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Impact of Biofilm Formation and Composition on Antibiotic Resistance in Environmental Isolates of Escherichia coli and Salmonella spp.

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Impact of Biofilm Formation and Composition on Antibiotic Resistance in Environmental Isolates of *Escherichia coli* and *Salmonella* spp.

by

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Abstract

Escherichia coli and *Salmonella* spp., like most bacteria, prefer to grow in biofilms. These biofilms provide bacteria with protection from harsh environmental factors (such as desiccation and changes in pH), aid in the evasion of host immune responses and provide increased antibiotic resistance. Biofilms are present in non-host environments (e.g. water pipes) as well as in mammalian hosts (in the healthy gastrointestinal microbiota and in over 65% of nosocomial infections). Two important components utilized by *E. coli* and *Salmonella* spp. to form biofilms are cellulose and curli fimbriae. Curli fimbriae mediate the attachment of bacteria to abiotic surfaces and host epithelial cells. The other component, cellulose, is an exopolysaccharide that provides many benefits such as water retention, tensile strength to the structure and masking of bacterial antigens from host lymphocytes. This research aims to better elucidate the association between host and non-host biofilms produced by *E. coli* and *Salmonella* spp.. Firstly, environmental isolates of *E. coli* and *Salmonella* spp. were profiled for biofilm formation and survival in host and non-host conditions. Then, biofilm composition (curli fimbriae and cellulose) was monitored under varying conditions in order to understand the correlation between expression of components and biofilm formation in host and non-host conditions. The isolates were examined for antibiotic resistance and acid tolerance in synthetic gastric juice. It was found that over 98% of isolates were able to form biofilms. Isolates produced the highest proportion of moderate biofilms at 23°C and 28°C with 38% and 42% of total isolates, respectively. Some biofilm-formers expressed curli fimbriae and cellulose components, with the highest proportion of components expressed at 37°C. Overall, the presence of biofilms increased isolates' ability to survive pH stress and antibiotic resistance. These results show that environmental bacteria possess characteristics that may allow them to infect a host.

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1. Introduction

1.1 *Escherichia coli* and *Salmonella* spp.

Escherichia coli and *Salmonella* spp. are leading causes of foodborne illness. Each year, in the United States alone, there are approximately 76 million cases of foodborne infections, which result in 325,000 hospitalizations and 5000 deaths (WHO, 2014). Approximately 1.2 million cases are due to *Salmonella* infections, and it is estimated that for every reported case of infection there are 38 unreported cases (Mead *et al.*, 1999; CDC, 2015). The cost of foodborne diseases caused by *Salmonella* is estimated to be as high as $1 billion dollars annually, due to medical costs, work absenteeism and economic loss of food industries (Todd, 2014). *Salmonella* spp. are encountered through the consumption of contaminated food such as undercooked beef, pork, poultry, seafood, eggs and milk (WHO, 2014). The first signs of infection appear 12-72 hours after ingestion and can last between four and seven days. The symptoms include fever, nausea, diarrhea and abdominal pain (WHO, 2014).

Most strains of *E. coli* are harmless, and are commonly found within the normal gastrointestinal microbiota of mammals. However, certain strains of *E. coli*, such as enterohemorrhagic *E. coli* (EHEC), or Shiga toxin-producing *E. coli* (STEC), produce toxins that are harmful to the host and can lead to life-threatening complications (WHO,

2014). *E. coli* can be transmitted through contaminated water or undercooked meat, raw milk products and various vegetables (*e.g.* bean sprouts) (WHO, 2014; Foodsafety, 2014). Food products are often recalled due to *E. coli* contamination. For example, beef products from XL Foods Inc. and cheese products from Gort's Gouda Cheese Farm were recalled in October 2012 and November 2013, respectively, both due to *E. coli* O157:H7 contamination (PHAC, 2012; PHAC, 2013). Ingesting food contaminated with pathogenic *E. coli* can result in symptoms of abdominal cramps, diarrhea, vomiting and nausea which generally resolve within 10 days (WHO, 2014).

Conversely, *Salmonella* spp. strains are not commensal and ingesting *Salmonella* spp. can cause salmonellosis with symptoms including diarrhea, vomiting, stomach cramps and fever (PHAC, 2015). Most *Salmonella* spp. infections are resolved within a few days, but complications can lead to chronic symptoms such as Reiter's Syndrome (reactive arthiritis), and in severe cases, death (PHAC, 2015). The *Salmonella* genus is composed of two species (*S. bongori* and *S. enterica*) and a total of 2,463 serotypes, which are identified based on O (somatic) and H (flagellar) antigens (Brenner *et al.,* 2000). The majority of the serotypes belong to the *S. enterica* species including *S.* Typhi (causative agent of typhoid fever), *S.* Typhimurium and *S.* Enteriditis (both of which cause food poisoning) (Brenner *et al.,* 2000). Other *Salmonella* spp. which cause food poisoning include but are not limited to, *S.* Heidleberg, *S.* Thompson, *S.* Braenderup, *S.* Hartford, *S.* Stanley and *S.* Schwarzengrund (CDC, 2013). *Salmonella* spp. and *E. coli* infections are an ongoing concern. Each year, in the United States, pathogenic *E. coli* cause 100,000 illnesses, 3,000

hospitalizations and 90 deaths (CDC, 2009). *Salmonella* spp. are estimated to cause more than 1.2million illnesses each year in the United States, resulting in 23,000 hospitalizations and 450 deaths (CDC, 2015).Therefore, it is important to understand the mechanisms of bacterial survival in the environment (*e.g.* food, water) in order better detect and treat the bacteria and consequently prevent outbreaks and infections.

Bacteria can survive in a host environment during infection or in a non-host environment (*e.g.*, food, water and soil). For the purpose of this thesis "host" environment will refer to conditions within a mammal, and a "non-host" environment refers to any conditions that the bacteria may encounter outside the mammalian host. The host and non-host environments present the bacteria with different challenges, and the bacteria must find ways to adapt and survive within these environments. Within a host, *E. coli* and *Salmonella* spp., occupy an environment where nutrients are readily available and temperature and pH are relatively constant, but in order to survive, the bacteria have to evade the constant onslaught of host immune responses (Winfield and Groisman, 2003). During transition between hosts, bacteria are able to overcome low nutrient availability and temperature changes in the non-host environment, and can be found in our food, soil and water (Winfield and Groisman, 2003). In order to overcome these challenges of the host and non-host environments, *E. coli* and *Salmonella*, similar to over 99% of bacteria, predominantly survive within multicellular communities termed, biofilms (Brown and John, 1999; Prakash, 2003).

1.2 Biofilms

Bacterial biofilms have been predominantly found in non-host, aquatic environments (e.g. waterpipes, rocks, ponds, rivers, streams and other water-associated bodies), host environments (e.g. teeth, mammalian intestinal tract) and can be associated with medical implants including catheters, joint replacements and heart valves (Mah and O'Toole, 2001; Fux *et al*., 2005). Biofilms are often beneficial to bacteria and can be helpful to the host. For example, biofilms that are present in the normal intestinal microbiota of animals can protect the host against harmful bacteria and can occasionally help with the digestion of food (Kudo *et al.,* 1986) *Bacteroides* present in the cow rumen aid in digestion by mediating cellulose degradation, and they can also prevent the establishment of pathogenic bacteria by competing for space and resources (Kudo *et al.*, 1986). If pathogenic bacteria manage to form biofilms, they can be damaging to the host. For example, biofilms formed on medical devices are the cause of more than 65% of nosocomial (hospital acquired) infections (Licking, 1999). In the United States, nosocomial infections are the fourth leading cause of death, with 2 million cases annually (Wenzel, 2007). Therefore, it is important to gain a better understanding of the mechanisms of biofilm formation on abiotic surfaces (such as medical devices) and biofilm survival within a host, so they can be effectively treated, or prevented.

Multiple studies have demonstrated that bacteria growing in a biofilm have increased resistance to environmental fluctuations (Scher *et al.,* 2005; Uhlich *et al.,* 2006). For

example, biofilms are known to protect bacteria from non-host conditions such as desiccation, changes in temperature (4-37°C), UV radiation (sunlight) changes in pH, and low nutrient availability (Olson *et al.,* 2002; Fux *et al.*, 2005). Biofilms formed in the non-host environment are generally composed of multiple species of microorganisms, which share genetic material and nutrients and are able to communicate intracellularly by diffusing quorum sensing molecules through the polymeric matrix (Watnik and Kolter, 2000). The structural materials of the biofilm are the components that provide increased protection to the bacterial communities growing in the biofilm. Biofilm bacteria are protected from desiccation because many biofilm exopolysaccharides are able to retain water, so the cells residing within the biofilm will be able to survive longer in areas with low water availability than planktonic (free-living) bacterial cells (Klemm, 2001). Biofilms increase bacterial survival in non-host environments by protecting them from unfavourable conditions, but they can also increase survival within a host.

Bacterial biofilms are known to be problematic for the host during infections and research has been performed on the role of specific biofilm components (Costerton *et al.,* 1999). While cellulose (exopolysaccharide often present in *E. coli* and *Salmonella* spp. biofilms) helps the bacteria evade host immune responses, curli fimbriae (protein that aids in irreversible attachment) can bind to the epithelium as well as host proteins such as fibronectin, laminin, plasminogen and complex class I molecules (Pozo and Patel, 2007; Olsen *et al*., 1989, 1998). Curli have also been suggested to activate cytokines during human sepsis (bacterial infection in the blood), and to mediate the formation of large

aggregates of *E. coli* during urinary tract infections (Bian *et al.*, 2009; Saldana *et al.*, 2009). Once established, the biofilm can help bacteria evade host defences since the biofilm matrix encompasses the bacteria; thereby covering bacterial ligands and antigens that elicit an immune response (Parsek and Singh, 2003). In addition to avoidance of the immune response, biofilm-related infections are frequently chronic because antibiotic therapy is often ineffective at treating the bacteria encased in the biofilm matrix material (Marrie *et al.*, 1982). Even with prompt treatment, the infection will show recurring symptoms, and may require surgical removal of the biofilm (Costerton *et al.*, 1995). A clear understanding of how bacterial biofilms are formed and compositional analysis of different biofilms is critical in aiding efforts to resolve and/or prevent chronic biofilm infections.

1.2.2. Biofilm Formation

Bacterial biofilms are formed through complex interactions between bacterial cells and the surrounding environment (as reviewed by White, 2007) (Figure 1.1). The first step in biofilm formation is the initial, reversible attachment of planktonic cells to a surface. Once contact with the surface is made, bacteria spread out on the surface forming a monolayer and using proteins such as curli fimbriae for irreversible attachment (Branda *et al.*, 2005). The cells then begin to aggregate and multiply and initiate the mass production of extracellular polymeric substances such as cellulose. The biofilm matures as it thickens and forms microcolonies (Branda *et al.*, 2005). Occasionally, the biofilm- associated

bacteria are able to detach from the biofilm matrix, disperse, and colonize on a new surface (Watnick, 2000).

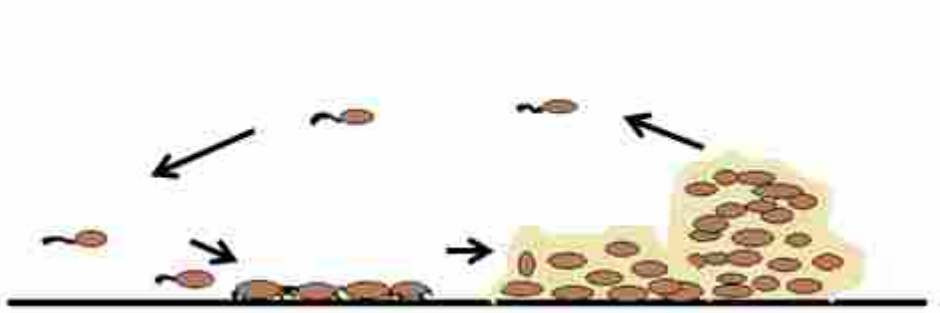


Figure 1.1. A schematic representation of the process of biofilm formation. Planktonic cells loosely attach to a surface after which a monolayer forms and irreversible attachment takes place. Once a certain density is reached, the mass production of exopolysaccharides is initiated. Cells continue to proliferate and the biofilm continues to grow. Cells may detach from the surface of the biofilm and colonize on a new surface (Branda *et al.*, 2005).

The bacterial cells and their self-produced matrix components create biofilms with complex three-dimensional structures (Fux *et al*., 2005). A high proportion of the matrix components are exopolysaccharides, but the biofilms can also be comprised of proteins and DNA (Pozo and Patel, 2007). Depending on the bacterial strains, the exopolysaccharides and proteins present in the biofilm can vary. For example, *E. coli* and *Salmonella* spp. are known to produce cellulose, while *Staphylococcus aureus* and *Staphylococcus epidermis* produce polysaccharide intracellular adhesin (PIA) or poly-N-acetylglucosamine (PNAG) polymer, and *Pseudomonas aeruginosa* produces the exopolysaccharide, alginate, during chronic infection of the cystic fibrosis lung (Branda *et al.*, 2005). In terms of proteins present in biofilms, *E. coli* and *Salmonella* spp. produce curli fimbriae (also known as

aggregative adherence pili) (Branda *et al.*, 2005). *E. coli* can also express Ag43 and type I pili to aid in adhesion, but the expression of these proteins is dispensable depending on conditions (Branda *et al.,* 2005). Other bacteria, such as *P. aeruginosa* use type IV pili or CupA fimbriae, which serve similar purposes as curli fimbriae (Branda *et al.,* 2005). *S. aureus* uses sortase for attachment, which is also a type of pili (Rice *et al*., 2007).

