, Ontario. The author, Justin Falardeau, submitted bacterial isolates to be tested and performed the analysis and interpretation of results.

Subsection 2.2.3.3.4, Serotyping of verotoxigenic *Escherichia coli* was conducted by Dr. Roger Johnson and Ms. Kim Ziebell at the *E. coli* Reference Laboratory, Laboratory for Foodborne Zoonoses, Public Health Agency of Canada, Guelph, Ontario. The author, Justin Falardeau, submitted bacterial isolates to be tested and performed the analysis and interpretation of results.

The rest of this research was completed solely by the author, Justin Falardeau, under the guidance of Dr. Siyun Wang.

The work in this thesis is original and has not been previously published.

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A_w – Water activity

χ^2 – Chi-square

r – Pearson correlation

r_{pb} – point-biserial correlation

r_s – spearman's rank correlation

List of Abbreviations

ALUI – Agricultural Land Use Inventory

BC – British Columbia

BCIG – 5-bromo-6-chloro-3-indolyl-β-D-glucuronide

CFU – colony forming units

DNA – deoxyribonucleic acid

dNTP – deoxynucleotide tri-phosphate

EPA – Environmental Protection Agency

FC – fecal coliforms

FDA – Food and Drug administration

FSMA – Food Safety Modernization Act

GAP – good agricultural practice

HC – haemorrhagic colitis

HGMF – hydrophobic grid membrane filter

HUS – haemolytic uremic syndrome

IMS – immunomagnetic separation

LEE – locus of enterocyte attachment

LSD – least significant difference

MFB – modified Fraser broth

MLST – multi-locus sequence typing

MPN – most probable number

mTSA-VC – modified tryptic soy agar with bile salts, vancomycin, and cefsulodin

mTSB-VC - modified tryptic soy broth with bile salts, vancomycin, and cefsulodin

MUG – 4-Methylumbelliferyl β-D-Glucuronide

NA-MUG – nutrient agar with 4-Methylumbelliferyl β-D-Glucuronide

OD – optical density

PAI – pathogenicity island

PBS – phosphate buffered saline

PBS-T – phosphate buffered saline with tween-20

PCR – polymerase chain reaction

PFGE – pulsed field gel electrophoresis

qPCR – quantitative polymerase chain reaction

RE-EM – Random effect – estimation method

RNA – ribonucleic acid

TC – total coliforms

TFC – total fecal coliforms

TSA – tryptic soy agar

TSB – tryptic soy broth

US – United States

USDA – United States Department of Agriculture

VT – verotoxin

VT-ELISA – verotoxin enzyme-linked immunosorbent assay

VT-IB – verotoxin immunoblot

VTEC – verotoxigenic *Escherichia coli*

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First and foremost, I would like to acknowledge Dr. Siyun Wang for her continuing supervision and mentorship. I would like to thank her for accepting me as her student, and allowing me to pursue my Masters of Science in the Wang Lab of Molecular Food Safety. Dr. Wang has always encouraged me to pursue any academic interests outside the scope of my project, and supported me, whenever possible, in attending workshops and conferences to improve my scientific foundations.

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Dedication

I would like to dedicate this work to Victoria Smar; her love is what drives me to accomplish great things.

Chapter 1 – Introduction and Literature Review

1.1 Introduction

Foodborne illness continues to be an issue for consumers in British Columbia, as well as the rest of Canada. In addition to health costs, foodborne illnesses can also lead to an economic burden due to lost productivity and wages among those affected (Thomas et al., 2006). Though most foodborne pathogens, such as *Listeria monocytogenes, Salmonella,* and verotoxigenic *Escherichia coli* (VTEC), are generally associated with meat, pathogen outbreaks resulting from the consumption of fresh produce have been increasing in prevalence. Produce accounted for more foodborne illnesses and outbreaks than any other food category in the United States between 2004 and 2013 (Fisher et al., 2015), while in Canada, there were 27 produce related outbreaks reported between 2001 and 2009 (Kozak et al., 2013). This is of special concern, as Health Canada recommends 7-10 servings of fruits and vegetables per day for adults (Health Canada, 2016). A likely source of contamination of fresh produce is through irrigation with contaminated water. The use of poor quality water for irrigation has been correlated with increased incidence of foodborne infections (Steele and Odumeru, 2004), and experiments have specifically shown the effective transmission and internalization of *E. coli* to lettuce through spraying with contaminated water (Solomon et al., 2002). Once attached, the bacteria are able to survive for long periods, and may not to be removed through washing (Berger et al., 2010), thus leading to considerable risks associated with any produce consumed raw, such as leafy greens and sprouts.

The objective of this project was therefore to investigate the occurrence and environmental factors that affect the presence of three foodborne pathogens in surface waters used for produce irrigation in the Lower Mainland of British Columbia: VTEC, *Salmonella,* and *L. monocytogenes*.

1.2 Literature review

1.2.1 Foodborne pathogens

1.2.1.1 Verotoxigenic *E. coli*

1.2.1.1.1 Characteristics and phylogeny

Escherichia coli is a gram-negative, rod-shaped bacterium of the *Enterobacteriaceae* family. It is characterized as oxidase-negative, both aerobic and facultative anaerobic, and motile or non-motile depending on the presence of peritrichous flagella. This bacterium will produce both acid and gas on commonly fermentable carbohydrates (Sheutz and Strockbine, 2005). Most strains are lactose fermenting, allowing them to be differentiated from *Salmonella* and *Shigella* on MacConkey agar, but exceptions do occur (Croxen et al., 2013). One important exception is the inability of *E. coli* O157:H7 to ferment sorbitol (March and Ratnam, 1986). *E. coli* are typically found in the small and large intestines of mammals, and as such prefer a temperature of 37°C to 42°C, but can grow under a wide range of temperatures (15°C to 48°C), and survive and persist in the natural environment under a range of adverse conditions as well (Welch, 2006).

The most commonly used serotype classification method for *E. coli* is based on the classification scheme developed by Kauffman, which relies primarily on the somatic "O" and flagellar "H" antigens (Nataro and Kaper, 1998). Currently there are 174 and 53 distinct *E. coli* O and H antigens, respectively, with non-motile strains having an H designation of NM due to lack of flagella (Croxen et al., 2013). Other typing methods, especially for outbreak investigations, include pulsed-field gel electrophoresis (PFGE) (Swaminathan et al., 2001) and multilocus

sequence typing (MLST) (Jenke et al., 2011), with whole genome sequencing beginning to become more common (Wang et al., 2016).

Verotoxigenic *E. coli* are loosely defined as any strain of *E. coli* containing the gene for producing either or both variants of the Shiga-like verotoxin (VT; vt_1 or vt_2), which is carried on a lambdoid bacteriophage. Only a subset of these, however, have been implicated in human illness (Croxen et al., 2013). A further subset of VTEC, and of primary concern is Enterohemorrhagic *E. coli* (EHEC), which is associated with hemorrhagic colitis (HC); the most well-known and commonly occurring member is *E. coli* O157:H7 (Nataro and Kaper, 1998). Other important non-O157 EHEC serotypes known to cause significant levels of human illness, termed the "Big Six", include the serotypes O26, O45, O103, O111, O121, and O145. (Brooks et al., 2005). Other VTEC serotypes are known to cause illness sporadically, including a large VTEC outbreak in Germany related to VTEC O104:H4 (Navarro-Garcia, 2014).

1.2.1.1.2 Pathogenicity of VTEC

Pathogenicity and virulence is highly varied across VTEC isolates, and is dependent on varying combinations of virulence factors contained on mobile elements such as plasmids, pathogenicity islands (PAI), and prophages (Croxen et al., 2013). There are many different factors leading to pathogenicity, but a select few have been shown to associate with increased severity of disease (Boerlin et al., 1999).

The most important virulence factor defining VTEC is the production of the VT, which has been associated with the development of hemolytic uremic syndrome (HUS) in infected individuals

(Mayer et al., 2012). There are two types of VT, VT1 and VT2, which are encoded on prophage inserted into the bacterial chromosome (*vt*$_1$ and *vt*$_2$, respectively), and each possessing various sub-types. VTEC can carry one or both types, or a combination of subtypes (Croxen et al., 2013). The VT1 variant is almost identical to that of *Shigella dysenteriae*, with VT2 being genetically and immunologically distinct (Scheutz et al., 2012). Of note, VTEC containing *vt*$_2$ are more often associated with severe human illness and HUS; though *vt*$_1$-containing isolates have also been known to lead to HUS (Boerlin et al., 1999). VT induces cell death after internalization into the cell where it removes an adenine from the 28S ribosomal RNA subunit, inhibiting protein synthesis (Gyles, 1992).

The pathogenicity of VTEC can be split across two groups based on the presence or absence of the locus of enterocyte effacement (LEE), a PAI responsible for the attachment and effacement lesions associated with Enteropathogenic *E. coli* (Croxen et al., 2013). The LEE contains the genes for effector proteins such as the type III secretion system, intimin, and the translocated intimin receptor, which are responsible for the creation of the attaching and effacing lesions that allow the bacterium to anchor to epithelial cells (Grant et al., 2011). Of importance is that the presence of the intimin producing gene of LEE, *eaeA*, has been associated with increased virulence in VTEC (Boerlin et al., 1999). Despite the association of LEE-positive VTEC with severe disease, not all HC and HUS causing strains contain this PAI. Of note is a 2011 outbreak of LEE-negative *E. coli* O104:H4 in Germany resulting in more than 3,800 cases of gastroenteritis and more than 800 cases of HUS (Frank et al., 2011).

A third virulence factor in VTEC is the plasmid bound hemolysin (*hlyA*). This pore-forming toxin has shown the ability to lyse sheep erythrocytes and may contribute to HUS development (Croxen et al., 2013). The association of this virulence factor with disease is somewhat dubious, however, as *hlyA* has been associated strongly with the presence of *eaeA* which may be confounding its association with disease in humans (Boerlin et al., 1999).

1.2.1.1.3 Presence of VTEC in the agricultural environment

Within the agricultural environment, VTEC may be found in various reservoirs, including animals, soil, water, and even on the produce itself. The primary reservoir for VTEC is cattle, with a global prevalence that can be as high as 74% and 70.1% in dairy and beef cattle, respectively, but can also vary significantly across herds (Hussein and Bollinger, 2005; Hussein and Sakuma, 2005). In Canada, VTEC O157 in cattle feces collected at abattoirs was observed to be at a level of 42.6% in Alberta (Van Donkersgoed et al., 1999), compared to 10.2% VTEC in cattle feces collected at Ontario abattoirs (Karama et al., 2008b). Cattle positive for VTEC may typically shed the pathogen in feces at levels as high as 1 - 4 log CFU/g feces, and may do so for as long as 10 weeks (Widiasih et al., 2004). Certain cattle on occasion may shed at levels greater than 4 CFU/g feces; these cattle are termed "super shedders" (Persad and Lejeune, 2014). Other animals that may carry VTEC include swine (Tseng et al., 2014), as well as a variety of other domesticated and feral ruminants (Persad and Lejeune, 2014). For example, VTEC isolates were recovered from 28.5% of fecal samples collected from swine across the United States (Fratamico et al., 2004), and the occurrence of VTEC in goats in the US was observed to be 14% (Jacob et al., 2013)

Surface waters are also a common place to find VTEC in the environment; though occurrence rates may vary depending on location. The occurrence of VTEC O157 in the surface waters of southern Alberta were observed to range from 1.7% to 2.3% across two surveys (Gannon et al., 2004; Jokinen et al., 2011), and a cross Canada survey found the national occurrence to be 3% for VTEC O157 (Edge et al., 2012). Of note, the occurrence of VTEC O157 was observed to be higher in waters of agricultural areas compared to waters collect from control regions not affected by agricultural activity (Edge et al., 2012). In New York State, the occurrence of VTEC was determined to be 2% for surface waters (Strawn et al., 2013a), compared to a study in California which observed occurrences of 8% and 11% for O157 and non-O157 VTEC, respectively (Cooley et al., 2014). It should be noted, however, that the California study sampled much larger volumes of water, leading to an increased likelihood of finding the pathogen when present in small concentrations. A more recent study in British Columbia and using a recently developed method for VTEC detection found an occurrence rate of 20% (Nadya et al., 2016).

VTEC may also find its way into the soils and onto the plants of produce farms. Soils collected from produce farms in California showed a 0.6% occurrence of the pathogen (Cooley et al., 2013) with a produce farm in New York State showing an occurrence of 2% in collected soil samples (Strawn et al., 2013a). On harvested produce, the Molecular Data Program conducted by the USDA found yearly estimates of VTEC occurrence to be as high as 0.6%, 0.5%, and 0.18% for spinach, cilantro, and lettuce samples, respectively (Feng, 2014). In the European Union, VTEC was found in 0.18% of produce samples tested (Feng 2014).

1.2.1.1.4 Epidemiology and produce-related foodborne outbreaks of VTEC

The global incidence of acute illness from VTEC has been estimated at 2.8 million cases annually (Majowicz et al., 2014). In Canada, the rate was estimated at 7 per 100,000 people in 2000/2001 (Thomas et al., 2006), but has been declining and was 1.8 per 100, 000 people in 2014 (BC Centre for Disease Control, 2017). Here in British Columbia, the rates of VTEC infection have been consistently higher than the national average, but recently, a 10-year low at 2.4 cases per 100,000 people was observed in 2015 (BC Centre for Disease Control, 2017). VTEC O157 has been the most common serotype observed in British Columbia, representing 35% and 47% of annual cases in 2014 and 2015, respectively (BC Centre for Disease Control, 2017). In Canada, VTEC O157 is responsible for 6% of hospitalizations and 8% of deaths resulting from foodborne infection (Public Health Agency of Canada, 2015a). Non-O157 serotypes also causing disease in British Columbia include O26, O121, O117, O103, and O111 (BC Centre for Disease Control, 2017).

A variety of foodborne outbreaks have been associated with produce; primarily leafy greens and sprouts. A selection of recent outbreaks is summarized in Table 1.1. In 2006, an outbreak of VTEC O157:H7 was traced back to spinach, that caused 192 cases, including one case in Canada. This outbreak resulted in 97 hospitalizations, 29 cases of HUS, and 4 deaths (Kozak et al., 2013; Sharapov et al., 2016). Between 2006 and 2008, VTEC O157:H7 was implicated in multiple outbreaks caused by lettuce, leading to at least 39 confirmed cases in Canada (Kozak et al., 2013; Michigan Department of Health, 2008), followed by another lettuce related outbreak in Canada of VTEC O157:H7 in 2012, resulting in 31 cases with one confirmed case of HUS (Tataryn et al., 2014). Other produce related outbreaks of VTEC in Canada include 235 cases resulting from Spanish onions contaminated with VTEC O157 (Kozak et al., 2013), and, perhaps the largest

outbreak, the 2011 outbreak of VTEC O104:H4 from sprouts, resulting in 4,068 cases with 908 cases of HUS and 50 deaths in Europe, along with 6 cases in the United States and 1 case in Canada (King et al., 2012; World Health Organization, 2011).

Table 1.1 - Recent produce-related outbreaks associated with verotoxigenic *E. coli*, including the number of confirmed cases in Canada, the United States, and Europe; the number of hospitalizations; number of cases of hemolytic uremic syndrome (HUS); and number of deaths.

Year	Food	Serotype	Cases	Hosp.	HUS	Deaths	Reference
2006	Spinach	O157:H7	1 (Can) 191 (US)	97	29	4	(Kozak et al., 2013; Sharapov et al., 2016)
2006	Lettuce	O157:H7	7 (Can)	NR	NR	NR	(Kozak et al., 2013)
2008	Lettuce	O157:H7	3 (Can) 47 (US)	21	1	0	(Michigan Department of Health, 2008)
2008	Lettuce (presumed)	O157:H7	29 (Can)	NR	NR	NR	(Kozak et al., 2013)
2008	Spanish Onions	O157:H7	235 (Can)	NR	NR	NR	(Kozak et al., 2013)
2010	Lettuce	O145	26 (US)	12	3	0	(Taylor et al., 2013)
2011	Sprouts	O104:H4	1 (Can) 6 (US) 4068 (Eur)	NR	908	50	(King et al., 2012; World Health Organization, 2011)
2011	Lettuce	O157:H7	58 (US)	34	3	0	(Slayton et al., 2013)
2012	Salad mix	O157:H7	33 (US)	15	2	0	(Marder et al., 2014)
2012	Sprouts	O26	29 (US)	7	0	0	(Centers for Disease Control, 2012)
2012	Lettuce	O157:H7	31 (Can)	13	1	0	(Tataryn et al., 2014)
2014	Sprouts	O121	19 (US)	7	0	0	(Centers for Disease Control, 2014)
2016	Sprouts	O157	11 (US)	2	0	0	(Centers for Disease Control, 2016a)

NR – not reported

1.2.1.1.5 Detection and isolation of VTEC from environmental samples

In Canada, a Health Canada approved method only exists for detection and isolation of VTEC O157. This method involves isolation using immunomagnetic separation (IMS) with beads coated in anti-*E. coli* O157 antibodies, followed by plating on selective agar media, such as Sorbitol MacConkey Agar, to capitalize on the inability of O157 isolates to ferment sorbitol (Health Canada, 2014). A similar procedure is standard in the United States, however IMS for the "Big Six" serotypes has also been approved. Samples are pre-screened for VT and serotype specific gene by the BAX® polymerase chain reaction (PCR) system to determine the appropriate IMS beads to use for isolation (United States Department of Agriculture, 2014). In both Canada and the United States, confirmation of isolates by VITEK 2 or equivalent is required.

Since IMS is serotype specific, and uncommon serotypes have been known to cause unexpected outbreaks (*e.g.*, VTEC O104:H4), other methods have been used to try and detect or isolate all VTEC serotypes. Some studies of surface water have used PCR to detect the presence of VT producing genes (Cooley et al., 2014; Strawn et al., 2013a), though this method may not produce isolates, making it difficult to confirm their presence as a result of viable VTEC. A variety of selective and differential media have also been developed (*e.g.*, CHROMagar, Rainbow agar O157), but these may not detect all VTEC serotypes and tend to result in a relatively high number of false positives (Parsons et al., 2016). Most recently, Johnson et al., (2014) developed a VT-immunoblot method (VT-IB) targeting all known variants of VT, and allowing for the identification of suspected VTEC colonies on hydrophobic grid membrane filters. This method has also proven to be quite sensitive, more than doubling the recovery of VTEC O157 compared to the standard IMS method (Johnson et al., 2014).

1.2.1.2 *Salmonella*

1.2.1.2.1 Characteristics and phylogeny

The genus *Salmonella*, a member of the *Enterobacteriaceae* family, is characterized as gram-negative, facultative anaerobic rods, usually motile due to the presence of peritrichious flagella. They are non-lactose fermenting, allowing them to be differentiated from *E. coli* on MacConkey agar, but produce gas from *D*-glucose. They also produce hydrogen sulfide on triple-sugar iron agar (Popoff and Le Minor, 2005). *Salmonella* are mesophilic, growing across a temperature range of 5 to 46°C and having an optimal range of 35 to 37°C. They cannot grow at A_w below 0.94 (Li et al., 2013).

The genus contains two distinct species, *Salmonella enterica* and *Salmonella bongeri*, with *S. enterica* being the most commonly associated with human infection (Jones et al., 2008). *S. enterica* is further divided into six subspecies (McQuiston et al., 2008), with subspecies I, (subsp. *enterica*) being the most clinically significant, accounting for 99% of human cases (Shi et al., 2013), and contains >1,500 of the >2,500 known *Salmonella* serovars (Li et al., 2013). The other subspecies are generally associated with cold-blooded animals (Brenner et al., 2000).

Identification of *Salmonella* serotypes has been traditionally done using the White-Kauffmann-Le Minor method, which uses antisera to determine the identity of somatic (O), flagellar (H), and capsular (Vi) antigens on the surface of the bacterium (Li et al., 2013). Molecular approaches (e.g. PFGE and MSLT) have been attempted with varying degrees of success; though while these

methods do not always accurately predict serotype, they do provide better resolution between individual strains/isolates, making them better suited for outbreak investigations (Shi et al., 2013).

1.2.1.2.2 Pathogenicity of *Salmonella*

The pathogenesis of *Salmonella* differs across serotypes. Typhoid and paratyphoid fever, caused by *S.* Typhi and *S.* Paratyphi, respectively, are more common in developing nations, accounting for less than 400 cases annually in the United States (Adams et al., 2016); primarily resulting from overseas travel (Lynch et al., 2009). On the other hand, foodborne non-typhoidal serotypes are believed to be responsible for an estimated one million illnesses annually in the United states (Scallan et al., 2011). Non-typhoidal *Salmonella* serovars show differences in disease severity. A survey of non-typhoidal *Salmonella* infections data collected by FoodNet between 1996 and 2006 found that the mortality rate, compared to the most common serotype in the United States, *S.* Typhimurium, was significantly higher in *S.* Dublin and significantly lower in *S.* Newport. Moreover, serotypes Heidelberg, Choleraesuis, and Dublin had a much higher rate of invasive disease (13%, 57%, and 64%, respectively), compared to Typhimurium (6%). Choleraesuis was also more likely to lead to hospitalization (Jones et al., 2008).

Disease typically occurs after the ingestion of >50,000 cells (Coburn et al., 2007), but can be as low as 1,000 cells (Public Health Agency of Canada, 2011). Symptoms can occur anywhere from 6 to 72 hours after exposure and include abdominal pain and cramping, and diarrhea (Coburn et al., 2007). *Salmonella* attach to the intestinal epithelial cells and are able to induce phagocytosis through bacterial-mediated endocytosis. This encloses the bacterial cells in large vesicles which internalizes them. This process is controlled by virulence factors injected using the type III

secretion system. Once inside the cellular environment, the bacterium can survive and multiply within the resulting vacuole, eventually lysing the cells and leading to inflammation and swelling of the infected area. During this time, a reduced uptake in Na^+ and an increased secretion of Cl^- leads to fluid loss and diarrhea (Ohl and Miller, 2001; Ray and Bhunia, 2008). In rare cases, invasive infections can also occur, which are more likely to lead to death compared to purely enteric infections (Jones et al., 2008).

1.2.1.2.3 Presence of *Salmonella* in the agricultural environment

The most common animal reservoir for *Salmonella* is poultry, which are known to be asymptomatic excreters of the pathogen (Park et al., 2008). *Salmonella* was detected from 31.2% of turkey carcasses in Quebec (Arsenault et al., 2007) and in 5.6% of fecal samples collected from poultry in North Carolina (Alali et al., 2010). Samples collected from various sources on poultry farms also found a 3% occurrence for *Salmonella* (Rodriguez et al., 2006). Domestic swine may also be prominent carriers, as 31.5% of samples collected from pig farms in Southern Ontario showed the presence of *Salmonella* (Farzan et al., 2010), and 10.1% of samples collected from pig farms in the United States (Rodriguez et al., 2006). *Salmonella* was found in the feces of dairy cattle at rates as high as 10% in the United States (Callaway et al., 2005), but only 0.08% of fecal samples were positive at an Alberta based abattoir (Van Donkersgoed et al., 1999). Once present in the agricultural environment, the pathogen tends to persist for long periods through cycling through animal hosts as a result of fecal contamination of water and feces on the farm (Jacobsen and Bech, 2012). In areas with high prevalence, wild birds may also become carriers, which can carry the pathogen long distances (Andrés et al., 2013).

Presence in local animal sources may also lead to contamination of surface waters in the area. The annual mean occurrence of *Salmonella* in waters of agriculture areas across Canada was estimated to be 11% (Edge et al., 2012). In southern Alberta, multiple surveys have shown occurrence in surface waters to range from 6.2% to 10.3% (Gannon et al., 2004; Johnson et al., 2003; Jokinen et al., 2011). South of the border, 9% of collected water samples in New York State were found to be positive for *Salmonella* (Strawn et al., 2013a), and a recovery as high as 65% was observed in California, although the California samples represented a much larger volume of water tested (Cooley et al., 2014).

Salmonella can also be routinely isolated from soil samples. On livestock farms, recovery ranging between 8.3% to 22.9% was observed (Rodriguez et al., 2006), while on produce farms, occurrence has ranged from 0.7% (Micallef et al., 2012) to 6.1% (Strawn et al., 2013b). This presence in soil suggests a risk for the pathogen to contaminate produce grown in the area. A survey of fresh produce in the United States found a 0.13% occurrence of *Salmonella* on the products sampled, with the highest occurrences being observed for cilantro (0.34%), parsley (0.29%), bagged spinach (0.29%), hot peppers (0.26%), and sprouts (0.25%). A total of 7% of products contaminated with *Salmonella* had been imported from Canada (Reddy et al., 2016).

1.2.1.2.4 Epidemiology and produce-related foodborne outbreaks of *Salmonella*

The global incidence of non-typhoidal *Salmonella* infection has been estimated at 93.8 million cases annually (Majowicz et al., 2010) In Canada, the rate was 19 illnesses per 100,000 people in 2000/2001 (Thomas et al., 2006), but this has been on the rise and increased to 21.5 and 25.2 cases per 100,000 people in Canada and British Columbia, respectively (BC Centre for Disease Control,

2017). *Salmonella* is attributed to 5% of illnesses, 24% of hospitalizations, and 16% of the deaths resulting from foodborne illness (Public Health Agency of Canada, 2016a). The most commonly observed serovars associated with disease in Canada have consistently been Enteritidis, Heidelberg, and Typhimurium (Public Health Agency of Canada, 2015a), with Enteritidis accounting for approximately 50% of infections observed in British Columbia in 2014 and 2015 (BC Centre for Disease Control, 2017).

A variety of produce related outbreaks have been associated with *Salmonella*. These have been summarized in Table 1.2. The most commonly observed vehicles are sprouts, cucumbers, and cantaloupe. Multiple *Salmonella* outbreaks from sprouts were responsible for over 360 illnesses in Canada between 1995 and 2001 (Honish and Nguyen, 2001; Sewell and Farber, 2001; Van Beneden et al., 1999). A major outbreak of *Salmonella* St. Paul from peppers resulted in 1500 cases across the United States and Canada, and resulting in 2 deaths (Barton Behravesh et al., 2011). *Salmonella* Brandenburg was responsible for a outbreak from cucumbers that affected 12 Canadians in 2004 (British Columbia Centre for Disease Control, 2005), and most recently in the United states, cucumbers were tied to outbreaks of *Salmonella* Newport and *Salmonella* Poona causing 275 and 907 cases respectively, which resulted in a total of 7 deaths (Angelo et al., 2015; Centers for Disease Control, 2016b). Outbreaks associated with *Salmonella* contaminated cantaloupes lead to 78, 22, and 9 illnesses in Canada in 1991, 1998, and 2008, respectively (Centers for Disease Control, 2008; Deeks et al., 1998; Sewell and Farber, 2001).

Table 1.2 - Recent produce-related outbreaks associated with *Salmonella*, including the number of confirmed cases in Canada and the United States, the number of hospitalizations, and number of deaths.

