Detecting pathogenic *Yersinia enterocolitica* in surface water from the Grand River watershed: An evaluation and comparison of methods

by

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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ABSTRACT

Yersinia enterocolitica are potentially pathogenic bacteria transmitted through the fecal-oral route. Typical disease symptoms include those associated with gastrointestinal disease, although infection can also lead to more serious and invasive illnesses, particularly in sensitive populations. Previous surveys have detected *Y. enterocolitica* in surface water in various parts of the world, and studies have found drinking untreated water to be a possible risk factor for *Y. enterocolitica* infection.

Methods available for the detection of *Y. enterocolitica* have been developed primarily for food and clinical samples and have not been tested extensively with water. More commonly used methods include culture-based isolation of *Yersinia* spp. and polymerase chain reaction (PCR)-based detection of *Y. enterocolitica*. Reports suggest that culture-based methods available for the isolation of *Y. enterocolitica* may not be effective for environmental samples. Strain isolation using culture-based methods is important, so that further subtyping information can be obtained for epidemiological investigations. In contrast, PCR-based detection is more rapid, of higher throughput, can be highly specific and can target pathogenic strains within a species.

The overall objective of this work was to evaluate culture-based and PCR-based methods for the detection of *Y. enterocolitica* in water, and to examine its prevalence in the Grand River watershed in Southwestern Ontario, Canada. Surface water in this watershed is used to provide all or part of the drinking water for approximately 500,000 people, as well as for recreational purposes. It is also one of the most heavily impacted watersheds in Canada by both agricultural and urban activities.

Culture-based studies compared two selective agars and four enrichment broths. Results showed that Cefsulodin-Irgasan-Novobiocin (CIN) agar and modified tryptic soy broth (mTSB) had greater potential for recovering *Y. enterocolitica* from surface water. Consequently, enrichment in mTSB followed by growth on CIN agar was used to isolate

Yersinia from the Grand River. *Yersinia* strains were isolated from 52 out of 200 (26 %) surface water samples collected over a 17-month period. No seasonal trends were observed in isolation rates. Species isolated were typically considered to be non-pathogenic species, although recent evidence suggests they may have potential virulence to humans. The majority of these strains have been found by other groups in surveys of aquatic environments.

PCR methods developed targeted two *Y. enterocolitica* virulence genes: the *ail* gene, located in chromosomal DNA; and the *yad A* gene, located on a virulence plasmid. In surface water collected from the Grand River, the *ail* gene target was detected in 121 samples out of 319 (38 %) over a 29-month period and the *yadA* gene target was detected in 44 samples out of 206 (21 %) over a 20-month period. Both genes were detected more frequently when the water temperatures were colder. PCR-based studies conducted were quantitative, which has not previously been done with water samples. The median and maximum concentrations in samples positive for the *ail* gene were 40 and 2,000 cells/100 mL, and in samples positive for the *yadA* gene were 32 and 3,276 gene copies/100 mL, respectively.

Overall results demonstrated that culture-based methods are less sensitive than PCR-based detection methods for specific detection of pathogenic *Y. enterocolitica*, suggesting that previous culture-based surveys may have underestimated their potential prevalence. Furthermore, potentially pathogenic *Y. enterocolitica* may be present in the Grand River watershed. While *Y. enterocolitica* is relatively easily inactivated by traditional disinfection methods used in drinking water treatment processes, it is possible their presence poses a concern for recreational users and individuals drinking untreated water. This study suggests that further investigation is necessary to evaluate possible health risks associated with the occurrence of potentially pathogenic *Y. enterocolitica* in the Grand River.

This work assists with the development of methods and information gathering for an emerging waterborne pathogen that has not been surveyed in the Grand River watershed, nor quantitatively surveyed in any water previously. Findings provide important information for drinking water providers and public health investigations.

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1 Introduction

Yersinia enterocolitica is an enteric bacterium that has been identified as an emerging waterborne pathogen (Theron *et al*., 2002; Sharma *et al*., 2003). Pathogenic *Y. enterocolitica* have been implicated in a few cases of waterborne illness in humans (Lassen, 1972; Keet, 1974; Highsmith *et al.*, 1977; Christensen, 1979; Tacket *et al.*, 1985; Thompson *et al.*, 1986) and case-controlled studies have identified drinking untreated water as a risk factor for *Y. enterocolitica* infection (Ostroff *et al.*, 1994; Leclerc *et al.*, 2002). Given that *Y. enterocolitica* is associated with animal hosts and shed in the feces of infected animals, it is reasonable to assume that waterborne transmission of *Y. enterocolitica* may be occurring, similar to other agriculturally important microbial pathogens. Nonetheless, there have been few studies reporting on the prevalence of pathogenic *Y. enterocolitica* strains in water and none that have enumerated *Y. enterocolitica* in water.

A major challenge to surveying water for the prevalence of *Y. enterocolitica* is detecting the organism. Commonly used detection methods for bacteria in various types of samples include culture-based and polymerase chain reaction-based (PCR-based) methods, although the latter has not been used extensively for detecting *Y. enterocolitica* in water samples. *Y. enterocolitica* have been isolated using culture-based methods from surface water samples from a variety of geographic locations around the world. In these surveys, isolation rates have been low. Recently, food and animal samples have been surveyed for *Y. enterocolitica* using both culture-based and PCR-based methods. Only one such comparison study has been conducted with water samples. Findings consistently demonstrated that PCR-based detection rates were significantly higher than culture-based detection rates, leading researchers to report that culture-based methods available for isolating *Y. enterocolitica* are not effective. This may be particularly true for environmental samples in which pathogens concentrations may be low relative to other indigenous organisms. This research evaluates and compares culture-based and PCR-based methods for detecting *Y. enterocolitica* is surface waters. Using PCR-based methods, *Y. enterocolitica* were not only detected, but also enumerated, which has never previously been done in surface water samples.

This investigation was conducted through a Canadian Water Network (CWN)-funded project whose aim was to survey pathogen occurrence in two watersheds, including the Grand River watershed in Southern Ontario. This CWN project was associated with an epidemiological study conducted by the Public Health Agency of Canada to monitor pathogen occurrence in water, food and humans within a certain geographical area. This project was entitled C-EnterNet, and Waterloo region was selected as the sentinel geographic site for the project. Waterloo region is located in the central portion of the Grand River watershed. A central component of these projects was to evaluate and implement detection methods for pathogens in source waters. Ultimately, the projects aimed to evaluate and standardize methods that could be used for routine monitoring. The list of pathogens for the C-EnterNet project included bacteria, viruses and protozoa. *Yersinia enterocolitica* was among the bacteria included in the study.

The Grand River watershed spans an area close to 7,000 km^2 and is the largest watershed in Southern Ontario. The watershed is used for several varied purposes. It is a drinking water source, an aquatic habitat, and is used for industrial, commercial, agricultural and recreational activities (Cooke, 2006). The total population living in the watershed is over 800,000 people, with projections to grow 37% over the next 20 years (Dorner *et al.*, 2004; Bellamy *et al.*, 2005). There are 26 wastewater treatment plants that discharge into the Grand River and its tributaries. The predominant land use is agriculture (Bellamy *et al.*, 2005) with close to 80% of the land being farmed (Dorner *et al.*, 2004; Cooke, 2006). Livestock found in the area (listed in order of decreasing prevalence) include chickens, pigs, cattle, sheep and horses (Dorner *et al.*, 2004).

Drinking water supplies originate from both groundwater and/or surface water sources. In the central portion of the watershed, there are three drinking water treatment plants. Of those three plants, the Mannheim Water Treatment Plant is located furthest north and part of its treated Grand River water is blended with groundwater before entering the distribution system, and the remainder is applied to an aquifer storage and recovery system for later introduction into the distribution system. The other two plants, Brantford and Oshwegan, only draw Grand River water (Dorner *et al.*, 2004; Dorner *et al.*, 2007). The water quality is

also poorest in this central portion of the Grand River, including the major tributaries: Canagagigue Creek, Conestogo River and the lower Speed River (Cooke, 2006). While the watershed is not regularly monitored for pathogens, a study by Dorner *et al.* (2007) monitored 36 sampling locations in the watershed and detected pathogens including *E. coli* O157:H7, human enteric viruses, (both by culture-based methods), *Campylobacter* spp., *Giardia* spp., and *Cryptosporidium* spp. (by PCR-based methods). The Grand River watershed has never previously been monitored for *Y. enterocolitica*.

There were four major objectives for this work:

i. To evaluate culture-based methods for the isolation of *Y. enterocolitica* from surface water,

ii. To evaluate quantitative PCR (Q-PCR)-based methods for the detection of *Y. enterocolitica* in surface water,

iii. To examine the occurrence of *Y. enterocolitica* in surface water from the Grand River watershed using both culture-based and PCR-based methods, and

iv. To compare the results of the culture-based and PCR-based surveys of the Grand River.

1.1 Thesis Structure

This thesis is organized into six chapters as described below. Experimental findings are summarized in two chapters (Chapters 3 and 4), which will be refined and submitted to peer-reviewed journals.

A literature review is provided in Chapter 2. The review addresses three key points: (1) *Y. enterocolitica* as a potential waterborne pathogen and its prevalence in water,

(2) culture-based detection methods available for isolating *Y. enterocolitica*, and (3) molecular biological-based detection methods for detecting *Y. enterocolitica*.

Culture-based studies are outlined in Chapter 3. Culture-based isolation methods for *Y. enterocolitica* were evaluated. Different enrichment methods commonly used for isolating *Y. enterocolitica* were compared. Results showed that one of the methods tested showed the most potential for recovery of *Yersinia*. Using this method, *Yersinia* strains were isolated from surface water samples from the Grand River watershed.

Molecular biological-based studies are outlined in Chapter 4. Quantitative polymerase chain reaction (Q-PCR) methods were used to detect two *Y. enterocolitica* virulence genes in surface water samples. Standard curves were developed for PCR-based assays, and the specificity of primers and probes and DNA extraction efficiency were each evaluated. Subsequently, methods were used to analyze surface water samples from the Grand River for the occurrence of both *Y. enterocolitica* virulence genes.

Key results and conclusions are summarized in Chapter 5. In this chapter, the results of the culture-based and PCR-based surveys for *Y. enterocolitica* are also compared. The thesis concludes with Chapter 6, which outlines some recommendations for future work.

2 Background

2.1 Waterborne Pathogens

Waterborne diseases are predominantly caused by enteric pathogenic microorganisms (Ashbolt, 2004), which include bacteria, viruses and protozoa. Typically, these pathogens originate from the feces of animals or humans carrying the organism and are introduced to water through disposal of waste water or agricultural waste and runoff (Theron *et al.*, 2002). Humans may subsequently ingest contaminated water or inhale contaminated aerosols through drinking water, foods washed with water, or during recreational use of water. Enteric pathogens cause gastrointestinal illness as well as a variety of other diseases. While incidents of waterborne outbreaks are relatively rare in developed countries, it is suspected that enteric waterborne pathogens are responsible for low-level incidences of disease (Theron *et al.*, 2002). Furthermore, according to a recent study, for every case of enteric illness reported in Canada, there are approximately 314 unreported cases (Majowicz *et al.*, 2004). Similar trends have been reported for other developed countries demonstrating that enteric disease remains a significant concern in the developed world (Payment *et al.*, 2006).

Changes in the developed world are influencing the occurrence and impact of waterborne pathogens. Improvements in health care have increased the average age of the population, as well as the number of immunocompromised individuals in the population, and hence increased the number of individuals who are more susceptible to waterborne disease. Furthermore, increased and more concentrated agricultural inputs and urban growth have increased the potential for contamination of our water (Theron *et al.*, 2002) and increased the challenges of water treatment facilities to deliver safe, high quality water.

Huck and Coffey (2004) highlight several elements necessary for providing safe drinking water, the first of which is a good source. Identifying good sources requires characterization of source waters through monitoring, including surveillance for pathogens. Furthermore, to evaluate the risks to human health posed by the presence of pathogens in water, it is necessary to understand their ecology and physiology (Szewzyk *et al.*, 2000). For example,

the occurrence of waterborne disease depends on the survival of pathogens in water as well as their infectious dose (Leclerc *et al.*, 2002). Therefore, evaluating the occurrence of pathogens in waters alone is not sufficient. It is essential to also characterize waterborne pathogens. These challenges become increasingly complicated when considering emerging waterborne pathogens.

Emerging pathogens include pathogenic organisms that: (1) have newly appeared in a population; (2) have always existed, but their prevalence has increased over recent years; or (3) have newly recognized routes of transmission (Theron *et al.*, 2002). In some cases the infectious agent may have been present historically, but due to advances in detection methods is only now being recognized as a relevant pathogen. Emerging pathogens present a significant health concern (Sharma *et al.*, 2003) and demand not only further investigation, but also sustained research efforts.

2.2 *Yersinia enterocolitica*

Yersinia enterocolitica is an emerging waterborne bacterial pathogen (Theron *et al.*, 2002; Sharma *et al.*, 2003). The bacteria are rod-shaped, non-spore forming, Gram-negative, facultatively anaerobic and will grow at a wide range of temperatures, from 4°C to 43°C (Wanger, 2007). *Y. enterocolitica* is a member of the genus *Yersinia*. There are two species within the genus that contain potentially pathogenic strains transmitted by the fecal-oral route: *Y. enterocolitica* and *Y. pseudotuberculosis* (Marenne *et al.*, 2004). However, *Y. enterocolitica* is more commonly isolated from patients (Bissett *et al.*, 1990). Also a member of the genus *Yersinia* is the infamous *Y. pestis*, which is the causative agent of bubonic plague. *Y. pestis* is primarily contained within a sylvatic reservoir and is not transmitted by water (Stenseth *et al.*, 2008).

It is important to note that there are several *Yersinia* spp. that are highly similar to *Y. enterocolitica*. Between 1980 and 1988, using DNA homology techniques, the species *Y. enterocolitica* was gradually divided as seven new separate species were distinguished: *Y. intermedia*, *Y. frederiksenii*, *Y. kristensenii*, *Y. aldovae*, *Y. rohdei*, *Y. bercovieri* and

Y. mollaretii (Schiemann, 1990; Sulakvelidze, 2000; Wanger, 2007). These re-classified species of *Yersinia* are often referred to as *Y. enterocolitica*-like spp. and are traditionally considered to be non-pathogenic species. However, this has been brought into question as strains from all of the *Y. enterocolitica*-like species, except *Y. aldovae*, have been isolated from patients displaying symptoms of gastrointestinal disease (Sulakvelidze, 2000). The species *Y. enterocolitica* is divided into six biogroups: 1A, 1B, 2 through 5; and into more than 50 serogroups (Wauters *et al.*, 1987). Pathogenicity has traditionally been associated with certain biogroups and serogroups, specifically: 1B/O:8, 2/O:5, 27, 2/O:9, 3/O:3 and 4/O:3 (Fredriksson-Ahomaa *et al.*, 2006).

Y. enterocolitica is traditionally associated with foodborne illness, typically related to pork or dairy products (Jones, 2007). Pigs have been found to be a major reservoir of human pathogenic strains (Fredriksson-Ahomaa *et al.*, 2006). It is only more recently that the organism has become recognized for its waterborne transmission potential and hence referred to as an emerging waterborne pathogen (Szewzyk *et al.*, 2000; Sharma *et al.*, 2003). Studies have reported the occurrence of *Yersinia* in environmental waters (Fredriksson-Ahomaa *et al.*, 2003), however, the majority of isolates from these studies were reported to be non-pathogenic strains (Fredriksson-Ahomaa *et al.*, 2003).

2.2.1 *Y. enterocolitica* Infection in Humans

Illness caused by *Y. enterocolitica* infection is referred to as yersiniosis and there are a wide variety of disease outcomes that can result. Typical disease symptoms include those associated with gastrointestinal disease, such as fever, abdominal pain and diarrhea (Bottone, 1997; Jones, 2007). However, the consequences of infection can be very serious, particularly in sensitive populations like the young, the elderly and the immunocompromised (Sharma *et al.*, 2003; Wanger, 2007). Infection causes a wide range of clinical symptoms depending on factors such as the patient's age and health, as well as the serotype of the strain. Symptoms range from mild self-limiting diarrhea (gastroenteritis) to inflammation of the small intestine (acute terminal ileitis) or inflammation of the mesenteric lymph nodes (acute mesenteric lymphademitis) that can lead to pseudoappendicitis (Bottone, 1997; Wanger, 2007). Infants

and young children usually acquire gastroenteritis, while older children and young adults are more likely to experience acute terminal ileitis (Bottone, 1997) or pseudoappendicitis (Fredriksson-Ahomaa *et al.*, 2006). Postinfection manifestations sometimes occur in adults, including reactive arthritis, inflammation of fat cells under the skin (erythema nodosum), inflammation of blood vessels in the kidneys (glomerulonephritis) and inflammation of the muscular tissue in the heart (myocarditis) (Bottone, 1997; Fredriksson-Ahomaa *et al.*, 2006). Although rare, certain patients may be predisposed to severe complications like septicemia (occurs when bacteria get into the bloodstream) (Fredriksson-Ahomaa *et al.*, 2006), which can then lead to other serious developments (Bottone, 1997). Patients susceptible to septicemia include the young, the elderly and the immunocompromised, in particular patients suffering from diseases associated with iron overload, cancer, liver disease and patients on steroid therapy (Wanger, 2007).

Incubation periods for *Y. enterocolitica* have been reported to range from as little as 24 to 48 hours (Jones, 2007) to 3 to 7 days (Hunter, 1997). The clinical course will run approximately 3 to 28 days for infants and for 7 to 14 days for adults (Bottone, 1997). However, patients may carry the organism in their gastrointestinal tracts for several months after symptoms resolve (Wanger, 2007). The infectious dose for a healthy human is reported to be very high, around 10^9 organisms (Morris *et al.*, 1976; Hunter, 1997).

The serogroup of the *Y. enterocolitica* strain will also impact the severity of disease symptoms (Fredriksson-Ahomaa *et al.*, 2003; Wanger, 2007). For example, infections with *Y. enterocolitica* O:8 have been found to cause more serious disease symptoms than *Y. enterocolitica* O:3 or O:9 (Bottone, 1997). And, although rare, infection leading to inflammatory bowel disease is more commonly associated with *Y. enterocolitica* from serogroup O:3 (Wanger, 2007).

In several European countries, yersiniosis is the third most common enteric disease (Fredriksson-Ahomaa *et al.*, 2007). According to a report by the United States Foodborne Diseases Active Surveillance Network investigating selected sites in the United States, approximately 0.3 to 0.8 culture-confirmed *Y. enterocolitica* infections per 100,000 persons

occurred in 1999 (Centers for Disease Control and Prevention, 2000). In a later study, the incidence rate observed across ten states was determined to be 0.36 cases per 100,000 persons in 2005 (Centers for Disease Control and Prevention, 2006).

In Canada, illness caused by *Y. enterocolitica* is not a notifiable disease (Public Health Agency of Canada, 2007) and national incidence rates are not available. However, there is some data available from studies investigating regions of Canada. In Ontario, over the course of 5 years spanning 1997 through 2001, the annual average incidence rate of disease caused by *Yersinia* spp. was 3.0 cases per 100,000 persons (Lee *et al.*, 2003). This compared to rates of 42.3 and 3.7 cases per 100,000 persons for disease caused by *Campylobacter* and verotoxigenic *E. coli*, respectively. Authors of this study did note that incidence of *Yersinia*-borne disease appeared to drop over the time period investigated. More recently, the occurrence of *Yersinia* infections has been monitored in the Region of Waterloo (Ontario) since June 2005. C-EnterNet is an enteric disease surveillance pilot project initiated by the Public Health Agency of Canada that monitors pathogen occurrence in humans, food, water, and food animals. The C-EnterNet 2006 annual report documented 17 cases of *Y. enterocolitica* infection in the sentinel site study area: the Regional Municipality of Waterloo (Public Health Agency of Canada, 2007). Disregarding one case that was determined to be travel related, the endemic incidence rate of *Y. enterocolitica* infection was calculated to be 3.3 cases per 100,000 person-years. In the same report, endemic incidence rates for *Campylobacter*, *Salmonella* and *Cryptosporidium* infections were 22.4, 12.4 and 3.1 cases per 100,000 person-years, respectively.

2.2.2 Routes of Transmission

Y. enterocolitica is transmitted via the fecal-oral route, which means that individuals become infected through ingesting food or water contaminated with fecal matter containing the bacteria. *Y. enterocolitica* is more commonly associated with foodborne illness, including pork products. Pigs are the major reservoir of human pathogenic strains (McNally *et al.*, 2004; Fredriksson-Ahomaa *et al.*, 2006) and are the only animals from which pathogenic strains have frequently been isolated (Fredriksson-Ahomaa *et al.*, 2006). Studies have

detected pathogenic *Y. enterocolitica* in both pig tissues (Doyle *et al.*, 1983), particularly in pig tonsils (Fukushima *et al.*, 1983), as well as in pig feces (Fredriksson-Ahomaa *et al.*, 2003; McNally *et al.*, 2004). While it has also been detected in other animals, including cattle (McNally *et al.*, 2004), sheep (McNally *et al.*, 2004), goats (Arnold *et al.*, 2006), and chickens (Kechagia *et al.*, 2007), strains associated with humans illness have not usually been isolated from these hosts.

Although, *Y. enterocolitica* is primarily considered a foodborne pathogen, pathogenic strains have not been frequently isolated from foods (Fredriksson-Ahomaa *et al.*, 2006). In fact, most cases of yersiniosis are sporadic and a source is rarely identified (Bottone, 1997). This is often attributed to difficulties associated with isolating the organism, in particular, pathogenic strains of the organism (Fredriksson-Ahomaa *et al.*, 2003). Pathogenic strains of *Y. enterocolitica* are, however, being detected more frequently in raw pork products since new molecular-based detection methods, like PCR, have become more widely available (Fredriksson-Ahomaa *et al.*, 2006). This has strengthened the suspected link between human illness and the ingestion of contaminated pork products. In contrast, there have been limited studies using molecular-based detection methods evaluating the occurrence of *Y. enterocolitica* in environmental waters. Consequently, this route of transmission has not yet been thoroughly investigated.

2.2.2.1 Survival in the Environment

Although *Y. enterocolitica* thrives in the intestines of warm-blooded animals, it also survives very cold temperatures and is considered psychotrophic, unlike other members of the family Enterobacteriaceae (Fredriksson-Ahomaa *et al.*, 2003), which includes many enteric pathogens. In preliminary tests, Harvey *et al.* (1976) demonstrated that *Y. enterocolitica* could survive in refrigerated water for 6 months. Another study found that the viable cell count for *Y. enterocolitica* increased over the first 72 hours of incubation in sterile distilled water at temperatures of 4, 25 and 37°C (Highsmith *et al.*, 1977). After 72 hours, viable cell counts leveled off but did not decrease, indicating the cells continued to survive for 216 hours.

Karapinar and Gonul (1991) compared growth and survival of *Y. enterocolitica* to *E. coli* in sterilized stream water at 4°C and found significant differences between these organisms. They demonstrated that *Y. enterocolitica* grew and survived better than *E. coli*. *Y. enterocolitica* viable cell counts increased during the first 3 weeks and at the end of the study, 64 weeks, viable cell counts corresponded to the level of the initial inoculum. In contrast, *E. coli* viable cell counts began to decrease after 1 week and after 13 weeks no viable cells were detected. Similar trends were observed when *Y. enterocolitica* and *E. coli* cells were mixed together.

Terzieva and McFeters (1991) also compared survival rates for *Y. enterocolitica* and *E. coli*, but did so in stream water that was not sterilized and changed the water daily. To do so, they used a membrane diffusion chamber. Tests were conducted at 6°C and 16°C. Survival rates were based on viable cell counts over a 14 day period. Although, survival rates for each organism were similar during the first 7 days, *Y. enterocolitica* showed greater persistence than *E. coli* at both temperatures over the 14 day study. However, unlike the previously discussed study, *Y. enterocolitica* growth was not observed.

A recent study followed the survival of several different bacterial pathogens in sterile distilled water over the course of many years (Liao *et al.*, 2003). Bacteria studied were strains isolated from fruits and vegetables. Experiments were conducted at room temperature in the dark. Two *Y. enterocolitica* strains were tested and both were found to survive for at least 5 years. For comparison, other bacterial strains were also able to survive for long periods, including *Salmonella* spp. for 5 years and *Pseudomonas* spp. for 12 to 16 years.

Consequently, it seems likely that *Y. enterocolitica* are likely to survive in surface waters. Furthermore, these observations suggest that *E. coli* may not provide a reliable indicator for *Y. enterocolitica* contamination in surface waters.

2.2.2.2 Effectiveness of Drinking Water Disinfection Processes

Y. enterocolitica appears to be inactivated by many traditional disinfection methods used in drinking water treatment practices, such as chlorination, UV irradiation and ozonation.

Y. enterocolitica has been described to have a similar sensitivity to chlorination as *E. coli* (American Water Works Association, 2006). However, studies show that *Y. enterocolitica* may be more resistant to chlorination than *E. coli*. In a study that evaluated the sensitivity of *Y. enterocolitica* to chlorination, a chlorine dose of 1 mg/L with an exposure time of 30 min (20°C, pH 7) was needed to achieve a 1.1-log (92%) reduction in viable cells (Paz *et al.*, 1993). No residual chlorine concentrations were provided, and hence no CT values could be calculated. In contrast, Bansal *et al.* (2000) reported a 1-log (90 %) reduction in viable *Y. enterocolitica* cell counts with chlorine doses of 1 mg/L over an exposure time of 20 s (25°C, pH 7.2) and a 4-log (99.99 %) decrease in viable counts with doses of 10 mg/L over 20 s (25°C, pH 7.2). (Again, no residual chlorine concentrations were provided to allow calculation of CT values.) The *Y. enterocolitica* strains tested above did not possess the pYV plasmid, a plasmid considered critical to pathogenicity. Interestingly, this study showed that *Y. enterocolitica* containing the pYV virulence plasmid (pYV^+), were more resistant to chlorine than *Y. enterocolitica* lacking the plasmid (pYV^-). This phenomenon was only observed when both pYV^+ and pYV^- strains were grown at 25°C. Conversely, when strains were grown at 37°C, no difference in susceptibility was observed.

A study by LeChevallier *et al.* (1985) compared chlorine susceptibilities of *Y. enterocolitica* to *E. coli*. Chlorine doses of 1.07 mg/L (4°C, pH 6.5-7) were needed to produce >90 % injured *Y. enterocolitica* compared to doses of only 0.33 to 0.38 mg/L to produce >90 % injured enterotoxigenic *E. coli* and coliform bacteria, respectively. Also, at a chlorine dose that caused coliform bacteria to be more than 90% injured, *Y. enterocolitica* cells were less than 20% injured. It is important to note that this study evaluated injury, and not viability. Percent injury was determined by calculating the percent difference between viable cell counts on selective and non-selective agar medium. This study was designed to reproducibly produce injured bacteria and not to evaluate inactivation by chlorine.

Overall, *Y. enterocolitica* is sensitive to chlorination, but appears to be less sensitive than *E. coli*. Sensitivity to chlorination may also depend on the presence of the pYV plasmid. However, there are insufficient studies on *Y. enterocolitica* inactivation by chlorine, in particular studies that report residual chlorine levels. For this reason, it is difficult to make direct comparisons on the effectiveness of chlorination on *Y. enterocolitica* and other microorganisms.

In contrast, *Y. enterocolitica* appears to be more sensitive to UV irradiation than *E. coli*. Butler *et al.* (Butler *et al.*, 1987) compared low pressure UV irradiation (254nm) doses required to inactivate *Y. enterocolitica* and *E. coli*. The doses necessary to achieve a 3-log (99.9%) reduction were 2.7 mJ/cm^2 for *Y. enterocolitica* and 5.0 mJ/cm^2 for *E. coli*. In this same study, they found no difference between *Y. enterocolitica* with and without the pYV virulence plasmid. *Y. enterocolitica* has been shown to exhibit similar sensitivity to ozonation as *E. coli* (Restaino *et al.*, 1995).

Interestingly, it has been demonstrated that *Y. enterocolitica* can be ingested by freshwater protozoa and in doing so may evade inactivation by chlorination (King *et al.*, 1988). However, the significance of this phenomenon to human health has yet to be thoroughly investigated.

2.2.2.3 Waterborne Illness

Drinking untreated water has been found to be a risk factor for *Y. enterocolitica* infection (Saebo et al, 1994; Ostroff *et al.*, 1994; Satterthwaite *et al.*, 1999), suggesting that *Y. enterocolitica* may be causing waterborne disease. Moreover, incidents of waterborne disease caused by *Y. enterocolitica* have been documented.

A frequently cited instance of what was thought to be a yersiniosis outbreak due to contaminated water occurred at a mountain resort during which an estimated 750 individuals became ill (Highsmith *et al.*, 1977). *Y. enterocolitica* was detected in the well water and was consequently implicated in the outbreak. Subsequent subtyping of the isolates identified them

to be non-pathogenic (Leclerc *et al.*, 2002). However, evidence is accumulating that suggests *Y. enterocolitica* subtyping analysis may not provide a reliable indication of pathogenicity (Bissett *et al.*, 1990; Grant *et al.*, 1998; Tennant *et al.*, 2003; Thoerner *et al.*, 2003; Bhagat *et al.*, 2007).

There have been confirmed waterborne *Y. enterocolitica* infections, including one incident in Ontario. A family outbreak of *Y. enterocolitica* infection was determined to be waterborne. *Y. enterocolitica* biotype 4, serotype O:3 was isolated from two family members with disease symptoms and from well water from the family residence (Thompson *et al.*, 1986). Other studies have also reported waterborne disease cases of *Y. enterocolitica* infection. In each case, the *Y. enterocolitica* serotype isolated from a human case matched the serotype isolated from well water (Lassen, 1972) or mountain stream water (Keet, 1974) used as a drinking water source by the case patients, or from well water used to prepare baby food for the case patient (Christensen, 1979).

Yersiniosis has also been associated with the ingestion of food washed with contaminated water. In a case-control study of an outbreak of 50 cases of gastrointestinal illness, it was found that the majority of patients had eaten a certain brand of tofu. Subsequent analysis isolated *Y. enterocolitica* serotype O:8 from patients, from the tofu and also from untreated water used in the tofu manufacturing plants (Tacket *et al.*, 1985).

While in the above cases a route of transmission was usually established, the causative agent and the route of transmission for most enteric disease incidents in Canada are rarely identified (Lee, 2003), indicating a need to improve surveillance and reporting for disease occurrence and transmission routes.

2.3 Watershed Monitoring

Traditionally, monitoring practices have utilized indicator bacteria to detect fecal matter and hence the potential for the presence of pathogenic enteric microorganisms. Unfortunately, recent studies are revealing that pathogenic organisms, including *Y. enterocolitica*, may be

present even when indicator bacteria are not detected (Theron *et al.*, 2002). Consequently, unless an alternative and more reliable indicator is found, monitoring for the presence of specific pathogenic bacteria is more reliable and may provide more useful information. Source water monitoring programs in Canada are limited and monitoring approaches are not standardized (Payment *et al.*, 2006).

2.4 Detecting Bacteria in Water

Commonly used methods available to detect bacteria in water can generally be placed into two categories: culture-based methods or molecular biological-based methods. Culture-based methods involve growing bacteria from a water sample in the laboratory, isolating the target bacteria, and then conducting tests to confirm the identity of the isolated bacteria. Molecular biological-based methods involve the direct detection of nucleic acids or more specifically deoxyribonucleic acid (DNA) sequences associated with the target bacteria.

2.4.1 Culture-based Isolation Methods

Traditionally, bacteria have been detected in samples through culture-based isolation techniques. Individual strains of the target bacteria are isolated from the sample through various culturing techniques. Typically, methods for isolating any target bacteria from water involve the following steps: concentration, enrichment, isolation and identification (Theron *et al.*, 2002).

First, the water sample is concentrated, usually by filtration or centrifugation. An optional pre-enrichment step is sometimes employed at this stage, which involves growing the bacteria in a non-selective broth. The use of a non-selective broth has been recommended to improve the recovery of bacteria from the environment because these bacteria can be stressed and hence vulnerable to selective agents (Koster *et al.*, 2003). Next, the sample is introduced into a selective growth media, usually referred to as an enrichment medium, which contains additives that either promote the growth of the target organism and/or inhibit the growth of non-target organisms. Subsequently, bacteria grown in the enrichment medium, referred to as

the enriched sample, are plated onto a selective growth agar. Under some circumstances, the concentrated sample can be applied directly to the selective growth agar, referred to as direct isolation. The agar medium, like the enrichment medium, may also contain additives which select for the growth of the target organism. Plating steps produce individual colonies, and hence pure strains, to be isolated. Furthermore, in addition to selective agents, the agar may contain additives that cause the target organism to display distinct colony morphologies different from other organisms. Such agar is referred to as differential medium. Once a presumptive target bacterial strain has been isolated, it must be tested to confirm that it possesses the characteristics of the target organism. For example, it may be tested for its ability to metabolize certain sugars.

Culture-based detection methods are highly laborious and take a long time to acquire results as they require time to grow the bacteria in the laboratory, then to conduct the necessary confirmation tests (Toze, 1999). Furthermore, some bacteria can exist in a viable but nonculturable state making culture-based isolation unfeasible (Theron *et al.*, 2002). While quantifying the number of pathogens in a sample using culturing methods is possible, it is very challenging (Toze, 1999).

2.4.1.1 Isolating *Y. enterocolitica* from the Environment

There are three major problems encountered when attempting to isolate *Y. enterocolitica* from environmental samples. First, there tends to be a very high concentration of background organisms (non-*Yersinia*) in environmental samples and *Y. enterocolitica* grow poorly in competition with other organisms (Schiemann *et al.*, 1984; Calvo *et al.*, 1986b). In fact, results from one study suggest that *Y. enterocolitica* growth may be impeded by bacteriocin-like agents (agents intended to kill closely related species) produced by *Y. frederiksenii*, *Y. kristensenii* and *Y. intermedia* (Calvo *et al.*, 1986a). Second, methods are not selective for pathogenic strains of *Y. enterocolitica* and will also isolate non-pathogenic strains of *Yersinia* (both non-pathogenic *Y. enterocolitica* as well as the other non-pathogenic *Yersinia* spp.), which appear to be prevalent in the environment (Fredriksson-Ahomaa *et al.*, 2003). Third, a virulence plasmid, which imparts certain phenotypic characteristics to

pathogenic strains that are used to identify these strains, is sometimes lost during culturing steps (Blais *et al.*, 1995). It is important to be aware of these challenges when evaluating and implementing *Y. enterocolitica* isolation methods. Many different methods have been published on the isolation of *Y. enterocolitica* from samples, the majority of them developed for isolation from clinical or food samples.

Enrichment

Yersinia are known to grow slowly and compete poorly with other organisms (Schiemann *et al.*, 1984; Calvo *et al.*, 1986b), which creates a challenge when trying to isolate *Yersinia*. To improve recovery of low levels of *Y. enterocolitica* in samples, various antimicrobial agents as well as bile salts have been used in enrichment broths to inhibit the growth of non-*Yersinia* organisms. Also, because *Yersinia* have been demonstrated to survive cold temperatures (Highsmith *et al.*, 1977; Karapinar *et al.*, 1991), cold incubations have been proposed to slow the growth of other non-*Yersinia* organisms in the sample that do not adapt as well to cold temperatures (Schiemann *et al.*, 1984). Table 2-1 is adapted from a table in a review by Fredriksson-Ahomaa and Korkeala (2003) and lists enrichment methods that have been developed for isolating *Y. enterocolitica*.

Cold incubations at 4°C for extended periods of time in phosphate-buffered-saline (PBS) have been used to isolate *Y. enterocolitica* (Johnson, 1998; Fredriksson-Ahomaa *et al.*, 2003). Many surveys for *Y. enterocolitica* in water have used some type of cold enrichment prior to isolation (Fukushima *et al.*, 1984; Massa *et al.*, 1988; Arvanitidou *et al.*, 1994). However, while the cold incubations did successfully isolate *Yersinia* strains, the majority of the isolates recovered in these studies were not *Y. enterocolitica*, but rather *Y. enterocolitica*-like strains. Cold incubations have been criticized because there are other bacteria in natural samples, including *Y. enterocolitica*-like spp. that can also resist cold temperatures (Fredriksson-Ahomaa *et al.*, 2003).

Table 2-1: Enrichment methods for *Y. enterocolitica* (adapted from Fredriksson-Ahomaa and Korkeala (2003))

Preenrichment	Enrichment	Serotypes targeted	Reference(s)
	PBS, 4°C, 2 weeks		(Johnson, 1998)
	PSB, 22°C, 6-10 days		(Weagant *et al.*, 1983)
	PSB, 10°C, 10 days		(Weagant *et al.*, 2001)
PBS, 4°C, 14 days	MRB, 22 °C, 4 days	O:3, O:9	(Schiemann, 1982)
YER, 4°C, 9 days	BOS, 22 °C, 5 days	O:3, O:8	(Schiemann, 1982)
TSB, 22°C, 1 day	BOS, 22°C, 3-7 days	O:3, O:8	(Schiemann, 1983b)
	ITC, 25°C, 2 days	O:3	(Wauters *et al.*, 1988)
	mTSB, 12°C, 2-3 days		(Bhaduri *et al.*, 1997; Bhaduri *et al.*, 1997)
	LB-BSI, 12°C, 3 days		(Hussein *et al.*, 2001)

Note: PBS = Phosphate buffered saline, PSB = Sorbitol-bile salts broth with peptone, MRB = Modified Rappaport broth, YER = Yeast extract-rose bengal broth, BOS = Bile-oxalate-sorbose broth, ITC = Irgasan-tricarcillan-chlorate, mTSB = Modified tryptic soy broth, LB-BSI = Luria-Bertani-bile salts-irgasan

Weagant and Kaysner (1983) compared sorbitol-bile salts broth with and without the addition of peptone (PSB and SB) at 22°C and found the addition of peptone significantly improved growth rates for *Y. enterocolitica* in the broth and also improved recovery of *Y. enterocolitica* from spiked water samples. A study by Schiemann and Olson (1984) suggested that incubation at 15°C would serve the purpose of allowing *Y. enterocolitica* to compete equally with other bacteria while decreasing the incubation time required compared with cold incubations carried out a 4°C. Weagant and Feng (2001) later proposed using a 10°C incubation period for enrichment of *Y. enterocolitica* in PSB as part of the online Bacteriological Analytical Manual for the US Food and Drug Administration (USFDA) (Johnson, 1998).

Modified rappaport broth (MRB) is particularly good at recovering *Y. enterocolitica* serotype O:3, but tends to be inhibitory towards serotype O:8 (Schiemann, 1982). Preenrichment in PBS followed by enrichment in MRB (PBS/MRB) has been used to isolate *Y. enterocolitica* from water samples in studies by Gonul and Karapinar (1991) and Brennhovd *et al.* (Brennhovd *et al.*, 1992). These surveys found *Yersinia* in 6% and 4% of the samples, respectively. Schiemann (1982) developed and compared several enrichment methods and

concluded that preenrichment in yeast extract-rose bengal (YER) broth followed by enrichment in bile-oxalate-sorbose (BOS) gave better recoveries than the PBS/MRB method and also reduced the pre-enrichment incubation time. Subsequently, Schiemann (1983b) developed a similar two-step enrichment method with an even shorter pre-enrichment incubation time in tryptic soy broth (TSB). Later still, irgasan-ticarcillan-chlorate (ITC) broth (Wauters *et al.*, 1988) was derived from MRB and was tested against several existing methods, including a modified BOS method and cold incubation in PBS followed by an alkaline treatment. It was concluded that ITC broth provided improved recovery for specific isolation of *Y. enterocolitica* serogroup O:3. Unless indicated otherwise, the above studies were conducted using food (and not water) samples.

While some methods are less effective for isolating all the different *Y. enterocolitica* serotypes, they are usually more effective for isolating one or two select serotypes, often including serotype O:3 (see Table 2-1). Nonetheless, these methods are still of value given that *Y. enterocolitica* 4/O:3 accounts for the majority of human cases in Europe, Japan, Canada and the USA (Bottone, 1999). The United States Department of Agriculture, Food Safety and Inspection Services (USDA/FSIS) (Johnson, 1998) microbiology laboratory guidebook recommends using three different enrichment methods (ITC, PBS and TSB/BOS) in parallel to ensure that a range of serotypes are recovered.

More recently, Bhaduri *et al.* (1997) developed an enrichment method that used modified TSB (mTSB). The method was intended to improve upon the cold enrichments that required long incubation periods. Similar to most cold enrichments, enrichment in mTSB was developed to isolate a wide range of serotypes, but do so in a shorter time period by incubating the broth at a slightly warmer temperature of 12°C. Furthermore, the selective antibiotic, irgasan, is not added at the beginning of the enrichment period. Instead, it is added at 24 hours, allowing injured bacteria in the sample to adapt and begin growing, which is the same concept as the non-selective pre-enrichment steps. Recently, a similar enrichment method using Luria Bertani-bile salts-irgasan (LB-BSI) broth, was described by Hussein *et al.* (2001).

Y. enterocolitica are known to be resistant to alkaline conditions, hence post-enrichment alkaline treatments have also been used kill other non-*Yersinia* in the sample (Aulisio *et al.*, 1980). Although Doyle and Hugdahl (1983) did find some variability in resistance to an alkaline treatment between different strains in a study of 22 *Y. enterocolitica* isolates, the authors concluded that all the strains studied should be recovered from alkaline treated enrichment cultures containing 10^4 organisms per mL before the treatment.

Isolation

Following an enrichment step, cultures are plated on selective agar media. A commonly used selective agar for the isolation of *Y. enterocolitica* is cefsulodin-irgasan-novobiocin (CIN) agar (Schiemann, 1979). CIN agar inhibits the growth of many types of organisms (Schiemann, 1979; Schiemann, 1982). The selective agents in CIN agar are sodium deoxycholate, cefsulodin, irgasan, and novobiocin (the latter three are antibiotics). Mannitol and a neutral red indicator dye in the CIN agar causes *Y. enterocolitica* colonies to have a deep red center with a transparent margin, often referred to as having a "bull's-eye" appearance (Chapin *et al.*, 2007). *Y. enterocolitica* ferments the mannitol in the medium, producing an acidic pH around the colonies. The colonies become transparent with a dark red centre where the red dye has absorbed. CIN agar has been shown to be superior to *Salmonella*-Shigella (SS) agar and MacConkey agar (Head *et al.*, 1982; Schiemann, 1982), which are other selective media that have been used for the isolation of *Y. enterocolitica*. Another study by Schiemann (1983b) compared five plating agars, including CIN. While results did not suggest that one agar was superior for recovery, CIN did yield the highest confirmation rate for presumptive colonies. It has been suggested that the growth of *Y. enterocolitica* biotype 3B/serotype O:3 is inhibited by CIN (Fukushima *et al.*, 1986). This study, however, incubated CIN agar plates at 32°C. It has since been shown that in the presence of inhibitors, pYV plasmid-bearing strains grow slower than plasmidless strains at 37°C, but have similar growth rates at 25°C (Logue *et al.*, 2006). Although, when all three antibiotics were present together, the growth rates were relatively similar. Therefore, incubation temperature plays a critical role in the recovery of plasmid bearing strains, and hence pathogenic strains.

A modification of *Salmonella*-Shigella agar has also been used to isolate *Y. enterocolitica*. SS agar with sodium deoxycholate and calcium chloride (SSDC) (Wauters, 1973; Johnson, 1998; Fredriksson-Ahomaa *et al.*, 2003) contains lactose and neutral red indicator dye. Lactose-positive bacterial colonies growing on SSDC agar are red coloured. *Y. enterocolitica* are lactose-negative and produce colourless colonies. Bile salts, deoxycholate, sodium citrate and brilliant green are also contained in the media (Wauters, 1973). Wauters *et al.* (1988) found that plating ITC enriched cultures on SSDC yielded better recoveries than plating on CIN for *Y. enterocolitica* serotype O:3.

CIN agar and SSDC agar are the two most commonly used agars for food samples (Fredriksson-Ahomaa *et al.*, 2003) and are both recommended in the USDA/FSIS methods for isolating *Y. enterocolitica* (Johnson, 1998).

Identification and confirmation

Once isolated from a selective agar medium, the bacterial strains are referred to as presumptive *Yersinia* isolates and need to be tested to confirm their identity. An isolate is likely to be *Yersinia* if it ferments glucose under anaerobic conditions, produces urease, is mobile at 25°C but not 37°C and lacks oxidase, phenylalanine deaminase, lysine decarboxylase and arginine dihydrolase activities (Bottone, 1997). It has been suggested that two key biochemical tests, Christensen urea agar and Kligler iron agar, are adequate for distinguishing *Yersinia* isolates from other bacteria that present similar colony morphologies on CIN agar (Devenish *et al.*, 1981). One or both of these key tests are commonly used in combination with at least one of the other tests listed above to identify presumptive *Y. enterocolitica* isolates from water (Fukushima *et al.*, 1984; Massa *et al.*, 1988; Brennhovd *et al.*, 1992; Arvanitidou *et al.*, 1994; Sandery *et al.*, 1996; Schaffter *et al.*, 2002). The USDA/FSIS Microbiology Laboratory Guidebook (Johnson, 1998) uses three tests for identifying *Y. enterocolitica*: Christensen urea agar, Kligler iron agar, and Simmon citrate agar Subsequently, *Y. enterocolitica* can be distinguished from other *Y. enterocolitica*-like species based on fermentation of sucrose, L-rhamnose, raffinose and melibiose as well as a Voges-Proskauer test at 25°C (Bottone, 1997).

Commercially produced kits that identify bacteria based on biochemical properties are also available; for example, the BIOLOG (BIOLOG, California, US) and API (bioMerieux, Nurtingen, Germany) systems. Results from these tests create a profile for the isolate which is compared to a database of bacterial species allowing probable identities for the isolate to be determined. These tests are somewhat expensive and identification systems based on biochemical profiles sometimes do not provide reliable identification at the species level (Fredriksson-Ahomaa *et al.*, 2006; Carroll *et al.*, 2007). Nonetheless, the API 20E identification system has been suggested to be a superior system for identifying *Y. enterocolitica* isolates (Neubauer *et al.*, 1998; Arnold *et al.*, 2004; Wanger, 2007).

Subtyping

Confirmed *Y. enterocolitica* are then typically subtyped. Traditional methods that are commonly used include biotyping, serotyping and antimicrobial susceptibility (Fredriksson-Ahomaa *et al.*, 2006). Biotyping is based on biochemical properties; serotyping is based on the presence of surface antigens; and antimicrobial susceptibility is based on susceptibility to various antimicrobial agents.

Biotyping and serotyping schemes have been developed for subtyping *Y. enterocolitica* isolates (Aleksic *et al.*, 1984; Wauters *et al.*, 1987; Wauters *et al.*, 1991). Biogroup and serogroup classification are typically used to evaluate the clinical significance of an isolate (Bottone, 1997). Human infections are more commonly associated with *Y. enterocolitica* of the following bioserogroups: 1B/O:8, 2/O:5, 27, 2/O:9, 3/O:3 and 4/O:3 (Fredriksson-Ahomaa *et al.*, 2006). Serogroups O:3 and O:9 are more commonly isolated in Europe and Canada, while O:8 and O:5, 27, and more recently O:3, are more common in the United States (Bissett *et al.*, 1990). Serogroup O:3 accounts for the majority of human cases in Europe, Japan, Canada and the USA (Bottone, 1999). However, recent studies have argued that biogroup and serogroup classifications may not be a reliable indicator of pathogenicity (Grant *et al.*, 1998; Thoerner *et al.*, 2003; Tennant *et al.* 2003; Bhagat and Virdi 2007). In one study of over 300 strains isolated from humans, many of the isolates from patients

displaying disease symptoms did not belong to serogroups traditionally associated with disease (Bissett *et al.*, 1990).

