The occurrence and sources of *Campylobacter* spp., *Salmonella enterica* and *Escherichia coli* O157:H7 in the Salmon River, British Columbia, Canada

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ABSTRACT

In this study, we wished to assess the prevalence and determine the sources of three zoonotic bacterial pathogens (*Salmonella*, *Campylobacter*, and *Escherichia coli* O157:H7) in the Salmon River watershed in southwestern British Columbia. Surface water, sewage, and animal faecal samples were collected from the watershed. Selective bacterial culture and PCR techniques were used to isolate these three pathogens and indicator bacteria from these samples and characterize them. *Campylobacter* was the most prevalent pathogen in all samples, followed by *Salmonella*, and *E. coli* O157:H7. *E. coli* O157:H7 and *Salmonella* isolation rates from water, as well as faecal coliform densities correlated positively with precipitation, while *Campylobacter* isolation rates correlated negatively with precipitation. Analysis of DNA extracted from water samples for the presence of *Bacteroides* host-species markers, and comparisons of *C. jejuni flaA*-RFLP types and *Salmonella* serovars from faecal and water samples provided evidence that human sewage and specific domestic and wild animal species were sources of these pathogens; however, in most cases the source could not be determined or more than one source was possible. The frequent isolation of these zoonotic pathogens in the Salmon River highlights the risks to human health associated with intentional and unintentional consumption of untreated surface waters.

Key words | *Bacteroides-Prevotella*, *Campylobacter*, *Escherichia coli* O157:H7, faecal coliforms, *Salmonella*, surface water

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TERMS AND ABBREVIATIONS

AU	animal units
BB	Bolton's broth
BGS	brilliant green sulpha agar
BPW	buffered peptone water
CCDA	charcoal cefoperazone deoxycholate agar

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CO	Coghlan Creek
DA	Davidson Creek
GITC	guanidium isothiocyanate
MIO	motility-indole-ornithine medium
MST	microbial source tracking
MSRV	modified semi-solid RV agar
PT	phage type
RFLP	restriction fragment length polymorphism
RV	Rappaport Vassiliadis broth
SF	Salmon Fish Counter
SH	Salmon Headwaters
TSI	triple sugar iron agar
TT	tetrathionate
UPGMA	unweighted pair group method with arithmetic mean

INTRODUCTION

Water contaminated with animal wastes has been linked to outbreaks of waterborne disease around the world (Hrudey *et al.* 2002). Well-water sources under the influence of contaminated surface water are responsible for a disproportionate number of cases of waterborne disease outbreaks (Blackburn *et al.* 2004; Smith *et al.* 2006). For example, in Walkerton Ontario, failure to adequately treat drinking water, intense rains, and surface water contaminated with cattle manure were responsible for a community outbreak of *E. coli* O157:H7 and *Campylobacter* infections which resulted in an estimated 2300 cases of gastrointestinal illness and seven deaths (Hrudey *et al.* 2002; Auld *et al.* 2004). In addition, the number of waterborne disease outbreaks associated with contaminated surface water used for recreational activities and irrigation of vegetable crops has been on the rise (Yoder *et al.* 2008*a*).

The Salmon River watershed is located in the township of Langley in the lower mainland of British Columbia and contains numerous small farms and settlements. Beef, dairy, and sheep farms, as well as "hobby farms" dominate the area. The township's wastewater treatment plant is located approximately 5 km northeast of the watershed; however, many residents rely on private septic systems rather than a municipal system. There are more than 3200 septic systems in the Salmon River watershed (Wernick *et al.* 1998). The aquifer-fed Salmon River passes through the township downstream of the Salmon River headwaters, and serves as a drinking water source for Langley residents. Although surface water used for drinking is generally treated, a large proportion of Langley residents drink untreated well water.

An understanding of the relative contributions of pathogenic bacteria in faeces of different animal species in this area would be a logical precursor to any source abatement strategy. In addition, the application of bacterial pathogen typing (Ertas *et al.* 2004; Takahashi *et al.* 2006; Wilson *et al.* 2008; Hannon *et al.* 2009; Huang *et al.* 2009; Sheppard *et al.* 2009) and direct detection of host-species specific bacterial DNA (Bernhard & Field 2000*b*; Dick *et al.* 2005*a*) could be useful in identifying sources (microbial source tracking; MST) of faecal contamination in surface waters. While library- and culture-independent MST methods are inexpensive, quick, and easy to use (Bernhard & Field 2000*b*), molecular subtyping methods for pathogens may provide confirmation, and in some cases greater discrimination in the identification of faecal sources of contamination. In this context, we undertook a detailed analysis of the prevalence and the characteristics of pathogens isolated from surface waters and animal faecal matter, and the distribution of various host-species specific MST markers in these surface waters. The specific purposes of this study were to: 1) assess the prevalence of the zoonotic bacterial pathogens *Salmonella* spp., *Campylobacter* spp., and *Escherichia coli* O157:H7 in these surface waters, 2) explore the spatial and temporal factors associated with the presence of these three pathogens in these surface waters, and 3) determine the animal sources of water contamination with these bacterial pathogens using pathogen typing and 16S rDNA *Bacteroides-Prevotella* host species-specific markers (Bernhard & Field 2000*a*; Dick *et al.* 2005*a*,*b*).

MATERIALS AND METHODS

Water and faecal sampling

Between September and November 2004–2006, 186 surface water samples were collected from four stations within the Salmon River watershed (Figure 1). Station SH (Headwaters $n = 30$) is located upstream of station SF (Fish Counter $n = 54$) on the main stem of the Salmon River.