Year	Food	Serotype	Cases	Hosp.	Deaths	Reference
1991	Cantaloupe	Poona	78 (Can.) >300 (US)	NR	NR	(Centers for Disease Control, 1991; Sewell and Farber, 2001)
1995	Alfalfa sprouts	Stanley	30 (Can.) 242 (US, Fin.)	NR	NR	(Mahon et al., 1997; Sewell and Farber, 2001)
1995-1996	Alfalfa sprouts	Newport	121 (Can.), >20,000 (US, Den.)	NR	NR	(Sewell and Farber, 2001; Van Beneden et al., 1999)
1997	Alfalfa sprouts	Meleagridis	124 (Can.)	NR	NR	(Sewell and Farber, 2001)
1998	Cantaloupe	Oranienburg	22 (Can.)	NR	NR	(Deeks et al., 1998)
2001	Mung bean sprouts	Enteritidis	84 (Can.)	6	0	(Honish and Nguyen, 2001)
2004	Cucumbers	Brandenburg	12 (Can.)	NR	NR	(British Columbia Centre for Disease Control, 2005)
2005-2006	Tomatoes	Multiple	459 (US)	58	0	(Centers for Disease Control, 2007)
2008	Cantaloupe	Litchfield	9 (Can.) 51 (US)	16	0	(Centers for Disease Control, 2008)
2008	Peppers	St. Paul	1500 (US and Can.)	>286	2	(Barton Behravesh et al., 2011)
2009	Onion sprouts	Cubana	20 (Can.)	NR	NR	(Kozak et al., 2013)
2009-2011	Alfalfa sprouts	Multiple	509 (US)	44	0	(Centers for Disease Control, 2016c)
2011	Papayas	Agona	106 (US)	10	0	(Centers for Disease Control, 2011)
2011-2012	Cantaloupe	Multiple	383 (US)	118	4	(Centers for Disease Control, 2016c)
2014	Cucumbers	Newport	275 (US)	48	1	(Angelo et al., 2015)
2015	Cucumbers	Poona	907 (US)	204	6	(Centers for Disease Control, 2016b)

NR – not reported

1.2.1.2.5 Detection and isolation of *Salmonella* from environmental samples

Both in Canada and the United States, the detection and isolation of *Salmonella* from environmental samples follows the same general procedure: Non-selective enrichment, selective enrichment, selective plating, and biochemical screening. The standard Health Canada approved method (MFHPB-20; Reid, 2009) mandates non-selective enrichment in either nutrient broth or buffered peptone water, followed by selective enrichment in Tetrathionate Brilliant Green broth and Rappaport-Vassiliadis Soya Peptone broth. This is similar to methods outlined by the USDA (MLG 4.09; U.S. Department of Agriculture, 2017) and the US FDA (Andrews et al., 2016), though the US EPA (Method 1200; U.S. Environmental Protection Agency, 2012) mandates enrichment in Tryptic Soy broth followed by selective plating directly onto modified semisolid Rappaport-Vassiliadis media to check for mobility. Selective plating of the selective enrichments on at least two media is recommended, with Health Canada recommending Bismuth Sulfite agar and Brilliant Green Sulfa agar (Reid, 2009). Other selective media, such as Xylose Lysine Deoxycholate agar are approved by American agencies as well (Andrews et al., 2016; U.S. Environmental Protection Agency, 2012). Finally, in all cases, suspected colonies are subjected to biochemical screening for carbohydrate utilization, H_2S production, and gas formation from dextrose utilization on Triple Sugar Iron agar; presence and absence of lysine decarboxylase and lysine deaminase, respectively, on Lysine Iron agar; and absence of urease on Christensen's Urea media (Andrews et al., 2016; Reid, 2009; U.S. Department of Agriculture, 2017a; U.S. Environmental Protection Agency, 2012).

1.2.1.3 *Listeria monocytogenes*

1.2.1.3.1 Characteristics and phylogeny

Listeria spp. are non-spore-forming, gram-positive, aerobic/facultative anaerobic rods. They are able to grow at temperatures ranging from < 0°C to 45°C, and a pH range between 6 and 9. They are also motile due to the presence of peritrichious flagella at temperatures below 30°C; however, no motility is observed at 37°C (McLaughlin and Rees, 2009). The species *L. monocytogenes*, in particular, is differentiated from the other species by its hemolytic ability and its production of acid from *L*-rhamnose and alpha-methyl-*D*-mannoside, but not *D*-xylose (Bille et al., 1992).

The species *L. monocytogenes* is separated into 13 distinct serotypes based on somatic (O) and flagellar (H) antigens; these are summarized in Table 1.3 (McLaughlin and Rees, 2009). Of these serotypes, 4b has been primarily responsible for outbreaks, while 1/2a and 1/2b are responsible for most of the sporadic illnesses observed (Wiedmann, 2002); though the largest outbreak to date was caused by strains of serotypes 1/2a and 1/2b and associated with cantaloupe (McCollum et al., 2013). Initial genotypic evaluation of the species has shown the existence of three distinct groups, or lineages: lineage I consisting of serotypes 1/2b, 3b, 4b, 4d, and 4e; lineage II consisting of serotypes 1/2a, 1/2c, 3a, and 3c; and lineage III consisting of serotypes 4a and 4c (Liu, 2006), with some atypical 4b strains also included in lineage III (Orsi et al., 2011). Recently however, a lineage III subgroup (IIIB) has been declared to be its own distinct lineage IV (Ward et al., 2008). Lineages I and II are considered to be the most common, with lineage I being highly clonal and highly adapted to host survival, while lineage II has shown evidence of greater horizontal gene transfer, leading to a more diverse, generalist group able to survive in diverse environments (Nightingale et al., 2005). Moreover, human isolates are predominantly from lineage I, whereas animal isolates

are associated more with lineage II, although all lineages are capable of causing human disease (Haase et al., 2014)

Table 1.3 - Antigens used for serotyping of *Listeria monocytogenes* (McLaughlin and Rees, 2009) and their respective lineages (Orsi et al., 2011).

Serovar	Somatic (O) antigens	Flagella (H) antigens	Lineage
1/2a	I, II, III	A, B	II
1/2b	I, II, III	A, B, C	I
1/2c	I, II, III	B, D	II
3a	II, III, IV, (XII), (XIII)	A, B	II
3b	II, III, IV, (XII), (XIII)	A, B, C	I
3c	II, III, IV, (XII), (XIII)	B, D	I
4a	III, (V), VII, IX	A, B, C	III, IV
4ab	III, V, VI, VII, IX, X	A, B, C	
4b	III, V, VI	A, B, C	I, III, IV
4c	III, V, VII	A, B, C	III, IV
4d	III, (V), VI, VIII	A, B, C	
4e	III, V, VI, (VIII), X	A, B, C	
7	III, XII, XIII	A, B, C	

1.2.1.3.2 Pathogenicity of *L. monocytogenes*

L. monocytogenes is unique among *Listeria* species in that it is pathogenic to humans. However, *L. ivanovii* has been shown to be an opportunistic pathogen in humans in a few rare cases (Guillet et al., 2010). In healthy individuals, infection is rare but may lead to febrile gastroenteritis, which includes symptoms similar to mild influenza and diarrhea. The mildness of these symptoms suggest that the number of infections may be drastically under-reported (McLauchlin et al., 2004). In immunocompromised populations, pregnant women, and neonates, invasive systemic disease may occur, potentially leading to meningitis, septicemia, and spontaneous abortions. Of particular concern is the mortality rate of these infections, which has been estimated to be between 20% and 40% (Public Health Agency of Canada, 2012), and survivors of severe infections will likely be left with life-long sequelae (Farber and Peterkin, 1991). Pathogenicity of *L. monocytogenes* results

from the presence of several virulence factors present on a PAI. The presence of these virulence factors allows the bacterium to invade epithelial cells of the intestine where it is able to translocate across cells through inducing of actin polymerization within the cytoplasm. The cells can also survive inside macrophage, allowing them to travel through the bloodstream to various organs, across the blood brain barrier, or through the placental barrier (Ray and Bhunia, 2008). The infectious dose is suggested to be high, ranging between 7 and 9 log CFU (Public Health Agency of Canada, 2012). The incubation period for listeriosis can range anywhere from 3 to 70 days, with an average of 1 to 3 weeks (McCollum et al., 2013), often making it difficult to trace the food source. Furthermore, novel food vehicles are continually being implicated in recent outbreaks.

1.2.1.3.3 Presence of *L. monocytogenes* in the agricultural environment

Cattle are considered to the be the primary animal reservoir for *L. monocytogenes*, but the bacterium can also be shed by chickens, horses, and wild animals and birds (Milillo et al., 2012). *L. monocytogenes* has been found in the feces of cattle farms at rates as high as 32% in New York State (Nightingale et al., 2004), 16.1% in Finland (Husu, 1990), and 9.3% in Spain (Vilar et al., 2007). Also in Spain, the pathogen was recovered from 26.5% of feces samples collected on poultry farms (Esteban et al., 2008)

Of concern from an agricultural perspective is the ubiquity of *L. monocytogenes* in the environment. The bacterium can routinely be isolated from soil, at rates ranging from 0.4% to 17% (Dowe et al., 1997; Locatelli et al., 2013a; MacGowan et al., 1994; Sauders et al., 2012; Strawn et al., 2013a), but also from surface waters. *L. monocytogenes* was isolated from all samples taken from seven rivers in the United Kingdom at levels ranging from 3 to >180 per litre of water

(Watkins and Sleath, 1981), and in 4% of surface water samples collected in Northern Greece (Arvanitidou et al., 1997). Closer to home, *L. monocytogenes* was isolated from 10% of samples collected from surface waters within a Southern Ontario watershed (Lyautey et al., 2007b), and in 33% of surface water samples collected from creeks and ponds near farms in New York State (Strawn et al., 2013a).

Irrigation events have been associated with increased prevalence of *L. monocytogenes* in agricultural soil samples (Weller et al., 2015), suggesting transfer of the bacterium from irrigation ditches by the irrigation sprinklers. This is of concern since *L. innocua*, an avirulent surrogate often used in *L. monocytogenes* studies (Milillo et al., 2012), has shown the bacterium to readily attach to lettuce leaves through irrigation with infected water (Oliveira et al., 2011). A review of studies investigating the occurrence of *L. monocytogenes* on raw vegetables found a global average of 11.4%, including a 2.2% occurrence of *L. monocytogenes* on cabbage in Canada, a 2.2% occurrence on cucumbers and 36.8% occurrence on radishes in the United States, and of greater concern, a rate of occurrence of 85.7% on bean sprouts in Malaysia (Beuchat, 1996).

1.2.1.3.4 Epidemiology and produce-related foodborne outbreaks of *L. monocytogenes*

L. monocytogenes is resistant to many of the control strategies used by food processors to minimize risks of foodborne pathogens. Unlike other common foodborne pathogens, *L. monocytogenes* is capable of growing at refrigerated temperatures (Walker et al., 1990), with computer models suggesting the possibility of growth at temperatures as low as -1.5°C (Tienungoon et al., 2000). Furthermore, the bacterium is resistant to freezing conditions in most foods (Palumbo and Williams, 1991). *L. monocytogenes* is also able to survive in acidic conditions at a pH as low as

four (Phan-Thanh and Montagne, 1998), A_w as low as 0.90 (Nolan et al., 1992), and in salt concentrations as high as 16% (Hudson, 1992).

Global estimates of listeriosis in 2010 were 23,150 cases, resulting in 5,463 deaths (de Noordhout et al., 2014). In Canada, listeriosis rates have remained steady between 2010 and 2013 at approximately 0.33 cases per 100,000 individuals, with similar statistics observed in British Columbia (BC Centre for Disease Control, 2017). Despite these low incidences compared to other foodborne pathogens, *L. monocytogenes* is responsible for 35% of foodborne illness related deaths in Canada (Public Health Agency of Canada, 2016a).

The first confirmed outbreak of foodborne listeriosis was in Canada and was due to coleslaw produced using contaminated cabbage (serotype 4b), leading to 41 cases and 18 deaths (Schlech et al., 1983). Since then, most outbreaks have been tied to ready-to-eat meats and cheeses (Cartwright et al., 2013), but recent outbreaks have highlighted concerns about fresh produce as a vehicle for *L. monocytogenes* transmission, as summarized in Table 1.4. Since 1998 in the US, sprouts have been implicated in three individual outbreaks, leading to 27 cases of illness and two deaths (Centers for Disease Control, 2016c). In 2010, celery was implicated in an outbreak in Texas hospitals leading to 10 cases of listeriosis that resulted in 5 deaths (Gaul et al., 2013). In 2011, the largest outbreak of listeriosis ever recorded resulted from contaminated cantaloupes from a single farm in Colorado. The outbreak led to 147 cases in 28 states, resulting in 33 deaths. This outbreak was also unique in that two distinct serotypes, 1/2a and 1/2b, were involved (McCollum et al., 2013). Continuing on the trend of novel vehicles, a major outbreak in 2014 resulting in 35 illnesses across 12 states, and 7 deaths was attributed to caramel apples (Centers for Disease

Control, 2015). In addition, one case associated with this outbreak was also confirmed in Canada (Public Health Agency of Canada, 2015b). Finally, most recently, an outbreak involving prepackaged salads led to 19 illnesses and one death in the US (Centers for Disease Control, 2016d), and 14 illnesses and three deaths in Canada (Public Health Agency of Canada, 2016b).

Table 1.4 - Recent produce-related outbreaks associated with *Listeria monocytogenes*, including the number of confirmed cases in Canada and the United States, the number of hospitalizations, and number of deaths.

Year	Food	Serotype	Cases	Hosp.	Deaths	Reference
2008	Sprouts	1/2a	20 (US)	16	0	(Centers for Disease Control, 2016c)
2010	Celery	1/2a	10 (US)	10	5	(Gaul et al., 2013)
2011	Cantaloupe	1/2a, 1/2b	147 (US)	143	33	(McCollum et al., 2013)
2014	Sprouts	4b	5 (US)	5	2	(Centers for Disease Control, 2016c)
2014	Caramel Apples	4b	1 (Can) 35 (US)	34	7	(Angelo et al., 2017)
2015-2016	Packaged Salads	4b	14 (Can) 19 (US)	33	4	(Centers for Disease Control, 2016d; Public Health Agency of Canada, 2016b)

1.2.1.3.5 Detection and isolation of *L. monocytogenes* from environmental samples

Approved methods for the detection and isolation of *L. monocytogenes* from environmental samples are similar across Health Canada (MFHPB-30; Pagotto et al., 2011), the USDA (MLG 8.10; U.S. Department of Agriculture, 2017b), and the US FDA (Hitchins et al., 2016). Samples are first enriched in *Listeria* enrichment broth, followed by further selective enrichment in modified Fraser broth (Pagotto et al., 2011) or the addition of a *Listeria* selective enrichment supplement (Hitchins et al., 2016; U.S. Department of Agriculture, 2017b). Enrichments are then plated on selective Oxford agar and a second selective agar medium, such as PALCAM or

modified Oxford to confirm the ability to hydrolyze esculin (Hitchins et al., 2016; Pagotto et al., 2011; U.S. Department of Agriculture, 2017b). Suspect colonies are then biochemically screened for hemolysis, motility, and carbohydrate utilization on mannitol, L-rhamnose, and D-xylose agars. Acid production should be observed from L-rhamnose, but not the other two carbohydrates (Pagotto et al., 2011). Confirmation through the use of VITEK 2, or equivalent is also recommended (Pagotto et al., 2011; U.S. Department of Agriculture, 2017b).

1.2.2 Irrigation water as a source for foodborne pathogens in fresh produce

Contaminated irrigation water is believed to be a source of produce related foodborne illnesses, as a higher incidence of foodborne illness exists in areas that use minimally treated wastewater for irrigation purposes (Steele and Odumeru, 2004). Furthermore, in two large outbreaks, the outbreak strain was isolated from the irrigation source water: A 2002 outbreak of *S.* Newport linked to tomatoes in Virginia (Greene et al., 2008), and a 2008 outbreak associated with peppers from Mexico caused by *S.* Saintpaul (Barton Behravesh et al., 2011). Spray and flood irrigation methods are considered to induce the highest risk since in both these methods the water comes into direct contact with the edible parts of the plant (Olaimat and Holley, 2012)

Foodborne pathogens have shown the ability to attach to the leaves of the produce. This has been shown for VTEC O157 on lettuce leaves (Seo and Frank, 1999) and for *S.* Senftenberg on various leafy greens (Berger et al., 2009). Once present, these pathogens are able to persist for significant lengths of time. When applied at high concentrations, VTEC O157 was still detectable on lettuce leaves for 5 weeks (Oliveira et al., 2012) and 177 days on parsley (Islam et al., 2004a). VTEC O157 was also detectable on onions and carrots 74 and 168 days post

irrigation with 5 log CFU/ml of the pathogen (Islam et al., 2005). At lower concentrations, (~1 log CFU/ml), VTEC O157 was still detectable on lettuce up to 30 days post-watering (Mootian et al., 2009); however, no viable cells of VTEC O157 were detectable on spinach after 24 hours when watered at concentrations below 126 CFU/100 ml (Ingram et al., 2011). *Salmonella* have also been shown to persist, surviving 203 days on carrots grown in soil treated initially with water containing 5 log CFU / ml of the pathogen. In this same experiment, *Salmonella* persisted 84 days on radishes (Islam et al., 2004b). *L. innocua*, a surrogate for *L. monocytogenes*, was shown to survive on lettuce for up to 9 weeks after being exposed to sprinkler irrigation with water contaminated with the bacterium at 7 log CFU/ml (Oliveira et al., 2011).

A potential method for survival is the internalization of the bacterial cells into the plant tissue. VTEC have been observed to internalize into the stomata of the plant leaf, and can be found in the intercellular space, as well as the vascular tissue (Saldaña et al., 2011). It has also been suggested that VTEC O157 is able to internalize into plants from contaminated soil through the root system, where it can travel to edible parts of the plant (Solomon et al., 2002), but other research has suggested that this is a rare phenomenon (Erickson et al., 2010).

The presence of these pathogens on greens, even at low concentrations, is a concern. Pre-packaged salads, when subjected to temperature abuses not uncommon in retail and transport, can sustain the growth of these pathogens to harmful levels (Bovo et al., 2015; Zeng et al., 2014)

1.2.3 Indicator organisms and water quality assessment

The testing of water for the presence of foodborne pathogens is too expensive and time consuming for routine analysis, so water quality assessment and regulation has been based on the concentrations of microbial indicators. These are bacteria that are non-pathogenic, but are accepted to correlate with the presence of foodborne pathogens (Pachepsky et al., 2011). Initial water quality standards were based on total coliform bacteria as an indicator. Coliforms can be loosely defined as members of the *Enterobacteriaceae* that ferment lactose to form acid and gas. Although commonly associated with feces, some are capable of growth in the environment and are therefore not completely predictive of fecal contamination (Tallon et al., 2005). More recently, total coliforms (TC) have been replaced with thermo-tolerant fecal coliforms (FC) which are a subset of coliforms able to fermenting lactose at 44.5-45.5°C, and more commonly associated with fecal contamination (Pachepsky et al., 2011). The majority of FC are strains of *E. coli*, which is now considered to be the best indicator of fecal contamination since other genera of FC can also be found in the environment (Steele and Odumeru, 2004). Other indicator organisms have been suggested, such as *Bacteriodes* spp., but little data currently exists to support their value as indicators of fecal pollution (Uyttendaele et al., 2015). Indeed, a study in the Salmon River of British Columbia found FC to give stronger correlations than *Bacteriodes* spp. with respect to the occurrence of *Salmonella*, *Campylobacter* and VTEC O157 (Jokinen et al., 2010).

Water quality guidelines in Canada recommend fewer than 1000 TC and 100 FC per 100 ml of water for surface waters used in crop irrigation (CanAgPlus, 2015; Steele and Odumeru, 2004). Some provinces have also instituted their own recommendations, and in British Columbia, the Ministry of the Environment recommends fewer than 200 FC per 100 ml, and fewer that 77 *E. coli*

per 100 ml in all waters used for irrigation of produce (Government of British Columbia, n.d.). These levels are all based on single measurements. Currently in Canada, however, no water quality standards for irrigation water have been legislated.

In the United States, recent legislation under the Food Safety Modernization Act has implemented the monitoring of trends for *E. coli* as a way to estimate water quality. The law requires a geometric mean of below 126 CFU of *E. coli* per 100 ml across 20 consecutive samples, but no sample should exceed 410 CFU of *E. coli* per 100 ml. Furthermore, no *E. coli* can be present in any water used for during or after harvest, or to irrigate sprouts (U.S. Food and Drug Administration, 2015).

In Canada, the recommended testing method for FC and *E. coli* in water samples is the Most Probable Number (MPN) method (MFHPB-19; Christensen et al., 2002). In this method, multiple tubes of EC broth with MUG (4-methylumbelliferyl-β-D-glucuronide) inoculated with three or more decimal dilutions of the water sample are incubated at 45°C for 24 hours. After this period, the production of gas due to fermentation of the lactose in the EC broth is indicative of the presence of fecal coliforms, while the presence of fluorescence under UV light is indicative of glucuronidase activity, suggesting the presence of *E. coli.* Using the number of tubes of each dilution positive for the indicator of interest, a statistical estimate of the concentration can be determined.

Alternatively, *E. coli* can be enumerated by a membrane filtration method on mTEC media containing BCIG (5-bromo-6-chloro-3-indolyl-β-D-glucuronide). A suitable volume of water sample is filtered through a 0.45 μm filter which is subsequently placed on the agar medium and

incubated at 45°C for 24 hours. The resulting colonies are considered to be FC. Strains of *E. coli* will hydrolyze the BCIG substrate, resulting in a red or magenta colour (Health Canada, 2012).

The use of fecal indicators as predictors for the presence of pathogenic bacteria has shown limited success in recent studies. A recent investigation by Pachepsky et al. (2014) reviewed 81 datasets comparing concentrations of FC and *E. coli* in surface waters with the concentrations of one or more foodborne pathogenic organisms and only found a significant relationship in 35% of the cases reviewed. Moreover, in a study in California, the concentration of *E. coli* was found to be not associated with the occurrence of VTEC O157 or *Salmonella* in either sediment or in surface water (Benjamin et al., 2013).

Correlations between indicator organisms and pathogenic bacteria are more often related to the probability of finding foodborne pathogens in the long-run more so than predicting their presence at a single point in time. For instance, a cross-Canada investigation of foodborne pathogen occurrence in surface waters found that the annual mean concentration of *E. coli* at a particular site was significantly correlated with the mean annual recovery of foodborne pathogens at the same site (Edge et al., 2012), and in Alberta, the mean seasonal concentration of FC in waters was correlated with the seasonal occurrences of VTEC O157 and *Salmonella* (Jokinen et al., 2010). Similarly, a study in Spain found that samples positive for VTEC or *Salmonella* had a higher average concentration of *E. coli* than negative samples (Truchado et al., 2016).

Elevated levels of indicators may or may not provide an early warning for the added risk of foodborne pathogens. For instance, a study in Belgium by Holvoet et al. (2014) found that 42%

of water samples with higher than 100 *E. coli* per 100 ml were positive for *Salmonella* or VTEC associated genes, compared to only 10% of the samples below this concentration of *E. coli*. On the other hand, a Canadian study by Edge et al. (2012) found that 80% of water samples with fewer than 100 *E. coli* per 100 ml were positive for at least one foodborne pathogen.

The correlations of indicators with the likelihood of finding pathogens in surface waters may result from similar factors affecting the presence of the various indicator organisms and the pathogens themselves. For instance, in the Salmon River of British Columbia, both FC and the pathogens VTEC O157 and *Salmonella* correlated significantly with precipitation. Furthermore, concentrations of *E. coli* and *Salmonella* were observed to be higher at sample sites in regions affected by animal agriculture when compared to non-agricultural reference sites (Edge et al., 2012).

1.2.4 Environmental factors associated with pathogen occurrence

The occurrence of foodborne pathogens in water samples has been observed to correlate with various environmental factors such as landscape, weather, and season. As mentioned above, a cross-Canada survey of surface waters found *Salmonella* to occur more frequently in areas affected by animal agriculture (Edge et al., 2012). Similarly, recovery of *L. monocytogenes* from surface water was correlated with proximity to the nearest upstream dairy farm in Ontario (Lyautey et al., 2007b), and proximity to land used for pasture in New York State (Chapin et al., 2014).

Pathogen occurrence has been observed to change over season, but not always consistently in different areas. The occurrence of VTEC O157 was observed to be higher during the summer

months in Alberta (Gannon et al., 2004) and Georgia (Gu et al., 2013), but was more common during the winter in California (Cooley et al., 2013). In British Columbia, VTEC was also more common during the winter months (Nadya et al., 2016). More consistency has been observed for *L. monocytogenes,* which is more prevalent during the winter months (Cooley et al., 2014; Strawn et al., 2013a), and *Salmonella,* which has been observed to be more common during the summer months (Haley et al., 2009; Jokinen et al., 2012).

Precipitation has also been observed to correlate with pathogen occurrence. In Georgia, higher precipitation was shown to correlate with higher occurrence of VTEC O157 (Gu et al., 2013) and *Salmonella* (Haley et al., 2009). Similarly, precipitation three days prior to sampling was significantly correlated with the occurrence of VTEC O157 in Alberta (Jokinen et al., 2012), VTEC in British Columbia (Nadya et al., 2016), and *Salmonella* in New York State (Jones et al., 2014).

1.3 Research purpose

To date, only one study has investigated the occurrence of VTEC in the surface waters of the Lower Mainland of British Columbia, and none have investigated the occurrence of *L. monocytogenes* or *Salmonella*. Furthermore, no studies in the area have investigated the relative occurrences these three pathogens together, or studied any geographical and environmental sources affecting occurrence. Therefore, the primary purpose of this study was to investigate the occurrence of VTEC, *Salmonella,* and *L. monocytogenes* in surface waters used for irrigation in the Lower Mainland of British Columbia, and to assess the usefulness of various predictors of their

presence. These results can be used to provide information to local produce growers to help them reduce the risk of fresh produce contamination with foodborne pathogens during irrigation.

Four hypotheses were tested during this study:

1. The three foodborne pathogens, VTEC, *Salmonella*, and *L. monocytogenes* are present in surface waters used for irrigation in the Lower Mainland of British Columbia.
2. The occurrence of VTEC, *Salmonella*, and *L. monocytogenes* in surface waters in the Lower Mainland is not uniform across space or time.
3. Fecal coliforms and/or generic *E. coli* are suitable indicators of the occurrence of VTEC, *Salmonella*, and *L. monocytogenes* in surface waters in the Lower Mainland.
4. The presence of VTEC, *Salmonella*, and *L. monocytogenes* in surface waters used for irrigation in the Lower Mainland is effected by geographical and environmental factors.

To test these hypotheses, three objectives were included in this study:

1. To determine the occurrence rates of VTEC, *Salmonella*, and *L. monocytogenes* in surface waters used for irrigation of fresh produce at multiple sites within two distinct water sheds in the Lower Mainland: the Sumas watershed, and the Serpentine watershed.
2. To evaluate the relationship between VTEC, *Salmonella*, and *L. monocytogenes* occurrence in surface waters with the concentrations of fecal coliforms and generic *E. coli* using two different methods.
3. To investigate the relationship between VTEC, *Salmonella*, and *L. monocytogenes* occurrence in surface waters with local weather patterns and the proximity to and density of upstream animal agriculture sources.

Chapter 2: Occurrence of Verotoxigenic *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* in the Irrigation Waters Used in Two Watersheds of the Lower Mainland of British Columbia

2.1 Introduction

Foodborne pathogens in the environment, specifically in waters used for irrigation of fresh produce, are a potential source of contamination for a variety of produce related foodborne illness outbreaks (reviewed in Uyttendaele et al. 2015). Studies have shown the presence of *Listeria monocytogenes*, *Salmonella*, and both O157 and non-O157 verotoxigenic *Escherichia coli* (VTEC) in various watersheds across Canada (Edge et al., 2012; Johnson et al., 2014; Lyautey et al., 2007b), including a recent investigation of the prevalence of VTEC in the surface waters of the Lower Mainland of British Columbia (Nadya et al., 2016).

Fresh produce is the vehicle for a significant amount of foodborne disease. It was the food type most associated with foodborne outbreaks in the United States between 2004 and 2013 (Fisher et al., 2015), and was responsible for 27 foodborne outbreaks in Canada between 2001 and 2009 (Kozak et al., 2013). Contaminated irrigation water has been suggested as a possible source for the transmission of foodborne pathogens to produce (Steele and Odumeru, 2004), and recent work has highlighted the potential for bacterial pathogens to attach and potentially internalize into produce during the preharvest stage (Erickson et al., 2010; Hintz et al., 2010; Mootian et al., 2009; Oliveira et al., 2011).

Therefore, the objective of this part of the project was to determine the occurrence of VTEC, *Salmonella*, and *L. monocytogenes* in the irrigation waters used in the Lower Mainland of British Columbia, and to investigate whether there is evidence for geographic or seasonal trends in their isolation.

2.2 Materials and methods

2.2.1 Site selection

Sampling sites were located in two distinct watersheds: The Serpentine watershed in the Cloverdale region of Surrey, BC, and the Sumas watershed on the Sumas prairie near Abbotsford, BC. Three surface water sites (*i.e.*, ditch, creek, or stream) within each watershed were chosen which represented water adjacent to vegetable growing fields (Figure 2.1). A fourth site (*i.e.*, Sumas 4) was added to the Sumas watershed during the first summer of sampling.

2.2.2 Sample collection

Water samples were collected from each site once per month from February 2015 to April 2015, then twice per month until August 2016, with the exception of October 2015 when only one sample was collected from each site. Sampling began in February 2015 for Sumas 1, March 2015 for Sumas 2 and Sumas 3, April 2015 for Serpentine 2, May 2015 for Serpentine 1 and Serpentine 3, and July 2015 for Sumas 4.

Surface water samples were collected from the ditches in 532 ml Stand-Up Whirl-Pak® bags using a telescopic sampling pole designed to hold the Whirl-Pak® bags (Nasco, Fort Atkinson, WI). The

sampling pole was rinsed thoroughly with 70% ethanol before and after sampling at each site. Five sample bags were collected at each site: two to be tested for *L. monocytogenes* and *Salmonella*, and two to be tested for VTEC. The fifth sample bag was used to measure the temperature of the sample water (Chapter 4).

2.2.3 Pathogen detection

Each sample was tested for the presence of *L. monocytogenes*, *Salmonella*, and VTEC in duplicate, as follows. If either replicate was positive for a pathogen, the site was considered positive for that pathogen on that date of sampling.

