Pathogen and indicator variability in a heavily impacted watershed

Sarah M. Dorner, William B. Anderson, Terri Gaulin, Heather L. Candon, Robin M. Slawson, Pierre Payment and Peter M. Huck

ABSTRACT

Water samples were collected from 36 locations within the Grand River Watershed, in Southwestern Ontario, Canada from July 2002 to December 2003 and were analyzed for total coliforms, fecal coliforms, *Escherichia coli*, *Escherichia coli* O157:H7, and thermophilic *Campylobacter* spp. A subset of samples was also analyzed for *Cryptosporidium* spp., *Giardia* spp., culturable human enteric viruses, and *Clostridium perfringens*. Storm and snowmelt events were sampled at two locations including a drinking water intake. For the majority of the events, the Spearman rank correlation test showed a positive correlation between *E. coli* levels and turbidity. Peaks in pathogen numbers frequently preceded the peaks in numbers of indicator organisms and turbidity. Pathogen levels sometimes decreased to undetectable levels during an event. As pathogen peaks did not correspond to turbidity and indicator peaks, the correlations were weak. Weak correlations may be the result of differences in the sources of the pathogens, rather than differences in pathogen movement through the environment. Results from this investigation have implications for planning monitoring programs for water quality and for the development of pathogen fate and transport models to be used for source water risk assessment.

Key words | *Cryptosporidium*, drinking water, *E. coli* O157:H7, pathogens, quantitative (real-time) PCR, watershed

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INTRODUCTION

Following the introduction of modern water treatment techniques more than a century ago, the incidence of waterborne diseases such as cholera and typhoid fever were greatly reduced in the developed world. Yet in 1993, an outbreak in Milwaukee, Wisconsin involving more than 400,000 cases of gastroenteritis caused by the protozoan parasite *Cryptosporidium parvum* was linked to the city's drinking water source (Mackenzie *et al.* 1994). Furthermore, in 2000, Walkerton, Ontario, Canada experienced a waterborne disease outbreak caused by the bacteria *Escherichia coli* O157:H7 and *Campylobacter jejuni* resulting in more than 2000 cases of gastroenteritis and seven deaths (O'Connor 2002). Both of these outbreaks followed periods of heavy rainfall, as have others (Hrudey *et al.* 2002; Hunter 2003), suggesting a link between watershed hydrology and waterborne disease outbreaks.

Zoonotic pathogens (pathogens transmissible between vertebrate animals and humans) comprise 75% of the emerging infectious diseases in humans (Bolin *et al.* 2004). Many of these emerging zoonoses are transmitted indirectly

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through food, water, or environmental contamination. Evidence suggests that zoonotic waterborne pathogens will continue to be recognized as an increasing public health concern worldwide due to a multitude of driving forces that include changing pattern in water use, climate change, severe weather events, increasingly concentrated livestock operations, and international trade in animal products. Pathogens may (re)emerge because of their ability to adapt, mutate, or recombine in response to changing environmental pressures (WHO 2004).

In the United States, *Giardia*, *Campylobacter*, *Cryptosporidium*, *Salmonella*, and *E. coli* have been the most commonly identified zoonotic agents of waterborne disease outbreaks from contaminated drinking water (Craun *et al.* 2004). From a study examining infectious doses, 88% of healthy individuals became infected following ingestion of 300 or more *Cryptosporidium* oocysts (Dupont *et al.* 1995). A mathematical model developed from the data of Dupont *et al.* (1995) estimated that 0.5% of individuals would become infected following the ingestion of a single oocyst (Haas *et al.* 1996). The average infective dose of *Giardia* can be as low as 10 cysts (Hunter 1997; Chin 2000). *E. coli* O157:H7 is also highly infectious in very small doses of only 10 to 100 cells (Tilden *et al.* 1996). Other strains of pathogenic *E. coli* have a higher infectious dose, typically in the range of 10^8 to 10^{10} cells (Hrudey & Hrudey 2004). Additionally, some *Campylobacter* species are infectious at relatively low doses with estimates of 500 cells observed as capable of causing human illness (Black *et al.* 1988). From the point of view of drinking water source protection, treatment, and distribution it is important that the concentration of a given pathogen reaching the consumer is ideally zero but in any case is below the infectious dose. Ideally, pathogen loading and persistence should be controlled through appropriate proactive measures at all points from source to tap.

An overall goal of this study was to assess the spatial and temporal variability of selected pathogens in a mixed-use watershed. The information gained will have the potential to positively influence the implementation of management practices and further the development of pathogen fate and transport models. The specific objectives of this investigation were to (1) develop real-time PCR methods for the quantitative assessment of select pathogens in environmental water samples (2) determine the presence and baseline numbers of key pathogens of concern to drinking water utilities within a system that is representative of rivers impacted by both urban regions and livestock operations, (3) identify the environmental conditions such as temperature, hydrologic conditions (i.e. precipitation and streamflow) that contribute to peak occurrences of pathogens and (4) develop a set of recommendations for the water treatment industry for assessing source waters for pathogenic contamination.

MATERIALS AND METHODS

Study area – the grand river watershed

The case study region is the Grand River Watershed located in Southwestern Ontario, Canada (Figure 1). With an area of close to 7000 km^2, the Grand River Watershed is the largest in Southern Ontario. The total population

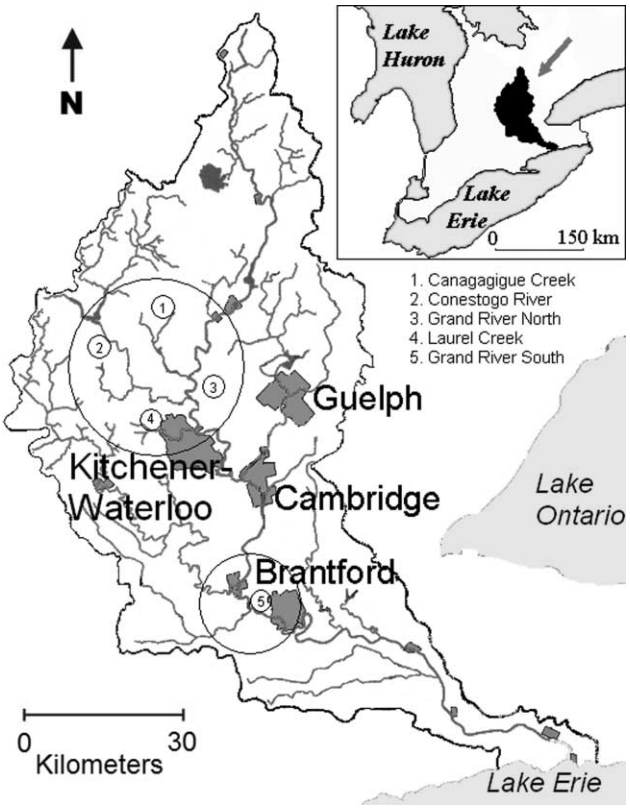

Figure 1 | Monitoring regions in the Grand River Watershed.

living in the watershed is greater than 800,000 and is expected to grow by 37% in the next 20 years. The watershed is intensively farmed with 80% of the land being used for agriculture. The central portion of the watershed is heavily urbanized with approximately 500,000 people living in five cities - Brantford, Cambridge, Guelph, Kitchener, and Waterloo. Forest covers only 14% of the land, but reaches 30% in the eastern portion of the watershed (GRCA 2004, 2005).