While the extracellular matrix can vary based on organism, it has also been demonstrated that even within the same organism, certain components can also be differentially expressed based on habitat. For example, *E. coli* and *Salmonella* spp. can produce curli and cellulose in both environmental and host-related biofilms but under different selective pressures (Saldana *et al*., 2009). Studies with *Salmonella* indicate that salt, pH <6, and higher temperatures (i.e. 42˚C vs 22˚C) can lead to the induction of biofilm formation (Solano *et al.*, 1998). When biofilm components were studied, *Salmonella* spp. were found to express both curli and cellulose at 28˚C, and only cellulose at 37˚C (Römling *et al.*, 2003). Studies have shown that in uropathogenic *E. coli,* induction of curli fimbriae expression was found to occur at 37˚C, when no salt is present, while other strains of *E. coli* can express cellulose or both components at 37˚C (Bokranz *et al.,* 2005; Saldana *et al.*, 2009; Monteiro *et al.,* 2009). These studies are limited but imply that cellulose and/or curli may be important components of biofilms formed in both host and non-host environments. Further research into this area will be important to provide a better understanding of whether there are common factors and biofilm components that could enhance persistence in (and transmission between) both host and non-host environments.

1.2 Curli Fimbriae

Curli fimbriae play a major role in the establishment of bacterial biofilms (Olsen *et al*., 1989, 1998). Curli are protein fibres that mediate the irreversible attachment of bacteria to host cells (*i.e. E. coli* aggregation in urinary tract infections), and help establish biofilm in a non-host environment (*i.e.* adherence to an abiotic surface) (Houdt, 2005; Saldana *et al*., 2009). Curli are coiled, aggregative fibres that protrude from the cell membrane and coat the exterior of the cell (Olsen *et al.*, 1989; Saldana *et al*., 2009). Curli fimbriae vary in length and have a diameter of about 2-5 nm (Olsen *et al.*, 1989; Saldana *et al*., 2009). Curli is a crucial structural component for multicellular community formation because they help bacterial cells attach to surfaces, but without production of other stabilizing or adhesive factors (*e.g.* exopolysaccharides), the resulting community structures will be very fragile (Solano *et al*., 1998).

1.3 Cellulose

Cellulose is an abundant polymer that is produced by plants, fungi, bacteria and some animals (Zogaj *et al*., 2001). In all cases, cellulose has the same simple chemical structure of β-1-4 linked linear glucose chains, but the inter-chain hydrogen bonding differs between bacterial and plant cellulose (Szymanska-Chargot *et al.,* 2011). While the molecular composition of plant and bacterial cellulose is the same, bacterial cellulose has an ultrafine network arrangement with higher moldability and better water retention than plant cellulose (Klemm *et al.,* 2001). Bacterial cellulose forms complex networks which provide microorganisms within the biofilm with mechanical and chemical protection from unfavourable environments. For example, cellulose can aid in inhibiting the recognition-based host immune response and avoidance of phagocytosis by covering the antigens on the bacterial surface, preventing the host immune response cells (T-cells, B lymphocytes) from detecting the presence of the invaders (Parsek and Singh, 2003; Fux *et al.,* 2005). During biofilm formation, cellulose is produced after the attachment of bacterial cells to a surface by curli fimbriae, and aids in the maturation of biofilms, avoiding the host immune response and providing increased antimicrobial resistance (Zogaj *et al*., 2001).

1.5 Antibiotic Resistance

It is becoming increasingly more difficult to treat bacteria with antimicrobial agents due to a rise in resistance to antimicrobial compounds that occurs either through the spread of resistance genes, generalized stress response mechanisms, or by the formation of a biofilm (Fux *et al.,* 2004; Mah and O'Toole, 2001). For example, bacteria can achieve resistance through the transfer of resistance genes for enzymes such as catalase and β-lactamase between species. Bacteria possessing these resistance genes can then produce enzymes to degrade specific antimicrobial compounds when exposed to these harmful substances (Fux *et al.,* 2004). Antibiotic resistance can also be a result of a more generalized bacterial stress response that occurs when bacteria detect the compounds that may be toxic or detrimental (*e.g.* antibiotics). The bacterial generalized stress response includes altering membrane permeability or use of an alternative metabolic pathway to avoid the uptake of harmful substances (Fux *et al.,* 2004). In addition to these mechanisms, bacteria can also use biofilms as protection from antimicrobial compounds. Biofilms can provide protection in a number of different ways. The exopolysaccharide present in the biofilm can act as a physical barrier, which can inhibit the entry of antimicrobial agents and antibodies into the biofilms. This could be due to the thickness of the biofilm, or because the compounds may bind to the matrix (Costerton *et al.,* 1995; Lewis, 2001; Mah and O'Toole, 2001). Antimicrobial compounds are also less effective on embedded biofilm bacteria because of

the microorganisms' low metabolic activity. Because of its three-dimensional structure, a mature biofilm is composed of numerous microniches. Bacterial cells that utilize oxygen and glucose reside within the outer layers of the biofilms (Fux *et al.,* 2004). The lower layers of the biofilm are anoxic and have low nutrient levels; these layers typically contain bacterial cells that have low metabolic activity (Fux *et al.,* 2004). Antimicrobial compounds are known to be more effective against rapidly growing cells, so within a biofilm, the bacteria residing in the outer layers are affected while the bacteria living in the inner layers will exhibit increased antibiotic resistance (Mah and O'Toole, 2001; Prakash *et al.,* 2003). Due to the close proximity of metabolically active cells, biofilms are also an ideal environment for the exchange of antibiotic resistance genes (Prakash *et al.,* 2003). Additionally, enzymes which degrade antibiotics, such as β-lactamase, may be concentrated in the biofilm matrix. Thus, when antibiotics enter the biofilm, the enzymes readily degrade the compounds (Prakash *et al.,* 2003). Overall, biofilms contribute to the inherent antibiotic resistance of bacteria in a number of significant ways.

Given the variety of methods that bacteria can employ to increase resistance to antimicrobial agents, it is important to understand which antibiotics work best against the bacteria of interest during infections. For example, *Salmonella* spp. infections are generally self-limiting and resolve within 2 to 7 days (WHO, 2013). If the infection becomes invasive then antimicrobial compounds are used for treatment (Foley and Lynne, 2008). Quinolones and 3rd generation cephalosporins are often used to treat *Salmonella* infections (Shea, 2004; Buyaye *et al.,* 2006). *Salmonella* can also be treated using chloramphenicol, ampicillin,

amoxicillin and trimethoprim-sulfamethoxazole, but they may exhibit limited effectiveness due to high levels of *Salmonella* antimicrobial resistance (WHO, 2013; Thomas, 2011). Most *E. coli* infections are also self-limiting (WHO, 2014b). A few examples of antibiotics occasionally used against *E. coli* (depending on the infection) are 3rd generation cephalosporins (*e.g*. Ceftriaxone, Cefotaxime), ampicillin, fluoroquinolones (*e.g.* Ciprofloxacin), Rifaximin and Aztreonam (Madappa, 2014). *E. coli* is often resistant to antibiotics used to treat common infections (penicillins, cephalosporins) and can even be resistant to the antibiotics used to treat more severe infections (fluoroquinolones) (Madappa, 2014). Like most bacteria, *E. coli* and *Salmonella* spp. are becoming increasingly resistant to antibiotics (see CLSI, 2007 for list of antibiotics, a sample table can also be found in Table A6 of Appendix B). Biofilm formation could play a major role in the exchange of the bacterial resistance genes for *E. coli* and *Salmonella* spp.. Alternatively, the components of some biofilms may be more effective at increasing resistance to antibiotics due to changes in charge, hydrophobicity and porosity of the biofilm when that component is expressed. Understanding the mechanisms of biofilm formation and possibly preventing the formation could have a great impact on decreasing antibiotic resistance (not only from the non-specific resistance the biofilm provides, but also decreasing chances of exchanging resistance genes).

1.6 Acid Tolerance

E. coli and *Salmonella* spp. infections often begin with the ingestion of contaminated food or water. After ingestion, the microorganisms must survive the selective pressures and unfavourable environment of the stomach in order to successfully colonize within the gastrointestinal tract. Bacteria have many mechanisms of acid tolerance, for example, *E. coli* uses resistance systems such as the acid-induced oxidative system, acid-induced arginine dependent system and glutamate-dependent system when faced with acidic environments (Lin *et al.*, 1996). *Salmonella* spp. survive in low pH by using the acid tolerance response (ATR) which is mediated by a number of proteins (Foster and Hall, 1990). Research has been conducted studying the ability of *E. coli* and *Salmonella* spp. to survive in acidic environments (Lin *et al.*, 1996; Foster and Hall, 1990; Xia *et al.*, 2009), but little information is available on the effects of acidic environments on biofilm-embedded cells. Previous research using gram positive bacteria in dental plaque found that biofilms protect bacteria from acidic environments (Svensater *et al.*, 2001). However, research on gram-negative biofilms and their protection against acidic environments is limited.

2. Research Need

Outbreaks of *E. coli* and *Salmonella* spp. due to contaminated food and water are an ongoing concern. Food products are often recalled in North America due to contamination of *E. coli* and *Salmonella* spp. (see CDC foodsafety) (CDC, 2014). Many foodborne outbreaks are dependent on attachment mechanisms. Bacteria are able to attach themselves to surfaces such as food, water pipes, rocks, as well as medical devices and form biofilms (Mah and O'Toole, 2001; Fux *et al*., 2004; Kumar, 1998). These biofilms protect bacteria from host immune responses, antimicrobial compounds and environmental factors, making them increasingly more difficult to treat in a host infection, as well as outside the host.

Since greater than 65% of nosocomial infections are a result of bacteria growing in a biofilm, it is important to better understand the conditions under which bacteria are able to produce biofilms, and the possible increased resistance the biofilm provides. Specifically, in-depth studies are needed on the capability of environmentally-isolated microorganisms to form biofilms, what components are present in these biofilms, and how these components help promote bacterial survival in various non-host and host-related situations. For example, studies have noted that individual pathogenic isolates of *E. coli* and *Salmonella* spp. are able to produce curli and cellulose-containing biofilms in situations that mimic the host environment (*i.e.* invasion of epithelial cells) and or non-host environments (*i.e.* abiotic surfaces) (Zogaj *et al.*, 2001; Saldana *et al.*, 2009). However, a direct linkage between host and non-host environments and biofilm formation has not yet been established.

2.1. Hypotheses

With the use of *E. coli* and *Salmonella* spp. as model organisms and a series of studies aimed at understanding biofilm formation, composition and antimicrobial resistance, a greater understanding of a potential linkage between biofilm formation and the persistence and transmissibility of pathogens from environmental reservoirs to hosts can be established.

My specific hypotheses are as follows:

1) More environmental isolates of *E. coli* and *Salmonella* spp. from predominantly host locations will form biofilms than isolates from non-host locations.
2) Biofilm forming isolates will be able to better survive in various environmental conditions and host-like conditions.
3) Biofilm formers will express curli fimbriae and cellulose biofilm components to various degrees that may affect the type and resilience of the biofilm formed.

2.2. Objectives

The overall objective of this project which aims to test the hypotheses of this research, is to better elucidate the association between non-host and host biofilms. To test the hypotheses, biofilm formation and antibiotic resistance of *E. coli* and *Salmonella* spp. environmental isolates were assessed and related back to their ability to survive under non-host, environmental conditions or under conditions that more closely resemble a host environment.

The overall objective was addressed by the following:

1) Isolate *E. coli* and *Salmonella* from environmental and waste-water sources and identify biofilm formers.

2) Probe linkages between common host and non-host environmental stressors for their impact on biofilm formation.

3) Perform compositional analysis among the isolates demonstrating the ability to form biofilms in order to understand the correlation between expression of curli fimbriae and cellulose and biofilm formation under host and non-host conditions.

3. Experimental Approach

The integrative nature of this project can be seen to some extent in the experimental approach (Figure 3.1.). A series of culture-based and molecular methods were used to assess biofilm formation in the presence of certain host stressors as well as non-host environmental exposures. Biofilm formation was studied at a community level, as well as at a cellular level to understand the expression of biofilm components.

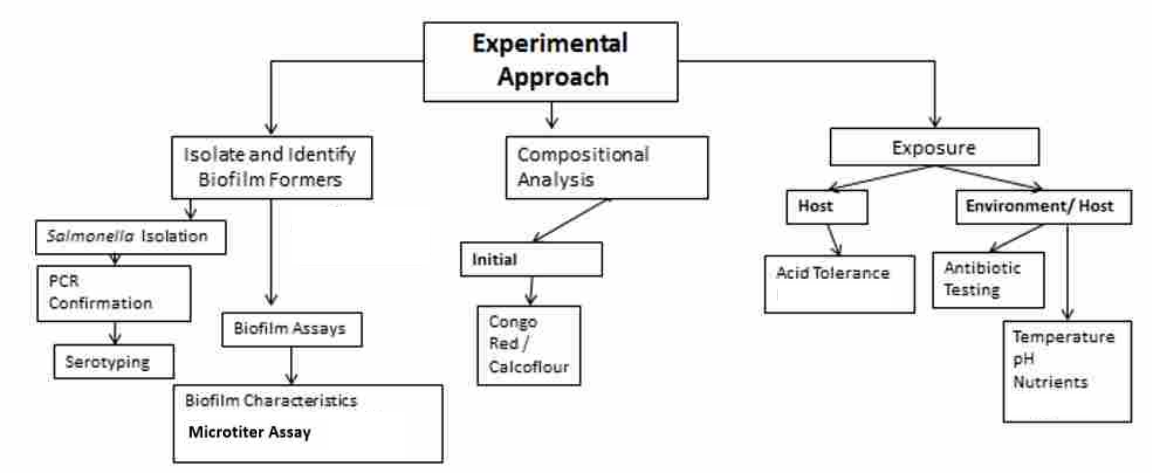


Figure 3.1. Schematic representation of experimental approach. This diagram details the specific experiments used to test a linkage between biofilm formation and the stressors that mimic host and/or non-host environments.

3.1. Isolates and Controls

In order to better understand patterns of biofilm formation and bacterial expression of biofilm components, it is important to have samples from a variety of different locations. *Salmonella* spp. and *E. coli* samples were each isolated from locations of wastewater effluent and freshwater locations. The isolates from the wastewater samples were expected to have recently been in a host environment, while the freshwater isolates were more likely in the environment for a longer period of time and were expected to be more accustomed/adapted to environmental conditions. The sampling locations were subsequently divided into predominantly host and predominantly non-host locations (see Table 3.1. for division of sites). The different sites were chosen in order to compare the biofilm-forming capabilities and resistance profiles of bacteria that dominate various host and non-host environmental conditions. Locations of isolation are described below, and specific methods of *E. coli* and *Salmonella* spp. isolation are outlined in sections 3.3 and 3.4.

