Coliform Bacteria From A Drinking Water Distribution System: Microbial Source Tracking, Characterization And Biofilm Formation

By

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ABSTRACT

A library of 18 coliform bacteria strains was obtained from different sampling points in the drinking water distribution system in Lexington, KY, over a three month period in 2006. To investigate the cause of the coliform occurrence we conducted a microbial source tracking study using phenotypic (API 20E, Biolog, and Vitek) and genotypic (pulsed field gel electrophoresis (PFGE) and ribotyping) analyses to determine the degree of genetic variation among isolates. Characterization of isolates by PFGE and ribotyping showed that coliform events in the distribution system were related and a regrowth problem may exist due to biofilm formation.

The ability of a persistent *Enterobacter cloacae* strain to adhere and form biofilm was found to depend on environmental conditions such as temperature, pipe material, soiled surface, chlorine and nutrient levels with higher temperatures and nutrient levels promoting adherence. Considerable variation in adherence and biofilm formation was observed among representative *Enterobacter* isolates.

LIST OF ABBREVIATIONS AND SYMBOLS USED

MST	Microbial source tracking
PFGE	Pulsed field gel electrophoresis
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
PCR	Polymerase chain reaction
PVC	Polyvinyl chloride
TC	Total coliforms
EPS	Extracellular polymeric substances
EPA	Environmental Protection Agency
TSA	Tryptic soy agar
TE	Tris-Ethylenediaminetetraacetic acid
TSB	Tryptic soy broth
PW	Peptone water
PS	Peptone saline
CUP	Carbon-source utilization profiling
SS	Stainless steel
CFU	Colony forming unit

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Chapter 1: INTRODUCTION

Microbial source tracking (MST) has become a well established tool to analyse microbes both in source and drinking water and to identify the origin of the microbial pollutants and potentially pathogenic bacteria that may impact downstream drinking water treatment plants and/or end-users at the tap. Microbial source tracking involves detection and characterization of waterborne bacterial contaminants for the purpose of tracing the event to a reservoir, a deficiency in the water treatment process, or point of intrusion into the distribution system. It is based on the concept that fecal contamination can be traced back to its original source using molecular, biochemical or immunological methods that can be library-dependent or library-independent (Scott *et al.,* 2002; Simpson *et al.,* 2002; Payne, 2007; Roslev & Bukh, 2011). These methods can be used to detect, characterize and confirm the identity of an isolate (Payne, 2007). In library-dependent studies, which use a collection of microbial isolates with a possible link to the case or environment under investigation, characterization of groups of microorganisms detects subtle differences present within these different groups and can subsequently be used to identify microorganisms or environments from which the microorganisms were derived (Scott *et al.,* 2002). Characterization can be conducted on isolates using either phenotypic or genotypic methods. Phenotypic methods can use carbon-source utilization profiling (CUP); this can be demonstrated using commercially available microplate systems that contain substrates for bacterial growth that generate patterns of substrate utilization of the isolates (Field & Samadpour, 2007). Three examples of such phenotypic methods include API 20E, Biolog and Vitek. These tests have been found useful in the identification of bacteria but also have the drawback that different species of enteric microorganisms can show very similar biochemical responses, potentially causing non-discriminating phenotypic fingerprints (Cimenti *et al.,* 2007). Genotypic analyses are based on some aspect of an organism's deoxyribonucleic acid (DNA) sequence. Genotypic methods differ by targeting sequences of specific genes or by measuring genetic differences in the genomic DNA (Anonymous, 2005). Pulsed field gel electrophoresis is a DNA fingerprinting tool that has been used successfully to determine genetic variability between clinical and environmental isolates belonging to the same bacterial species and this technique has proven to be more discriminatory than other molecular MST methods as well as highly reproducible (Meays *et al.,* 2004).

Each MST method has a unique set of advantages and limitations; thus, no standard MST method has been established and a multi-tiered approach is recommended (Noble *et al.,* 2006; Roslev & Bukh, 2011). Payne (2007) has suggested combining a number of methods in order to enhance discrimination or provide confirmation of results. Although MST has been used to identify sources of contaminants in a watershed, application of MST to waterborne organisms following drinking water treatment appears to be limited even though it could be a useful investigative tool in relation to the coliform episodes documented to occur in distribution systems (Blanch *et al.,* 2007). Coliforms can be introduced into the distribution system by source water combined with inadequate disinfection at the water treatment plant or through a breach in distribution system integrity such as a main break. Reoccurring coliform events may also be due to regrowth in established biofilm communities found in distribution system pipes. Coliforms may migrate to different points in the distribution system by cycles of forming and detaching of bacteria from biofilms (Stoodley, 2001). In this study, coliform isolates from a distribution system were compared for their relatedness using phenotypic and genotypic methods as well as characterized for their ability to adhere and form biofilm under a number of environmental conditions. This thesis includes reference to a number of publications where the terms adherence and attachment are used interchangeably. However, in this thesis the terms “adhere” and “adherence” will be used consistently throughout the thesis to describe cells adhering to surfaces in such a way that they cannot be rinsed off by use of water only.

Many drinking water utilities have reported coliform occurrences considered to be attributed to regrowth in drinking water distribution systems. Low concentrations of carbon a nutrient-based source in the water flowing through the distribution system may encourage bacterial adherence to the pipe surface and formation of biofilms. This may be due to the insufficient usable organic carbon available to support the proliferation of planktonic bacteria in the distribution system. Once established, various conditions can result in the detachment of bacteria from these biofilms and the reseeding of the system. Tracing the location of these bacterial biofilms in the drinking water distribution system is an extremely difficult task as biofilms are not present in any specific or uniform pattern. *Ent. cloacae*, which is the bacteria mainly used in this study, is commonly detected in the drinking water distribution system and tends to frequently be involved in biofilm and regrowth events (Camper *et al.,* 1996).

A biofilm is a community of microbes adhering to a surface (Varnam & Evans, 2000). It is defined as an assemblage of microbial cells that are not removed by gentle rinsing and are irreversibly adhered with a surface and enclosed in a matrix of extracellular polymeric substances (EPS) (Carpentier & Cerf, 1999; Donlan, 2002). Extracellular polymeric substances can consist of polysaccharides, proteins, nucleic acids and heterogeneous polymeric compounds (Manz *et al.,* 1999). The quantity of these components differs depending on the microorganisms, the nutrients available and the environment (Ells, 2007). The process of biofilm formation is considered complex and has been acknowledged as occurring through the stages listed below (Percival *et al.,* 2000; Lindsay & von Holy, 2006). The five stages include: 1) development of a surface conditioning film, 2) events that bring the organism in close proximity with the surface such as fluid dynamics or bacterial motility, 3) adhesion of microorganisms to the surface including reversible and irreversible adherence where the irreversible physical adherence may be mediated by EPS, 4) maturation of the biofilm involving growth and division of adhered cells to colonize the surface, leading to microcolony formation and biofilm formation, and 5) detachment (Percival *et al.,* 2000; Chavant *et al.,* 2002). Many environmental factors have been known to influence the adherence of cells to a surface and biofilm formation. If a bacterium such as *Ent. cloacae* is provided with the right conditions for growth it can increase in numbers in the distribution system. Environmental factors that can influence the numbers of adhered cells to a surface include disinfectants, nutrients available, material surface, temperature, environmental strain, and soiling of a surface also used interchangeably throughout this thesis with preconditioning of a surface (Momba & Makala, 2004). These factors are significant in the control of total coliforms (TC), fecal coliforms and biofilms in the distribution system to provide safe drinking water to consumers.

1.1. Objectives and Hypothesis

The first objective of this research was to determine whether a series of coliform occurrences within a drinking water distribution system were of the same or unrelated origin, by characterization of 18 coliform isolates using phenotypic and genotypic MST tools. These MST tools were selected and their usefulness as an investigative tool in drinking water distribution systems tested. Microbial source tracking methods were assessed based on their ability to identify and distinguish the contaminating microorganisms and their relatedness. It was

hypothesized that the genotypic methods would better distinguish the relatedness between isolates compared to the phenotypic methods. As PFGE is the main MST tool used in investigation of foodborne outbreak cases in Canada and the USA, it was hypothesized that this tool could also determine relatedness between the environmental coliform isolates.

The second objective of this research was to compare the ability of 5 of the 18 coliform strains to adhere and form biofilm. It was hypothesized that different strains of a species (*Ent. cloacae* and *Enterobacter asburiae*) would vary in their cell adherence and ability as biofilm formers. In particular, genotyping showed that one *Ent. cloacae* strain persisted in the distribution system as it was repeatedly isolated in a number of different areas within the distribution system. The adherence and biofilm formation of this strain was further evaluated under a number of environmental conditions including temperature, material surface, presence of residual free chlorine, presence of nutrients as organic material (peptone), and presence of organic material on the surface prior to the adherence assay. Cellular adherence was assessed by both microscopic examination and the plate count technique. It was hypothesized that higher temperatures and nutrient levels would increase the number of adhered cells to the surface over time. It was also hypothesized that higher numbers of adhered cells would be observed for hydrophobic surfaces such as polyvinyl chloride (PVC) when compared to more hydrophilic surfaces, and that a soiled surface would lead to greater numbers of adhered cells compared to a non-soiled surface. The presence of residual free chlorine (0.44 mg/l) was hypothesized to decrease cell adherence and biofilm formation and the overall effect of a number of treatments was hypothesized to have a synergistic effect.

Chapter 2: LITERATURE REVIEW

2.1. Public Health Issues in Drinking Water

Water authorities are devoted to ensure that the drinking water that leaves a treatment plant is safe for consumers' consumption (September *et al.,* 2007). Although these water authorities have to adhere to restrictions and regulations for water leaving a treatment plant, the quality of the water may decline dramatically from the time it leaves a plant to the time it arrives to the consumer having travelled through the distribution system (September *et al.,* 2007). This decrease in water quality is commonly associated with taste and odour changes caused by corrosion and microorganisms in the water (Beech & Sunner, 2006). The main public health concern deals with the presence of pathogenic protists, bacteria and viruses in the water. This could be attributed to the recovery and growth of injured or damaged bacteria due to treatment or distribution system deficiencies. Also, the presence of biofilms, that can harbour bacteria, pathogens, protists and viruses, in the distribution system can result in cells being separated from the biofilm and released into the drinking water; thereby contaminating the water that reaches consumers (September *et al.,* 2007).

While many bacteria in a drinking water distribution system are of no public health concern, some opportunistic microorganisms and pathogens, when released into the water, can be harmful and cause waterborne disease if the contaminated water is ingested (Geldreich, 1996; Power & Nagy, 1999; Percival *et al.,* 2000). Opportunistic pathogenic bacteria, *Klebsiella* spp*., Ent. cloacae* and *Citrobacter freundii*, are some of the most successful coliform colonizers as they can grow in low nutrient environments and are capable of survival in unfavourable environments (Geldreich, 1996). These opportunistic microorganisms can cause health problems including gastroenteritis, causing symptoms such as diarrhea, cramps, nausea and vomiting, due to colonization of the body after ingestion of the contaminated water (Geldreich, 1996; Camper *et al.,* 1999; Anonymous, 2006). Gastroenteritis is usually not serious for a healthy individual but it can lead to more serious problems for individuals with immunocompromised systems, young children, newborns and pregnant women.

Bacterial pathogens including *Legionella pneumophila, Campylobacter* spp*., Escherichia coli* O157:H7 and *Vibrio cholera* have been found in water systems (Donlan, 2002). When opportunistic microorganisms are able to adhere to the surface of a pipe in a distribution system

and form biofilms, pathogens, such as those listed above, may be able to exist in these biofilms (Beech & Sunner, 2006). These biofilms can act as safe havens for the bacteria and help facilitate the pathogens' survival and growth within the biofilm and may assist in aiding genetic transfer of antibiotic resistance, as well as virulence factors within the bacterial community (Anonymous, 2006; Beech & Sunner, 2006). One problem that exists is that pathogens may be able to live and flourish in these protective biofilms until detachment (Anonymous, 2006). When the pathogens later become detached from the biofilm and enter the distribution system, they become a serious concern as they may able to reach consumers' taps. The presence of chlorine residual in drinking water is supposed to help prevent bacteria from reaching the consumers' taps. Chlorine residual in drinking water is correlated with the absence of organisms causing waterborne disease. However, when chlorine is added to the water it reacts with organic materials and metals found in the water. This reaction can use up all of the added chlorine and reduce its disinfection efficacy. This loss of active disinfectant is referred to as the chlorine demand of the water. The remaining chlorine is called free chlorine and is the chlorine measured in this study. Free chlorine is available to inactivate microorganisms including those causing waterborne diseases (Anonymous, 2011a).

There is some controversy regarding the contribution of biofilms to the presence of waterborne pathogens in the distribution system under normal conditions. Some researchers believe that biofilms are not common habitats for pathogens as they appear incapable of extensive growth in biofilms (Donlan, 2002; Blanch *et al.,* 2007). These researchers suggest that waterborne pathogens enter the distribution system in some other way and do not come from biofilms. The model system used in this study evaluated the cell adherence of an opportunistic pathogen in a very low nutrient environment and in many cases at a low temperature of 10°C. The bacterial cells were able to irreversibly adhere to the surface and in some cases initial biofilm stages likely formed. This may help provide evidence to suggest the contribution of biofilms to the presence of opportunistic pathogens in the drinking water distribution system.

Other researchers believe that microorganisms in biofilms tend to become more resistant to treatment with disinfectants, and that the biofilms can become reservoirs for the subsequent spread of pathogenic and opportunistic microorganisms (September *et al.,* 2007). If this is the case, then this can become a problem for the water utility and water regulating organizations in an area. The sessile biofilm bacteria are generally more resistant to many antimicrobial agents

compared to the same bacteria growing in a planktonic state and this resistance of bacteria in biofilms can lead to persistent infections in the human body (Kolari, 2003). Some research has indicated that in order for antibiotics to be effective against biofilm bacteria, concentrations need to be 500 to 5000 times greater than those required for planktonic cells from the same species (Wellman *et al.,* 1996).

2.2. Sources of Microbial Contamination into the Drinking Water Distribution System

The presence of coliform bacteria in drinking water suggests that there is a problem with the treatment system either due to inadequate disinfection at the water treatment plant (Stoodley, 2001) or to a problem in the distribution system transferring treated water from the treatment plant to consumers' homes (Anonymous, 2002). A review of the literature reporting on pathways found to breach distribution system integrity proposes a few mechanisms of coliform occurrence. These mechanisms include the two mentioned above: (a) inadequate treatment as coliforms from the source water supply break through the treatment process (this will not be further discussed), (b) intrusion into the distribution system downstream from a treatment plant resulting in a recontamination of the treated water, and (c) coliform regrowth occurrences typically due to the presence of biofilms in the system (Besner *et al.,* 2002). Intrusion can occur as a result of transitory low-pressure events, cross-connections, and pipe repairs (Anonymous, 2002; Besner *et al.,* 2002). The intrusion may be very localized in time and space making it difficult to narrow down this event specifically as being the cause of coliform occurrence. Total coliforms and/or a number of pathogens can become adhered to a surface or become entangled in pre-existing biofilms on pipe walls; this is another mechanism of coliform occurrence in distribution systems (Anonymous, 2002). Many pathogens have been found to survive and proliferate in these biofilms on pipe surfaces where the sessile cells are better protected from disinfectants than planktonic cells (Anonymous, 2002). Over time, coliform bacteria may detach from the biofilm causing persistent recurring TC detections (Anonymous, 2002). Pathogens may also be included in the detached material and may result in waterborne diseases (Anonymous, 2002).

2.3. Microbial Source Tracking

Microbial source tracking is a tool used to analyse microorganisms in both source and drinking water (Meays *et al.,* 2004). Microbial source tracking focuses on identifying the source(s) of contaminants and potentially pathogenic bacteria impacting a water system (Anonymous, 2005). It is based on the concept that fecal contamination can be traced back to its original source. Methods used in MST include both library-dependent and library-independent methods, which can consist of molecular, biochemical or immunology based techniques (Scott *et al.,* 2002; Simpson *et al.,* 2002; Ahmed 2005; Payne, 2007; Anonymous, 2011h). Library-dependent MST analysis requires that a collection or library of isolates exists to characterize. Library-independent MST analysis does not require such a library or collection of isolates and uses detection of host-specific markers in DNA samples from the source (Roslev & Bukh, 2011). Both library-dependent and library-independent methods can be used to detect, characterize and confirm the identity of an isolate (Payne, 2007). This study focused on characterization of the bacterial isolates using library-dependent phenotypic and genotypic methods. Characterization of groups of microorganisms as was carried out in this study detects subtle differences present within different groups and these groupings of microorganisms can subsequently be used to identify the microorganisms or environments from which the microorganisms were derived and possible inter-relatedness (Scott *et al.,* 2002).

Library-dependent phenotypic methods include carbon substrate utilization and antibiotic resistance analysis tests (Simpson *et al.,* 2002; Hagedorn *et al.,* 2003; Ahmed 2005; Anonymous, 2005; Anonymous, 2011h). Carbon-source utilization profiling uses commercially available microplate systems containing substrates for bacterial growth that are used to generate patterns of substrate utilization by the isolates (Field & Samadpour, 2007). Three examples of commercially available phenotypic CUP test systems include API 20E, Biolog and Vitek. Each test system contains a selection of substrates, and after incubation a positive or negative score is obtained depending on whether the substrate was utilized or not by the microorganism. This scoring system is used to generate a numeric profile and a probable identification based on a database provided by the manufacturer. API 20E uses 20 different substrates, while Vitek and Biolog use 30 and 95 different substrates, respectively. These tests have been compared in this study for their suitability for use to characterize the coliform library. Although these tests have been found useful in the identification of many bacterial isolates they also have the drawback

that different species of enteric microorganisms can show very similar biochemical responses, potentially resulting in non-unique phenotypic fingerprints (Cimenti *et al.,* 2007). Antibiotic resistance analysis is conceptually based on bacteria in the intestinal flora demonstrating an increase in antibiotic resistance when the host animal is treated with antibiotic. A library of antibiotic resistance patterns for known bacterial strains from human and animal sources has been created. When bacteria is found it is grown on a set of antibiotic containing media and based on the bacterial strain's response, resistance patterns are developed and the patterns of bacteria from known sources compared to those of unknown sources (Anonymous, 2011h).

Library-dependent genotypic methods can characterize isolates using genetic methods such as PFGE, ribotyping, and repetitive extragenic palindromic polymerase chain reaction to name a few of the methods available (Anonymous, 2011h). Other library-dependent genotypic methods exist but will not be discussed here. Genotypic analyses are based on an aspect of an organism's DNA sequence. Genotypic methods differ by targeting specific genes or by measuring genetic differences in the genome (Anonymous, 2005). Ribotyping is a DNA fingerprinting method that involves the digestion of bacterial DNA, separation of restriction fragments by gel electrophoresis, hybridization with a probe specific for a conserved region of the ribosomal ribonucleic acid (rRNA) genes, and observation of the banding patterns using autoradiography or chemiluminescence and digital imaging equipment. Genetic variability in the specific portions of the rRNA genes generates different fragment band profiles known as banding patterns that can be used to differentiate bacterial strains as was demonstrated in this study (Scott *et al.,* 2002; Foley *et al.,* 2009; Anonymous, 2011h). Repetitive extragenic palindromic polymerase chain reaction uses the polymerase chain reaction (PCR) and specific primers to amplify repetitive segments of the bacterial DNA. The DNA fragments are then separated by gel electrophoresis to produce a banding pattern. These DNA fingerprints are then compared to one another to determine genetic relatedness (Ahmed, 2005; Foley *et al.,* 2009; Anonymous, 2011h). Pulsed field gel electrophoresis, a genetic method used in the characterization of isolates in this thesis, is a DNA fingerprinting technique that utilizes restriction enzymes to digest bacterial DNA embedded in agarose plugs followed by placement of these into wells of an agarose gel. The large genomic fragments are then separated by subjecting them to alternately pulsed, perpendicularly oriented electrical fields. Following

staining of the gels, banding patterns can be seen, which again allows for the determination of strain relatedness (Maier *et al.,* 2000; Scott *et al.,* 2002; Meays *et al.,* 2004, Foley *et al.,* 2009).

The PFGE method has been described as the superior method for molecular typing and referred to as the "gold standard" (Anonymous, 2005; Foley *et al.,* 2009). The Centers for Disease Control and Prevention has implemented the use of this method for their National Molecular Subtyping Network for Foodborne Disease Surveillance in order to discriminate strains of *E. coli* O157:H7 and other foodborne pathogens involved in outbreaks (Anonymous, 2005). The PulseNet network and website was developed to enable researchers and scientists from health fields around the globe to immediately compare molecular PFGE genotypic data (Anonymous, 2005). This network has been used to rapidly compare any PFGE profiles of samples that are being investigated, with those already deposited in the national database. Health Canada's Bureau of Microbial Hazards and the Public Health Agency of Canada's Laboratory for Foodborne Zoonoses are governmental agencies that participate in PulseNet Canada, a Canadian national network that is harmonized with the PulseNet USA. PulseNet Canada plays an important role in the surveillance and investigation of foodborne illness outbreaks and can play a part in investigating outbreaks associated with fecally contaminated drinking water or water irrigation systems resulting in foodborne illness. One of the main reasons for the creation of PulseNet Canada was to assist in epidemiological investigations to differentiate outbreak from sporadic cases and to help identify the source of outbreaks (Anonymous, 2011f). Molecular typing methods such as PFGE can be used to link isolates from food or water with isolates from sick individuals. In a pathogen outbreak, PFGE analysis of bacterial strains isolated from sick individuals can be compared with bacterial strains isolated from suspected sources and compared for genetic relatedness. This helps to distinguish outbreak cases from sporadic cases.

In an outbreak connected to a farm, manure was suspected of leaking into a well that served a county fair and infected the general public visiting, PFGE analysis was carried out on isolates taken from sick individuals and compared to isolates from the suspected water source. Pulsed field gel electrophoresis analysis showed that 63% of the patient isolates and 63% of the isolates from the water source displayed an identical PFGE profile. Whereas 27% of the isolates from patients displayed a PFGE profile differing by two bands, this suggests the outbreak strain may have persisted long enough to produce variants (Barrett *et al.,* 2006). In the present study, PFGE was used to characterize isolates from a drinking water distribution system to compare

their genetic relatedness. The PFGE profiles were then compared to their respective ribotype as well as to API, Vitek and Biolog profiles.

Multiple-locus variable number tandem repeat analysis is another molecular typing method that was not used in this particular study. This technique uses the variation in the number of tandem repeated DNA sequences found in many different loci in the genome of microorganisms. It is able to compare molecular DNA fingerprints of bacteria. Many DNA sequence analysis programs have the ability to scan genome sequences to identify repeat regions known as tandem repeats. When these regions are discovered, PCR primers are designed so they will allow amplification of the tandem repeats. After amplification PCR products are separated and the product sizes determined to detect the number of tandem repeats; this may be done using an automatic DNA sequencer. Differences in the number of tandem repeats allows for the discrimination between different bacterial strains (Foley *et al.*, 2009). As a result of a number of bacterial genomes having been fully sequenced over the past 10 years, multiple-locus variable number tandem repeat analysis is becoming more common as a typing method.

Sequence variability is observed within specific genes and can be used in the characterization of bacterial isolates to determine the relatedness of the bacteria. Multi-loci sequence typing is a molecular typing technique that uses particular nucleotide base changes. It can involve both housekeeping and/or virulence genes. For a number of bacterial species, internet-based multi-loci sequence typing databases exist (Foley *et al.*, 2009).

Single nucleotide polymorphism analysis can be used to characterize and differentiate bacterial strains. Strains of a bacterial species differ genetically from one another due to deletion or addition of genetic sequences, which can take place by way of horizontal gene transfer, recombination events or nucleotide mutations. By comparing multiple single nucleotide polymorphisms the relatedness of bacterial strains can be observed (Foley *et al.*, 2009).

The need to create a strain library is the main disadvantage of a library-dependent MST method. This may be a reason why researchers are investigating library-independent MST techniques. Library-independent methods are either culture-dependent or culture-independent. Library-independent methods identify sources based on host-specific characteristics of the bacteria. Two common library-independent culture-based methods include bacteriophages and bacterial methods (Field & Samadpour, 2007). Methods identifying host-specific characteristics of bacteria aim to find specific markers unique for the source, e.g., *Bacteroidales* markers

associated with humans, cows, pigs, etc. (Roslev & Bukh, 2011). This approach is based on PCR detection of *Bacteroidales*, a group of anaerobic bacteria that are often found in abundance in the gut of warm blooded mammals. These bacteria are present in larger numbers than indicator bacteria in feces but are more challenging to culture due to their anaerobic nature (Anonymous, 2011h). This method detects sequences also known as DNA markers that are relatively easy to detect in water, are highly specific to the host, can be associated with recent fecal pollution because they will not survive for long periods of time in the environment due to environmental conditions, and do not require culturing of bacteria (Lee *et al.,* 2010). Polymerase chain reaction detection of specific virulence genes that are clinically important is another technique that may provide a better indication of microbial water quality. Methods have been developed that use biomarkers targeting the clinically important *E. coli* as compared to the non-pathogenic *E. coli* in the intestine based on enterotoxin genes for *E. coli* (Ahmed, 2005). Overall each MST method appears to have distinct advantages and disadvantages and as a result there is no single method that is capable of identifying specific sources of fecal pollution in the environment with absolute certainty (Scott *et al.,* 2002; Meays *et al.,* 2004). Payne (2007) has suggested combining a number of methods in order to enhance discrimination or provide confirmation of results. Use of a multi-tiered approach has been recommended (Roslev & Bukh, 2011).

2.4. Biofilms in the Drinking Water Distribution System

In this study, isolates were characterized using phenotypic and genotypic methods followed by an examination of their biofilm forming capabilities under a number of environmental conditions. The definition of the word biofilm remains a topic of debate by many researchers, most argue that bacteria must be embedded in a matrix of EPS to be a biofilm, but others state that EPS can be absent (Carpentier & Cerf, 1999). After a review of the literature offering definitions of a biofilm, the majority of the literature suggests a biofilm to be a community of microbes adhering to a surface (Varnam & Evans, 2000). It is defined as an assemblage of microbial cells that are not removed by gentle rinsing and are irreversibly adhered with a surface and in some cases enclosed in a matrix of EPS (Carpentier & Cerf, 1999; Donlan, 2002). Extracellular polymeric substances can consist of polysaccharides, proteins, nucleic acids, and heterogeneous polymeric compounds (Manz *et al.,* 1999). The quantity of these components differs depending on the microorganisms, the nutrients available and the environment (Ells,

2007). The EPS produced by the microorganisms forms a slimy, highly hydrated matrix that can act as glue and provide a protective barrier for the biofilm (Manz *et al.,* 1999).

The process of biofilm formation is considered complex and occurs through a number of stages (Percival *et al.,* 2000; Lindsay & von Holy, 2006). Most researchers agree on the overall sequence of events involved in biofilm formation; adherence, growth and detachment. The difference seen in the number of stages defined by researchers as being part of biofilm formation is due to varying descriptions of what is included in each stage. Mostly four to five stages of biofilm formation are described. The five step process includes: 1) development of a surface conditioning film, 2) events that bring the organism in close proximity with the surface such as fluid dynamics or bacterial motility, 3) adhesion of microorganisms to the surface including reversible and irreversible adherence where the irreversible physical adherence may be mediated by EPS, 4) maturation of the biofilm involving growth and division of adhered cells to colonize the surface, leading to microcolony formation and biofilm formation, and 5) detachment (Percival *et al.,* 2000; Chavant *et al.,* 2002).

2.4.1. Formation of Biofilms: Cell Adherence, Microcolony Development and Detachment

The solid-liquid interface between a solid surface such as stainless steel (SS) and a liquid such as water provides an ideal environment for the adherence and growth of microorganisms (Donlan, 2002). Under natural conditions, a pipe surface in a water distribution system may become covered with organic and inorganic nutrients (Varnam & Evans, 2000; Donlan, 2002; Nikolaev & Plakunov, 2007). The formation of this layer of molecules is the first step prior to the adherence of the bacteria (Nikolaev & Plakunov, 2007). It is known as a conditioning film, and can form in only minutes (Varnam & Evans, 2000). This conditioning film provides a higher concentration of nutrients at the surface when compared with the nutrients found throughout the bulk water in the distribution system (Palmer *et al.,* 2007). This conditioning of the surface can also alter the physicochemical properties of the surface, influence the adsorption of other molecules, and affect bacterial adhesion (Carpentier & Cerf, 1999; Manz *et al.,* 1999; Palmer *et al.,* 2007).

The adhesion of bacteria to a surface can be active or passive depending on cell motility (Chmielewski & Frank, 2003; Kolari, 2003). Passive transport of bacteria to a surface is driven

by gravity, random Brownian motion, diffusion and fluid dynamic forces (Manz *et al.,* 1999; Chmielewski & Frank, 2003; Lindsay & von Holy, 2006). This initial transport by fluid dynamic forces can involve convective currents in which cells are brought to the surface by the movement of the bulk liquid (McLean & Decho, 2002). In active transport, the bacterial cell surface facilitates initial adherence and is mediated by flagellar activity (McLean & Decho, 2002; Chmielewski & Frank, 2003; Palmer *et al.,* 2007).

Once contact with a surface has been made, bacteria must develop an interaction with that surface (McLean & Decho, 2002). In reversible adherence, it is initially a weak interaction that involves three types of forces; namely van der Waals, electrostatic forces and hydrophobic interactions (Carpentier & Cerf, 1999; Chmielewski & Frank, 2003; Palmer *et al.,* 2007). During this contact, bacteria still exhibit Brownian motion and are easily removed by the application of a mild shear force such as simple rinsing (Chmielewski & Frank, 2003; Palmer *et al.,* 2007). This is one of the reasons the reversible adherence stage is considered the weakest link in the chain of events connecting bacterial cells to the conditioned surface (Lindsay & von Holy, 2006).

The transition from reversible to irreversible adherence involves bonding between bacterial appendages including pili, flagella and adhesion proteins and the surface (Chmielewski & Frank, 2003). This switch in adherence is carried out by various short range forces including covalent bonding, hydrogen bonding, and hydrophobic interactions (Manz *et al.,* 1999; Palmer *et al.,* 2007). This bonding usually occurs within a few hours of contact with the surface (Chmielewski & Frank, 2003).

Irreversibly adhered bacterial cells produce EPS due to stimulation of membrane-bound sensory proteins in the bacterial cell wall, and the resulting EPS establishes cell-to-cell bridges and acts as a glue to cement the cells to the surface (Manz *et al.,* 1999; Chmielewski & Frank, 2003; Lindsay & von Holy, 2006). At the end of this stage, strong physical or chemical forces are required to remove the bacteria from the surface including scraping, scrubbing and/or chemical cleaners (Palmer *et al.,* 2007). This stage marks the transformation of adhered planktonic cells to sessile cells having a true biofilm physiology (McLean & Decho, 2002).

Once bacteria have become irreversibly adhered to a surface, the process of biofilm maturation begins (Dunne, 2002). This stage consists of the growth of bacteria; development of microcolonies referred to as the basic organizational units of a biofilm and the recruitment of other bacteria and their colonization of the surface (Percival *et al.,* 2000; Lindsay & von Holy,

2006). During this stage of adhesion, planktonic microorganisms can also stick to each other or to different species of bacteria that are adhered to the surface already. These are called secondary colonizers and result in the aggregation of bacterial cells on the surface (Dunne, 2002; Chmielewski & Frank, 2003; Nikolaev & Plakunov, 2007). This is often accompanied by additional production of EPS (Chmielewski & Frank, 2003). The overall density and complexity of the biofilm increase when adhered cells start to actively replicate and die (Dunne, 2002). It is also affected by the extracellular components produced by the bacterial cells adhered to the surface and their interaction with organic and inorganic molecules to construct a biofilm matrix (Dunne, 2002).

The structure of mature biofilms in natural and laboratory systems is as diverse as the habitats themselves (Manz *et al.,* 1999; Chmielewski & Frank, 2003). The structure of these biofilms ranges from thin monolayers of scattered cells in extremely oligotrophic habitats, to patchy arrangements of matrix-embedded cell clusters intermingled with water-filled channels and to the layered densely packed biofilms forming bacterial mats (Manz *et al.,* 1999; Chmielewski & Frank, 2003). Simultaneously with an increase in thickness of the biofilms, maturation and the development of complex architecture with channels, pores and a redistribution of bacteria away from the surface occurs (McLean & Decho, 2002; Kolari, 2003; Lindsay & von Holy, 2006; Nikolaev & Plakunov, 2007). Under favourable conditions, this stage of build up of mature biofilms continues for a long time (Nikolaev & Plakunov, 2007).

Detachment is a common term used to describe the removal of individual or groups of cells from a biofilm (McLean & Decho, 2002). Five different processes known to be involved in the removal of bacterial cells from a biofilm include erosion, sloughing, abrasion, human intervention, and predator grazing (Percival *et al.,* 2000; McLean & Decho, 2002). Three of the processes are physical processes: erosion or shearing, sloughing, and abrasion. Erosion is caused by the force of flowing water known as the shear effect and results in the continuous removal of small portions of the biofilm (Percival *et al.,* 2000; Donlan, 2002). Sloughing is more random than erosion and involves hydrodynamic forces, which influence the detachment of large particles of biofilm (Manz *et al.,* 1999; Percival *et al.,* 2000; Donlan, 2002). This tends to occur more often in the thicker biofilms obtained in nutrient rich environments (Percival *et al.,* 2000). Abrasion is the result of the collision of solid particles from the water with the biofilm, whereas detachment of the biofilm by human intervention involves physically detaching the biofilm

and/or the addition of chemicals to detach cells (Percival *et al.,* 2000; Donlan, 2002). Predator grazing involves the consumption of biofilms by organisms such as protists, snails and worms (Percival *et al.,* 2000). These detachment processes may seem passive in comparison to cells that actively leave biofilms through cell division using a few different dispersal strategies (Manz *et al.,* 1999). During cell division, daughter cells are shed from the biofilm while other actively growing parental cells remain firmly adhered to the surface keeping the biofilm intact (Manz *et al.,* 1999; Donlan, 2002).

Once microcolonies or individual biofilm cells are released into the flowing water in a distribution system they are able to return to the planktonic state (Donlan, 2002; Kolari, 2003; Lindsay & von Holy, 2006; Nikolaev & Plakunov, 2007). These individual former biofilm cells can act as seeds in search of a new home; thus contributing to the colonization of new surfaces (Manz *et al.,* 1999; Lindsay & von Holy, 2006; Blaschek *et al.,* 2007). Biofilms are also known to be self-regulating because as a biofilm grows, pieces break off and disperse into the flowing water through the detachment process then enabling new cells to adhere and more biofilm matrices to develop (Hood & Zottola, 1995).

Detachment of individual cells or groups of cells from a biofilm is a cause for concern to public health as mentioned above (Hood & Zottola, 1995). The introduction of harmful bacteria through detachment from biofilms into the water system can have ramifications on the formation of additional biofilms and contamination of the drinking water supply, which potentially can have vast implications on the public health system (Percival *et al.,* 2000).