The annual average ambient air temperature is approximately 6.7°C. Long term average annual precipitation in the Grand River Watershed ranges from 892 to 940 mm with average annual snowfall ranging from 101 to 169 mm (Canadian Climate Normals 1971–2000).

Water supply in the watershed is from both groundwater and surface water. In the northern region of the watershed, groundwater is used exclusively. In the central and southern regions, three water treatment plants draw water from the Grand River. The Mannheim water treatment plant supplies blended Grand River water with groundwater for Kitchener-Waterloo, and several smaller communities. In 1993, a *Cryptosporidium* outbreak occurred in Kitchener-Waterloo shortly following the commissioning of this plant (Welker *et al.* 1994). The Brantford and Oshwegan treatment plants obtain their entire water supply from the Grand River.

There are 26 wastewater treatment plants servicing approximately 680,000 people that discharge into the Grand River and its tributaries. All sewage receives secondary treatment, with 19% treated to the tertiary level. Wastewater may be a source of pathogenic contamination during spills or sewage bypasses.

Three subwatersheds, and two regions of the Grand River were selected for initial baseline sampling. On average, 7 or 8 samples were collected and analyzed weekly from one of the regions of the Grand River Watershed on a rotating basis. The quasi-random baseline sampling began in July 2002, and ended in October 2003. Each sampling location was sampled every 5 weeks, and over the course of the study, approximately 10 samples were collected per location. Figure 1 also shows the monitoring regions selected for the rotating weekly sampling with detailed region descriptions provided in Table 1. Additionally, two locations, one at the outlet of Canagagigue Creek, and the other at the Mannheim Water Treatment Plant intake were selected for frequent sampling (approximately every 6 to 12 hours) during snowmelt and/or precipitation events.

Enumeration of indicators and pathogens

Enumeration of total and fecal coliforms, *E. coli*, and *E. coli* O157:H7

Total coliform, fecal coliform, and *E. coli* levels were determined to confirmation according to methods published in Standard Methods for Water and Wastewater Treatment (APHA, AWWA & WEF 1998). Total coliforms were enumerated using Method 9221 and Method 9020B:9. Positive tubes were scored using the most probable number (MPN) Table 9221.IV in Standard Methods and reported as MPN/100 ml. Fecal coliform levels were determined by Method 9222D, and *Escherichia coli* levels were determined by Method 9213D:3 and 9221F which includes a confirmation step using EC-MUG medium (4-methylumbelliferyl-B-D-glucuronide). For all coliform analyses, *E. coli* was used as a positive control, and *Pseudomonas aeruginosa, Enterobacter aerogenes, and Klebsiella pneumonia* were used as negative controls for total coliforms, fecal coliforms, and *E. coli*, respectively. Fecal coliform and *E. coli* results are reported as Colony Forming Units (CFU) per 100 ml.

Escherichia coli O157:H7 levels were determined to confirmation in a five-step procedure including Method 9260F in Standard Methods (APHA, AWWA & WEF 1998) with modifications to incorporate a membrane filtration procedure in order to make the method quantitative. A detailed description of the method is available in Anderson *et al.* (2003).

Enumeration of *Campylobacter*, *Cryptosporidium*, and *Giardia*

Thermophilic *Campylobacter*, *Cryptosporidium*, and *Giardia* densities were determined using quantitative real-time PCR methods developed in our laboratory.

Strains and species. Standard curves were generated using *Campylobacter jejuni* subsp. *jejuni* (American Type

Table 1 | Description of sampled regions of the Grand River Watershed

Subwatershed or Region	Description	Wastewater Inputs	Average Streamflow (February – October 2003)
Canagagigue Creek	Agricultural region (~130 km^2) with highest livestock density in Grand River Watershed. Approximately 60% of agricultural land is tile drained.	Receives treated wastewater from 7,848 residents with average daily flow of 4,157 m^3/day (Region of Waterloo 2004).	1 m^3/s
Conestogo River	Agricultural region with high livestock density. More than 60% of agricultural land is tile drained. Streamflow less influenced by groundwater than other regions due to geology consisting mostly of lower permeability till (GRCA 2001).	Receives treated wastewater from 1,378 residents, averaging 887 m^3/day (Region of Waterloo 2004).	3 m^3/s
Laurel Creek	80% of drainage area (~74 km^2) within urban environment. Upland regions are either forested or agricultural. Water flows through natural and concrete channels, and constructed reservoirs.	No direct wastewater discharges	0.1 m^3/s
Grand River North	Region immediately upstream and including Mannheim Water Treatment Plant. Conestogo River is the largest upstream tributary. Downstream of both agricultural and urban areas.	Largest of discharges has an average flowrate of 40,781 m^3/day (Region of Waterloo 2004).	19 m^3/s
Grand River South	Region of watershed immediately upstream and including the Holmdale Water Treatment Plant. Sole source of municipal drinking water for the City of Brantford.	Region is downstream of all of the largest wastewater discharges in the Grand River Watershed.	49 m^3/s

Culture Collection [ATCC] # 35920). The freeze-dried strain was reconstituted as outlined by ATCC (Manassas, VA) and incubated at 37°C in a gas jar prepared with a Campy Pak Plus® gas packet (BBL Becton Dickinson, Sparks, MD) in order to generate a microaerobic atmosphere. Other *Campylobacter* used to generate melt curve profiles included *C. lari* (ATCC # 35221) and *C. coli* (ATCC # 33559), also reconstituted as outlined by ATCC and grown at 37°C under microaerobic conditions.

Live pure samples of *Cryptosporidium parvum*, *Cryptosporidium muris*, *Giardia lamblia*, and *Giardia muris* were obtained from Waterborne Inc. (New Orleans, LA). All oocysts and cysts were obtained live in phosphate buffered saline (PBS) with antibiotics (penicillin, streptomycin, gentamicin, and Amphotericin B) and 0.01% Tween 20. Upon arrival all strains were stored at 4°C for no more than 2 months.

DNA extraction and preparation of quantification standards for real-time PCR. *Campylobacter* DNA was extracted from cultures of known concentration by a phenol chloroform method, previously described by Savill *et al.* (2001). The purified DNA was resuspended in 100 µl of ultra-pure water and stored at −20°C. A quantification curve of target DNA was prepared in ultra-pure water using 10-fold dilutions ranging from 1 to 1×10^6 cells. Dilutions used to generate the curve were stored at 3°C for up to 3 months.

For *Cryptosporidium* and *Giardia*, the number of expected cysts or oocysts was confirmed by using a Petroff-Haussser Sperm Bacteria Hemacytometer. In a sterile microfuge tube, 1 ml of the cysts or oocysts was centrifuged at 2,500 × g for 10 min. The supernatant was removed and the pellet was resuspended in 1 ml of sterile

1X PBS. The sample was pelleted again at 2,500 × g for 10 min. Cells were washed with 1 ml ultra-pure sterilized water and re-pelleted via centrifugation at 2,500 × g for 10 min. Chelex-100, to bind inhibitors, was added directly to sample at 25% wt/volume with 1 ml sterile ultra-pure water and vortexed (Johnson *et al.* 1995). The sample was then subjected to 4 cycles of a freeze-thaw lysis by transferring samples between −196°C liquid nitrogen (5 min) and boiling water (5 min). After lysis, cells were centrifuged at 5,000 × g for 10 minutes to pellet cell debris and Chelex-100. The supernatant was transferred to a sterile 1 ml centrifuge tube and the pellet was discarded. Serial dilutions were prepared in ultra-pure water to generate a standard curve of DNA. An aliquot of 20 μl was used as the template DNA in qPCR analysis.