Table 3.1. Locations of *E. coli* and *Salmonella* spp. Isolation Divided by Predominantly Non-Host, and Predominantly Host Locations.

Predominantly Non-Host	**Predominantly Host (suspected to recently have been in a host environment)**
Clair Lake	**Fleming**
28 *Salmonella* spp. isolates	46 *Salmonella* spp. isolates
Grand River	**Nunavut**
22 *E. coli* isolates	25 *E. coli* isolates
Grand Bend	8 *Salmonella* spp. isolates
10 *E. coli* isolates	**Coboconk/ Gull Lagoon**
Mill Street	18 *E. coli* isolates
7 *Salmonella* spp. isolates	6 *Salmonella* spp. isolates
20 *E. coli* isolates	**Omeemee**
Coboconk/ Gull River	3 *Salmonella* spp. isolates
10 *E. coli* isolates	**Water Treatment Center**
	8 *Salmonella* spp. isolates

Isolation site 1: Fleming College. Lindsay, Ontario (wetland)

Forty six *Salmonella* spp. isolates were previously isolated by Robyn Morrison (Morrison, 2013) using the swab method, as detailed in section 3.3. The samples were collected from an on-site constructed wetland from the Frost Campus of Fleming College in Lindsay, Ontario. The wetland was constructed to treat septic waste from the Frost Campus. The

wetland has four test vaults (V1, V2, V3 and V4) as well as a polishing pond (PP) which holds water before it is recycled back into the wetland or discharged into municipal sewers (CAWT, 2013) (schematic representation of wetland available in Figure A1 of Appendix B). The *Salmonella* spp. isolates used in this study were collected from V1, V2, V3 and PP.

Isolation site 2: Clair Lake. Waterloo, Ontario

Twenty eight *Salmonella* spp. isolates were retrieved from Clair Lake in Waterloo, Ontario. Clair Lake is an urban pond located in North Waterloo impacted by storm water and waterfowl. Isolates retrieved from this location are expected to have been in the environment longer than samples from the other wastewater sources.

Isolation site 3: Grand River. Waterloo, Ontario

E. coli isolates from this location were previously isolated by Robyn Morrison and Patricia Jarosz from the Slawson Lab (Morrison, 2013 unpublished). There are 22 *E. coli* isolates that were retrieved from the Grand River in Waterloo, Ontario. The samples were taken approximately 5-8 km upstream of the Waterloo Wastewater Treatment plant. The Grand River Watershed flows over a distance of 300 km and spans from Dufferin County to Port Maitland on Lake Erie. Because of the large area which it covers, Grand River waters are impacted by natural, municipal and agricultural activities. Samples retrieved from the Grand River may have originated from any number of these activities.

Isolation site 4: Nunavut

E. coli was also isolated from primary wastewater samples which were collected from an undisclosed location in Nunavut. These 25 isolates represent bacteria which have most recently been in a host environment but are exposed to more extreme environmental conditions.

Isolation site 5: Grand Bend

Ten *E. coli* isolates were taken from the Grand Bend location of Lake Huron during the summer of 2014. Samples were taken from water approximately 3 meters from the shoreline. At the time of the sampling event, the beach was deemed unsafe for swimming due to high levels of *E. coli* (Boyce, 2014).

Isolation site 6: Wastewater Treatment Plant (Burlington, ON)

Eight of the *Salmonella* spp. isolates were previously isolated by Cassandra Helt (Helt, 2012) from the Wastewater Treatment Center (WTC). The WTC is a pilot plant which is designed for research and evaluation of various treatment technologies for wastewater. The treatment center receives raw effluent from the Skyway Wastewater Treatment Plant which serves the Burlington region.

Isolation site 7: Coboconk Lagoon

Coboconk Lagoon is a constructed lagoon which is designed to filter and trap nutrients from storm and wastewater, protecting downstream waters from harmful contaminants (WaterTap, 2013). Samples were taken from Floating Treatment Wetlands (FTW) located in the Coboconk Lagoon. The FTWs are composed of plants growing on mats floating on the surface of a water basin, allowing their roots to float freely in the water. Microbial biofilms typically form on the roots of the plants. The plants and microbial communities help the water treatment process through the uptake and transformation of the nutrients and contaminants in the water. Samples were taken of the roots and the area surrounding the FTWs. From this location, 18 *E. coli* and 6 *Salmonella* spp. isolates were retrieved.

Isolation site 8: Gull River

Ten *E. coli* isolates were retrieved from the Gull River in Coboconk, Ontario. The Gull River is located downstream from the discharge of the Coboconk Lagoon. Samples were taken from the area surrounding FTWs located in this river.

Isolation site 9: Scugog River

Samples were taken from the Mill Street location of Scugog River located in Lindsay, Ontario. There are FTWs at this location and samples were taken from the roots and the surrounding water. In total, there were 27 *Salmonella* spp. and 20 *E. coli* isolates that were successfully isolated from this location.

Isolation site 10: Omeemee Water Pollution Control Plant

The Omemee Water Pollution Control Plant is composed of 2 lagoons, which have a designed capacity of 608 m^3 per day. The wastewater is mainly treated through microbial decomposition, which accounts for approximately 90% of the reduction in organic nutrients (Cambium Environmental, 2015). From this location, 3 *Salmonella* spp. isolates were retrieved.

Control samples:

In addition to the environmental isolates, four *Salmonella enterica* lab strains were used as biological controls for assessing the presence of biofilm components. These controls were generously supplied by Dr. Ute Römling, Karolinska Institute, Sweden (Römling *et al.*, 2003). The controls are identified by their strain designations, UMR1, MAE14, MAE299 and MAE775. The controls consist of one wild-type strain (UMR1) which expresses both cellulose and curli fimbrae. The remainder of the controls are mutants which were altered to express only cellulose (MAE 14), only curli (MAE299) or neither component (MAE775).

3.2. General Growth Conditions

E. coli and *Salmonella* spp. strains were routinely maintained on Luria-Bertani (LB, recipe in Appendix A) agar at 37°C and LB broth at 37°C. The isolates were grown at

temperatures of 10°C, 21-23°C, 28°C and 37°C when testing for biofilm formation and expression of biofilm components. These temperatures were chosen to represent their non-host and/or host environments and for optimal expression of biofilm components. Temperatures of 10°C and 21°C are often encountered in the environment, 37°C is a mammalian host temperature and 28°C is the optimal temperature for expression of curli fimbriae and cellulose for the *S. enterica* controls (Römling *et al.*, 2003). Growth assays were performed in LB broth with and without salt (recipe in Appendix A). The medium with no salt represents many external environmental conditions, such as freshwater, where salinity is low. Medium containing salt is more representative of a host environment.

Objective 1: Isolate E. coli and Salmonella from environmental and waste-water sources and identify biofilm formers.

3.3 *Salmonella* spp. isolation

Salmonella spp. were collected from the environment using a swab collection technique as specified by Standard Methods (APHA, 2005). A swab was constructed using sterilized cheesecloth, and placed under water using a pig-tailed spike. After 3 days, the swab was collected along with approximately 100mL of water. Processing of the samples began the same day of collection. The *Salmonella* isolation protocol used is similar to the procedure described by Rybolt *et al.* (2004). All media used for the isolation of *Salmonella* spp., outlined in sections 3.3 and 3.3.1, was purchased from BD Difco™ Missisauga, ON. Upon

arrival to the lab, 90 mL of the samples were inoculated in 10 mL of 10x buffered peptone water (BPW), which allows for non-selective recovery of *Salmonella* spp. by acting as a buffer and providing a nutrient rich environment (BD™a, 2015). The flasks were then incubated in an incubator-shaker (Fisher Scientific, Whitby, Canada) at 37°C for 24 hrs at 140 rpm.

Following the recovery period, samples underwent selective enrichment using Tetrathionate Broth (TB) with 2% (v/v) iodine solution. The media promotes *Salmonella* spp. growth because of their ability to reduce tetrathionate, while the iodine and oxgall in the medium prevents the growth of coliforms (BD™b, 2015). From the BPW sample solution, 1 mL was inoculated into 9 mL of the TB with 2% (v/v) iodine. As a negative control, 1mL of *E. coli* BL21 grown in LB broth was inoculated into the TB media, and *S. enteritidis* grown in LB was inoculated as a positive control. These controls were carried out throughout the rest of the isolation process. The isolation step was performed in duplicate, and samples were incubated in the incubator-shaker for 24 hrs at 37°C and 170 rpm.

The next selective enrichment medium was Modified Semisolid Rapaport-Vassiliadis (MSRV). This media is for the detection of motile *Salmonella* spp., to distinguish this organism from the closely related, non-motile *Shigella* (BD™c, 2015). From the TB-Sample mix, 100 µL was inoculated into the center of the MSRV plate, in triplicate, and

incubated with the agar-side facing up at 44°C for 24hrs. If samples were motile, they would leave a halo of growth around the spot of inoculation.

The third selective enrichment medium used was MacConkey (MAC) agar. This medium allows for the differentiation between lactose fermenters and non-lactose fermenting organisms such as *Salmonella* spp. (BD™d, 2015). On this agar, *Salmonella* spp. are expected to show up as clear colonies, while lactose fermenters, such as *E. coli*, would appear pink. Using a small micropipette tip, about 2µL of culture were picked up from the most outer edge of the halo on MSRV. The culture was then transferred to MAC, in duplicate, and quadrant streaked using a flame-sterilized loop, and incubated at 37°C for 24hrs.

3.3.1 Biochemical Testing

Following the selective enrichment procedures, three biochemical tests were performed in parallel in order to determine which environmental isolates were presumptive *Salmonella* spp. isolates (isolates yielding characteristics of *Salmonella* spp.). The three biochemical tests were carried out using Triple Sugar Iron (TSI), Lysine Iron Agar (LIA) and Urea broth. All three were inoculated using a single colony picked from MAC. The TSI agar was inoculated using a stab and streak method, and a positive *Salmonella* spp. result was determined by the formation of H_2S precipitate, hydrogen gas and dextrose fermentation. LIA was inoculated using the double stab and streak method. *Salmonella* spp. also produces

H_2S in this medium. Finally, isolates were inoculated into Urea broth. *Salmonella* spp. do not contain the urease enzyme, therefore, a negative Urea broth test result is considered positive for *Salmonella* spp.. A positive *Salmonella* spp. control was used for each test, and *Klebsiella pneumoniae* was used as a positive control for the Urea broth test. All inoculated tests were incubated for 48 hours at 37°C.

3.3.2. PCR Colony Confirmation

The presumptive *Salmonella* spp. isolates were confirmed using colony PCR. The primers used for *Salmonella* spp. confirmation are genus specific primers of the *invA* invasion protein Sal-F 5′CGTTTCCTGCGGTACTGTTAATT 3′ and Sal-R 5′AGACGGCTGGTACTGATCGATAA 3′ (Shannon *et al.*, 2007). DNA was isolated using Insta-Gene Matrix (Bio-Rad Laboratories, CA, United States) as described by the Insta-Gene protocol. The PCR master mix was prepared for the 25 µL reactions using a kit from Promega (Madison, WI, USA). Each reaction contained 1x Go-Taq™ Flexi Green PCR Buffer, 1.5 µM $MgCl_2$, 0.5 µM of Sal-F and Sal-R, 200µM dNTP, Milli-Q water (enough to complete 25 µL reaction) and 0.2 µL Go-Taq™ Flexi. The PCR Reaction was carried out using the PTC-100 Programmable Thermal Controller (Bio-Rad, CA, United States). The PCR conditions began with an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 20 sec at 94°C and 1 min at 65°C and a final extension step of 7 minutes at 72°C. Once the reaction ended, the PCR products were held at 4°C until they

were stored at -20°C. *S. enteritidis* and *E. coli* were used as the positive and negative controls, respectively. To observe the PCR products, 7 µL were then loaded onto a 2% (w/v) agarose gel in 1X tris-acetate-EDTA (TAE) (recipe in Appendix A) buffer and run for 60 min at 100V. The gels were then stained in ethidium bromide solution for 20 min, and decolourized in water for 5 min. The gels were imaged using a BioRad™ GelDoc™ XR (CA, United States). *Salmonella* spp. isolates were confirmed by the presence of an 82 bp band.

3.3.3. Serotyping

A group of *Salmonella* spp. isolates were streaked onto LB slants (see Appendix A) and sent to the Public Health Agency of Canada's Laboratory of Foodborne Zoonoses in Guelph, ON for serotyping. Serotyping involves the detection of O antigens on the cell surface, H (flagellar) antigens and Vi (capsular) antigens (Imen, 2012). This is done using a slide agglutination test using antisera (Imen, 2012).

3.4. *E. coli* Isolation

E. coli was isolated using the membrane filtration technique as described by the American Public Health Association (APHA, 1998). Samples were diluted to 10^{-1} and 10^{2} and 10mL were filtered through 0.45 µm, 47 mm mixed cellulose filters (Difco, Fisher Scientific;

Ottawa, Canada). Additionally, 1 mL and 10 mL of the undiluted samples were also filtered. As a positive control, 100 µL of an *E. coli* culture was filtered. The filters were then placed onto mFC media (Difco, Fischer Scientific) supplemented with 100µg/L BCIG (5-bromo-4-chloro-3-indolyl-beta-D-glucuronide) (Medox Diagostics Ottawa, Canada). The plates were then incubated at 44± 0.5°C for 24 hrs in a hot water bath, or incubator with a large, full beaker of water. After incubation, colonies were checked for colour. Blue colonies were considered to be *E. coli.* The selected isolates were streaked onto LB agar for storage.