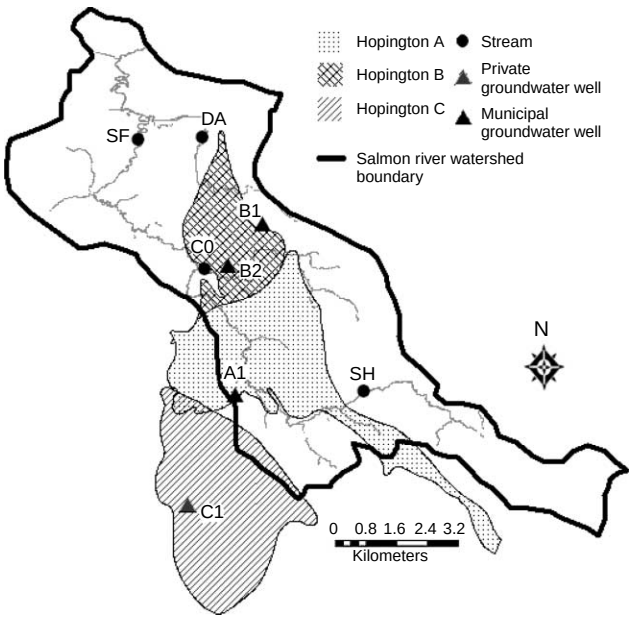

Figure 1 | Map of the Salmon River watershed and the Hopington aquifer. Stream sampling locations include SF (Salmon Fish Counter), DA (Davidson), and CO (Coghlan Creek) for north Langley, and SH (Salmon Headwaters) for central Langley. A1 = municipal groundwater well; B1 and B2 = private groundwater wells. The septic systems sampled are dispersed throughout Hopington aquifers A and B.

Stations CO (Coghlan Creek $n = 71$) and DA (Davidson Creek $n = 31$) enter the Salmon River downstream of station SH and upstream of station SF. Water samples were collected through the water column at a depth of 30–100 cm, using sterile polyethylene glycol autoclavable water bottles. Raw sewage ($n = 11$) was sampled to represent potential human sources of faecal contamination. Six of the raw sewage samples were collected over three months from the Langley sewage treatment plant located approximately 5 km north-east of the watershed. The remaining five raw sewage samples were composites from eight different local septic systems located within Hopington aquifers A and B (Figure 1). Animal faecal samples were obtained fresh from species found within the watershed (alpaca $n = 9$, cattle $n = 33$, dog $n = 17$, chicken $n = 17$, duck $n = 23$, horse $n = 23$, goat $n = 13$, goose $n = 13$, llama $n = 10$, sheep $n = 42$, pig $n = 5$, rabbit $n = 11$, rat $n = 1$). Water, sewage, and faecal samples were placed on ice upon collection, shipped to the Lethbridge, Alberta laboratory, and analysed within 24 h.

To estimate animal numbers in the watershed, we used the sub-division census data for north (SF, DA, CO) and central (SH) Langley (Statistics Canada 2007). Data from these regions most accurately match the watershed, covering approximately 70% of its total area. Animal units (AU) for each subdivision were approximated by multiplying the number of animals by an animal unit factor for the specific type of animal (Table 1). Animal unit factors are based on the amount of manure produced by an animal of a certain average weight. An adult cow weighing approximately 1,000 lb or 454 kg has an AU factor of 1; therefore, to calculate AU factors for other animals, the average weight of that animal in pounds or kilograms was divided by 1,000 or 454, respectively (Delaware Department of Agriculture 2001; Minnesota Department of Agriculture 2007). The types of animals (e.g. roasting versus broiler chickens) were not publicly available; therefore, some assumptions were made for the calculation of AU. It was assumed that the proportion of cattle to calves would be similar in north and central Langley, so an AU factor of 1 was used for all cattle. Similarly, the AU factor used for hens and chickens was that which refers to chickens weighing more than 2.3 kg, and the AU factor used for swine was that which refers to pigs weighing more than 136 kg (Minnesota Department of Agriculture 2007).

Faecal coliform enumeration

A 100 ml aliquot of each water sample was filtered through a 0.45 μm membrane filter and cultured on M-FC Agar at

Table 1 | Numbers of animal livestock units present in central and north Langley. Animal unit factors used: hens and chickens over 2.3 kg = 0.005, pigs over 136 kg = 0.40, sheep and lambs = 0.10, goats = 0.17, llamas = 0.35, horses = 1.00, cattle and calves = 1.00, dairy cows = 1.40. Stream sampling locations include SF (Salmon Fish Counter), DA (Davidson), and CO (Coghlan Creek) for north Langley, and SH (Salmon Headwaters) for central Langley

Animal type	Animal units/ha SH (Central Langley)	CO, DA, SF (North Langley)
Hens & Chickens	0.69	0.17
Pigs	0.0080	0.24
Sheep & Lambs	0.022	0.017
Goats	0.010	0.014
Llamas	0.011	0.0035
Horses	0.32	0.50
Cattle & Calves	0.86	0.69
Dairy Cows	0.18	0.31
Total units/ha	2.11	1.95

44.5°C for 22–26 h. Faecal coliforms were enumerated by counting colonies with various shades of blue on the filter (Eaton *et al.* 2005).

