

Using Host-Specificity of *Cryptosporidium* to Understand Contaminant Sources, Seasonality, and Human Health Risk in Three Watersheds of Differing Land-Use

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Received 18 January 2016; accepted 23 February 2016; published 26 February 2016

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Abstract

Three tributaries of the Grand River watershed (Ontario, Canada), each representing different watershed types (urban, agricultural/rural, and mixed land-use) were examined to understand the spatial, temporal, and host-source distribution of the waterborne pathogen, *Cryptosporidium*. *Cryptosporidium* was frequently found throughout the study (73%, 65/89) with occurrence and concentrations observed to be similar among the varying watershed types. However, applying advanced genotyping techniques, marked differences in dominant host sources could be observed in each watershed. The agricultural/rural and mixed land-use watersheds were dominated by genotypes typically associated with cattle (*i.e.*, *C. andersoni*), while the urban watershed had the highest diversity of *Cryptosporidium* genotypes with a variety of wildlife as the common source of contamination (e.g., muskrat and cervine genotypes). A similar seasonal trend observed in the urban, agricultural, and mixed land-use watershed suggests that factors beyond specific land use activities (e.g. autumn manure spreading) may influence the timing and concentration of *Cryptosporidium* in these streams. Corresponding genotyping results provided additional insight into source inputs during these seasonal peaks, indicating that wildlife may be important seasonal contributors to *Cryptosporidium* contamination in these streams. Despite the abundance of *Cryptosporidium* in these watersheds, most of the genotypes observed were of limited human health importance. This study provides evidence regarding the significance of including genotyping results into studies examining waterborne *Cryptosporidium*. Using this technique can provide a greater un-

How to cite this paper: Thomas, J.L., Pintar, K.D.M., Wallis, P.M. and Neumann, N.F. (2016) Using Host-Specificity of *Cryptosporidium* to Understand Contaminant Sources, Seasonality, and Human Health Risk in Three Watersheds of Differing Land-Use. *Journal of Environmental Protection*, 7, 372-381. <http://dx.doi.org/10.4236/jep.2016.73033>

derstanding of the risk to the population using water sources, as well as provide insight into the probable sources and timing of contamination. This ancillary information can contribute to implementation of targeted management strategies to further protect sources of drinking water and recreation areas.

Keywords

Cryptosporidium, Genotyping, Source Tracking, Health Risk, Water Contamination

1. Introduction

Cryptosporidium is a well-recognized waterborne pathogen that has been implicated in drinking water and recreational water outbreaks in North America and around the world [1]–[4]. As part of its life cycle, *Cryptosporidium* produces an environmentally robust oocyst stage that is shed in very large numbers in the feces of infected animals and humans, thereby increasing environmental contamination. Agricultural animals, particularly cattle, are well recognized as a source of *Cryptosporidium* [4]–[7]. However, a wide range of host animals can be infected and excrete *Cryptosporidium* in their feces, including domestic animals, various wildlife, and humans [6] [8]–[11]. Given the host range of *Cryptosporidium*, many fecal sources can be a source of contamination to water, including sewage treatment discharges, run-off from cropland following manure application, and direct access of domestic and wild animals to water sources.

Many *Cryptosporidium* species are host-adapted, meaning that they specifically infect certain types of animals over others. For example, *C. andersoni* is commonly associated with post-weaned cattle and *C. baileyi* predominantly infects birds [7] [11]. Two species of *Cryptosporidium* are primarily responsible for human infections: *C. hominis* and *C. parvum*. *C. hominis* is host-adapted and found almost exclusively in humans [11]. *C. parvum* is less host-adapted and can infect a range of animals including humans and cattle [12]–[14].

Routine detection and enumeration of *Cryptosporidium* from water, particularly for regulatory compliance, are conducted microscopically using standard methods such as US EPA Method 1622 and 1623 [15] [16]. These methods allow for the detection and enumeration of total *Cryptosporidium* oocysts in a water sample; however, they do not differentiate between specific species/genotypes. Over the past decade, some researchers have applied advanced molecular methodologies to examine the genotypes in water samples. Distinguishing between genotypes can provide a refined estimation of the potential human health risk that this contamination poses to the public. In addition, due to the host-adapted nature of many *Cryptosporidium* species this additional information can provide a better understanding of the predominant sources of this contamination in a watershed, which can assist in source tracking this type of contamination and provide additional insight into future management and protection strategies for specific water sources.