2.2.3.1 *L. monocytogenes*

2.2.3.1.1 Detection and isolation

The presence of *L. monocytogenes* was detected in water samples by Silliker JR Laboratories (Burnaby, BC) using the MFHPB-30 method from Health Canada (Pagotto et al., 2011). Briefly, 25 ml of sample was enriched in *Listeria* enrichment broth for 24 and 48 hours at 35°C before being subsequently used to inoculate modified Fraser Broth (MFB) and incubated for 24-26 hours at 35°C. Positive enrichments in MFB were then streaked onto Oxford agar and PALCAM agar and incubated for up to 48 hours at 35°C. A minimum of five typical colonies from each plating were then tested for hemolysis, motility, and carbohydrate utilization to confirm the identity as *L. monocytogenes*. Positive isolates were then recovered and submitted for serotyping analysis.

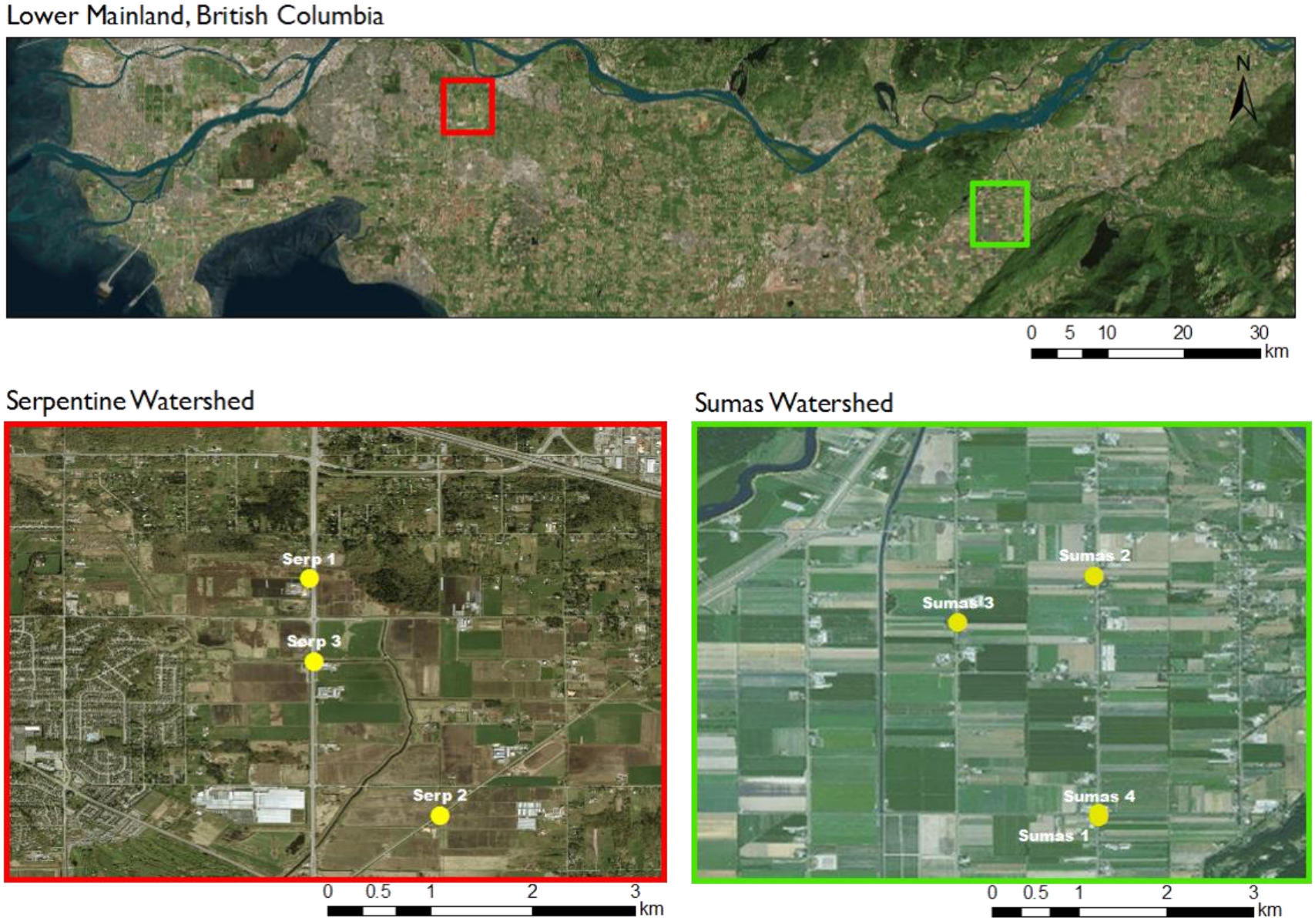

Figure 2.1 – Location of sampling sites in the Serpentine (red border) and Sumas (green border) watersheds of the Lower Mainland of British Columbia

2.2.3.1.2 Serotyping and pulsed-field gel electrophoresis characterization

Recovered *L. monocytogenes* isolates were characterized by serotyping and pulsed-field gel electrophoresis (PFGE) by Dr. Franco Pagotto, Karine Hébert, and Kevin Tyler at the Listeriosis Reference Service, Health Canada, Ottawa, Ontario. Serotype was only predicted from ribotype due to a defective serology kit at the time of analysis. PFGE analysis was conducted separately after digestion with the restriction endonucleases AscI and ApaI.

2.2.3.2 *Salmonella*

2.2.3.2.1 Detection and isolation

The presence of *Salmonella* was detected in the water samples by Silliker JR Laboratories (Burnaby, BC) using the MFHPB-20 method from Health Canada (Reid, 2009). Briefly, 25 ml of sample was non-selectively enriched for 18 to 24 hours at 35°C in buffered peptone water, followed by selective enrichment of these cultures in Tetrathionate Brilliant Green broth and Rappaport-Vassiliadis Soya Peptone broth for 24 ± 2 hours at 42.5°C. Selective enrichment cultures were then streaked onto Bismuth Sulfite agar and Brilliant Green Sulfa agar and incubated for 24 ± 2 hours at 35°C. Suspected colonies were subjected to biochemical screening for carbohydrate utilization, H_2S production, and gas formation on Triple Sugar Iron agar; presence and absence of lysine decarboxylase and lysine deaminase, respectively, on Lysine Iron agar; and absence of urease on Christensen's Urea agar. Positive isolates were then recovered and submitted for serotyping analysis.

2.2.3.2.2 Serotyping

Recovered *Salmonella* isolates were serotyped by Dr. Gitanjali Arya and Dr. Cornelis Poppe at the OIE Reference Centre for Salmonellosis, National Microbiology Laboratory, Public Health Agency of Canada, Guelph, Ontario.

2.2.3.3 Verotoxigenic *Escherichia coli* detection and isolation

The presence of VTEC in water samples was determined using the verotoxin immunoblot (VT-IB) method previously described by Johnson et al. (2014). A volume of 25 ml of each sample was filtered through a 0.45 μm Hydrophobic Grid Membrane Filter (HGMF; Neogen Corp., Lansing, MI). The HGMF were then incubated on VT-IB membranes to detect presence of VT production. Suspected colonies were confirmed using a VT Enzyme Linked Immunosorbent Assay (VT-ELISA). The VT-ELISA confirmed isolates were then confirmed as *E. coli* using polymerase chain reaction (PCR) for the *gadA* gene, and tested to confirm the presence of either of the VT producing genes *vt1* and *vt2*, as well as two other virulence factors, *eaeA* and *hlyA*, also using PCR. These methods are elaborated below.

2.2.3.3.1 Verotoxin immunoblot

After vacuum filtration of water samples through HGMF, the filters were over-layed on VT-capture membranes and incubated for 18 – 24 hours at 37°C on tryptic soy agar (BD, Mississauga, ON) containing 1.5 g/L bile salts No.3, 10 μg/ml vancomycin, and 10 μg/ml cefsulodin (Sigma, Oakville, ON) (mTSA-VC).

The VT-capture membranes consisted of 0.2 μm nitrocellulose filter membranes (Biotrace, Pall Life Sciences, Mississauga, ON) coated with rabbit anti-VT antibodies specific to all known variants of verotoxin (LFZ, Guelph, ON), and were blocked using 2% bovine serum albumin (MP Biomedicals, Solon, OH, USA) in phosphate buffered saline (PBS; Amresco, Solon, OH, USA). After incubation, a series of needle pricks were used to mark the relative position of the HGMF on the VT-capture membrane for later re-orientation. The VT-capture membranes were then probed using indirect detection at room temperature for the presence of VT using a set of four monoclonal mouse anti-VT antibodies (2 μg/ml; LFZ), followed by alkaline-phosphatase labelled anti-mouse IgG (0.02 μg/ml; Jackson ImmunoResearch Inc., West Grove, PA, USA) and visualized using the substrate nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Mandel Scientific, Guelph, ON). Membranes were washed three times at each step using a wash buffer composed of PBS and Tween-20 (MP Biomedicals) (PBS-T).

Dark purple spots on the membrane suggested the presence of VT. Relative location of the suspected VT-producing colony on the HGMF was measured using a transparent copy of the HGMF aligned on the VT-capture membrane using the needle pricks. Up to eight suspected colonies were selected from the HGMF and streaked onto MacConkey agar (BD) and incubated at 37°C for 18-24 hours in preparation of confirmation of VT production using a VT-ELISA.

2.2.3.3.2 Verotoxin enzyme linked immunosorbent assay

Up to six colonies of each morphology were collected from each MacConkey plate, and grown in 400 μl of Tryptic Soy agar (BD) containing 1.5 g/L bile salts No.3, 10 μg/ml vancomycin, and 10 μg/ml cefsulodin (Sigma) (mTSB-VC) for 18-27 hours at 37°C with shaking at ~150 rpm. One

hundred microliters of each resulting culture where then transferred, in duplicate, to a 96-well microplate (Fisher Scientific, Ottawa, ON) pre-coated with rabbit anti-VT antibodies specific to all known variants of verotoxin (LFZ) and blocked using 2% bovine serum albumin (MP Biomedicals) in PBS, followed by incubation at room temperature for 30 minutes. Wells were then probed for the presence of bound VT using indirect detection at room temperature for 30 minutes using 100µl each of a set of four monoclonal mouse anti-VT antibodies (2 µg/ml; LFZ), followed by horseradish peroxidase labelled anti-mouse IgG (0.2 µg/ml; Jackson ImmunoResearch Inc.). The wells were washed five times with PBS-T between each step, and seven times afterwards. The presence of bound VT was then visualized by incubating with the substrate tetramethylbenzidine (Sigma) at room temperature with agitation at ~150 rpm for 10 minutes, followed by addition of 100 µl of 0.2 M sulfuric acid (Sigma). The absorbance of the wells was measured using a SpectraMax M2 Microplate Reader (MTX Lab Systems, Inc., US) at a dual wavelength of 450/620 nm and air as a blank. Wells were considered positive if the optical density (OD) was greater than two times that of the negative control. If the OD of both replicate wells of a particular isolate were greater than 1.5 times the negative control, but below twice that of the negative control, they were scored as suspicious and were tested a second time. Presumptive VTEC isolates found to be positive by VT-ELISA were simultaneously streaked onto TSA to confirm them as a pure culture, and into TSB in preparation for final confirmation using PCR.

2.2.3.3.3 Polymerase chain reaction for confirmation of *Escherichia coli* and detection of virulence genes

Overnight cultures (18-24 hours) of presumptive VTEC were confirmed as *E. coli* using a monoplex PCR for the *gadA* gene as described by Doumith et al. (2012). They were also confirmed for both variants of the VT producing gene (i.e., *vt1*, *vt2*) and two other virulence determinants: *eaeA* and *hlyA*

by a multiplex PCR previously described by Paton and Paton (1998), and modified by Ms. Kim Ziebell of the Public Health Agency of Canada *E. coli* Reference Lab (Personal communication).

The DNA was extracted from lysates using a boiling method. A 360 µl aliquot of overnight culture was combined with 40 µl of 10X PBS, pH 7.2 (Amresco) in a 1.5 ml microcentrifuge tube (Fisher Scientific) and incubated for ten minutes in a heating block at 96°C, followed by cooling on ice for five minutes. The tubes were then centrifuged (Microcentrifuge 5415 R, Eppendorf, Mississauga, ON) at 13,300 rpm for 5 minutes, and the supernatant, containing the DNA, was reserved and stored at -20°C until use.

Monoplex PCR for the confirmation of the isolates as *E. coli* was conducted using 2 µl of DNA lysate in a 25 µl reaction mixture containing 1X buffer solution, 1X Coral Dye, 5 µl Q-solution, and 0.625 units of TopTaq DNA polymerase (Qaigen, Toronto, ON), along with 50 µM dNTP's (Invitrogen, Thermo Fisher Scientific, Ottawa, ON) and 1 µM each of the *gadA* primers (Table 2.1). The PCR reaction was carried out in a C1000 Touch™ Thermal Cycler (BioRad Laboratories, Mississauga, ON) under the following conditions: a 4 min denaturation at 94°C, followed by 30 cycles of 94°C for 30 sec, 65°C for 30 sec, and 72°C for 30 sec, and a final extension step at 72°C for 5 min. Products were held at 4°C until visualization.

Multiplex PCR to determine the presence of virulence determinants was conducted using 2 µl of DNA lysate in a 25 µl reaction mixture containing 1X buffer solution, 1X Coral Dye, 5 µl Q-solution, and 0.625 units of TopTaq DNA polymerase (Qaigen), along with 50 µM dNTP's (Invitrogen) and 1 µM each of the primers for *eaeA* and *hlyA*, and 0.4 µM of primers for *stx1*, *stx2*, and *stx2e* (Table 2.1). The PCR reaction was carried out in a C1000 Touch™ Thermal Cycler

(BioRad) under the following conditions: a 3 min denaturation at 95°C, followed by 35 cycles consisting of a denaturation step at 95°C for 1 min; an annealing step at 65°C for 2 min for the first 10 cycles, decrementing 1°C per cycle to 60°C by cycle 15; and an elongation step at 72°C for 1.5 min, incrementing to 2.5 min from cycles 25 to 35; and a final extension step at 72°C for 5 min. Products were held at 4°C until visualization.

Table 2.1 – Sequences of primers used for confirmation of verotoxigenic *E. coli* isolates

Gene	Primer	Sequence (5'-3')	Amplicon Size (bp)	Reference
gadA	forward	GATGAAATGGCGTTGGCGCAAG	373	(Doumith et al., 2012)
	reverse	GGCGGAAGTCCCAGACGATATCC		
stx1	forward	ATAAATCGCCATTCGTTGACTAC	180	(Paton and Paton, 1998)
	reverse	AGAACGCCCACTGAGATCATC		
stx2	forward	GGCACTGTCTGAAACTGCTCC	255	(Paton and Paton, 1998)
	reverse	TCGCCAGTTATCTGACATTCTG		
eaeA	forward	GACCCGGCACAAGCATAAGC	384	(Paton and Paton, 1998)
	reverse	CCACCTGCAGCAACAAGAGG		
hlyA	forward	GCATCATCAAGCGTACGTTCC	534	(Paton and Paton, 1998)
	reverse	AATGAGCCAAGCTGGTTAAGCT		
stx2e	forward	GAACAGATGGAATTTGCAGCCA	112	(Dr. Kim Ziebell, Personal communication)
	reverse	TAAACTTCACCTGGGCAAAGCC		

Products of the both the monoplex and multiplex PCR reactions were visualized on 2% agarose gel stained with SYBR® Safe (Invitrogen) after electrophoresis in TAE buffer (Invitrogen) at 90V for 45 min. Expected amplicon sizes are shown in Table 2.1. A positive control of *E. coli* O157:H7 possessing *vt1*, *vt2*, *eaeA*, and *hlyA* was donated by Linda Hoang (BC Centre for Disease Control, Vancouver, BC). Samples positive for *gadA* were confirmed to be *E. coli*, and *E. coli* positive for any of *vt1*, *vt2*, or *vt2e* were confirmed to be VTEC.

2.2.3.3.4 Serotyping

Recovered VTEC isolates were serotyped by Dr. Roger Johnson and Ms. Kim Ziebell at the *E. coli* Reference Laboratory, Laboratory for Foodborne Zoonoses, Public Health Agency of Canada, Guelph, Ontario.

2.2.4 Statistical analysis

Statistical analysis of results was conducted using R version 3.2.3 (R Core Team, 2015; http://www.R-project.org). Uniformity of occurrence within and between watersheds was completed using either the χ^2 test when appropriate, or the Fisher's Exact test when frequencies of occurrence less than five were present in the calculations.

2.3 Results

2.3.1 Pathogen occurrence

A total of 223 water samples were collected between the Sumas and Serpentine watersheds; of those, 16.6% were positive for at least one of the three pathogens tested. The occurrence of pathogens in the water samples is outlined in Table 2.2. The most commonly occurring pathogen

was *L. monocytogenes* which was found in 11.2% of the samples, followed by VTEC and *Salmonella* with 4.9% and 2.7% occurrence, respectively.

The recovery of any of the three pathogens was more common in the Serpentine watershed compared to the Sumas watershed, with 24.5% compared to 10.9% occurrence, respectively (χ^2; p = 0.0119). This difference was primarily due to the increased occurrence of *L. monocytogenes* in the Serpentine watershed compared to the Sumas watershed: 20.2% and 4.65%, respectively (χ^2; p = 0.0006). Neither VTEC nor *Salmonella* showed evidence for differences in occurrence between watersheds (χ^2; p > 0.10), although all but one occurrence of *Salmonella* was derived from the Sumas watershed. Site specific differences were observed for VTEC occurrence within both the Serpentine watershed (Fisher's exact test; p = 0.046) and the Sumas watershed (Fisher's exact test; p = 0.075), with all but one positive sample in each watershed coming from a single location. Moreover, VTEC was never recovered at 1 and 2 sites in the Serpentine and Sumas watersheds, respectively. Similarly, *Salmonella* showed some evidence of site-specific occurrence in the Sumas watershed (Fisher's exact test; p = 0.097), especially when taken into account that both sites where the bacterium was recovered were in close proximity (Figure 2.1 and Table 2.2), thereby suggesting a potential for a common source of contamination. No significant site-specific differences were observed for *L. monocytogenes* in either watershed, though two thirds of positive samples from the Sumas watershed came from a single site.

Table 2.2 – Occurrence of verotoxigenic *E. coli* (VTEC), *L. monocytogenes*, and *Salmonella* in irrigation water collected from seven sites in the Sumas and Serpentine watersheds.

Site	# of Samples	Number of positive samples (% of positive samples)			
		VTEC	***L. monocytogenes***	***Salmonella***	**Any Pathogen**
Sumas 1	35	4 (11.4%)$^{\alpha}$	1 (2.86%)	3 (8.57%)$^{\alpha}$	6 (17.1%)
Sumas 2	34	0 $^{\beta}$	1 (2.94%)	0 $^{\beta}$	1 (2.94%)
Sumas 3	34	1 (2.94%)$^{\beta}$	4 (11.8%)	0 $^{\beta}$	5 (14.7%)
Sumas 4	26	0 $^{\beta}$	0	2 (7.69%) $^{\alpha}$	2 (7.69%)
Serpentine 1	30	0 $^{\beta}$	6 (20.0%)	1 (11.1%)	6 (20.0%)
Serpentine 2	34	5 (14.7%)$^{\alpha}$	9 (26.5%)	0	12 (35.3%)
Serpentine 3	30	1 (3.33%)$^{\beta}$	4 (13.3%)	0	5 (16.7%)
Total Sumas	129	5 (3.88%)	6 (4.65%)^{A}	5 (3.88%)	14 (10.9%)^{A}
Total Serpentine	94	6 (6.38%)	19 (20.2%)^{B}	1 (1.06%)	23 (24.5%)^{B}
Total	223	11 (4.93%)	25 (11.2%)	6 (2.69%)	37 (16.6%)

^{A,B} Pathogens at watersheds with different letters represent a significant difference in pathogen occurrence between the two watersheds (χ^2; $p < 0.05$)

$^{\alpha,\beta}$ Pathogens at sites with different letters represent a significant difference in pathogen occurrence between those sites within their respective watershed (Fisher's exact test; $p < 0.05$)

The monthly occurrence of pathogens in the water samples is outlined in Figure 2.2. The most positive samples were collected during February and September. This increase in recovery is primarily the result of *L. monocytogenes* which showed a marked increase in occurrence during the cooler months. No temporal trends for VTEC can be seen, though February is the only month with two positive recoveries of the pathogen. Also, *Salmonella* was recovered twice in September and twice in February, representing the bulk of the occurrence save for a single recovery in August 2016.

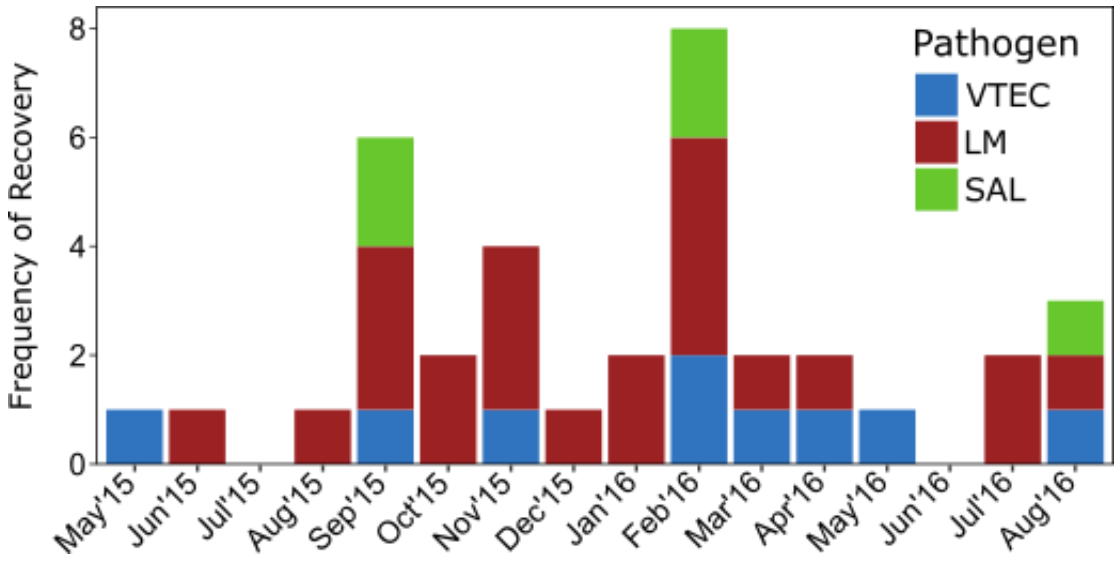

Figure 2.2 – Monthly recovery of verotoxigenic *E. coli* (VTEC), *L. monocytogenes* (LM), and *Salmonella* (SAL) between May 2015 and August 2015 from irrigation water collected at three sites each in the Sumas and Serpentine watersheds of the Lower Mainland of British Columbia.

To investigate seasonal changes in pathogen occurrence, relative occurrence was investigated for winter (December to February), spring (March to May), summer (June to August), and fall (September to November) months. These results are summarized in Table 2.3. The recovery of any pathogen was more common during the fall and winter months with 28.6% and 23.3% positive samples, respectively, compared to 15.6% for spring and 8.6% for summer (χ^2; $p = 0.033$). This was likely driven by differences in recovery of *L. monocytogenes* which showed 22.9% occurrence in the fall and 16.3% occurrence in the winter, compared to 7.8% and 6.2% for spring and summer, respectively (Fisher exact test; $p = 0.038$). No significant difference in occurrence of VTEC or *Salmonella* was observed, though the lowest occurrence for both pathogens was observed to be during the summer.

Table 2.3 – Seasonal occurrence [number of positive samples/total number of samples collected (percent recovery)] of verotoxigenic *E. coli* (VTEC), *L. monocytogenes*, *Salmonella*, or any of the three in water collected from irrigation ditches at three sites each in the Sumas and/or Serpentine watersheds in the Lower Mainland of British Columbia

	Winter (Dec-Feb)	Spring (Mar-May)	Summer (Jun-Aug)	Fall (Sep-Nov)
Sumas Watershed				
VTEC	2/25 (8%)	2/39 (5.1%)	0/45 (0%)	1/20 (5%)
L. monocytogenes	2/25 (8%)	1/39 (2.6%)	2/45 (4.4%)	1/20 (5%)
Salmonella	2/25 (8%)	1/39 (2.6%)	1/45 (2.2%)	1/20 (5%)
Any	5/25 (20%)	4/39 (10.3%)	3/45 (6.7%)	2/20 (10%)
Serpentine Watershed				
VTEC	1/18 (5.6%)	3/25 (12%)	1/36 (2.8%)	1/15 (6.7%)
L. monocytogenes[α]	5/18 (27.8%)	4/25 (16%)	3/36 (8.3%)	7/15 (46.7%)
Salmonella	0/18 (0%)	0/25 (0%)	0/36 (0%)	1/15 (6.7%)
Any[α]	5/18 (27.8%)	6/25 (24%)	4/36 (11.1%)	8/15 (53.3%)
Both Watersheds				
VTEC	3/43 (7%)	5/64 (7.8%)	1/81 (1.2%)	2/35 (5.7%)
L. monocytogenes[α]	7/43 (16.3%)	5/64 (7.8%)	5/81 (6.2%)	8/35 (22.9%)
Salmonella	2/43 (4.7%)	1/64 (1.6%)	1/81 (1.2%)	2/35 (5.7%)
Any[α]	10/43 (23.3%)	10/64 (15.6%)	7/81 (8.6%)	10/35 (28.6%)

[α] Significant seasonal variation was observed for this pathogen(s) within respective watershed(s) (Fisher exact test; $p < 0.05$)

Significant seasonal differences were only observed for the Serpentine watershed. These differences echoed those of both watersheds together, with significant differences being observed for *L. monocytogenes* (Fisher exact test; $p = 0.016$) or any of the three pathogens (Fisher exact test; $p = 0.018$), but not for VTEC or *Salmonella*. The occurrence of any of the three pathogens in the Serpentine watershed was highest during the fall with 53.3% occurrence, compared to 27.8%, 24%, and 11.1% for winter, spring, and summer, respectively. Similarly, the occurrence of *L. monocytogenes* was greater in the fall at 46.7% occurrence compared to 27.8% occurrence in the winter, 16% occurrence in the spring, and only 8.3% occurrence in the summer.

2.3.2 Pathogen characteristics

Of the 11 positive VTEC samples, eight distinct serotypes were observed, though isolates from two positive samples were not obtained for serotyping. These results are summarized in Table 2.4. There was no occurrence of the same serotype being observed at different sites; however, VTEC O69:H11 was observed on two consecutive sampling dates at Serpentine 2. Also of note was the recovery of O103:H2, a member of the "Big Six" non-O157 VTEC associated with human disease.

Table 2.4 – Serotype and virulence genes of verotoxigenic *E. coli* isolates collected from irrigation water at three sites each in the Sumas and Serpentine watersheds of the Lower Mainland of British Columbia

Serotype	Site	Month collected	*vt1*	*vt2*	*eaeA*	*hlyA*
O103:H2	Sumas 1	Feb 2015	+	-	+	+
O109:H5	Sumas 1	Mar 2015	+	-	-	-
O116:H25	Serpentine 2	May 2015	-	+	+	+
Unknown 1[a]	Serpentine 2	Sep 2015	-	+	+	+
O153:NM	Sumas 1	Nov 2015	-	+	+	-
O76:H19	Sumas 1	Feb 2016	+	-	-	+
O69:H11	Serpentine 2 Serpentine 2	Feb 2016 Mar 2016	+	-	+	+
Unknown 2[a]	Serpentine 2	Apr 2016	-	+	+	+
O34:H2	Sumas 3	May 2016	-	+	+	-
O22:H8	Serpentine 3	May 2016	+	-	-	+

[a]These strains were not isolated for serotyping

In order to further characterize the recovered isolates, the presence of four genes associated with virulence were investigated: the toxin producing genes *vt1* and *vt2*; the intimin producing gene *eaeA*, responsible for host-cell attachment; and *hlyA*, producing the pore-forming hemolysin toxin. The results are summarized in Table 2.4. There was an equal occurrence of both toxin genes, and no recovered isolate showed the presence of both toxin genes. The virulence genes *eaeA* and *hlyA* were each present in 8 of the eleven VTEC isolates recovered, with six isolates showing the presence of both.