Novel subtyping methods are being developed that are DNA-based, known as molecular typing. Molecular identification methods are being researched for a variety of organisms, including *Y. enterocolitica* (Buchrieser *et al.*, 1994; Wannet *et al.*, 2001; Hallanvuo *et al.*, 2006). Thus far, they have not proven useful due to the high genetic similarity between strains (Fredriksson-Ahomaa *et al.*, 2006). Consequently, traditional subtyping methods, discussed above, continue to be used by laboratories that specialize in subtyping *Yersinia* spp..

Virulence Testing

Certain biogroups and serogroups have been more commonly associated with human disease. However, serogroup testing requires access to a laboratory equipped with the appropriate antisera (Bottone, 1997). Alternatively, phenotyping tests may be used to evaluate whether an isolate is potentially pathogenic including calcium dependent growth at 37°C, Congo red binding, autoagglutination testing, and serum resistance testing (Bottone, 1997). However, each test is limited by the fact that they depend on the presence and expression of virulence genes located on the pYV plasmid associated with pathogenic *Yersinia* spp. (Thoerner *et al.*, 2003), and *Y. enterocolitica* cells can lose the plasmid when cultured above 30°C for an extended period of time, or when passaged repeatedly (Blais *et al.*, 1995). Consequently, testing for pathogenicity in cultured isolates can be challenging. Furthermore, the validity of the above virulence tests for *Y. enterocolitica* has been questioned. Prpic *et al.* (1985), using a mouse lethality model, found that while all virulent strains were calcium dependent, not all calcium dependent strains were virulent. A study by Noble *et al.* (1987) found a lack of agreement between tests. Moreover, the authors found that no individual virulence tests or group of tests could be consistently associated with symptomatic patients. A review by Bottone (1997) outlines numerous studies that have evidence that results from virulence testing may not be a reliable indicator of whether an isolate is of clinical significance to humans. It has been hypothesized that isolates (both *Y. enterocolitica* isolates as well as

isolates belonging to other species of *Yersinia*) lacking the classic virulence characteristics may still be of clinical concern to humans and possess different, uncharacterized pathogenic mechanisms (Bottone, 1997; Grant *et al.*, 1998; Sulakvelidze, 2000).

2.4.2 Molecular-based Detection Methods

Over the last two decades, researchers have been developing molecular biological-based methods for detecting microorganisms in the environment (Lemarchand *et al.*, 2004). These methods have several advantages over culture-based methods in that they are specific, sensitive, and quick. Molecular biological-based methods typically involve detecting nucleic acid sequences unique to the targeted organism. There are a few different types of these nucleic acid-based detection methods including fluorescence in situ hybridization (FISH), DNA microarrays, and polymerase chain reaction (PCR) (Koster *et al.*, 2003).

FISH permits detection of bacteria in their natural habitat (in situ) using oligonucleotide probes that penetrate intact cells and bind to target nucleic acids, usually ribosomal RNA (rRNA) (Moter *et al.*, 2000). A detailed explanation of FISH techniques is provided in a review by Moter and Gobel (2000). Unfortunately, there are several disadvantages to using FISH for detecting bacteria in water. Bacteria in the environment are often starved or stressed. Starved or stressed cells are difficult to detect because such cells are less reactive and smaller (Koster *et al.*, 2003). Also, there needs to be a high copy number of the nucleic acid target to enable detection of a cell by FISH and rRNA levels in starved cells is lower than in growing cells (Lemarchand *et al.*, 2004). Finally, FISH is not appropriate for detecting bacteria at low concentrations (Koster *et al.*, 2003).

DNA microarrays are a fairly new technology still being developed for environmental applications (Koster *et al.*, 2003). A DNA microarray chip is constructed by immobilizing oligonucleotide probes in a grid pattern (or array) on a solid support that is typically the size of a glass microscope slide (Lemarchand *et al.*, 2004). Thousands to millions of oligonucleotides can be arranged on a single chip, enabling the detection of many targets in a single sample (Straub *et al.*, 2003). DNA microarrays have great flexibility in their design

and hence have numerous applications. Nonetheless, the use of microarrays for detecting microorganisms is less well studied and there are many challenges yet to overcome, including complex chemical procedures and quality control of the printed chip. These challenges as well as others are discussed in detail in a review by Lemarchand *et al.* (Lemarchand *et al.*, 2004). Regardless, DNA microarrays show great promise as a detection tool. In fact, Maynard *et al.* (2005) recently developed a microarray prototype for detecting multiple pathogens in wastewater. While problems were encountered due to variable sensitivity between organisms, results did suggest that research into microarray-based detection of pathogens is worthwhile pursuing. Nevertheless, much research is still required to improve specificity, sensitivity, and quantitation (Kostic *et al.*, 2007) before microarray-based detection can be widely accepted and integrated into watershed monitoring programs.

The PCR technique makes copies of (or amplifies) targeted DNA sequences in a sample. Amplifying a specific DNA sequence facilitates detecting that sequence in a mixture of DNA (Steffan *et al.*, 1991), which can be useful for detecting pathogens that are often at low concentrations in the environment (Lemarchand *et al.*, 2004). Consequently, research into the use of PCR-based methods for detecting various pathogens in environmental samples has become widespread (Theron *et al.*, 2002).

2.4.2.1 PCR-based Detection

The PCR technique amplifies targeted DNA sequences. Hence, if a DNA sequence unique to a particular organism is targeted for amplification, then successful amplification indicates the potential presence of that organism. A detailed explanation of the principles of DNA amplification by PCR is provided in a review by Steffan and Atlas (1991).

PCR-based detection can be highly specific due the ability to selectively amplify a DNA sequence that is unique to a particular species, or even subgroups within a species; for example, genes that are responsible for imparting pathogenicity to a bacterial strain. The amplified DNA products can subsequently be verified for specificity (*i.e.* tested to confirm

that the correct target was amplified) through probe hybridization, restriction enzyme digestion or sequencing (Olsen *et al.*, 1995). In addition to being highly specific, PCR techniques have several major advantages over culture-based methods. They are highly sensitive, rapid, accurate and can detect very small amounts of target DNA in a sample (Toze, 1999). There are, however, also disadvantages associated with using PCR as a detection method for pathogens in the environment, namely the presence of substances that are inhibitory to the DNA amplification reaction and problems in distinguishing DNA from viable and non-viable cells.

Substances introduced into the sample from the environment may inhibit DNA amplification (Wilson, 1997). These include humic compounds, divalent cations at high concentrations and salts (Toze, 1999). There are several sample processing methods proposed for removing these inhibitors (Toze, 1999). Fortunately, water samples are suggested to be the easiest environmental sample from which to extract DNA (Steffan *et al.*, 1991; Bej *et al.*, 1992). It is recommended that processed samples are tested for DNA amplification inhibition (Zhang *et al.*, 2006). This can be accomplished through the use of a control DNA amplification reaction. When designing a control reaction, it is important that the control DNA is not amplified preferentially over the target DNA (Sandery *et al.*, 1996).

Using standard PCR assays, it is not possible to distinguish between DNA amplified from viable and from non-viable cells (Lemarchand *et al.*, 2004; Wolffs *et al.*, 2005). This has been specifically been demonstrated to be problematic in water samples (Josephson *et al.*, 1993). Some methods have employed a culture-based enrichment step upstream of DNA extraction and amplification. This is thought to dilute the non-viable cells in the sample and hence reduce the false-positive signal generated by non-viable cells (Theron *et al.*, 2002). However, this approach is potentially less useful for strains that are not easy to culture and some enrichment broths contain components that are inhibitory to DNA amplification. Furthermore, advanced PCR methods can be quantitative, but this feature is lost if an enrichment step is employed. Another possible solution involves using reverse-transcriptase PCR (RT-PCR), which instead detects messenger RNA (mRNA). mRNA is less stable than DNA and potentially a good indication of the presence of viable cells (Toze, 1999; Koster *et*

al., 2003; Lemarchand *et al.*, 2004). However, due to the transient nature of mRNA, selecting appropriate mRNA targets for detecting microorganisms in the environment presents a significant challenge. Generally, RT-PCR is a challenging technique to implement in a laboratory due to several complicating factors that are outlined in reviews by Bustin (2000) and Bustin and Nolan (2004). Furthermore, some evidence suggests that detection of mRNA may also not be a reliable indicator of viability (Bej *et al.*, 1992). Recent studies have employed flotation to process samples prior to DNA amplification to detect *Y. enterocolitica* in food (Thisted Lambertz *et al.*, 2000; Wolffs *et al.*, 2004). Flotation uses density gradient centrifugation to separate matrix particles and microorganisms of different buoyant densities. Wolffs *et al.* (2004) selected the method to test removal of PCR inhibitors and reduction of false-positives due to DNA from dead cells. It was concluded that the approach effectively attained those goals. However, while this study did confirm that no signal was generated in samples spiked with naked DNA from *Y. enterocolitica*, no experiments were conducted that involved spiking non-viable cells into samples.

2.4.2.2 Real Time PCR Methods

Traditionally, DNA amplification products are detected by separating the amplified products by gel electrophoresis, then visualizing products with a stain that binds DNA. This traditional PCR technique is termed end-point detection and is usually employed as a presence/absence test. A more recent development, quantitative real time PCR technology, enables quantification of the original DNA in the sample (prior to amplification). This is accomplished through the use of a fluorescent reporter that allows the experimenter to monitor the rate of DNA amplification over the course of the PCR cycles (Toze, 1999). Figure 2-1 shows typical results obtained when monitoring DNA amplification over the course of several PCR cycles. The fluorescence signal, which monitors DNA amplification, is plotted on the vertical axis against the PCR cycle number on the horizontal axis. The fluorescence curves shown are for standard DNA samples that were prepared from enumerated cell cultures.

The threshold cycle (CT) is the cycle at which DNA amplification is in a logarithmic growth phase (Zhang *et al.*, 2006). The DNA amplification rate is monitored through fluorescence and the cycle at which the fluorescence exceeds the background fluorescence (*i.e.* the cycle at which amplified DNA products are first detected) is taken as the CT. The threshold fluorescence level is indicated by the horizontal orange line in Figure 2-1. In practice, the point at which the fluorescence signal hits this threshold line is recorded as the CT. Theoretically, the higher the initial amount of target DNA, the sooner the amplified DNA products will be detected, and the smaller the CT. Specifically, the CT value is inversely related to the logarithm of the concentration of initial DNA target (Zhang *et al.*, 2006). Consequently, CT values measured for DNA standards of known concentration can be used to generate a standard curve as shown in Figure 2-2.

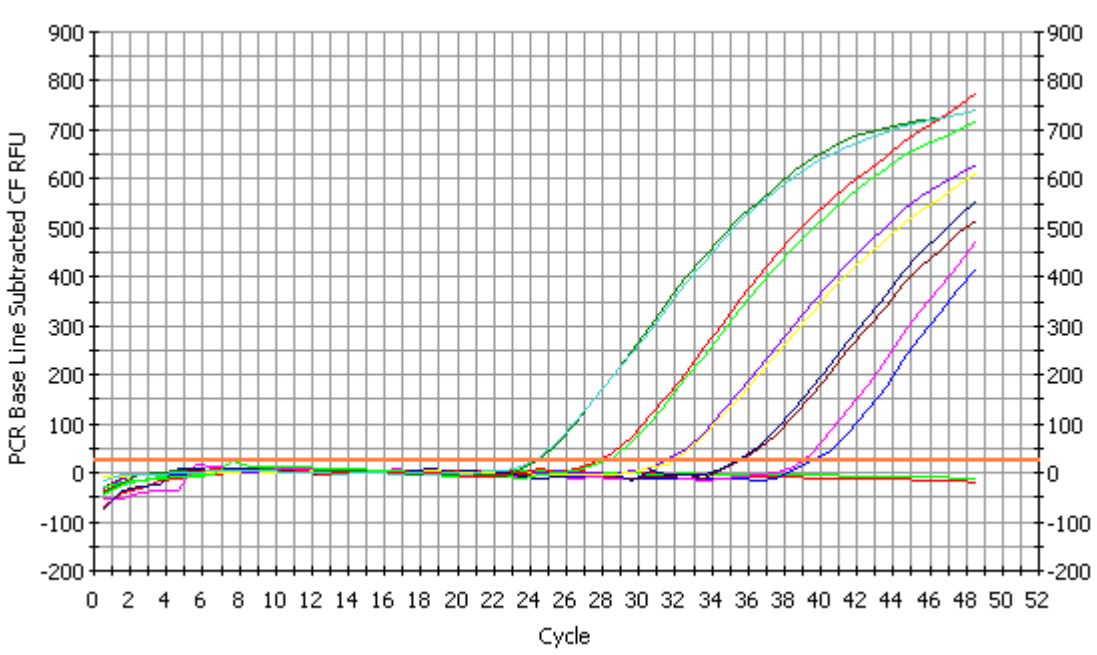


Figure 2-1: DNA amplification of DNA standard samples. Each trace represents a different sample. The samples are DNA standards that were prepared by extracting DNA from an enumerated cell culture. Shown from left to right are DNA amplification curves for standard DNA samples with starting concentrations of 1×10^5, 1×10^4, 1×10^3, 1×10^2 and 1×10^1 cells per reaction).

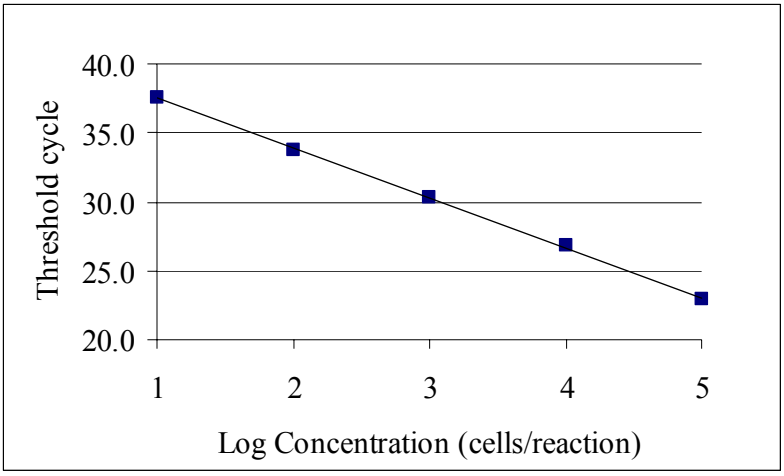


Figure 2-2: DNA amplification standard curve. The threshold cycle, calculated based on the curves in Figure 2-1, is plotted against the log concentration of the standard DNA samples.

Real time PCR facilitates automation and quantification of DNA samples and reduces the time required to obtain results (Fredriksson-Ahomaa *et al.*, 2006). There are different types of real time PCR methodologies that have been developed that utilize different types of fluorescent reporters. There are three types that are most commonly used with environmental samples: SYBR® Green reagent (Invitrogen, Carlsbad, California), Taqman® probes (Applied Biosystems, Foster City, CA) and Molecular Beacons (Zhang *et al.*, 2006).

SYBR® Green is a fluorescent dye whose fluorescence intensity increases 200-fold relative to its autofluorescence when it binds double-stranded DNA (dsDNA) (Zhang *et al.*, 2006). Hence, as more dsDNA is synthesized the fluorescent signal will increase. However, the dye has no specificity for the target sequence and may bind non-specifically amplified dsDNA. Consequently, SYBR® Green assays require extensive optimization and further validation tests post-amplification (*i.e.*, melting point curve).

Taqman® probes are dual-labelled oligonucleotide probes that are designed to anneal to a region between the forward and reverse primers used for DNA amplification (Heid *et al.*, 1996). The dual-labelled probe is 5' labeled with a fluorescent dye and 3' labeled with a quencher molecule. When in proximity to one another, the fluorescent dye transfers energy to

the quencher molecule, preventing fluorescence. When the probe is incorporated into a newly synthesized DNA stand during amplification, the fluorescent dye molecule is cleaved and released into solution generating a fluorescence signal (Heid *et al.*, 1996). A disadvantage to using Taqman® probes is that the technique is limited to detected DNA targets that are less than 150 base pairs (Zhang *et al.*, 2006). Nevertheless, unlike SYBR® Green stain, Taqman® probes are specific to the DNA target, providing additional confirmation that the correct target is being detected. Furthermore, Taqman® RT-PCR is considered significantly more sensitive than traditional PCR methods (Boyapalle *et al.*, 2001; Foulds *et al.*, 2002).

Molecular beacon probes are also dual labeled probes, but function through a slightly different mechanism than Taqman® probes. This mechanism requires that the molecular beacon probe hybridizes perfectly to the target (Zhang *et al.*, 2006). Consequently, designing a molecular beacon probe and selecting suitable PCR conditions is highly challenging. Moreover, this approach tends to be more suitable to other very specific applications, and its application to environmental samples remains limited (Zhang *et al.*, 2006).

The fact that PCR-based (or real time PCR-based) detection methods can be highly selective is a major advantage over culture-based detection methods. The selectivity of a PCR-based method depends on the DNA sequence being targeted. Consequently, a critical step in developing a PCR-based detection method is selecting the DNA target (Olsen *et al.*, 1995).

2.4.2.3 Virulence Genes in *Y. enterocolitica*

Genes that are typically targeted in PCR-based methods for detecting pathogenic *Y. enterocolitica* include the *yadA*, *virF*, *ail*, *inv* and *yst* genes, which are all virulence genes (Fredriksson-Ahomaa *et al.*, 2006). Virulence genes in bacteria code for proteins that play a role in pathways that lead to infection of a host. There are usually a very large number of genes that each play an important role in the pathways to infection. It is important that the experimenter pick genes that are critical to pathogenicity, and that are present exclusively in pathogenic strains and not in non-pathogenic strains.

The biochemical pathways in *Y. enterocolitica* that lead to infection are complicated and involve many different genes located on chromosomal DNA and the pYV virulence plasmid (Revell *et al.*, 2001). The pYV plasmid is a low copy number plasmid found in virulent strains of both *Y. enterocolitica* and *Y. pseudotuberculosis* (and *Y. pestis*), but is absent in avirulent strains of *Yersinia* (Robins-Browne *et al.*, 1989). Several plasmid encoded virulence genes have been identified that play a role in virulence pathways in pathogenic *Yersinia*. In *Y. enterocolitica*, the transcription of many of these virulence genes is regulated by the VirF protein. The *virF* gene is located on the virulence plasmid and is thermally induced. Consequently, many plasmid encoded virulence factors are expressed at 37°C, but not at 25°C (Portnoy *et al.*, 1981a; Portnoy *et al.*, 1981b; Cornelis *et al.*, 1998). This feature is thought to help the organism adapt to the environment inside the intestines of warm blooded mammals and prepare for infection. Among the genes regulated by VirF are the *yop* genes and the *yadA* gene (Cornelis *et al.*, 1998).

The *yadA* gene is located on the pYV plasmid and codes for a protein that has been demonstrated to be essential to *Y. enterocolitica* virulence (Cornelis *et al.*, 1998). The protein is involved in promoting adherence to mucus layers, attachment to host cells and enhancing serum resistance (Bottone, 1997). Although the pYV plasmid is reported to only be hosted by *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis*, a recent study detected the *yadA* gene in a *Y. intermedia* strain (Kechagia *et al.*, 2007).

There are eight different *Yersinia* outer proteins (Yops) that work together to inhibit host cell responses to infection, primarily counteracting defense mechanisms of phagocytes and inducing programmed cell death in phagocytes (Cornelis *et al.*, 1998; Viboud *et al.*, 2005). By mediating attachment to host cells, *yadA* (discussed above) facilitates secretory systems that inject (Yops) into phagocytes causing paralysis (Roggenkamp *et al.*, 2003). There is some uncertainty about which Yops factors are critical, although three, YopH, YopM and YopE, have been proposed to be more important for virulence (Viboud *et al.*, 2005). A mutant lacking *yopH* and *yopE* expression showed the same reduced level of resistance to phagocytosis as a strain lacking the plasmid entirely. The *yop* genes are almost identical in *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis* (Cornelis *et al.*, 1998).

While it is clear that the pYV plasmid is essential to imparting full virulence to *Y. enterocolitica*, it has been demonstrated that the plasmid alone does not confer virulence (Schiemann *et al.*, 1982; Heesemann *et al.*, 1984). Chromosomal genes have also been identified that contribute to *Y. enterocolitica* virulence.

The first step of the infection process involves invading intestinal epithelial cells. Consequently, the ability to attach to and invade cells is crucial to pathogenicity (Wachtel *et al.*, 1995). As mentioned above, *yadA* has been implicated in attachment to cells. Two additional genes important to enabling invasion of cells have been identified: *inv* and *ail* (Miller *et al.*, 1988). The *inv* and *ail* genes are located in chromosomal DNA (Miller *et al.*, 1989) and have been suggested to be good gene targets that indicate pathogenicity (Olsen *et al.*, 1995).

The *inv* gene codes for the protein invasin that initiates cell penetration by binding cell receptors (Isberg *et al.*, 1990). Expression of the *inv* gene is regulated by RovA (Revell *et al.*, 2001; Ellison *et al.*, 2004). The *inv* gene, however, has been found in both pathogenic and non-pathogenic *Y. enterocolitica* serotypes (Miller *et al.*, 1989). The *ail* gene plays a role in attachment and invasion as well as serum resistance (Bottone, 1997). In a study by Wachtel and Miller (1995), a mutant *Y. enterocolitica* strain that could not produce the *ail* protein had a reduced ability to invade cells. The *ail* gene is thought to be only found in *Y. enterocolitica* serotypes that are associated with disease (Miller *et al.*, 1989; Revell *et al.*, 2001; Howard *et al.*, 2006). However, one study identified two *Y. enterocolitica* 1A strains that possessed the *ail* gene (Thoerner *et al.*, 2003).

The *yst* virulence gene is also located on chromosomal DNA and codes for a heat-stable enterotoxin found to play a role in virulence (Revell *et al.*, 2001). However, in one study, *yst* gene probes developed to detect pathogenic subtypes of *Y. enterocolitica* cross reacted with strains of *Y. intermedia*, *Y. enterocolitica* biotype 1A and five other species in the Enterobacteriaceae family (Kwaga *et al.*, 1992). In some strains of *Y. intermedia* and *Y. kristensenii*, a gene homologous to *yst* or an inactive *yst* gene has been found and its presence has not always correlated with toxin production (Grant *et al.*, 1998; Sulakvelidze,

2000). In an earlier study, an enterotoxin-negative *Y. enterocolitica* strain was found to produce diarrhea in mice that had ingested the bacteria. *Y. enterocolitica* was also recovered from the feces of those infected mice (Schiemann, 1981). Conversely, isolates that produce these enterotoxins have been found that are not positive for classical virulence characteristics and did not produce diarrhea in mice (Schiemann *et al.*, 1982).

The virulence factors selected for discussion are among the better understood and more thoroughly studied. However, there are many more genes thought to be involved in pathogenicity. Plasmid encoded factors are thoroughly discussed in a review by Cornelis *et al.* (1998) and chromosomal factors in a review by Revell and Miller (2001). The genes discussed above each play an important role in the virulence pathways for *Y. enterocolitica* infections. DNA amplification of some of these genes has been used to detect potentially pathogenic strains of *Y. enterocolitica* in samples.

2.4.2.4 PCR-based Methods for Detecting *Y. enterocolitica*

Several PCR-based methods have been developed to investigate the prevalence of pathogenic *Y. enterocolitica*. A review by Fredriksson-Ahomaa *et al.* (2006) provides a summary of the genes targeted as well as the sample processing methods used in 24 different PCR-based detection method studies. The genes targeted include: *ail, virF, yadA,* yst, *inv, yopT* and the 16s rRNA gene, with the *ail* gene being targeted most frequently. Only three of the cited methods were used to detect *Y. enterocolitica* in water, and each of these methods included an enrichment step prior to the PCR assay (Kapperud *et al.*, 1993; Sandery *et al.*, 1996; Waage *et al.*, 1999). In fact, a majority of the studies employed an enrichment step prior to PCR. One purpose of the enrichment step is to improve sensitivity of the methods.

Recently developed real time PCR methods are highly sensitive and may provide an alternative method that does not require an upstream enrichment step. Real time PCR-based methods developed for detecting *Y. enterocolitica* are summarized in Table 2-2.

Table 2-2: Virulence gene targets used in real time PCR assays developed for detecting pathogenic *Y. enterocolitica* in samples (adapted from Fredriksson-Ahomaa *et al.* 2006)

Gene target(s)	Sample type	Detection system	Reference
ail	food, feces	Taqman® PCR	(Jourdan *et al.*, 2000)
	food	Taqman® PCR	(Boyapalle *et al.*, 2001)
	food, feces	Taqman® PCR	(Bhaduri *et al.*, 2005)
yst	food	Taqman® PCR	(Vishnubhatla *et al.*, 2000)
	food	Taqman® PCR	(Wu *et al.*, 2004)
yadA	feces	SYBR® Green PCR	(Fukushima *et al.*, 2003)
	food	SYBR® Green PCR	(Wolffs *et al.*, 2005)

2.5 Comparing Culture-based and PCR-based Detection Methods

The advantages and disadvantages of culture-based and PCR-based detection methods are listed and contrasted side-by-side in Table 2-3. It is interesting to note that the advantages and disadvantages of culture-based and PCR-based methods complement one another. Furthermore, their individual strengths and weaknesses suggest that, at this point in method development, neither approach used independently will provide complete information. Therefore, depending on the objectives of a study, it may be valuable to use both culture-based and PCR-based methods to acquire comprehensive results.

Table 2-3: Advantages and disadvantages of culture-based and PCR-based detection methods. Advantages and disadvantages are listed in the grey squares and white squares, respectively.

Culture-based methods	PCR-based methods
▪ Time-consuming ▪ Non-culturable strains exist ▪ Less selective ▪ Quantitation is laborious ▪ Poor sensitivity	▪ Quick, high-throughput ▪ Does not rely on culturing ▪ Highly selective ▪ Quantitative can be simple ▪ Good sensitivity
▪ Isolates strains ▪ Selectively detects viable cells	▪ No strain isolation (at present) ▪ Cannot distinguish between viable and non-viable cells

2.6 Previous Surveys for *Y. enterocolitica* in Water

Surveys for *Y. enterocolitica* in surface water, well water and other water sources have been conducted in many different regions of the world. These studies have found a variety of *Yersinia* spp. in the waters investigated, but limited evidence for the presence of pathogenic *Y. enterocolitica* strains in these waters (Table 2-4).

Table 2-4: Surveys of water for *Y. enterocolitica* in different geographic regions

Location	Water type	*Yersinia* spp. (%) [a]	Fraction of isolates that were *Y. enterocolitica* [b]	Pathogenic subtypes? [c]	Reference
Norway	well, unchlorinated public	20 %	-	-	(Lassen, 1972)
California, US	surface	29 %	-	-	(Harvey *et al.*, 1976)
Colorado and Wisconsin, US	surface	9 %	-	-	(Saari *et al.*, 1979)
New York, US	surface	24 %	0.16	no	(Shayegani *et al.*, 1981)
Wisconsin, US	surface	8 %	n.a. [e]	-	(Meadows *et al.*, 1982)
Washington, US	surface	77 %	-	-	(Weagant *et al.*, 1983)
Norway	untreated drinking, sewage sludge	53 %	0.09	no	(Langeland, 1983)
Matsue, Japan	surface	81 %	0.18	yes	(Fukushima *et al.*, 1984)
Ontario	surface, groundwater	17 % (surface) 1 % (well)	0.63	no	(Vajdic, 1985)
Germany	well, treated drinking	n.a. [d]	0.82	no	(Aleksic *et al.*, 1988)
Italy	surface	22 %	0.46	-	(Massa *et al.*, 1988)
Izmir City, Turkey	public, well, spring, bottled	6 %	n.a. [e]	-	(Gonul *et al.*, 1991)
Norway	surface, well	4 %	0.50	no	(Brennhovd *et al.*, 1992)
Northern Greece	chlorinated and unchlorinated drinking	4 %	0.08	no	(Arvanitidou *et al.*, 1994)
Australia	surface	1 % (Culture) 10 % (PCR)	-	yes	(Sandery *et al.*, 1996)
Switzerland	surface	6 %	0.67	-	(Schaffter *et al.*, 2002)
Brazil	several types	n.a. [d]	0.47	yes	(Falcao *et al.*, 2004)

[a] Frequency of samples positive for *Yersinia* spp. by culture-based methods unless indicated otherwise.
[b] This fraction was calculated by dividing the number of *Y. enterocolitica* isolates by the total number of *Yersinia* isolates.
[c] This column outlines whether the study claimed to have isolated pathogenic strains (based on subtyping analysis).
[d] Information not available because survey results were not included in the paper. These studies focused on characterization of *Yersinia* strains isolated from water.
[e] Information not available because these surveys only included results for *Y. enterocolitica* isolates and did not discuss other *Yersinia* spp. isolates. It is unknown whether this is because no other *Yersinia* spp. isolates were found or whether they were omitted.

It should be noted that in most surveys, all *Yersinia* spp., including the non-pathogenic species (*i.e. Y. aldovae*, *Y. intermedia*, etc.), are often included. This is because isolation methods available do not selectively isolate *Y. enterocolitica*. The 4th column from the left in the table shows the number of *Y. enterocolitica* isolates divided by the total number of *Yersinia* spp. isolates to provide a clearer picture of the actual occurrence of *Y. enterocolitica* documented in these studies. A problem with studies conducted prior to 1988 is that many of the non-pathogenic *Yersinia* spp., often referred to as *Y. enterocolitica*-like species, were discovered between 1980 and 1988 (Schiemann, 1990; Bergey *et al.*, 1994). Hence, it is possible that isolates identified as *Y. enterocolitica* in studies prior to 1988 were in fact one of the non-pathogenic *Yersinia* spp. that were later identified. The isolation rates of *Y. enterocolitica* in most of the cited studies are quite low. With the exception of one study by Sandery *et al.* (1996), these surveys utilized culture-based methods and not PCR-based methods. Given that culture-based methods are known to have poor sensitivity, it is probable that they are underestimating prevalence rates.

A review by Fredriksson-Ahomaa *et al.* (2006) lists several studies that compare the detection rates of pathogenic *Y. enterocolitica* using culture-based and PCR-based methods in natural samples, mostly in pig tissue and food samples. The detection rates by PCR-based methods were consistently and sometimes significantly higher than culture-based methods. Boyapalle *et al.* (2001) consistently detected pathogenic *Y. enterocolitica* by PCR in four different samples types, including pig tonsils, feces, mesenteric lymph nodes and ground pork, but never isolated any pathogenic strains using culture methods. In an examination of surface water from creeks and reservoirs, both culture-based and PCR-based detection methods were employed (Sandery *et al.*, 1996). In this study, samples enriched in a culture-based enrichment method were plated to isolate *Y. enterocolitica* strains and were also processed for DNA extraction. PCR methods targeted the *ail* gene and served two purposes. (1) To screen DNA extracted from enrichment cultures. (2) To screen *Y. enterocolitica* isolates recovered from plated samples. Only one of the *Y. enterocolitica* isolates recovered was positive for the *ail* gene. Results showed detection rates of 1% and 10% for culture-based and PCR-based detection, respectively.

2.7 Summary

This review describes the bacterial pathogen *Y. enterocolitica*, including information regarding its occurrence in environmental water and its potential as a waterborne pathogen. Methods used to detect *Y. enterocolitica* along with their associated advantages and disadvantages are summarized. Several surveys have found *Yersinia* strains in surface waters in various parts of the world, however, the majority of these isolates have traditionally been classified as non-pathogenic. Yet, recent studies show evidence that these strains may be potentially pathogenic and of significance to human health. Meanwhile, with the recent advances in molecular biology techniques, PCR-based methods for detecting bacteria are becoming widespread and are proving to be significantly more sensitive in detecting pathogens at higher frequencies than culture-based methods, including pathogenic *Y. enterocolitica*. The concepts outlined in this review provided the framework on which research for this thesis was based and a context for the discussion of the outcomes and findings of the study.

3 An Evaluation of Culture-based Methods for the Isolation of *Yersinia enterocolitica* in Surface Water from the Grand River

3.1 Abstract

Methods available for the detection of *Yersinia enterocolitica* have been developed primarily for food and clinical samples and may not be effective for use with environmental samples. The objective of this study was to evaluate and compare the ability of four different enrichment broths (ITC, mTSB, LB-BSI, and PBS) and two selective agars (CIN and SSDC) to isolate *Y. enterocolitica* from surface waters. The effect of an alkaline treatment protocol was also evaluated. Methods were compared using surface water spiked with a pure culture of *Y. enterocolitica* (ATCC 700822) and with non-spiked surface water samples. Results showed that the methods did not adequately inhibit other bacteria from the surface water matrix. Consequently, none of the methods were effective for recovering *Y. enterocolitica* spiked into surface water samples. Naturally occurring *Yersinia* spp. were also isolated from non-spiked surface water samples collected over a 17-month period. Of 200 samples analyzed, *Yersinia* spp. were isolated from 52 samples (26%). Only eight samples contained *Y. enterocolitica*, and were all biotype 1A, which is typically considered non-pathogenic. Other *Yersinia* spp. isolated included: *Y. aldovae*, *Y. bercovieri*, *Y. frederiksenii*, *Y. intermedia*, *Y. kristensenii* and *Y. mollaretii*.

3.2 Introduction

Y. enterocolitica is an emerging waterborne pathogen (Theron *et al.*, 2002; Sharma *et al.*, 2003) that has the potential to cause gastrointestinal disease as well as a wide variety of other diseases (Bottone, 1997). There are six *Y. enterocolitica* biogroups and more than 50 serogroups, however, human infections are more commonly associated with bioserogroups 1B/O:8, 2/O:5, 27, 2/O:9, 3/O:3 and 4/O:3 (Fredriksson-Ahomaa *et al.*, 2006). There are several *Yersinia* spp. that are highly similar to *Y. enterocolitica*, including: *Y. intermedia*, *Y. frederiksenii*, *Y. kristensenii*, *Y. aldovae*, *Y. rohdei*, *Y. bercovieri*, and *Y. mollaretii* (Sulakvelidze, 2000). These newly classified species of *Yersinia* are often referred to as *Y. enterocolitica*-like spp. and have traditionally been considered to be non-pathogenic species (Sulakvelidze, 2000; Fredriksson-Ahomaa *et al.*, 2003).

Y. enterocolitica is more commonly associated with foodborne illness (Fredriksson-Ahomaa *et al.*, 2003) and pigs are a major reservoir of human pathogenic strains (McNally *et al.*, 2004; Fredriksson-Ahomaa *et al.*, 2006). However, most cases of *Y. enterocolitica* infection are sporadic and a source is rarely identified (Bottone, 1997). This has been attributed to difficulties associated with isolating the organism, in particular pathogenic strains of the organism (Fredriksson-Ahomaa *et al.*, 2003).

Although reports of waterborne disease caused by *Y. enterocolitica* are rare, studies have documented the occurrence of various *Yersinia* spp. in environmental waters (Brennhovd *et al.*, 1992; Leclerc *et al.*, 2002; Fredriksson-Ahomaa *et al.*, 2003). The majority of *Yersinia* isolates recovered from water are considered non-pathogenic *Y. enterocolitica* subtypes or *Y. enterocolitica*-like strains. However, recent studies have suggested that *Y. enterocolitica* subtyping analyses may not be a reliable indication of pathogenicity (Grant *et al.*, 1998; Thoerner *et al.*, 2003; Bhagat *et al.*, 2007). *Y. enterocolitica* strains from bioserogroups not traditionally considered pathogenic have been isolated from patients displaying symptoms of gastrointestinal illness (Bissett *et al.*, 1990), including strains belonging to biotype 1A (Tennant *et al.*, 2003), although their contribution to symptoms is unclear. Similarly, all of

the *Y. enterocolitica*-like species, except *Y. aldovae*, have been isolated from patients displaying disease symptoms (Sulakvelidze, 2000).

There are three major problems encountered when attempting to isolate *Y. enterocolitica* from environmental samples. First, there tends to be a very high concentration of background organisms (non-*Yersinia*) in environmental samples and *Y. enterocolitica* are known to grow poorly in competition with other organisms (Schiemann *et al.*, 1984; Calvo *et al.*, 1986b). Second, methods developed are not selective for pathogenic strains of *Y. enterocolitica*, and will also isolate non-pathogenic strains of *Yersinia*, which appear to be prevalent in the environment (Fredriksson-Ahomaa *et al.*, 2003). Third, a virulence plasmid, which imparts certain phenotypic characteristics that are used to identify pathogenic strains, is sometimes lost during culturing steps (Blais *et al.*, 1995). It is important to be aware of these challenges when evaluating and implementing *Y. enterocolitica* isolation methods.

There are three major steps typically involved in isolating *Y. enterocolitica*: (1) enrichment in selective broth, (2) isolation on selective agar and (3) identification. Two selective agars and several identification tests were first evaluated independently. Multiple enrichment methods were then compared along with the previously evaluated selective agar and identification tests.

The objective of this research was to evaluate and compare culture-based methods for isolating *Y. enterocolitica* from surface waters. Four different enrichment methods, including Irgasan-Ticarcillan-Chlorate (ITC) (Wauters *et al.*, 1988), modified tryptic soy broth (mTSB) (Bhaduri *et al.*, 1997), Luria-Bertani-Bile Salts-Irgasan (LB-BSI) (Hussein *et al.*, 2001), and cold enrichment in phosphate-buffered saline (PBS) (Johnson, 1998), were compared for the recovery of *Y. enterocolitica* added to Grand River surface water samples. The ability of a post-enrichment alkaline treatment (Aulisio *et al.*, 1980; Johnson, 1998) to kill other indigenous non-*Yersinia* organisms from surface water samples was also evaluated. Enrichment in mTSB, followed by alkaline treatment and plating on CIN agar, was then selected to survey *Yersinia* from surface water collected at five locations in the Grand River watershed over a 17-month period.

The Grand River watershed spans an area close to 7,000 km^2 and is the largest watershed in Southern Ontario, Canada. Although the watershed is not regularly monitored for pathogens, a study of the watershed by Dorner *et al.* (2007) detected several different enteric pathogens that are common in other surface waters. *Y. enterocolitica*, however, was not among the pathogens surveyed. The watershed is a drinking water source and is also used for industrial, commercial, agricultural and recreational activities (Cooke, 2006). There are 26 wastewater treatment plants that discharge into the Grand River and its tributaries. Close to 80 % of the land is used for agriculture (Dorner *et al.*, 2004; Bellamy *et al.*, 2005; Cooke, 2006).

3.3 Materials and Methods

3.3.1 Laboratory Cultures

Laboratory strains of *Yersinia* spp. used as positive controls are listed in Table 3-1 and were obtained from either the American Type Culture Collection (ATCC) (Manassas, VA) or provided by the Ontario Ministry of Health and Long-Term Care, Central Public Health Laboratory (Etobicoke, ON). Non-*Yersinia* strains used as negative controls are listed in Table 3-2 and were obtained from ATCC or provided by Drs. Lee and Trevors from the University of Guelph, ON.

Table 3-1: *Yersinia* laboratory control strains

Strain ID	Species [a]	Biogroup	Serogroup
1	*Y. enterocolitica*	1A	O:7, 13
2	*Y. enterocolitica*	1A	O:41,42
3	*Y. enterocolitica* ATCC [b] 9610	1B	O:8
4	*Y. enterocolitica*	1B	O:8
5	*Y. enterocolitica*	1B	O:8
6	*Y. enterocolitica*	1B	O:21
7	*Y. enterocolitica*	2	O:9
8	*Y. enterocolitica*	2	O:5, 27
9	*Y. enterocolitica*	3	O:1, 2,3
10	*Y. enterocolitica*	4	O:3
11	*Y. enterocolitica* ATCC 800722	4	O:3
12	*Y. pseudotuberculosis*		III
13	*Y. pseudotuberculosis*		I
14	*Y. frederiksenii*		
15	*Y. intermedia*		
16	*Y. kristensenii*		
17	*Y. mollaretti*		
18	*Y. rodher*		

[a] All strains were obtained from the Ontario Ministry of Health and Long-Term Care, Central Public Health Laboratory (Etobicoke, ON), with the exception of strains 3 and 11.
[b] ATCC, American Type Culture Collection (Manassas, VA)

Table 3-2: Non-*Yersinia* laboratory control strains

Strain ID	Species
19	*Aeromonas hydrophila* ATCC [a] 7966
20	*Citrobacter freundii* ATCC 8090
21	*Escherichia coli* ATCC 11229
22	*Escherichia coli* ATCC 15597
23	*Escherichia coli* O157:H7 ATCC 43895
24	*Pseudomonas aeruginosa* UG2Lr [b]
25	*Proteus mirabilis* ATCC 43071
26	*Staphylococcus aureus* ATCC 12600
27	*Salmonella enterica* ATCC 13311

[a] ATCC, American Type Culture Collection (Manassas, VA)
[b] Strain was provided by Drs. Lee and Trevors, University of Guelph, ON.

Yersinia spp. were maintained on tryptic soy agar (TSA) (BD, Oakville, ON) plates and grown at 28°C for 16-24 h. Non-*Yersinia* strains were maintained on nutrient agar (BD, Oakville, ON) plates and grown at 37°C for 16-24 h. All cultures were stored at 4°C and sub-cultured every two weeks.

For long term storage, glycerol frozen stocks of all strains were prepared as follows. Overnight cultures of *Yersinia* were grown in tryptic soy broth (TSB) (BD, Oakville, ON) at 28°C and non-*Yersinia* in nutrient broth (BD, Oakville, ON) at 37°C for 16-24 h. One mL of each culture was added to 0.5 mL sterile 80 % glycerol solution in a 2 mL freezer vial and mixed well. Glycerol stocks were stored at -80°C.

3.3.2 Evaluation of Selective Agar Media

Two selective agar media were tested for isolating *Y. enterocolitica*: (1) Cefsulodin-Irgasan-Novobiocin (CIN) agar (BD, Oakville, ON) with *Yersinia* antimicrobiotic supplement CN (BD, Oakville, ON) and (2) *Salmonella*-Shigella agar (BD Oakville, ON) with sodium deoxycholate and calcium chloride (SSDC agar) (Johnson, 1998).

CIN and SSDC agar plates were streaked with *Yersinia* and non-*Yersinia* control strains (Table 3-1 and Table 3-2) and incubated at 28°C for 24 h. The presence or absence of growth was recorded along with a description of colony morphology if growth was observed.

3.3.3 Evaluation of Identification Tests

Identification tests are used to confirm the identity of bacterial isolates by testing the physical or biochemical properties of the organism. Identification tests commonly used to identify *Yersinia* were evaluated as described in the following sections and summarized in Table 3-3, using bacterial controls listed in Table 3-1 and Table 3-2.

Table 3-3: Summary of identification tests

Test	Agar or Reagent	Expected result for *Yersinia* spp.
Lactose fermentation	MacConkey agar plates	Lactose negative (colourless colonies)
Oxidase production	Oxidase reagent	Oxidase negative (no colour change)
Urea utilization	Urea agar slants	Urease positive (pink slant)
Citrate utilization	Simmon's citrate agar slants	Citrate negative (green slant)
Kligler Iron Agar (KIA) Test	Kligler iron agar slants	Lactose negative (red slant) Glucose positive (yellow butt) No gas, no H_2S production

3.3.3.1 Lactose Fermentation

Bacteria were streaked onto MacConkey agar (Difco BD, Oakville, ON) plates and incubated at 28°C for 24 h. Colonies were observed for the appearance of colour. If colonies were white, the organism was positive for lactose fermentation and if colonies were pink, the organism was negative for lactose fermentation. *Yersinia* spp. are lactose negative. MacConkey agar also inhibits the growth of Gram-positive bacteria.

3.3.3.2 Oxidase Test

Oxidase reagent (BD, Oakville, ON) was transferred directly onto filter paper and allowed to soak in. Using a sterile wooden stick, a small amount of bacteria was transferred from an agar plate to the filter paper soaked with oxidase reagent. If the bacterial smear turned purple, the strain was oxidase positive. If no colour change was observed, the strain was oxidase negative. *Yersinia* spp. are oxidase negative.

3.3.3.3 Urease Test

Bacteria were streaked onto Urea agar slants (OXOID, Nepean, ON) supplemented with 2 % urea (Difco, Oakville, ON) and incubated at 28°C for 24 h. If the slant turned pink-red, the isolate was urease positive. If the slant remained yellow, the isolate was urease negative. *Yersinia* spp. are urease positive.

3.3.3.4 Simmons Citrate Agar Test

Isolates were streaked onto Simmons Citrate agar (OXOID, Nepean, ON) slants and incubated at 28°C for 24 h. If the slant turned blue, the isolate was citrate positive. If the slant remained green, the isolate was citrate negative. *Yersinia* spp. are citrate negative.

3.3.3.5 Kligler Iron Agar Test

Bacteria were streaked and stabbed on Kligler Iron agar (KIA) (OXOID, Nepean, ON) slants and incubated at 28°C for 16-24 h. The agar was observed for a colour change from red (alkaline conditions) to yellow (acidic conditions) on the slant and in the butt to indicate lactose and glucose fermentation, respectively. Slants were also observed for black precipitate in the agar, indicating hydrogen sulfide production. Lastly, slants were observed for gas production. If an organism formed gas from glucose or lactose fermentation, it was demonstrated by bubbles or large cracks in the agar. *Yersinia* spp. have an alkaline slant (red), an acidic butt (yellow) and do not produce gas or hydrogen sulfide.

3.3.4 Evaluation of Enrichment Methods

Enrichment methods were compared in three experiments, A, B and C. The objective of experiment A was to compare the growth of *Y. enterocolitica* ATCC 70822 in each broth without other organisms from surface water; the objective of experiment B was to compare the inhibition of indigenous bacteria from surface water from the Grand River in each broth; and the objective of experiment C was to compare the recovery of *Y. enterocolitica* ATCC 700822 spiked into surface water from the Grand River.

A pure culture of *Y. enterocolitica* was added to different enrichment broths with and without concentrated surface water from the Grand River. Methods were evaluated for *Y. enterocolitica* recovery and for inhibition of other indigenous bacteria from the Grand River.

3.3.4.1 *Y. enterocolitica* Inoculum

Y. enterocolitica bioserogroup 4/O:3 (ATCC 700822) was grown in TSB (BD, Oakville, ON) at 28°C for 16-20 h. The culture was enumerated using a Petroff-Hausser Counting Chamber (Hausser Scientific, Horsham, PA) and a Zeiss Axioskop 2 Microscope (Empix Imaging Inc., Mississauga, ON). Serial dilutions were prepared in phosphate-buffered water (PBW) (0.3 mM KH_2PO_4, 2 mM $MgCl_2 \cdot H_2O$, pH 7.2), and were used to inoculate enrichment broths in the recovery experiments. Serial dilutions were also plated on TSA (BD, Oakville, ON) to obtain viable cells counts. After incubation at 28°C for 16-20 h, colonies were counted.

3.3.4.2 Enrichment Broths

Four different enrichment methods were compared in this study (listed in Table 3-4): (1) Irgasan-Ticarcillan-Chlorate (ITC) (Wauters *et al.*, 1988); (2) Luria-Bertani-Bile Salts-Irgasan (LB-BSI) broth (Hussein *et al.*, 2001); (3) modified tryptic soy broth (mTSB) (Bhaduri *et al.*, 1997); and (4) cold enrichment in phosphate-buffered saline (PBS) (Johnson, 1998). The source or reference used to prepare each broth is as follows. ITC broth was purchased from Fluka (Steinheim, Switzerland). PBS was purchased from EMD (Darmstadt, Germany). LB-BSI was prepared as described by Hussein *et al.* (2001). mTSB was prepared as described by Bhaduri *et al.* (1997). Ten mL aliquots of each broth were added to 20-mL glass test tubes, then sterilized by autoclaving at 121°C for 20 mins and stored at 4°C. After broths were inoculated as described in the recovery experiments below, they were incubated as described in Table 3-4. For LB-BSI and mTSB cultures, irgasan was added to a final concentration of 4 µg/mL after 24 h. A 4 mg/mL stock solution of irgasan (Fluka, Steinheim, Switzerland) was prepared by dissolving 40 mg in 10 mL of methanol and was stored at 4°C.

