Canadian Journal of Microbiology
Revue canadienne de de microbiologie

**Temporal variation in the prevalence and species richness
of *Campylobacter* spp. in a prairie watershed impacted by
urban and agricultural mixed inputs**

Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2015-0710.R2
Manuscript Type:	Article
Date Submitted by the Author:	13-Jan-2016
Complete List of Authors:	Tambalo, Dinah D.; University of Regina, Biology Department Boa, Tyler; University of Regina, Biology Department Aryal, Bijaya; University of Regina, Biology Department Yost, Christopher; Dept. of Biology,
Keyword:	Campylobacter, pathogen prevalence, surface water, agricultural watershed, water quality

SCHOLARONE™
Manuscripts

Temporal variation in the prevalence and species richness of *Campylobacter* spp. in a prairie watershed impacted by urban and agricultural mixed inputs

Dinah D. Tambalo^{*}, Tyler Boa, Bijaya Aryal, Christopher K. Yost

Biology Department, University of Regina, Regina, Saskatchewan, Canada S4S 0A2

^{*}Corresponding author:

Biology Department

University of Regina

3737 Wascana Parkway, Regina, Saskatchewan, Canada S4S 0A2

E-mail address: Dinah.Tambalo@uregina.ca

Keywords: *Campylobacter*, pathogen prevalence, surface water, agricultural watershed, water quality

Abstract

Campylobacter spp. are a substantial cause of gastroenteritis worldwide. Human infection can result from ingestion of contaminated food or water from a variety of sources, including the consumption of fresh produce that is contaminated with the pathogen via the use of contaminated irrigation water. Using molecular methods, we investigated the occurrence of *Campylobacter* in the Qu'Appelle River watershed, an important source of irrigation water for vegetable producers in Southern Saskatchewan, Canada. Water samples were collected from 7 sampling sites, from April to September 2009 (145 samples), and from 5 sampling sites from May to October 2013 (116 samples). *Campylobacter* was detected in 57% and 16% of the samples collected in 2009 and 2013, respectively. *Campylobacter* detection was highest in May and June for both sampling years. In 2009, the predominant species were *Campylobacter lari* and *Campylobacter jejuni*, with prevalences of 84% and 41%, respectively. Other *Campylobacter* spp. were detected less frequently. Only *C. lari* was detected in 2013. The results in 2009 demonstrate the species richness of *Campylobacter* in water sources within the watershed. The occurrence of *Campylobacter* in the study area also underscores the importance of monitoring irrigation water used to irrigate fresh produce from a public health prospective.

Introduction

Campylobacter spp. are a leading cause of food-borne illness worldwide and are the most commonly implicated bacterial cause of gastroenteritis (WHO 2011). In Canada, a total of 118 cases were reported in 2009, with the majority (84%) classified as endemic (Public Health Agency of Canada 2012). The total number of infections is likely much larger because *Campylobacter* infections are generally self-limiting and therefore likely under-reported (Flint et al. 2004; Majowicz et al. 2005; Thomas et al. 2008). *Campylobacter* infections have been frequently associated with the consumption of contaminated raw milk (Taylor et al. 1982; Potter et al. 1984; Headrick et al. 1998; Neimann et al. 2003; Huang et al. 2015), poultry (Harris et al. 1986; Huang et al. 2015), and drinking water (Millson et al. 1991; Pitkanen 2013; Huang et al. 2015). Fresh fruits and vegetables that are irrigated with pathogen-contaminated water can also be an alternative exposure pathway for human infection (Kumar et al. 2001; Chai et al. 2007; Pachepsky et al. 2011; Verhoeff-Bakkenes et al. 2011).

The occurrence of *Campylobacter* in surface waters located in Canada has been reported for the provinces of Alberta (Jokinen et al. 2011; Khan et al. 2014), British Columbia (Jokinen et al. 2010; Khan et al. 2014), Ontario (Van Dyke et al. 2010; Schmidt et al. 2013; Khan et al. 2014), Quebec (St-Pierre et al., 2009; Khan et al. 2014), and Nova Scotia (Ridley et al. 2014). These studies have highlighted the potential of surface water as an important source of human pathogenic *Campylobacter* species. In this study, we investigated the occurrence of *Campylobacter* spp. during two irrigation seasons in 2009 and 2013 in the Qu'Appelle River and its tributary, Wascana Creek located in southern Saskatchewan, Canada. The Qu'Appelle River is an important source of irrigation water for vegetable growers in the province. We have

previously reported high levels of *Escherichia coli* in the watershed, exceeding the Canadian Council of the Ministers of the Environment's recommendation of <100 *E. coli*/100 ml for irrigation of fresh produce (Fremaux et al. 2009; Tambalo et al. 2012). The study area is impacted by both agricultural operations (mainly cattle production and vegetable production) and urban wastewater treatment. Urban contributions are from the City of Regina wastewater treatment plant (Hall et al. 1999) via Wascana Creek, as well as sewage lagoons in the towns of Craven and Lumsden. Given the evidence for fecal inputs into the water sources, this study was conducted to investigate the prevalence of pathogens such as *Campylobacter*, in the watershed during the vegetable growing season. Previous studies in agriculturally impacted watersheds have generally focused on *C. jejuni*, *C. coli*, and *C. lari* (Jokinen et al. 2010; Van Dyke et al. 2010; Jokinen et al. 2011; Schmidt et al. 2013; Khan et al. 2014) largely due to these three species contributing significantly to human gastroenteritis (Huang et al. 2015). However, other *Campylobacter* species may also be present in these watersheds, although at a lower frequency (Jokinen et al. 2010; Jokinen et al. 2011; Khan et al. 2014). These infrequently detected *Campylobacter* species are considered emerging pathogens that can cause diarrhoeal illness in humans as well as serious extra-intestinal diseases (Lynch et al. 2010; On 2013). Hence, we also performed speciation of *Campylobacter* to identify the dominant species in the watershed and to investigate possible sources of contamination.