Three distinct serotypes of *L. monocytogenes* were observed over the course of this study and are summarized in Table 2.5. No positive isolate was obtained for serotyping in two positive samples. The serotype 1/2a was the most common (15 occurrences) followed by 4b (10 occurrences), 1/2b (2 occurrences), and 4c (1 occurrence). Within the Serpentine watershed, serotype 1/2a and 4b were equally common with 10 recoveries each, along with one recovery each of 4c and 1/2b. This is in contrast with the Sumas watershed, where only serotype 1/2a was ever recovered except for one occurrence of 1/2b. Also of note is that on five separate sampling dates, two different serotypes were observed at the same site. This occurred four times for sample site Serpentine 2, and once for Serpentine 1.

Table 2.5 – Serotype and PFGE patterns of *L. monocytogenes* isolates collected from irrigation water at three sites each in the Sumas and Serpentine watersheds of the Lower Mainland of British Columbia

Serotype	PFGE-AscI	PFGE-ApaI	Site	Date
1/2a	LMACI.0172 (03-7760)	LMAAI.0217	Serpentine 2	Mar, 2015
	LMACI.0906	LMAAI.0909 (PNC_9089)	Sumas 3	Mar, 2015
	LMACI.0084 (BOM_4)	LMAAI.0531	Sumas 3	Jun, 2015
	LMACI.0216	LMAAI.1338	Serpentine 1	Sep, 2015
	LMACI.0041	LMAAI.0033	Serpentine 3	Sep, 2015
	LMACI.0011	LMAAI.0015	Serpentine 1	Sep, 2015
	LMACI.0118/221/681	LMAAI.0213	Serpentine 2	Oct, 2015
	LMACI.0543	LMAAI.0524	Serpentine 3	Oct, 2015
	LMACI.0195	LMAAI.0252	Sumas 2	Feb, 2016
	LMACI.0155	LMAAI.0165	Sumas 3	Feb, 2016
				Jul, 2016
	LMACI.0122 (02-2448)	LMAAI.0003	Serpentine 2	Feb, 2016
	LMACI.0738	LMAAI.1076	Serpentine 2	Mar, 2016
	LMACI.0044 (PNC_08-5757)/LMACI.0616 (ON_10PF0153)	LMAAI.0193/0818	Serpentine 2	Jul, 2016
	New	New	Serpentine 2	Feb, 2016
	New	New	Serpentine 2	Feb, 2016
1/2b	New	New	Sumas 1	Nov, 2015
	New	LMAAI.0548	Serpentine 2	Dec, 2015
4b	LMACI.0003 (PNC_08-2076)	LMAAI.0019	Serpentine 2	Mar, 2015
			Serpentine 1	Jan, 2016
	LMACI.0051	LMAAI.0048	Serpentine 2	Apr, 2015
				Sep, 2015
				Feb, 2016[a]
				Feb, 2016[a]
			Serpentine 1	Jan 2016
	LMACI.0071	LMAAI.0022	Serpentine 3	Nov, 2015
	LMACI.0822 (PNC_15-0050)	LMAAI.1234	Serpentine 2	Dec, 2015
	LMACI.0009 (03-7263)	LMAAI.0112	Serpentine 3	Apr, 2016
4c	LMACI.0051	LMAAI.0048	Serpentine 1	Sep, 2015

[a]These represent two different sampling dates within the same month

Within the 25 water samples found positive for *L. monocytogenes*, there were 19 unique PFGE fingerprints observed. Three sample sites showed recurring PFGE fingerprints on multiple sampling dates. The same PFGE fingerprint was observed twice at Serpentine 1, four times at Serpentine 2, (the same PFGE fingerprint all four times), and twice at Sumas 3. Furthermore, two PFGE fingerprints were observed at two different sampling sites within the Serpentine watershed: Serpentine 1 and Serpentine 2. Of particular interest, one of these multi-site PFGE fingerprints was also a recurring fingerprint, being recovered twice at Serpentine 1, and four times at Serpentine 2.

Of the six *Salmonella* isolates recovered, four distinct serotypes were observed, summarized in Table 2.6. The most common, and only recurring serotype was *S.* Enteritidis, which was recovered three separate times. Interestingly, these recoveries were at two different sites; however, these two sites are in close proximity, so it is likely that this may have come from a single source.

Table 2.6 – Serotypes of *Salmonella* isolates collected from irrigation water at three sites each in the Sumas and Serpentine watersheds of the Lower Mainland of British Columbia

Serotype	Site	Month
Typhimurium	Sumas 1	Apr 2015
Enteritidis	Sumas 4	Sep 2015
	Sumas 1	Feb 2016
	Sumas 4	Aug 2016
Daytona	Serpentine 1	Sep 2015
Heidelberg	Sumas 1	Feb 2016

2.4 Discussion

Foodborne pathogens in water used to irrigate food crops have the potential to contaminate pre-harvest produce and lead to foodborne illness in consumers (Steele and Odumeru, 2004); therefore, it is important to understand the occurrence, prevalence, and spatial/ temporal spread of these pathogens in the water used for local produce production. The objective of this portion of the study was to investigate the occurrence of three foodborne pathogens from the irrigation waters used in the Lower Mainland of British Columbia: VTEC, *Salmonella*, and *L. monocytogenes*.

The most commonly observed pathogen was *L. monocytogenes*, which was expected due to its relative ubiquity in the natural environment (Vivant et al., 2013), followed by VTEC then *Salmonella*. This trend differs from two previous studies which looked at the occurrence of these three specific pathogens in surface waters from agricultural areas: one from New York State (Strawn et al., 2013a), and one from California (Cooley et al., 2014). Strawn et al. (2013a) found *L. monocytogenes* to be more prevalent than the other two pathogens, but a higher occurrence of *Salmonella* compared to VTEC was also reported. Similarly, Cooley et al. (2014) also reported *Salmonella* to occur more often than VTEC, but also more often than *L. monocytogenes*. The reduced recovery of VTEC in these two studies can partially be explained by differences in detection methods, as discussed below. The observed occurrence of *L. monocytogenes* by Cooley et al. was not unusual, but the recovery of *Salmonella* (65%) was higher than in other studies. This was likely due to a higher volume of water sampled, as discussed below.

2.4.1 VTEC occurrence

2.4.1.1 Environmental occurrence of VTEC

The overall recovery of VTEC during the course of this experiment was 4.93%; however, recovery ranged from 3.9% to 6.4% between watersheds. At the site level, three of seven sites showed consistently no occurrence of VTEC. Of the remaining four sites, recovery ranged from 2.9% to 14.7%. Few other studies in Canada have investigated the occurrence of non-O157 VTEC, but one investigation of an Ontario watershed reported a recovery of 32% for all VTEC (Johnson et al., 2014). Similarly, a previous project investigating surface waters in the Lower Mainland found the occurrence for VTEC to be in the range of 20.3% (Nadya et al., 2016). Both of these studies reported higher levels of VTEC recovery than found during this project. Similarly, a study in California observed an 8% occurrence for VTEC O157, and 11% occurrence for non-O157 VTEC (Cooley et al., 2014), and in New York State, VTEC occurrence in surface water was found to be 2.3% (Strawn et al., 2013a). Previous surface water studies in Canada focused on VTEC O157, indicated that the relative occurrence ranged from 1% to 3% (Edge et al., 2012; Gannon et al., 2004; Johnson et al., 2003; Jokinen et al., 2011). Of particular note is that, unlike any of these previous studies, no VTEC O157 was recovered during this project.

Possible reasons for the range of differences in VTEC recovery may be due to differences in sampling volume or method. For example, both Johnson et al. (2014) and Nadya et al. (2016) filtered 70-100 ml of water in duplicate samples for the VT-IB assay. For the sake of consistency with the detection and isolation methods used for *Salmonella* and *L. monocytogenes*, as well as filter performance, a smaller volume (25 ml) of water from duplicate samples was chosen in this study. The relative recovery in the present study was on the order of one quarter that of Nadya et

al., suggesting similar results were obtained considering the similar area of sampling. Alternatively, Cooley et al. (2014) made use of a submersed Moore swab for 24 hours, which does not allow comparison for sample volume. Instead, it is assumed that large quantities of water are filtered through the swab over the 24-hour period, implying the potential for a significantly higher recovery. However, the overall recovery, only about 19%, was lower than expected with such a high volume. Similarly, Strawn et al. (2013) also tested a larger volume of water (250 ml) and found a lower overall recovery for VTEC.

The lower relative recovery by Cooley et al. (2014) and Strawn et al. (2013) compared to relative sample volumes may have also stemmed from the difference in detection method for VTEC. Both groups used sample enrichment in TSB prior to detection of *vt* genes by real-time PCR. Previous work by Johnson et al. (2014) showed that increased recovery of VTEC, from 7.5% to 32%, was observed by switching to smaller volumes of water with no enrichment. It could be suggested that this is due to other microorganisms out-competing VTEC during the non-selective enrichment. If the differences in recovery are indeed due to this updated detection procedure, then the occurrence of VTEC in agricultural waters may be much higher than previously believed.

2.4.1.2 Spatial and temporal distribution of VTEC

While no significant difference was observed in the occurrence of VTEC between watersheds, there was a clear site dependence within watersheds. In both watersheds, all but one occurrence of VTEC was from a single site. Furthermore, in the Sumas watershed, the two sites where VTEC was recovered are in close proximity to each other and share a common source, meaning these two sites are likely not independent of each other. This site-specific trend is consistent with previous

studies (Cooley et al., 2014; Johnson et al., 2014; Nadya et al., 2016) and implies that there is a geographical dependence on the likelihood of finding VTEC, such as an increase in occurrence in agricultural areas compared to control sites located away from agriculture and waste areas (Edge et al., 2012). These differences may be attributed to proximity to a host source, such as livestock (Chapter 4) or the application of manure to neighbouring fields.

No significant difference in the occurrence of VTEC across seasons was observed, however recoveries were noticeably lower during the summer months at only 1.2% compared to 5.7%, 7%, and 7.8% for fall, winter, and spring, respectively. The seasonal effect on VTEC occurrence has varied between different studies. A study of VTEC O157 occurrence in Southern Alberta found that peak isolation happened in July, and lower recoveries were observed in the spring and fall (Gannon et al., 2004). Conversely, Cooley et al. (2014) found higher occurrence of VTEC in the winter and spring, compared to the summer and fall in California, and Nadya et al. (2016) observed higher recoveries for VTEC during the winter than in the other three seasons in the Lower Mainland of British Columbia. No seasonal differences were observed by Johnson et al. (2014) in Southern Ontario. Interestingly, it is accepted that the shedding of VTEC O157 by cattle, a common reservoir for this bacterium, is greater during the summer months (Hancock et al., 2001), though a more recent study found the occurrence of the VTEC O157 in beef cattle feces reported no seasonal variation (Alam and Zurek, 2006). These different observations may imply that seasonality is not the cause of the observed differences and perhaps is confounded with a more indicative factor such as changes in agricultural practices or weather patterns during these seasons. For instance, cooler months on the West Coast of Canada are often associated with higher rates of

precipitation (Chapter 4), and during the summer months, damning of the irrigation ditches results in a stopping or reducing of water flow.

2.4.1.3 Serotype and pathotype of recovered VTEC

Of the VTEC recovered during this study, no recurring serotypes were observed, save for O69:H11, which was recovered twice at the same site (Serpentine 2) on successive samplings. Interestingly, two isolates recovered from the sample site Serpentine 2 on two different occasions showed the same genotypic virulence pattern (*i.e.*, *vt2*, *eaeA*, and *hlyA*) as a VTEC O116:H25 isolated from Serpentine 2 earlier in the study, but were not retained for serotyping. This is disappointing since we may have found evidence of the recurrence of this serotype. No other recovered serotype showed this same virulence factor pattern. The same virulence factors were shared between an O103:H2 isolate and an O69:H11 isolate, however, suggesting that the virulence genotype may be shared across serotype, and is insufficient to link untyped isolates.

No occurrence of VTEC O157:H7 was observed during this study, which was interesting since every other recent survey of water in Canada has shown the presence of VTEC O157:H7, although always at relatively low levels between 1% and 3% (Edge et al., 2012; Gannon et al., 2004; Johnson et al., 2003; Jokinen et al., 2010). Of the serotypes that were observed herein, only one, O103:H2, is a member of the "Big 6" non-O157 serotypes associated with a high proportion of severe human illness (Croxen et al., 2013). VTEC O103:H2 has been isolated from human cases of illness both in North America (Karama et al., 2008a), and Europe (Mariani-Kurkdjian et al., 1993; Prager et al., 2002), and was responsible for an outbreak at a nursery school in Japan (Muraoka et al., 2007). Furthermore, serotype O103 is commonly associated with illness in British

Columbia (Wang et al., 2013). Other disease associated isolates include O76:H19 which was responsible for a household outbreak of bloody diarrhea in Spain (Sanchez et al., 2014), and O22:H8 which has been associated with bloody diarrhea in Germany (Bielaszewska et al., 2006) and isolated from patients exhibiting symptoms of HUS (Constantiniu, 2002). Members of serogroup O69 and O153 have also been isolated with patients exhibiting diarrhea in India (Gazal et al., 2014)

Of the serotypes recovered during this study, five had previously been isolated from surface waters of the Lower Mainland: O22:H8, O69:H11, O76:H19, O103.H2, and O116:H25 (Nadya et al., 2016). Other serotypes not previously recovered in this area were O34:H2, O109:H5, and O153:NM. Common serotypes between studies, however, did not guarantee identical virulence genotypes.

Genotype analysis of VTEC showed an even proportion of isolates possessing *vt1* and *vt2*, with no recovered isolates possessing both toxin producing genes. This is in contrast with the results of Nadya et al. (2016), where there was a 50% greater number of *vt1* possessing isolates compared to *vt2*. Nadya et al. also observed a 10% occurrence of isolates which had both variants of the toxin producing gene. The intimin gene, *eaeA*, was observed in seven of the nine observed serotypes, and was more commonly associated with the *vt2* variant, which is of concern since the presence of these two genes has been most associated with EHEC symptoms, (*i.e.*, enterohemorrhagic colitis, hemolytic-uremic syndrome) in humans (Boerlin et al., 1999). Observed serotypes with *vt2* and *eaeA* included O116:H25, O153:NM, O34:H2, and both of the un-serotyped VTEC isolates. On the other hand, *hlyA* was also present in seven of the observed

serotypes, but was more commonly associated with *vt1* which is similar to observations by Nadya et al. No other work has been done to investigate virulence factors of VTEC recovered from surface waters, but a recent survey of VTEC isolated from produce by the US FDA found that *vt2* was over five times more common than *vt1* in those isolates, with the presence of *eaeA* and *hlyA* being 9% and 61%, respectively (Feng and Reddy, 2013).

Similar VTEC serotypes recovered from a previous study in the same area did not necessarily share the same virulence factors. The O116:H25 isolates recovered by Nadya et al. (2016) in the nearby Nicomekl watershed were positive for *vt2*, but not *eaeA*, where an O116:H25 possessed both of these virulence factors in the present study. Furthermore, O22:H8 isolates recovered by Nadya et al. were only positive for *vt2* and not the other three virulence factors, whereas the O22:H8 isolate recovered in this study was positive for *vt1*, and *hlyA*. This discrepancy between virulence factors and serotype begs the question as to whether serotype or genotype is a better predictor of human pathogenicity, since serotype is considered to be a reasonable predictor of pathotype (Boerlin et al., 1999). The investigation of genetic markers for molecular risk assessment of potential EHEC strains has shown promise, and that the presence of *eae* in combination with a variety of other virulence determinants is a strong indicator for severe human pathogenicity (Bugarel et al., 2010). Care should be taken, however, as a major outbreak in Germany and France was the result of serotype O104:H4 which acquired the *vt2* gene, but did not have any other virulence factors typically associated with VTEC or EHEC. Instead, this strain was a member of the enteroaggregative *E. coli* and possessed completely different genetic mechanisms of intestinal colonization (Navarro-Garcia, 2014). The EHEC O104:H4 outbreak also highlighted the transient nature of the Shiga-toxin producing prophage, and reminded us that new VTEC

serotypes are likely to develop in the future, and that targeting of virulence factors may be the best way to monitor for VTEC/EHEC occurrence in the environment compared to serotype specific methods such as immunomagnetic separation.

2.4.2 *Salmonella* occurrence

2.4.2.1 Environmental occurrence of *Salmonella*

The overall recovery of *Salmonella* across all sites during this study was 2.69%, but ranged from 7.7% to 11.1% across the three sites that were positive for this pathogen at least once. Four sample sites, two in each watershed, showed no occurrence of *Salmonella*. These recoveries are lower than those from a previous cross-Canada study, which included the Sumas river, where the annual mean occurrence for *Salmonella* was 11% (Edge et al., 2012), and another study in the Salmon River watershed of British Columbia where a mean occurrence of 13% was observed (Jokinen et al., 2010), though these two studies both made use of 500 ml samples of water, much higher than the 25 ml sample volume used in this study. Two studies in Southern Alberta observed the relative occurrence of *Salmonella* in surface water to be 10.3% (Gannon et al., 2004) and 6.2% (Johnson et al., 2003), respectively, but both studies observed differences in recoveries between years. Both of these studies used 90 ml water samples for analysis, while a later study in the same watershed used 500 ml water samples and observed a relative occurrence of 8.5% for *Salmonella* in water samples (Jokinen et al., 2011). Similarly, two studies in New York State found the occurrence of *Salmonella* in agricultural waters ranged between 9% and 11% (Strawn et al., 2013a, 2013b). Interestingly, a study of agricultural water in California found a relative occurrence of *Salmonella* to be 65%; much higher than other studies (Cooley et al., 2014). It should be noted, however, that 24-hour Moore swabs were used for sampling, meaning an indeterminately large volume of water

could have been "filtered" through the swab. The authors also acknowledged that the swab will also capture sediment particles, where *Salmonella* are more common.

2.4.2.2 Spatial and temporal occurrence of *Salmonella*

No significant difference in occurrence for *Salmonella* was observed between watersheds, though only one occurrence was observed in the Serpentine watershed compared to five occurrences in the Sumas. Site dependence within watersheds was observed, but the difference was only significant for the Sumas watershed. It should also be noted that the two sites in the Sumas watershed where *Salmonella* was isolated were in close proximity and could be considered as a single site. This suggests that site dependence is strong within the Sumas watershed. Previous studies have also shown differences in *Salmonella* occurrence across various sampling sites. Cooley et al. (2014) observed specific "hotspots" where recovery of *Salmonella* was as high 96%, and agricultural sites have been observed to show significantly higher levels of *Salmonella* compared to sites not in proximity to agricultural activity (Edge et al., 2012), which implies that proximity to a contamination source such as livestock likely plays a role in the probability of occurrence. A significantly higher recovery of *Salmonella* from smaller streams, rather than larger streams in agricultural areas has also been observed (Edge et al., 2012).

Due to the limited recovery of *Salmonella* in this study, no seasonal effect on occurrence could be observed. Previous studies have documented a significant increase in the occurrence of *Salmonella* in the spring compared to the summer (Jokinen et al., 2010) while others have seen an increase in recovery during the summer time (Cooley et al., 2014; Gannon et al., 2004). This lack of consistent seasonal trend may suggest that apparent seasonal differences may be due to

confounding factors within the sampling area, such as changes in precipitation or farming practices (*i.e.*, manure application) during that time.

2.4.2.3 Serotype analysis of recovered *Salmonella*

Over the course of this study, four different *Salmonella* serotypes were recovered. The only one recovered more than once was *S.* Enteritidis, which was observed at two sites in the Sumas watershed. It should also be noted that these two sites are in close proximity and connected, suggesting the possibility that this recurrence may be from a single source. *S.* Enteritidis has been implicated previously in outbreaks involving mung bean sprouts (Honish and Nguyen, 2001), and alfalfa sprouts (Centers for Disease Control, 2016c). *S.* Typhimurium was also recovered, which as been implicated in outbreaks involving tomatoes (Centers for Disease Control, 2007) and a recent outbreak associated with cantaloupe (Centers for Disease Control, 2016c). Also recovered was *S.* Heidelberg, which has not been implicated in any produce related outbreaks, but is commonly associated with poultry and considered to be one of the top serovars associated with human disease, and often leads to invasive infection (Foley et al., 2011). Finally, *S.* Daytona has not been implicated in any foodborne outbreaks, to the best of the author's knowledge. *S.* Enteritidis, *S.* Typhimurium, and *S.* Heidelberg were also recovered from surface water in Southern Alberta; however, these serotypes were relatively rare compared to *S.* Rubislaw which made up 52% of the recovered isolates (Gannon et al., 2004). A previous study in the Salmon River of British Columbia found *S.* Typhimurium to be the most common serotype observed (Jokinen et al., 2010).

2.4.3 *Listeria monocytogenes* occurrence

2.4.3.1 Environmental occurrence of *Listeria monocytogenes*

The overall occurrence for *L. monocytogenes* in this study was 11.2%, but differed significantly between the two watersheds, ranging from 4.7% to 20.2%. The relative occurrence was similar to that observed during a study from Southern Ontario where *L. monocytogenes* was recovered from 10% of surface water samples (Lyautey et al., 2007b). It should be noted, however, that this previous study used the same detection method as used in our study, but with a greater volume of water sampled, meaning that our observations may imply a higher concentration of *L. monocytogenes* in the Lower Mainland water. A higher occurrence (28%-30%) of the pathogen in agricultural waters in New York State (Strawn et al., 2013a, 2013b), and higher still in agricultural waters in California with an occurrence of 43% (Cooley et al., 2014). Both of these American studies made use of larger volumes of water sampled, which may partly explain the disparity in observed results.

2.4.3.2 Spatial and temporal occurrence of *Listeria monocytogenes*

There was a significant difference in *L. monocytogenes* occurrence between watersheds, with occurrence in the Serpentine watershed being four times greater than that of the Sumas watershed. Differences between watersheds have also been reported in California, as well as the presence of "hotspots" where recovery for the pathogen was as high as 96% (Cooley et al., 2014). These differences in occurrence and the presence of "hotspots" suggests geographical or environmental factors can have an effect of the presence and persistence of *L. monocytogenes*. Indeed, previous work has observed *L. monocytogenes* to be more frequently recovered from uncultivated compared to cultivated agricultural soils (Vivant et al., 2013).

Seasonal dependence was also observed for *L. monocytogenes* occurrence, with fall and winter showing higher recovery compared to both spring and summer. Winter and spring were found to show a higher occurrence of *L. monocytogenes* in California (Cooley et al., 2014), suggesting a trend that at least the cooler months show greater occurrence of *L. monocytogenes* compared to the heat of the summer. Temperature may be a driving force of this phenomenon, as survival of *L. monocytogenes* in a water environment has been shown to double at 4°C when compared to 20°C (Budzińska et al., 2012), meaning that any contamination of the water will persist for longer periods during cooler months. Unfortunately, many other studies did not investigate occurrence during winter due to below freezing temperatures in the study area.

2.4.3.3 Serotyping and PFGE analysis of *L. monocytogenes* isolates

Over the course of this study, the recovered isolates of *L. monocytogenes* were overwhelmingly members of the serotypes 1/2a and 4b except for one isolate deemed to be serotype 4c and one isolate 1/2b. This is of concern since the serotypes 1/2a, 1/2b, and 4b are responsible for the majority of human listeriosis cases (McLauchlin et al., 2004). Most recently, a large multi-state outbreak related to cantaloupes was caused by strains from serotypes 1/2a and 1/2b (McCollum et al., 2013), and outbreaks related to caramel apples (Angelo et al., 2017) and packaged salads (Chen et al., 2017) were the result of strains from serotype 4b. To the best of the author's knowledge, no outbreaks associated with serotype 4c have been reported.

Serotype 1/2a was the most common serotype observed, representing 54% of the recovered isolates. This increased representation of 1/2a, however, is directly tied to the Sumas watershed

where this serotype was the only one recovered. In the Serpentine watershed, equal recoveries of 1/2a and 4b were observed at all three sampling sites. Previous studies in Canada only used PCR to identify serogroups, but found that the serogroup consisting of 1/2a and 3a represented 49% and 68% of recovered isolates in watersheds in Ontario (Lyautey et al., 2007b) and Nova Scotia (Stea et al., 2015), respectively. Similarly, in Austria, two thirds of isolates from soil and water were found to cluster into lineage II, which includes 1/2a, compared to only one third from lineage I, which includes 4b and 1/2b (Linke et al., 2014). In contrast, surveys in California found serotype 4b isolates to make up >85% of recovered *L. monocytogenes* compared to 1/2a which only represented 7-8% of recovered isolates (Cooley et al., 2014; Gorski et al., 2014). It has been suggested that isolation methods may be biased, with lineage II isolates outcompeting lineage I isolates in during enrichment (Bruhn et al., 2005); however in this study we saw isolation of both 1/2a and 4b serotypes from single samples suggesting both were enriched successfully. Instead, these differences suggest that geography or the local environment may affect the occurrence of various serotypes. For instance, serotype 1/2a has been observed to be more common overall within the natural environment (Schaffter et al., 2004), whereas serotype 4b has been more commonly recovered from the feces of wild animals (Orsi et al., 2011) and cattle (Schaffter et al., 2004). Therefore, the occurrence of serotype 4b in the Serpentine watershed could be the result of an animal reservoir in the area with the observed 1/2a isolates spread across the natural environment.

Persistence of certain strains was also observed using PFGE analysis of the recovered *L. monocytogenes* isolates. Multiple recoveries of isolates showing the same PFGE pattern were observed at three sites. Isolates of serotype 1/2a showing the same PFGE pattern were recovered

twice at Sumas 3, five months apart, suggesting a potential upstream point source for the bacterium. More importantly, however, isolates of serotype 4b showing a single identical PFGE pattern were recovered four times from Serpentine 2 and once from Serpentine 1 over an 11-month period, suggesting not only persistence but the potential for a mobile source of this bacterium (*i.e.*, wildlife) since neither site is upstream of the other. This same PFGE fingerprint was observed a second time at Serpentine 1, but interestingly was serotyped as 4c, a member of a completely different lineage (lineage III). Lineage III is considered a related group to lineage I, and atypical isolates for 4b can be members of this group (Nightingale et al., 2005). Therefore, it is possible that all these isolates are related, but that the five recurring 4b isolates are actually members of lineage III rather than lineage I. Lineage III isolates are overrepresented in animal cases of listeriosis compared to human cases (Orsi et al., 2011). One other PFGE fingerprint relating to serotype 4b was also shared between Serpentine 1 and Serpentine 2, but only occurred once at each site. Taken together, these data suggest that wild animals may be a reservoir for transporting *L. monocytogenes* within the Serpentine watershed, but it remains to be seen whether this poses a risk to human health. Whole genome sequencing of these isolates in the future may provide more insight into their genetic relatedness.

2.4.4 Effect of sampling and detection methodologies

At least some of the variation observed between this study and other similar studies, both in Canada and the United States, can be attributed to differences in either sampling or pathogen detection methodology. Already discussed above is the increased recovery of VTEC without the use of pre-enrichment (Johnson et al., 2014). It remains to be seen if this same trend will hold for other

pathogens, and will require the development of more sensitive assays to detect these pathogens at relatively low concentrations within the water environment.

Another major difference is in the volume of water sampled. Including this study, sample volumes for compared studies range from 25 ml as high as 500 ml, and that does not include the use of Moore swabs which allow 24 hours of water flow to be measured. Since these methods ultimately lead to a qualitative result, the volume of water tested will have a significant effect on the likelihood of observing the presence of a pathogen in low concentrations. One solution to this is to convert relative recovery of pathogens into a ratio of occurrence per volume of sample. This method was used by Edge et al. (2012) to compare the relative occurrence of pathogens in waters collected from agricultural areas to water collected from non-agricultural areas when there was a large discrepancy in the number of sampling sites within each category. The relative occurrence per volume method, however, still does not account for the qualitative nature of pathogen detection, where two positive surface water samples may have vastly different concentrations of the pathogen of interest. This also makes it difficult to define the level of risk. At what sample volume does the presence of foodborne pathogens in the irrigation water become a hazard? In the future, this question will need to be answered to not unnecessarily put too high a burden on growers as our ability to detect pathogens becomes more and more sensitive. Therefore, consistent and sensitive pathogen detection methods should be developed and agreed upon in order to better understand the spread of pathogens world-wide, as well as to quantitatively estimate the risks associated with certain waters.

2.5 Conclusion

The foodborne pathogens VTEC, *L. monocytogenes*, and *Salmonella* are clearly present in waters used for irrigation in the Lower Mainland of British Columbia, with *L. monocytogenes* being the most common, followed by VTEC and *Salmonella*. Furthermore, serotypes previously shown to cause both illnesses and outbreaks were recovered, suggesting a public health risk may exist with using these waters for irrigation of fresh produce. Recovery of pathogens was higher during the cooler months, and location specific trends were observed for their occurrence. These observed trends suggest the potential for determining physical and environmental factors which may predict the presence of these pathogenic bacteria, allowing growers to assess water quality before irrigating their crops.