3.5. Biofilm Formation

3.5.1. Microtiter Plate Biofilm Assay

The microtiter biofilm assay was performed as described by Merrit and colleagues (2011), with some modifications. This assay allowed for qualitative and quantitative observation of the amount of biofilm formed. Bacterial isolates were grown in LB broth overnight. The samples were then diluted 1:100 in fresh medium and 100 µL of each sample were transferred in quadruplicate to a 96 well microtiter plate (Non- Tissue Culture Treated, Flat Bottom with Low Evaporation Lid, Corning Inc., NY, USA). Each of the isolates were inoculated in four different plates for incubation at various temperatures. The plates were then incubated; at 37°C for 16-24 hrs, 28°C for 36-42 hrs, 23°C for 48-54 hrs and 10°C for 5 days. After incubation, the microtiter plates were washed twice with distilled water and

dried. The plates were then stained with 125 µL of 0.1% (w/v) Crystal Violet solution. The crystal violet stains the bacterial cells that are adhered to the sides of the wells; these cells are those that typically produce extracellular substances (*i.e.*, proteins and exopolysaccharides) which facilitate attachment to the surface and can lead to biofilm formation. The crystal violet in the wells was then solubilized using 200µL 30% (v/v) acetic acid and measured for absorbance at 600nm in a HT microplate reader (Biotek, VT, United States). A high absorbance reading corresponded to a high amount of biofilm and, low absorbance readings indicated scarce biofilm formation (Merrit *et al.*, 2011). This experiment was repeated 3 times to ensure results were consistent between trials.

3.5.2. Biofilm Assessment

After determining the absorbance values of the crystal violet bound to attached cells, the degree of biofilm formation was assessed. The biofilms were characterized as previously described by Stepanovic and coworkers (2000). The values were classified as no biofilm, weak biofilm, moderate biofilm and strong biofilm, by comparing the absorbance of the crystal violet solubilized in 30% acetic acid (v/v) (AB) to the negative control, or blank, which was 30% acetic acid (v/v) (AB_c). The categories are outlined in Table 3.2.

Table 3.2. Biofilm Classification System

No Biofilm	$AB^* \leq AB_c^{**}$
Weak Biofilm	$AB_c < AB \leq 2x\ AB_c$
Moderate Biofilm	$2xAB_c < AB \leq 4x\ AB_c$
Strong Biofilm	$4x\ AB_c < AB$

*AB= absorbance at 600nm reading of crystal violet solubilized in 30% acetic acid (v/v)

**AB_c= absorbance at 600nm reading of the negative control, or blank (30% acetic acid (v/v)

Objective 2: Probe linkages between common host and non-host environmental stressors for their impact on biofilm formation. Attempts will also be made to correlate isolates to known cultures by serotyping isolates.

3.6. Antibiotic Testing

Antibiotics for susceptibility testing were chosen based on suggested groupings by the Clinical and Laboratory Standards Institute (2007) of antibiotics generally used for *E. coli* and *Salmonella* spp. infections, and previous research on *Salmonella* susceptibility completed by Janis Thomas in the Slawson Lab (Thomas, 2011).

Table 3.3. Susceptibility Testing Antimicrobial Compounds

Subclass of Antimicrobial Drug	Antimicrobial Drug	Drug Code	Disk content (µg)	Concentrations Tested for MBEC (µg/ml)	Action*
Penicillins	Ampicillin[a]	A	10	16-1024	bactericidal
	Amoxicillin/ Clauvulanic acid	AmC	20/10	16-1024	bactericidal
Cephalosporins	Cefotaxime[a]	Ctx	30	16-1024	bactericidal
Aminoglycosides	Streptomycin[a]	S	10	16-1024	bactericidal
Tetracyclines	Oxytetracycline[a]	T	30	16-1024	bacteriostatic
Fluoroquinolones	Ciprofloxacin	Cip	5	16-1024	bactericidal
Folate pathway inhibitors	Trimethoprim-sufamethoxazole	SxT	1.25/23.75	16-1024	bacteriostatic
Monobactam	Aztreonam	Atm	30	16-1024	bactericidal

* (Liofilchem,2015)

[a] Antibiotics used for the Minimum Biofilm Eradication Concentration assay

3.6.1. Disk- Diffusion Assay

Environmental isolates were tested for antimicrobial susceptibility using antibiotic discs (BD) listed in Table 3.3. Antibiotic resistance was determined as described by the Clinical and Laboratory Standards Institute, using the Kirby-Bauer disk diffusion method (CLSI, 2007). Isolates were grown in LB Broth to a 0.5 McFarland standard of turbidity, after which 100 µL were transferred onto Mueller-Hinton (MH) agar. Disks of antibiotics listed in Table 3.3 (purchased from BD, MD, USA) were then placed on the inoculated plates using sterilized tweezers. Following incubation at 37˚C for 16-24 hours, the zones of inhibition were measured and used to categorize the isolates as susceptible, intermediately resistant, or resistant according to the CLSI zone diameter interpretive standards for each antibiotic (CLSI, 2007).

3.6.2. Minimum Biofilm Eradication Concentration

The minimum biofilm eradication concentration (MBEC) assay was performed similar to the method described by Ceri *et al.* (1999) using the Calgary Biofilm Device (CBD). However, the methods were slightly altered for the use of microtiter plates instead of the CBD. The antibiotics used in this assay were: Ampicillin (Sigma-Aldrich, MO, USA),

Cefotaxime (Fisher Scientific, Ottawa, Canada), Streptomycin (Sigma-Aldrich, MO, USA) and Oxytetracycline (listed in Table 3.3). Only four out of the previously listed eight antibiotics were used due to time constraints, and the large number of isolates used in this study. Isolates were grown in overnight (~16h) cultures in LB broth, after which they were diluted 1:100 and 100 µL were transferred to microtiter plates. The plates were incubated overnight at 37°C to allow for biofilm formation. After incubation, the broth was removed and the wells were washed twice using sodium-free phosphate buffer. The wells were then filled with cation-adjusted Mueller-Hinton broth (CAMHB) (Fluka Analytical, Sigma-Aldrich, MO, USA) supplemented with antibiotics at two-fold increments ranging from 16 -1024 µg/mL. The antibiotic containing plates were then incubated for 24 hrs at 37°C. Following incubation, the wells were rinsed twice with 125 µL of sodium-free phosphate buffer (recipe in Appendix A). The biofilm was scraped off the sides of the well using a sterile micropipette tip by circling the well four times and mixed into the buffer in the wells, and 10 µL of the solution were plated onto LB plates and incubated at 37°C to check for survival. If isolates grew they were considered to be resistant to that particular concentration of antibiotic. For example, if isolates did not grow after incubation in 1024 µg/mL antibiotics, but did after incubation in 512 µg/mL, the isolate was considered to have a MBEC of 512 µg/mL. If isolates grew after incubation in a concentration of 1024 µg/mL of an antibiotic, they were considered to have a MBEC >1024 µg/mL. The isolates were tested at each antibiotic concentration in triplicate. A positive control for each isolate was included in antibiotic-free broth.

3.7. Exposure Trials

Isolates were tested for biofilm formation and survival under a variety of different conditions which were designed to mimic the host or external environment, respectively. The host and non-host assays include the conditions explained above in General Growth Conditions and Microtiter Plate Biofilm Assay, in medium with salt and no salt and at temperatures of 37°C, 28°C, 23°C and 10°C.

3.7.1. Host Condition: Acid Tolerance Testing

Acid tolerance testing was performed as described by Beumer *et al.* (1992) and Xia *et al.* (2009) to test for the isolates' ability to survive in synthetic gastric juice (pH 3.0). Often, *Salmonella* and *E. coli* infections begin by ingestion of contaminated food or water which ultimately causes gastroenteritis. In order for the bacteria to establish themselves and infect, they must first endure the unfavourable conditions of the stomach. Isolates were grown overnight in LB after which they were diluted 1:200 and incubated in a synthetic gastric juice preparation (recipe in Appendix A) pre-warmed to 37°C. The isolates were incubated for 2 hours at 37°C after which they were plated and tested for survival. This assay was performed in triplicate.

3.7.2. Host Condition: Biofilm Acid Tolerance Testing

The acid tolerance test described by Xia *et al.* (2009) was altered for testing the ability of biofilm-encased bacteria to survive in synthetic gastric juice. Isolates were grown overnight in LB broth after which they were diluted 1:100 in fresh medium and transferred to 96 well microtiter plates (100 µL sample/ well). The plates were incubated overnight at 37°C after which the wells were washed out twice with sodium-free phosphate buffer, and replaced with 125 µL pre-warmed synthetic gastric juice. After a 2 hour incubation at 37°C, the wells were washed out twice with buffer. The biofilms were then scraped off and plated to confirm survival or eradication of cells as explained in the MBEC assay. Each isolate was tested in triplicate.

Objective 3: Perform compositional analysis among the isolates demonstrating the ability to form biofilms in order to understand the correlation between expression of curli fimbriae and cellulose and biofilm formation under host and non-host conditions.

3.8. Cellulose and Curli Detection

Calcofluor-infused agar plates were used for the initial detection of cellulose, or similar exopolysaccharides. Cultures were inoculated on LB plates containing 0.025% (w/v) calcofluor white (fluorescent brightener 28, Sigma-Aldrich, MO, USA). If an

exopolysaccharide, such as cellulose, is present it binds to the calcofluor dye and fluoresces under UV light (Römling *et al.*, 2003). The isolates were streaked onto the agar and incubated at temperatures of 10°C, 21°C, 28°C and 37°C. After incubation, the colonies were observed for fluorescence under 300 nm UV light using a Hoefer Scientific Mighty Bright UV light box. For positive and negative controls, UMR1, MAE14, MAE299 and MAE775 were plated and incubated at 28°C. Each isolate was tested in triplicate.

Congo red (Fluka Analytical, Sigma Aldrich, MO, USA and Fisher Scientific, Ottawa, Canada) and coommassie blue (Fisher Scientific) infused LB agar (final concentrations of 40µg ml^{-1}, 15µg ml^{-1}, respectively) were used to detect the presence of curli fimbriae and cellulose produced by bacterial colonies (Römling *et al.*, 2003). After incubation, the colonies exhibit different morphotypes based on the presence of curli and/or cellulose. The rdar (red, dry and rough) morphotype is expressed by colonies that have both curli fimbriae and cellulose as part of their extracellular matrix, the pdar (pink, dry and rough) morphotype expresses only cellulose, bdar (brown, dry and rough) only curli, and the saw (smooth and white) morphotype does not express either component (Römling *et al.*, 2003). Similar to the calcofluor plates, isolates were incubated on congo red plates at different temperatures. The previously mentioned controls, UMR1, MAE14, MAE299 and MAE775 were plated and incubated at 28°C and used as a guide while comparing morphotypes of environmental isolates. This assay was completed in triplicate.

4. Results

As described previously, *E. coli* and *Salmonella* spp. isolates were extracted from 10 different locations which were subsequently categorized as predominantly host, or predominantly non-host locations. Sampling locations were classified as predominantly host if it contained microorganisms that may have recently been in contact with a mammalian host. Locations were classified as non-host if the microorganisms present are likely to have been in the environment for longer periods of time. The isolates were tested for their ability to form biofilms and survive host and non-host stressors. Table 4.1 provides a summary of the total number of *E. coli* and *Salmonella* spp. that were successfully isolated from each sample location, along with the assigned code which will be used throughout the rest of the data presentation.

Table 4.1. Summary of Total Number of *Salmonella* spp. and *E. coli* Isolates.

Total Number of Environmental Isolates

Sampling Site	**Sampling Date**	***E. coli***	***Salmonella* spp.**	**Code**
Clair Lake	October, 2013	0	28	CLS
Fleming*	August 24, 2012	0	47	FS*
Water Treatment Plant*	June 18, 2012	0	7	WTS*
Gull River	June 25th, 2014 Oct 8th, 2014	11	7	GS GE
Coboconk Lagoon*	June 25th,2014 Oct 8th, 2014	18	5	CS* CLE*
Scugog River	June 25, 2014 Oct 8th, 2014	20	6	SRS SE
Omeemee Water Pollution Treatment Pond	Oct 8th, 2014	0	3	OS*
Grand Bend	July 2014	10	0	GBE
Grand River	July 4th, 2012	22	0	GRE
Nunavut*	August, 2013	26	0	NE*

*Primarily host locations

4.1. Biofilm formation

Isolates were tested for their ability to form biofilms in the absence or presence of salt at four different temperatures. The degree of biofilm formation was determined based on the absorbance of bound crystal violet. Using predetermined thresholds (Table 3.2), the absorbance values were subsequently categorized as no biofilm, weak, moderate and strong biofilm. Each isolate was tested between 4 and 12 times (an example of the raw data can be found in Table A1 of Appendix B).

Figure 4.1 depicts the percentage of overall biofilm formation for all *Salmonella* spp. (panel A) and all *E. coli* isolates (panel B). Most *Salmonella* spp. isolates were able to form biofilms at all temperatures tested. A small percentage of isolates were unable to form biofilms at 28°C and 10°C. Conversely, *E. coli* isolates exhibited some non-biofilm formers at all temperatures. The highest proportion of strong biofilms was observed at 23°C and 28°C for both *Salmonella* spp. and *E. coli*. At all temperatures, more moderate and strong biofilms were observed in media with no salt compared to salt containing media, with the exception of *E. coli* isolates at 10°C.