2.4.2. Factors Affecting Biofilm Formation

The present study investigates how a number of environmental conditions influence cell adherence and biofilm formation of *Enterobacter* spp. isolates from a drinking water distribution system. In order to better understand coliform bacteria and their cell adherence and biofilm formation in the distribution system, the conditions tested included temperature, environmental isolate variance, material surface, soiling of the surface, chlorine, and nutrient availability. A number of these environmental factors have been proven to influence cell adherence and biofilm formation for other bacterial species.

2.4.2.1. Temperature

Temperature is an important factor in the adherence and biofilm formation by many bacteria. A study by Kim *et al*. (2006) assessed the effect of temperature on the adherence of what is now known as *Cronobacter sakazakii* but was formerly *Ent. sakazakii* to SS surfaces. *Ent. sakazakii* strains were grown at two different temperatures, 12 and 25°C, in the same media. Two strains of *Ent. sakazakii* adhered to SS in significantly ($P<0.05$) higher numbers at 25°C compared to 12°C. Three other strains similarly tended to adhere better at 25°C than at 12°C, however, this was not significant. Several studies have shown that the temperature of the water in the pipes of a water distribution system change with the season resulting in a seasonal effect (Percival *et al.,* 2000; Donlan, 2002). This seasonal temperature variation was reported by a number of water utilities that had issues with coliform regrowth in their distribution systems (Geldreich, 1996). These water utilities noted that increases in bacterial population typically occurred in late spring, summer, and autumn where warmer water temperatures existed (Geldreich, 1996). This seasonal effect would also be observed in the Lexington, Kentucky, and Halifax, Nova Scotia, drinking water distribution systems. This seasonal effect would likely display temperatures similar to those observed by LeChevallier *et al.* (1996) ranging from approximately 5°C in February to 23°C in August, September and even in October. Due to geographic differences and therefore climate temperature variances it is likely that the temperatures in the Lexington, Kentucky drinking water distribution system might be slightly warmer than those in Halifax. Warmer water temperatures have been suggested to play an important role in the proliferation of coliforms (Camper *et al.,* 1999) and in the rate of microbial adherence to pipe surfaces in the distribution system (Donlan, 2002). Water temperatures above 15°C are known to speed up the growth of adapted microorganisms persisting in the water distribution system (Geldreich, 1996). This may ultimately lead to biofilm formation (Donlan, 2002).

Geier (2008) suggests that the optimal growth temperature for a bacterium is not necessarily optimal for biofilm formation for that bacterium. It is proposed that temperatures above the optimal growth temperature prompt a range of bacterial stress responses that can enhance biofilm formation. Since the water temperature in a distribution system would rarely reach above the optimum growth temperature for human pathogens or fecal microorganisms (>37°C), this stress response associated with high temperatures may be of little importance in the

drinking water distribution system. Of more relevance to the water distribution system, temperatures lower than optimal growth temperatures of bacteria have also been suggested to be linked with biofilm formation (Geier, 2008).

An example, of where biofilm formation is enhanced at a lower temperature than the bacteria's optimal growth temperature, is with *L. monocytogenes*. *L. monocytogenes* optimal growth temperature is 37°C but its optimal biofilm formation temperature has been determined to be 20°C for a number of strains (Geier, 2008). Similar to these findings, Norwood & Gilmour (2001) found adherence of *L. monocytogenes* to SS surfaces was greatest at 18°C when compared to both 4 and 30°C. Greater cell adherence and biofilm formation observed at a lower than optimal growth temperature is also observed with other bacteria such as *Le. pneumophila* and *E. coli*, whose optimal growth temperatures are 36 and 37°C, respectively, while their optimal biofilm formation temperatures are 20 and 23°C, respectively (Geier, 2008). In *E. coli*, 23°C was revealed to be a cue for biofilm formation (Geier, 2008). Similar to the higher temperatures inducing stress responses in a number of bacteria as mentioned above, a reason for greater adherence observed at lower than optimal growth temperatures may be due to induction of a number of stress-related genes. In Gram-negative and Gram-positive bacteria, this can be done under the control of the general sigma factor regulators of the stress response RpoS and SigB, respectively.

Chauret *et al.* (2001) also found that seasonal temperatures affect cell adherence and in a study observed greater cell adherence by *Aeromonas* spp. with increasing temperatures in the range from 5 to 20°C. Smoot & Pierson (1998) found a similar trend of greater cell adherence of *L. monocytogenes* on both SS and Buna-N rubber surfaces with increasing temperature from 10, 30 to 45°C. Perhaps if they had used 20°C, which has been found to be optimal for biofilm formation for many *L. monocytogenes* strains, they might have found that adherence at 30 and 45°C were lower than at 20°C similar to the report by Geier (2008).

Other reasons that might account for the variances in the effect of temperature may be associated with temperature affecting the physiological characteristic of the microorganism and its surface properties related to adherence (Geier, 2008). Temperature has been found to regulate the production of fibrils, flagella and EPS which in turn affects biofilm formation based on their functions to triumph over the electrostatic repulsion forces between bacterial cells and a surface (Chen, 2009). Norwood & Gilmour (2001) construed two possible explanations for greater cell

adherence at 18°C, which is below the optimal growth temperature for *L. monocytogenes*: one) *L. monocytogenes* produces EPS at 21°C but not at 10 or 35°C, or two) *L. monocytogenes* possesses numerous flagella at 20°C but only a small number of flagella at higher temperatures such as 37°C (Norwood & Gilmour, 2001). The production of EPS, higher numbers of flagella and greater cell adherence to surfaces at lower than optimal growth temperatures may also be found in other bacteria such as *Le. pneumophila*, *E. coli* and *Ent. cloacae*.

2.4.2.2. Strain Variation

Various species and strains of bacteria differ in their cell adherence and biofilm formation. A study by Norwood & Gilmour (1999) found that persistent *L. monocytogenes* strains adhered in significantly ($P<0.05$) higher numbers with a mean value of 5.67 $\log_{10}$ colony forming unit (CFU)/cm^2 than sporadic strains, which adhered with a mean value of 5.45 $\log_{10}$CFU/cm^2. Trachoo (2007) demonstrated that mean adherence of *Pseudomonas aeruginosa* was significantly ($P<0.05$) higher than *E. coli*, with the mean adherence of *P. aeruginosa* being 1.2 $\log_{10}$CFU/cm^2 higher than *E. coli*. This study also found a significant strain effect when comparing different strains within a species. Marouani-Gadri *et al.* (2009) found *E. coli* 0157:H7 pathogenic and non-pathogenic strains to vary in their cell adherence and biofilm formation to surfaces and that this strain variability may be affected by a combination of factors including temperature, material and medium. Vivas *et al.* (2008) demonstrated strain-related variability in the cell adherence and biofilm formation among strains of *Hafnia alvei*. It seemed as though strains from different sources displayed varying capabilities to adhere to surfaces and form biofilms. A study by Iversen *et al.* (2004) provides additional evidence that strain variance exists; this study found denser biofilms by a capsulated strain versus a non capsulated strain of what is now known as *Cronobacter sakazakii* (formerly *Ent. sakazakii)*. Reisner *et al.* (2006) showed that a number of naturally occurring *E. coli* strains varied in their biofilm forming abilities. In this study other factors such as growth medium composition were shown to have an effect on biofilm forming ability.

In another study by Lunden *et al.* (2000) persistent strains from a poultry plant were 2.7 to 4.6 fold higher in their initial cell adherence than most of the non-persistent strains. However, after prolonged cell adherence and 72 hours incubation several of the non-persistent strains

reached cell adherence levels comparable to that of the persistent strains suggesting contact time as a contributing factor (Lunden *et al.,* 2000). Chae & Schraft (2001) compared two *L. monocytogenes* strains with respect to cell adherence and biofilm formation. They found that one of the *L. monocytogenes* strains displayed both a higher initial cell adherence and faster growth rate than the other strain. In contrast, other studies have shown that different strains of bacteria had similar cell adherence in $\log_{10}$CFU/cm^2, regardless of their origin. A study by Harvey *et al.* (2007) found 36 persistent and 32 sporadic *L. monocytogenes* strains to be similar in their biofilm forming abilities when assessed after incubation for 48 hours at 20°C in a microtitre plate assay.

Some reasons for this could be attributed to the difference in motility or colony morphology. Lunden *et al.* (2000) found that *L. monocytogenes* serotype 1/2c had good adhering ability to surfaces; they suggest that this may be attributed to the difference in its flagellar antigen compared to the other serotypes. When observing motility and cell adherence, a non motile strain exhibited the poorest adhering ability to surfaces (Lunden *et al.,* 2000). Flagella have continually demonstrated their role in the initial adherence of bacteria such as *E. coli*, to surfaces (Lunden *et al.,* 2000). Todhanakasem & Young (2008) found that flagellar motility was a contributing factor to biofilm formation in a study using the static-microtitre-plate assay. However, they also found that loss of flagellar motility alters the dynamics of *L. monocytogenes* biofilm development when using a flow cell experimental design. Examination of motility mutants where there was deletion of flagellar genes showed an initial lag in surface adherence of bacteria compared to a wild-type strain with no deletion of flagellar genes. Over time the mutants progressively formed surface-adhered communities to a greater extent than the wild-type strain which did not have the deletion of flagellar genes. This study suggests the loss of flagellar motility may lead to the formation of hyperbiofilms which are defined as surface adhered communities of high density (Todhanakasem & Young, 2008).

2.4.2.3. Test Material Surface

Throughout the world different materials are used in construction of pipes for water distribution systems, these include copper, stainless steels, galvanized steels, plastics, lead and cast iron (Parizzi *et al.,* 2004; Beech & Sunner, 2006). These materials can be susceptible to

microbial colonization under both a flowing and static regime (Beech & Sunner, 2006). The type of pipe material used in the system has been argued to affect the microbial colonization of the surface including, cell adherence, microbial growth and biofilm formation (Geldreich, 1996; Vatanyoopaisarn *et al.,* 2000; Skinner, 2003; Tam & Conner, 2007). Most investigators have found that microorganisms adhere more rapidly to hydrophobic, nonpolar surfaces such as Teflon and other plastics than to hydrophilic materials such as glass or metals (Donlan, 2002). This finding agrees with Ryu *et al.* (2004a) who tested adherence to two surfaces, SS which is a metal and moderately hydrophilic and PVC which is a hydrophobic plastic. Camper *et al.* (1999) stated that iron surfaces in particular are prone to substantial microbial colonization and have been implicated as a key component in microbial regrowth distribution systems with iron pipes. Kim *et al.* (2006) reported that *Enterobacter* spp. including *Ent. sakazakii* was able to adhere to and form biofilms on silicon, latex, polycarbonate, SS, glass, and PVC. *Ent. sakazakii* adhered to the highly hydrophobic PVC feeding tubes in higher numbers than to the hydrophilic SS. Research has generally shown most bacteria adhere more freely to hydrophobic surfaces.

In another study by Iversen *et al.* (2004), *Ent. sakazakii* formed a biofilm on latex, silicon and to a lesser extent SS surfaces associated with production of infant formula. This study showed a variation in the adherence of microorganisms depending on the surface material. The hydrophobicity of the surfaces was not given in the study but perhaps the latex and silicon were more hydrophobic than the SS. Chavant *et al.* (2002) showed that the nature of the surface was one of the main factors that statistically affected adhesion and colonization of *L. monocytogenes*. In another study, *L. monocytogenes* was found to adhere to PVC in much higher numbers than to SS, and this was inferred to be due to different hydrophobicities of the surfaces (Djordjevic *et al.,* 2002). Furthermore, when rougher SS was compared to electron-polished non rough SS the rougher SS attracted 1.4 times more microorganisms (Momba & Makala, 2004).

In contrast to the findings of Kim *et al.* (2006), no differences were observed in the number of microorganisms adhered to both hydrophobic electron polished SS and PVC (Momba & Makala, 2004). This suggests material surface did not affect the number of adhered cells to the surface although the SS surface was recorded as hydrophobic electron polished. Similar results were published by Parizzi *et al.* (2004) who reported that the number of adhering *L. innocua* or *Staphylococcus aureus* cells were the same regardless of the surface material.

2.4.2.4. Nutrient Availability

The level of nutrients available for microorganisms in a water distribution system affects the adhesion of bacteria to the pipe material (Geldreich, 1996; Vatanyoopaisarn *et al.,* 2000; Donlan, 2002; Tam, 2006; Tam & Conner, 2007). Furthermore, when bacterial cells have become irreversibly adhered to a pipe surface forming a biofilm, its growth potential is affected by the nutrients available in its direct environment (Percival *et al.,* 2000; Dunne, 2002). The effect of nutrient availability on bacterial adherence has also been found to be species-specific and can differ depending on bacterial strain (Tam, 2006; Tam & Conner, 2007; Geier, 2008).

Some studies have shown increased bacterial cell adherence in low nutrient environments (Tam, 2006; Tam & Conner, 2007). These low nutrient oligotrophic environments seem to support biofilm growth in drinking water, more than they support planktonic free floating cells (Carpentier & Cerf, 1999; Beech & Sunner, 2006). Nutrient availability is known to have an effect on biofilm formation and can encourage changes in cell physiology and composition (Allan *et al.,* 2002). *E. coli* begins forming biofilms in response to nutrient limitation as several macromolecules and nutrients hoard together on the surface (Geier, 2008). Hood & Zottola (1995) also noted the higher concentration of nutrients near the pipe surface as opposed to the flowing water in a distribution system and that this contributes to increased cell adherence during nutrient limitation. Moreover Skinner (2003) made the point that in a low nutrient environment, extracellular polymers assist in accumulating nutrients from the free flowing water to the surface of the biofilm. *Ent. cloacae* can grow well below the nutrient concentrations necessary for *E. coli* (Geldreich, 1996). This helps to demonstrate that cell adherence and biofilm formation may contribute to the survival of the microorganism in a low nutrient environment. In this study nutrient availability was evaluated and although an increase in nutrients resulted in a greater number of adhered cells to a surface, very low levels of nutrients were compared. The nutrients in tap water alone were compared to the nutrients in tap water with the addition of 0.1 % peptone. Tap water is generally considered to have a low number of nutrients.

In contrast, Donlan (2002) states that an increase in nutrient levels up to a critical limit may result in increased adherence. Other studies have shown increasing bacterial cell detachment in a low nutrient environment (Tam, 2006; Tam & Conner, 2007). Skinner (2003) argues that the presence of nutrients in drinking water distribution systems encourage microbial growth, meaning bacteria flourish in a more nutrient rich environment. For some species, starvation is a

signal for the cells to detach from a biofilm and spread out in search for a more nutritious environment (Geier, 2008). *P. aeruginosa* is an example of a bacteria species that forms biofilms in nutrient-rich environments. When grown in M9 medium, *P. aeruginosa* forms a thick, multilayer biofilm; whereas when grown in the less nutritious FAB medium, the bacteria only formed a sparse monolayer (Geier, 2008). In this study increasing the nutrients available in the model system resulted in greater numbers of adhered cells to the surface. This agrees with the studies by Donlan (2002), Skinner (2003) and Geier (2008).

Other evidence to suggest that nutrients have an effect on cell adherence and biofilm formation was found in a study by Kim *et al.,* (2008), where results indicated that infant formula provided greater protection for adhered cells, as well as cells in a biofilm when compared to M9 medium. They concluded that the medium had an effect on cell adherence and biofilm formation. This compares with Geier (2008) who states that the composition of the growth medium is an important factor in the biofilm formation observed during biofilm studies in the laboratory.

2.4.2.5. Preconditioning of the Surface

An important phenomenon in the initial adhesion of bacteria to pipe and other surfaces is surface conditioning (Kolari, 2003; Trachoo, 2007). This term is used to describe how when a clean material surface is exposed to an aqueous medium, interactions between the surface and the medium will begin immediately to "condition" the surface (Donlan, 2002; Kolari, 2003). This was demonstrated by a study from Loeb & Neihof (1975) who discovered that conditioning films on surfaces exposed in seawater formed within minutes and continued to grow for several hours. This interaction between the medium and the surface may involve the surface adsorbing polymers, inorganic salts, proteins, glycoproteins and humic compounds to the surface (Donlan, 2002; Kolari, 2003). The thickness of these conditioning films has been found to range from 30 to 80 nm (Kolari, 2003). Pre-treatment of a surface by soiling the surface with nutrients to produce a conditioning film, as was done in the present study, has been suggested to affect the rate and extent of microbial adherence. It provides an ideal environment for the adherence and growth of microorganisms (Donlan, 2002). In general bacterial cell adherence will occur more often on surfaces that are rougher, more hydrophobic and coated by these conditioning films (Donlan, 2002).

2.4.2.6. Time

A relationship between bacterial cell adherence and biofilm formation, and incubation time has been identified. Longer incubation periods have been found to result in greater bacterial cell adherence and biofilm formation. This may be a result of the time required to enable the bacteria to adhere to a surface and form a biofilm (Chen, 2009). In a study by Herald & Zottola (1988) the influence of incubation time on cell adherence to SS was observed. In their experiment, the number of adhered cells increased with increasing incubation time. Moreover when examining the SS pieces of metal, single cells were seen at earlier stages of the incubation period whereas pairs and smaller groups of cells were seen later in the incubation period. This is comparable to the results found in a study by Fletcher (1979) who found that the number of adhered cells rose with increasing incubation time to a polystyrene surface. This can be explained by a longer incubation time offering more time for cells to adhere to the surface and collide with one another creating a biofilm as suggested above by Chen (2009). In the study by Lunden *et al.* (2000), adherence of *L. monocytogenes* cells after 72 hours was 100 to 1000 fold higher than after shorter contact times. When comparing persistent strains and non-persistent strains adherence was initially much higher for the persistent strains when incubated up to 72 hours. Whereas after 72 hours there was no difference between the groups indicating that strain differences in cell adherence to surfaces may depend on the length of the contact time (Lunden *et al.,* 2000). Hu *et al.* (2008) also found that the number of bacterial cells adhering to aluminum increased over time.

2.4.2.7. Chlorination

Disinfection is used to treat drinking water in order to protect consumers against waterborne disease (Lee & Nam, 2005). The effective use of these disinfectants and minimization of consumer exposure to any by-products is an area of concern (Lee & Nam, 2005). Disinfectants that have found use in drinking water treatment include chlorine (Cl_2) either in gaseous or liquid form, chloramines, chlorine dioxide and other chlorine compounds (Batte *et al.,* 2003; Lee & Nam, 2005; Anonymous, 2011i). These disinfectants are useful for controlling

fecal coliforms, TC and heterotrophic bacteria which are often found in water. There are two types of disinfection, primary disinfection at a treatment plant and secondary disinfection, which entails adding free chlorine or total chlorine to water after it has left a treatment plant (Krentz, 2008). This study focused on secondary disinfection and the disinfectant residual free chlorine in the water from a drinking water distribution system in Halifax, Nova Scotia that was taken directly from a consumers tap. This water was used as it was unable to obtain the water from the Lexington, Kentucky water distribution system. The residual free chlorine that was measured in the distribution system would vary slightly from time to time of sampling. Chlorine and free chlorine work by limiting bacterial regrowth (Percival *et al.,* 2000; Batte *et al.,* 2003). The mechanism where free chlorine inactivates microorganisms has not been elucidated. A few factors that may play a role in the inactivation of the microorganisms include oxidation of sulfhydryl enzymes and amino acids; ring chlorination of amino acids; loss of intracellular contents; decreased uptake of nutrients; inhibition of protein synthesis and decreased oxygen (Anonymous, 2011j). The chlorine injures bacteria preventing growth and the multiplication of bacteria in both the planktonic or biofilm state (Batte *et al.,* 2003). If water in a distribution system contains silt and organic matter bacteria may 'hide' and become protected from chlorine; chlorine is only effective if it comes into immediate contact with bacteria (Anonymous, 2011d). Chlorine may also react with the pipe material and not be available for disinfection. Any remaining chlorine that is not used up after reaction with metals and organic material is referred to as total chlorine. This total chorine may, however, react with nitrates and become unavailable for disinfection. If any remaining chlorine exists after reaction with nitrates it is referred to as free chlorine which is available to disinfect organisms (Anonymous, 2011a).

As mentioned above the inactivation of bacteria usually involves the use of disinfectants such as free chlorine which exists in equilibrium between undissociated hypochlorous acid (HOCl) and dissociated hypochlorite ion (OCl^-). Hypochlorous acid is a weak acid that is a 100-fold more effective as a disinfectant when compared to the hypochlorite ion. Factors that can affect the efficiency of disinfection with chlorine include pH, which is a measure of the hydrogen ion concentration and measured on a scale of 0 to 14 with 7 being neutral. Over 90% of the free chlorine at pH 6 is found in the form of hypochlorous acid whereas only 2.9% is in the form of hypochlorous acid at pH 9 with the remaining occurring in the form of hypochlorite ions. At a pH of 7.5, which is similar to the pH-values found in drinking water from both the Lexington

(Kentucky) and Halifax (Nova Scotia) water distribution systems, there will be a relatively equal ratio of hypochlorous acid to the hypochlorite ion. Hence pH affects chlorine's disinfection efficiency due to its effect on the dissociation of hypochlorous acid (Galal-Gorchev, 1996; Anonymous, 2011i, Anonymous, 2011j). The lower the pH the lower the CT value (defined as disinfectant concentration (C) multiplied by contact time (T)) needed for inactivation of the microorganisms in turn due to the presence of increasing concentrations of the undissociated form of hypochlorous acid (Krentz, 2008, Friedman *et al.,* 2009).

Some evidence to suggest that chlorine affects cell adherence and biofilm formation is discussed in the following paragraphs. Camper *et al.* (1999) stated that the structure of biofilms that underwent chlorine treatment were different compared to biofilms not exposed to a chlorine treatment. Cells in chlorinated biofilms were stated to be smaller in size, more rounded in shape and patchier with clumps. This implies a physically different appearance of the chlorinated biofilm. Chu *et al.* (2003) found heterotrophic plate counts of biofilm bacteria to be roughly two to three fold higher in the absence of chlorine than in the presence of any level of chlorine; this study did not investigate the effect of nutrient content on cell numbers adhered to surfaces and chlorine. No significant (P>0.05) difference was observed when comparing plate count numbers from water with a low or high level of chlorine (Chu *et al.*, 2003). Similar to the results reported by a number of researchers, Lomander *et al.* (2004) observed that the presence of chlorine in water decreased biofilm formation. When a low level of chlorine was added to water this solution killed significant (P<0.05) amounts of bacteria in the biofilm compared to controls, thus suggesting chlorine can reduce bacteria in biofilms. In a study by van der Wende *et al.* (1989), chlorine was found to reduce biofilm accumulation. In conclusion, literature suggests that the number of adhered cells on a surface or the amount of accumulated biofilm on a surface should decrease with higher levels of chlorine in the water. Chlorine in a number of cases has been found to reduce biofilm formation.

2.5. Strategies for Removal of Biofilms in the Drinking Water Distribution System

It is not feasible to completely prevent the adherence of bacteria to surfaces in the drinking water distribution system (Lindsay & von Holy, 2006). Biofilms form in distribution systems and other places, such as food processing plants, and are in general difficult to remove and eliminate (Carpentier & Cerf 1999). Strategies to eliminate biofilms include, cleaning and

disinfection (Carpentier & Cerf 1999; Corry & Allen 2000; Lindsay & von Holy, 2006). Cleaning entails mechanical force using brushes, high-pressure water jets, sprayers, and scrubbers, or high velocity of cleaning solution to increase shear stress in pipes and on material surfaces (Carpentier & Cerf 1999; Lindsay & von Holy, 2006). Carpentier & Cerf (1999) suggest that this physical biofilm removal strategy produces immediate maximal effects and any prolonging treatment will not improve results. Disinfection used in conjunction with cleaning will further improve removal and is often used after cleaning in order to inactivate or remove any organisms that remain on the surface (Carpentier & Cerf 1999; Lindsay & von Holy, 2006). The main goal of disinfection is to inactivate the microorganisms although some microorganisms in biofilms have a high resistance to disinfectants and antimicrobial agents (Carpentier & Cerf 1999; Corry & Allen 2000; Lindsay & von Holy, 2006). Such microorganisms may be more difficult to remove from the system. If the disinfection strategy is used frequently it is more likely to inactivate and kill new bacteria and pathogenic organisms introduced into the biofilm as these bacteria may be less resistant to the disinfectants than older "persistent" microorganisms left behind from previous treatments (Carpentier & Cerf 1999).

2.6. Current Drinking Water Regulations in Canada and the United States of America

Both Canada and the United States consider microbiological water quality to be of great importance in the safety of drinking water that reaches consumers' taps across both nations. Therefore, Drinking Water Guidelines have been set by both the Canadian and American governments, which recommend the guidelines being adopted by the provinces and states (Payne, 2007).

In Canada, the microbial quality of drinking water is tested by the presence of TC and *E. coli*. Canadian drinking water guidelines state that the maximum acceptable concentration of *E. coli* or TC in water leaving a treatment plant in a public, semi-public and private supply system is less than one cell per 100 ml of water sampled. The Health Canada Drinking Water Guidelines are established by the Federal-Provincial-Territorial Committee on Drinking Water. These guidelines are used in every jurisdiction in Canada and are the basis of drinking water requirements. Each province or territory is generally responsible for regulating their own drinking water and enforcing legislation (Anonymous, 2011b). Municipalities are usually

responsible for the treatment and distribution of the water. In Nova Scotia, drinking water regulators have implemented the Guidelines for Canadian Drinking Water Quality published by Health Canada and established by the Committee on Drinking Water as legally binding standards for regulated public drinking water supplies and recommended these guidelines for private well owners (Anonymous, 2011g). These regulations require public drinking water supply owners to test their water on a regular basis and to inform customers and Nova Scotia Environment if a problem occurs and take the proper corrective action to address the issue. The number, frequency, and location of samples for TC and *E. coli* testing will differ depending on the type and size of the distribution system and jurisdictional requirements. When less than 10 water samples are collected in a specified sampling period no TC should be present. When more than 10 samples are collected in a specified sampling period no consecutive samples from a sampling location should have TC present. Also, less than 10 percent of all water samples should be positive for TC or *E. coli* (Anonymous, 2011g).

The drinking water guidelines set by Health Canada are similar to those set by the United States. In the United States the quality of drinking water is regulated by the Environmental Protection Agency (EPA) under the Safe Drinking Water Act. The National Primary Drinking Water Regulations are the drinking water standards set and enforced to protect the public from different levels of contaminants in drinking water including microorganisms such as TC. Under the Safe Drinking Water Act the EPA allows individual states the opportunity to set and enforce their own drinking water standards as long as they meet the minimum requirements of the EPA. The EPA sets a maximum contaminant health goal which is zero TC; however, this is not enforced. The EPA also sets a maximum contaminant level, which is the legal limit for TC. The maximum contaminant level states that for water systems no more than five percent of samples tested may be positive for TC in a month and when collecting less than 40 routine samples in a month no more than one of these samples tested may test positive for TC. If the maximum contaminant level is exceeded and more than five percent of samples tested contain TC water system operators must report their results to the state authority and the general public. The presence of more than five percent TC positive samples may indicate treatment failure or a problem in the distribution system warranting a number of corrective actions to avoid or eliminate contamination. These measures could include system repair, flushing the distribution system, repairing treatment equipment, or endorsing source water protection and prevention

programs. When a positive TC is found a sample must be repeated and taken within 24 hours and tested for the presence of fecal coliforms or *E. coli*. If the sample is confirmed as being positive for the presence of fecal coliforms or *E. coli* it is considered a violation and the state and public are to be contacted immediately. The number and frequency of sampling a water system operator must carry out is based on the population served by the utility (Anonymous, 2011e).

2.7. Coliform Bacteria as the Indicator Organism of Water Quality in the Distribution System

Total coliforms are facultative anaerobic, Gram-negative, non-spore forming and rod shaped bacteria that ferment lactose (Cohn *et al*., 1999; Ashbolt *et al*., 2001; Rompre *et al*., 2002) and are oxidase negative (Ashbolt *et al*., 2001) and catalase positive (Percival *et al*., 2000). Total coliforms are capable of growth at 37°C and possess β-galactosidase which is used to ferment lactose to produce acid and gas (Percival *et al*., 2000).

Total coliforms are part of the *Enterobacteriaceae* family; they are a group of closely related bacteria that have been used for years as the fecal contamination indicator of choice for drinking water (Cohn *et al*., 1999). The use of bacteria as indicators of the sanitary quality of water most likely dates back to the 1800's (Ashbolt *et al*., 2001) yet, the use of coliforms as an indicator of the potential presence of enteric pathogens in aquatic systems has been a topic of scientific debate for many years (Rompre *et al.,* 2002, Skinner, 2003). The concept behind use of TC as indicator organisms is the assumption that coliforms and fecal coliforms (*E. coli*) in drinking water indicates possible fecal contamination and therefore the potential presence of other enteric pathogens (Montville & Matthews, 2006). However, Ashbolt *et al*. (2001) proposes that the use of coliforms as an indicator comes with some concerns as the presence of a number of microorganisms in the TC group and a few fecal coliforms may not be specifically tied to fecal contamination. For example, species of *Klebsiella* and *Enterobacter*, and even a few strains of *E. coli* have been shown to grow in a natural aquatic environment (Ashbolt *et al*. 2001). Another limitation in the use of TC as an indicator organism of external fecal contamination is that under specific situations TC may proliferate in a biofilm, where the bacteria may be more resistant to disinfection as compared to planktonic cells (Cohn *et al*., 1999; Anonymous, 2006). Also, fecally derived viruses and protists may be even more resistant to disinfection than the fecal indicator bacteria (Cohn *et al*., 1999; Anonymous, 2006). This suggests that TC may not be

good indicators for predicting the presence of pathogenic viruses and protists due to differences in biological resistance to disinfectants and the potential protection offered in biofilms. Nonetheless, TC and *E. coli* remain the legal indicators of drinking water microbial quality in both Canada and the United States (Cohn *et al*., 1999; Montville & Matthews, 2006; Health Canada, 2011c; Anonymous, 2006).

The coliform group consists of a broad diversity in terms of genus and species. Most definitions of coliforms are based on common biochemical characteristics, which can differentiate genus and species (Rompre *et al*., 2002). The TC group consists of a number of genera including, but not exclusive to: *Enterobacter*, *Citrobacter*, *Klebsiella*, *Serratia* and *E. coli* (Cohn *et al*., 1999; Percival *et al*., 2000). Of the genera mentioned above, several species: *Ent. cloacae*, *Klebsiella oxytoca*, *C. freundii*, *Enterobacter agglomerans*, *Klebsiella pneumoniae*, and *E. coli,* have been found to have the ability to grow in biofilms on pipe surfaces (Geldreich, 1996; Anonymous, 2006). These and other TC may occur in animal feces, naturally in soils, decaying plant material and may proliferate in water rich in nutrients (Geldreich, 1996; Percival *et al*., 2000). Environmental conditions that are considered favourable by Anonymous (2006) to the colonization of TC on surfaces and to biofilm formation in a distribution system are water temperatures above 15°C, neutral pH, and adequate assimilable organic carbon concentrations.

2.7.1. Biology of the Coliform *Enterobacter* Genus

2.7.1.1. Morphology, Physiology and Ecology

Enterobacter are coliform bacteria that are phylogenetically grouped in the family *Enterobacteriaceae* (Bergey & Holt, 1994; Huber, 2000). They are Gram-negative, facultative anaerobic straight rods that are approximately 0.6 - 1.0 μm in width and 1.2 - 3.0 μm in length (Bergey & Holt, 1994; Ray & Bhunia, 2008). Almost all species are motile by peritrichous flagella and are mesophiles that grow favourably between 30 to 37°C, with an optimal temperature of 30°C (Bergey & Holt, 1994; Grimont & Grimont, 2006). Currently there are 11 known species compiling the genus (formerly 12 with *E. sakazakii*), including both *Ent. cloacae*, and *Ent. asburiae* (Grimont & Grimont, 2006). The genus differs from other *Enterobacteriaceae* biochemically (Bergey & Holt, 1994). Most *Enterobacter* are positive for the Voges-Proskauer

test (a method for detecting the production of acetoin), Simmons citrate and ornithine tests (Bergey & Holt, 1994; Huber, 2000; Grimont & Grimont, 2006). Most are negative for hydrogen sulfide production from thiosulfate, and in the urea and lysine decarboxylase tests (Bergey & Holt, 1994; Huber, 2000; Grimont & Grimont, 2006). *Enterobacter* spp. can also be distinguished from many *E. coli* using an indole test where *Enterobacter* spp. are indole negative (Bergey & Holt, 1994; McLellan *et al*., 2001).

Information gathered from research dating back to the 1950's has suggested that most or all of the 11 species (formerly 12 species) of *Enterobacter* are widely dispersed in nature (Janda & Abbott, 2006). The genus has been found in the environment in habitats such as water, sewage, vegetables, and soil (Grimont & Grimont, 2006). It has been frequently isolated from fresh and frozen produce including lettuce, cabbage, cucumbers, tomatoes, celery, radishes, sprouts and fresh fruit (Huber, 2000; Janda & Abbott, 2006). The genus has also been isolated from many vertebrate and invertebrate hosts including pinnipeds, buzzards, raptors and reptiles (Janda & Abbott, 2006). *Ent. cloacae* is ubiquitous in nature, it is found in both the environment and in clinical isolates (Janda & Abbott, 2006). It is part of the normal flora of the gastrointestinal tract of 40 – 80% of humans (Keller *et al.,* 1998) and it has also been isolated from soil, sewage and water (Stolp, 1988). Of the *Enterobacter* spp., *Ent. cloacae* and *Enterobacter aerogenes* are the most common species of the genus encountered in clinical, food and environmental samples (Huber, 2000).

2.7.1.2. Clinical Significance

There is some uncertainty regarding the clinical significance and pathogenicity of all *Enterobacter* spp. Some researchers state that certain species of the genus *Enterobacter* are clearly opportunistic pathogens and rarely cause disease in a healthy individual (Stolp, 1988; Sanders & Sanders, 1997). Before the extensive use of antibiotics in treating infections, *Enterobacter* spp. were not often found as opportunistic pathogens, but these organisms are now increasingly found to be causing many nosocomial infections (Huber, 2000; Grimont & Grimont, 2006; Janda & Abbott, 2006).

In particular, *Ent. cloacae* has been associated with a variety of infections such as endocarditis, ventriculitis, meningitis, arthritis or osteomyelitis, urinary tract infections,

bloodstream infections and pneumonia (Huang *et al.*, 2001; Janda & Abbott 2006; Grimont & Grimont, 2006). The role of *Enterobacter* spp. in causing cases of hospital acquired pneumonias has rapidly increased over the past 20 years (Janda & Abbott, 2006). *Enterobacter* spp., including *Ent. cloacae*, can also be isolated from wound infections including from polymicrobic infections of the skin, muscle, subcutaneous tissues, fascia and have been associated with infections of the bone (Janda & Abbott, 2006). The importance of *Enterobacter* spp. as an opportunistic pathogen may be attributed to its increased resistance to antibiotics. For example, meningitis caused by *Enterobacter* spp. is now frequently complicated by the resistance of many *Enterobacter* spp. isolates to third generation cephalosporins and poor central nervous system penetration of other antibiotics (Huang *et al*., 2001). This may be in part due to *Enterobacter* spp. commonly containing chromosomally mediated, depressed type I β-lactamases (Huang *et al*., 2001). The clinical significance of *Ent. asburiae*, another *Enterobacter* spp. studied in this thesis, is unknown (Grimont & Grimont, 2006).