Sample preparation for pathogen enrichment and *Bacteroides* 16S marker detection

Five grams of faecal matter were added to 5 ml of phosphate buffered saline (137 mmol l^{-1} NaCl, 8.1 mmol l^{-1} Na_2HPO_4, 1.5 mmol l^{-1} KH_2PO_4, 2.7 mmol l^{-1} KCl, pH 7.4) and mixed well. One millilitre of the mixture was added to 20 ml of buffered peptone water (BPW; Oxoid Ltd., Basingstoke, Hampshire, England) for the pre-enrichment of *E. coli* O157:H7 (Chapman *et al.* 1994) and *Salmonella* spp. (D'Aoust & Purvis 1998), and also to 20 ml of Bolton's broth (BB; Oxoid Ltd., Basingstoke, Hampshire, England) for the enrichment of *Campylobacter* spp. (Diergaardt *et al.* 2004).

Three–500 ml aliquots of water samples were vacuum-filtered through 0.45 μm sterile nitrocellulose filters (Pall Corporation, Ann Arbor, MI). One filter was immersed into 20 ml of BPW for the pre-enrichment of *E. coli* O157:H7 (Chapman *et al.* 1994) and *Salmonella* spp. (D'Aoust & Purvis 1998). A second filter was immersed into 20 ml of BB for the enrichment of *Campylobacter* spp. (Diergaardt *et al.* 2004). The third filter was placed into a 15 ml Falcon tube (Becton Dickinson Labware, Franklin Lakes, NJ) and coated with 500 μl of guanidium isothiocyanate buffer (GITC; 5 mol l^{-1} guanidine isothiocyanate, 100 mmol l^{-1} ethylenediamine tetraacetic acid, 0.5% sarkosyl) to preserve nucleic acids for culture-independent analyses. Filters in GITC buffer were incubated in a 70°C water bath for 20 min to lyse bacteria and destroy nucleases, and then frozen at − 20°C until DNA could be extracted. The culture-independent analyses were performed on water samples collected during 2006 only.

Pathogen isolation and detection in water and faecal samples

A combination of the methods of D'Aoust & Purvis (1998) and of DeSmedt & Bolderdijk (1987) was employed for the isolation of *Salmonella*. The BPW cultures described above were incubated for 18–20 h at 37°C, and 1 ml of the culture was then inoculated into 9 ml of tetrathionate (TT) broth (Oxoid Ltd., Basingstoke, Hampshire, England), and 0.1 ml was inoculated into 9.9 ml of Rappaport Vassiliadis broth (RV; Oxoid Ltd., Basingstoke, Hampshire, England). The RV and TT broths were incubated at 42°C for 24 h. Approximately 0.02 ml of both broth cultures were then inoculated onto the surface of brilliant green sulpha agar (BGS; Becton Dickinson, Franklin Lakes, NJ), and 0.02 ml of RV broth culture was pipetted onto the surface of modified semi-solid RV agar (MSRV; Oxoid Ltd., Basingstoke, Hampshire, England DeSmedt & Bolderdijk 1987; Poppe *et al.* 2004; USEPA 2006). Following incubation of the MSRV culture, a loop of motile bacteria from the outer edge of the MSRV inocula was streaked onto BGS agar plates. Both BGS and MSRV agar plates were incubated at 42°C for 24 h. Presumptive *Salmonella* spp. were subcultured onto MacConkey agar (Oxoid Ltd., Basingstoke, Hampshire, England) and incubated at 37°C for 24 h, and used to inoculate the confirmatory tests Christensen's urea and triple sugar iron (TSI; Oxoid Ltd., Basingstoke, Hampshire, England) agars, lysine decarboxylase broth and motility-indole-ornithine medium (MIO; Becton Dickinson, Sparks, MD; Davis & Morishita 2005). Isolates were confirmed as *Salmonella enterica* subsp. *enterica* by PCR detection of the virulence genes *invA* and *spvC* (Chiu & Ou 1996). Serotyping was performed by the Public Health Agency of Canada (PHAC), Laboratory for Foodborne Zoonoses (LFZ), at the Office International des Épizooties *Salmonella* Reference Laboratory in Guelph, ON.

For the isolation of *E. coli* O157:H7, the BPW enrichment cultures were incubated for 18–20 h at 37°C. One millilitre of the culture was then inoculated into 9 ml of modified trypticase soy broth containing 20 mg l^{-1} of novobiocin (Padhye & Doyle 1991) and incubated at 42°C for 6 h. Immuno-magnetic capture of *E. coli* O157:H7 using paramagnetic beads coated with O157 antibody (Dynal, Oslo, Norway) was carried out using the manufacturer's instructions. Bead suspensions were removed from the cultures and spread onto sorbitol MacConkey agar containing 50 mg l^{-1} cefixime and 2.5 mg l^{-1} tellurite (Chapman *et al.* 1994) and incubated for 24 h at 42°C. Suspect colonies were tested by slide agglutination using an O157 latex agglutination test (Difco, Detroit, MI). Bacterial colonies positive in the agglutination assay were tested using an

E. coli O157:H7-specific PCR assay targeting the *fliC*, *eaeA*, and verotoxin genes (Gannon *et al.* 1997). For serotyping and verotoxin detection, isolates were sent to the Verotoxin-Producing *E. coli* (VTEC) Reference Laboratory in Guelph, ON, at PHAC, LFZ.

