Efficacy of bacteria and caffeine as indicators of anthropogenic waste in freshwater systems

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Abstract

With urban sprawl and intensified agriculture, nutrient loading of waterways has become a pressing issue across southern Ontario. Domestic wastes are more likely than other wastewater sources to be associated with the presence of human pathogens, and therefore they may present a greater public health risk in watersheds. Field studies were performed in the watersheds of the County of Simcoe, ON to assess the efficacy of tracing nutrient loads directly attributable to domestic waste using caffeine as an indicator, and comparing caffeine patterns with those seen with conventional bacterial assessments such as heterotrophic plate count, total coliforms, fecal coliforms, and *E.coli* counts. Seasonal fluctuations in the levels of caffeine in these systems, both in water and surface sediments, were also studied. In a controlled experiment in the laboratory, a one-week study investigated the efficacy of standard bacterial tests and caffeine measurements, both individually and in combination, as predictors of the relative contribution of domestic waste to wastewater. Trace concentrations of caffeine in water and sediment samples were measured using gas chromatography ion-trap tandem mass spectrometry. In a series of step-wise multiple regression analyses, caffeine was found to be a useful and significant factor that helped to predict the extent of domestic land use and domestic waste concentrations in the environmental samples ($p<0.05$) , and in the laboratory study examining conditions in various blends of domestic and agricultural waste ($p < 0.05$). In a multiple analysis of covariance, caffeine levels showed a significant correlation with seasonally varying water parameters ($p < 0.05$). The use of caffeine as a contributing indicator of the relative contribution of domestic wastes in waterways is supported by the present study.

Lay Summary

Nutrient loading is a major issue promoting eutrophication and related problems in the Lake Simcoe watershed and surrounding areas (Winter et al., 2007). "Nutrient loading is determined primarily by surface and subsurface transport from the contributing landscape and varies significantly as a function of weather and landscape characteristics such as soils, topography, and land use" (U.S. EPA, 2008). In previous research, caffeine has been used as a "fingerprint" for contamination with domestic waste in freshwater systems. The current research was carried out to improve our understanding of the relationship between caffeine levels in local freshwater system and independent estimates of the relative inputs of domestic (human) wastes. We also sought to better differentiate domestic waste contamination from agricultural (livestock) waste contamination. A better understanding of the sources of local nutrient loads may improve the waste management strategies and thus the ecological health of freshwater systems in the County of Simcoe. Three major research questions were investigated. 1. Can a combination of caffeine and bacterial analysis indicate domestic nutrient loads on a local scale? 2. Does the usefulness of caffeine as an anthropogenic marker change seasonally in these freshwater systems? 3. Do caffeine concentrations in sediment provide greater accuracy than concentrations in water for tracking domestic waste in this freshwater system? Our results showed that caffeine is generally present in higher concentrations where raw domestic waste occurs. A combination of caffeine and bacterial counts provided a better model for estimating the relative contribution of domestic waste than either metric alone. Caffeine concentations do vary with season, with the highest concentrations being seen in winter. Caffeine concentrations were higher in sediment than in water, but the results varied greatly so

more studies of sediment are required before a definite conclusion can be made. This study provides insight on improved methods for tracking domestic waste in our region that combine bacterial and caffeine assessments. This suggests it may be useful to develop a caffeine-based water quality assessment procedure for use on a larger scale.

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Chapter 1: Introduction and Background

1.1 Overview

Aquatic health is a critically important area of research. Water quality and monitoring programs are an important aspect of environmental conservation as well as public health (Bartram 2003; Winter et al. 2007). Indeed, globally, at least 2 billion people use a drinking water source contaminated with feces, and contaminated water can transmit diseases such diarrhea, cholera, dysentery, typhoid, and polio. In addition, contaminated drinking water is estimated to cause 502,000 deaths from diarrhea each year (WHO 2018). Conventional indicators of aquatic health can be seen in the literature pertinent to the topic, and are generally measurements of physical, chemical or biological attributes (TRCA, 2000). The proposed research involves collection of freshwater and sediment samples for analyses of caffeine and microbial indicators. This information will be used in parallel with microbial tracking to monitor water quality and generate predictions of the likelihood of contamination with domestic waste. There are many important factors to consider and much needs to be understood about the chemical and microbial parameters of this study.

1.2 Nutrient Loading

1.2.1 What is Nutrient Loading

Nutrient loading is a major issue in the Lake Simcoe watershed and surrounding areas (Winter et al., 2007). According to the US Environmental Protection Agency, "Nutrient loading is determined primarily by surface and subsurface transport from the contributing landscape and varies significantly as a function of weather and landscape characteristics such as soils, topography, and land use" (U.S. EPA, 2008). It can be

problematic for a freshwater system as excess concentrations of nitrogen and phosphorous often lead to a eutrophic ecosystem, which may heavily impact many of the organisms present, harm the quality of drinking water downstream, and negatively affect any industry that relies on the freshwater system (Winter et al., 2007). Two of the main contributing factors to nutrient loading in the Lake Simcoe region are discharges of agricultural and domestic waste (Kurissery et al., 2012; Winter et al., 2007).

1.2.2 Seasonal changes in Freshwater Systems

Previous research in this area suggest that nutrient loadings peak during rainfall events, and that their ecological impacts on Lake Simcoe differ with seasonality (Evans et at, 2011; Nicholls, 1995; Winter, 2007). While rainfall is usually greater in spring and fall, large summer storms can be associated with contributions of considerable nutrient loads (Nicholls, 1995). Other research has suggested that winter and spring snowmelt (the Freshet) is the dominant cause of agricultural surface runoff in Canada (Jensen et al., 2011). It can be challenging to accurately quantify the total nutrient load of a single event. Earlier studies have suggested that minor weather events, where there is only moderate velocity increase in streams and rivers, can allow nutrients in the water column to settle into the sediment. However, larger single storm events can re-suspend previously deposited sediments and nutrients (Beck, 1985).

Clearly rainfall and stream velocity will affect nutrient transport in streams, but in addition seasonality plays an important role in the process of nutrient mixing in lakes (Salonen et al., 2009). In these temperate latitudes, whether a lake is deep enough to stratify, the formation of ice on the lake surface can greatly reduce lake mixing (Salonen et al., 2009). In deeper lakes, during midsummer stratification, not only are the three

separate layers (epilimnion, metalimnion and hypolimnion) prevented from mixing, but benthic mixing is also reduced (Winter et al., 2007).

1.2.3 Nutrient Loading in the County of Simcoe

A thorough study of phosphorous inputs to the Lake Simcoe watershed was conducted from 1990 to 2003, and the overall quality of the Lake Simcoe watershed was assessed (Winter et al., 2007). This article provides an excellent variety of background information on the ecological health of various parts of the Lake Simcoe watershed.

The watershed is approximately 3,557 km^2 in area with 80% of it being terrestrial. The terrestrial portion of the watershed consists of 47% agricultural land, 40% natural heritage systems (wetland and forest), and 12% urban land (Winter et al., 2007). The urban population increased by 30% over the decade (1991-2001), and is now a total of 30,586 people (Winter et al. 2007; Government of Canada 2012). One of the major issues faced by the Lake Simcoe Region Conservation Authority (in terms of watershed management), is phosphorous loading. This is said to be largely the result of tributary discharge, direct run-off from urban subsurface, wastewater treatment plants (WWTPs) and septic tanks (Winter et al., 2007).

Based on samples collected between 1998-2004, Winter et al. (2007) estimated the total phosphorous (TP) loading for Lake Simcoe to be 53-57 t/yr. At this time, the phosphorus concentrations for Lake Simcoe were above the provincial guidelines (0.03mg/L) (MOECC, Government of Canada, 2012). As a result, stricter regulations of TP entering Lake Simcoe, through waste water treatment facilities have been implemented (LSEMS, 2010, Winter et al. 2007).

The study by Winter et al. (2007) also highlighted the importance of concentrations of dissolved oxygen (DO) in the hypolimnion. Higher total phosphorous (TP) levels are suspected to be resulting in extreme low end-of-summer oxygen levels, due to active decomposition of the large organic biomass resulting from eutrophication. Levels of DO are critical for cellular respiration in organisms such as fish; a minimum concentration of 7 mg/L for hypolimnetic DO, is required to sustain a self-recruiting population of an important species to the fisheries industry, lake trout or *Salvelinus namaycush* (Evans et al. 2006). The Lake Simcoe Environmental Management Strategy (LSEMS) had set a goal of 5mg/L in 2009, as an achievable target for end-of-summer hypolimnetic DO concentrations in Lake Simcoe (Lake Simcoe Protection Act 2008). Unfortunately, recent reports suggest that Lake Simcoe does not yet stay above this threshold year-round.

Land areas can be classified in terms of human use, as being used for recreation, agriculture, or urban uses (NRC, 2015). When there are plans to change land use in an area, formal land use applications are required; these involve baseline mapping and subsequent monitoring in efforts to balance conservation, any conflicting use, and an assessment of developmental pressures (NRC, 2015). The Lake Simcoe Regional Conservation Authority initiated a Natural Heritage and Land Use Mapping Program in 2000; this now covers the entire Lake Simcoe Watershed (LSRCA, 2007). Land use is classified into the categories of: intensive agriculture, non-intensive agriculture, urban development, rural development, estate residential, manicured open space, institutional, rail, and roads (LSRCA, 2007).

Previous studies have directly identified patterns of land usage as indicators of ecosystem health and community dynamics (Crosbie & Chow-Fraser, 1999; Danielson, 2002; Delattre et al., 1992; D. Evans et al., 2011; Foley, 2005). Relating specifically to the topic of nutrient loading, Land use classification has been previously studied as an possible indicator of phosphorous removal, sediment quality, and overall aquatic health in the Great Lakes Basin, where percentage of wetland cover was a highly correlated factor (Crosbie & Chow-Fraser, 1999).

Only part of Simcoe County is regulated by the LSRCA, but similar support maps are available for the remainder of the county through Nottawasaga Valley Conservation Authority (NVCA), the Couchiching Conservancy, and the Severn Sound Environmental Association which must abide to the Conservation Authorities Act, meeting standardized guidelines lines (R.S.O., 1990).

1.2.4 Stormwater Drainage

Stormwater drainage refers to the removal of runoff water from impervious surfaces in an urban area (Bryan, 1972). In contrast naturalized areas provide penetrable soil, whether they are loams or wetlands, and there surface runoff and sheet flow are greatly reduced (Bryan, 1972). Urban areas are often composed of concrete, asphalt and large buildings where water cannot permeate the surface. Therefore these surface flows must be removed via catch basins, sewers, and ditches (Bryan, 1972).

Stormwater may carry some nitrate and phosphate from fertilized lawns and ornamental gardens, but the greatest environmental impact is often heavy metals, road salt, and suspended sediments (Casey et al., 2007; LSRCA, 2012). Stormwater management (SWM) ponds have been incorporated in new developments in many urban

areas over the past four decades; their primary role is for sediment removal. However, SWM ponds are not always effective at removing all contaminants and they have not been retroactively installed in most older urban developments (Casey et al., 2007). An example is the Barrie Creeks Subwatershed of Lake Simcoe. Hotchkiss Creek and Lover's Creek run through downtown Barrie, where much of the stormwater runs directly into the creeks (LSRCA, 2012). In recent studies, the creeks have exhibited exceedingly high conductivity and chloride levels that are suspected to be attributable to winter road salt and sanding practices (LSRCA, 2012).

1.2.5 Domestic Waste Processing

Municipal wastewater is often referred to as domestic waste, but it is actually an aggregate of all water used and disposed of in a community. These potentially high waste loads will vary in composition according to population density and type of industry that is present (Ferreira, 2005).

In 2013, the City of Orillia released the *2012 Master Plan Update*. This document includes information regarding the operation of Orillia's domestic Waste Water Treatment Centre (WWTC). The system includes 163km of sanitary sewers, 20 pumping stations, a septic sewage receiving facility, and a 27,300 m^3/d capacity waste water treatment plant (WWTP). Industry, commercial development, and institutional sewage represent 43% of total flow of the influent; the remainder could be described as "domestic wastes". The effluent from the Orillia WWTC is discharged to a small tributary creek, known as Ben's Ditch, that feeds into Lake Simcoe. Lake Simcoe in turn discharges to an adjacent waterbody, Lake Couchiching, which eventually connects to the Severn River, feeding Severn Sound and the Midland Harbor. The City of Orillia was

required to reduce their phosphorous discharge to meet Provincial Standards, in accordance with the *Lake Simcoe Protection Plan* and *Lake Simcoe Phosphorous Reduction Strategy* (Lake Simcoe Protection Act 2008), and the *Reduction Strategy for the Lake Simcoe Watershed* (2010). The current provincial limit for phosphorus concentrations in effluent is set at 0.1 mg/L and 996kg/yr as of June 2015 (Lake Simcoe Protection Act 2008). An Environmental Compliance Approval was issued to the City of Orillia by the Ministry of Environment (MOE) in 2012. From 2007-2011, average concentrations of phosphorous in treated effluent were 0.15mg/L, and total loading averaged 968kg/yr with notable reductions in the most recent years. The WWTP effluent from the City of Orillia discharges at the Orillia Narrows and flows northwest along the Trent-Severn Waterway.

The Trent-Severn Waterway northwest of Orillia has been recognized as part of the Severn Sound Area of Concern (AOC) identified by the International Joint Commission (Figure 1-1). In recent years, Lake Couchiching and Sparrow Lake have not been able to meet water quality objectives or water quality standard guidelines (IJC, 2003). This highlights the direct impact of municipal wastes contributed by the Orillia area, and underscores the importance of managing municipal waste and monitoring the concentrations of nutrients such as nitrates and phosphates in all areas of adjoining waterbodies and interpreting their sources and movement in light of important factors such as directional flow (AECOM and Parks Canada, 2011).

Figure 1-1: The Trent Severn Waterway from Lake Simcoe to Georgian Bay with an indication of directional flow (arrows).

The Orillia WWTC resembles many of other WWTCs in the Lake Simcoe watershed, in terms of their treatment process. This involves primary treatment (grit removal, screening, and settling), followed by secondary treatment (activated sludge tanks with aeration, and secondary clarifiers). Tertiary treatment at the Orillia WWTC currently consists of UV disinfection (Phillips et al., 2012).

The Orillia WWTP is one of 14 sewage facilities within the Lake Simcoe watershed. These facilities include some secondary treatment plants, some tertiary treatment plants and some lagoon-based systems (W.E.A.O. 2010). While there is a wide variety of treatment processes, Orillia uses conventional activated sludge methods which are relatively common (W.E.A.O. 2010). Numerous studies have confirmed the value of

activated sludge treatment as a way to remove chemical contaminants as well as nutrients from sewage (Rodríguez et al., 2003; Sabaliunas et al., 2003; Ternes, 1998).

1.2.6 Combined Sewer Overflow (CSO)

In some, typically older, urban areas, storm sewers and domestic sewers may be combined, that is, served by the same system of pipes, draining to the wastewater treatment plant (WWTP). A CSO event can occur when there is an extreme weather event, causing a surge in stormflow which exceeds the capacity of a waste water treatment plant (WWTP). As a result, excess untreated sewage, combined with storm water runoff in an urban storm water drainage system, may be released directly into nearby rivers or bodies of water. This is a common phenomenon in 700 cities across the USA (Phillips et al., 2012), and while there is limited information on Canadian CSO events, it does occur in many towns and cities in Ontario. Other areas reporting CSOs include areas on the East Coast, around the Laurentian Great Lakes, and the Pacific North West, and such events have been linked to major inputs of polycyclic aromatic hydrocarbons (PAHs), organochlorine compounds, nutrients, and increased chemical oxygen demand (COD) (Gasperi et al., 2008 and Eganhouse et al., 2001). At times, a combined sewer overflow event may be an issue in the Lake Simcoe watershed and southern Ontario (LSRCA, 2013).

At a WWTP in Burlington, Vermont, USA, the contents of water bypassing the plant in CSOs were assessed. CSOs accounted for only 10% of annual water discharge, but they contributed 40-90% of hormones and wastewater micropollutants (such as caffeine, ibuprofen, estradiol, etc.) which have high (>90%) removal in WWTP. This means that although the WWTP can remove 90% of these contaminants through effluent

treatment, this only represents 10-60% of the total lake contamination. The conclusion is that the rest of the contamination in Lake Simcoe is a product of CSO events and other point-sources of untreated domestic waste (Phillips et al., 2012).

This finding shows that CSO discharges are very important contributors to environmental contamination since this small portion, about 10% of domestic waste seems to be responsible for as much as 90% of some lake contaminants. This highlights the importance of recording CSO occurrences and relating them to the time and location of sampling sites.

While CSO events alone may be major point sources of nutrient loading in southern/central Ontario and the Great Lakes region (LSRCA, 2013), regular Sanitary Sewer Overflows (SSOs) are often overlooked because several facilities often bypass treatment at the same time and the contributions of individual systems are often difficult to distinguish (Tibbetts, 2005). An overflow from SSOs is more common in cities with older infrastructure. Often, inadequate, heavily infiltrated, or congested sanitary sewers cause a surcharge higher than the sewer pipe and benching in upstream manhole access points. These manholes are equipped with various types of simple bypass systems where an open pipe is installed at a higher elevation. This pipe is a direct bypass to a nearby Stormwater sewer (figure 1-2). Many cities across Ontario contain numerous SSO systems; some of these are equipped with ultrasound depth, velocity, and pressure sensors so that these events can be anticipated and quantified (Lanning & Peterson, 2012). Such events, however remain poorly understood, and are underreported. Give the specificity of the locations of each event, it may also be more challenging to address releases from such sources.

With the introduction of new high volume WWTP designs and sewage retention basins, it seems that CSO problems may be solved more readily than those involving SSOs, which are an inevitable consequence of the design of outdated sewer infrastructure (Tibbetts, 2005).

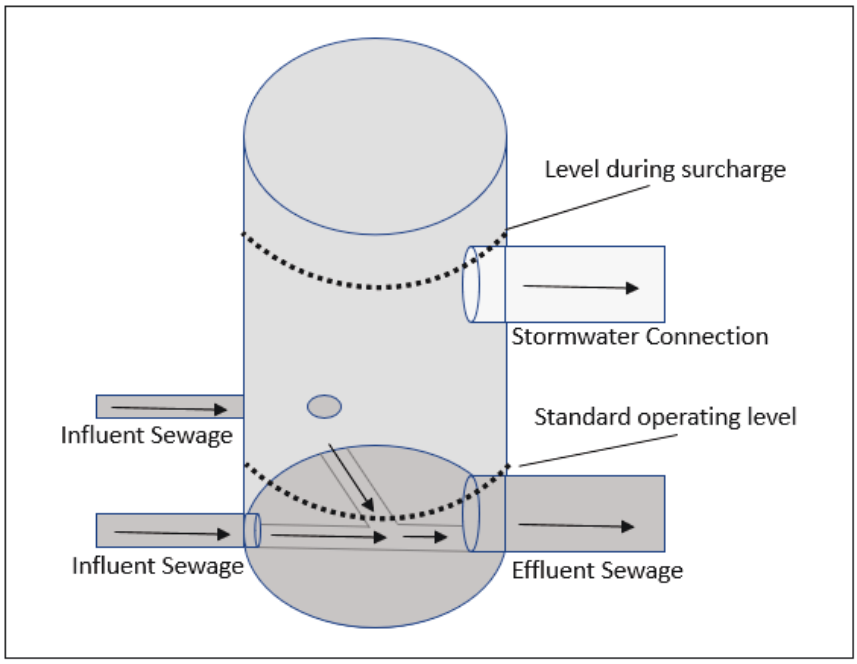

Figure 1-2: Depiction of a sanitary manhole chamber equipped with a combined sewer connection to Stormwater.

1.2.7 Septic System Leakage

There are some additional potential sources of untreated sewage contamination in more rural areas, including septic tanks and leachfields or other soil-based treatment systems. A recent study was made of household septic systems and nearby soils, where all septic systems were between eleven and twenty-eight years of age (Eveborn et al., 2014). At all sites, soils were contaminated by the adjacent septic systems. The soil parameters that had changed due to contamination include pH (decreased) and phosphorous content increased Phosphorous levels reached as much as 6 mg/L in nearby soils, and although natural phosphorous sinks were found to exist at many sites, nutrient

discharge into subsurface water flow was still apparent, and sites that were close to water bodies would, in fact, be likely to contribute to detrimental effects on the aquatic environment.

1.3 General Indicators of Aquatic Health

1.3.1 Nutrient Analysis

Phosphorous plays a key role in the eutrophication of freshwater lakes and rivers (Winter et al., 2007). The Canadian Council of Ministers of the Environment (CCME, 1999) suggests that while natural phosphorous is released from phosphate rock and biota, there are many anthropogenic sources that need to be taken into account when examining total phosphorus (TP) loadings. Phosphorous plays a role in biological metabolism (Correll, 1998; Winter et al., 2007).When compared to other macronutrients for primary producers in most freshwater systems, phosphorous tends to be present at the lowest concentrations, and is usually the major factor limiting growth (Correll, 1998). Most organisms are approximately 0.3% phosphorous in terms of dry mass (Horne and Goldman, 1994). Phosphorous in the aquatic environment is not all immediately available to plants and algae; it is typically found in three natural forms: inorganic phosphorous (typically phosphate ions which are highly available), particulate organic phosphorous (POP) and dissolved organic phosphorous (DOP). The phosphorous that is readily absorbed by macrophytes can be measured as inorganic phosphate, specifically orthophosphate (PO_4^{3-}). However the chemical breakdown of organic forms of phosphorous can be relatively rapid, so generally assessment of total phosphorous gives the most useful estimate of phosphorus availability in the system (Johnston et al., 2005)

1.3.2 Indicator Species

There have been numerous studies on organisms with sensitivities and/or preferences in relation to specific water parameters, known as indicator species (Gaufin et al., 1952; Omar and Maznah 2010). For example, macroinvertebrates can be used as biotic, aquatic indicators of water quality (Gaufin & Tarzwell, 1952). In a pertinent study, the key aspect was to create standard field monitoring procedures using aquatic invertebrates to assess water quality. Groups of organisms that were considered were monophyletic groups such as *Trichoptera, Odonata, and Crustaceae* (Gaufin & Tarzwell, 1952). An interesting finding in this study was the unexpected discovery of certain organisms that appeared to thrive in the poorest conditions. For example, a mosquito species, *Culex pipiens*, a beetle, *Tropisternus sp.*, and two sludgeworm species, *Limnodrilus sp.* and *Tubiflex sp*. all grew better under the poorest of conditions, including high nutrient loading and low levels of dissolved oxygen. There was one site, in particular, where they were doing well was near a sewage effluent discharge! This reinforces the point that organisms are quite diverse, and chemical, physical and biological requirements differ among species. Consequently, if organisms are to be used as indicators of water quality parameters, there is great value in identifying not only to the level of order and phylum, but also to the level of genus, and, ideally, species, where possible.

Another North American study, using aquatic indicators, focused on two distinct measurement techniques in relation to diatom species. First the relationship between diatoms and nutrients were modeled as symmetrical unimodal distributions and second, as complex asymmetrical response curves. Under both methodologies, the principal idea

was that diatom population and community dynamics could be used as a general form of water quality testing when evaluating their species response curve to environmental changes. Most diatom species can be observed and fit to a symmetrical unimodal pH and total phosphorus (TP) model. *Gophoneis heruleana* and *Achanthidium sp*. were most useful as indicators of low TP conditions (Potapova et al, 2004). Complications in this study included the high variability in samples and the uncertainty of the multiple environmental factors that influence populations of diatoms. Additionally, this study outlines the importance of finding an appropriate model fit, when evaluating indicators of aquatic health.

1.4 Microbiology

1.4.1 Bacterial Indicators of Aquatic Health

Bacterial communities in aquatic ecosystems can be extremely diverse, especially in wetlands, shorelines and throughout the sediments and saturated soil (Shange et al., 2013). Even in the most remote regions of Canada, in Arctic lakes, 2987 taxonomic units were identified and counted in a study using 16S rRNA gene sequencing (Wang et al., 2016). Other studies have compared communites in surface sediments with those in water; findings generally indicate higher cell populations, but lower biological diversity within communities than was the case for communities of planktonic cells in the surface water (Shange et al., 2013.; Tamaki et al., 2005).

Assessment of the total diversity of bacterial communities can be a relatively intensive procedure using methods of 16S rRNA gene sequencing (Shange et al., 2013.; Tamaki et al., 2005; Wang et al., 2016). In recent years, studies have been using a library based system of genetic markers to identify bacterial indicators of fecal contamination

down to their strand and origin (Stoeckel & Harwood, 2007). In other research, bacterial counting methods have been used to estimate population size as indicators of aquatic health (Glassmeyer et al., 2005; Kirchman, 1994; Raj and Dhala, 1965; White et al., 1991). Often methods include direct cell counts, which involve the use of an epifluorescence microscope with ethidium bromide staining, or the incubations and culturing of colonies on nutrient agar (Glassmeyer et al., 2005; Kirchman, 1994; Raj and Dhala, 1965; White et al., 1991).

Enumeration of colony forming units (CFUs), is a relatively simple and cost effective semi-quantitative method for estimating the size of a bacterial population (Kaspar, 2006). Different media can capture a wide assortment of bacteria, such as heterotrophic plate counts (HPC) using R2A agar. This medium (R2A agar) was formulated by Reasoner and Geldreich to be used as an indicator of aerobic and facultative bacteria in potable drinking water, and it is recommended for this purpose by the American Public Health Association (APHA) (APHA, 1999). The term "heterotrophic bacteria" includes all bacteria that utilize nutrients for growth (i.e. it excludes photosynthetic bacteria), making it an excellent representation of the "microbial loop" community (Allen et al., 2004). In addition, several selective and differential media are available for the enumeration of coliform bacteria, fecal coliform bacteria, and *E. coli* as indicators of fecal contamination.

1.4.2 Coliform Bacteria

Coliform bacteria are rod shaped, gram-negative, and non-spore forming bacteria (Dufour, 1977). The genera *Citrobacter, Enterobacter, Hafnia, Klebsiella,* and *Escherichia* are often present in environmental samples, although *Escherichia* is

considered to be essentially confined to the intestinal tracts of endotherms (Dufour, 1977; Jin et al., 2004). Fecal coliform (*E. coli*) have been suggested as an ideal indicator of fecal contamination in water, and therefore an indicator of the possible presence of human pathogens (Edwards et al., 1997; Hai & Hongdao, 1982; Ishii et at., 2006; Jin et al., 2004; Pachepsky and Shelton, 2011).

1.4.3 *Escherichia coli* in the Environment

The efficacy of cultivating heterotrophic planktonic bacteria using varying media compared to that of a standard one (recovery efficiency = ca. 1%) has been explored in terms of nutrient broth concentrations (nutrient broth + yeast extract) and further, how subsequent population sizes may be affected (Bussmann et al., 2001). Lowered oxygen levels in a liquid (broth) substrate were shown to reduce the estimated number of bacterial populations. In contrast, when N-acyl homoserine was added to the media, more accurate estimates were obtained. The optimal conditions for cultivation in terms of total bacterial counts were: low substrate concentrations (0.03-0.06% (w/v)), 21% atmospheric oxygen, 16 degrees Celsius, for a duration of 4 weeks. The key finding of this study is that adjusting culture methods - even slightly - can yield significantly better results. It also suggests that slight adjustments in nutrient agar, temperature and duration of incubation can be optimized to yield more accurate results.

1.4.4 Collecting Microbial Sediment Samples

Different procedures for isolating and culturing microorganisms from freshwater sediments were tested by dos Santos et al (2000). In this study, surface sediment samples were collected from the upper-most section of the core (0-5cm). Bacterial abundance was estimated using a staining method and fluorescence microscopy. The authors

assessed the effects of sonication, centrifugation, and fixation by formalin, as well as centrifugation speed. In diluted sediment, one minute of sonication reduced counts by 47%, and with the addition of percoll after sonication and centrifugation, population counts were significantly reduced (dos Santos et al., 2000).

Microbial Source Tracking

Microbial source tracking can be defined as any means where bacterial cultivation or microbiological techniques can be used to track the source of water contamination. Some of the major disadvantages of using microbial source tracking include the additional time required for incubation, and the inability to discriminate between human and animal fecal sources (Glassmeyer et al., 2005). In parallel, a complementary approach, using chemical monitoring, was examined; the potential advantage was that it would not require the lengthy incubation time, and it would allow differentiation of the source of contaminants (Glassmeyer et al., 2005). This aspect of the research involved collecting samples from points downstream, and upstream of WWTPs in ten different locations within the Lake Simcoe watershed. In addition, two "reference locations" where it was assumed there would be minimal human impact, were included for comparison. The results of this work suggested that a specific chemical might be useful for tracing human wastewater discharge, and that there were advantages in using this approach rather than microbial source tracking. This chemical is caffeine, the stimulant present in coffee, tea, cola drinks, etc., and it was reportedly found at detectable levels in 70% of samples, at concentrations ranging from 0-7.99 μg/L (Glassmeyer et al., 2005).

Microbial source tracking is typically used to investigate whether animal waste is present and at what quantity (Ishii & Sadowsky, 2008). *Escherichia coli* and other

coliform bacteria can be used for source tracking since they often originate from animal intestinal tracts (Ishii et al., 2006). Conventionally, total coliforms (TC) are used to monitor drinking water, and fecal coliform counts (FC)/*E. coli* are used to assess recreational waters (Ishii et al., 2006). *Escherichia coli* is normally present as a commensal in the intestines of warm-blooded animals, and because of this, *E. coli* in the environment is an indicator of inputs of animal wastes. A major issue with this concept is the potential naturalization of *E. coli* in freshwater systems and the reduced accuracy of microbial source tracking when this occurs (Ishii et al., 2006).

Most monitoring of fecal contamination is carried out to assess drinking water, groundwater, and recreational water. Characteristics of bacteria that are directly associated with fecal contamination include: presence in intestinal tracts of warm-blooded animals; presence when pathogens are also present; presence in greater numbers than the pathogen; ability to survive similarly in the environment; inability to multiply in the natural environment; detectable and quantified by easy, rapid, inexpensive methods; and non-pathogenic (Ishii & Sadowsky, 2008). While the microbial-source tracking discussed is referring to its use for assessing the safety of drinking water and avoidance of pathogens, it may also be readily linked to levels of phosphorous loading (Ishii & Sadowsky, 2008). These general characteristics should be considered when looking at other, alternative forms of source tracking. Overall a good indicator will be highly correlated with the public health risk of water-borne and water-contact diseases (Ferreira, 2005). These target organisms must be native to animal intestinal tracts, enter water through fecal discharge, and be found in the presence of other enteric pathogens, thus

reducing the costs and technical constraints of testing for all potential pathogens (Ferreira, 2005).

Many problems have been associated with using bacteria from fecal sources (e.g. *E. coli)* and non-fecal sources (e.g. *Klebsiella spp.*) for source tracking (Ferreira, 2005; Ishii et al., 2006; Ishii & Sadowsky, 2008). Given this challenge, further studies of bacterial indicators of fecal contamination in relation to phosphorous loading, from a wide variety of point and non-point sources, should be conducted in the near future.

1.4.5 Heterotrophic Plate Count (HPC)

Prior to 1985, standard plate count agar was generally accepted and used to assess water and wastewater (Reasoner & Geldreich, 1985). However, this medium does not necessarily allow the growth of all possible bacterial indicator species in water and wastewater. For this reason, R2A medium was developed as a specific test for these materials, in relation to monitoring drinking water and wastewater treatment plants (Reasoner & Geldreich, 1985). The use of heterotrophic plate counts is widely recognized as a useful indirect indicator of the possible presence of pathogens of concern in drinking water (W.H.O., 2003). According to the World Health Organization (WHO), the term heterotroph is broadly classified as microorganisms which require carbon for growth, including heterotrophic bacteria and fungi. The set of simple culture-based tests that are intended to identify the presence of a wide range of microorganisms is typically referred to as heterotrophic plate counts (HPCs). These offer a simple and effective means of identifying the degree of microbial and bacterial colony forming units (CFUs) in a sample. Unfortunately, this methodology cannot be used to distinguish bacterial taxa.