Other evidence suggests that some *Enterobacter* spp. may be more than opportunistic pathogens. Although there is little evidence of *Enterobacter* spp. as a primary animal pathogen, the species can be recovered from the brain, liver or lungs suggesting a contributory role in the disease process (Janda & Abbott, 2006). Water is often the vector for transmission of *Enterobacter* spp. because the bacteria are found in the environment and easily transferred into water, where the bacteria's survival may be supported (Huber, 2000).

Since the 1980's the clinical awareness of the potential of *Ent. cloacae* strains to cause disease has been reflected in the increasing number of epidemiological studies of these microorganisms showing that they are a cause of nosocomial Gram-negative infections (Keller *et al.,* 1998). *Ent. cloacae*, isolated from an operating drinking water system, were able to multiply under the conditions found in municipal water systems (Huber, 2000). Furthermore these researchers conducted survival studies of *Ent. cloacae* in water and found a slow death rate causing the organisms to remain viable for long periods of time. Also, in one outbreak the source of *Ent. cloacae* bacteraemia was traced to tap water having contaminated the pressure-monitoring equipment used in open heart surgery while another outbreak that resulted in 129 hospital *Ent. cloacae* acquired infections, was traced to a drain pipe leaking water (Huber, 2000).

Chapter 3: MATERIALS AND METHODS

3.1. Site Description

Lexington-Fayette county has an estimated population of 266,798, as of 2003 (Anonymous, 2007) and is served by the Kentucky-American Water Company. The Kentucky River is the main water supply source and the Jacobson Reservoir is used as an alternate source. Kentucky-American Water Company operates two treatment facilities. The Kentucky River Station has a treatment capacity of approximately 151,400,000 litres per day, and uses a conventional process of chemical treatment, flocculation, settling and filtration. Final treatment steps include chloramination, fluoridation, and addition of zinc orthophosphate for corrosion control. The second treatment plant, Richmond Road Station, has a treatment capacity of approximately 94,600,000 litres per day and can be supplied by the Jacobson Reservoir or the Kentucky River. The Richmond Road Station also uses conventional chemical treatment, flocculation, settling, filtration through granular activated carbon filters, post-treatment chloramination, fluoridation, and addition of zinc orthophosphate. The drinking water distribution system is more than 2,626 km long, and the pipes range in diameter from 50 mm to 915 mm. Pipe material consists of copper, gray cast-iron, ductile iron, asbestos cement, PVC, and pre-stressed concrete. There are seven in-line booster stations and eight pressure-monitoring vaults in the pipe network. A routine monitoring program regularly samples water from 16 different sites in the distribution system. Samples are analysed for a range of chemical and microbiological parameters, including the content of TC (Table 1). For this study, we obtained a collection of 18 coliform strains isolated over a two month period in 2006. Table 2 provides information about where and when the coliform isolates were obtained from the distribution system as well as the chlorine concentration at the site. In the evaluation of factors affecting cell adherence and biofilm formation of *Ent. cloacae* in this study it was not practical to obtain water from this Lexington-Fayette county utility. However, as a number of water parameters were relatively similar, Halifax tap water was used as a readily available alternate. The typical characteristics of drinking water from Lexington, Kentucky and Halifax, Nova Scotia are provided in Table 1.

Table 1: Water quality characteristics of Halifax, Nova Scotia and Lexington, Kentucky drinking water.

Parameter	Unit	Lexington, Kentucky	Halifax, Nova Scotia
Chlorine (Total/ Free)	mg/l	2.7	0.44
Alkalinity	mg/l	67.5	17
Chloride	mg/l	28.1	8.5
Iron	mg/l	0	<0.020
Sulfate	mg/l	76.8	8
% of samples positive for TC	cfu/ml	2	0.04
pH	Units	7.4	7.3
Turbidity	NTU	0.2	<0.1
Total organic carbon	ppm/ mg/l	1.09	1.4

(Friedman *et al.,* 2009; Anonymous, 2011k; Anonymous, 2011l; Anonymous, 2011m)

The water temperatures observed in the Lexington, Kentucky, and Halifax, Nova Scotia, drinking water distribution systems would likely display temperatures similar to those observed by LeChevallier *et al.* (1996) ranging from approximately 5°C in February to approximately 23°C in August, September and 17°C in October. Due to geographic differences and therefore climate temperature variances it is likely that the temperatures in the Lexington, Kentucky drinking water distribution system might be slightly warmer than those in Halifax.

Table 2: Origin of coliform isolates from the drinking water distribution system in Lexington, KY.

Isolate	Site[a]	City	Sample Date	Sample Time	Total chlorine concentration (mg/l)
KY 1	A	Lexington	10/10/2006	10:35	3.2
KY 2	B	Lexington	9/20/2006	8:50	3.1
KY 3	B	Lexington	9/21/2006	9:50	3.1
KY 4	C	Lexington	9/14/2006	9:55	2.6
KY 5	D	Lexington	9/8/2006	9:00	2.4
KY 6	E	Lexington	10/11/2006	13:55	2.8
KY 7	E	Lexington	10/12/2006	13:25	2.9
KY 8	F	Lexington	10/11/2006	9:10	3.6
KY 9	G	Lexington	10/12/2006	13:15	3.2
KY 10	H	Lexington	9/6/2006	11:10	2.7
KY 11	I	Lexington	8/8/2006	13:40	3.4
KY 12	J	Georgetown	9/19/2006	11:15	2.7
KY 13	K	Georgetown	9/5/2006	10:10	2
KY 14	L	Lexington	10/17/2006	9:45	3
KY 15	M	Lexington	10/17/2006	9:50	2.9
KY 16	N	Lexington	10/10/2006	8:55	2.6
KY 17	O	Lexington	10/17/2006	13:00	3.1
KY 18	P	Lexington	10/12/2006	13:10	3.2

[a] Letter designations refer to sampling points labelled on the map shown in Figure 1

3.2. Isolation of Waterborne Coliform Bacteria

Water samples of 100 ml were passed through a 0.45 μm filter. The filter was placed on a mEndo agar plate and incubated for 24 hours at 37°C to detect and enumerate TC. Distribution system water samples from Lexington, KY were initially analysed in a 100 ml Colilert presence-absence test for TC. Coliform isolates from positive Colilert tests were further subcultured, characterized and identified using the API 20E system (bioMérieux, St. Laurent, QC). A total of 18 coliform isolates from Lexington, KY were sent to Dalhousie University on agar slants and used to prepare stock cultures with 20% glycerol that were maintained frozen at - 77°C until further use.

3.3. Identification and Biochemical Characterization

3.3.1. API 20E

Enteric Gram-negative isolates were identified using the API 20E system (bioMérieux). Isolates were plated on Tryptic soy agar (TSA, Oxoid, Nepean, ON) and grown overnight at 37°C. Individual colonies from each strain were analyzed according to the manufacturer's instructions. The reactions were scored as positive or negative and used to generate a numeric profile, which was searched against the API 20E database to determine the most probable identity of the isolate.

3.3.2. Biolog

Biolog GN2 (Biolog, Hayward, CA) microplates are specific to Gram-negative bacteria. They use different carbon sources in 95 of the 96 wells of the microplate to produce a phenotypic profile and a most probable identity of the isolate. Each isolate was initially streaked onto sheep blood agar. The sheep blood agar was prepared by adding sheep blood provided by Hemostat Laboratories (Dixon, CA) to TSA (Oxoid) to a concentration of 5% (w/v) sheep blood. Plates were incubated at 37°C for 24 hours.

Cultures were then suspended in GN/GP inoculating fluid (0.40% NaCl, 0.03% Pluronic F-68, 0.02% Gellan Gum) (Biolog) containing 1 ml of 5 mM sodium thioglycolate. The absorbance was read using a turbidity meter. Isolates were then matched to the manufacturer's GN-ENT turbidity standard which was 178 Nephelometric Turbidity Units. The suspension was then poured into a sterile petri dish, and 150 µl was dispensed into the wells of the Biolog microplate using an eight-tip multi-channel pipette. These microplates were then incubated at 35°C for 16 hours. Individual wells contained media which either turned purple, indicating substrate utilization or a positive reaction, or remained colourless, indicating no growth or a negative reaction. The pattern of positive and negative results was entered into the Microlog computer program automatically by the Biolog reader and a database search was conducted to determine the most probable identity of the isolate.

3.3.3. Vitek

The GNI+ card (bioMérieux) was developed for the identification of certain species of aerobic and facultative anaerobic Gram-negative bacteria. All coliform isolates were streaked onto eosin methylene blue agar (Oxoid) made according to the manufacturer's instructions and incubated for 24 hours at 37°C. A 1.0 McFarland suspension of each organism was prepared in a volume of 0.45% (w/v) sterile saline to inoculate the GNI+ card. The saline was then divided into separate tubes for the inoculation of the individual cards. All cards were inoculated within 20 minutes of inoculum preparation, and a portion of each bacterial suspension was streaked onto a TSA plate to check for inoculum purity. The use of the transfer tubes, filling module, sealing module, and loading of the GNI+ cards into the reader/incubator tray were performed according to the Vitek operator's manual. The reactions were scored as positive or negative and used to generate a numeric profile, which was searched against the Vitek database to determine the most probable identity of the isolate.

3.4. Genotypic Characterization

3.4.1. Ribotyping

Ribotyping was conducted on the isolates at the Laboratory for Microbial Typing located at Cornell University, Ithaca, NY. The ribotyping was done according to the procedure described by Jeffers *et al.* (2001) using a RiboPrinter Microbial Characterization System (Du Pont Qualicon, Wilmington, DE). The process is automated and involves cell lysis and DNA digestion using the restriction enzyme *Eco*RI. DNA fragments were separated using gel electrophoresis and the DNA was analyzed using a modified Southern blot hybridization technique. The DNA fragments were transferred to a membrane and hybridized with a labelled universal ribosomal RNA operon probe, which was derived from *E. coli*. The location of bands containing any fragment of 16S or 23S ribosomal RNA genes that had hybridized with the probe was determined using a chemiluminescent substrate, and the bands were visualized using a customized charge-couple device camera. Ten of the 18 samples were provided blindly as duplicates to evaluate method accuracy equalling a total of 28 "unknown" samples.

3.4.2. Pulsed Field Gel Electrophoresis

Another standard genotyping method, PFGE, was used to generate a DNA fingerprint for the bacterial isolates. Bacteria were cultured overnight on TSA at 37°C. Cells were scraped off the plate using a sterile pipette tip and suspended into 1.5 ml of cell suspension buffer (100 mM Tris: 100 mM EDTA, pH 8.0). Cell density was adjusted until the sample absorbance at 610 nm was 1.4 ± 0.1. A small volume (120 µl) of the cell suspension was transferred to a microcentrifuge tube and mixed with 6 µl of 20 mg/ml Proteinase K solution and 120 µl of agarose solution (1% SeaKem Gold: 1% sodium-dodecyl-sulphate) in Tris-Ethylenediaminetetraacetic acid (TE, 10 mM Tris: 1 mM EDTA, pH 8.0) buffer. The mixture was immediately dispensed into the well of a disposable plug mold and allowed to solidify. Agarose plugs were placed in a tube containing 5 ml of cell lysis buffer (50 mM Tris: 50 mM EDTA, pH 8.0; 1% Sarcosyl) and 25 µl of 20 mg/ml Proteinase K solution. Samples were incubated at 54°C for two hours. Agarose plugs were washed twice in 10 ml of pre-warmed deionized water for 10 minutes at 50°C, and three times in 10 ml of pre-warmed TE buffer for 10 minutes at 50°C. A 2 mm slice of the agarose plug was digested with XbaI (Roche Applied Sciences, Laval, QC) for two hours. Plug slices were washed once with 200 µl of 0.5X Tris-Borate EDTA and loaded into the wells of a 1% SeaKem Gold gel. The wells were sealed with a 1% SeaKem Gold solution and the gel was run in 0.5X Tris-Borate EDTA for 19 hours at 14°C using a Chef Mapper (Bio-Rad Laboratories, Mississauga, ON). The Chef Mapper program used was an auto algorithm with a low molecular weight of 30 kb and high molecular weight of 600 kb, and initial and final switch times of 2.16 seconds and 54.17 seconds, respectively. *Salmonella* Braenderup H9812 was used as a size standard while the *Ent. cloacae* strain (ATCC 23355) was used as a control to monitor reproducibility of gel runs. The gels were stained using ethidium bromide and photographed under UV light.

3.5. Analyses of Biochemical and Genotypical Characteristics

3.5.1. Biochemical Characterization: Cluster Analysis of API 20E Results

Cladograms were constructed by scoring all API 20E reactions as either a one (positive reaction) or zero (negative reaction). The data was entered into Systat 11 (Systat Software Inc.,

Chicago, IL) and a multivariate cluster hierarchical analysis was performed. The resulting cladogram showed percent difference between the phenotypic profiles for all isolates.

3.5.2. Genotypic Characterization: Ribotyping Results

Ribotyping DNA fragments displayed as bands were visualized and the image manipulated to normalize each sample lane according to a standard marker set. The RiboPrint™ pattern was analysed using RiboExplorer software and compared to previously run isolates to determine isolate similarity.

3.5.3. Genotypic Characterization: Cluster Analysis of Pulsed Field Gel Electrophoresis Results

To determine the molecular size of each DNA fragment (band in the electrophoresis gel), the *Salmonella* Braenderup H9812 strain (with a known XbaI fragmentation pattern) was used to construct a standard curve relating electrophoretic bands of known sizes to the distance travelled through the PFGE gel. The distance travelled of bands from samples with the same PFGE profiles on different gels were averaged in order to account for slight variations between gels. For each isolate, all possible band sizes were identified. A library of possible band sizes was constructed such that each band size fit into one discrete group, and a group could not include two separate bands from the same sample isolate. The lower and upper limit were less than or equal to one standard deviation from each sample isolate. In most cases the spacing between groups (= bands) was at least five kb. Finally, each band size was used to generate the template for similarity scoring. The presence of a band size was given a positive score of one, and the absence of a band size was given a negative score of zero. This data was entered into Systat 11 (Systat Software Inc.) and a multivariate cluster hierarchical analysis was performed. The resulting cladogram showed percent difference between the genotypic profiles for all the isolates.

3.6. Bacterial Culture Preparation and Inoculation of Test Surfaces

Prior to each experimental trial, the bacterial isolate(s) taken from frozen stock cultures were grown overnight in Tryptic Soy Broth (TSB) (Oxoid) at 35°C to the stationary phase resulting in a concentration of 10^8–10^9 CFU/ml. The culture was then streaked onto TSA and incubated at 35°C for 24 hours to affirm purity. An isolated colony was taken from this TSA plate, inoculated into 1 ml of TSB, and incubated overnight at 35°C resulting again in a concentration of 10^8–10^9 CFU/ml. Ten µl of this bacterial suspension was then added to 5 ml of TSB and incubated at 35°C for 24 hours to yield 10^9 CFU/ml. Subsequently, the bacterial suspension was serially diluted in peptone saline ((PS), 0.1% peptone and 85% NaCl) and used to inoculate the test surfaces in the adherence assays.

3.7. Preparation of Stainless Steel and Polyvinyl Chloride Test Surfaces

Stainless steel coupons (2 cm by 1.5 cm by 0.005 cm) made of type 304 steel with no. 4 finish (Valley Metals, Kentville, NS), were used. The coupons were boiled for 5 to 10 minutes in a solution of 1% (w/v) sodium-dodecyl-sulphate and then rinsed in 100% isopropanol to remove any residual detergent. The coupons were then stored in 95% ethanol overnight and sonicated the following day for one hour in 5% (v/v) CiDecon (Decon Labs Ltd., Bryn Mawr, PA) solution to remove any organic matter. The coupons were then sterilized at 121°C for 15 minutes. Coupons were stored in 95% ethanol and further sterilized by flaming and allowed to cool prior to use.

In one experiment, PVC coupons (2 cm by 1.5 cm by 0.12 cm) provided by the Department of Civil and Resource Engineering at Dalhousie University were used. The coupons were prepared by immersion in a 5% (v/v) CiDecon solution followed by gentle mixing. To remove any remaining detergent, coupons were rinsed five times in autoclaved distilled water (dH_2O). Coupons were air-dried at room temperature followed by immersion in 90% ethanol for 10 seconds. Polyvinyl chloride coupons were then heated for 20 minutes at 80°C and kept in an empty sterile container until further use.

3.8. Adherence Assay and Coupon Transfer

Sterile SS coupons were placed into the wells of a sterile 12 x 6 ml polystyrene tissue culture plate (Corning, Fisher Scientific, Nepean, ON) containing 5 ml of 0.1% peptone (Oxoid) water and incubated at 10°C for 24 hours in order to allow the formation of an organic conditioning film. Following this surface conditioning, the treated SS coupons were removed and placed into the wells of a new sterile 12 x 6 ml polystyrene tissue culture plate (Corning) containing 5 ml of Halifax municipal tap water per well. This protocol allowed each side of the coupon to be accessible for bacterial adherence and for the entire coupon area to be covered by liquid. Appropriate volumes of the bacterial suspension were placed approximately in the center of each well already containing the coupon and municipal water followed by careful mixing. In order to verify initial concentration, the bacterial suspension was serially diluted in PS, spread plated and enumerated on TSA after incubation at 35°C as described above. Successive transfers of SS coupons into 5 ml of fresh municipal tap water were done every 24 hours for each coupon by moving the coupons into wells of a new sterile 12 x 6 polystyrene tissue culture plate. Six coupons were pulled on each sampling day (one, three and seven), three for the microscopic analysis and three for enumeration by the plate count method.

Experimental parameters that were investigated in this model system for the adherence and biofilm formation of *Enterobacter* spp. strains were: the soiling of the surface of SS or PVC coupons coated with 0.1% peptone prior to inoculation, the presence of chlorine disinfectant in the tap water, the presence of additional organic material (0.1% peptone) in the water, different strains and incubation temperatures ranging from 4-30°C.

3.8.1. Effect of Temperature on Adherence of *Ent. cloacae* KY 1 to Stainless Steel

The *Ent. cloacae* KY 1 isolate was inoculated into the model system containing soiled (0.1% peptone water (PW) for 24 hours at 10°C) SS coupons placed in wells filled with tap water with a chlorine residual (0.44 mg/l). The tissue culture plates were incubated at 4, 10, 21 and 30°C and coupons sampled on days one, three and seven as described above.

3.8.2. Comparison of the Adherence of Five Environmental Coliform Isolates to Stainless Steel

In this experiment adherence of five coliform environmental isolates (KY 1, KY 4, KY 6, KY 11, and KY 13) to SS was compared at 10°C. The five strains were selected to represent both the most persistent and non-persistent strains and included common pheno- and genotypes isolated from the different sampling sites in the Kentucky water distribution system. The remaining experimental conditions were the same as in section 3.8.

3.8.3. Effect of Surface Material on the Adherence of *Ent. cloacae* KY 1

In this set of the experiments the adherence to SS and PVC coupons of *Ent. cloacae* KY 1 was compared. The experimental design followed that outlined in section 3.8. with the following exceptions: PVC coupons were also used, and polystyrene tissue culture plates were incubated aerobically at 10°C.

3.8.4. Effect of Soiling of the Surface on the Adherence of *Ent. cloacae* KY 1 to Stainless Steel

To determine the effect of soiling of the surface by preconditioning, the adherence of *Ent. cloacae* KY 1 to SS coupons with a surface preconditioning treatment achieved by soaking the coupons in 0.1 % PW for 24 hours at 10°C as described in section 3.8 was compared to coupons that had not received the preconditioning treatment. The experimental design followed that described in section 3.8. with plates incubated aerobically at 10°C.

3.8.5. Effect of Chlorine on the Adherence of *Ent. cloacae* KY 1 to Stainless Steel

In this set of the experiments the adherence of *Ent. cloacae* KY 1 to SS coupons in tap water with two levels of residual free chlorine were tested at 10°C. The residual free chlorine that was measured in tap water taken directly from the distribution system was 0.44 mg/l. The residual free chlorine that was measured after partial removal of the chlorine by aging the water

in 500 ml bottles for 24 hours at 5°C was 0.2 mg/l. The residual free chlorine concentration was measured using the DPD Method (Hach Company, Loveland, CO). This method has a detection range of 0.01 to 2.0 mg/l. Briefly, a free chlorine powder tablet was added to 10 ml of each of the water samples and mixed. Then the absorbance at 530 nm was measured and used to compute the concentration of free chlorine. Coupons were not preconditioned.

3.8.6. Effect of Organic Material on the Adherence of *Ent. cloacae* KY 1 to Stainless Steel

The effect of organic material was tested by comparing adherence of *Ent. cloacae* KY 1 to SS coupons in the presence or absence of 0.1% (w/v) peptone in the tap water during aerobic incubation at 10°C.

3.8.7. Effect of Different Combinations of Factors (Soiled Surface, Chlorine, Organic Material) on *Ent. cloacae* KY 1 Adherence to Stainless Steel

These experiments were designed to study how different combinations of surface conditioning (section 3.8.4), chlorine concentrations (section 3.8.5), and presence of organic material (0.1% peptone) in the tap water (section 3.8.6) impacted adherence of *Ent. cloacae* KY 1 to SS. The experiment was conducted at 10°C as described in section 3.8. Table 3 shows an overview of the experimental conditions, which were tested:

Table 3: Factorial experimental design to determine the combined effect of surface soiling, residual chlorine and presence of organic material in the water on adherence of *Ent. cloacae* KY 1 to SS after one, three and seven days of incubation at 10°C.

Presence of soiled surface (0.1% PW for 24 hours at 10°C)	Presence of residual free chlorine (0.44 mg/l)	Presence of organic material (0.1% peptone)
-	-	-
-	-	+
-	+	-
-	+	+
+	-	-
+	-	+
+	+	-
+	+	+

3.9. Staining of Stainless Steel Coupons for Epifluorescence Light Microscopy

After incubation of the coupons for one, three and seven days, coupons were removed from the wells and non-adhering cells were rinsed off by gently dipping the coupon in sterile dH_2O for 10 seconds. Coupons were then stained using the LIVE/DEAD BacLight bacterial viability stain (Molecular Probes, Burlington, ON) solution. The dye solution consisted of 800 µl of PS (PS, 0.1% w/v peptone and 0.85% w/v NaCl) and 200 µl of a stock stain solution comprised of 1.5 µl of SYTO 9 and 1.5 µl of propidium iodine in 1 ml of dH_2O. One hundred and fifty µl of the dye solution was added to the surface of each coupon and placed in the dark for 15 minutes. Coupons were then dabbed near the edge of the coupon with paper towel to collect surplus stain without disruption of adhering bacterial cells. Two hundred µl of dH_2O was then added to each of the coupons. Coupons were then dabbed again with paper towel near the edge of the coupon in order to remove any excess stain. Coupons were left at room temperature in a biosafety cabinet to dry for approximately 10 minutes. Coupons were then placed on glass slides for observation under the microscope.

3.10. Epifluorescence Light Microscopy

Adhesion of bacterial cells and biofilm formation on the stained SS coupons was examined using a Nikon Eclipse 80i upright light microscope (Nikon Canada, Mississauga, ON) equipped for epifluorescence microscopy. Five randomly selected view fields for each SS coupon were examined at 1000x magnification using a Nikon triple band DAPI-FITC-Texas Red Filter (Excitation 395-410 nm DAPI, 490-505 nm FITC, 560-580 nm Texas Red; Emission 450-470 nm DAPI, 515-545 nm FITC, 600-650 nm Texas Red). This filter allows for observation of both the green and red signal simultaneously as excitation/emission wavelengths are 490/520 nm for SYTO 9, and 495/635 nm for propidium iodine, respectively. Images were taken using a high-resolution Nikon DS-Fil camera connected to a computer running NIS-Elements BR 2.30 image analysis software where images are stored and analysed. Bacterial cells in each picture were counted to provide a number for live (green) cells and dead/damaged (red) cells. When using the LIVE/DEAD BacLight stain to stain the bacteria, the green fluorescence derived from SYTO 9 indicates the presence of live cells demonstrating cytoplasmic membrane integrity (Fuster-Valls *et al.,* 2008), whereas the red fluorescence derived from propidium iodine indicates the presence of dead or damaged cells with compromised membrane integrity (Fuster-Valls *et al.,* 2008).

Cell numbers were then calculated using the actual size of the viewing area of the picture (frame area) to determine the average number of cells per cm^2 for each coupon. These cell numbers were then transformed to $\log_{10}$cells/cm^2 for statistical analysis. The limit of detection was determined to be 3.23 $\log_{10}$cells/cm^2.

3.11. Removal of Bacteria from Test Surfaces and the Plate Count Method

Sampling and rinsing of coupons were performed as described above (section 3.9). Coupons were then immediately placed into sterile centrifuge tubes with 10 ml PS and vortexed at maximum speed for two minutes. For enumeration, the detached bacteria were serially diluted in PS and 0.1 ml of each dilution was immediately spread plated on mEndo agar and incubated at 35°C for 24 hours. The bacterial counts were counted and converted into $\log_{10}$CFU/cm^2. The limit of detection was determined to be 1.52 $\log_{10}$CFU/cm^2.

3.12. Statistical Analysis for Microscopy and the Plate Count Method

An Analysis of Variance was used to quantitatively assess the significance of environmental factors (temperature, material surface, preconditioning of the surface, residual free chlorine, organic material, strains) on the adherence of coliform isolates from the distribution system. The Bonferroni method identified the significant differences in pairs of means. Analysis of variance was also used to quantitatively assess the significance of the interactions between environmental factors (preconditioning of surface, residual free chlorine and organic material) and cellular adherence. Results were considered significant if $P<0.05$. All computations were performed using the Systat 11 software package (Systat Software Inc.). Graphs were plotted using Sigma Plot 8.0 (Systat Software Inc.).

Chapter 4: RESULTS

4.1. Microbial Source Tracking: Phenotypic and Genotypic Strain Characterization

Eighteen coliform bacterial isolates were obtained from 16 different sample sites throughout a water distribution system in Lexington, KY. API 20E identified 14 of the isolates as *Enterobacter* spp. and four as *C. freundii*. Of the 14 *Enterobacter* spp. 11 were identified as *Ent. cloacae* and three as *Ent. asburiae*. Six different phenotypic API 20E profiles were observed among *Ent. cloacae* isolates (Table 4). When *Ent. cloacae* isolates were examined for genetic similarity by PFGE analysis; it was shown that there were five distinct groups of isolates (Table 4).

Four *C. freundii* isolates were identified by API 20E, and two different phenotypic profiles were observed. However, all *C. freundii* isolates had identical PFGE profiles. These results show that a group of isolates with identical PFGE profiles may have phenotypic profiles that differ from one another. In several cases, isolates originating from different sample sites showed identical PFGE profiles (Figure 4). Genotypic characterization and grouping of isolates by PFGE and ribotyping showed similar results for all isolates with the exception of isolate KY 6. Pulsed field gel electrophoresis and ribotyping resulted in the same band profile for isolates KY 1, 2, 3, 7, 8, 9 and 16, with all strains being classified as PFGE type 5 (Figure 4) and ribotype V (Table 4). However, while isolate KY 6 exhibited the same ribotype (V) as the aforementioned strains it had a unique PFGE profile (7) (Figure 4). Phenotypic characterization by API 20E resulted in numerical profiles for isolates KY 6 and 16 that were different from those of KY 1, 2, 3, 7, 8 and 9 (Table 4, Figure 2). Vitek, which uses many of the same carbon sources as API 20E, gave the same numerical profile numbers for isolates KY 1, 2, 6, 7, 8, 9 and 16 (Table 4). Figure 3 shows the cluster analysis of *Citrobacter* spp. isolated from the Lexington, KY distribution system using API 20E. Figure 4 shows the cluster analysis for *Enterobacter* and *Citrobacter* spp. based on the PFGE profiles.

Table 4: Phenotypic and genotypic characterization of coliform isolates obtained from the water distribution system in Lexington, KY.

Isolate	API Identification[a]	API 20E profile number[b]	Vitek profile number[c]	PFGE Type[d]	Ribotype[e]
KY 1	*Ent. cloacae*	3205663	6614571270	5	V
KY 2	*Ent. cloacae*	3205663	6614571270	5	V
KY 3	*Ent. cloacae*	3205663	6634571270	5	V
KY 4	*Ent. asburiae*	3305523	7614775230	4	IV
KY 5	*Ent. asburiae*	3305523	7634775232	4	IV
KY 6	*Ent. cloacae*	3205463	6614571270	7	V
KY 7	*Ent. cloacae*	3205663	6614571270	5	V
KY 8	*Ent. cloacae*	3205663	6614571270	5	V
KY 9	*Ent. cloacae*	3205663	6614571270	5	V
KY 10	*Ent. cloacae*	2005573	6454770630	1	I
KY 11	*Ent. cloacae*	2305133	6444750632	3	V
KY 12	*Ent. asburiae*	2304523	7614775232	4	IV
KY 13	*Ent. cloacae*	2105572	6044772632	2	II
KY 14	*C. freundii*	1404572	7014774730	6	VII
KY 15	*C. freundii*	1404572	7014774730	6	VII
KY 16	*Ent. cloacae*	3204463	6614571270	5	V
KY 17	*C. freundii*	3604572	7014774730	6	VII
KY 18	*C. freundii*	1404572	7014774730	6	VII

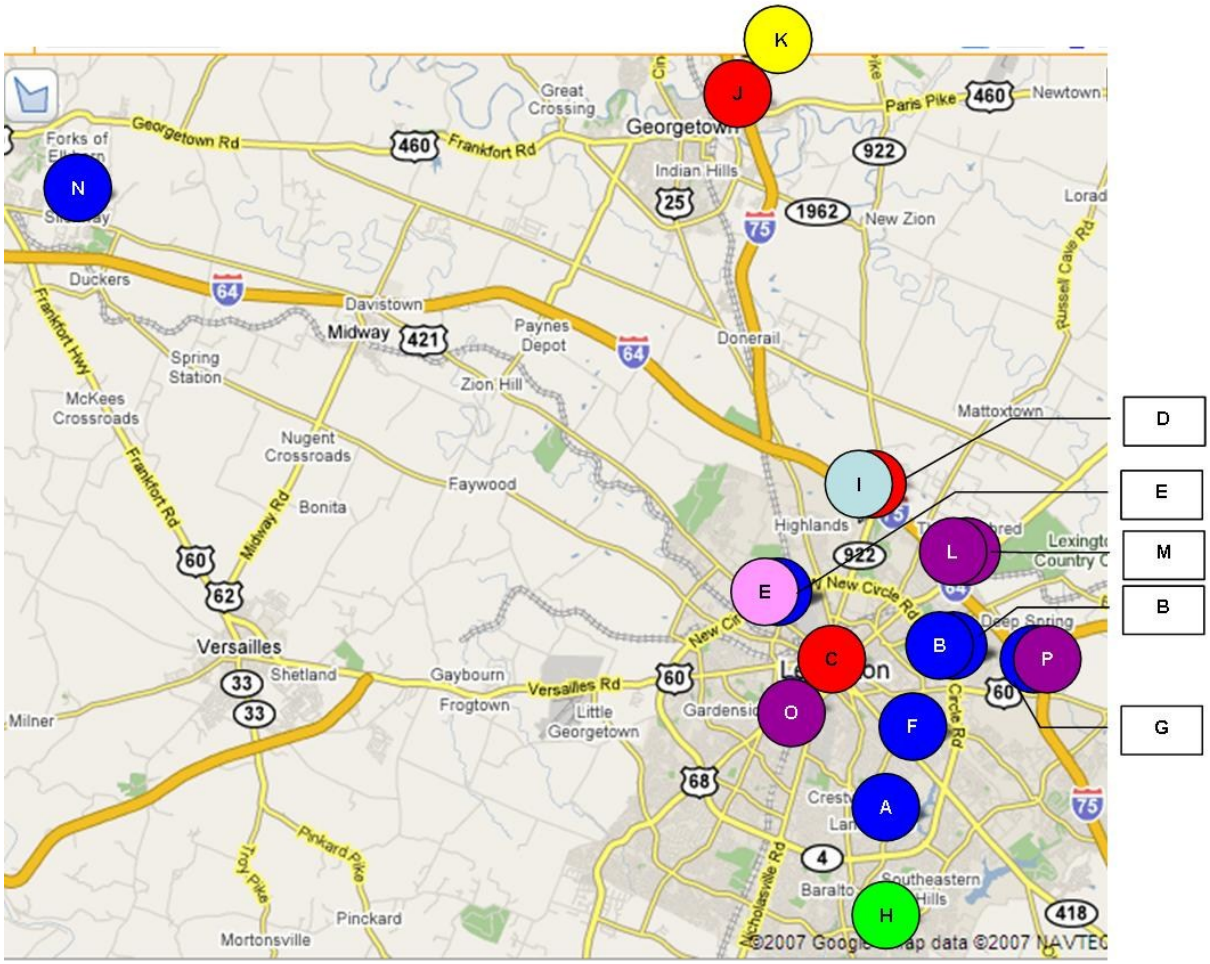
[a] Bacterial identification based on the API database (BioMerieux)

[b] Numeric API profile based on the pattern of positive and negative results in 20 biochemical tests

[c] Numeric Vitek profile based on the pattern of positive and negative results in 30 biochemical tests

[d] PFGE profile based on the electrophoretic band patterns of XbaI restricted DNA

[e] Ribotype based on the electrophoresis/Southern hybridization pattern of rRNA genes



PFGE Type	1	2	3	4	5	6	7
Isolate(s)	KY 10	KY 13	KY 11	KY 4, 5, 12	KY 1, 2, 3, 7, 8, 9, 16	KY 14, 15, 17, 18	KY 6
Sample Date(s)	9/6/06	9/5/06	8/8/06	9/14/06, 9/08/06, 9/19/06	10/10/06, 9/20/06, 9/21/06, 10/12/06, 10/11/06, 10/12/06, 10/10/06	10/17/06, 10/17/06, 10/17/06, 10/12/06	10/11/06

Figure 1: Sampling sites displayed by PFGE type with the date of sampling within the drinking water distribution system in Lexington, KY. Circles indicate sites where coliform bacteria were found, and circles of the same colour indicate isolates with the same PFGE profile (Google Maps, 2007).