The objectives of this study were to compare the prevalence of *Cryptosporidium* in three watersheds impacted by differing land-uses (agricultural/rural, mixed land-use, and urban-dominated watersheds) and apply advanced genotyping techniques to: 1) understand the dominant host sources of this pathogen in each watershed and among seasons; and, 2) determine the prevalence of species that may pose a risk to human health.

2. Methods

2.1. Sampling Sites

Three sub-watersheds within the Grand River Watershed (Ontario, Canada) with varying dominant land-uses were chosen for sampling (Figure 1). Schneider Creek is a highly urban watershed with dense roads and residential/industrial areas. No point sources of contamination exist on this stream, such as sewage treatment discharges, however; this stream is impacted by storm water discharges throughout the year. The sample location on the Nith River is in the headwaters of this sub-watershed which is agriculturally-dominated. Canagagigue Creek is a mixed land-use sub-watershed, with intensive agriculture and a small urban area with a sewage treatment discharge outfall located upstream of the sample collection point (see Table 1).

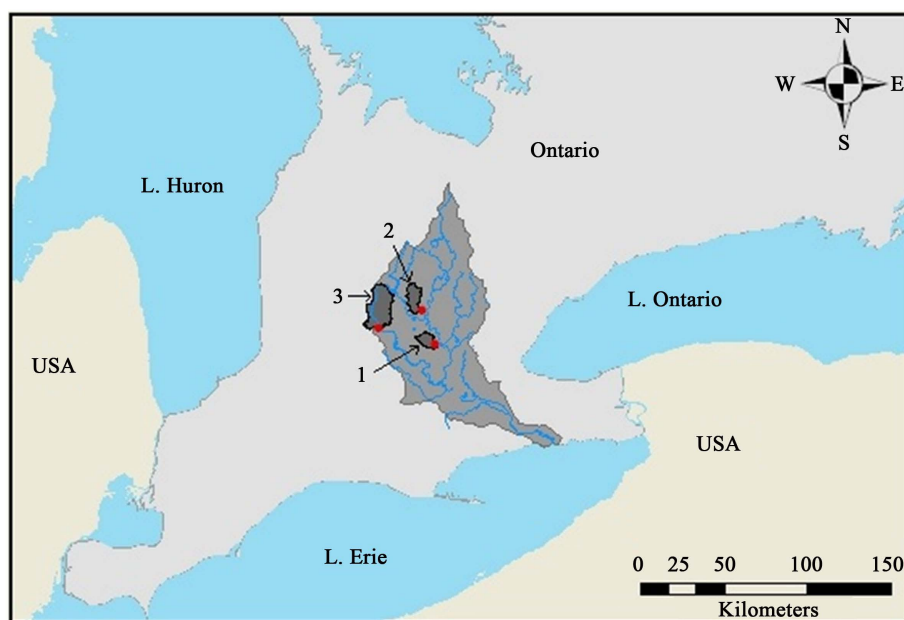


Figure 1. Grand River watershed (in light grey) Southwestern Ontario, Canada that discharges into Lake Erie and three sub-watersheds (dark grey) and sample locations included in this study; 1) Schneider Creek; 2) Canagagigue Creek; and 3) Nith River.

Table 1. Watershed characterization and *Cryptosporidium* oocysts occurrence in three tributaries of the Grand River watershed.

Stream name	Watershed information			Number of samples taken	<i>Cryptosporidium</i> oocysts		
	Watershed area (km ²)	% Agriculture ^a	% Urban ^a		Number of positive samples	Occurrence (%)	Concentration. range (above DL ^c) per 100 L
Schneider Creek	71	33	57	32	19	59.4	1.6 - 80.4
Canagagigue Creek	111	87	4 ^b	38	32	84.2	1.0 - 282.8
Nith River	313	91	0	19	14	73.7	1.0 - 500.0

^aData obtained from the Ontario Ministry of Natural Resources Land Cover Data Base (2000 edition); ^bSewage treatment discharge located 5.3 km upstream of site, facility provides tertiary treatment including UV disinfection; ^cDL = theoretical detection limit (1.0 to 45.5 oocysts per 100 L).