Chapter 3: Generic *Escherichia coli* and Total Fecal Coliforms as Indicator Organisms for the Presence of Verotoxigenic *Escherichia coli*, *Salmonella*, and *Listeria monocytogenes* in Irrigation Water

3.1 Introduction

Water quality in agriculture is important for food safety, but testing for individual pathogens is both expensive and time consuming, making pathogen testing an unrealistic expectation of growers and regulators. Instead, easily detected and enumerated indicators are often used to assess the risk of the presence of foodborne pathogens. Since these pathogens are primarily of fecal origin, indicators are bacteria associated with the intestines of warm blooded animals such as fecal coliforms, *Enterococcus,* and *Escherichia coli* (Pachepsky et al., 2014). Qualities that make a suitable indicator organism include easy and relatively quick detection and enumeration, a strong correlation with pathogen occurrence, and the same or slightly longer survival in the water environment than the pathogens they are used to predict (Uyttendaele et al., 2015).

In Canada, recommended water quality guidelines for good agricultural practice (GAP) suggest surface water testing three times per season, and that less than 1000 total coliforms and 100 FC per 100 ml of water should be present (CanAgPlus, 2015; Steele and Odumeru, 2004). Some provinces in Canada have implemented their own recommendations, and the British Columbia Ministry of the Environment has set a guideline of less than 200 FC, and less than 77 *E. coli* in 100 ml of all water used for irrigation of produce (Government of British Columbia, n.d.). Recent legislation in the United States under the Food Safety Modernization Act requires that all surface

water used for irrigation of fresh produce is based solely on levels of *E. coli* and requires a geometric mean of 126 CFU per 100 ml across 20 consecutive samples, with no sample exceeding 410 CFU per 100 ml. Furthermore, any water used to irrigate spouts or used during or after harvest must contain zero *E. coli,* and no untreated surface water can be used for either activity (U.S. Food and Drug Administration, 2015).

Recent studies have shown that many correlations between fecal indicators and pathogen occurrence are either weak, or non-existent (Pachepsky et al., 2014), and pathogens have been observed even when indicator levels are low (Jokinen et al., 2012). Furthermore, not all foodborne pathogens are necessarily of fecal origin (*e.g.*, *Listeria monocytogenes*). Also of interest, different methods exist for quantifying indicator organisms, but little work has been done to compare how methods may differ in their ability to predict pathogen presence. The objective of this part of the project was therefore to investigate the suitability of two different methods of fecal coliform and *E. coli* enumeration to predict the occurrence of three foodborne pathogens in the irrigation waters around the Lower Mainland of British Columbia.

3.2 Materials and methods

3.2.1 Enumeration of generic *E. coli* and total fecal coliforms

Generic *E. coli* and total fecal coliforms (TFC) were enumerated in each sample using two distinct methods: 1) membrane filtration followed by plating on selective differential media, and 2) 3M™ Petrifilm™ *E. coli*/Coliform Count Plates.

3.2.1.1 Membrane filtration method

A 25 ml aliquot of each water sample was vacuum filtered through an 85 mm, 0.45 μm pore size GN-6 Metricel® membrane filter disc (Pall Laboratory, St. Laurent, PQ), which was subsequently transferred to m-FC agar medium (Hardy Diagnostics, Santa Maria, CA) containing 1% rosolic acid (Hach Canada Ltd., London, ON) and incubated at 44.5 ± 1°C for 18-24 hours (Myers et al., 2014). All resulting colonies with a blue colour were counted as fecal coliforms. The filter membranes were then transferred from the m-FC media to nutrient agar medium containing 4-methylumbelliferyl β-D-glucuronide (NA-MUG; Hardy Diagnostics) and incubated at 37°C for 4-6 hours. Colonies producing fluorescence under long wave UV light (365 nm) were assumed to be generic *E. coli* (Myers et al., 2014).

3.2.1.2 Enumeration on 3M™ Petrifilm™

A 1 ml aliquot of each water sample was applied to an *E. coli*/Coliform Count Petrifilm™ (3M Science, London, ON) following the manufacturers directions, then subsequently incubated at 44.5 ± 1°C for 18-24 hours. All blue and red colonies showing evidence of gas production were assumed to be fecal coliforms, and all gas-producing blue colonies *E. coli*.

3.2.2 Pathogen occurrence

The occurrence of verotoxigenic *E. coli* (VTEC), *Salmonella*, and *L. monocytogenes* were measured as described and discussed in Chapter 2. Only samples collected during or after June 2014 were used as that is when consistent enumeration of TFC and generic *E. coli* was completed using both methods. Samples from sample site Sumas 4 were not included in the point-biserial correlations due to insufficient sampling and its close proximity to Sumas 1.

3.2.3 Statistical analysis

All statistical analysis was conducted using R (version 3.3.2; R Core Development Team, Vienna, Austria; http://www.R-project.org). Correlations between indicator concentrations and pathogens were calculated using the point-biserial method, which correlates a continuous variable with a dichotomous variable (Gu et al., 2013), using the ltm software package (http://www.jstatsoft.org/v17/io5/). Regression trees were constructed using the Random Effect-Estimation Method (RE-EM) with the REEMtree package (version 0.90.3). The sample sites were set as the random variable, and the pathogen occurrence and indicator concentration were chosen as the dependent and independent variables, respectively. This method was used since the data was longitudinal and the results from repeated sampling at the same sites were not independent (Jones et al., 2014). Figures were produced using the ggplot2 package (http://ggplot2.org) and edited using Inkscape (version 0.91; http://inkscape.org).

3.3 Results

3.3.1 Comparison of methods and indicator concentrations

Data collected using the two different enumeration methods were compared to determine if they provide similar estimates of TFC and *E. coli* in water samples. The resulting slopes and correlations are shown in Figure 3.1. A significant correlation was observed between both methods for both TFC ($r = 0.60$; $n = 190$; $p < 0.001$) and generic *E. coli* ($r = 0.77$; $n = 176$; $p < 0.001$); however, there was no consensus between methods on the values as the slopes deviated from the expected slope of 1.00 (slope = 0.28 and slope = 0.49 for TFC and generic *E. coli*,

respectively). The resulting slopes of less than one indicated that higher concentrations of the indicators were observed when using the Petrifilm™ method over the membrane filtration method.

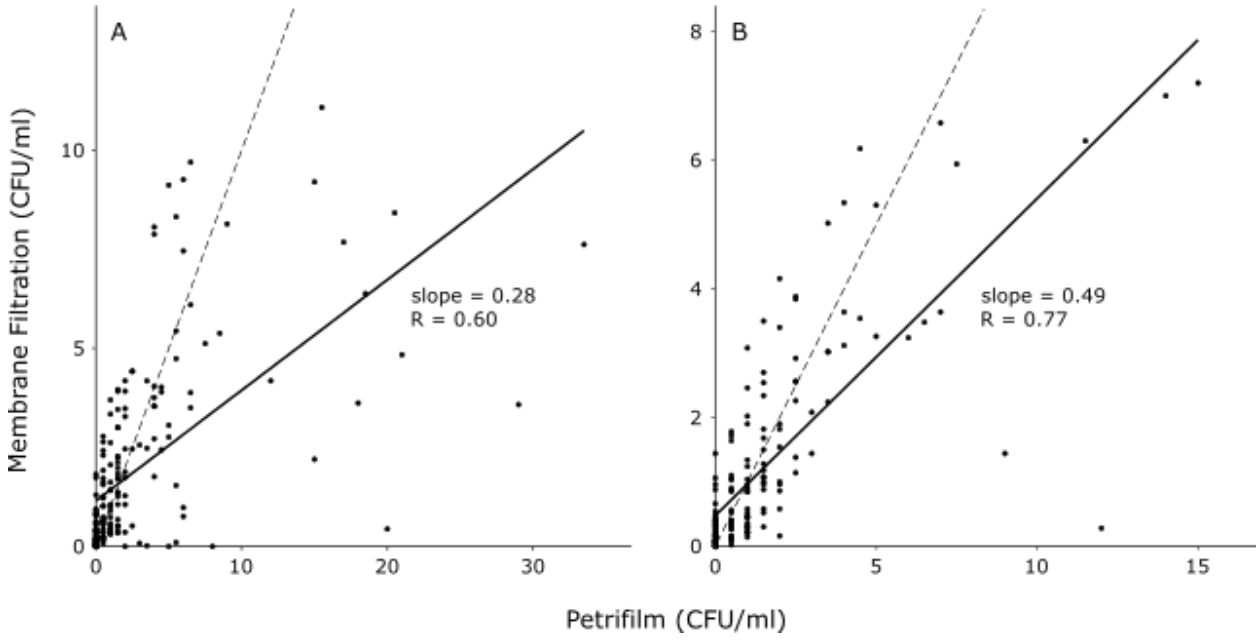

Figure 3.1 – Correlation between the membrane filtration and petrifilm methods for enumeration of total fecal coliforms (A) and generic *Escherichia coli* (B). The slope of the resulting trendlines (solid line) is compared to the expected slope of 1.0 (dashed line).

The observed concentrations of both indicator bacteria were also compared to determine if the concentrations were related. The concentrations of TFC and generic *E. coli* were strongly correlated when measured using the membrane filtration method ($r = 0.882$; $n = 176$; $p < 0.001$), but slightly less so than when measured using the Petrifilm™ method ($r = 0.710$; $n = 221$; $p < 0.001$). Moreover, all individual sites which showed a significant correlation with generic *E. coli* by membrane filtration were also significantly correlated with TFC by membrane filtration, but not necessarily the other way around (Table 3.1).

Table 3.1 – Point-biserial correlation coefficients (r_{pb}) of total fecal coliforms and generic *Escherichia coli* with the occurrence of verotoxigenic *E. coli* (VTEC), *Salmonella*, and *Listeria monocytogenes* in water samples collected from three sites each in two watersheds of the Lower Mainland of British Columbia.

	Total Fecal Coliforms		Generic *E. coli*	
	Membrane Filtration	3M™ Petrifilm™	Membrane Filtration	3M™ Petrifilm™
VTEC				
Sumas Watershed	0.312***	0.084	0.309**	0.132
Sumas 1	0.573**	0.159	0.529**	0.442*
Sumas 2	-	-	-	-
Sumas 3	-0.074	-0.120	-0.087	-0.063
Serp.Watershed	0.242*	0.166	0.318**	0.012
Serpentine 1	-	-	-	-
Serpentine 2	0.582***	0.461*	0.670***	0.425*
Serpentine 3	-0.227	-0.119	-0.211	-0.077
Both Watersheds	0.273***	0.266***	0.320***	0.092
Salmonella				
Sumas Watershed	0.291**	0.122	0.289**	0.089
Sumas 1	0.481**	0.018	0.462*	0.175
Sumas 2	-	-	-	-
Sumas 3	-	-	-	-
Serp.Watershed	0.025	0.146	0.050	-0.027
Serpentine 1	0.089	0.225	0.174	-0.035
Serpentine 2	-	-	-	-
Serpentine 3	-	-	-	-
Both Watersheds	0.097	0.042	0.099	-0.017
L. monocytogenes				
Sumas Watershed	0.169	0.049	0.144	0.118
Sumas 1	0.102	0.135	0.057	0.270
Sumas 2	0.620***	0.041	0.616***	0.143
Sumas 3	0.280	-0.024	0.323	0.060
Serp.Watershed	0.363***	0.072	0.342**	0.00
Serpentine 1	0.440*	0.170	0.305	-0.059
Serpentine 2	0.213	-0.024	0.357	0.374
Serpentine 3	0.582**	0.075	0.465*	0.005
Both Watersheds	0.389***	0.132*	0.374***	0.061
Any pathogen				
Sumas Watershed	0.297**	0.101	0.277**	0.125
Sumas 1	0.462*	0.096	0.419*	0.308
Sumas 2	0.620***	0.041	0.616***	0.143
Sumas 3	0.189	-0.092	0.217	0.013
Serp.Watershed	0.419***	0.137	0.388***	-0.013
Serpentine 1	0.440*	0.170	0.305	-0.059
Serpentine 2	0.468*	0.241	0.549**	0.393*
Serpentine 3	0.422*	0.011	0.323	-0.033
Both Watersheds	0.431***	0.234***	0.408***	0.074

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

3.3.2 Predictive ability of total fecal coliforms and generic *E. coli*

In order to determine if either method for either indicator was a reliable predictor of pathogen presence, point-biserial correlations (r_{pb}) were calculated for each indicator/method combination with respect to each pathogen. These correlations are summarized in Table 3.1. The occurrence of any of the three pathogens was significantly correlated with both TFC ($r_{pb} = 0.431$; $n = 190$; $p < 0.001$), and generic *E. coli* ($r_{pb} = 0.408$; $n = 176$; $p < 0.001$) when measured using the membrane filtration method, but only with TFC ($r_{pb} = 0.234$; $n = 221$; $p < 0.001$) when measured using the Petrifilm™ method; moreover, the correlation coefficient was much lower for the Petrifilm™ method.

For individual pathogens, TFC were significantly correlated with the occurrence of VTEC using both the membrane filtration method ($r_{pb} = 0.273$; $n = 190$; $p < 0.001$) and by the Petrifilm™ method ($r_{pb} = 0.266$; $n = 221$; $p < 0.001$). Generic *E. coli* were only significantly correlated when measured using the membrane filtration method ($r_{pb} = 0.320$; $n = 176$; $p < 0.001$). The same pattern was observed for *L. monocytogenes* where the occurrence was significantly correlated with TFC by both the membrane filtration method ($r_{pb} = 0.389$; $n = 190$; $p < 0.001$) and by Petrifilm™ ($r_{pb} = 0.132$; $n = 221$; $p = 0.049$), but only significantly correlated with generic *E. coli* by the membrane filtration method ($r_{pb} = 0.374$; $n = 176$; $p < 0.001$). No significant correlations were observed for either indicator by either method for the occurrence of *Salmonella*.

Within individual watersheds, only the membrane filtration method showed any significant correlations, but these differed between the two watersheds. Both TFC and generic *E. coli* were more strongly correlated with the occurrence of any of the three pathogens in the Serpentine

watershed (r_{pb} = 0.419; n = 81; $p < 0.001$ and r_{pb} = 0.388; n = 75; $p < 0.001$, respectively) compared to the Sumas watershed (r_{pb} = 0.297; n = 109; p = 0.002 and r_{pb} = 0.277; n = 101; p = 0.005, respectively). For the occurrence of *L. monocytogenes*, significant correlations were observed in the Serpentine watershed for both TFC (r_{pb} = 0.363; n = 81; $p < 0.001$) and generic *E. coli* (r_{pb} = 0.342; n = 75; p = 0.003), but no significant correlations were observed in the Sumas watershed. For the occurrence of VTEC, the correlations were more similar between the Serpentine and Sumas watersheds for both TFC (r_{pb} = 0.242; n = 81; p = 0.028 and r_{pb} = 0.312; n = 109; $p < 0.001$, respectively) and generic *E. coli* (r_{pb} = 0.318; n = 75; p = 0.005 and r_{pb} = 0.309; n = 101; p = 0.002, respectively). Significant correlations of both TFC and generic *E. coli* with the occurrence of *Salmonella* in the Sumas watershed were observed, specifically at sample site 1 (r_{pb} = 0.481; n = 28; $p < 0.001$, and r_{pb} = 0.462; n = 26; $p < 0.001$, respectively), which was the only site in the Sumas watershed where *Salmonella* was recovered.

In order to further characterize the potential of TFC and generic *E. coli* by membrane filtration to predict the occurrence of these three foodborne pathogens, regression trees were produced. The resulting splits are shown in Figure 3.2. Across all sites tested, TFC measured by the membrane filtration method showed a single split with ≥ 7.54 CFU/ml in a sample showing an 83.3% chance of pathogen occurrence, compared to a 15.3% occurrence for samples showing < 7.54 CFU/ml TFC. A root node only was observed for generic *E. coli* suggesting less predictive power across the seven sites compared to TFC. Within the Serpentine watershed, a node split was observed for TFC with ≥ 8.23 CFU/ml showing a 100% occurrence of pathogens, and < 8.23 CFU/ml showing only a 17.5% occurrence. Similarly, for generic *E. coli* in the Serpentine watershed a node split was observed with ≥ 3.59 CFU/ml showing a 75.0% chance of occurrence and < 3.59 CFU/ml

showing a 17.5% chance of pathogen occurrence. No node splits were observed for either indicator in the Sumas watershed, and no nodes were observed in either or both watersheds for any individual pathogen for either indicator.

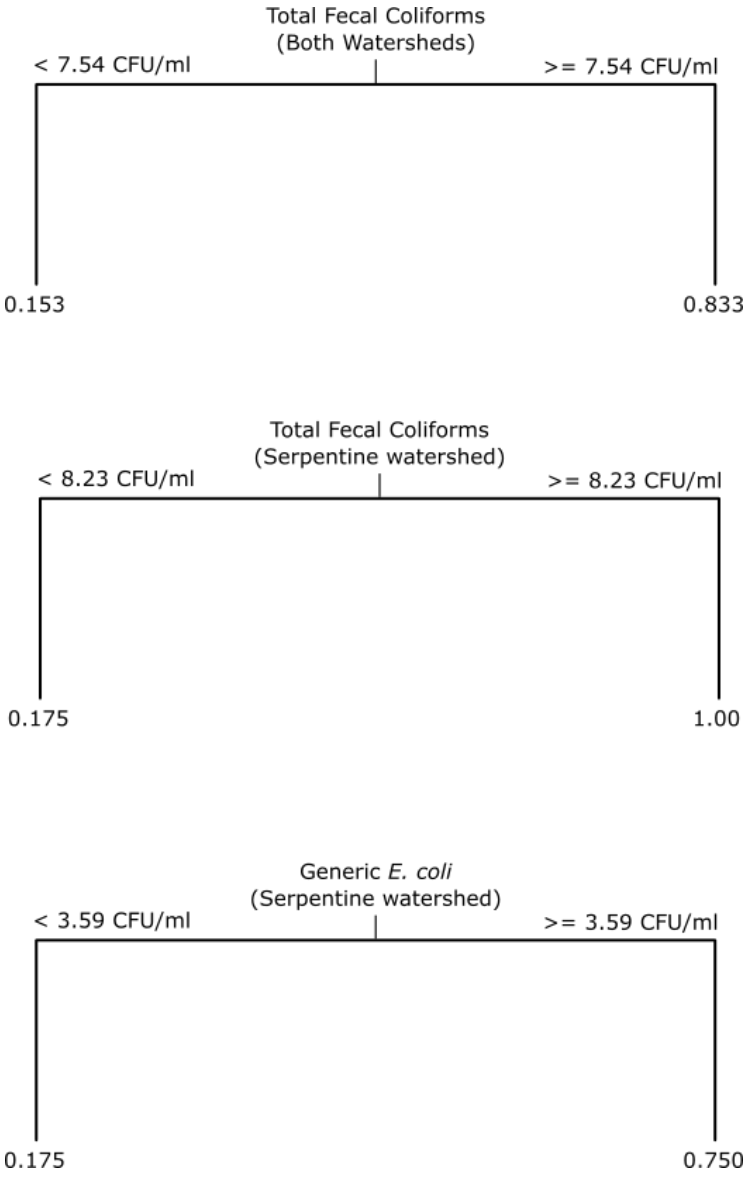

Figure 3.2 – Random effect – estimation method regression trees predicting the occurrence of veroxigenic *E. coli*, *Salmonella*, and *L. monocytogenes* relative to the concentrations of the fecal indicators, fecal coliforms and generic *E. coli*, within two watersheds in the Lower Mainland of British Columbia.

In order to better visualize how the occurrence of pathogens related to indicator organism trends at the six primary sampling sites, the observed levels of indicator organisms measured by membrane filtration were plotted over time along with dates where these three foodborne pathogens were recovered and are shown in Figure 3.3. As can be seen, many sites showed a wide variation in the observed levels of both indicator organisms, but the Sumas watershed seems to have overall lower levels of these indicators when compared to the Serpentine watershed. Sumas 2 and Sumas 3 sites, showed notably lower indicator concentrations compared to the other four sites. These two sites also had the lowest occurrences of pathogens. Similarly, the Serpentine watershed, which showed much higher overall concentrations of both indicator organisms had a higher occurrence of these three pathogens than Sumas 2 and Sumas 3. Also observed in Figure 3.3, the occurrence of pathogens often coincided with a spike in indicator organism concentration, resulting in the mean concentration of both fecal indicators to be, on average, higher in samples positive for the presence of a pathogen than samples where pathogens were not recovered (Wilcoxon rank sum test; $p < 0.0001$). Often times, however, these pathogens also occurred when the indicators were at their lowest levels.

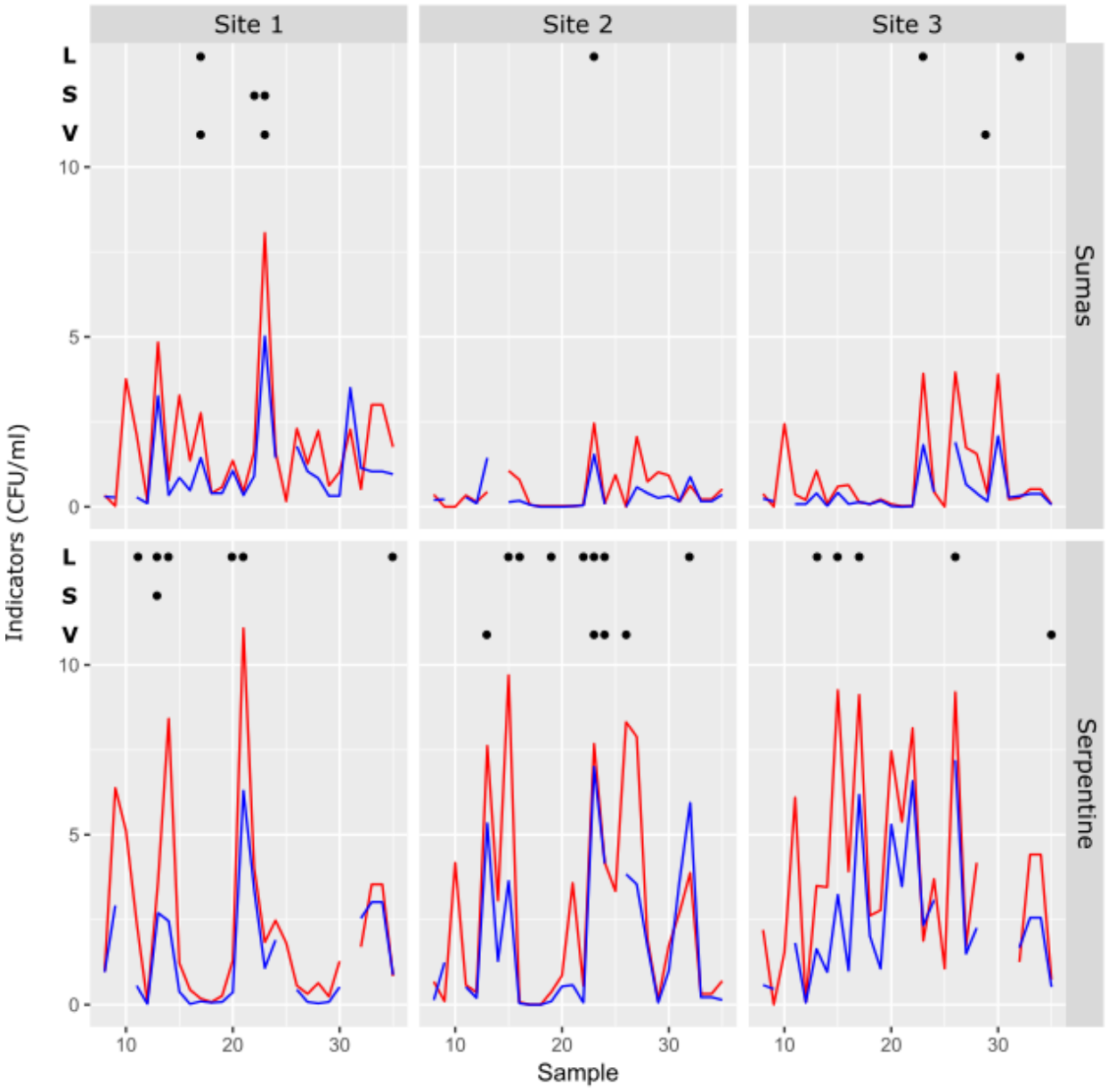

Figure 3.3 – Plot of the concentrations of total fecal coliforms (red) and generic *E. coli* (blue) observed for each sample site through the membrane filtration method, as well as samples that were positive for verotoxigenic *E. coli* (V), *Salmonella* (S), and *L. monocytogenes* (L). Only samples eight through thirty-five are shown since the membrane filtration method was not used during the first seven samplings.

The mean and maximum levels of each indicator measured using the membrane filtration method were also calculated for each primary sampling site and are shown in in Table 3.2. The concentrations of both indicators were significantly greater in the Serpentine watershed than the Sumas watershed (Wilcoxon rank sum test; $p < 0.001$). Similarly, within each watershed, site-specific differences in indicators concentrations were also observed (Kruskal-Wallace test; $p < 0.05$).

Table 3.2 – Average total fecal coliform and generic *E. coli* concentrations for the three sites sampled at each watershed during this experiment.

Sample Site	Mean F. coliforms (CFU/100ml)	Max. F. coliforms (CFU/100ml)	Mean Generic *E. coli* (CFU/100ml)	Max. Generic *E. coli* (CFU/100ml)
Sumas 1	185	806	111	502
Sumas 2	49	246	30	154
Sumas 3	85	396	42	208
Serpentine 1	239	1108	136	630
Serpentine 2	267	970	174	700
Serpentine 3	393	926	251	720

Significantly higher mean concentrations of both indicators were observed in the Serpentine watershed than in the Sumas watershed (Wilcoxon rank sum test; $p < 0.001$)

3.4 Discussion

The detection of foodborne pathogens in irrigation water is both time consuming and impractical due to costs. Therefore, the quality of irrigation water in Canada, the United States, and around the world is primarily assessed using indicators such as fecal coliforms and generic *E. coli* (Uyttendaele et al., 2015). Significant debate currently exists, however, on whether or not measurements based on these organisms truly predict the risk of foodborne pathogens, with a variety of studies showing both positive and negative correlations (Pachepsky et al., 2014). The

purpose of this section was to investigate the ability of TFC and generic *E. coli* enumerations to predict risk of pathogen occurrence when measured using two different techniques.

3.4.1 Comparison of enumeration methods

When comparing between the two methods, the numbers of TFC and generic *E. coli* observed were significantly correlated but variation was still present with r = 0.60 and r = 0.77 for TFC and generic *E. coli*, respectively. This is similar to a previous study that compared *E. coli* concentrations from a culture method and qPCR method and found a similar correlation value (r = 0.72) (Truchado et al., 2016). The variation between the two methods in this study may have been the result of different sampling volumes, since the 25 ml volume used for the membrane filtration, allowed for a more accurate enumeration. Indeed, previous work comparing the Petrifilm™ and membrane filtration methods of generic *E. coli* enumeration has shown a stronger correlation and similarity (r > 0.9; slope = 0.9-1.0) when the volumes sampled were the same between methods (Vail et al., 2003).

The difference in volume, then, could further explain the deviation from a slope of 1.00 and intercept of 0. At lower indicator concentrations, the small sample volume on the Petrifilm™ reduces the likelihood of observing the bacteria and errors will tend to be on the lower end since the cells in the sample are not likely to be concentrated in a random spot. On the other hand, the larger volume of the membrane filtration method will give a more representative concentration. This results in a shift of the trend line up the membrane filtration axis. Since the bulk of the observations are in this area, the shift actually skews the tend line to give a reduced slope. This is further evidenced by the fact that a greater number of observations exist on the membrane filtration

side of the trendline after the initial low concentration cluster (Figure 3.1). It might also be possible that the Petrifilm™ may be more favourable to growth of injured cells and may give greater, and more accurate enumerations of TFC and generic *E. coli*; however, this did not lead to an improved ability to predict the occurrence of foodborne pathogens.

3.4.2 Correlation and prediction of pathogen occurrence

Relative to the ability of each method to predict the occurrence of any of the three pathogens used in this study, the membrane filtration method showed a greater correlation when compared to the Petrifilm™ method. The Petrifilm™ method was only significantly correlated on a non-site-specific basis for TFC, and at that, the correlation coefficients were reduced compared to the membrane filtration method. Generic *E. coli* enumerated by Petrifilm™ significantly correlated with pathogen occurrence only on a site-specific basis. Further, any significant correlations observed using the Petrifilm™ method were also evident with the membrane filtration method, and in all cases, the membrane filtration method produced a higher correlation coefficient. As stated above, it is reasonable to suggest that the increased predictive power of the membrane filtration method is due to the increased volume sampled since similar results are expected when the sample volumes are consistent (Vail et al., 2003). To the best of my knowledge, no other study has investigated the effect that sample volume has on the correlation of pathogens with microbial indicators. For the remainder of this discussion, references to TFC and generic *E. coli* enumerations will be in relation to the membrane filtration method.