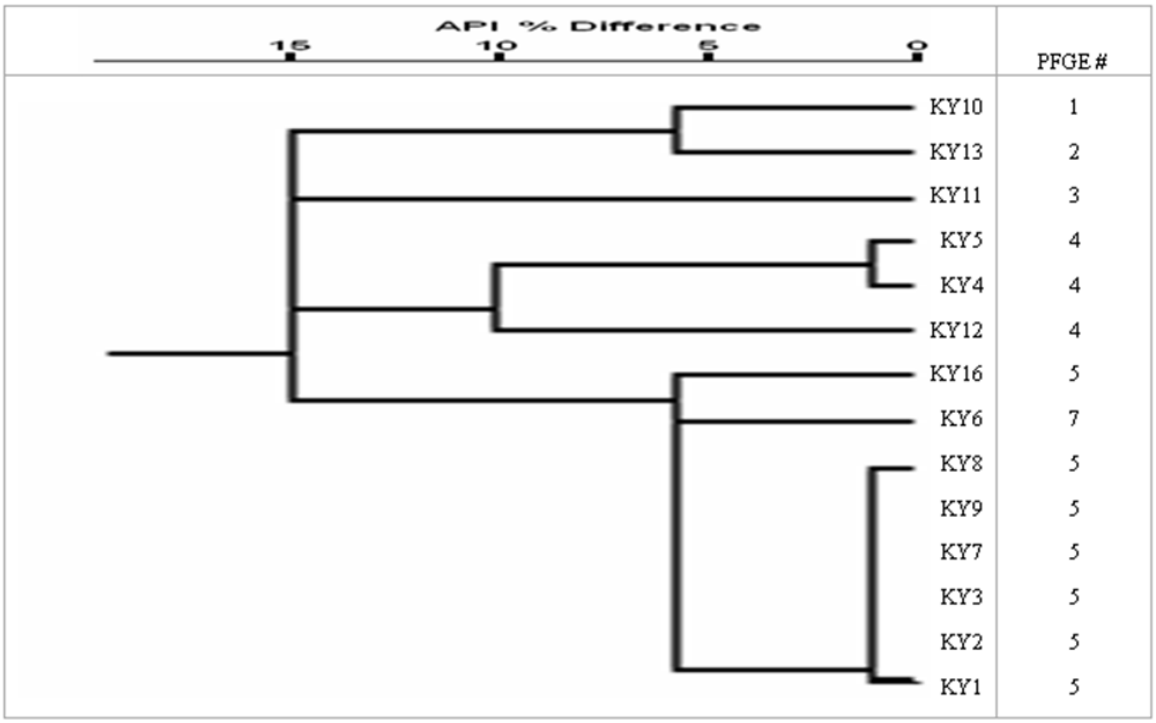


Figure 2: Phenotypic characterization: Cluster analysis of *Enterobacter* spp. isolated from the water distribution system in Lexington, KY. The cladogram shows the phenotypic relationships between isolates determined by the API system. Isolates in the same group showed identical API profiles and each five percent difference represents one different result for a particular API reaction.

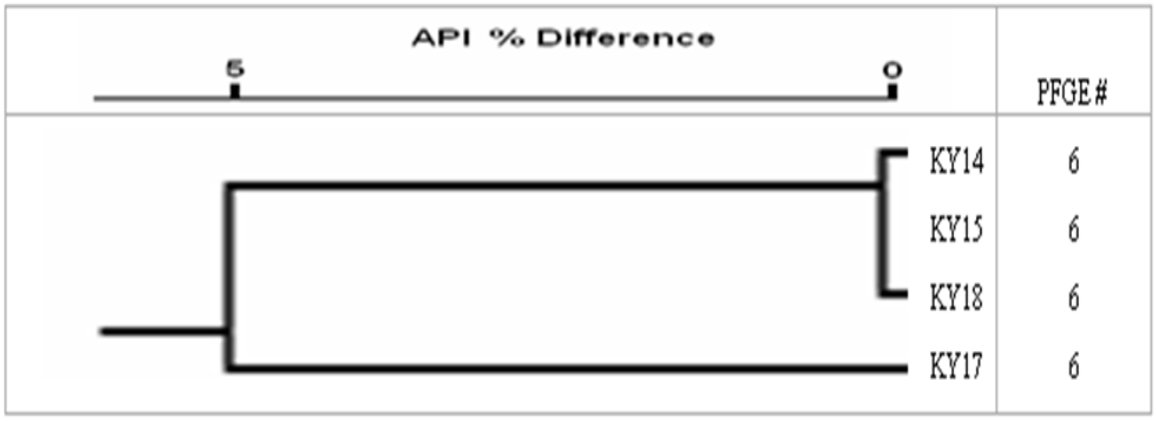


Figure 3: Phenotypic characterization: Cluster analysis of *Citrobacter* spp. isolated from the water distribution system in Lexington, KY. The cladogram shows the phenotypic relationships between isolates determined by the API system. Isolates in the same group showed identical API profiles and each five percent difference represents one different result for a particular API reaction.

The Biolog system was unable to identify the *Ent. cloacae* ATCC 23355 control strain in this study. The phenotypic profiles obtained from the Lexington bacterial isolates were mostly without matches in the database. In addition, there were cases in which positive reactions were observed in the negative control well with no carbon source. Neither addition of sodium thioglycolate nor sodium salicylate was able to prevent bacteria-mediated oxidation of capsule polysaccharides and subsequent reduction of the colour-producing agent. Biolog profile groupings differed from those obtained with API 20E, Vitek and PFGE. Eighteen different Biolog profiles were observed among the 18 bacterial isolates from Lexington, KY. Isolates KY 12, 4 and 5, identified as *Ent. asburiae* by API 20E testing, showed some similarities in the groupings created in both the API 20E and Biolog cladograms (data not shown). The most probable identity of KY isolates 14, 15, 17 and 18 was found to be *C. freundii* by both API 20E and Biolog. The remaining 11 bacterial isolates, assigned as *Ent. cloacae* by API 20E, were not identified or even a probable identity in many cases by the Biolog system.

Vitek was able to identify 10 out of 18 isolates; only six of these identifications were similar to those found using API 20E, these included four *C. freundii* and two *Ent. cloacae*. The other four identifications were neither *Enterobacter* nor *Citrobacter* spp. and included *Yersinia enterocolitica*, *Kluyvera* spp. and *Serratia plymuthica*. Among the 18 bacterial isolates nine different Vitek numerical profiles were observed. The API 20E, Vitek and PFGE assays pointed to isolates KY 1, 2, 7, 8, and 9 being identical (Table 4). Vitek was not able to identify any of these isolates (KY 1, 2, 7, 8 and 9).

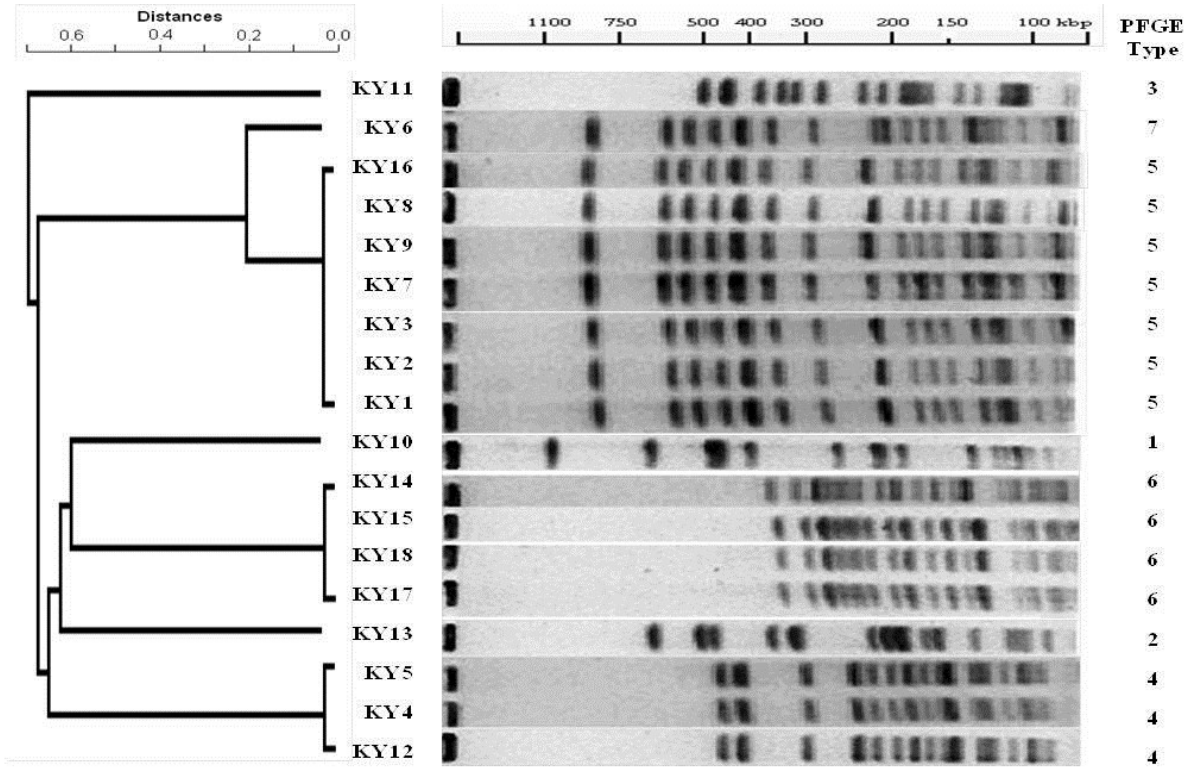


Figure 4: Genotypic characterization: Cluster analysis of *Enterobacter* and *Citrobacter* spp. isolated from the drinking water distribution system in Lexington, KY. The cladogram shows the genotypic relationships between isolates determined by PFGE analysis.

All Kentucky isolates were analysed using ribotyping and PFGE. The Ribotyping identified six groupings of isolates with some groups of coliform strains displaying identical profiles regardless of the fact that they were isolated from samples obtained from different sites on different dates in the drinking water distribution system. These ribotype groupings were similar to those found by PFGE with the exception of isolate KY 6 as mentioned above as six different genotypic ribotyping profiles but seven different PFGE profiles were observed (Table 4, Figure 4). Isolate KY 6 had the same ribotyping profile as isolates KY 1, 2, 3, 7, 8, 9 and 16 but exhibited a slightly different PFGE profile (Table 4). Overall, ribotyping and PFGE gave very similar results and were reproducible when isolates were analysed in duplicate.

4.2. Influence of Different Environmental Conditions on Cell Adherence, Cell Survival and Biofilm Formation of *Ent. cloacae*

4.2.1. Effect of Temperature on Adherence of *Ent. cloacae* KY 1 to Stainless Steel and Biofilm Formation

Microscopic observation revealed that initial cell adherence ranged from 5.16 to 6.09 $\log_{10}$cells/cm^2 (Figure 5) and was significantly (P<0.05) lower at 4°C than at 10, 21 and 30°C (Table A.1). Initial cell adherence of the *Ent. cloacae* KY 1 strain at 10°C was also lower than at 21 and 30°C suggesting warmer temperatures may result in greater initial cell adherence, defined as higher $\log_{10}$cells/cm^2 adhered to SS after one day of incubation.

After three days of incubation at the various temperatures, the *Ent. cloacae* KY 1 strain continued to adhere in higher $\log_{10}$cells/cm^2 at warmer temperatures compared to lower temperatures. Specifically, cell adherence after three days of incubation at 21°C was significantly (P<0.05) higher than at 4 and 10°C and was over a log higher than at 30°C although this difference was not significant (P>0.05). Cell adherence after three days of incubation at 30°C was also significantly (P<0.05) higher than at 4°C and over a log higher than at 10°C, however, this difference was not statistically significant (P>0.05) (Table A.1).

After seven days of incubation a similar trend was observed at the four incubation temperatures, where cell adherence at 21°C was again found to be significantly (P<0.05) higher than at 4, 10 and 30°C. The cell adherence at 30°C was also significantly (P<0.05) higher than at 4 and 10°C (Table A.1). At this time, the adhered number of cells of the *Ent. cloacae* KY 1 strain varied from 3.43 $\log_{10}$cells/cm^2 at 10°C to 7.23 $\log_{10}$cells/cm^2 at 21°C, representing an increase at 21°C while bacterial numbers decreased at all other temperatures (Figure 5).

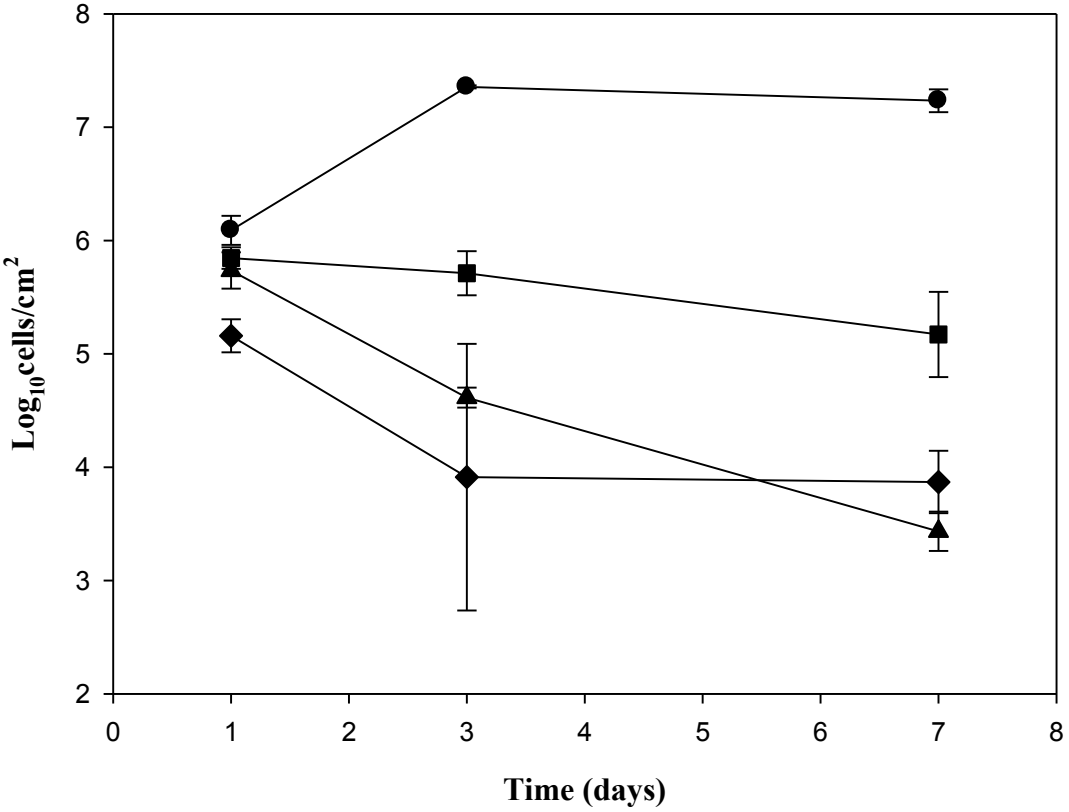


Figure 5: Effect of incubation temperature (4, 10, 21 and 30°C) on cellular adherence and survival of the *Ent. cloacae* KY 1 on SS after one, three and seven days incubation. The number of cells was enumerated by epifluorescence microscopy using the LIVE/DEAD BacLight stain. Error bars represent standard deviation for n=3. Symbols: (♦) 4°C; (▲) 10°C; (●) 21°C; (■) 30°C.

Representative microscopic images of the development of cell adherence by *Ent. cloacae* KY 1 at 4, 10, 21, and 30°C after one, three and seven days incubation on SS coupons are shown in Figures 6 and 7. The images in Figure 6 clearly show the decreasing number of cells adhering to SS coupons as a function of incubation time at 4 and 10°C. At these low temperatures adherence consisted mostly of single cells adhered to the SS surface with no indication of biofilm formation. In contrast, the appearance of single colonies, cells in pairs, chains and clusters as well as what might be the initial phases of biofilm formation was observed on the SS surface during incubation at 21°C (Figure 7). After seven days, most cells at 21°C stained red, indicating some injury to the cells. At 30°C the number of cells was visibly far fewer in between and no biofilm formation was observed at that temperature.

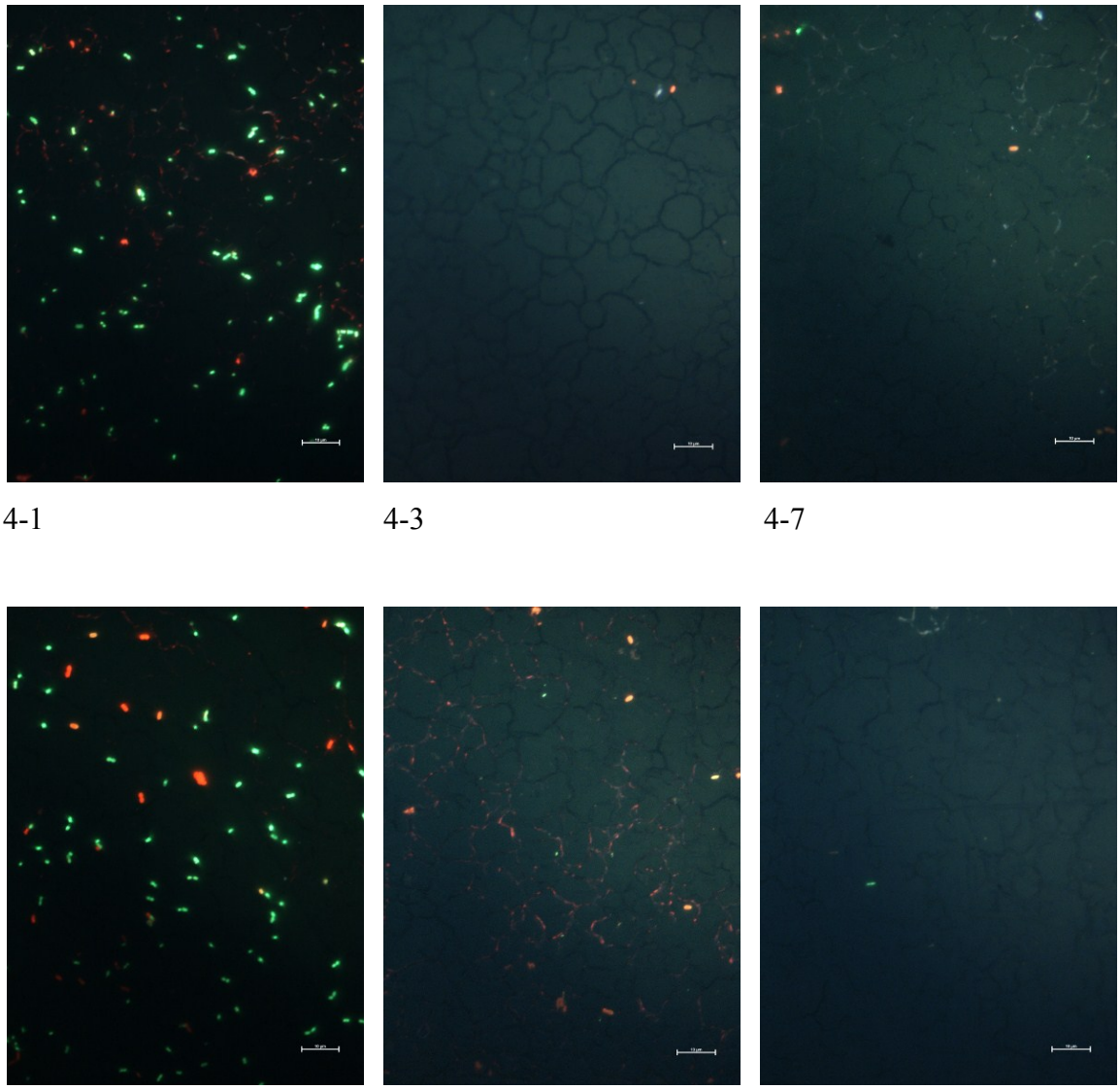
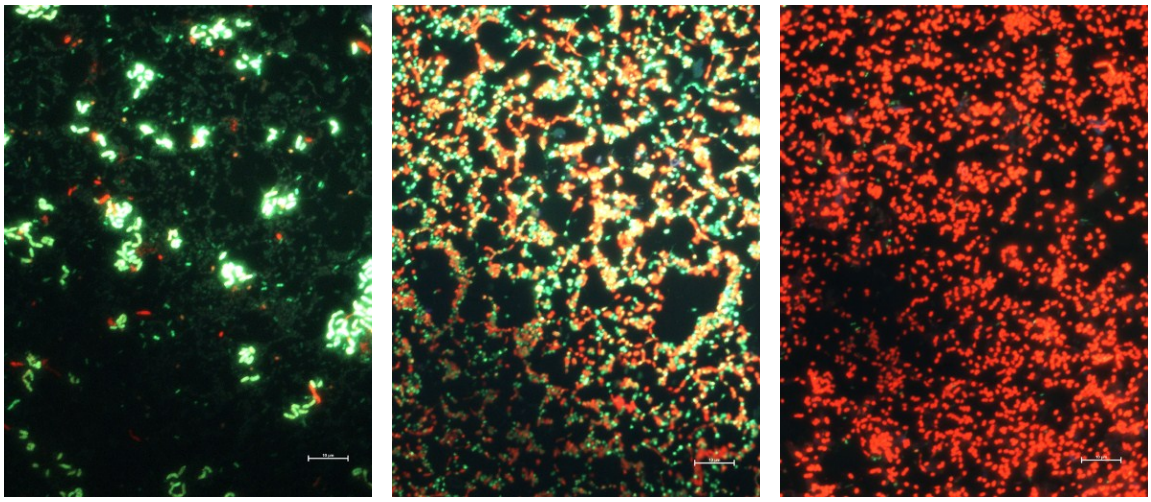


Figure 6: Micrographs of the cell adherence by *Ent. cloacae* KY 1 on SS coupons during growth in water obtained from a drinking water distribution system in Halifax, Nova Scotia. Bacteria were stained with LIVE/DEAD BacLight and observed by epifluorescence microscopy using a 1000 x oil-immersion objective: (4-1), after one day at 4°C; (4-3), after three days at 4°C; (4-7), after seven days at 4°C; (10-1), after one day at 10°C; (10-3), after three days at 10°C; (10-7), after seven days at 10°C. Scale bars, 10.0 µm.



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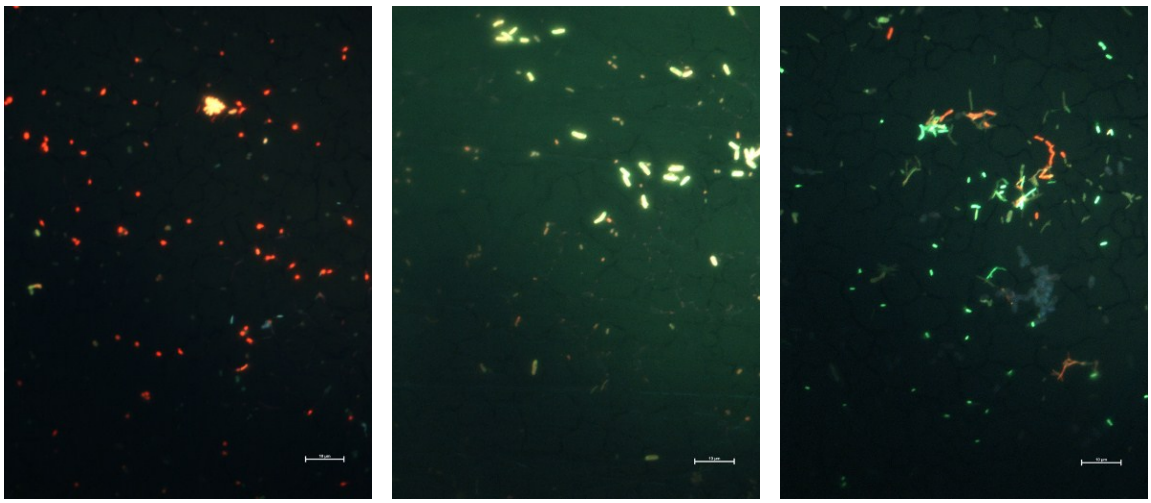


30-1 30-3 30-7

Figure 7: Micrographs of the cell adherence by *Ent. cloacae* KY 1 on SS coupons during growth in water obtained from a drinking water distribution system in Halifax, Nova Scotia. Bacteria were stained with LIVE/DEAD BacLight and observed by epifluorescence microscopy using a 1000 x oil-immersion objective: (21-1), after one day at 21°C; (21-3), after three days at 21°C; (21-7), after seven days at 21°C; (30-1), after one day at 30°C; (30-3), after three days at 30°C; (30-7), after seven days at 30°C. Scale bars, 10.0 µm.

Enumeration of culturable bacteria by the plate count method showed that after one day of incubation, the numbers of adhered *Ent. cloacae* KY 1 cells ranged from 5.68 to 7.36 $\log_{10}$CFU/cm^2 for the four temperatures (Figure 8). These numbers approximately corresponded to those obtained by the microscopic *in situ* examination method, indicating that at this time

point most cells were viable and culturable. However, after three days of incubation the plate count numbers decreased dramatically to levels around or below the detection limit of 1.52 $\log_{10}$CFU/cm^2 for 4, 10 and 30°C whereas the plate count number for 21°C decreased to 5.02 $\log_{10}$CFU/cm^2 (Figure 8). After seven days of incubation, the plate counts for all temperatures were below the detection limit of 1.52 $\log_{10}$CFU/cm^2. Clearly, there is a discrepancy between the microscopic and plate counts seen in Figures 5 and 8, respectively. This may be related to the fact that the components of the LIVE/DEAD BacLight stain detect DNA and membrane integrity whereas the plate count method determines whether cells are culturable in a particular bacteriological media under a defined set of incubation conditions.

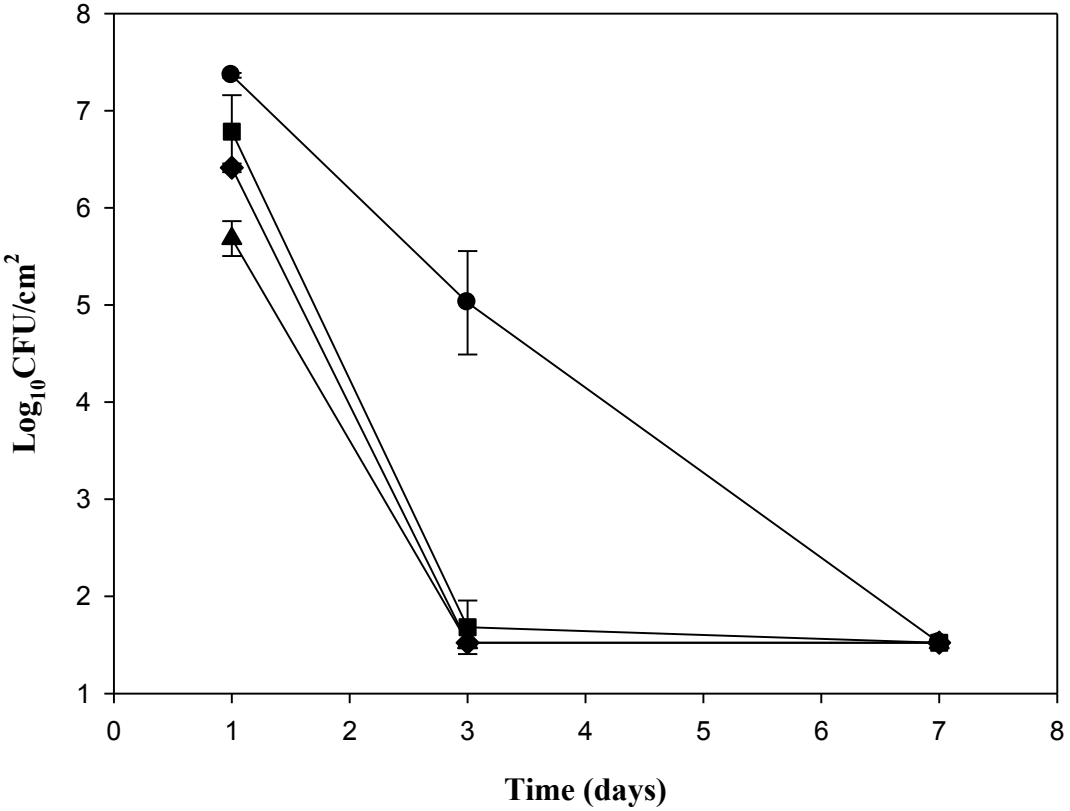


Figure 8: Enumeration of adhered culturable *Ent. cloacae* KY 1 on SS after one, three and seven days incubation at four different temperatures. The values displayed for each point are the average of three samples from one experiment for each time period (one, three and seven day's incubation) at each temperature. The error bars represent the standard deviation for the three samples. The temperatures used were 4°C (♦), 10°C (▲), 21°C (●), and 30°C (■).

4.2.2. Comparison of the Cell Adherence, Cell Survival and Biofilm Formation Among Different Environmental Isolates

After one day of incubation, the five different environmental isolates were shown to adhere in the following order from greatest to least cell adherence: *Ent. asburiae* KY 4, *Ent. cloacae* KY 6, *Ent. cloacae* KY 1, *Ent. cloacae* KY 11 and *Ent. cloacae* KY 13. These five environmental isolates were compared under sub-optimal temperature conditions of 10°C for cell adherence and included: the most persistent strain, (isolate KY 1 that was chosen from a number of isolates that gave identical genetic PFGE profiles), an isolate that was very genetically closely related to isolate KY 1 by PFGE and identical by ribotyping (KY 6), the second most persistent strain (isolate KY 4 was chosen among three isolates with identical genetic PFGE profiles), and two non-persistent strains (isolates KY 11 and 13 were only detected once in the distribution system).

Microscopic observation revealed that the initial cell adherence ranged from 4.13 to 6.74 $\log_{10}$cells/cm^2 (Figure 9). Environmental isolates KY 4 and KY 6 adhered in significantly (P<0.05) greater numbers than the *Ent. cloacae* KY 1 followed by KY 11 and KY 13 (Table A.2). This suggests more persistent strains and strains genetically closely related to persistent strains exhibit greater initial cell adherence to surfaces than non-persistent strains. However, the *Ent. cloacae* KY 1 deemed to be most persistent due to its isolation from multiple sites in the MST part of this study did not display the greatest initial cell adherence.

After three days of incubation, the persistent environmental isolate KY 4 was found to adhere in significantly (P<0.05) greater numbers compared to all other isolates. Adherence of the environmental isolate KY 6 was also significantly (P<0.05) greater when compared to isolates KY 1, KY 11 and KY 13. The variation in the number of adhered cells ranged from 3.43 to 6.70 $\log_{10}$cells/cm^2 after seven days, and while levels of all other strains remained stable, the number of adhered *Ent. cloacae* KY 1 cells decreased significantly (P<0.05) (Figure 9). This may suggest that the sub-optimal temperature conditions of 10°C under which the isolates were compared were not ideal for the *Ent. cloacae* KY 1 strain especially as compared to other strains tested.

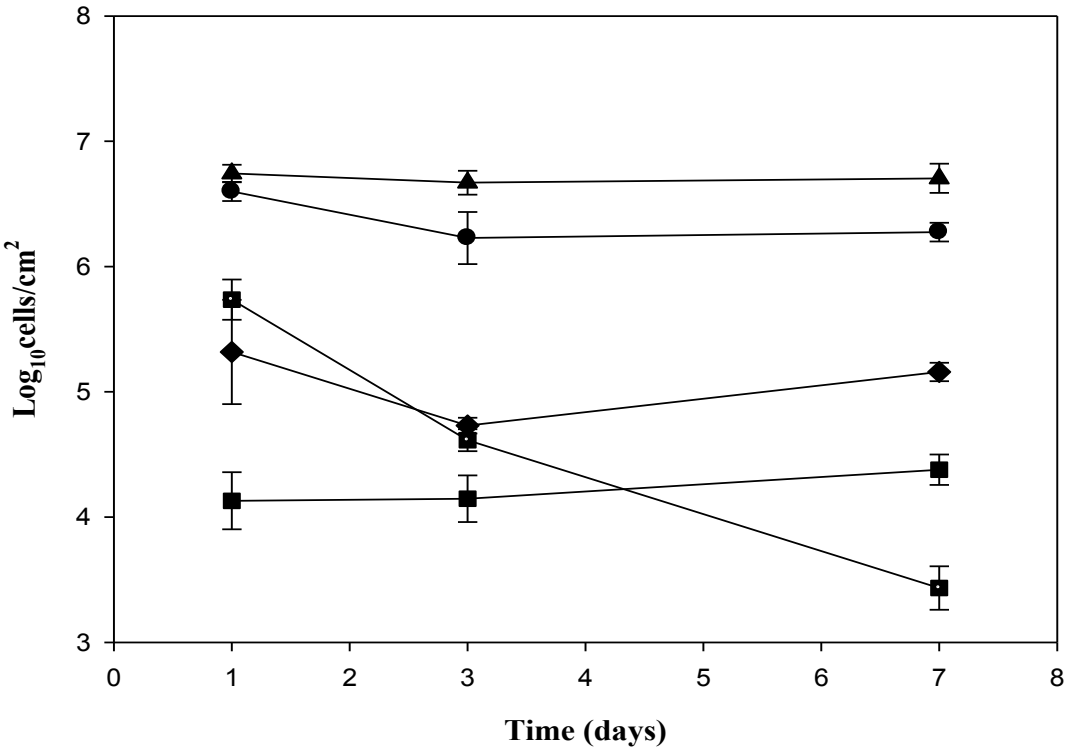
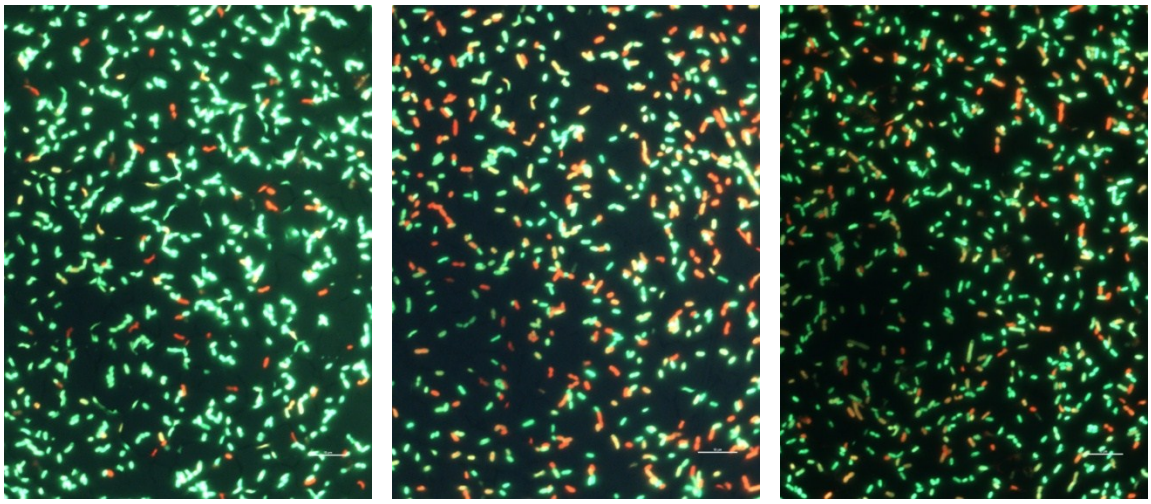


Figure 9: Comparison of cellular adherence of five different environmental isolates: *Ent. asburiae* KY 4, *Ent. cloacae* KY 6, *Ent. cloacae* KY 1, *Ent. cloacae* KY 11, and *Ent. cloacae* KY 13 as measured by epifluorescence microscopy to SS coupons in Halifax tap water and incubated at 10°C over a seven day period. Error bars represent standard deviation for n=3. Symbols: (▲) KY 4; (●) KY 6; (◘) KY 1; (♦) KY 11; (■) KY 13.