Materials and Methods

Water sample collection

Seven sites along Qu'Appelle River (Site C, Sites 5 and 6 with 22 samples each) and Wascana Creek (Sites 1-4 with 17, 20, 20, and 22 samples, respectively) were sampled weekly

from April to September 2009 for a total of 145 samples (Tambalo et al. 2012; Fig. 1). These sites were previously selected to assess sources and the magnitude of faecal contamination in irrigation water sources within the Qu'Appelle River watershed (Tambalo et al. 2012). Site C which has minimal agricultural and anthropogenic inputs served as a reference site. Sites 1 to 4 were selected to investigate the influence of the cattle pastures along Wascana Creek and of the effluent coming from the City of Regina sewage treatment plant. Biweekly samples from three irrigation pump sites along Wascana Creek (IP1 and IP2 with 20 samples each) and the Qu'Appelle River (IP3 with 20 samples) were collected from May to October 2013; Site C (18 samples) and Site 3 (18 samples) were also sampled for a total of 116 samples in 2013. IP1, which is downstream of Site 4, draws water from Wascana Creek while IP2 and IP3, which are upstream and downstream of Site 5, respectively, draw water from the Qu'Appelle River. Water samples (500 ml) collected at about 20 cm depth were transported on ice and were processed within 3 hours of collection.

Enrichment of *Campylobacter* spp. and DNA extraction

The presence of *Campylobacter* in the water samples was investigated following the method described by Walters et al. (2007). Briefly, 200 ml of the water sample was filtered through a 0.45 µm Millipore membrane and the filter was placed in 30 ml of Bolton Enrichment Broth (Oxoid) containing selective supplement (Oxoid SR183E) and 5% (v/v) laked horse blood (Oxoid). The broth cultures were incubated under microaerophilic conditions (5-15% oxygen) using a GasPak EZ Campy container system (BD Diagnostic Systems, New Jersey), at 37°C overnight (to allow resuscitation of cells), then at 42°C for 48 h. A 500 µl aliquot of the enriched

culture was used for DNA extraction using the Power Soil DNA kit (MoBio, Laboratories, Inc.). The enriched cultures were also grown on Blood-free *Campylobacter* selective agar (Oxoid) followed by incubation at 42°C under microaerophilic conditions as described above for 48 hours. Presumptive *Campylobacter* colonies were identified based on colony morphology, *i.e.* grey and moist colonies.

Detection of *Campylobacter* in water samples using real-time PCR

The presence of *Campylobacter* in the enriched samples (not individual colonies) was investigated by real-time PCR using primers and probes (Table 1) that target the 16S rDNA gene *C. coli*, *C. jejuni*, *C. lari*, *C. helveticus*, *C. hyointestinalis*, and *C. upsaliensis* (Lund et al. 2004). The PCR results are reported as absence or presence since an enrichment step was included in the protocol. PCR assays were conducted using either an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories) or an Applied Biosystems StepOne Real-Time PCR System (Lifetech). A 25-µl reaction was prepared containing the following: 12.5 µl of iQ Supermix (Bio-Rad Laboratories) or TaqMan Environmental Master Mix (Life Technologies), 4 µl of template DNA, 5 µg bovine serum albumin (BSA, Promega, WI, USA), 0.6 µM of each primer, and 0.2 µM probe. The amplification program used was as follows: 95°C for 6 minutes (10 minutes on StepOne RT-PCR) followed by 40 cycles (50 cycles on StepOne RT-PCR) of 95°C for 15 s and 60°C for 1 min. A standard curve was generated using 10-fold dilutions of a recombinant plasmid DNA containing known copies of the target region. The linear range of detection was from 100 copies to 10⁷ copies per reaction and accepted amplification efficiency was set at 85%-110%. Controls for the PCR assay (no template control), and extraction negative controls (filtration step

and DNA isolation method) were included in each PCR run. Internal amplification controls were not included in the assays and the DNA was not quantified, which could have potentially led to false negatives in this study. Samples with Ct values below 34 and lower than the negative controls were considered positive for *Campylobacter*.

Identification of *Campylobacter* spp. present in *Campylobacter*-positive samples

The presence of 14 different *Campylobacter* spp. in the *Campylobacter*-positive samples was investigated following the method described by Chaban et al. (2009), but using conventional PCR. The DNA used previously for the *16S rDNA* assay was also used in this experiment hence one DNA sample may contain several *Campylobacter* species. *Campylobacter* species-specific PCR primers (Table 1) targeting the *cpn60* gene, which encodes a conserved chaperonin, were used. Conventional PCR was performed using the following reaction: 1X PCR buffer (BioBasic Canada), 5 µg BSA, 0.2 mM MgSO₄, 0.2 mM dNTPS, 0.2 mM each of the forward and the reverse primers, 2.5 U Taq Polymerase (BioBasic Canada), and 2 µl of DNA template. The cycling condition used for PCR amplification included the following: 95°C for 5 min; 40 cycles of 94°C for 30 s, 30 s at the appropriate annealing temperature (Table 1), and 72°C for 30 s; and final extension at 72°C for 10 minutes. PCR products were visualized on 2% agarose gels following standard techniques. Crude DNA extracts from *E. coli* strains harboring plasmids with the *cpn60* target region in each *Campylobacter* spp. tested were included as positive controls in the PCR assays and in the gel electrophoresis. The *E. coli* strains were kindly provided by Drs. Janet Hill and Bonnie Chaban from the University of Saskatchewan. Crude DNA extracts were prepared using a boiling lysis method. Briefly, cell suspensions were incubated at 99°C for 10

minutes followed by centrifugation at 13,000 rpm for 10 s. The supernatant was used as template for PCR amplification.

Cloning and/or DNA sequencing

To confirm correct amplification of the target region, the *cpn60* PCR amplicons/clones of the predominant species (*C. lari*, *C. coli*, *C. jejuni*, and *C. showae*) were sequenced. PCR amplicons for *C. showae cpn60* were cloned into pGEM-T Easy (Promega) following the manufacturer's instructions. DNA sequencing was performed by Quintara Biosciences (CA, USA) and the sequences were analyzed using BLAST searches at NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>; accessed on November 6, 2013) and at the chaperonin sequence database (<http://www.cpnadb.ca/cpnDB/seqComp.php>; accessed on November 6, 2013).

Statistical Test

Using Graphpad Prism, a Student t-test was conducted to compare the detection frequency of *Campylobacter* between sites that are located in Wascana Creek and in the Qu'Appelle River. .