DNA extraction from environmental samples. For *Campylobacter*, 200 ml aliquots were centrifuged at 6,500 × g for 20 min., the pellet was re-suspended in 1 ml of sterile ultra-pure water, and centrifuged for 5 min at 6,500 × g. The pellet was then re-suspended in 200 μl of sterile ultra-pure water and boiled for 12 min. Cell debris was pelleted at 11,100 × g for 10 min., and 20 μl of supernatant was used for qPCR and melt curve analysis for detection of *Campylobacter* spp.

Cryptosporidium and *Giardia* samples were filtered in the field through an Envirochek sampling capsule (Pall Corporation, Mississauga ON). Depending on turbidity of the water, as measured using a 2100P Hach Turbidimeter (Loveland, CO, USA), 20 L – 40 L were filtered. In 7 instances during the spring and the fall, when turbidity levels were exceptionally high, fewer than 20 L were filtered. All capsules were stored from 0–8°C from time of filtration to time of analysis, within 24 hours. The capsules were filled with an elution buffer (1 g Laureth-12; 10 ml 1 M Tris pH 7.4; 2 ml 0.5 M EDTA, 2Na, pH 8.0; 150 μl Antifoam A; 1 L ultra-pure water), shaken at the 9 o'clock position (determined by position of bleed valve) for 45 minutes, then decanted. Elution was repeated as before, but with shaking at the 12 o'clock position. The resulting sample from the addition of both decanted volumes was centrifuged at 1,500 × g for 20 minutes. The supernatant was decanted and the pellet was washed with 100 ml of 1X PBS. Samples were centrifuged as before and subsequently washed 2 more times with sterile ultra-pure water. The final pellet was resuspended in 10 ml of ultra-pure water. If the pellet was greater than 0.5 g the pellet was resuspended and separated into two volumes of 10 ml. The 10 ml volumes were transferred into Dynal L10 tubes and Dynalbeads GC-Combo (Dynal Bio-tech, Oslo, Norway) were used to simultaneously separate *Cryptosporidium* spp. and *Giardia* spp. from the water sample concentrate. A volume of 1.4 ml ASL, a buffer supplied in a QIAamp DNA Stool Mini Kit (Qiagen, Mississauga ON), was added to the resulting immunomagnetic separation (IMS) concentrate. DNA extraction by freeze-thaw lysis was accomplished as previously described. The resulting DNA was purified using a modified protocol as set out by QIAamp DNA Stool Mini Kit with the exception that the lysis steps were omitted as the cells were already lysed. The eluted purified DNA product was subjected to qPCR and melt curve analysis for detection.

Real time quantitative PCR. All PCR amplification was conducted using an iCycler iQ real time PCR detection system (Bio-Rad, Hercules, CA, USA) in a final sample volume of 50 μl per reaction. Each sample volume consisted of 1.5 μl of each primer (10 μm; Sigma Genosys, Oakville, ON), 20 μl of sample supernatant, and 25 μl iQ SYBR Green Supermix I (2 × PCR Buffer; 400 nm each dATP, dCTP, dTTP, dGTP; 6 mm MgCl2; 50 U/ml iTaq DNA polymerase; SYBR Green I; 20 nm fluoresin) (Bio-Rad, CA), and 2 μl of ultra-pure water. Negative controls were prepared as described above, with 20 μl of ultra-pure water in place of extracted DNA. The sequences for the primer sets are presented in Table 2. Primers used for the detection and enumeration of thermophilic *Campylobacter* were chosen based on extensive specificity information originally collected by Eyers *et al.* (1993, 1994) and later supplemented by Savill *et al.* (2001). The specificity of the primer set for *Giardia* spp., was investigated by Mahbubani *et al.* (1991, 1992). No amplification was seen with any non-*Giardia* spp. tested. In addition, primer set CPB-DIAGF and CPB-DIAGR were chosen based on the specificity information composed by Johnson *et al.* (1995).

The PCR protocol for *Campylobacter* consisted of a primary denaturation step at 95°C for 30 seconds followed by 55 cycles of denaturation at 95°C for 1 min, annealing at

Table 2 | qPCR primers for pathogen detection and quantification

Oligonucleotide			
Primer pair	**Name**	**Target**	**Sequence**
Campylobacter Eyers *et al.* (1993, 1994)	THERM-1 THERM-2	23S rRNA	5′-TATTCCAATACCAACATTAGT-3′ 5′-CGGTACGGGCAACATTAG-3′
Giardia (Baker *et al.* 1988; Mahbubani *et al.* 1991)	20 mer GGL 639–658 21 mer GGR 789–809	B-giardin gene	5′-AAGTGCGTCAACGAGCAGCT-3′ 5′-TTAGTGCTTTGTGACCATCGA-3′
Cryptosporidium (Johnson *et al.* 1995)	21 mer CPB-DIAGF 601–621 21 mer CPB-DIAGR 1015–1035	18 s rRNA	5′-AAGCTCGTAGTTGGATTTCTG-3′ 5′-TAAGGTGCTGAAGGAGTAAGG-3′

54°C for 1 min and at 72°C for 1 min. The protocol was completed with a final extension cycle at 72°C for 8 min. The PCR protocol for *Cryptosporidium* and *Giardia* was as follows: 55°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 s and 60°C for 1 minute. Annealing temperature of 55°C occurred for 10 s with an increase in temperature after cycle 2 by 0.5°C for melt curve data collection and analysis. An extra 5-min extension at 72°C was added to the amplification reaction when an electrophoresis gel was being run.

Standard curves used for quantification of unknowns were generated by performing a linear regression analysis using the iCycler iQ software. The standard curves for *Campylobacter*, *G. lamblia*, and *C. parvum* produced slopes of −3.807 (PCR efficiency of 83.1%), −3.342 (PCR efficiency of 99.2%), and −3.32 (PCR efficiency of 100%), respectively.

Sensitivity. Real-time PCR amplification was carried out on 20 μl aliquots of DNA that was subjected to 10 fold serial dilutions following extraction from a culture confirmed to contain 5×10^9 *C. jejuni* cells/ml by direct plating. The detection limit was determined to be 1 to 10 copies of target DNA per PCR reaction as shown in Figure 2. The lowest threshold cycle (C_T) value (the point at which the fluorescence crosses the defined threshold) was 9.6–9.7 corresponding to the reaction containing 1×10^8 copies of target, while the highest C_T value was 43.4 corresponding to the reaction containing 1 copy of target. Samples containing one copy showed detection 59% of the time. The sample containing 10 copies of target was consistently detected with a C_T value of 35.1. The sample containing 1 target copy was prepared by serial dilution; therefore, PCR reactions that did not show amplification may not have actually contained the single target copy required for detection. These experiments determine that the limits of detection fall between 1 and 10 target copies per PCR reaction. No target amplification was observed for negative controls. Recovery efficiency as determined by spiking environmental water samples from Laurel Creek was determined to be 79% (equivalent of 722 cells recovered out of 910 spiked in 200 ml of water).