1.4.6 *Fecal Coliform*: *Fecal Streptococcus* (FC: FS) ratio

Many attempts have been made to identify and distinguish human fecal waste from animal fecal waste (REFERENCE 1969, Sargeant 1999). For example, in terms of the ratio of fecal coliform (FC) to fecal streptococci (FS), a ratio of 4:1 or greater is considered human fecal contamination, a ratio of less than 1:0.7 suggests non-human sources (Edwards et al., 1997). While this technique appeared to work in laboratory trials, in the natural environment the accuracy of this test was unreliable (Sargeant, 1999). One issue was the finding that there is a high degree of variance in survival among species in the streptococcus group. For example, *Streptococcus bovis* and *S. equinus* do not survive under most environmental freshwater conditions, while *S. faecalis* and *S. faecium* can survive in freshwater systems for some time (Sargeant, 1999). Other complications include the disinfection of wastewater and survival and cultivability of these bacteria during the assessment procedure.

In terms of conditions in the natural environment, for FC:FS technique to be properly carried out, the pH of the water body must be between pH 4.0 and 9.0; sampling must occur within 24 hours of the release event being monitored (e.g. a CSO event); sampling must be carried out as close as possible to the contamination source; fecal streptococcal counts must be less than 100/100mL; and samples should only be counted in waters where regrowth cannot occur (where there are less than ideal conditions for environmentally persistent streptococcal species). In addition, large numbers of samples would be needed to provide sufficient accuracy in terms of the conclusions (Geldreich and Kenner, 1969; Coyne and Howell, 1994). Thus, taking together these various

concerns, FC: FS ratios are not a recommended technique for source determination (APHA, 1998).

1.4.7 Coliforms as Indicators in Water and Wastewater

The bacterium *Eschericia coli (E. coli)* has been recognized as an indicator of fecal contamination in water since the late 1800s (Edberg et al., 2000). Early methods for testing water quality included total coliform counts. However, other related bacteria, that might be included as members of the coliform community, can be of non-fecal origin (e.g. *Citrobacter, Enterobacter, Hafnia,* and *Klebsiella*) (Cohen & Shuval, 1973). Methodologies improved over time, and by the 1970s similar media were used to distinguish and culture thermo-tolerant coliforms (fecal coliforms, typical of endotherms or "warm-blooded organisms) (Dufour, 1977). By the 1980s, differential media became available, allowing for the enumeration of total *coliforms* and *E.coli* in a single test (Edberg et al., 2000). *Escherichia coli* are found at high concentrations in mammalian (and bird) fecal matter and are capable of surviving for weeks in natural freshwater systems, without actually multiplying (Edberg et al., 2000; Ishii et al., 2006). For the reasons stated above, *E.coli* remains the major tool used as an indicator of fecal contamination in water, found, for example, in the *Guidelines for Drinking-Water Quality* (W.H.O., 1996)

1.5 Chemical and Pharmaceutical Indicators of Aquatic Health

In recent years, there has been an increasing effort to identify and mitigate nutrient loading in order to improve human health and environmental conservation (*Lake Simcoe Protection Act 2008* [S.O] c. 23). Recent studies have suggested that pharmaceuticals and

other chemical compounds associated with human consumers may be used to distinguish and track domestic waste in natural freshwater systems (Daneshvar et al., 2012; Halling-Sørensen et al., 1998; Khan & Ongerth, 2004; Spongberg & Witter, 2008; Stackelberg et al., 2004). Several recent studies have, for example, identified and proposed compounds such as nicotine, sucralose, triclosan and caffeine as ideal indicators of domestic waste based on their abundance and specificity to human consumers (Bruton et al., 2010; Buerge et al, 2003; Chen et al., 2002; Daneshvar et al., 2012; Ferreira, 2005; Halling-Sørensen et al., 1998; Khan & Ongerth, 2004; Kurissery et al., 2012; Spongberg & Witter, 2008; Stackelberg et al., 2004). Other factors that influence the efficacy of a domestic waste tracer must also be considered. It is important to have a good understanding of the fate of these compounds over time, if they are to be coherently and consistently interpreted as indicators (Bruton et al., 2010; Halling-Sørensen et al., 1998; Sabaliunas et al., 2003). For example, if a substance has a high octanol-water partitioning coefficient (log K_{ow}), it is likely to leave the water column and be absorbed by biota and bioaccumulate, potentially biomagnifying through the food chain. However, if it has a low log K_{ow}, it will more likely persistent in the aqueous phase of the freshwater system (Bruton et al., 2010). It is also important to consider the rate of natural breakdown, either through chemical or microbial processes (Bruton et al., 2013). If a compound degrades very slowly, i.e. if it is a persistent contaminant, which is most likely in the case of synthetic compounds, its concentration in the environment will continuously increase, but the chemical may not produce the visible peaks during loading events that might serve as an indicator of pulses of domestic wastewater. For example synthetic compounds such as sucralose show very low degradation in the environment (Stackelberg et al., 2004).

1.5.1 Caffeine

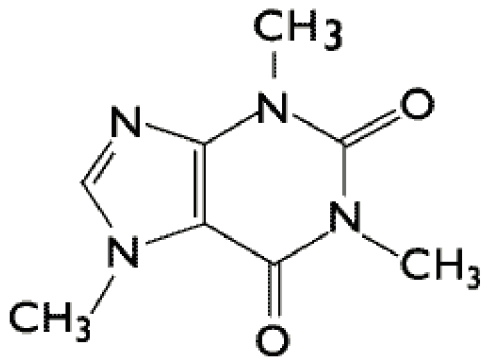

Figure 1-3

1.5.2 Properties of Caffeine

Caffeine is a natural secondary chemical, an alkaloid present in over 30 species of plants; it evolved as a substance that would repel insect pests that would otherwise eat coffee beans; also the residue of dead material around coffee plants inhibits the germination of other plant species. Caffeine, also identified as 1,3,7-trimethylxanthine (Figure 1-3) has a hydrophobic methyl group, hydrophilic parent chain and a molecular weight of 194.2 g/mole (Ahmad, 2014). It is a very stable compound with high solubility and low volatility, making it persistent in environmental conditions (Seiler et al., 1999). It is much used in the food and pharmaceutical industries and therefore is able to make its way into public WWTPs (Bruton et al., 2010; Ferreira, 2005; Kurissery et al., 2012). Caffeine is rarely targeted for removal in the wastewater treatment process (Spongberg & Witter, 2008). Additionally, periods of combined sewer overflow (CSO), during large storm events allow for the release of untreated human waste products into local inland water systems (Phillips et al., 2012).

Although, in nature, caffeine would be released from the breakdown of plants that contain it, it is typically below detectable limits in water bodies where there is little anthropogenic influence (Peeler et al., 2006). Caffeine has been used effectively to demonstrate surface and ground water contamination by domestic waste in other parts of the world including the United States, Switzerland, Rio de Jeneiro other countries (Buerge et al., 2003; Peeler et al., 2006; Seiler et al.,1999; Standley et al., 2000). However, to this point there have been very few studies conducted in North America using this compound as a "tracer" (Kurissery et al., 2012).

1.5.3 Caffeine in the Environment

Correlations between caffeine and more traditional water quality parameters has previously been examined (Ferreira, 2005). It is suggested that humans metabolize most of the caffeine they consume, but still excrete 0.5-10% (Ferreira, 2005). It has been suggested that anthropogenic activity may be closely linked to both nutrient loading and elevated caffeine levels in freshwater (Buerge et al., 2003; Chen et al., 2002; Ferreira, 2005; Kurissery et al., 2012). It has also been proposed that accidental sewage leakages may be one of the primary sources of this occurrence (Phillips et al., 2012). Caffeine has been used to trace domestic waste based on its useful qualities including: anthropogenic nature, distinctive origin, environmental destination, and elevated consumption. A single person is capable of consuming hundreds to thousands of milligrams of caffeine per day. Caffeine shows persistence in water, as it has high solubility (13.5g/L), and a low octanol-water partition coefficient (log K_{OW} = 0.01) combined with very low volatility. For all of these reasons, it is reasonable to propose that caffeine would be a good candidate for chemical source tracking as it is suspected to be relatively stable in the

environment and consistently found in WWTP effluent. A better understanding of the chemical properties of compounds such as caffeine will be beneficial in predicting the environmental persistence and likely fate of caffeine in the environment.

1.5.4 Caffeine in Waste Water Treatment Facilities

The fate of pharmaceuticals in wastewater effluent has been a topic of increasing interest in recent years (Thomas & Foster, 2005). The alkaloid caffeine is a common ingredient in headache and cold remedies, coffee, tea, chocolate and soft drinks, particularly “energy drinks” (Buerge et al., 2003; Ferreira, 2005; Kurissery et al., 2012). Other routes whereby caffeine can enter the environment include solid waste disposal of unused medications, landfill runoff, veterinary use, and application of manure and human biosolids (dried sludge from water treatment plants) to fields (Halling-Sørensen et al., 1998). Caffeine influent variables appear to be dependent upon location, socioeconomic factors, pharmaceutical cost and demographic data (Thomas & Foster, 2004). The majority of caffeine removal from domestic wastewater occurs during secondary treatment, but typically concentrations of 13-56 ng/L remain, indicating removal rates of approximately 44-100% (Rodríguez et al., 2003; Sabaliunas et al., 2003; Ternes, 1998) when secondary treatment involves processes of sorption to sludge and promotion of biodegradation (Clark, Henry, & Mackay, 1995).

The US Geological Survey and Centre for Disease control and Prevention collected a series of water samples from drinking water treatment facilities, WWTPs, and natural water systems, and confirmed that caffeine and similar compounds likely originate from domestic and/or industrial wastewaters (Stackelberg et al., 2004). They also mentioned that most WWTPs are designed for the removal of suspended solids,

oxygen-demanding substances, and in many cases the removal of inorganic constituents like phosphate (Spongberg & Witter, 2008). However, the process is not specifically designed to remove organic pharmaceuticals that are present at trace levels in most WWTPs, despite the fact that these substances can have significant environmental impacts. In the USA, caffeine in treated effluent water ranged from concentrations of 0.014-0.119 µg/L (Stackelberg et al., 2004). Indeed, an Ohio WWTP reported having caffeine concentrations as high as 3.361ug/L in the influent, and 0.0130ug/L in the effluent (Spongberg & Witter, 2008). This indicates that relatively low concentrations are being found in WWTP effluents, although these levels are still likely higher than background levels that might be attributed to natural sources.

Another concern is the potential bioaccumulation of caffeine in aquatic systems. Although it influx occurs in generally low quantities, and caffeine exhibits a low octanol-water partition coefficient (log K_{ow} = 0.01) (Wang & Gardinalli, 2012). Some reports suggest caffeine may have an effect on organisms in most urbanized freshwater systems (Peake et al., 2015). Short term acute toxicity of caffeine to *Daphnia sp.* was reported as occurring at 177.49 mg/L (with a 48-hour lethal concentration (LC_{50-48})); however such high concentrations are closer to those found in, for example a cup of coffee than levels typically found in streams, rivers, and other waterbodies (Murray & Laredo, 2014).

1.5.5 Degradation of Caffeine

Conventional methods that can be used for caffeine degradation in a waste water treatment facility include a photo-Fenton pre-treatment, or an advanced oxidation process (Trovó et al., 2013). Several factors can work synergistically alongside UV light assisting in the degradation of caffeine including: higher temperature, higher iron concentration,

addition of H_2O_2, and pH of lower than 3 or higher than 8 (Trovó et al., 2013). These parameters have been closely examined in order to determine optimal conditions for this degradation process (Alam et al., 2013). Solar pretreatment was conducted in a small-scale pilot test with an average solar irradiance of 49.7 ±3.9 Wm^{-2} where 50L of caffeine solution was circulated for 180 minutes. Caffeine had mineralized to below the quantification limit after 20 minutes in EasyPure water (a commercial water purification system from Barnstead (Dubuque, IA, USA)) and 40 minutes in sewage water. The authors found that the highest mineralization (breakdown) of caffeine occurred when caffeine, Fe^{2+}, and H_2O_2 were at concentrations of 52.0mg/L, 10.0mg/L^{-1}, and 42.0mg L^{-1} respectively. Furthermore, they demonstrated that the photo-Fenton process can be successfully applied to the degradation of caffeine, even when the contaminant is present in complex samples such as sewage water and WWTP effluents (Trovó et al., 2013). This background information is important when considering the potential for persistence of caffeine in natural environments.

The disposal of coffee waste is highly problematic in countries where coffee beans are produced (Ahmad, 2014). Bioremediation of coffee waste can be carried out by caffeine bio-degrading organisms such as *Pseudomonas, Alcaligenes, Aspergillus, Serratia, Penicillium, Klebsiella, Stemphylium, Rhizopus, Rhodococcus, Brevibacterium, Bacillus sp.,* and *Phanerochaete,* which readily metabolize caffeine for its carbon and nitrogen content (Ahmad, 2014). Prior to the 1970s, it was generally believed that caffeine was toxic to bacteria based on its effect on enzymes (Summers et al., 2011; Yu et al., 2009). Caffeine concentrations above 0.0025g/ml inhibit microbial growth, although the authors also reported caffeine biodegradation was occurring (Raj & Dhala, 1965).

Since then, studies have shown caffeine to be a potential energy source for microorganisms, primarily bacteria of the genus *Pseudomonas, Serratia, Rhodococcus* and *Klebsiella* (Ahmad, 2014; Dash & Gummadi, 2012; Vogels & Van der Drift, 1976). Iron concentrations (0.04% Fe^{2+}) have also been identified as a co-factor in the production of dymethylating enzymes (Dash & Gummadi, 2012; Ramarethinam & Rajalakshmi, 2004). Understanding the role that iron plays in the biodegradation of caffeine highlights the importance of taking conductivity readings when collecting samples, as well as assessing the concentration of iron in the water.

Pseudomonas sp., in particular, show the highest growing rates and ability to degrade caffeine at a rate of 90.00 mg/hr under ideal conditions and supplemented with external sources of nitrogen (Vogels & Van der Drift, 1976). Anaerobic degradation of caffeine does occur, but at much slower rates (e.g. 13-63% reported after 100 days compared to 100% aerobically after 14 days (Dash & Gummadi, 2012; Ramarethinam & Rajalakshmi, 2004). These factors are affected by temperature, pH, initial caffeine concentrations, and additional nutrient sources (Dash & Gummadi, 2012). It is important to understand chemical parameters of caffeine and the likelihood of persistence of this chemical under natural environmental conditions, in contrast to its persistence or breakdown in laboratory tests. It appears that there are several factors that may influence the persistence of caffeine in respect to biological processes depending on the availability of oxygen; degradation of caffeine is slower via anaerobic pathways, than aerobic processes (Dash & Gummadi, 2012; Ramarethinam & Rajalakshmi, 2004).

1.5.6 Degradation Pathways of caffeine

The biodegradation of caffeine is a well-studied area of research (Dash & Gummadi, 2012; Mazzafera, 2004; Yu et al., 2009). However, most studies involve the biodegradation of coffee pulp in compost and soil, where microbial communities differ from those in aquatic environments (Mazzafera, 2004). In the bacterial species, *Pseudomonas putida* and *Serratia marcescens*, the major pathway is from 7-methylxanthine to xanthine and methyluric acids (Mazzafera, 2004). Studies indicate that the fungal species, *Penicillium commune* and *Aspergillus tamarii*, initiate pathways which similarly result in the production of xanthine which eventually undergoes purine metabolism (Yu et al., 2009). While the terrestrial biodegradation of caffeine is relatively well studied, there is an apparent gap in knowledge regarding the aqueous biodegradation of caffeine.

1.6 Project Designs

1.6.1 Pertinent Research

A recent study compared caffeine and other chemicals as "source-trackers" of phosphorus loading (Ferreira, 2005). Five sampling stations were established in Guanabara Bay, and the Leopoldina Basin of Rio de Janeiro. These sites were chosen based on proximity to external sources of organic matter to the aquatic system. Sampling occurred from July until August 2004. The procedure involved collecting water samples in sterile 1 L containers at a depth of 4-8cm below the water surface, with the opening pointed in an upstream direction. Samples were placed on ice, stored at approximately 5^{o}C, and analyzed within 6 hours of sampling. Analyses included assessment of microbiological, physicochemical, and caffeine content. Thermo-tolerant coliforms and

total coliforms were assessed. In the present study, the same procedures were followed, using membrane filtration of the sample and growth of microorganisms on selective media at 45ºC for approximately 24 hours. Physico-chemical parameters including DO, pH, nitrite, nitrate and total nitrogen (TN) were calculated using an AutoAnalyzer II Technicon. Dissolved organic nitrogen and phosphorous (DON and DOP) were calculated using the photo-oxidation technique. The biological oxygen demand (BOD) was also calculated. Caffeine was analyzed using liquid chromatography. In terms of statistical analysis, the microorganisms were the dependent or predicted variable, assessed in relation to the measured caffeine content of the water sample.

1.6.2 Caffeine Analysis

Caffeine analysis can be carried out in a variety of ways. One method involves using a basic spectrophotometric determination in the UV-visible region, which is an inexpensive and a simple procedure, although alternative procedures such as gas chromatography (GC) and (HPLC) can give lower detection limits (and more precise measurements) (Ahmad Bhawani et al., 2015). Verenitch and Mazumder (2008) provide detailed procedures for caffeine analysis. In their study, 1 Litre water samples were collected at sites where it was expected that water quality would be affected by different levels of human activity and domestic effluent. Since sample concentrations were typically <100 ug/L, they suggested that photo-spectrometry would not be suitable for the assessment of trace concentrations of caffeine in environmental samples and that methodologies with lower detection limits, such as ion-trap mass spectrometry, would be necessary (Verenitch & Mazumder, 2008).

1.6.3 Previous Research in this Region

A report on water quality in Farlain Lake, Tiny Township, Ontario was published in 1973 by the Ministry of the Environment (MOE). This article reflected on the key attributes of Farlain Lake, with particular attention to the sudden and rapid development of personal residences and cottages around the lake. The lake was evaluated using bacteriological tests including total coliforms and specific tests for enterococci. They described a "rainfall effect", where the lake showed low microbial counts most of the summer, but higher counts just after a rainfall event. This suggested that outhouse and septic systems may have been a contributing factor, but the proposed explanation could not be confirmed. This suggests that Farlain Lake would be a good candidate site for using caffeine to "source track" domestic waste.

Another potential sampling location is the Holland River. In 2000, the Lake Simcoe Regional Conservation Authority (LSRCA) developed the "*East Holland River sub-watershed management plan"* (L.S.R.C.A 2000); this discussed issues of land use and water allocation in the area. The water flows mainly through forested and agricultural areas prior to entering Holland Marsh. The marsh itself includes both wetland and intensively farmed areas. In addition, the city of Bradford releases treated wastewater effluent to this river.

Most of the original wetland of Holland Marsh has now been drained for agriculture. Water is constantly draining through canals to pumping stations, and from there it is released into the Holland River. A sample location near one of these pumping stations could provide data on the quantity of nutrient loading that can be attributed to Holland landing and the agricultural activities in the area. In the 2009 Watershed Report

Card, the river received a "D-grade" based on its elevated phosphorous levels; these were were reported to be about 25 µg/L at the mouth of the river in Cook's Bay (L.S.R.C.A 2009). The authors of that report were concerned about the high degree of human activity in the area and suggested that some domestic waste may be reaching the lake, potentially leading to microbial contamination and excessive growth of algae, macrophytes, and potentially cyanobacteria. The ecological surveys in the present study were designed to evaluate: lake shore development; the distribution and abundance of bacteria; changes in temperature and DO; and concentrations of plant nutrients. Given the potential of this lake as a sample location, there is inherent value in knowing the background history of cottage development and growing anthropogenic influences that have affected local water quality over time.

A recent study of the Lake Simcoe watershed focused on the use of caffeine as an indicator of anthropogenic impacts (Kurissery et al., 2012). From May to December 2010, water samples and sediment samples were collected from five locations in South-Eastern Lake Simcoe. This study provided a very useful background for the present study in terms of characterizing the range of caffeine concentrations that might be expected in the region. All sampling sites had measurable concentrations of caffeine, measured using gas chromatography ion-trap tandem mass spectrometry at the University of Victoria, following the procedures outlined in Verenitch and Mazumder (2008). Water samples from the site with the highest anthropogenic influence had caffeine concentrations of 5.1-76.8 µg/L. Caffeine concentrations in the surface sediment were much higher, reaching up to 6.9 ng/g of sediment.

1.7 Gaps and objectives of the present study

Many studies have identified the impacts that nutrient loading as well as animal and fecal contamination can have on natural freshwater systems e.g. Winter et al. (2007). It is also clear that we need to better distinguish different sources of nutrient loading and contamination so that targeted measures can be taken to mitigate their effects (Gardinali & Zhao, 2002; Peeler et al., 2006). For decades, methods involving microbial source tracking have been used to identify fecal contamination in freshwater systems, but many of these methods cannot readily distinguish whether the waste is of domestic or agricultural origin (Hai & Hongdao, 1982). While there are some procedures fecal indicator bacteria capable of distinguishing animal from human waste (Stoeckel & Harwood, 2007), they have not been widely accepted as standard practice in public health, presumably due to the cost of sample analyses. More recently, chemical tracers such as caffeine have been used to track human fecal contamination from wastewater treatment plants and other domestic sources (Gardinali & Zhao, 2002; Kurissery et al., 2012; Peeler et al., 2006).

The fate and occurrence of caffeine in freshwater systems is still a relatively new topic, and much is still unknown, particularly on a local scale (Kurissery et al., 2012). The current research is designed to address gaps in information regarding the value of caffeine as an indicator of domestic waste in the County of Simcoe and in the Lake Simcoe Watershed.

In the field component of this study, the goal was to combine methods of bacterial indicators and caffeine as methods to evaluate the domestic influence of sampling locations on a local scale. In addition, the study examined levels of caffeine at selected sampling locations, over the course of a full year, in order to assess the effect of

seasonality on caffeine concentrations in the freshwater environment. We will also assess the value of taking near-shore surface sediment samples of bacteria and caffeine, in order to determine whether sediment samples offer advantages over surface water samples, or indeed if both are necessary for a full understanding of loadings (Chapter 3).

In addition to these field studies, a laboratory experiment was conducted to evaluate the efficacy of combined source tracking (bacterial and caffeine) of domestic waste in a controlled setting where known proportions of agricultural and domestic waste were present. This experiment was also designed to determine the stability of caffeine and bacterial testing over time (Chapter 4).

Chapter 2: Methods and Materials

2.1 Microbial Assessment: Heterotrophic Plate Count

Water and sediment samples were analyzed for the total count of viable heterotrophic bacteria using standard pour-plating techniques. The plating was carried out under a biohood under sterile condition and following appropriate aseptic technique (Bykowski & Stevenson, 2005). Water samples were shaken vigorously for 20 seconds before 1 mL was pipetted into 9 mL of sterilized DI water. Following standard serial dilution methods (Ben-David & Davidson, 2014), each test-tube was then vortexed and a 1 mL subsample used to inoculate the next dilution step in the series. This was procedure repeated until a 10^{-5} dilution was reached.

Surface sediment samples were processed in a similar manner. In this case, 1 g of sediment was added to the first test tube containing 9 mL of sterile DI water. After vigorous shaking for 1 min, 1 mL of the sample was added to the next test tube with 9 mL of sterile DI water; the dilution procedure was repeated until a 10^{-5} dilution was obtained.

Three replicate individual petri dishes were then each inoculated with 1 mL of each of the dilution series that were considered to produce between 30 and 300 colony forming units (CFUs) (WHO, 2003). This was followed by adding approximately 15 mL of cooled, but molten sterile R2A agar medium, following the standard pour-plating techniques. When the agar solidified, plates were inverted and incubated for 48 hours at 25± 3° C (WHO, 2003).

2.2 Membrane Filtration: Total Coliform, Fecal Coliform & *E. coli*

Differential coliform (DC) and fecal coliform agar media were prepared following standard aseptic techniques. Petri dishes were then filled with 15 mL of this DC medium and allowed to solidify. 1 mL and 10 mL of each sample were then filtered through 0.45 mm sterile filter papers, using a filtration unit connected to an 18 PSIG (pounds per square inch gauge) vacuum pump (Bykowski & Stevenson, 2005). For sediment samples, 1 g of sediment was added to 9 mL of sterile DI water in a test tube and after shaking well for about 1 min it was centrifuged at 2500 rpm for 5 mins in a Sorvall ST8 centrifuge. From the supernatant, 0.1 mL and 1 mL samples were filtered through 0.45 µm filter paper as described above. In order to avoid contamination between samples, the filtration unit was cleaned with 70% ethanol and sterile distilled water between filtration runs.

Using sterile forceps, the filter papers were removed and placed carefully on the DC media plates under the biohood. The labelled Petri dishes were placed in an incubator for 24 hours at 34 ± 2 °C (for total coliforms and *E. coli*), and for 24 hours at 44.5 ± 2 °C (for fecal coliforms) following the Environmental Protection Agency (EPA) approved Hach methodology Number 10029 (Hach, 1999; EPA, 2002).

2.3 Total Suspended Solids (TSS)

TSS were determined using the 1 L samples collected in the field. These 1 L samples were filtered through a pre-weighed 47 mm membrane 0.7 µm pore filter paper. This was then placed on a sheet of aluminum foil and dried in an incubator at 55° C for approximately 24 hours. The filter papers were then re-weighed and the difference in mass allowed calculation of the mass of total suspended solids (APHA, 2012). This process was repeated for all samples.

2.4 Nitrate Estimation

All glassware was rinsed with a 10% solution of hydrochloric acid, followed by a rinse in DI water. Next, 15 mL of each sample was transferred into test tubes. A pillow of Nitraver 6 was added to each sample, then the tube was capped and shaken consistently for 3 mins. Samples were then left to rest for at least 3 mins. At that point, 10 mL of each sample was transferred with a pipette into a new test tube. One Nitraver3 pillow was then added to each tube; they were capped and shaken gently for an additional 30 seconds, then left to settle for a minimum of 15 mins. At that point, any samples containing nitrate had become pink in colour. Approximately 3 mL of each sample was then transferred into a 10 mL cuvette and the absorbance was measured in a spectrophotometer at 507 nm wavelength. The reading was then compared to the standard curve for nitrate in order to determine the nitrate concentrations in mg/L (APHA, 2012).

2.5 Total Phosphorous (TP) Estimation

All glassware was rinsed with a 10% solution of hydrochloric acid, followed by a rinse in DI water. Next, 50 mL of each sample was placed in a 150 mL beaker. Additional beakers were filled with DI water to provide a control and act as the blank for the spectrophotometer. One drop of phenolphthalein was added to each sample, and the solution was neutralized with 1 mL of a 30% solution of sulfuric acid. This was done to ensure all samples were at or below pH 7.

To each of the beakers, 0.4 g of ammonium persulfate was added, and the beakers were placed on a hotplate to simmer until the volume remaining in the beaker was concentrated down to 10 mL. The samples were then diluted with 20 mL of DI water and mixed with a stirring rod for 15 seconds. One drop of phenolphthalein was then added to

each sample to provide an indication of pH. A 30% solution of sodium hydroxide was then added very gradually until the solution was at pH 7 (neutral).

10 mL of each of sample were then transferred to test tubes, using a pipette. One pillow of Phospher 3 reagent was then added to each of the samples, which were then capped and mixed for 15 seconds. A portion of each sample was then transferred to a 3 mL cuvette, and absorbance measured in a spectrophotometer at 880 nm. The absorbance values were compared to the standard curve to calculate total phosphorus (TP) concentrations in mg/L.

2.6 Filtration and Extraction of Caffeine from Water Samples

Clean plastic bottles, each containing 1 L of water, were wrapped in aluminum foil and stored at -5º C in a freezer. The samples then were shipped in plastic coolers to the Bob Wright Centre at the University of Victoria, BC for caffeine analysis.

Water samples were filtered using Whatman 934-AH membrane filter paper. All filters were pre-washed in DI water for quality control purposes. All samples were neutralized with sodium hydroxide prior to analysis. Quality control measures were undertaken to ensure the accuracy of caffeine analysis, in that, for every 10 samples prepared, a procedural blank and standard spikes were used. For the blank, 1 L of EasyPure water was used. For the spike, 1 L of EasyPure water was mixed with 200 – 400 ng of caffeine standard. For each one-litre sample, 10 µL of 2000 pg/µL (ppb) solution of the surrogate internal standard was added and shaken to mix thoroughly.

The Oasis hydrophilic-lipophilic-balanced (HLB) reversed-phased sorbent was conditioned with 3 mL of methyl-tert-butyl ether (MTBE), followed by 3 mL of methanol

(MeOH) combined with 3 mL of EasyPure water. The extraction tubes were rinsed with MeOH acetone and dichloromethane (DCM), respectively. The samples were passed through the Oasis HLB solid-phase-extraction (SPE) cartridge at approximately 10 mL/min. Each bottle was thoroughly rinsed three times with 10 mL EasyPure water to ensure as much as possible of the sample's caffeine had been extracted. Each Cartridge was then rinsed with 2 mL of 25% MeOH to remove any polar co-extractives. The Cartridges were dried under vacuum for 10 mins, and the sample extraction tubes were rinsed with MeOH, Acetone, and DCM before storage. The analytes were eluted from the cartridges and transferred to culture tubes using MeOH followed by MeOH/MTBE (1:9, v/v). Cartridge extractants were then concentrated to near dryness under an N_2 atmosphere (no oxygen was present). Then, 3 mL of DCM and Na_2SO_4 were added to the culture tubes. Samples were concentrated to 500 µL and 1 mL of DCM was added to each culture tube, then the tubes were vortexed and transferred to the 10 mL Kuderna-Danish (KD) centrifuge certified vials. Samples were concentrated to 100 µL, then, using a 250 µL Hamilton syringe, they were transferred to a gas chromatography (GC) certified vial insert and the meniscus was marked. Care was taken to avoid cross-contamination of samples by wiping the bottom of the needle and bottom of the syringe with methanol between transfers. The samples were then ready for the gas chromatography (GC) procedure.

2.7 Filtration and Extraction of Caffeine from Sediment Samples

10 g of sediment samples were weighed and placed in a 50 mL conical tube. 10 µL of ^{13}C labeled Internal standard (2.063 ppm) was added. Needles were rinsed with DI water. Each conical tube was then filled to 37 mL with EasyPure water and vortexed until

well mixed and homogenized. The samples were then left overnight. The following morning, samples were sonicated for 45 mins, then centrifuged at room temperature at 5000 rpm.

The supernatant was decanted into 150 mL Nalgene bottles, which were then topped up to 37 mL with DI water, well mixed and then sonicated for 45 mins. The steps of sonication, centrifugation, and decanting were repeated 2 more times (3 times total).

2.8 Analysis of Prepared Aqueous Caffeine Samples

Frozen samples were left to thaw overnight, and glass fiber filters, glassware, and Na_2S0_4, were prepared the day before. The following day, 10 μL of ^{13}C caffeine stock solution was added to each 1 L sample and was well shaken. The needles were wiped with MeOH after each sample to avoid cross-contamination. The samples were then filtered; meantime the Oasis HLB was conditioned with 3 mL MTBE and then 3 mL of MeOH, then rinsed with 3 mL of EasyPure water. The extracted sample was passed through the Oasis HLB SPE cartridges at 10 mL/min and the cartridges were then dried in a full vacum for 10 mins. The analytes were eluted from the HLB SPE cartridges to culture tubes by rinsing with MeOH and MTBE solution. Oasis extracts were concentrated further using nitrogen gas. The samples were dried to provide a concentrated solution of 100 uL. Using a Hamilton syringe, the samples were tranferred to a GC vial and the meniscus was marked with pen. The caffeine samples were now fully extracted and prepared for mass spectrometry. The prepared samples were cycled through a Varian CP 3800 gas chromatograph with a 30 m × 0.25 mm film thickness CP-SIL 8CB-MS capillary column interfaced to a Varian Saturn 2200 ion-trap mass spectrometer using the methods outlined above (Verenitch & Mazumder, 2008).