Results

Prevalence of *Campylobacter* in the watershed

Campylobacter spp. was detected by molecular methods in 57% (82/145) and 16% (19/116) of the water samples collected in 2009 (Tambalo et al. 2012) and 2013, respectively (Table 2). The occurrence of *Campylobacter* in the sites along Wascana Creek was comparable

to those sites along the Qu'Appelle River (Student t-test; $p=0.9229$). The detection frequency ranged from 50% at site 4 to 64% at site 6 in 2009; and from 10% at sites IP1 and IP2 to 25% at IP3 in 2013. The growth of *Campylobacter*-like colonies from the enrichment culture suggests that live *Campylobacter* cells may be present in the water samples.

Seasonal differences in *Campylobacter* prevalence

Our observations from both 2009 and 2013 sampling detected a higher *Campylobacter* frequency in May and June of each year compared with July and August (Fig. 2). There was no correlation between detection frequency and the total monthly rainfall within the study area (rainfall data was obtained from an Environment Canada climate station, Regina Gilmour station, located near IP1; data not shown). The only data available on air temperature is from an Environment Canada monitoring station that is close to Sites C and IP1. However, we were unable to perform correlation analysis because of our low sample numbers from these two sites.

Species Richness of *Campylobacter* spp. within the water samples

Seventy-four DNA samples from *Campylobacter* positive samples that were archived from the 2009 sampling season were processed for *Campylobacter* speciation. The 19 *Campylobacter*-positive water samples from 116 samples in 2013 were also included in the speciation analysis (Table 2). Sample of agarose gels for each *Campylobacter* species are shown in Figure S1. We observed false positive detection for *C. showae* using the *cpn60* PCR assay. The sequenced amplicons obtained from three different sites were not homologous to *C. showae* *cpn60* but were homologous to *Aeromonas 2-isopropylmalate synthase* genes (94-96% identity); hence results for *C. showae* were excluded in the subsequent analysis. Amplicons for *C. coli*, *C.*

jejuni, and *C. lari* were confirmed with sequencing results (Fig. S1). A number of the *Campylobacter*-positive water samples collected in 2009 contained multiple *Campylobacter* spp. (. Thirty percent of the 2009 samples contained two species (50% of which contained both *C. lari* and *C. jejuni*) while 22% had more than two species. One sample in Site 4 and Site C contained as many as 6 and 5 different species, respectively. Forty three percent of the samples contained only one *Campylobacter* spp., the majority of which contained *C. lari* (81%) (data not shown). Within the study area, the predominant species in 2009 were *C. lari* (84%) and *C. jejuni* (41%) (Table 3). *C. lari* and *C. jejuni* were detected in all the sampling sites in 2009, ranging from 58% to 92% and from 15% to 67%, respectively. In addition, *C. jejuni* was found in 39% of samples that contained *C. lari*. *C. coli* was not detected in site 2, but it was detected at least once in all the other sampling sites. *C. consisus* was only detected in sites C and 4, while *C. curvus* was detected once in the reference site. *C. coli* and *C. consisus* were each detected in 14% of the 2009 samples. All of the 13 *Campylobacter* spp. tested in the study were undetected in 4 samples (5%) from Site C, Site 2, Site 3, and Site 6. Notably, and in contrast to the results in 2009, only *C. lari* was detected in the water samples collected in 2013.

Monthly variations in *Campylobacter* species richness

The number of *Campylobacter* species detected per month was also determined for the samples in 2009 (Table 4). The highest number of *Campylobacter* spp. was detected in April 2009 (8 species) and then decreased to 6 species in May, followed by 5 species in June, July, and August; the lowest species richness was detected in September with 3 *Campylobacter* species. *C. jejuni* detection frequency also decreased from 64% in May and 53% in June 2009, to an average of 25% from July to September 2009 (Table 4). In contrast, *C. lari* was detected at comparable

frequencies during the entire sampling season (75% to 93%). There was no significant correlation between the number of *Campylobacter* species detected per month and the total monthly rainfall ($r_s = -0.5768$; $p = 0.2417$).

Discussion

The detection frequency of *Campylobacter* in 2009 in this study is comparable to previous reports (25% to 70%) in other river basins across Canada (Wilkes et al. 2009; Van Dyke et al. 2010; Jokinen et al. 2011; Khan et al. 2014; and Huang et al. 2015). The river systems investigated in these studies are also influenced by agriculture and livestock operations (*i.e.* cattle or poultry). Our results further support the common occurrence of *Campylobacter* in agricultural basins in Canada (Khan et al. 2014). The different rates of detection in 2009 and 2013 could be attributed to differences in the density of potential sources of faecal contamination, and year-to-year variations in physico-chemical parameters (e.g. water temperature, dissolved oxygen, light, and nutrients) that may affect *Campylobacter* survival (Thomas et al. 1999; Pitkanen et al. 2013). In addition, although the sampling sites used in 2009 and 2013 were relatively close in proximity they were slightly different, e.g. Sites 1, 2, 4, 5, 6 were excluded and new sites (IP1-IP3) were included in 2013.

C. lari is the predominant species in the Qu'Appelle River watershed, followed by *C. jejuni*. Both species are also widely distributed in the study area. Potential sources of these two species are wild birds that are present in the watershed. Previous studies have identified *C. jejuni* as the most frequently isolated *Campylobacter* species from surface waters in watersheds in Canada (Jokinen et al. 2011; Public Health Agency of Canada 2013; Schmidt et al. 2013; Khan et al. 2014), USA (Carter et al. 1987), and UK (Kemp et al. 2005). Similar to the results obtained

from the present study, Van Dyke et al. (2010) also detected *C. lari* more frequently than *C. jejuni* in the Grand River watershed (Waterloo, Ontario), which is influenced by swine and cattle operations. In a recent study, Khan et al. (2014) reported different profiles of either *C. jejuni* or *C. coli* as the predominant *Campylobacter* species in four river basins across Canada. *Campylobacter* carriage in different animal species in the watersheds and the persistence characteristics of *Campylobacter* spp. (Obiri-Danso et al. 2001; Van Dyke et al. 2010; Pitkanen 2013) may have contributed to the different predominant species in each watershed. The low number of *Campylobacter* species detected in 2013 could be due to the lower occurrence of *Campylobacter* during the sampling months that year.