Representative microscopic images of the development of cell adherence by KY 4, KY 6, KY 1, KY 11 and KY 13 at 10°C after one, three and seven days incubation on SS coupons are shown in Figures 10, 11, and 12, respectively. Adherence of KY 4 and 6 remained constant over the seven days of incubation or showed a very slight decrease as a function of increasing incubation time (Figure 10). For environmental isolate KY 4 adherence consisted mostly of single and pairs of cells adhered to the SS surface. A few small clusters of cells and cells in chains were observed. Cells adhered to the SS surface by isolate KY 4 seemed to be more evenly distributed over the surface of the coupon as compared to isolate KY 6 where groups of small clusters were observed resulting in more unpopulated area on the entire surface of the coupon. In contrast to the cellular adherence of KY 4, KY 6 appeared to form closely connected networks of multilayered cells where a formation of an EPS matrix and biofilm may have been in the initial stages.



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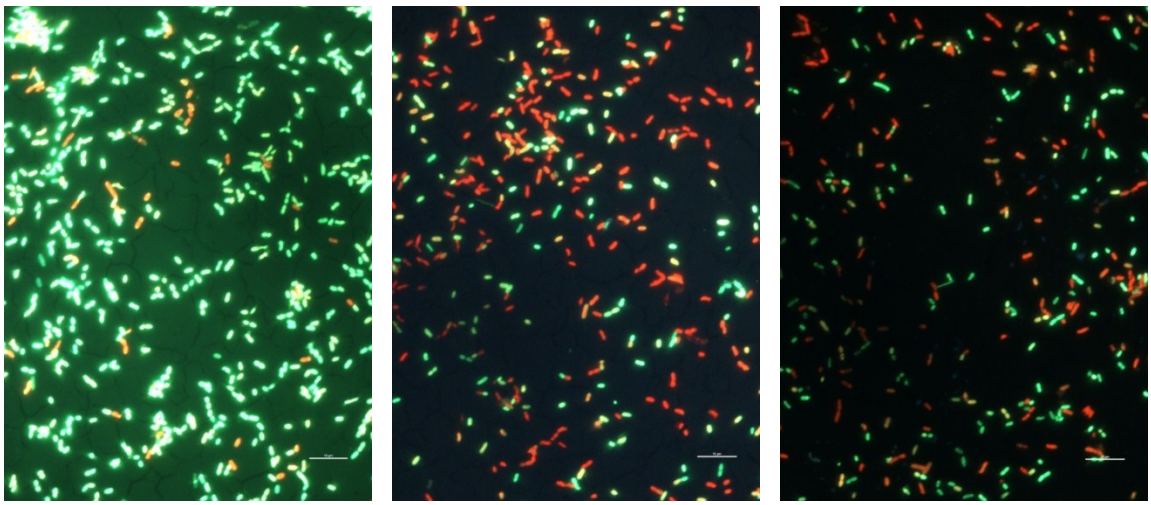
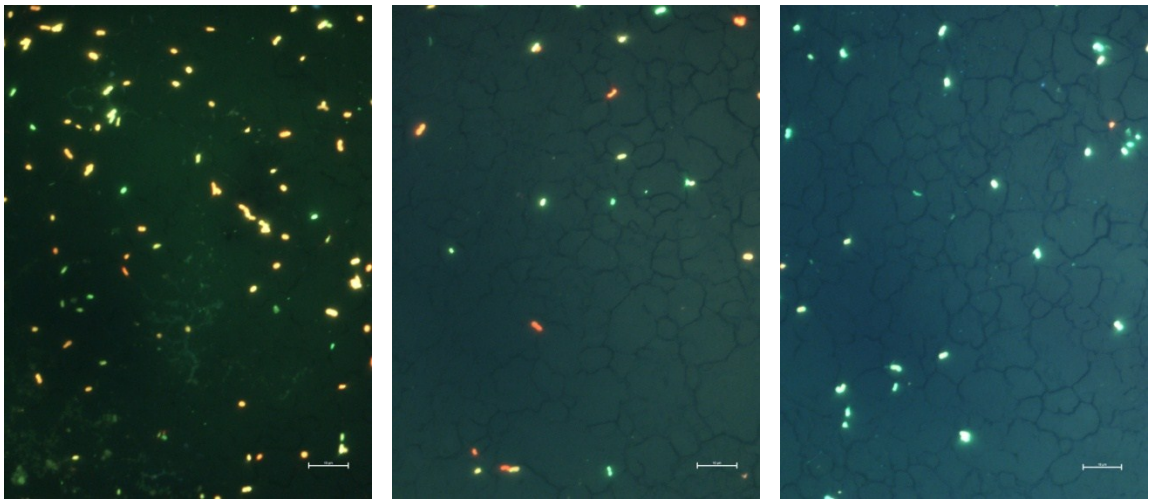


6-1 6-3 6-7

Figure 10: Micrographs of the cell adherence formed by *Ent. asburiae* KY 4 and *Ent. cloacae* KY 6 on SS coupons during growth in water obtained from a drinking water distribution system in Halifax, Nova Scotia at 10°C. Bacteria were stained with LIVE/DEAD BacLight and observed using a 1000 x oil-immersion objective: (4-1), KY 4 after one day; (4-3), KY 4 after three days ; (4-7), KY 4 after seven days; (6-1), KY 6 after one day; (6-3), KY 6 after three days; (6-7), KY 6 after seven days. Scale bars, 10.0 µm.

The images in Figure 11 show the decreasing adherence to SS coupons as a function of incubation time of isolates KY 1 and KY 11, which consisted mostly of single cells adhered to the SS surface with no indication of biofilm formation. Compared to isolates KY 4 and KY 6 (Figure 10), more unpopulated area was observed between cells on the coupon surface.



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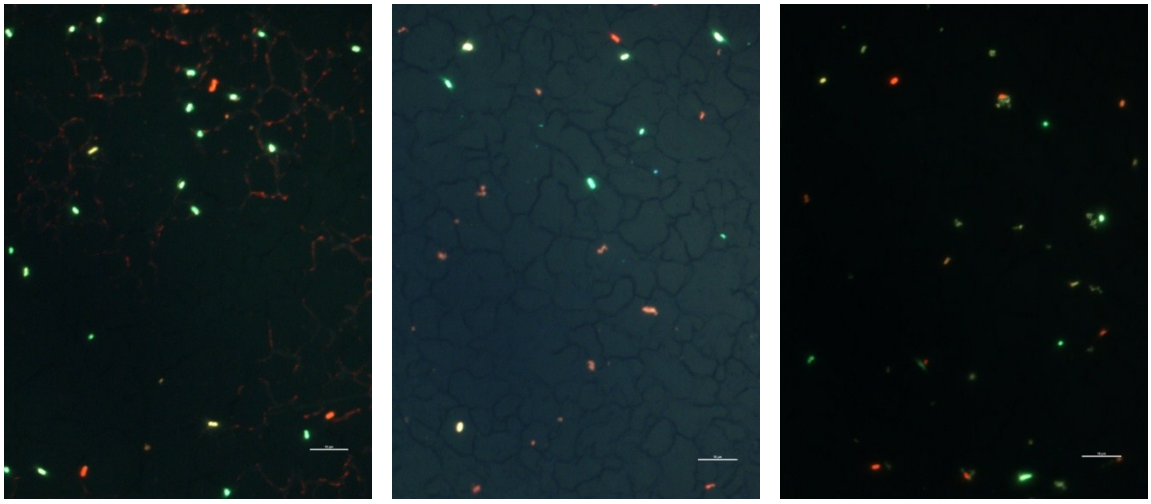
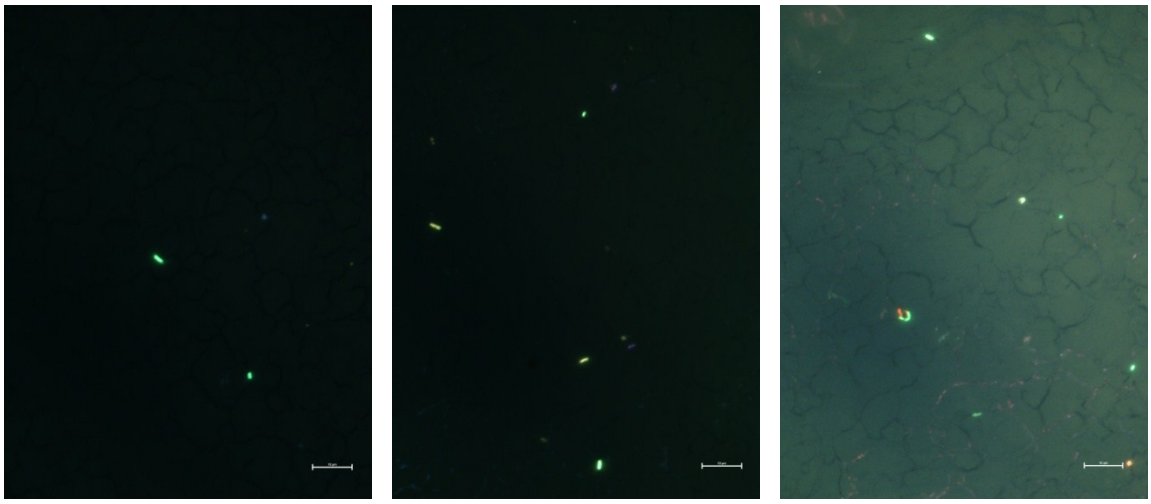


11-1 11-3 11-7

Figure 11: Micrographs of the cell adherence formed by *Ent. cloacae* KY 1 and *Ent. cloacae* KY 11 on SS coupons during growth in water obtained from a drinking water distribution system in Halifax, Nova Scotia at 10°C. Bacteria were stained with LIVE/DEAD BacLight and observed using a 1000 x oil-immersion objective: (1-1), KY 1 after one day; (1-3), KY 1 after three days ; (1-7), KY 1 after seven days; (11-1), KY 11 after one day; (11-3), KY 11 after three days; (11-7), KY 1 after seven days. Scale bars, 10.0 µm.

The images in Figure 12 show very limited adherence of isolate KY 13 consisting only of single cells adhered to the SS surface with no indication of biofilm formation.



13-1 13-3 13-7

Figure 12: Micrographs of the cell adherence formed by *Ent. cloacae* KY 13 on SS coupons during growth in water obtained from a drinking water distribution system in Halifax, Nova Scotia at 10°C. Bacteria were stained with LIVE/DEAD BacLight and observed using a 1000 x oil-immersion objective: (13-1), KY 13 after one day; (13-3), KY 13 after three days; (13-7), KY 13 after seven days. Scale bars, 10.0 µm.

After one day of incubation, the numbers of viable and culturable cells ranged from 5.36 to 6.63 $\log_{10}$CFU/cm^2 at 10°C for the five environmental isolates (Figure 13). These numbers correspond quite well to those obtained by the microscopic *in situ* examination method, suggesting that at this time point most cells were viable and culturable. However, after three days of incubation the plate count numbers decreased dramatically to levels around or below the detection limit of 1.52 $\log_{10}$CFU/cm^2 for all environmental isolates with the exception of the *Ent. cloacae* KY 1, which maintained a slightly greater viable adhering cell population (Figure 13). Following seven days of incubation, the plate counts for all environmental isolates decreased below the detection limit of 1.52 $\log_{10}$CFU/cm^2. Clearly, there is a discrepancy between the microscopic and plate count results reported in Figures 9 and 13, respectively. This may again be related to the fact that the two enumeration methods are conceptually different, with the detection of microbial DNA and membrane integrity for the microscopic *in situ* examination whereas the plate count method determines whether cells are culturable in a particular bacteriological media under a defined set of incubation conditions.

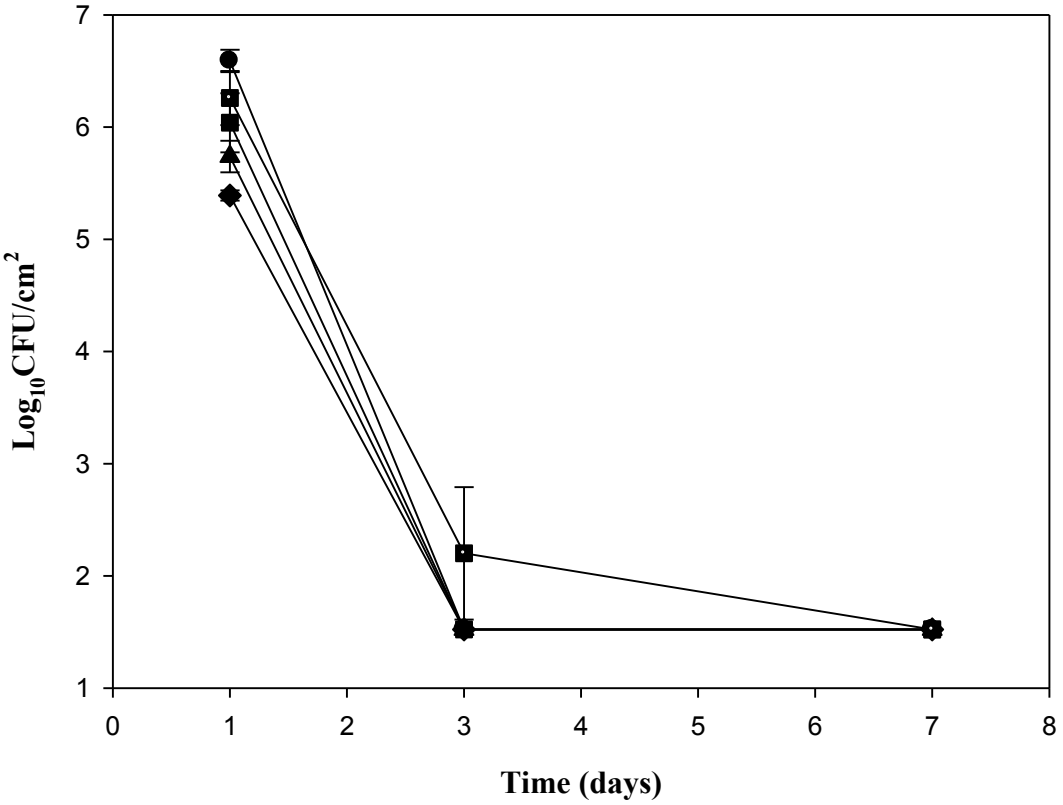


Figure 13: Adhered cells of *Ent. asburiae* KY 4, *Ent. cloacae* KY 6, *Ent. cloacae* KY 1, *Ent. cloacae* KY 11, and *Ent. cloacae* KY 13 on SS coupons for seven days at 10°C. The values displayed for each point are the mean of three samples from one experiment for each sample period (one, three and seven days). The error bars represent the standard deviation for the three samples. Symbols: (▲) KY 4; (●) KY 6; (◘) KY 1; (♦) KY 11; (■) KY 13.

4.2.3. Effect of Surface Material on Cell Adherence

After one day of incubation, the numbers of adhered *Ent. cloacae* KY 1 cells on SS (5.68 $\log_{10}$CFU/cm^2) were significantly (P<0.05) lower than on the PVC surface (6.87 $\log_{10}$CFU/cm^2). After three and seven days of incubation the plate count numbers decreased dramatically to levels around or below the detection limit of 1.52 $\log_{10}$CFU/cm^2 for both surfaces (Figure 14).

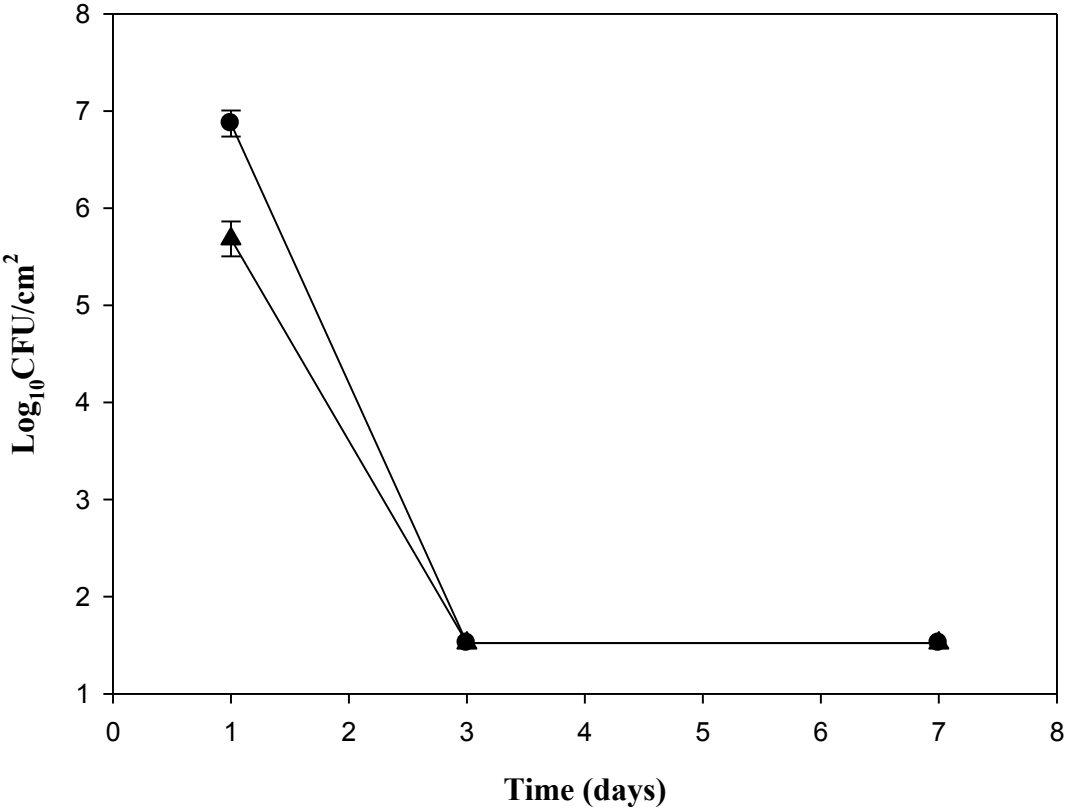


Figure 14: Adhered cell numbers of *Ent. cloacae* KY 1 on SS and PVC coupons for seven days at 10°C. The values displayed for each point are the average of three samples from one experiment for each time period (one, three and seven day's incubation). The error bars represent the standard deviation for the three samples. Symbols: (▲) SS (●) PVC.

4.2.4. Effect of a Soiled Surface on the Cellular Adherence of *Ent. cloacae* KY 1 to Stainless Steel

Microscopic observation showed the initial ability of *Ent. cloacae* KY 1 to adhere to SS coupons in the presence and absence of a soiled surface ranging between 5.42 to 5.59 $\log_{10}$cells/cm^2 with no significant difference (P<0.05) between the surface treatments (Figure 15). The cell adherence dropped after three days of incubation to approximately 5 $\log_{10}$cells/cm^2, regardless of the presence or absence of soil. However, after seven days, cell adherence in the presence of a soiled surface was higher than on the unsoiled surface with levels of 4.64 and 4.03 $\log_{10}$cells/cm^2, respectively (Table A.3).

Initial plate counts showed that *Ent. cloacae* KY 1 adhered in cell numbers of approximately 6 $\log_{10}$CFU/cm^2 in both soiled and unsoiled surfaces after one day of incubation. After three days, however, the number of culturable cells had dropped below the detection limit for both surfaces (Table A.3). These numbers again do not exactly correspond to those obtained by microscopic *in situ* examination due to the differences in the methods themselves; this will be discussed later.

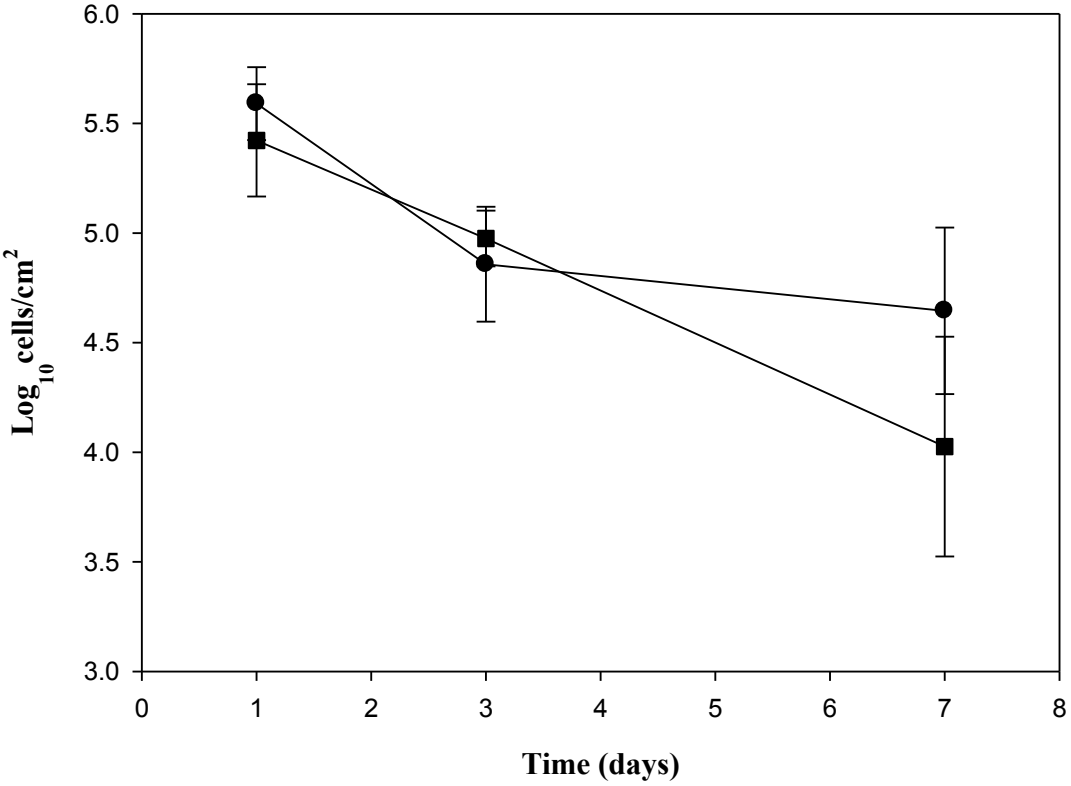
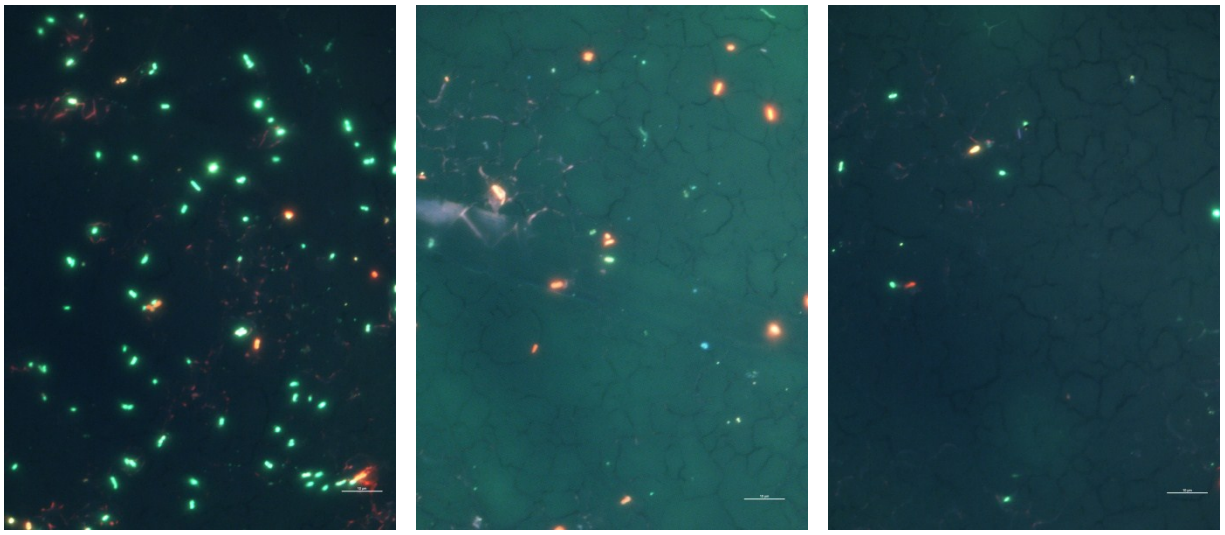


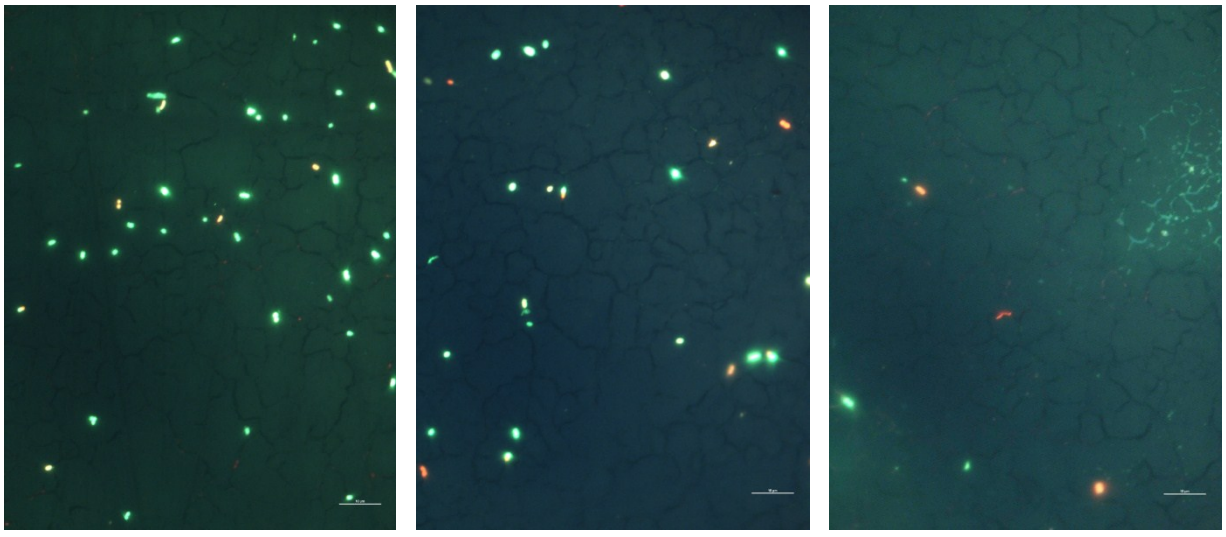
Figure 15: Effect of preconditioning of the surface (soiled surface) on cellular adherence and survival of *Ent. cloacae* KY 1 on SS coupons over seven days. The number of cells was enumerated by epifluorescence microscopy using the LIVE/DEAD BacLight stain. Error bars represent standard deviation for n=3. Symbols: (●) soiled surface; (■) unsoiled surface.

Representative microscopic images of the development of cell adherence by *Ent. cloacae* KY 1 in the presence and absence of a soiled surface after one, three and seven days of incubation on SS coupons at 10°C clearly show the decreasing number of cells adhering to SS coupons as a function of incubation time (Figure 16). The number of adhered cells on the soiled

surface decreased more slowly over time. For both surface treatments, adherence consisted mostly of single cells adhered to the SS surface with no indication of biofilm formation.



PC-1 PC-3 PC-7



NPC-1 NPC-3 NPC-7

Figure 16: Micrographs of the cell adherence by *Ent. cloacae* KY 1 on SS coupons submersed in water obtained from a drinking water distribution system in Halifax, Nova Scotia. Bacteria were stained with LIVE/DEAD BacLight and observed by epifluorescence microscopy using a 1000 x oil-immersion objective: (PC-1), after one day in the presence of a soiled surface; (PC-3), after three days in the presence of a soiled surface; (PC-7), after seven days in the presence of a soiled surface; (NPC-1), after one day in the absence of a soiled surface; (NPC-3), after three days in the absence of a soiled surface; (NPC-7), after seven days in the absence of a soiled surface. Scale bars, 10.0 µm.

4.2.5. Effect of Chlorine on the Cellular Adherence of *Ent. cloacae* KY 1 to Stainless Steel

When subjected to the stressful environmental and nutrient-poor conditions *Ent. cloacae* KY 1 adhered in higher $\log_{10}$cells/cm^2 in the presence of a residual free chlorine concentration of 0.44 mg/l (Figure 17). Initial cell adherence ranged between 5.42 to 5.82 $\log_{10}$cells/cm^2. However, after three and seven days of incubation the number of adhered cells decreased to 4.03 and 5.16 $\log_{10}$cells/cm^2 in the unchlorinated samples (in this study: 0.2 mg/l residual free chlorine after removal of chlorine by aging the water) and chlorinated samples (0.44 mg/l), respectively.

After one day of incubation, the numbers of viable and culturable cells were very similar with cell densities of approximately 6 $\log_{10}$CFU/cm^2 whereas after three days of incubation no cells were culturable using the plate count method (Table A.4).

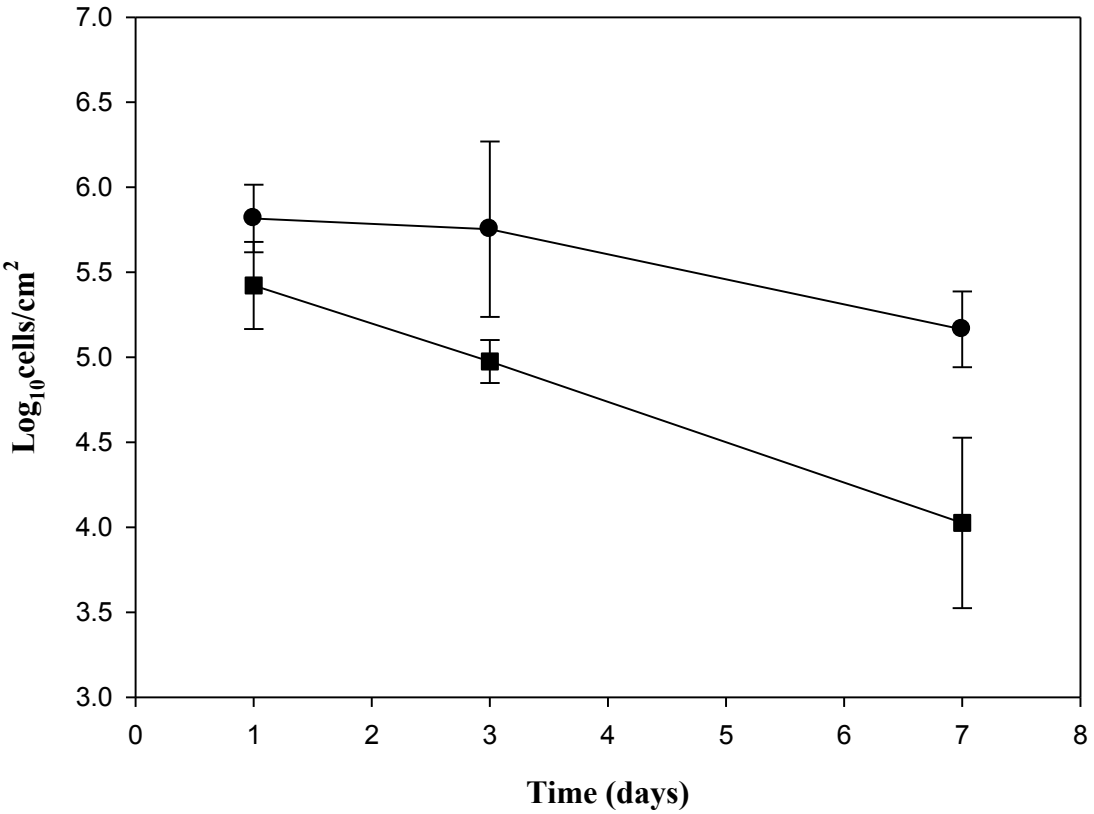


Figure 17: Effect of residual free chlorine on cellular adherence and survival of *Ent. cloacae* KY 1 on SS coupons over seven days. The number of cells was enumerated by epifluorescence microscopy using the LIVE/DEAD BacLight stain. Error bars represent standard deviation for n=3 in duplicate. Symbols: (●) presence of residual free chlorine (0.44 mg/l); (■) absence of chlorine (0.2 mg/l).

Representative microscopic images of the development of cell adherence by *Ent. cloacae* KY 1 in the presence and absence of residual free chlorine as defined above show the decreasing number of cells adhering to SS coupons as a function of incubation time (one, three and seven days, Figure 18). The number of adhered cells decreased more slowly in the presence of chlorine. In both the presence and absence of chlorine, adherence consisted mostly of single cells adhered to the SS surface with no indication of biofilm formation (Figure 18).

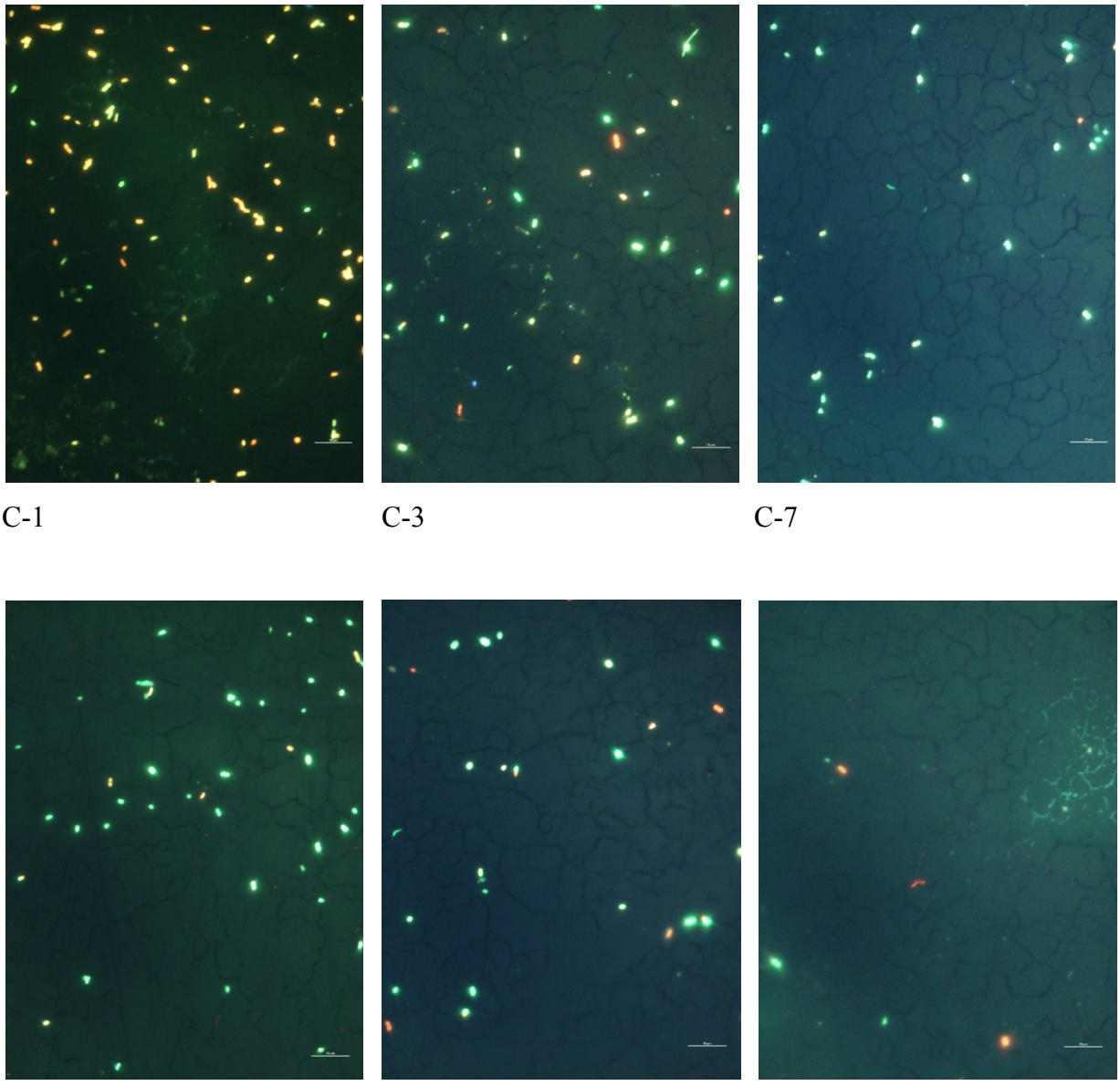


Figure 18: Micrographs of the cell adherence by *Ent. cloacae* KY 1 on SS coupons during immersion in water obtained from a drinking water distribution system in Halifax, Nova Scotia. Bacteria were stained with LIVE/DEAD BacLight and observed by epifluorescence microscopy using a 1000 x oil-immersion objective: (C-1), after one day in the presence of chlorine; (C-3), after three days in the presence of chlorine; (C-7), after seven days in the presence of chlorine; (NC-1), after one day in the absence of chlorine; (NC-3), after three days in the absence of chlorine; (NC-7), after seven days in the absence of chlorine. Scale bars, 10.0 µm.