Chapter 3: Field Study

3.1 Introduction

Trace amounts of caffeine have been found in surface water samples throughout the world, from open oceans to inland freshwater systems (Bradley et al., 2007; Bruton et al., 2010; Chen et al., 2002; Dash et al., 2012; Edwards et al., 2015; Kurissery et al., 2012; Mazzafera, 2004; Siegener et al., 2002). Previous studies have suggested that trace caffeine concentrations may be used to identify waste loadings from domestic sources (Chen et al., 2002; Kurissery et al., 2012).

Although these studies have recognized the value of using "tracers" such as caffeine to differentiate domestic nutrient loading and nutrient loading from other sources, these methodologies have yet to be incorporated in the general guidelines for water quality monitoring in North America. It is clear that elevated caffeine concentrations can be closely linked to domestic waste, and that caffeine can be an ideal indicator for the trace determination of domestic waste (Chen et al., 2002; Furtado et al., 2000; Gardinali et al., 2002; Kurissery et al., 2012; Peeler et al, 2006; Thomas et al., 2005). Also, there have been few studies of caffeine in freshwater systems in Canada. A comprehensive investigation of caffeine concentrations and their persistence in freshwater systems should provide more clarity on the efficacy of using caffeine as a tracer compound to detect domestic waste on a regional scale.

3.1.1 Objectives

The primary objective of this study was to contribute to the existing knowledge on nutrient source tracking in freshwater systems using caffeine as a tracer molecule (Buerge et al., 2003; Kurissery et al., 2012; Peeler et al., 2006; Thomas et al., 2005). To

do this, data on caffeine concentrations were collected for one year to determine the utility of caffeine as a means to track the relative contribution of domestic waste in freshwater systems and its variability with seasons. Previous studies have suggested that the survival of *E. coli* and other bacteria (microbial tracers of domestic waste) is largely dependent on water temperature, so the winter months are a sub-optimal time to conduct microbial-based source tracking to identify nutrient loading (Ishii et al., 2006). In contrast, caffeine as a tracer of domestic nutrient loading should offer a robust and stable method that can be used year-round (Oliver et al., 2016).

The second objective of this study was to determine if caffeine in surface sediment samples can be used as a more stable method for tracing nutrient loading. It has been suggested that caffeine may remain more stable and less likely to degrade in sediment than in the overlying water, in the aquatic environment (Ahmad, 2014; Bruton et al., 2010; Kurissery et al., 2012; Trovó et al., 2013).

Thus, this study investigated three research questions.

1. Do caffeine concentrations fluctuate over different seasons of the year?

2. Do caffeine concentrations in surface sediment provide a more stable indicator of domestic waste loading than do concentrations in surface waters?

3. Would the combination of microbial source tracking and caffeine analysis provide a more accurate method for distinguishing nutrient loading *via* domestic waste than an assessment based on microbial source tracking alone?

3.2 Materials and Methods

3.2.1 Study Sites

Eight sampling locations were selected within the County of Simcoe (Figure 3-1). Six of these sites were in the Lake Simcoe watershed, and two were in the Georgian Bay watershed. These sites were chosen to represent various land use patterns within a variety of sub watersheds and drainage areas. The following coordinates are represented in NAD 83 (North American Datum 1983) UTM 17 (Universal Traverse Mercador Zone 17). The sites were: Holland River (HR), Bradford, ON (4891010.67 m N, 618249.47 m E); Hotchkiss Creek (HC), Barrie, ON (4914492.17 m N, 604506.64 m E); Lake Simcoe (LS), Hawkstone, ON (4927601.58 m N, 622242.84 m E); Bluff's Creek (BF), Orillia, ON (4936300.42 m N, 624974.56 m E); Kettle's Lake (KL), Awenda Provincial Park, ON (4966201.51 m N, 581166.71 m E); Farlaine Lake (FL), Tiny, ON (4964520.05 m N, 581111.36 m E); Ben's Ditch above the Orillia WWTP (BDa), Orillia, ON (4938822.29 m N, 625336.84 m E); and Ben's Ditch below the Orillia WWTP (BDb), Orillia, ON (4938578.38 m N, 626135.86 m E).

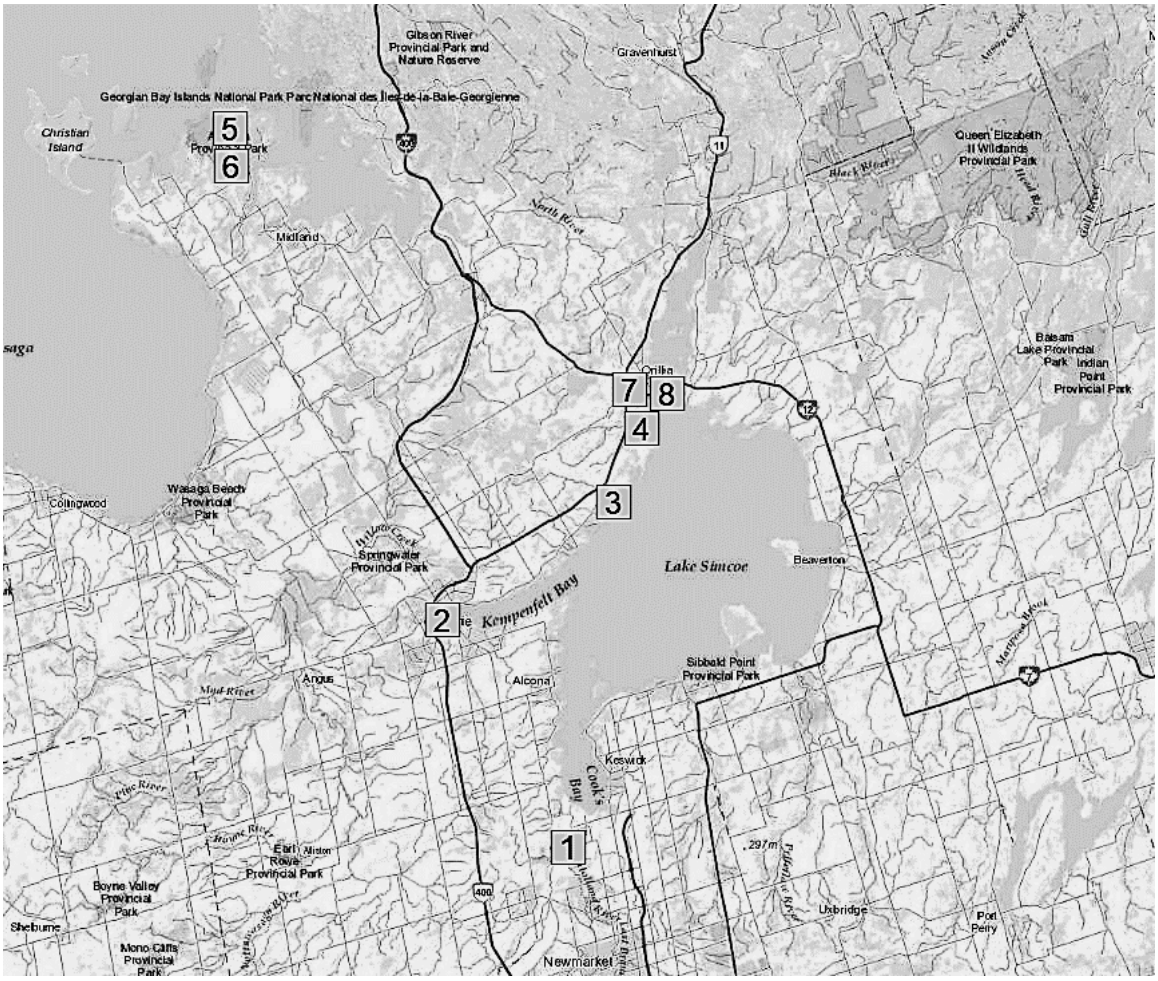

Figure 3-1: Map of the study area showing the eight sampling locations. Base map from Esri, 2016.

3.2.1.1 Site 1: West Holland Landing (HR)

The first sampling location was on the bank of the West Holland River Subwatershed, Lake Simcoe Watershed in Innisfil, ON (Figure 3-2). The watershed includes the agricultural lands of Holland Landing, and the city of Bradford, and covers a total of 354 km^2 located to the southeast of Cook's Bay, Lake Simcoe. While the river flows through the town of Bradford, it is surrounded by the Holland Marsh polder, a series of channels, pump-stations and tilled fields which considerably affects the hydrology of this area (LRSCA, 2010). Approximately 57% of the subwatershed comprises agricultural land and only 3% is urban area.

From an ecological perspective, the West Holland river is in sub-optimal condition. Most of the natural riparian zone and natural floodplain have been removed, and densely tile-drained farmland has created sheet flow and low percolation through the soils. These factors have reduced groundwater base flow and increased surface runoff, which in this agricultural area is impacting the river system.

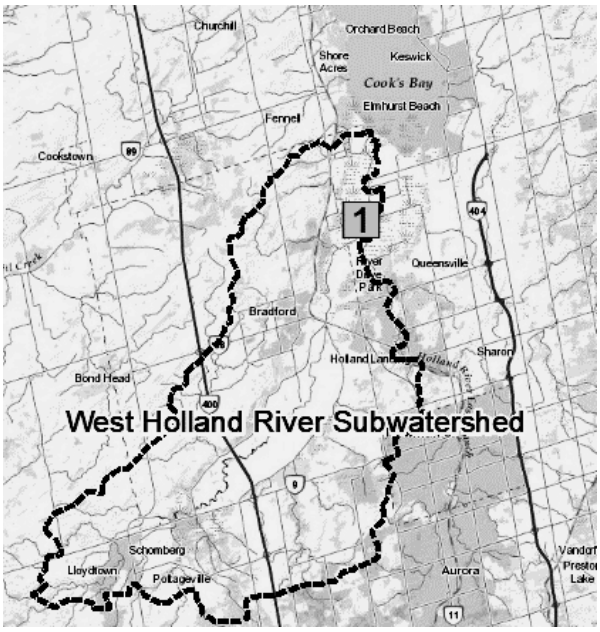

Figure 3-2: Map of sample location in the West Holland River Subwatershed of Lake Simcoe (sampling location HR). Base map from Esri, 2016.

3.2.1.2 Site 2: Mouth of Hotchkiss Creek, Kempenfelt Bay (HC)

The second sampling location was at the mouth of Hotchkiss Creek, in the Barrie Creeks subwatershed of the Lake Simcoe Watershed in Barrie, ON (Figure 3-3). The Barrie Creeks Subwatershed covers approximately 37 km^2 , and is an area comprising 75% urban development and less than 4% agricultural land use. The Hotchkiss river is significantly influenced by development activities in the city of Barrie. Low surface permeability and poor floodplain characteristics result in high surface runoff during wet weather events (reference?). Large deposits of sand and sediment at the mouth of

Hotchkiss Creek may also indicate impacts associated with stormwater and nutrient loading (LSRCA, 2012). Hotchkiss Creek shows significantly higher levels of total phosphorous and chloride compared to other stream systems in the Lake Simcoe Watershed (LSRCA, 2012). Although the waste water treatement plant (WWTP) is located alongside Hotchkiss Creek, its effluent is primarily discharged into Kempenfelt Bay. However, as a contingency plan, sanitary manholes in the city were designed with combined sewage overflow (CSO) bypass connections to stormwater sewers. Hotchkiss Creek feeds directly into Kempenfelt Bay of Lake Simcoe.

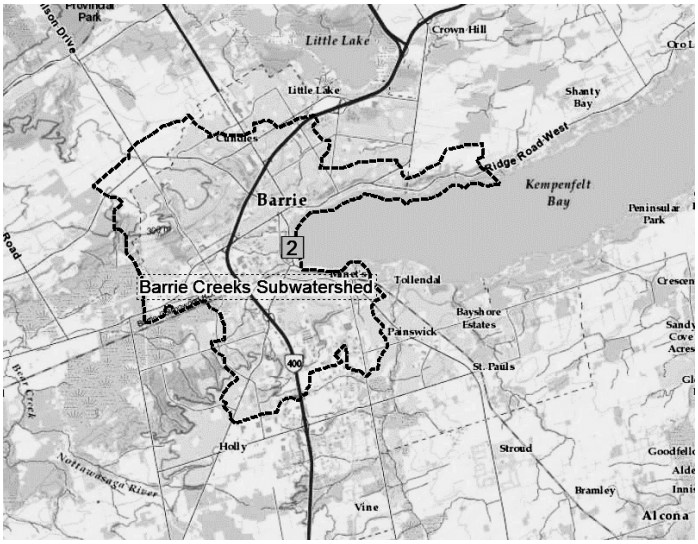

Figure 3-3: Map of sample location in the Barrie Creeks Subwatershed of Lake Simcoe (sampling location HC). Base map from Esri, 2016.

3.2.1.3 Site 3: Hawkstone, Oro Medonte (LS)

This sampling site was at Hawkstone Creek, Hawkstone Creeks Subwatershed, Lake Simcoe Watershed in Oro Medonte, ON (Figure 3-4). Samples were collected at the mouth of the creek, directly adjacent to the shoreline of the western end of Lake Simcoe. The subwatershed covers an approximate area of 48 km^2. It has 57% natural cover, comprising forested areas and wetland and 24% of the area is agricultural land (LSRCA,

2013). This site represents a blend of natural systems and agricultural land use, with minimal domestic (urban settlement) impacts.

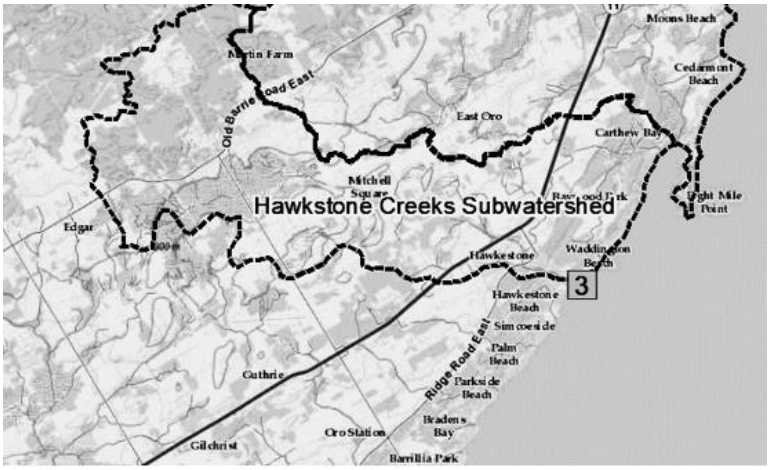

Figure 3-4: Map of sample location in the Hawkstone Creeks Subwatershed of Lake Simcoe (sampling location LS). Base map from Esri, 2016.

3.2.1.4 Site 4: Bluffs Creek, Orillia (BC)

This site was at the mouth of Bluffs Creek in the Oro Creeks North Subwatershed, Lake Simcoe Watershed in Orillia, ON (Figure 3-5). The subwatershed covers an approximate area of 46 km^2 with 46% has natural land use and 35% agricultural, although, Bluffs Creek more specifically, appears to be more naturalized when compared to the other subwatershed creeks. This site was chosen as a natural creek that was relatively close to the sites downstream and upstream of Ben's Ditch (BDa and BDb respectively).

3.2.1.5 Site 5: Kettle's Lake, Awenda Provincial Park (KL)

The sampling site was on Kettle's Lake in the Kettle's Lake Subwatershed of the Georgian Bay Watershed in Tiny, ON (Figure 3-5). It is an 11 km^2 drainage area with 90% natural cover, 8% domestic, and less than 2% agricultural land use. Kettle's Lake, located within Awenda Provincial Park, has a light recreational trail around the perimeter

of the lake. Policies are in place to restrict the use of motorized boats (MNR, 1990). Kettle's lake is of similar size and morphology to Farlain Lake . The Kettle's Lake (KL) sampling locations exemplify a very naturalized region with minimal human activity, and serves as an excellent contrast with Farlain Lake (FL), see below.

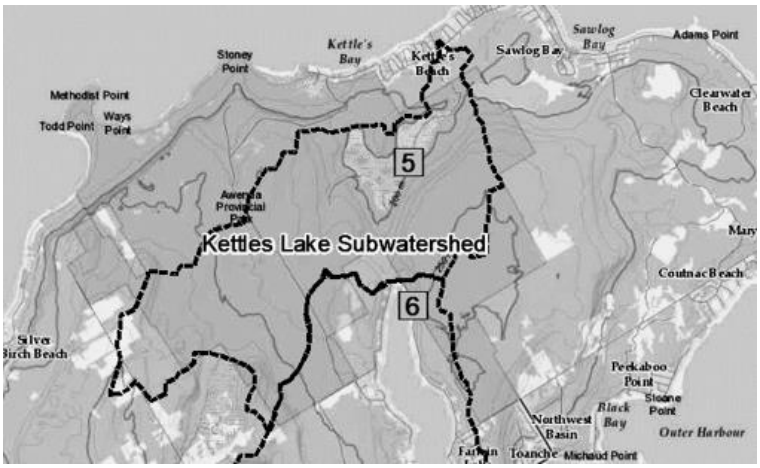

Figure 3-5: Map of sample location five in the Kettles Lake Subwatershed of Georgian Bay (sampling location KL). Base map from Esri, 2016.

3.2.1.6 Site 6: Farlain Lake, Tiny Township (FL)

This sampling site was at the north end of Farlain Lake, in the Farlain Lake Subwatershed of the Georgian Bay Watershed in Tiny, ON (Figure 3-6). This subwatershed drains a 14 km^2 area with 83% natural cover, 7% agricultural, and 10% domestic land use. Farlaine Lake is considered a heavily used recreational waterbody with most of the shoreline occupied by residential homes and cottages. The lake is approximately 2.5 kilometers long, and runs north to south. It is a natural, spring fed kettle lake (on a glaciated plain). In the past, there have been reports of bacterial growth in Farlain Lake after heavy rainfall events, raising concerns about nutrient loading (MOE, 1973); specifically overloaded cottage septic systems and outhouses are suspected of being the major contributors of nutrients (MOE, 1973).

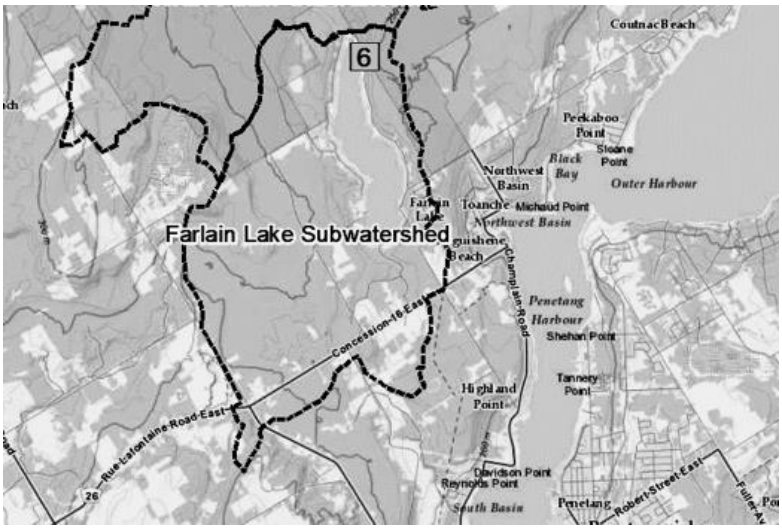

Figure 3-6: Map of sample location in the Farlain Lake Subwatershed of Georgian Bay (sampling location FL). Base map from Esri, 2016.

3.2.1.7 Site 7 and Site 8: Ben's Ditch (BDa and BDb)

Ben's Ditch is located in the Oro Creeks North Subwatershed of the Lake Simcoe Watershed in Orillia, ON (Figure 3-7). The subwatershed is approximately 75 km^2 area and consists of 46% natural cover, 35% agriculture and 10% urban area (LSRCA, 2013). More specifically, Ben's Ditch is an extension of Mill's Creek, a relatively naturalized stream with little obvious anthropogenic disturbance. However, Ben's Ditch is a man-made channel that serves as a primary drainage system for Orillia's stormwater run off (Aquafor Beech Ltd., 2016). The characteristics of this sampling location were useful as it had some similarities to Hotchkiss Creek (HC), but the two sites had contrasting levels of urban development in their watersheds. In Ben's Ditch, two sample locations were chosen, one upstream of the Orillia WWTP and one downstream of its outlet (labeled as BDa and BDb respectively).

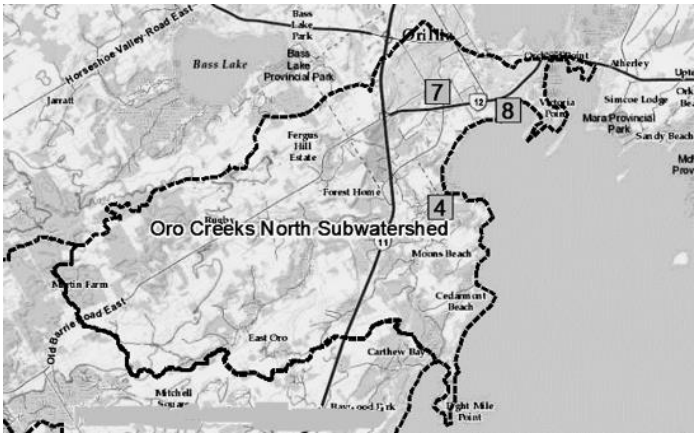

Figure 3-7: Map of sample locations in the Oro Creeks North Subwatershed of Lake Simcoe, including; Bluff's Creek (Sample site BC); and areas upstream and downstream of the wastewater treatment plant effluent to Ben's Ditch (sampling locations BDa and BDb). Base map from Esri, 2016.

Table 3-1: Summary of sampling locations and their land usage attributes, including natural cover, agricultural cover, urban cover, and rank in terms of domestic influence (Rank).

Name	Abbreviation	Coordinates (NAD 83 UTM 17)	% Natural cover	% Agricultural cover	% Urban cover (subwatershed)	% Urban cover (1 km)	Anthropogenic influence (50 m)	Evaluation Score	Rank
Kettle's Lake	KL	4966201.51 m N, 581166.71 m E	90%	2%	8%	2%	1	2.0	1
Hawkstone Shore (Lake Simcoe)	LS	4927601.58 m N, 622242.84 m E	57%	24%	19%	10%	2	4.9	2
Bluff's Creek	BC	4936300.42 m N, 624974.56 m E	46%	35%	10%	30%	2	6.0	3
Ben's Ditch (upstream of WWTP)	BDa	4938822.29 m N, 625336.84 m E	46%	35%	10%	30%	3	7.0	4
Farlain Lake	FL	4964520.05 m N, 581111.36 m E	83%	7%	10%	30%	5	9.0	5
West Holland River	HR	4891010.67 m N, 618249.47 m E	40%	57%	3%	25%	7	9.8	6
Hotchkiss Creek	HC	4914492.17 m N, 604506.64 m E	21%	4%	75%	60%	2	15.5	7
Ben's Ditch (downstream of WWTP)	BDb	4938578.38 m N, 626135.86 m E	46%	35%	10%	60%	9	16.0	8

Sites were ranked from lowest to highest in terms of the anticipated degree of urban or domestic land impacts. Several factors were taken into consideration to evaluate the sampling sites. First, the total subwatershed drainage area was assessed (Table 3-1). Second, land use in the vicinity of the sampling locations (within, approximately, a 300

m radius) was assessed, and any unique attributes of the area were noted. Three criteria were evaluated to generate a score for ranking. First the percent of urban cover in the subwatershed (available through Lake Simcoe Regional Conservation Authority and Severn Sound Environmental Association) was converted to a value out of ten. Second, the percent of urban land use within a 1 km radius was converted to a value out of ten (estimated by evaluating arial photographs) (Google, 2017). Third, the immediate sampling location (50 m radius) was given a value of anthropogenic disturbance out of ten (observed and estimated visually at sampling location). The three values were added together to generate the evaluation score (out of a maximum possible score of 30) and then the sampling locations were ranked accordingly (Table 3.2-1).

3.2.2 Sampling Methods

Monthly sampling was conducted from October, 2015 to September, 2016. To ensure no accidental contamination of samples or materials occurred while working with samples in the field or laboratory, the investigator limited his personal use and consumption of caffeine related products during this period of study. For the microbial sample collection standard aseptic techniques were followed (Bykowski et al., 2005). Chest waders and fresh nylon gloves were worn at each sampling event. During each sampling episode, data on the site condition, weather, animal activity and any other factors that could affect the data were recorded. Methodology that is explicitly related to the monthly field collection of samples is described below; further information on analytical methods used in the laboratory are given in Chapter 4, below (Laboratory Procedures).

3.2.2.1 Collection of Bacterial Samples

Bacterial samples were always collected first as they are readily cross-contaminated. Water samples were collected in pre-sterilized sampling bottles at approximately 10 cm below surface and from a water column of approximately 40 cm in depth. Sediment samples were collected with a D-core which was wiped with 70% ethanol prior to collection. Microbial samples for heterotrophic plate count (HPC), total coliform, and *E. coli* enumeration (from both water and surface sediment), were stored in sterile sampling bottles in a cooler and processed in the laboratory immediately upon arrival, following the standard procedures (Health Canada, 2014). All samples were partitioned into three (replicate) subsamples.

3.2.2.2 Collection of Water Samples

Water samples for the analysis of total suspended solids (TSS) were collected from 10 cm below the water surface in clean 1 L bottles. Samples for nutrient analysis (total phosphorus (TP), and nitrate) were collected from 10 cm below the water surface in clean 250 mL bottles, and were stored in a freezer (<-4ºC) until processing. All samples were analyzed as three replicate subsamples.

3.2.2.3 Collection of Caffeine Samples

Water samples for caffeine analysis were collected in clean 1 L bottles that were previously washed with 10% hydrochloric acid solution and rinsed in deionized (DI) water (Balance & Bartram, 1998). Samples were collected from 10 cm below the water surface. The bottles were then wrapped in tinfoil and stored at <0°C. From the core samples collected, only the upper 3 cm of surface sediment was used for caffeine analysis. Sediment samples were wrapped in a clean tinfoil, properly labelled and stored

in freezer bags at <0°C until analysis. All caffeine samples were analyzed as two replicate subsamples.

3.2.2.4 Additional Data Collection

A multiparameter Hydrolab (VWR symphony, SB90M5) and Hach, HQ40d were used to record dissolved oxygen (DO), conductivity, pH, and water temperature at each sampling location at each sampling event. In each case two replicate measurements were taken.

3.2.2.5 Statistical Analysis

3.2.2.5.1 Seasonal Comparisons

To study the variation of caffeine concentration with respect to other variables recorded, the following factors were considered. Sampling location and month of sampling as independent variables, and water temperature as a co-variate since temperature is very closely related to seasonality (Gan, 1995). All other parameters such as bacterial counts, caffeine concentration and other water parameters were treated as multiple response variables, and are listed in Table 3.2-2.

Table 3-2: Factors, covariates and response variables in the statistical analysis.

Factors:	**Covariates:**	**Continuous Response Variables:**
Sampling Location	Water Temperature	Bacteria - Water Samples: – *E. coli* (CFU/100 mL) – Total Coliforms (CFU/100 mL) – Heterotrophs (CFU/100 mL)
Domestic Land-Use (%)	Month of sampling	Bacteria - Sediment Samples: – *E. coli* (CFU/100 mL) – Total Coliforms (CFU/100 mL) – Heterotrophs (CFU/100 mL)
		Water parameters: – TSS (mg/L) – TP (mg/L) – Nitrate (mg/L)
		Caffeine
		pH
		Dissolved oxygen (mg/L)
		Conductivity (µS/mL)

In order to study any seasonal variation in caffeine concentration in relation to all other independent and other parameters mentioned in Table 3.2-1, a multivariate analysis of covariance (MANCOVA) was carried out (Quinn et al., 2002). The MANCOVA test will help to determine any effects of sampling date (seasonality) on the combination of measured response variables, treating water sampling location as a covariate.

Before proceeding with the MANCOVA, a series of assumptions were tested such as missing data, outliers, normality and linearity among variables and covariates, homogeneity of variance/covariance and homogeneity of regression slopes.

3.2.2.5.2 Comparing Sampling locations

A model was constructed to predict land use pattern based on the recorded hydrologic and sediment sampling parameters. Land use information was available

through local conservation authorities and subwatershed plans and other documentation (LSRCA, 2010; LSRCA, 2012; SSEA, 2015). The percentage of domestic land use was assessed in each upstream area of subwatersheds associated with each of the sampling areas. In addition, land use in the immediate area of each sampling site was assessed. Based on this, sampling sites were ranked in order from the lowest to the highest degree of anticipated domestic influence. The land use ranked sampling locations were used as outcome variables for a stepwise multiple regression analysis. The rank order of the sites from lowest to highest domestic influence were KL, LS, BC, BDa, FL, HR, HC, and BDb. A series of step-wise multiple regressions were used to generate models with different combinations of predictor variables to estimate domestic land use and site characteristics following the guidelines provided in Quinn & Keough (2002).

Analysis of variance (ANOVA) was used to identify the differences among data from BDa and BDb, as well as FL and KL. Both paired sampling site comparisons were chosen for their close geographic proximity to each other as well as their contrasting levels of anthropogenic influence. All data were subjected to appropriate tests of assumptions (normality and variance) following standard procedures (Quinn & Keough, 2002). Sampling sites were tested to determine any differences among heterotrophic plate count (HPC), total coliforms (TC), and *E. coli* and caffeine from water and sediment as well as nitrate and Total Phosphate.

3.3 Results

3.3.1 Seasonal Comparisons

3.3.1.1 Water Parameters

Water temperature showed high seasonal variability; the lowest mean temperatures were in January (0.74°C, SE ± 0.61) and highest in August (26.65°C, SE ± 0.81) (Figure 3-8). Mean pH was relatively consistent over the course of study, but was lowest in March (7.38, SE ± 0.21) and highest in December (8.26, SE ± 0.28) (Figure 3-9). Mean dissolved oxygen concentrations were lowest in September (84% Saturation, 7.16 mg/L, SE ± 0.81) and highest in April (99% saturation, 12.92 mg/L, SE ± 0.51) (Figure 3-10). Mean TSS fluctuated over the year but were lowest in April (3.08 mg/L, SE ± 0.70) and highest in November (9.65 mg/L, SE ± 3.83) (Figure 3-11). Mean conductivity was lowest in May (500.13 µS/L, SE ± 176.82) and highest in August (936.13 µS/L, SE ± 330.97) (Figure 3-12). Mean TP concentrations were higher in spring, and lowest in late fall. The lowest average TP concentration was recorded in September (38.34 mg/L, SE ± 8.71) and the highest was in May (372.26 mg/L, SE ± 36.09) (Figure 3-13). Nitrate concentrations peaked in late summer and early fall, with the highest concentration in August (1582.52 mg/L, SE ± 934.44) and lowest in December (217.18 mg/L, SE ± 117.12) (Figure 3-13).

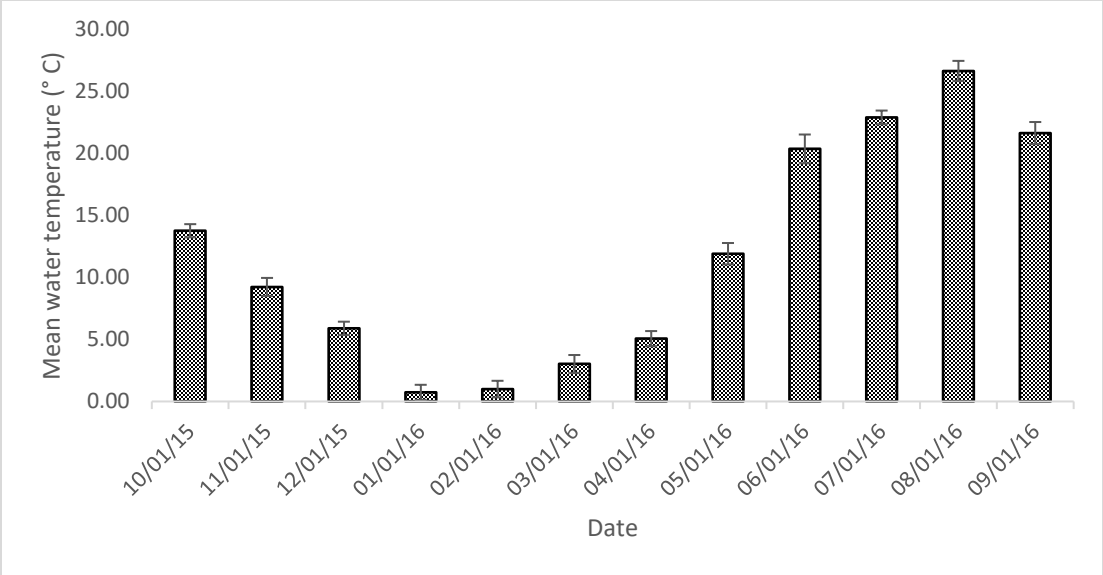

Figure 3-8: Mean water temperature (° C) recorded over the period of study in all sampling locations. Error bars indicate standard error.