The two PCR methods used in this study have primers that bind to different targets, *16S rDNA* (Lundt et al. 2004) and *cpn60* (Chaban et al. 2009). While there is a single copy of *cpn60* gene per *Campylobacter* genome (Hill et al. 2004), the *16S rDNA* is generally present in 3 copies in a *Campylobacter* genome (Stoddard et al. 2015). Hence, the *16S rDNA* assay would be more sensitive. This could be reflected in our result where 4 samples were positive for *Campylobacter* using the *16S rDNA* primers but *Campylobacter* spp. were not detected in the same samples using the *cpn60* primers. However, the *16srDNA* assay is limited to detecting 6 *Campylobacter* species; this could have led to false negative results for samples that contained other *Campylobacter* species. Sewage effluents from the City of Regina, septic systems from rural communities along the watershed, cattle pastures along Wascana Creek (a major tributary), and wildlife within the watershed are potential sources of *Campylobacter* bacteria. However, it is noteworthy that the reference site (Site C) had comparable frequency of occurrence with the agriculturally and anthropogenically impacted sampling sites (Sites 1-6, IP1, IP2, and IP3). Eight different species (*C. lari*, *C. jejuni*, *C. helveticus*, *C. gracilis*, *C. curvus*, *C. coli*, *C.*

252 *consisus*, *C. hyointestinalis*) were identified in Site C during the sampling season in 2009,
253 exhibiting high species richness. Additionally, one water sample collected in April 2009
254 contained as many as 5 *Campylobacter* species (*C. lari*, *C. jejuni*, *C. helveticus*, *C. gracilis*, and
255 *C. curvus*). Further studies are needed to investigate the potential sources of background
256 *Campylobacter* population in the watershed, as demonstrated in the reference site. Wildlife
257 species, including migratory birds are potential contamination sources.

258 *C. mucosalis*, which is found in pigs (On 2013), was undetected in the study site. The
259 study site is not influenced by swine operations, which could explain the absence of this species
260 in the water samples. *C. upsaliensis*, found in dogs and cats (Chaban et al. 2009; On 2013) was
261 undetected in the sampling sites. *C. fetus* is infrequently isolated from cattle feces (Inglis and
262 Kalischuk 2003; Inglis et al. 2003; Inglis and Kalischuk 2004; Inglis et al. 2004) and was also
263 undetected. Survival and consequently the detection of the non-thermotolerant *Campylobacter*
264 spp. (*C. sputorum*, *C. fetus*, *C. hyointestinalis*, *C. consisus*, *C. curvus*, *C. gracilis*, and *C. rectus*)
265 (Nothaft et al. 2012) may have been affected by the incubation at 42°C, when cells were initially
266 present at lower densities. Nevertheless, the detection of *C. curvus*, *C. consisus*, and *C. gracilis*
267 in the study sites suggests anthropogenic inputs into the Qu'Appelle River since these species
268 have been mainly associated with human microflora (Macuch and Tanner 2000).

269 The prevalence of *Campylobacter* in aquatic ecosystems can display seasonal patterns
270 (St-Pierre et al., 2009; Jokinen et al. 2010; Van Dyke et al. 2010; Tambalo et al. 2012). In this
271 study, *Campylobacter* was detected more frequently in the colder months of April to June in
272 2009, and in May and June in 2013. This is in contrast to the results reported by St-Pierre and
273 colleagues (2009) from their study on rivers and streams in Quebec whereby they detected
274 *Campylobacter* more frequently in the fall. This difference could be explained by different

amounts of surface runoff during spring in Saskatchewan and Quebec, and different sources of *Campylobacter* contamination. Other factors that could have affected the presence and survival of different *Campylobacter* species in the two provinces are light, oxygen concentrations, biotic interactions, and nutrient availability (Thomas et al. 1999). Temperature is also considered a key factor in the survival of *Campylobacter* in aquatic environments (Thomas et al. 1999; Obiri-Danso et al. 2001; Rodriguez and Araujo 2010; Van Dyke et al. 2010). However, we were unable to perform correlation analysis between temperature and the frequency of *Campylobacter* detection due to lack of temperature data.

The presence of *Campylobacter* in Wascana Creek and the Qu'Appelle River highlights the potential risks associated with the use of contaminated irrigation water in fresh produce, and the importance of protecting these water sources from microbial threats. Future studies are needed to identify sources of *Campylobacter* in the study area and to understand further the seasonal effects on persistence of *Campylobacter* and factors that lead to annual variations in persistence of *Campylobacter*.

Acknowledgements

We acknowledge the financial support of the Saskatchewan Agriculture, Agriculture Development Fund (Grant number 20110096) and a Canada Research Chair to C.K. Yost. We also thank Drs. Janet Hill and Bonnie Chaban at the University of Saskatchewan for providing clones for the *cpn60* amplicons.

Conflict of Interest

No conflict of interest declared.