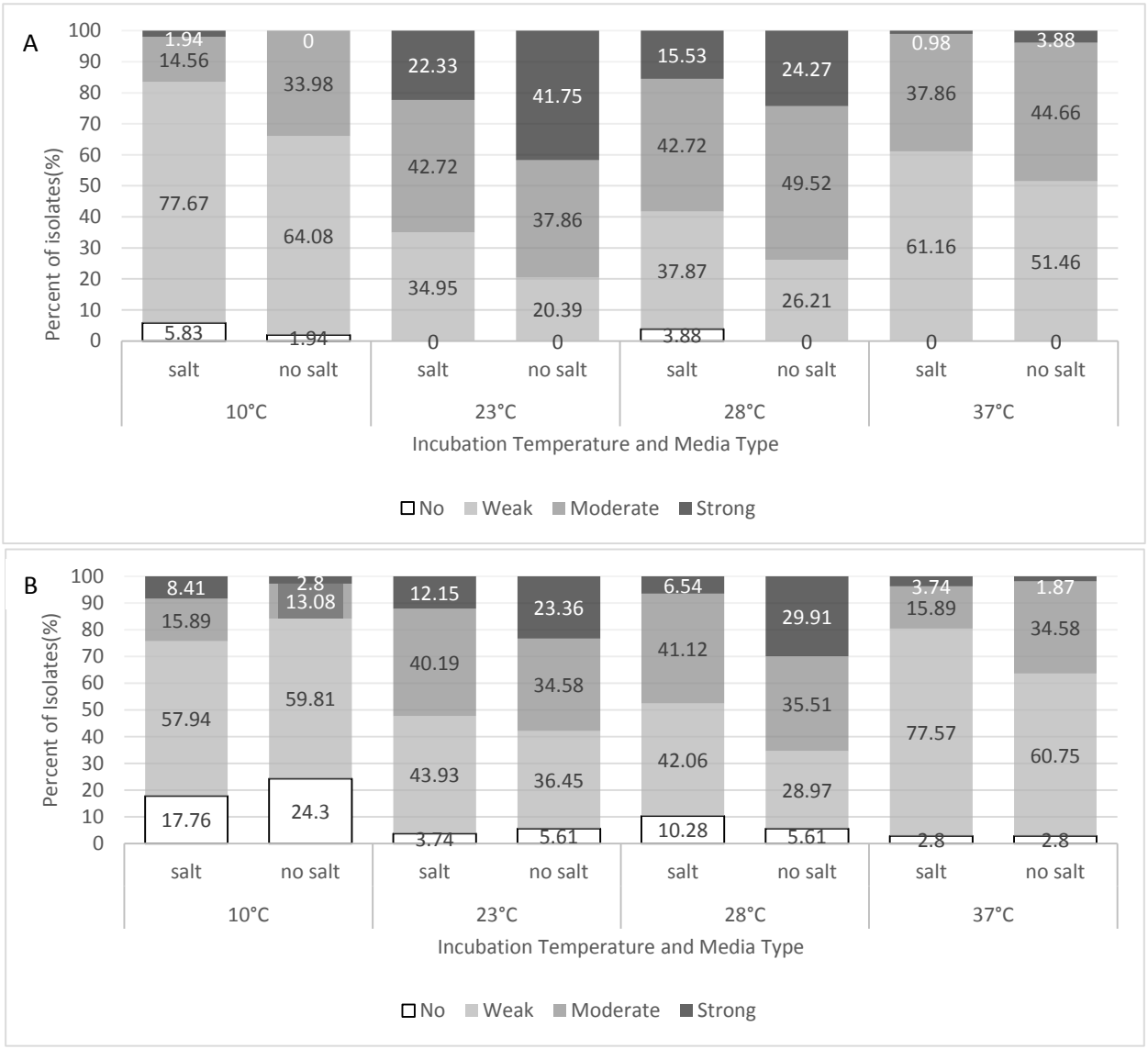


Figure 4.1. Percentages of overall biofilm formation of *Salmonella* spp. and *E. coli* isolates. Panel A depicts all *Salmonella* spp. isolates (N=103) and Panel B depicts all *E. coli* isolates (N=100) at various temperatures in media with salt and no salt. The legend denotes the shading corresponding to the biofilm strength categories. This data represents numbers from at least four replicates. The standard error across all replicates was equal to or less than 0.2 for absorbance values ranging from 0 to 1.8.

Observations were made of biofilm formation by *E. coli* and *Salmonella* spp. isolates based on sample location (Figures 4.2- 4.5). *E. coli* isolates from Grand Bend (GBE) and the Gull River (GE) locations were able to form biofilms at all the temperatures tested. Isolates from the Gull River produced a higher proportion (27%) of strong biofilms at 10°C than any other temperature. All isolates from Nunavut (predominantly host location, NE*) were able to form biofilms at 37°C, with the highest proportion (34.6% in media with no salt) of strong biofilms at 28°C (Figure 4.2). The isolates from the other predominantly host location, Coboconk Lagoon (CLE*), had a small proportion (5.6% in media with no salt, 11.1% in salt) of non-biofilm formers at 37°C and similar to Nunavut isolates, had most strong biofilms (38.9% in media with no salt) at 28°C. Sample locations generally exhibited a preference for stronger biofilm formation in media with no salt, with the exception of Gull River isolates at 28°C, 23°C and 10°C.

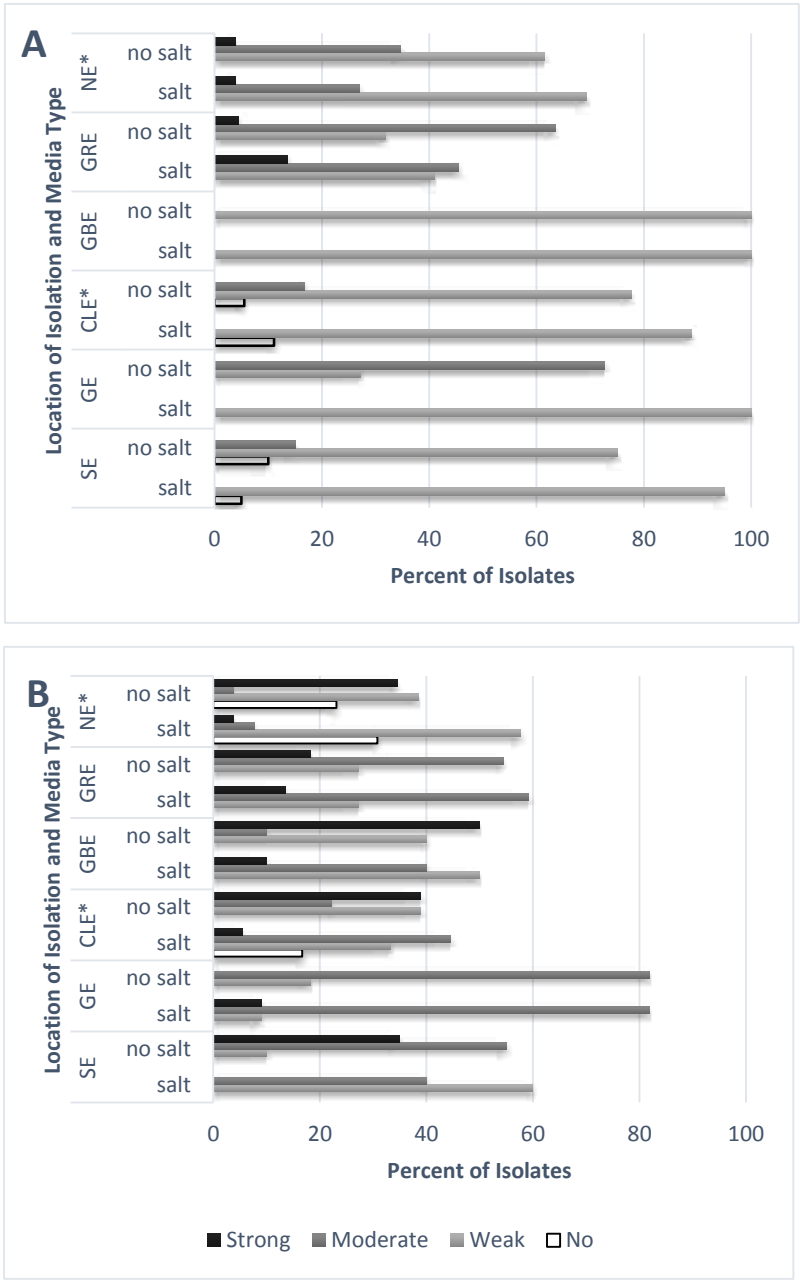


Figure 4.2. Summary of biofilm formation of *E. coli* isolates based on sampling location. Panel A depicts the accumulation of results at 37°C and panel B depicts results at 28°C. The legend denotes shading corresponding with strong, moderate, weak and no biofilm formation. This data represents numbers from at least four replicates. The standard error across all replicates was equal to or less than 0.2 for absorbance values ranging from 0 to 1.8. *Indicates predominantly host locations.

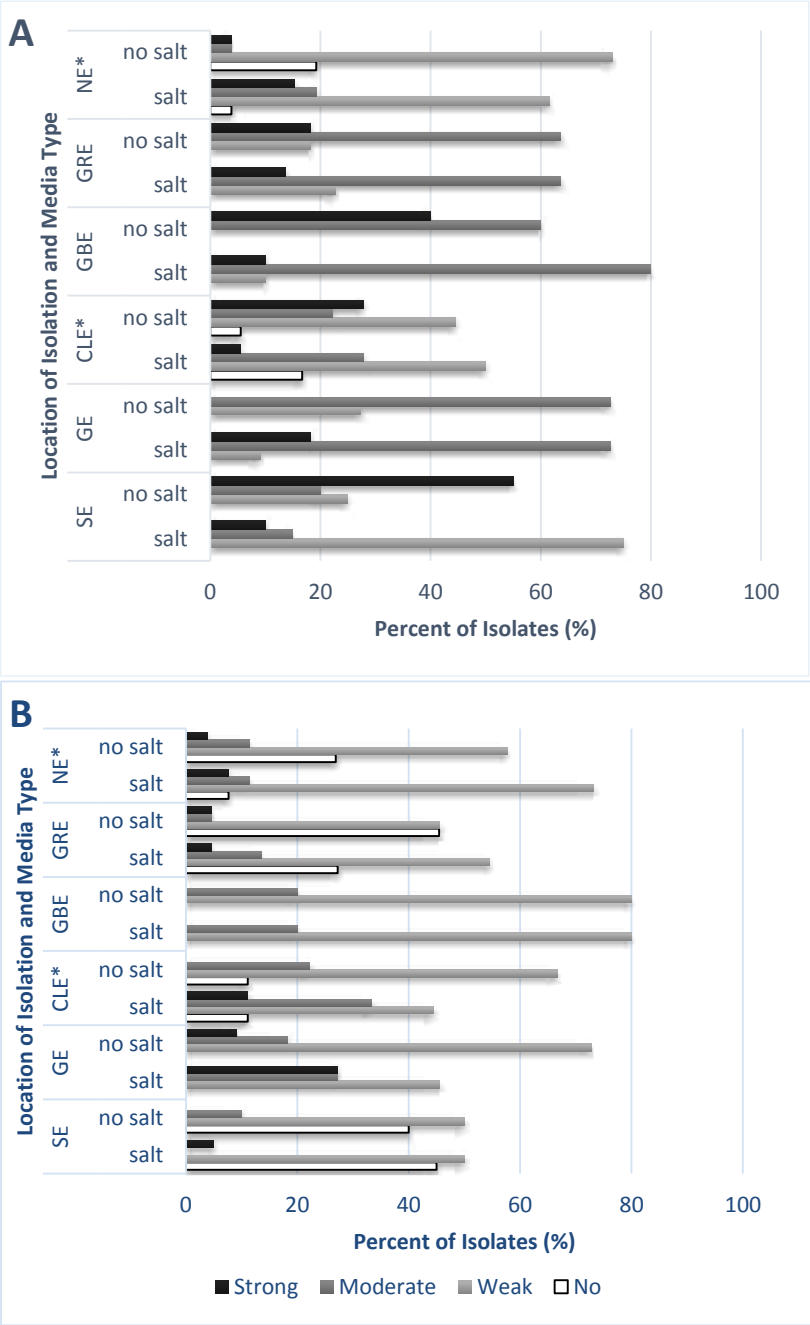


Figure 4.3. Summary of biofilm formation of *E. coli* isolates based on sampling location. Panel A depicts biofilm formation at 23°C and panel B depicts biofilm formation at 10°C. The legend denotes shading corresponding with strong, moderate, weak and no biofilm formation. This data represents numbers from at least four replicates. The standard error across all replicates was equal to or less than 0.2 for absorbances ranging from 0 to 1.8. *Indicates predominantly host locations.

Observations of biofilm formation of *Salmonella* spp. isolates based on sample locations were also made (Figure 4.4. and Figure 4.5). All *Salmonella* spp. isolates were able to produce biofilms at 37°C. The highest proportion of strong biofilms was observed at 23°C (Figure 4.5 (A)). Isolates from the Fleming location (FS*) were able to form some strong biofilms at all the temperatures tested (Figure 4.4, Figure 4.5). At 10°C, some isolates from the water treatment center (WTS*), Fleming (FS*), and Clair Lake (CLS) locations were unable to form biofilms (significant growth was noted in the culture tubes of non-biofilm formers). Some Clair Lake isolates were unable to form biofilms at 28°C. At 37°C and 10°C, most *Salmonella* spp. isolates formed weak biofilms (Figure 4.4 (A), Figure 4.5(B)). A higher prevalence of strong and moderate biofilms was observed at 23°C and 28°C.

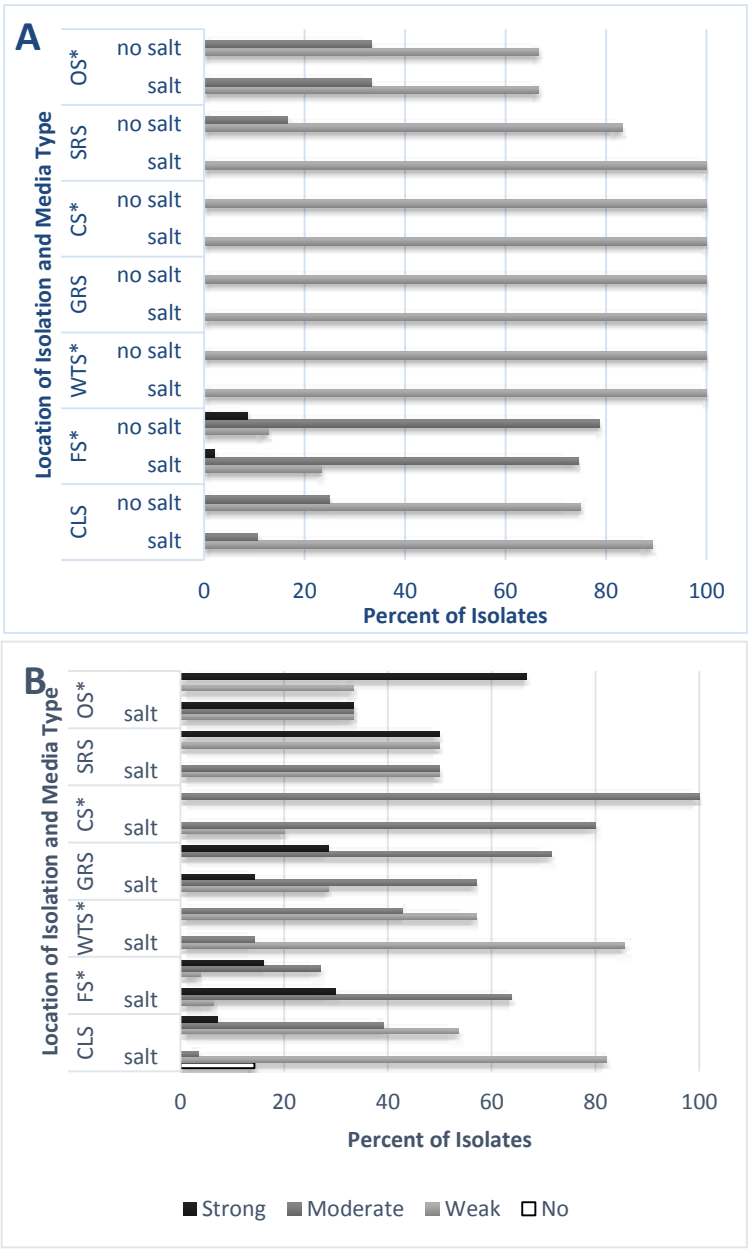


Figure 4.4. Summary of biofilm formation of *Salmonella* spp. isolates based on sampling location. Panel A represents biofilm formation at 37°C and panel B represents biofilm formation at 28°C. The figure legend shows shading corresponding with strong, moderate, weak and no biofilm formation. This data represents numbers from at least four replicates. The standard error across all replicates was equal to or less than 0.2 for absorbances ranging from 0 to 1.8. * Indicates predominantly host locations.