Similarly, a limit of detection for *Cryptosporidium* and *Giardia* was as low as 1 cyst or oocyst per qPCR reaction (not shown). The C_T value that corresponded to 1 copy of target DNA generated C_T values of 36.0 for *Cryptosporidium* spp. The standards that contained one copy of DNA for amplification did not consistently detect for the same reasons described above for *Campylobacter*. All negative controls showed no amplification.

Culturable human enteric viruses and Clostridium perfringens

Clostridium perfringens spores were enumerated by a membrane filtration method using m-CP medium as described by Armon & Payment (1988).

Human enteric viruses were concentrated from 12 liters of water using organic flocculation methods and enumerated on MA-104 cells using an immunoperoxidase assay (Payment *et al.* 2000). Viruses were concentrated by organic flocculation by adding 1.5% of beef extract to the

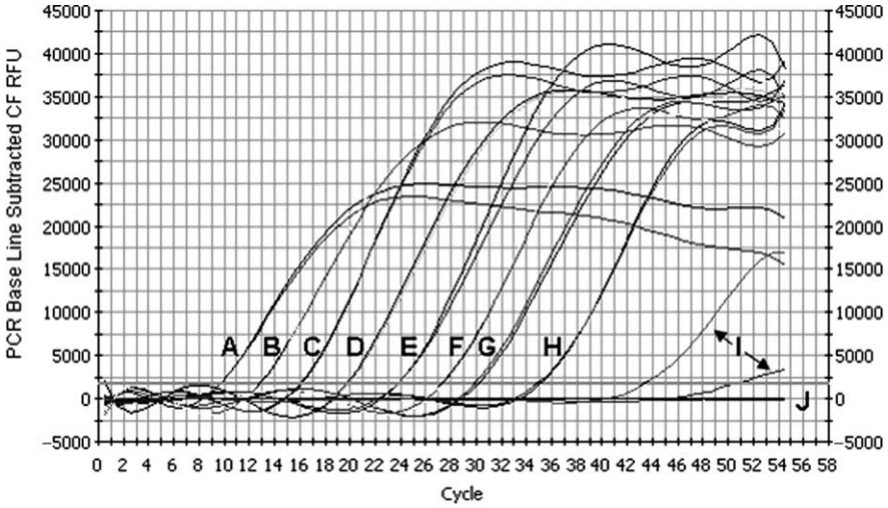

Figure 2 | Real-time qPCR amplification cycle for 10 fold serial dilutions of *C. jejuni* DNA as standards for the determination of assay detection limits (in duplicate) [PCR, polymerase chain reaction; CF, curve fit; RFU, relative fluorescent unit]. [A] = 1×10^8 copies, [B] = 1×10^7 copies, [C] = 1×10^6 copies, [D] = 1×10^5 copies, [E] = 1×10^4 copies, [F] = 1×10^3 copies, [G] = 1×10^2 copies, [H] = 1×10^1 copies, [I] = 1×10^0 copies, [J] = negative control.

sample, acidification at pH 3.5 with 0.1N HCl and 0.005 M ferric chloride. After 30 minutes, the floc was collected by centrifugation at 3000 × g for 30 minutes in 1-liter bottles in a J-6 refrigerated centrifuge (Beckman Instruments) without brake. Concentrates were stored at 70°C until assayed. All samples were assayed on MA-104 cells using aliquots (1 ml) of concentrates or dilutions that were inoculated into 25 cm^2 tissue culture flasks containing near confluent monolayers of cells and using 10 flasks per sample or dilution of the sample. Briefly, flasks were incubated at 37°C for 7 to 10 days, freeze-thawed at 20°C and repassaged on the same cell line using 24 well plates (one well for each first passage flask). After another incubation period of 7–10 days, monolayers were fixed with absolute methanol containing 1% hydrogen peroxide. The fixed monolayers were submitted to an immunoperoxidase immunoassay developed at the Institut Armand-Frappier laboratory using human immune serum globulins to detect viruses in infected cells. Reactions were evaluated with an inverted microscope: infected cells were stained dark brown and were easily detected even at low or medium magnification. The number of viruses in the original sample was calculated using the number of immunoperoxidase positive and negative wells after the second passage and estimating the most probable number of infectious units per unit per liter as described previously (Payment *et al.* 1987).

Statistical analyses

Statistical analyses were performed in MS Excel™, following an inspection for missing data points.

RESULTS AND DISCUSSION

The first series of data from 36 locations was collected from July 2002 to December 2003 to determine baseline pathogen and indicator densities. Furthermore, two of the locations were intensely sampled during snowmelt and/or precipitation events between March 2003 and May 2004. Statistical analysis of baseline pathogen and indicator densities did not include event data, as these data were examined separately. Data were log-transformed ($\log_{10}(x+1)$) for many of the statistical analyses as they followed non-Gaussian distributions.

Spatial variability between subwatersheds

An analysis of variance on $\log_{10}$-transformed data revealed that fecal coliforms and *E. coli* concentrations were significantly different ($P < 0.05$) between most regions of the Grand River Watershed. Bonferroni t-Tests were performed to compare multiple means. Paired comparisons were made in descending order, with most individual tests revealing significant differences. A drawback of using the

Bonferroni t-Test is that the overall probability of making a Type I error, α', (rejecting the null hypothesis that means are equal, when the null hypothesis is true) is greater than α, and is unknown.

From a comparison of means, it was determined that *E. coli* concentrations were marginally higher in the Canagagigue Creek (CAN) Watershed, as compared to the Laurel Creek (LC) Watershed ($P < 0.1$). A detailed description of potential environmental loadings of pathogens from livestock in the Grand River Watershed is provided by Dorner *et al.* (2004). The two watersheds, although comparable in size are very different in terms of land use. The Canagagigue Creek Watershed drains an agricultural region, and during sampling, the presence of cattle in the creek was occasionally observed. In contrast, although the Laurel Creek Watershed originates in an agricultural region, it also passes through an urban region, with large numbers of ducks and geese observed (>40 ducks at one location alone) near many of the sampling locations. When water samples were collected, the numbers of ducks and geese observed were also documented. Ducks and geese were observed more frequently and in higher numbers in the Laurel Creek Watershed than in any other part of the Grand River Watershed.

Figures 3 and 4 present boxplots of *E. coli* and fecal coliform concentrations, respectively, of the sampled regions of the Grand River Watershed. For all boxplots, no values were considered explicitly as outliers, thus the

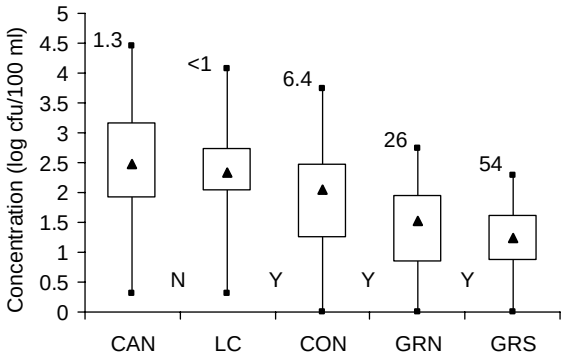

Figure 3 | *E. coli* densities in sampled regions of the Grand River Watershed. CAN = Canagagigue Creek, LC = Laurel Creek, CON = Conestogo River, GRN = Grand River North, GRS = Grand River South. Numbers above the boxplots provide the average streamflow for each location from 1998–2001 (m^3/s). N = no significant differences between regions, Y = differences significant ($P < 0.05$).