Draft

References

- Bolton, F.J., Hutchinson D.N., and Parker, G. 1987. Isolation of *Campylobacter*: What are we missing? J. Clin. Pathol. **40**:702-703.
- Carter, A.M., Pacha, R.E., Clark, G.W., and Williams, E.A. 1987. Seasonal occurrence of *Campylobacter* spp. in surface waters and their correlation with standard indicator bacteria. Appl. Environ. Microbiol. **53**:523-526.
- Chaban, B., Musil, K.M., Himsworth, C.G., and Hill, J.E. 2009. Development of cpn60-based real-time quantitative PCR assays for the detection of 14 *Campylobacter* species and application to screening of canine fecal samples. Appl. Environ. Microbiol. **75**:3055-3061.
- Chai, L.C., Robin, T., Ragavan, U.M., Gunsalam, J.W., Bakar, F.A., Ghazali, F.M., Radu, S., and Kumar, M.P. 2007. Thermophilic *Campylobacter* spp. in salad vegetables in Malaysia. Int J Food Microbiol. **117**:106-111.
- Flint, J.A., Dore, K., Majowicz, S.E., Edge, V.L., and Sockett, P. 2004. From stool to statistics: reporting of acute gastrointestinal illnesses in Canada. Can. J. Public Health. **95**:309-313.
- Fremaux, B., Gritzfeld, J., Boa, T., and Yost, C.K. 2009. Evaluation of host-specific *Bacteroidales* 16S rRNA gene markers as a complementary tool for detecting fecal pollution in a prairie watershed. Water Res. **43**:4838-4849.
- Hall, R.I., Leavitt, P.R., Quinlan, R., Dixit, A.S., and Smol, J.P. 1999. Effects of agriculture, urbanization, and climate on water quality in the Northern Great Plains. Limnol. Oceanogr. **44**:739-756.
- Harris, N.V., Weiss, N.S., and Nolan, C.M. 1986. The role of poultry and meats in the etiology of *Campylobacter jejuni/coli* enteritis. Am. J. Public Health. **76**:407-411.
- Headrick, M.L., Korangy, S., Bean, N.H., Angulo, F.J., Alterjuse, S.F., Potter, M.E., and Klontz, K.C. 1998. The epidemiology of raw milk-associated foodborne disease outbreaks reported in the United States, 1973 through 1992. Am. J. Public Health **88**:1219-1221.
- Hill, J.E., Penny, S.L., Crowell, K.G., Han Goh, S., Hemmingsen, S.M. 2004. cpnDB: A chaperonin sequence database. Genome Research. **14**:1669-1675.
- Huang, H., Brooks, B.W., Lowman, R., and Carillo, C. 2015. *Campylobacter* species in animal, food, and environmental sources, and relevant testing programs in Canada. Can. J. Microbiol. **61**: 701-721.
- Inglis, G.D. and Kalischuk, L.D. 2003. Use of PCR for direct detection of *Campylobacter* species in bovine feces. Appl. Environ. Microbiol. **69**:3435-3447.

- Inglis, G.D. and Kalischuk, L.D. 2004. Direct quantification of *Campylobacter jejuni* and *Campylobacter lanienae* in feces of cattle by real-time quantitative PCR. Appl. Environ. Microbiol. **70**:2296-2306.
- Inglis, G.D., Kalischuk, L.D., and Busz, H.W. 2003. A survey of *Campylobacter* species shed in faeces of beef cattle using polymerase chain reaction. Can. J. Microbiol. **49**:655-661.
- Inglis, G.D., Kalischuk, L.D., and Busz, H.W. 2004. Chronic shedding of *Campylobacter* species in beef cattle. *J Appl Microbiol* **97**, 410-420.
- Jokinen, C., Edge, T.A., Ho, S., Koning, W., Laing, C., Mauro, W., Medeiros, D., Miller, J., Robertson, W., Taboada, E., Thomas, J.E., Topp, E., Ziebell, K., and Gannon, V.P. 2011. Molecular subtypes of *Campylobacter* spp., *Salmonella enterica*, and *Escherichia coli* O157:H7 isolated from faecal and surface water samples in the Oldman River watershed, Alberta, Canada. Water Res. **45**:1247-1257.
- Jokinen, C.C., Schreier, H., Mauro, W., Taboada, E., Isaac-Renton, J.L., Topp, E., Edge, T., Thomas, J.E., and Gannon, V.P. 2010. The occurrence and sources of *Campylobacter* spp., *Salmonella enterica* and *Escherichia coli* O157:H7 in the Salmon River, British Columbia, Canada. J. Water Health. **8**:374-386.
- Kemp, R., Leatherbarrow, A.J., Williams, N.J., Hart, C.A., Clough, H.E., Turner, J., Wright, E.J., and French, N.P. 2005. Prevalence and genetic diversity of *Campylobacter* spp. in environmental water samples from a 100-square-kilometer predominantly dairy farming area. Appl. Environ. Microbiol. **71**:1876-1882.
- Khan, I.U., Gannon, V., Jokinen, C.C., Kent, R., Koning, W., Lapen, D.R., Medeiros, D., Miller J., Neumann, N.F., Phillips, R., Schreier, H., Topp, E., van Bochove, E., Wilkes, G., and Edge, T.A. 2014. A national investigation of the prevalence and diversity of thermophilic *Campylobacter* species in agricultural watersheds in Canada. Water Res. **61**:243-252.
- Kumar A., Agarwal, R.K., Bhilegaonkar, K.N., Shome, B.R., Bachhil, Vand .N. 2001. Occurrence of *Campylobacter jejuni* in vegetables. Int J Food Microbiol. **67**:153–155.
- Lund, M., Nordentoft, S., Pedersen, K., and Madsen, M. 2004. Detection of *Campylobacter* spp. in chicken fecal samples by real-time PCR. J. Clin. Microbiol. **42**:5125-5132.
- Lynch, O.A., Cagney, C., McDowell, D.A., and Duffy, G. 2010. A method for the growth and recovery of 17 species of *Campylobacter* and its subsequent application to inoculated beef. J. Microbiol. Methods. **83**:1-7.
- Macuch, P.J., and Tanner, A.C. 2000. *Campylobacter* species in health, gingivitis, and periodontitis. J. Dent. Res. **79**:785-792.