Table 3-4: Enrichment methods summary

Enrichment Broth	Incubation Temperature	Incubation Period	Reference
ITC	25°C	2 days	Wauters *et al.* (1988)
LB-BSI	12°C	3 days	Hussein *et al.* (2001)
mTSB	12°C	3 days	Bhaduri *et al.* (1997)
PBS	4°C	14 days	Johnson *et al.* (1998)

Each method was conducted with and without a post-enrichment alkaline treatment, then plated on CIN agar plates. The alkaline treatment (Johnson, 1998) involved adding 0.5 mL of enrichment culture to 4.5 mL of alkaline solution (0.25 % potassium hydroxide, 0.5 % sodium chloride; prepared as described by Johnson (1998)), vortexing for 2-3 s, then plating the alkaline treated culture on CIN agar.

3.3.4.2.1 Recovery Experiment A

The growth of *Y. enterocolitica* with each enrichment method was compared by inoculating *Y. enterocolitica* cells into each broth and enumerating the cultures on CIN agar at the end of the incubation period.

One hundred µL of a 1000 CFU/mL inoculum (section 3.3.4.1) of *Y. enterocolitica* ATCC 700822 (100 CFU total) was added to 10 mL of each enrichment broth, with the exception of PBS to which 100 µL of a 3500 CFU/mL inoculum (350 CFU total) were added. Preliminary experiments showed that, due to limited cell growth, PBS needed to be inoculated higher to enable subsequent cell counts. Cultures were incubated as described in Table 3-4.

At the end of the incubation period, each enrichment culture was serially diluted in PBW and spread plated on CIN agar. Concurrently, enrichment cultures were alkaline treated (as described above), then immediately serially diluted in PBW and spread plated on CIN agar. Plates were incubated at 28°C for 24 h and colonies were counted to enumerate *Y. enterocolitica*.

3.3.4.2.2 Recovery Experiment B

The growth of indigenous bacteria from the Grand River with each enrichment method was compared by inoculating concentrated Grand River water samples into each broth and enumerating the cultures on CIN agar at the end of their incubation periods.

Surface water was collected from the Grand River just upstream of a drinking water treatment plant intake in Kitchener, ON (see map in Figure 3-1). Water was collected in 25 L plastic carboys and stored at 4°C for up to 3 days. A 500-mL water sample was concentrated by filtration through a 0.45 µm GN-6 Metricel® (47 mm diameter) filter (Pall Corporation, Mississauga, ON) using a sterile filter unit (Nalgene, Rochester, NY) and a vacuum pump under ~500 mM Hg pressure. Filters were rolled such that sample residue was on the inside, and then placed into 10 mL of each broth. Each broth was incubated, treated and plated as described in experiment A. Colonies on each CIN agar plate were classified and counted in two categories: (1) presumptive *Yersinia* and (2) non-*Yersinia* bacteria. Presumptive *Yersinia* colonies were ~0.5-2 mm in diameter, with a red bulls-eye centre surrounded by an entire or undulated, transparent edge. All presumptive *Yersinia* colonies were restreaked on TSA plates and screened with the tests outlined in section 3.3.3 to confirm whether they were *Yersinia* isolates.

3.3.4.2.3 Recovery Experiment C

The ability of each method to recover *Y. enterocolitica* in the presence of indigenous bacteria from the Grand River was compared by inoculating a pure culture of *Y. enterocolitica* and concentrated Grand River water samples into each broth, then enumerating *Y. enterocolitica* on CIN agar at the end of the incubation period. Experiment C compared only the first three enrichment broths listed in Table 3-4 (ITC, LB-BSI and mTSB).

For each method tested, two 10 mL tubes of broth were inoculated with concentrated surface water samples (as described in experiment B), and one tube was also inoculated with 100 µL of a 2000 CFU/mL inoculum of *Y. enterocolitica* ATCC 700822 (200 CFU total). All cultures were incubated, treated, plated and counted as described in experiment B.

3.3.5 Culture-Based Detection of *Y. enterocolitica* in the Grand River

The previous section compared different methods for isolating *Y. enterocolitica* from surface water. This section outlines the methods that were used to survey surface water from the Grand River watershed for the presence of *Y. enterocolitica*. For the survey, the mTSB enrichment method (Bhaduri *et al.* 1997) with and without an alkaline treatment (Johnson, 1998) was used.

3.3.5.1 Sampling Sites

Sites for sampling surface water in the Grand River watershed were selected based on a previous study by Dorner *et al.* (2007). Five sampling sites were selected in the Grand River watershed in the areas surrounding the cities of Kitchener and Waterloo (Figure 3-1). Sites included a point in the Grand River north of Kitchener-Waterloo, the Canagagigue Creek and Conestogo River just before each tributary meets the Grand River, and in the Grand River just downstream of a wastewater treatment plant effluent and just upstream of a drinking water treatment plant intake.

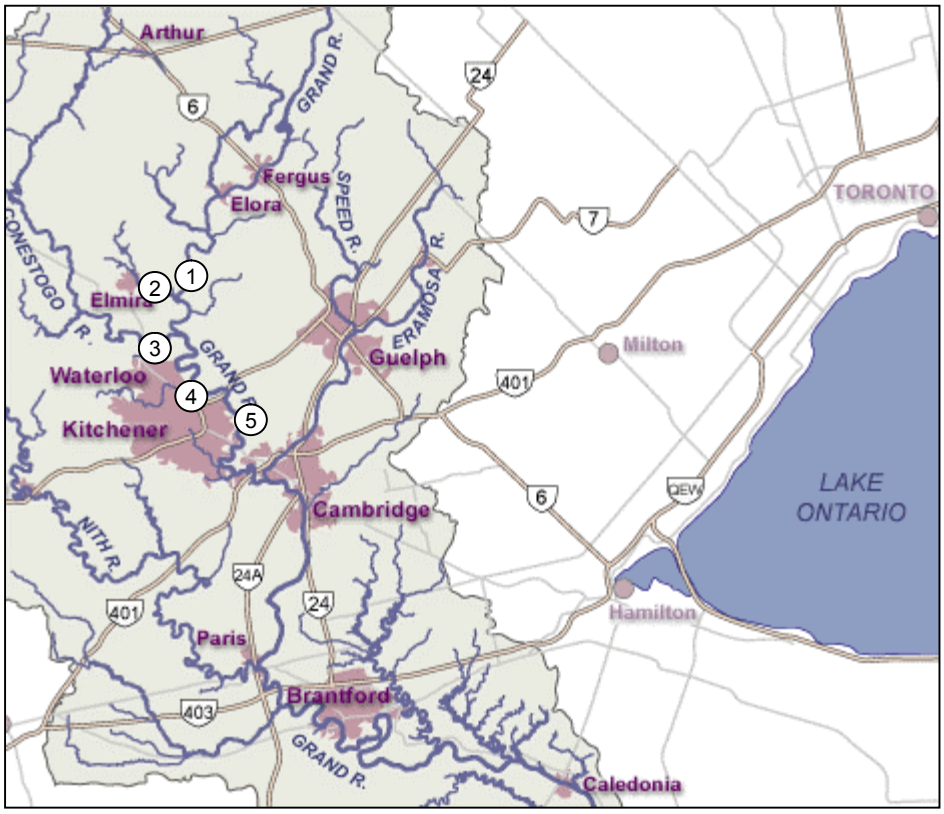


Figure 3-1: Grand River watershed sampling locations. Surface water was collected from five sampling locations: (1) Grand River (North), (2) Canagagigue Creek, (3) Conestogo River, (4) Grand River (WW) (downstream of a wastewater treatment plant effluent), and (5) Grand River (IN) (upstream of a drinking water treatment plant intake).

3.3.5.2 Surface Water Collection

Surface water samples were collected from all five sampling sites every other week and also, when possible, following precipitation events including heavy rainfall and spring snow melt. Water samples were collected approximately 2-3 m away from the edge of the river and approximately 10-20 cm below the surface from a fast flowing portion of the river. Surface water was collected in sterile 1 L polypropylene, wide-mouth bottles (VWR, Mississauga, ON) containing 0.5 mL of 0.1 N sodium thiosulfate, transported on ice and stored at 4°C. Samples were analyzed within 24 h of collection. Since one sampling point was downstream

of a wastewater effluent discharge, sodium thiosulfate was added to bottles, as described, to neutralize any residual chlorine that may be in the water.

3.3.5.3 Sample Concentration

Surface water samples were concentrated by filtering 500 mL water through 0.45 µm GN-6 Metricel® (47 mm diameter) filters (Pall Corporation, Cat. No. P/N 66191), using a vacuum pump under ~500 mmHg pressure using a sterile filter unit (Nalgene).

3.3.5.4 Sample Enrichment

Using sterilized forceps, filters containing the residue from Grand River water samples were rolled such that sample residue was on the inside. Rolled filters containing Grand River filtered residue were placed into test tubes containing 10 mL mTSB and were incubated as described in Table 3-4 with shaking at 100 rpm. At 24 h, 10 µL of an irgasan stock solution (4 mg/mL in methanol) was added to each enrichment culture, to achieve a final concentration of 4 µg/mL.

3.3.5.5 Isolating and Identifying *Yersinia* Strains

After the enrichment period, the mTSB broth was plated on CIN agar plates with and without an alkaline treatment. Fifty µL of non-alkaline treated enrichment broth was transferred to CIN agar and streaked for isolated colonies. Enrichment cultures were also treated with an alkaline solution (as described by Johnson 1998) and 100 µL of the alkaline treated culture was transferred to CIN agar streaked for isolated colonies. Plates were incubated at 28°C for 16-24 h.

After incubation, the plates were observed for colonies displaying typical *Y. enterocolitica* morphology. Up to eight representative colonies were selected as presumptive *Yersinia* isolates, streaked onto TSA plates and incubated at 28°C for 16-24 h. Presumptive *Yersinia* isolates were screened with the identification tests described in section 3.3.3.

Isolates that gave expected results for *Yersinia* spp. for all of the identification tests were subsequently screened with the BIOLOG MicroLog Microbial Identification System. The BIOLOG system classifies bacterial isolates based on their ability to oxidize 95 different organic carbon sources. Isolates were tested as recommended by the manufacturer (BIOLOG, Hayward, CA). BIOLOG plates were read visually and results were compared to the GN database using the BIOLOG MicroLog software, version 4.20. Duplicate glycerol frozen stocks of the isolates identified as *Yersinia* by BIOLOG screening were prepared as described in section 3.3.1.

3.3.5.6 Biotyping and Serotyping

BIOLOG confirmed *Yersinia* isolates were shipped to the Ontario Ministry of Health and Long-Term Care, Central Public Health Laboratory (Etobicoke, ON) for further testing. The API-20E Biochemical Identification kit (bioMerieux, Inc., Hazelwood, MO) was used to identify the species of the Grand River *Yersinia* isolates. Isolates were also biotyped based on methods developed by Wauters *et al.* (1987) and the online Bacteriological Analytical Manual for the USFDA (Weagant *et al.*, 2001) and serotyped based on methods developed by Wauters *et al.* (1991) and Aleksic and Bockemuhl (1984).

3.4 Results and Discussion

Several culture-based methods for isolating *Y. enterocolitica* from surface water were evaluated and compared. One of the methods tested was selected and used to conduct a survey of the Grand River watershed for *Yersinia* spp..

3.4.1 Comparison of *Yersinia* Selective Agars

MacConkey agar, CIN agar and SSDC agar are the most commonly used agars for isolating *Y. enterocolitica* (Fredriksson-Ahomaa *et al.*, 2003). CIN agar has been shown to be superior to MacConkey agar (Head *et al.*, 1982; Schiemann, 1983b). Both CIN and SSDC agar are recommended in the USDA/FSIS methods for isolating *Y. enterocolitica* (Johnson, 1998).

The growth of laboratory bacteria on CIN agar and SSDC agar were compared. Results are summarized in Table 3-5 and Table 3-6.

Yersinia strains were grown on CIN and SSDC agar to confirm that strains of *Y. enterocolitica* from a variety of biogroups and serogroups would grow and to evaluate the growth of other *Yersinia* spp. as well. All of the *Yersinia* strains tested grew well on CIN agar with the exception of the two strains of *Y. pseudotuberculosis* (Table 3-5). This was consistent with previous reports that *Y. pseudotuberculosis* is inhibited by CIN agar (Fukushima *et al.*, 1986). All *Yersinia* strains grew on SSDC agar, but colonies tended to be very small after 24 h (Table 3-6). Colonies growing on SSDC agar were generally smaller than on CIN agar.

Non-*Yersinia* organisms were also tested under the same conditions to evaluate the ability of CIN and SSDC agar to inhibit the growth of other laboratory bacterial strains. CIN agar is supposed to inhibit many of the organisms from the family Enterobacteriaceae (Wanger, 2007), including *E. coli* and *P. mirabilis*, and also inhibits *P. aeruginosa* (Schiemann, 1979). Most of the non-*Yersinia* organisms tested belonged to the family Enterobacteriaceae and were selected to confirm claims that CIN agar will inhibit their growth and to evaluate their growth on SSDC agar. The non-*Yersinia* organisms tested can also be found in water. (*S. aureus* was also tested to include a Gram-positive organism for subsequent tests with MacConkey agar, which inhibits Gram-positive organisms.)

Only two of the non-*Yersinia* organisms tested were able to grow on CIN agar: *C. freundii* and *P. aeruginosa* (Table 3-5). *C. freundii* colonies did not have the bull's eye appearance of *Yersinia* colonies and *P. aeruginosa* did not grow very well on CIN agar and produced very small colonies. In contrast, eight of the nine non-*Yersinia* organisms tested grew on SSDC agar and at least four of those yielded colonies that were difficult to differentiate from *Yersinia* colonies grown on SSDC agar (Table 3-6).

Table 3-5: Comparing the growth of different laboratory bacteria on CIN agar

Strain ID	Species	Colony diameter	Colony form, colour	Colony margin
1-11	*Y. enterocolitica*	0.5-1 mm	circular or irregular, red center	transparent, entire or undulated
12 -13	*Y. pseudotuberculosis*	poor growth	irregular, pink-red	*
14	*Y. frederiksenii*	1 mm	circular, red center	transparent, undulated
15	*Y. intermedia*	0.5 mm	circular, red center	transparent, entire
16	*Y. kristensenii*	1 mm	circular, red centre	transparent, undulated
17	*Y. mollaretti*	0.5 mm	irregular, red center	transparent, undulated
18	*Y. rodher*	0.5 mm	circular, red center	transparent, undulated
19	*A. hydrophila*	no growth		
20	*C. freundii*	1 mm	circular, pink-red	translucent, undulated
21	*E. coli*	no growth		
22	*E. coli*	no growth		
23	*E. coli* O157:H7	no growth		
24	*P. aeruginosa*	poor growth	irregular, white	*
25	*P. mirabilis*	no growth		
26	*S. aureus*	no growth		
27	*S. enterica*	no growth		

* Could not be assessed because colonies were extremely small.

Table 3-6: Comparing the growth of different laboratory bacteria on SSDC Agar

Strain ID	Species	Colony diameter	Colony form, colour	Colony margin
1-11	*Y. enterocolitica*	≤0.5 mm	circular, white	Undulated
12-13	*Y. pseudotuberculosis*	<0.5 mm	White	*
14	*Y. frederiksenii*	<0.5 mm	White	*
15	*Y. intermedia*	0.5 mm	circular, white	Undulated
16	*Y. kristensenii*	<0.5 mm	White	*
17	*Y. mollaretti*	<0.5 mm	White	*
18	*Y. rodher*	<0.5 mm	White	*
19	*A. hydrophila*	<0.5 mm	White	*
20	*C. freundii*	<0.5 mm	Pink	*
21	*E. coli*	<0.5 mm	Pink	*
22	*E. coli*	0.5 mm	Pink	Entire
23	*E. coli* O157:H7	<0.5 mm	Pink	*
24	*P. aeruginosa*	<0.5 mm	White	*
25	*P. marbilis*	<0.5 mm	White	*
26	*S. aureus*	no growth		
27	*S. enterica*	1 mm	white / black [§]	Entire

* Could not be assessed because colonies were very small.
[§] Individuals colonies were white. However, in areas of dense growth colonies were black.

Comparing the growth of various *Yersinia* and non-*Yersinia* organisms on CIN and SSDC agar showed that CIN agar inhibited more non-*Yersinia* organisms and yielded *Yersinia* colonies that were far easier to differentiate from non-*Yersinia* colonies. Although it has been suggested CIN agar inhibits the growth of *Y. enterocolitica* 3B/O:3 (Fukushima *et al.*, 1986), these authors incubated samples at 32°C and this may have impacted the growth rates of the *Y. enterocolitica* strains studied. A subsequent study has shown that at 37°C *Y. enterocolitica* strains with the pYV plasmid grew slower compared to strains without the plasmid, but no differences in growth rates were observed at 25°C (Logue *et al.*, 2000; Logue *et al.*, 2006).

A survey of the literature indicates that CIN agar is commonly the only selective agar used to isolate *Y. enterocolitica* from samples. CIN has been identified as an effective and preferred agar for isolating *Y. enterocolitica* primarily for two reasons: CIN is better at inhibiting non-*Yersinia* organisms and has a higher confirmation rate when compared with other agars that are used to select for *Y. enterocolitica* (Head *et al.*, 1982; Schiemann, 1983b). Results

from the current study indicated that SSDC did not inhibit any of the Gram-negative bacteria tested. Additionally, on SSDC non-*Yersinia* bacteria produced colonies that were very difficult to distinguish from *Yersinia* colonies. This is partially because *Yersinia* colonies did not produce particularly distinctive colonies, however they did match the expected description (as provided by Johnson (1998)). This suggested that differentiating *Yersinia* colonies on an agar plate containing a mixture of non-*Yersinia* colonies from environmental samples would likely be very challenging. Consequently, CIN agar was selected for use in subsequent experiments for isolating *Yersinia* from samples.

3.4.2 Evaluation of *Yersinia* spp. Identification Tests

In order to confirm the identity of presumptive *Yersinia* isolated from CIN agar plates, it is necessary to conduct biochemical identification tests. Two biochemical tests using urea agar and Kligler iron agar (KIA), have been recommended for distinguishing *Yersinia* isolates from other bacteria that present similar colony morphologies on CIN agar (Devenish *et al.*, 1981). At least one of these tests is usually used in combination with one or more other biochemical tests to identify presumptive *Y. enterocolitica* isolates from water (Fukushima *et al.*, 1984; Massa *et al.*, 1988; Brennhovd *et al.*, 1992; Arvanitidou *et al.*, 1994; Sandery *et al.*, 1996; Schaffter *et al.*, 2002). The USDA/FSIS Microbiology Laboratory Guidebook (Johnson, 1998) suggests testing isolates with urea agar, KIA, and Simmon's citrate agar for identifying *Yersinia* strains. In addition, we tested all presumptive isolates for oxidase reaction and growth on MacConkey agar. Growth on MacConkey agar is useful as this agar inhibits Gram-positive organisms and tests for lactose fermentation. To further identify the species of *Yersinia*, additional testing is required. For our study, the species and genotype of *Yersinia* isolates were determine by the Ontario Ministry of Health and Long-Term Care, Central Public Health Laboratory (as described in section 3.3.5.6).

Laboratory control strains were used to test a series of identification tests, including: growth on MacConkey plates, an oxidase test, urea utilization on urea slants, citrate utilization on Simmon's citrate slants, and lastly, glucose and lactose fermentation, gas production and H_2S production on KIA slants (Table 3-7).

Table 3-7: Identification tests results for different laboratory bacteria controls

Strain ID	Species	Growth on MacConkey	Oxidase	Urease	Citrate	Lactose	Glucose	Gas prod.	H_2S prod.
1-18	*Yersinia* spp.	+	-	+	-	-	+	-	-
19	*A. hydrophila*	+	+	-	-	-	+	-	-
20	*C. freundii*	+	-	+	+	+	+	+	+
21	*E. coli*	+	-	-	-	+	+	-	-
22	*E. coli*	+	-	-	-	+	+	-	-
23	*E. coli* O157:H7	+	-	-	-	+	+	-	-
24	*P. aeruginosa*	+	+	-	+	-	-	-	-
25	*P. mirabilis*	+	-	+	-	-	+	-	+
26	*S. aureus*	-	-	+	-	-	+/-*	-	-
27	*S. enterica*	+	-	-	-	-	+	+	+

* The butt of the KIA agar slant was difficult to interpret.

The general purpose of testing positive and negative control strains with each identification test was to ensure that each agar or reagent used gave the correct negative and positive results and to confirm that *Yersinia* strains gave the expected results. All laboratory strains of *Yersinia* yielded the expected results for *Yersinia* spp. and none of the non-*Yersinia* control bacteria completely matched *Yersinia* for all the tests conducted. When these identification tests were used in preliminary screening of Grand River samples (data not shown) it was determined that these tests were able to successfully screen out non-*Yersinia* isolates from the Grand River that were picked from CIN agar. It was also determined that the subsequent use of the BIOLOG (California, USA) identification system screened out a small number of additional non-*Yersinia* isolates. The series of identification tests in combination with the BIOLOG identification system always successfully identified *Yersinia* spp.. Consequently, these tests were used in subsequent experiments for identifying *Yersinia* strains.

3.4.3 Comparison of Enrichment Methods

Four different enrichment methods were compared to determine which method yielded maximum recovery of *Y. enterocolitica* from surface water samples. Two of the four methods, enrichment in PBS and ITC, are recommended in the USDA/FSIS methods for *Y. enterocolitica* (Johnson, 1998). Cold enrichment (4°C) in PBS, has often been used to isolate *Y. enterocolitica*, which is a psychotrophic bacteria (Fredriksson-Ahomaa *et al.*, 2003). The cold incubation is intended to inhibit and kill other bacteria in the sample that are less tolerant of cold temperatures. However, cold treatment methods require long incubation periods (up to 4 weeks (Fredriksson-Ahomaa *et al.* 2003)). In contrast, ITC broth is incubated at 25°C for only 2 days and contains both chemical agents and antibiotics, to which *Yersinia* is resistant, intended to inhibit other bacteria. The authors of the present study also tested two other enrichment methods, including mTSB and LB-BSI. Both of these methods utilize (1) milder selective agents, (2) delayed addition of antibiotics, (3) an incubation temperature of 12°C. The antibiotic, irgasan, is not added to enrichment cultures until after 24 h of incubation. Bhaduri *et al.* (1997) found that inclusion of irgasan in the broth from the beginning of the incubation period suppressed the growth of *Y. enterocolitica* containing the pYV plasmid. The authors found that adding irgasan at 24 h provided *Y. enterocolitica* a

period of time to adapt to the conditions in the enrichment broth and begin growing. In addition, Schiemann and Olson (1984) suggested that incubation at temperatures closer to 15°C would enable *Y. enterocolitica* to compete better with other bacteria while also reducing the incubation time required compared to most cold incubations.

Enrichment methods were evaluated in three experiments. In the first experiment (A), *Y. enterocolitica* cells were added to each enrichment broth and enumerated at the end of the incubation period. In the second experiment (B), concentrated surface water from the Grand River was added to each enrichment broth and indigenous bacteria were enumerated at the end of the incubation periods. Lastly, in the third experiment (C), *Y. enterocolitica* cells and concentrated Grand River water were both added to each enrichment broth, and methods were evaluated for their ability to recover the *Y. enterocolitica* cells. The effect of an alkaline treatment step (Johnson, 1998) was also evaluated in combination with each enrichment method, as *Y. enterocolitica* are known to be tolerant of alkaline conditions (Aulisio *et al.*, 1980).

The laboratory strain used to spike samples in the recovery experiments was *Y. enterocolitica* ATCC 700822. This strain was selected because it is bioserotype 4/O:3, which is responsible for most of the human cases in Europe, Japan, Canada and the USA (Bottone, 1999). *Y. enterocolitica* ATCC 700822 also contains the pYV virulence plasmid (determined in Chapter 4), considered crucial to imparting full virulence to *Y. enterocolitica* (Schiemann *et al.*, 1982; Heesemann *et al.*, 1984) and also demonstrated to influence growth of *Y. enterocolitica* strains (Logue *et al.*, 2000; Logue *et al.*, 2006).

In experiment A, each method was evaluated for the ability to support the growth of a laboratory *Y. enterocolitica* strain. The ability of *Y. enterocolitica* cells to survive an alkaline treatment was also evaluated. Approximately 100 *Y. enterocolitica* CFU were inoculated into 10 mL of each broth (or 10 CFU/mL), except PBS to which approximately 350 CFU were added (35 CFU/mL). A higher inoculum was added to PBS samples to facilitate culture enumeration, since this enrichment method does not promote cell growth. At the end of each incubation period, enrichment cultures were spread plated on CIN agar and enumerated.

Table 3-8: Comparison of the growth of *Y. enterocolitica* in each enrichment broth without the addition of surface water

Media [ab]	Alkaline treatment	*Yersinia* (log CFU/mL) [c] Mean (range)
ITC	-	8.0 (7.4-8.5)
	+	8.0 (7.4-8.5)
mTSB	-	6.1 (5.4-6.6)
	+	5.1 (3.8-6.6)
LB-BSI	-	4.1 (3.9-4.3)
	+	3.6 (3.0-4.1)
PBS	-	2.1 (1.9-2.2)
	+	2.0 (1.5-2.5)

[a] For each media, n=2, except mTSB, n=3
[b] The starting concentration in all broths was ~1.0 log CFU/mL prior to incubation, with the exception of PBS, which had a starting log concentration of ~1.5 log CFU/mL.
[c] Log concentrations at the end of the incubation period.

Results from experiment A (Table 3-8) demonstrated that *Y. enterocolitica* grew in most enrichment methods tested. *Y. enterocolitica* ATCC 700822 concentrations at the end of the incubation period (without an alkaline treatment) were the highest in ITC (8.0 log CFU/mL), followed by mTSB (6.1 log CFU/mL), then LB-BSI (4.1 log CFU/mL), and were lowest in PBS (2.1 log CFU/mL). Since the starting concentration in PBS was 1.5 log CFU/mL, there was only a 0.5 log increase following incubation at 4°C. Given that PBS does not contain any nutrients, cells were not expected to grow in PBS, but simply to survive. It is possible that some of the growth observed may be attributable to cells that were in the exponential phase of growth (phase in which cells are rapidly dividing) when they were transferred to PBS, or due to the carry over of nutrients from the diluted TSB culture used as the inoculum. According to one study, *Y. enterocolitica* was able to grow by over 1 log after 72 hours in sterile distilled water at 4°C, after which viable cell concentrations leveled off but were maintained for an additional 6 days (Highsmith *et al.*, 1977).

Y. enterocolitica remained at high levels following a post-enrichment alkaline treatment. However, in mTSB and LB-BSI, *Y. enterocolitica* counts did show a decrease after an alkaline treatment of about 0.5 log in mTSB and 1 log in LB-BSI.

Results show that *Y. enterocolitica* grows best in ITC broth. *Y. enterocolitica* concentrations were likely higher because ITC broth was incubated at a higher temperature (25°C) compared to mTSB and LB-BSI (12°C) and although *Y. enterocolitica* are tolerant of cold temperatures, they still grow faster at warmer temperatures. However, an ideal enrichment method must also effectively inhibit the growth of non-*Yersinia* bacteria.

In experiment B, each method was evaluated for the ability to inhibit the growth of indigenous bacteria from the Grand River. Concentrated samples of Grand River water (500 mL) were inoculated into 10 mL of each broth. At the end of the incubation periods, cultures were spread plated on CIN agar and enumerated.

Table 3-9: Comparison of each enrichment broth and alkaline treatment on the recovery of indigenous bacteria from concentrated Grand River samples

Media [a]	Alkaline treatment	Indigenous bacteria [b] (log CFU/mL) Mean (range)
ITC	-	8.5 (8.3 8.6)
	+	8.4 (8.3 8.5)
mTSB	-	7.8 (7.5 8.1)
	+	4.4 (4.2 4.6)
LB-BSI	-	7.9 (7.7 8.0)
	+	5.2 (3.9 6.4)
PBS	-	6.0 (5.8 6.2)
	+	4.0 (3.9 4.1)

[a] For each media, n=2, except mTSB, n=3
[b] Log concentrations at the end of the incubation period.

Results from experiment B (Table 3-9) suggest that indigenous bacteria are not inhibited by the enrichment broths and furthermore can grow in the broths and on CIN agar. At the end of the incubation periods, the concentration of indigenous bacteria (without an alkaline treatment) was highest in ITC (8.5 log CFU/mL), followed by mTSB (7.8 log CFU/mL), LB-BSI (7.9 log CFU/mL), and was lowest in PBS (6.0 log CFU/mL). Although an alkaline treatment did not reduce the concentration of indigenous bacteria growing in ITC, the treatment was effective in reducing indigenous bacteria in the other broths by 3.5 log in

mTSB, 2.5 log in LB-BSI and 2 log in PBS. Naturally occurring *Yersinia* (non-spiked) were not detected in this experiment using any of the enrichment methods. Results from experiment B showed that indigenous bacteria from the Grand River were at lower concentrations in mTSB and LB-BSI compared with ITC. This is likely due to the lower incubation temperature (12°C) used in the mTSB and LB-BSI methods compared to the ITC method (25°C). High levels of indigenous bacteria present in PBS after a cold incubation confirms that other bacteria from the Grand River can also survive cold temperatures. This challenge has been noted previously by Fredriksson-Ahomaa and Korkeala (2003). Also, the carry-over of nutrients into the enrichment media from the concentrated river water samples may have enabled some growth of indigenous bacteria in PBS. It was concluded that cold enrichment in PBS is not an appropriate enrichment method for surface water samples from the Grand River. Consequently, PBS was not tested in recovery experiment C.

In experiment A, *Y. enterocolitica* was inoculated at levels that were low to mimic conditions in which the organism is present at very low levels in the environment. This was intended to allow an approximate comparison of the final concentrations of *Y. enterocolitica* (Table 3-8) to the final concentrations of indigenous bacteria (Table 3-9). Recall that approximately 100 CFU were added to each broth in experiment A (with the exception of PBS to which 350 CFU were added) and that 500 mL of surface water was concentrated and added to each enrichment broth in experiment B. If final CFU counts in experiment A and B are compared, the concentrations of indigenous bacteria were usually higher than concentrations of *Y. enterocolitica* grown in the same enrichment broths. However, one exception was mTSB samples treated with an alkaline solution. With this enrichment method, *Y. enterocolitica* grew to concentrations that were slightly higher than indigenous bacteria (just over 0.5 log higher). In LB-BSI and PBS samples indigenous bacteria concentrations (without an alkaline treatment) were 3.8 log and 4 log higher than *Y. enterocolitica* concentrations. Even after an alkaline treatment, indigenous bacteria concentrations were about 1.5 log (LB-BSI) and 2 log (PBS) higher than *Y. enterocolitica*.

Under most conditions tested, indigenous bacteria levels (in experiment B) exceeded pure culture *Y. enterocolitica* levels (in experiment A). An ideal enrichment method must also effectively inhibit the growth of non-*Yersinia* bacteria. We, therefore, next tested the ability to recover *Y. enterocolitica* from these same river samples.

In experiment C, the growth of *Y. enterocolitica* in competition with non-*Yersinia* bacteria from the Grand River was evaluated for ITC, mTSB and LB-BSI enrichment methods. Approximately 200 *Y. enterocolitica* CFU were added together with indigenous bacteria from 500 mL of river water. At the end of the incubation periods, cultures were spread plated on CIN agar, enumerated and evaluated for *Y. enterocolitica* recovery. In experiment C, indigenous bacterial concentrations were relatively similar in non-spiked and spiked broths (Table 3-10), and were similar to results from recovery experiment B (Table 3-9). Indigenous bacterial concentrations in non-alkaline treated samples were highest in ITC (8.8-9.0 log CFU/mL), followed by mTSB (7.9-8.3 log CFU/mL) and LB-BSI (7.7-7.9 log CFU/mL). Results also demonstrated that *Y. enterocolitica* ATCC 700822 was very difficult to recover from Grand River samples. The only media tested which recovered *Yersinia* strains from spiked samples was LB-BSI combined with an alkaline treatment. However, upon careful examination of the colony morphology of the *Yersinia* isolated from spiked samples, it was concluded these isolates were not the same *Y. enterocolitica* strain (ATCC 700822) that had been spiked into samples. Regardless, *Yersinia* was more frequently detected in non-spiked samples. This showed that indigenous *Yersinia* are present in the Grand River, which was expected. It had been predicted, however, that final *Y. enterocolitica* (ATCC 700822) concentrations in spiked samples would be sufficiently above the background levels of indigenous *Yersinia* in non-spiked samples to yield a noticeable difference between spiked and non-spiked samples. This, however, was not the case, as the methods tested were not effective for recovering *Y. enterocolitica* (ATCC 700822). This strain was selected because it is bioserotype 4/O:3, responsible for most of the human cases of enteric disease (Bottone 1999), and because it contains the pYV plasmid (see Chapter 4), considered crucial to imparting full virulence to *Y. enterocolitica* (Schiemann *et al.*, 1982; Heesemann *et al.* 1984) and influential to the growth of *Y. enterocolitica* in selective media (Logue *et al.* 2000; Logue *et al.* 2006).

Table 3-10: Comparison of *Y. enterocolitica* recovery using different enrichment broths with and without an alkaline treatment. *Y. enterocolitica* were added to broths (spiked samples) with indigenous bacteria from concentrated Grand River samples. Control samples (non-spiked samples) contained indigenous bacteria from concentrated Grand River samples, but no *Y. enterocolitica* inoculum.

		Non-spiked samples [b]		Spiked samples [bc]	
Media [a]	**Alkaline treatment**	***Indigenous Yersinia* (log CFU/mL)**	**Indigenous bacteria (log CFU/mL)**	***Recovered Yersinia* (ATCC 700822) (log CFU/mL)**	**Indigenous bacteria (log CFU/mL)**
ITC	-	n.d.[d] – 6[e]	9.0 (8.9-9.0)	n.d.	8.8 (8.7-8.9)
	+	n.d.	8.9 (8.7-9.0)	n.d.	8.8 (8.7-8.9)
mTSB	-	n.d. – 6[e]	7.9 (7.9-7.9)	n.d.	8.3 (8.1-8.4)
	+	n.d.	6.5 (6.2-6.7)	n.d.	6.7 (6.4-6.9)
LB-BSI	-	n.d.	7.7 (7.6-7.7)	n.d.	7.9 (7.8-7.9)
	+	n.d. – 4[e]	6.4 (6.2-6.5)	n.d.	6.1 (6.0-6.1)

[a] For each media, n=2
[b] Log concentrations at the end of the incubation period.
[c] Approximately 200 *Y. enterocolitica* ATCC 700822 CFU were spiked into each 10 mL (~1.3 log CFU/mL) broth sample before the incubation period.
[d] n.d. = not detected
[e] Approximate values based on low CFU counts (*i.e.* 1-2 confirmed *Yersinia* colonies detected on one plate in a dilution series).

It is important to note that in Table 3-10 *Yersinia* concentrations are based on very low CFU counts, meaning that only one or two confirmed *Yersinia* colonies were detected on one plate in a dilution series. Normally, less dilute samples are plated to obtain higher and more reliable CFU counts. However, in this case, agar plates from lower dilutions were completely overgrown with other bacteria from the Grand River.

The objective of this experiment had been to enumerate levels of *Yersinia* and non-*Yersinia* organisms in the enriched samples. However, the high concentration of non-*Yersinia* bacteria from the Grand River that grew in the enrichment broths and on CIN agar made it difficult to detect and enumerate low levels of *Yersinia*. Furthermore, there were many non-*Yersinia* bacteria from the Grand River that formed colonies on CIN agar that looked very similar to *Yersinia* colonies. Consequently, many presumptive colonies that were screened were not confirmed as *Yersinia* after identification tests were conducted. Although CIN agar has been identified as a preferred agar compared to other selective agar available for isolating *Y. enterocolitica* (Head *et al.*, 1982; Schiemann, 1983b), it was not effective enough for isolating *Y. enterocolitica* from surface waters tested in this study.

Results from experiment C did not reveal that any one enrichment method was superior at recovering *Y. enterocolitica* spiked into surface water. Results did show that the culture-based methods tested may not be effective for recovering low levels of *Y. enterocolitica* from Grand River water, and would likely not be effective for isolation from other surface water matrices. Overall, results demonstrated that the problems encountered when trying to recover *Y. enterocolitica* can likely be attributed to the prolific growth of indigenous bacteria from the Grand River in the enrichment broths as well as on the CIN agar. *Y. enterocolitica* tend to grow poorly in competition with other organisms (Schiemann *et al.*, 1984; Calvo *et al.*, 1986b), which also likely contributed to challenges in recovering *Y. enterocolitica* from water samples. One study found that *Y. enterocolitica* growth may be impeded by bacteriocin-like agents produced by *Y. frederiksenii*, *Y. kristensenii* and *Y. intermedia* (Calvo *et al.*, 1986a), organisms that have previously been detected in surface waters (Fredriksson-Ahomaa *et al.*, 2003).

It is important to note the variable impact of the alkaline treatment on indigenous bacteria concentrations observed in experiments B (Table 3-9) and C (Table 3-10). While the concentration of indigenous bacteria without an alkaline treatment remained constant in experiments B and C, the concentrations of the alkaline treated samples were slightly higher in experiment C by about 0.4 log in ITC, 2 log in mTSB and 1 log in LB-BSI. This is likely due to the difficulty in reproducing the alkaline treatment consistently each time. According to the method described by Johnson (1998), the enriched culture is to be mixed with the alkaline solution for 2-3 seconds. However, in practice it is difficult to reproduce a 2-3 second treatment period consistently from one sample to the next. The inconsistent results obtained using a post-enrichment alkaline treatment has been noted in previous studies (Schiemann, 1983a). Also, it has previously been demonstrated that resistance to alkaline conditions vary between *Y. enterocolitica* strains (Doyle *et al.*, 1983). Nonetheless, the use of an alkaline treatment seems likely to help in the recovery of *Y. enterocolitica* from samples. Plating both alkaline treated, as well as non-treated, enrichment cultures on *Yersinia* isolation agar is recommended in the USDA/FSIS Microbiology Laboratory Guidebook (Johnson, 1998).

Comparisons can be drawn from the results of experiments A and B. Under nearly all of the conditions tested, indigenous bacteria from Grand River water grew to similar or higher levels than *Y. enterocolitica* grown in pure culture, with the exception of cultures enriched in mTSB treated with an alkaline solution. This suggests that the mTSB enrichment method in combination with an alkaline treatment may perform better than the other methods tested in isolating *Y. enterocolitica* from the Grand River.

3.4.4 Grand River Survey

Surface water was collected from sampling sites in the Grand River watershed every two weeks, and analyzed for the presence of *Yersinia* using the culture-based method involving enrichment in mTSB (Bhaduri *et al.*, 1997), followed by plating on CIN agar with and without an alkaline treatment (Johnson, 1998).

Between April 2006 and August 2007, 200 surface water samples were collected and analyzed. *Yersinia* spp. were detected in 52 samples (26 %) and *Y. enterocolitica* isolates were detected in 8 (4 %) of those 200 samples (Table 3-11). In previous studies that isolated *Yersinia* from surface water, a seasonal trend has been observed (Meadows *et al.*, 1982; Fukushima *et al.*, 1984; Massa *et al.*, 1988). However, no seasonal trends were observed in *Yersinia* isolation rates from the Grand River.

Table 3-11: *Yersinia* occurrence at each sampling location between April 2006 and August 2007

Sampling Location	Samples analyzed	Samples positive for *Yersinia*	*Yersinia* isolates [e]	*Y. enterocolitica* isolates
Grand River (North) [a]	40	15	32	4
Canagagigue Creek [b]	40	11	17	4
Conestogo River [b]	40	10	14	1
Grand River (WW) [c]	40	9	16	0
Grand River (IN) [d]	40	7	18	2
All locations	200	52	97	11

[a] Sampled from the Grand River at a location upstream of the other four sites
[b] Canagagigue Creek and Conestogo River are tributaries of the Grand River
[c] Sampled from the Grand River downstream of a wastewater treatment plant discharge
[d] Sampled from the Grand River upstream of a drinking water treatment plant intake
[e] *Yersinia* isolate counts include *Y. enterocolitica* isolates

From the 52 water samples positive for *Yersinia*, a total of 97 *Yersinia* isolates were collected (Table 3-12). A detailed list of the Yersinia isolates from the Grand River watershed is provided in Appendix A. The distribution of these isolates among sampling locations is outlined in Table 3-11. It should be noted that multiple isolates of the same subtype were sometimes isolated from one sample and it is possible these isolates originated from a single strain in the river.

Table 3-12: *Yersinia* strains isolated from surface water samples

Species	Biotype	Serotype	Number of isolates
Y. enterocolitica	1A	O:5	2
Y. enterocolitica	1A	O:5, 27	1
Y. enterocolitica	1A	O:7,8	4
Y. enterocolitica	1A	O:7, 13	1
Y. enterocolitica	1A	O:41,43	1
Y. enterocolitica	1A	O:rough	1
Y. enterocolitica	1A	O:Untypeable	1
Y. aldovae			11
Y. bercovieri			9
Y. frederiksenii			16
Y. intermedia	1		37
Y. intermedia	4		4
Y. kristensenii			2
Y. mollaretii			7
All species			97

The majority of isolates (89 %) were *Y. enterocolitica*-like species, including *Y. aldovae* (11 %), *Y. bercovieri* (9 %), *Y. frederiksenii* (16 %), *Y. intermedia* (42 %), *Y. kristensenii* (2 %) and *Y. mollaretii* (7 %). These species are traditionally considered non-pathogenic. Meanwhile, *Y. enterocolitica* strains accounted for 11 % of the *Yersinia* isolates. All of the *Y. enterocolitica* isolates belonged to biogroup 1A, which is also traditionally considered to be a non-pathogenic biotype. *Y. frederiksenii*, *Y. intermedia*, and *Y. kristensenii* have all been isolated from water previously (Shayegani *et al.*, 1981; Fukushima *et al.*, 1984; Aleksic *et al.*, 1988; Massa *et al.*, 1988; Brennhovd *et al.*, 1992; Arvanitidou *et al.*, 1994; Schaffter *et al.*, 2002; Falcao *et al.*, 2004). However, the Grand River survey was the first study to isolate *Y. bercovieri* and *Y. mollaretii* from water. *Y. enterocolitica* 1/O:5, 1/O:5,27, and 1/O:7,8 have also previously been isolated from water (Fukushima *et al.*, 1984; Aleksic *et al.*, 1988; Massa *et al.*, 1988; Arvanitidou *et al.*, 1994). These early studies did not, unfortunately, distinguish between biotype 1A and 1B, and typed strains simply as biotype 1. A more recent study by Falcao *et al.* (2004) identified several isolates from water that were *Y. enterocolitica*, including bioserotype 1A/O:5, which was also found in the Grand River.

Although all of the *Yersinia* isolates from the Grand River watershed are typically considered non-pathogenic, the classic use of subtyping analysis to differentiate between pathogenic and non-pathogenic *Yersinia* strains has been questioned. Strains traditionally classified as non-pathogenic have been isolated from patients displaying gastrointestinal disease symptoms, including *Y. enterocolitica* 1A (Tennant *et al.*, 2003), as well as all of the *Y. enterocolitica*-like species except *Y. aldovae* (Sulakvelidze, 2000). *Y. enterocolitica* biotype 1A strains isolated from patients displaying symptoms have been studied for the presence of virulence genes found in other *Y. enterocolitica* subtypes that are traditionally classified as pathogenic (Grant *et al.*, 1998; Thoerner *et al.*, 2003; Bhagat *et al.*, 2007). These studies suggest that biotype 1A may not be completely non-pathogenic. A review by Tennant *et al.* (2003) lists *Y. enterocolitica* 1A clinical isolates that have been associated with disease. This list includes 1A strains of serotype O:5 and O:7,8, both of which were also isolated in our survey of the Grand River (Table 3-12).

Pigs are a major reservoir for *Y. enterocolitica* strains of clinical importance to humans (Fredriksson-Ahomaa *et al.*, 2006). In a study related to the current research, *Y. enterocolitica* were isolated from pig feces from farms in the Grand River watershed in 2006 and 2007. *Y. enterocolitica* were isolated from 14 (6%) out of 240 samples. While the majority of the strains isolated were *Y. enterocolitica* 4/O:3, *Y. enterocolitica* biotype 1A strains were also isolated, including one *Y. enterocolitica* 1A/O:5 strain (Public Health Agency of Canada, 2007). *Y. enterocolitica* 1A/O:5 strains were also isolated from Grand River surface water (Table 3-12). Since close to 80% of the land in the Grand River watershed is farmed and pigs are the second most prevalent livestock found in the watershed (Dorner *et al.*, 2004), it seems reasonable that *Yersinia* may be entering the watershed through agricultural run-off. However, occurrence rates in other animals, including wildlife, is still needed to evaluate all possible sources of *Yersinia* in the watershed.

3.5 Conclusions

This study demonstrates that current culture-based methods are not sufficiently optimized for isolating pathogenic *Y. enterocolitica* from surface water samples. Culture-based methods need to be improved to isolate low levels of *Y. enterocolitica* from a surface water matrix containing diverse bacterial species. The poor sensitivity of current methods suggests that published culture-based survey results are likely underestimating the prevalence of *Y. enterocolitica* in water. Culture-based surveys conducted previously have found similar strains as those detected in this study, however, isolation rates were often low. Despite limitations of the methods, indigenous *Y. enterocolitica* 1A and other *Yersinia* spp. were successfully isolated from surface water from the Grand River. While the isolated strains are generally considered non-pathogenic, reports have implicated these strains in human gastrointestinal cases. Previous occurrence data for *Yersinia* was not available for the Grand River watershed, which is used both as a drinking water source and for recreational activities. Pathogenicity studies involving the *Yersinia* strains isolated from the Grand River are needed to assess the risks to human health.

4 Evaluation of Quantitative PCR Methods for Detecting *Yersinia enterocolitica* in Surface Water from the Grand River

4.1 Abstract

Both culture-based and PCR-based methods for the detection of *Y. enterocolitica* are available. Studies have shown that culture-based methods may not be effective for detecting *Y. enterocolitica* environmental samples and PCR-based detection has been demonstrated to be more sensitive than culture-based detection. In this study, Taqman® quantitative PCR-based methods for enumerating *Y. enterocolitica* in surface water were developed and evaluated and used to assess the prevalence of pathogenic *Y. enterocolitica* in the Grand River watershed in Southwestern Ontario. Methods were developed that targeted two virulence genes in two separate PCR assays. The genes targeted were *ail*, a chromosomal gene, and *yadA*, a plasmid-borne gene. Standard curves were evaluated and detection limit, specificity and recovery studies were conducted to assess the performance of each PCR assay. Grand River surface water samples were subsequently analyzed with each PCR assay. Between March 2005 and August 2007 the *ail* gene target was detected in 121 samples out of 319 (38 %), and between January 2006 and August 2007 the *yadA* gene target was detected in 44 samples out of 206 (21 %). A trend was observed which showed a higher frequency of detection when water temperatures were colder. The median and maximum concentrations in samples positive for the *ail* gene were 40 and 2,000 cells/100 mL, respectively, and in samples positive for the *yadA* gene were 32 and 3,276 gene copies/100 mL, respectively.