Total fecal coliforms showed a marginally higher correlation with total pathogen occurrence than generic *E. coli*, and was the only indicator able to produce a split using RE-EM tree analysis. This

was not necessarily the case for the occurrence of specific pathogens. Generic *E. coli* was a better predictor for the occurrence of VTEC, while *L. monocytogenes* correlated slightly better with TFC. Neither indicator was significantly correlated with the presence of *Salmonella*, but this may be the result of the overall lower occurrence of the bacterium, primarily being recovered at only one sampling site. It makes intuitive sense that generic *E. coli* would be a better predictor of VTEC, a subset of the indicator species, although Gu et al. (2013) found no correlation between generic *E. coli* and VTEC O157 occurrence, while a weak but significant correlation was observed with TFC. Overall, however, there was very little difference in predictive power, and a high correlation was observed between the two indicators when measured by the membrane filtration method ($r = 0.88$). This correlation between the two indicators is similar to $r = 0.82$ (Wilkes et al., 2009) and $r = 0.92$ (Holvoet et al., 2014) observed in previous studies and suggests that using multiple indicators will not increase the prediction of pathogen presence. Furthermore, TFC and generic *E. coli* have been found to be more robust indicators than others, such as total coliforms, *Enterococcus*, and *Clostridium perfringens*, with generic *E. coli* suggested to be the most predictive overall (Wilkes et al., 2009).

Correlations with individual pathogens were lower than correlations for the pathogens as a group. No overall correlation was observed with the occurrence of *Salmonella*, but this is likely due the rarity of its recovery. Indeed, a significant site-specific correlation for TFC and generic *E. coli* was observed for the presence of *Salmonella* at the site Sumas 1, where the highest recovery of the pathogen occurred. Resutls from previous studies indicated that seasonal trends in *Salmonella* occurrence correlate to corresponding seasonal trends in indicator concentrations (Jokinen et al., 2010), and Wilkes et al. (2009) observed a regression tree split where 88.9% of samples with > 89

CFU/ml *E. coli* were positive for *Salmonella* despite only a weak correlation with the indicator (r = 0.162). On the other hand, Benjamin et al. (2013) measured the correlation between *Salmonella* occurrence and sample *E. coli* concentration, as was done in this study, and found that there was no association between the two.

Significant correlations were observed for both indicators by membrane filtration with the occurrence of VTEC. Site specific correlations were indeed stronger than across all sites together, and coincide mostly with Sumas 1 and Serpentine 2, which were the two sites where VTEC was most commonly recovered. Most previous studies have only investigated the occurrence of VTEC O157 and found weak (Gu et al., 2013) or insignificant (Benjamin et al., 2013) correlations. Interestingly, occurrences of VTEC O157 were higher (14.6% and 13.8%, respectively) than were found for all VTEC in this study. This emphasizes that low correlation coefficients cannot necessarily be attributed to low occurrence values. It should also be noted, however, that generic *E. coli* was enumerated by the most probably number method in both of these studies, and used 100 ml of sample for each enumeration. Over the long run, however, a strong correlation (r = 0.859) was observed between the average seasonal concentration of TFC and the seasonal occurrence of VTEC O157 in the Salmon River of British Columbia (Jokinen et al., 2010). Indicator concentration and pathogen occurrence in the Salmon River study both also correlated with total seasonal precipitation which may suggest a confounded link between the two. Similarly, Duris et al. (2013) compared the occurrence of VTEC related genes (*e.g.*, *stx1*, *stx2*, *eaeA*, etc.) in water samples that met or exceeded the recreation water quality standards in Pennsylvania, (298 CFU generic *E. coli* per 100 ml), and found the presence of VTEC genes was observed more often

in water exceeding the quality standards (χ^2-test; $p < 0.05$). However, the genes were also found in a number of water samples that were within the recommended safe levels of the *E. coli* indicator.

A significant correlation between the occurrence of *L. monocytogenes* and both indicator organisms when measured by the membrane filtration method was observed. This is inconsistent with previous studies that have found no relationship between this indicators and the pathogen (Arvanitidou et al., 1997; Wilkes et al., 2009). Stronger correlations were found to exist at only certain sites, and while these site-specific correlations for the two other pathogens coincided with sites that had relatively high occurrences for the pathogen, this was not the same for *L. monocytogenes*. Indeed, no correlation was observed between either indicator organism and *L. monocytogenes* presence for Serpentine 2, which had the highest occurrence of the bacterium over the course of this study, and the highest correlation was observed for Sumas 2, where only one occurrence of *L. monocytogenes* was observed, but happened to coincide with a spike in indicator concentrations (Figure 3.3). One possibility is that these observations are driven by chance and in the long run will not continue to show a correlation. The other possibility is that the source of *L. monocytogenes* occurrence differs between the sites. *L. monocytogenes* is commonly associated with fecal shedding by wild life and livestock (Lyautey et al., 2007a), but can also be present and survive in environmental sources such as soil (Vivant et al., 2013). The significant correlations observed at individual sites may point to a fecal source of contamination, and more sporadic recoveries, compared to a site with high occurrence but low correlation which suggests an environmental "point-source" reservoir which provides more consistent contamination of the water.

While significant correlations were observed for pathogen occurrence, recoveries of pathogens often occurred in water samples with low levels of indicators. Indeed, just under 50% of pathogen occurrence occurred when fecal indicators were near their lowest levels (Figure 3.3). Interestingly, though, the highest peaks for each site always correlated with the occurrence of at least one pathogen, which is likely the driver of any observed significant correlation, and leading to higher average concentrations of fecal indicators when pathogens are present. Previous studies have shown higher mean concentrations of indicators when pathogens are present, but not necessarily in all cases of pathogen occurrence (Duris et al., 2013; Jokinen et al., 2012). Furthermore, higher indicator concentrations have been previously observed in agricultural areas than in water not affected by agriculture, which also correlated with the presence of pathogens (Edge et al., 2012). Similarly, higher indicator concentrations were observed in the Serpentine watershed, which correlated with overall increased pathogen recovery. Counter-intuitively, however, the highest indicator occurrence in the Serpentine watershed was observed at site 3, which also showed the lowest pathogen recovery within the watershed. Within the Sumas watershed, which showed a lower average level of indicators and in turn a lower recovery of pathogens, higher indicator concentrations were observed at site 1, which coincided with higher overall pathogen recovery. These results show that high levels of indicator organisms over the long-run may signal an increased risk for pathogen presence, but low levels of indicators do not necessarily signal an absence of foodborne pathogens. Further, it emphasizes that long term trends may be more predictive than single samplings when it comes to pathogen prediction in surface waters for agricultural use.

Despite low correlations between pathogen occurrence and indicator organism concentrations, RE-EM tree analysis emphasized that a there is a significantly increased risk of pathogen occurrence when indicator organisms are in higher concentrations (Figure 3.2). These concentrations, however, are much higher than current guidelines for water quality analysis. In British Columbia, recommended water quality assessment guidelines suggest that irrigation waters should contain TFC and generic *E. coli* concentrations below 100 CFU/100 ml and 77 CFU/100 ml, respectively (Government of British Columbia, n.d.). Of interest is that only two of the six primary sites sampled during this study meet that criteria. Furthermore, all the sites were above these criteria on multiple single samplings. This may add a complication if mandated regulations are ever put in place with the same criteria. Alternatively, the Health Canada regulations for recreational water mandate less than 200 *E. coli* per 100 ml geometric mean over at least five samplings, and no sample showing a concentration greater than 400 *E. coli* per 100 ml. By these regulations, all but one sample site (Serpentine 3) fit the criteria for mean occurrence; however, four sites showed at least one sample above the maximum threshold. While these criteria are not currently mandated for agricultural waters, the Food Safety Modernization Act (FSMA) in the United States has mandated criteria that will need to be met for any growers hoping to ship their product to our southern neighbour. The FSMA regulations (U.S. Food and Drug Administration, 2015) require a geometric mean of *E. coli* of less than 126 CFU/100 ml, which was not met by any of the three Serpentine sample sites, and no single sample to have greater than 410 CFU of *E. coli* per 100 ml, which was, again, not met by any site in the Serpentine watershed or one site in the Sumas watershed.

3.5 Conclusion

Total fecal coliforms and generic *E. coli* show some correlation with pathogen occurrence in the irrigation waters of the Lower Mainland of British Columbia, but they do not tell a complete story. Higher concentrations of indicators at individual sites were associated with increased presence of foodborne pathogens at those particular sites, but pathogens were also recovered at sites with lower average indicator concentrations. Moreover, indicator concentrations in individual samples provided little insight into the likelihood of pathogens being present in those samples.

Additionally, the method of indicator enumeration may have an effect on how predictive indicator organisms may be. Using a membrane filtration method of enumeration with a higher volume of sample was more predictive of pathogen occurrence than using a smaller volume with 3M™ Petrifilm™.

Finally, many of the sites sampled for this study were found to be outside the recommended limits for water quality indicators suggested by CanadaGAP™ and mandated by FSMA.

Chapter 4 – Environmental Factors Associated with the Occurrence of Verotoxigenic *Escherichia coli*, *Salmonella*, and *Listeria monocytogenes*

4.1 Introduction

Much research has demonstrated that the occurrence of foodborne pathogens in surface waters is not uniform across temporal and spatial lines, and that the presence of pathogens can be affected by season (Gu et al., 2013; Haley et al., 2009; Jokinen et al., 2012; Strawn et al., 2013a), location (Chapin et al., 2014; Lyautey et al., 2007b), and weather (Gu et al., 2013; Jokinen et al., 2012; Jones et al., 2014). An understanding of how these factors may relate to, and therefore predict, the presence of these pathogens in the waters used for irrigation may allow for a reduction in the risk of contamination of fresh produce by applying control strategies at the right times and places. Therefore, the objective of this section is to determine any physicochemical or environmental factors which may correlate and/or predict the presence of verotoxigenic *Escherichia coli* (VTEC), *Salmonella*, or *Listeria monocytogenes* in the irrigation waters of the Lower Mainland of British Columbia.

4.2 Materials and methods

4.2.1 Water temperature, pH, and total dissolved solids

The temperature of the water samples was measured at the time of sampling using an alcohol thermometer. The thermometer was inserted into the sample within one minute of collection, and allowed to equilibrate for at least 20 seconds before reading. The pH of the samples was measured

within 36 hours after collection, at the laboratory in duplicate using an Accumet digital pH meter (Fisher Scientific, Ottawa, ON).

Total dissolved solids (TDS) were measured for each sample by oven drying. Briefly, foil weigh boats were dried over night at 120°C before pre-weighing. A 25 ml aliquot of each water sample was added to a pre-dried and pre-weighed weigh boat in duplicate after the water samples were allowed to settle for > 2 hours. The aliquots were dried overnight (~16 hours) at 120°C and allowed to cool at room temperature in a desiccator before determining the final weight of the remaining TDS.

4.2.2 Collection of weather data

Weather data was collected from the Environment Canada website for the sampling areas on each sampling date. Data for the Serpentine watershed sampling sites were collected from the Pitt Meadows CS weather station (49°12'29.964" N; 122°41'24.076" W), and data for the Sumas watershed sampling sites were collected from the Sumas Canal weather station (49°06'48.008" N; 122°06'35.004" W) with missing data being filled in from the Mission West Abbey weather station (49°09'09.002" N; 122°16'14.001" W). The weather data collected included total precipitation and average temperature on the date of sampling as well as on each of the three days prior to sampling.

4.2.3 Collection of geographical data

Water flow direction at the sample sites was determined by visual inspection during each sampling. The upstream water sources were determined using the Drainage Mains and Drainage Open Channels datasets collected from the City of Surrey's Open Data Site (http://data.surrey.ca), the

City of Abbotsford Map Viewer (maps.abbotsford.ca), and the City of Chilliwack Webmap (maps.chilliwack.com). Livestock information was collected from the Agricultural Land Use Inventories (ALUI) for Surrey (2010), Abbotsford (2012), and Chilliwack (2012), retrieved from the Government of British Columbia (http://www2.gov.bc.ca/gov/content/industry/agriculture-seafood/agricultural-land-and-environment/strengthening-farming/planning-for-agriculture/agricultural-land-use-inventories/south-coast).

4.2.4 Analysis of geographical data

Maps were produced based on the collected geographic data (section 4.2.3) using ArcMap (ArcGIS, version 10.2.2; http://www.arcgis.com). Upstream water sources were determined up to three kilometers from each sample site. Upstream water sources were considered to be any waterway directly connected to the sample site where the water was determined, either from retrieved geographic data or direct observation, to flow to the sample site. If livestock was present on a property on the ALUI, that whole property was considered to be positive for the presence of that livestock type. Livestock was considered to be cow (*i.e.*, dairy or beef), poultry, or other (*i.e.*, swine, sheep, goat). Livestock were considered to be upstream if the property directly bordered on to the connecting waterway. Livestock properties separated from the waterway by a road were not considered to be bordering on the waterway.

Using the measurement tool in ArcMap, the upstream distance to the nearest livestock of each type was measured for each livestock type. Also measured were the number of bordering livestock properties 1 km, 2 km, and 3 km upstream, and the total length of livestock associated property bordering the upstream waterways up to a distance of 1 km, 2 km, and 3 km. Any lengths of

upstream waterway that passed through a livestock property were counted twice to account for bordering on two banks of the waterway.

4.2.5 Pathogen occurrence

The occurrence of VTEC, *Salmonella*, and *L. monocytogenes* were measured and are shown and discussed above in Chapter 2. Samples from sample site Sumas 4 were not included due to insufficient sampling and its close proximity to Sumas 1.

4.2.6 Statistical analysis

Statistical analysis of results was conducted using R version 3.2.3 (R Core Team, 2015; http://www.R-project.org). Fisher's LSD tests were conducted using the agricolae software package (version 1.2-4; https://CRAN.R-projects.org/package=agricolae). Correlations between weather or water characteristics and pathogen occurrence were calculated using the point-biserial method, which correlates a continuous variable with a dichotomous variable (Gu et al., 2013), using the ltm software package (http://www.jstatsoft.org/v17/io5/). Correlations between relative pathogen occurrence at each site (*i.e.*, number of positive samples vs. total number of samples collected at that site) and livestock proximity and density were calculated using Spearman's rank correlation test.

4.3 Results

4.3.1 Water temperature, pH, and total dissolved solids

Water temperature, pH, and TDS were measured for each site and are summarized in Table 4.1. No significant differences were observed in the temperature of the water between sites over the

course of sampling, but a significantly lower pH was observed in the Serpentine watershed compared to the Sumas watershed, specifically at Serpentine 2 (Fisher's LSD; $p < 0.05$). In addition, the mean pH at all sites was alkaline, but acidic pH was occasionally observed at Serpentine 2. A greater amount of TDS was also observed at Serpentine 2 compared to the other five sample sites tested (Fisher's LSD; $p < 0.05$).

Table 4.1 – Water temperature, pH, and total dissolved solids (TDS) observed at the six primary sampling sites monitored during this study

Site	Temperature (°C)	pH	TDS (mg/ml)
Sumas 1	14.3 ± 5.4	7.64 ± 0.32^{a}	0.254 ± 0.224^{a}
Sumas 2	15.3 ± 5.8	7.59 ± 0.28^{a}	0.212 ± 0.097^{a}
Sumas 3	15.5 ± 6.4	7.72 ± 0.30^{a}	0.276 ± 0.126^{a}
Serpentine 1	12.7 ± 4.4	7.35 ± 0.43^{b}	0.205 ± 0.095^{a}
Serpentine 2	14.5 ± 5.5	7.11 ± 0.83^{c}	0.382 ± 0.232^{b}
Serpentine 3	13.9 ± 4.8	7.56 ± 0.33^{ab}	0.219 ± 0.283^{a}

Values in the same column with different superscripts indicated a significant difference (Fisher's LSD; $p < 0.05$)

Correlations between pathogen occurrence and water characteristics were measured using the point-biserial correlation method; the resulting correlation coefficients are shown in Table 4.2. Water temperature significantly correlated negatively with pathogen occurrence ($r_{pb} = -0.217$; $n = 216$; $p = 0.001$), as did the pH of the water samples ($r_{pb} = -0.274$; $n = 214$; $p < 0.001$), implying that pathogen occurrence was greater when the water was cooler and closer to a neutral pH. Neither of these correlations are particularly strong, however. No correlation was observed between the total dissolved solids and pathogen occurrence.

Table 4.2 – Point-biserial correlations (r_{pb}) of the occurrence of verotoxigenic *E. coli* (VTEC), *Salmonella*, and *L. monocytogenes* with physicochemical characteristics of the irrigation water samples collected from three sites each from the Sumas and Serpentine watersheds in the Lower Mainland of British Columbia

	Temperature	pH	Total Dissolved Solids
VTEC			
Sumas Watershed	-0.152	-0.031	-0.147
Sumas 1	-0.370*	-0.158	-0.238
Sumas 2	-	-	-
Sumas 3	0.041	0.085	-0.116
Serp.Watershed	-0.062	-0.113	0.046
Serpentine 1	-	-	-
Serpentine 2	-0.219	-0.062	-0.063
Serpentine 3	0.126	-0.025	-0.056
Both Watersheds	-0.115	-0.104	-0.020
Salmonella			
Sumas Watershed	-0.093	-0.093	-0.106
Sumas 1	-0.347*	-0.225	-0.153
Sumas 2	-	-	-
Sumas 3	-	-	-
Serp.Watershed	0.001	0.035	-0.037
Serpentine 1	0.046	0.083	-0.022
Serpentine 2	-	-	-
Serpentine 3	-	-	-
Both Watersheds	-0.059	-0.005	-0.080
L. monocytogenes			
Sumas Watershed	-0.081	-0.056	-0.023
Sumas 1	-0.224	-0.114	-0.147
Sumas 2	-0.200	-0.148	-0.134
Sumas 3	-0.029	-0.108	-0.006
Serp.Watershed	-0.323**	-0.341***	0.027
Serpentine 1	-0.183	0.108	-0.029
Serpentine 2	-0.510**	-0.569***	0.034
Serpentine 3	-0.209	-0.200	-0.090
Both Watersheds	-0.214**	-0.316***	0.033
Any pathogen			
Sumas Watershed	-0.148	-0.062	-0.112
Sumas 1	-0.493**	-0.184	-0.247
Sumas 2	-0.200	-0.148	-0.134
Sumas 3	-0.007	-0.057	-0.061
Serp.Watershed	-0.279**	-0.313**	0.062
Serpentine 1	-0.183	0.108	-0.029
Serpentine 2	-0.486**	-0.471**	0.068
Serpentine 3	-0.129	-0.195	-0.110
Both Watersheds	-0.217**	-0.274***	0.011

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

In relation to correlations with individual pathogens, both water temperature and pH only correlated significantly with the occurrence of *L. monocytogenes* (r_{pb} = -0.214; n = 216; p = 0.002 and r_{pb} = -0.316; n = 214; p < 0.001, respectively). These correlations were primarily observed within the Serpentine watershed, specifically at sample site 2. This is also the site where the highest number of samples positive for *L. monocytogenes* were recovered. Water temperature was significantly negatively correlated with VTEC and *Salmonella*, but only on a site-specific basis at the Sumas 1 sample site in the Sumas watershed, with r_{pb} = -0.370 (n = 34; p = 0.029) and r_{pb} = -0.347 (n = 34; p = 0.041) for VTEC and *Salmonella*, respectively.

4.3.2 Correlations with weather factors

4.3.2.1 Precipitation

Correlations between pathogen occurrence and levels of precipitation were measured using the point-biserial correlation method with the resulting correlation coefficients are shown in Table 4.3. Pathogen occurrence was most strongly correlated with higher levels of precipitation the day before sampling (r_{pb} = 0.203; n = 223; p = 0.002). Less strongly correlated were precipitation levels three days before sampling (r_{pb} = 0.175; n = 216; p = 0.010), and the total amount of precipitation over the three days prior to sample collection (r_{pb} = 0.188; n = 223; p = 0.005). At a site-specific level, correlations of pathogen occurrence with total precipitation the day prior to sample collection were only observed in the Sumas watershed (r_{pb} = 0.352; n = 129; p < 0.001), with all three sample sites within that watershed showing significant correlations. The Serpentine watershed did not show any watershed specific correlation between pathogen occurrence and precipitation one day prior to sample collection, but did show a correlation between pathogen occurrence and precipitation three days prior to sample collection (r_{pb} = 0.257; n = 91; p = 0.013).

Table 4.3 – Point-biserial correlations (r_{pb}) of the occurrence of verotoxigenic *E. coli* (VTEC), *Salmonella*, and *L. monocytogenes* in the Sumas and Serpentine watersheds with the levels of precipitation three days prior (P3), two days prior (P2), one day prior (P1), the day of (P0), and the total volume over the three days prior (P1-3) to sample collection.

	P3	P2	P1	P0	P1-3
VTEC					
Sumas Watershed	0.041	0.016	0.266**	-0.053	0.144
Sumas 1	0.168	0.108	0.613***	-0.070	0.391*
Sumas 2	-	-	-	-	-
Sumas 3	-0.116	-0.124	-0.098	-0.095	-0.129
Serp.Watershed	0.160	0.039	0.175	-0.046	0.156
Serpentine 1	-	-	-	-	-
Serpentine 2	0.374*	0.132	0.394*	-0.037	0.379*
Serpentine 3	-0.100	-0.089	-0.088	-0.082	-0.126
Both Watersheds	0.093	0.024	0.205**	-0.053	0.135
Salmonella					
Sumas Watershed	0.096	0.145	0.072	-0.049	0.121
Sumas 1	0.119	0.286	0.137	-0.096	0.212
Sumas 2	-	-	-	-	-
Sumas 3	-	-	-	-	-
Serp.Watershed	0.318**	0.057	0.010	-0.045	0.176
Serpentine 1	0.550	0.095	0.015	-0.082	0.303
Serpentine 2	-	-	-	-	-
Serpentine 3	-	-	-	-	-
Both Watersheds	0.158*	0.114	0.075	-0.039	0.142*
L. monocytogenes					
Sumas Watershed	0.096	0.015	0.300***	-0.025	0.178*
Sumas 1	-0.114	-0.127	0.016	-0.097	-0.075
Sumas 2	0.271	0.192	0.396*	-0.022	0.348*
Sumas 3	0.168	0.022	0.509**	0.005	0.306
Serp.Watershed	0.236*	0.130	0.158	0.087	0.222*
Serpentine 1	0.261	-0.088	-0.036	0.387*	0.051
Serpentine 2	0.247	0.464**	0.309	-0.027	0.475**
Serpentine 3	0.210	-0.066	0.187	-0.126	0.087
Both Watersheds	0.147*	0.066	0.147*	0.008	0.141*
Any pathogen					
Sumas Watershed	0.129	0.102	0.352***	-0.063	0.247**
Sumas 1	0.110	0.221	0.452**	-0.120	0.337*
Sumas 2	0.271	0.192	0.396*	-0.022	0.348*
Sumas 3	0.097	-0.039	0.417*	-0.040	0.217
Serp.Watershed	0.257*	0.106	0.116	0.048	0.204
Serpentine 1	0.261	-0.088	-0.036	0.387*	0.051
Serpentine 2	0.364*	0.427*	0.238	-0.076	0.481**
Serpentine 3	0.133	-0.103	0.129	-0.155	0.018
Both Watersheds	0.175**	0.090	0.203**	-0.028	0.188**

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

With respect to individual pathogens, the amount of precipitation on the day before sample collection significantly correlated with the occurrence of VTEC (r_{pb} = 0.205; n = 223; p = 0.002) and *L. monocytogenes* (r_{pb} = 0.147; n = 223; p = 0.028). On the other hand, *Salmonella* occurrence was most strongly correlated with precipitation three days prior to sample collection (r_{pb} = 0.158; n = 216; p = 0.020). For the precipitation level the day prior to sampling, the strongest correlations with respect to VTEC were at the Sumas 1 sampling site (r_{pb} = 0.613; n= 35; p < 0.001), and the Serpentine 2 sampling site (r_{pb} = 0.394; n = 34; p = 0.019), which were the two sites with the highest occurrence of the pathogen. For *L. monocytogenes*, higher precipitation the day before sampling was strongly correlated for Sumas 3 (r_{pb} = 0.509; n = 34; p = 0.002), which was the most common site of *L. monocytogenes* recovery in the Sumas watershed. Alternatively, this was not observed for any sites in the Serpentine watershed where *L. monocytogenes* was more common. Interestingly, the strongest correlation in the Serpentine watershed for *L. monocytogenes* was at site 2 and was with the total level of precipitation two days prior to sampling (r_{pb} = 0.464; n = 34; p = 0.005) and total precipitation over the three days prior to sample collection (r_{pb} = 0.475; n = 33; p = 0.004).

4.3.2.2 Air temperature

Correlations between pathogen occurrence and mean temperature were measured using the point-biserial correlation method, with the resulting correlation coefficients shown in Table 4.4. Pathogen occurrence significantly correlated negatively with the mean air temperature on the day of sampling (r_{pb} = -0.155; n = 217; p = 0.022), implying increased pathogen prevalence on cooler days. The correlation was only weak, however, and could really only be seen for Sumas 1 (r_{pb} = -0.384; n = 35; p = 0.021) and for Serpentine 2 (r_{pb} = -0.376; n = 32; p = 0.031), but not for any of

the other sampling sites. Additionally, Sumas 1 was significantly correlated with lower temperatures on any of the three days prior to sampling, and also with the average temperature of those three days.

Table 4.4 – Point-biserial correlations (r_{pb}) of the occurrence of verotoxigenic *E. coli* (VTEC), *Salmonella*, and *L. monocytogenes* in the Sumas and Serpentine watersheds with average temperatures three days prior (T3), two days prior (T2), one day prior (T1), the day of (T0), and the average temperature over the three days prior (T_{avg}) to sample collection.

	T3	T2	T1	T0	T_{avg}
VTEC					
Sumas Watershed	-0.085	-0.115	-0.125	-0.087	-0.110
Sumas 1	-0.274	-0.270	-0.297	-0.248	-0.286
Sumas 2	-	-	-	-	-
Sumas 3	0.150	0.012	0.010	0.083	0.060
Serp.Watershed	0.055	0.005	-0.022	-0.077	0.015
Serpentine 1	-	-	-	-	-
Serpentine 2	-0.048	-0.092	-0.113	-0.207	-0.087
Serpentine 3	0.355	0.253	0.172	0.135	0.276
Both Watersheds	-0.019	-0.059	-0.073	-0.081	-0.051
Salmonella					
Sumas Watershed	-0.028	-0.027	-0.043	-0.085	-0.033
Sumas 1	-0.299	-0.267	-0.224	-0.307	-0.270
Sumas 2	-	-	-	-	-
Sumas 3	-	-	-	-	-
Serp.Watershed	0.039	0.034	0.001	-0.001	0.026
Serpentine 1	0.064	0.055	-0.002	-0.006	0.042
Serpentine 2	-	-	-	-	-
Serpentine 3	-	-	-	-	-
Both Watersheds	-0.007	-0.012	-0.032	-0.061	-0.017
L. monocytogenes					
Sumas Watershed	-0.045	-0.061	-0.082	-0.115	-0.063
Sumas 1	-0.232	-0.203	-0.217	-0.267	-0.222
Sumas 2	-0.170	-0.138	-0.122	-0.121	-0.147
Sumas 3	0.088	0.015	-0.040	-0.090	0.024
Serp.Watershed	-0.127	-0.151	-0.191	-0.222*	-0.162
Serpentine 1	0.098	0.033	-0.053	-0.052	0.030
Serpentine 2	-0.321	-0.297	-0.290	-0.386*	-0.314
Serpentine 3	-0.128	-0.147	-0.210	-0.197	-0.165
Both Watersheds	-0.092	-0.098	-0.128	-0.157*	-0.108
Any pathogen					
Sumas Watershed	-0.043	-0.082	-0.112	-0.130	-0.079
Sumas 1	-0.381*	-0.367*	-0.364*	-0.384*	-0.379*
Sumas 2	-0.170	-0.138	-0.122	-0.121	-0.147
Sumas 3	0.152	0.019	-0.032	-0.042	0.050
Serp.Watershed	-0.055	-0.114	-0.169	-0.198	-0.117
Serpentine 1	0.098	0.033	-0.053	-0.052	0.030
Serpentine 2	-0.258	-0.286	-0.300	-0.376*	-0.292
Serpentine 3	0.075	-0.012	-0.109	-0.103	-0.017
Both Watersheds	-0.052	-0.091	-0.132	-0.155*	-0.093

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

With respect to individual pathogens, only *L. monocytogenes* was correlated with air temperature, and only on the day of sample collection (r_{pb} = -0.157; n = 217; p = 0.020), but this was only evident at the Serpentine 2 sample site (r_{pb} = -0.386; n = 32; p = 0.027).