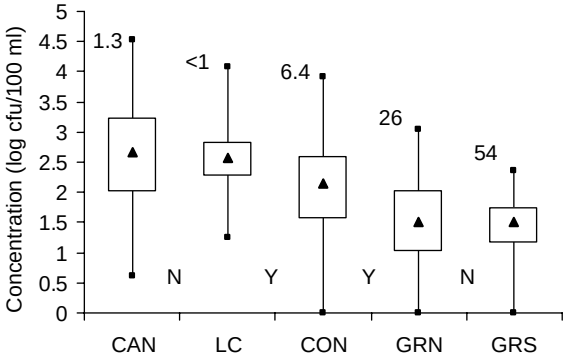

Figure 4 | Fecal coliform densities in sampled regions of the Grand River Watershed. CAN = Canagagigue Creek, LC = Laurel Creek, CON = Conestogo River, GRN = Grand River North, GRS = Grand River South. Numbers above the boxplots provide the average streamflow for each location from 1998–2001 (m^3/s). N = no significant differences between regions, Y = differences significant ($P < 0.05$).

"whiskers" represent the minimum and maximum observed concentrations (i.e. the range of measured values). The measure of central tendency was the median, and the upper and lower bars of the "box" represent the 75th and 25th percentiles, respectively. The highest *E. coli* concentrations observed were in an intensively farmed region of the watershed, followed closely by an urban region of the watershed with large observed numbers of ducks and geese. *E. coli* concentrations were higher in the Conestogo River as compared to the Grand River North section, which were again higher than the Grand River in the southern region (see Figure 3). Therefore, the results strongly suggest that *E. coli* densities decrease in the downstream direction at a watershed scale. The results are in contrast to many investigations of *E. coli* densities that have either concluded that no relationship existed between subcatchment size (e.g. Crowther *et al.* 2002), or that *E. coli* concentrations increased with increasing stream order (or increased in the downstream direction) (e.g. Irvine & Pettitbone 1996; Byappanahalli *et al.* 2003). Differences likely exist between the Grand River Watershed and the watersheds investigated by others. A key difference from a microbial perspective is likely the nature of the sources, and their location within a watershed.

Differences in fecal coliform concentrations between regions of the watershed were less obvious, perhaps reflecting the greater contribution of environmental (non-fecal) sources of fecal coliforms which are ubiquitous. However, the fecal coliforms had a lower density in lower order streams (i.e. smaller streams) within the watershed.

Tables 3 to 5 summarize the pathogen results by region sampled. A total of 338 water samples were analyzed for *E. coli* O157:H7, and 237 water samples were analyzed for *Campylobacter*. Fewer samples were collected for *Cryptosporidium* and *Giardia* ($n = 80$ and 89, respectively) as it involves a more elaborate collection method that requires filtering large volumes of water in the field.

For the pathogenic microorganisms, the underlying assumptions for performing analysis of variance are not valid. The log-transformed data also do not follow normal distributions, and the variances are not equal. In order to determine if there were differences in *Campylobacter* concentrations between regions of the watershed, a non-parametric test – the Kruskal-Wallis test was performed. The Kruskal-Wallis test revealed that differences in the distributions of *Campylobacter* among regions were significant ($P < 0.001$).

Cryptosporidium was detected in 11 of 79 samples (14%); however, recovery efficiencies for the quantitative polymerase chain reaction (PCR) were highly variable (including no recovery during some spiking tests). The probability of a false negative may be large, but it is unknown. The collection of protozoan samples involved filtering approximately 20 to 40 L which concentrates the oocysts, but also concentrates possible PCR inhibitors. It is possible that detections occurred only when very high concentrations were present in the samples. It is clear that an important research need is the development of reliable molecular methods for the sensitive and specific detection and enumeration of *Cryptosporidium* from environmental water samples.

The units typically used to describe *Cryptosporidium* in water samples is number observed per 100 L, but it is

Table 3 | *E. coli* O157:H7 in sampled regions of the Grand River Watershed

Watershed region	Number positive: number sampled	Median of positive samples (#/100 ml)	Density range of positive samples (CFU/100 ml)
Canagagigue Creek	5:75	100	100 to 110
Laurel Creek	4:72	100	41 to 200
Conestogo River	1:66	10	10
Grand River North	2:59	10	10
Grand River South	1:66	100	100
Total	13:338	100	10 to 200

Table 4 | *Campylobacter* in sampled regions of the Grand River Watershed

Subwatershed	Number positive: number sampled	Median of positive samples (#/100 ml)	Density range of positive samples (#/100 ml)
Canagagigue Creek	24:48	63	2 to 1.2×10^6
Laurel Creek	33:61	123	1 to 6.7×10^3
Conestogo River	21:46	52	1 to 6.5×10^5
Grand River North	16:36	39	1 to 9.4×10^2
Grand River South	25:46	46	1 to 5.5×10^2
Total	119:237	66	1 to 1.2×10^6

Table 5 | *Giardia* in sampled regions of the Grand River Watershed

Subwatershed	Number positive: number sampled	Median of positive samples (#/100L)	Density range of positive samples (#/100L)
Canagagigue Creek	7:20	22	2 to 1.0×10^4
Laurel Creek	4:13	81	6 to 1.4×10^3
Conestogo River	6:20	313	20 to 2.2×10^3
Grand River North *	5:13	439	3 to 3.0×10^3
Grand River South	12:23	92	7 to 7.2×10^2
Total	34:89	92	2 to 1.0×10^4

*includes wastewater stream discharging into Grand River.

generally not possible to filter 100 L because of issues with filter clogging. It has been generally accepted that it is necessary to filter large volumes of water to observe the presence of *Cryptosporidium*. Yet, the trade-offs are potentially severe, particularly for PCR methods if inhibitors are adsorbed in the filtration process. If the observed concentrations are true, it may not be necessary to concentrate such large volumes of water for *Cryptosporidium* PCR methods, particularly when high numbers are expected, thereby reducing potential interferences.

Giardia was detected in 34 of 89 samples (38%). As with *Cryptosporidium*, the recovery efficiency for *Giardia* was variable, and results must be interpreted with caution. A Kruskal-Wallis test examining differences in the distribution of *Giardia* concentrations among regions of the Grand River Watershed found that significant differences existed ($P < 0.001$). It is interesting to note that trends evident for the indicator bacteria, such as decreasing densities in the downstream direction were not evident for *Campylobacter* and *Giardia*. The highest percentage of positive samples observed for *Giardia* was for the region upstream of and including the Brantford drinking water treatment plant intake (GRS, the region the furthest downstream). Ongerth *et al.* (1995) found lower *Giardia* concentrations traveling in the downstream direction, but observations such as these are likely to be watershed-specific, and related to sources of pathogens in the watershed, and watershed drainage area.