- Majowicz, S.E., Edge, V.L., Fazil, A., McNab, W.B., Doré, K.A., Sockett, P.N., Flint, J.A., Middleton, D., McEwen, S.A., and Wilson, J.B. 2005. Estimating the under-reporting rate for infectious gastrointestinal illness in Ontario. *Can. J. Public Health*. **96**:178-181.
- Millson, M., Bokhout, M., Carlson, J., Spielberg, L., Aldis, R., Borczyk, A., and Lior, H. 1991. An outbreak of *Campylobacter jejuni* gastroenteritis linked to meltwater contamination of a municipal well. *Can. J. Public Health*. **82**:27-31.
- Neimann, J., Engberg, J., Molbak, K., and Wegener, H.C. 2003. A case-control study of risk factors for sporadic *Campylobacter* infections in Denmark. *Epidemiol. Infect.* **130**:353-366.
- Nothaft, H., Scott, N.E., Vinogradov, E., Liu, X., Hu, R., Beadle, B., Fodor, C., Miller, W.G., Li, J., Cordwell, S.J., and Szymanski, C.M. 2012. Diversity in the protein N-glycosylation pathways within the *Campylobacter* genus. *Mol. Cell. Proteomics*. **11**:1203-19.
- Obiri-Danso, K., Paul, N., and Jones, K. 2001. The effects of UVB and temperature on the survival of natural populations and pure cultures of *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter lari* and urease-positive thermophilic campylobacters (UPTC) in surface waters. *J. Appl. Microbiol.* **90**:256-267.
- On, S.L. 2013. Isolation, identification and subtyping of *Campylobacter*: where to from here? *J. Microbiol. Methods*. **95**:3-7.
- Pachepsky, Y., Shelton, D.R., McLain, J.E.T., Patel, J., and Mandrell, R.E. 2011. Irrigation water as a Source of Pathogenic Microorganisms in Produce: A Review. In *Advances in Agronomy* ed. Sparks, D.L. pp. 73-138. Burlington: Academic Press.
- Pitkanen, T. 2013. Review of *Campylobacter* spp. in drinking and environmental waters. *J. Microbiol. Methods*. **95**:39-47.
- Potter, M.E., Kaufmann, A.F., Blake, P.A., and Feldman, R.A. 1984. Unpasteurized milk. The hazards of a health fetish. *J.A.M.A.* **252**:2048-2052.
- Public Health Agency of Canada. 2013. The Chief Public Health Officer's Report on the State of Public Health in Canada, 2013: Infectious Diseases-The Never-ending Threat. p. 51.
- Public Health Agency of Canada. 2012. C-Internet 2009 Annual Report-National Integrated Enteric Pathogen Surveillance Program. In. Public Health Agency of Canada, p 19.
- Ridley, C.M., Jamieson, R.C., Truelstrup Hansen, L., Yost, C.K., and Bezanson, G.S. 2014. Baseline and storm event monitoring of *Bacteroidales* marker concentrations and enteric pathogen presence in a rural Canadian watershed. *Water Res.* **60**:278-288.

- Rodríguez, S. and Araujo, R. 2010. Occurrence of thermotolerant *Campylobacter* species in surface waters of a Mediterranean area and in its prevailing pollution sources. *J. Appl. Microbiol.* **109**:1027-1034.
- Schmidt, P.J., Pintar, K.D., Fazil, A.M., Flemming, C.A., Lanthier, M., Laprade, N., Sunohara, M.D., Simhon, A., Thomas, J.L., Topp, E., Wilkes, G., and Lapen, D.R. 2013. Using *Campylobacter* spp. and *Escherichia coli* data and Bayesian microbial risk assessment to examine public health risks in agricultural watersheds under tile drainage management. *Water Res.* **47**:3255-3272.
- St-Pierre, K., Levesque, S., Frost, E., Carrier, N., Arbeit, R.D., and Michaud, S. 2009. Thermotolerant Coliforms are not a good surrogate for *Campylobacter* spp. in environmental water. *Appl. Environ. Microbiol.* **75**:6736-6744.
- Stoddard S.F, Smith B.J., Hein R., Roller B.R.K. and Schmidt T.M. 2015. *rrnDB*: improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development. *Nucleic Acids Res.* 2014; doi: 10.1093/nar/gku1201.
- Tambalo, D.D., Fremaux, B., Boa, T., and Yost, C.K. 2012. Persistence of host-associated *Bacteroidales* gene markers and their quantitative detection in an urban and agricultural mixed prairie watershed. *Water Res.* **46**:2891-2904.
- Taylor, D.N., Porter, B.W., Williams, C.A., Miller, H.G., Bopp, C.A., and Blake, P.A. 1982. *Campylobacter* enteritis: a large outbreak traced to commercial raw milk. *West. J. Med.* **137**:365-369.
- Thomas, C., Hill, D.J., and Mabey, M. 1999. Evaluation of the effect of temperature and nutrients on the survival of *Campylobacter* spp. in water microcosms. *J. Appl. Microbiol.* **86**:1024-1032.
- Thomas, M.K., Majowicz, S.E., Pollari, F., and Sockett, P.N. 2008. Burden of acute gastrointestinal illness in Canada, 1999-2007: interim summary of NSAGI activities. *Can. Commun. Dis. Rep.* **34**:8-15.
- Van Dyke, M.I., Morton, V.K., McLellan, N.L., and Huck, P.M. 2010. The occurrence of *Campylobacter* in river water and waterfowl within a watershed in southern Ontario, Canada. *J. Appl. Microbiol.* **109**:1053-66.
- Verhoeff-Bakkenes, L., Jansen, H.A.P.M., in 't Veld, P.H., Beumer, R.R., Zwietering, M.H., and van Leusden F.M. 2011. Consumption of raw vegetables and fruits: A risk factor for *Campylobacter* infections. *Int. J. Food Microbiol.* **144**:406-412.
- Walters, S.P., Gannon, V.P., and Field, K.G. 2007. Detection of *Bacteroidales* fecal indicators and the zoonotic pathogens *E. coli* 0157:H7, *Salmonella*, and *Campylobacter* in river water. *Environ. Sci. Technol.* **41**:1856-62.

- 473
474 Wilkes, G., Edge, T., Gannon, V., Jokinen, C., Lyautey, E., Medeiros, D., Neumann, N., Ruecker,
475 N., Topp, E., and Lapen, D.R. 2009. Seasonal relationships among indicator bacteria,
476 pathogenic bacteria, *Cryptosporidium* oocysts, *Giardia* cysts, and hydrological indices for
477 surface waters within an agricultural landscape. Water Res. **43**:2209-23.
478
479 World Health Organization (2011) Campylobacter-Factsheet N°225.
480 <http://www.who.int/mediacentre/factsheets/fs255/en/>. Accessed on November 12, 2013.
481
482
483

Table 1- PCR primers and probe sequences used to detect *Campylobacter*.