4.2.6. Effect of Organic Material on the Cellular Adherence of *Ent. cloacae* KY 1 to Stainless Steel

Initial cell adherence was found to be similar with cell densities of approximately 5.3 $\log_{10}$cells/cm^2 (Figure 19); no significant difference was observed between the two treatments (Table A.5). *Ent. cloacae* cells adhered in higher $\log_{10}$cells/cm^2 in the presence of organic material than in the absence of organic material after three days of incubation. After seven days similar results were found where cell adherence in the presence of organic material was significantly (P<0.05) higher than in the absence of organic material, ranging from 5.98 to 4.03 $\log_{10}$cells/cm^2, respectively (Figure 19).

Plate counts of adhered *Ent. cloacae* KY 1 cells were regardless of the treatment approximately 6.4 $\log_{10}$CFU/cm^2 after one day. However, after three and seven days the number of culturable cells decreased below the detection limit in the absence of organic material whereas only a small decline in adhered cell numbers was observed in the presence of organic material (Table A.5).

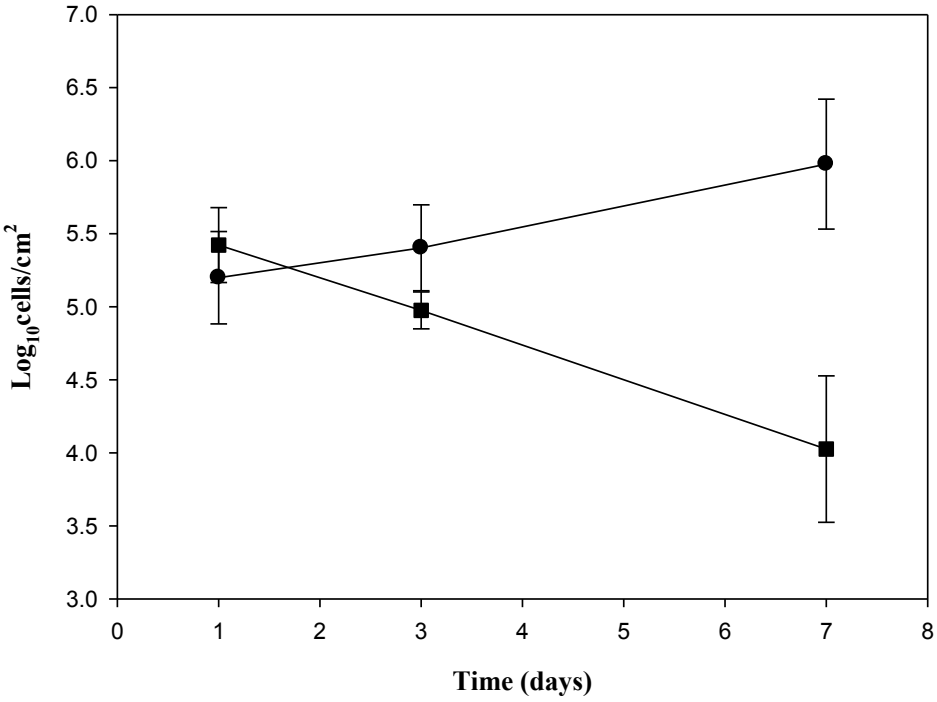
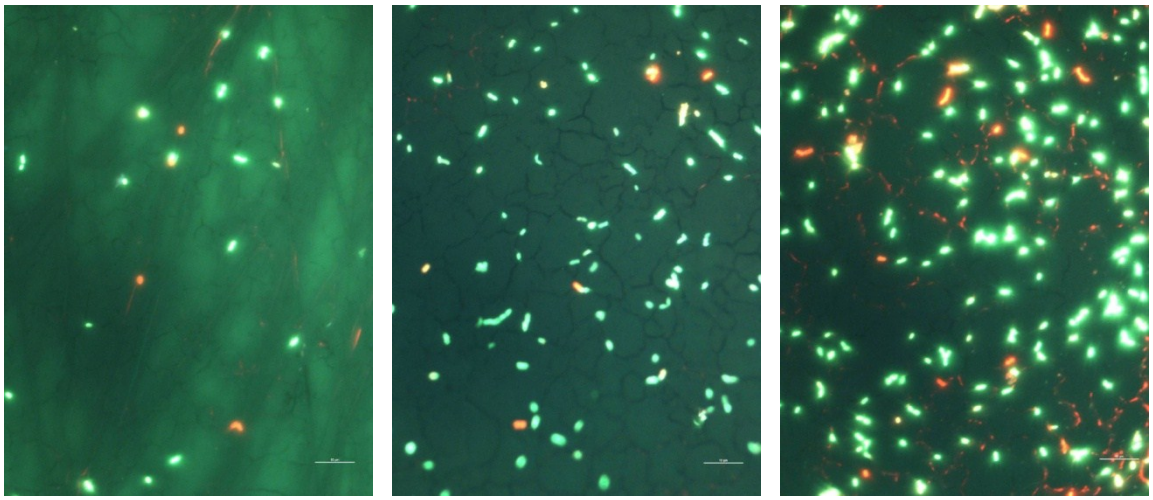
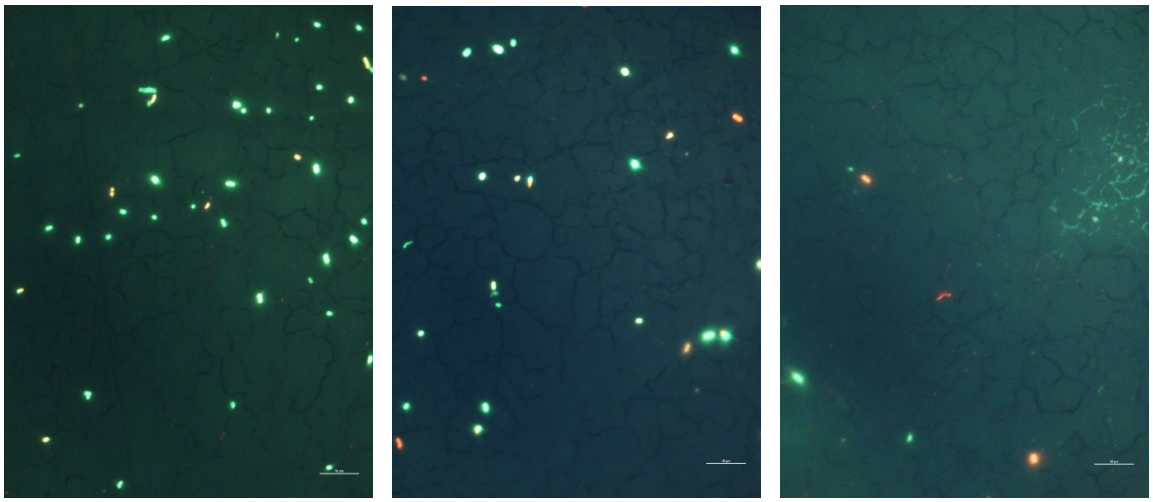


Figure 19: Effect of organic material on cellular adherence and survival of *Ent. cloacae* KY 1 on SS coupons over seven days. The number of cells was enumerated by epifluorescence microscopy using the LIVE/DEAD BacLight stain. Error bars represent standard deviation for n=3. Symbols: (●) presence of organic material; (■) absence of organic material.

Representative microscopic images in Figure 20 clearly show the decreasing number of cells of *Ent. cloacae* KY 1 adhering to SS coupons in the absence of organic material. In contrast, the presence of organic material promoted adherence of cells over time. While mostly single cells adhered to the SS surface in the absence of organic material, presence of organic material promoted the formation of chains and small clusters of cells after three and seven days incubation (Figure 20). This may be indicative of the initial stages of biofilm formation.



OM-1 OM-3 OM-7



NOM-1 NOM-3 NOM-7

Figure 20: Micrographs of the cell adherence by *Ent. cloacae* KY 1 on SS coupons during growth in water obtained from a drinking water distribution system in Halifax, Nova Scotia. Bacteria were stained with LIVE/DEAD BacLight and observed by epifluorescence microscopy using a 1000x oil-immersion objective: (OM-1), after one day in the presence of organic material; (OM-3), after three days in the presence of organic material; (OM-7), after seven days in the presence of organic material; (NOM-1), after one day in the absence of organic material; (NOM-3), after three days in the absence of organic material; (NOM-7), after seven days in the absence of organic material. Scale bars, 10.0 µm.

4.2.7. Effect of a Number of Factors (Soiled Surface, Chlorine and Organic Material) and their Treatment Combinations on the Cellular Adherence of *Ent. cloacae* KY 1 to Stainless Steel

The effect of eight treatment combinations on the cell adherence and biofilm formation of *Ent. cloacae* KY 1 on SS coupons after one, three and seven days incubation was evaluated by microscopic *in situ* examination and the plate count technique. Treatments included (also see Table 3):

- presence of a soiled surface
- presence of a soiled surface and chlorine
- presence of a soiled surface and organic material
- presence of a soiled surface and chlorine and organic material
- presence of chlorine
- presence of organic material
- presence of chlorine and organic material
- no treatment (no soiled surface, no chlorine and no additional organic material added)

The initial cell adherence for all treatments ranged from 5.19 to 5.96 $\log_{10}$cells/cm^2 and from 5.98 to 6.52 $\log_{10}$CFU/cm^2 as enumerated using microscopy and the plate count method, respectively (Table 5). The treatment combination of a soiled surface, residual free chlorine and organic material displayed the greatest overall initial cell adherence at 5.96 $\log_{10}$cells/cm^2 (microscopy) and second greatest overall initial cell adherence at 6.42 $\log_{10}$cells/cm^2 (plate count) indicating the combination of the three treatments may have a slightly synergistic effect on the initial cell adherence (Table 5).

After three days of incubation, a slight decline was observed for each treatment in the cell numbers adhered to the SS surface as enumerated by microscopy; however, no significant difference was observed among treatments (Table 5). Treatments with organic material both alone or in combination with other experimental factors were found to always yield significantly (P<0.05) greater plate count numbers compared to treatments without organic material (Table 5). After three and seven days of incubation, no cells were culturable for four out of eight of the

treatments: soiled surface, soiled surface and chlorine, no treatment and presence of chlorine (Table 5). After three days of incubation, the other four treatments all of which included the presence of organic material ranged from 5.77 to 6.20 $\log_{10}$CFU/cm^2 and displayed no significant differences from one another (Table 5). After seven days of incubation, an increase in cell numbers was observed for four treatments; this suggests the addition of organic material has a major effect on cell adherence, cell survival and biofilm formation (Table 5).

After seven days of incubation, the adhered cell numbers for the same four treatments ranged from 3.76 to 6.47 $\log_{10}$CFU/cm^2. The combination of a soiled surface and organic material displayed the highest number of adhered cells (6.47 $\log_{10}$CFU/cm^2) and was significantly (P<0.05) higher than the number of adhered cells obtained for the treatment with organic material alone (Table 5). The combination of a soiled surface, organic material and chlorine displayed the second highest cell adherence with 5.90 $\log_{10}$CFU/cm^2 followed by organic material and organic material plus chlorine at 5.60 and 4.98 $\log_{10}$CFU/cm^2, respectively.

Table 5: Effect of a number of factors (soiled surface, residual chlorine and organic material) and a combination of these factors on the cellular adherence of *Ent. cloacae* KY 1 to SS after one, three and seven days of incubation at 10°C as measured by epifluoresence microscopy and using the standard plate count method on mEndo agar.

Treatment conditions	Incubation time (day)	Live bacteria	Dead/damaged bacteria	Total bacteria	Plate count numbers
Soiled surface	1	5,50 ± 0,19 (a,b)	4,67 ± 0,51 (a,b)	5,59 ± 0,17 (a,b)	6,14 ± 0,53 (a)
Soiled surface & organic material	1	5.64 ± 0.26 (a,b)	5.16 ± 0.29 (a)	5.78 ± 0.24 (a,b)	6.4 ± 0.73 (a)
Soiled surface & chlorine	1	5.48 ± 0.38 (a,b)	4.69 ± 0.43 (a,b)	5.55 ± 0.39 (a,b)	6.08 ± 0.44 (a)
Soiled surface & chlorine & organic material	1	5.85 ± 0.58 (b)	5.16 ± 0.25 (a)	5.96 ± 0.48 (a)	6.42 ± 0.47 (a)
No treatment	1	5.37 ± 0.27 (a,b)	4.39 ± 0.29 (b)	5.42 ± 0.26 (a,b)	6.26 ± 0.44 (a)
Organic material	1	5.14 ± 0.34 (a,b)	4.17 ± 0.53 (b)	5.2 ± 0.32 (b)	6.52 ± 0.19 (a)
Chlorine	1	5.8 ± 0.21 (b)	4.26 ± 0.27 (b)	5.82 ± 0.2 (a,b)	5.98 ± 0.26 (a)
Chlorine and organic material	1	5.04 ± 0.64 (a)	4.43 ± 0.28 (b)	5.19 ± 0.53 (b)	6.27 ± 0.29 (a)
Soiled surface	3	4,24 ± 0,27 (a,b)	4,71 ± 0,32 (a,b)	4,86 ± 0,26 (a)	1,52 ± 0 (a)
Soiled surface & organic material	3	5.28 ± 1.19 (b)	5.21 ± 0.59 (a,b)	5.63 ± 0.91 (a)	6.2 ± 0.41 (b)
Soiled surface & chlorine	3	3.61 ± 0.51 (a)	4.81 ± 0.25 (a,b)	4.84 ± 0.28 (a)	1.52 ± 0 (a)
Soiled surface & chlorine & organic material	3	4.03 ± 0.94 (a,b)	5.19 ± 1.12 (a,b)	5.56 ± 0.76 (a)	5.77 ± 0.08 (b)
No treatment	3	4.78 ± 0.19 (a,b)	4.44 ± 0.25 (a,b)	4.98 ± 0.13 (a)	1.52 ± 0 (a)
Organic material	3	5.3 ± 0.26 (b)	4.65 ± 0.52 (a,b)	5.4 ± 0.29 (a)	5.89 ± 0.3 (b)
Chlorine	3	4.04 ± 0.94 (a,b)	5.41 ± 0.89 (a)	5.75 ± 0.52 (a)	1.52 ± 0 (a)
Chlorine and organic material	3	4.91 ± 0.65 (a,b)	4.09 ± 0.65 (b)	4.99 ± 0.62 (a)	6.17 ± 0.31 (b)
Soiled surface	7	4.42 ± 0.39 (a,b)	4.23 ± 0.39 (a,b)	4.64 ± 0.38 (a,b)	1.52 ± 0 (a)
Soiled surface & organic material	7	5.74 ± 1.04 (a)	5.02 ± 0.6 (a,b)	5.85 ± 0.96 (b)	6.47 ± 0.89 (c)
Soiled surface & chlorine	7	4.4 ± 1.36 (a,b)	4.17 ± 1.17 (a,b)	4.53 ± 1.37 (b)	1.52 ± 0 (a)
Soiled surface & chlorine & organic material	7	4.56 ± 1.26 (a,b)	5.71 ± 0.43 (c)	5.97 ± 0.19 (a)	5.9 ± 1.14 (b,c)
No treatment	7	3.77 ± 0.36 (b)	3.77 ± 0.5 (a)	4.03 ± 0.5 (b)	1.52 ± 0 (a)
Organic material	7	5.82 ± 0.55 (a)	5.3 ± 0.28 (b,c)	5.98 ± 0.44 (a)	5.6 ± 0.6 (b)
Chlorine	7	4.25 ± 0.76 (a,b)	4.89 ± 0.47 (a,b,c)	5.16 ± 0.22 (a,b)	1.52 ± 0 (a)
Chlorine and organic material	7	4.86 ± 0.96 (a,b)	4.58 ± 0.92 (a,b,c)	5.06 ± 0.94 (a,b)	4.98 ± 1.12 (b,c)

[a] Within each column, and comparing the same incubation day, values followed by the same letters (a)-(c) are not significantly different from each other ($P > 0.05$).

[b] Cell numbers are given in average $\log_{10}$cells/cm^2 where (n=6) or $\log_{10}$CFU/cm^2 where (n=6) ± standard deviation.

Enumeration by microscopy similarly revealed the numbers of adhering cells after one day to be significantly ($P<0.05$) higher in treatments involving a soiled surface and/or presence of organic material. After seven days, adherence was favoured significantly ($P<0.05$) in treatments with combinations of soiled surface, chlorine and organic material.

Chapter 5: DISCUSSION

5.1. Microbial Source Tracking: Phenotypic and Genotypic Strain Characterization

API 20E, Biolog and Vitek systems provide a phenotypic profile of the bacteria's ability to utilize specific carbon sources. Phenotypic profiling has a number of limitations. Firstly, only microorganisms capable of growing under the given experimental conditions may be characterized. According to environmental microbiologists, less than 2% of bacteria can be cultured *in vitro* (Wade, 2002). Also, these methods favour fast growing microorganisms and are sensitive to inoculum density. Lastly, interpretation of test results may be subjective (Kirk *et al.,* 2004). However, phenotypic tests such as API 20E are advantageous because they are generally easy to use and give a result in 18 to 24 hours.

The Kentucky isolates were analysed by the API 20E, Biolog and Vitek systems. Kentucky isolates were identified by API 20E as *Ent. cloacae*, *Ent. asburiae* or *C. freundii*. Although the virulence properties of these bacteria in water have not been fully elucidated, *Ent. cloacae* and *C. freundii* are considered to be opportunistic pathogens and may cause nosocomial infections in immunocompromised individuals (Stolp, 1988; Janda & Abbott, 2006). Although the Biolog system has been successful in identifying organisms isolated from foods and beverages (Praphailong *et al.,* 1997), profiles obtained in the present study resulted in inconclusive database identifications. This may be attributed to the fact that the substrate utilization profiles of the environmental isolates tested may have differed significantly from the species entered into the Biolog database. This corresponds with the findings of Klingler *et al.* (1992), who suggested that rapid automated identification systems designed for identifying bacteria from clinical specimens often have trouble identifying environmental isolates. Klingler *et al.* (1992) also found that the potential of Biolog to identify Gram-negative bacteria was highly dependent on incubation time and the optimal incubation times varied depending on the bacteria being analysed. Miller & Rhoden (1991) found that Biolog was able to correctly identify *Citrobacter* species more consistently than *Enterobacter* species after a 24 hour incubation period. Difficulty in identifying *Enterobacter* spp. may be due to the high degree of similarity in phenotypic profile amongst species.

In this study, the Biolog method was not able to identify any of the *Ent. cloacae* isolates previously identified as such by API 20E. In order to improve identification success for

Enterobacter isolates, the Biolog instructions recommended adding sodium salicylate to the inoculation fluid instead of sodium thioglycolate to prevent occurrence of false positive reactions in the control well (no carbon substrate). In this study, neither the use of sodium salicylate nor sodium thioglycolate supplemented media resulted in the correct identification of *Ent. cloacae* (ATCC 23355) after four to six hours or 24 hours of incubation. However, interestingly the *C. freundii* most probable result from the Biolog system for isolates KY 14, 15, 17, and 18 agreed with the API 20E identification. This suggests that Biolog has some potential to identify certain environmental species. Biolog identified some bacteria, but other *Enterobacteriaceae* genera including *Klebsiella, Enterobacter*, and *Serratia* spp. were also not easily identified in another study. Miller & Rhoden (1991) suggested this to be due to the species being so active that most of the carbon sources are utilized, giving too many positive reactions and no identification. The Vitek system yielded substrate utilization profiles but was only able to identify some of the Lexington and Georgetown coliform isolates. Expansion of species identification databases to represent a broader range of both clinical and environmental strains of bacteria would strengthen the position of phenotypic identification systems as water quality monitoring tools. The results of this study show that some phenotypic profiling systems such as Biolog and Vitek systems may be useful in differentiation rather than identification of environmental species.

For the reasons mentioned above phenotypic profiling can only be used to obtain a rough microbial source tracking profile mostly finding use as a first level characterization method. In some cases API 20E, Vitek and Biolog are also used as identification systems. In this study Vitek and Biolog displayed poor identification of the environmental bacterial isolates whereas API 20E identified all of the environmental bacterial isolates. These methods tend to have a lower discriminatory power when compared to genetic methods; genotyping allows the discrimination of finer differences among strains belonging to the same species. Pulsed field gel electrophoresis has a higher discriminatory power, broad application and is the "gold standard" for typing bacterial isolates (Shi *et al.,* 1996; Barrett *et al.,* 2006).

Characterization of bacterial isolates is important because it aids epidemiologists in determining outbreak strains from sporadic strains. When a food has been implicated as a potential cause of an outbreak, characterization of microbial isolates by typing using one of the many methods available helps provide confirmation of the events being linked (Barrett *et al.,* 2006). In a pathogen outbreak, PFGE analysis of bacterial strains isolated from sick individuals

can be compared with bacterial strains isolated from suspected sources and compared for genetic relatedness. For example in an outbreak connected to a farm, manure was suspected of leaking into a well that supplied a county fair with its drinking water. Visitors became infected and PFGE analysis done on isolates from sick individuals and the suspected water source, showed that 63% of both the human and drinking water isolates displayed an identical PFGE profile. Twenty-seven % of the human isolates displayed a PFGE profile differing by two bands, suggesting the outbreak strain may have persisted long enough to produce variants (Barrett *et al.,* 2006).

Our results show that PFGE can discriminate between two isolates that based on enzymatic tests appear to be the same. This may be due to the fact that genetic differences can exist between two strains that express the same phenol- or biotype. It should be noted that genotypic tests are commonly favoured over phenotypic tests because genotypic profiles are more stable (Simpson *et al.,* 2002). The genotyping results discriminating between bacterial isolates were similar for PFGE and ribotyping with the exception of one bacterial isolate (Table 4). Ribotyping and PFGE have previously been used for the typing of *Ent. cloacae* with high discriminatory potential and good reproducibility (Shi *et al.,* 1996). Overall a disadvantage to ribotyping occurs when there are a limited number of rRNA genes within a bacterial species. This limitation has decreased the discriminatory level as compared to other methods such as PFGE (Foley *et al.,* 2009). A number of researchers have observed higher discriminatory power with the PFGE method as compared to ribotyping (Pfaller *et al.*, 1996; Hollis *et al.*, 1999; Botes *et al.*, 2003; Rodriguez-Calleja *et al*., 2006). The discriminatory power of ribotyping has specifically been stated to be lower than that of PFGE for *E. coli* (Foley *et al.,* 2009). Although PFGE generally has a higher discriminatory power than ribotyping it has a few disadvantages including being labour intensive, requiring two to four days from start to finish and working best if linked to a MST library database. Multiple restriction enzymes have been used to optimize the cutting frequency so a change such as a genetic event may be identified thereby increasing the discriminatory power of the method (Foley *et al.,* 2006).

Figure 1 shows that the Kentucky isolates were sampled from geographically separated sites in the Lexington and Georgetown distribution system and that several of these isolates had similar PFGE profiles, despite the fact they originated from different sites and/or dates (Table 2 and Table 4). This wide distribution of the “same” strain suggests that the species or genotype

had become ubiquitous in the water distribution system. Furthermore, the similar PFGE profiles suggest the bacterial isolates were genetically linked and most likely came from a common source. This indicates that a regrowth problem may exist within the Kentucky distribution system and that a permanent biofilm flora may have become established in the system. Regrowth is illustrated by the persistent occurrence of coliform bacteria in drinking water. The issue with biofilms in water distribution systems is that they may act as reservoirs for microorganisms including bacteria, viruses and protists (Blanch *et al.,* 2007). In this case study, PFGE turned out to be a useful tool to track a series of coliform events associated with the distribution system as the results pointed toward there being coliform regrowth/biofilm problem. Consequentially, it is suggested that the water utility strive toward some type of remediation with a focus on controlling the occurrence of biofilm and coliform regrowth in the system.

A collection of 18 coliform bacterial strains were isolated from a specific area of the drinking water distribution system. All our study strains were isolated during the months of August, September and October. This led to the suspicion that the occurrence of coliform bacteria in this Lexington, Kentucky drinking water distribution system was linked to possible cross contamination from a commercial food service outfit. During the same period, in many cases when taps from the distribution system were flushed rapidly during sampling, the water initially came out clear but then turned black for many seconds before changing back to clear again (Friedman *et al.,* 2009). It may be the case that biofilms had formed in the water pipes possibly due to backflow from other devices used in the commercial food location (e.g., soft drink dispensers). Other observations, such as the release of several pieces of a rubber gasket during vigorous flushing of the tap, would indicate that a back flow problem existed which could have introduced both coliform bacteria and nutrients into the drinking water system. In this study nutrients and a soiled surface were shown to promote cell adherence and biofilm formation of *Ent. cloacae* KY 1, which is one of the persistent *Enterobacter* genotypes isolated from the distribution system.

Other tools that may be useful in tracking a series of coliform events associated with a distribution system but not tested in this study include library-independent methods that identify sources based on host-specific characteristics of the bacteria. A main advantage of these methods is that they do not require library databases or collection of isolates. Methods identifying host-specific characteristics of bacteria aim to find specific markers unique for the source. Polymerase

chain reaction based detection of *Bacteroidales*, a genus of anaerobic bacteria that are often found in abundance in the gut of warm blooded mammals, is one of these techniques. These bacteria are present in larger numbers than indicator bacteria in feces but are challenging to culture due to their anaerobic nature (Anonymous, 2011h). Polymerase chain reaction detection of specific virulence genes that are clinically important is another technique that may provide a better indication of microbial water quality. Methods that use biomarkers targeting the clinically important *E. coli* as compared to the non-pathogenic *E. coli* in the intestine based on enterotoxin genes for *E. coli* have been developed (Ahmed, 2005). Overall each MST method appears to have distinct advantages and disadvantages and as a result there is no single method that is capable of identifying specific sources of fecal pollution in the environment with absolute certainty (Scott *et al.,* 2002; Meays *et al.,* 2004). Researchers have suggested combining a number of methods in order to enhance discrimination or provide confirmation of results hence the use of a multi-tiered approach is recommended (Payne, 2007; Roslev & Bukh, 2011).

5.2. Influence of a Number of Environmental Conditions (Temperature, Strain, Material Surface, Soiling of Surface, Chlorine and Organic Material) on the Cellular Adherence, Cell Survival and Biofilm Formation of *Ent. cloacae* and *Ent. asburiae*

5.2.1. Effect of Temperature on the Cell Adherence and Biofilm Formation of *Ent. cloacae* KY 1

Numbers of adhered cells of *Ent. cloacae* KY 1 to SS at warmer incubation temperatures (21 and 30°C) was found to be significantly (P<0.05) higher than at colder incubation temperatures (4 and 10°C) in this study suggesting greater cell adherence at warmer temperatures. This is in agreement with a study by Kim *et al*. (2006) who found that two strains of *E. sakazakii* adhered to SS in significantly (P<0.05) higher numbers at 25°C compared to 12°C. Three other strains exhibited a similar trend with greater cell adherence at 25°C when compared to 12°C but this was not significant. This is likely due to the variation in cell adherence by different strains. In other studies authors reported similar findings, Di Bonaventura *et al.* (2008) observed the ability of *L. monocytogenes* strains to adhere and produce biofilm on SS which proved to be significantly (P<0.05) greater at 22 and 37°C when compared with 4 and

12°C. Similarly, many water utilities observe increases in bacterial numbers in the drinking water distribution systems during late spring, summer and early fall coinciding with warmer than average water temperatures (Geldreich, 1996). Evidence suggests that warmer water temperatures above 15°C speed up the growth of microorganisms that are persisting in the water distribution system (Geldreich, 1996). The warmer incubation temperatures offer better environmental conditions for the growth of coliform bacteria such as *Ent. cloacae*, whose optimal growth temperature ranges between 30 and 37°C (Bergey & Holt, 1994).

When the number of adhered cells of *Ent. cloacae* KY 1 was compared at the various temperatures, the greatest cell adherence to SS was observed at 21°C. A water temperature of 21°C commonly occurs during the months of August, September and even into October in both the Lexington and Halifax drinking water distribution systems. Also, 21°C was the only temperature, of the four temperatures used in the study where the initial stages of biofilm formation may have been observed. This suggests that while the optimal growth temperature for most *Ent. cloacae* is between 30-37°C (Bergey & Holt, 1994), this temperature range may not be the optimal for biofilm formation of this persistent strain of *Ent. cloacae*. This observation of biofilm formation by a bacterial spp. at a lower than optimal growth temperature has been demonstrated in numerous studies and is not a surprising finding. This observation is in agreement with a study where cell adherence and growth of *Ent. cloacae* to plastic material surfaces was greatest at the mid-range temperatures, where the bacteria displayed good growth at 15, 20, 25 and 30°C and moderate and poor growth at 37 and 45°C respectively (McEldowney & Fletcher, 1988). A study by Stepanovic *et al.* (2003) also found cell adherence and biofilm formation to be greatest at 21°C for a *Salmonella* spp. after 48 hours of incubation when compared with incubation at 30 and 37°C. This adds additional evidence that the optimal temperature for cell adherence and biofilm formation is lower than the optimum growth temperature. Vivas *et al.* (2008) found that the most favourable temperature for biofilm formation for all *Hafnia alvei* strains examined in their study was 25°C as opposed to 37°C. Biofilm formation is seen more frequently at a lower temperature than the bacteria's optimal growth temperature, as has been observed for *L. monocytogenes, Le. pneumophila* and *E. coli*, whose optimal growth temperatures are 37, 36 and 37°C, respectively, while their optimal biofilm formation temperatures have been found to be 20, 20 and 23°C, respectively (Norwood & Gilmour, 2001; Geier 2008).

It has also been proposed that temperatures above the optimal growth temperature may prompt a range of bacterial stress responses that could be associated with biofilm formation. However, this phenomenon was not observed in the study by McEldowney & Fletcher (1988) with the bacterium *Ent. cloacae*. The other studies mentioned above, did not include temperatures above the optimal growth temperature for the bacterium. Since the water temperature in the distribution systems mentioned in this study would rarely reach temperatures above the optimal growth temperature of coliforms, existence of this stress response associated with these temperatures may be of little importance in the drinking water distribution systems. Of more relevance to the drinking water distribution systems discussed in this study are temperatures that are lower than the optimal growth temperature of the bacteria which as mentioned above have been linked to greater numbers of adhering cells and more biofilm formation (Geier, 2008).

Similar to the hypothesis regarding the stress responses induced by temperatures above the optimal growth temperature for the bacterium causing an increase in cell adherence and biofilm formation, perhaps the reason for greater adherence at lower than optimal growth temperatures may be found in the induction of a number of stress-related genes (Geier, 2008; Landini, 2009). Geier (2008) has stated that in both Gram-positive and Gram-negative bacteria induction of stress-related genes, which are controlled by the common stress response regulators, SigB and RpoS, respectively, might account for the differences associated with temperature. This may be due to the physiological characteristics of the microorganism and ensuing effect on adherence. Temperature has also been found to regulate the production of fibrils, flagella and EPS to in turn affect biofilm formation based on their influence on the electrostatic repulsion forces between bacterial cells and a surface as reviewed by Chen (2009). Landini (2009) indicated that lower than optimal growth temperatures stimulate production of curli fibers which are adhesion factors that are known as thin aggregative fimbriae involved in the link between the stress response and biofilm formation in *Enterobacteriaceae.* These fibers have previously been shown to be involved in bacterial adherence and biofilm formation, and furthermore, expression of curli fibers in a number of *E. coli* strains from the *Enterobacteriaceae* family were found to be temperature dependent and occurring at 28°C but not 37°C (Ong *et al.,* 2008). Norwood & Gilmour (2001) construed two possible explanations for the observed greater cell adherence at 18°C, which is below the optimal growth temperature for *L. monocytogenes*: 1) *L.*

monocytogenes produces EPS at 21°C but not at 10 or 35°C, or 2) *L. monocytogenes* possesses numerous flagella at 20°C but only a small number of flagella at higher temperatures such as 37°C (Norwood & Gilmour, 2001). The influence of the production of EPS, and higher numbers of flagella, and their effect on enhancing cell adherence to surfaces at lower than optimal growth temperatures may also be observed in other bacteria including *Le. pneumophila*, *E. coli* and *Ent. cloacae* (Trachoo, 2007). Taken together, this body of knowledge points to a mechanism where temperature affects motility and in turn cell adherence and biofilm formation as flagella are motility organelles that participate in the initial adhesion stage of biofilm formation (Ong *et al.,* 2008)

In contrast, other studies have shown increased cell adherence with increasing incubation temperature. For example, the number of adhered bacterial cells to SS in both nutrient-rich and nutrient-poor media was found to increase with increasing temperature with the exception of 42°C where it was found to be lower (Tam & Conner, 2007). Similarly, Pan *et al.* (2010) also observed increasing biofilm densities of *L. monocytogenes* strains with increasing incubation temperatures (22.5, 30 and 37°C). This indicates that the connection between temperature and biofilm formation may be rather complex and difficult to predict due to the use of different strains and experimental conditions. Theoretically approximately 10^7 CFU of bacterial suspension was spiked into the well with the coupon. Microscopic examination and the plate counts were not carried out at time zero. The rate of adherence was not measured in this study as most cell adherence occurred within 24 hours of incubation. Only the temperature of 21°C and the addition of nutrients played a role in increasing cell adherence after one day of incubation. After 24 hours of incubation the bacterial population was generally stable or declining unless additional nutrients were supplied.