2.2. Sample Collection and Water Quality Analysis

Samples were collected between September 2007 and August 2009. Samples were taken once to several times per month so as to obtain samples representing a range of hydrological conditions including base-flow and storm-flow (rain events and snow melt).

Surface water samples were filtered on-site, up to a maximum of 100 litres (L), using IDEXX filters (comprised of compressed foam discs to capture particulate) and filtration housings (IDEXX Laboratories Ltd., Markham, ON). Following filtration, each filter was placed in a sterile whirl pack bag and shipped on ice overnight to the laboratory (Hyperion Research Ltd., Medicine Hat, AB) for analysis using US EPA method 1623 [16] and processed within 72 hours of collection. US EPA 1623 method involves the elution and concentration of debris/trapped material from each filter. The *Cryptosporidium* oocysts are separated from this debris using magnetic beads coated with antibodies specific for the antigen proteins on the outside wall of oocysts (GC-Combo magnetic beads, Invitrogen, Oslo, Norway). Once separated and washed, the cells were treated with monoclonal antibodies with CY3 fluorochrome conjugates (Waterborne Inc, New Orleans, LA) to cause oocysts to fluoresce under ultra-violet (UV) light during microscopic examination (Zeiss Axioskop, Carl Zeiss Canada Ltd, Canada). Cells were also counter-stained with 4',6-diamidino-2-phenylindole (DAPI) and examined under differential interference contrast (DIC) microscopy for confirmation. During this study, recovery of spiked sam-

ples was within the range specified by US EPA Method 1623 [16], with recoveries reported at 38.5% (Residual Standard Deviation; RSD, 30.3) for *Cryptosporidium* oocysts.

In preparation for genotyping, the material on each microscope slide, was prepared and subject to cell lysis and DNA extractions, as described by [17]. This method involves the lysis of cells following several freeze/thaw cycles (2 minutes (min.) in liquid nitrogen and 5 min. at 65°C) followed by DNA extraction (DNeasy Kit, Qiagen, Mississauga, ON).

The extracted DNA was sent to the Alberta Provincial Health Laboratory (Calgary, AB) where repetitive nested-PCR(polymerase chain reaction), restriction fragment length polymorphism (RFLP) analysis, and sequencing were performed as described in [17] [18]. A total of five replicate PCR reactions were performed per sample to determine if more than one species were present in a sample. PCR products were sequenced (Sun Center of Excellence for Visual Genomics, University of Calgary, Calgary, AB) and results were compared to known *Cryptosporidium* sequences in a reference database maintained at the University of Calgary prior to assigning a genotype name to a specific sample.

2.3. Data Analysis and Statistical Testing

Non-parametric statistical methods were used as the microbiological data were not-normally distributed. All data were analyzed using Excel (Microsoft Office) and SigmaPlot (version 12.1). Fisher's exact and Chi square tests were applied to test for significant spatial differences ($P < 0.05$) in occurrence of *Cryptosporidium* oocysts among streams and between seasons (Winter: Dec-Feb, Spring: Mar-May, Summer: Jun-Aug, and Fall: Sep-Nov). Kruskal-Wallis testing (nonparametric ANOVA on ranks) was conducted to examine differences ($P < 0.05$) in densities among streams and seasons, with significant differences further identified using Dunn's multiple comparison test, which is an appropriate nonparametric pairwise multiple comparison procedure when a Kruskal-Wallis test is rejected.

Cryptosporidium were considered present if the value reported was above the method detection limit (1.0 to 45.5 oocysts per 100 L). In a few instances ($n = 8$), samples were also considered positive for *Cryptosporidium* when detected through PCR (following genotyping analysis), but were below the detection limit following microscopic detection. These PCR positive, microscopic negative results were only used for occurrence analysis.

3. Results

Cryptosporidium oocysts were detected in 73% (65/89) of the water samples. Fifty-seven samples were positive following microscopic analysis, with 8 additional samples positive following molecular analysis. The proportion of samples positive for *Cryptosporidium* in each stream is shown in Table 1. Significant differences in *Cryptosporidium* oocysts occurrences were not detected among streams ($P = 0.7$).