4.3.3 Correlations with proximity and density of upstream livestock

Correlations between pathogen occurrence and nearby upstream livestock were calculated using the Spearman's rank correlation test (r_s) and the results are summarized in Table 4.5. Pathogen occurrence correlated significantly with proximity to the nearest upstream cow or poultry farm (r_s = -0.886; n = 6; p = 0.033), showing that sample sites with close proximity to livestock were more likely to be positive for the pathogens of interest. Pathogen occurrence also correlated significantly with the number of upstream cow farms (r_s = 0.828; n = 6; p = 0.042) as well as the total length of cow farms bordering upstream waterways to a distance of 1 km (r_s = 0.845; n = 6; p = 0.034). Interestingly, stronger correlations were observed when poultry farms were also included, (r_s = 0.878; n = 6; p = 0.021 and r_s = 0.941; n = 6; p = 0.005 for number and length, respectively), despite little to no correlation observed with poultry itself. No correlations with total pathogen occurrence were observed at the 2 km and 3 km distances for either number or length of bordering livestock.

Table 4.5 – Spearman rank correlation coefficients (r_s) for the occurrence of verotoxigenic *E. coli* (VTEC), *Salmonella*, and *L. monocytogenes* with proximity and density of upstream livestock.

	VTEC	*Salmonella*	*L. monocytogenes*	Any Pathogen
Nearest Livestock[A]				
Cow[B]	-0.377	-0.135	-0.714	-0.829
Poultry[C]	-0.464	-0.507	0.200	-0.429
Cow or Poultry	-0.696	-0.439	-0.371	-0.886*
Any[D]	-0.493	-0.034	-0.200	-0.371
Livestock Number (1 km)[E]				
Cow	0.105	0.122	0.828*	0.828*
Poultry	0.399	0.775	-0.655	0.131
Cow or Poultry	0.396	0.693	0.293	0.878*
Any	0.315	0.490	0.207	0.621
Livestock Number (2 km)[F]				
Cow	0.626	-0.146	0.123	0.278
Poultry	0.626	0.237	-0.185	0.339
Cow or Poultry	0.721	0.017	-0.058	0.319
Any	0.672	-0.104	0.000	0.265
Livestock Number (3 km)				
Cow	0.579	0.018	-0.247	0.093
Poultry	0.585	0.359	-0.334	0.273
Cow or Poultry	0.627	0.261	-0.383	0.177
Any	0.662	0.257	-0.348	0.203
Livestock Border (1 km)				
Cow	0.257	0.020	0.845*	0.845*
Poultry	0.399	0.775	-0.655	0.131
Cow or Poultry	0.431	0.395	0.577	0.941**
Any	0.397	-0.034	0.551	0.609
Livestock Border (2 km)				
Cow	0.609	-0.439	0.771	0.543
Poultry	0.308	0.359	-0.577	-0.030
Cow or Poultry	0.812*	-0.372	0.543	0.486
Any	0.812*	-0.372	0.543	0.486
Livestock Border (3 km)				
Cow	0.377	-0.778	0.200	-0.200
Poultry	0.585	0.359	-0.334	0.273
Cow or Poultry	0.812*	-0.304	0.143	0.257
Any	0.638	-0.304	-0.029	0.029

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

[A]Nearest distance for surface water to travel

[B]Includes both dairy and beef cattle

[C]Includes chicken and turkey

[D]Includes bovine and poultry, as well as swine, sheep, or goats

[E]Number of properties bordering the surface water within a given distance upstream

[F]Total length of waterway bordered by properties containing livestock within a given distance

Looking at each pathogen individually, the occurrence of VTEC correlated significantly with the length of upstream water way that bordered on cow or poultry farms to a distance of 2 km (r_s = 0.812; n = 6; p = 0.05) and 3 km (r_s = 0.812; n=6; p = 0.05); however, no significant correlation was observed at the 1 km range. Similar to the occurrence of any pathogen, the occurrence of *L. monocytogenes* was correlated with both the number (r_s = 0.828; n=6; p = 0.042) and border length (r_s = 0.845; n=6; p = 0.034) of upstream cow farms to a distance of 1 km, but unlike with the occurrence of any of the three pathogens, no significant correlation was observed when poultry was included. No significant correlations were observed for the occurrence of *Salmonella* with nearby livestock, but some evidence was observed of a correlation with both the number and border length of poultry farms up to a distance of 1 km (r_s = 0.775; n=6; p = 0.07 for both).

4.4 Discussion

The presence of foodborne pathogens in irrigation water has been suggested to be nonuniform across environmental variables. In this part of the project, I looked at how physicochemical, meteorological, and geospatial factors may correlate with, and potentially predict the presence of VTEC, *Salmonella*, and *L. monocytogenes*.

4.4.1 Geographical characteristics

The strongest correlations were observed with proximity to and the density of upstream livestock. The occurrence of any of the three pathogens correlated strongly with the proximity to the nearest upstream cow or poultry source, as well as the number and total length of cow and poultry sources bordering on the upstream connected water ways to a distance of 1 km. This is consistent with a previous Canadian survey of foodborne pathogens in surface waters which found that the

occurrence of pathogens was higher in agricultural areas compared to control sites not affected by upstream agricultural practices (Edge et al., 2012). Since these pathogens are primarily associated with agricultural livestock reservoirs (Nightingale et al., 2004; Persad and Lejeune, 2014; Rodriguez et al., 2006), it follows that their occurrence would be correlated with nearby proximity to these sources. No correlations with total pathogen occurrence were observed outside of a 1 km radius, suggesting that nearby farms have the largest impact on the occurrence of these pathogens in surface waters. It should be noted, however, that livestock associated pathogens (*i.e.*, VTEC O157, *Salmonella*, and *Campylobacter*) can still be isolated from water sources not affected by livestock agriculture, reminding us that these are not the only sources of these bacteria (Edge et al., 2012).

Conversely, significant correlations were observed with upstream cow or poultry density and VTEC up to 2 km and 3 km, but not at 1 km. This is possibly due to the fact that Sumas 1, which accounted for a large proportion of VTEC occurrence (36%) does not have any livestock within 1 km upstream, but has a relatively large density after a distance of 1 km (Figure 4.1). Interestingly, the significant correlation was only observed for cow and poultry together, and not with cow individually, despite the fact that cattle is considered the primary reservoir (Hussein and Bollinger, 2005; Hussein and Sakuma, 2005), with minimal occurrence of VTEC associated with domestic poultry (Persad and Lejeune, 2014). The driving force for the inclusion of poultry in the correlation is likely Sumas 1, which accounted for 36% of recovered VTEC isolates but is surrounded primarily by poultry farms (Figure 4.1). Serpentine 2, on the other hand, accounted for 45% of VTEC isolates and is surrounded primarily by cow farms (Figure 4.2). Therefore, it is likely that a high level of VTEC at Sumas 1 may be coming from a small number of upstream cow farms. It

should be also noted that a significantly larger amount of water flow was present at Sumas 1 compared to some other sites which could also be responsible for carrying VTEC from locations further upstream. For instance, a previous study in California found that VTEC O157 was only carried downstream 135 meters in a low flow creek, but at times of high rains, flooding, and high water flow, transport as far as 32 km was observed (Cooley et al., 2007).

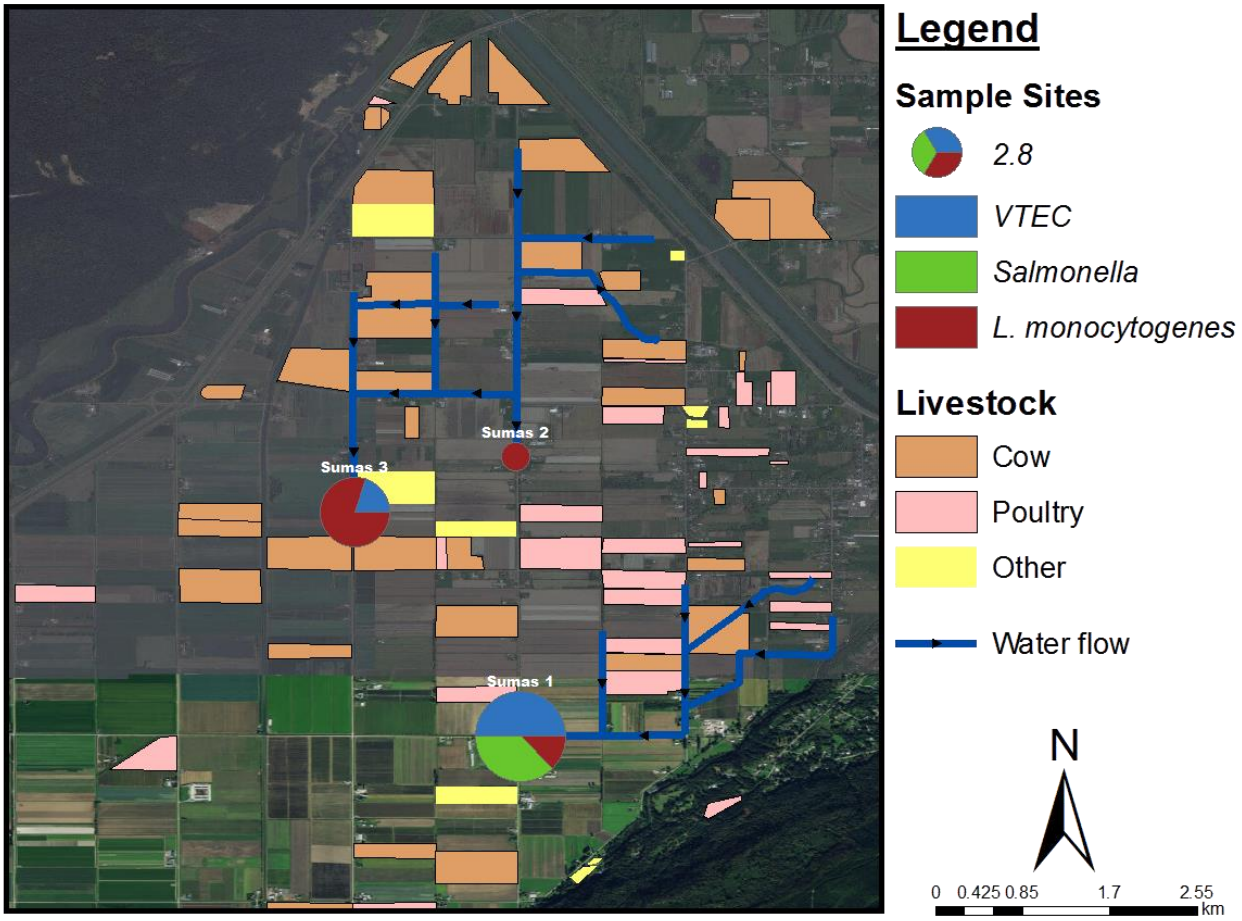

Figure 4.1 – Map of the sampling area within the Sumas watershed. Pie charts represent the sample sites, with their size being proportional to the total number of pathogens recovered and their divisions representing the individual pathogens. Coloured polygons represent properties where livestock are present. Upstream water flow is shown to a distance of 3 km from each sample site.

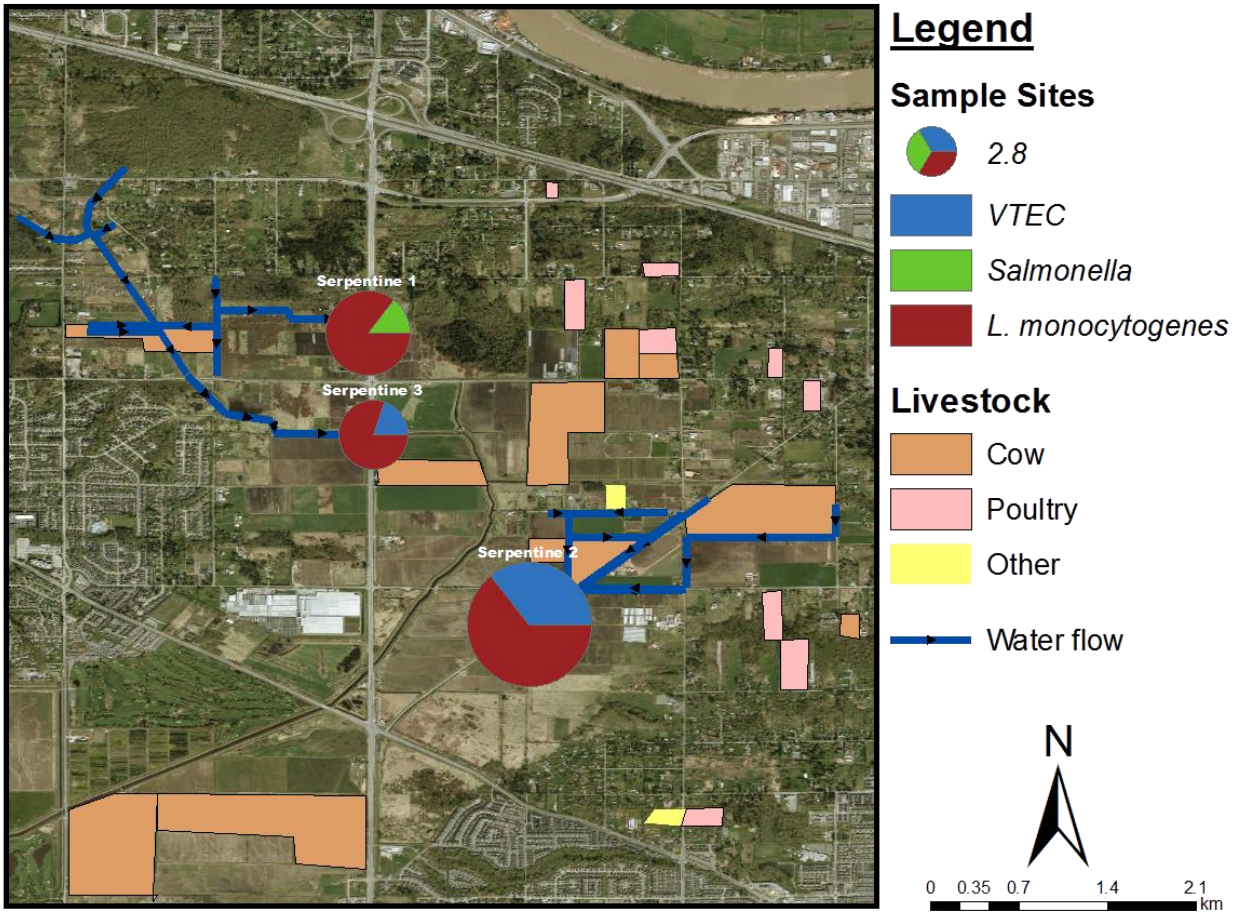

Figure 4.2 – Map of the sampling area within the Serpentine watershed. Pie charts represent the sample sites, with their size being proportional to the total number of pathogens recovered and their divisions representing the individual pathogens. Coloured polygons represent properties where livestock are present. Upstream water flow is shown to a distance of 3 km from each sample site.

The occurrence of *L. monocytogenes* correlated significantly with the density of cow farmland within 1 km upstream of sampling. Domestic cattle are known to be a common carrier of *L. monocytogenes* (Lyautey et al., 2007a; Nightingale et al., 2004), and previous studies have found the occurrence of *L. monocytogenes* to correlate with proximity to pasture (Chapin et al., 2014) and upstream dairy farms (Lyautey et al., 2007b). Not all sources of *L. monocytogenes* are related to livestock, however. The bacterium is routinely isolated and shown to survive in soils (Vivant

et al., 2013), and uncultivated fields and meadows have shown a higher prevalence than agricultural land (Dowe et al., 1997).

Weaker correlation were observed between the occurrence of *Salmonella* and poultry farms, which reaffirms the understanding of poultry to be a primary reservoir for this pathogen (Park et al., 2008). It should be noted, though, that three quarters of *Salmonella* recoveries were made at Sumas 1 which has a high concentration of upstream poultry facilities (Figure 4.1). The other recovery of this pathogen occurred at Serpentine 1, a site with no presence of any upstream poultry, but rather was downstream of a cow farm, which is a reminder that cows can also be a reservoir for *Salmonella* (Rodriguez et al., 2006), as well as the potential for it to be carried by wild birds (Andrés et al., 2013).

Taken together, these results suggest that the proximity to a livestock farm, a common source of these foodborne pathogens, increases the risk of foodborne pathogens being present in the irrigation water.

4.4.2 Weather patterns

The occurrence of any of the three pathogens was significantly correlated with precipitation on the day prior, and over the three days leading up to sample collection. This is consistent with previous studies that have found pathogen recovery to be higher after precipitation events (Gu et al., 2013; Haley et al., 2009; Jokinen et al., 2012; Jones et al., 2014; Nadya et al., 2016). The effect of rainfall is suggested to increase the transport of pathogens into surface waters (O'Shea and Field, 1992), and carry them longer distances downstream (Cooley et al., 2007); though it has been suggested that pathogens surviving in sediment reservoirs may also be released due to heavy rainfall (Cooley et al., 2007).

In this study, the correlation with precipitation was specifically observed within the Sumas watershed: Sumas 1 showed strong correlation between VTEC occurrence and precipitation the day before sample collection, and Sumas 2 and Sumas 3 showed a strong correlation between *L. monocytogenes* occurrence and precipitation before sample collection. In the Serpentine watershed, Site 2 showed significant correlation of both VTEC and *L. monocytogenes* with the total precipitation over the three days prior to sample collection. These pathogen specific correlations coincide with the pathogens most commonly recovered from their respective sample sites, implying that if an upstream reservoir exists (*e.g.*, shedding livestock), then precipitation may contribute to contamination.

Pathogen occurrence was also significantly correlated with lower average temperatures; though this observation was mostly site specific for Sumas 1 and Serpentine 2. It is difficult to determine

if these observations were actually affected by temperature or if they were related to other seasonal factors (*e.g.*, higher precipitation during the fall and winter), since, as discussed in chapter 2, seasonal variation in pathogen occurrence was also observed. Other studies have also pointed to seasonal and temperature related differences in recovery, but as higher occurrence of *L. monocytogenes* in the agricultural environment has repeatedly been observed during cooler periods (Cooley et al., 2014; Strawn et al., 2013a), the summer season and higher temperatures have been previously associated with increases in VTEC O157 (Gu et al., 2013) and *Salmonella* (Haley et al., 2009; Jokinen et al., 2012) occurrence from surface waters. This is of interest since temperature (Table 4.3) and seasonal differences (Chapter 2) were observed only for *L. monocytogenes* when reviewed on a pathogen specific level. It is possible that trends for the other two pathogens were not observed do to lower recovery for VTEC and *Salmonella* of only 4.9% and 2.7%, respectively compared to 11.2% for *L. monocytogenes*. The correlation observed for *L. monocytogenes* with temperature may also be confounded by precipitation since there was a significant correlation between the two factors ($r = 0.373$; $p < 0.001$), and precipitation was found to be highest during the fall and winter compared to the Spring and Summer (Fisher's LSD; $p < 0.05$). Another seasonally confounding variable, as previously mentioned in Chapter 2, is that during the summer growing season, dams are put in place to fill up the irrigation ditches, thereby reducing the water flow. As water flow has previously been suggested to be an important method for downstream dissemination (Cooley et al., 2007; O'Shea and Field, 1992), this fact cannot be discounted.

Overall, no definitive conclusions can be made about the relationship between precipitation and temperature, but as a logical mechanism for pathogen occurrence exists with precipitation, I would

suggest that rainfall is the dominant factor in relation to weather patterns. The potential for increased pathogen prevalence during colder temperatures cannot be discounted, but further study will be needed to tease these two factors apart in the future.

4.4.3 Water characteristics

The occurrence of any of the three pathogens was correlated with lower water temperatures at the time of sampling; however, there is no reason to believe that this observation is independent of the weather temperature correlation since we see the same trend: site specific correlations observed at Sumas 1 and Serpentine 2. It should be noted that water temperature at Sumas 1 also correlated with VTEC and *Salmonella* recovery whereas these correlations were not observed for the average air temperature. Since these two pathogens have been previously shown to occur more frequently in the warmer months, but also after significant precipitation in previous studies, (Gu et al., 2013; Haley et al., 2009; Jokinen et al., 2012; Jones et al., 2014), it is likely that the higher precipitation observed during the cooler temperatures in this study confounds this correlation.

Finally, pH was found to significantly correlate negatively with pathogen occurrence. On a pathogen and site specific level, however, this correlation was only significant with respect to *L. monocytogenes* occurrence at the Serpentine 2 sample site. The mean pH across sampling sites ranged from 7.11 to 7.72, but Serpentine 2 was singular in having a significantly lower mean pH than all of the other sample sites (Fisher's LSD; $p < 0.05$). Serpentine 2 also had the highest recovery of *L. monocytogenes*; likely driving this observed correlation. Closer to neutral pH has been shown to improve the survival of *L. monocytogenes* in the soil environment (Locatelli et al., 2013b; McLaughlin et al., 2011), but it remains to be seen if this trend holds for surface waters as

well. Furthermore, since the spread of pH across the samples did not vary largely, it could be suggested that this observed relationship is likely a coincidence or a correlation of minimal importance for pathogen prediction.

4.5 Conclusion

Correlations between pathogen occurrence and environmental factors were observed, which may allow for the potential prediction of pathogen occurrence in surface waters used for irrigation. While proximity and density of upstream livestock is a strong predictor of the likelihood of recovering VTEC, *Salmonella*, or *L. monocytogenes*, other sources such as wild animals or soil/sediment reservoirs cannot be discounted. Given the presence of an upstream reservoir for these pathogens, recent significant precipitation and/or cooler temperatures may increase the probability of their presence in the water. As temperature and precipitation were correlated together in this study, further investigation is required to determine if one or both of these factors is truly tied the presence of these pathogens; perhaps by only collecting samples after a minimum level of precipitation. With these data, further understanding of the mechanisms leading to these bacteria infecting the surface waters may be developed.

Chapter 5 – Conclusion and Future Directions

5.1 Conclusion

The occurrence and environmental factors affecting the presence of three foodborne pathogens (*i.e.*, verotoxigenic *Escherichia coli* (VTEC), *Salmonella*, and *Listeria monocytogenes*) in the surface waters used for produce irrigation were investigated in the Lower Mainland of British Columbia. All three pathogens were recovered from these waters, with *L. monocytogenes* being the most common with a 11.2% recovery, followed by VTEC and *Salmonella*, with 4.93% and 2.69% recoveries, respectively. Of concern is that serotypes commonly associated with human illnesses in British Columbia were recovered for all three pathogens. These results confirm the first hypothesis that VTEC, *Salmonella*, and *L. monocytogenes* are present in the surface waters used for irrigation in the Lower Mainland of British Columbia.

The second hypothesis was that the occurrence of these foodborne pathogens was not uniform across space or time. The occurrence of *L. monocytogenes* was observed to be higher in the Serpentine watershed (20.2%) compared to the Sumas watershed (4.65%), while a significant difference in the recovery of VTEC at individual sites was observed in both watersheds, and significant difference in occurrence of *Salmonella* was observed at individual sites within the Sumas watershed. Seasonally, *L. monocytogenes* was observed to occur more frequently during the fall (22.9%) and winter (16.3%) compared to the spring (7.8%) and summer (6.2%). These findings suggest that we can accept the hypothesis that pathogen occurrence is not uniform. Furthermore, they suggest that it may be possible to predict the occurrence of these pathogens based on local environmental factors such as weather and land use.

The third hypothesis was that higher numbers of total fecal coliforms (TFC) and/or generic *E. coli* can predict the presence of foodborne pathogens in surface waters. Statistically significant correlations with pathogen occurrence and indicator concentrations were observed, but primarily only when a 25-ml volume of water was sampled using a filtration method. The commonly used 1 ml method on 3M™ Petrifilm™ *E. coli*/Coliform count plates showed either reduced or non-existent correlations when compared to the filtration method. Water samples that were high for either indicator organism were more likely to be positive for the presence of any of the three pathogens, but pathogens were also commonly recovered from water samples containing low levels of indicators. The results suggest that high levels of indicators organisms indeed predict a higher risk for the presence of foodborne pathogens over the long run, but cannot accurately predict the presence or absence of foodborne pathogens at any particular point in time.

The final hypothesis was that environmental factors such as landscape and weather can predict the presence of foodborne pathogens in surface waters used for irrigation. Proximity and density of upstream animal agriculture (*i.e.*, cow or poultry) correlated highly with the occurrence of these three foodborne pathogens, emphasizing that domestic animals are common reservoirs of these bacteria. Precipitation within three days prior to sample collection also correlated with the occurrence of pathogens, especially at sites with overall higher pathogen occurrence, suggesting that rain may act as a catalyst for the contamination of waterways and transport downstream. Overall, these results suggest that being in close proximity to livestock, especially after a recent significant rainfall, may increase the risk of foodborne pathogens being present in the water.

In summary, foodborne pathogens associated with human illness are present in the waters used to irrigate fresh produce in British Columbia. These bacteria are most commonly present during the fall and winter months, but can also be found during the growing and harvesting seasons. The long-term trends of indicator organisms, as well as proximity and density of upstream sources of animal agriculture may provide an estimate of relative risk for a particular site. Water from high risk sites should be used with caution when irrigating crops, especially after significant rainfall over the three days beforehand.

5.2 Future directions

The present work revealed that pathogen occurrence is correlated to environmental factors affecting surface waters in the Lower Mainland of British Columbia, such as proximity to animal agriculture and recent precipitation, but further research is required to test whether these correlations are causative, and which may be confounded with other factors. Hypothesis driven studies designed to investigate each factor should be conducted before any definitive conclusions can be drawn.

One primary example was that it was not possible to separate correlations between seasonal occurrence and precipitation, as rainfall and pathogen occurrence were both more common during the fall and winter months. Precipitation provides a potential mechanism for transporting the pathogens downstream, but seasonal factors may also play a roll, such as the damming of waterways, and reducing water flow during the growing season. Whether one or both of these are dominant factors affecting pathogen occurrence remains to be determined.

While this study showed the presence of foodborne illness causing organisms in the waters used for irrigation in the Lower Mainland of British Columbia, it remains to be determined at what levels these organisms pose a significant risk to human health. To date, methods for detecting and isolating these pathogens are qualitative, often requiring a pre-enrichment step, and do not provide any information about pathogen density in water. Furthermore, many previous studies sampled different volumes of water, making comparisons between studies difficult. To this end, the development of novel methods that allow for the quantification of pathogens within a sample may help to better understand how concentration may vary across spatial and temporal lines, and at what concentrations these pathogens pose an actual risk to human health. New technologies able to quantify small concentrations of DNA, such as digital droplet PCR, may hold the key to developing new quantitative methods for these pathogens.

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Appendix

Appendix A – Water and geographical data used for statistical analysis

Table A.1 – Measured data from each collected samples, including the presence of verotoxigenic *Escherichia coli* (V), *Listeria monocytogenes* (L), *Salmonella* (S), or any of the three (A); the temperature, pH, and total dissolved solids (TDS) of the water samples; and the concentration of fecal indicators generic *E. coli* (EC) and fecal coliforms (FC) measured using 3M™ Petrifilm (PF) and a membrane filtration method (MF).