It is possible that the microorganisms that are more easily inactivated in the environment, such as *E. coli* will decrease in numbers more readily in the downstream direction, whereas microorganisms that are more environmentally resistant, such as *Giardia* or *Clostridium perfringens* may not be inactivated in the same numbers. The maximum concentrations of *E. coli*, fecal coliforms, *Campylobacter*, and *Giardia* were observed in Canagagigue Creek. The greatest peaks may be observed in regions closest to potential sources, but for pathogens which are not as widely distributed in the environment as indicators such as *E. coli* the probability of observing them may increase in the downstream direction. However, substances that are inhibitory to the polymerase chain reaction procedure may be present in lower concentrations farther downstream as a result of dilution (additional groundwater inputs with less organic matter). The potential impact of different water matrices on PCR results should be considered in more detail in future investigations.

Although some of the *Campylobacter* numbers appear to be high (close to what could be expected from a direct fecal sample), it is possible that on occasion, a given water sample had a large amount of fecal matter in it. In Canagagigue Creek, the highest levels of *Campylobacter* were recorded near a location where cattle were wandering through the creek. The highest numbers of *Campylobacter* in a sample from the Conestogo River were recorded at a sampling location at a bridge where a large number of barn swallows (>20) were roosting at the time.

Samples were collected in each region to determine the presence of culturable human enteric viruses, indicative of human fecal contamination (see Table 6). Even with a limited number of samples (34), viruses were found in all regions at relatively high frequency and at levels suggestive of significant human fecal pollution. Wastewater treatment plants, combined sewer overflows and leaky sewerage lines are obvious sources and viruses can be transported over long distances. Viruses also survive better in cold water in

Table 6 | Culturable human enteric viruses in sampled regions of the Grand River Watershed

Subwatershed	Number positive (>0.2/L): number sampled	Median of positive samples (mpniu/L)	Concentration range (mpniu/L)
Canagagigue Creek	3:7	1.8	<0.2 to 2
Laurel Creek	2:6	3.4	<0.2 to 6.4
Conestogo River	1:4	0.9	<0.2 to 0.9
Grand River North *	1:9	0.65	<0.2 to 4.3†
Grand River South	2:8	0.6	<0.2 to 0.6
Total	9:34	0.8	<0.2 to 6.4

*Includes wastewater stream discharging into Grand River.
†Measured upstream of the wastewater treatment plant mpniu – most probable number of infectious units.

the absence of biological activity in the water. Local septic systems also contribute to the occurrence of viruses in a watershed. *Clostridium perfringens* results provide similar data as both indicates human fecal pollution and will survive for long periods. The data obtained is consistent with observations made earlier in the Saint-Laurence river watershed (Payment *et al.* 2000).

All samples collected and analyzed for *Clostridium perfringens* were positive for the microorganism (Table 7). *Clostridium perfringens* is an excellent indicator of human fecal pollution and can also serve as conservative tracer for remote sources as its sporulated form survives for long periods in water. It is also present at lower concentrations in the feces of farm animals (Pipes 1982). The non-parametric Kruskal-Wallis test demonstrated that the distribution of *Clostridium perfringens* concentrations were significantly different between regions of the Grand River Watershed ($P < 0.05$). The highest measured point concentration of *C. perfringens* was from a direct sample of wastewater effluent; however wastewater effluent data was not considered in the Kruskal-Wallis test. *C. perfringens* data suggest that sole reliance on indicator bacteria such as *E. coli* which do not persist in the environment may be misleading in terms of water quality from a microbial perspective.

Table 7 | *Clostridium perfringens* in sampled regions of the Grand River Watershed

Subwatershed	Number sampled	Mean (± s.d.)	Geometric mean	Concentration range (#/L)
Canagagigue Creek	7	33 (± 17)	25	2.7 to 55
Laurel Creek	6	78 (± 37)	74	50 to 147
Conestogo River	4	42 (± 35)	29	11 to 82
Grand River North *	9	101 (± 203)	37	6 to 642†
Grand River South	8	97 (± 73)	72	14 to 217

*includes wastewater stream discharging into Grand River.
†Measured in the effluent of the wastewater treatment plant.

Within watershed variability

Canagagigue Creek subwatershed

Figure 5 presents sampled *E. coli* densities along Canagagigue Creek below the Woolwich dam. The creek passes through both agricultural and urban areas as it travels downstream. No statistically significant differences ($P > 0.05$) were observed among coliform concentrations along Canagagigue Creek. Larches Creek (CAN_3), a tributary of Canagagigue Creek had significantly lower coliform concentrations than Canagagigue Creek ($P < 0.05$). Five of 75 (6.67%) samples were positive for *E. coli* O157:H7 and 24 of 48 (50%) of samples were positive for *Campylobacter*. *E. coli* O157:H7 was detected only in the region of the subwatershed downstream of the town of Elmira at CAN_5, CAN_6, CAN_7, and CAN_8. The Elmira wastewater treatment plant discharges between CAN_4 and CAN_5, and was not observed to have any effect on monitored bacterial concentrations. CAN_4, located within the town of Elmira had the highest median *E. coli* levels. A large uncontained manure pile located within 30 metres of Canagagigue Creek immediately upstream of the town of Elmira was observed during routine sampling. However, small numbers of ducks (<5) were also often observed near the CAN_4 sampling location.

The distributions of *Campylobacter* spp. densities along Canagagigue Creek, as seen in Figure 6 were found to be significantly different ($P < 0.05$) among sampling points using a Kruskal-Wallis test. Although it is not possible to

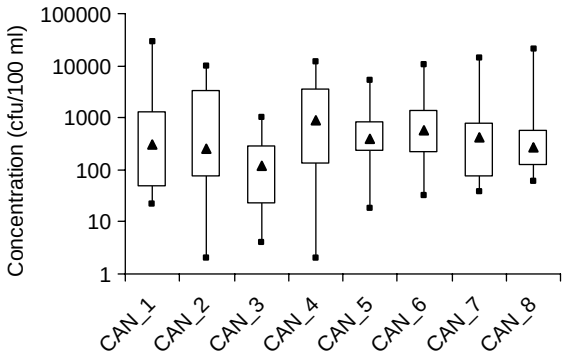

Figure 5 | *E. coli* densities at various sampling locations within the Canagagigue Creek. Samples are ordered as traveling downstream. CAN_3 and CAN_7 are tributaries to Canagagigue Creek.

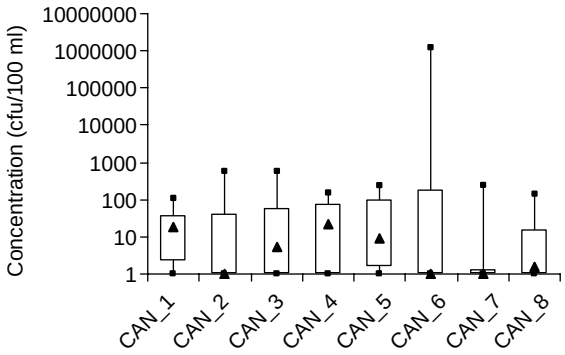

Figure 6 | *Campylobacter* densities at various sampling locations along the Canagagigue Creek. Samples are ordered as traveling downstream. CAN_3 and CAN_7 are tributaries to Canagagigue Creek.