For *Campylobacter* spp. isolation, a modification of the procedures described by Diergaardt *et al.* (2004) was used. BB enrichment cultures were incubated at 42°C for 24 h in a CO_2 incubator (Binder Inc., Great River, NY) under a microaerophilic atmosphere of 5% O_2, 10% CO_2, and 85% N_2 to select for the most common human pathogenic *Campylobacter* spp. (i.e. *C. jejuni* and *C. coli*). Blood-free charcoal cefoperazone deoxycholate agar (CCDA; Oxoid Ltd., Basingstoke, Hampshire, England) with selective supplement was streaked with approximately 0.02 ml of BB culture, and incubated microaerophilically at 42°C for 48 h (Bolton *et al.* 1984). *Campylobacter*-like colonies were subcultured onto blood agar plates and incubated microaerophilically at 42°C for 24 h. Following incubation, colonies were screened using oxidase and catalase tests. Isolates positive for both of these tests were confirmed using a multiplex PCR assay for the detection of *Campylobacter*-specific 16S rRNA gene sequences, as well as the *mapA*, and the *ceuE* genes specific to *C jejuni* and *C. coli*, respectively (Denis *et al.* 1999). RFLP typing of the *flaA* gene of *Campylobacter* spp. was performed using the methods of Nachamkin *et al.* (1993) and Harrington *et al.* (2003). A cluster analysis of the *flaA*-RFLP profiles was performed with the Bionumerics software (Version 4.1; Applied Maths, BVBA, Austin, TX) using the Jaccard binary coefficient and UPGMA dendrogram algorithm. A cluster was defined as a group of two or more *C. jejuni* isolates with identical RFLP profiles of the *flaA* gene.

DNA extraction and *Bacteroides* 16S host-species specific PCR

DNA was extracted from GITC-stabilized filters using the Qiagen DNeasy kit for cultured animal cells according to the manufacturer's instructions with the following modifications (Qiagen Inc. Mississauga, ON). GITC was used in place of proteinase K (Pitcher *et al.* 1989), 500 µl of Buffer AL (Qiagen Inc. Mississauga, ON) was used to bring the sample volume to 1 ml, two additional AW2 (Qiagen Inc. Mississauga, ON) washes were used to reduce PCR inhibition due to phenolic compounds, and half of the elution buffer was used to increase the concentration of DNA.

Two microlitres of extracted DNA were used as template in the PCRs using previously published primer pairs. Primer pairs used for the generic and ruminant-, human-, pig-, horse-, and dog-specific PCRs were BAC32F/BAC708R, CF128F/BAC708R, HF134F/HF654R (Bernhard & Field 2000*a*), PF163F/BAC708R, HoF597F/BAC708R (Dick *et al.* 2005*a*), and DF475F/BAC708R (Dick *et al.* 2005*b*), respectively. The detection of PCR products using these primers is indicative of faecal pollution (the *Bacteroides* genus) from ruminant, human, pig, horse, or dog faecal sources. Each 50 µl reaction mixture contained 1X *Taq* polymerase buffer (Qbiogene, Carlsbad, CA), 2.75 mmol l^{-1} of $MgCl_2$ for the genus-specific PCRs, and 3.5, 3.25, and 2.5 mmol l^{-1} of $MgCl_2$ for the ruminant-, human- and pig-, and horse- and dog-specific PCRs, 0.2 µmol l^{-1} of each primer pair, 200 µmol l^{-1} of each dNTP, and 2U of Fisherbrand *Taq* enzyme (Qbiogene, Carlsbad, CA). The reaction mixtures were initially held at 94°C for 2 min. This was followed by 37 cycles of amplification consisting of denaturation at 94°C for 1 min, annealing at 53°C, 63°C, 62°C, 54°C, 62°C, and 57°C for the genus-, ruminant-, human-, pig-, horse-, and dog-specific PCRs, respectively for 45s, and extension at 72°C for 45s. At the end of cycling, a final extension at 72°C for 6 min was used. PCR products were held at 4°C until they could be visualized in ethidium bromide stained 1.2–2% agarose gels following electrophoresis using 1X TBE buffer (pH 8.0) at 110 V for 35 min. Amplicon sizes produced for the generic and ruminant-, human-, pig-, horse-, and dog-specific PCRs were 677, 595, 520, 563, 129, and 220 bp, respectively. *Bacteroides vulgatus* and known host-source faecal DNA was used as positive controls for the genus-specific and host-species specific primers, respectively.

Statistical analyses

Pearson's correlation coefficient (r) was applied to measure the significance of seasonal relationships between pathogen isolations, indicator counts, and *Bacteroides-Prevotella* host-species marker prevalence. Fisher's exact test

($P < 0.05$) with Bonferroni's adjustment was applied to test for significant differences between bacterial isolation rates and *Bacteroides-Prevotella* marker prevalence.

RESULTS

Spatial and seasonal distribution of pathogens and *Bacteroides* 16S markers

Although there were no statistically significant spatial differences within the watershed with respect to the frequency of pathogen detection and the density of faecal coliform bacteria (Table 2), *Campylobacter* spp. isolation rates were higher at the upstream site SH than at the downstream site SF (56.7 versus 31.5%, respectively), and were similar between the two tributaries CO and DA (42.3 and 38.7%, respectively). In contrast to *Campylobacter* spp., *Salmonella* was isolated at the greatest frequency from the downstream site SF (16.7%) compared with the upstream sites SH (13.3%), CO (11.3%) and DA (9.7%). Like *Campylobacter* spp., *E. coli* O157:H7 isolation rates were higher at the upstream site SH than at the downstream site SF (6.7 versus 0%, respectively) and were similar between the two tributaries DA and CO (3.1 and 2.8%, respectively). Average faecal coliform counts ranged from 334 to 1191 CFU/100 ml.