Target/Species	Primer/ Probe	Primer Sequence (5' to 3')	PCR Annealing Temp (°C)	PCR product size (bp)	Reference
<i>Campylobacter</i> spp.	campF2	CACGTGCTACAATGGCATAT	58	NA	Lund et al. 2004
	campR2	GGCTTCATGCTCTCGAGTT			
	campP2	FAM-CAGAGAACAATCCGAACTGGGACA-BHQ1			
<i>C. coli</i>	JH0041	TGACGGTAGAACTTTCAAATCC	64.6	148	Chaban et al. 2009
	JH0042	GCAAGTGCTTCACCTTCGATA			
<i>C. concisus</i>	JH0023	GGCTCAAAAGAGATCGCTCA	64.6	158	Chaban et al. 2009
	JH0024	CCCTCAACAACGCTTAGCTC			
<i>C. curvus</i>	JH0027	CTGATAGCTGATGCGATGGA	64.6	162	Chaban et al. 2009
	JH0028	CTCGACCTGCATTTTCTCG			
<i>C. fetus</i>	JH0087	TGAGGCTGTTACAAGCGAGTTA	62.5	100	Chaban et al. 2009
	JH0088	TGAGCTATCGCTATTTGCTGAA			
<i>C. gracilis</i>	JH0021	AAGAGATCGCACAGGTTGCT	64.6	162	Chaban et al. 2009
	JH0022	CAAACCTGCATTCCCTCGACT			
<i>C. helveticus</i>	JH0017	TGAAGCGATTGTTGATGAGC	64.6	151	Chaban et al. 2009
	JH0018	AACGCCATCTTTTCCAACCTC			
<i>C. hyointestinalis</i>	JH0033	GGGGCAAATCCTATTGAGGT	62.5	137	Chaban et al. 2009
	JH0034	TCGCTATTTGCAGAGATCGTAG			
<i>C. jejuni</i>	JH0039	GAGCTTTCAAGCCCTTATATCC	62.5	174	Chaban et al. 2009
	JH0040	AAGAACACCGCGAAGTTTATTT			
<i>C. lari</i>	JH0015	TCTGCAAATTCAGATGAGAAAA	54.3	180	Chaban et al. 2009
	JH0016	TTTTTCAGTATTTGTAATGAAATATGG			
<i>C. mucosalis</i>	JH0029	TGCTGATGCAATGGAGAAAG	64.6	152	Chaban et al. 2009
	JH0030	CCTGCATTTTCTCGGTGTTT			
<i>C. rectus</i>	JH0013	AGCTATCATCGCCGAGCTAA	64.6	177	Chaban et al. 2009
	JH0014	TGAATGGATTTTGCCTCCTC			
<i>C. showae</i>	JH0037	GCCACTATCTCGGCAAATTC	60	125	Chaban et al. 2009
	JH0038	ACGTTTAGCTCGTCGTGGAT			
<i>C. sputorum</i>	JH0025	CAAAGTTGCTGAGGCAATCA	64.6	118	Chaban et al. 2009
	JH0026	TGATCCAACAGCCTCATCTG			
<i>C. upsaliensis</i>	JH0019	CGTTTTGGCACACGCTATTT	64.6	110	Chaban et al. 2009
	JH0020	CATCAACAATAGCCTCACAAGC			

Table 2 - Frequency of *Campylobacter* detection from sampling sites in the Qu'Appelle River Watershed*.

Sampling Site	Detection Frequency (%)
2009	
Site C (Reference Site)	12/22 (55)
<i>Wascana Creek Sites</i>	
Site 1	9/17 (53)
Site 2	11/20 (55)
Site 3	12/20 (60)
Site 4	11/22 (50)
<i>Qu'Appelle River Sites</i>	
Site 5	13/22 (59)
Site 6	14/22 (64)
Total	82/145 (57)
2013	
Site C	5/28 (18)
Site 3	5/28(18)
IP1 (Wascana Creek; upstream of site 4)	2/20 (10)
IP2 (Qu'Appelle River; upstream of site 5)	2/20 (10)
IP3 (Qu'Appelle River; downstream of site 5)	5/20 (25)
Total	19/116 (16)

**Campylobacter* was detected by qPCR using *Campylobacter* 16S rDNA primers (Lund et al. 2004).

Table 3 - Detection of *Campylobacter* spp. at different sampling sites in the Qu’Appelle River watershed in 2009 and 2013*.

Number of samples that tested positive (%)																		
Site	N†															Negative Number for all of		
		<i>C. coli</i>	<i>C. concisus</i>	<i>C. curvus</i>	<i>C. fetus</i>	<i>C. gracilis</i>	<i>C. helveticus</i>	<i>C. hyointestinalis</i>	<i>C. jejuni</i>	<i>C. lari</i>	<i>C. mucosalis</i>	<i>C. rectus</i>	<i>C. showae</i> ††	<i>C. sputorum</i>	<i>C. upsaliensis</i>	<i>Campylobacter</i> spp. detected	Species tested	
2009																		
Site C	12	1 (8)	1 (8)	1 (8)	0	4 (33)	2 (17)	1 (8)	5 (42)	11 (92)	0	0	0	0	0	1	8	
Site 1	5	1 (20)	0	0	0	2 (40)	0	0	2 (40)	4 (80)	0	0	0	0	0	0	4	
Site 2	9	0	0	0	0	0	0	0	6 (67)	7 (78)	0	0	0	0	0	1	2	
Site 3	12	1 (8)	0	0	0	0	1 (8)	0	5 (42)	7 (58)	0	0	0	0	0	1	4	
	11																	
Site 4		3 (27)	9 (82)	0	0	1 (9)	2 (18)	1 (9)	4 (36)	10 (91)	0	0	0	0	0	0	8	
Site 5	12	2 (17)	0	0	0	0	0	3 (25)	6 (50)	11 (92)	0	0	0	2 (17)	0	0	5	
Site 6	13	2 (15)	0	0	0	0	0	1 (8)	2 (15)	12 (92)	0	0	0	1 (8)	0	1	5	
	74	10 (14)	10 (14)															
Total																		
		)	)		1 (1)	0	7 (9)	5 (7)	6 (8)	30 (41)	62 (84)	0	0	0	3 (4)	0	4 (5)	10
2013																		
Site C	5	0	0	0	0	0	0	0	0	5 (100)	0	0	0	0	0	0	1	
IP1	2	0	0	0	0	0	0	0	0	2 (100)	0	0	0	0	0	0	1	
IP2	2	0	0	0	0	0	0	0	0	2 (100)	0	0	0	0	0	0	1	
IP3	5	0	0	0	0	0	0	0	0	5 (100)	0	0	0	0	0	0	1	
Site 3	5	0	0	0	0	0	0	0	0	5 (100)	0	0	0	0	0	0	1	
	19	0	0	0	0	0	0	0	0	19	0	0	0	0	0	0	1	
Total																		
										(100)								

* *Campylobacter* spp. were identified by conventional PCR using *cpn60* primers (Chaban et al. 2009).