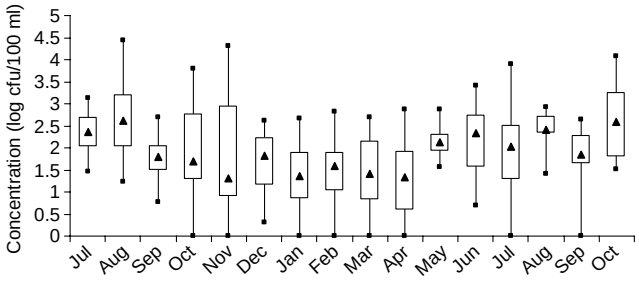

Figure 7 | *Escherichia coli* densities from July 2002 to October 2003.

determine the sources of *Campylobacter* spp. with certainty, it is interesting to note that the maximum concentration of *Campylobacter* spp. measured was immediately downstream of an area where cattle were frequently observed in the stream, and severe streambank erosion at the site appeared to be caused by cattle with access to the creek.

One sample of 20 (5%) was positive for *Cryptosporidium*. Seven of 20 samples (35%) were positive for *Giardia*. The maximum *Giardia* densities varied considerably and ranged from 22 to 10,036 per 100 L for the sites tested. It should be noted that the recovery efficiencies for *Giardia* detection and enumeration were highly variable, which leads to greater uncertainty with respect to the results.

Long-term temporal variability

More than one year of data should be collected before determining the significance of seasonal effects on microbial occurrence. The period for which quasi-random data are available is July 2002 to October 2003. Lower densities of *E. coli* were generally observed during the winter/early spring months (Figure 7). However, the opposite appears to be true for *Campylobacter*, where higher median observed densities were observed during the late winter/ early spring months (Figure 8). Although more data are needed to determine possible seasonal effects, it is possible that cooler temperatures may assist the survival or preservation of *Campylobacter* cells during the winter and spring periods. Others have observed higher *Campylobacter* densities in the winter as compared to the summer (e.g. Obiri-Danso & Jones 1999). These results are also comparable to those of Eyles *et al.* (2003) who used an MPN method to detect and enumerate thermophilic *Campylobacter* spp. from river samples in New Zealand. They observed that the number of positive samples ranged from 55% ($n = 60$) in the autumn to 91% ($n = 80$) in the winter. Although more samples were positive in the winter, in that study, these authors also found that the summer had the highest occurrence of samples with densities >11 MPN/100 ml.

No discernable long term temporal trend was observed for *Giardia* densities. *Cryptosporidium* was detected in water samples only during the period from February (when *Cryptosporidium* sampling began) to May 2003 (not shown). As the recovery efficiencies were variable, the reliability of the data is questionable with a high likelihood of false negatives.

Bodley-Tickell *et al.* (2002) found *Cryptosporidium* to be present throughout the year during the monitoring of surface waters in the UK, but most frequently detected in

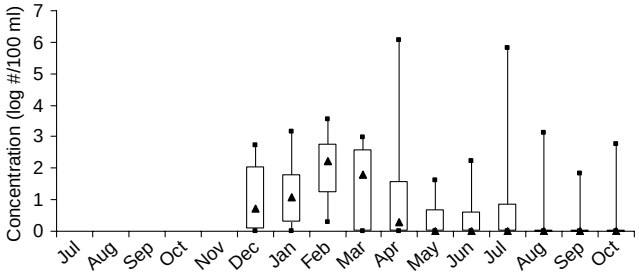

Figure 8 | *Campylobacter* densities from December 2002 to October 2003.

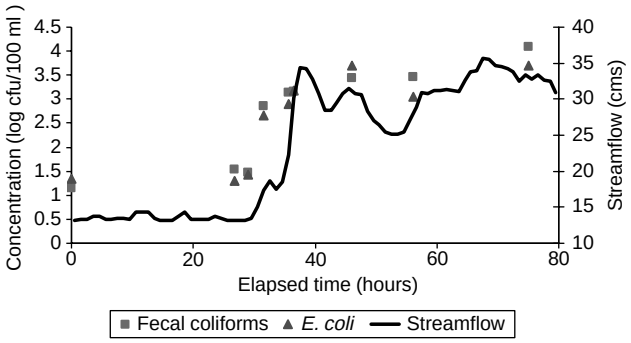

Figure 9 | October 13–16, 2003. Fecal coliforms and *E. coli* at the Mannheim Raw Water Intake, and their relationship to streamflow.

the autumn and winter with the highest densities also observed during that period. Ong *et al.* (1996) also found higher densities in the winter months.

In an investigation of *Giardia* and *Cryptosporidium* occurrence that included the Grand River, LeChevallier *et al.* (2000) observed that the majority of protozoan detections were between October and April (99 samples were collected). However, data were only collected for one year and at one location – the intake of one of the water treatment plants.

Short-term temporal variability (wet weather events)

Figures 9 to 12 present monitoring results for events in 2003 and 2004 from two separate sampling locations. Note that the y-axis scales are different for each event as the objective was to determine relationships between environmental factors during an event rather than event-to-event comparisons. Differences between events are evident with some storms generating much larger increases in turbidity. Total coliforms, fecal coliforms, and *E. coli* were present in all event samples collected (including the pre-event samples that were taken before each storm). One fall event for the raw drinking water intake is shown in Figures 9 and 10. Measured peak coliform densities at the intake were comparable (similar order-of-magnitude) to peak densities in Canagagigue Creek but the Canagagigue Creek experienced greater fluctuations in turbidity levels.

Samples were not analyzed for *Cryptosporidium* spp. and *Giardia* spp. during events of the spring of 2003. *Cryptosporidium* spp. and *Giardia* spp. were not detected in any event samples collected in the fall of 2003 or spring of 2004. *E. coli* O157:H7 was detected (concentration = 4 cfu/100 ml) in one of the event samples collected in February. Manure spreading on snow was observed in the watershed a day prior to the major snowmelt period.

Generally, the peak *Campylobacter* density precedes the peak densities of coliform bacteria and turbidity before dropping off to zero (e.g. see Figure 10). Although additional event data would help in confirming the trend, it appears that *Campylobacter* and potentially other pathogens are limited in supply and are flushed out of the stream before the coliform bacteria tail off. However, if one is concerned about the peak pathogen density arriving at a drinking water treatment plant intake, it may potentially occur very soon after the beginning of the event, before turbidity reaches its peak level. In fact, the peak pathogen concentration may occur before turbidity has increased

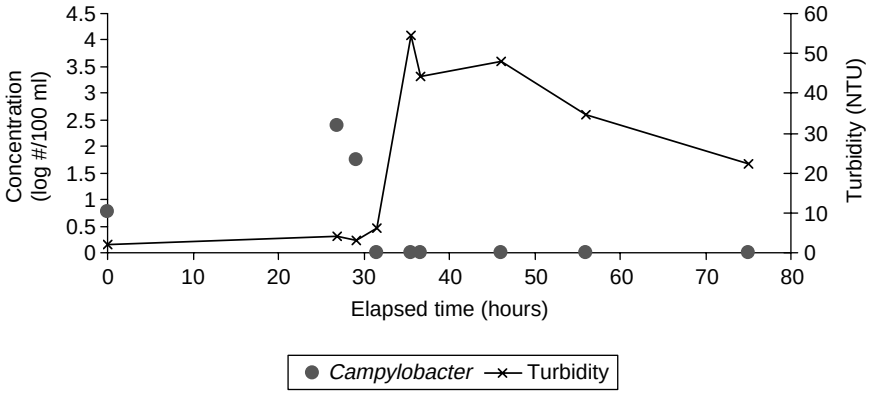

Figure 10 | October 13–16, 2003. *Campylobacter* spp. at the Mannheim Raw Water Intake, and relationship to turbidity.