There were marked seasonal differences in the detection of pathogens and faecal coliform bacteria. *E. coli* O157:H7 was isolated during the winter, spring, and fall, but not the summer (Table 2). Seasonal isolation rates of this pathogen were not significantly different; however, there was a strong positive relationship between seasonal *E. coli* O157:H7 isolation rates and average faecal coliform counts ($r = 0.859$). *Salmonella* spp. and *Campylobacter* spp. were isolated during each of the four seasons. Significantly more *Salmonella* spp. were isolated during the spring (20%) than in the summer (2%), and isolation rates tended to be higher when average faecal coliform densities were higher ($r = 0.649$). *Campylobacter* isolation rates were above 34% during each of the four seasons. The lowest faecal coliform densities and isolation rates of *E. coli* O157:H7 (0%) and *Salmonella* (2%), but not *Campylobacter* spp. (41.2%), occurred during the summer. The summer months also received the lowest amount of cumulative precipitation (232 mm). *E. coli* O157:H7 ($r = 0.888$) and *Salmonella* ($r = 0.564$), as well as faecal coliform density ($r = 0.688$) correlated positively with total seasonal precipitation, while *Campylobacter*

Table 2 | Spatial and seasonal bacterial isolation rates, 16S *Bacteroides* marker detection rates, average faecal coliform (Avg. FC) density, and total precipitation

	Site % (no.)				Season % (no.)			
	SH ($n = 30$)	CO ($n = 71$)	DA ($n = 31$)	SF ($n = 54$)	Winter ($n = 32$)	Spring ($n = 35$)	Summer ($n = 51$)	Fall ($n = 62$)
E. coli O157:H7	6.7 (2)	2.8 (2)	3.1 (1)	0 (0)	3.1 (1)	2.9 (1)	0 (0)	4.8 (3)
Salmonella	13.3 (4)	11.3 (8)	9.7 (3)	16.7 (9)	12.5 (4)	20 (7)*	2 (1)	17.7 (11)
Campylobacter	56.7 (17)	42.3 (30)	38.7 (16)	31.5 (17)	40.6 (13)	54.3 (19)	41.2 (21)	35.5 (22)
Generic *Bacteroides*†	39.1 (9)	29.1 (7)	17.4 (4)	37.5 (9)	56.3 (9)*	45.5 (10)*	0.0 (0)	41.7 (10)*
Ruminant *Bacteroides*†	4.3 (1)	4.2 (1)	4.3 (1)	0.0 (0)	18.8 (3)	0.0 (0)	0.0 (0)	0.0 (0)
Human *Bacteroides*†	30.4 (7)‡	12.5 (3)	4.3 (1)	4.2 (1)	37.5 (6)*,§	22.7 (5)§	3.1 (1)	0.0 (0)
Pig *Bacteroides*†	8.7 (2)	8.3 (2)	26.1 (6)‡	0.0 (0)	6.3 (1)	4.5 (1)	15.6 (5)	12.5 (3)
Dog *Bacteroides*†	4.3 (1)	0.0 (0)	4.3 (1)	4.2 (1)	6.3 (1)	4.5 (1)	0.0 (0)	4.2 (1)
Horse *Bacteroides*†	8.7 (2)	4.2 (1)	0.0 (0)	4.2 (1)	6.3 (1)	4.5 (1)	0.0 (0)	8.3 (2)
Avg. FC (CFU 100 ml − 1)	641	334	1191	776	437	532	313	989
Total Precipitation (mm)	NA	NA	NA	NA	1442	673	232	1559

Site: SH $n = 23$, CO $n = 24$, DA $n = 23$, SF $n = 24$, Season: winter $n = 16$, spring $n = 22$, summer $n = 32$, fall $n = 24$; "NA" Not Applicable; Fisher's Exact with Bonferroni's adjustment, $P < 0.05$.

*Significantly higher detection rate during this season than during the summer season.

†*Bacteroides* analysis was performed on samples collected during 2006 only.

‡Significantly higher detection rate at this site than at site SF.

§Significantly higher detection rate during this season than during the fall season.

spp. isolation rates ($r = -0.497$) correlated negatively with cumulative seasonal precipitation.

Several host species-specific *Bacteroides* markers were detected within the watershed. The genus-specific *Bacteroides* marker was detected at the greatest frequency, followed by the human, pig, horse, and dog and ruminant markers (Table 2). The *Bacteroides* genus-specific and human-specific markers were the only markers detected at all of the sites. The frequency of detection of the generic marker was not significantly different between sites; however, detection of the human-specific marker was significantly higher at the upstream site SH than at the downstream site SF (30.4 versus 4.2%, respectively), and the pig marker was detected at a significantly higher rate at site DA than at site SF (26.1 versus 0%, respectively). No other significant differences in the spatial distribution of host-species specific markers were observed.

The frequency of detection of the various *Bacteroides* markers also varied markedly with season (Table 2). Significantly more of the genus-specific *Bacteroides* marker was detected during winter (56.3%), spring (45.5%), and fall (41.7%) than during summer (0%). Significantly more of the human marker was detected during the winter (37.5%) than during the summer (3.1%) or fall (0%), and also during the spring (22.7%) than during the fall (0%). The ruminant marker detections occurred only during the winter (18.8%) and not in any other season. The lowest frequencies of the generic, ruminant, human and dog *Bacteroides* markers were detected during the summer and the greatest frequencies of these markers was detected during the winter. The pig marker frequency, in contrast, was highest in the summer and fall (15.6 and 12.5%, respectively) and lowest in the winter and spring (6.2 and 4.5%, respectively).