[†]N=number of *Campylobacter*-positive samples used for speciation.

^{††}PCR amplicons were not homologous to *C. showae* *cpn60*, hence reported as undetected.

Table 4 - Number of samples that tested positive for *Campylobacter* spp. as a function of month (2009)*.

Month	Number (%) of positive samples															Total # of spp. detected
	N	<i>C. coli</i>	<i>C. consisus</i>	<i>C. curvus</i>	<i>C. fetus</i>	<i>C. gracilis</i>	<i>C. helveticus</i>	<i>C. hyointestinalis</i>	<i>C. jejuni</i>	<i>C. lari</i>	<i>C. mucosalis</i>	<i>C. rectus</i>	<i>C. showae</i> [†]	<i>C. sputorum</i>	<i>C. upsaliensis</i>	
April	10	4 (40)	2 (20)	1 (10)	0	3 (30)	5 (50)	4 (40)	3 (30)	8 (80)	0	0	0	0	0	8
May	14	0	3 (21)	0	0	2 (14)	0	1 (7)	9 (64)	13 (93)	0	0	0	1 (7)	0	6
June	19	1 (5)	2 (10)	0	0	0	0	0	10 (53)	16 (84)	0	0	0	2 (10)	0	5
July	9	0	1 (11)	0	0	1 (11)	0	1 (11)	2 (22)	8 (89)	0	0	0	0	0	5
August	14	3 (21)	2 (14)	0	0	1 (7)	0	0	4 (29)	11 (79)	0	0	0	0	0	5
September	8	2 (25)	0	0	0	0	0	0	2 (25)	6 (75)	0	0	0	0	0	3

* *Campylobacter* spp. were identified by conventional PCR using *cpn60* primers (Chaban et al. 2009).

[†]PCR amplicons were not homologous to *C. showae cpn60*, hence reported as undetected.

Figure Captions

Fig. 1. Location of sampling sites along the Qu’Appelle River and Wascana Creek. Figure was revised from Tambalo et al. 2012.

Fig. 2. Monthly detection frequency of *Campylobacter* in the study area in 2009 and 2013. Detection frequency in 2009 was obtained from Tambalo et al. 2012.

Draft

155x136mm (300 x 300 DPI)

Figure S1. Sample agarose gels of *Campylobacter cpn60* amplicons. The positive controls are indicated as * and positive samples are labelled with +. The bands for *C. showae* are false positives, based on sequencing results.

A. *Campylobacter coli*

CATCGTTGTACCCAGTGGTTTTCTGTTTTTTGATTTGTTCTAAAACCGGTAACAAAT
CTTTTAAATTTGCAATTTTTTTATCAAAAAGCAAGATATAAGGATTTGAAAGTTCTAC
CGTCAAGTTGTTGAAATCTCGCTTTTTGATAAAAAAATTGCAAATTTCAAAGATTTA
TTAGGGTTTTAAAAAATTAAAAGCGGAAGCCCCCTTTTGATCTCGGTGAAACTTCGAA
GTCAAGCGTTGCCACG

B. *Campylobacter jejuni*

TGGGTGGCGGCAGTCGCTTCCCACACCTATCTTCTGCGATAATTACACGTGGTTTGC
CTGTTTTTTGAATTCGACAAAAACCGGTAATAAATCTTTTAAATTTGTAATTTTTTTA
TCAAAAAGCAGGATATAAGGGCTTGAAAGCTCCCATATTTGGGGTTGGTTTTTCACT
TTTTGGGGCGGGTAAATAAAAAACAGGCGGGGGTAACAAAAAAACAGGCGGGGT
AATTTAAATACCGGCGGGGGGAAAAAAGGGGGGGGTGTATAAAAAACG
GGGCGGTGTTATAAAAAACCGGGGGGTTCGATAAAAAACACTTCCGTCGTGTA
AACAACTTTCCCCTTTTATAAACCCCCCTCTCTCTTTTCCTTTTAGG

C. *Campylobacter lari*

GAGCTATTCTCTATCAATTGCATACCTTCAACTACATTTAATTCATCATTGATTGAT
TTTGCTTCTTCAACAGTAATAACACCATCTTTTCCAACCTTTTCCATAGCATCAGCGA
TTAAATTTCCGATTTTCTCATCTGAATTTGCAGACAAGAGAAGATCGGAAATTTAAT
CGCTAATGCTATGGAAAAAGTGAAAAAGATGGTGTATTACTGTTGAAGAAGCAA
AATAATCGGGATAAAATAAATGTACTTGAAGGTATGCATTTGAATAGAAGGATATTT
AAACCTATGTATTAAAATCAGAAAAA

D. *Campylobacter showae* *cpn60* clone 1:

ACGTTTAGCTCGTCGTGGATACGTTTAGCTCGTCGTGGATCTGGCTGAAGAAGGCCA
GCGCTTCGAGGTCGTAGTCGAATACCTGCCCTTCTTGTCGGCCAGGGTCAGGAATT
TGCCGAGA

E. *Campylobacter showae* *cpn 60* clone 2:

ACGTTTAGCTCGTCGTGGATCTGGCTGAAGAAGGCCAGCGCTTCGAGGTCGTAGTCG
AACACCTGCCCCCTTCTTGTCGGCCAGGGTCAGGAATTTGCCGAGA

F. *Campylobacter showae* *cpn60* clone 3:

ACGTTTAGCTCGTCGTGGATCTGGCTGAAGAAGGCCAGCGCTTCGAGGTCGTAGTCG
AATACCTGCCCCCTTCTTGTCGCCAGGGTCAGGAATTTGCCGAGA

Figure S2. Sequences of representative *cpn60* amplicons for (A) *C. coli*, (B) *C. jejuni*, and (C) *C. lari*. For *C. showae*, three clones were sequenced and the *cpn60* sequences are shown in D, E, and F. Blastn results for *C. showae* demonstrated homology (94%-96% identity) to *Aeromonas* (2-isopropylmalate synthase).