4.2 Introduction

Yersinia enterocolitica is an enteric bacterium that has the potential to cause gastrointestinal disease as well as a wide variety of other diseases (Bottone, 1997) and has been classified as an emerging waterborne pathogen (Theron *et al.*, 2002; Sharma *et al.*, 2003). The species *Y. enterocolitica* is divided into six biogroups: 1A, 1B, 2 through 5; and into more than 50 serogroups (Wauters *et al.*, 1987). *Y. enterocolitica* is a well known foodborne pathogen

(Fredriksson-Ahomaa *et al.*, 2003) and pigs have been identified as a major reservoir of human pathogenic strains (McNally *et al.*, 2004; Fredriksson-Ahomaa *et al.*, 2006), however, most cases of *Y. enterocolitica* infection are sporadic and a source is rarely identified (Bottone, 1997). Although there have been few reported incidences of waterborne disease, previous studies have found drinking untreated water was a risk factor for *Y. enterocolitica* infection (Saebo et al, 1994; Ostroff *et al.*, 1994; Satterthwaite *et al.*, 1999). Using culture-based methods, some studies have isolated various *Yersinia* spp. in environmental waters (Brennhovd *et al.*, 1992; Leclerc *et al.*, 2002; Fredriksson-Ahomaa *et al.*, 2003). While these studies generally report low occurrence rates in water, it has been argued that culture-based isolation rates are likely an underestimation of true prevalence due to difficulties with isolating pathogenic *Y. enterocolitica* from the environment (Fredriksson-Ahomaa *et al.*, 2006).

Difficulties encountered when isolating this organism from environmental samples are generally attributed to the low concentration of *Y. enterocolitica*, the high concentration of other indigenous bacteria, and the fact that *Y. enterocolitica* compete poorly when grown in competition with other organisms (Schiemann *et al.*, 1984; Calvo *et al.*, 1986b). Moreover, the presence of the pYV plasmid, which codes for several virulence factors, is often used to identify pathogenic *Y. enterocolitica* isolates. This plasmid is sometimes lost during culturing steps in the laboratory (Blais *et al.*, 1995) and hence may not be a reliable indicator of pathogenicity in cultured strains. In contrast, the detection rates of *Y. enterocolitica* in various samples by PCR-based methods have been demonstrated to be consistently higher than those measured by culture-based methods (Fredriksson-Ahomaa *et al.*, 2006).

The objective of this study was to develop and evaluate quantitative-PCR (Q-PCR)-based methods for detecting *Y. enterocolitica* virulence genes in surface water samples. Q-PCR methods were then used to assess the prevalence of pathogenic *Y. enterocolitica* in surface water from the Grand River. Both chromosomal genes as well as genes located on the pYV virulence plasmid have been identified to play critical roles in *Y. enterocolitica* virulence pathways (Schiemann *et al.*, 1982; Heesemann *et al.*, 1984). Consequently, two *Y. enterocolitica* virulence gene targets were selected including *ail,* a chromosomal gene, and

yadA, a gene located on the pYV virulence plasmid. The *yadA* gene codes for a protein that promotes adherence to mucus layers, attachment to host cells and enhances serum resistance (Bottone, 1997; Cornelis *et al.*, 1998). The chromosomal gene, *ail,* plays an important role in enabling invasion of host cells (Miller *et al.*, 1988) and has been suggested to be a good gene target for the detection of pathogenic strains of *Y. enterocolitica* (Olsen *et al.*, 1995), as it is found uniquely in serotypes associated with disease (Miller *et al.*, 1989; Howard *et al.*, 2006).

The study site for this project was the Grand River watershed in Southern Ontario, Canada. Surface water in this watershed is used to provide all or part of the drinking water for approximately 500,000 people, as well as for industrial, commercial, agricultural and recreational uses (Cooke, 2006). It is also one of the most heavily impacted watersheds in Canada and receives inputs from both agricultural and urban activities (Dorner *et al.*, 2004; Bellamy *et al.*, 2005; Cooke, 2006). The watershed is not routinely monitored for pathogens and there is currently no information available on the occurrence of *Y. enterocolitica*. However, several different enteric pathogens regularly found in other surface waters were detected in the samples from Grand River in a study by Dorner *et al.* (Dorner *et al.*, 2007). The Grand River watershed was monitored for the presence of potentially pathogenic *Y. enterocolitica* over the course of 29 months by screening surface water samples using PCR assays developed in our laboratory which targets the *ail* and *yadA* virulence genes.

4.3 Materials and Methods

4.3.1 Bacterial Strains

The laboratory strains of *Yersinia* spp. used as controls were the same as those used in Chapter 3 (Table 3-1). The non-*Yersinia* bacterial strains used as negative controls in this study are listed in Table 4-1. Bacterial strains were maintained and stored as described in the Chapter 3 (section 3.3.1).

Table 4-1: Non-*Yersinia* bacterial control strains

Strain ID	Species
19	*Aeromonas hydrophila* ATCC 7966
20	*Campylobacter coli* ATCC 43478
21	*Escherichia coli* ATCC 11229
23	*Escherichia coli* O157:H7 ATCC 43895
24	*Legionella pneumophila* ATCC 33152
25	*Pseudomonas aeruginosa* UG2Lr *
26	*Salmonella enterica* ATCC 13311

*Provided by Drs. H. Lee and J.T. Trevors, University of Guelph (Guelph, ON)

4.3.2 DNA Extraction of Pure Cultures

Genomic DNA was prepared by growing broth cultures of *Yersinia* spp. in tryptic soy broth (TSB) (BD, Oakville, ON) at 28°C and non-*Yersinia* spp. in nutrient broth (BD, Oakville, ON) at 37°C for 16-20 h. Genomic DNA was extracted from 1 mL of each broth culture and using a Qiagen DNeasy kit (Qiagen, Mississauga, ON) eluted in 400 µL of AE buffer (according to the manufacturer's instructions).

4.3.3 Design of Primers and Probes

The design of the primers and probe for the *ail* gene target were from a study by Bhaduri *et al.* (2005) and amplified a 91-base pair (bp) fragment. The study by Bhaduri *et al.* (2005) had an error in the published sequence for the *ail* reverse primer. The authors were contacted to obtain the correct sequence. The sequences for the *ail* primers and probe are provided in Table 4-2.

Table 4-2: Primers and probes for the *ail* and *yadA* PCR assays

Oligonucleotide	Sequence
ail forward primer	5'-GGTCATGGTGATGTTGATTACTATTCA-3'
ail reverse primer	5'-CGGCCCCCAGTAATCCATA-3'
ail probe	5'-[FAM]-CATCTTTCCGCATCAACGAATATGTTAGC-[BHQ1]-3'
yadA forward primer 1	5'-GTATCCATTGGTCATGAAAGCCTT-3'
yadA reverse primer 1a	5'-CTTTCTTTAATTGCGCGACATTCA-3'
yadA reverse primer 1b	5'-CGCGACATTCACTGCATCAG-3'
yadA probe 1	5'-[FAM]-TTGACACATCTTGCGGCTGGCACT-[BHQ1]-3'
yadA forward primer 2	5'-CAATTGGGGATCGTTCTAAAACTG-3'
yadA reverse primer 2	5'-TTTCTTTCTTTAATTGCGCGACAT-3'
yadA probe 2	5'-[FAM]-TCAGTGTCTTTAGTGCCAGCCGCA-[BHQ1]-3'

Table 4-3: Primer and probe sets for the *yadA* DNA target

Set	Forward primer	Reverse primer	Probe	Length of amplified DNA fragment
A	1	1a	1	97bp
B	1	1b	1	84bp
C	2	2	2	138bp

The second gene target was the *yadA* gene. Several primer and probe sets were designed for this target. The primer and probe sets for the *yadA* gene target were designed by M. Van Dyke at the University of Waterloo (Waterloo, ON). The primers and probe were designed using Beacon Designer 2.1 software (Bio-Rad, Mississauga, ON) together with sequence alignment data of *yadA* genes from *Y. enterocolitica* (National Center for Biotechnology Information (NCBI) accession numbers: NC_005017, NC_004564.1, NC_002120.1, AF336309, AY150843, AF102990, AF056092, X13882) and from *Y. pseudotuberculosis* (X13883, BX936399). The specificity of the primers and probes was assessed using the Basic Local Alignment Search Tool (BLAST) software (Madden *et al.*, 1996). The sequences for the *yadA* primers and probe can be found in Table 4-2. Primers and probes were tested as shown in Table 4-3. All primers and probes were purchased from Sigma-Genosys (Oakville, ON). Each probe was 5'-labeled with 6-carboxyfluorescein (FAM) that fluoresces at 530 nM and 3'-labelled with a Black Hole Quencher-1™ (BHQ1) molecule (Sigma-Genosys, Oakville, ON).

4.3.4 Quantitative PCR Assay Conditions

Each 50 µL PCR reaction contained DNA template, 300 nM of forward primer, 300 nM of reverse primer, 100 nM of probe, 3.5 mM $MgCl_2$, 1 x PCR buffer without $MgCl_2$ (10 mM Tris-HCl [pH 8.3], 50 mM KCl), 1.25 units of Jumpstart Taq polymerase, and 200 µM deoxynucleotide triphosphates (dNTPs). All PCR reagents were purchased from Sigma-Aldrich (Oakville, ON).

PCR cycling conditions for the *ail* assay were as follows: one cycle at 95°C for 3 min, 50 cycles at 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s; and one cycle at 72°C for 10 min. PCR cycling conditions for the *yadA* assay were as follows: one cycle at 95°C for 3 min, 50 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s; and one cycle at 72°C for 10 min. PCR reactions were performed in a 96-well plate (Bio-Rad, Mississauga, ON) using a Bio-Rad iCycler iQ Real-Time PCR Detection System. Bio-Rad iCycler iQ software (version 3.1) was used to analyze the data. The threshold fluorescence was set manually above the background fluorescence levels. For each assay no template controls (negative controls) were run, and DNA standards (positive controls) were run to generate a standard curve (section 4.3.5) for samples enumeration.

4.3.5 Standard Curves

To quantify *Y. enterocolitica* in samples, standard DNA samples were needed to generate a standard curve. A standard curve for the *ail* PCR assay was generated using genomic DNA obtained from *Y. enterocolitica* ATCC 700822. Three different methods were tested for developing a standard curve for the *yadA* PCR assay, including: (A) genomic DNA extracts, (B) plasmid DNA extracts and (C) a synthetic oligonucleotide.

4.3.5.1 Genomic DNA Standards

A culture of *Y. enterocolitica* ATCC 700822 was inoculated into 10 mL of TSB and incubated at 28°C for 16-20 h. Genomic DNA was extracted from 1 mL of the overnight culture as described in section 4.3.2. An aliquot of the overnight culture was also preserved in 2.2 % formalin and stored at 4°C for 24-48 h. The concentration of the formalin-fixed culture was determined by direct microscopic cell count using SYBR-gold (Invitrogen, Burlington, ON) to stain the cells (see Appendix B). Serial dilutions of the extracted DNA were prepared in autoclaved MilliQ® water to generate standards of concentrations ranging from 1×10^3 to 1×10^7 cells/mL. 10 µL of each genomic DNA standard was tested with the *ail* primers and probe in PCR reactions as described in section 4.3.4. The *yadA* primer and probe set A (Table 4-3) was also tested with genomic DNA standards in PCR reactions as described above, but resulted in a poor detection limit.

4.3.5.2 Plasmid DNA Standards

The *yadA* gene is located on the *Y. enterocolitica* pYV virulence plasmid. To determine if plasmid loss resulted in the poor detection level found in section 4.3.5.1, a pYV plasmid DNA extraction was tested as a DNA standard for the *yadA* PCR assay. A 10-mL starter culture of *Y. enterocolitica* (ATCC 700822) was grown in TSB at 28°C for 10 h. Two 250-mL volumes of TSB were inoculated with 1 mL each of the starter culture, and were incubated at 28°C with shaking at 80 rpm for 12 h. Plasmid DNA was extracted from the 500 mL (2 x 250 mL) of culture using a PureLink™ HiPure Plasmid Filter Maxiprep Kit (Invitrogen, Burlington, ON) according to the manufacturer's instructions. The concentration of the pYV plasmid preparation was determined using a Quant-it™ PicoGreen® dsDNA Reagent and Kit (Invitrogen, Burlington, ON) according to the manufacturer's instructions. The pYV plasmid preparation was then diluted in autoclaved MilliQ® water to generate standards of concentrations ranging between 1×10^3 to 1×10^7 plasmid copies/mL. The *yadA* primer and probes sets A through C (Table 4-3) were tested with the plasmid DNA standards in PCR reactions as described in section 4.3.4, except two different annealing temperatures were tested (57°C and 60°C) and two different magnesium chloride concentrations (3.5 mM

and 4.5 mM) were tested. Results using the pYV DNA plasmid standards yielded a poor detection, similar to results observed using genomic DNA standards.

4.3.5.3 Synthetic Oligonucleotide Standards

The third approach involved using a synthesized oligonucleotide molecule to make DNA standards. A DNA oligonucleotide synthesized by Integrated DNA Technologies (Coralville, IA) was designed to match the *yadA* gene sequence (NCBI accession number X13882) between and including the *yadA* forward and reverse primers plus 30 additional base pairs at each end that also matched the *yadA* gene sequence. The resulting sequence of the *yadA* oligonucleotide template was: 5'-GAT CGT TCT AAA ACT GAC CGA GAA AAT AGT GTA TCC ATT GGT CAT GAA AGC CTT AAT CGC CAA TTG ACA CAT CTT GCG GCT GGC ACT AAA GAC ACT GAT GCA GTG AAT GTC GCG CAA TTA AAG AAA GAA ATT GAA AAA ACA CAG GAA AAT ACA AAT A-3'. Serial dilutions of the *yadA* oligonucleotide template were prepared in sterile MilliQ® water to generate standards of concentrations ranging from 1×10^3 to 1×10^7 gene copies/mL (gene copies refers to the number of copies of the *yadA* gene target and hence the number of oligonucleotide molecules). Synthetic oligonucleotide standards were tested with *yadA* primer and probe set A (Table 4-3) in PCR reactions performed, as described previously, and yielded results with a lower detection limit, similar to that observed for the *ail* assay. Consequently, synthetic oligonucleotide standards were used to generate *yadA* standard curves in subsequent experiments.

4.3.5.4 Detection Limit of Standards

The lower limit of purified DNA necessary to generate a fluorescent signal above the background was determined by diluting the genomic DNA standards to concentrations ranging from 2.5×10^3 to 5×10^2 cells/mL and analyzing 10 µL in ten replicate *ail* PCR reactions. The oligonucleotide DNA standards were diluted to concentrations ranging from 5×10^3 to 8×10^2 cells/mL and 10 µL of each was analyzed in ten replicate *yadA* PCR reactions.

4.3.6 PCR Specificity

The *ail* primer and probe set and the *yadA* primer and probe set A (Table 4-3) were tested with DNA from various *Yersinia* spp. and non-*Yersinia* spp.. Genomic DNA preparations were prepared as described above and diluted 100-fold in sterile MilliQ® water before analysis by PCR.

4.3.7 Surface Water Collection

Five sampling locations in the Grand River watershed were selected for sampling and are outlined in section 3.3.5.1. Surface water samples were collected from these sites every other week from March 2005 to August 2007, as described in section 3.3.5.2.

4.3.8 Water Quality Parameter Testing

The following parameters were measured for each water sample: temperature, turbidity, nitrate, ammonia and total *E. coli* concentrations. Temperature was measured on site immediately after sampling the water. The remaining parameters were measured in the laboratory. Turbidity was analyzed using a Hach 2100P turbidity meter (Hach, Winnipeg, MB). Nitrate and ammonia were measured using a Hach DR 2500 spectrophotometer following filtration through a 0.45 µm cellulose acetate filter. Nitrate was analyzed using the NitraVer 5 reagent kit (Hach) and ammonia was analyzed using the AmVer Test'N Tube Ammonia kit (Hach). Total *E. coli* concentrations were determined by membrane filtration onto mFC-BCIG agar (Ciebin *et al.*, 1995).

4.3.9 DNA Extraction and Quantitative PCR of Surface Water Samples

The surface water DNA extraction method was based on methods by Pitcher *et al.* (1989) and Boom *et al.* (1990). 1500-mL volumes of surface water were concentrated by filtering water through 0.45 µm Supor®-450 (47 mm diameter) membrane filters (Pall Corporation, Mississauga, ON) using a vacuum pump under ~500 mmHg pressure and a sterile filter unit

(Nalgene, Rochester, NY). Two filters were used to concentrate each 1500-mL sample (approximately 750 mL of water per filter). If water samples were very turbid, the volume that could be concentrated with each filter was reduced, and less than 1500 mL was sometimes analyzed (exact volumes analyzed are indicated in Appendix C). Each filter containing concentrated surface water was rolled and placed into separate 3 mL microcentrifuge tubes containing 1.5 mL of guanidine isothiocyanate (GITC) extraction buffer (Pitcher *et al.* 1989; 5 M GITC, 0.1 M ethylenediaminetetraacetic acid (pH 8.0), 5 g/L N-laurylsarcosine). Immediately after placing a filter inside a tube, the tube was shaken manually to loosen the material from the filter surface and then stored at -20°C. The remaining sample processing steps were performed within 2 weeks. Next, the tubes were removed from -20°C and were rotated for at least 1 hour at room temperature using a Dynal Biotech Sample mixer (Dynal Biotech Inc., Lake Success, NY). Extraction buffer from each tube was transferred to 2-mL screw-capped centrifuge tubes (VWR, Mississauga, ON). The samples in 2 mL centrifuge tubes were centrifuged at 13,000×g for 5 minutes. Next, a 600 µL aliquot of each supernatant was passed through DNeasy purification columns (Qiagen, Mississauga, ON) according to the manufacturer's instructions. The flow-through was discarded and another 600 µL aliquot of each lysate was passed through the same column. This was repeated until the supernatant from the entire sample was passed through the column. In accordance with the manufacturer's instructions, the columns were washed and then eluted using 200 µL of AE buffer (Qiagen, Mississauga, ON). DNA preparations were stored at -80°C until PCR analysis. For each sampling event, a negative control was prepared using the same reagents and solutions as were used for the Grand River samples.

Subsequently, Grand River DNA extracts were analyzed in quantitative *ail* and *yadA* PCR assays as described in section 4.3.4. 13.3 µL of DNA extract, corresponding to 100 mL of surface water, was added to each PCR reaction. Gene targets were quantified by analyzing DNA standards (in duplicate) with final concentrations of $1x10^1$, $1x10^2$, $1x10^3$, $1x10^4$, and $1x10^5$ cells or gene copies/PCR reaction and generating a standard curve. After Q-PCR analyses, PCR reaction products were stored at -80°C.

4.3.10 Analyzing Surface Water Extracts for PCR Inhibition

The efficiency of all PCR reactions is sensitive to inhibitors that may be present in extracted surface water samples (Wilson, 1997; Toze, 1999). To monitor the inhibition in an extracted sample, the Grand River DNA preparations were tested in an external control reaction using a *luxB* PCR assay. *P. aeruginosa* UG2Lr (listed in Table 4-1) is a genetically engineered strain that has been marked with the *luxB* gene from *Vibrio harveyi* (Flemming *et al.*, 1994). The *luxB* gene was selected because *V. harveyi* is a marine bacteria not found in river water, and the *luxB* gene target would therefore not be present in the Grand River samples. The *luxB* PCR reactions contained both: (1) *P. aeruginosa* UG2Lr DNA containing the *luxB* gene and (2) aliquots of Grand River DNA. Simultaneously, a control *luxB* PCR reaction was run without the addition of a Grand River water sample. If the signal generated by the PCR reaction containing Grand River water sample was weaker than the control, the corresponding DNA preparation was further purified as described below.

The methods for the *luxB* PCR were as follows. The primers and probe for the *luxB* gene target (Table 4-4) were designed by M. Van Dyke at the University of Waterloo (Waterloo, ON). The primers and probe were designed using Beacon Designer 2.1 software (Bio-Rad, Mississauga, ON) together with the sequence data for the *luxB* gene from *V. harveyi* (NCBI accession number E12410). The probe was 5'-labelled with hexachloro-6-carboxyfluorescein (HEX), a reporter dye, and 3'-labelled with a BHQ1 molecule (Sigma-Genosys, Oakville, ON), the same quencher dye used with the *ail* and *yadA* probes.

Table 4-4: The primer and probes for the *luxB* PCR assay

Oligonucleotide name	Sequence
luxB forward primer	5'-GGGTACTGCCATCCAAACAATGA-3'
luxB reverse primer	5'-TTCTTTGCTCGTCGCATTCACA-3'
luxB probe	5'-[HEX]-CGCAGGACCGCCTTCAGTGAACGC-[BHQ1]-3'

The template DNA for the *luxB* gene was obtained by extracting genomic DNA from *P. aeruginosa* UG2Lr as described previously (section 4.3.2) and diluting an aliquot of the DNA preparation with MilliQ® water to 5×10^4 cells/mL. Each *luxB* PCR reaction contained

10 µL of the diluted DNA preparation, corresponding to 5×10^2 cells per reaction. Each 50 µL PCR reaction contained *luxB* DNA template, 300 nM of each primer, 100 nM of probe, 3.5 mM $MgCl_2$, 1 x PCR buffer without $MgCl_2$ (10 mM Tris-HCl [pH 8.3], 50 mM KCl), 1.25 units of Jumpstart Taq polymerase, and 200 µM dNTPs. All PCR reagents were purchased from Sigma-Aldrich (Oakville, ON). Reactions to test PCR inhibition also contained a 14 µL aliquot from Grand River DNA preparations. Also, a control reaction was run in triplicate without the addition of a Grand River water sample. PCR cycling conditions for the *luxB* assay were as follows: one cycle at 95°C for 3 min, 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s; and one cycle at 72°C for 10 min. PCR reactions were performed in a 96-well plate (Bio-Rad, Mississauga, ON) using a Bio-Rad iCycler iQ Real-Time PCR Detection System.

The PCR amplification curves for the *luxB* PCR with and without Grand River samples were compared. If the PCR amplification signal was inhibited, indicated by a reduced C_T value or reduced signal intensity, this was an indication that the Grand River DNA preparation was inhibiting the DNA amplification and needed to be further purified. When necessary, DNA preparations were purified for a second time using the Qiagen DNeasy purification kit. During this second purification, the volume of AE elution buffer was adjusted to account for sample used for the *luxB* PCR assay.

4.3.11 Recovery of *Y. enterocolitica* DNA from Grand River Samples

To evaluate DNA extraction efficiency from river water, known quantities of *Y. enterocolitica* cells were added to Grand River water samples, and subsequently concentrated, processed for DNA extraction and analyzed with each PCR assay.

Inoculum was prepared by adding *Y. enterocolitica* ATCC 700822 to 10 mL TSB and incubated at 28°C for 24 h. The overnight culture was enumerated using a hemacytometer counter with a Petroff-Hausser Counting Chamber (Hausser Scientific, Horsham, PA) and a Zeiss Axioskop 2 Microscope (Empix Imaging Inc., Mississauga, ON). Based on the hemacytometer count, the culture was serially diluted in PBW to approximately $2x10^1$, 2×10^2, 2×10^3, 2×10^4, and 2×10^5 cells/mL.

Control samples were prepared, in triplicate, by extracting genomic DNA from 1 mL of each dilution using the Qiagen DNeasy kit and eluted in 200 µL of AE buffer (Qiagen, Mississauga, ON). DNA preparations were stored at -20°C until analysis. Samples used to evaluate recovery were also prepared in triplicate as follows. One mL of each diluted *Yersinia* inoculum ($2x10^1$, $2x10^2$, $2x10^3$, $2x10^4$, and $2x10^5$ cells/mL) was spiked into 1-L surface water samples. Grand River surface water was collected from a point just upstream of a drinking water treatment plant intake (refer to section 3.3.5.1) in 25-L plastic carboys and kept at 4°C for up to 3 days. 1-L aliquots of the river water were distributed into 1-L sterile polypropylene, wide-mouth bottles (VWR, Mississauga, ON). After each *Yersinia* inoculum was added, water samples were mixed by inverting and shaking the bottle manually. Each 1-L spiked river water sample was concentrated and processed for DNA extraction as described in section 4.3.9. Triplicate 1-L samples of unspiked Grand River samples (no *Y. enterocolitica* cells added) were also concentrated and processed in the same manner. During sample concentration, two filters were used to concentrate each 1-L spiked or non-spiked sample (approximately 500 mL of water per filter).

DNA from control samples and surface water samples were analyzed in quantitative *ail* and *yadA* PCR assays (in duplicate PCR reactions) as described in section 4.3.9. Twenty µL aliquots of each DNA extraction were analyzed.

4.3.12 Confirmation of PCR Assay Results

Real-time PCR results were confirmed by analyzing DNA amplification products with agarose gels and by sequence analysis.

Selected Grand River water samples analyzed by *ail* and *yadA* PCR assays were further analyzed by agarose gel electrophoresis. Twenty two *ail* positive samples, 26 *ail* negative samples, 14 *yadA* positive samples, and 13 *yadA* negative samples were selected. Five µL aliquots of the amplification products from the selected samples were each mixed with DNA loading buffer (Bio-Rad, Mississauga, ON). These samples were then analyzed on 2 % agarose gels at a constant voltage of 100 V in 1 x TRIS-Acetate-EDTA buffer (EMD, Darmstadt, Germany). The agarose gels were stained in a 0.5 µg/mL ethidium bromide solution and visualized with a Bio-Rad Universal Hood II transilluminator using Quantity One 4.6.2 software.

PCR products (two *ail* positive reactions and two *yadA* positive reactions) were further analyzed through sequence analysis. PCR products from the selected samples were cloned into TOPO® cloning vectors (Invitrogen, Burlington, ON) and chemically transformed into One Shot® TOP10 competent cells (Invitrogen) according to the manufacturer's instructions. Transformed cells were grown in Luria-Bertani (LB) broth (BD, Oakville, ON) containing 50 µg/mL of ampicillin at 37°C for 48 h. Plasmid DNA was extracted from the LB cultures using a PureLink™ Quick Plasmid miniprep kit (Invitrogen) according to the manufacturer's instructions. One or two cloned plasmids from each of the selected PCR reactions were sent to Laboratory Services at the University of Guelph (Guelph, ON) for sequencing using the Universal M13 Reverse primer (5'-CAG GAA ACA GCT ATG AC-3'). PCR amplification product from an *ail* positive PCR reaction was also sent directly for sequencing (without conducting the above cloning procedure). In this case, two sequencing reactions were carried out: the first using the *ail* forward primer and the second using the *ail* reverse primer.

4.4 Results and Discussion

In this study, Q-PCR-based methods for detecting *Y. enterocolitica* in surface water were evaluated that targeted two different gene targets, a chromosomal gene (*ail*) and a plasmid-borne gene (*yadA*). Q-PCR-based methods were then used to enumerate each gene target in Grand River surface water samples.

The *ail* and *yadA* genes were selected because they have been identified to play critical roles in the pathogenic pathways for *Y. enterocolitica*. The *yadA* gene is located on the pYV plasmid and codes for a protein that has been demonstrated to be critical to *Y. enterocolitica* virulence as it is necessary for survival and multiplication in the host (Cornelis *et al.*, 1998). The *yadA* protein plays several roles including attachment to host cells (Bottone, 1997). The pYV plasmid has been described to only be carried by pathogenic strains (Robins-Browne *et al.*, 1989; Fredriksson-Ahomaa *et al.*, 2006). Though the pYV plasmid is considered essential to imparting full virulence to *Y. enterocolitica*, it has been demonstrated that the plasmid alone does not necessarily confer virulence (Schiemann *et al.*, 1982; Heesemann *et al.*, 1984). Chromosomal genes have also been identified that are important for virulence. The ability to attach to and invade cells is crucial to *Y. enterocolitica* pathogenicity (Wachtel *et al.*, 1995). There are two chromosomal genes that play an important role in cell invasion (Miller *et al.*, 1988). One of these is the *ail* gene, which is only found in *Y. enterocolitica* serotypes that are associated with disease (Miller *et al.*, 1989; Revell *et al.*, 2001). In a study of 140 *Y. enterocolitica* strains by Thoerner *et al.* (2003), the *ail* gene was detected in nearly all of the traditional pathogenic biotypes studied.

4.4.1 Testing DNA Standards

Standard DNA samples of known concentration were used to generate a standard curve and subsequently enumerate unknown quantities of DNA in samples using Q-PCR. Genomic DNA standards used for the *ail* PCR assay were prepared by extracting genomic DNA from an enumerated culture of *Y. enterocolitica*. As a result, the unit of measurement for the *ail* PCR assay was cells per unit volume. To confirm that the preparation of DNA standards

using the methods described was reproducible, three different DNA extractions were prepared. Duplicate dilutions (A and B) were then prepared from each of the three DNA extractions. The standard curves generated by each set of standards are compared in Table 4-5 and Figure 4-1.

Table 4-5: Threshold cycle values resulting from amplification of the *ail* target with multiple sets of DNA standards.

Log concentration of DNA standard (cells/reaction)	Threshold cycle values for each DNA dilution series*					
	1A	**1B**	**2A**	**2B**	**3A**	**3B**
5	22.2	22.6	23.7	23.6	22.7	22.9
4	26.6	26.6	27.3	27.0	26.8	26.6
3	30.3	30.6	30.5	30.4	30.4	30.1
2	33.6	34.1	34.0	33.8	33.7	33.5
1	37.4	38.8	36.5	38.2	37.6	37.1

*DNA preparations 2 and 3 were extracted from the same culture. From each DNA preparation (1, 2 and 3), two dilution series were prepared (A and B).

The six different sets of DNA standards yielded highly similar curves using the *ail* PCR assay, showing the methods used to make *ail* DNA standards were reproducible. Furthermore, the standard curves yielded R^2 values of 0.98 or greater with slopes that ranged between -3.2 and -3.6. A reliable Q-PCR standard curve should have an R^2 value of more than 0.95 and a slope between -3.0 and -3.9 (Zhang *et al.*, 2006). The value of the slope of the standard curve is important because it is a measure of the PCR efficiency. Based on these guidelines, the standard curves in Figure 4-1 are reliable. Standard samples with concentrations as low as 10 cells per reaction consistently resulted in DNA amplification, indicating that the detection limit is likely below 10 cells per reaction. The standard curves were also linear over a 5-log dilution series.

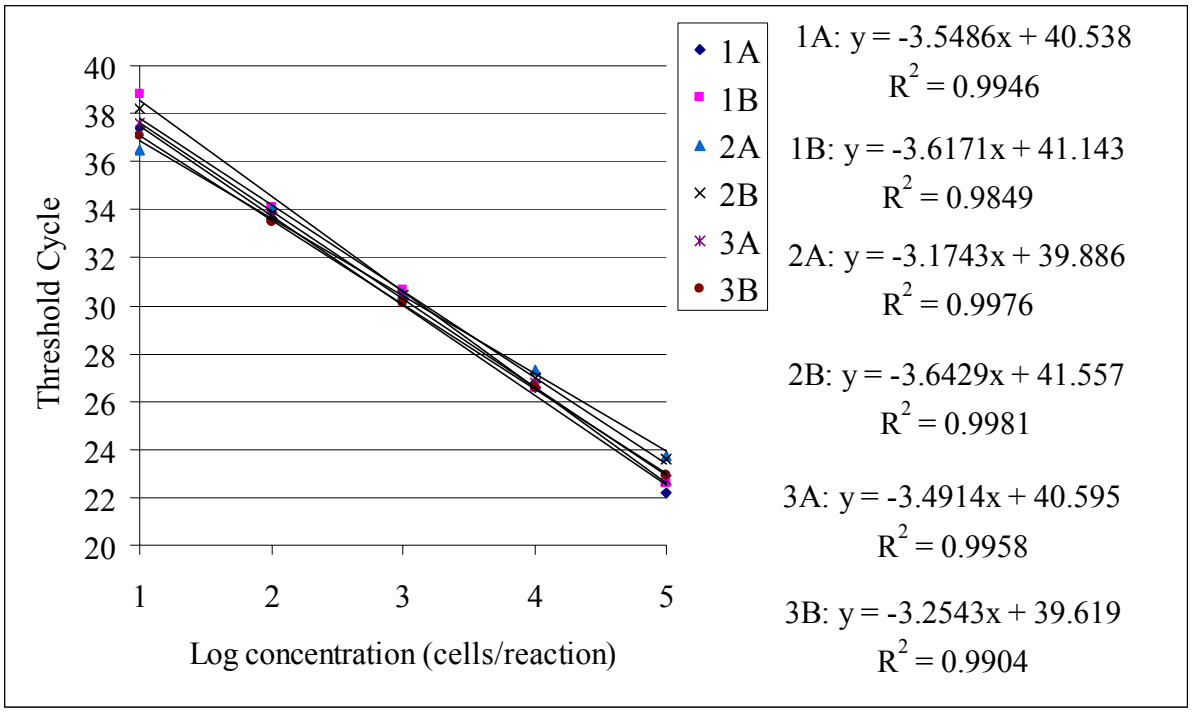


Figure 4-1: Standard curves for the amplification of the *ail* target (using the standard curve data in Table 4-5).

Three different approaches were employed to make DNA standards for the *yadA* PCR assay. The first approach (A) used the same methods that were used for the *ail* PCR assay. Genomic DNA standards were tested with *yadA* primer and probe set A (see Table 4-3). The threshold cycle (C_T) values obtained for the *yadA* assay were significantly higher than those of the *ail* PCR assay (Table 4-6). The C_T value is a reflection of the initial concentration of target DNA, and a larger C_T value indicates that there was less target DNA in the initial sample. Also, unlike the *ail* assay, no amplification of the *yadA* target was observed in DNA standards with a concentration of 10 cells per reaction (Table 4-6), suggesting that the detection limit of the *yadA* target using genomic DNA standards was above 10 cells per reaction.

Table 4-6: Comparison of representative threshold cycle (C_T) values for the amplification of genomic DNA standards with the *ail* and *yadA* (primer and probe set A) assays

	Threshold cycle value	
Concentration of genomic DNA standards (cells/reaction)	***ail***	***yadA***
1×10^5	23.0	27.0
1×10^4	26.8	30.9
1×10^3	30.4	34.7
1×10^2	33.8	37.0
1×10^1	37.6	no amplification

In our laboratory, other Q-PCR assays have been developed to detect different gene targets found in other bacteria. In those studies, the C_T values and detection limits recorded were all similar to those observed with the *ail* DNA standards. Although C_T values are expected to vary with different DNA targets, the difference between the *ail* and *yadA* standard curve results was noticeably larger than differences observed in standard curves for other Q-PCR assays studied in our laboratory.

The larger C_T values and poor detection limit observed for the *yadA* target with genomic DNA standards suggested that while this strain was being maintained in culture, some cells may have lost their pYV plasmid, which contains the *yadA* gene. It has been described previously that *Y. enterocolitica* may loose the pYV virulence plasmid when cultured at temperatures above 30°C for long periods or after repeating sub-culturing (Blais *et al.*, 1995; Bottone, 1997). If this were the case, the culture could not be used to make DNA standards for the *yadA* gene target because only a fraction of the cells enumerated would contain the target DNA. The procedure was repeated several times with culture grown from the original frozen cell stocks and the same results were obtained. Other researchers have also experienced difficulties in obtaining sufficient quantities of pYV plasmid DNA for their work and also attributed the problem to plasmid loss (Robins-Browne *et al.*, 1989).

The second approach (B) used to make standards for the *yadA* PCR assay involved a pYV plasmid DNA extraction rather than a genomic DNA extraction. In this method, the plasmid DNA preparation was quantified by measuring the amount of DNA in the purified sample

using the Quant-it™ PicoGreen® dsDNA reagent and kit (Invitrogen, Burlington, ON). The pYV plasmid is reported to be a low copy number plasmid (Robins-Browne *et al.*, 1989). A low copy number plasmid means there are generally less than five plasmid copies per cell (Glick *et al.*, 1998). Hence, enumerating the pYV plasmid copies in a sample provided a general approximation of the number of *Y. enterocolitica* cells.

Initially, plasmid DNA standards were tested with *yadA* primer and probe set A (Table 4-3). This yielded similar results to those seen using genomic DNA. However, in this case, the poor detection limit could not be attributed to plasmid loss. Some of the parameters of the DNA amplification reaction were varied in attempts to improve the detection limit of the *yadA* target. Two other primer and probe sets (sets B and C in Table 4-3) were also tested, using two different annealing temperatures (60°C and 57°C) and two different magnesium chloride concentrations (3.5 mM and 4.5 mM). These modifications, however, did not improve the detection limit, and yielded similar results to those obtained using primer and probe set A (Table 4-3) under the original PCR conditions used. Further investigations are necessary to elucidate possible explanations.

The third and final approach (C) involved using a synthetic DNA oligonucleotide matching the sequence of the *yadA* gene target. The unit of measurement for the *yadA* PCR assay was gene copies per unit volume. Gene copies referred to the number of copies of the *yadA* gene target. Oligonucleotide DNA standards were tested with *yadA* primer and probe set A. The resulting standards yielded C_T values similar to those observed with the *ail* PCR assay (Figure 4-2). Moreover, a signal was detected for the oligonucleotide DNA standard with a concentration of 10 gene copies per reaction, indicating an improved detection limit over results seen with both the genomic and plasmid DNA standards. Also, like the standard curve obtained for the *ail* PCR, the improved *yadA* standard curve was linear over a 5-log dilution series. The *yadA* primer and probe set A was used with the oligonucleotide DNA standards in subsequent enumeration studies targeting the *yadA* gene.

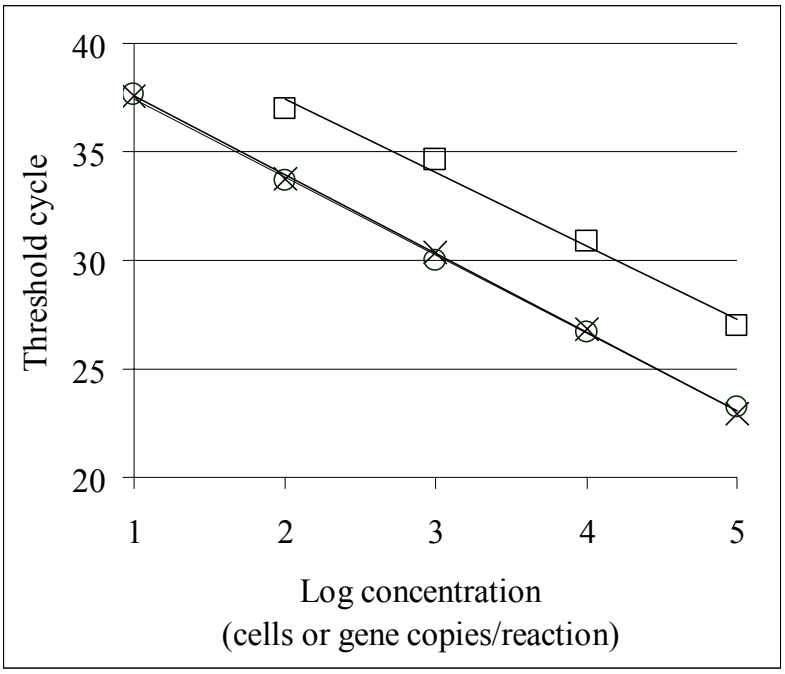


Figure 4-2: Representative standard curves for the *ail* and *yadA* PCR assays. Genomic DNA standards from *Y. enterocolitica* ATCC 700822 were tested using the *ail* (×) or *yadA* (□) PCR assays. Synthetic oligonucleotide standards (○) were tested with the *yadA* PCR assay.

4.4.2 Detection Limit of DNA Standard Samples

Ten replicate DNA amplification reactions were carried out with DNA standards at concentrations ranging from 5 to 100 cells per reaction to determine an approximate detection limit for the Q-PCR assays. The results for the *ail* and the *yadA* Q-PCR assays are shown in Table 4-7 and Table 4-8, respectively.

Table 4-7: Detection limit of the *ail* Q-PCR assay using genomic DNA standards

Replicate	Threshold cycle values for each genomic DNA standard (cells/reaction)			
	100	**10**	**8**	**5**
1	37.1	41.0	42.2	43.3
2	37.0	40.0	43.3	46.8
3	37.7	41.1	41.9	42.4
4	36.6	41.6	40.7	41.8
5	36.4	38.4	42.2	42.2
6	36.1	43.0	41.2	41.5
7	36.8	40.7	40.1	no amplification
8	38.7	41.9	40.6	45.6
9	37.0	40.8	40.1	no amplification
10	36.6	43.2	n.a.[b]	42.1
Average [a]	37.0	41.2	41.4	43.2
St. Dev. [a]	0.739	1.40	1.10	1.94

[a] Average and standard deviation calculations only include samples in which DNA amplification was detected.

[b] A result is not available because the fluorescence signal produced an atypical amplification curve that could not be used to measure the threshold cycle value.

The amplification of the *ail* DNA target was detected in all ten replicates for the DNA standards at 100 and 10 cells per reaction, in nine replicates for the DNA standard at 8 cells per reaction and in eight replicates for the standard at 5 cells per reaction. As expected, the standard deviation in the C_T values increased as the DNA concentration decreased, a phenomenon also observed in quantitative PCR experiments by Yang *et al.* (2003). According to Behets *et al.* (2007), a quantitative PCR detection limit is the minimum DNA quantity that yields a fluorescence signal in at least 90 % of the positive controls. Based on this definition, the above results indicate that the detection limit for the *ail* gene target for *Y. enterocolitica* is between 5 and 8 cells per PCR reaction.

Table 4-8: Detection limit of the *yadA* Q-PCR assay using oligonucleotide DNA standards

Replicate	Threshold cycle value for each oligonucleotide DNA standard (gene copies/reaction)			
	100	**50**	**25**	**10**
1	34.2	35.4	35.7	37.7
2	33.8	36.0	36.7	38.1
3	33.6	34.8	36.7	no amplification
4	34.3	35.9	36.8	38.9
5	34.7	34.1	37.8	37.7
6	34.5	35.3	35.9	36.5
7	34.5	34.4	36.3	38.3
8	34.0	34.5	36.5	36.0
9	34.0	35.6	no amplification	38.1
10	33.8	35.6	35.7	36.9
Average [a]	34.1	35.2	36.5	37.6
St. Dev. [a]	0.360	0.665	0.664	0.932

[a] Average and standard deviation calculations only include samples in which DNA amplification was detected.

Amplification of the *yadA* DNA target was detected in all ten replicates for the DNA standards at 100 and 50 cells per reaction and in nine out of ten replicates for the DNA standard at 25 and 10 cells per reaction (Table 4-8). As seen with the *ail* PCR assay, the standard deviation in the C_T values increased as the DNA concentration decreased and approached the detection limit. In prior *yadA* PCR reactions, it was found that when 10 cells were added per reaction, it was common that a signal was only detected in one of the duplicate runs. Consequently, 10 cells per reaction was chosen as the lower limit to test in this study. In contrast to the *ail* primers and probe, the *yadA* primers and probe have not been tested previously. Based on the definition for detection limit used above, our results indicate that the detection limit for the *yadA* gene target for *Y. enterocolitica* is at or below 10 gene copies per PCR reaction.

4.4.3 Specificity of Primers and Probes

When designing and evaluating the *ail* and *yadA* primers and probes, the NCBI genomic DNA database was searched to confirm that these oligonucleotides were specific for

Y. enterocolitica ail and *yadA* genes. The DNA amplification reactions carried out below were conducted to confirm the specificity of the primer and probe design.

Genomic DNA from a variety of bacteria, including *Yersinia* spp. and non-*Yersinia* spp., was added to PCR reactions designed to amplify the *ail* and *yadA* DNA targets. The results are shown in Table 4-9. A positive result (+) indicates that DNA amplification was detected and a negative result (−) indicates that no DNA amplification was detected.

Table 4-9: DNA amplification of *ail* and *yadA* targets in laboratory bacteria

Strain ID	Species	Biogroup	Serogroup	*ail* result	*yadA* result
1	*Y. enterocolitica*	1A	O:7, 13	−	−
2	*Y. enterocolitica*	1A	O:41,42	+	+
3	*Y. enterocolitica* ATCC 9610	1B	O:8	−	−
4	*Y. enterocolitica*	1B	O:8	+	+
5	*Y. enterocolitica*	1B	O:8	−	−
6	*Y. enterocolitica*	1B	O:21	+	−
7	*Y. enterocolitica*	2	O:9	+	+
8	*Y. enterocolitica*	2	O:5, 27	+	+
9	*Y. enterocolitica*	3	O:1, 2,3	+	+
10	*Y. enterocolitica*	4	O:3	−	−
11	*Y. enterocolitica* ATCC 700822	4	O:3	+	+
12	*Y. pseudotuberculosis*		III	−	+
13	*Y. pseudotuberculosis*		I	−	+
14	*Y. frederiksenii*			−	−
15	*Y. intermedia*			+	+
16	*Y. kristensenii*			−	−
17	*Y. mollaretii*			−	−
18	*Y. rohdei*			−	−
19	*A. hydrophila*			−	−
20	*C. coli*			−	−
21	*E. coli*			−	−
23	*E. coli* O157:H7			−	−
24	*L. pneumophila*			−	−
25	*P. aeruginosa* UG2Lr			−	−
26	*S. enterica*			−	−

DNA amplification of both DNA targets was observed in six of the eleven *Y. enterocolitica* strains. The *Y. enterocolitica* 1B, O:21 strain (strain ID number 6) only yielded a signal for the amplification of the *ail* target. It is possible that the strain simply lost the pYV virulence plasmid containing the *yadA* gene in culture, as this is known to occur (Blais *et al.*, 1995; Bottone, 1997). The *Y. enterocolitica* ATCC 9610 strain was negative for both the *ail* and *yadA* genes, which has been observed previously (Blais *et al.*, 1995; Thoerner *et al.*, 2003).

DNA amplification results indicate that *Y. enterocolitica* 1A/O:41,42 (strain ID number 2) possessed both the *ail* and *yadA* genes. *Y. enterocolitica* belonging to biogroup 1A are traditionally considered non-pathogenic and considered not to possess the pYV plasmid or *ail* gene (Tennant *et al.*, 2003). However, other studies have isolated *Y. enterocolitica* 1A from patients displaying disease symptoms (Grant *et al.*, 1998). While it has been suggested that these disease-causing biotype 1A strains possess a different set of virulence genes, one study found one *Y. enterocolitica* 1A strain that possessed the *yadA* gene and two that possessed the *ail* gene (Thoerner *et al.*, 2003).

The *ail* target did not amplify in either *Y. pseudotuberculosis* strain. Although, there is an *ail* homolog in *Y. pseudotuberculosis* (Yang *et al.*, 1996), the *ail* primers were designed specifically to target the *ail* gene from *Y. enterocolitica* (Bhaduri *et al.*, 2005), and did not result in DNA amplification when tested with other *Y. pseudotuberculosis* strains previously (Jourdan *et al.*, 2000). Conversely, the *yadA* gene target did amplify in both *Y. pseudotuberculosis* strains, demonstrating that this primer and probe set were specific to both *Y. enterocolitica* and *Y. pseudotuberculosis*.