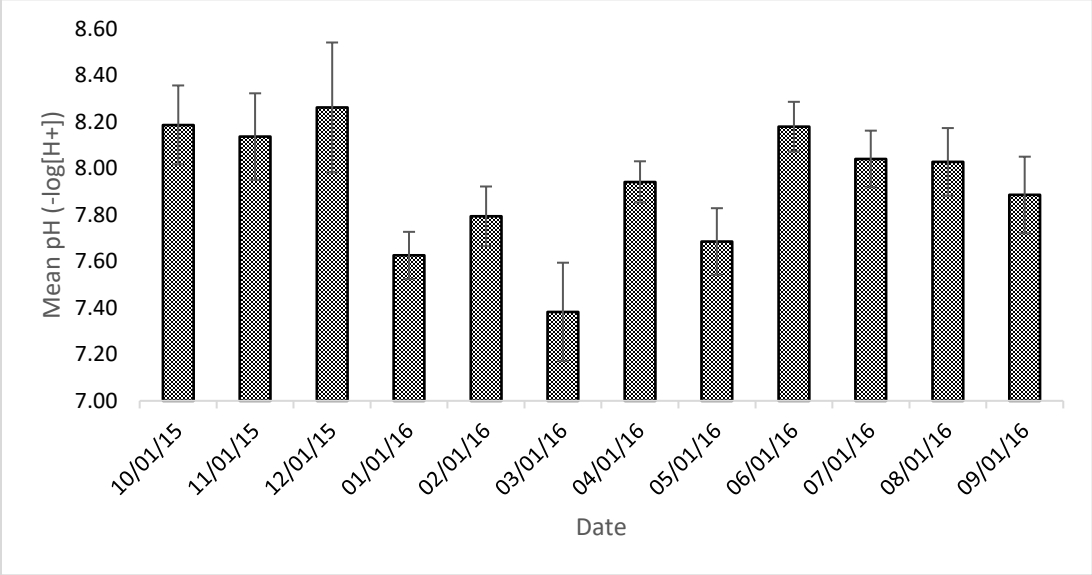

Figure 3-9: Mean pH (-log[H+]) values over the period of study in the sampling locations. Error bars indicate standard error.

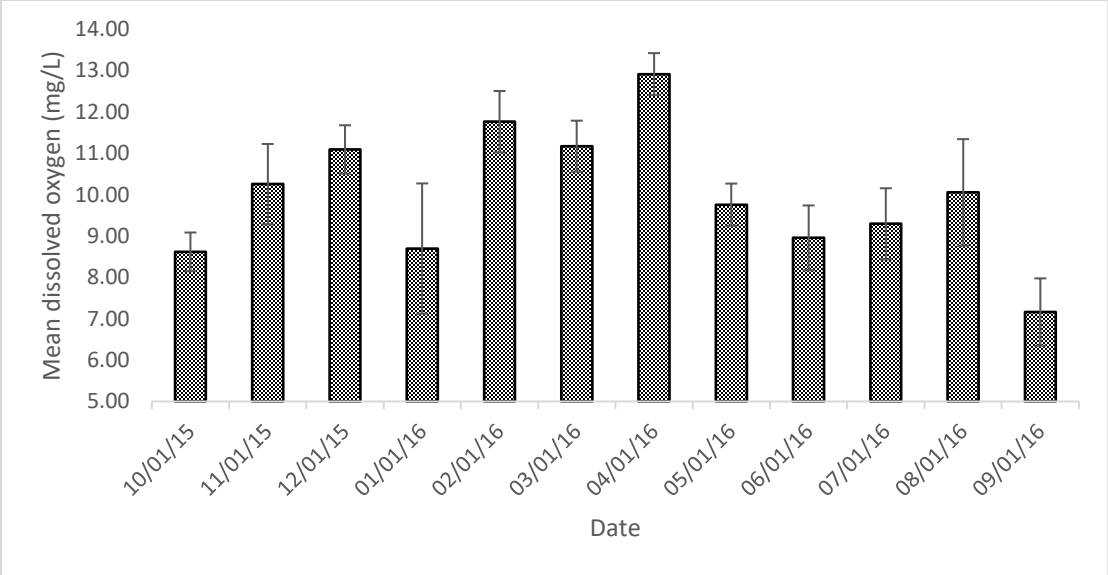

Figure 3-10: Mean dissolved oxygen concentrations(mg/L) recorded over the period of study in all sampling locations. Error bars indicate standard error.

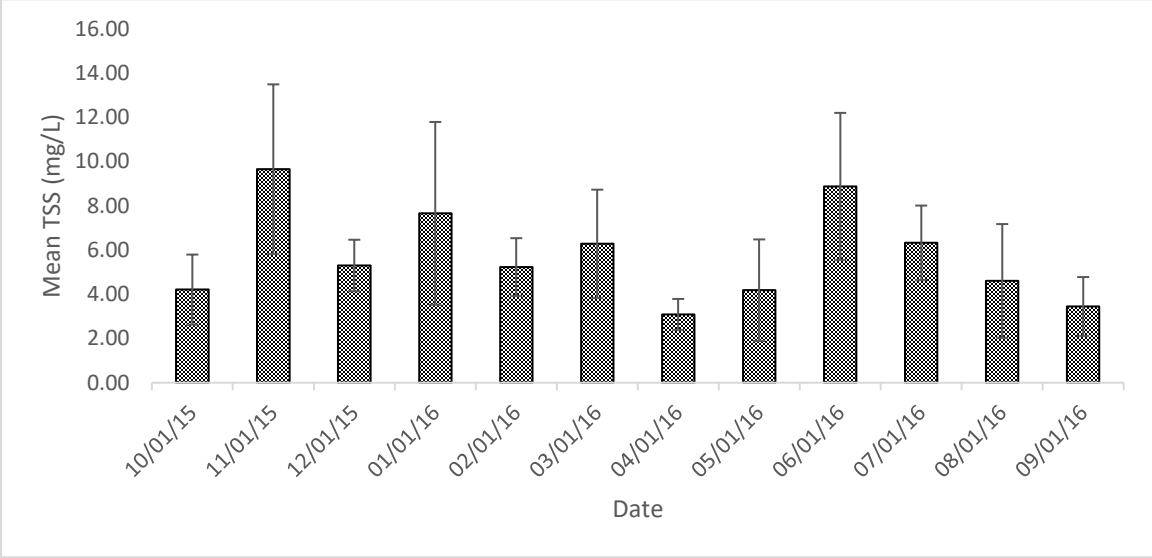

Figure 3-11: Mean TSS (mg/L) recorded over the period of study in all sampling locations. Error bars indicate standard error.

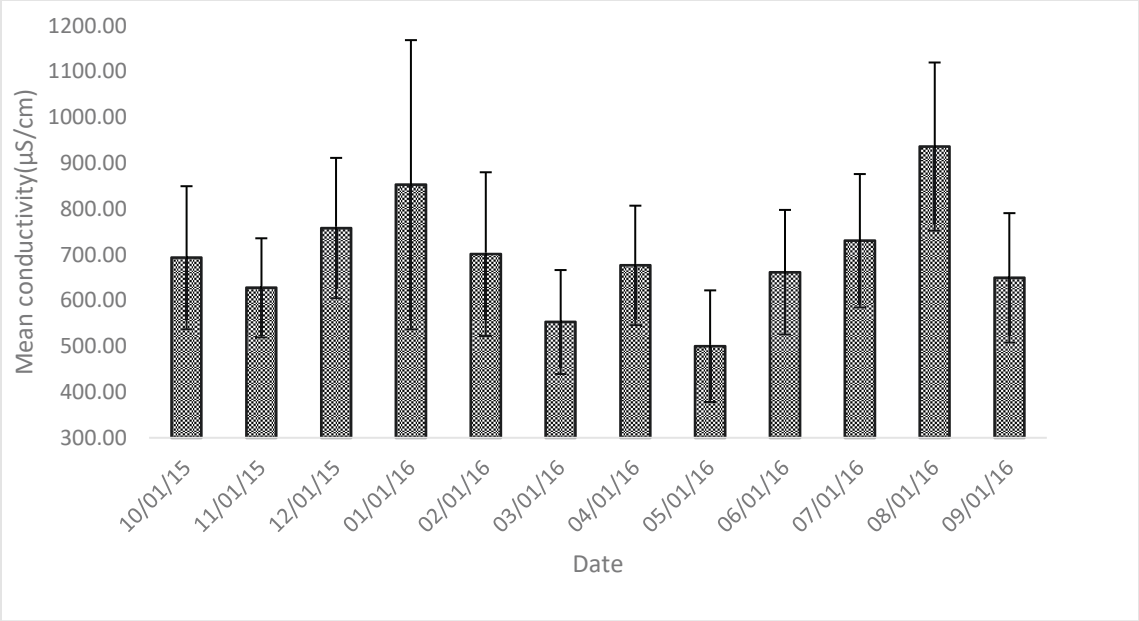

Figure 3-12: Mean conductivity(µS/cm) recorded over the period of study in all sampling locations. Error bars indicate standard error.

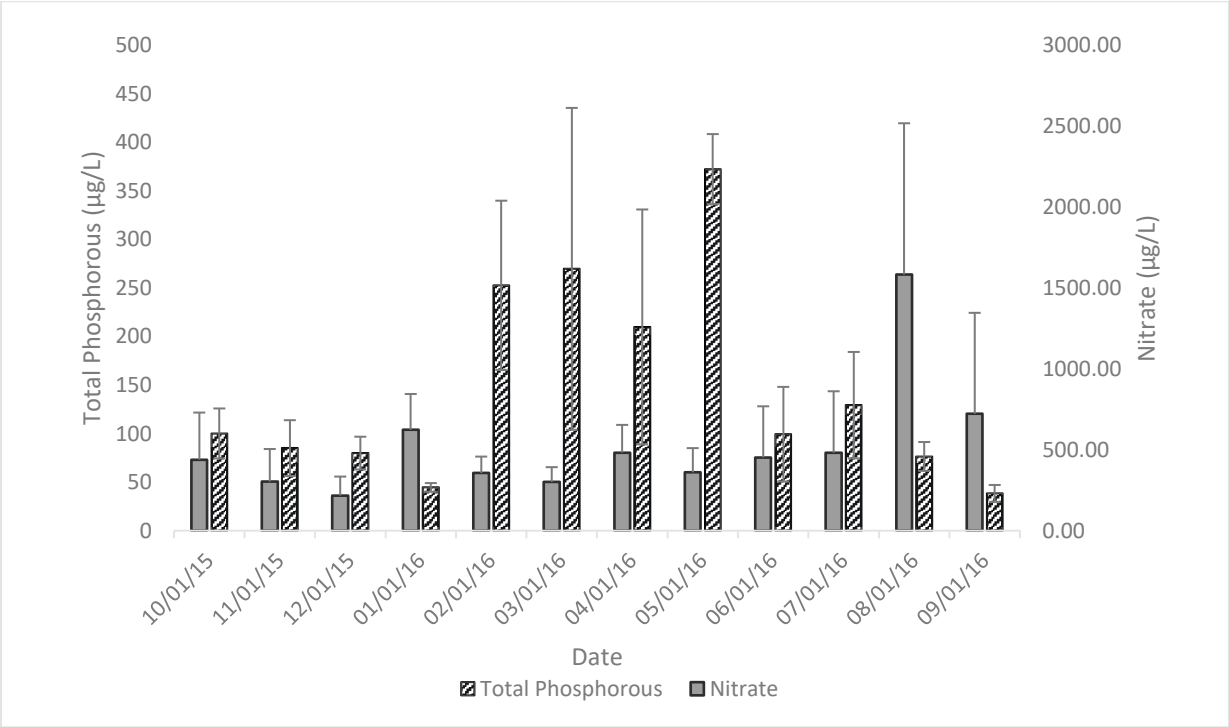

Figure 3-: Mean nitrate and TP (µg/L) recorded over the period of study in all sampling locations. Error bars indicate standard error.

3.3.1.2 Bacterial Counts

In the water samples, the mean heterotrophic plate counts (HPC) were consistently higher than other indicator bacterial counts throughout the year of sampling. Mean HPC was lowest in June (2.88 $\log_{10}$ cfu/100 mL, SE ± 0.14) and highest in August (7.47 $\log_{10}$ cfu/100 mL, SE ± 0.04) (Figure 3-14). Mean total coliform count (TC) was lowest in March (1.60 $\log_{10}$ cfu/100 mL, SE ± 0.23) and highest in April (2.92 $\log_{10}$ cfu/100 mL, SE ± 0.06) (Figure 3-14). Mean *E.coli* counts were lowest in February (0.46 $\log_{10}$ cfu/100 mL, SE ± 0.31) and highest in May (2.65 $\log_{10}$ cfu/100 mL, SE ± 0.13) (Figure 3-14).

In sediment samples, the mean counts of heterotrophic bacteria were consistently higher than the other types of bacterial counts throughout the year of study. Lowest mean HPC (in sediment) were recorded in June (3.55 $\log_{10}$ cfu/g, SE ± 0.78) and highest in October (5.10 $\log_{10}$ cfu/g, SE ± 0.14 (Figure 3-15). The mean counts for TC were lowest in March (0.55 $\log_{10}$ cfu/g, SE ± 0.36) and highest in November (1.89 $\log_{10}$ cfu/g, SE ± 0.30) (Figure 3-15). The mean counts of *E. coli* were below detectable limits during the months of February, March, and April, and highest in October (1.14 $\log_{10}$ cfu/g, SE ± 0.36) (Figure 3-15).

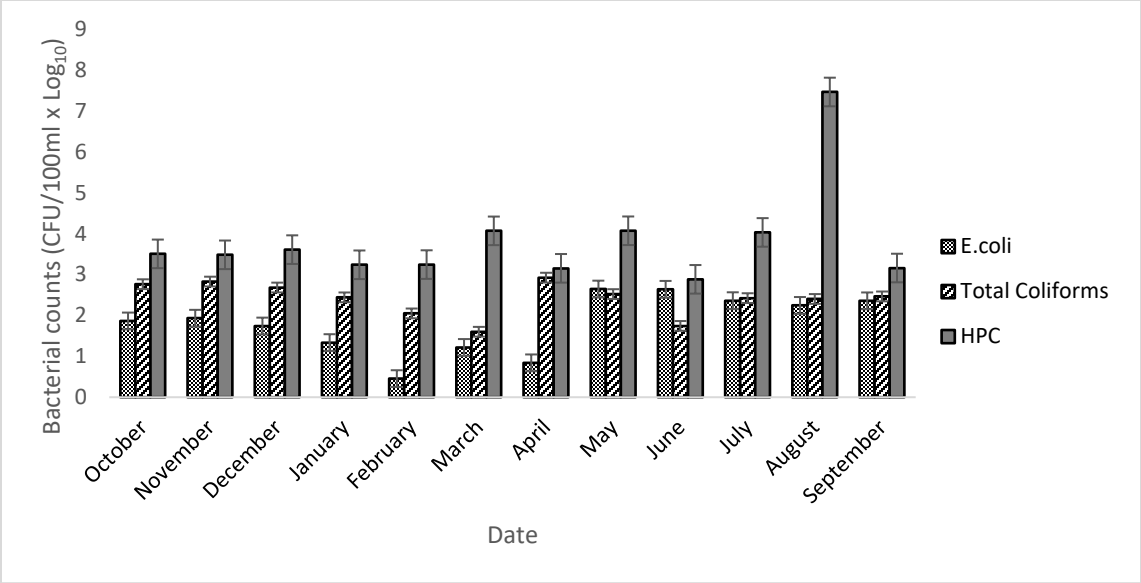

Figure 3-14: Mean bacterial counts in water samples (log_{10} cfu/100 mL) over the period of study. Error bars indicate standard error.

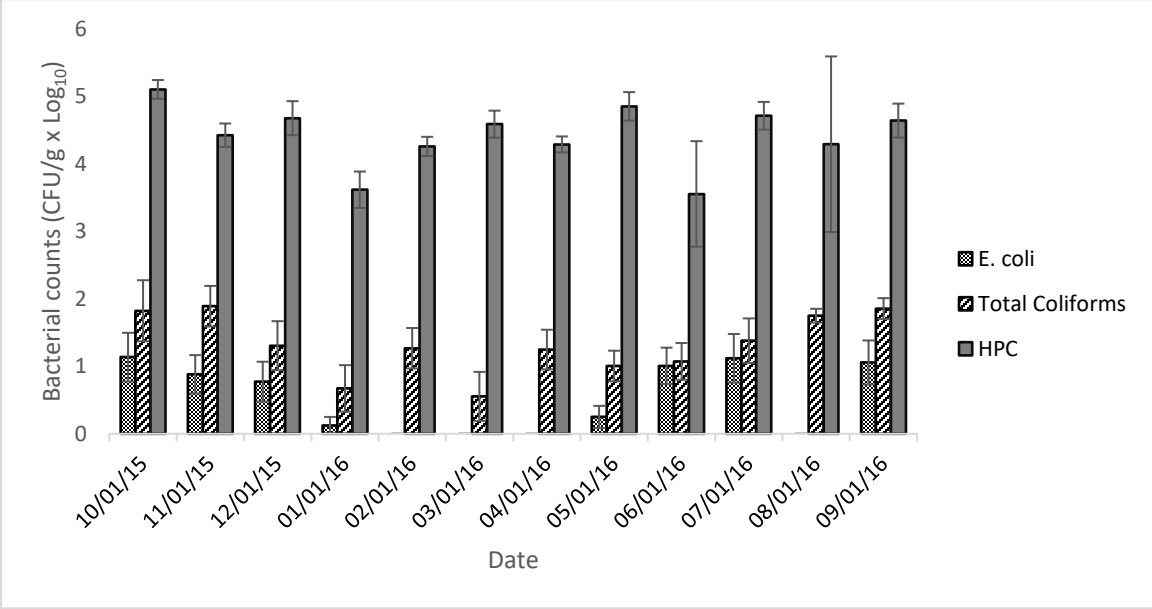

Figure 3-15: Mean bacterial counts in surface sediment samples (log_{10} cfu/g) over the period of study. Error bars indicate standard error.

3.3.1.3 Caffeine Concentrations

The mean caffeine concentration in water samples was lowest in May (14.75 ng/L SE ± 4.77) and higher during the winter months, peaking in January (102.75 ng/L, SE ± 39.64) (Figure 3-16) (14750 ppt, SE ± 4770 and 102750 ppt, SE ± 39640 respectively) . Mean caffeine concentrations in sediment samples were lowest in July (0.16 ng/g, SE ± 0.16) and highest in March (2.81 ng/g, SE ± 1.03) (160 ppt, SE ± 160 and 2810 ppt, SE ± 1030 respectively) (Figure 3-17).

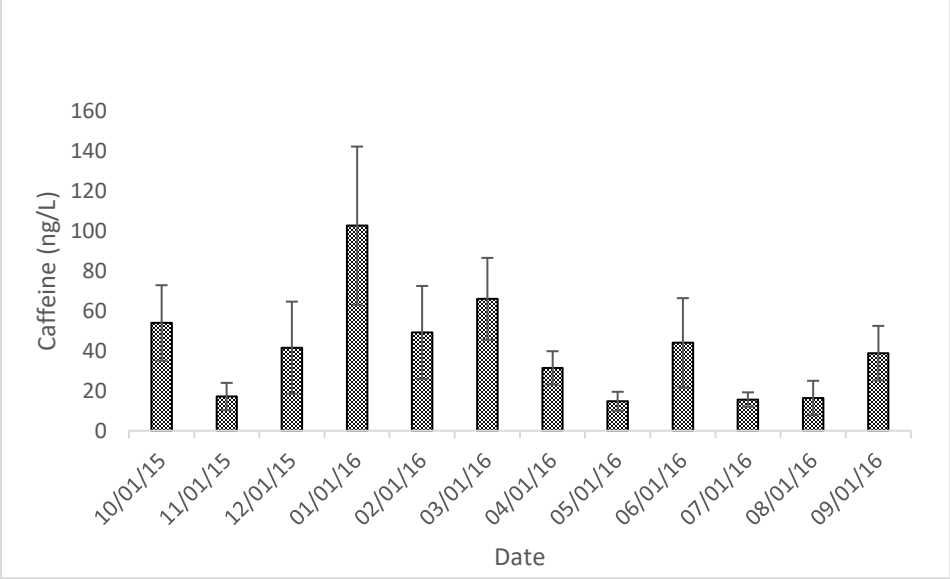

Figure 3-16: Mean caffeine concentrations in water samples (ng/L) over the period of study. Error bars indicate standard error.

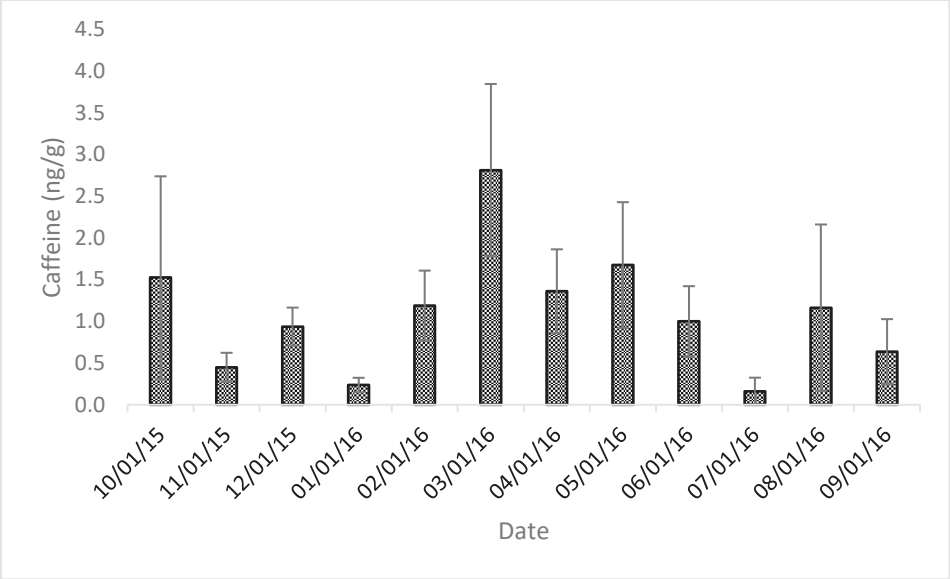

Figure 3-17: Mean surface sediment caffeine concentrations (ng/g) over the period of study. Error bars indicate standard error.

3.3.2 Comparing Sampling Locations

3.3.2.1 Water Parameters

The lowest recorded mean shoreline water temperature was at site BDa (10.63°C, SE ± 2.47) while the highest temperature was recorded at the near site BDb (13.64°C, SE ± 2.28) (Figure 3-18). pH was lowest at sampling location BDa (7.33, SE ± 0.07) and highest at HC (8.30, SE ± 0.07) (Figure 3-19). The lowest recorded dissolved oxygen concentrations were found at sampling location BDa, (83% saturation, 8.38 mg/L, SE ± 0.62) while the highest was at BDb (99% saturation, 11.63 mg/L, SE ± 0.90) (Figure 3-20). Mean TSS levels were lowest at sampling location KL (1.93 mg/L, SE ± 0.61) and highest at HC (9.94 mg/L, SE ± 3.45) mg/L (Figure 3-21). Mean conductivity was lowest at site KL (286.34 µS/L, SE ± 146.08) and highest at BDa, (1255.75 µS, SE ± 54.07) (Figure 3-22). Mean nitrate concentration was lowest at site LS (82.29 µg/L, SE ± 3.41) and highest at BDa (1671 µg/L, SE ± 860.84), while mean TP concentration was lowest

at FL (72.56 µg/L, SE ± 35.56) and highest at HR (380.35 µg/L, SE ± 126.33) (Figure 3-23).

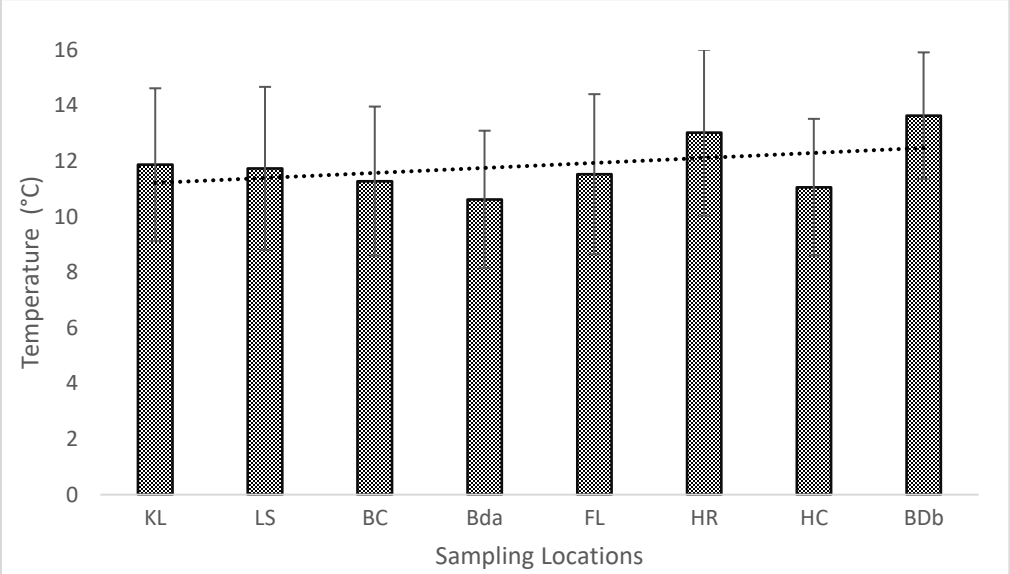

Figure 3-18: Mean temperature (degrees Celsius) recorded at sampling locations ranked in the order of domestic land use (percentage) in corresponding subwatershed (least to greatest) with trendline. Error bars indicate standard error.

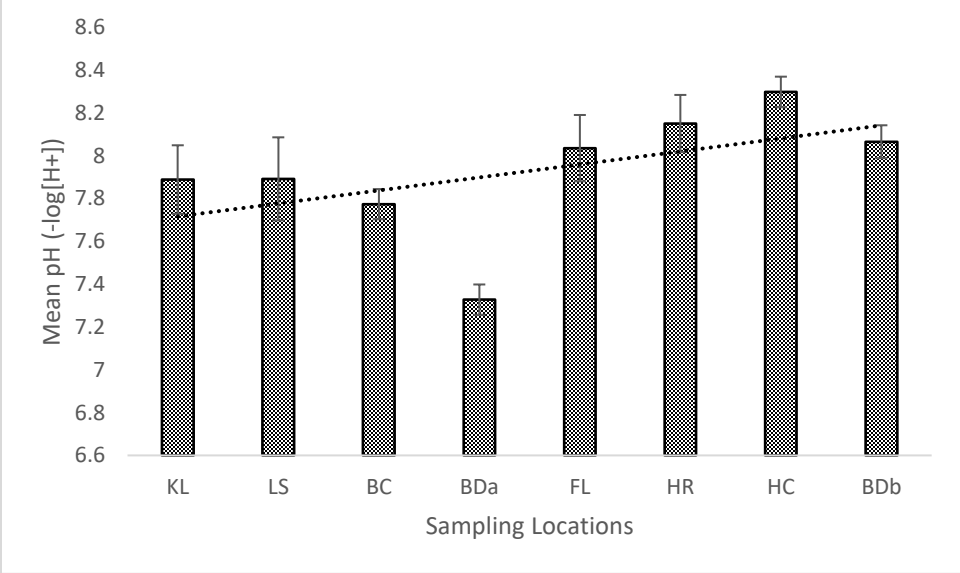

Figure 3.3-19: Mean pH values recorded at different sampling locations ranked in the order of domestic land use (percentage) in corresponding subwatershed (least to greatest) with trendline. Error bars indicate standard error.

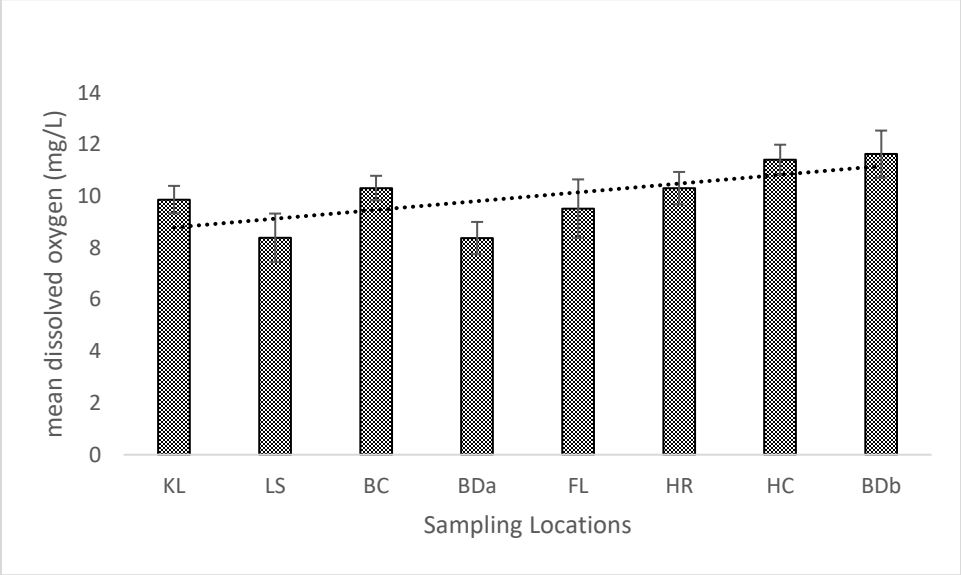

Figure 3.3-20: Mean dissolved oxygen concentrations (mg/L) recorded at different sampling locations ranked in the order of domestic land use (percentage) in corresponding subwatershed (least to greatest) with trendline. Error bars indicate standard error.

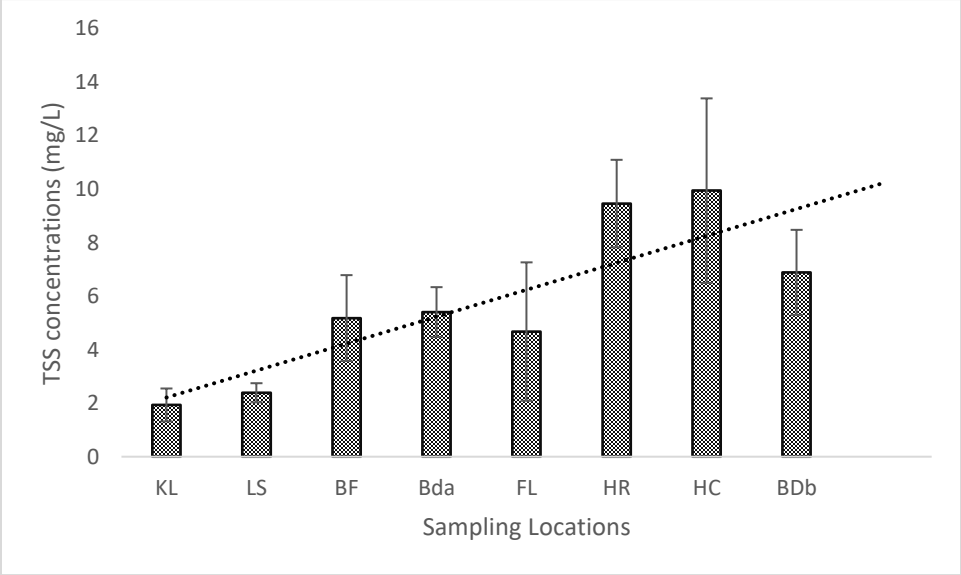

Figure 3.3-21: Mean TSS concentrations (mg/L) recorded at different sampling locations ranked by order of domestic land use (percentage) in corresponding subwatershed (least to greatest) with trendline. Error bars indicate standard error.