The median concentration of *Cryptosporidium* was 3.3 oocysts/100 L, with values ranging from less than the method detection limit to 500 oocysts/100 L. Differences were not observed ($P = 0.25$) in the median concentration of *Cryptosporidium* among streams. A wide range of *Cryptosporidium* concentrations were observed in all streams, with the greatest variability in the Nith River (Figure 2) and the lowest in Schneider Creek.

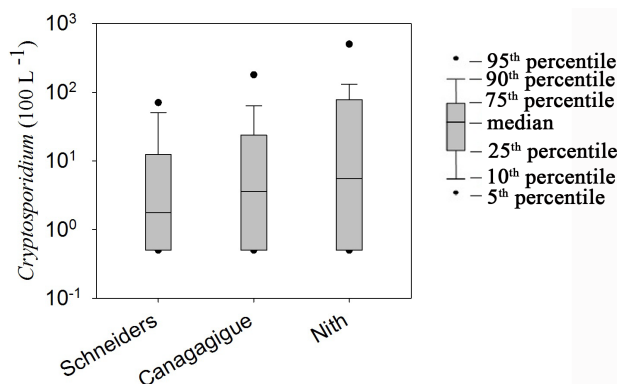


Figure 2. *Cryptosporidium* concentrations in three streams in the Grand River watershed.

Significant differences ($P < 0.05$) in the occurrence of *Cryptosporidium* were observed with season. Overall, fall months (September to November) revealed the greatest frequency of *Cryptosporidium* occurrence (19/20) which was significantly different ($P < 0.001$) from the levels observed in both winter (13/24) and spring (12/28). Differences ($P > 0.05$) were not observed between occurrences in the summer (12/17) and the other seasons. This trend was apparent in all three streams, with the fall months showing the greatest occurrence of *Cryptosporidium*. In many instances, the summer months showed high occurrence levels as well (Figure 3).

Similar to occurrence levels, differences in median concentrations for *Cryptosporidium* were observed among seasons ($P < 0.001$). In general, higher median values were observed in the summer and fall for *Cryptosporidium*, compared to the winter and spring (Figure 4). The highest median concentration was observed in the summer, which was significantly different to the median concentrations observed in the winter and spring, but not the fall, following a pairwise multiple comparisons test. Spring revealed the greatest portion of non-detectable samples. Although the lowest median concentration for *Cryptosporidium* occurred in the spring months, this season showed great variability and the highest reported level for this parasite for the entire study period at 500 oocysts per 100 L.

A total of 26 water samples generated positive genotyping results. Of these samples, 9 distinct *Cryptosporidium* genotypes were found (Table 2). The most common species detected was *C. andersoni*, which was found in 19 water samples. In most instances, only one genotype was detected, however, 19% (5/26) of the samples revealed more than one different genotype in a single sample.

Samples from Schneider Creek showed the most diverse range of genotypes (total of 8 different genotypes), followed by Canagagigue Creek (4 different genotypes). Samples from the Nith River only revealed one genotype, *C. andersoni*, throughout the study period (Table 2). Although positive microscopic detection of *Cryptosporidium* oocysts occurred throughout all of these seasons (Figure 3), positive genotype results (through molecular analysis) were more commonly reported in samples taken in the summer and fall months (Table 2).

Table 2. *Cryptosporidium* genotypes observed in each watershed and with season, associated health risk, and sources.

Stream name	Genotypes detected	Human health risk class ^b	Common host-sources ^c	Proportion of genotypes obtained in stream	No. water samples positive for genotype	Season genotype detected			
						Winter	Spring	Summer	Fall
Nith River	<i>C. andersoni</i>	Low	Ruminants (predominantly adult cattle, >3 months)	100%	3	■			■
	<i>C. andersoni</i>	Low	Ruminants (predominantly adult cattle, >3 months)	80%	16	■	■	■	■
Canagagigue Creek	W12 genotype	None	Unknown-likely wildlife	10%	2			■	■
	<i>C. baileyi</i>	None	Avian	5%	1				■
	W25 genotype	None	Unknown-likely wildlife	5%	1			■	
	<i>C. baileyi</i>	None	Avian	22%	2			■	■
Schneider Creek	<i>C. parvum</i>	High	Humans, ruminants (in particular pre-weaned calves < 3 months)	11%	1			■	
	Cervine genotype II ^a	Medium	Deer, sheep	11%	1			■	
	Deer mouse genotype III	None	Deer mouse	11%	1				■
	Muskrat genotype	None	Muskrat, vole	11%	1				■
	Skunk genotype	Low	Skunk, raccoon	11%	1			■	
	W12 genotype	None	Unknown-likely wildlife	11%	1				■
	W25 genotype	None	Unknown-likely wildlife	11%	1			■	

^a*C. ubiquitum*; ^bReference [19]; ^cReferences [7] [11] [19] [20].