No DNA amplification was observed with the *ail* primers and probes, nor the *yadA* primers and probes for the genomic DNA from other *Yersinia* spp. (strain ID numbers 14-18), with a single exception. DNA amplification of both target genes occurred in reactions with genomic DNA from *Y. intermedia*. It has been suggested in the literature that this traditionally non-pathogenic *Yersinia* spp. may, in fact, contain pathogenic strains (Sulakvelidze, 2000). Furthermore, two plasmid-borne genes, *virF* and *yadA,* as well as the chromosomal gene *yst*, were recently detected in a *Y. intermedia* strain (Kechagia *et al.*, 2007). And, in another

study, a DNA probe targeting a region of the *ail* gene hybridized to colonies of one of the *Y. intermedia* strains tested (Robins-Browne *et al.*, 1989). It would have been interesting to further investigate this *Y. intermedia* strain, however, it was outside the scope of this thesis project.

As expected, no DNA amplification was detected in reactions with genomic DNA from any of the non-*Yersinia* spp., which provided additional assurances that the primers and probes were specific to *Y. enterocolitica*. Although *ail* homologs have been found in both *E. coli* (Mecsas *et al*., 1995) and in *S. enterica* (Heffernan *et al*., 1992; Heffernan *et al*., 1994), the *ail* primers and probe did not match these homologs and were not expected to yield DNA amplification in either species. The non-*Yersinia* organisms used in this study were selected because they are bacteria that are also found in water. The above DNA amplification results provide further evidence to support the specificity of the primer and probe design.

4.4.4 Recovery Studies

Pathogens in surface water are typically present in very low concentrations (Koster *et al*., 2003). In order to detect bacteria at low concentrations by PCR analysis, surface water samples must be concentrated. In this study, membrane filtration was used for concentration of samples. Subsequently, DNA is extracted and purified from concentrated samples. DNA purification is critical because components of river water, including humic materials, clay and organics (Bej *et al.*, 1992) will affect the DNA amplification efficiency. During concentration, extraction, and purification steps, DNA from the sample may be lost. To evaluate the recovery of the surface water DNA extraction methods, a *Y. enterocolitica* (ATCC 700822) inoculum was added to river water samples that were subsequently processed using the surface water DNA extraction methods and enumerated by Q-PCR. Concurrently, DNA was extracted directly from the *Y. enterocolitica* inoculum and enumerated by Q-PCR. The direct extraction of the inoculum was the control to which spiked samples were compared. Equivalent volumes of the original *Y. enterocolitica* inoculum were added to PCR reactions for both control and spiked samples. Five different inoculum concentrations were tested. These steps are outlined in Figure 4-3.

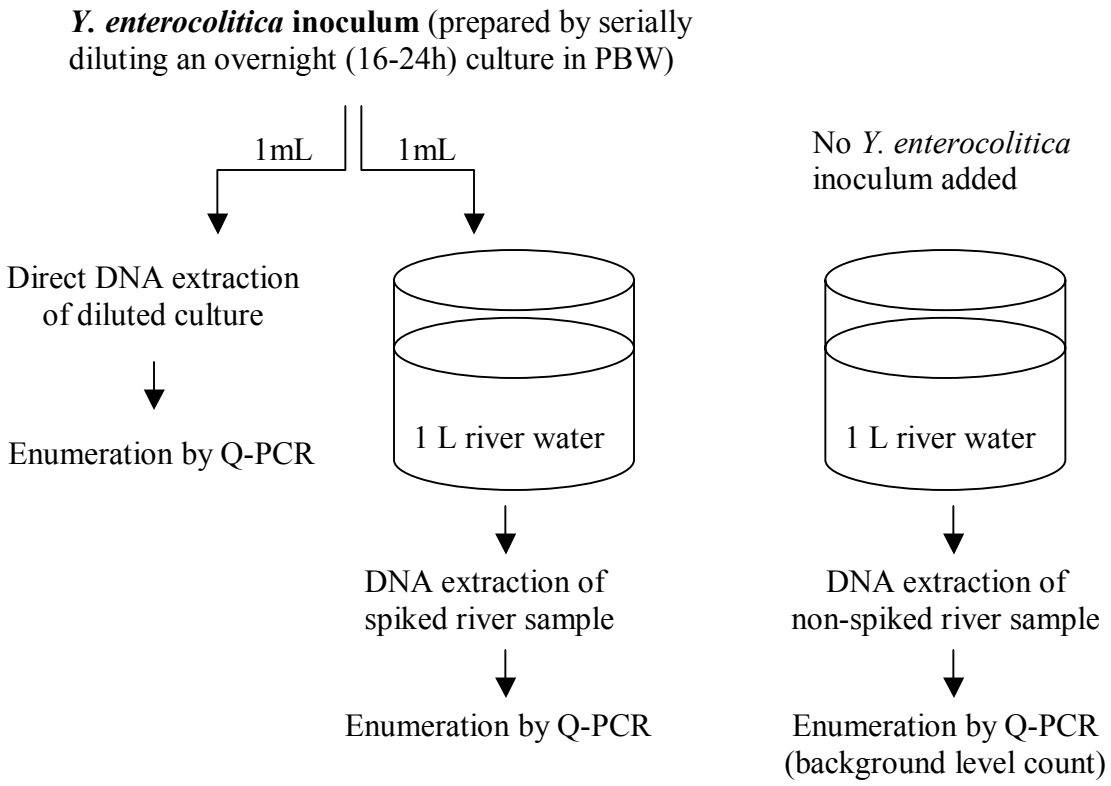


Figure 4-3: Schematic of the recovery experiment. Five different *Y. enterocolitica* inoculum concentrations were tested for recovery from river water, and each treatment was done in triplicate. DNA extracted from controls and from river water were analyzed by both the *ail* and *yadA* Q-PCR assays.

All samples were prepared and processed in triplicate, then analyzed in duplicate PCR reactions. Mean and standard deviations were calculated using all six values. Percent recoveries were calculated by dividing the enumeration results for the spiked samples by the control samples. Non-spiked surface water samples were also processed for DNA extraction and analyzed by PCR and used to measure the background level *ail* and *yadA* signal. No amplification was detected in the non-spiked river samples. Hence, in the water samples collected for this experiment, there was no detectable naturally occurring *ail* or *yadA* genes in the river water samples, yielding a background level of zero. The final results are summarized in Table 4-10 and Table 4-11 for the *ail* and *yadA* PCR assays, respectively.

Table 4-10: Results for the enumeration of control and spiked river water samples using the *ail* gene target for enumeration by Q-PCR. For each sample, DNA extract was eluted in 200 µL. Then, 20 µL of the eluted samples was added to each PCR reaction. This was equivalent to adding 100 mL of surface water to each PCR reaction.

Inoculum level	Concentration of sample measured by the *ail* PCR (cells/100 mL)		Recovery
	Control sample	Spiked river water sample	
1	9.3×10^0 ($\pm$ 6.1×10^0)	n.d.[a] – 4.1×10^0 [b]	0 – 44 % [b]
2	4.9×10^1 ($\pm$ 1.4×10^1)	1.2×10^1 ($\pm$ 3.6×10^0)	24 %
3	3.6×10^2 ($\pm$ 8.1×10^1)	1.1×10^2 ($\pm$ 3.2×10^1)	30 %
4	2.1×10^3 ($\pm$ 5.3×10^2)	7.1×10^2 ($\pm$ 1.5×10^2)	34 %
5	1.2×10^4 ($\pm$ 2.8×10^3)	4.3×10^3 ($\pm$ 2.7×10^3)	37 %

[a] n.d. = not detected

[b] A range is reported because DNA amplification was not consistently detected in PCR replicates.

Enumeration results for spiked river water samples were consistently lower than the enumeration results for control samples (Table 4-10). The percent recoveries observed ranged from 24 % to 37 %, with an average of 31 %. This excludes the lowest inoculum level tested. However, results for spiked samples at the lowest inoculum level were highly variable. At this level, DNA amplification was only detected in half of the replicates. Not unexpectedly, percent recoveries decreased with decreasing concentrations. Results also suggest that the *ail* PCR assay was sensitive enough to detect *Y. enterocolitica* cells in river water at concentrations in the order of 10^1 cells in 100 mL of surface water.

Table 4-11: Results for the enumeration of control and spiked river water samples using the *yadA* gene target for enumeration by Q-PCR. For each sample, DNA extract was eluted in 200 µL. Then 20 µL of the eluted sample was added to each PCR reaction. This was equivalent to adding 100 mL of surface water to each PCR reaction.

Inoculum level	Concentration of sample measured by PCR (gene copies/100 mL)		Recovery
	Control sample	Spiked river water sample	
1	n.d. [a]	n.d.	-
2	n.d.	n.d.	-
3	7.3×10^1 ($\pm$ 3.6×10^1)	3.7×10^1 ($\pm$ 1.4×10^1)	50 %
4	4.4×10^2 ($\pm$ 1.0×10^2)	1.3×10^2 ($\pm$ 6.0×10^1)	29 %
5	4.6×10^3 ($\pm$ 5.1×10^2)	1.2×10^3 ($\pm$ 2.2×10^2)	25 %

[a] n.d. = not detected

Similar to *ail* results, the *yadA* PCR enumeration results for spiked river water samples were consistently lower than controls (Table 4-11), showing that some DNA loss did occur using the surface water DNA extraction methods. The percent recoveries observed when comparing spiked samples to controls ranged from 25 % to 50 %, with an average of 34 %. In contrast to the *ail* results, percent recovery increased with decreasing concentrations. At the lower inoculum levels tested, *yadA* DNA amplification was only detected in at most one of the six replicates. This is contrary to results obtained for the *ail* PCR assay. This is likely a result of some of the *Y. enterocolitica* cells having lost the pYV plasmid in culture (Blais *et al.*, 1995) (discussed previously in section 4.4.1). Similar to the *ail* PCR assay, the *yadA* PCR assay was able to detect *Y. enterocolitica* cells in river water at concentrations in the order of 10^1 gene copies in 100 mL of river water.

DNA loss can be attributed to various steps involved in the DNA extraction of surface water samples. Cell lysis may have been incomplete as a result of other substances in the river water, or due to the large number of cells introduced from the river water. Some of the extracted DNA may have bound to membrane filters or to cell debris that was centrifuged and discarded. Some DNA was likely lost during DNA purification steps, which may have been negatively impacted by substances in the river water competing for or blocking binding sites on the purification columns from the Qiagen DNeasy DNA extraction kit. It is also possible there was some carry over of PCR inhibitors, however, samples are analyzed for PCR inhibition and this factor should have been minimal.

While there have been other studies that have used PCR and real time PCR to detect *Y. enterocolitica* genes in water samples, these studies were not quantitative and, rather, monitored only the presence or absence of *Y. enterocolitica*. In previous presence/absence studies by Kapperud *et al.* (1993) and Waage *et al.* (1999), the detection of *Y. enterocolitica* spiked into water samples was evaluated by a PCR method that targeted the *yadA* gene. Both studies employed an upstream enrichment step in non-selective TSB broth and Kapperud *et al.* (1993) also tested an immunomagnetic separation step to improve the sensitivity of the method. Both studies utilized the same nested PCR assay, which was also intended to improve the sensitivity. Kapperud *et al.* (1993) reported a detection level of 10 to 30 CFU of

Y. enterocolitica in 100 mL surface water. Waage *et al.* (1999) reported a detection level of 8 to 17 CFU of *Y. enterocolitica* in 100 mL of various types of water samples. The sensitivities reported in each of these studies is comparable to that found in our study, which demonstrated that both the *ail* and *yadA* PCR assays could detect approximately 10^1 *Y. enterocolitica* cells or gene copies in 100 mL of river water. In our investigation, we did not use a preenrichment step, an immunomagnetic separation step or a nested PCR to improve sensitivity. Instead quantitative Taqman® PCR methods were used, which have been shown to improve sensitivity versus traditional PCR methods (Boyapalle *et al.*, 2001; Foulds *et al.*, 2002). To our knowledge, this is the first study to enumerate *Y. enterocolitica* cells in river water samples using Q-PCR and to determine the associated recovery rates observed for Q-PCR-based detection methods for surface water samples. This study found that the average percent recovery observed for the *ail* PCR assay was 31 % and for the *yadA* PCR assay was 35 %. These recovery rates correspond to a loss of less than a log between spiked samples and controls.

4.4.5 Grand River Watershed Survey

Surface water was collected from sampling sites in the Grand River watershed every two weeks. Several parameters were measured for each water sample: temperature, turbidity, nitrate, ammonia and *E. coli* concentrations. Samples were also screened by PCR for the *ail* and *yadA* gene targets. The results for the water quality parameters measured and the PCR assays are shown in Appendix C. PCR values reported were not adjusted for recovery efficiency. A summary of the results from the PCR-based survey of the Grand River is provided in Table 4-12.

Table 4-12: PCR results for the Grand River Watershed samples

Sampling Location	Total number of samples analyzed		Frequency of positive samples		Median value* (cells or gene copies/100 mL)		Maximum value (cells or gene copies/100 mL)	
	ail	*yadA*	***ail***	*yadA*	***ail***	*yadA*	***ail***	*yadA*
Grand River (North[a])	**64**	42	**47 %**	21 %	**42**	20	**2000**	53
Canagagigue Creek[b]	**64**	42	**50 %**	26 %	**39**	25	**576**	76
Conestogo River[b]	**64**	41	**27 %**	18 %	**51**	45	**296**	3296
Grand River (Wastewater[c])	**66**	41	**32 %**	22 %	**23**	42	**730**	91
Grand River (Intake[d])	**61**	40	**34 %**	20 %	**37**	38	**775**	277
Overall	**319**	206	**38 %**	21 %	**40**	32	**2000**	3276

*Median values were calculated from the samples that tested positive only

[a] Sampled from the Grand River at a location upstream of the other four sampling sites

[b] Canagagigue Creek and Conestogo River are tributaries of the Grand River

[c] Sampled from the Grand River downstream of a wastewater treatment plant discharge

[d] Sampled from the Grand River upstream of a drinking water treatment plant intake

Both *ail* and *yadA* DNA targets were detected in all five sites examined in the Grand River watershed (Table 4-12). Overall, the frequencies of *ail* and *yadA* genes were 38 % and 21 %, respectively. There were 31 samples (15 %) that were positive for both gene targets. The frequency of detecting the *ail* target was consistently higher than the *yadA* target at all five sampling sites. The differences observed in detection rates of the two gene targets may have been due to the difference between the occurrences of each gene target in the population of *Y. enterocolitica* from the Grand River. The median values observed across the watershed for the *ail* and *yadA* targets were 40 cells/100 mL and 32 gene copies/100 mL for the *ail* and *yadA* DNA targets, respectively.

This is the first Q-PCR-based survey of surface water samples. Previous surveys for *Y. enterocolitica* in surface water have been culture-based, and consequently report detection rates for all the *Yersinia* spp., as pathogenic subtypes have rarely been isolated from water. In the culture-based survey described in chapter 3, the isolation rate of *Y. enterocolitica* was 4 %, however, the isolates were type 1A, which is traditionally a non-pathogenic strain. One study used non-quantitative PCR to survey for *Y. enterocolitica* in water (Sandery *et al.*, 1996). This study used a preenrichment step prior to conducting a PCR assay that targeted the *ail* gene. The authors examined 105 surface waters samples from creeks and reservoirs in Victoria, Australia and found 11 (10 %) samples that were positive for the *ail* gene. Interestingly, they also conducted a concurrent culture-based survey which found only one (1 %) of the samples tested positive for pathogenic *Y. enterocolitica* (cultured *Y. enterocolitica* isolates were screened using the *ail* PCR to confirm pathogenicity). In another study, *Y. enterocolitica* strains isolated from various water sources in Brazil were characterized using PCR (Falcao *et al.*, 2004). Several isolates possessed the *ail* gene and a few possessed the pYV plasmid-borne gene, *virF*.

In the Grand River survey, both targets were detected most frequently at the Canagagigue Creek sampling location, with a frequency of 50 % and 26 % for the *ail* and *yadA* targets, respectively. *Campylobacter*, *Salmonella* and *E. coli* O157:H7 were also monitored in the Grand River in a related study over the same time frame using similar Q-PCR-based methods and the same sampling locations (summary results are provided in Appendix D). In this

concurrent study, *Campylobacter* was detected more frequently in Canagagigue Creek and the Grand River sampling site located furthest upstream. Canagagigue Creek samples also showed the second highest *median* concentration for indicator *E. coli*, with the highest levels at the site in the Grand River downstream of a wastewater treatment plant effluent (Appendix E). High concentrations of indicator *E. coli* in Canagagigue Creek have been noted in an earlier Grand River watershed survey (Dorner *et al.*, 2007).

The higher detection rates for *Y. enterocolitica* and *Campylobacter* in Canagagigue Creek may result from agricultural inputs to the tributary. The Canagagigue Creek is known to be heavily impacted by agriculture (Dorner *et al.*, 2007) and pigs are the second most abundant livestock, following hens and chickens, in the watershed (Dorner *et al.*, 2004). The Canagagigue Creek sub-watershed was located in a region with the highest livestock density in the watershed (Dorner *et al.*, 2007), and where estimates for daily manure production were also highest (Dorner, 2004). In a study related to the current research, *Y. enterocolitica* were isolated from pig feces from farms in the Grand River watershed in 2006 and 2007. Although isolation rates were low (14 samples were positive out of 240 (6%)), the majority of the isolates were *Y. enterocolitica* 4/O:3 (Public Health Agency of Canada, 2007), which is commonly recognized as a potential pathogen. In a study by Thoerner *et al.* (2003), 100 % of the biotype 4 strains investigated (26 strains) possessed the *ail* gene and 42 % (11 strains) possessed the *yadA* gene, although authors suggest that the reduced occurrence of the *yadA* gene may have been due to plasmid loss during culturing. All but one of the biotype 4 strains studied by Thoerner *et al.* (2003) were serotype O:3.

Both *ail* and *yadA* targets were detected least frequently in the Conestogo River (Table 4-12). This trend was also observed for *Campylobacter* rates (Appendix D). This was somewhat unexpected as the Conestogo River sub-watershed is also a region with high livestock density. It would be interesting to compare the Canagagigue Creek and Conestogo River sub-watersheds for types of livestock found, agricultural practices and other parameters related to the surface water flows of each region.

It is interesting to note the *Salmonella* and *E. coli* O157:H7 were both detected at significantly higher frequencies at the Grand River sampling site just downstream of the wastewater treatment plant effluent (Appendix E). However, *Y. enterocolitica* was detected relatively infrequently at this site (Table 4-12), suggesting that it is not being introduced in high amounts from municipal wastewater effluent. This is not unexpected, given that the incidence rate for *Y. enterocolitica* infection in the Waterloo Region in 2006 was relatively low at 3.3 cases per 100,000 person-years (Public Health Agency of Canada, 2007).

The Grand River watershed is located in Southern Ontario, a region which experiences large temperature swings between the winter and summer seasons (0-30°C) (Appendix E). The occurrence of *ail* and *yadA* DNA targets was tracked over the course of the sampling period and plotted against the river water temperature (Figure 4-4 through Figure 4-8). At all five sampling sites there was a higher frequency of *Y. enterocolitica ail* and *yadA* detection when water temperatures were colder.

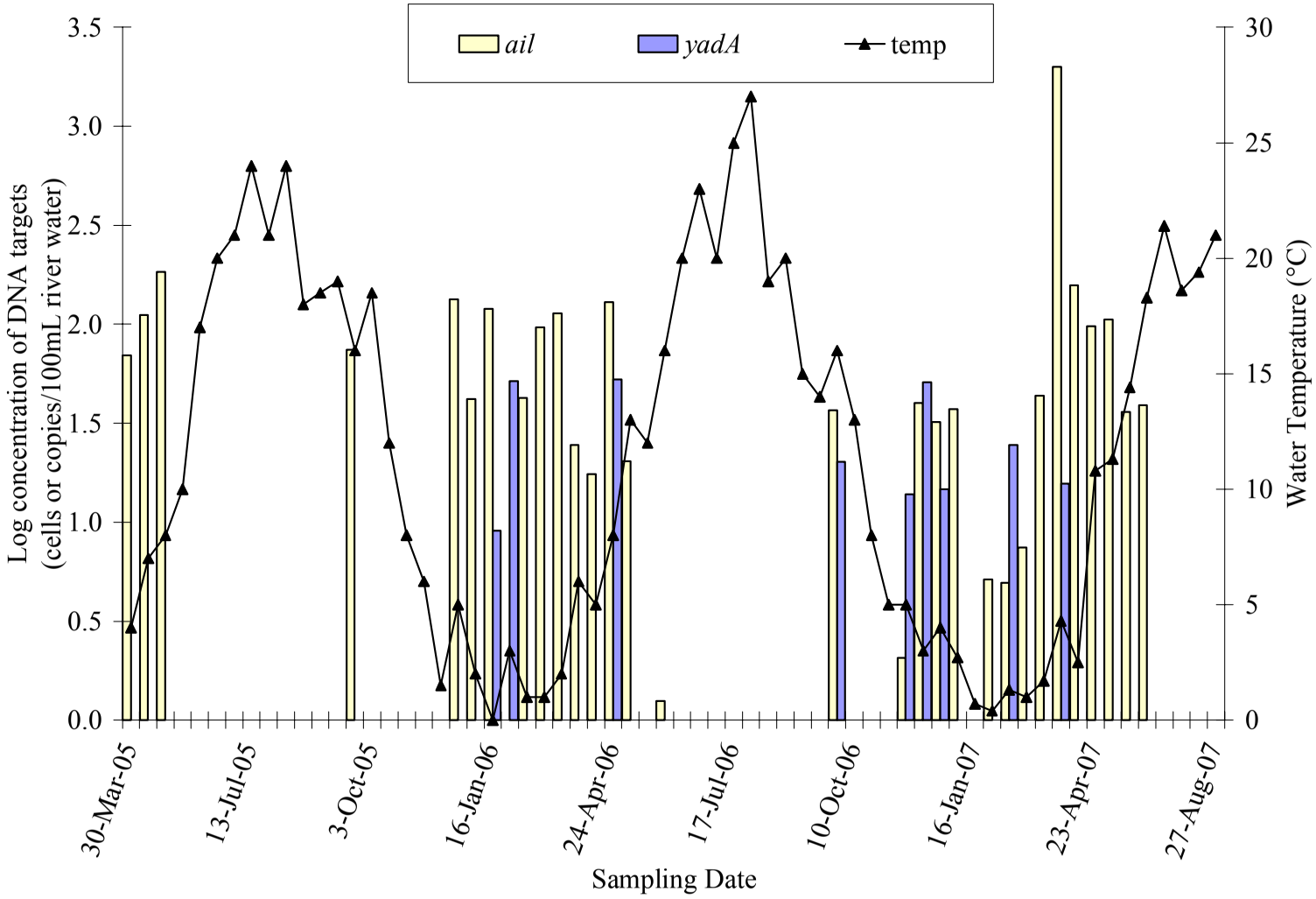


Figure 4-4: Relationship between the presence of the *Y. enterocolitica ail* and *yadA* genes and water temperature at the Grand River (north) site. This sampling location was located upstream of the other four sampling sites. Screening samples for the *yadA* gene target began in January 2006. Note that each tick along the horizontal axis represents a sampling date.

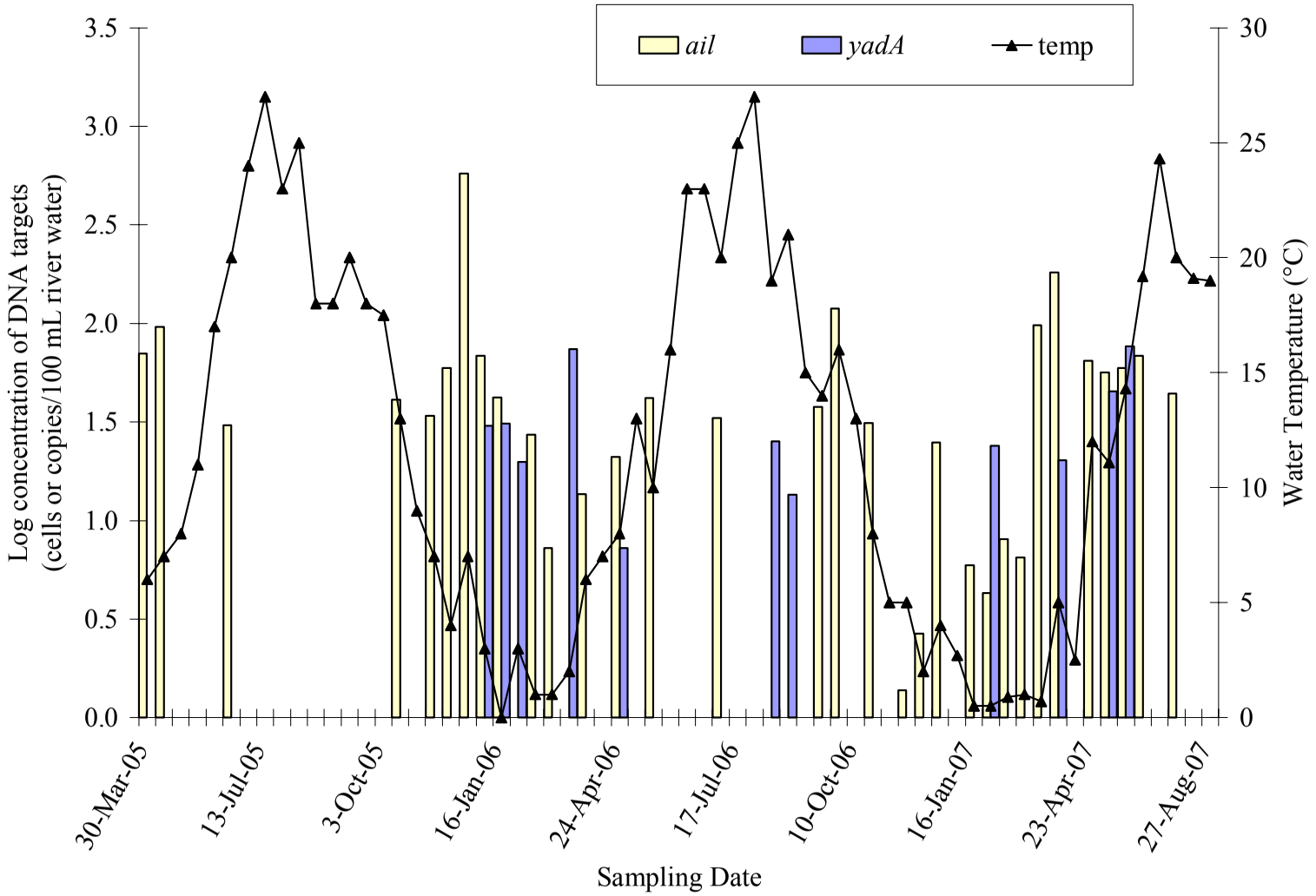


Figure 4-5: Relationship between the presence of *Y. enterocolitica ail* and *yadA* genes and the water temperature at the Canagagigue Creek site. The Canagagigue Creek is a major tributary of the Grand River. Screening samples for the *yadA* gene target began in January 2006. Note that each tick along the horizontal axis represents a sampling date.

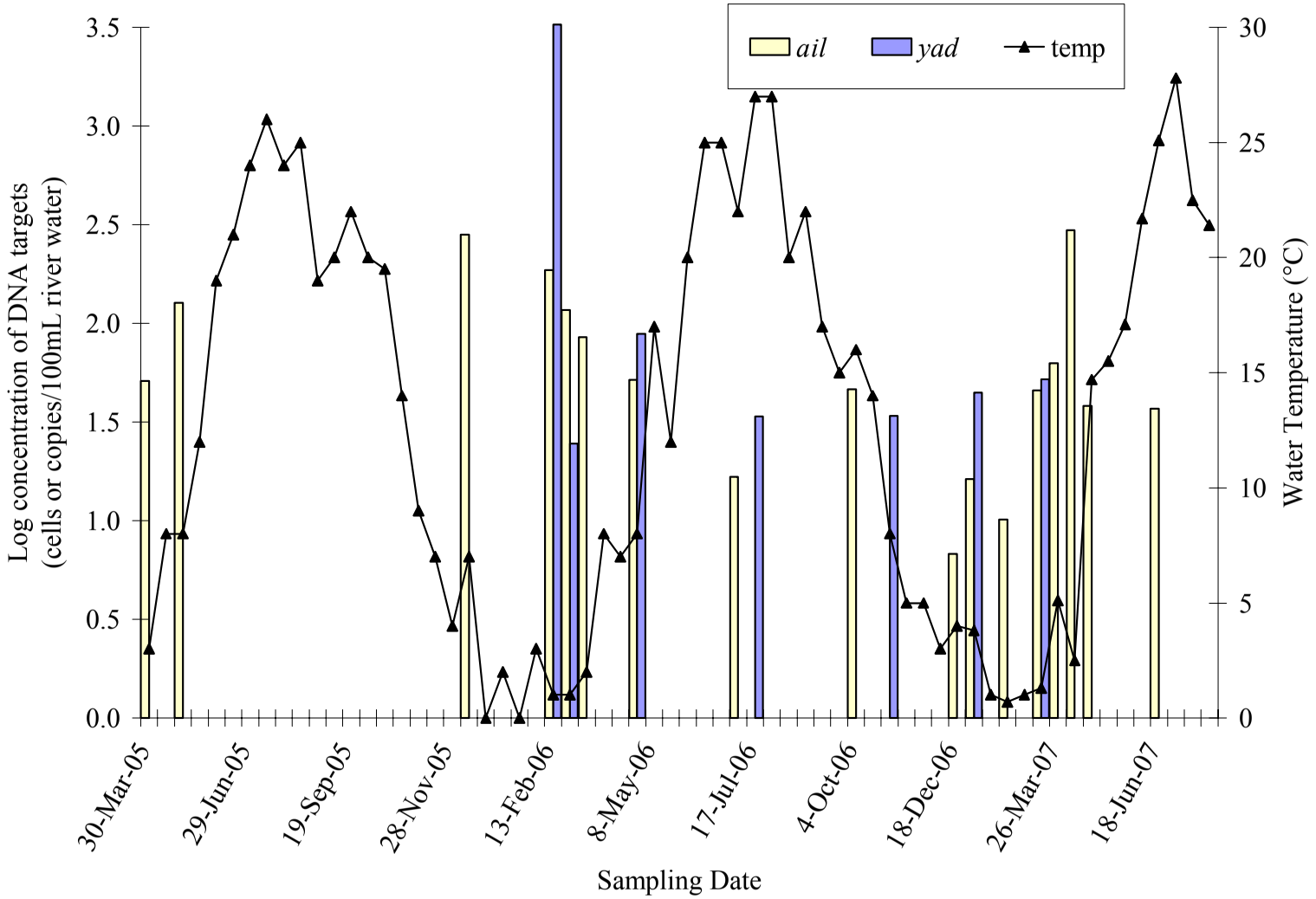


Figure 4-6: Relationship between the presence of *Y. enterocolitica ail* and *yadA* and the water temperature at the Conestogo River site. The Conestogo River is major tributary of the Grand River. Screening samples for the *yadA* gene target began in January 2006. Note that each tick along the horizontal axis represents a sampling date.

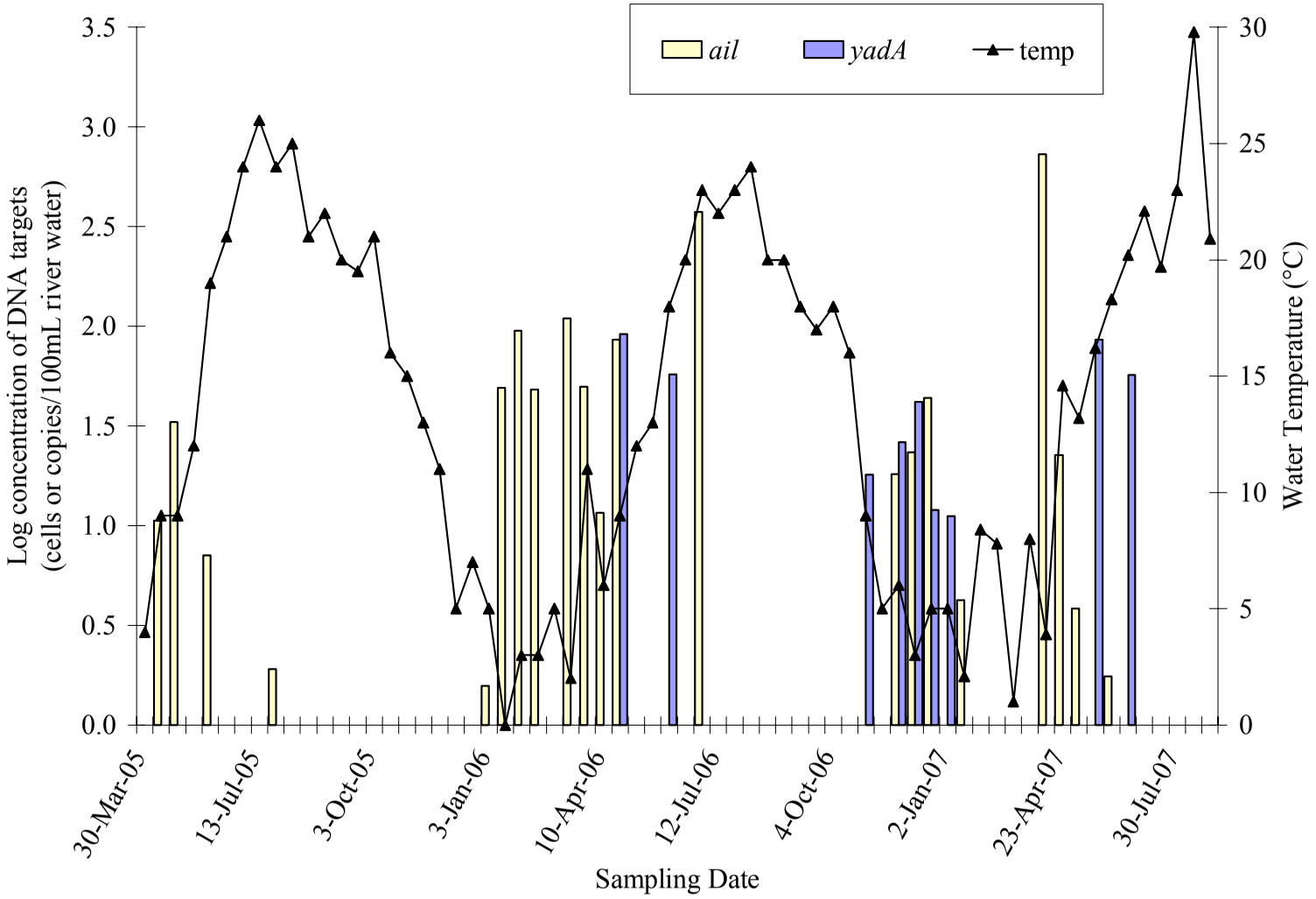


Figure 4-7: Relationship between the presence of *Y. enterocolitica ail* and *yadA* and the water temperature at a sampling site in the Grand River located downstream of a wastewater treatment plant effluent discharge. Screening samples for the *yadA* gene target began in January 2006. Note that each tick along the horizontal axis represents a sampling date.

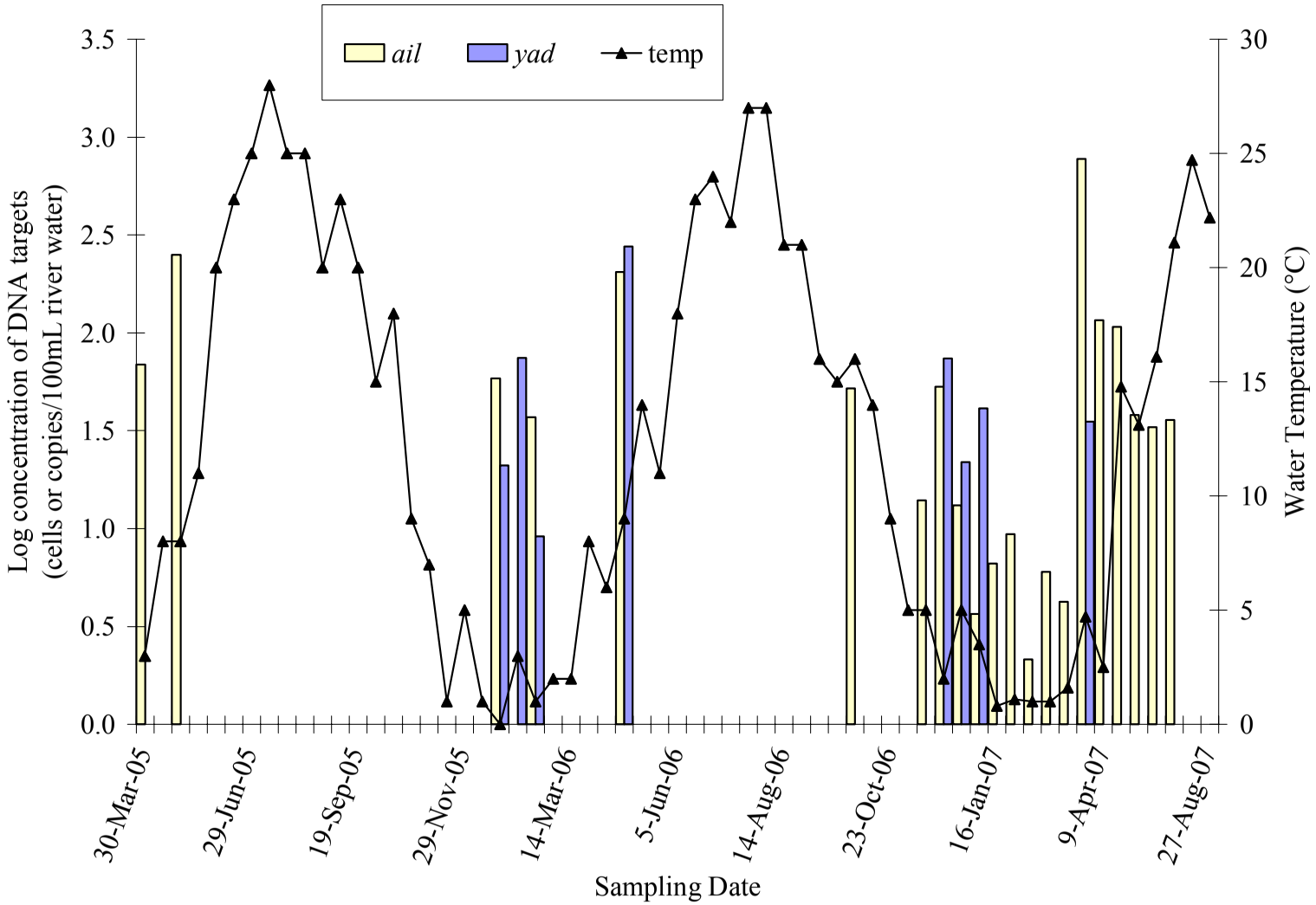


Figure 4-8: Relationship between the presence of *Y. enterocolitica ail* and *yadA* and the water temperature at a sampling site in the Grand River upstream of a drinking water treatment plant intake. Screening samples for the *yadA* gene target began in January 2006. Note that each tick along the horizontal axis represents a sampling date.

The seasonal trend can be further illustrated by looking at the frequency of detection within various temperature ranges as shown in Figure 4-9 and Table 4-13. The frequency of positive samples for the *ail* and *yadA* genes increased as water temperature decreased. For samples taken at temperatures below 5°C, 67% of samples were positive for the *ail* gene and 35% were positive for the *yadA* gene. In contrast, at temperature above 20°C, the virulence genes were detected in less than 12% of samples.

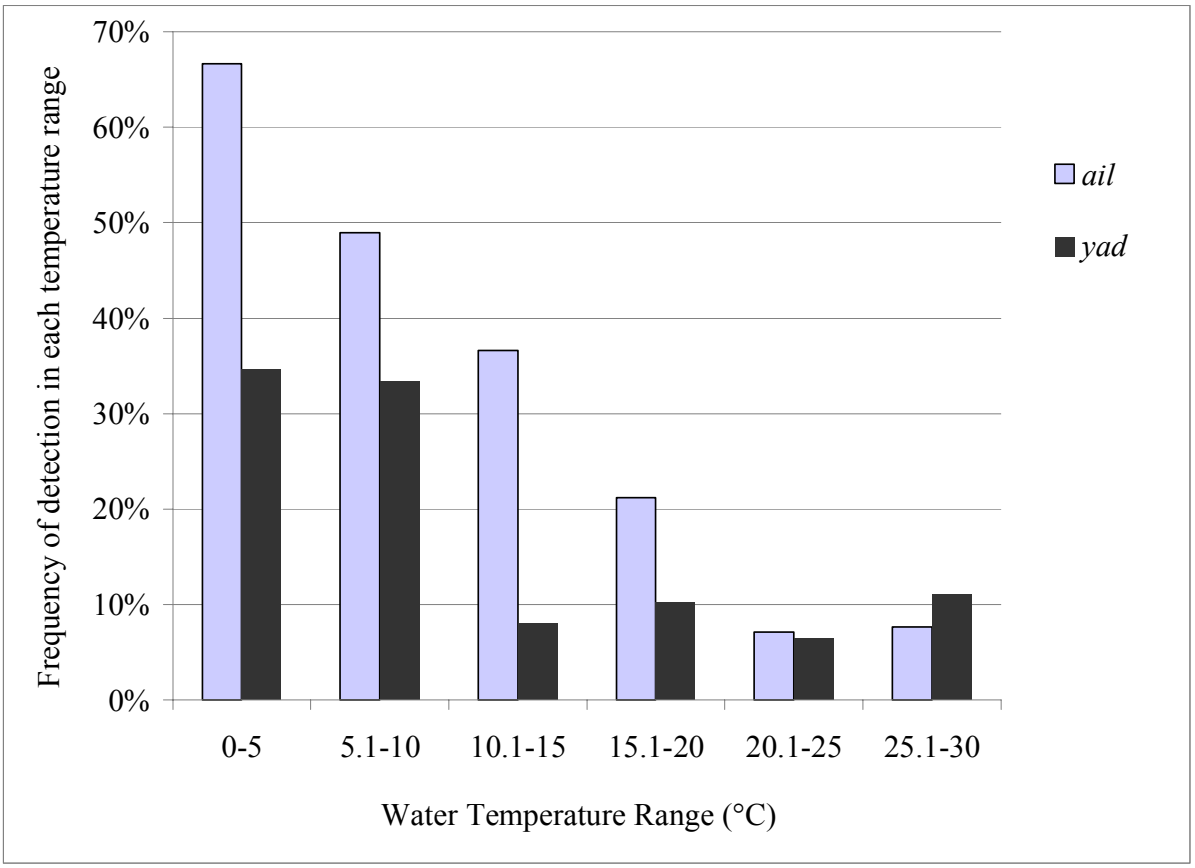


Figure 4-9: Relationship between *Y. enterocolitica* gene occurrence and water temperature. The total number of samples (n value) analyzed that fell into each temperature range is provided in Table 4-13.

Table 4-13: Frequency of detecting *Y. enterocolitica ail* and *yadA* genes at different water temperature ranges

Temperature range (°C)	Frequency of detection in each temperature range (n value*)	
	***ail* target**	***yadA* target**
0-5	67% (96)	35% (78)
5.1-10	49% (47)	33% (24)
10.1-15	37% (41)	8% (25)
15.1-20	21% (66)	10% (39)
20.1-25	7% (56)	6% (31)
25.1-30	8% (13)	11% (9)

* The n value indicates the total number of samples analyzed that fell into each temperature range.

A relationship between water temperature and occurrence of *Yersinia* spp. has been observed previously. Massa *et al*. (1988) isolated *Y. enterocolitica* and other *Yersinia* spp. from surface waters in Italy and found most isolates during the colder months of the year when the water was between 5°C and 10°C. Two other culture-based surveys for *Yersinia* isolates in water also noted a higher rate of isolation during colder months of the year (Meadows *et al.*, 1982; Fukushima *et al.*, 1984). This temperature trend has also been observed in a study investigating *Y. enterocolitica* prevalence in pigs in the United States, and detected the *ail* gene in pig feces at a higher rate during colder months of the year (Bhaduri *et al.*, 2005). Furthermore, *Y. enterocolitica* seems to be more frequently isolated from humans living in countries with cooler climates (Kapperud *et al.*, 1991). Unlike other members of the family Enterobacteriaceae, *Y. enterocolitica* survives cold temperatures (Fredriksson-Ahomma *et al*., 2003), which may explain the observed relationships between *Y. enterocolitica* incidence rates and colder temperatures. Moreover, Schiemann and Olson (1984) demonstrated that while *Y. enterocolitica* grew poorly in competition with other organisms at higher temperatures (25°C and 32°C), *Y. enterocolitica* growth rates were no longer impeded by other organisms when grown at 15°C. Fukushima *et al.* (1984) also postulated that the unique growth characteristics of *Y. enterocolitica* may contribute to the observed temperature trends. A culture-based survey conducted concurrently with the above PCR-based survey did not show a temperature trend for the isolation of *Yersinia* from the Grand River (see section 3.4.4). This difference may be related either to differences in the sensitivities of each method

or to differences in the survival rates of different strains of *Yersinia*, as most of the isolates from the Grand River were not *Y. enterocolitica*.

Previous culture-based surveys of water samples have noted the lack of correlation between the presence of *Y. enterocolitica* or *Yersinia* spp. isolates and the presence of indicator organisms, including total and fecal coliform counts (Meadows *et al.*, 1982; Massa *et al.*, 1988). Indicator *E. coli* was detected in 99 % of the samples tested in this study. As such, in this particular watershed, *E. coli* would be a poor indicator from a *source tracking* point of view.

Relationships between *ail* and *yadA* gene occurrence and the monitored water quality parameters, including indicator *E. coli*, turbidity, nitrate and ammonia, were evaluated (Appendix F). Results do not show any clear trends between *ail* or *yadA* gene occurrence and these water quality parameters. It sometimes appeared as though there may be a relationship between gene target occurrence and ammonia concentrations, as both increased during the winter months. However, ammonia concentrations in surface water in the winter are higher due to intermittent ice cover preventing dissolved ammonia from volatilizing into the atmosphere (the river has a high surface to volume ratio). It is unlikely *Y. enterocolitica* gene occurrence is related directly to ammonia concentration.

4.4.5.1 Reproducibility of Grand River Sample Measurements

In this experiment, six replicate *ail* DNA amplification reactions were carried out on two Grand River water samples (Table 4-14). Samples were selected for this experiment that had concentrations in the same range as the overall median values determined above. The relative standard deviations for the two samples were 45 % and 51 %. This highlights a limitation of Q-PCR. Although real time PCR enables quantification of DNA in a sample, this study demonstrates that results from analyzing the same sample will vary. Nonetheless, Q-PCR methods still provide a semi-quantitative measure of the concentration of pathogens in surface water and improve the detection of gene targets at low concentrations, both of which are highly valuable.