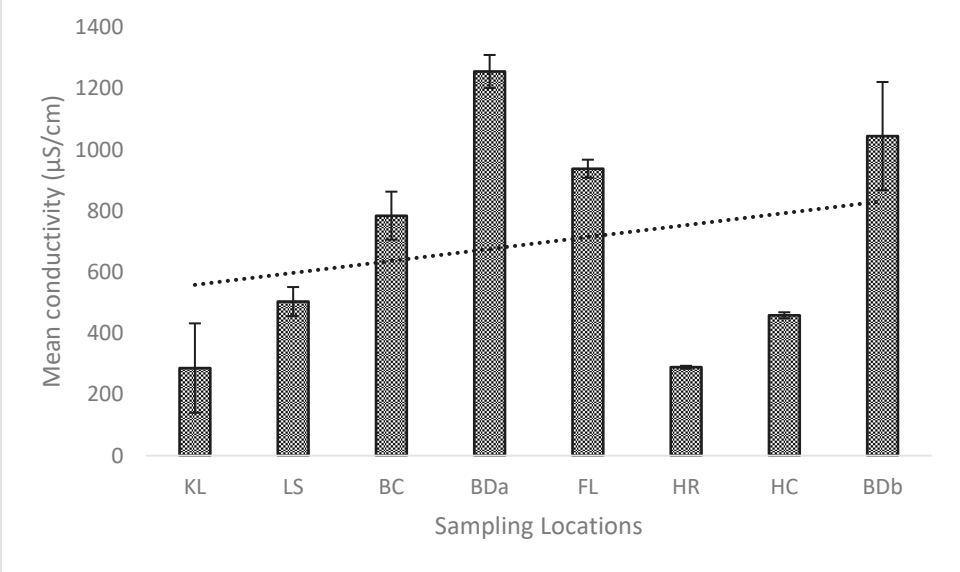

Figure 3-22: Mean conductivity (µS/cm) recorded in different sampling locations ranked in the order of domestic land use (percentage) in corresponding subwatershed (least to greatest) with trendline. Error bars indicate standard error.

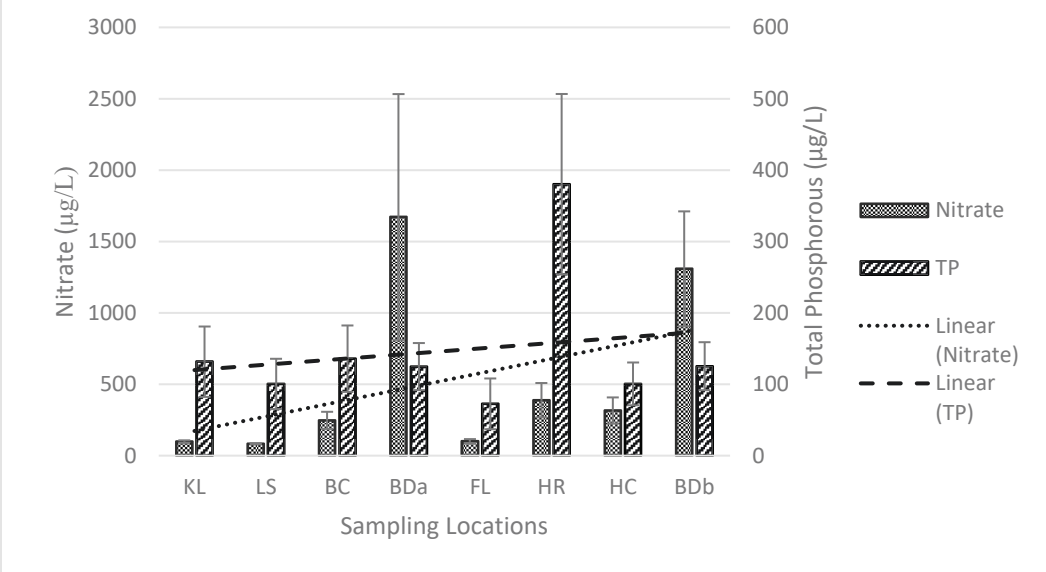

Figure 3.3-23: Mean nitrate and TP concentrations (µg/L) recorded in different sampling locations ranked in the order of domestic land use (percentage) in corresponding Subwatershed (least to greatest) with trendline. Error bars indicate standard error.

3.3.2.2 Bacterial Counts

In water samples, at all sampling locations, the mean HPC was greater than bacterial counts of TC and *E. coli*. HPC was lowest at sampling location BDa (6.39 $\log_{10}$ cfu/100 mL, SE ± 1.20) and highest at KL (6.63 $\log_{10}$ cfu/100 mL, SE ± 2.07) (Figure 3-24). Mean TC counts were lowest at KL (2.27 $\log_{10}$ cfu/100 mL, SE ± 0.65) and highest at BDb (3.02 $\log_{10}$ cfu/100 mL, SE ± 0.86) (Figure 3-24). Mean *E. coli* counts were lowest at KL (1.70 $\log_{10}$ cfu/100 mL, SE ± 0.55) and highest at BDb (2.92 $\log_{10}$ cfu/100 mL, SE ± 0.82) (Figure 3-24).

In sediment samples, mean HPC was lowest at FL (4.45 $\log_{10}$ cfu/g, SE ± 1.27) and highest at HC (6.47 $\log_{10}$ cfu/g, SE ± 2.00) (Figure 3-25). Mean TC count was lowest at LS (1.55 $\log_{10}$ cfu/g, SE ± 0.52) and highest at BDa (2.16 $\log_{10}$ cfu/g, SE ± 0.68) (Figure 3-25). *E. coli* densities in sediment samples ranged from below detection limits in sites LS and HC to the highest mean count at HR (1.59 $\log_{10}$ cfu/g, SE ± 0.55) (Figure 3-25).

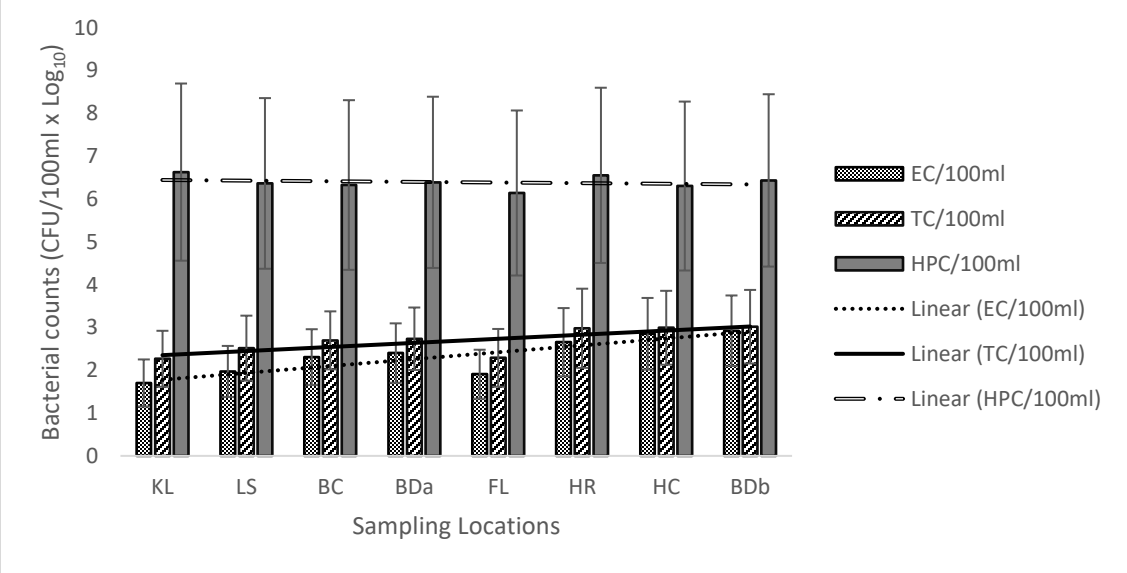

Figure 3.3-24: Mean bacterial count from water samples ($\log_{10}$ cfu/100 mL) recorded from different sampling locations ranked in the order of domestic land use (percentage) in corresponding subwatershed (least to greatest). Trendlines

show a weak positive relationship between domestic land use and TC as well as E.coli. Error bars indicate standard error.

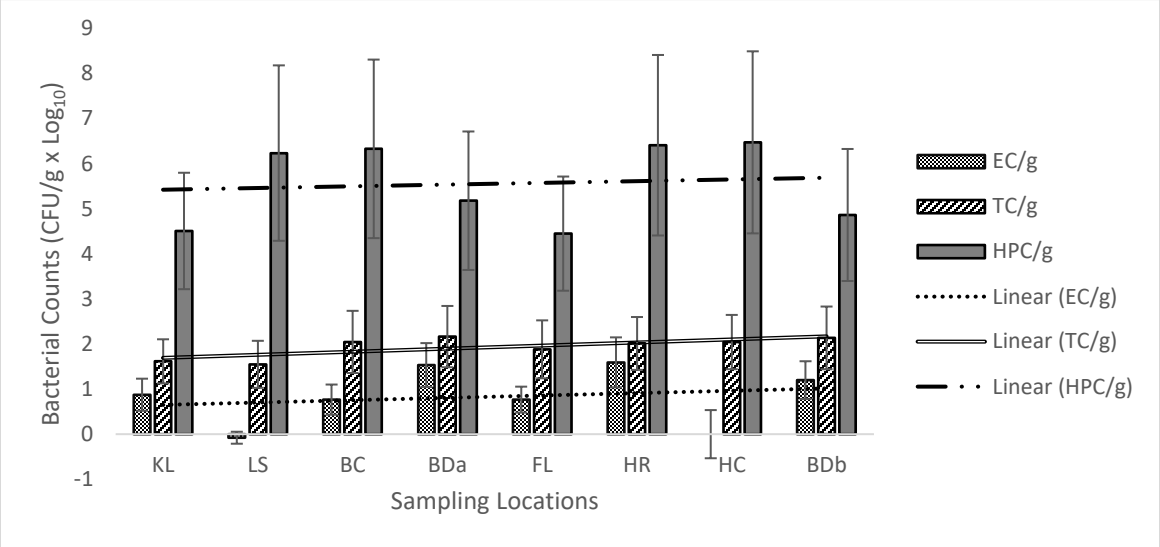

Figure 3.3-25: Mean bacterial count from sedimentary samples (log10 cfu/g) recorded in different sampling locations ranked in the order of domestic land use (percentage) in corresponding subwatershed (least to greatest). Trendlines show a weak positive relationship between domestic land use and bacterial parameters.

3.3.2.3 Caffeine Concentration

The mean caffeine concentration among water samples was lowest at site BC (11.20 ng/L, SE ± 5.62) and highest in BDb (66.28 ng/L, SE = ± 25.48) (11200 ppt, SE ± 5620 and 66280 ppt, SE ± 25480 respectively) (Figure 3-26). Mean caffeine concentration in sediment samples was lowest at BDb (0.47 ng/g, SE ± 0.26) and highest at KL (2.40 ng/g, SE ± 0.81) (470 ppt, SE ± 260 and 2400 ppt, SE ± 810 respectively) (Figure 3-27).

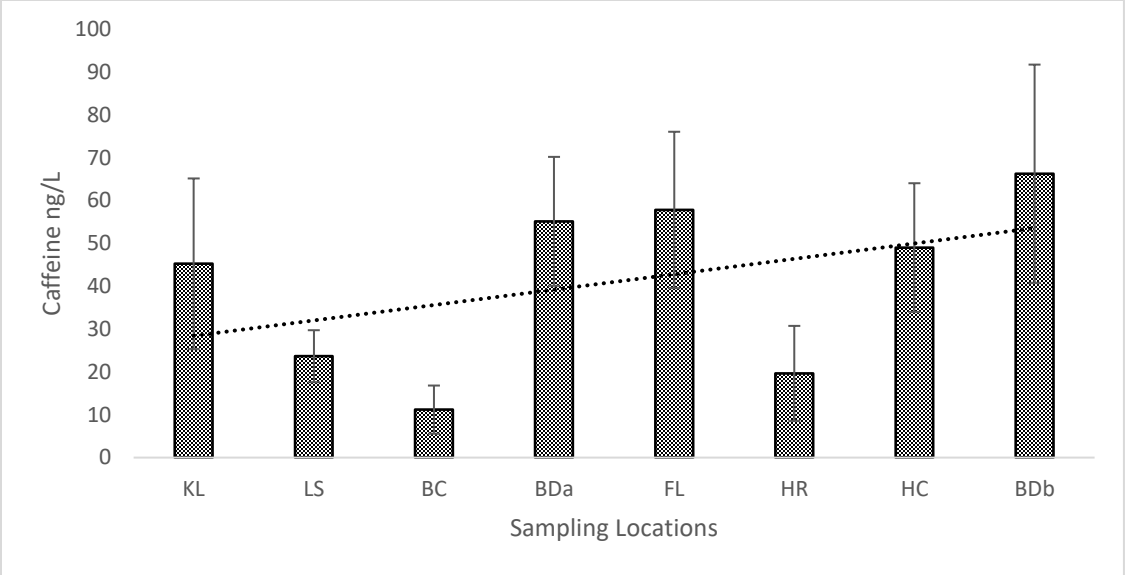

Figure 3.3-26: Mean caffeine concentrations in water samples (ng/L) recorded at different sampling locations ranked by the order of domestic land use (percentage) in corresponding subwatershed (least to greatest) with trendline. Error bars indicate standard error.

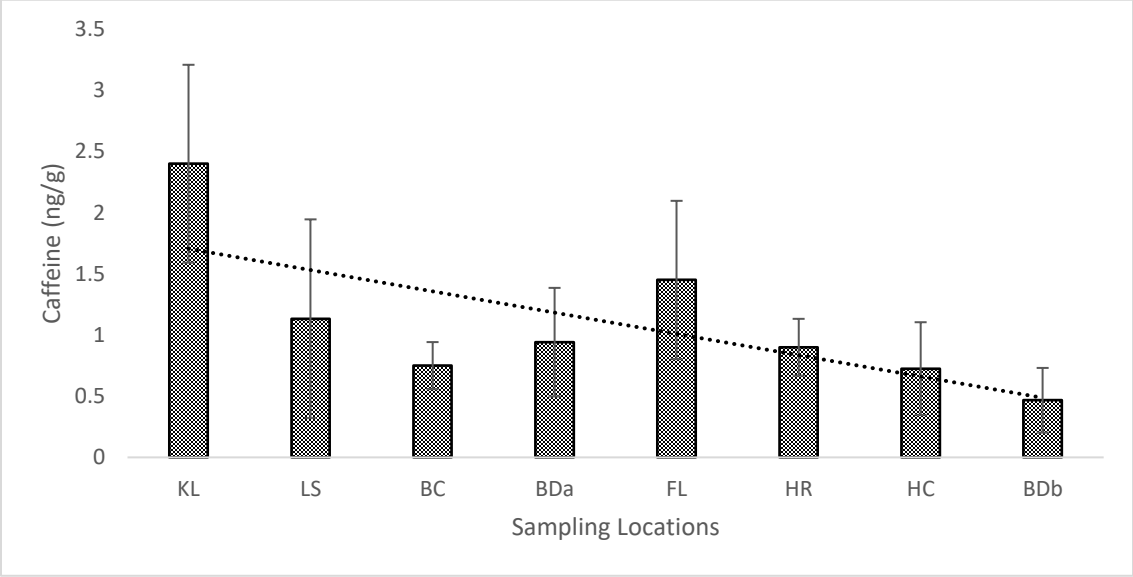

Figure 3.3-27: Mean caffeine concentrations in surface sediment (ng/g) samples recorded at different sampling locations ranked by the order of domestic land use (percentage) in corresponding subwatershed (least to greatest) with trendline. Error bars indicate standard error.

3.3.3 Trends Analysis

3.3.3.1 Seasonal trends of caffeine in relation to other parameters

A MANCOVA was used to determine relationships between seasonality and the parameters measured, while removing the covariate effects of sampling location. Data were normalized using the Box-Cox methodology as described in Tabachnick & Fidell (1996). This method uses both the Box-Cox transformation and log transformation to normalize the data. The response variables and covariate were also tested for homogeneity of variance. This was done using Levine's test for homogeneity. This indicates that the variables show homoscedasticity and should be further transformed or tested under non-parametric measures. The response variables and covariate were also tested for homogeneity of regression slopes, and there were no concerns.

Overall there were significant relationships between the sampling date (seasonality) and variables such as temperature, pH, DO, conductivity, TSS, bacterial counts, and caffeine concentration ($F_{14,78} = 9.377$, $p < 0.01$). In the analysis of between-subject effects, relationships were assessed among temperature, pH, DO, conductivity, TSS, bacterial parameters, and caffeine. Many of the recorded water parameters were significantly affected by seasons. This includes conductivity ($F_{22,73} = 2.524$, $p < 0.01$), TSS ($F_{22,73} = 3.288$, $p < 0.01$), and TP ($F_{22,73} = 3.494$, $p < 0.01$). Officially, no significance was found between seasons and DO ($p = 0.059$), pH ($p = 0.18$) or nitrate ($p = 0.223$).

Significant relationships were found among bacterial samples from water and seasonality. This includes HPC ($F_{22,73} = 32.298$, $p < 0.01$), TC ($F_{22,73} = 2.792$, $p < 0.01$), and *E. coli* ($F_{22,73} = 2.240$, $p < 0.05$). A significant relationship was also found between

seasons and bacterial counts in sediment samples. This includes HPC ($F_{22,73} = 2.618$, $p < 0.01$), TC ($F_{22,73} = 2.708$, $p < 0.01$), and *E. coli* ($F_{22,73} = 2.836$, $p < 0.01$).

In Leven's test, caffeine from water samples showed a significantly lower variance within data than caffeine from sediment samples ($p < 0.01$). The results of ANOVA showed caffeine from sediment samples were of significantly higher concentration than caffeine from water samples overall ($F_{1,190} = 31.177$, $p < 0.05$). Seasonality significantly affected the caffeine concentrations in sediment samples as it varied significantly with seasons ($F_{22,73} = 2.152$, $p < 0.05$). There was no significant relationship between seasonality and caffeine in water samples ($F_{22,73} = 1.758$, $p = 0.07$).

3.3.3.2 Land Use Trends

The eight different sampling locations and their respective sub-watersheds were assessed and ranked from lowest to highest degree of domestic land use (Table 3.2-1). The ranking of sampling locations was tested for its relationship to the monitored parameters using a step-wise multiple regression analysis. The resulting correlation matrix showed positive relationships between land use and two of the sampled water parameters: pH ($R^2 = 0.322$, $p < 0.01$) and nitrate ($R^2 = 0.332$, $p < 0.01$). Among the bacterial counts in water, a positive relationship was found between land use and *E. coli* ($R^2 = 0.308$, $p < 0.01$). However, in the sediment samples, a positive relationship was found between land use and HPC ($R^2 = 0.322$, $p < 0.01$) as well as TC ($R^2 = 0.264$, $p < 0.01$).

A multiple regression analysis using bacterial counts (HPC, TC, and *E. coli* from water and sediment) was significantly able to predict land use pattern ($F_{6,89} = 4.211$, $R^2 =$

0.221, $p < 0.01$). A second model that tested a combination of bacterial counts and caffeine concentration resulted in a slightly stronger relationship ($F_{8,87} = 3.861$, $R^2 = 0.262$, $p < 0.01$) than the bacterial counts alone. Using a step-wise multiple regression, starting with all parameters, it was determined that the combination of pH TP, HPC (water samples), TC (sediment samples), *E. coli* (water samples), and caffeine (water and sediment samples) was the most effective combination of parameters to predict land use in the study ($F_{7,95} = 8.096$, $R^2 = 0.397$, $p < 0.01$). The results indicated a significant predictability of land use from pH ($p < 0.05$), nitrate ($p < 0.01$), *E. coli* from water ($p < 0.01$) and TC from sediment ($p < 0.01$). Caffeine from sediment samples was not significantly associated with land use ($p = 0.086$) in the sites surveyed in this study.

3.3.3.3 Direct Comparisons of Select Locations

There was overall significance in an ANOVA comparing all sampling locations and caffeine samples from water, $F_{7,88} = 4.112$, $p < 0.01$. In a Dunnett T3 follow-up test, significance was found between locations HR and FL ($p < 0.05$), between LS and FL ($p < 0.05$), between BC and FL ($p < 0.05$), between FL and BDb ($p < 0.05$), and between FL and BDa ($p < 0.05$). No significant relationship was found between locations FL and KL ($p = 0.131$).

Overall Significance was observed in an ANOVA between all sampling locations and caffeine samples from water ($F_{7,88} = 4.219$, $p < 0.01$). A post-hoc Bonferroni test revealed significant relationships between locations KL and BDb ($p < 0.01$), as well as FL and BDb ($p < 0.01$). No significant relationships were found between locations HR and KL, HC and KL, or between LS and BDb ($p = 0.10$).

The ANOVA comparing all sampling locations with in terms of their level of caffeine in sediment samples was statistically significant ($F_{7,88} = 3.407$, $p < 0.01$) as was a comparison of sites in terms of TP ($F_{7,88} = 2.866$, $p < 0.01$). The ANOVA results comparing sampling locations in relation to nitrate levels was not statistically significant ($p = 0.06$).

3.4 Discussion

3.4.1 Seasonal comparisons

Various factors are known to influence the degradation or residence time of caffeine in water and sediment (Rapoula, 2003; Trovó et al., 2013, 2013; Vogels et al., 1976). Degradation of caffeine by light could be influenced by turbidity, water depth, presence of vegetation and many other variables in the water (Trovó et al., 2013).

Water temperature was highly variability over time, with mean temperatures ranging from 0.74° C (SE ± 0.61) in winter, to 26.65° C (SE ± 0.81) in summer. During the winter months, ice was present on all shorelines, and many sampling locations were completely covered with surface ice. The temperature fluctuations and freezing effects observed were consistent with previous reports addressing the local climate and freshwater systems in the area (Salonen et al., 2009; Winter et al., 2007). pH remained relatively consistent over the period of study, showing a slight decrease over the winter months. This finding is consistent with previously reports on the wastershed (Nicholls, 1995). Mean DO concentration was lowest in September and highest in April. The low dissolved oxygen in late summer is regularly seen in the Lake Simcoe Watershed and has been shown to have on-going negative impacts on aquatic environmental health (Nicholls, 1995, Winter et al., 2007). The high DO in spring may be a simple function of

the greater solubility of oxygen in colder, and aeration due to the mixing of layers in freshwater systems at this latitude (spring overturn) (Bengtsson, 1996; Salonen et al., 2009). TSS fluctuated over the year with no obvious seasonal pattern. Chapman et al. (2017) have suggested that high TSS may be observed as a direct response to high precipitation and increased water velocity in streams. They also suggested that high TSS levels may be an excellent indicator of short-term weather events and low TSS concentrations may be indicative of stable weather. Mean conductivity readings seem to show a bimodal pattern, with the highest peaks in January and August. Adams & Lasenby (1985) and Faithful (2016) have suggested that ice cover and low mixing conditions (during winter stratification) may be related to high conductivity levels, while spring seasonal dilution of dissolved ions in water (resulting in lower conductivity) can be attributed to snow-melt. Other reports suggested that high conductivity may be attributable to road salting in winter (Bant, 2009; Novotny et al., 2007). TP concentrations and nitrate concentrations followed similar patterns (to each other) but peaked in different months of the year. TP was highest in May (372.26 mg/L, SE ± 36.09) and nitrate was highest in August (1582.52 mg/L, SE ± 934.44). These data are consistent with earlier studies that show nutrient loading and processes associated with eutrophication most intense over the summer months (Evans et al., 2011; Nicholls, 1995; Winter et al., 2007).

During all sampling periods, water and sediment samples were assessed for an assortment of bacterial populations. HPC was generally higher than TC or *E. coli* counts, which has been seen also in other studies (WHO, 2003). HPC counts were present in all water and sediment samples, supporting the concept that heterotrophic bacteria are

common in most freshwater systems (WHO, 2003). TC were less abundant than HPC counts and showed a more seasonal pattern of abundance. TC counts was lowest in March for water samples, and lowest in January for sediment samples. *E. coli* densities followed similar trends to TC counts, an observation that has also been made in previous studies (Cohen et al., 1973; Ishii et al., 2006; Lin, 1974; Noble et al., 2010), with the lowest counts being recorded during winter. As mentioned earlier, in regard to the environmental persistence of *E. coli* and other enterococci, these mesophilic bacterial communities show peak growth at temperatures approximating those of the (endothermic) mammalian body, around 36°C, and they do not generally thrive under colder conditions (Ishii et al., 2006; Ishii et al., 2008). In surface sediment samples, *E. coli* densities were considerably lower than was expected. Unden et al (1994) have suggested that *E. coli* are facultative anaerobes, that is, bacteria that can use either aerobic or anaerobic respiration, utilizing electron acceptors other than oxygen (nitrate, nitrite, trimethylamine N-oxide, dimethyl sulfoxide, or fumarate) (Unden & Bongaerts, 1997). Among the bacterial populations studied in this study, *E. coli* has demonstrated the most specificity to human/animal digestive tracts and has been incorporated in local monitoring procedures tracking water quality and assessment for public health (SMDHU, 2016).

Over the course of this study, caffeine from water and sediment samples was monitored alongside other water parameters. Caffeine in water samples peaked in January and was generally found in higher concentrations during periods of freezing and surface ice. While it is unclear why the concentrations of caffeine were higher in winter months, it is possible that the normal degradation process may be impacted by low temperature

(Ahmad, 2014). Previous studies have suggested that sunlight may play a major role in caffeine degradation (Rapoula, 2003; Trovó et al., 2013). Clearly light intensity is lower in winter in the northern hemisphere, and the presence of ice cover would further reduce light intensity in the water column and the surface sediments below (Salonen et al., 2009). Other studies have shown that bacterial metabolism and chemical processes may play a role in caffeine degradation, and these in turn are also influenced by water temperature (Bradley et al., 2007; Dash et al., 2012; Yu et al., 2009). Caffeine in surface sediment samples followed a different pattern to that seen in water, peaking in March. Previous studies have shown that spring snow-melt has an effect on nutrient loading. Additionally, processes of inflow and infiltration of sanitary systems peak at this time of the year (EPA, 2014). Thus, the factors outlined above, that might prompt bypassing of WWTPs, may be acting as strong factors affecting caffeine levels in surface sediment in spring.

3.4.2 Comparing Sampling Locations

Sampling locations were selected based on the distinctive differences among them. The primary objective of this component was to explore how caffeine and other parameters might differ among sampling locations that are differentially influenced by anthropogenic waste.

Mean water temperatures were lowest at BDa, and highest at BDb. Ben's ditch is fed by Mill Creek of the Oro North Sub-watershed, which is relatively cold, and spring-fed as it passes through Scout's Valley, Orillia (LSRCA, 2013a). The warming effect seen at the downstream site, BDb, could be partially caused by a change in flow rate, velocity, the absence of a riparian zone, and contributions of stormwater and treated

wastewater effluent (City of Orillia, 2017; LSRCA, 2013; LSEMS, 2003). The pH was lowest at sampling site BDa and highest at BDb. pH levels at BDb were consistent with the data from the City of Orillia. The City of Orillia WWTP works within the guidelines of their Environmental Compliance Approval (ECA) certification, which states that the effluent must, at all times, have a pH in the range of 6.0-9.5 (City of Orillia, 2017). Mean DO concentration was also highest at sampling location BDa and lowest at BDb. In other studies, factors such as high biological/chemical oxygen demand and warmer temperatures have been shown to be highly associated with low DO concentrations (Jianlong et al., 2004; Tadesse et al, 2004). TSS concentrations were generally lower at sampling locations that were along lake shorelines (LS, KL, FL) than they were in creeks or the river. Earlier studies have suggested that TSS can be related to nutrient loading, turbidity, and fast moving water; these are all factors more closely associated with (lotic, or flowing) river systems than with the (more lentic) lakes (Chapman et al., 2017; Nasrabadi et al, 2016). Conductivity was generally higher at sampling location BDb than BDa, which suggests that the stormwater drainage and WWTP discharge are contributing ions that increase the conductivity of the water.

Discharges from WWTPs generally have high heavy metal content, and this would be reflected in elevated conductivity values (Azizi et al., 2016). As mentioned above, road salt has been shown to influence conductivity values in the area (LSRCA, 2012). Water from sampling location FL had consistently higher conductivity values than those from KL, which may also illustrate the effects of sewage and urban land use on conductivity in freshwater systems. Total Phosphate (TP) and nitrate were very variable

among sampling locations, but mean concentrations were relatively consistent across sampling locations. Samples from creeks and rivers had higher concentrations of nitrate; this is consistent with other reports on the level of nutrient loading that is occurring in the Lake Simcoe watershed (Correll, 1998; Heisler et al., 2008; Romshoo, 2011).

Heterotrophic plate counts cover a wide range of bacteria and are considered a suitable general representation of the bacterial community as a whole (WHO, 2003; Staff et al., 2003). Total Coliforms (TC) and *E. coli* counts in water samples followed similar trends, but *E. coli* counts were generally lower than the TC counts, suggesting that other coliform organisms were present in the water system (Dufour, 1977; Jin et al., 2004; Noble et al., 2003).

In sediment samples, HPC remained more abundant than the other bacterial populations studied, but the data appeared to be more variable among sampling locations. For example, sampling locations LS and HC had extremely low counts of *E. coli* in sediment. Other sites appeared to show a greater difference between TC counts and *E. coli* counts suggesting that many other coliform bacteria besides *E. coli* were present in the sediment (Dufour, 1977; Jin et al., 2004; R. Noble et al., 2003).

Caffeine in water samples showed relatively high variability among sampling locations. Sampling location KL showed relatively higher caffeine concentrations than anticipated while sampling location HR showed considerably lower caffeine concentrations than anticipated. Previous studies had shown similar results and have suggested that possible explanations for such variation would include biodegradation, photo-degradation, and dilution factors that may vary from one to other location (Ashton et al., 2004; Rapoula, 2003). Although wastewater treatment facilities do not directly

address all organic compounds, caffeine may, to some extent, be incidentally removed by physical, chemical and bacterial processes that occur during water treatment (Baalbaki et al., 2016; Thomas et al., 2005).

Caffeine concentrations from sediment samples did not closely parallel the corresponding concentrations of caffeine in water samples. Sampling locations KL and FL had the highest concentrations of caffeine in sediment while sampling location BDb, downstream of a wastewater effluent discharge, had the lowest. While these results were not what had been hypothesized earlier, several factors may account for these differences. A previous study suggested that the bacterial community present in the surface sediment may result in substantial biodegradation of caffeine, but that the process is highly dependent on the redox status of that sediment (Bradley et al., 2007). These authors suggested that factors such as sediment composition can have a major influence on the diffusion of oxygen and composition of bacterial communities, which in turn would impact caffeine concentrations over time. For future studies on sedimentary caffeine as a tracer for domestic waste, it is recommended that estimates of redox potential and sediment composition be incorporated in the experimental design, due to their potential impact on caffeine levels in the sediment. It would also be beneficial to include sedimentary analysis, including total organic compounds and other factors influencing the absorption of caffeine as well as degradation (Bruton et al., 2010).

In the present study, significant variation in caffeine concentrations with season was observed. Caffeine concentrations increased in winter, particularly at sites with surface ice cover. It is possible that processes such as lower temperatures and light intensity due to ice cover may have reduced the degradation of caffeine (Rapoula, 2003;

Trovó et al., 2013). In addition, the dimictic pattern of overturn typical of freshwater lakes in a temperate area like this may also have influenced the caffeine concentration. With the exception of groundwater flow, the overall mixing of water in a freshwater system is significantly reduced during periods of freezing and sub-zero conditions (Bengtsson, 1996; Salonen et al., 2009). The increased caffeine concentrations in winter contrast with the lower bacterial counts at that time. As suggested by Jones et al (1987) *E. coli* and other mesothermic coliforms that thrive at 37°C, but have limited tolerance to hypothermic conditions, undergo cold shock and therefore slow reduced growth below 15°C. The results of the statistical analysis are in support of the hypothesis that caffeine fluctuates with season.