Relationships between pathogen subtypes isolated from faecal and water sources

Campylobacter was isolated at the greatest frequency from animal faecal samples and sewage (22%), followed by *Salmonella* (4.8%; Table 3). *E. coli* O157:H7 was not isolated from animal faecal matter during this study. *Campylobacter* was present in the faeces of 11/13 animal species tested, as well as in sewage. Although there were no statistically significant differences between *Campylobacter* spp. isolation rates among the sources tested in the current study, the highest rates were observed in sewage (36.4%), as well as pig (80%), duck (52.2%), and goose (38.5%) faecal samples. *C. jejuni* was isolated from all of the 11 host species, as well as from sewage, *C. coli* was isolated from pig, chicken, and goose faeces, and other *Campylobacter* spp. were isolated from pig faeces only (Table 4). *C. jejuni* was also detected at the greatest frequency in water samples (72/76 or 93.4%), followed by *C. coli* (7/76 or 9.2%) and other *Campylobacter* spp. (1/76 or 1.3%)

Table 3 | Host species distribution of *Campylobacter* and *Salmonella* isolated from animal faecal and sewage samples. Fisher's Exact with Bonferroni's adjustment, $P < 0.05$

Source	% *Salmonella* (No. Pos.)	% *Campylobacter* (No. Pos.)
Rabbit ($n = 11$)	0 (0)	0 (0)
Rat ($n = 1$)	0 (0)	0 (0)
Horse ($n = 23$)	0 (0)	4.3 (1)
Dog ($n = 17$)	5.9 (1)	5.9 (1)
Llama ($n = 10$)	0 (0)	10.0 (1)
Alpaca ($n = 9$)	0 (0)	11.1 (1)
Sheep ($n = 42$)	4.8 (2)	11.9 (5)
Goat ($n = 13$)	0 (0)	23.1 (3)
Cow ($n = 33$)	0 (0)	24.2 (8)
Chicken ($n = 17$)	0 (0)	29.4 (5)
Sewage ($n = 11$)	54.5 (6)*	36.4 (4)
Goose ($n = 13$)	15.4 (2)	38.5 (5)
Duck ($n = 23$)	0 (0)	52.2 (12)
Pig ($n = 5$)	0 (0)	80.0 (4)
Totals ($n = 228$)	4.8 (11)	22.0 (50)

*Significant differences between this source and horse, sheep, cow, and duck.

Cluster analyses of *flaA*-RFLP profiles were performed on 24 *C. jejuni* isolates of faecal origin and 42 *C. jejuni* isolates of water origin (Table 5). Of the 66 isolates examined, 27 fell within nine clusters, while the remaining 39 were singletons. Four clusters contained both faecal- and waterborne isolates, four clusters contained water isolates only, and one cluster contained isolates of chicken and cow origin only. The *flaA*-RFLP profiles of *C. jejuni* isolated from stations CO, SH, and SF matched profiles of *C. jejuni* isolated from sheep, llama, and cow faeces. Where more than one water isolate clustered, four of these clusters

contained isolates from the same sampling site over different sampling periods. However, identical *flaA*-RFLP profiles were also obtained from water isolates from different sampling sites and sampling periods. Three of nine clusters contained identical *flaA*-RFLP profiles from more than one host species.

Five *Salmonella enterica* serovars were isolated from the faeces of 3/13 animal species and sewage, and 12 different serovars were detected in surface water samples (Table 6). *Salmonella* serovars Typhimurium, Typhimurium Copenhagen, Mbandaka, and Agona were isolated more than once from different surface water sites. *Salmonella* serovars isolated from water and faecal sources varied considerably. *Salmonella* Typhimurium and I:4,5,12:i:- were the only serovars isolated from both water and faeces. The *Salmonella* serovar Typhimurium isolated from surface water had the same phage type (PT) as the serovar Typhimurium isolated from goose faeces (Table 6).

DISCUSSION

To our knowledge, this study is the first to present information on the distribution and characteristics of the three most important enteric bacteria associated with waterborne disease from both surface waters and animal sources in the same watershed. Most other reports have provided information on only water or faecal isolates of these pathogens (Johnson *et al.* 2003; Stanley & Jones 2003; Brown *et al.* 2004; Dorner *et al.* 2004; Jay *et al.* 2007; Walters *et al.* 2007).

There were some similarities between seasonal isolation rates of the pathogens and MST markers in the study; however, isolation rates were variable overall. More importantly, average faecal coliform counts were high at each of the four sites examined. Although the use of faecal coliforms, among other faecal indicator organisms, as predictors of faecal contamination has been one of debate, these organisms and other organisms such as *E. coli* are still used as indicators of water quality for lack of a better substitute (Tortorello 2003). While *E. coli* may be considered a more suitable indicator of faecal contamination than faecal coliforms, a high correlation between *E. coli* and faecal coliform densities in agricultural watersheds has been observed in previous studies, supporting their use in the current study (Wilkes *et al.* 2009; unpublished data). The prevalence of the 16S rDNA genus-specific *Bacteroides* marker was examined and compared to pathogen isolation rates and faecal coliform densities in order to assess its usefulness as an alternative indicator of faecal pollution. Stronger positive correlations were observed between pathogen isolation rates (with the exception of *Campylobacter*) and faecal coliform densities than between pathogen isolation rates and detection rates of the genus-specific *Bacteroides* marker. Negative correlations were observed between *Campylobacter* isolations and faecal coliform