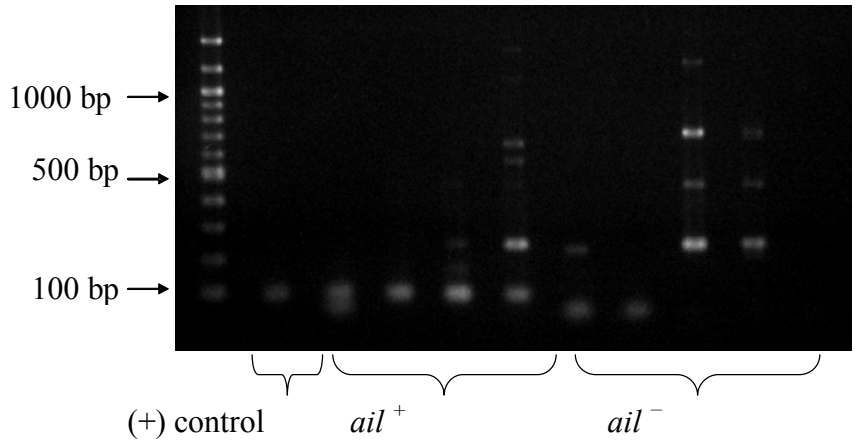
Table 4-14: Reproducibility of enumerating the *ail* gene target in Grand River samples using Q-PCR methods

Replicate	Concentration (cells/100 mL river water)	
	Sample # 07-0049	Sample # 07-0051
1	39	17
2	38	47
3	81	22
4	22	30
5	78	51
6	55	15
Average	52	30
Standard deviation	24	16
Relative standard deviation	45 %	51 %

4.4.5.2 Confirmation of Real-Time PCR Results

Two tests were run to confirm that the DNA amplification was specific for the desired DNA targets from *Y. enterocolitica*. The first test used agarose gel electrophoresis to confirm that the DNA fragments amplified by PCR were the expected size. The amplified DNA fragments from selected reactions were separated on agarose gels. Figure 4-10 shows representative agarose gels for the *ail* PCR assay. Grand River samples that produced a fluorescent signal for the *ail* DNA amplification reactions are denoted as *ail* $^{+}$, whereas samples for which no fluorescence was detected are denoted as *ail* $^{-}$. The positive control marked in each figure refers to a DNA amplification reaction using standard control DNA as the template. The *ail* PCR amplifies a DNA fragment 91-bp in length. The *ail* $^{+}$ sample lanes consistently show a distinct band just below the 100-bp marker that matched the band in the positive control lane. In some of these samples, other larger bands were also observed, which indicates that some non-specific DNA amplification occurred. The *ail* $^{-}$ samples do not show a 91-bp band. These samples also often show several larger bands and occasionally smaller bands, indicating that non-specific DNA amplification occurred in those samples; however, a fluorescence signal was not detected.

(a)



(b)

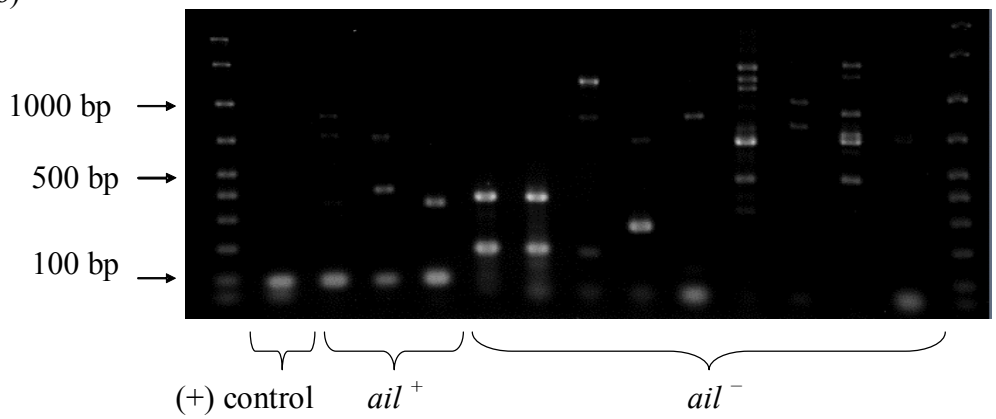


Figure 4-10: DNA amplification of the *ail* gene target in Grand River samples. (a) and (b) show two representative gels that were run to visualize the *ail* DNA amplification products. The positive (+) control is a DNA amplification of the *ail* target using *Y. enterocolitica* genomic DNA as a template. The *ail* $^{+}$ samples are Grand River samples that generated a fluorescent signal in the *ail* DNA amplification reaction. The *ail* $^{-}$ samples are Grand River samples that did not produce a fluorescent signal. The first lane in figure (a), and the first and last lanes in figure (b) are DNA molecular weight markers. Note that sample was only loaded into every other lane in both (a) and (b).

The agarose gel showing the DNA amplification products for the *yadA* PCR is shown in Figure 4-11. Grand River samples that produced a fluorescent signal for the *yadA* DNA amplification reactions are denoted as *yadA* $^{+}$, whereas samples for which no fluorescence was detected are denoted as *yadA* $^{-}$. The *yadA* PCR amplifies a DNA fragment 97-bp in length. Although it is sometimes difficult to see, a 97-bp band does appear in the *yadA* $^{+}$ sample lanes. However, it was difficult to determine whether all of the *yadA* $^{-}$ samples lack a 91-bp band as they would be expected to. Many of the *yadA* DNA amplification reactions (both *yadA* $^{+}$ and *yadA* $^{-}$) contained numerous non-specifically amplified bands, far more than those observed in the *ail* reaction products. This higher rate of non-specific amplification may have contributed to the lower frequencies of detection of the *yadA* gene target in the Grand River survey compared to the *ail* target.

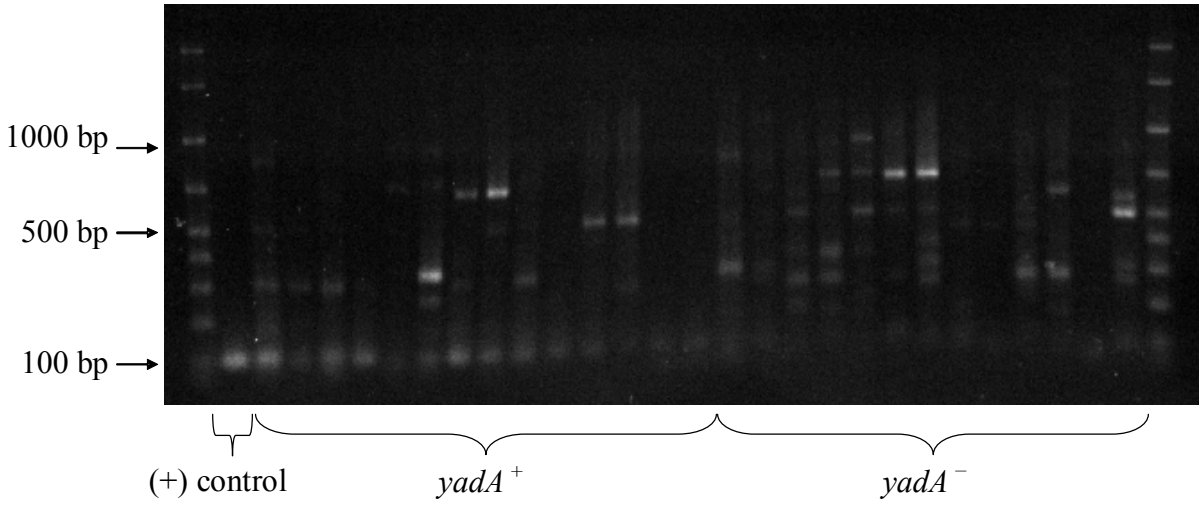


Figure 4-11: DNA amplification of the *yadA* gene target in Grand River samples. The positive (+) control is a DNA amplification of the *yadA* target using synthetic oligonucleotide DNA as a template. The *yadA* $^{+}$ samples are Grand River samples that generated a fluorescent signal in the *yadA* DNA amplification reaction. The *yadA* $^{-}$ samples are Grand River samples that did not produce a fluorescent signal. The first and last lanes are molecular weight markers.

The second confirmation test used to validate PCR positive results involved sequencing the amplified DNA fragments to verify that they matched the targeted gene sequence. DNA amplification products were cloned into sequencing vectors. The cloned samples are summarized in Table 4-15. For sample 06-0038, clone ailC4 (Table 4-15) was sequenced and amplification products were also sequenced directly without cloning.

Table 4-15: The sequenced *ail* and *yadA* clones

Gene target	Grand River sample	Clones
ail	06-0038	06-0038-*ail*C4
	06-0127	06-0127-*ail*C2 and 06-0127-*ail*C6
yadA	06-0041	06-0041-*yadA*C4
	06-0053	06-0053-*yadA*C4 and 06-0053-*yadA*C5

All *ail* samples (the three cloned samples and the non-cloned sample) sequences were identical (100 % homology). This sequence was searched using the NCBI basic local alignment search tool (BLAST), which finds regions of local similarity between sequences. The NCBI BLAST results for the *ail* PCR fragments are summarized in Table 4-16. The only sequences found to align with the *ail* clones belonged to *Y. enterocolitica*. No other sequences were found to have good homology. For example, according to the NCBI BLAST results, the next best match in the database had 87% homology with a 31-bp fragment within the 91-bp *ail* cloned fragment. Although *ail* sequence data was obtained for only a limited number of samples, results showed that the *ail* Q-PCR assay can amplify the desired gene target from *Y. enterocolitica* strains in the surface water samples.

Table 4-16: Sequence in the NCBI database that matched the sequences of the *ail* clones (clones 06-0038-*ail*C4, 06-0127-*ail*C2, 06-0127-*ail*C6)

NCBI accession no.	NCBI sequence description	Homology (%) of *ail* clones
emb|AM286415.1	*Y. enterocolitica* subsp. *enterocolitica* 8081 complete genome	100
gb|DQ157767.1	*Y. enterocolitica* attachment invasion locus protein (*ail*) gene, partial cds	100
gb|M29945.1|YEPAIL	*Y. enterocolitica* attachment invasion locus (*ail*) gene and 2 ORFs, complete cds	100
gb|AY004311.1	*Y. enterocolitica* attachment invasion locus protein (*ail*) gene, partial cds	95
emb|AJ605740.1	*Y. enterocolitica* (type 0:3) pseudogene for transposase (partial), pseudogene for hypothetical protein, *ail* gene, and second pseudogene for transposase (partial)	95
gb|DQ003329.1	*Y. enterocolitica* (type 0:3) attachment invasion locus protein (*ail*) gene, partial cds	95

Two of the *yadA* clones, 06-0041-*yadA*C4 and 06-0053-*yadA*C5, had 100 % sequence homology. The third clone, 06-0053-*yadA*C4, differed from the other clones by only one base pair, and was missing the base at position number 12 compared with the other two clones. Both sequences were searched using NCBI BLAST and results are summarized in Table 4-17. The only sequences from the database found to align with the *yadA* clones belonged to *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis*. Although three of the matches listed in Table 4-17 were described as sequences for the *yopA* gene, *yadA* was previously called *yopA* (Skurnik *et al.*, 1989; Michiels *et al.*, 1991). The *yadA* gene is found in all three species and prior screening of the *yadA* primers and probe revealed that they amplified DNA targets in *Y. pseudotuberculosis* strains. Consequently, it is possible that the *yadA* PCR assay was detecting *Y. pseudotuberculosis* in addition to *Y. enterocolitica* strains in the Grand River. This may explain why some Grand River samples were positive for the *yadA* gene target, but not the *ail* gene target. No other sequence alignments were found to have good homology with significant portions of the *yadA* clones. The next best match in the database had 85 % homology with a 49-bp fragment within the 97-bp *yadA* cloned fragment. Results suggest that the *yadA* Q-PCR assay is detecting *Yersinia* in surface water samples. However, due to the high homology between the pYV plasmids of *Y. enterocolitica* and *Y. pseudotuberculosis*, it is possible that both are being detected. It is unlikely that *Y. pestis* strains are being detected, as *Yersinia pestis* is primarily contained within a sylvatic reservoir and is not transmitted by water (Stenseth *et al.*, 2008).

Table 4-17: Sequences in the NCBI database that matched the sequences of the *yadA* clones

NCBI accession no.	NCBI sequence description	Homology (%) of *yadA* clones:	
		07-0041-*yadA*C4 07-0053-*yadA*C5	07-0053-*yadA*C4
emb|AM286416.1	*Y. enterocolitica* subsp. *Enterocolitica* 8081 plasmid pYVe8081 complete genome	98	96
gb|AF336309.1	*Y. enterocolitica* plasmid pYVe8081, complete sequence	98	96
gb|AY150843.2	*Y. enterocolitica* strain A127/90 plasmid pYVa127/90, complete sequence	98	96
emb|X13882.1|YEYOPA64	*Y.enterocolitica yadA* gene for *YadA*	98	96
gb|AF102990.1|AF102990	*Y. enterocolitica* plasmid pYVe227, complete sequence	98	96
gb|AF056092.1|AF056092	*Y. enterocolitica* plasmid pYV adhesin (*yadA*) and genes, complete cds	98	96
emb|X13881.1|YEYOPA80	*Y. enterocolitica* virulence plasmid pYV8081 *yopA* gene for yop1 protein	98	96
emb|X13883.1|YPYOPA	*Y. pseudotuberculosis* virulence plasmid pIBI *yopA* gene for Yop1 protein	98	96
emb|BX936399.2|	*Y. pseudotuberculosis* IP32953 pYV plasmid, complete sequence	96	98
gb|CP000722.1	*Y. pestis* CA88-4125 plasmid pCD1, complete sequence	98	96
gb|CP000669.1	*Y. pestis* Pestoides F plasmid CD, complete sequence	98	96
gb|CP000311.1	*Y. pestis* Antiqua plasmid pCD, complete sequence	98	96
gb|AE017043.1	*Y. pestis* biovar Microtus str. 91001 plasmid pCD1, complete sequence	98	96
gb|AF074612.1	*Y. pestis* KIM plasmid pCD1, complete sequence	98	96
gb|AF053946.1	*Y. pestis* KIM plasmid pCD1, complete sequence	98	96
emb|AL117189.1|YPCD1	*Y. pestis* CO92 plasmid pCD1	98	96
gb|CP000902.1|	*Y. pestis* Angola plasmid new_pCD, complete sequence	97	95
emb|X13880.1|YP*YOPA*NF	*Y. pestis* virulence plasmid pYV019 *yopA* pseudogene	97	95

4.5 Conclusions

The methods evaluated in this study demonstrate that Taqman Q-PCR-based methods can be effective and sensitive tools for enumerating *Y. enterocolitica* in water samples. While prior real time PCR-based methods have been used to detect *Y. enterocolitica* in water, these studies used an upstream preenrichment step and therefore were not quantitative. This is the first study to use Q-PCR methods to enumerate water samples and to subsequently survey for the pathogen over an extended period of time. The Q-PCR-based survey of the Grand River watershed confirmed that *Y. enterocolitica ail* and *yadA* genes are both present in surface water samples, suggesting that potentially pathogenic *Y. enterocolitica* may be present. Moreover, gene targets were more prevalent in colder water. Given that similar seasonal trends have been observed in other studies, it may be of interest to further examine this phenomenon to elucidate factors influencing *Y. enterocolitica* occurrence in the environment.

This work assists with the development of methods and information gathering for an emerging waterborne pathogen for which limited PCR-based surveys exist and for which no occurrence data is available for the Grand River watershed. This watershed is used both as a drinking water source and for recreational activities. There is evidence that *Y. enterocolitica* is sensitive to chlorination, UV irradiation and ozonation. However, inactivation studies are limited and additional research may be necessary to support these data. Further investigation is also necessary to evaluate whether the presence of pathogenic *Y. enterocolitica* in surface waters is a risk for individuals drinking non-treated water or for recreational water users. Given that *Y. enterocolitica* virulence genes are detected more frequently in the winter, it seems likely that risk of exposure to recreational users is low.

5 Summary of Results and Conclusions

This chapter integrates findings from the two main focuses of the study: culture-based detection and PCR-based detection of *Y. enterocolitica* in surface water from the Grand River watershed in Southern Ontario, Canada.

5.1 Culture-based Detection of *Y. enterocolitica*

The objectives of the culture-based study were to evaluate and compare culture-based methods for isolating *Y. enterocolitica* from surface water, then to use the best culture-based method, to isolate indigenous *Y. enterocolitica* from Grand River surface water. In the evaluation and comparison studies, two selective agars and four enrichment methods were compared. Subsequently, one of the culture-based methods was used to survey the Grand River. The key findings are summarized below:

- The growth and growth inhibition of various bacteria, including *Yersinia* and non-*Yersinia* spp. on SSDC and CIN agar were compared. While CIN agar effectively inhibited the growth of 7 out of the 9 non-*Yersinia* enteric bacteria tested, SSDC only inhibited 1 of 9 species. Furthermore, on SSDC the non-*Yersinia* bacteria produced colonies that were very difficult to differentiate from *Yersinia* colonies. It was concluded that CIN agar was better for isolating *Yersinia* strains from surface water samples.

- The ability of four enrichment broths, ITC, mTSB, LB-BSI broth and PBS, to recover a *Y. enterocolitica* strain from surface water samples was evaluated. Results demonstrated that non-*Yersinia* bacteria indigenous to Grand River surface water were not effectively inhibited by the enrichment broths tested. None of the enrichment methods tested were effective for recovering a *Y. enterocolitica* strain spiked into surface water from the Grand River. However, based on post-enrichment concentrations of indigenous bacteria from Grand River samples and post-enrichment concentrations of *Y. enterocolitica* grown in pure culture, it was concluded that

enrichment in mTSB was a better method than the others tested for isolating *Yersinia* from surface water samples.

- A survey of the Grand River was conducted using the selected enrichment method (mTSB) and demonstrated that *Yersinia* spp. are present in the watershed. *Yersinia* strains were isolated from 52 (26%) samples and *Y. enterocolitica* strains were isolated from 8 (4%) samples out of 200 samples that were collected.

- The 97 *Yersinia* strains isolated included traditionally non-pathogenic strains: *Y. enterocolitica* biotype 1A (11 %), *Y. aldovae* (11 %), *Y. bercovieri* (9 %), *Y. frederiksenii* (16 %), *Y. intermedia* (42 %), *Y. kristensenii* (2 %) and *Y. mollaretii* (7 %).

In conclusion, *Yersinia* are present in the Grand River watershed. An evaluation of the culture-based methods, however, would suggest that non-*Yersinia* bacteria indigenous in surface water make it difficult to recover *Yersinia* from samples. Hence, it seems likely that *Yersinia* in the Grand River is more prevalent than this culture-based survey may suggest.

5.2 PCR-based Detection of *Y. enterocolitica*

The objective of the PCR-based component of this study was to evaluate PCR-based methods that target the *ail* and *yadA* virulence genes for detecting *Y. enterocolitica* from surface water. These genes have been associated with pathogenic species of *Y. enterocolitica*. Each PCR assay was evaluated by assessing the resulting standard curves, detection limit, specificity and recovery. The *ail* and *yadA* PCR assays were then used to survey the Grand River for pathogenic *Y. enterocolitica*. The key findings from this study are summarized below:

- Genomic DNA extracted from a *Y. enterocolitica* strain served as the DNA standards for the *ail* assay and yielded linear standard curves with a detection limit between 5 and 8 cells per PCR reaction.

- Genomic DNA and plasmid DNA extracted from the same *Y. enterocolitica* strain did not serve as useful DNA standards for the *yadA* assay due to a poor detection limit. A synthetic DNA oligonucleotide did prove to be useful as a DNA standard for the *yadA* assay, as it yielded linear standard curves with a detection limit around 10 cells per PCR reaction.

- The *ail* and *yadA* PCR assays were tested with a variety of *Yersinia* and non-*Yersinia* bacteria. The primers and probe targeting the *ail* gene were specific to *Y. enterocolitica*, while those targeting the *yadA* gene were found to amplify the *yadA* gene in both *Y. enterocolitica* and *Y. pseudotuberculosis*. Unexpectedly, both genes were detected in a laboratory strain of *Y. intermedia*. The significance of this finding needs to be further investigated.

- The DNA extraction efficiency of *Y. enterocolitica* from surface water was evaluated by each PCR assay. The recovery observed using the *ail* PCR assay ranged from 24 % to 37 % and using the *yadA* PCR assay from 25 % to 50 %.

- In a survey of the Grand River watershed, *Y. enterocolitica ail* and *yadA* genes were present in 38 % and 21 % of the surface water samples, respectively. While both genes were detected at all five sampling locations, they were also detected most frequently in samples from a small tributary of the Grand River, Canagagigue Creek.

- Pathogenic *Y. enterocolitica* were detected more frequently when water temperatures were cold. For samples taken at water temperatures below 5°C, 67% of samples were positive for the *ail* gene and 35% were positive for the *yadA* gene. Whereas, at temperature above 20°C, the virulence genes were detected in less than 12% of samples.

- In addition to temperature, several other surface water parameters were monitored including, turbidity, nitrate, ammonia, and indicator *E. coli* concentrations. However, neither the presence of the *ail* gene, nor the presence of the *yadA* gene correlated with these parameters.

In conclusion, the *Y. enterocolitica ail* and *yadA* genes are both present in Grand River surface water and appear to be more prevalent when the water temperatures are colder. The presence of these virulence genes suggests that pathogenic *Y. enterocolitica* may be present in the watershed. Based on a review of the literature, it was determined that pigs are a major reservoir for human pathogenic strains of *Y. enterocolitica*. Given that the predominant land use in the watershed is agriculture, and that pigs are the second most prevalent form of livestock, it is possible that agricultural run-off may be introducing *Y. enterocolitica* into surface water in the watershed.

5.3 Comparing the Culture-based and PCR-based Findings

The Grand River was surveyed for *Y. enterocolitica* using both culture-based and PCR-based methods. The prevalence rates using each method were calculated over slightly differing time frames. Monitoring samples for the *ail* gene target started in March 2005, the *yadA* gene in January 2006 and culture-based isolation in April 2006. All monitoring ended in August 2007. To conduct a comparison of all three methods, the prevalence rates for each gene target were calculated for the time period when all three surveys were being simultaneously employed (April 2006 to August 2007). Over this time period, the *ail* and *yadA* gene targets were detected in 43% and 19% of the samples, respectively. In contrast, the culture-based isolation rate of *Y. enterocolitica* biotype 1A was 4 %. Although *Y. enterocolitica* 1A is not traditionally a pathogenic strain, recent evidence suggests that traditionally non-pathogenic subtypes may in fact be pathogenic, and *Y. enterocolitica* 1A strains have been found that possess the *ail* and/or *yadA* genes. Regardless of whether the 1A strains are considered potentially pathogenic, the detection rate of *Y. enterocolitica* in the Grand River is significantly higher using PCR-based methods than culture-based methods. Earlier, conclusions drawn from the evaluation of culture-based methods suggested that the incidence

rate of the culture-based survey of the Grand River was an underestimate of the true prevalence of *Y. enterocolitica*. A comparison of culture-based and PCR-based methods supports this conclusion.

Also, the PCR-based survey demonstrated a seasonal trend with a higher frequency of detecting *Y. enterocolitica* genes during the winter months, which has been observed in other studies. However, the culture-based survey did not show any seasonal trends in isolation rates. Recall that the culture-based study did not isolate traditionally pathogenic strains, and hence, cannot necessarily be compared directly to the PCR-based results.

5.4 Implications for Water Treatment Providers and Regulators

This work assists with the development of methods for an emerging waterborne pathogen for which few water-related studies exist. While both culture-based and PCR-based methods for detecting *Y. enterocolitica* have been tested extensively with food and clinical samples, limited studies have been conducted to evaluate these methods for water samples. Consequently, standardized detection methods for water are not available. This work also contributes to information gathering for a waterborne pathogen for which no occurrence data is available for the Grand River watershed, used both as a drinking water source and for recreational activities. Although Grand River surface water is used as a drinking water source, results showed that *Y. enterocolitica* were detected in surface water samples at concentrations below those detected for indicator *E. coli*. Furthermore, *Y. enterocolitica* has demonstrated similar sensitivity as *E. coli* to disinfection technologies used in drinking water treatment processes, including; chlorination, UV irradiation, and ozonation. However, only a limited number of inactivation studies have been conducted with *Y. enterocolitica* and additional research is necessary to support these data. Consequently, for surface water from the Grand River watershed, indicator *E. coli* may provide an indication of the presence of *Y. enterocolitica* in treated water, however this needs further research to be confirmed. The presence of pathogenic *Y. enterocolitica* may still pose a concern for individuals drinking non-treated water or for recreational users.

These findings provide important information for both drinking water providers and public health investigations. The presence of potentially pathogenic *Y. enterocolitica* in surface water at this study site suggests the organism may be present in other surface waters. A limited number of culture-based surveys conducted previously have found *Y. enterocolitica*, however, isolations rates were often low and strains were usually non-pathogenic. These findings highlight the limitations of culture-based detection for surveying surface water and suggest that prevalence rates were likely higher than reported in prior culture-based studies.

6 Recommendations

Recommendations for future studies, including method evaluation and comparison studies as well as future surveys for *Y. enterocolitica*, are provided below.

6.1 Future Culture-based Studies

Findings indicated that culture-based methods tested were not effective for recovering *Y. enterocolitica* (ATCC 700822) added to surface water. A *Y. enterocolitica* strain isolated from the Grand River may better compete against other bacteria indigenous to the Grand River in an enrichment broth compared to a laboratory strain of *Y. enterocolitica*, as was used in this study. This was not attempted in this study because *Y. enterocolitica* strains were not isolated from the Grand River until quite late in the study, and the species of these isolates was not confirmed until the end of the study. Consideration should be given to performing recovery experiments with one of the indigenous *Y. enterocolitica* strains isolated from the specific surface water to be surveyed.

Another challenge encountered in the culture-based recovery study was associated with evaluating results. *Yersinia* colonies were sometimes detected in only one of two replicate samples. Typically, replicate values were averaged. However, if *Yersinia* was not detected, the sample was recorded as a "non-detect" and not as a zero value. This was because it was possible that *Yersinia* was present, but in concentrations below the detection limit due to the high concentration of non-*Yersinia* bacteria indigenous to the Grand River. A possible solution to this problem could be to instead evaluate *Y. enterocolitica* recovery using the most probable number method for enumerating organisms in a sample. This solution, however, is not likely to improve results if the isolation methods cannot effectively inhibit non-*Yersinia* growth.

Given the challenges associated with the available culture-based methods for *Y. enterocolitica*, it may be worthwhile considering an upstream immunocapture step. This technique captures the bacteria of interest on a solid surface (*i.e.*, magnetic beads) to which antibodies are fixed that bind antigens on the surface of target bacteria. Thus far, development of such methods for *Y. enterocolitica* have had limited success. However, any small improvement to recovery over current methods, which have very poor recovery, could be useful and should be considered. Immunocapture steps have also been used upstream of PCR-based methods.

6.2 Future PCR-based Studies

Several non-pathogenic *Yersinia* strains were isolated from the Grand River, many of them from water samples that were also positive for the *ail* and/or *yadA* genes. Limited reports have suggested that some of these traditionally non-pathogenic strains may in fact possess the same virulence genes as other *Y. enterocolitica*, including the *ail* and *yadA* genes. It would be very interesting to screen such *Yersinia* isolates for the presence of the *ail* and *yadA* gene targets using the PCR assays evaluated in this study.

It is important to note that PCR-based methods are at risk of yielding false-positives due to signals generated by either non-viable cells or naked DNA. An experiment could be conducted to determine whether this risk is a concern for detecting *Y. enterocolitica*. Inactivated *Y. enterocolitica* cells and naked DNA from *Y. enterocolitica* could be added to surface water samples and incubated for varying amounts of time, then analyzed by PCR. Previous studies have used chlorination and UV irradiation to inactivate cells, and used boiling steps to lyse cells and release naked DNA.

It is also important to confirm PCR results to ensure DNA amplification was specific to the desired target. In this study, DNA amplification products were examined on DNA agarose gels and were sequenced to confirm their identity. DNA sequencing requiring that samples are cloned is more labour intensive and time consuming and hence, very few samples were confirmed. A faster alternative method for confirming PCR results is to separate the DNA

amplification products on an agarose gel and to excise the target DNA fragment from the gel and purify the DNA. This excised and purified DNA is used as template DNA in a second confirmatory PCR designed to amplify a smaller fragment within the original target. This confirmation method was used in another PCR-based survey of surface waters for *Y. enterocolitica*. Alternatively, due to the real time nature of the PCR method utilized in this study, there may be an even simpler method still. After the first PCR it may not be necessary to excise and purify the targeted DNA fragment from a gel. Rather, one could use an aliquot of the non-purified products from the first reaction as the template DNA in the second confirmation PCR. One would expect the C_T value for positive samples to be small because the starting concentration of target DNA should be larger at the end of the first PCR. Given the short length of the DNA targets in the *ail* and *yadA* PCR in this study, it may be difficult to design a second PCR that targets a smaller region within the first target.

6.3 Future *Y. enterocolitica* Surveys

Findings from this work suggest that PCR-based methods are better than culture-based methods for detecting *Y. enterocolitica*. However, this does not take into account all of the advantages and disadvantages of each approach. Culture-based methods isolate viable strains that can be further studied, which is critical for epidemiology studies. Strains isolated may be subtyped and compared to strains isolated from other sources, including humans, to evaluate potential modes of transmission or to investigate disease outbreaks. Hence, to acquire a complete picture of both *Y. enterocolitica* occurrence and transmission, it is recommended to use both culture-based and PCR-based detection methods in combination, as was done in this research.

Most importantly, from the viewpoint of drinking water providers, the findings of this work suggest that potentially pathogenic *Y. enterocolitica* are present in surface water in the Grand River watershed. Moreover, the organism appears to be more prevalent during colder months of the year. As discussed previously, *Y. enterocolitica* is relatively easy to inactivate using disinfection technologies used in drinking water treatment processes. However, it should be noted that disinfection is less effective at colder temperatures. It may be of interest to

investigate the survival of *Y. enterocolitica* in Grand River surface water collected during both cold and warm seasons. Beyond survival studies, further investigation is still required to determine how to interpret survey results as it is important to base conclusions on comprehensive information, particularly in regards to assessing risks to human health.

APPENDIX A: *Yersinia* Isolates from the Grand River

This appendix lists the *Yersinia* strains isolated from Grand River water samples. The sample date column indicates the date that the river water was sampled. The isolate ID is composed of 3 parts. The first part is a 2 digit number corresponding to the year the sample was collected. The second part is a 4 digit number corresponding to the sample number for that year. The third part is a 2 digit number following the letters, YE, for *Yersinia*. Each presumptive *Yersinia* strain isolated from a sample was assigned a 2 digit number. The *Y. enterocolitica* isolates are listed in bold text.

Sample Date	Isolate ID	Location	Species and subtype
10-Apr-06	06-0047-YE02	Intake	*Y. aldovae*
5-Jun-06	06-0072-YE01	Grand River North	*Y. intermedia* 1
5-Jun-06	06-0072-YE02	Grand River North	*Y. intermedia* 1
19-Jun-06	06-0076-YE06	Conestogo River	*Y. intermedia* 1
19-Jun-06	06-0077-YE04	Canagagigue Creek	*Y. aldovae*
19-Jun-06	06-0078-YE01	Grand River North	*Y. aldovae*
19-Jun-06	06-0078-YE02	Grand River North	*Y. aldovae*
19-Jun-06	06-0078-YE03	Grand River North	*Y. aldovae*
4-Jul-06	06-0083-YE03	Canagagigue Creek	*Y. bercovieri*
4-Jul-06	06-0085-YE02	Waste Water Effluent	*Y. intermedia* 4
4-Jul-06	06-0085-YE03	Waste Water Effluent	*Y. intermedia* 4
4-Jul-06	06-0086-YE01	Intake	*Y. intermedia* 4
4-Jul-06	06-0086-YE02	Intake	*Y. intermedia* 4
12-Jul-06	06-0088-YE02	Conestogo River	*Y. frederiksenii*
12-Jul-06	06-0090-YE01	Grand River North	*Y. aldovae*
12-Jul-06	06-0090-YE02	Grand River North	*Y. aldovae*
12-Jul-06	06-0090-YE03	Grand River North	*Y. aldovae*
12-Jul-06	06-0090-YE05	Grand River North	*Y. aldovae*
17-Jul-06	06-0097-YE03	Canagagigue Creek	*Y. intermedia* 1
17-Jul-06	06-0097-YE05	Canagagigue Creek	*Y. intermedia* 1
1-Aug-06	06-0102-YE01	Conestogo River	*Y. intermedia* 1
1-Aug-06	06-0102-YE02	Conestogo River	*Y. intermedia* 1
1-Aug-06	06-0103-YE01	Canagagigue Creek	*Y. intermedia* 1
1-Aug-06	06-0103-YE02	Canagagigue Creek	*Y. intermedia* 1
1-Aug-06	06-0105-YE02	Waste Water Effluent	*Y. frederiksenii*
28-Aug-06	06-0115-YE02	Canagagigue Creek	*Y. intermedia* 1
2-Jan-07	07-0001-YE01	Conestogo River	*Y. intermedia* 1
2-Jan-07	07-0001-YE02	Conestogo River	*Y. bercovieri*
2-Jan-07	07-0003-YE01	Grand River North	*Y. aldovae*
2-Jan-07	07-0004-YE02	Waste Water Effluent	*Y. bercovieri*

Sample Date	Isolate ID	Location	Species and subtype
2-Jan-07	07-0004-YE03	Waste Water Effluent	*Y. intermedia* 1
2-Jan-07	07-0005-YE01	Intake	*Y. mollaretii*
2-Jan-07	07-0005-YE04	Intake	*Y. bercovieri*
29-Jan-07	07-0013-YE03	Conestogo River	*Y. bercovieri*
29-Jan-07	07-0014-YE01	Canagagigue Creek	*Y. intermedia* 1
29-Jan-07	07-0014-YE03	Canagagigue Creek	*Y. intermedia* 1
29-Jan-07	07-0015-YE02	Grand River North	*Y. aldovae*
29-Jan-07	07-0015-YE03	Grand River North	*Y. intermedia* 1
29-Jan-07	07-0015-YE04	Grand River North	*Y. intermedia* 1
29-Jan-07	07-0015-YE05	Grand River North	*Y. intermedia* 1
29-Jan-07	07-0016-YE05	Waste Water Effluent	*Y. intermedia* 1
12-Feb-07	07-0020-YE03	Canagagigue Creek	*Y. mollaretii*
12-Feb-07	07-0021-YE02	Grand River North	***Y. enterocolitica* 1A, O:7, 8**
12-Feb-07	07-0021-YE03	Grand River North	*Y. intermedia* 1
12-Feb-07	07-0021-YE04	Grand River North	***Y. enterocolitica* 1A, O:5, 27**
12-Feb-07	07-0021-YE05	Grand River North	***Y. enterocolitica* 1A, O:7, 8**
12-Feb-07	07-0022-YE02	Waste Water Effluent	*Y. frederiksenii*
12-Feb-07	07-0023-YE01	Intake	*Y. intermedia* 1
12-Feb-07	07-0023-YE03	Intake	***Y. enterocolitica* 1A, O:7, 8**
12-Feb-07	07-0023-YE04	Intake	*Y. intermedia* 1
12-Feb-07	07-0023-YE05	Intake	*Y. kristensenii*
26-Feb-07	07-0025-YE06	Conestogo River	*Y. intermedia* 1
26-Feb-07	07-0025-YE07	Conestogo River	*Y. bercovieri*
26-Feb-07	07-0026-YE03	Conestogo River	*Y. intermedia* 1
26-Feb-07	07-0026-YE06	Conestogo River	*Y. intermedia* 1
26-Feb-07	07-0027-YE04	Grand River North	*Y. intermedia* 1
26-Feb-07	07-0027-YE05	Grand River North	*Y. intermedia* 1
26-Feb-07	07-0027-YE06	Grand River North	*Y. mollaretii*
26-Feb-07	07-0027-YE08	Grand River North	*Y. mollaretii*
26-Feb-07	07-0029-YE01	Intake	*Y. kristensenii*
26-Feb-07	07-0029-YE02	Intake	*Y. bercovieri*
26-Feb-07	07-0029-YE03	Intake	*Y. intermedia* 1
26-Feb-07	07-0029-YE04	Intake	*Y. intermedia* 1
26-Feb-07	07-0029-YE05	Intake	*Y. intermedia* 1
16-Jul-07	07-0031-YE03	Grand River North	*Y. bercovieri*
26-Feb-07	07-0033-YE04	Conestogo River	*Y. intermedia* 1
16-Jul-07	07-0038-YE02	Canagagigue Creek	*Y. mollaretii*
16-Jul-07	07-0038-YE04	Canagagigue Creek	***Y. enterocolitica* 1A, O:7, 13**
16-Jul-07	07-0039-YE04	Grand River North	*Y. mollaretii*
16-Jul-07	07-0039-YE05	Grand River North	***Y. enterocolitica* 1A, O:Untypable**
16-Jul-07	07-0040-YE01	Waste Water Effluent	*Y. intermedia* 1
16-Jul-07	07-0040-YE02	Waste Water Effluent	*Y. intermedia* 1
16-Jul-07	07-0041-YE02	Intake	*Y. intermedia* 1
16-Jul-07	07-0041-YE04	Intake	*Y. bercovieri*

Sample Date	Isolate ID	Location	Species and subtype
16-Jul-07	07-0054-YE01	Canagagigue Creek	***Y. enterocolitica* 1A, O:7, 8**
16-Jul-07	07-0054-YE02	Canagagigue Creek	*Y. intermedia* 1
16-Jul-07	07-0055-YE01	Grand River North	*Y. mollaretii*
16-Jul-07	07-0060-YE01	Canagagigue Creek	*Y. intermedia* 1
3-Jul-07	07-0085-YE01	Conestogo River	*Y. intermedia* 1
3-Jul-07	07-0087-YE02	Grand River North	*Y. frederiksenii*
10-Jul-07	07-0093-YE01	Grand River North	*Y. frederiksenii*
10-Jul-07	0700-93-YE02	Grand River North	*Y. frederiksenii*
10-Jul-07	07-0094-YE01	Waste Water Effluent	*Y. frederiksenii*
10-Jul-07	07-0094-YE02	Waste Water Effluent	*Y. frederiksenii*
10-Jul-07	07-0095-YE01	Intake	*Y. intermedia* 1
10-Jul-07	07-0095-YE02	Intake	***Y. enterocolitica* 1A, O:5**
16-Jul-07	07-0099-YE01	Grand River North	*Y. frederiksenii*
16-Jul-07	07-0099-YE02	Grand River North	*Y. frederiksenii*
30-Jul-07	07-0109-YE01	Grand River North	*Y. frederiksenii*
13-Aug-07	07-0114-YE01	Canagagigue Creek	***Y. enterocolitica* 1A, O:5**
13-Aug-07	07-0114-YE02	Canagagigue Creek	***Y. enterocolitica* 1A, O:41, 43**
13-Aug-07	07-0115-YE01	Grand River North	*Y. frederiksenii*
13-Aug-07	07-0116-YE01	Waste Water Effluent	*Y. frederiksenii*
13-Aug-07	07-0116-YE02	Waste Water Effluent	*Y. frederiksenii*
13-Aug-07	07-0116-YE03	Waste Water Effluent	*Y. frederiksenii*
27-Aug-07	07-0119-YE01	Conestogo River	***Y. enterocolitica* 1A, O:rough**
27-Aug-07	07-0122-YE02	Waste Water Effluent	*Y. frederiksenii*

APPENDIX B: Direct Microscopic Cell Counts

This method allows for the direct enumeration of the total number of cells in a sample (viable and non-viable). Cells in a sample are stained with SYBR-Gold nucleic acid stain (Invitrogen, Burlington, ON) and are enumerated by fluorescence microscopy. Total cell counts were obtained for cultures used to prepare a standard DNA samples to generate a standard curve for the PCR targeting the *ail* gene.

All Milli-Q® water and solutions used were filtered through a 0.22 μM filter then autoclaved. Also, all glassware was rinsed with filtered Milli-Q® water and then autoclaved. Mounting media used to prepare slides below was prepared by combining 45 mL glycerol, 2.5 mL 1 M filtered Tris (pH 8.0), 2.5 mL filtered Milli-Q® water and 1.25g of 1,4-diazabicyclo(2,2,2,)octane (DABCO) (Sigma cat. no. D25322), then warming the solution to 70°C to dissolve the DABCO.

Then the sample was fixed by adding 180 μL of filter-sterilized 37 % formalin to 3 mL of sample. The sample was stored at 4°C and analyzed (in less than 3 weeks, but typically in less than 1 week) as follows. The fixed sample was diluted using phosphate-buffered water (PBW) (0.3 mM KH_2PO_4, 2 mM $MgCl_2 \cdot H_2O$, pH 7.2) to an approximate concentration of 10^5 to 10^6 cells/mL (overnight cultures generally grew to between 10^8 and 10^9 cells/mL). 1.2 mL of diluted sample was transferred to a sterile tube to which 1 μL of 10,000x SYBR-Gold reagent was added, and then mixed by vortexing. The sample was incubated with the SYBR-Gold stain in the dark for at least 5 minutes at room temperature. A black, polycarbonate 0.2 μM filter (Nucleopore, Millipore Corporation, Bedford, MA) was placed on a small glass filter unit using sterile forceps. Two mL of PBW was added to the filter unit and let sit for 5 minutes. It was then passed through the black filter using a vacuum (the vacuum was operated at approximately 300 mmHg throughout this procedure). Once the PBW water had passed through the unit, the resulting vacuum inside the filter unit was relieved and 1 mL of the SYBR-Gold stained sample was added to the filter unit. The sample was pulled through the filter using a vacuum and the filter was rinsed with 10 mL of

Milli-Q® water three times. The black filter was aseptically removed from the filter unit and mounted on a glass slide between drops of mounting media and covered with a glass coverslip. The slides were immediately viewed through a Zeiss Axioskop 2, Routine Microscope (Empix Imaging Inc., Mississauga, ON). Cells within an ocular grid area of 9.216×10^{-3} mm^2 were enumerated manually at 1,000X magnification in at least 20 different fields of view. The concentration of cells in the original sample (C) was calculated from the mean number of cells counted in the ocular grid area (N), dilution factor (d), filtration area (A_f), ocular grid area (A_g) and the volume of sample filtered (V), according to the following equation:

$$C = N \cdot A_f / d \cdot A_g \cdot V$$

APPENDIX C: Grand River Survey Raw Data

This appendix summarizes the data collected for the Grand River Water Samples. The date column indicates the date that the river water was sampled. The culture results indicated whether *Y. enterocolitica* were isolated, and hence present, or not isolated, and hence absent from each samples. The "Water Filtered for PCR" column indicates the volume of water filtered and processed for PCR analysis. The *ail* and *yadA* columns show the results obtained for each real-time PCR assay. The sampling site acronyms are as follows: Conestogo River (CON), Canagagigue Creek (CAN), Grand River North (GRN), downstream of a wastewater treatment plant effluent (WW) and upstream of a drinking water treatment plant intake (IN). (Note: n.d. = not done)

Date	Sample ID	Site	Temp (°C)	Turbidity (NTU)	Nitrate (mg/L)	Ammonia (mg/L)	*E. coli* (CFU/ 100mL)	*Yersinia* Culture Result (500 mL)	Water Filtered for PCR (mL)	*ail* (cells/ 100 mL)	*yadA* (copies/ 100 mL)
30-Mar-05	05-0001	CON	3.0	39.6	2.1	n.d.	169	n.d.	1000	51	n.d.
30-Mar-05	05-0002	CAN	6.0	12.3	4.1	n.d.	120	n.d.	1000	70	n.d.
30-Mar-05	05-0003	GRN	4.0	30.1	2.1	n.d.	178	n.d.	1000	70	n.d.
30-Mar-05	05-0004	WW	4.0	39.5	1.4	n.d.	179	n.d.	1000	0	n.d.
30-Mar-05	05-0005	IN	3.0	51.6	2.2	n.d.	165	n.d.	1000	69	n.d.
13-Apr-05	05-0006	CON	8.0	11.1	3.7	n.d.	0	n.d.	1000	0	n.d.
13-Apr-05	05-0007	CAN	7.0	2.6	5.5	n.d.	0	n.d.	1000	96	n.d.
13-Apr-05	05-0008	GRN	7.0	3.1	2.2	n.d.	5	n.d.	1000	111	n.d.
13-Apr-05	05-0009	WW	9.0	5.1	2.1	n.d.	2	n.d.	1000	11	n.d.
13-Apr-05	05-0010	IN	8.0	6.2	2.2	n.d.	3	n.d.	1000	0	n.d.
27-Apr-05	05-0011	CON	8.0	9.5	3.6	n.d.	1092	n.d.	1000	127	n.d.
27-Apr-05	05-0012	CAN	8.0	4.6	4.7	n.d.	1288	n.d.	1000	0	n.d.

Date	Sample ID	Site	Temp (°C)	Turbidity (NTU)	Nitrate (mg/L)	Ammonia (mg/L)	*E. coli* (CFU/ 100mL)	*Yersinia* Culture Result (500 mL)	Water Filtered for PCR (mL)	*ail* (cells/ 100 mL)	*yadA* (copies/ 100 mL)
27-Apr-05	05-0013	GRN	8.0	3.8	1.8	n.d.	273	n.d.	1000	184	n.d.
27-Apr-05	05-0014	WW	9.0	5.9	2.3	n.d.	6	n.d.	1000	33	n.d.
27-Apr-05	05-0015	IN	8.0	5.1	1.8	n.d.	22	n.d.	1000	251	n.d.
17-May-05	05-0016	CON	12.0	3.7	0.9	0.00	30	n.d.	1000	0	n.d.
17-May-05	05-0017	CAN	11.0	1.7	0.0	0.00	79	n.d.	1000	0	n.d.
17-May-05	05-0018	GRN	10.0	2.1	0.2	0.00	23	n.d.	1000	0	n.d.
17-May-05	05-0019	WW	12.0	2.4	1.2	10.80	7	n.d.	1000	0	n.d.
17-May-05	05-0020	IN	11.0	2.1	0.4	0.33	17	n.d.	1000	0	n.d.
1-Jun-05	05-0021	CON	19.0	3.8	0.3	0.00	36	n.d.	1000	0	n.d.
1-Jun-05	05-0022	CAN	17.0	1.8	0.5	0.00	79	n.d.	1000	0	n.d.
1-Jun-05	05-0023	GRN	17.0	2.4	0.2	0.00	44	n.d.	1000	0	n.d.
1-Jun-05	05-0024	WW	19.0	2.5	3.5	10.10	13	n.d.	1000	7	n.d.
1-Jun-05	05-0025	IN	20.0	2.7	0.2	0.18	32	n.d.	1000	0	n.d.
15-Jun-05	05-0026	CON	21.0	10.5	0.6	0.01	23000	n.d.	1500	0	n.d.
15-Jun-05	05-0027	CAN	20.0	6.6	0.0	0.13	12400	n.d.	1500	30	n.d.
15-Jun-05	05-0028	GRN	20.0	1.9	0.0	0.00	1400	n.d.	1500	0	n.d.
15-Jun-05	05-0029	WW	21.0	11.7	1.0	10.70	45	n.d.	1500	0	n.d.
15-Jun-05	05-0030	IN	23.0	13.8	0.3	0.35	1900	n.d.	1500	0	n.d.
29-Jun-05	05-0031	CON	24.0	3.0	0.7	0.04	106	n.d.	1500	0	n.d.
29-Jun-05	05-0032	CAN	24.0	3.1	1.9	0.02	254	n.d.	1500	0	n.d.
29-Jun-05	05-0033	GRN	21.0	2.2	1.1	0.00	256	n.d.	1500	0	n.d.
29-Jun-05	05-0034	WW	24.0	4.5	4.2	5.30	77	n.d.	1500	0	n.d.
29-Jun-05	05-0035	IN	25.0	2.6	1.4	0.05	157	n.d.	1500	0	n.d.
13-Jul-05	05-0036	CON	26.0	2.1	1.1	0.06	210	n.d.	1500	0	n.d.
13-Jul-05	05-0037	CAN	27.0	1.9	1.7	0.03	2000	n.d.	1500	0	n.d.