5.2.2. Comparison of the Cell Adherence and Biofilm Formation of Five Different Environmental Isolates to Stainless Steel

This present study shows that strains belonging to the same bacterial species differ in their cell adherence and biofilm formation. This result is similar to the findings of (Trachoo, 2007; Vivas *et al.,* 2008) who demonstrated variability in cell adherence and biofilm formation by different strains of bacteria within a species. A study by Iversen *et al.* (2004) provides

additional evidence that strain variance exists; this study found denser biofilms by a capsulated strain versus a non capsulated strain of *Ent. sakazakii*. Reisner *et al.* (2006) showed that a number of naturally occurring *E. coli* strains varied in their biofilm forming abilities. Also, in their study, other factors such as growth medium composition was shown to have an effect on biofilm forming ability (Reisner *et al.*, 2006). Marouani-Gadri *et al.* (2009) found variability in the cell adherence and biofilm formation by *E. coli* 0157:H7 strains on surfaces. A combination of factors including temperature, medium and surface material was found to play a role in this variability in cell adherence. Many other researchers observed strain variability when comparing the cell adherence and/or biofilm formation of bacterial strains to surfaces (Norwood & Gilmour, 1999; Lunden *et al.,* 2000; Chae & Schaft, 2001; Borucki *et al.,* 2003; Reisner *et al.,* 2006; Wood *et al.,* 2006; Marouani-Gadri *et al.,* 2009; Pui *et al.,* 2011). This demonstrates that bacterial strains of the same species under identical growth and environmental conditions adhere to SS surfaces in a way that is strain-specific. This finding precludes a trend observed in the present study toward greater cell adherence by a persistent strain and a closely related strain with the exception of *Ent. cloacae* KY 1. This trend towards greater cell adherence by a persistent strain is in agreement with studies of other bacteria. A study by Norwood & Gilmour (1999) found that persistent *L. monocytogenes* strains adhered in significantly (P<0.05) higher numbers with a mean value of 5.67 $\log_{10}$CFU/cm^2 than sporadic strains with a mean value of 5.45 $\log_{10}$CFU/cm^2. In another study by Lunden *et al.* (2000) persistent *L. monocytogenes* strains from a poultry plant exhibited initial cell adherence levels, which were 2.7 to 4.6 fold higher than those of most of the non-persistent strains. Although after prolonged cell adherence and 72 hours incubation more of the non-persistent strains reached cell adherence levels comparable to that of the persistent strains (Lunden *et al.,* 2000). Additional evidence that supports persistent strains being more adhering and better biofilm formers was found in a study of bulk milk sample isolates (Boruki *et al.,* 2003). Here they reported that strains that were considered poor biofilm formers adhered to both PVC and SS surfaces in low numbers whereas other strains considered to be good biofilm formers adhered in higher numbers to both PVC and SS resulting in the formation of dense biofilms.

In our study, environmental isolate *Ent. cloacae* KY 1 appears to be persistent in the drinking water distribution system in Lexington, KY, however, it was less adhering to SS surfaces compared to the KY 4 and KY 6 isolates at 10°C. The results may have been different

had another assay temperature (e.g., 21°C) been used. The water temperature might have been higher than 10°C at the time of sampling in Lexington, KY as the sampling dates were in the months of August, September and October where air temperatures likely range from 20-25°C. A warmer water temperature of 21°C in the distribution system would provide a more optimal temperature for cell adherence and biofilm formation of the *Ent. cloacae* strain as was observed in section 4.2.1. Therefore, temperature may play a role in the persistence of a bacterial strain and may explain why the most persistent strain *Ent. cloacae* KY 1, did not display the greatest number of adhered cells in the assay at 10°C. The results may also have been different due to the assay using Halifax tap water where nutrient conditions may differ slightly from the drinking water in the Lexington system.

The reason as to why bacterial strains of the same species may differ in their cell adherence to surfaces under identical growth and environmental conditions may in part be attributed to the motility of the bacterial strain. This has been proposed to play a role in the variability in biofilm formation of *Listeria* strains (Djordjevic *et al.,* 2002). In a study by Wood *et al.* (2006) bacterial strains that were found to be the best biofilm formers exhibited the highest motility whereas the strains that displayed poor biofilm forming abilities exhibited impaired motility. Motility appendages known as flagella have been known to contribute to adhesion of bacterial cells to surfaces (Vatanyoopaisarn *et al.,* 2000; Lemon *et al.,* 2007). In the study by Vatanyoopaisarn *et al.* (2000) *Listeria* spp. flagella were shown to be involved in the adherence of cells to the surface at 22°C but not at 37°C where the flagella were not expressed. A study by Lemon *et al.* (2007) similarly showed the critical nature of flagella motility in formation of biofilms at 30°C. In contrast, a study by Tresse *et al.* (2009) found naturally aflagellated and wild-type motile *L. monocytogenes* strains comparable in their cellular adherence to surfaces with *flaA* mutant non-motile strains.

Throughout our study, a discrepancy was observed between the microscopic and plate counts as for example seen in Figures 9 and 13, respectively. Initially the plate count numbers were similar to the microscopy method suggesting that after one day most cells were viable and culturable. After seven days of incubation, however, all the plate counts for all environmental isolates fell below the detection limit of 1.52 $\log_{10}$CFU/cm^2. This indicates that while the cells may have maintained membrane integrity and are observable by microscopy, they were, however, dead, injured or stressed to the point that they were no longer culturable in the

particular bacteriological media under the given set of incubation conditions. In the case of the stressed or injured cells they are commonly referred to as viable but nonculturable. mEndo agar was chosen over a non selective agar to selectively enumerate coliform bacteria in the non-sterile Halifax water samples. Microscopic examination of coupons immersed in unspiked Halifax tap water revealed indigenous bacterial levels to be below the detection limit of 3.23 $\log_{10}$cells/cm^2. In Nova Scotia, drinking water regulators have implemented the federal Guidelines for Canadian Drinking Water Quality published by Health Canada and established by the Committee on Drinking Water as legally binding standards for regulated public drinking water supplies. These guidelines state that the maximum acceptable concentration of *E. coli* or TC in water leaving a treatment plant in public, semi-public and private supply systems is less than one cell per 100 ml of water. In this study, coliform bacteria were never detected in unspiked Halifax water samples indicating compliance to the regulations by the utility.

5.2.3. Effect of Material Surface on the Cellular Adherence of *Ent. cloacae* KY 1

The number of adhering *Ent. cloacae* KY 1 was significantly (P<0.05) higher on PVC than on the SS surface, suggesting that adherence is affected by the material surface. Kim *et al.* (2006) similarly found greater numbers of *Ent. sakazakii* (now known as *Cr. Sakazakii*) to adhere to PVC feeding tubes than to SS surfaces. This influence of material surface on the adherence of bacteria has also been observed by a number of other researchers (Speers & Gilmour, 1985; Hallam *et al.,* 2001; Djordjevic *et al.,* 2002; Momba & Makala, 2004; Simoes *et al.,* 2006). Speers & Gilmour (1985) found that numbers of adhered cells were higher on SS surfaces when compared to glass and rubber surfaces. In agreement with our study Djordjevic *et al.* (2002) found higher numbers of adhered cells on PVC than on SS surfaces for all strains tested. In a study by Momba & Makala (2004) higher numbers of adhered cells were found on plastic-based material surfaces relative to cement-based materials. Similarly, Simoes *et al.* (2006) added evidence in support of PVC providing a material surface that generally allows bacterial cells to adhere in greater numbers than SS.

Hydrophobicity of the surface may play an important role in the adherence of bacteria as PVC is known to be hydrophobic apparently enabling bacteria to adhere more readily to the surface than to the hydrophilic SS surface. Cell wall hydrophobicity of the strains and charge of

the bacterial cell wall have also been suggested to play a role (Djordjevic *et al.,* 2002). This helps explain why greater cell adherence is observed on the PVC surface than on SS. This is in agreement with a study by Iversen *et al.* (2004) that found two strains of *Ent. sakazakii* to adhere to silicon, latex and polycarbonate in greater numbers than to SS when grown in infant formula milk. A study by Ryu *et al.* (2004b) hypothesized that the production of curli by *E. coli* would increase hydrophobicity of the surface of the cells consequently impeding the adherence of the bacteria to SS. However, the production of curli by *E. coli* 0157:H7 had no effect on the cell adherence to SS but helped allow bacterial cells to form biofilm. Based on these results the authors concluded that the adherence of cells to a surface is affected by a number of complicated mechanisms impacted by the cell surface, the surface material and the medium (Ryu *et al.,* 2004b).

Smoothness of a surface has also been proposed to be a factor in the initial adherence of bacteria to a surface. Biofilms have been suggested to form at a slower initial rate on smoother surfaces (Momba & Makala, 2004). The smoothness of the material may depend on the finishing of the surface; this was not recorded in this study but may have played a role in cell adherence.

In contrast, Zacheus *et al.* (2000) observed no difference in terms of cellular adherence to the surface or biofilm formation when comparing three surfaces: PVC, polyethylene and SS. In a study by Pedersen (1990) the number of adhered cells of total microorganisms as determined on surfaces using epifluorescent microscopy after a period of 167 days did not differ significantly between PVC and SS surfaces.

In the current study after both three and seven days of incubation the plate count numbers decreased dramatically to levels below the detection limit of 1.52 $\log_{10}$CFU/cm^2 for both surfaces. This was likely due to the stressful conditions that included the low incubation temperature and an environment low in nutrients. If more nutrient rich conditions and a warmer temperature that would be more supporting of growth were used in the experiment, it would have been possible to establish whether a difference was observed between the PVC and SS under these conditions. Under the harsh conditions used in our study the *Ent. cloacae* KY 1 was not able to survive at detectable levels on either surface as indicated above. Simoes *et al.* (2006) have suggested that other factors can play a role in promoting bacterial cell adherence and survival on the material surface including environmental conditions to which they are exposed. This may help explain why some researchers may have not observed a significant difference in

cellular adherence for some surfaces and why no differences were observed in viable numbers of adhering cells after three and seven days incubation in our study.

5.2.4. Effect of a Soiled Surface on the Cellular Adherence of *Ent. cloacae* KY 1

To imitate the conditioning films formed on surfaces in aquatic environments, SS coupons were immersed in the water containing 0.1% peptone and incubated at 10°C for 24 hours before the addition of the bacterial culture. A study from Loeb & Neihof (1975) discovered that conditioning films on surfaces exposed to seawater formed immediately but could continue to grow for several hours. Surface conditioning has been known to affect the adhesion of bacteria to pipe and other surfaces (Barnes *et al.,* 1999; Murga *et al.,* 2001; Kolari, 2003; Chen *et al.,* 2010; Dat *et al.,* 2010; Takahashi *et al.,* 2010). In this study, the numbers of adhered cells was slightly higher after one day of incubation when the surface was previously conditioned with 0.1% peptone. This slight increase in adhering cells to the surface may be due to the surface conditioning of the SS coupon and more nutrients being available near the surface and in the suspending medium. After seven days of incubation the number of adhered cells in the presence of a soiled surface was higher than for the non-soiled surface, suggesting that the presence of a soiled surface may increase adherence.

A study by Murga *et al.* (2001) also observed increased adherence of cells to the preconditioned surface for all three organisms tested as compared to non-conditioned surfaces. Greater biofilm formation was observed on the human blood preconditioned surface after 14 days when compared to the non-conditioned surface (Murga *et al.,* 2001). Additional evidence to support our results was observed by Takahashi *et al.* (2010) who found greater survival of *L. monocytogenes* and *S. aureus* on food soiled SS surfaces than on non-food soiled surfaces. In a study by Dat *et al.* (2010) the numbers of adhered bacteria increased when SS surfaces were soiled with milk that had a neutral or slightly alkaline pH. Overall, numbers of adhered bacteria were higher on conditioned surfaces than on the non-conditioned surfaces similar to this study. Barnes *et al.* (1999) observed that bacterial adherence increased when SS surfaces were soiled with milk as compared to non-soiled surfaces. It was implied that this may be a consequence of ferrous ions in solution which due to their interaction with surface-adsorbed protein may act as a

bridging cation between protein and the bacteria or by cross-linking the protein molecules lessening their probability for contact with the bacteria.

It has been proposed that when a clean surface such as a SS coupon is placed in an aqueous environment such as water (in this study 0.1 % peptone), it will likely result in a quicker adsorption of inorganic and organic substances to the SS coupon producing the conditioning film before the initial adhesion of the microorganisms (Chen *et al.* 2010; Ortega *et al.,* 2010). This interaction between the medium, water with 0.1 % peptone in our study, and the surface, may involve the surface becoming conditioned by polymers with adsorption of inorganic salts, proteins, glycoproteins and humic compounds to the surface (Donlan, 2002; Kolari, 2003) as conditioning films consist of organic molecules including carbon sources, complex polysaccharides, inorganic components including mineral salts and metallic hydroxides (Chen *et al.,* 2010). Organic and inorganic material is moved toward the SS surface either by diffusion or turbulent flow (Palmer *et al.,* 2007). In this static experiment diffusion would likely be the cause of organic material being carried towards the surface. The adsorption of organic material and molecules such as those mentioned above to SS surfaces play an important role in the production of a conditioning film by altering the physicochemical properties of the surface varying the capability of bacteria to be attracted to the surface (Barnes *et al.,* 1999; Palmer *et al.,* 2007; Dat *et al.,* 2010; Mafu *et al.,* 2011). Variable physicochemical factors include surface free energy, hydrophobicity and electrostatic charges (Dat *et al.,* 2010). In addition, physicochemical properties of bacterial cell surfaces vary with species and physiological state, and such variability in turn affects the compatibility with the preconditioning organic molecules. This compatibility depends on the organic material used (Ortega *et al.,* 2010).

This accumulation of molecules at the solid-liquid interface leads to a higher concentration of nutrients at the surface compared with the liquid phase, offering a rich fresh nutrient source for newly adhering cells. There is likely a natural tendency of planktonic cells to move toward the nutrient-concentrated surface (Palmer *et al.,* 2007; Chen *et al.,* 2010). The rough film resulting from surface conditioning might also play a role in allowing the surface to harbour bacteria (Dat *et al.,* 2010). Also, the increased hydrophobicity of the conditioning film, along with the improvement of cation-bridging interactions and the impact of the film imply that the initial stages of cellular adherence should be considered as the combined outcome of multiple factors (Chen *et al.,* 2010).

Any differences observed by researchers regarding bacterial cell adherence to soiled surfaces may be due to varying laboratory conditions, use of different strains of bacteria, surface materials and organic material in forming the conditioning film (Palmer *et al.,* 2007).

Conceivably, the use of 0.1% peptone as the organic material to soil the surface constituted a very low nutrient level. It is proposed that if another substance was used to soil the surface or a higher nutrient level was used for the preconditioning then we may have seen a larger effect on initial cell adherence and biofilm formation.

5.2.5. Effect of Chlorine as a Disinfectant

In the present study we observed contrary to our initial hypothesis that higher numbers of cells adhered to SS in the presence of 0.44 mg/l residual free chlorine.

A number of researchers have observed the effect of chlorine on bacterial cell adherence to surfaces. Camper *et al.* (1999) observed differences in the structure of biofilms that were treated with chlorine and not treated with chlorine; thereby demonstrating chlorine had an effect on biofilms. Van der Wende *et al.* (1989) found that chlorine concentrations lower than 0.2 mg/l reduced biofilm accumulation in the reactor. In agreement with the survival of *Enterobacter* spp. observed in this study, Williams *et al.* (2003) showed that *E. coli* and *Listeria* spp. were able to survive in drinking water with 0.2 mg/l free chlorine.

However, in contrast to the results reported in our study, Zhou *et al.* (2009) found that the number of effluent heterotrophic bacteria was lower from AR reactors fed with chlorine (0.6 mg/l) containing water than from ARs fed with chlorine-free water. The water in Zhou *et al.,* (2009) study was reported as containing high concentrations of nutrients, which in comparison to this study where nutrient content was minimal may not demonstrate the effect of chlorine alone as a factor. Chu *et al.* (2003) found heterotrophic plate counts of biofilm bacteria to be roughly two to three fold higher in the absence of chlorine than in the presence of any level of chlorine but they did not investigate the effect of nutrient content. No significant ($P>0.05$) difference was observed when comparing plate count numbers from water with a low or high level of chlorine (Chu *et al.,* 2003). Similar to the results found by a number of researchers, Lomander *et al.* (2004) observed that the presence of chlorine in water decreased biofilm formation. When the low level of chlorine was added to the water this solution killed significantly more bacteria in the

biofilm than water with no chlorine, demonstrating the killing effect of chlorine. Additional evidence supporting the inhibitory effect of chlorine was offered in a study by Tsai (2006), where the presence of chlorine in the water decreased the number of bacteria in the biofilm due to the bactericidal effect of the chlorine. Chlorine and free chlorine has been stated to generally work by limiting bacterial regrowth. The chlorine in these instances likely injures bacteria thereby preventing growth and potentially restricting the production of bacteria in both the planktonic or biofilm states (Percival *et al.,* 2000; Batte *et al.,* 2003). The power of chlorine to act as a disinfectant occurs due to its oxidative interactions with the sulfhydryl groups on specific enzymes in the cell membrane of bacteria or protoplasts, leading to cellular proteins becoming permanently damaged (Lomander *et al.,* 2004). These authors also suggest that chlorine encourages irreversible decarboxylation reactions and that a specific amount of free chlorine should be used in the disinfectant solution to counter the inactivation caused by organic material.

Considering the usual antimicrobial effect of chlorine, the number of surface adhered cells or amount of accumulated biofilm would be hypothesized to decrease with increasing levels of chlorine in the water, yet this was not the case in the present study. The environmental stress appeared to have promoted adherence, however, the reason for this is not known but may be related to chlorine induced changes to the cell surface or the stress response. It is also possible that there was greater detachment of biofilm cells in the absence of free chlorine resulting in higher numbers of planktonic cells in the water; however, this was not measured in the present study. Another possible reason may be that temperature may have affected the cells' adherence to SS as water was refrigerated to remove chlorine and was likely colder than the water with chlorine taken from the tap directly. This cold stress to the cells may have affected the results.

As previously mentioned chlorine based inactivation of bacteria is related to the concentration of hypochlorous acid (HOCl), which in turn is related to the pH of the water. This means variations in pH and chlorine concentration could potentially also affect adherence and biofilm formation although this study did not look at these factors.

5.2.6. Effect of Organic Material on the Cellular Adherence of *Ent. cloacae* KY 1

The present study demonstrated that organic material in drinking water affects the adherence of the *Ent. cloacae* KY 1 strain to SS surfaces at 10°C. The presence of organic material as 0.1% peptone resulted in significantly (P<0.05) higher numbers of cells adhering to the SS surface. This observation agrees with numerous reports showing that the level of nutrients available for microorganisms in a water distribution system, affects adhesion of the bacteria to the pipe material as well as biofilm formation (Geldreich, 1996; Percival *et al.,* 2000; Vatanyoopaisarn *et al.,* 2000; Donlan, 2002; Dunne, 2002; Tam, 2006; Tam & Conner, 2007). Donlan (2002) suggested that an increase in the number of adhered cells to a surface was correlated to an increase in nutrient concentration. This is in agreement with a study by Kim *et al.* (2006) who concluded that the type and availability of nutrients plays a function in the process that leads to biofilm formation. In their study, *Ent. sakazakii* biofilms formed on the PVC feeding tubes immersed in infant formula milk at 25°C, in sterile TSB and in lettuce juice. These media vary in their nutrient levels with infant formula milk and TSB containing greater levels of nutrients than lettuce juice. Infant formula milk may contain certain proteins that have the potential to enhance bacterial adherence to surfaces more so than TSB and may be a reason why it was the only medium in which a biofilm was formed. The lack of nutrients in lettuce juice and an observed reduction in pH during incubation may have contributed to the smaller number of surface adhered cells in that medium (Kim *et al.,* 2006). Additional evidence that supports the role of nutrients in cell adherence was observed by Vivas *et al.* (2008), who found that conditions rich in nutrients (TSB and Brain Heart Infusion Broth) favoured biofilm formation. Tam (2006) similarly reported that the adherence of *L. monocytogenes* to SS was greater when cultured in Brain Heart Infusion Broth compared to a medium low in nutrients that consisted of 10% naturally derived pond microcosm and 90% water. *P. aeruginosa* is another example of a bacterium which preferentially forms biofilms in environments rich in nutrients. When grown in M9 medium, *P. aeruginosa* forms a thick, multilayer biofilm; whereas when grown in the less nutritious FAB medium, the bacteria only formed a sparse monolayer (Geier, 2008).

Other evidence to suggest that nutrients have an effect on cell adherence and biofilm formation was found in a study by Simoes *et al.* (2006) who established that the addition of nutrients to water resulted in a greater number of total and culturable bacteria in biofilms. A study by Dewanti & Wong (1995) demonstrated that increasing the concentration of a carbon

source such as glucose from 0.01% to 0.04% led to higher numbers of cells adhering to the surface. Similarly, in studies by Marouani-Gadri *et al.* (2009), and Chen *et al.* (2010), a decrease in basic carbon nutrients (glucose, proteins), mineral salts and trace elements led to a decrease in the rate of early biofilm formation, indicating the direct importance of nutrient availability on cell adherence and subsequent growth into a biofilm. Comparable to this study, cell adherence still occurred under very low nutrient conditions and was also noted to be influenced by a number of other factors including strain variation. These studies compare with our experimental results in that more nutrients led to higher numbers of surface adhering cells. This may be due to more nutrients being available to encourage changes in cell physiology and composition thus enhancing cell adherence (Allan *et al.,* 2002). A higher concentration of nutrients would also be able to hoard together on the pipe surface as opposed to in the flowing water in a distribution system; this contributes to increased cell adherence and biofilm formation (Hood & Zottola, 1995; Geier, 2008).

Low nutrient or oligotrophic environments as was used in this study (Halifax tap water), seem to support cell adherence and biofilm growth in drinking water, more than they may support planktonic free floating cells (Carpentier & Cerf, 1999; Beech & Sunner, 2006). *E. coli* has been found to begin forming biofilms in response to nutrient limitation as several macromolecules and nutrients hoard together on the surface (Geier, 2008). Moreover, Skinner (2003) mentioned that extracellular polymers assist in accumulating nutrients from the free flowing water to the surface of the biofilm. In this study, nutrient availability was evaluated and although an increase in nutrients resulted in a greater number of adhered cells to a surface, relatively speaking very low levels of nutrients were compared. The low nutrient levels in the Halifax tap water (total organic carbon of 1.4) were augmented by the addition of 0.1% peptone, which still contained a very low amount of nutrients compared to studies using standard bacteriological media in their biofilm assays.

For example, Dewanti & Wong (1995) found higher numbers of adhered cells to surfaces where TSB was diluted 1/5 compared to undiluted TSB. Microscopic *in situ* examination displayed dramatic differences in biofilms formed on SS surfaces with differing levels of nutrient media. Stress from starvation was suggested to aid in biofilm cell production of additional EPS (Pan *et al.,* 2010). In another study by Oh *et al.* (2007) cellular adherence of bacteria and biofilm formation was observed using both a nutrient rich (Luria-Bertani Broth) and poor (M9) medium.

These authors found cells in the Luria-Bertani Broth medium displayed poor initial cell adherence and seemed to detach from the surface. Also, biofilms in Luria-Bertani Broth medium produced less EPS than those in the M9 medium. These results are likely due to the stress response exhibited by bacteria subjected to stressful low nutrient conditions.

5.2.7. Effect of Multiple Factors (Soiled Surface, Chlorine and Organic Material) Used in a Number of Treatment Combinations on the Cellular Adherence of *Ent. cloacae* KY 1 at 10°C for Seven Days Incubation

In this study the influence of a soiled surface, chlorine and nutrients on the cellular adherence of *Ent. cloacae* KY 1 to SS was evaluated. The fluorescent microscopic counts showed that the combination of a soiled surface, chlorine and organic material resulted in the greatest initial cell adherence, suggesting the combination of the three treatments together may have a slightly synergistic effect on the initial cell adherence. In contrast, plate count results do not necessarily suggest any synergistic effect of these experimental factors on initial adherence of viable and culturable cells to surfaces. Perhaps the chlorine injured the cells making them non-culturable.

After three days of incubation, no significant difference was observed in the adhered cell numbers as enumerated by microscopy but when comparing the plate count results, treatments with organic material (alone or in combinations) were found to yield significantly ($P<0.05$) greater adhered cell numbers compared to treatments without organic material. In fact, cells could not be cultured from treatments with no organic material after three and seven days. After seven days of incubation, the addition of organic material to the water was found to result in an increase in adhered cell numbers observable by microscopy, indicating that addition of organic material is the single most important factor to influence cell adherence, cell survival and biofilm formation as also discussed above.

Among the treatments with organic material, the combination of a soiled surface and organic material displayed the greatest culturable cell adherence numbers at 6.47 $\log_{10}$CFU/cm^2 after seven days, suggesting that this treatment provided the best environmental conditions for bacteria to adhere to the surface. It should be noted that in this experiment soiling of the surface was done by immersing the coupon in 0.1 % PW for 24 hours followed by incubation in fresh 0.1

% PW each day for seven days thereby replenishing the nutrients. This combination of two combined nutrient sources was found to have a synergistic effect.

The combination of a soiled surface, organic material and chlorine displayed the second greatest cell adherence at 5.90 $\log_{10}$CFU/cm^2. This decrease in cell adherence may be due to the addition of chlorine which may have injured and/or inhibited some cells. This would agree with the hypothesis that chlorine reduces numbers of adhered cells or biofilm formation.

Simoes *et al.* (2006) also studied interactions between multiple factors and found strong interactions between flow regime and the material surface, and also between flow regime and nutrients. Cellular adherence increased under combined turbulent water flow and nutrient-rich conditions. With the addition of nutrients, turbulent flow and PVC as the material surface total biofilm accumulation was increased.

Chapter 6: CONCLUSIONS

The results of this study show that phenotypic profiling systems such as Biolog and Vitek may be useful in differentiation rather than identification of environmental species. API 20E identified more of the isolates than Biolog and Vitek and proved more useful as an identification tool. Expansion of species identification databases to represent a broader range of both clinical and environmental strains of bacteria would strengthen the position of phenotypic identification systems as water quality monitoring tools.

Results from this study showed that the genotypic PFGE method gave a clearer indication of relatedness amongst isolates than the enzyme-based tools. This may be due to the fact that genetic differences can exist between two strains that express the same pheno- or biotype. Based on the degree of relatedness among coliforms sampled from different sampling sites throughout the distribution system, it was determined that coliform events in the Lexington distribution system appear to be related and a regrowth problem may therefore exist and that a permanent biofilm flora may have become established in the system.

Water utilities have frequently reported unexplained occurrences of coliform bacteria in drinking water distribution systems. The testing of water at different sampling sites and the occurrence of the same bacteria at these various sampling sites has been attributed to regrowth and biofilm formation. Control of environmental conditions, which can limit the presence of coliforms and biofilms in the drinking water distribution system, is important for water utilities and other organizations in the drinking water industry. Consequentially, microbial biofilm control and eradication strategies are important areas of interest for water utilities, regulators and for food processing facilities that use water from the drinking water distribution system.

In this study, warmer incubation temperatures were found to offer better environmental conditions for cell adherence and biofilm formation as compared to colder temperatures. The optimal temperature for a persistent *Ent. cloacae* strain to adhere to surfaces and form biofilms was found to be lower than the optimal growth temperature for the bacteria in suspension. The ability of different *Enterobacter* spp. strains to adhere to surfaces and form biofilms is an important factor in the development of persistent strains within a water distribution system and also within food processing facilities. Surface material was observed to influence cell adherence with greater adherence on PVC than SS. Over time the number of adhered *Ent. cloacae* cells to SS surfaces preconditioned with soil increased. Increasing the level of nutrients (0.1% peptone)

led to greater numbers of adhered cells of the persistent *Ent. cloacae* strain compared to water with no addition of peptone. Nutrient levels were found to be extremely important and considered the single most important factor for cell adherence and biofilm formation leading to the conclusion that maintaining low nutrient levels in a drinking water distribution system may help in controlling regrowth in water distribution systems. The presence of chlorine in drinking water from a water distribution system resulted in differing results depending on other nutrient conditions simulated in the study. In a low nutrient environment the presence of chlorine did not decrease the number of adhered cells; however, the opposite was true for the nutrient-rich environment. When a combination of treatments was used in some cases a synergistic effect on cell adherence was observed.

To the best of our knowledge, this study is the first to characterize a collection of coliform bacteria from a drinking water distribution system using biochemical and molecular MST methods in combination with a study of the cell adherence and biofilm forming capabilities of these strains under a number of environmental conditions. This study has demonstrated the appropriateness of using MST to characterize coliforms isolated from various points in the drinking water distribution systems and to elucidate the causes of their presence in the water. Such microbial tools can ultimately be used to enhance quality control strategies that are implemented by water treatment utilities. It is expected that an improved understanding of how microbial events are linked will refine or even redefine preventative and/or corrective actions taken by utilities to address coliform occurrences. This study also highlights the need for a more thorough understanding of how a number of factors alone and in combination affect cell adherence and biofilm formation. This present study improved our understanding of the cell adherence and biofilm formation of coliform bacteria in drinking water distribution systems.

REFERENCES

1. Allan, V. J. M., M. E. Callow, L. E. Macaskie, and M. Paterson-Beedle. 2002. Effect of nutrient limitation on biofilm formation and phosphatase activity of a *Citrobacter* spp. Microbiol. 148:277-288.

2. Ahmed, W. 2005. Host species-specific metabolic fingerprint database for tracking the sources of faecal contamination in surface waters. Australia: The University of the Sunshine Coast (Australia). 153 p.

3. Ashbolt, N. J., W. O. K. Grabow, and M. Snozzi. 2001. Indicators of microbial water quality. In Water Quality: Guidelines, Standards and Health. Assessment of Risk and Risk Management for Water-related Infectious Disease. IWA Publishing, London. 289-316.

4. Anonymous. 2002. U.S. Environmental Protection Agency website. Health Risks from Microbial Growth and Biofilms in Drinking Water Distribution Systems. Web page accessed August 2011. http://www.epa.gov/ogwdw/disinfection/tcr/pdfs/whitepaper_tcr_biofilms.pdf.

5. Anonymous. 2005. U.S. Environmental Protection Agency website. Microbial Source Tracking Guide Document. Web page accessed August 2011. http://www.epa.gov/nrmrl/pubs/600r05064/600r05064.pdf

6. Anonymous. 2006. U.S. Environmental Protection Agency website. Distribution System Indicators of Drinking Water Quality. Web page accessed August 2011. http://www.epa.gov/ogwdw/disinfection/tcr/pdfs/issuepaper_tcr_indicators.pdf.

7. Anonymous. 2007. Lexington-Fayette, Kentucky. U.S. Census Bureau: State and County Quickfacts. Web page accessed October 2007. http://quickfacts.census.gov/qfd/states/21/2146027.html

8. Anonymous. 2011a. Centers for Disease Control and Prevention website. Chlorine Residual Testing. Web page accessed August 2011. http://www.cdc.gov/safewater/publications_pages/chlorineresidual.pdf.

9. Anonymous. 2011b. Health Canada website. Canadian Drinking Water Guidelines. Web page accessed August 2011. http://www.hc-sc.gc.ca/ewh-sent/water-eau/drink-potab/guide/index-eng.php.

10. Anonymous. 2011c. Health Canada website. Guidelines for Canadian Drinking Water Quality-Summary Table. Web page accessed August 2011. http://www.hc-sc.gc.ca/ewh-semt/pubs/water-eau/2010-sum_guide-res_recom/index-eng.php.

11. Anonymous. 2011d. World Health Organization website. How to measure chlorine residual in water. Web page accessed August 2011. http://www.searo.who.int/LinkFiles/List_of_Guidelines_for_Health_Emergency_How_to_measure_chlorine_residual.pdf.

12. Anonymous. 2011e. U.S. Environmental Protection Agency website. Water: Laws and Regulations. Web page accessed August 2011. http://www.water.epa.gov/lawsregs/rulesregs/sdwa/tcr/basicinformation.cfm.

13. Anonymous. 2011f. Public Health Agency of Canada website. National Microbiology Laboratory: PulseNet-Overview. Web page accessed July 2011. http://www.nml-lnm.gc.ca/PulseNet/overview-apercu-eng.htm.

14. Anonymous. 2011g. Nova Scotia Environment and Labour website. A Drinking Water Strategy for Nova Scotia: A Comprehensive Approach to the Management of Drinking Water. Web page accessed August 2011. http://www.gov.ns.ca/nse/water/docs/NSWaterStrategy.pdf.

15. Anonymous. 2011h. Using Microbial Source Tracking to support TMDL development and implementation. Web page accessed August 2011. http://www.epa.gov/region10/pdf/tmdl/mst_for_tmdls_guide_04_22_11.pdf

16. Anonymous. 2011i. Centers for Disease Control and Prevention website. Guideline for Disinfection and Sterilization in Healthcare Facilities, 2008. Web page accessed November 2011. http://www.cdc.gov/hicpac/disinfection_sterilization/6_0disinfection.html

17. Anonymous. 2011j. Health Canada website. Microbiological Characteristics. Web page accessed November 2011. http://www.hc-sc.gc.ca/ewh-semt/pubs/water-eau/temperature/index-eng.php

18. Anonymous. 2011k. Typical Analysis of Pockwock/ Lake Major Water 2007-2008. Web page accessed November 2011. http://www.halifax.ca/hrwc/documents/AnnualReport2008_final.pdf

19. Anonymous. 2011l. Halifax Water 2007/2008 Water Quality Analysis Results. Web page accessed November 2011. http://www.halifax.ca/hrwc/documents/HalifaxWaterReport2007and2008.pdf

20. Anonymous. 2011m. Annual Water Quality Report: Water Quality Results. Web page accessed November 2011. http://www.amwater.com/files/KY_0340250_CCR.pdf

21. Barnes, L.-M., M. F. Lo, M. R. Adams, and A. H. L. Chamberlain. 1999. Effect of milk proteins on adhesion of bacteria to stainless steel surfaces. Appl. Environ. Microbiol. 65:4543-4548.

22. Barrett, T. J., P. Gerner-Smidt, and B. Swaminathan. 2006. Interpretation of Pulsed-Field Gel Electrophoresis Patterns in Foodborne Disease Investigations and Surveillance. Foodborne Pathog. Dis. 3:20-31.

23. Batté, M., B. M. R. Appenzeller, D. Grandjean, S. Fass, V. Gauthier, F. Jorand, L. Mathieu, M. Boualam, S. Saby, and J. C. Block. 2003. Biofilms in drinking water distribution systems. Rev. Environ. Sci. Biotechnol. 2:147-168.

24. Beech, I. B., and J. A. Sunner. 2006. Chapter 14: Biocorrosion in drinking water distribution systems. In: G. Newcombe and D. Dixon. Ed. Interface Science in Drinking Water Treatment. Elsevier Ltd. Amsterdam, Netherlands. .pp. 245-255.