Over the course of one year, caffeine concentrations fluctuated greatly. It was expected that caffeine concentrations in sediment would be more consistent over time than the corresponding concentrations in water. However, this was not the case. Caffeine concentrations in sediment showed significantly higher variance than caffeine concentrations in water ($p < 0.01$. The literature suggests that the processes of caffeine degradation will be less effective in sediment (Bradley et al., 2007). It is possible that the variance observed in sediment could be due to the sampling procedure, given that samples were collected from the top three centimeters of sediment, a region that is prone to disturbance by, and exchange with, the overlying water column. The present study suggests that caffeine in sediment is often more concentrated than it is in the overlying water. It also shows that caffeine concentrations in sediment show significantly higher variance than those in the water column. Thus, the hypothesis that assessments of

caffeine in sediment would provide a more reliable measure of domestic waste inputs than caffeine in water samples was not supported by the results of this study.

3.4.2.1 Domestic Land Usage Trends

The model created to predict domestic land-use using bacterial indicators gave a significant R^2 value ($F_{6,89} = 4.211$, $R^2 = 0.221$, $p < 0.01$), but note that the relatively low R^2 value means that bacterial indicators were therefore explaining only about 22% of the variance. The addition of caffeine data to this model raised the R^2 value to 26% ($F_{8,87}=3.861$, $R^2 = 0.262$, $p < 0.01$). This suggests that inclusion of data on caffeine levels improves the predictive power of bacterial assessments in terms of predicting the relative impact of domestic land use.

The result of the backward selection multiple regression helped to generate a list of eight predictor variables that produce a strong correlation with land-use pattern, accounting for nearly 40% of the variation ($F_{7,95} = 8.096$, $R^2 = 0.397$, $p < 0.01$. However, individually, no single predictor variable was significant ($p > 0.05$) as a predictor of the land-use pattern. The data thus suggest that a combination of variables is needed to produce a reasonably good predictor of land-use pattern in a freshwater ecosystem, and we cannot turn to caffeine as the sole factor used to assess the relative impact of domestic land use in a watershed.

In some instances, the data from the sediment samples gave a more accurate prediction of the land use pattern. This may be due to the stable, more long-term persistence of tracer compounds in sediment compared to surface waters (Ahmad, 2014; Bradley et al., 2007; Dash et al., 2012; Rapoula, 2003).

This aspect of the study was designed to determine whether combining microbial source tracking with caffeine samples would more accurately identify domestic sources of nutrient loading than would bacterial indicators alone. Bacterial counts were significant predictors of domestic land use at the sampling locations but caffeine alone was not statistically significant ($F_{2,93} = 2.762$, $R^2 = 0.056$, $p = 0.068$). However, the combination of bacterial and caffeine tracking yielded a stronger correlation and better overall predictability ($F_{8,87} = 3.861$, $R^2 = 0.262$, $p < 0.01$). These finding agree with earlier reports that support the use of caffeine as well as bacterial assessments as a tracer molecule for domestic waste (Buerge et al., 2003; Edwards et al., 2015; Gardinali et al., 2002; Kurissery et al., 2012; Linden et al., 2015; Peeler et al., 2006; Siegener et al., 2002b; Thomas et al., 2005)

3.4.2.2 Comparisons of Selected Sampling Sites

Sites KL and FL are interesting to compare; KL is located in Awenda Provincial Park and the Kettle's Lake sub-watershed, an area with some recreational camping activities, but that otherwise has only limited public access. It was expected that this site would have much lower caffeine concentrations overall, compared to Farlain lake, which has higher recreational activities and areas of domestic land use adjacent to its shoreline. This hypothesis was not supported, in that caffeine concentrations were significantly lower in KL than in FL (see Figure 3-26 and 3-27, ($p = 0.131$)).

Previous studies had suggested that *E. coli* counts would be greater in Farlain Lake than in Kettle's Lake (MOE, 1973; SSEA, 2016). However, the present study did not support this. Sediment samples from FL had marginally higher *E. coli* counts than those from KL, but the reverse was true for counts in the water samples. The difference

between the *E. coli* counts in water from KL and FL was not statistically significant ($p > 0.05$).

In another comparison, sites BDa and BDb were selected to quantify the changes in water parameters attributable to inputs from the Orillia WWTP in terms of its effluent discharge and its effect on caffeine concentration. Contrary to expectations, sites BDa and BDb did not show any significant differences in caffeine, *E. coli,* or nutrient concentrations. Additionally, it is possible that WWTPs, although not specifically engineered to remove caffeine, have been able to significantly reduce concentrations of caffeine as wastewater goes through their primary, secondary and, if applicable, tertiary treatment processes (Dash et al., 2012; Rapoula, 2003; Trovó et al., 2013).

3.5 Chapter Conclusions

Seasonal changes in Lake Simcoe, Kettles Lake and Farlain Lake watersheds were investigated, and significant fluctuations in caffeine and bacterial levels were observed over the study period. The general trend observed in the study was that *E. coli* and other bacteria were greatly reduced in abundance in the cold season compared to other times of year. Meantime, in winter, higher concentrations of caffeine were observed in water samples. The elevated presence of caffeine during periods when bacteria are reduced suggests that it may be helpful to substitute chemical tracking for microbial source tracking during the winter months in these freshwater systems.

It was proposed earlier that caffeine concentrations in sediment samples might be more useful and consistent than those in water. The variance among replicate (triplicate) sediment samples was high, which reduced the precision of these estimates. It was also noted that caffeine concentrations were significantly higher in sediment, suggesting that

if samples could be collected with lower variance, sediment collection for caffeine could potentially be more useful for the detection and prediction of domestic waste inputs in future studies.

Microbial source tracking is well proven as an effective tool to identify nutrient loading in natural freshwater systems (Glassmeyer et al., 2005; Ishii et al., 2006; Peeler et al., 2006). The consistent presence of *E. coli* in environmental samples has been demonstrated, but it had not always been an effective tool for differentiating domestic and agricultural waste. In the present study, the addition of caffeine monitoring to microbial tracking improves the accuracy of the model that predicts the presence of domestic waste in the water, and the addition of more chemical and physical parameters for the samples significantly increased the accuracy of the model.

Chapter 4: Laboratory Procedures

4.1 Introduction

Excessive nutrient loading can have devastating ecological impacts on freshwater systems (Evans et al., 2011; Winter et al., 2007). Also, several human health hazards can be attributed to source loading and an increase in phosphorous in inland waters. For example, higher nutrient loadings may result in eutrophication and toxic algal blooms which can cause many concerns in terms of water quality and the ecological community in freshwater systems (Correll, 1998; Heisler et al., 2008). Source loading can also bring pathogens from agricultural and domestic waste, introducing virulent microorganisms to water bodies used for recreational and drinking water (Pandey et al., 2014).

Understanding the nature of nutrient loading, particularly, at the source or confluence of freshwater systems can be helpful in identifying and remediating the source and therefore protecting the receiving water body (Romshoo, 2011). Bacterial indicators of nutrient loading have been used in the fields of public health and environmental monitoring for years to identify contamination sources (Edberg et al., 2000; Ishii et al., 2008). Microbial source tracking (MST) includes tracking of broader group of microorganisms such as heterotrophic bacteria and total coliform bacteria (Edberg et al., 2000). However, since the 1990s, *Escherichia coli* (*E. coli*) has been recognized as the ideal tracer of fecal waste (and potentially the associated human pathogens) based on its abundance in the gastrointestinal tracts of endotherms and its environmental persistence (Ishii et al., 2008).

While microbial source tracking can be effective in identifying fecal contamination, there are a number of concerns raised to its scope of application and efficacy over time (Ravaliya et al., 2014). Some of the major ones are: 1. While *E. coli* can display environmental persistence, there have been studies demonstrating the potential naturalization of these bacteria when conditions are favorable for survival thereby making it difficult to differentiate the naturalized ones from those arriving as new contaminants (Ishii et al., 2006). 2. While there are techniques for differentiating domestic and animal waste such as the ratio of fecal coliform to fecal streptococci (FC:FS), there is uncertainty as to their accuracy as well because these microorganisms have different biological requirements and rates of survival under environmental conditions (Geldreich et al., 1969; Wang et al, 2004). As a result, the World Health

Organization (WHO) does not recommended this method for the assessment under most circumstances (Fewtrell et al., 2001; Howell et al., 1995).

New techniques for differentiating animal (agricultural) waste from domestic have been emerging in recent time around the world, most prominently, the utilization of caffeine as an indicator (Kurissery et al., 2012; Peeler et al., 2006; Thomas et al., 2005; Verenitch et al., 2008).

4.1.1 Overview of objectives

While several studies have been conducted to investigate the possible use of caffeine to identify domestic waste, its usefulness is not entirely clear. Since only a limited number of studies have focused on chemical source tracking using caffeine, this method has not yet been adopted as standard practice. Thus, it is very important to provide more evidence of the potential use of caffeine as a tracking tool of domestic waste in aquatic ecosystems.

A major objective of the present study is to quantify the relationship between caffeine and domestic waste in a controlled environment. With a limited number of field samples, it is difficult to clearly compare microbial source tracking and caffeine analysis and their possible correlations with human and animal fecal waste based on field samples alone. Also, many additional environmental factors (including some that may not have been measured) may be affecting observations in the field. We can more readily control environmental variables under laboratory conditions, so alternative sources of variance may be limited (Krajewski, 1990); also higher levels of replication can give a more precise assessment of any correlations.

Another objective of this laboratory-based study was to determine if caffeine analysis provides a more long-term indicator of human fecal waste in freshwater habitats than microbial source tracking. Previous studies have reported that the efficacy of microbial source tracking falls off rapidly due to high mortality rates under sub-optimal conditions, while others suggest that caffeine is relatively stable due to slow degradation and therefore is a more dependable tracer for the detection of domestic waste in an aquatic environment (Baalbaki et al., 2016; Rapoula, 2003; Thomas et al., 2005; Trovó et al., 2013).

4.2 Materials and Methods

4.2.1 Experimental Design

This experiment was designed to assess the value of caffeine as an indicator to differentiate domestic waste from agricultural waste under controlled environmental conditions. Under controlled laboratory conditions, a series of flasks containing mixtures of agricultural and domestic wastes (from stock solutions prepared from these wastes) were sampled and monitored over the course of one week. Erlenmeyer flasks (500 mL) were filled with 200 mL of sterile deionized (DI) water and 100 mL of stock solution (agricultural stock solution, and domestic stock solution are described in section 4.2.2). Of the 100 mL stock solutions added to each flask, different ratios of agricultural and domestic stock were blended to represent different nutrient loading events. The first sets of flasks contained 100 mL of agricultural stock to represent an exclusively agricultural nutrient loading and 1:0 ratio of agricultural to domestic stock. The second set of flasks contained 80 mL of agricultural stock and 20 mL of domestic stock to represent a mixed source nutrient load with most being agricultural waste and a

ratio of 4:1 (agricultural waste to domestic waste). The third set of flasks contained 60 mL of agricultural stock and 40 mL of domestic stock to represent a mixed nutrient load with slightly more agricultural input than domestic input and a 6:4 ratio (agricultural waste to domestic waste). The fourth set of flasks contained 40 mL of agricultural stock and 60 mL of domestic stock to represent a mixed nutrient load with slightly more domestic waste than agricultural waste, and a ratio of 4:6 (agricultural waste to domestic waste). The fifth set of flasks contained 20 mL of agricultural stock and 80 mL of domestic stock to represent a mixed nutrient load consisting of mostly domestic waste and a 1:4 ratio (agricultural waste to domestic waste). The final set of flasks contained 100 mL of domestic stock and were intended to represent a nutrient load comprising only domestic waste (with no agricultural waste influence) with a 0:1 ratio (agricultural waste to domestic waste). As a control, flasks were set up with 300 mL of DI water. To reduce the number of external variables influencing the study, nutrients (nitrate and total phosphate) were supplemented to achieve approximately the same nutrient concentrations in all flasks and series of the experiment. To achieve this, all flasks were spiked with a micro-pipette using 4427 μg/mL nitrate standard solution and 20 μg/mL phosphorous standard solution (80 mg/L ± SE 1.97 of nitrate and 95 mg/L ± 3.62 of total phosphorous).

The inoculated Erlenmeyer flasks were sealed with sterile cotton and cheesecloth to allow the diffusion of oxygen. Flasks were swirled and agitated gently each day for 1 min to facilitate oxygen diffusion. The flasks were stored in a transparent container on a ledge in the laboratory window where moderate light intensity was available. The sample flasks were rotated three times daily to allow for a more even

exposure to light. The pH of all flasks was checked daily to ensure it remained at 7.5 ±0.5 SE throughout the study. The water temperature was checked daily to ensure it was at 21°C± 1 SE throughout the study.

A total of 63 flasks (each 300 mL) were used in this study which ran from January 18, 2017 to January 25, 2017. At the outset, 9 replicates of each combination, including the control (a total of seven treatments) were set up, but three replicates of each treatment were "harvested" on each of three occasions (at the beginning of study, 48 hours into the study, and after one week, that is, at 0, 2, and 7 days, respectively). On each occasion, levels of caffeine, nutrients (nitrate and total phosphorus), and bacterial composition were recorded.

Based on the objectives stated previously, a series of hypotheses were developed:

H_1: Caffeine concentrations provide a more accurate prediction of domestic waste content than do bacterial counts.

H_2: The combination of caffeine concentrations and bacterial counts will be more accurate at distinguishing domestic waste from agricultural waste than either marker alone.

H_3: Caffeine concentrations will degrade at a slower rate than bacterial counts so caffeine is a better "long-term" indicator of contaminant sources than are the bacterial counts.

4.2.2 Stock Solutions

Stock solutions were produced to represent purely domestic, and purely agricultural fecal contamination in a freshwater ecosystem. Various proportions of each

of these solutions were then used to inoculate the subsequent experimental treatments in this experiment.

To represent agricultural discharge, fresh manure samples were collected from J&K Farms Limited., Springwater Township, ON; five manure samples were collected from Charolais and Limousin mixed-breed beef cattle. Samples were collected with a sterile scoopula from three locations on each of three manure piles and stored in sterile 200 cc plastic bottles in a cooler with ice for transport.

In the laboratory, 1 g of each manure sample (total of 5 g) was weighed on an analytical scale and diluted in an Erlenmeyer flask with 1 L of sterile deionized (DI) water. The samples were mixed vigorously for 30 mins on a mixer and then vacuum filtered through Whatman 0.7 µm pore, 47 mm microfiber membrane filters. Samples were collected, the parent stock solution was produced, and experimental samples were inoculated in the same 12-hour period. The stock solution was intended to represent cattle manure runoff as would be found in nutrient loading events.

On the same day, domestic sewage was collected from the Orillia Waste Water Treatment Plant (WWTP). The 1 L grab samples were collected in triplicate from the main sewer trunk that services the City of Orillia drainage area. Samples were collected where the water discharges from the sewer and enters the primary treatment facility. The samples were collected with a sterile 1 L plastic bottle fastened to an extension pole. Samples were stored in a cooler with ice for transport. In the laboratory, the sample was left undisturbed for 1 hour, allowing sedimentation to occur. The samples were vacuum filtered through Whatman 0.7 µm pore, 47mm microfiber membrane filters to remove particulate matter. Samples were collected, the parent stock solution was produced, and

experimental samples were inoculated in the same 12-hour period. The stock solution was intended to represent raw sewage runoff as it is found in nutrient loading events, and to provide this material at concentrations comparable levels in other research studies (Li et al, 2014; Vadas et al., 2009; Wang et al., 2004).

4.2.3 Water Parameters

At each sampling interval (0, 2, and 7 days) a series of flasks (3 replicates of the nine flasks representing each treatment combination) were removed and analyzed in terms of the following parameters. From each flask, 100 mL was removed with a sterile pipette for the nutrient and bacterial analyses. The remaining 200 mL was used for caffeine analysis. More details on the methods used to analyze water quality parameters, bacterial counts and caffeine analysis are provided in the general methodology chapter (Chapter 2). The tests on each flask therefore included total phosphorous, nitrate, heterotrophic plate counts, total coliform, fecal coliform and *E. coli* enumeration, as well as caffeine concentration.

4.2.4 Statistical analysis

A series of multiple regressions were used to compare the data in a blocked and step-wise pattern. The data were subjected to tests of assumptions associated with multiple regression to ensure normal distribution, linear relationship and homoscedasticity (Osborne et al., 2002). Initially, the variables used for modelling to predict the source of waste materials in the water were: heterotrophic plate counts (HPC), total coliforms (TC), fecal coliforms (FC) and *E. coli* (EC). As the second step, in addition to the above parameters, caffeine concentrations were added to the model. In the third step, nutrient concentrations were also added to the model. Finally, a reverse

stepwise multiple regression was used to determine the most effective combination of variables to predict domestic waste concentrations. Since the flasks contained various proportions of both agricultural and domestic waste, a major objective of this experiment was to test the power of the model to monitor the relative concentrations of domestic waste and agricultural waste.

4.3 Results

4.3.1 Predictors of Domestic Waste Concentrations

4.3.1.1 Water Quality Parameters

Nutrient concentrations decreased over the week of the study, causing an increase in variance over time. By the end of the week, the mean nitrate concentration was 54.09 mg/L ± 3.55 SE and TP was 70.22 mg/L ± 3.81 SE (Figure 4-1).

The mean HPC counts did not appear to fluctuate greatly across all waste mixtures. The lowest mean HPC count was observed in 1:0 (agricultural to domestic waste ratio) flasks (5.62 cfu/100 mL Log^{10}, SE ± 0.25) while the highest count was in the blend of 3:2 (agricultural: domestic) flasks (5.97 cfu/100 mL Log^{10}, SE ± 0.08). The lowest mean TC count was observed in 1:4 flasks (4.32 cfu/100 mL Log^{10}, SE ± 0.62) while the highest count was in 1:0 flasks (4.76 cfu/100 mL Log^{10}, SE ± 0.43). For FC, similar results to those for the TC counts were observed, with the lowest mean count in 0:1 flasks (3.59 cfu/100 mL Log^{10}, SE ± 0.79) and the highest count in 1:0 flasks (4.48 cfu/100 mL Log^{10}, SE ± 0.48). The lowest mean *E. coli* count was observed in 1:4 flasks (3.65 cfu/100 mL Log^{10}, SE ± 0.74) and the highest count was in 1:0 flasks (4.53 cfu/100

mL Log^{10}, SE ± 0.54) (Figure 4-2). So, overall, none of the microbial counts allowed the different blends of agricultural and domestic wastes to be distinguished.

Caffeine concentrations were relatively low in 1:0 (agricultural: domestic waste) flasks (26.89 ng/L, SE ± 1.57). Caffeine concentrations show a positive relationship with domestic waste content and was highest in flasks with a 1:4 (agricultural: domestic) waste blend (5003.45 ng/L, SE ± 20.41) (Figure 4-3).

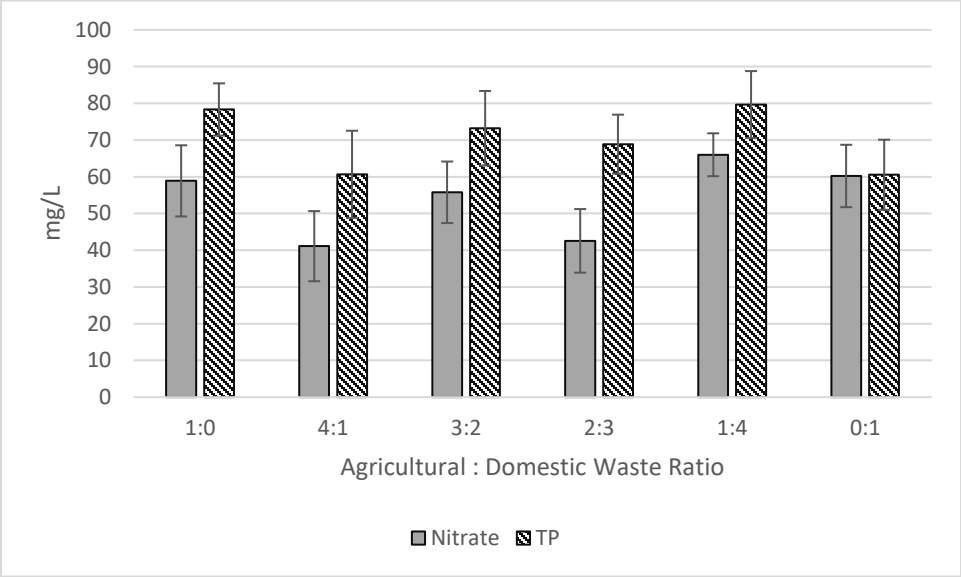

Figure 4-1: Mean nitrate and total phosphorous concentrations (mg/L) from sample groups with varying agricultural and domestic stock concentrations from 1:0 to 0:1 (10A, 8A2B, 6A4B, 4A6B, 2A8B, 10B respectively). Error bars show standard error.

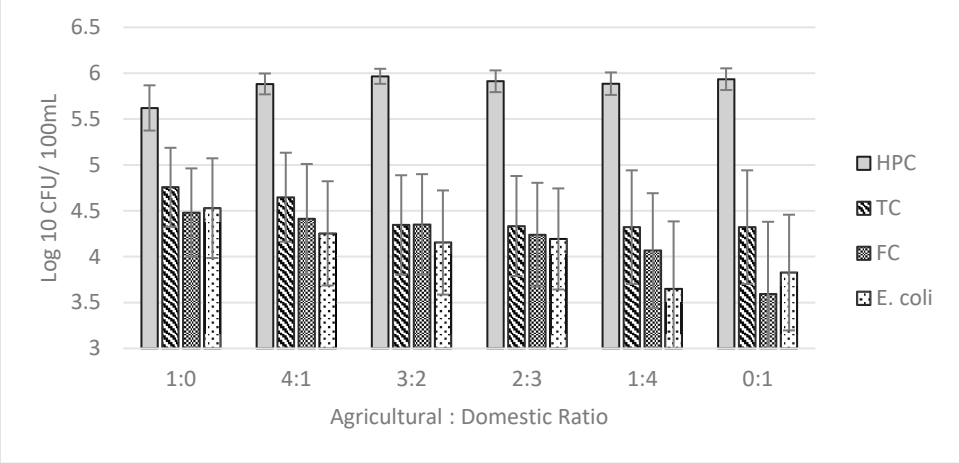

Figure 4-2: Mean HPC, E. coli, TC, FC bacterial populations (CFU/100 mL [Log10]) from sample groups with varying agricultural and domestic stock concentrations from 1:0 to 0:1 (10A, 8A2B, 6A4B, 4A6B, 2A8B, 10B respectively). Error bars show standard error.

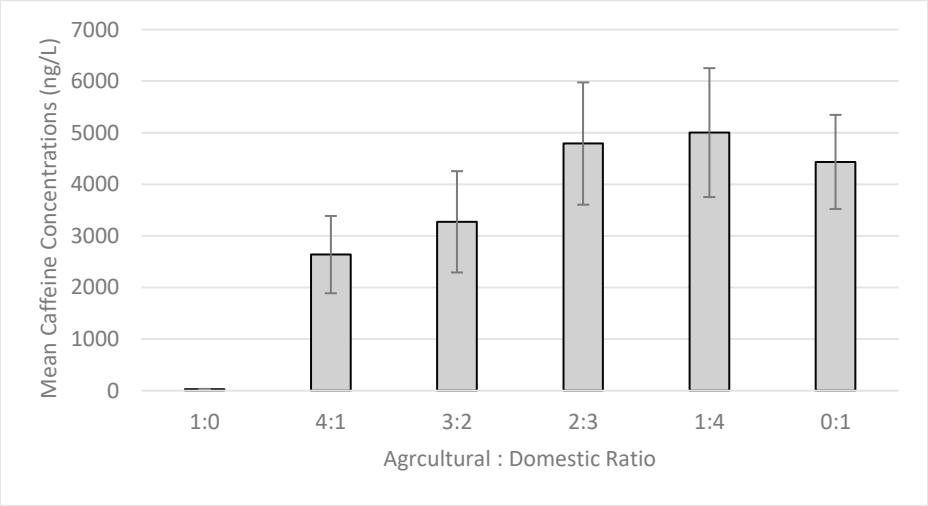

Figure 4-3: Mean caffeine concentrations (ng/ L) from sample groups with varying agricultural and domestic stock concentrations from 1:0 to 0:1 (10A, 8A2B, 6A4B, 4A6B, 2A8B, 10B respectively). Error bars show standard error.

4.3.1.2 Trend Analysis

A Pearson correlation matrix showed a strong relationship between caffeine concentration and domestic waste concentration ($R^2 = 0.612$, $p < 0.01$. Other parameters TP ($R^2 = 0.041$, $p = 0.38$), nitrate ($R^2 = 0.127$, $p = 0.18$), HPC ($R^2 = 0.169$, $p = 0.11$), TC ($R^2 = 0.089$, $p = 0.26$), FC ($R^2 = 0.156$, $p = 0.13$), and *E. coli* ($R^2 = 0.149$, $p = 0.14$) were not significantly correlated with the proportion of domestic waste.

A model was constructed using the various bacterial counts as predictors of domestic waste concentrations, but no significant relationship was found ($p = 0.12$). However, when caffeine was added to the model, there was a significant improvement in the model's fit ($R^2 = 0.46$, $F_{5, 48} = 8.172$, $p < 0.01$ in terms of predicting the relative contribution of domestic waste in a variety of stock solution blends designed to simulate various mixed nutrient loads.

4.3.2 Reliability of indicators over time

4.3.2.1 Water Quality Parameters

Nutrient concentrations decreased, and increased in variability over the week of the study. Mean nitrate concentration began at 77.94 mg/L, SE ± 2.37, but after the first 48 hours they had dropped to 43.22 mg/L, SE ± 5.46, and by the seventh day, average nitrate concentrations were 41.11, SE ± 5.64 (Figure 4-4). Total Phosphorus followed similar patterns with the highest mean concentrations at the beginning of study (98.67 mg/L, SE ± 1.78). After 48 hours, TP concentrations had decreased to 60.11 mg/L, SE ± 5.25, and after one week they had dropped somewhat to 51.89 mg/L, SE ± 5.62 (Figure 4-4). It appears that nutrient consumption is most rapid at the beginning of the one-week study, slowing down after the first two days.

The cell counts for HPC changed the least over the week, starting from the initial mean count of 6.22 cfu/100 mL Log^{10} (SE ± 0.04). After 48 hours, we saw 5.59 cfu/100 mL Log^{10}, SE ± 0.13, and after one week, the mean HPC changed very slightly, increasing to 5.79 cfu/100 mL Log^{10}, SE ± 0.06 (Figure 4-5). The values for Total Coliforms changed more over the course of the study, starting at 6.51 cfu/100 mL Log^{10}, SE ± 0.03, and after 48 hours dropping to 3.94 cfu/100 mL Log^{10}, SE ± 0.09. After one

week, they had dropped further to 2.95 cfu/100 mL Log^{10}, SE ± 0.10. The values for Fecal Coliforms followed a similar pattern with the highest mean counts at the beginning of the study (6.43 cfu/100 mL Log^{10}, SE ± 0.03), a drop after 48 hours (3.65 cfu/100 mL Log^{10}, SE ± 0.16), and a further decrease after one week (2.49 cfu/100 mL Log^{10}, SE ± 0.16) (Figure 4-5). *Escherichia coli*, at the outset, showed the lowest mean counts of all bacterial forms assessed (6.34 cfu/100 mL Log^{10}, SE ± 0.03). This dropped further after 48 hours (3.54 cfu/100 mL Log^{10}, SE ± 0.14) and after one week went down to (2.42 cfu/100 mL Log^{10}, SE ± 0.17) (Figure 4-5).

Caffeine concentrations decreased by an order of magnitude over the one-week study. The initial mean concentration was 5102 ng/L, SE ± 838.37. After 48 hours, the mean concentrations had dropped a little, but not significantly, to 4344.70 ng/L, SE ± 617.25, and after one week they had dropped further to 636 ng/L, SE ± 112.45 (Figure 4-6).

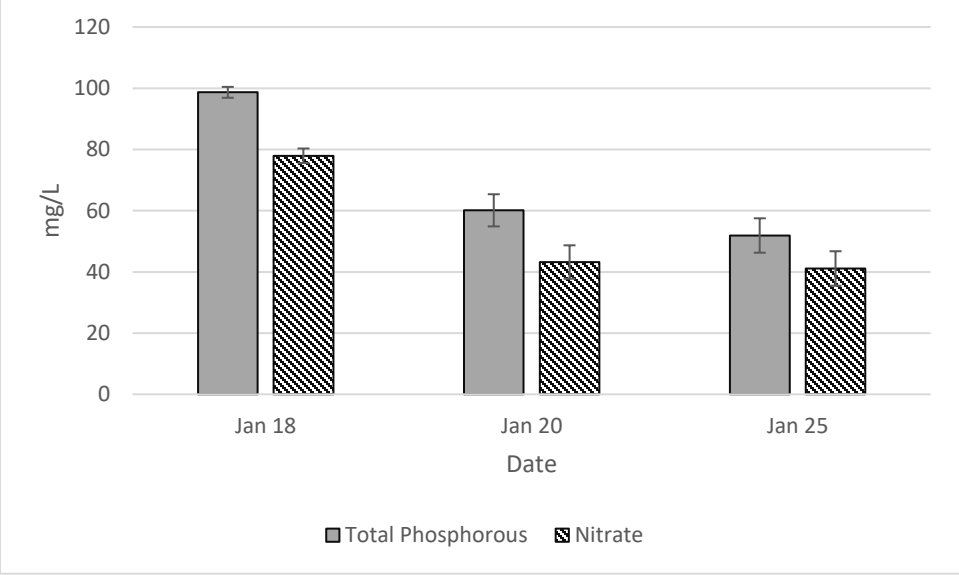

Figure 4-4: Mean nitrate and TP concentrations (mg/ L) over the period of study. Error bars show standard error.

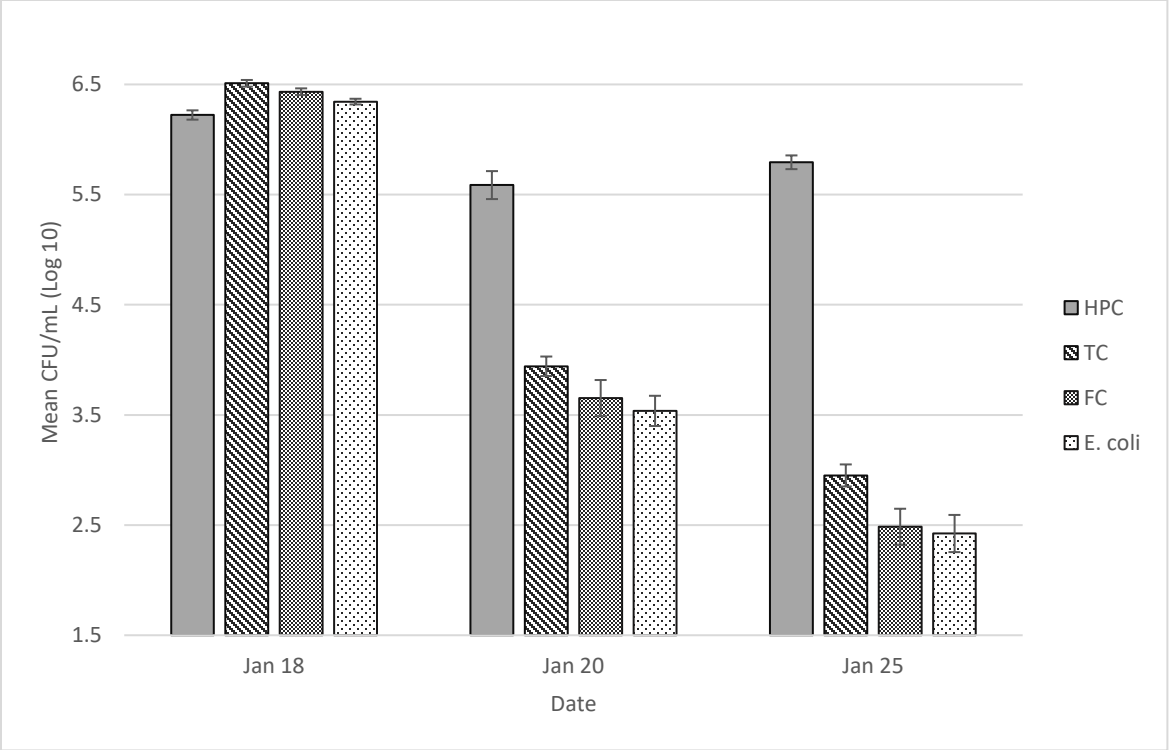

Figure 4-5: Mean HPC, TC, FC, and E. coli concentrations (CFU/100 mL [Log10]) over the period of study. Error bars show standard error.