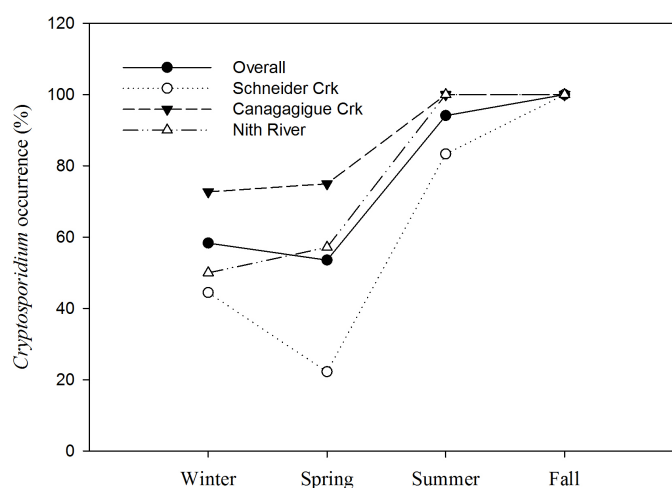


Figure 3. Seasonal trends in the occurrence of *Cryptosporidium* overall (data for all streams combined) and within each stream studied. Connecting lines shown for clarity.

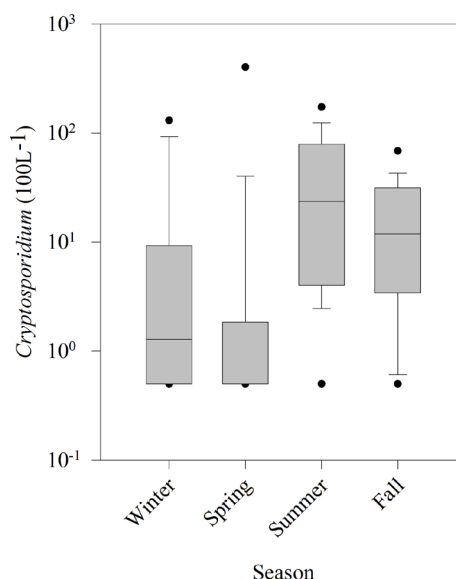


Figure 4. Median and range of *Cryptosporidium* concentrations throughout all seasons.

4. Discussion

This study examined the spatial and temporal distribution of *Cryptosporidium* within three tributaries of the Grand River watershed, with the objective to apply advanced genetic techniques to identify predominant host sources of *Cryptosporidium* contamination within each stream and among seasons, as well as to determine the prevalence of genotypes that may pose a risk to human health.

Overall, there was frequent detection of *Cryptosporidium* (73%) throughout this two year study. Similar levels and concentrations of *Cryptosporidium* were observed among the urban, agricultural, and mixed land-use watersheds, however, genotyping results revealed discernible differences in host sources among watersheds. *C. andersoni* was the most frequently detected genotype in this study, where it was the dominant genotype observed in the agricultural watershed (Nith River) and in the mixed land-use watershed (Canagagigue Creek), which is reflective of the livestock intensity and production of manure in these two streams. This genotype is commonly associated with adult cattle [6] [7] and is frequently reported in watersheds when agricultural activi-

ties are occurring on the landscape [18] [19] [21] [22]. Despite the dominance of this genotype in this study, *C. andersoni* is considered low risk to human health [12].

In contrast to the Nith River and Canagagigue Creek, no *C. andersoni* was observed in the urban watershed (Schneider Creek), which is reflective of the limited occurrence of agricultural activities on the landscape. Overall, the urban watershed had the highest diversity of *Cryptosporidium* genotypes with a variety of wildlife as the common source of contamination (e.g., muskrat, skunk, and cervine genotypes [20]). A similar finding was reported by [23] who observed a large diversity of wildlife-associated *Cryptosporidium* species in urban storm water samples in three New York watersheds.