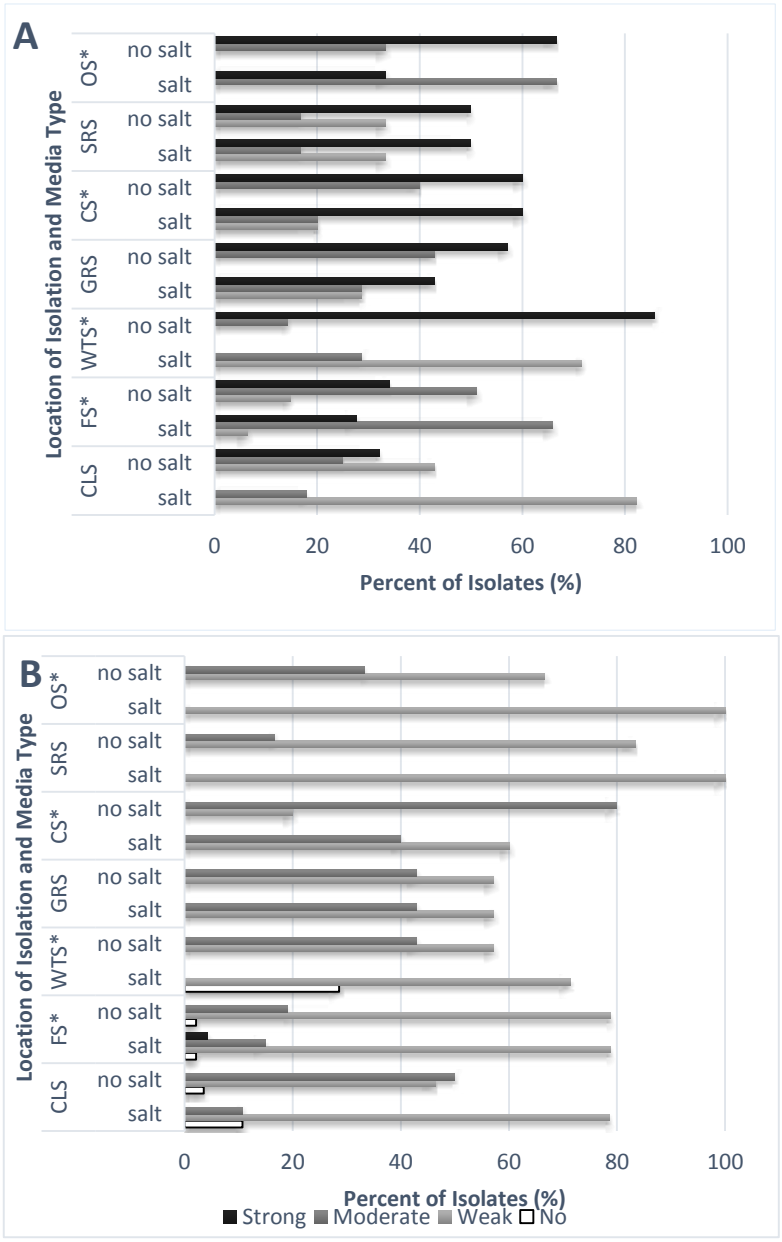


Figure 4.5. Summary of biofilm formation of *Salmonella* spp. isolates based on sampling location. Panel A represents biofilm formation at 23°C and panel B represents biofilm formation at 10°C. The legend denotes shading corresponding to strong, moderate, weak and no biofilm formation. This data represents numbers from at least four replicates. The standard error across all replicates was equal to or less than 0.2 for absorbances ranging from 0 to 1.8. * Indicates predominantly host locations.

To visualize the above data in an alternate way biofilm formation was summarized based on predominantly host and predominantly non-host isolates (depicted in Figures 4.6. and 4.7). This figure does not show the percentage of no biofilm formation, only weak, moderate and strong biofilms. The following paragraphs explain the predominant trends in results according to data observed at each temperature.

At 37°C predominantly host and predominantly non-host *Salmonella* spp. produced the highest percentage (77.4% and 78.5%, respectively) of weak biofilms in media with salt. Alternatively, in media with no salt, these isolates produced the most moderate biofilms, with 43.9% of the non-host *Salmonella* spp. and 27.4% of the total host *Salmonella* spp.. Overall, at 37°C, biofilm formation patterns for all predominantly non-host and host *E. coli* and *Salmonella* spp. isolates were similar, with the majority of the isolates being weak biofilm formers. Moderate biofilm formation for total host and total non-host isolates was observed more in media with no salt with 22.6% of predominantly host isolates and 24% of non-host isolates. When strong biofilms were observed at 37°C, they were found in higher proportions in media with salt. Of the total isolates, 5.6% of host isolates and 4.8% of non-host isolates produced strong biofilms in media with salt as opposed to 0.94% and 1.92% in no salt, respectively.

At 28°C isolates formed more moderate and strong biofilms, when compared to 37°C (Figure 4.6). Over 43% of predominantly host isolates formed moderate biofilms in media with salt, and 31% in media with no salt. These isolates formed more strong biofilms in

media with no salt (31%) compared to in the presence of salt (20.8%). Predominantly non-host isolates had a similar pattern of biofilm formation; around 40% of isolates formed moderate biofilms in both types of growth media, while 13.5% and 33.6% formed strong biofilms in media with salt and no salt, respectively. Predominantly host *Salmonella* spp. produced more moderate and strong biofilms compared to predominantly host *E. coli*, which was reversed in the case of predominantly non-host *E. coli* and *Salmonella* spp..

Biofilm formation patterns at 23°C were similar to those at 28°C, with a slight variation in percentages. Most predominantly host, and non-host isolates were moderate biofilm formers. Predominantly host *E. coli* had the highest proportion of strong biofilms, with 36% in media with no salt, while predominantly host *Salmonella* spp. produced the most moderate biofilms with 58% in media with salt. Weak biofilm formation was observed in predominantly non-host *Salmonella* spp. in media with salt.

At 10°C, most isolates were weak biofilm formers, with the exception of predominantly host *Salmonella* spp. which formed ~60% moderate biofilms. Predominantly host isolates had a higher proportion of moderate and strong biofilms than the predominantly non-host isolates. All groups were able to form some strong biofilms with the exception of predominantly non-host *Salmonella* spp., which in turn formed the most biofilms classified as weak.

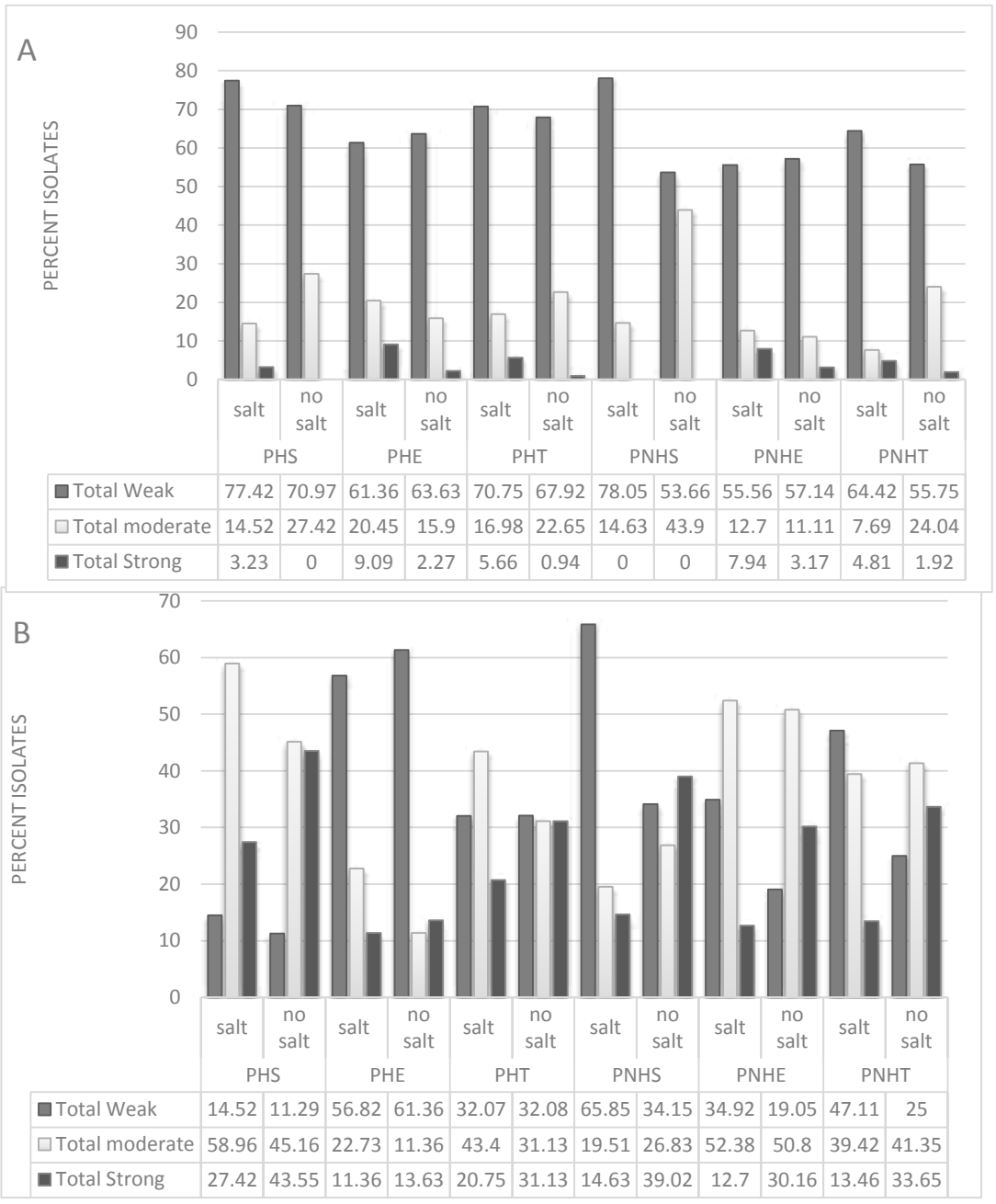
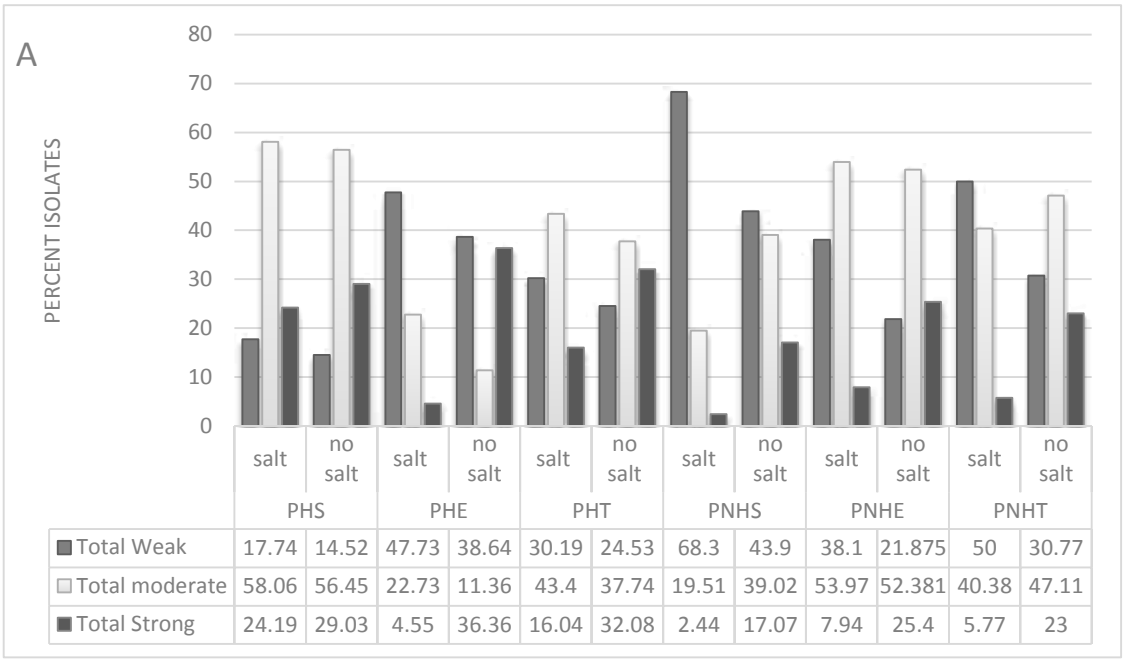
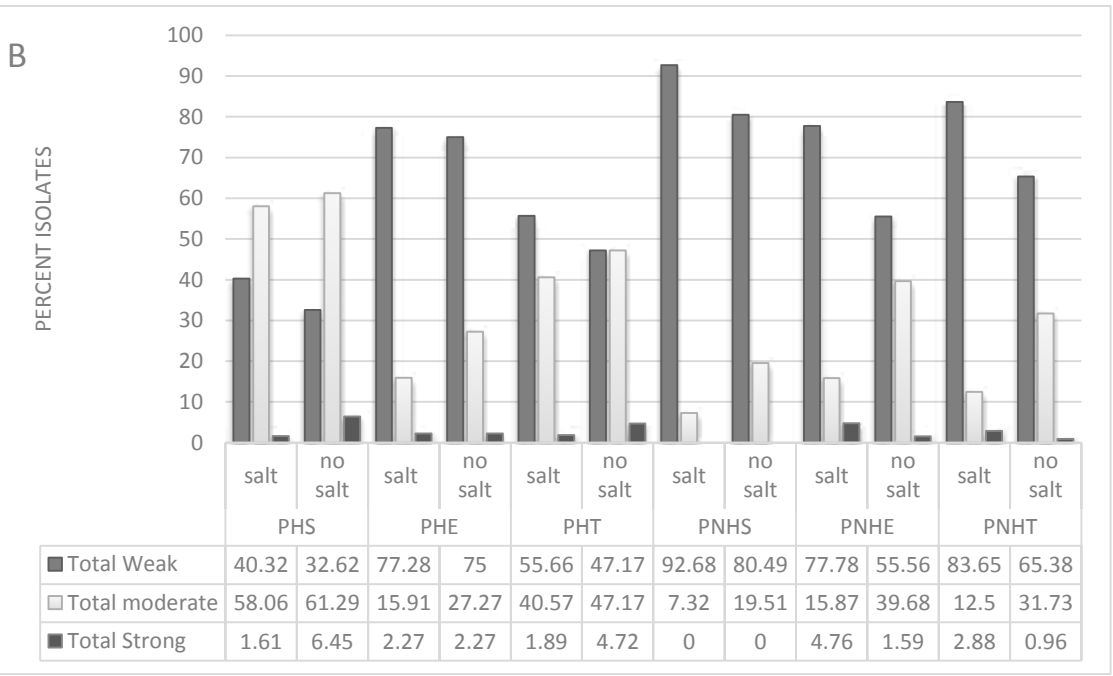