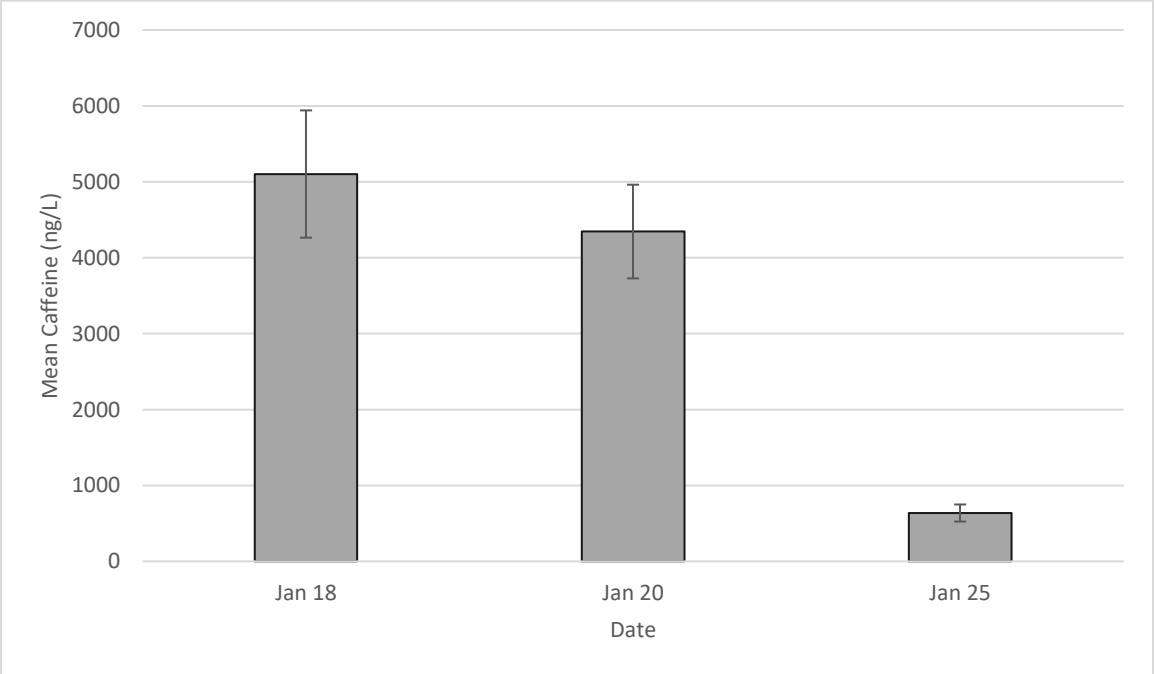

Figure 4-6: Mean caffeine concentrations (ng/L) over the period of study. Error bars show standard error.

4.3.2.2 Trend Analysis

All parameters tested exhibited a negative relationship with time, that is, they decreased over the week of the study. In a correlation matrix, the variables: *E. coli* counts ($R^2 = 0.843$, $p < 0.01$, Fecal Coliform counts ($R^2 = 0.844$, $p < 0.01$), and Total Coliform counts ($R^2 = 0.861$, $p < 0.01$ showed the strongest negative relationships with time. The variables; nitrate ($R^2 = 0.533$, $p < 0.01$ and Total Phosphorus ($R^2 = 0.624$, $p < 0.01$ showed moderate negative relationships with time. Caffeine concentrations ($R^2 = 0.280$, $p < 0.01$ and HPC ($R^2 = 0.278$, $p < 0.01$ showed the weakest negative relationships with time.

A multiple regression showed that bacterial indicators drop off significantly in density over the one-week period of study, and that their numbers were good predictors of the time elapsed since the initial inoculation of the wastewater ($R^2 = 0.773$, $F_{4, 49} = 41.664$, $p < 0.01$. The addition of caffeine to the model, although it, alone, gave a weak correlation, produced a significant improvement in the R^2 value ($R^2 = 0.824$, $F_{5, 48} = 45.073$, $p < 0.01$. The addition of nutrient data did not significantly improve the model or resulted in a significant change in the R^2 value.

4.4 Discussion

4.4.1 Predictors of Domestic Waste Concentrations

Conventional methods used in microbial source tracking (MST) have become a proven and widely accepted means to identify nutrient loading from human and animal fecal contamination (Dufour and W.H.O, 2012; Glassmeyer et al., 2005). However, in most environmental monitoring studies, MST does not seem to be very useful for differentiating human and animal fecal contamination (Howell et al., 1995). In this chapter of the study, simulated water samples containing known blends of agricultural and domestic wastes, were assessed using a variety of potential "candidate" indicators. Bacterial communities were assessed at different "levels" of discrimination, from broad-spectrum tests intended to identify all heterotrophic bacteria (HPC), to all *coliforms* which are the array of bacteria that are found in soil as well as in animal guts (TC), to those coliforms that are an indicator of the presence of wastes from animal intestinal tracts (FC), to the particular species, *E. coli*, a species which originates in the intestines of endotherms (Cohen et al., 1973; Ishii et al., 2008).

As expected, the more specific indicators, TC, FC, and *E. coli* were present at lower counts than HPC, as these tests are more selective indicators (Allen et al., 2004; Jin et al., 2004). The TC tally is inclusive of all coliforms, bacteria capable of fermenting lactose at 35-37°C but not necessarily of fecal origin; these showed higher counts than FC and *E. coli* in domestic, mixed, and agricultural waste. This makes sense since the FC and *E. coli* tests are specific to varieties of *E. coli* (fecal coliforms). Since all of these counts (for HPC, TC, FC and *E. coli*) were abundant in domestic, mixed and agricultural waste

they could not be used to discriminate between the different wastewater blends in each series of (3 replicate) flasks.

Caffeine concentrations were assessed as predictors of wastewater blends in this study (the relative concentrations of agricultural and domestic waste). In the present study, levels of Total Phosphate and nitrate were examined, but these are not likely to indicate mixture composition, since the stock solutions were supplemented to reach approximately the same starting concentrations of both these nutrients.

4.4.1.1 Water Quality Parameters

TP and nitrate showed a greater decline in concentration over the first 48 hours of study than over the remainder of the week-long study period (Figure 4-4).The uptake of nutrients by autotrophic organisms, could have played a major role in nutrient reduction (Fouilland et al, 2007; Kirchman, 1994). There were no obvious differences between nutrient concentrations in different wastewater mixtures showed no major trend.

For some time, HPC counts have been used as a means to test drinking water quality on the principle that heterotrophic bacteria could be an indicator of the presence of human pathogens (Staff et al., 2003). The HPC test has proven an effective indicator as these bacteria are extremely abundant in most environments (Allen et al., 2004). In the present study, heterotrophic plate counts were very high (5.59-6.22 cfu/100 mL Log^{10}, SE ± 0.08), suggesting that pathogens may be present in all flasks of each series, but this test is not able to provide insight on the types of pathogen that may be present or their sources (Staff et al., 2003). Heterotrophic bacteria were found at similar densities in all mixtures of agricultural and domestic waste. Previous studies have reported similar difficulties in

differentiating source loading, looking only at bacterial indicators (Ashton et al., 2004; Daneshvar et al., 2012; Kurissery et al., 2012; Peeler et al., 2006; Seiler et al, 1999).

In contrast to the bacterial parameters examined, caffeine concentrations showed a positive relationship with domestic waste concentrations (Figure 4-3). This finding supports earlier work that suggested that caffeine levels are higher in domestic waste than in agricultural waste (Daneshvar et al., 2012; Ferreira, 2005; Kurissery et al., 2012; Seiler et al., 1999).

Low caffeine concentrations were seen in samples containing only agricultural waste. Possible sources of contamination could include the laboratory procedures, sample collection, or attributes of the agricultural land where sampling occurred. No caffeine was observed in the control flasks, which might rule out contamination in the lab. However, the farm where manure was sampled also uses sludge from a Wastewater Treatment Plant as a fertilizer in many of their fields. Earlier reports in the literature suggested that sewage sludge from the primary removal stage in wastewater treatment plants can contain caffeine at concentrations as high as 446 mg/kg dry mass; this might account for the presence of caffeine, even in the 100% agricultural wastewater (Martín et al, 2012).

4.4.1.2 Trend analysis

Bacterial counts were not sufficient to differentiate the various combinations of wastewaters in each sample ($p > 0.05$). Several factors may contribute to this. The microbial communities tested, although abundant, were not specifically associated with either domestic or agricultural waste. While some organisms have been used as tracers of animal waste (such as Fecal *Streptococci*), they do not typically survive for long in the

natural environment such that they would be useful for environmental monitoring (Howell et al., 1995).

On a more general scale, HPC can be used as an indicator of most bacterial pathogens and biologically contaminated drinking water. For these reasons, it has been widely accepted as a means to test water and wastewater (WHO, 2003). The communities of HPC are abundant in both domestic and agricultural wastes however they are not significant predictors of nutrient loading ($p = 0.11$). More specific indicators of wastewater loading were tested (TC, FC, and *E. coli*) with similar results. Coliform bacterial counts were high in both domestic and agricultural stock solutions.

Caffeine was a significant predictor of domestic waste and displayed a strong overall relationship with domestic waste ($R^2 = 0.46$, $F_{5, 48} = 8.172$, $p < 0.01$. This result supports earlier findings, but the strength of the relationship is difficult to determine given that several different variables affect caffeine concentrations in the environment (Ferreira, 2005; Kurissery et al., 2012; Peeler et al., 2006; Seiler et al., 1999). A better understanding of the strength of the correlation between caffeine and anthropogenic waste in a controlled laboratory environment can provide an insight on the strength of external environmental factors in inland freshwater systems. This study supports the hypothesis that caffeine concentrations can more accurately predict domestic waste content than bacterial counts (the first hypothesis, H_1). Of the multiple regressions performed, the combination of caffeine and MST produced the strongest model ($R^2 = 0.46$, $F_{5, 48} = 8.172$, $p < 0.01$. This supports the hypothesis that the combination of caffeine concentrations and bacterial counts will be more accurate at identifying and

differentiating domestic waste from agricultural waste than either assessment alone (the second hypothesis, H_2).

4.4.2 Precision of Indicators over Time

4.4.2.1 Water Quality Parameters

Over the week of study, nutrient concentrations dropped significantly. A number of factors could play a role in the depletion of nitrate and TP concentrations, including processes such as denitrification and nutrient assimilation by autotrophic microorganisms (Fouilland et al, 2007; Kirchman, 1994).

Heterotrophic bacteria maintained relatively stable counts over the week of study. HPC counts began at a mean concentration of 6.22 cfu/100 mL Log^{10} (SE ± 0.04) and after one week were still averaging counts of 5.79 cfu/100 mL Log^{10} (SE ± 0.06). Other studies support the identification of HPC as a robust indicator of community size, as a large range of organisms can grow in the R2A media (WHO, 2003; Staff et al., 2003).

The coliform measures, TC, FC and *E. coli* all followed similar patterns. Earlier studies have offered a number of explanations for the rapid mortality of coliform bacteria in laboratory experiments (van Elsas, et al, 2011; Wang et al., 2004). Coliform bacteria, particularly *E.coli,* survive best in the intestinal tracts of endotherms (van Elsas et al, 2011). These authors suggested that, in the natural environment, a similar decline in population also occurs, and that *E. coli* can survive for periods ranging from hours to as long as a year (van Elsas et al., 2011). Major factors influencing the survival of coliforms in their secondary habitats include available energy sources (glucose, lactose, etc.), low oxygen levels, unfavorable temperatures, pH, and osmolarity (Habteselassie et al., 2008). Based on earlier studies and the results of the present experiment, it is possible

that competition for nutrients and oxygen with other heterotrophs and colder water temperatures (21°C, SE ± 2 °C) may explain the rapid decline of coliforms observed in the experiment.

In this experiment caffeine degraded from an initial concentration of 5102 ng/L, SE ± 838.37 to 4344.70 ng/L, SE ± 617.25 after 48 hrs. After one week caffeine concentration reduced to 636 ng/L, SE ± 112.45. Previous studies suggested that the major processes of caffeine degradation could include biodegradation by bacteria and photo-degradation (Ahmad, 2014; Dash et al., 2012; Rapoula, 2003, 2003; Trovó et al., 2013; Vogels et al., 1976). Since both bacteria and sunlight were present in the flasks, it is not possible to distinguish between these possible factors in the present study, so to fully resolve this issue, further laboratory studies are needed. However, in a natural system, both factors would be occurring in an ongoing way.

4.4.2.2 Trend Analysis

Over a one-week period, a drop in bacterial density, nutrients and caffeine concentrations were observed. A good understanding of the effects of time on indicators of nutrient loading is extremely important if we are to adopt them as metrics of relative inputs from different sources. In the natural environment, it is difficult to collect a sample immediately after contaminants enter the system; sample collection may occur many hours, days or weeks after a release occurs. For these reasons, it is important to know how long factors that can act as indicators will persist.

The bacterial counts were high, but not specific to either domestic or agricultural wastes. Bacterial counts dropped significantly over the one week period of the study. A rapid decline in cell numbers and shift in microbial communities can be observed (Figure

4-5). Counts of HPC did not drop off as rapidly, but these data characterize a diverse microbial community, and therefore they may be more robust to stressors in general (Turner et al., 2007). Other bacterial data showed very rapid die-off over the one-week period. The bacterial data support the rationale behind using time-sensitive guidelines for rapid detection of TC, FC, and *E. coli* in freshwater systems (Cabral, 2010; Dufour et al., 2012; Edberg et al., 2000).

Caffeine concentrations did degrade over time, but at a slower rate than bacterial parameters (Figure 4-5 and 4.3-6). Both caffeine and bacterial parameters exhibited a significant negative relationship with time. Caffeine exhibited slower, consistent, and significant degradation compared to bacterial tests ($R^2 = 0.280$, $p < 0.01$), supporting the hypothesis that caffeine concentrations will degrade at a slower rate than bacterial death rates so that caffeine lasts longer, and is a better indicator of the contaminants than the bacterial populations (supporting the third hypothesis, H_3).

This information is useful in determining the value of indicator tests after a nutrient loading event has contaminated freshwater systems. It also clarifies how the length of time elapsed from a discharge event to the response and sample collection can affect the accuracy and usefulness of sample analyses.

4.5 Chapter Conclusions

The monitoring of caffeine concentrations offers an effective means of identifying domestic waste under a variety of conditions where samples were mixed with agricultural waste. This research supports all three of the hypotheses tested: H_1: Caffeine concentrations can more accurately predict combination of wastes than the bacterial counts; H_2: The combination of caffeine concentrations and bacterial counts will be more

accurate at predicting waste combination than either parameter alone; H_3: Caffeine concentrations will degrade at a slower rate than bacterial numbers.

Nutrient loading and eutrophication are major environmental concerns in terms of the degradation of freshwater systems (Correll, 1998; Heisler et al., 2008).. In this study, a laboratory experiment was conducted to assess the value of caffeine as an indicator of domestic waste, and evaluate how caffeine monitoring may be used in conjunction with conventional, accepted methods of microbial source tracking (MST). In Chapter 3, environmental samples were assessed, but the high variability of the results, likely due to additional environmental factors that were not measured, may have reduced the apparent value of these proposed new source tracking procedures. In the present chapter, a controlled laboratory experiment provided a very convincing demonstration of the value of a combined microbially-based and caffeine-based analysis for differentiating domestic waste and agricultural waste. Future studies should be designed to bridge the gap between these experiments by controlling, and/or recording in the field, a greater number of environmental factors so that the combined protocol can be further tested in field situations.

Chapter 5: General Discussion and Conclusions

Excess nutrient loadings resulting from human activities have long been recognized as having negative impacts on environmental health (Edberg et al., 2000; Winter et al., 2007). Nutrient loading, particularly loading of phosphorous, has been recognized as a major factor for eutrophication (Nicholls, 1995; Winter et al., 2007). Animal and human fecal wastes can also introduce pathogens to recreational and drinking waters (Cohen & Shuval, 1973; Glassmeyer et al., 2005; Wang et al., 2004). To this point, bacterial indicators of fecal contaminations have proven effective for the monitoring and assessment of freshwater systems (WHO, 2003; Cohen & Shuval, 1973). However, several studies have discussed the need to be able to differentiate nutrient load sources (domestic or agricultural), and to this point bacterial indicators have not proven sufficient to determine the nature of the waste source under varying environmental conditions (Cohen & Shuval, 1973; Edwards et al., 1997).

Chemical indicators such as caffeine have shown some promise for the distinguishing of domestic wastes in a water-body, as they would seem to be useful indicators of human fecal contamination (Buerge et al., 2003; Kurissery et al., 2012; Peeler et al. 2006). Some specific attributes of caffeine make it an ideal candidate for the differentiation of anthropogenic waste from other animal sources. Caffeine is not a substance naturally found in freshwater systems in Canada (Bruton et al., 2010; Buerge et al., 2003; Kurissery et al., 2012) and its presence can be closely linked to human excrement (Peeler et al., 2006). Caffeine shows low volatility and is reported as showing slow degradation over time (Bradley et al., 2007; Trovó et al., 2013). In the present study, using eight sampling locations and a series of laboratory experiments, it was established

that caffeine can be used to identify sewage from human waste in water, and can differentiate human sewage waste from cattle manure contamination under controlled conditions.

The objectives of this study were addressed over two separate chapters, the field component (Chapter 3) and the laboratory experiment (Chapter 4). First, a twelve-month field study was conducted to better understand the relationships that caffeine and bacterial communities have with seasonality and land use in the County of Simcoe and the Lake Simcoe Watershed. In addition, caffeine and bacteria were sampled from both surface waters and sediment at each site to assess the key differences between these two types of samples from the same sampling location. Second, a week-long laboratory experiment was conducted to understand the relationships that different blends of domestic waste and agricultural waste have with the parameters studied (heterotrophic plate count, total coliforms, *E.coli* counts, and caffeine concentrations). In addition, the laboratory experiment investigated the effects of time (as a proxy for degradation time) and cell growth/death over the course of one week in a controlled environment.

It was expected that environmental samples would display high variability in terms of various parameters (biological, chemical and physical) on a spatial and temporal scale, while in the laboratory studies much of natural environmental variance can be controlled. It was hypothesized that caffeine would show greater persistence over a wide range of environmental conditions and hydrologic parameters than bacterial communities, and that its concentration would be highly representative of the quantity of anthropogenic waste. It was hypothesized that bacterial communities would be abundant in most sampling locations, but their abundance and density would depend on temperature and

other environmental parameters. It was anticipated that fecal coliforms (*E. coli)* would be indicative of fecal contamination, but caffeine would be exclusively indicative of domestic contamination (as opposed to agricultural wastewater). Additionally, it was expected that the eight sites, ranked in terms of their degree of domestic land use, would positively correlate with caffeine concentrations (KL, LS, BC, BDa, FL, HR, HC, and BDb respectively).

The field study showed that caffeine in environmental samples was highly variable on a temporal and spatial scale. Caffeine was not, on its own, a significant predictor of watershed land use, but it did make a significant contribution to the model overall when combined with microbial source tracking. Caffeine varied significantly over the year, showing its highest concentrations in winter, when lakes were ice-covered. Surface sediment samples had higher caffeine concentrations than surface water, but they, too were highly variable. Comparing sites, as expected, KL showed lower mean caffeine concentrations than FL, but the comparison of two sites upstream and downstream of effluent release from a wastewater treatment plant, between BDa and BDb did not show a significant difference.

In the laboratory study, caffeine concentrations were positively correlated with the proportion of domestic waste (stock solution) in a flask, but not the stock solution containing agricultural wastes. Caffeine was effectively able to predict domestic waste concentrations throughout the study. Bacterial indicators were present at similar concentrations in both domestic and agricultural waste, so they did not, on their own, provide a way to distinguish between domestic and agricultural waste inputs. Bacterial indicators died off quite quickly over the week of the study.

Overall, the major findings of the study suggest that caffeine is an effective tracer for domestic waste in conditions where external variables are controlled, or more are recorded. Caffeine assessment, in conjunction with conventional methods of MST, offers a more effective model for predicting the relative contribution of anthropogenic waste in freshwater. Caffeine in sediment, although it is found at higher concentrations, showed higher variance, so larger sample sizes may be needed to provide more reliable data. Caffeine concentrations tend to increase in winter under ice cover; this suggests that, in winter, when fecal indicator bacteria cannot survive, caffeine may offer a superior indicator of anthropogenic fecal contamination.

Earlier studies have suggested that caffeine may be an ideal tracer molecule for domestic waste due to its specificity and environmental persistence (Chen et al., 2002; Kurissery et al., 2012; Peeler et al., 2006; Rapoula, 2003; Seiler et al., 1999; Thomas & Foster, 2005). Overall, the present study supports this position, and suggests that caffeine analysis for the trace determination of domestic waste, combined with traditional MST, could provide valuable information for water quality monitoring in the County of Simcoe. Although sediment samples had high caffeine concentrations, they were also very variable; this suggests that while sediment sampling of caffeine may be better, in the long run, than measurements made in the water column, a larger sample size or subsampling procedures should be used in future. The higher caffeine levels measured in the water column under ice cover may be a result of either winter stratification, or lower rates of (microbial) degradation (Bruton et al., 2010; Dash & Gummadi, 2012; Peake et al., 2015; Salonen et al., 2009). The use of caffeine as an indicator of domestic waste could be particularly beneficial in winter when microbial indicators are often inviable

(Ishii & Sadowsky, 2008; Oliver & Page, 2016). The use of caffeine to distinguish human fecal contamination, could be used in the public health sector along with standard methods of microbial water quality indicators. Understanding the origin of fecal contamination can provide valuable insight on what pathogens could be impacting human health (Cabral & João, 2010). Given the rapid expansion of urban areas and the inexorable conversion of natural and agricultural land to seasonal or year-round residential use, the issues involved with managing municipal sewage and identifying problems related to inflow, infiltration and sewage overflow, are becoming increasingly important, and highly relevant to environmental protection and drinking water source protection (LSRCA, 2016).

List of References

Adams, W. P., & Lasenby, D. C. (1985). The roles of snow, lake ice and lake water in the distribution of major ions in the ice cover of a lake. *Annals of Glaciology*, *7*, 202–207.

AECOM, & Parks Canada. (2011). Trent Severn Waterway: Water Management Study Review of Water Management Systems and Models. Retrieved April 24, 2018, from http://cewf.typepad.com/TSW_WMS_Part_2.pdf

Ahmad, S. A. (2014). Bacterial Degradation of Caffeine: A Review. *Asian Journal of Plant Biology (e-ISSN 2289-5868)*, *2*(1). Retrieved from http://journal.hibiscuspublisher.com/index.php/AJPB/article/view/84

Allen, M. J., Edberg, S. C., & Reasoner, D. J. (2004). Heterotrophic plate count bacteria—what is their significance in drinking water? *International Journal of Food Microbiology*, *92*(3), 265–274. https://doi.org/10.1016/j.ijfoodmicro.2003.08.017

APHA. (2012). Standard Methods for the Examination of Water and Wastewater 22nd Ed®. Retrieved November 22, 2017, from http://secure.apha.org/imis/ItemDetail?iProductCode=978-087553-0130&CATEGORY=BK

Aquafor Beech Limited. (2016). City of Orillia Comprehensive Stormwater Management Master Plan.

Ashton, D., Hilton, M., & Thomas, K. V. (2004). Investigating the environmental transport of human pharmaceuticals to streams in the United Kingdom. *Science of The Total Environment*, *333*(1–3), 167–184. https://doi.org/10.1016/j.scitotenv.2004.04.062

Azizi, S., Kamika, I., & Tekere, M. (2016). Evaluation of Heavy Metal Removal from Wastewater in a Modified Packed Bed Biofilm Reactor. *PLoS ONE*, *11*(5). https://doi.org/10.1371/journal.pone.0155462

Baalbaki, Z., Sultana, T., Maere, T., Vanrolleghem, P. A., Metcalfe, C. D., & Yargeau, V. (2016). Fate and mass balance of contaminants of emerging concern during wastewater treatment determined using the fractionated approach. *Science of The Total Environment*, *573*, 1147–1158.

Bant, C. (2009). *Ecological effects of road salt: the effects of road salt on the composition of macroinvertebrate fauna in three different streams receiving highway runoff* (Master's Thesis). [C. Bant].

Ballance, R., & Bartram, J. (Eds.). (1998). *Water Quality Monitoring: A Practical Guide to the Design and Implementation of Freshwater Quality Studies and Monitoring Programmes*. Spon Press. https://doi.org/10.4324/9780203476796

Beck, M. B. (1985). Lake eutrophication: Identification of tributary nutrient loading and sediment resuspension dynamics. *Applied Mathematics and Computation*, *17*(4), 433–458. https://doi.org/10.1016/0096-3003(85)90044-X

Ben-David, A., & Davidson, C. E. (2014). Estimation method for serial dilution experiments. *Journal of Microbiological Methods*, *107*(Supplement C), 214–221. https://doi.org/10.1016/j.mimet.2014.08.023

Bengtsson, L. (1996). Mixing in ice-covered lakes. *Hydrobiologia*, *322*(1), 91–97.

Bradley, P. M., Barber, L. B., Kolpin, D. W., McMahon, P. B., & Chapelle, F. H. (2007). Biotransformation of caffeine, cotinine, and nicotine in stream sediments: implications for use as wastewater indicators. *Environmental Toxicology and Chemistry*, *26*(6), 1116–1121.

Bruton, T., Alboloushi, A., de la Garza, B., Kim, B.-O., & Halden, R. U. (2010). Fate of Caffeine in the Environment and Ecotoxicological Considerations. In *Contaminants of Emerging Concern in the Environment: Ecological and Human Health Considerations* (Vol. 1048, pp. 257–273). American Chemical Society. Retrieved from http://dx.doi.org/10.1021/bk-2010-1048.ch012

Bryan, E. H. (1972). Quality of Stormwater Drainage from Urban Land1. *JAWRA Journal of the American Water Resources Association*, *8*(3), 578–588. https://doi.org/10.1111/j.1752-1688.1972.tb05180.x

Buerge, I. J., Poiger, T., Müller, M. D., & Buser, H.-R. (2003). Caffeine, an Anthropogenic Marker for Wastewater Contamination of Surface Waters. *Environmental Science & Technology*, *37*(4), 691–700. https://doi.org/10.1021/es020125z

Bussmann, I., Philipp, B., & Schink, B. (2001). Factors influencing the cultivability of lake water bacteria. *Journal of Microbiological Methods*, *47*(1), 41–50.

Bykowski, T., & Stevenson, B. (2005). Aseptic Technique. In *Current Protocols in Microbiology*. John Wiley & Sons, Inc. https://doi.org/10.1002/9780471729259.mca04ds11

Cabral, J. P. S. (2010). Water Microbiology. Bacterial Pathogens and Water. *International Journal of Environmental Research and Public Health*, *7*(10), 3657–3703. https://doi.org/10.3390/ijerph7103657

Canadian Council of Ministers of the Environment (Ed.). (1999). *Canadian environmental quality guidelines*. Hull, QC: CCME.

Casey, R. E., Simon, J. A., Atueyi, S., Snodgrass, J. W., Karouna-Renier, N., & Sparling, D. W. (2007). Temporal Trends of Trace Metals in Sediment and Invertebrates from Stormwater Management Ponds. *Water, Air, and Soil Pollution*, *178*(1–4), 69–77. https://doi.org/10.1007/s11270-006-9132-z

Chapman, P. M., Hayward, A., & Faithful, J. (2017). Total Suspended Solids Effects on Freshwater Lake Biota Other than Fish. *Bulletin of Environmental Contamination and Toxicology*, *99*(4), 423–427. https://doi.org/10.1007/s00128-017-2154-y

Chen, Z., Pavelic, P., Dillon, P., & Naidu, R. (2002). Determination of caffeine as a tracer of sewage effluent in natural waters by on-line solid-phase extraction and liquid chromatography with diode-array detection. *Water Research*, *36*(19), 4830–4838. https://doi.org/10.1016/S0043-1354(02)00221-X

City of Orillia. (2012). 2012 WWMP Update Report: Wastewater System Master Plan Update. Retrieved April 21, 2016, from http://www.orillia.ca/en/livinginorillia/resources/Environmental_Services/Wastewater_System_Master_Plan_Update.pdf

City of Orillia. (2017). WWTC Annual Compliance Report. Retrieved from https://www.orillia.ca/en/living-here/resources/Environmental_Services/2016-WWTC-Annual-Compliance-Report.pdf

Clark, B., Henry, G. L. H., & Mackay, D. (1995). Fugacity Analysis and Model of Organic Chemical Fate in a Sewage Treatment Plant. *Environmental Science & Technology*, *29*(6), 1488–1494. https://doi.org/10.1021/es00006a009

Cohen, J., Shuval, H. I., & Ministry of the Environment. (1973). Coliforms, fecal coliforms, and fecal streptococci as indicators of water pollution. Retrieved from http://link.springer.com/article/10.1007/BF00572392

Correll, D. L. (1998). The Role of Phosphorus in the Eutrophication of Receiving Waters: A Review. *Journal of Environment Quality*, *27*(2), 261. https://doi.org/10.2134/jeq1998.00472425002700020004x

Crosbie, B., & Chow-Fraser, P. (1999). Percentage land use in the watershed determines the water and sediment quality of 22 marshes in the Great Lakes basin. *Canadian Journal of Fisheries and Aquatic Sciences*, *56*(10), Daneshvar, A., Aboulfadl, K., Viglino, L., Broséus, R., Sauvé, S.,

Madoux-Humery, A.-S., Prévost, M. (2012). Evaluating pharmaceuticals and caffeine as indicators of fecal contamination in drinking water sources of the Greater Montreal region. *Chemosphere*, *88*(1), 131–139. https://doi.org/10.1016/j.chemosphere.2012.03.016

Danielson, T. J. (2002). *Methods for evaluating wetland condition 1 introduction to wetland biological assessment.* DIANE Publishing. Retrieved from https://books.google.com/books?hl=en&lr=&id=aYda_4Yn5-sC&oi=fnd&pg=PR5&dq=%22all.+A+list+of+the+inaugural+set+of+20+modules+can+be+found+at+the+end+of+this%22+%22information+about+wetland+biological+assessments+is+available+at+the%22+&ots=xF4nR8njJ3&sig=8hle90f64EHEV668z1sc28gWKGk

Dash, S. S., & Gummadi, S. N. (2012). Biotechnological Approach to Caffeine Degradation: Current Trends and Perspectives. In Anil Prakash, T. Satyanarayana, & B. N. Johri (Eds.), *Microorganisms in Sustainable Agriculture and Biotechnology* (pp. 435–451). Dordrecht: Springer Netherlands. Retrieved from http://www.springerlink.com/index/10.1007/978-94-007-2214-9_20

Delattre, P., Giraudoux, P., Baudry, J., Musard, P., Toussaint, M., Truchetet, D., … Quéré, J.-P. (1992). Land use patterns and types of common vole (Microtus arvalis) population kinetics. *Agriculture, Ecosystems & Environment*, *39*(3), 153–168. https://doi.org/10.1016/0167-8809(92)90051-C

Dufour, A. P. (1977). Escherichia coli: The Fecal Coliform. https://doi.org/10.1520/STP34817S

Dufour, Alfred P., World Health Organization, & United States (Eds.). (2012). *Animal waste, water quality and human health*. London: Published on behalf of the World Health Organization by IWA Publishing.