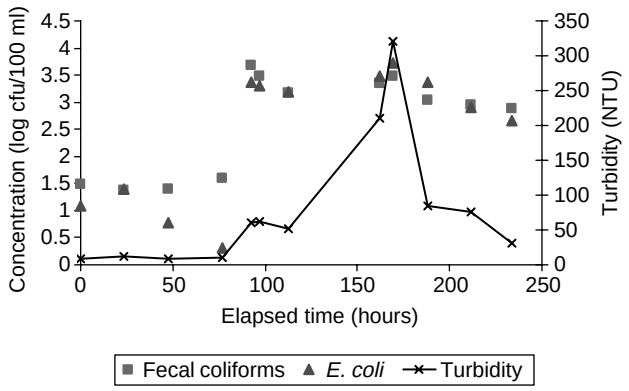

Figure 11 | February 27–March 8, 2004. Fecal coliforms and *E. coli* in Canagagigue Creek during period of snowmelt. *E. coli* correlated to turbidity – Spearman Rank Correlation Coefficient, Rs = 0.96.

noticeably from baseline values, suggesting that other rapidly measurable parameters such as UV_{254} could be investigated to provide an indication of peak occurrence. Total organic carbon has been correlated with UV_{254} (MacCraith *et al.* 1993) and correlations may also exist between total organic carbon and the presence of pathogenic microorganisms. Particles associated with turbidity may interfere with microbial analyses. Therefore it is useful to note that *Campylobacter* was detected during a period of very high turbidity (greater than 180 NTU) as seen in Figure 12. A lack of correlation between *Campylobacter* levels and turbidity has been observed by others (e.g. Eyles *et al.* 2003).

In the events sampled, once precipitation began, the densities of total and fecal coliforms and *E. coli* generally rose rapidly. A long tailing was observed, particularly for larger events. Densities of the coliform bacteria frequently

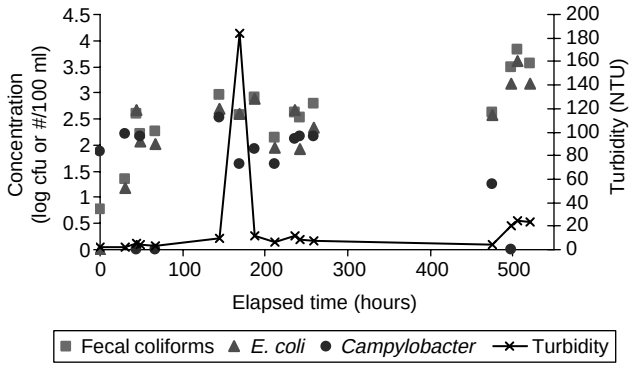

Figure 12 | April 11 – May 3, 2004. Fecal coliforms, *E. coli*, and *Campylobacter* in Canagagigue Creek during period of snowmelt. *E. coli* correlated to turbidity – Spearman Rank Correlation Coefficient, Rs = 0.90.

increase by more than one or two orders of magnitude. During precipitation events of low intensity and short duration, stream coliform densities varied little, and correlations with turbidity were poor. Good correlations with turbidity were evident for larger events.

Microbial data can be accompanied by large variances, depending on the detection method efficiency, especially when compared to other measurements such as turbidity. For the study areas tested, samples collected in short succession (for example on a half hour basis) did not generally show a clear increasing or decreasing trend in microbial numbers. Given that a limited number of samples can be processed in any given week, trends were more easily observed when the time step was increased to every 4 to 6 hours and the same numbers of samples were collected over a longer time frame. Trends were also more easily observed in larger events (more intense precipitation, longer duration).

Sampled snowmelt and storm events at two locations within the Grand River Watershed in Southwestern Ontario showed that turbidity generally correlates with the presence of *E. coli* in stream samples, particularly for larger precipitation events. This has also been observed by Nagels *et al.* (2002). These results suggest a mechanistic linkage between the mobilization of sediments and the mobilization of bacteria, but it does not suggest a possible source of the bacteria. Nagels *et al.* (2002) observed that during both an artificial flood event created by the release of a reservoir, and a natural flood event that *E. coli* and turbidity were closely correlated, but that both peaked before peak flow. They concluded that processes other than wash off from land-based sources are of similar or greater importance.

Turbidity was not correlated with the presence of the potentially pathogenic microorganisms sampled (Dorner *et al.* 2005). During the events, the density of total and fecal coliforms, and *E. coli* often increased by more than 2 orders of magnitude, and their peak densities frequently coincided with peak turbidity measurements. However, peak densities of pathogens may precede the peak densities of coliform bacteria and turbidity because they are more limited in supply and can be diluted to undetectable levels during higher flows (see Figure 10). Crowther *et al.* (2002) also observed a greater than 10-fold increase in geometric mean

fecal indicator densities during high flow as compared to low flow. They found that the high flow geometric mean density had significant positive correlations to land use and management variables associated with livestock farming whereas no significant correlation was identified with developed regions.

CONCLUSIONS

Significant differences in the distribution of indicator and pathogen densities were observed between regions of the Grand River Watershed. Differences within sampled regions were not as evident. Upland regions with high livestock density and urban development had the highest levels of coliform indicators, and the greatest variability of pathogen densities. Densities of coliform bacteria were lower in the lower reaches of the Grand River as compared to the upland regions. However, this trend was not observed for the more environmentally-resistant bacterium *Clostridium perfringens*, a common indicator of sewage contamination, or *Giardia* suggesting that the sole use of *E. coli* as an indicator of microbial water quality may not always be reliable for downstream reaches of a watershed.

When raw water quality is being monitored, a greater emphasis should be placed on monitoring wet-weather events which may carry greater pathogenic loads. During wet weather events, turbidity and *E. coli* were correlated, whereas pathogens were not. Differences in the origins of pathogens within a watershed from variations in host populations (e.g. Dorner *et al.* 2005) may explain why pathogens are not correlated to turbidity or *E. coli*. During storm events that may carry pathogen loads, the peak pathogen concentration may arrive earlier than the peak turbidity level because pathogens are generally not widespread within a watershed, and are more likely to be clustered in space and in time.

Analyzing a minimum of 12 samples per year for *Cryptosporidium* may not provide a water utility with sufficient data for assessing raw water quality. The natural variability of oocyst occurrence is high, and even a highly impacted water source may produce a large number of non-detects. Many years of data would be required to characterize the *Cryptosporidium* variability in the raw water source if samples are collected solely on a monthly basis. A watershed-based approach for assessing raw water quality may be more appropriate than limited monitoring. In a watershed-based approach, potential sources of pathogens upstream of a drinking water treatment plant would be identified and quantified.

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