Figure 4.6. Biofilm formation based on predominantly host and non-host locations .Panel A represents biofilm formation at 37°C and Panel B at 28°C. Isolates were subdivided as follows, PHS= Predominantly Host *Salmonella* spp., PHE= Predominantly Host *E.coli*, PHT= Predominantly Host Total, PNHS= Predominantly Non-Host *Salmonella* spp. PNHE= Predominantly Non- Host *E. coli* and PNHT= Predominantly Non-Host Total. This data represents numbers from at least four replicates. The standard error across allreplicates was equal to or less than 0.2 for absorbances ranging from 0 to 1.8.



	PHS		PHE		PHT		PNHS		PNHE		PNHT	
	salt	no salt	salt	no salt	salt	no salt	salt	no salt	salt	no salt	salt	no salt
Total Weak	17.74	14.52	47.73	38.64	30.19	24.53	68.3	43.9	38.1	21.875	50	30.77
Total moderate	58.06	56.45	22.73	11.36	43.4	37.74	19.51	39.02	53.97	52.381	40.38	47.11
Total Strong	24.19	29.03	4.55	36.36	16.04	32.08	2.44	17.07	7.94	25.4	5.77	23



	PHS		PHE		PHT		PNHS		PNHE		PNHT	
	salt	no salt	salt	no salt	salt	no salt	salt	no salt	salt	no salt	salt	no salt
Total Weak	40.32	32.62	77.28	75	55.66	47.17	92.68	80.49	77.78	55.56	83.65	65.38
Total moderate	58.06	61.29	15.91	27.27	40.57	47.17	7.32	19.51	15.87	39.68	12.5	31.73
Total Strong	1.61	6.45	2.27	2.27	1.89	4.72	0	0	4.76	1.59	2.88	0.96

Figure 4.7. Biofilm formation based on predominantly host and non-host locations. Panel A represents biofilm formation at 23°C and Panel B at 10°C. Isolates were subdivided as follows, PHS= Predominantly Host *Salmonella* spp., PHE= Predominantly Host *E.coli*, PHT= Predominantly Host Total, PNHS= Predominantly Non-Host *Salmonella* spp. PNHE= Predominantly Non- Host *E. coli* and PNHT= Predominantly Non-Host Total. This data represents numbers from at least four replicates. The standard error across all replicates was equal to or less than 0.2 for absorbances ranging from 0 to 1.8.

While it was observed that media components (*i.e.*, salt or no salt) had an effect on biofilm formation, the data was also plotted against only temperature to gather a better understanding as to what effect this variable had on biofilm formation. Table 4.2 summarizes overall biofilm formation at the four different temperatures tested. The temperature that led to the highest proportion isolates that did not form biofilms (10%) was 10°C. Weak biofilm formation was most prevalent at 37°C, with 76% of the isolates, and lowest at 23°C, with 28% of the total isolates. Isolates produced the highest proportion of moderate biofilms at 23°C and 28°C with 38% and 42% of total isolates, respectively. The greatest occurrence of strong biofilms was observed at 23°C (31% of isolates).

Table 4.2. Summary of Biofilm Formation of all Isolates at the Four Temperatures Tested.

	Percent of Total Isolates (%)			
	10°C	23°C	28°C	37°C
No Biofilm Formation	10.06	1.74	3.33	1.22
Weak Biofilm Formation	64.44	28.82	35.9	76.33
Moderate Biofilm Formation	22.52	38.01	42.53	21.05
Strong Biofilm Formation	2.98	31.43	18.24	1.41
Total Biofilm Formation	89.94	98.26	96.67	98.78

4.2. Antibiotic Resistance

Antibiotic resistance patterns were determined using the disk diffusion assay against a panel of eight antibiotics (an example of the raw data can be found in Table A2 of Appendix B). A MBEC assay was also performed in order to test the resistance of biofilm forming isolates using four antibiotics (an example of the raw data can be found in Table A3 of Appendix B). Antibiotics were chosen based on CLSI- suggested groupings of antibiotics for *Salmonella* spp. and *E. coli* and encompass commonly administered antibiotics to treat infections caused by this bacteria. Both assays were conducted at 37°C, in triplicate.

4.2.1 Disk-Diffusion

Percent resistance to antibiotics was determined based on the location of isolation of *E. coli* and *Salmonella* spp.. As portrayed in Tables 4.3 and 4.4 the greatest level of resistance was exhibited towards Ampicillin (as noted with the majority of the bolded numbers in each of the tables). With the exception of Clair Lake (CS) *Salmonella* spp. and Fleming (FS*) *Salmonella* spp., all other locations had isolates that were resistant to Ampicillin. *E. coli* isolates from Coboconk Lagoon (CLE*) and Grand River (GRE) had some level of

resistance to each of the antibiotics tested. Overall, *E. coli* isolates had a wider range of resistance to the variety of antibiotics tested. *Salmonella* spp. from at least three and up to six locations were fully susceptible to Cefotaxime, Ciprofloxacin, Streptomycin, Trimethoprim-Sulfamethoxazole and Oxytetracycline (Table 4.4.). *Salmonella* spp. from Clair Lake (CS) were the most sensitive to all of the antibiotics assayed, with the least resistance among these (only 3.6% of isolates) to Aztreonam and Amoxicillin /Clavulanic acid. The widest range of resistance (*i.e.* resistance to six out of the eight antibiotics tested) was observed in *Salmonella* spp. from the Water Treatment Plant.

Table 4.3. Percentage of Resistance to Antibiotics in *E. coli* Isolates Based on Location of Isolation

E. coli	**Location of Isolation[a]**					
	SGE N[b]=17	GE N=11	CLE* N=18	GRE N=21	NE* N=26	GBE N=10
Antibiotic	%[c]	%	%	%	%	%
Aztreonam 30µg	35.3	16.7	38.8	14	38.3	40
Amoxicillin/ Clavulanic acid 20/10µg	41.2	16.7	38.9	40.9	15.3	70
Ampicillin 10µg	**82.3**	**100**	**83.3**	33.3	**42.2**	**90**
Cefotaxime 30µg	11.7	16.7	27.7	**57.1**	19.2	0
Ciprofloxacin 5µg	17.7	8.35	16.7	4.7	7.6	0
Streptomycin 10µg	47.1	41.7	**83.3**	19.1	38.5	20
Sulfamethoxazole/Trimethoprim 23.75/1.25µg	0	0	16.6	14.2	11.5	10
Oxytetracycline 30µg	0	0	5.5	9.5	0	10

[a]SGE= Scugog River, GE=Gull River N, CLE= Coboconk Lagoon, GBE= Grand Bend, GRE= Grand River, NE= Nunavut

[b]N= number of isolates from a particular location

[c]%= the percentage of isolates from that location resistant to the level of antibiotic tested

*indicates predominantly host in origin

Bolded numbers represent the antibiotic with the greatest level of resistance from a particular location

Table 4.4. Percentage of Resistance to Antibiotics in *Salmonella* spp. Isolates Based on Location of Isolation

***Salmonella* spp.**	**Location of Isolation[a]**						
	CLS N[b]=28	WTS* N=7	FS* N=46	GRS N=7	CS* N=5	SRS N=7	OS* N=3
Antibiotic	%	%	%	%	%	%	%
Aztreonam 30µg	**3.6**	**57.1**	8.7	28.6	40	28.6	33.3
Amoxicillin/ Clavulanic acid 20/10µg	**3.6**	0	8.7	**100**	80	**85.7**	**100**
Ampicillin 10µg	0	0	**91.3**	**100**	**100**	**85.7**	**100**
Cefotaxime 30µg	0	**57.1**	0	0	0	0	66.67
Ciprofloxacin 5µg	0	14.3	0	0	0	0	0
Streptomycin 10µg	0	71.4	0	71.4	80	42.9	0
Sulfamethoxazole/ Trimethoprim 23.75/1.25µg	0	28.6	2.2	0	0	0	0
Oxytetracycline 30µg	0	28.6	0	0	0	0	0

[a] CLS= Clair Lake, FS= Fleming, WTS= Wastewater Treatment Plant, GRS= Gull, CS= Coboconk Lagoon, SRS= Scugog River, OS= Omeemee Water Pollution Treatment Pond

[b]N= number of isolates from a particular location

[c]%= the percentage of isolates from that location resistant to the level of antibiotic tested

*indicates predominantly host in origin

Bolded numbers represent the antibiotic with the greatest level of resistance from a particular location.

Resistance patterns were alternatively summarized in Table 4.5., in order to determine whether the host/non-host locations had an effect on the antimicrobial resistance of isolates. Overall, total predominantly host isolates exhibited more resistance to antibiotics compared to total predominantly non-host isolates. Predominantly host *Salmonella* spp. showed some resistance to all antibiotics, while non-host *Salmonella* spp. were completely susceptible to half of the antibiotics tested (Cefotaxime, Ciprofloxacin, Trimethoprim-Sulfamethoxazole and Oxytetracycline). Predominantly host *Salmonella* spp. were most resistant to Ampicillin (82%), while predominantly non-host *Salmonella* spp. showed most resistance towards Amoxicillin/Clavulanic acid (33.3%). Least resistance for predominantly host *Salmonella* spp. was observed towards Ciprofloxacin (1.6%). Resistance patterns for predominantly host and non-host *E. coli* were similar, which were both most resistant to Ampicillin (59.1% and 71.2%, respectively) and least resistant to Oxytetracycline (2.3% and 5.1%, respectively).

Table 4.5. Percentage of Resistance to Antibiotics Based on Total Predominantly Host and Non-Host Isolates.

	Percent Resistance %						
Antibiotic	Predominantly Host			Predominantly Non-Host			
	E.coli	*Salmonella* spp.	Total	*E.coli*	*Salmonella* spp.	Total	**Total[a]**
Aztreonam 30µg	38.6	18	26.7	25.4	11.9	19.8	23.3
Amoxicillin/ Clavulanic acid 20/10µg	25	18	21	40.7	**33.3**	37.6	29.1
Ampicillin 10µg	**59.1**	**82**	**72.3**	**71.2**	31	**54.4**	**63.6**
Cefotaxime 30µg	22.7	9.8	15.2	27.1	0	15.8	15.5
Ciprofloxacin 5µg	11.4	1.6	5.7	8.5	0	5	5.3
Streptomycin 10µg	56.8	14.8	32.4	32.2	19.04	26.7	29.6
Sulfamethoxazole/ Trimethoprim 23.75/1.25µg	13.6	4.9	8.6	6.8	0	4	6.3
Oxytetracycline 30µg	2.3	3.3	2.9	5.1	0	3	2.9

[a]Total represents total *E. coli* and *Salmonella* spp. resistance

Bolded numbers represent the antibiotic with the greatest level of resistance from a particular location.

4.2.2 Minimum Biofilm Eradication Concentration

The minimum biofilm eradication concentration assay was conducted in order to test the effects of biofilm formation on bacterial survival in the presence of antimicrobials. Figure 4.8. Shows the MBEC of *E. coli* isolates to the four antibiotics and the variety of concentrations of these antibiotics that were tested. Overall, isolates were most sensitive to Cefotaxime and had the greatest level of resistance to Ampicillin. Most isolates had a MBEC of >1024 µg/mL towards all of the antibiotics. The notable exceptions to this trend were the isolates from Gull River (GE) and Scugog River (SE), which had some isolates with a MBEC of 512 µg/mL towards all antibiotics. Four percent of isolates from Grand River had a MBEC of 128 µg/mL to Oxytetracycline.