Edberg, S. c., Rice, E. w., Karlin, R. j., & Allen, M. j. (2000). Escherichia coli: the best biological drinking water indicator for public health protection. *Journal of Applied Microbiology*, *88*(S1), 106S–116S. https://doi.org/10.1111/j.1365-2672.2000.tb05338.x

Edwards, D. R., Coyne, M. S., Vendrell, P. F., Daniel, T. C., Moore, P. A., & Murdoch, J. F. (1997). *Fecal Coliform and Streptococcus Concentrations in Runoff from Grazed Pastures in Northwest Arkansas*. Wiley Online Library. Retrieved from http://onlinelibrary.wiley.com/doi/10.1111/j.1752-1688.1997.tb03520.x/abstract

Edwards, Q. A., Kulikov, S. M., & Garner-O'Neale, L. D. (2015). Caffeine in surface and wastewaters in Barbados, West Indies. *SpringerPlus*, *4*(1), 57. https://doi.org/10.1186/s40064-015-0809-x

Eganhouse, R. P., Cozzarelli, I. M., Scholl, M. A., & Matthews, L. L. (2001). Natural Attenuation of Volatile Organic Compounds (VOCs) in the Leachate Plume of a Municipal Landfill: Using Alkylbenzenes as Process Probes. *Ground Water*, *39*(2), 192–202. https://doi.org/10.1111/j.1745-6584.2001.tb02300.x

Environmental Protection Agency. (2014). Guide for Estimating Infiltration and Inflow. Retrieved from https://www3.epa.gov/region1/sso/pdfs/Guide4EstimatingInfiltrationInflow.pdf

EPA. (2002). Analytical Methods Approved for Compliance Monitoring under the Revised Total Coliform Rule. Retrieved from http://linkinghub.elsevier.com/retrieve/pii/0048969794903328

EPA. (2008). Methods for evaluating wetland condition: using vegetation to assess environmental conditions in wetlands. *EPA 843-B-00-0002j*. Retrieved from https://works.bepress.com/siobhan_fennessy/16/

Eveborn, D., Gustafsson, J. P., Elmefors, E., Yu, L., Eriksson, A.-K., Ljung, E., & Renman, G. (2014). Phosphorus in soil treatment systems: accumulation and mobility. *Water Research*, *64*, 42–52. https://doi.org/10.1016/j.watres.2014.06.034

Evans, D., Nicholls, K. H., Allen, Y., & McMurtry, M. J. (2011). Historical land use, phosphorus loading, and loss of fish habitat in Lake Simcoe, Canada. *Canadian Journal of Fisheries and Aquatic Sciences*, *53*, 194–218. https://doi.org/10.1139/cjfas-53-S1-194

Evans, D. O., & others. (2006). *Effects of hypoxia on scope-for-activity of lake trout: defining a new dissolved oxygen criterion for protection of lake trout habitat*. Habitat and Fisheries Unit, Aquatic Research and Development Section, ARDB. Retrieved from http://www.web2.mnr.gov.on.ca/mnr/ebr/lake_trout/_fr/report.pdf

Faithful, J. W. (2016). Physico-chemical changes in two northern headwater lakes in the Northwest Territories, Canada, during winter to spring seasonal transitions. *Journal of Great Lakes Research*, *42*(2), 166–172. https://doi.org/10.1016/j.jglr.2016.01.004

Ferreira, A. P. (2005). Caffeine as an environmental indicator for assessing urban aquatic ecosystems. *Cadernos de Saúde Pública*, *21*(6), 1884–1892. https://doi.org/10.1590/S0102-311X2005000600038

Fewtrell, L., & Bartram, J. (Eds.). (2001). *Water quality: guidelines, standards, and health: assessment of risk and risk management for water-related infectious disease*. Geneva: World Health Organization.

Foley, J. A. (2005). Global Consequences of Land Use. *Science*, *309*(5734), 570–574. https://doi.org/10.1126/science.1111772

Fouilland, E., Gosselin, M., Rivkin, R. B., Vasseur, C., & Mostajir, B. (2007). Nitrogen uptake by heterotrophic bacteria and phytoplankton in Arctic surface waters. *Journal of Plankton Research*, *29*(4), 369–376. https://doi.org/10.1093/plankt/fbm022

Furtado, A. L., & Casper, P. (2000). Different methods for extracting bacteria from freshwater sediment and a simple method to measure bacterial production in sediment samples. *Journal of Microbiological Methods*, *41*(3), 249–257.

Gan, T. Y. (1995). Trends in air temperature and precipitation for Canada and north-eastern USA. *International Journal of Climatology*, *15*(10), 1115–1134. https://doi.org/10.1002/joc.3370151005

Gardinali, P. R., & Zhao, X. (2002). Trace determination of caffeine in surface water samples by liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (LC–APCI–MS). *Environment International*, *28*(6), 521–528. https://doi.org/10.1016/S0160-4120(02)00080-6

Gasperi, J., V., R., & Moilleron, R. (2008). Priority Pollutants in Wastewater and Combined Sewer Overflow. Science of the Total Environment, 407, 263-272. - References - Scientific Research Publishing. Retrieved February 28, 2018, from http://www.scirp.org/(S(351jmbntvnsjt1aadkposzje))/reference/ReferencesPapers.aspx?ReferenceID=1325715

Gaufin, A. R., & Tarzwell, C. M. (1952). Aquatic invertebrates as indicators of stream pollution. *Public Health Reports*, *67*(1), 57–64.

Geldreich, E. E., & Kenner, B. A. (1969). Concepts of Fecal Streptococci in Stream Pollution. *Journal (Water Pollution Control Federation)*, *41*(8), R336–R352.

Glassmeyer, S. T., Furlong, E. T., Kolpin, D. W., Cahill, J. D., Zaugg, S. D., Werner, S. L., Meyer, M.T., Kryak, D. D. (2005). Transport of Chemical and Microbial Compounds from Known Wastewater Discharges: Potential for Use as Indicators of Human Fecal Contamination. *Environmental Science & Technology*, *39*(14), 5157–5169. https://doi.org/10.1021/es048120k

Google Maps. (2017). Retrieved May 3, 2018, from https://www.google.ca/maps/place/Simcoe+County,+ON/@44.4630225,-80.8837099,222329m/data=!3m2!1e3!4b1!4m5!3m4!1s0x4cd53ae43148b1b5:0xbb0d02ca24aa3e89!8m2!3d44.4716525!4d-79.8296743

Government of Ontario. (1990). Conservation Authorities Act, R.S.O. 1990, c. C.27 [Text]. Retrieved March 20, 2018, from https://www.ontario.ca/laws/view

Government of Ontario. (2008). Lake Simcoe Protection Act, 2008, S.O. 2008, c. 23. Retrieved February 25, 2018, from https://www.ontario.ca/laws/statute/08l23

Government of Ontario. (2012). 5-6-2-ministers-report-on-simcoe-2011-12. Retrieved March 19, 2018, from https://dr6j45jk9xcmk.cloudfront.net/documents/869/5-6-2-ministers-report-on-simcoe-2011-12-en.pdf1781–1791.

H. Nicholls, K. (1995). Some recent water quality trends in Lake Simcoe, Ontario: Implications for basin planning and limnological research. *Canadian Water Resources Journal - CAN WATER RESOUR J*, *20*, 213–226. https://doi.org/10.4296/cwrj2004213

Habteselassie, M., Bischoff, M., Blume, E., Applegate, B., Reuhs, B., Brouder, S., & Turco, R. F. (2008). Environmental Controls on the Fate of <Emphasis Type="Italic">Escherichia coli</Emphasis> in Soil. *Water, Air, and Soil Pollution*, *190*(1–4), 143–155. https://doi.org/10.1007/s11270-007-9587-6

Hach. (1999). Coliforms, Total and E. coli. Retrieved from https://www.hach.com/asset-get.download-en.jsa?id=7639984023

Hai, L., & Hongdao, C. (1982). Significance of fecal coliform and fecal streptococcus in water pollution monitoring. *Acta Academiae Medicinae Wuhan*, *2*(4), 251. https://doi.org/10.1007/BF02858867

Halling-Serensen, B., Nielsen, S. N., Lanzky, P. F., Ingerslev, F., Lutzhoeft, H. C. H., & Joergensen, S. E. (Section of E. C. (1998). Occurrence, fate and effects of pharmaceutical substances in the environment - a review. *Chemosphere (United Kingdom)*. Retrieved from http://agris.fao.org/agris-search/search.do?recordID=GB1997038993

Health Canada. (2014). *Guidelines for Canadian drinking water quality: guideline technical document - Escherichia coli*. Retrieved from http://www.deslibris.ca/ID/241734

Heisler, J., Glibert, P., Burkholder, J., Anderson, D., Cochlan, W., Dennison, W., … Suddleson, M. (2008). Eutrophication and Harmful Algal Blooms: A Scientific Consensus. *Harmful Algae*, *8*(1), 3–13. https://doi.org/10.1016/j.hal.2008.08.006

Horne, A. J., & Goldman, C. R. (1994). *Limnology*. McGraw-Hill.

Howell, J. M., Coyne, M. S., & Cornelius, P. (1995). Fecal bacteria in agricultural waters of the bluegrass region of Kentucky. *Journal of Environmental Quality*, *24*(3), 411–419.

International Joint Commission. (2003). Status of Restoration Activities in the Great Lakes Areas of Concern: a Special Report. Retrieved March 19, 2018, from http://ijc.org/files/publications/aoc_report-e.pdf

Ishii, S., Ksoll, W. B., Hicks, R. E., & Sadowsky, M. J. (2006). Presence and Growth of Naturalized Escherichia coli in Temperate Soils from Lake Superior Watersheds. *Applied and Environmental Microbiology*, *72*(1), 612–621. https://doi.org/10.1128/AEM.72.1.612-621.2006

Ishii, S., & Sadowsky, M. J. (2008). Escherichia coli in the Environment: Implications for Water Quality and Human Health. *Microbes and Environments / JSME*, *23*(2), 101–108.

Jensen, T., Tiessen, K., Salvano, E., Kalischuk, A., & Flaten, D. N. (2011). Spring snowmelt impact on phosphorus addition to surface runoff in the Northern Great Plains. *Better Crops*, *95*(1), 28–31.

Jianlong, W., & Ning, Y. (2004). Partial nitrification under limited dissolved oxygen conditions. *Process Biochemistry*, *39*(10), 1223–1229. https://doi.org/10.1016/S0032-9592(03)00249-8

Jin, G., Jeng, H.-W., Bradford, H., & Englande, A. J. (2004). Comparison of E. coli, enterococci, and fecal coliform as indicators for brackish water quality assessment. *Water Environment Research: A Research Publication of the Water Environment Federation*, *76*(3), 245–255.

Johnston, A. E., Dawson, C. J., & Agricultural Industries Confederation. (2005). *Phosphorus in agriculture and in relation to water quality*. Peterborough: Agricultural Industries Confederation.

Jones, P. G., VanBogelen, R. A., & Neidhardt, F. C. (1987). Induction of proteins in response to low temperature in Escherichia coli. *Journal of Bacteriology*, *169*(5), 2092–2095.

Kaspar, H. M. de, Kreutzer, T. C., Klauss, V., & Kampik, A. (2006). Counts of Colony Forming Units as an Effective Semi Quantitative Method to Specify Changes in Amounts of Bacteria on the Conjunctiva. *Investigative Ophthalmology & Visual Science*, *47*(13), 1884–1884.

Khan, S. J., & Ongerth, J. E. (2004). Modelling of pharmaceutical residues in Australian sewage by quantities of use and fugacity calculations. *Chemosphere*, *54*(3), 355–367. https://doi.org/10.1016/j.chemosphere.2003.07.001

Kirchman, D. L. (1994). The uptake of inorganic nutrients by heterotrophic bacteria. *Microbial Ecology*, *28*(2), 255–271. https://doi.org/10.1007/BF00166816

Krajewski, P. (1990). Heterogeneity of variance in field experiments: some causes and practical implications. *The Journal of Agricultural Science*, *115*(1), 83–93. https://doi.org/10.1017/S0021859600073950

Kurissery, S., Kanavillil, N., Verenitch, S., & Mazumder, A. (2012). Caffeine as an anthropogenic marker of domestic waste: A study from Lake Simcoe watershed. *Ecological Indicators*, *23*, 501–508. https://doi.org/10.1016/j.ecolind.2012.05.001

Lake Simcoe Environmental Management Strategy. (2003). State of the Lake Simcoe Watershed. Retrieved March 19, 2018, from https://www.lsrca.on.ca/Shared%20Documents/reports/lsems/state_lake.pdf

Lanning, A., & W. Peterson, E. (2012). Evaluating Subdivisions for Identifying Extraneous Flow in Separate Sanitary Sewer Systems. *Journal of Water Resource and Protection*, *04*(06), 334–341. https://doi.org/10.4236/jwarp.2012.46037

Li, G., Li, H., Leffelaar, P. A., Shen, J., & Zhang, F. (2014). Characterization of Phosphorus in Animal Manures Collected from Three (Dairy, Swine, and Broiler) Farms in China. *PLoS ONE*, *9*(7). https://doi.org/10.1371/journal.pone.0102698

Lin, S. D. (1974). *Evaluation of Methods for Detecting Coliforms and Fecal Streptococci in Chlorinated Sewage Effluents* (Vol. 78). Illinois State Water Survey. Retrieved from https://www.ideals.illinois.edu/bitstream/handle/2142/77788/ISWSRI-78.pdf?sequence=2

Linden, R., Antunes, M. V., Heinzelmann, L. S., Fleck, J. D., Staggemeier, R., Fabres, R. B., … Spilki, F. R. (2015). Caffeine as an indicator of human fecal contamination in the Sinos River: a preliminary study. *Brazilian Journal of Biology*, *75*(2), 81–84. https://doi.org/10.1590/1519-6984.0513

LSRCA. (2007). Natural Heritage System for the Lake Simcoe Watershed. Retrieved from https://www.lsrca.on.ca/Shared%20Documents/reports/natural_heritage_2007.pdf

LSRCA. (2009). Lake Simcoe Watershed Report Card. Retrieved March 20, 2018, from https://www.lsrca.on.ca/Shared%20Documents/reports/watershed_report_card_2009.pdf

LSRCA. (2010a). East Holland River Subwatershed Management Plan. Retrieved from https://www.lsrca.on.ca/Shared%20Documents/reports/east-holland-subwatershed-plan.pdf

LSRCA. (2010b). Lake Simcoe Phosphorous Reduction Strategy. Retrieved March 19, 2018, from https://dr6j45jk9xcmk.cloudfront.net/documents/872/5-6-4-lake-simcoe-phosphorus-reduction-en-pdf.pdf

LSRCA. (2012). Barrie Creeks, Lovers Creek, and Hewitt's Creek Subwatershed Plan. Retrieved from https://www.lsrca.on.ca/Shared%20Documents/reports/barrie_subwatershed_plan_2012.pdf

LSRCA. (2013a). Lake Simcoe Watershed Report Card 2013. Retrieved from https://www.lsrca.on.ca/Shared%20Documents/reports/watershed_report_card_2013.pdf

LSRCA. (2013b). Oro Creeks North Subwatershed Plan. Retrieved from https://www.lsrca.on.ca/Shared%20Documents/reports/oro_hawkestone_subwatershed_plan.pdf

LSRCA. (2016). Technical Guidelines for Stormwater Management. Retrieved March 19, 2018, from https://www.lsrca.on.ca/Shared%20Documents/permits/swm_guidelines.pdf

Martín, J., Camacho-Muñoz, D., Santos, J. L., Aparicio, I., & Alonso, E. (2012). Occurrence of pharmaceutical compounds in wastewater and sludge from wastewater treatment plants: Removal and ecotoxicological impact of wastewater discharges and sludge disposal. *Journal of Hazardous Materials*, *239–240*, 40–47. https://doi.org/10.1016/j.jhazmat.2012.04.068

Mazzafera, P. (2004). Catabolism of caffeine in plants and microorganisms. *Frontiers in Bioscience: A Journal and Virtual Library*, *9*, 1348–1359.

Ministry of the Environment. (1973). Report of water quality in Farlain Lake, Tiny Twp., Simcoe County, 34.

Murray, C., & Laredo, T. (2014). Effect of Home Grinding on Properties of Brewed Coffee. *Journal of Food Research*, *4*(1), 77. https://doi.org/10.5539/jfr.v4n1p77

Nasrabadi, T., Ruegner, H., Sirdari, Z. Z., Schwientek, M., & Grathwohl, P. (2016). Using total suspended solids (TSS) and turbidity as proxies for evaluation of metal transport in river water. *Applied Geochemistry*, *68*, 1–9. https://doi.org/10.1016/j.apgeochem.2016.03.003

Natural Resources Canada. (2015). Land Cover & Land Use. Retrieved February 21, 2018, from http://www.nrcan.gc.ca/node/9373

Noble, R. T., Blackwood, A. D., Griffith, J. F., McGee, C. D., & Weisberg, S. B. (2010). Comparison of Rapid Quantitative PCR-Based and Conventional Culture-Based Methods for Enumeration of Enterococcus spp. and Escherichia coli in Recreational Waters. *Applied and Environmental Microbiology*, *76*(22), 7437–7443. https://doi.org/10.1128/AEM.00651-10

Novotny, E., Murphy, D., & Stefan, H. (2007). Road Salt Effects on the Water Quality of Lakes in the Twin Cities Metropolitan Area.

Oliver, D. M., & Page, T. (2016). Effects of seasonal meteorological variables on E. coli persistence in livestock faeces and implications for environmental and human health. *Scientific Reports*, *6*(1). https://doi.org/10.1038/srep37101

Omar, W. M. W. (2010). Perspectives on the Use of Algae as Biological Indicators for Monitoring and Protecting Aquatic Environments, with Special Reference to Malaysian Freshwater Ecosystems. *Tropical Life Sciences Research*, *21*(2), 51–67.

Osborne, J. W., & Waters, E. (2002). Multiple Regression Assumptions. ERIC Digest.

Pachepsky, Y. A., & Shelton, D. R. (2011). Escherichia Coli and Fecal Coliforms in Freshwater and Estuarine Sediments. *Critical Reviews in Environmental Science and Technology*. Retrieved from http://agris.fao.org/agris-search/search.do?recordID=US201400168706

Pandey, P. K., Kass, P. H., Soupir, M. L., Biswas, S., & Singh, V. P. (2014). Contamination of water resources by pathogenic bacteria. *AMB Express*, *4*, 51. https://doi.org/10.1186/s13568-014-0051-x

Peake, B. M., Braund, R., Tong, A., & Tremblay, L. A. (2015). *The Life-Cycle of Pharmaceuticals in the Environment*. Elsevier.

Peeler, K. A., Opsahl, S. P., & Chanton, J. P. (2006). Tracking Anthropogenic Inputs Using Caffeine, Indicator Bacteria, and Nutrients in Rural Freshwater and Urban Marine Systems. *Environmental Science & Technology*, *40*(24), 7616–7622. https://doi.org/10.1021/es061213c

Phillips, P. J., Chalmers, A. T., Gray, J. L., Kolpin, D. W., Foreman, W. T., & Wall, G. R. (2012). Combined Sewer Overflows: An Environmental Source of Hormones and Wastewater Micropollutants. *Environmental Science & Technology*, *46*(10), 5336–5343. https://doi.org/10.1021/es3001294

Potapova, M. G., Charles, D. F., Ponader, K. C., & Winter, D. M. (2004). Quantifying species indicator values for trophic diatom indices: a comparison of approaches. *Hydrobiologia*, *517*(1–3), 25–41.

Quinn, G. P., & Keough, M. J. (2002). *Experimental design and data analysis for biologists*. Cambridge University Press. Retrieved from https://books.google.com/books?hl=en&lr=&id=VtU3-y7LaLYC&oi=fnd&pg=PP17&dq=%22Samples+and%22+%22Interpretation+of+con%EF%AC

%81dence+intervals+for+population%22+%22Prior+knowledge+and%22+%22Type+I+and+II%22+%22Standard+errors+for+other%22+%22Classical+statistical+hypothesis%22+%22Spread+or%22+%22ML+vs+OLS%22+&ots=cztq4sjqjC&sig=VMdTgCBCUoLqnFTQ0YZTvgzmrL8

Raj, C. V., & Dhala, S. (1965). Effect of Naturally Occurring Xanthines on Bacteria. I. Antimicrobial Action and Potentiating Effect on Antibiotic Spectra. *Applied Microbiology*, *13*, 432–436.

Ramarethinam, S., & Rajalakshmi, N. (2004). Caffeine in tea plants [Camellia sinensis (L) O. Kuntze]: in situ lowering by Bacillus licheniformis (Weigmann) Chester. *Indian Journal of Experimental Biology*, *42*(6), 575–580.

Rapoula, S. (2003). Photodegradation of caffeine by UV radiation and H2O2. Retrieved from http://dspace.cc.tut.fi/dpub/handle/123456789/2018

Ravaliya, K., Gentry-Shields, J., Garcia, S., Heredia, N., Fabiszewski de Aceituno, A., Bartz, F. E., … Jaykus, L.-A. (2014). Use of Bacteroidales Microbial Source Tracking To Monitor Fecal Contamination in Fresh Produce Production. *Applied and Environmental Microbiology*, *80*(2), 612–617. https://doi.org/10.1128/AEM.02891-13

Reasoner, D. J., & Geldreich, E. E. (1985). A new medium for the enumeration and subculture of bacteria from potable water. *Applied and Environmental Microbiology*, *49*(1), 1–7.

Rodríguez, I., Quintana, J. B., Carpinteiro, J., Carro, A. M., Lorenzo, R. A., & Cela, R. (2003). Determination of acidic drugs in sewage water by gas chromatography-mass spectrometry as tert.-butyldimethylsilyl derivatives. *Journal of Chromatography. A*, *985*(1–2), 265–274.

Romshoo, S. (2011). Geospatial modeling for assessing the nutrient load of a Himalayan lake. *Environmental Earth Sciences*, *64*(5), 1269–1282.

Sabaliunas, D., Webb, S. F., Hauk, A., Jacob, M., & Eckhoff, W. S. (2003). Environmental fate of Triclosan in the River Aire Basin, UK. *Water Research*, *37*(13), 3145–3154. https://doi.org/10.1016/S0043-1354(03)00164-7

Salonen, K., Leppäranta, M., Viljanen, M., & Gulati, R. D. (2009). Perspectives in winter limnology: closing the annual cycle of freezing lakes. *Aquatic Ecology*, *43*(3), 609–616. https://doi.org/10.1007/s10452-009-9278-z

Sarjeant, D. (1999). Fecal Contamination Source Identification Methods in Surface Water. Retrieved April 26, 2016, from https://fortress.wa.gov/ecy/publications/summarypages/99345.html

Seiler, R. L., Zaugg, S. D., Thomas, J. M., & Howcroft, D. L. (1999). Caffeine and Pharmaceuticals as Indicators of Waste Water Contamination in Wells. *Ground Water*, *37*(3), 405–410. https://doi.org/10.1111/j.1745-6584.1999.tb01118.x

Severn Sound Environmental Association. (2015). Evaluation of Natural Heritage Conditions in the Township of Tiny. Retrieved from https://www.tiny.ca/Shared%20Documents/Planning/Evaluation%20of%20Natural%20Heritage%20Conditions%20in%20the%20Township%20of%20Tiny.pdf

Severn Sound Environmental Association. (2016). Summary of Farlain Lake Well Survey. Retrieved March 19, 2018, from https://www.severnsound.ca/Shared%20Documents/Reports/2016_Farlain_Lake_Well_Survey_Summary_final_w_appx_20170106.pdf

Shange, R., Haugabrooks, E., Ankumah, R., Ibekwe, A., Smith, R., & Dowd, S. (2013). Assessing the Diversity and Composition of Bacterial Communities across a Wetland, Transition, Upland Gradient in Macon County Alabama. *Diversity*, *5*, 461–478.

Siegener, R., & Chen, R. F. (2002). Caffeine in Boston Harbor seawater. *Marine Pollution Bulletin*, *44*(5), 383–387. https://doi.org/10.1016/S0025-326X(00)00176-4

Simcoe-Muskoka District Health Unit. (2016). 2016 Annual Report. Retrieved February 14, 2018, from https://www.simcoemuskokahealth.org/docs/default-source/hu-library/reports/2016-annual-report-web.pdf?sfvrsn=2

Spongberg, A. L., & Witter, J. D. (2008). Pharmaceutical compounds in the wastewater process stream in Northwest Ohio. *Science of The Total Environment*, *397*(1–3), 148–157. https://doi.org/10.1016/j.scitotenv.2008.02.042

Stackelberg, P. E., Furlong, E. T., Meyer, M. T., Zaugg, S. D., Henderson, A. K., & Reissman, D. B. (2004). Persistence of pharmaceutical compounds and other organic wastewater contaminants in a conventional drinking-water-treatment plant. *Science of The Total Environment*, *329*(1–3), 99–113. https://doi.org/10.1016/j.scitotenv.2004.03.015

Staff, W. H. O., Organization, W. H., (Organization), N. I., Staff, N. S. F., & Association, I. W. (2003). *HPC and Drinking-Water Safety: The Significance of Heterotrophic Plate Counts for Water Quality and Human Health*. IWA Publishing.

Standley, L. J., Kaplan, L. A., & Smith, D. (2000). Molecular Tracers of Organic Matter Sources to Surface Water Resources. *Environmental Science & Technology*, *34*(15), 3124–3130. https://doi.org/10.1021/es991381n

Stoeckel, D. M., & Harwood, V. J. (2007). Performance, Design, and Analysis in Microbial Source Tracking Studies. *Applied and Environmental Microbiology*, *73*(8), 2405–2415. https://doi.org/10.1128/AEM.02473-06

Summers, R. M., Louie, T. M., Yu, C. L., & Subramanian, M. (2011). Characterization of a broad-specificity non-haem iron N-demethylase from Pseudomonas putida CBB5 capable of utilizing

several purine alkaloids as sole carbon and nitrogen source. *Microbiology (Reading, England)*, *157*(Pt 2), 583–592. https://doi.org/10.1099/mic.0.043612-0

Tabachnick, B. G., & Fidell, L. S. (1996). Using Multivariate Statistics (3rd ed.). New York: Harper Collins. Retrieved October 1, 2017, from http://www.scirp.org/(S(czeh2tfqyw2orz553k1w0r45))/reference/ReferencesPapers.aspx?ReferenceID=1887286

Tadesse, I., Green, F. B., & Puhakka, J. A. (2004). Seasonal and diurnal variations of temperature, pH and dissolved oxygen in advanced integrated wastewater pond system treating tannery effluent. *Water Research*, *38*(3), 645–654.

Tamaki, H., Sekiguchi, Y., Hanada, S., Nakamura, K., Nomura, N., Matsumura, M., & Kamagata, Y. (2005). Comparative Analysis of Bacterial Diversity in Freshwater Sediment of a Shallow Eutrophic Lake by Molecular and Improved Cultivation-Based Techniques. *Applied and Environmental Microbiology*, *71*(4), 2162–2169. https://doi.org/10.1128/AEM.71.4.2162-2169.2005

Ternes, T. A. (1998). Occurrence of drugs in German sewage treatment plants and rivers1. *Water Research*, *32*(11), 3245–3260. https://doi.org/10.1016/S0043-1354(98)00099-2

Thomas, P. M., & Foster, G. D. (2005). Tracking acidic pharmaceuticals, caffeine, and triclosan through the wastewater treatment process. *Environmental Toxicology and Chemistry*, *24*(1), 25–30. https://doi.org/10.1897/04-144R.1

Tibbetts, J. (2005). Combined Sewer Systems: Down, Dirty, and Out of Date. *Environmental Health Perspectives*, *113*(7), A464–A467.

Toronto and Regional Conservation Authority (TRCA). (2000). Aquatic Habitat and Species Monitoring: A Discussion Paper in Support of the Development of A Regional Watershed Monitoring Network. Retrieved April 24, 2018, from http://www.trca.on.ca/dotAsset/114187.pdf

Trovó, A. G., Silva, T. F. S., Gomes Jr., O., Machado, A. E. H., Neto, W. B., Muller Jr., P. S., & Daniel, D. (2013). Degradation of caffeine by photo-Fenton process: Optimization of treatment conditions using experimental design. *Chemosphere*, *90*(2), 170–175. https://doi.org/10.1016/j.chemosphere.2012.06.022

Turner, W. R., Brandon, K., Brooks, T. M., Costanza, R., Fonseca, D., B, G. A., & Portela, R. (2007). Global Conservation of Biodiversity and Ecosystem Services. *BioScience*, *57*(10), 868–873. https://doi.org/10.1641/B571009

Unden, G., Becker, S., Bongaerts, J., Schirawski, J., & Six, S. (1994). Oxygen regulated gene expression in facultatively anaerobic bacteria. *Antonie Van Leeuwenhoek*, *66*(1–3), 3–22.

Unden, G., & Bongaerts, J. (1997). Alternative respiratory pathways of Escherichia coli: energetics and transcriptional regulation in response to electron acceptors. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1320*(3), 217–234. https://doi.org/10.1016/S0005-2728(97)00034-0

Vadas, P. A., Good, L. W., Moore, P. A., & Widman, N. (2009). Estimating phosphorus loss in runoff from manure and fertilizer for a phosphorus loss quantification tool. *Journal of Environmental Quality*, *38*(4), 1645–1653. https://doi.org/10.2134/jeq2008.0337

van Elsas, J. D., Semenov, A. V., Costa, R., & Trevors, J. T. (2011). Survival of Escherichia coli in the environment: fundamental and public health aspects. *The ISME Journal*, *5*(2), 173–183. https://doi.org/10.1038/ismej.2010.80

Verenitch, S. S., & Mazumder, A. (2008). Development of a methodology utilizing gas chromatography ion-trap tandem mass spectrometry for the determination of low levels of

caffeine in surface marine and freshwater samples. *Analytical and Bioanalytical Chemistry*, *391*(7), 2635–2646. https://doi.org/10.1007/s00216-008-2174-x

Vogels, G. van der, & Van der Drift, C. (1976). Degradation of purines and pyrimidines by microorganisms. *Bacteriological Reviews*, *40*(2), 403.

Wang, C., & Gardinali, P. R. (2012). Comparison of multiple API techniques for the simultaneous detection of microconstituents in water by on-line SPE-LC-MS/MS: API determination of PPCPs by on-line SPE-LC/MS/MS. *Journal of Mass Spectrometry*, *47*(10), 1255–1268. https://doi.org/10.1002/jms.3051

Wang, L., Mankin, K. R., & Marchin, G. L. (2004). Survival of fecal bacteria in dairy cow manure. *Transactions of the ASAE*, *47*(4), 1239.

Wang, N. F., Zhang, T., Yang, X., Wang, S., Yu, Y., Dong, L. L., … Zang, J. Y. (2016). Diversity and Composition of Bacterial Community in Soils and Lake Sediments from an Arctic Lake Area. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.01170

Water Environment Association of Ontario (WEAO). (2010). review of phosphorus removal to lake simcoe. Retrieved June 20, 2016, from http://www.weao.org/assets/docs/resources-links/reports/review-of-phosphorus-removal-to-lake-simcoe.pdf

White, P. A., Kalff, J., Rasmussen, J. B., & Gasol, J. M. (1991). The effect of temperature and algal biomass on bacterial production and specific growth rate in freshwater and marine habitats. *Microbial Ecology*, *21*(1), 99–118. https://doi.org/10.1007/BF02539147

Winter, J., Eimers, C., Dillon, P., Scott, L., Scheider, W., & Willox, C. (2007). Phosphorus Inputs to Lake Simcoe from 1990 to 2003: Declines in Tributary Loads and Observations on Lake Water Quality. *Journal of Great Lakes Research*, *33*(2), 381–396. https://doi.org/doi: 10.3394/0380-1330(2007)33[381:PITLSF]2.0.CO;2

World Health Organisation. (2003). *Heterotrophic plate counts and drinking-water safety: the significance of HPCs for water quality and human health*. London: IWA Publ.

Yu, C. L., Louie, T. M., Summers, R., Kale, Y., Gopishetty, S., & Subramanian, M. (2009). Two distinct pathways for metabolism of theophylline and caffeine are coexpressed in Pseudomonas putida CBB5. *Journal of Bacteriology*, *191*(14), 4624–4632. https://doi.org/10.1128/JB.00409-09