Table 4 | Distribution of *C. jejuni*, *C. coli*, and other *Campylobacter* spp. isolated from animal faeces, sewage, and surface water samples

Source	No. pos. samples	*C. jejuni*	*C. coli*	Both	Unknown spp.
Ruminant	18	18	0	0	0
Pig	4	2	1	0	1
Chicken	5	4	1	0	0
Goose	5	4	0	1	0
Duck	12	12	0	0	0
Sewage	4	4	0	0	0
Dog	1	1	0	0	0
Horse	1	1	0	0	0
SH	17	15	4	2	0
CO	30	28	2	0	0
DA	12	12	0	0	0
SF	17	16	0	0	1

Table 5 | Clusters of *flaA* restriction fragment length polymorphisms for *Campylobacter* spp. isolated from animal faeces and surface water samples

Cluster	Faecal source (no. isolates)	Water station (no. isolates)
1	Duck (1)	SF (1)
2	–	SF (2)
3	–	CO (1), SF (1)
4	Canada Goose (2), sheep (1)	CO (2)
5	–	DA (1), SF (1)
6	Donkey (1), Llama (1), Cow (1), Sheep (1)	SH (1), SF (2)
7	Goat (1)	SH (1), SF (1)
8	–	CO (2)
9	Chicken (1), Cow (1)	–

Table 6 | Frequency of *Salmonella enterica subsp. enterica* serovars and phagetypes (PT) isolated from A: animal faecal and sewage samples, and B: surface water samples

***A. Salmonella* serovar**	**PT**	**Dog (*n* = 1)**	**Goose (*n* = 2)**	**Sheep (*n* = 2)**	**Sewage (*n* = 6)**	**Serovar totals**
Heidelberg	19	1	0	0	0	1
Heidelberg	6	0	0	0	6	6
I:4,5,12:i:-	–	0	1	0	0	1
I:ROUGH-O:-:-	–	0	1	0	0	1
IIIb:61:-:1,5	–	0	0	2	0	2
Typhimurium	U284	0	2	0	0	2
Source totals	–	1	4	2	6	13
***B. Salmonella* serovar**	**PT**	**SH (*n* = 4)**	**CO (*n* = 8)**	**DA (*n* = 3)**	**SF (*n* = 9)**	**Serovar totals**
Agona	–	0	1	0	1	2
Daytona	–	0	0	0	1	1
Derby	–	0	0	0	2	2
I:4,5,12:-:-	–	0	1	0	0	1
I:4,5,12:i:-	120	2	0	0	0	2
Mbandaka	–	1	0	1	0	2
Montevideo	–	0	0	1	0	1
Saintpaul	–	0	2	0	0	2
Seftenberg	–	0	0	0	2	2
Thompson	–	0	0	0	1	1
Typhimurium	U292	0	1	0	0	1
Typhimurium	U284	0	4	0	2	6
Typhimurium	121	0	0	0	1	1
Typhimurium	99	1	0	0	0	1
Typhimurium var. Copenhagen	UT5	0	0	1	0	1
Typhimurium var. Copenhagen	U284	0	1	0	0	1
Site totals	–	4	10	3	10	27

densities, as well as between *Campylobacter* isolations and detection of the genus-specific *Bacteroides* marker. One possible explanation is that the main sources of *Campylobacter* are already in or near the water, whereas the main sources for *E. coli* O157:H7 and *Salmonella* are on the land and must be transported to the river. While manure application practises and precipitation may facilitate the transport of indicator organisms and pathogens to the river waters, rainfall may also dilute pathogens already in the water. Wilkes *et al.* (2009) also found an inverse relationship between indicator bacteria densities and the presence of *Listeria monocytogenes*. Relationships between indicator and pathogenic bacteria may be influenced by the presence or absence of pathogen host sources or reservoirs (Lyautey *et al.* 2007; Ruecker *et al.* 2007), seasonal variations in pathogen shedding rates (Jones *et al.* 1999), rates of surface run-off from rainfall (Auld *et al.* 2004), as well as the transport and fate (i.e. death, proliferation, or particle settling) of these pathogens and indicator bacteria, underlining the need for a greater understanding of these factors.

The presence of *Salmonella*, *Campylobacter*, and *E. coli* O157:H7 in this watershed highlights the potential risk to human health through consumption of untreated water, irrigated raw vegetables, and through recreational use of surface water. Wildlife and livestock that have direct, uncontrolled access to surface waters, as well as heavy rainfall are means whereby pathogens in manure may be

transported to surface waters. Although waterborne disease outbreaks have occurred after inadequate treatment of contaminated municipal drinking water following intense rain (Hrudey *et al.* 2002), the risk of waterborne disease in populations that drink untreated well water is also very high (Yoder *et al.* 2008*b*). In Langley, more than 25% of the residents drink untreated well water, and previous research attributed elevated nitrate levels in both groundwater and surface water in this watershed to a high density of animal production units and septic systems (Wernick *et al.* 1998). Although the Salmon River does not have designated beaches, it is popular for fishing and it is not uncommon for children to play in the water. Considering these possible routes of transmission of pathogens from land to surface water or groundwater, as well as from surface water or groundwater to humans, the level of risk would be expected to be high based on the fact that more than 50% of the water samples analysed were positive for at least one of the three pathogens, and also because of their potentially low infectious doses (Blaser & Newman 1982; Tauxe *et al.* 1992; Paton & Paton 1998; Kothary & Babu 2001). All three of these pathogens are capable of causing severe enteric disease that may lead to death. Campylobacteriosis continues to be the leading cause of gastroenteritis in North America, and both *Campylobacter* and *E. coli* O157:H7 were responsible for illness and the loss of life in the waterborne disease outbreak in Walkerton, Ontario (Hrudey *et al.* 2002; Auld *et al.* 2004).