25. Bergey, D. H., and J. G. Holt. 1994. Bergey's manual of determinative bacteriology. Williams & Wilkins, Baltimore. pp. 178.

26. Besner, M.-C., V. Gauthier, P. Servais, and A. Camper. 2002. Explaining the occurrence of coliforms in distribution systems. J. Am. Water Works Assoc. 94:95-109.

27. Blanch, A. R., B. Galofre, F. Lucena, A. Terradillos, X. Vilanova, and F. Ribas. 2007. Characterization of bacterial coliform occurrences in different zones of a drinking water distribution system. J. Appl. Microbiol. 102:711-721.

28. Blaschek, H. P., H. H. Wang, and M. E. Agle (ed.). 2007. Biofilms in the food environment. Blackwell Publishing/IFT Press, Ames, IA. pp. 4.

29. Borucki, M. K., J.D. Peppin, D. White, F. Loge, and D. R. Call. 2003. Variation in biofilm formation among strains of *Listeria monocytogenes*. Appl. Environ. Microbiol. 69:7336–7342

30. Camper, A. M. 1996. Factors limiting microbial growth in distribution systems: Laboratory and pilot-scale experiments. Denver, CO: AWWARF and AWWA.

31. Camper, A., M. Burr, B. Ellis, P. Butterfield, and C. Abernathy. 1999. Development and structure of drinking water biofilms and techniques for their study. J. Appl. Microbiol. 85:1S-12S.

32. Carpentier, B., and O. Cerf. 1999. Biofilms. In Encyclopedia of Food Microbiology, vol. 1. ed. Robinson, R.K., Batt, C.A. and Patel, P.D. pp. 252–259. London: GBR Academic Press.

33. Chae, M. S., and H. Schraft. 2001. Cell viability of *Listeria monocytogenes* biofilms. Food Microbiol. 18:103-112.

34. Chauret, C., C. Volk, R. Creason, J. Jarosh, J. Robinson, and C. Warnes. 2001. Detection of *Aeromonas hydrophila* in a drinking water distribution system: a field and pilot study. Can. J. Microbiol. 47:782-786.

35. Chavant, P., B. Martinie, T. Meylheuc, M. N. Bellon-Fontaine, and M. Hebraud. 2002. *Listeria monocytogenes* LO28: Surface physicochemical properties and ability to form biofilms at different temperatures and growth phases. Appl. Environ. Microbiol. 68:728-737.

36. Chen, M.-Y., M.-J. Chen, P.-F. Lee, L.-H. Cheng, L.-J. Huang, C.-H. Lai, and K.-H. Huang. 2010. Towards real-time observation of conditioning film and early biofilm formation under laminar flow conditions using a quartz crystal microbalance. Biochem. Eng. J. 53:121-130.

37. Chen, Y. 2009. Insertional mutagenesis in *Listeria monocytogenes* 568 reveals genes that may impact biofilm formation. M.Sc. Thesis. Dalhousie University, Halifax, NS; 144 p.

38. Chmielewski, R. A. N., and J.F. Frank. 2003. Biofilm formation and control in food processing facilities. Compr. Rev. Food. Sci. F. 2:22-32.

39. Chu, C., C. Lu, C. Lee, and C. Tsai. 2003. Effects of chlorine level on the growth of biofilm in water pipes. J. Environ. Sci. Health, Part A: Toxic/Hazardous Substances & Environmental Engineering 38:1377-1388.

40. Cimenti, M., A. Hubberstey, J. K. Bewtra, and N. Biswas. 2007. Alternative methods in tracking sources of microbial contamination in waters. Water S. A. 33:183-194.

41. Cohn, P. D., M. Cox, and P. S. Berger. 1999. Health and aesthetic aspects of water quality. In: Water Quality and Treatment. 5th ed. McGraw-Hill, New YorkCorry, J. E. L., and V. Allen. 2000. Biofilm formation and machine cleaning. Int Food Saf News. 9:2-5.

42. Dat, N. M., D. Hamanaka, F. Tanaka, and T. Uchino. 2010. Surface conditioning of stainless steel coupons with skim milk solutions at different pH values and its effect on bacterial adherence. J. Food Cont. 21:1769-1773.

43. Dewanti, R., A. C. L. Wong. 1995. Influence of culture conditions on biofilm formation by *Escherichia coli* O157:H7. Int. J. Food Microbiol. 26:147-164.

44. Di Bonaventura, G., R. Piccolomini, D. Paludi, V. D'Orio, A. Vergara, M. Conter, and A. Ianieri. 2008. Influence of temperature on biofilm formation by *Listeria monocytogenes* on various food-contact surfaces: relationship with motility and cell surface hydrophobicity. J. Appl. Microbiol. 104:1552-1561.

45. Djordjevic, D., M. Wiedmann, and L. A. McLandsborough. 2002. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. Appl. Environ. Microbiol. 68:2950-2958.

46. Donlan, R. M. 2002. Biofilms: Microbial life on surfaces. Emerging Infectious Diseases 8:881-890.

47. Dunne, W. M. Jr. 2002. Bacterial adhesion: Seen any good biofilms lately? Clin. Microbiol. Rev. 15:155-166.

48. Ells, T. C. 2007. Important factors that influence the survival of *Listeria monocytogenes* during the minimal thermal processing of freshly shredded cabbage. Ph.D. Thesis. Dalhousie University, Halifax, NS; 290 p.

49. Field, K. G., and M. Samadpour. 2007. Fecal source tracking, the indicator paradigm, and managing water quality. Water Res. 41:3517-3538.

50. Fletcher, M. 1979. Influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces. Appl. Environ. Microbiol. 37: 67

51. Foley, S. L., A. M. Lynne, and R. Nayak. 2009. Molecular typing methodologies for microbial source tracking and epidemiological investigations of Gram-negative bacterial foodborne pathogens. Infect. Genet. Evol. 9:430-440.

52. Friedman, M., A. Hanson, K. Dewis, G. Kirmeyer, M. LeChevallier, G. Gagnon, L. Truelstrup Hansen, C. Krentz, M. Mosher, S. J. Payne, J. Rosen, T. Hargy, J. Sobrinho, M.-C. Besner and M. Prevost. 2009. Strategies for Managing Total Coliform and *E. coli* in Distribution Systems [Project #3116]. AWWA Research Foundation and American Water Works Association. Denver, CO.

53. Fuster-Valls, N., M. Hernández-Herrero, M. Marín-de-Mateo, and J. J. Rodríguez-Jerez. 2008. Effect of different environmental conditions on the bacteria survival on stainless steel surfaces. J. Food Cont.19:308-314.

54. Galal-Gorchev, H. 1996. Chlorine in Water Disinfection. Pure & Appl Chem. 68: 1731-1735.

55. Geier, H. 2008. Environmental and genetic factors leading to *Mycobacterium avium* biofilm formation. Ph.D. Thesis. Montana State University, Bozeman, MT; 121 p.

56. Geldreich, E. E. 1996. Microbial quality of water supply in distribution systems. CRC Lewis Publishers, Boca Raton FL.

57. Grimont, F., and P. A. D. Grimont. 2006. The Genus *Enterobacter.* Prokaryotes. 6:197–214.

58. Hagedorn, C., J. B. Crozier, K. A. Mentz, A. M. Booth, A. K. Graves, N. J. Nelson, and R. B. Reneau Jr. 2003. Carbon source utilization profiles as a method to identify sources of fecal pollution in water. J. Appl. Microbiol. 94:792-799.

59. Hallam, N. B., J. R. West, C. F. Forster, and J. Simms. 2001. The potential for biofilm growth in water distribution systems. Water Res. 35:4063-4071.

60. Harvey, J., K. P. Keenan, and A. Gilmour. 2007. Assessing biofilm formation by *Listeria monocytogenes* strains. Food Microbiol. 24:380-392.

61. Herald, P. J., and E. A. Zottola. 1988. Adherence of *Listeria monocytogenes* to stainless steel surfaces at various temperatures and pH values. J. Food Sci. 53:1549-1562.

62. Hollis, R. L., J. L. Bruce, J. S. Fritschel, and M. A. Pfaller. 1999. Comparative evaluation of an automated ribotyping instrument versus pulsed-field gel electrophoresis for epidemiological investigation of clinical isolates of bacteria. Diagn. Microbiol. Infect. Dis. 34, 263–268.

63. Hood, S. K., and E. A. Zottola. 1995. Biofilms in food processing. Food Control 6:9-18.

64. Hu, T.-L., C. Lin, and W.-Y. Chen. 2008. Modeling of biofilm in the water distribution system. Water Sci. Technol. 8:513-518.

65. Huang, C. R., C. H. Lu, and W. N. Chang. 2001. Adult *Enterobacter* meningitis: a high incidence of coinfection with other pathogens and frequent association with neurosurgical procedures. Infection 29:75-79.

66. Huber, T. W. 1999. Enterobacter. Medical Microbiology and Immunology, Texas A & M College of Medicine Temple, USA. pp. 598-603.

67. Iversen, C., M. Lane, and S. J. Forsythe. 2004. The growth profile, thermotolerance and biofilm formation of *Enterobacter sakazakii* grown in infant formula milk. Lett. Appl. Microbiol. 38:378-382.

68. Janda, J. M., and S. L. Abbott. 2006. The *enterobacteria*. ASM Press, Washington, DC.

69. Jeffers, G. T., J. L. Bruce, P. L. McDonough, J. Scarlett, K. J. Boor, and M. Wiedmann. 2001. Comparative genetic characterization of *Listeria monocytogenes* isolates from human and animal listeriosis cases. Microbiol. 147:1095-1104.

70. Keller, R., M. Z. Pedroso, R. Ritchmann, and R. M. Silva. 1998. Occurrence of virulence-associated properties in *Enterobacter cloacae*. Infect. Immun. 66:645-649.

71. Kim, H., J. Bang, L. R. Beuchat, and J.-H. Ryu. 2008. Fate of *Enterobacter sakazakii* attached to or in biofilms on stainless steel upon exposure to various temperatures or relative humidities. J. Food Prot. 71:940-945.

72. Kim, H., J.-H. Ryu, and L. R. Beuchat. 2006. Adherence of and biofilm formation by *Enterobacter sakazakii* on stainless steel and enteral feeding tubes. Appl. Environ. Microbiol. 72:5846-5856.

73. Kirk, J. L., L. A. Beaudette, M. Hart, P. Moutoglis, J. N. Klironomos, H. Lee, and J. T. Trevors. 2004. Methods of studying soil microbial diversity. J. Microbiol. Methods 58:169-188.

74. Klingler, J. M., R. P. Stowe, D. C. Obenhuber, T. O. Groves, S. K. Mishra, and D. L. Pierson. 1992. Evaluation of the Biolog automated microbial identification system. Appl. Environ. Microbiol. 58:2089-2092.

75. Kolari, M. 2003. Adherence mechanisms and properties of bacterial biofilms on non-living surfaces. Ph.D. Thesis. University of Helsinki, Helsinki, Finland; 79 p.

76. Krentz, C. 2008. Identification and disinfection tolerance of coliform bacteria in water treatment. M.Sc. Thesis. Dalhousie University, Halifax, NS; 107 p.

77. Landini, P. 2009. Cross-talk mechanisms in biofilm formation and responses to environmental and physiological stress in *Escherichia coli*. Res. Microbiol. 160:259-266.

78. LeChevallier, M. W., N. J. Welch, and D. B. Smith. 1996. Full Scale Studies of Factors Related to Coliform Regrowth in Drinking Water. Appl. Environ. Microbiol. 62: 2201-2211.

79. Lee, Y., and S. Nam. 2005. Comparative Study of Chemical Disinfection in Drinking Water Supplies. Water Conditioning & Purification. 1-6.

80. Lee, J. E., S. Lee, J. Sung, and G. Ko. 2011. Analysis of human and animal fecal microbiota for microbial source tracking. ISME J. 5:362-365.

81. Lemon, K. P., D. E. Higgins, and R. Kolter. 2007. Flagellar Motility is critical for *Listeria monocytogenes* biofilm formation. J. Bacteriol. 189:4418-4424.

82. Lexington-Fayette, Kentucky. Map. Google Maps. Google, 19 August 2007. Web. August 2007.

83. Lindsay, D., and A. v. Holy. 2006. What food safety professionals should know about bacterial biofilms. Br. Food J. 108:27-37.

84. Loeb, G. I., and R. A. Neihof. 1975. Marine conditioning films. pp. 319-335. *In* R. E. Baier (ed.) Applied Chemistry at Protein Interfaces, vol. 145. Am. Chem. Soc., Washington.

85. Lomander, A., P. Schreuders, E. Russek-Cohen, and L. Ali. 2004. Evaluation of chlorines' impact on biofilms on scratched stainless steel surfaces. Bioresour. Technol. 94:275-283.

86. Lundén, J. M., M. K. Miettinen, T. J. Autio, and H. J. Korkeala. 2000. Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surface after short contact times. J. Food. Prot. 63:1204-1207.

87. Mafu, A. A., C. Plumety, L. Deschênes, and J. Goulet. 2011. Adhesion of pathogenic bacteria to food contact surfaces: Influence of pH of Culture. Int. J. Food Microbiol. 2011:1-10.

88. Maier, R. M., and G. Soberón-Chávez. 2000. *Pseudomonas aeruginosa* rhamnolipids: biosynthesis and potential applications. Appl. Microbiol. Biotechnol. 54:625-633.

89. Manz, W., K. Wendt-Potthoff, T. R. Neu, U. Szewzyk, and J.R. Lawrence. 1999. Phylogenetic composition, spatial structure, and dynamics of lotic bacterial biofilms investigated by fluorescent *in situ* hybridization and confocal laser scanning microscopy. Microb. Ecol. 37:225-237.

90. Marouani-Gadri, N., D. Chassaing, and B. Carpentier. 2009. Comparative evaluation of biofilm formation and tolerance to a chemical shock of pathogenic and nonpathogenic *Escherichia coil* 0157:H7 strains. J. Food Prot. 72:157-164.

91. McEldowney, S., and M. Fletcher. 1988. Effect of pH, temperature, and growth conditions on the adhesion of a gliding bacterium and three nongliding bacteria to polystyrene. Microb. Ecol. 16:183-195.

92. McLean, R. J. C., and A.W. Decho (ed.). 2002. Molecular ecology of biofilms. Scientific Press. Norfolk.

93. Meays, C. L., K. Broersma, R. Nordin, and A. Mazumder. 2004. Source tracking fecal bacteria in water: a critical review of current methods. J. Environ. Manage. 73:71-79.

94. Miller, J. M., and D. L. Rhoden. 1991. Preliminary evaluation of Biolog, a carbon source utilization method for bacterial identification. J. Clin. Microbiol. 29:1143-1147.

95. Momba, M. N. B., and N. Makala. 2004. Comparing the effect of various pipe materials on biofilm formation in chlorinated and combined chlorine-chloraminated water systems. Water S. A. 30:175-182.

96. Montville, T. J., and K. R. Matthews. 2006. Food microbiology: an introduction. ASM Press, Washington, D.C.

97. Murga, R., J. M. Miller, and R. M. Donlan. 2001. Biofilm formation by Gram-negative bacteria on central venous catheter connectors: effect of conditioning films in a laboratory model. J. Clin. Microbiol. 39:2294-2297.

98. Nikolaev, Yu. A., and V. K. Plakunov. 2007. Biofilm "City of microbes" or an analogue of multicellular organisms? Microbiol. 76:125-138.

99. Noble, R. T., J. F. Griffith, A. D. Blackwood, J. A. Fuhrman, J. B. Gregory, X. Hernandez, X. Liang, A. A. Bera, and K. Schiff. 2006. Multitiered approach using quantitative PCR to track sources of fecal pollution affecting Santa Monica Bay, California. Appl. Environ. Microbiol. 72:1604-1612.

100. Norwood, D. E., and A. Gilmour. 2001. The differential adherence capabilities of two *Listeria monocytogenes* strains in monoculture and multispecies biofilms as a function of temperature. Lett. Appl. Microbiol. 33:320-324.

101. Norwood, D. E., and A. Gilmour. 1999. Adherence of *Listeria monocytogenes* strains to stainless steel coupons. J. Appl. Microbiol. 86:576-582.

102. Oh, Y. J., W. Jo, Y. Yang, and S. Park. 2007. Influence of culture conditions on *Escherichia coli* O157:H7 biofilm formation by atomic force microscopy. Ultramicroscopy. 107:869-874.

103. Ong, C.-L. Y., G. C. Ulett, A. N. Mabbett, S. A. Beatson, R. I. Webb, W. Monaghan, G. R. Nimmo, D. F. Looke, A. G. McEwan, and M. A. Schembri. 2008. Identification of type 3 fimbriae in uropathogenic *Escherichia coli* reveals a role in biofilm formation. J. Bacteriol. 190:1054-1063.

104. Ortega, M. P., T. Hagiwara, H. Watanabe, and T. Sakiyama. 2010. Adhesion behavior and removability of *Escherichia coli* on stainless steel surface. Food Control 21:573-578.

105. Palmer, J., S. Flint, and J. Brooks. 2007. Bacterial cell adherence, the beginning of a biofilm. J. Ind. Microbiol. Biotechnol. 34:577-588.

106. Pan, Y., F. Breidt Jr., and L. Gorski. 2010. Synergistic effects of sodium chloride, glucose, and temperature on biofilm formation by *Listeria monocytogenes* serotype 1/2a strains and 4b strains. Appl. Environ. Microbiol. 76:1433-1441.

107. Parizzi, S. Q. F., N. J. de Andrade, C. A. de Sá Silva, N. F. F. Soares, and E. A. M. da Silva. 2004. Bacterial adherence to different inert surfaces evaluated by epifluorescence microscopy and plate count method. Braz. Arch. Biol.Technol. 47:77-83.

108. Payne, S. J. 2007. Tools for microbial detection and characterization in drinking water distribution systems. M.Sc. Thesis. Dalhousie University, Halifax, NS; 141 p.

109. Percival, S. L., J. T. Walker, and P. R. Hunter. 2000. Microbiological aspects of biofilms and drinking water. CRC Press, Boca Raton, FL.

110. Pfaller, M. A., C. Wendt, R. J. Hollis, R. P. Wenzel, S. J. Fritschel, J. J. Neubauer, and L. A. Herwaldt. 1996. Comparative evaluation of an automated ribotyping instrument versus pulsed-field gel electrophoresis for epidemiological typing of clinical isolates of *Escherichia coli* and *Pseudomonas aeruginosa* from patients with recurrent Gram-negative bacteremia. Diagn. Microbiol. Infect. Dis. 25: 1–8.

111. Power, K. N., and L. A. Nagy. 1999. Relationship between bacterial regrowth and some physical and chemical parameters within Sydney's drinking water distribution system. Water Res. 33:741-750.

112. Praphailong, W., M. Van Gestel, G. H. Fleet, and G. M. Heard. 1997. Evaluation of the Biolog system for the identification of food and beverage yeasts. Lett. Appl. Microbiol. 24:455-459.

113. Pui, C. F., W. C. Wong, L. C. Chai, H. Y. Lee, J. Y. H Tang, A. Noorlis, M. G. Farinazleen, Y. K. Cheah, and R. Son. 2011. Biofilm formation by *Salmonella* Typhi and *Salmonella* Typhimurium on plastic cutting board and its transfer to dragon fruit. Food Res Int. 18:31-38.

114. Ray, B., and A. K. Bhunia. 2008. Fundamental food microbiology. CRC Press, Boca Raton, FL.

115. Reisner, A., K. A. Krogfelt, B. M. Klein, E. L. Zechner, and S. Molin. 2006. *In vitro* biofilm formation of commensal and pathogenic *Escherichia coli* strains: Impact of environmental and genetic factors. J. Bacteriol. 188:3572-3581.

116. Rodríguez-Calleja, J. M., I. García-López, J. A. Santos, A. Otero, and M.-L. García-Lopez. 2006. Molecular and phenotypic typing of *Staphylococcus aureus* isolates from rabbit meat. Res. Microbiol. 157:496-502.

117. Rompré, A., P. Servais, J. Baudart, M.-R. de-Roubin, and P. Laurent. 2002. Detection and enumeration of coliforms in drinking water: current methods and emerging approaches. J. Microbiol. Methods 49:31-54.

118. Roslev, P., and A. S. Bukh. 2011. State of the art molecular markers for fecal pollution source tracking in water. Appl. Microbiol. Biotechnol. 89:1341-1355.

119. Ryu, J.-H., H. Kim, and L. R. Beuchat. 2004a. Adherence and biofilm formation by *Escherichia coli* O157:H7 on stainless steel as influenced by exopolysaccharide production, nutrient availability, and temperature. J. Food Prot. 67:2123-2131.

120. Ryu, J.-H., H. Kim, J. F. Frank, and L. R. Beuchat. 2004b. Adherence and biofilm formation on stainless steel by *Escherichia coli* O157:H7 as affected by curli production. Lett. Appl. Microbiol. 39:359-362.

121. Sanders, W. Jr., and C. C. Sanders. 1997. *Enterobacter* spp.: pathogens poised to flourish at the turn of the century. Clin. Microbiol. Rev. 10:220-241.

122. Scott, T. M., J. B. Rose, T. M. Jenkins, S. R. Farrah, and J. Lukasik. 2002. Microbial source tracking: Current methodology and future directions. Appl. Environ. Microbiol. 68:5796-5803.

123. September, S. M., F. A. Els, S. N. Venter, and V. S. Brözel. 2007. Prevalence of bacterial pathogens in biofilms of drinking water distribution systems. J. Water. Health. 5:219-227.

124. Shi, Z-Y., P. Y-F. Liu, Y-J. Lau, Y-H. Lin, and B-S. Hu. 1996. Epidemiological typing of isolates from an outbreak of infection with multidrug-resistant *Enterobacter cloacae* by repetitive extragenic palindromic unit b1-primed PCR and pulsed-field gel electrophoresis. J. Clin. Microbiol. 34:2784-2790.

125. Simões, L. C., N. Azevedo, A. Pacheco, C. W. Keevil, and M. J. Vieira. 2006. Drinking water biofilm assessment of total and culturable bacteria under different operating conditions. Biofouling 22:91-99.

126. Simpson, J. M., J. W. Santo Domingo, and D. J. Reasoner. 2002. Microbial Source Tracking: State of the Science. Environ. Sci. Technol. 36:5279-5288.

127. Skinner, D. M. 2003. Impact of water quality changes in the distribution system on disinfection efficacy. M.Sc. Thesis. Dalhousie University, Halifax, NS; 143 p.

128. Smoot, L. M., and M. D. Pierson. 1998. Influence of environmental stress on the kinetics and strength of adherence of *Listeria monocytogenes* Scott A to buna-N rubber and stainless steel. J. Food Prot. 61:1286-1292.

129. Speers, J. G. S., and A. Gilmour. 1985. The influence of milk and milk components on the adherence of bacteria to farm dairy equipment surfaces. J. Appl. Microbiol. 59:325-332.

130. Stepanovic, S., I. Cirkovic, V. Mijac, and M. Svabic-Vlahovic. 2003. Influence of the incubation temperature, atmosphere and dynamic conditions on biofilm formation by *Salmonella* spp. Food Microbiol. 20:339-343.

131. Stolp, H. 1988. Microbial Ecology: Organisms, Habitats, Activities. Cambridge University Press, Cambridge.

132. Stoodley, P., S. Wilson, L. Hall-Stoodley, J. D. Boyle, H. M. Lappin-Scott, and J. W. Costeron. 2001. Growth and detachment of cell clusters from mature mixed-species biofilms. Appl. Environ. Microbiol. 67: 5608-5613.

133. Tam, M. L. P. Adherence of *Listeria monocytogenes* to austenitic stainless steel. United States – Alabama: Auburn University (USA); 2007. 112 p.

134. Tam, M. L. P., and D. E. Conner. 2007. Effect of temperature and growth media on the adherence of *Listeria monocytogenes* to stainless steel. Int. J. Food Microbiol. 120:282-286.

135. Takahashi, H., S. Kuramoto, S. Miya, and B. Kimura. 2011. Desiccation survival of *Listeria monocytogenes* and other potential foodborne pathogens on stainless steel surfaces is affected by different food soils. Food Control 22:633-637.

136. Todhanakasem, T., and G. M. Young. 2008. Loss of flagellum-based motility by *Listeria monocytogenes* results in formation of hyperbiofilms. J. Bacteriol. 190:6030-6034.

137. Trachoo, N. 2007. Adherence of *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Pseudomonas aeruginosa* on food soiled plastic surfaces. Pak. J. Biol. Sci. 10:1918-1921.

138. Tresse, O., V. Lebret, D. Garmyn, and O. Dussurget. 2009. The impact of growth history and flagellation on the adhesion of various *Listeria monocytogenes* strains to polystyrene. Can. J. Microbiol. 55:189-196.

139. Tsai, Y-P. 2006. Interaction of chlorine concentration and shear stress on chlorine consumption, biofilm growth rate and particle number. Bioresour. Technol. 97:1912-1919.

140. van der Wende, E., W. G. Characklis, and D. B. Smith. 1989. Biofilms and bacterial drinking water quality. Water Res. 23:1313-1322.

141. Varnam, A. H., and M. G. Evans. 2000. Environmental Microbiology. Manson Publishing Ltd., London.

142. Vatanyoopaisarn, S., A. Nazli, C. E. R. Dodd, C. E. D. Rees, and W. M. Waites. 2000. Effect of flagella on initial adherence of *Listeria monocytogenes* to stainless steel. Appl. Environ. Microbiol. 66:860-863.

143. Vivas, J., D. Padilla, F. Real, J. Bravo, V. Grasso, and F. Acosta. 2008. Influence of environmental conditions on biofilm formation by *Hafnia alvei* strains. Vet. Microbiol. 129:150-155.

144. Wade, W. 2002. Unculturable bacteria the uncharacterized organisms that cause oral infections. J. R. Soc. Med. 95:81-83.

145. Wellman, N., S. M. Fortun, and B. R. McLeod. 1996. Bacterial biofilms and the bioelectric effect. Antimicrobial Agents Chemother. 40:2012-2014.

146. Williams, M. M., and E. B. Braun-Howland. 2003. Growth of *Escherichia coli* in model distribution system biofilms exposed to hypochlorous acid or monochloramine. Appl. Environ. Microbiol. 69:5463-5471.

147. Wood, T. K., A. F. G. Barrios, M. Herzberg, and J. Lee. 2006. Motility influences biofilm architecture in *Escherichia coli*. Appl. Microbiol. Biotechnol. 72:361-367.

148. Zacheus, O. M., E. K. Iivanainen, T. K. Nissinen, M. J. Lehtola, and P. J. Martikainen. 2000. Bacterial biofilm formation on polyvinyl chloride, polyethylene and stainless steel exposed to ozonated water. Water Res. 34:63-70.

149. Zhou, L., Y. Zhang, and G. Li. 2009. Effect of pipe material and low level disinfectants on biofilm development in a simulated drinking water distribution system. J Zhejiang Univ. Sci. A. 10:725-731.

APPENDIX: ADDITIONAL TABLES

Table A. 1: Microscopic counts of live, dead/damaged and total bacteria of *Ent. cloacae* KY 1 adhering to SS after incubation for one, three and seven days incubation at 4, 10, 21 and 30°C.

Incubation time (days)	Temperature (°C)	Live bacteria		Dead/damaged bacteria		Total bacteria	
1	4	5.2 ± 0.1	(a)	3.5 ± 0.5	(a)	5.2 ± 0.2	(a)
	10	5.7 ± 0.1	(b)	4.9 ± 0.4	(b)	5.7 ± 0.2	(b)
	21	6 ± 0.1	(b)	5.3 ± 0.1	(b)	6.1 ± 0.1	(b)
	30	4.4 ± 0.2	(c)	5.8 ± 0.1	(b)	5.9 ± 0.1	(b)
3	4	3.2 ± 0	(a)	3.9 ± 1.2	(a)	3.9 ± 1.2	(a)
	10	3.2 ± 0	(a)	4.6 ± 0.1	(a)	4.6 ± 0.1	(a,c)
	21	7 ± 0.1	(b)	7 ± 0.2	(b)	7.4 ± 0	(b)
	30	5.5 ± 0	(c)	5.3 ± 0.4	(a,b)	5.7 ± 0.2	(b,c)
7	4	3.6 ± 0.2	(a)	3.8 ± 0.2	(a,c)	3.9 ± 0.3	(a)
	10	3.3 ± 0.2	(a)	3.2 ± 0	(a)	3.4 ± 0.2	(a)
	21	5.3 ± 0.1	(b)	7.2 ± 0.1	(b)	7.2 ± 0.1	(b)
	30	5.1 ± 0.4	(b)	4.4 ± 0.5	(c)	5.2 ± 0.4	(c)

[a] Within the column, at each time point, values followed by the same letters (a-c) are not significantly different from each other ($P > 0.05$)

[b] Cell numbers are given in average $\log_{10}$cells/cm^2 where (n=3) ± standard deviation (n-1).

Table A. 2: Microscopic counts of live, dead/damaged and total bacteria of *Ent. cloacae* KY 1 and four other environmental isolates adhering to SS after one, three and seven days incubation at 10°C.

Incubation time (days)	Environmental isolate #	Live bacteria		Dead/damaged bacteria		Total bacteria	
1	KY 1	5.7 ± 0.1	(a,c)	4.9 ± 0.4	(a,c,b)	5.7 ± 0.2	(a)
	KY 4	6.7 ± 0.1	(b)	5.5 ± 0.2	(b)	6.7 ± 0.1	(b)
	KY 6	6.5 ± 0.1	(a,b)	5.8 ± 0.1	(b,d)	6.6 ± 0.1	(b)
	KY 11	5.2 ± 0.3	(c)	4.3 ± 1	(a,c,b)	5.3 ± 0.4	(a)
	KY 13	3.7 ± 0.6	(d)	3.7 ± 0.4	(a,c)	4.1 ± 0.2	(c)
3	KY 1	3.2 ± 0	(a)	4.6 ± 0.1	(a)	4.6 ± 0.1	(a)
	KY 4	6.3 ± 0.2	(b)	6.4 ± 0.1	(b)	6.7 ± 0.1	(b)
	KY 6	5.8 ± 0.2	(c)	6 ± 0.3	(b)	6.2 ± 0.2	(c)
	KY 11	4.5 ± 0.1	(d)	4.3 ± 0.2	(a)	4.7 ± 0.1	(a)
	KY 13	4.1 ± 0.2	(d)	3.2 ± 0	(c)	4.2 ± 0.2	(d)
7	KY 1	3.3 ± 0.2	(a)	3.2 ± 0	(a)	3.4 ± 0.2	(a)
	KY 4	6.6 ± 0.1	(b)	6.1 ± 0.2	(b)	6.7 ± 0.1	(b)
	KY 6	5.9 ± 0.1	(c)	6 ± 0.1	(b)	6.3 ± 0.1	(c)
	KY 11	4.9 ± 0	(d)	4.8 ± 0.1	(c)	5.2 ± 0.1	(d)
	KY 13	4.2 ± 0.2	(e)	3.9 ± 0	(d)	4.4 ± 0.1	(e)

[a] Within the column, at each time point, values followed by the same letters (a)- (c) are not significantly different from each other ($P > 0.05$)

[b] Cell numbers are given in average $\log_{10}$cells/cm^2 where (n=3) ± standard deviation (n-1).

Table A. 3: The effect of preconditioning of a surface on cellular adherence and cell survival of *Ent. cloacae* KY 1 after one, three and seven days incubation at 10°C. The number of cells was enumerated by epifluorescence microscopy using the LIVE/DEAD BacLight stain and the plate count method.

Treatment conditions	Incubation time (days)	Live bacteria	Dead/damaged bacteria	Total bacteria	Plate count numbers
Soiled surface	1	5.5 ± 0.2	4.67 ± 0.51	5.59 ± 0.2	6.14 ± 0.53
	3	4.24 ± 0.3	4.71 ± 0.32	4.86 ± 0.3	1.52 ± 0
	7	4.42 ± 0.4	4.23 ± 0.39	4.64 ± 0.4	1.52 ± 0
No treatment (unsoiled surface)	1	5.37 ± 0.3	4.39 ± 0.29	5.42 ± 0.3	6.26 ± 0.44
	3	4.78 ± 0.2	4.44 ± 0.25	4.98 ± 0.1	1.52 ± 0
	7	3.77 ± 0.4	3.77 ± 0.5	4.03 ± 0.5	1.52 ± 0

[a] Cell numbers are given in average $\log_{10}$cells/cm^2 where (n=6) or $\log_{10}$CFU/cm^2 where (n=6) ± standard deviation (n-1).

Table A. 4: The effect of residual chlorine on cellular adherence and cell survival of *Ent. cloacae* KY 1 on SS after one, three and seven days incubation at 10°C under stressful low nutrient conditions. The number of cells was enumerated by epifluorescence microscopy using the LIVE/DEAD BacLight stain and the plate count method.

Treatment conditions	Incubation time (days)	Live bacteria	Dead/damaged bacteria	Total bacteria	Plate count numbers
Presence of residual chlorine (0.44 mg/L)	1	5.80 ± 0.21	4.26 ± 0.27	5.82 ± 0.20	5.98 ± 0.26
	3	4.04 ± 0.94	5.41 ± 0.89	5.75 ± 0.52	<1.52 ± 0.00
	7	4.25 ± 0.76	4.89 ± 0.47	5.16 ± 0.22	<1.52 ± 0.00
After removal of residual chlorine by aging (0.2 mg/L)	1	5.37 ± 0.27	4.39 ± 0.29	5.42 ± 0.26	6.26 ± 0.44
	3	4.78 ± 0.19	4.44 ± 0.25	4.98 ± 0.13	<1.52 ± 0.00
	7	3.77 ± 0.36	3.77 ± 0.50	4.03 ± 0.50	<1.52 ± 0.00

[a] Cell numbers are given in average $\log_{10}$cells/cm^2 where (n=6) or $\log_{10}$CFU/cm^2 where (n=6) ± standard deviation.

Table A. 5: The effect of organic material on cellular adherence and cell survival of *Ent. cloacae* KY 1 on SS after one, three and seven days incubation at 10°C. The number of cells was enumerated by epifluorescence microscopy using the LIVE/DEAD BacLight stain and the plate count method.

Treatment conditions	Incubation time (days)	Live bacteria	Dead/damaged bacteria	Total bacteria	Plate count numbers
Presence of organic material	1	5.14 ± 0.3	4.17 ± 0.5	5.2 ± 0.3	6.52 ± 0.2
	3	5.3 ± 0.3	4.65 ± 0.5	5.4 ± 0.3	5.89 ± 0.3
	7	5.82 ± 0.6	5.3 ± 0.3	6 ± 0.4	5.6 ± 0.6
No treatment (no addition of organic material)	1	5.37 ± 0.3	4.39 ± 0.3	5.4 ± 0.3	6.26 ± 0.4
	3	4.78 ± 0.2	4.44 ± 0.3	5 ± 0.1	1.52 ± 0
	7	3.77 ± 0.4	3.77 ± 0.5	4 ± 0.5	1.52 ± 0

[a] Cell numbers are given in average $\log_{10}$cells/cm^2 where (n=6) or $\log_{10}$CFU/cm^2 where (n=6) ± standard deviation.