Samples from Canagagigue Creek also showed the occurrence of several wildlife-associated genotypes (e.g., *C. baileyi*, and W12 and W25 genotypes), demonstrating that wildlife plays a role in the fecal contamination in this watershed in addition to livestock. Recently [19] showed the utility of using genotyping techniques to identify that wildlife, not just livestock, are important contributors to *Cryptosporidium* contamination in mixed land-use watersheds.

In Canagagigue and Schneider Creeks a single wildlife species did not predominate and the diversity of genotypes obtained reflects the range of wildlife that can be a source of contamination to these watersheds. Wildlife have been shown to carry diverse *Cryptosporidium* species, although the prevalence of carriage is largely unknown and tends to vary by animal-type [8] [9] [20]. Following the analysis of scat samples from small wild animals, [9] reported a range of *Cryptosporidium* prevalence rates with the lowest detection in beavers (0%) and the highest detection in muskrats (12%), with few species of human health significance observed. A study by [20] reported a 20.5% prevalence rate of *Cryptosporidium* in various wildlife species with a higher prevalence in small mammals (including deer mice and squirrels), lower carriage in wild birds, and no detection in fish.

Schneider Creek was the only stream where genotypes that infect humans were found, including the detection of *C. parvum* (high risk to human health) and the cervine genotype (also called *C. ubiquitum*), which can be considered a moderate risk to human health. The cervine genotype has been detected in a range of wildlife [20]. Although most often associated with infections in humans and in pre-weaned cattle, *C. parvum* is considered to have a broad host range. Wildlife such as squirrels and Canada geese have been shown to carry this species at a low frequency, although its prevalence in many wildlife animals remains elusive [8] [20]. The lack of host specificity for this species indicates that its occurrence in the urban stream cannot be associated with any particular animal source in this tributary. It does, however, indicate that there may be an elevated risk to people that might be exposed to this urban water source (*i.e.*, accidental ingestion while wading/playing in the water) compared to the other streams included in this study.

Despite the fact that there was a sewage treatment plant (STP) effluent discharge upstream of the sample location on Canagagigue Creek, no strains that are commonly associated with human infections (*C. parvum* and *C. hominis*) were detected. This finding indicates that during the time of this study the sewage treatment outfall was contributing minimal *Cryptosporidium* contamination to the stream in comparison to livestock activities in the area, which is likely related to the treatments process, including UV disinfection, at this STP.

Peaks in *Cryptosporidium* abundance and densities were observed in the summer and fall in this study. Similar trends have been reported in other watersheds with increases in the fall months generally attributed to agricultural inputs [19] [24], although seasonal trends are not always consistently observed among studies e.g., [22].

Seasonal trends in *Cryptosporidium* abundance in surface water have been suggested to correspond to changes in the source of contamination e.g., [19]. These seasonal differences can include changes in land-use practices such as seasonal manure-application to fields and potential seasonal variations in host animal shedding intensity. In addition, changes in hydrologic events, including increased precipitation during certain seasons, may lead to enhanced transport of *Cryptosporidium* from land-based fecal material to a water course [18] [19] [24] [25].

While seasonal differences in agricultural activities may account for some portion of the seasonal differences in oocysts abundance in the agriculturally-dominated watersheds, this does not explain why similar seasonal trends were observed in the urban watershed where agricultural activities are minimal. This observation suggests that factors beyond specific land use activities (e.g. autumn manure spreading) may influence the timing and concentration of *Cryptosporidium* in these streams. The genotyping results provided additional insight into the host animals responsible for peak contamination levels during the summer and fall months. In the Nith River and Canagagigue Creek, cattle-associated *C. andersoni* were observed in many different seasons. However, the

genotyping data revealed a greater detection of wildlife-associated species in the summer and fall seasons. These data suggest that seasonal peaks in occurrence and abundance of *Cryptosporidium* may be related to increased inputs from wildlife.