		Pathogens				Water Characteristics			Fecal Indicators			
Sampling Date	**Site**	**V**	**L**	**S**	**A**	**Temp. (°C)**	**pH**	**TDS (mg/ml)**	**EC PF (CFU/ml)**	**EC MF (CFU/ml)**	**FC PF (CFU/ml)**	**FC MF (CFU/ml)**
16/02/15	Sumas 1	+	-	-	+	10.4	/	/	/	/	/	/
16/03/15	Sumas 1	+	-	-	+	8.8	7.78	0.111	5.0	/	27.5	/
	Sumas 2	-	-	-	-	10.0	7.53	0.198	0.0	/	0.5	/
	Sumas 3	-	+	-	+	12.7	7.56	0.274	0.0	/	1.5	/
	Serpentine 2	-	+	-	+	10.8	6.66	0.111	3.5	/	15.5	/
08/04/15	Sumas 1	-	-	+	+	9.3	/	0.289	1.0	/	1.5	/
	Sumas 2	-	-	-	-	8.9	/	0.377	0.0	/	0.0	/
	Sumas 3	-	-	-	-	9.5	/	0.107	0.0	/	0.0	/
	Serpentine 2	-	+	-	+	8.5	/	0.244	2.5	/	8.0	/

/ = data point not measured for that particular sample

Table A.1 - Continued

		Pathogens				Water Characteristics			Fecal Indicators			
Sampling Date	**Site**	**V**	**L**	**S**	**A**	**Temp. (°C)**	**pH**	**TDS (mg/ml)**	**EC PF (CFU/ml)**	**EC MF (CFU/ml)**	**FC PF (CFU/ml)**	**FC MF (CFU/ml)**
08/20/15	Sumas 1	-	-	-	-	14.9	/	0.120	0.0	/	4.0	/
	Sumas 2	-	-	-	-	20.0	/	0.173	0.0	/	0.5	/
	Sumas 3	-	-	-	-	20.5	/	0.416	0.5	/	2.5	/
	Serpentine 2	-	-	-	-	16.5	/	0.127	0.0	/	12.0	/
04/05/15	Sumas 1	-	-	-	-	15.0	7.77	0.347	4.0	/	5.5	/
	Sumas 2	-	-	-	-	15.9	7.41	0.346	0.0	/	2.0	/
	Sumas 3	-	-	-	-	18.5	7.64	0.192	2.0	/	2.0	/
	Serpentine 2	+	-	-	+	17.0	7.39	0.663	17.5	/	27.5	/
19/05/15	Sumas 1	-	-	-	-	19.0	7.29	0.192	0.0	/	4.0	/
	Sumas 2	-	-	-	-	21.2	7.35	0.326	0.0	/	3.0	/
	Sumas 3	-	-	-	-	22.1	7.52	0.324	1.0	/	3.5	/
	Serpentine 1	-	-	-	-	19.0	7.22	0.359	0.5	/	3.5	/
	Serpentine 2	-	-	-	-	24.5	7.34	0.252	1.5	/	6.5	/
	Serpentine 3	-	-	-	-	19.2	7.62	0.167	0.0	/	4.5	/
01/06/15	Sumas 1	-	-	-	-	20.0	7.75	0.541	2.5	/	11.5	/
	Sumas 2	-	-	-	-	21.2	7.70	0.255	1.0	/	4.5	/
	Sumas 3	-	+	-	+	21.5	8.00	0.319	2.0	/	5.0	/
	Serpentine 1	-	-	-	-	17.1	7.23	0.401	2.0	/	18.5	/
	Serpentine 2	-	-	-	-	20.9	7.59	0.191	1.0	/	2.5	/
	Serpentine 3	-	-	-	-	16.8	7.83	1.171	1.0	/	11.5	/

Table A.1 - Continued

		Pathogens				Water Characteristics			Fecal Indicators			
Sampling Date	**Site**	**V**	**L**	**S**	**A**	**Temp. (°C)**	**pH**	**TDS (mg/ml)**	**EC PF (CFU/ml)**	**EC MF (CFU/ml)**	**FC PF (CFU/ml)**	**FC MF (CFU/ml)**
22/06/15	Sumas 1	-	-	-	-	25.7	8.89	0.166	1.5	0.3	1.5	0.3
	Sumas 2	-	-	-	-	23.1	8.02	0.303	0.0	0.2	2.0	0.4
	Sumas 3	-	-	-	-	23.5	7.94	0.375	0.0	0.2	1.5	0.4
	Serpentine 1	-	-	-	-	15.3	7.68	0.350	1.0	1.0	6.0	1.0
	Serpentine 2	-	-	-	-	18.5	7.68	0.185	0.5	0.1	1.5	0.7
	Serpentine 3	-	-	-	-	19.0	7.77	1.222	2.0	0.6	15.0	2.2
07/07/15	Sumas 1	-	-	-	-	23.5	7.73	0.210	0.0	0.3	3.5	0.0
	Sumas 2	-	-	-	-	24.1	7.73	0.311	0.0	0.2	2.0	0.0
	Sumas 3	-	-	-	-	24.5	7.85	0.366	0.0	0.2	8.0	0.0
	Serpentine 1	-	-	-	-	15.5	7.74	0.350	2.5	2.9	18.5	6.4
	Serpentine 2	-	-	-	-	19.2	7.62	0.304	1.0	1.2	5.5	0.1
	Serpentine 3	-	-	-	-	20.8	7.70	0.214	1.0	0.5	5.0	0.0
21/07/15	Sumas 1	-	-	-	-	21.2	8.10	1.069	3.5	/	4.0	3.8
	Sumas 2	-	-	-	-	23.0	7.93	0.168	0.0	/	0.0	0.0
	Sumas 3	-	-	-	-	23.0	8.21	0.446	0.5	/	4.5	2.4
	Sumas 4	-	-	-	-	21.1	8.11	0.370	2.0	/	2.0	3.5
	Serpentine 1	-	-	-	-	13.9	8.10	0.338	4.5	/	7.5	5.1
	Serpentine 2	-	-	-	-	19.1	8.29	0.288	7.0	/	12.0	4.2
	Serpentine 3	-	-	-	-	18.6	8.06	0.173	3.5	/	5.5	1.5

Table A.1 - Continued

		Pathogens				Water Characteristics			Fecal Indicators			
Sampling Date	**Site**	**V**	**L**	**S**	**A**	**Temp. (°C)**	**pH**	**TDS (mg/ml)**	**EC PF (CFU/ml)**	**EC MF (CFU/ml)**	**FC PF (CFU/ml)**	**FC MF (CFU/ml)**
10/18/15	Sumas 1	-	-	-	-	20.0	7.45	0.652	1.0	0.3	1.5	2.1
	Sumas 2	-	-	-	-	20.5	7.38	0.159	0.5	0.3	1.0	0.3
	Sumas 3	-	-	-	-	21.9	7.72	0.170	0.0	0.1	0.5	0.4
	Sumas 4	-	-	-	-	19.8	7.49	0.292	0.0	0.1	0.0	0.8
	Serpentine 1	-	+	-	+	15.0	7.72	0.372	0.5	0.6	0.5	2.4
	Serpentine 2	-	-	-	-	19.9	7.84	0.106	0.5	0.5	1.0	0.6
	Serpentine 3	-	-	-	-	19.0	7.77	0.174	2.0	1.8	6.5	6.1
24/08/15	Sumas 1	-	-	-	-	19.1	7.98	0.776	0.5	0.1	0.5	0.2
	Sumas 2	-	-	-	-	20.2	7.99	0.166	0.0	0.1	0.0	0.1
	Sumas 3	-	-	-	-	20.9	8.11	0.143	0.0	0.1	0.0	0.2
	Sumas 4	-	-	-	-	19.1	8.01	0.210	0.0	0.0	0.0	0.1
	Serpentine 1	-	-	-	-	14.0	8.11	0.164	0.5	0.0	0.5	0.1
	Serpentine 2	-	-	-	-	19.0	7.95	0.142	1.0	0.2	0.5	0.4
	Serpentine 3	-	-	-	-	18.1	7.97	0.327	0.0	0.1	0.0	0.1
03/09/15	Sumas 1	-	-	-	-	15.0	7.59	0.429	5.0	3.3	21.0	4.8
	Sumas 2	-	-	-	-	15.8	7.62	0.117	9.0	1.4	20.0	0.4
	Sumas 3	-	-	-	-	16.5	7.92	0.137	0.0	0.4	2.0	1.1
	Sumas 4	-	-	+	+	16.0	7.81	0.207	0.0	0.3	6.0	0.8
	Serpentine 1	-	+	+	+	13.8	7.54	0.194	1.5	2.7	18.0	3.6
	Serpentine 2	+	-	-	+	15.0	7.37	0.134	4.0	5.3	33.5	7.6
	Serpentine 3	-	+	-	+	14.4	7.51	0.222	0.5	1.6	6.5	3.5

Table A.1 - Continued

		Pathogens				Water Characteristics			Fecal Indicators			
Sampling Date	**Site**	**V**	**L**	**S**	**A**	**Temp. (°C)**	**pH**	**TDS (mg/ml)**	**EC PF (CFU/ml)**	**EC MF (CFU/ml)**	**FC PF (CFU/ml)**	**FC MF (CFU/ml)**
23/09/15	Sumas 1	-	-	-	-	13.1	7.75	0.132	0.0	0.3	0.0	0.8
	Sumas 2	-	-	-	-	14.3	7.64	0.121	/	/	/	/
	Sumas 3	-	-	-	-	13.3	8.01	/	0.0	0.0	3.0	0.1
	Sumas 4	-	-	-	-	14.8	7.83	/	1.0	1.3	3.0	2.6
	Serpentine 1	-	+	-	+	9.8	7.76	/	1.0	2.5	20.5	8.4
	Serpentine 2	-	-	-	-	13.6	7.50	/	1.5	1.3	5.0	3.1
	Serpentine 3	-	-	-	-	12.1	7.69	/	0.0	1.0	1.5	3.5
13/10/15	Sumas 1	-	-	-	-	13.9	7.75	/	2.0	0.9	2.0	3.3
	Sumas 2	-	-	-	-	15.2	7.82	/	1.0	0.1	1.0	1.1
	Sumas 3	-	-	-	-	15.3	7.59	/	0.0	0.4	0.0	0.6
	Sumas 4	-	-	-	-	14.8	7.79	/	1.0	0.3	0.5	2.6
	Serpentine 1	-	-	-	-	13.6	7.63	/	0.5	0.4	0.5	1.2
	Serpentine 2	-	+	-	+	14.1	7.43	/	7.0	3.6	6.5	9.7
	Serpentine 3	-	+	-	+	14.1	7.51	/	6.0	3.2	6.0	9.3
09/11/15	Sumas 1	-	-	-	-	9.0	7.74	0.073	0.0	0.5	1.5	1.4
	Sumas 2	-	-	-	-	8.8	7.76	0.124	0.0	0.2	0.0	0.8
	Sumas 3	-	-	-	-	7.8	8.05	0.166	0.5	0.1	0.5	0.6
	Sumas 4	-	-	-	-	7.9	7.85	0.269	0.0	0.3	0.0	0.4
	Serpentine 1	-	-	-	-	8.2	7.28	0.167	0.0	0.0	0.0	0.4
	Serpentine 2	-	+	-	+	8.9	3.78	1.222	0.0	0.0	0.0	0.1
	Serpentine 3	-	-	-	-	9.0	7.57	0.067	2.0	1.0	2.0	3.9

Table A.1 - Continued

		Pathogens				Water Characteristics			Fecal Indicators			
Sampling Date	**Site**	**V**	**L**	**S**	**A**	**Temp. (°C)**	**pH**	**TDS (mg/ml)**	**EC PF (CFU/ml)**	**EC MF (CFU/ml)**	**FC PF (CFU/ml)**	**FC MF (CFU/ml)**
24/11/15	Sumas 1	+	+	-	+	7.4	7.44	0.067	3.0	1.4	5.0	2.8
	Sumas 2	-	-	-	-	7.1	7.57	0.103	0.0	0.1	0.0	0.1
	Sumas 3	-	-	-	-	4.9	7.88	0.154	0.0	0.1	0.0	0.1
	Sumas 4	-	-	-	-	5.1	7.60	0.224	1.0	0.9	1.0	0.9
	Serpentine 1	-	-	-	-	6.2	7.01	0.148	0.0	0.1	0.0	0.2
	Serpentine 2	-	-	-	-	6.3	6.26	0.456	0.0	0.0	0.0	0.0
	Serpentine 3	-	+	-	+	6.8	7.21	0.072	4.5	6.2	5.0	9.1
01/12/15	Sumas 1	-	-	-	-	/	7.81	0.255	0.0	0.4	0.0	0.4
	Sumas 2	-	-	-	-	/	7.68	0.092	0.0	0.0	0.0	0.0
	Sumas 3	-	-	-	-	/	7.99	0.307	0.0	0.1	0.0	0.1
	Sumas 4	-	-	-	-	/	7.83	0.174	0.0	0.3	0.0	0.4
	Serpentine 1	-	-	-	-	/	7.68	0.068	0.0	0.1	0.0	0.1
	Serpentine 2	-	-	-	-	/	7.50	0.492	0.0	0.0	0.0	0.0
	Serpentine 3	-	-	-	-	/	8.03	0.279	1.0	2.0	1.0	2.6
15/12/15	Sumas 1	-	-	-	-	7.2	7.61	0.090	0.0	0.4	0.0	0.6
	Sumas 2	-	-	-	-	9.9	7.88	0.189	0.0	0.0	0.0	0.0
	Sumas 3	-	-	-	-	8.0	7.84	0.256	0.0	0.2	0.0	0.2
	Sumas 4	-	-	-	-	7.5	7.57	0.135	0.0	0.0	0.0	0.2
	Serpentine 1	-	-	-	-	7.5	7.33	0.113	0.0	0.1	0.0	0.3
	Serpentine 2	-	+	-	+	7.9	5.60	0.444	0.0	0.1	0.0	0.4
	Serpentine 3	-	-	-	-	7.9	7.39	0.078	0.0	1.1	0.5	2.8

Table A.1 - Continued

		Pathogens				Water Characteristics			Fecal Indicators			
Sampling Date	**Site**	**V**	**L**	**S**	**A**	**Temp. (°C)**	**pH**	**TDS (mg/ml)**	**EC PF (CFU/ml)**	**EC MF (CFU/ml)**	**FC PF (CFU/ml)**	**FC MF (CFU/ml)**
12/01/16	Sumas 1	-	-	-	-	5.9	7.34	0.165	1.5	1.1	1.5	1.4
	Sumas 2	-	-	-	-	5.0	7.09	0.081	0.0	0.0	0.0	0.0
	Sumas 3	-	-	-	-	2.7	7.38	0.207	0.0	0.0	0.0	0.1
	Sumas 4	-	-	-	-	4.2	7.52	0.208	2.5	3.9	5.5	5.4
	Serpentine 1	-	+	-	+	5.0	6.65	0.124	0.0	0.4	0.0	1.3
	Serpentine 2	-	-	-	-	4.8	6.50	0.335	0.0	0.5	0.0	0.9
	Serpentine 3	-	-	-	-	5.2	7.00	0.171	5.0	5.3	6.0	7.5
26/01/16	Sumas 1	-	-	-	-	7.8	7.62	0.117	0.5	0.3	0.5	0.4
	Sumas 2	-	-	-	-	8.3	7.86	0.179	0.0	0.0	0.0	0.0
	Sumas 3	-	-	-	-	6.8	7.96	0.104	0.0	0.0	0.0	0.0
	Sumas 4	-	-	-	-	6.9	7.70	0.195	0.0	0.1	0.0	0.2
	Serpentine 1	-	+	-	+	8.1	7.79	0.135	11.5	6.3	15.5	11.1
	Serpentine 2	-	-	-	-	7.2	7.55	0.288	1.5	0.6	29.0	3.6
	Serpentine 3	-	-	-	-	8.0	7.76	0.079	6.5	3.5	8.5	5.4
02/02/16	Sumas 1	-	-	+	+	6.8	7.49	0.070	0.5	0.9	1.0	1.6
	Sumas 2	-	-	-	-	7.1	7.44	0.155	0.0	0.0	0.0	0.1
	Sumas 3	-	-	-	-	4.8	7.66	0.193	0.0	0.0	0.0	0.0
	Sumas 4	-	-	-	-	5.2	7.38	0.114	0.0	0.2	0.0	0.2
	Serpentine 1	-	-	-	-	6.2	6.74	0.103	2.0	3.4	4.5	4.0
	Serpentine 2	-	+	-	+	6.2	6.55	0.322	0.0	0.1	0.5	0.6
	Serpentine 3	-	-	-	-	6.9	7.46	0.081	7.0	6.6	9.0	8.1

Table A.1 - Continued

		Pathogens				Water Characteristics			Fecal Indicators			
Sampling Date	**Site**	**V**	**L**	**S**	**A**	**Temp. (°C)**	**pH**	**TDS (mg/ml)**	**EC PF (CFU/ml)**	**EC MF (CFU/ml)**	**FC PF (CFU/ml)**	**FC MF (CFU/ml)**
16/02/16	Sumas 1	+	-	+	+	8.9	7.24	0.077	3.5	5.0	4.0	8.1
	Sumas 2	-	+	-	+	8.8	7.36	0.139	2.0	1.5	2.0	2.5
	Sumas 3	-	+	-	+	8.9	7.36	0.088	1.5	1.8	1.5	3.9
	Sumas 4	-	-	-	-	8.8	7.14	/0.016	4.0	3.1	5.5	4.7
	Serpentine 1	-	-	-	-	7.5	6.51	0.031	1.5	1.1	1.5	1.8
	Serpentine 2	+	+	-	+	8.1	6.82	0.167	14.0	7.0	17.0	7.7
	Serpentine 3	-	-	-	-	8.1	7.29	0.064	1.5	2.3	2.0	1.9
0/03/16	Sumas 1	-	-	-	-	8.0	7.07	0.122	0.0	1.4	0.5	1.6
	Sumas 2	-	-	-	-	7.0	7.12	0.187	0.0	0.1	0.0	0.1
	Sumas 3	-	-	-	-	7.5	6.98	0.301	1.0	0.4	1.0	0.4
	Sumas 4	-	-	-	-	7.8	6.91	0.212	0.0	0.1	0.0	0.1
	Serpentine 1	-	-	-	-	7.9	6.81	0.215	2.0	1.9	3.5	2.5
	Serpentine 2	+	+	-	+	7.9	6.22	0.298	2.0	4.2	2.0	4.2
	Serpentine 3	-	-	-	-	8.0	6.86	0.105	1.0	3.1	1.0	3.7
30/03/16	Sumas 1	-	-	-	-	12.1	7.35	0.122	0.5	/	0.5	0.2
	Sumas 2	-	-	-	-	12.1	6.75	0.129	0.0	/	0.0	0.9
	Sumas 3	-	-	-	-	15.8	7.03	0.178	0.0	/	0.0	0.0
	Sumas 4	-	-	-	-	16.0	7.06	0.161	0.0	/	0.0	0.4
	Serpentine 1	-	-	-	-	9.0	6.84	0.163	0.0	/	0.0	1.8
	Serpentine 2	-	-	-	-	9.8	6.59	0.360	0.5	/	1.0	3.3
	Serpentine 3	-	-	-	-	10.9	7.08	0.136	1.5	/	1.5	1.1

Table A.1 - Continued

		Pathogens				Water Characteristics			Fecal Indicators			
Sampling Date	**Site**	**V**	**L**	**S**	**A**	**Temp. (°C)**	**pH**	**TDS (mg/ml)**	**EC PF (CFU/ml)**	**EC MF (CFU/ml)**	**FC PF (CFU/ml)**	**FC MF (CFU/ml)**
14/04/16	Sumas 1	-	-	-	-	10.1	7.72	0.144	0.5	1.8	0.5	2.3
	Sumas 2	-	-	-	-	10.4	7.37	0.165	0.0	0.0	0.0	0.0
	Sumas 3	-	-	-	-	10.5	7.62	0.530	1.0	1.9	1.5	4.0
	Sumas 4	-	-	-	-	11.0	7.67	0.191	0.5	1.1	0.5	1.7
	Serpentine 1	-	-	-	-	9.8	7.17	0.191	0.0	0.4	0.5	0.6
	Serpentine 2	+	-	-	+	10.4	7.17	0.480	2.5	3.8	5.5	8.3
	Serpentine 3	-	+	-	+	10.2	7.37	0.141	15.0	7.2	15.0	9.2
26/04/16	Sumas 1	-	-	-	-	14.9	7.66	0.156	1.0	1.0	0.5	1.3
	Sumas 2	-	-	-	-	15.1	7.39	0.149	1.0	0.6	1.0	2.1
	Sumas 3	-	-	-	-	22.4	8.21	0.535	0.0	0.7	0.0	1.7
	Sumas 4	-	-	-	-	13.6	7.51	0.137	1.0	0.9	1.0	1.0
	Serpentine 1	-	-	-	-	14.9	6.95	0.216	0.0	0.1	0.0	0.3
	Serpentine 2	-	-	-	-	16.2	7.30	0.643	4.5	3.5	4.0	7.9
	Serpentine 3	-	-	-	-	12.7	7.75	0.141	1.5	1.5	1.5	1.7
02/05/16	Sumas 1	-	-	-	-	15.9	7.37	0.114	1.0	0.8	1.5	2.2
	Sumas 2	-	-	-	-	17.1	7.31	0.125	0.5	0.4	0.5	0.7
	Sumas 3	-	-	-	-	19.5	7.25	0.156	0.5	0.4	0.5	1.6
	Sumas 4	-	-	-	-	14.7	7.07	0.118	1.5	1.0	1.5	2.0
	Serpentine 1	-	-	-	-	15.2	6.84	0.207	0.0	0.0	0.0	0.6
	Serpentine 2	-	-	-	-	18.0	7.01	0.691	0.5	1.7	0.5	1.9
	Serpentine 3	-	-	-	-	16.0	7.43	0.155	2.5	2.3	3.5	4.2

Table A.1 - Continued

		Pathogens				Water Characteristics			Fecal Indicators			
Sampling Date	**Site**	**V**	**L**	**S**	**A**	**Temp. (°C)**	**pH**	**TDS (mg/ml)**	**EC PF (CFU/ml)**	**EC MF (CFU/ml)**	**FC PF (CFU/ml)**	**FC MF (CFU/ml)**
17/05/16	Sumas 1	-	-	-	-	15.9	7.83	0.143	0.5	0.3	0.5	0.6
	Sumas 2	-	-	-	-	17.7	7.92	0.191	0.5	0.3	0.5	1.0
	Sumas 3	+	-	-	+	17.0	7.87	0.195	0.0	0.2	0.0	0.4
	Sumas 4	-	-	-	-	15.3	7.72	0.137	0.0	0.2	0.0	0.3
	Serpentine 1	-	-	-	-	13.6	7.29	0.213	0.0	0.1	0.5	0.2
	Serpentine 2	-	-	-	-	15.8	7.43	0.706	0.0	0.1	0.0	0.1
	Serpentine 3	-	-	-	-	14.0	7.82	0.135	42.0	/	42.0	/
07/06/16	Sumas 1	-	-	-	-	21.2	7.61	0.325	0.0	0.3	0.5	1.0
	Sumas 2	-	-	-	-	22.1	7.62	0.218	0.0	0.3	0.0	0.9
	Sumas 3	-	-	-	-	19.9	7.71	0.316	3.0	2.1	4.5	3.9
	Sumas 4	-	-	-	-	19.9	7.57	0.123	0.5	0.2	1.5	0.5
	Serpentine 1	-	-	-	-	16.0	7.27	0.235	1.5	0.5	2.0	1.3
	Serpentine 2	-	-	-	-	20.2	7.23	0.520	1.5	1.0	2.0	1.7
	Serpentine 3	-	-	-	-	18.9	7.59	0.135	3.5	2.2	4.0	4.0
20/06/16	Sumas 1	-	-	-	-	14.9	7.50	0.296	1.5	3.5	1.5	2.3
	Sumas 2	-	-	-	-	17.1	7.71	0.378	2.0	0.2	0.0	0.2
	Sumas 3	-	-	-	-	15.4	7.66	0.339	12.0	0.3	0.0	0.2
	Sumas 4	-	-	-	-	17.5	7.36	0.107	1.5	1.2	0.0	0.5
	Serpentine 1	-	-	-	-	19.6	7.38	0.193	71.5	/	49.0	/
	Serpentine 2	-	-	-	-	21.0	7.42	0.438	4.0	3.6	4.0	2.7
	Serpentine 3	-	-	-	-	16.5	6.85	0.133	60.5	/	25.5	/

Table A.1 - Continued

		Pathogens				Water Characteristics			Fecal Indicators			
Sampling Date	**Site**	**V**	**L**	**S**	**A**	**Temp. (°C)**	**pH**	**TDS (mg/ml)**	**EC PF (CFU/ml)**	**EC MF (CFU/ml)**	**FC PF (CFU/ml)**	**FC MF (CFU/ml)**
05/07/16	Sumas 1	-	-	-	-	16.9	7.47	0.113	2.5	1.1	2.5	0.5
	Sumas 2	-	-	-	-	17.3	7.67	0.345	1.5	0.9	0.5	0.6
	Sumas 3	-	+	-	+	17.0	7.63	0.415	1.0	0.3	0.5	0.3
	Sumas 4	-	-	-	-	16.9	7.48	0.135	1.0	0.5	1.0	0.4
	Serpentine 1	-	-	-	-	14.4	7.75	0.180	1.5	2.5	1.5	1.7
	Serpentine 2	-	+	-	+	17.2	7.31	0.357	7.5	5.9	6.5	3.9
	Serpentine 3	-	-	-	-	16.0	7.97	0.079	1.5	1.7	1.5	1.3
19/07/16	Sumas 1	-	-	-	-	19.2	7.57	0.170	0.5	1.0	1.5	3.0
	Sumas 2	-	-	-	-	20.9	7.79	0.430	0.0	0.2	0.5	0.2
	Sumas 3	-	-	-	-	20.5	7.75	0.471	0.0	0.4	1.5	0.5
	Sumas 4	-	-	-	-	18.1	7.60	0.144	0.5	0.9	1.0	1.4
	Serpentine 1	-	-	-	-	15.8	7.67	0.180	3.5	3.0	4.0	3.5
	Serpentine 2	-	-	-	-	17.1	7.67	0.289	1.0	0.2	1.0	0.3
	Serpentine 3	-	-	-	-	18.8	7.80	0.158	2.5	2.6	2.5	4.4
08/08/16	Sumas 1	-	-	-	-	15.8	7.57	0.419	0.5	1.0	1.5	3.0
	Sumas 2	-	-	-	-	20.2	7.79	0.254	0.0	0.2	0.5	0.2
	Sumas 3	-	-	-	-	18.8	7.75	0.308	0.0	0.4	1.5	0.5
	Sumas 4	-	-	-	-	21.1	7.60	0.111	0.5	0.9	1.0	1.4
	Serpentine 1	-	-	-	-	21.7	7.67	0.159	3.5	3.0	4.0	3.5
	Serpentine 2	-	-	-	-	22.2	7.67	0.533	1.0	0.2	1.0	0.3
	Serpentine 3	-	-	-	-	19.5	7.80	0.113	2.5	2.6	2.5	4.4

Table A.1 - Continued

		Pathogens				Water Characteristics			Fecal Indicators			
Sampling Date	**Site**	**V**	**L**	**S**	**A**	**Temp. (°C)**	**pH**	**TDS (mg/ml)**	**EC PF (CFU/ml)**	**EC MF (CFU/ml)**	**FC PF (CFU/ml)**	**FC MF (CFU/ml)**
22/08/16	Sumas 1	-	-	-	-	19.8	7.72	0.320	2.0	1.0	4.0	1.8
	Sumas 2	-	-	-	-	20.9	7.81	0.340	1.0	0.4	1.0	0.5
	Sumas 3	-	-	-	-	20.2	7.54	0.348	0.0	0.1	0.0	0.1
	Sumas 4	-	-	+	+	19.8	7.37	0.131	2.5	1.4	2.5	2.5
	Serpentine 1	-	+	-	+	15.1	7.21	0.170	0.0	0.9	0.0	0.8
	Serpentine 2	-	-	-	-	17.8	7.39	0.441	0.5	0.1	0.5	0.7
	Serpentine 3	+	-	-	+	17.1	7.52	0.136	1.0	0.5	1.0	0.7

Table A.2 – Data used to measure proximity and density of upstream livestock.

	Serpentine			Sumas		
	Site 1	**Site 2**	**Site 3**	**Site 1**	**Site 2**	**Site 3**
Nearest Livestock (km)[A]						
Cow[B]	0.93	0.423	1.46	1.17	1.79	1.15
Poultry[C]	-	1.34	-	0.93	1.48	3.10
Other[D]	-	-	-	-	-	0.08
Number within 1 km[E]						
Cow	1	1	1	0	0	0
Poultry	0	0	0	1	0	0
Other	0	0	0	0	0	1
Number within 2 km						
Cow	1	3	1	2	1	3
Poultry	0	2	0	2	1	0
Other	1	0	0	0	0	1
Number within 3 km						
Cow	1	3	1	3	2	3
Poultry	0	2	0	4	1	0
Other	1	0	0	0	0	1
Border within 1 km[F] (km)						
Cow	0.214	1.153	0	0	0	0
Poultry	0	0	0	0.078	0	0
Other	0	0	0	0	0	0.318
Border within 2 km (km)						
Cow	0.214	2.36	1.838	0.450	0.368	1.465
Poultry	0	0.119	0	0.719	0.152	0
Other	0	0	0	0	0	0.318
Border within 3 km (km)						
Cow	0.214	2.904	1.905	1.133	1.203	3.008
Poultry	0	0.412	0	0.792	0.141	0
Other	0	0	0	0	0	0.318

[A]Nearest distance for surface water to travel
[B]Includes both dairy and beef cattle
[C]Includes chicken and turkey
[D]Includes swine, sheep, or goats
[E]Number of properties bordering the surface water within a given distance upstream
[F]Total length of waterway bordered by properties containing livestock within a given distance