Elucidation of potential sources of enteric, waterborne pathogens is important because this information may suggest ways in which we can help reduce risks to human health associated with these pathogens. The *Bacteroides* culture- and library-independent MST methods applied in the current study are attractive because there is neither the need for culture nor is it necessary to develop a reference library. Unfortunately the majority of host-species specific *Bacteroides* appear to be non-culturable, and while culture-independent methods are quicker and more cost efficient, these types of MST assays are generally less sensitive than those that are culture-based. Serotyping and phage typing of *Salmonella* spp. isolated from water and faecal samples could be a useful tool used in conjunction with other MST methods in providing clues as to the likely sources of contamination; however, because many different *Salmonella* serovars exist that span a broad host range, this decreases the utility of the method as a single means for source attribution. *Campylobacter* spp. were frequently isolated from surface waters in this and other studies and have been isolated from a variety of animal hosts (Stanley & Jones 2003; Brown *et al.* 2004; Dorner *et al.* 2004). In this study, RFLP analysis of the *flaA* gene of *Campylobacter* was used to determine the sources of this pathogen in the watershed. The RFLP cluster analysis demonstrated that identical *Campylobacter flaA* profiles were present in many different environmental samples, while others were present only in specific environmental samples. Pathogen subtyping is used by public health laboratories around the world to determine sources of pathogens in foodborne disease outbreak investigations (Swaminathan *et al.* 2001). In this study we have applied these methods not only to provide evidence as to which animal species shed the organism, but by using these and more advanced subtyping methods with higher levels of discrimination (Lyautey *et al.* 2007; Laing *et al.* 2008; Hannon *et al.* 2009), we may be able to pin-point specific sites and groups of animals responsible for contamination of water by these pathogens. This may be more helpful and effective in watershed management than simply determining the animal species responsible for contamination. The latter has been the rather elusive goal of the science of microbial source tracking.

Relationships were observed between the pathogen subtyping methods and the *Bacteroides* MST PCR method. For example, high levels of the *Bacteroides* pig-specific marker (Table 2), as well as *Salmonella* spp. serovars Mbandaka and Typhimurium Copenhagen (Table 6), which are commonly isolated from pigs (Davies *et al.* 1999; Gebreyes *et al.* 2004*a*,*b*; Rajic *et al.* 2005), were more prevalent in areas with greater pig population densities (Table 1). The *Bacteroides* ruminant-specific marker (Table 2), *C. jejuni* isolates with *flaA-RFLP* profiles that matched those of ruminant animals (Table 5), and *E. coli* O157:H7 (Table 2), which are commonly associated with ruminant faeces (Walters *et al.* 2007; La Ragione *et al.* 2009), were isolated from water sites SF, CO and SH. Many possible domestic and wild ruminant sources of contamination exist at each of these sites (Table 1). Although no culture-independent bird-specific PCR-based assay was

used in the current study, *Salmonella* and *Campylobacter* spp. typing methods, as well as the high isolation rates of these pathogens from goose and duck faeces (Table 3) suggest that these birds may be another significant source of enteric pathogens in the Salmon River. Untreated human sewage was the highest source of *Salmonella* spp. (54.5% of the sewage samples were positive for this pathogen), and it was among the top four sources of *Campylobacter* spp. (36.4% of the sewage samples were positive for this pathogen). While none of the *Salmonella* spp. serovars or the *Campylobacter* spp. *flaA*-RFLP profiles obtained from untreated human sewage samples were isolated from water samples, the human *Bacteroides* signature was detected at each of the four surface water sites, and was detected more often than the other *Bacteroides* signatures. This suggests that human sewage may also be an important source of contamination of these surface waters.

The three molecular methods (*Salmonella* spp. serotyping and phage typing, *flaA*-RFLP profiling of *Campylobacter* spp., and the detection of host-species specific *Bacteroides* signatures) applied in the current study revealed humans, agriculture, and wildlife as contributing sources of faecal contamination of these surface waters. Specifically pigs, ruminants, geese, as well as septic systems and sewage were identified as potential contributors of faecal contamination in this mixed watershed. Available land-use information was helpful in understanding the potential spatial origin of pathogens; however, there were also instances where available land-use information could not help explain the presence of certain pathogens.

CONCLUSIONS

In this study, the frequent isolation of bacteria associated with drinking water and recreational outbreaks of waterborne disease (campylobacters, salmonellae and *E. coli* O157:H7) underlines the human health risks associated with untreated surface waters. The high prevalence of these enteric bacterial pathogens in animal faecal and sewage samples suggests that faecal wastes from many sources may contribute to this pathogen load. *Salmonella* serotyping and molecular subtyping of *C. jejuni* revealed that host species-specific and location-specific subtypes could be isolated from water. This information may be useful in pin-pointing sources of water contamination and implementing best management practises.

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