These seasonal trends in wildlife-associated strains may be related to changes in infections/excretion of *Cryptosporidium* in wildlife during these seasons, although this remains unknown. The influx of migratory animals, in particular birds, during these seasons may also account for some portion of the observed seasonal trend. For example, the summer and fall were the only seasons where *C. baileyi* was detected, which is considered to be of avian origin [11]. A similar suggestion was made by [24] while examining un-impacted streams in Texas. Although no genotyping data were reported, they suggested that seasonal *Cryptosporidium* peaks observed in a stream located in a wildlife refuge may be the result of migratory birds that frequent this area during this time-frame. Reference [26] reported a similar finding in two agricultural streams in Massachusetts where they observed birds as a source of oocysts and reported higher occurrence in the summer and fall months that corresponded to an influx of migratory bird in these areas.

Unfortunately, the genotyping method used in this study is not a quantitative evaluation; therefore changes in the density of wildlife-associated species with season cannot be made. However, this genotyping method tends to identify the predominant species in a water sample [27] and therefore suggests that wildlife-associated species are in high abundance during the summer and fall months.

These results imply that agricultural-activities may not be the only factor contributing impacts throughout the year. Few studies have included genotyping analysis along with seasonal investigations of *Cryptosporidium* occurrence in water [19]. Data of this type may be important for understanding which host animals are major seasonal contributors to contamination in watersheds and could lead to targeted strategies for mitigation in the future.

In addition to understanding the sources of contamination, applying advanced molecular genotyping analysis can provide additional insight into the health importance of *Cryptosporidium* species present in the water. This information can provide a more accurate assessment about the potential health risks associated with contamination in a specific watershed. Based on microscopic results alone, which is the method commonly used for regulatory compliance in various countries, the risk to human health could be overestimated. With the addition of genotyping analysis, few *Cryptosporidium* species of human health significance were observed in the study watersheds (<10% of the genotypes). Two species are predominantly responsible for infections in humans: *C. hominis* and *C. parvum* [12]. *C. parvum*, a species commonly associated with infections in humans and young cattle [12]–[14], was rarely observed in this study. *C. hominis*, which is only associated with human disease, was never detected in the three streams over the study period, although it has been detected in the Grand River watershed previously [6] [22]. When genotyping data are considered, there may be a reduced risk to the population using these waters as a source of drinking water and for recreation.

As more studies include genotyping techniques, there is a greater indication that genotypes of significant risk to the population are not as predominant as once assumed. Microscopic detection alone, without applying advanced genotyping analysis, could result in an over estimation of the health risk.

Although the genotypes observed in this stream are considered of limited human health importance, fecal contamination from both livestock and wildlife can carry other pathogens that have the ability to infect humans, including bacteria such as *Campylobacter*, *Salmonella* and pathogenic *E. coli* [6] [28]. As well, there is a possibility that pathogenic strains of *Cryptosporidium* could be undetected if not the predominant genotype in these streams. While the genotyping method used in this study is suitable for detecting multiple species of *Cryptosporidium* in a sample, as was observed in this study, it has been shown to favour the detection of the predominant species in a sample [27].

5. Conclusion

To better manage and protect sources of drinking water and recreation areas, an understanding of the occurrence and probable sources of pathogen contamination is needed. This study demonstrated that *Cryptosporidium* was ubiquitous in watersheds that were impacted by a range of land-uses, including urban, agricultural, and mixed land uses. Occurrence data alone could not determine the potential sources of *Cryptosporidium*; however, applying advanced molecular genotyping analysis proved to be a useful tool for understanding the dominant sources of *Cryptosporidium* in each watershed, as well as provided greater insight into seasonal contaminant sources. Knowledge of the various genotypes can also provide additional insight into the health importance of

Cryptosporidium present in the water, therefore providing a more accurate assessment about the potential health risks associated with this contamination. Despite the abundance of *Cryptosporidium* in these watersheds, most of the genotypes observed were of limited human health importance. This study provides evidence regarding the significance of including genotyping results into studies examining waterborne *Cryptosporidium*. Collecting this type of information can lead to targeted management strategies to further protect sources of drinking water and recreation areas.

Acknowledgements

Special thanks to staff at the Ontario Ministry of the Environment and Climate Change that were involved with sample collection, particularly Dave Supper and Beth Gilbert, and Georgina Kaltenecker for GIS support.

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