Date	Sample ID	Site	Temp (°C)	Turbidity (NTU)	Nitrate (mg/L)	Ammonia (mg/L)	*E. coli* (CFU/ 100mL)	*Yersinia* Culture Result (500 mL)	Water Filtered for PCR (mL)	*ail* (cells/ 100 mL)	*yadA* (copies/ 100 mL)
13-Jul-05	05-0038	GRN	24.0	3.2	0.9	0.04	138	n.d.	1500	0	n.d.
13-Jul-05	05-0039	WW	26.0	4.4	2.5	14.30	2600	n.d.	1500	0	n.d.
13-Jul-05	05-0040	IN	28.0	2.3	1.8	0.08	26	n.d.	1500	0	n.d.
20-Jul-05	05-0041	CON	24.0	9.0	1.0	0.05	113	n.d.	1500	0	n.d.
20-Jul-05	05-0042	CAN	23.0	4.6	2.1	0.04	228	n.d.	1500	0	n.d.
20-Jul-05	05-0043	GRN	21.0	2.1	1.1	0.06	84	n.d.	1500	0	n.d.
20-Jul-05	05-0044	WW	24.0	11.0	1.7	11.50	1600	n.d.	1500	2	n.d.
20-Jul-05	05-0045	IN	25.0	7.6	2.0	0.07	58	n.d.	1500	0	n.d.
8-Aug-05	05-0046	WW	25.0	4.1	6.8	10.60	49	n.d.	1500	0	n.d.
8-Aug-05	05-0047	IN	25.0	3.7	1.2	0.00	32	n.d.	1500	0	n.d.
10-Aug-05	05-0049	CON	25.0	3.9	0.5	0.00	58	n.d.	1500	0	n.d.
10-Aug-05	05-0050	CAN	25.0	3.1	1.7	0.00	179	n.d.	1500	0	n.d.
10-Aug-05	05-0051	GRN	24.0	3.2	0.6	0.00	135	n.d.	1500	0	n.d.
24-Aug-05	05-0052	CON	19.0	7.1	0.3	0.02	96	n.d.	1500	0	n.d.
24-Aug-05	05-0053	CAN	18.0	5.8	1.2	0.00	1000	n.d.	1500	0	n.d.
24-Aug-05	05-0054	GRN	18.0	4.5	0.1	0.00	37	n.d.	1500	0	n.d.
24-Aug-05	05-0055	WW	21.0	8.4	1.6	5.50	2500	n.d.	1500	0	n.d.
24-Aug-05	05-0056	IN	20.0	12.0	0.8	0.07	95	n.d.	1500	0	n.d.
6-Sep-05	05-0058	CON	20.0	5.1	0.3	0.00	82	n.d.	1500	0	n.d.
6-Sep-05	05-0059	CAN	18.0	8.0	1.2	0.01	100	n.d.	1500	0	n.d.
6-Sep-05	05-0060	GRN	18.5	7.0	0.0	0.01	82	n.d.	1500	0	n.d.
6-Sep-05	05-0061	WW	22.0	4.7	3.0	17.00	18	n.d.	1500	0	n.d.
6-Sep-05	05-0062	IN	23.0	8.4	1.1	0.02	46	n.d.	1500	0	n.d.
19-Sep-05	05-0063	CON	22.0	n.d.	0.1	0.03	100	n.d.	1500	0	n.d.
19-Sep-05	05-0064	CAN	20.0	n.d.	1.4	0.00	500	n.d.	1500	0	n.d.

Date	Sample ID	Site	Temp (°C)	Turbidity (NTU)	Nitrate (mg/L)	Ammonia (mg/L)	*E. coli* (CFU/ 100mL)	*Yersinia* Culture Result (500 mL)	Water Filtered for PCR (mL)	*ail* (cells/ 100 mL)	*yadA* (copies/ 100 mL)
19-Sep-05	05-0065	GRN	19.0	n.d.	0.0	0.03	30	n.d.	1500	0	n.d.
19-Sep-05	05-0066	WW	20.0	n.d.	1.7	14.00	65	n.d.	1500	0	n.d.
19-Sep-05	05-0067	IN	20.0	n.d.	1.5	0.03	100	n.d.	1500	0	n.d.
27-Sep-05	05-0069	CON	20.0	12.2	0.7	0.02	9700	n.d.	1500	0	n.d.
27-Sep-05	05-0070	CAN	18.0	19.7	0.9	0.06	31000	n.d.	1500	0	n.d.
27-Sep-05	05-0071	GRN	16.0	6.4	0.4	0.03	7800	n.d.	1500	74	n.d.
27-Sep-05	05-0072	WW	19.5	10.1	1.6	19.40	400	n.d.	1500	0	n.d.
27-Sep-05	05-0073	IN	15.0	12.0	0.8	0.22	3300	n.d.	1500	0	n.d.
3-Oct-05	05-0077	CON	19.5	8.1	1.4	0.02	119	n.d.	1500	0	n.d.
3-Oct-05	05-0078	CAN	17.5	7.0	1.4	0.01	1000	n.d.	1500	0	n.d.
3-Oct-05	05-0079	GRN	18.5	6.8	0.6	0.02	51	n.d.	1500	0	n.d.
3-Oct-05	05-0080	WW	21.0	7.4	1.0	18.30	1100	n.d.	1500	0	n.d.
3-Oct-05	05-0081	IN	18.0	6.4	0.7	0.05	82	n.d.	1500	0	n.d.
18-Oct-05	05-0084	CON	14.0	5.4	0.7	0.00	100	n.d.	1500	0	n.d.
18-Oct-05	05-0085	CAN	13.0	15.6	1.6	0.00	2300	n.d.	1500	41	n.d.
18-Oct-05	05-0086	GRN	12.0	2.9	0.4	0.00	84	n.d.	1500	0	n.d.
18-Oct-05	05-0087	WW	16.0	3.7	3.8	12.70	47	n.d.	1500	0	n.d.
18-Oct-05	05-0088	IN	13.0	4.2	1.0	0.04	76	n.d.	n.d.	n.d.	n.d.
31-Oct-05	05-0090	CON	9.0	3.0	1.6	0.00	90	n.d.	1500	0	n.d.
31-Oct-05	05-0091	CAN	9.0	7.0	2.3	0.02	187	n.d.	1500	0	n.d.
31-Oct-05	05-0092	GRN	8.0	2.1	0.8	0.01	33	n.d.	1500	0	n.d.
31-Oct-05	05-0093	WW	15.0	2.5	1.5	13.00	45	n.d.	1500	0	n.d.
31-Oct-05	05-0094	IN	9.0	2.6	1.1	0.05	26	n.d.	1500	0	n.d.
14-Nov-05	05-0097	CON	7.0	3.0	0.9	0.02	2	n.d.	1500	0	n.d.
14-Nov-05	05-0098	CAN	7.0	2.8	2.1	0.09	129	n.d.	1500	34	n.d.

Date	Sample ID	Site	Temp (°C)	Turbidity (NTU)	Nitrate (mg/L)	Ammonia (mg/L)	*E. coli* (CFU/ 100mL)	*Yersinia* Culture Result (500 mL)	Water Filtered for PCR (mL)	*ail* (cells/ 100 mL)	*yadA* (copies/ 100 mL)
14-Nov-05	05-0099	GRN	6.0	2.0	0.7	0.01	32	n.d.	1500	0	n.d.
14-Nov-05	05-0100	WW	13.0	3.9	2.4	15.50	900	n.d.	1500	0	n.d.
14-Nov-05	05-0101	IN	7.0	3.0	1.4	0.22	83	n.d.	1500	0	n.d.
28-Nov-05	05-0103	CON	4.0	17.5	1.3	0.14	143	n.d.	1500	0	n.d.
28-Nov-05	05-0104	CAN	4.0	9.9	2.1	0.48	1100	n.d.	1500	60	n.d.
28-Nov-05	05-0105	GRN	1.5	2.7	0.3	0.04	39	n.d.	1500	0	n.d.
28-Nov-05	05-0106	WW	11.0	7.0	0.8	17.00	208	n.d.	1500	0	n.d.
28-Nov-05	05-0107	IN	1.0	14.4	0.9	0.60	162	n.d.	1500	0	n.d.
29-Nov-05	05-0109	CON	7.0	523.0	3.3	0.26	18600	n.d.	250	282	n.d.
29-Nov-05	05-0110	CAN	7.0	688.0	2.9	0.41	16500	n.d.	300	576	n.d.
29-Nov-05	05-0111	GRN	5.0	112.0	0.9	0.09	3900	n.d.	400	134	n.d.
29-Nov-05	05-0112	WW	5.0	265.0	0.8	0.80	3400	n.d.	400	0	n.d.
29-Nov-05	05-0113	IN	5.0	183.0	0.7	0.25	1100	n.d.	550	0	n.d.
12-Dec-05	05-0118	CON	0.0	6.9	2.0	0.08	31	n.d.	1500	0	n.d.
12-Dec-05	05-0119	WW	7.0	4.5	2.6	15.60	21	n.d.	1500	0	n.d.
3-Jan-06	06-0001	CON	2.0	16.5	1.7	0.18	317	n.d.	1500	0	0
3-Jan-06	06-0002	CAN	3.0	7.8	2.7	0.38	141	n.d.	1500	68	30
3-Jan-06	06-0003	GRN	2.0	2.7	1.8	0.04	44	n.d.	1500	42	0
3-Jan-06	06-0004	WW	5.0	23.4	2.1	4.60	2	n.d.	1500	2	n.d.
3-Jan-06	06-0005	IN	1.0	11.5	0.9	0.28	110	n.d.	1500	0	0
16-Jan-06	06-0007	CON	0.0	13.2	6.1	0.06	139	n.d.	1500	0	0
16-Jan-06	06-0008	CAN	0.0	14.2	5.1	0.19	290	n.d.	1500	42	31
16-Jan-06	06-0009	GRN	0.0	4.2	3.1	0.03	111	n.d.	1500	120	9
16-Jan-06	06-0010	WW	0.0	7.7	4.1	0.05	126	n.d.	1500	49	n.d.
16-Jan-06	06-0011	IN	0.0	7.1	2.7	0.13	74	n.d.	1500	58	21

Date	Sample ID	Site	Temp (°C)	Turbidity (NTU)	Nitrate (mg/L)	Ammonia (mg/L)	*E. coli* (CFU/ 100mL)	*Yersinia* Culture Result (500 mL)	Water Filtered for PCR (mL)	*ail* (cells/ 100 mL)	*yadA* (copies/ 100 mL)
30-Jan-06	06-0013	CON	3.0	81.4	4.7	0.32	5400	n.d.	450	0	0
30-Jan-06	06-0014	CAN	3.0	38.2	6.8	0.36	3800	n.d.	800	0	20
30-Jan-06	06-0015	GRN	3.0	42.5	3.2	0.16	2100	n.d.	800	0	51
30-Jan-06	06-0016	WW	3.0	66.1	4.4	0.26	4200	n.d.	600	95	0
30-Jan-06	06-0017	IN	3.0	77.6	3.8	0.44	5200	n.d.	400	0	75
13-Feb-06	06-0019	CON	1.0	19.1	5.2	0.34	25	n.d.	1500	186	3276
13-Feb-06	06-0020	CAN	1.0	12.6	3.8	0.43	49	n.d.	1500	27	0
13-Feb-06	06-0021	GRN	1.0	1.9	3.0	0.33	12	n.d.	1500	42	0
13-Feb-06	06-0022	WW	3.0	13.2	3.7	5.20	149	n.d.	1500	48	0
13-Feb-06	06-0023	IN	1.0	8.2	4.0	0.58	34	n.d.	1500	37	9
27-Feb-06	06-0025	CON	1.0	7.0	3.7	0.31	11	n.d.	1500	117	25
27-Feb-06	06-0026	CAN	1.0	1.9	6.0	0.41	71	n.d.	1500	7	0
27-Feb-06	06-0027	GRN	1.0	3.0	3.2	0.30	8	n.d.	1500	96	0
27-Feb-06	06-0028	WW	5.0	3.4	4.1	12.60	2	n.d.	1500	0	0
27-Feb-06	06-0029	IN	2.0	8.9	3.9	0.75	3	n.d.	1500	0	0
14-Mar-06	06-0031	CON	2.0	56.4	2.8	0.45	800	n.d.	500	85	0
14-Mar-06	06-0032	CAN	2.0	49.4	4.6	0.62	1700	n.d.	500	0	74
14-Mar-06	06-0033	GRN	2.0	42.8	1.6	0.38	400	n.d.	500	113	0
14-Mar-06	06-0034	WW	2.0	63.4	2.4	0.40	300	n.d.	500	109	0
14-Mar-06	06-0035	IN	2.0	62.6	3.1	0.45	700	n.d.	400	0	0
27-Mar-06	06-0037	CON	8.0	7.7	3.3	0.32	9	n.d.	1000	0	0
27-Mar-06	06-0038	CAN	6.0	2.4	5.1	0.37	74	n.d.	1500	14	0
27-Mar-06	06-0039	GRN	6.0	2.7	2.2	0.32	6	n.d.	1500	25	0
27-Mar-06	06-0040	WW	11.0	3.6	5.4	14.30	0	n.d.	1000	50	0
27-Mar-06	06-0041	IN	8.0	3.3	3.9	0.70	3	n.d.	1500	0	0

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10-Apr-06	06-0043	CON	7.0	12.5	2.9	0.07	9	absent	1000	0	0
10-Apr-06	06-0044	CAN	7.0	3.6	4.8	0.08	214	absent	1500	0	0
10-Apr-06	06-0045	GRN	5.0	n.d.	n.d.	n.d.	12	absent	1500	18	0
10-Apr-06	06-0046	WW	6.0	7.1	2.7	1.29	9	absent	1500	12	0
10-Apr-06	06-0047	IN	6.0	9.9	2.9	0.07	20	present	1500	0	0
24-Apr-06	06-0049	CON	8.0	37.7	4.1	0.15	4600	absent	1000	52	88
24-Apr-06	06-0050	CAN	8.0	12.9	4.2	0.05	2600	absent	1500	21	7
24-Apr-06	06-0051	GRN	8.0	23.9	3.6	0.09	2000	absent	1500	130	53
24-Apr-06	06-0052	WW	9.0	38.6	3.2	0.19	7100	absent	1000	86	91
24-Apr-06	06-0053	IN	9.0	16.6	2.7	0.21	7900	absent	1000	204	277
8-May-06	06-0057	CON	17.0	8.4	1.9	0.02	0	absent	1500	0	0
8-May-06	06-0058	CAN	13.0	2.0	3.8	0.06	36	absent	1500	0	0
8-May-06	06-0059	GRN	13.0	2.1	1.3	0.03	4	absent	1500	20	0
8-May-06	06-0060	WW	12.0	3.1	7.1	7.60	228	absent	1500	0	0
8-May-06	06-0061	IN	14.0	4.3	2.3	0.08	1	absent	1500	0	0
23-May-06	06-0064	CON	12.0	4.6	2.6	0.02	16	absent	1500	0	0
23-May-06	06-0065	CAN	10.0	3.9	5.7	0.02	5	absent	1500	42	0
23-May-06	06-0066	GRN	12.0	2.3	1.3	0.01	7	absent	1500	0	0
23-May-06	06-0067	WW	13.0	4.6	5.1	0.50	63	absent	1500	0	0
23-May-06	06-0068	IN	11.0	4.4	6.4	0.08	2	absent	1500	0	0
5-Jun-06	06-0070	CON	20.0	18.3	2.8	0.09	500	absent	1500	0	0
5-Jun-06	06-0071	CAN	16.0	4.0	4.8	0.02	240	absent	1500	0	0
5-Jun-06	06-0072	GRN	16.0	3.5	1.3	0.03	52	present	1500	1	0
5-Jun-06	06-0073	WW	18.0	7.9	4.7	4.60	340	absent	1000	0	57
5-Jun-06	06-0074	IN	18.0	25.2	1.9	0.09	650	absent	1500	0	0

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19-Jun-06	06-0076	CON	25.0	11.6	1.5	0.01	60	present	1500	0	0
19-Jun-06	06-0077	CAN	23.0	3.8	3.1	0.00	120	present	1500	0	0
19-Jun-06	06-0078	GRN	20.0	4.6	1.0	0.00	92	present	1500	0	0
19-Jun-06	06-0079	WW	20.0	8.2	4.7	4.80	480	absent	1500	0	0
19-Jun-06	06-0080	IN	23.0	20.2	1.4	0.04	130	absent	1500	0	0
4-Jul-06	06-0082	CON	25.0	14.8	1.8	0.05	140	absent	1500	0	0
4-Jul-06	06-0083	CAN	23.0	3.4	1.9	0.13	8400	present	1000	0	0
4-Jul-06	06-0084	GRN	23.0	2.1	1.2	0.03	280	absent	1500	0	0
4-Jul-06	06-0085	WW	23.0	6.3	6.2	7.40	1800	present	1000	374	0
4-Jul-06	06-0086	IN	24.0	5.1	1.1	0.05	520	present	1500	0	0
12-Jul-06	06-0088	CON	22.0	59.2	1.4	0.15	1800	present	1000	17	0
12-Jul-06	06-0089	CAN	20.0	13.7	1.8	0.13	5800	absent	1000	33	0
12-Jul-06	06-0090	GRN	20.0	4.9	1.3	0.00	210	present	1500	0	0
12-Jul-06	06-0091	WW	22.0	123.0	1.4	3.10	2700	absent	1000	0	0
12-Jul-06	06-0092	IN	22.0	24.8	1.3	0.10	200	absent	1000	0	0
17-Jul-06	06-0096	CON	27.0	n.d.	1.1	0.00	56	absent	1500	0	34
17-Jul-06	06-0097	CAN	25.0	2.7	2.1	0.00	130	present	1500	0	0
17-Jul-06	06-0098	GRN	25.0	5.0	0.9	0.01	48	absent	1500	0	0
17-Jul-06	06-0099	WW	23.0	5.4	6.1	8.60	170	absent	1500	0	0
17-Jul-06	06-0100	IN	27.0	7.3	1.1	0.06	20	absent	1500	0	0
01-Aug-06	06-0102	CON	27.0	6.6	0.8	0.01	76	present	1500	0	0
01-Aug-06	06-0103	CAN	27.0	2.4	2.3	0.01	110	present	1500	0	0
01-Aug-06	06-0104	GRN	27.0	n.d.	1.8	0.03	48	absent	1500	0	0
01-Aug-06	06-0105	WW	24.0	2.6	4.2	8.10	310	present	1500	0	0
01-Aug-06	06-0106	IN	27.0	11.1	1.4	0.04	36	absent	1500	0	0

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14-Aug-06	06-0108	CON	20.0	15.5	1.3	0.03	151	absent	1500	0	0
14-Aug-06	06-0109	CAN	19.0	5.0	2.4	0.06	104	absent	1500	0	25
14-Aug-06	06-0110	GRN	19.0	4.8	0.7	0.00	76	absent	1500	0	0
14-Aug-06	06-0111	WW	20.0	7.2	6.3	6.20	22	absent	1500	0	0
14-Aug-06	06-0112	IN	21.0	15.7	1.3	0.65	24	absent	1500	0	0
28-Aug-06	06-0114	CON	22.0	19.1	1.5	0.00	130	absent	1500	0	0
28-Aug-06	06-0115	CAN	21.0	6.4	2.3	0.02	130	present	1500	0	14
28-Aug-06	06-0116	GRN	20.0	5.7	0.8	0.05	60	absent	1500	0	0
28-Aug-06	06-0117	WW	20.0	5.2	4.4	5.60	1600	absent	1500	0	0
28-Aug-06	06-0118	IN	21.0	13.5	1.8	0.03	42	absent	1500	0	0
11-Sep-06	06-0120	CON	17.0	6.7	1.2	0.04	130	absent	1500	0	0
11-Sep-06	06-0121	CAN	15.0	8.0	2.6	0.02	230	absent	1500	0	0
11-Sep-06	06-0122	GRN	15.0	5.7	0.6	0.03	40	absent	1500	0	0
11-Sep-06	06-0123	WW	18.0	3.7	4.3	0.10	570	absent	1500	0	0
11-Sep-06	06-0124	IN	16.0	13.5	1.6	0.10	30	absent	1500	0	0
25-Sep-06	06-0126	CON	15.0	6.5	1.2	0.01	160	absent	1500	0	0
25-Sep-06	06-0127	CAN	14.0	8.4	2.1	0.02	400	absent	1500	38	0
25-Sep-06	06-0128	GRN	14.0	3.9	0.6	0.03	60	absent	1500	0	0
25-Sep-06	06-0129	WW	17.0	2.9	2.0	13.20	1100	absent	1500	0	0
25-Sep-06	06-0130	IN	15.0	10.0	1.7	0.16	130	absent	1500	0	0
4-Oct-06	06-0134	CON	16.0	20.5	1.3	0.02	1500	absent	1500	46	0
4-Oct-06	06-0135	CAN	16.0	68.4	2.4	0.13	> 3000	absent	1000	119	0
4-Oct-06	06-0136	GRN	16.0	7.2	0.5	0.05	160	absent	1500	37	20
4-Oct-06	06-0137	WW	18.0	20.3	2.0	13.80	2000	absent	1000	0	0
4-Oct-06	06-0138	IN	16.0	19.1	1.4	0.18	300	absent	1500	52	0

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10-Oct-06	06-0140	CON	14.0	4.0	0.9	0.04	120	absent	1500	0	0
10-Oct-06	06-0141	CAN	13.0	7.8	2.7	0.05	410	absent	1500	0	0
10-Oct-06	06-0142	GRN	13.0	2.4	0.8	0.01	4	absent	1500	0	0
10-Oct-06	06-0143	WW	16.0	2.7	4.8	8.80	1300	absent	1500	0	0
10-Oct-06	06-0144	IN	14.0	7.2	1.3	0.07	64	absent	1500	0	0
23-Oct-06	06-0146	CON	8.0	39.1	2.9	0.22	2900	absent	1000	0	34
23-Oct-06	06-0147	CAN	8.0	16.9	3.5	0.20	1500	absent	1500	31	0
23-Oct-06	06-0148	GRN	8.0	5.4	2.9	0.03	230	absent	1500	0	0
23-Oct-06	06-0149	WW	9.0	19.0	2.6	2.00	880	absent	1500	0	18
23-Oct-06	06-0150	IN	9.0	11.6	2.3	0.17	380	absent	1500	0	0
06-Nov-06	06-0152	CON	5.0	12.0	4.0	0.08	160	absent	1500	0	0
06-Nov-06	06-0153	CAN	5.0	4.6	4.8	0.21	68	absent	1500	0	0
06-Nov-06	06-0154	GRN	5.0	2.1	2.8	0.02	< 4	absent	1500	0	0
06-Nov-06	06-0155	WW	5.0	9.1	3.2	2.70	240	absent	1500	0	0
06-Nov-06	06-0156	IN	5.0	9.6	3.0	0.14	56	absent	1500	0	0
20-Nov-06	06-0158	CON	5.0	21.5	2.7	0.07	250	absent	1500	0	0
20-Nov-06	06-0159	CAN	5.0	4.2	4.1	0.20	560	absent	1500	1	0
20-Nov-06	06-0160	GRN	5.0	4.7	1.8	0.01	130	absent	1500	2	14
20-Nov-06	06-0161	WW	6.0	14.6	2.1	2.00	360	absent	1500	18	26
20-Nov-06	06-0162	IN	5.0	9.5	2.3	0.12	210	absent	1500	14	0
04-Dec-06	06-0164	CON	3.0	56.6	3.1	0.16	2400	absent	1000	0	0
04-Dec-06	06-0165	CAN	2.0	33.8	5.0	0.16	2300	absent	1000	3	0
04-Dec-06	06-0166	GRN	3.0	17.0	1.6	0.06	560	absent	1000	40	51
04-Dec-06	06-0167	WW	3.0	48.2	2.4	1.30	1100	absent	1000	23	42
04-Dec-06	06-0168	IN	2.0	45.3	2.7	0.12	840	absent	1000	53	74

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18-Dec-06	06-0170	CON	4.0	20.4	4.3	0.08	140	absent	1500	7	0
18-Dec-06	06-0171	CAN	4.0	9.0	4.7	0.10	70	absent	1500	25	0
18-Dec-06	06-0172	GRN	4.0	3.8	2.0	0.04	88	absent	1500	32	15
18-Dec-06	06-0173	WW	5.0	15.1	3.0	0.86	110	absent	1500	44	12
18-Dec-06	06-0174	IN	5.0	13.8	3.4	0.16	90	absent	1500	13	22
2-Jan-07	07-0001	CON	3.8	20.6	2.3	0.09	1700	present	1500	16	45
2-Jan-07	07-0002	CAN	2.7	7.2	2.5	0.14	220	absent	1500	0	0
2-Jan-07	07-0003	GRN	2.7	4.8	1.6	0.05	76	present	1500	37	0
2-Jan-07	07-0004	WW	5.0	16.6	2.6	2.60	1400	present	1500	0	11
2-Jan-07	07-0005	IN	3.5	13.7	1.9	0.16	1000	present	1500	4	41
16-Jan-07	07-0007	CON	1.0	12.9	2.0	0.32	30	absent	1500	0	0
16-Jan-07	07-0008	CAN	0.5	4.9	2.0	0.36	48	absent	1500	6	0
16-Jan-07	07-0009	GRN	0.7	2.2	1.2	0.26	24	absent	1500	0	0
16-Jan-07	07-0010	WW	2.1	8.0	2.5	7.60	230	absent	1500	4	0
16-Jan-07	07-0011	IN	0.8	7.4	2.0	0.51	12	absent	1500	7	0
29-Jan-07	07-0013	CON	0.7	4.9	1.9	0.05	<4	present	1500	10	0
29-Jan-07	07-0014	CAN	0.5	3.9	1.4	0.16	160	present	1500	4	24
29-Jan-07	07-0015	GRN	0.4	1.7	1.1	0.03	48	present	1500	5	0
29-Jan-07	07-0016	WW	8.4	3.3	1.4	23.30	210	present	1500	0	0
29-Jan-07	07-0017	IN	1.1	2.1	1.0	0.52	12	absent	1500	9	0
12-Feb-07	07-0019	CON	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
12-Feb-07	07-0020	CAN	0.9	5.6	1.0	0.28	200	present	1100	8	0
12-Feb-07	07-0021	GRN	1.3	1.7	0.1	0.09	16	present	1500	5	24
12-Feb-07	07-0022	WW	7.8	4.6	0.9	23.20	1400	present	1100	0	0
12-Feb-07	07-0023	IN	1.0	2.0	1.9	1.20	81	present	1500	2	0

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26-Feb-07	07-0025	CON	n.d.	3.7	0.4	0.03	60	present	1500	0	0
26-Feb-07	07-0026	CAN	1.0	2.6	0.6	0.19	420	present	1500	6	0
26-Feb-07	07-0027	GRN	1.0	4.4	0.7	0.05	20	present	1500	7	0
26-Feb-07	07-0028	WW	1.0	4.2	1.0	17.50	200	absent	1500	0	0
26-Feb-07	07-0029	IN	1.0	1.9	0.3	0.95	8	present	1500	6	0
12-Mar-07	07-0031	CON	1.3	4.4	0.9	0.41	390	present	1500	46	52
12-Mar-07	07-0032	CAN	0.7	12.0	0.6	0.79	320	absent	1500	98	0
12-Mar-07	07-0033	GRN	1.7	2.2	0.7	0.08	170	present	1500	44	0
12-Mar-07	07-0034	WW	8.0	4.9	0.9	11.90	300	absent	1050	0	0
12-Mar-07	07-0035	IN	1.6	9.2	0.9	0.81	84	absent	1500	4	0
26-Mar-07	07-0037	CON	5.1	28.1	0.7	0.19	150	absent	1500	63	0
26-Mar-07	07-0038	CAN	5.0	11.3	0.3	0.25	400	present	1500	182	20
26-Mar-07	07-0039	GRN	4.3	13.7	1.2	0.06	68	present	1500	2000	16
26-Mar-07	07-0040	WW	3.9	13.5	0.9	0.10	160	present	1500	730	0
26-Mar-07	07-0041	IN	4.7	15.6	1.0	0.18	60	present	1500	775	35
9-Apr-07	07-0047	CON	2.5	10.2	0.9	0.07	24	absent	1500	296	n.d.
9-Apr-07	07-0048	CAN	2.5	4.4	1.1	0.08	40	absent	1500	0	n.d.
9-Apr-07	07-0049	GRN	2.5	5.6	0.5	0.01	100	absent	1500	158	n.d.
9-Apr-07	07-0050	WW	2.5	12.4	0.5	0.40	320	absent	1500	n.d.	n.d.
9-Apr-07	07-0051	IN	2.5	7.3	1.1	0.24	20	absent	1500	116	n.d.
23-Apr-07	07-0053	CON	14.7	4.5	0.9	0.01	4	absent	1500	38	n.d.
23-Apr-07	07-0054	CAN	12.0	1.0	1.2	0.01	36	present	1500	64	n.d.
23-Apr-07	07-0055	GRN	10.8	2.8	0.9	0.01	4	present	1500	98	n.d.
23-Apr-07	07-0056	WW	14.6	4.6	1.2	13.60	3000	absent	1500	23	n.d.
23-Apr-07	07-0057	IN	14.8	4.3	0.8	0.42	40	absent	1500	108	n.d.

Date	Sample ID	Site	Temp (°C)	Turbidity (NTU)	Nitrate (mg/L)	Ammonia (mg/L)	*E. coli* (CFU/ 100mL)	*Yersinia* Culture Result (500 mL)	Water Filtered for PCR (mL)	*ail* (cells/ 100 mL)	*yadA* (copies/ 100 mL)
7-May-07	07-0059	CON	15.5	3.6	0.7	0.01	24	absent	1500	0	0
7-May-07	07-0060	CAN	11.1	1.0	1.1	0.01	20	present	1500	56	45
7-May-07	07-0061	GRN	11.3	2.3	0.4	0.01	32	absent	1500	106	0
7-May-07	07-0062	WW	13.2	6.7	1.0	9.70	3000	absent	1500	4	0
7-May-07	07-0063	IN	13.1	7.9	0.8	0.12	24	absent	1500	38	0
22-May-07	07-0066	CON	17.1	6.9	0.2	0.02	84	absent	1500	0	0
22-May-07	07-0067	CAN	14.3	1.8	0.4	0.01	84	absent	1500	59	76
22-May-07	07-0068	GRN	14.4	2.4	0.8	0.01	8	absent	1500	36	0
22-May-07	07-0069	WW	16.2	5.9	1.3	7.30	7600	absent	1500	0	86
22-May-07	07-0070	IN	16.1	2.0	0.4	0.08	48	absent	1500	33	0
4-Jun-07	07-0072	CON	21.7	5.1	0.3	0.05	150	absent	1500	0	0
4-Jun-07	07-0073	CAN	19.2	4.3	0.3	0.08	480	absent	1500	68	0
4-Jun-07	07-0074	GRN	18.3	1.6	0.5	0.02	240	absent	1500	39	0
4-Jun-07	07-0075	WW	18.3	3.6	0.4	12.80	6400	absent	1500	2	0
4-Jun-07	07-0076	IN	21.1	4.1	0.3	0.18	150	absent	1500	36	0
18-Jun-07	07-0079	CON	25.1	6.1	0.2	0.07	20	absent	1500	37	0
18-Jun-07	07-0080	CAN	24.3	1.5	0.8	0.07	92	absent	1500	0	0
18-Jun-07	07-0081	GRN	21.4	1.9	0.3	0.02	120	absent	1500	0	0
18-Jun-07	07-0082	WW	20.2	3.7	1.3	5.20	5200	absent	1500	0	57
18-Jun-07	07-0083	IN	24.1	10.6	0.3	0.07	8	absent	1500	n.d.	n.d.
3-Jul-07	07-0085	CON	22.1	6.0	1.2	0.01	96	present	1500	n.d.	n.d.
3-Jul-07	07-0086	CAN	19.2	2.1	1.5	0.02	72	absent	1500	n.d.	n.d.
3-Jul-07	07-0087	GRN	18.3	2.1	1.1	0.01	140	present	1500	n.d.	n.d.
3-Jul-07	07-0088	WW	18.1	3.2	2.6	8.70	3800	absent	1500	n.d.	n.d.
3-Jul-07	07-0089	IN	20.5	9.7	0.4	0.01	44	absent	1500	n.d.	n.d.

Date	Sample ID	Site	Temp (°C)	Turbidity (NTU)	Nitrate (mg/L)	Ammonia (mg/L)	*E. coli* (CFU/ 100mL)	*Yersinia* Culture Result (500 mL)	Water Filtered for PCR (mL)	*ail* (cells/ 100 mL)	*yadA* (copies/ 100 mL)
10-Jul-07	07-0091	CON	27.8	4.8	0.2	0.13	130	absent	1500	0	0
10-Jul-07	07-0092	CAN	24.7	4.4	0.3	0.28	680	absent	1500	n.d.	n.d.
10-Jul-07	07-0093	GRN	25.4	2.0	0.5	0.27	60	present	1500	n.d.	n.d.
10-Jul-07	07-0094	WW	22.1	5.1	1.8	9.30	92	present	1500	0	n.d.
10-Jul-07	07-0095	IN	24.7	4.3	1.1	0.41	13000	present	1000	0	0
16-Jul-07	07-0097	CON	22.5	6.5	0.8	0.03	120	absent	1500	0	0
16-Jul-07	07-0098	CAN	20.0	3.8	0.3	0.03	120	absent	1500	44	0
16-Jul-07	07-0099	GRN	18.6	2.7	0.2	0.02	52	present	1500	0	0
16-Jul-07	07-0100	WW	19.7	3.3	1.9	1.04	520	absent	1500	0	0
16-Jul-07	07-0101	IN	20.2	10.9	0.7	0.01	80	absent	1500	n.d.	n.d.
30-Jul-07	07-0107	CON	22.4	12.7	0.8	0.01	150	absent	1500	n.d.	n.d.
30-Jul-07	07-0108	CAN	20.0	1.8	0.2	0.01	88	absent	1500	n.d.	n.d.
30-Jul-07	07-0109	GRN	20.6	2.9	0.2	0.01	56	present	1500	n.d.	n.d.
30-Jul-07	07-0110	WW	23.0	3.3	0.5	1.20	280	absent	1500	0	0
30-Jul-07	07-0111	IN	23.1	3.4	0.2	0.02	16	absent	1500	n.d.	n.d.
13-Aug-07	07-0113	CON	21.3	12.1	0.2	0.03	210	absent	1500	n.d.	n.d.
13-Aug-07	07-0114	CAN	19.1	7.3	0.2	0.01	180	present	1500	0	0
13-Aug-07	07-0115	GRN	19.4	3.4	0.1	0.08	56	present	1500	0	0
13-Aug-07	07-0116	WW	29.8	7.5	1.3	4.00	830	present	1500	0	0
13-Aug-07	07-0117	IN	24.3	8.5	0.2	0.05	72	absent	1500	n.d.	n.d.
27-Aug-07	07-0119	CON	21.4	6.7	0.5	0.26	140	absent	1500	0	0
27-Aug-07	07-0120	CAN	19.0	2.8	0.9	0.25	96	absent	1500	0	0
27-Aug-07	07-0121	GRN	21.0	4.5	0.1	0.26	52	absent	1500	0	0
27-Aug-07	07-0122	WW	20.9	5.9	2.1	10.00	4100	absent	1500	0	0
27-Aug-07	07-0123	IN	22.2	9.1	0.8	0.28	76	absent	1500	0	0

APPENDIX D: Summary of Pathogen Surveys in the Grand River

The two tables in this appendix summarize Q-PCR-based survey results as well as culture-based survey results for enteric bacterial pathogens surveyed as part of a larger, comprehensive study of the Grand River watershed. Results for *Campylobacter*, *Salmonella*, *E. coli* O157:H7 were provided by M. Van Dyke (University of Waterloo, Waterloo, ON) (personal communication). These results summarize data collected between March 2005 and August 2007, with two exceptions. (1) *Y. enterocolitica yadA* PCR data was collected between January 2006 and August 2007. (2) *Y. enterocolitica* culture data was collected between April 2006 and August 2007.

Organism	**Q-PCR Results**			**Culture Results**
	Median (cells/100 mL)	**Maximum (cells/100 mL)**	**Frequency**	**Frequency**
Campylobacter	7	130	68%	10%
Salmonella	3	200	25%	12%
E. coli O157:H7	4	112	27%	1%
Y. enterocolitica (*ail*)	41	2000	38%	4%
Y. enterocolitica (*yad*)	32	3276	21%	4%

Organism	Frequency of detection using Q-PCR at each sampling location				
	Grand River (north [a])	**Canagagigue Creek [b]**	**Conestogo River [b]**	**Grand River (Wastewater [c])**	**Grand River (Intake [d])**
Campylobacter	78%	78%	56%	70%	59%
Salmonella	16%	17%	23%	44%	22%
E. coli O157:H7	8%	25%	9%	70%	20%
Y. enterocolitica (*ail*)	47%	50%	27%	32%	34%
Y. enterocolitica (*yad*)	21%	26%	18%	22%	20%

[a] Sampled from the Grand River at a location upstream of the other four sites.

[b] Canagagigue Creek and Conestogo River are tributaries of the Grand River.

[c] Sampled from the Grand River downstream of a wastewater treatment plant discharge

[d] Sampled from the Grand River upstream of a drinking water treatment plant intake

APPENDIX E: Summary of Water Quality Parameter Data for the Grand River

The water quality parameters, turbidity, nitrate, ammonia, indicator *E. coli* and temperature were measured for all samples collected from the Grand River watershed. This table summarizes the mean and average values as well as the range for each parameter at all sampling locations.

	Parameter mean / average (range)				
Sampling Location	**Turbidity (NTU)**	**Nitrate (mg/L)**	**Ammonia (mg/L)**	**Indicator *E. coli* (CFU/100 mL)**	**Temperature (°C)**
Grand River (North [a])	3.1 / 7.5 (1.6-112)	0.9 / 1.1 (0.0-3.6)	0.03/ 0.06 (0.00-0.38)	60 / 355 (4-7,800)	12.0 /11.9 (0.0-27.0)
Canagagigue Creek [b]	4.6 / 18.8 (1.0-688)	2.1 / 2.4 (0.0-6.8)	0.07 / 0.14 (0.00-0.79)	193 / 1617 (0-31,000)	13.0 /12.3 (0.0-27.0)
Conestogo River [b]	9.0 / 22.2 (2.1-523)	1.3 / 1.7 (0.1-6.1)	0.05 / 0.09 (0.00-0.45)	125 / 1235 (0-23,000)	14.7 / 13.4 (0.0-27.8)
Grand River (Wastewater [c])	5.9 / 15.9 (2.4-265)	2.4 / 2.7 (0.0-7.0)	7.6 / 8.18 (0.05-23.30)	305 / 1131 (0-7,600)	14.8 /13.7 (0.0-29.8)
Grand River (Intake [d])	8.7 / 14.5 (1.9-183)	1.3 / 1.6 (0.2-6.4)	0.14 / 0.23 (0.00-1.20)	72 / 614 (1-13,000)	14.0 / 13.1 (0.0-28.0)

[a] Sampled from the Grand River at a location upstream of the other four sites

[b] Canagagigue Creek and Conestogo River are tributaries of the Grand River

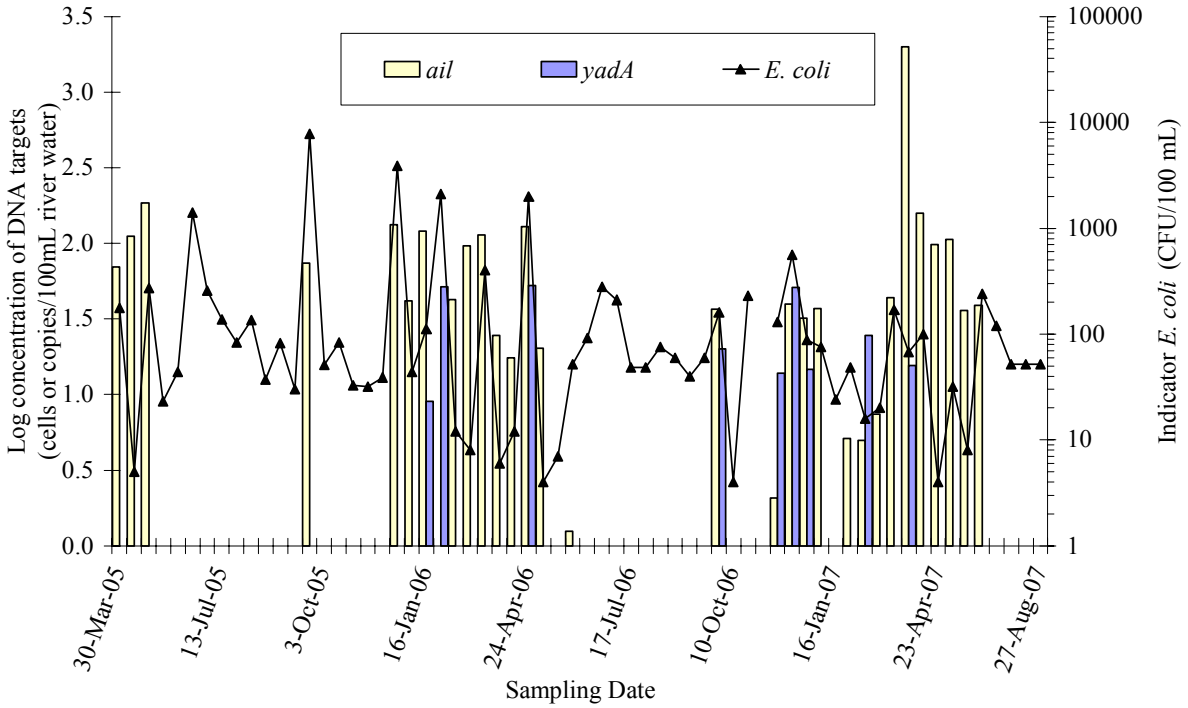
[c] Sampled from the Grand River downstream of a wastewater treatment plant discharge

[d] Sampled from the Grand River upstream of a drinking water treatment plant intake

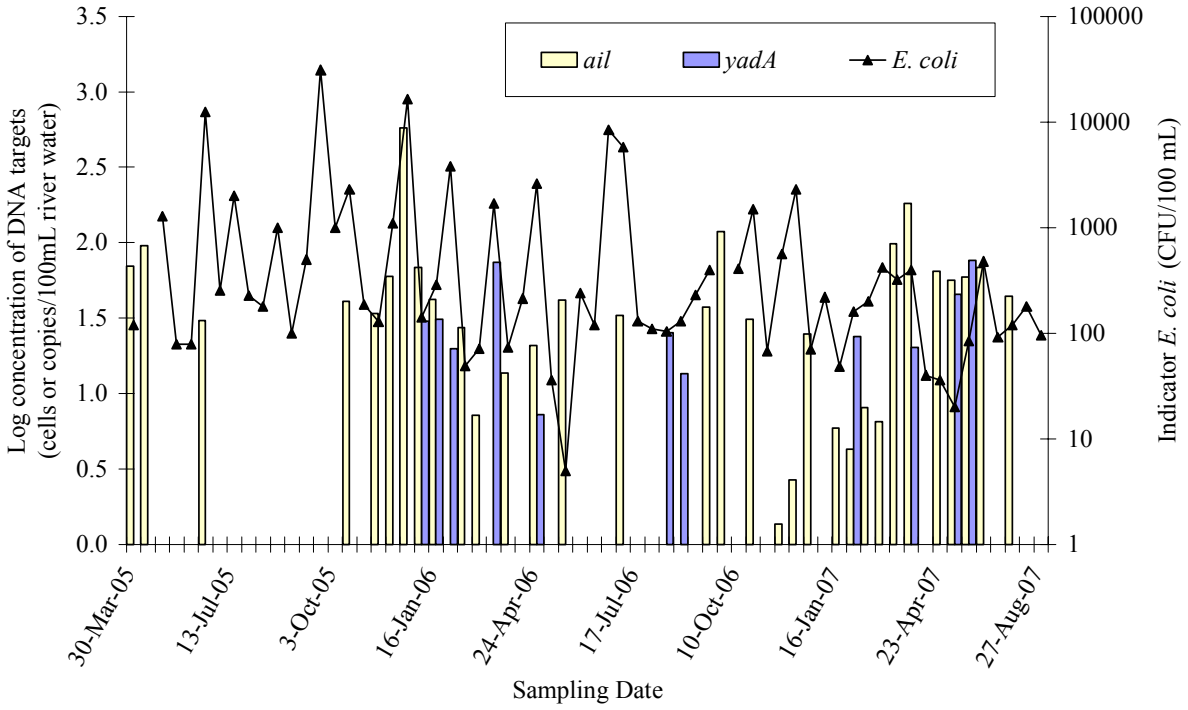
APPENDIX F: Relationships between *ail* and *yadA* Occurrence and Water Quality Parameters

The graphs in this appendix display the relationship between the presence of the *Y. enterocolitica ail* and *yadA* gene targets detected by PCR analysis and the various water quality parameters measured, including: indicator *E. coli*, turbidity, nitrate and ammonia concentrations. Data for each sampling location was plotted on separate graphs. The Grand River (north) sampling site is located upstream of the other four sampling sites. The Canagagigue Creek and Conestogo River are major tributaries of the Grand River. The Grand River (downstream of wastewater) sampling site is downstream of a wastewater treatment plant effluent. The Grand River (upstream of intake) sampling site is upstream of a drinking water treatment plant intake.

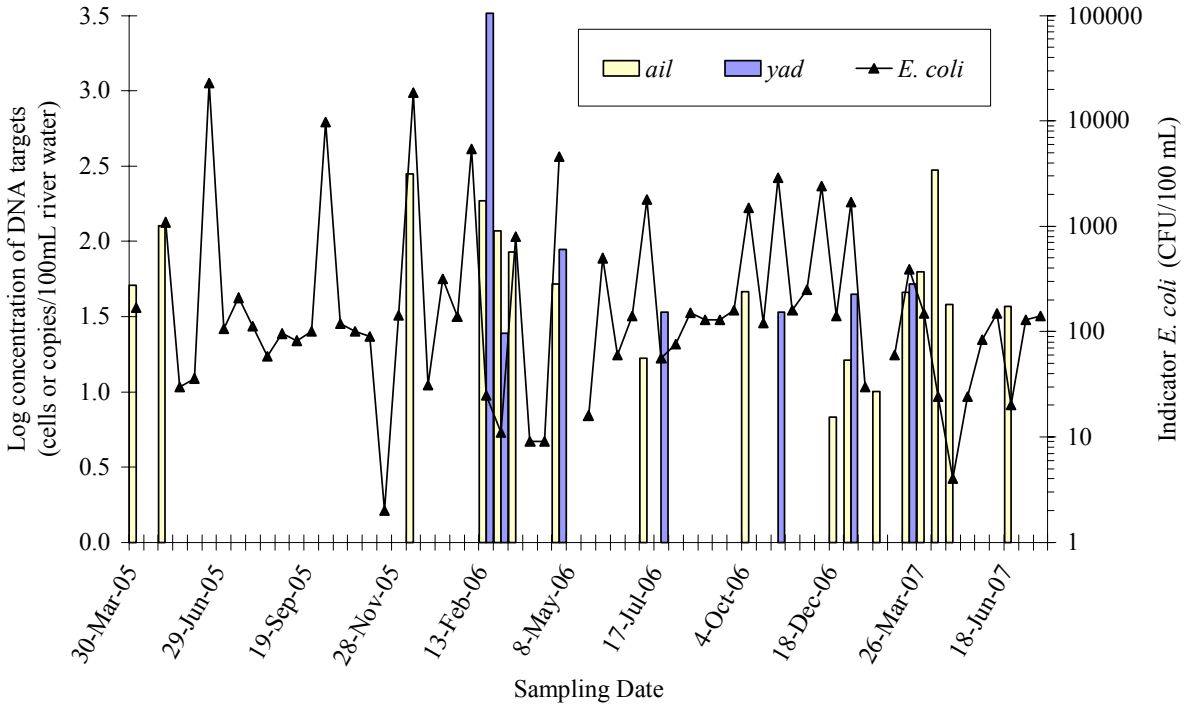
Grand River (north)



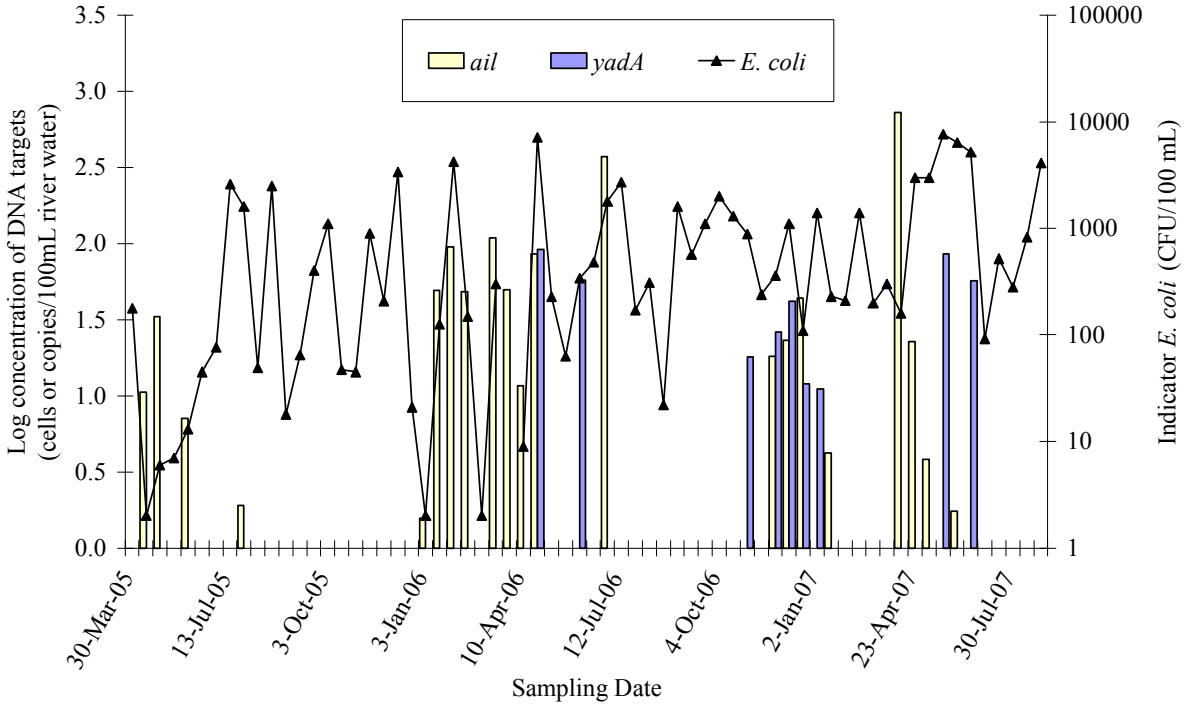
Canagagigue Creek



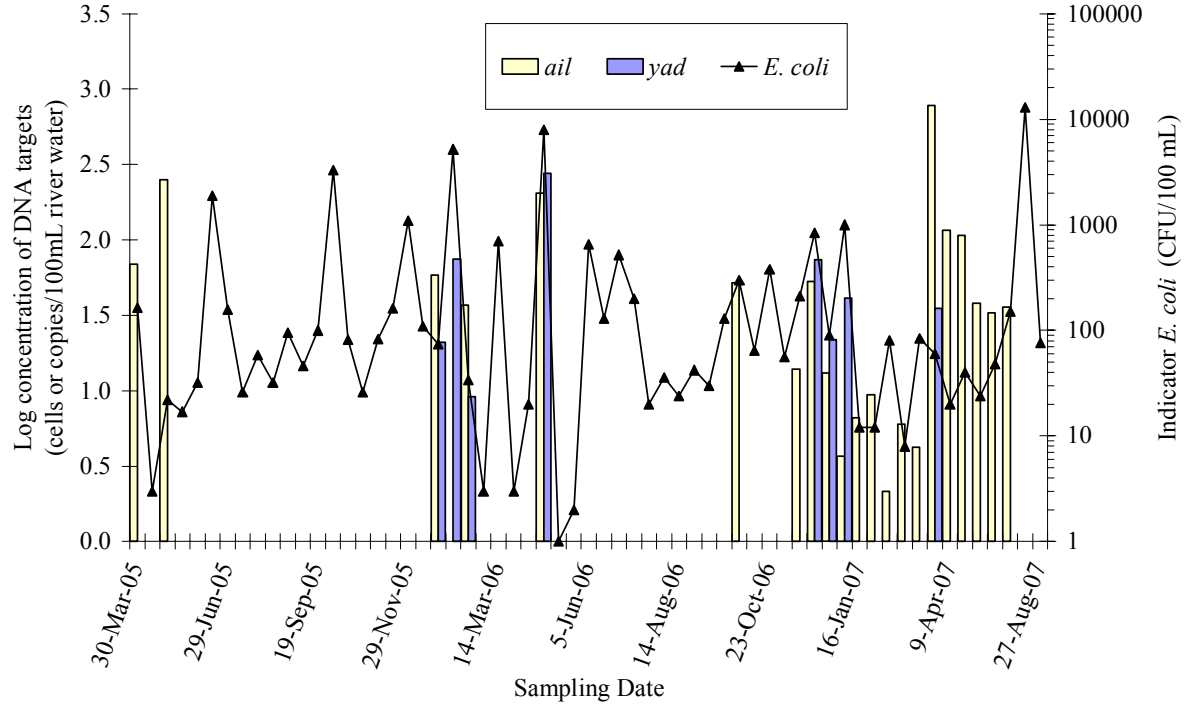
Conestogo River



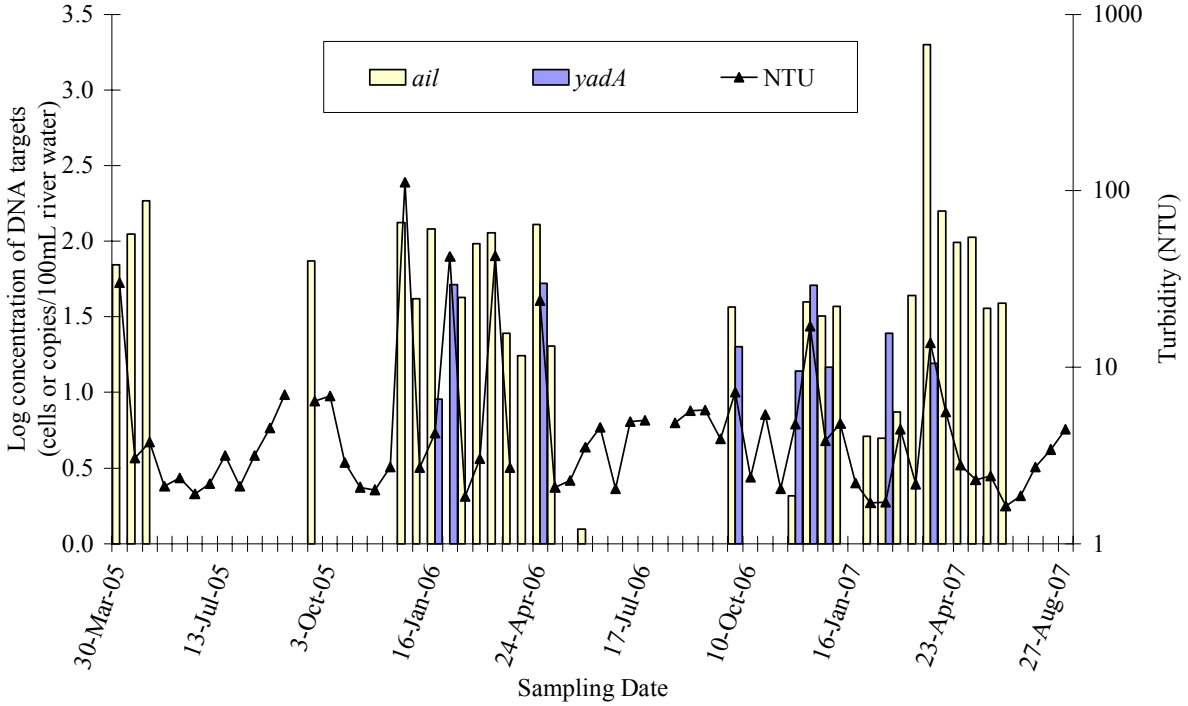
Grand River (downstream of wastewater)



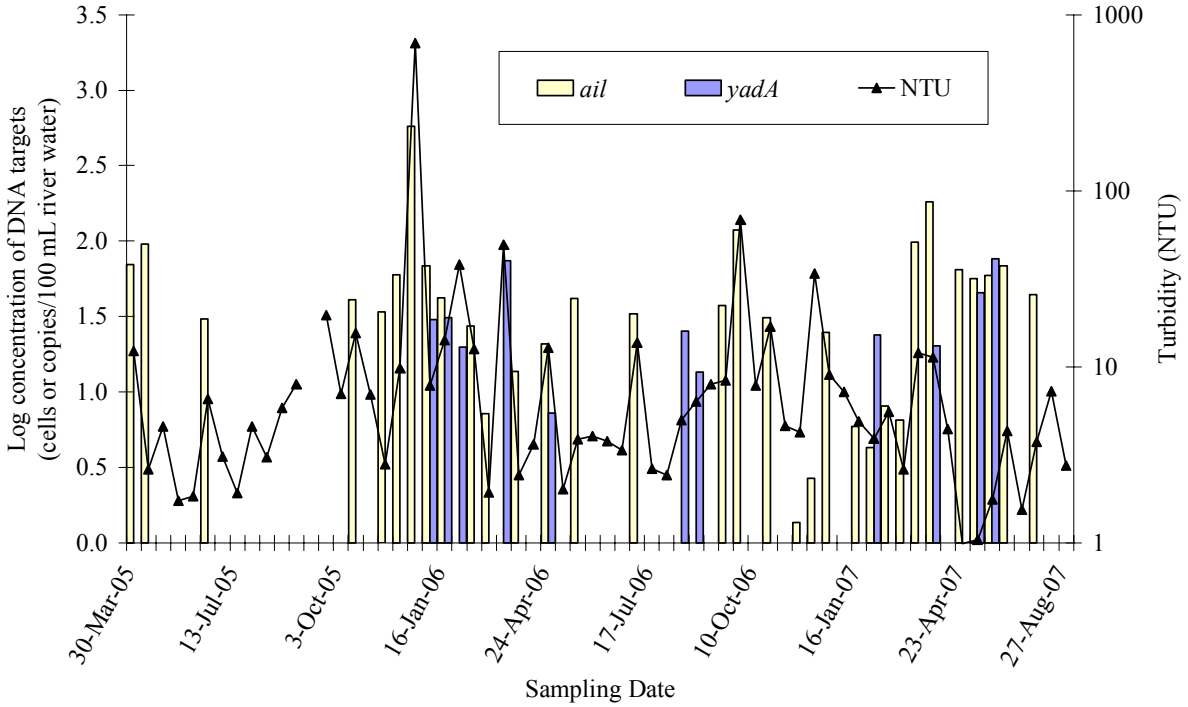
Grand River (upstream of intake)



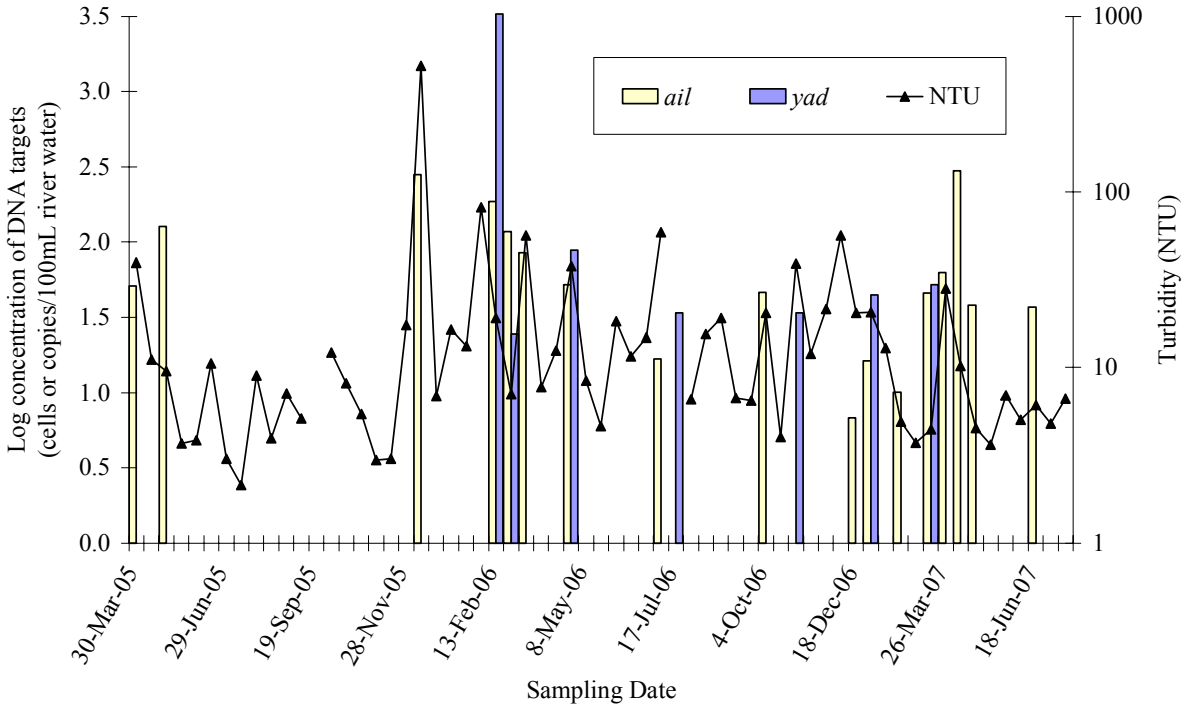
Grand River (north)



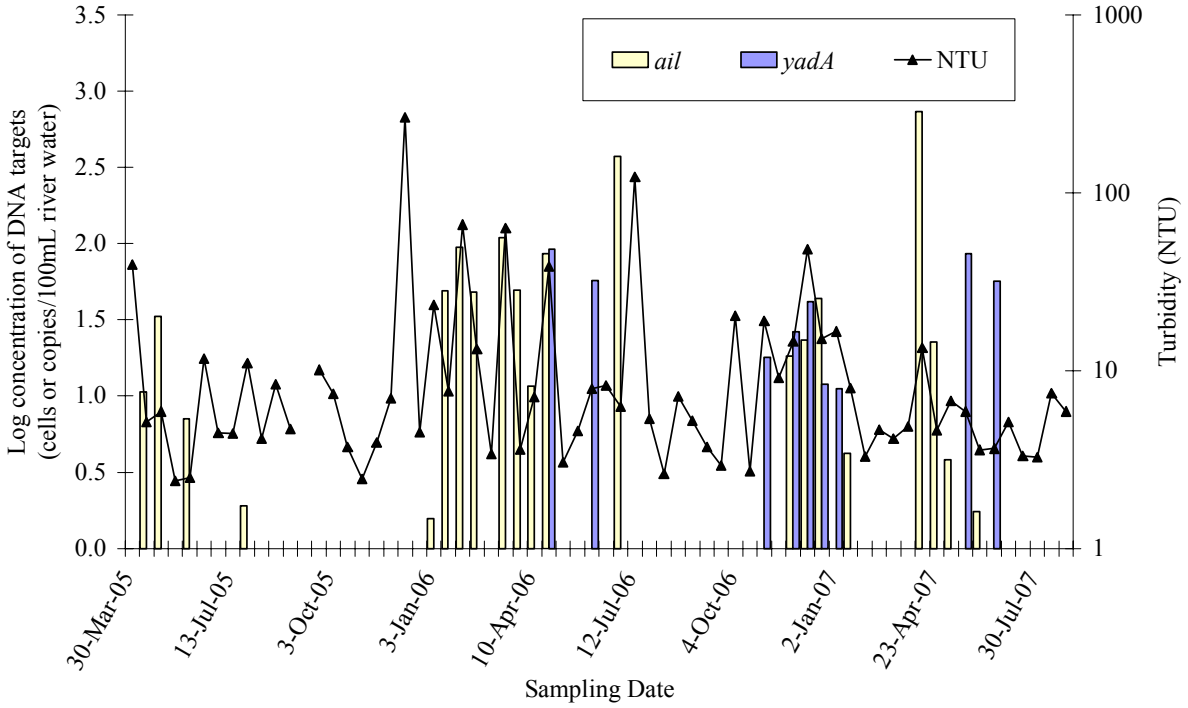
Canagagigue Creek



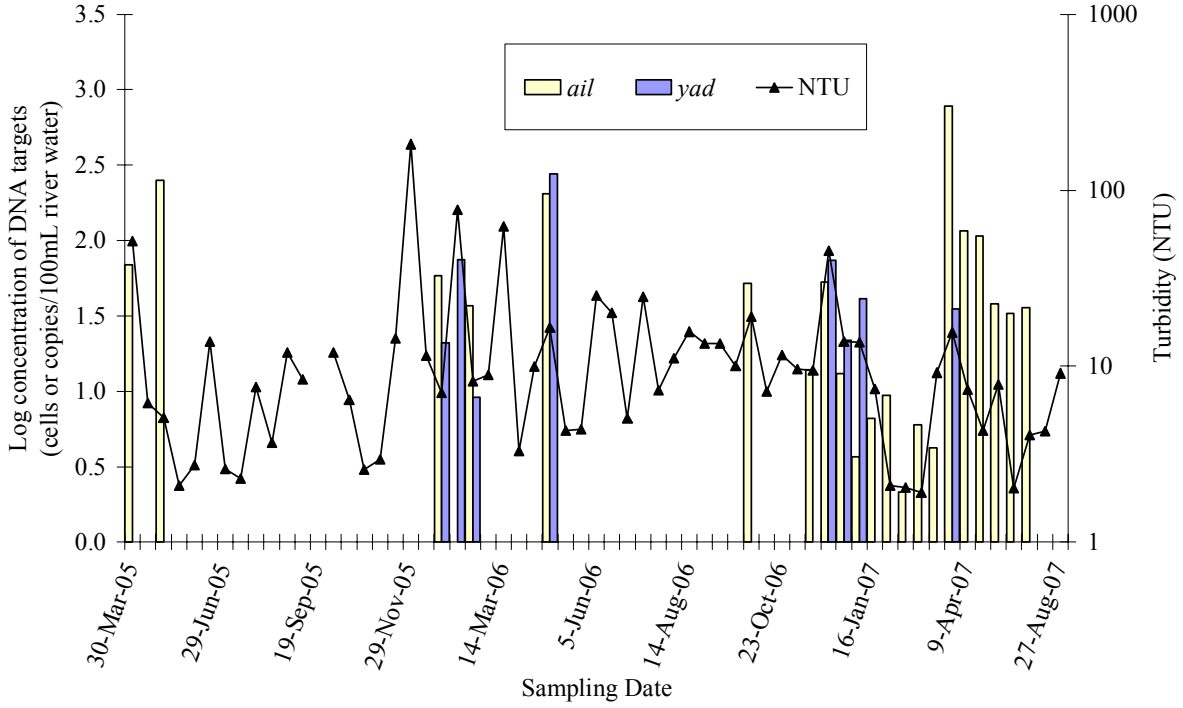
Conestogo River



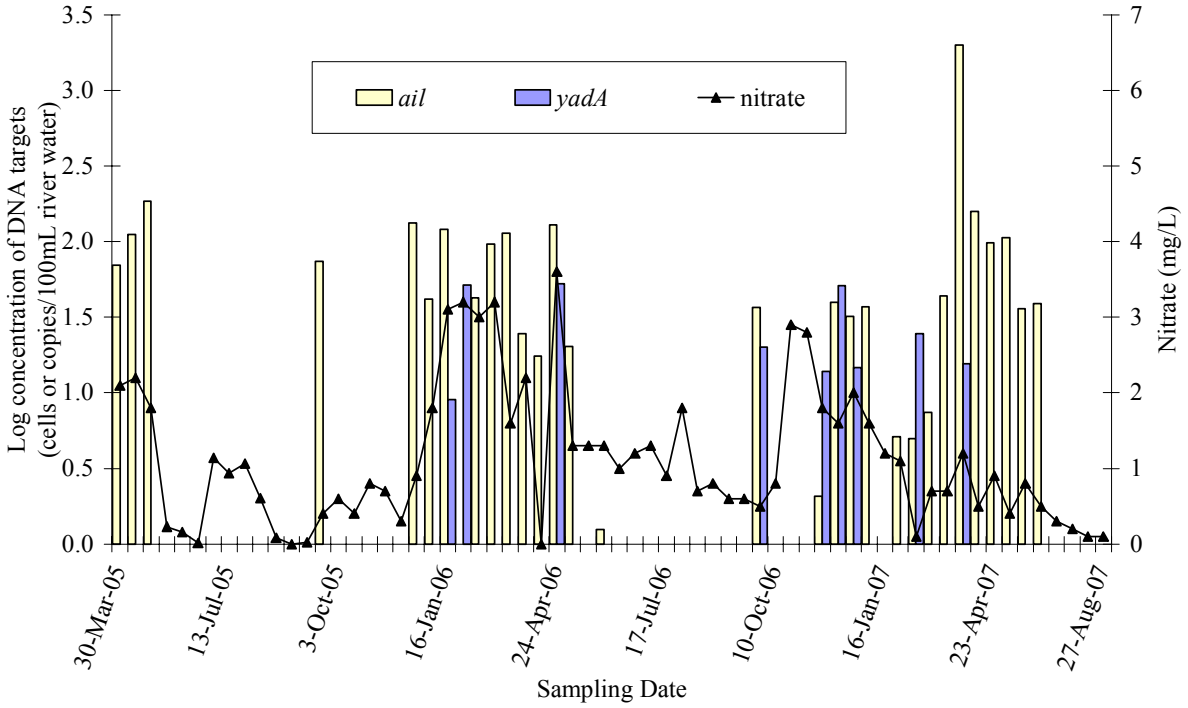
Grand River (downstream of wastewater)



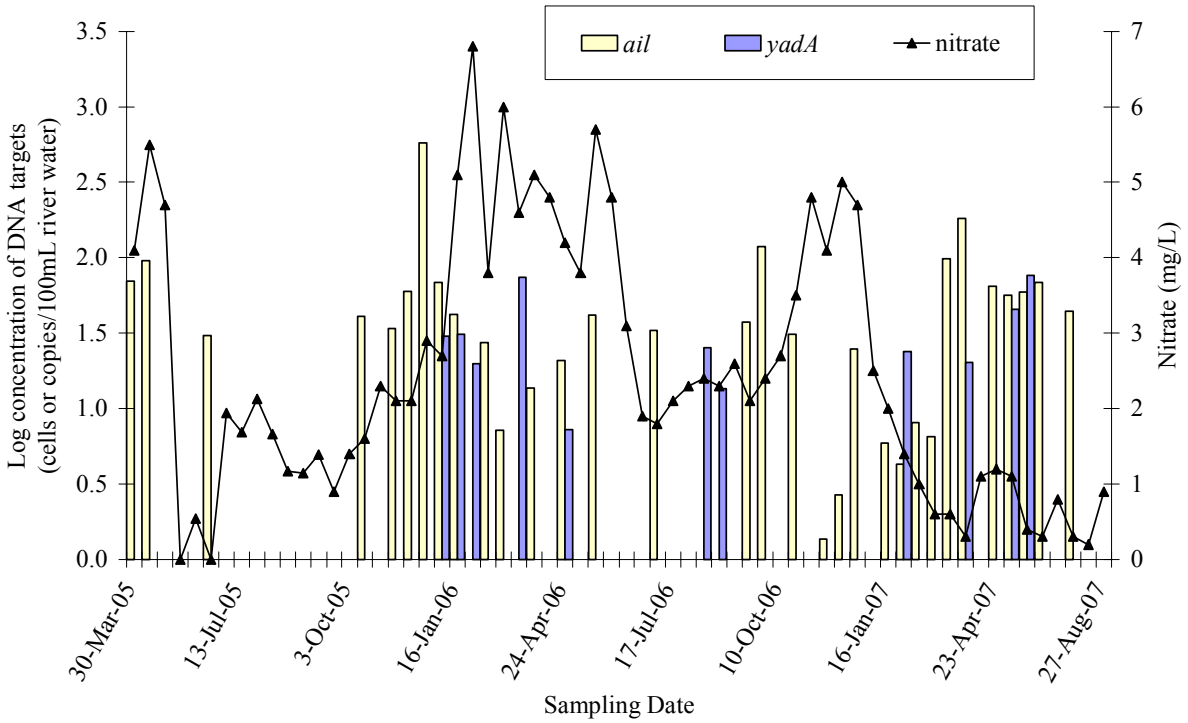
Grand River (upstream of intake)



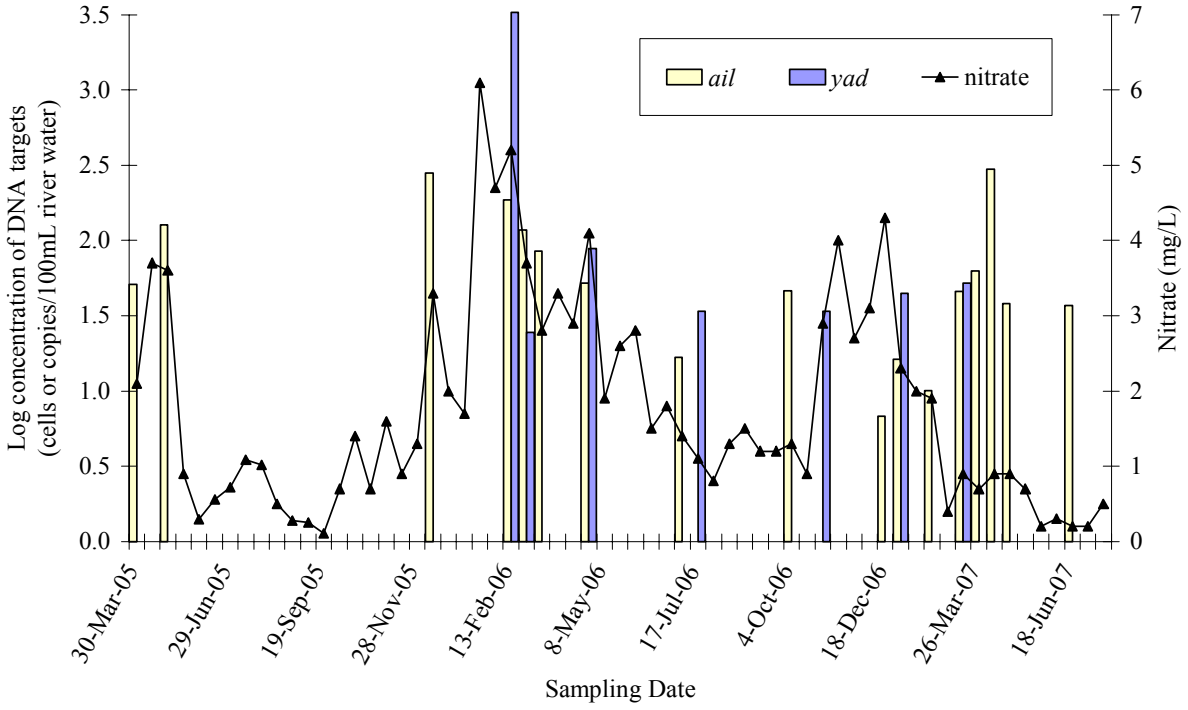
Grand River (north)



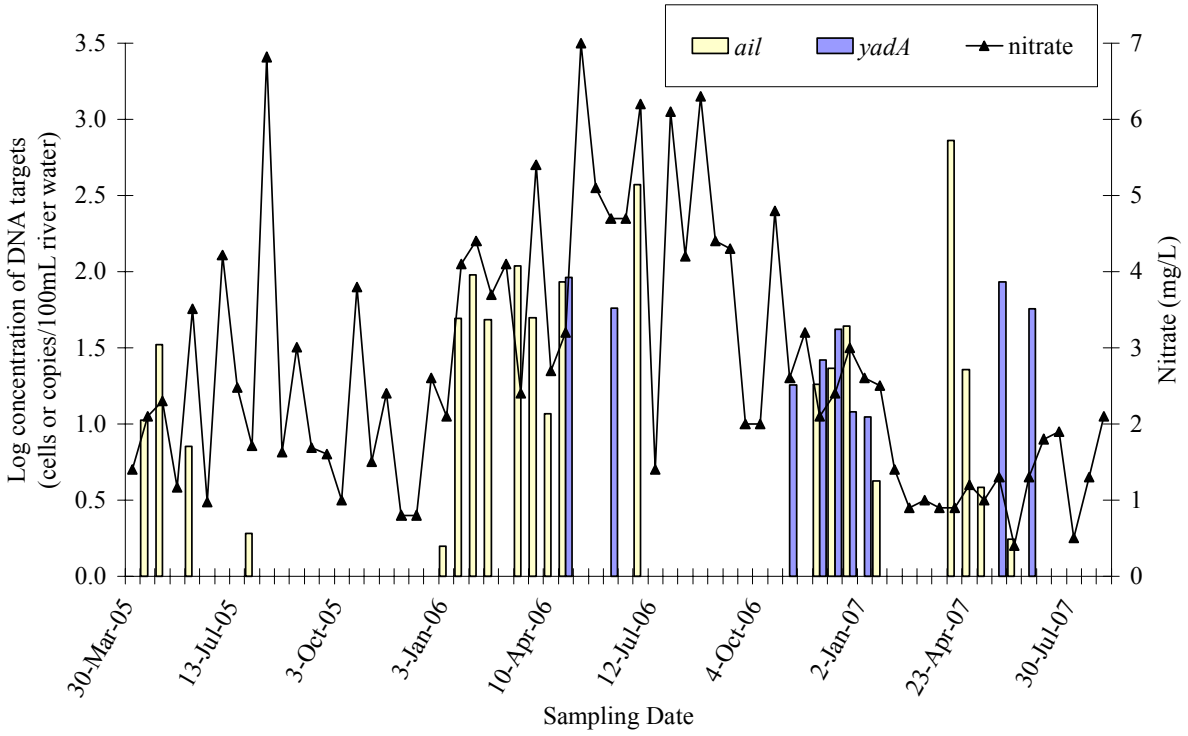
Canagagiague Creek



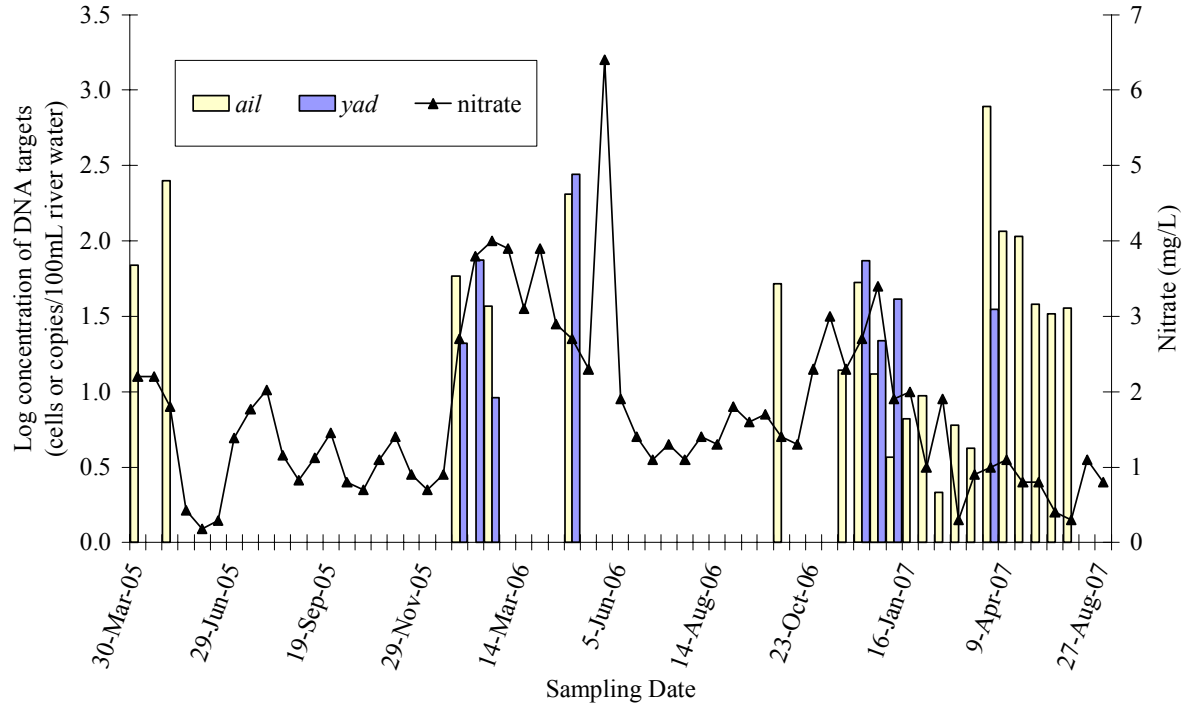
Conestogo River



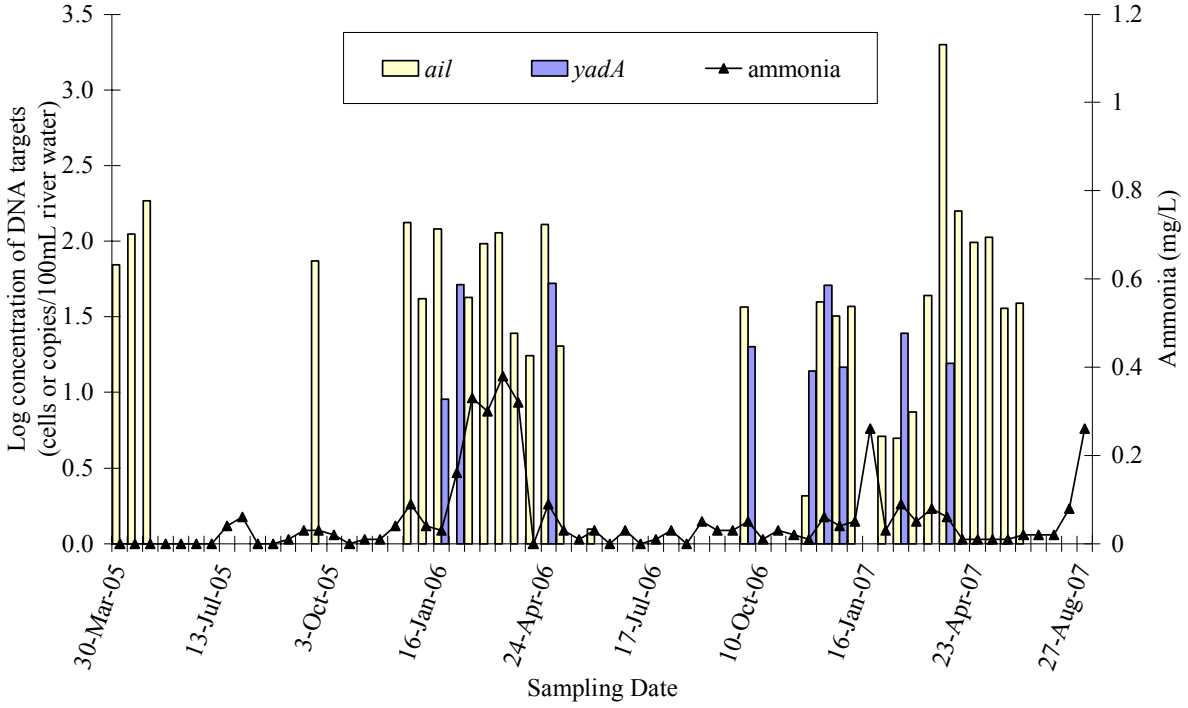
Grand River (downstream of wastewater)



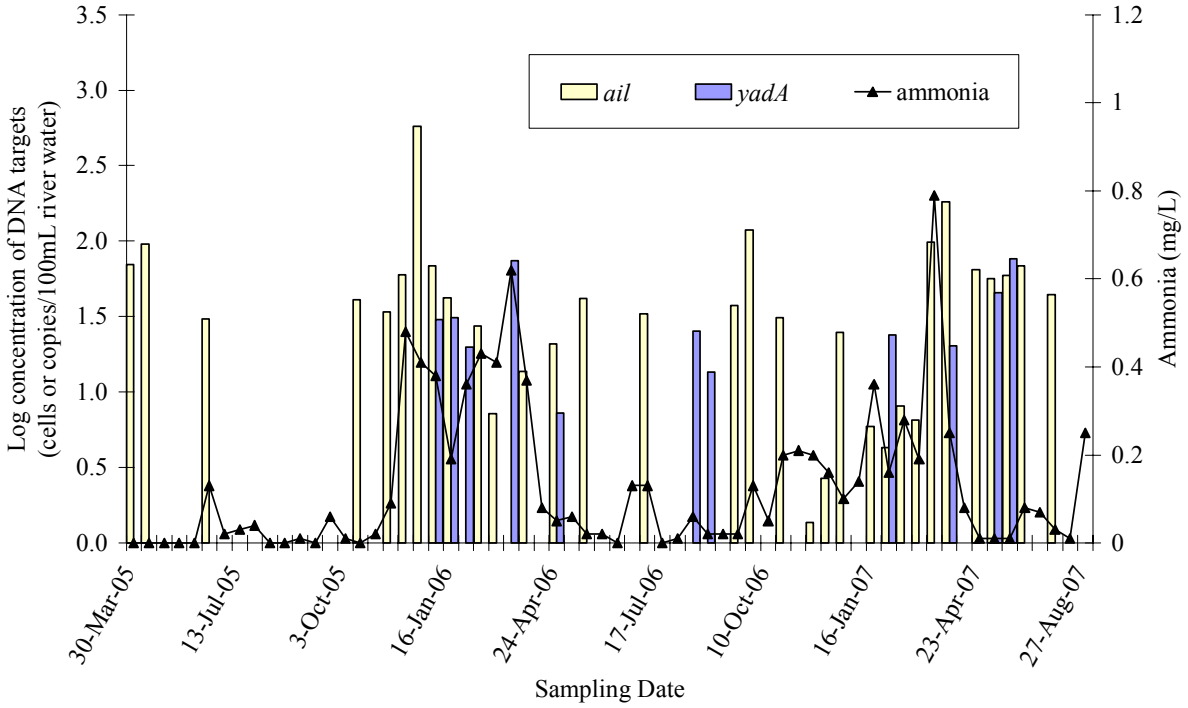
Grand River (upstream of intake)



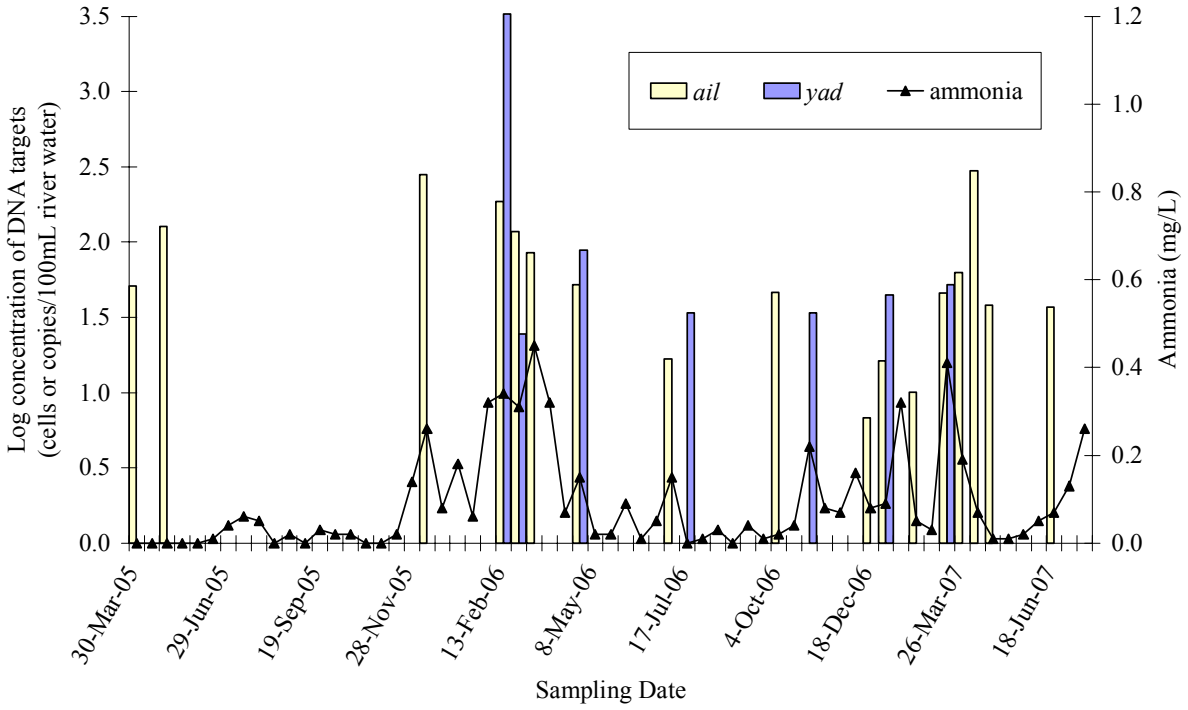
Grand River (north)



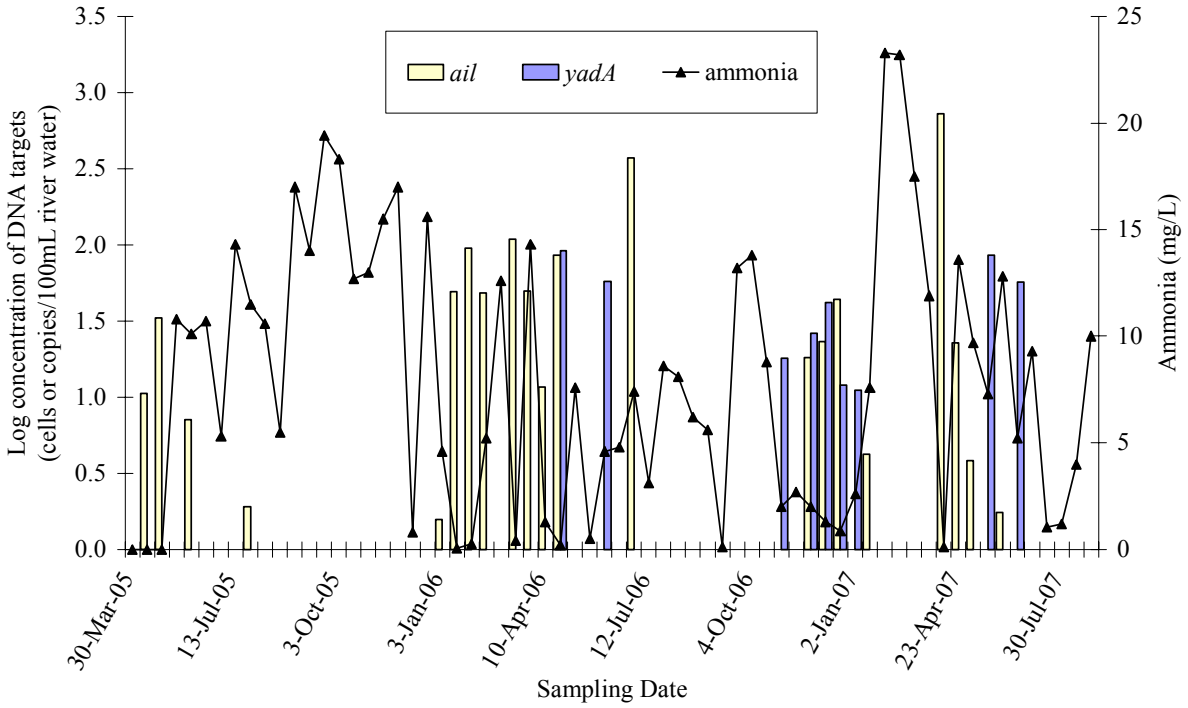
Canagagigue Creek



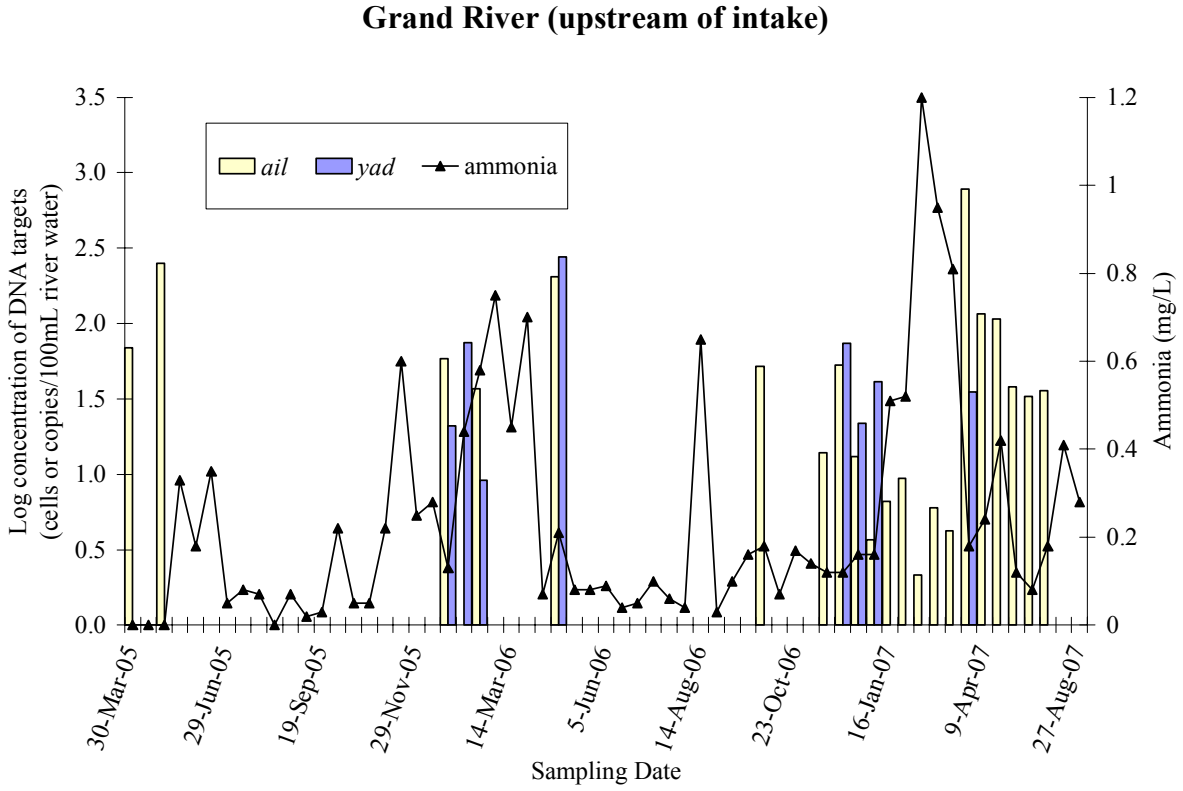
Conestogo River



Grand River (downstream of wastewater)



Note the different scale used for the axis indicating ammonia concentration (compared to other sites).


Grand River (upstream of intake)
ail
yad
ammonia
Log concentration of DNA targets (cells or copies/100mL river water)
Ammonia (mg/L)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1.2
1
0.8
0.6
0.4
0.2
0
30-Mar-05
29-Jun-05
19-Sep-05
29-Nov-05
14-Mar-06
5-Jun-06
14-Aug-06
23-Oct-06
16-Jan-07
9-Apr-07
27-Aug-07
Sampling Date

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