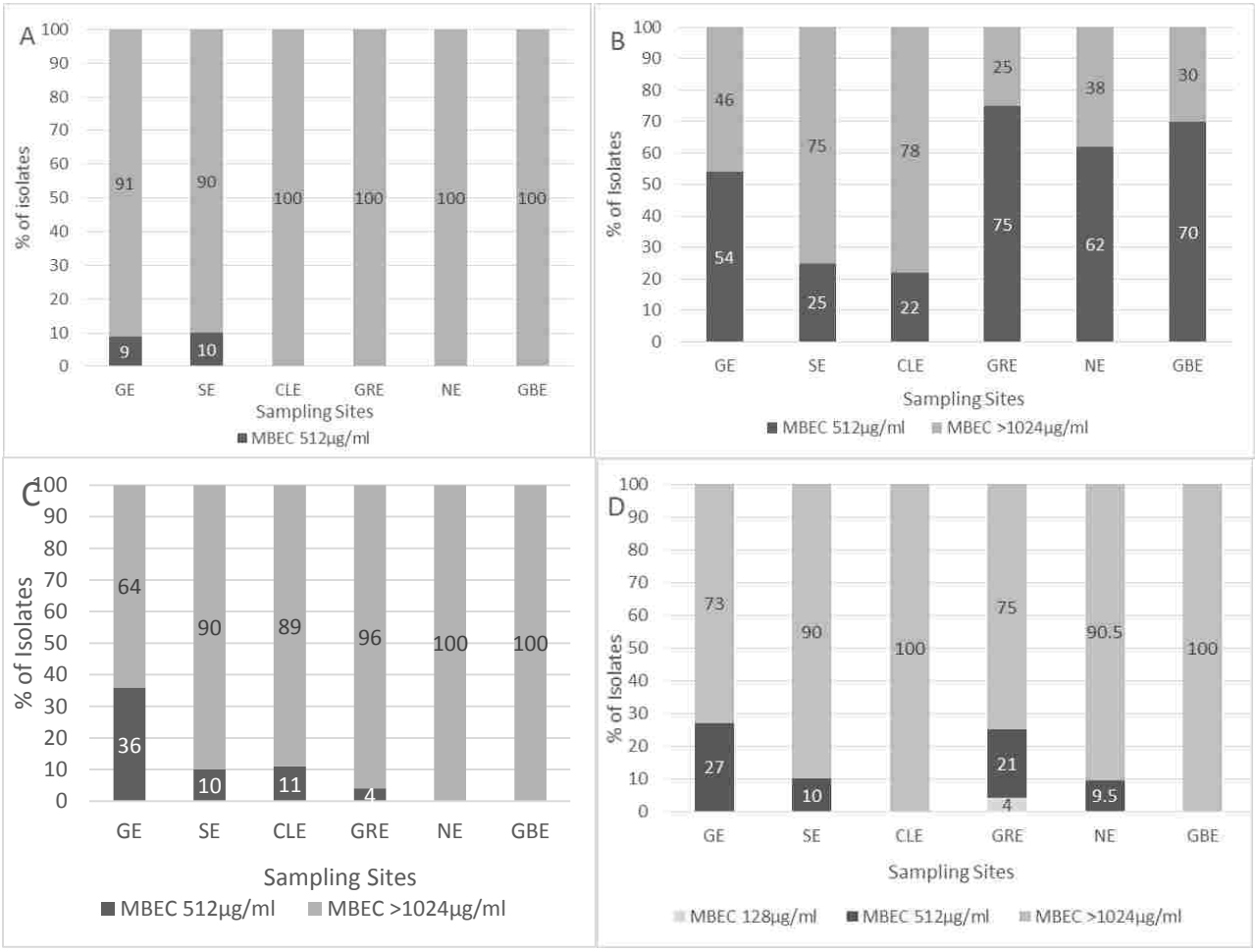


Figure 4.8. Minimum biofilm eradication concentrations of *E. coli* isolates based on location of isolation. Isolates were tested for resistance to (A) Ampicillin, (B) Cefotaxime, (C) Streptomycin and (D) Oxytetracycline. The experiment was performed in triplicate. Locations of isolation are as follows: GE= Scugog River, GE=Gull River N, CLE*= Coboconk Lagoon, GBE= Grand Bend, GRE= Grand River, NE*= Nunavut.

Similar to *E. coli* isolates (Figure 4.8), a large proportion of *Salmonella* spp. isolates exhibited MBECs >1024 µg/mL (Figure 4.9.) *Salmonella* spp. isolates were most sensitive to Cefotaxime, and had relatively equal levels of resistance to Ampicillin, Streptomycin and Oxytetracycline (Figure 4.6). Isolates from Gull River (GRS) were most sensitive to Cefotaxime with 75% of the isolates having a MBEC of 512 µg/mL and 12.5% with a MBEC of 256 µg/mL. The Clair Lake (CLS) location had between 36% and 46% of isolates with a MBEC of 512 µg/mL towards all antibiotics.

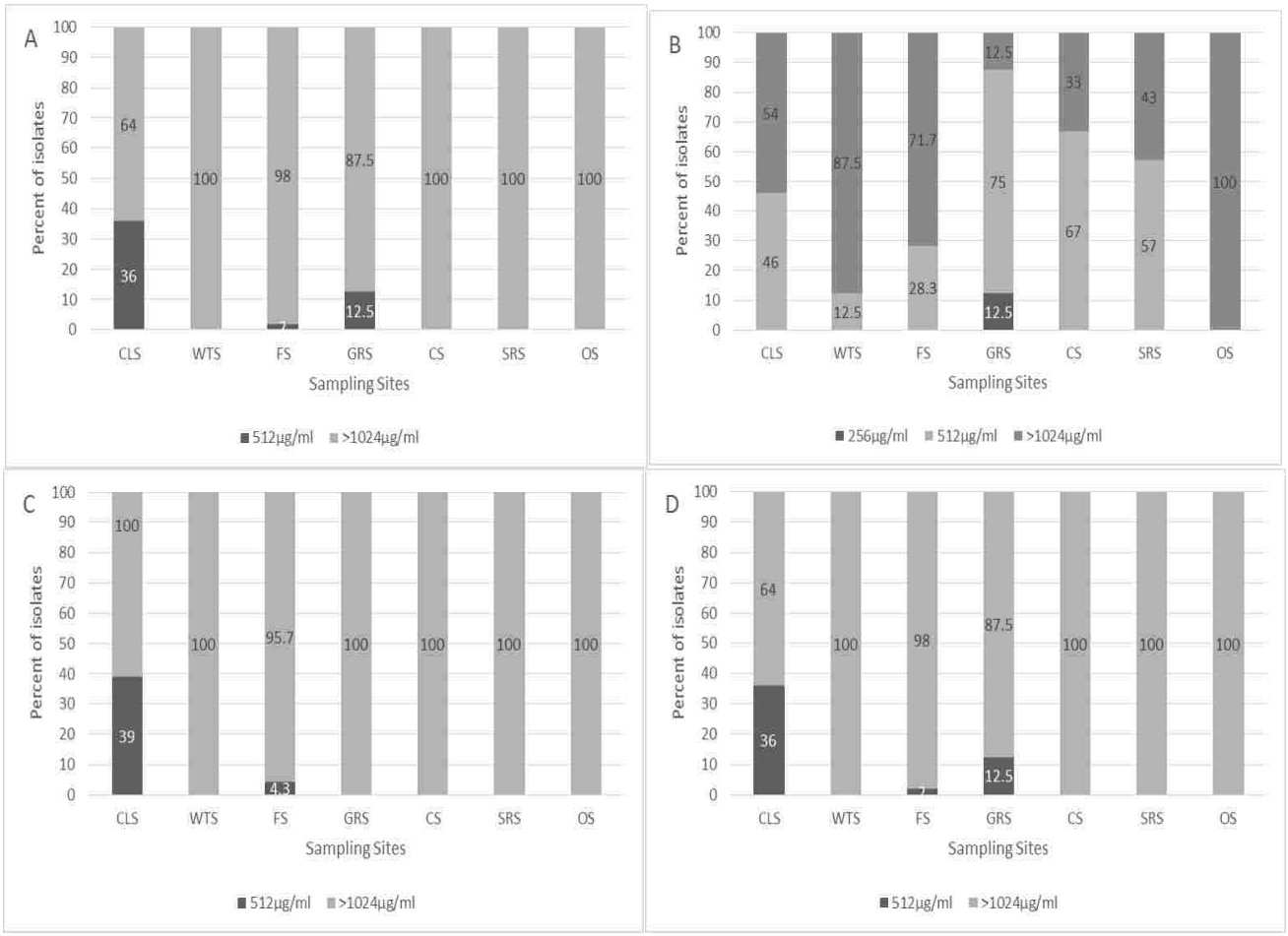


Figure 4.9. Minimum biofilm eradication concentrations of *Salmonella* spp. isolates based on location of isolation. Isolates were tested for resistance to (A) Ampicillin, (B) Cefotaxime, (C) Streptomycin and (D) Oxytetracycline. Each isolate had three replicates. Locations of isolation were as follows: CLS= Clair Lake, FS*= Fleming, WTS*= Wastewater Treatment Plant, GRS= Gull River, CS*= Coboconk Lagoon, SRS= Scugog River, OS*= Omeemee Water Pollution Treatment Pond.

The effects of biofilm formation on antibiotic resistance were also summarized based on predominantly host and predominantly non-host locations (Figure 4.10). Isolates from predominantly non-host locations were more sensitive to the tested antibiotics than isolates from the predominantly host locations (Figure 4.10). There was a larger prevalence of MBECs of >1024 µg/mL in the predominantly host groups compared to the predominantly non-host groups. The MBECs of all *E. coli* and *Salmonella* spp. isolates are summarized in Table 4.6. Overall, a higher percentage of *E. coli* isolates had MBECs >1024 µg/mL when tested against Ampicillin and Streptomycin compared to *Salmonella* spp.. In turn, a higher proportion of *Salmonella* spp. isolates had a MBEC >1024 µg/mL when tested against Cefotaxime. Resistance patterns to Oxytetracycline were similar for *E. coli* and *Salmonella* spp..

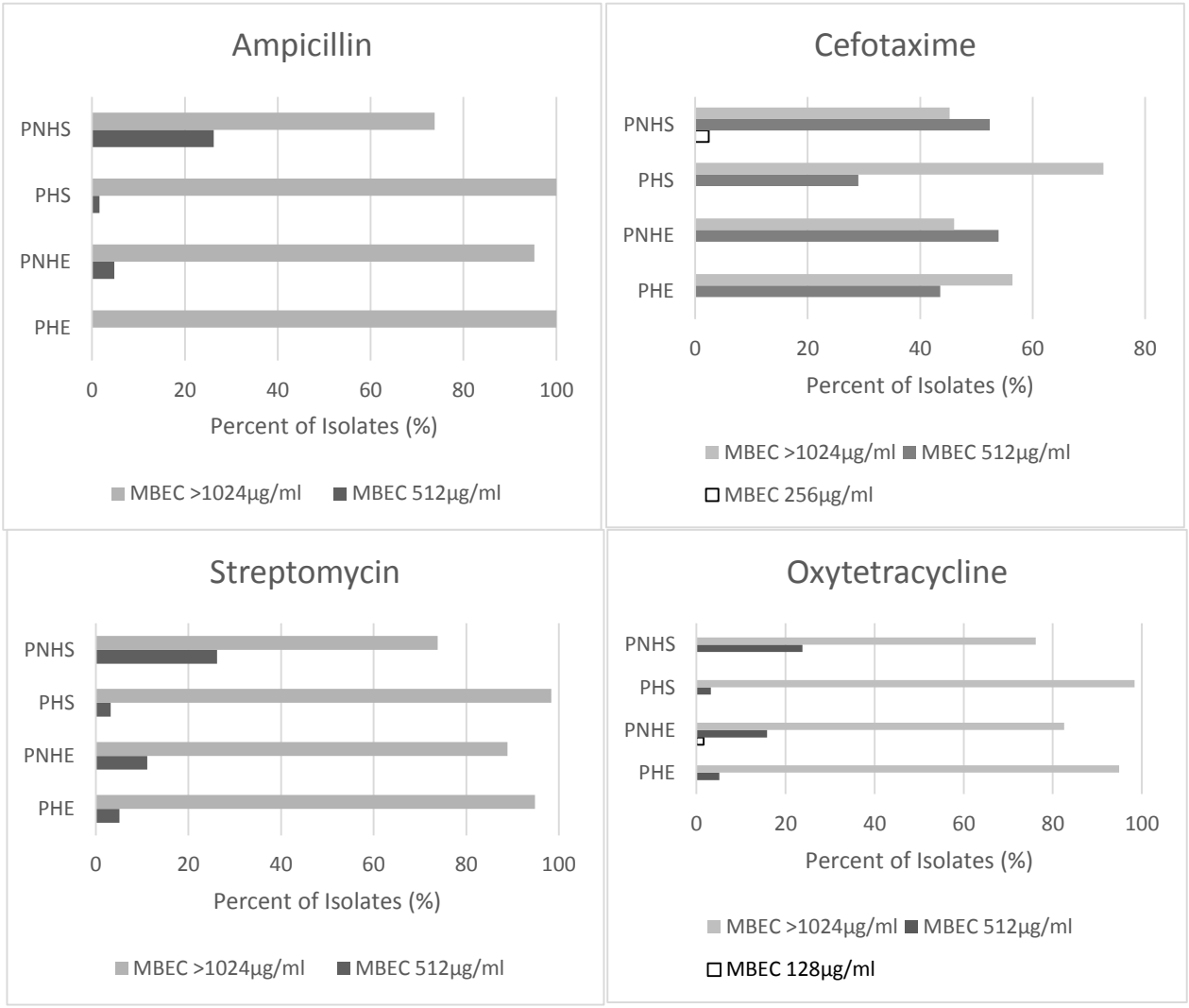


Figure 4.10. MBEC of isolates subdivided based on host and non-host locations. Subdivisions were PNHS= Predominantly Non-Host *Salmonella* spp., PHS= Predominantly Host *Salmonella* spp., PNHE= Predominantly Non-Host *E. coli*, PHE= Predominantly Host *E. coli.* The legends depict the shading representative of the MBEC values. Each isolate had three replicates.

Table 4.6. Summary of Minimum Biofilm Eradication Concentration for all *E. coli* and *Salmonella* spp. Depicted in Percentages of the Isolates

	Ampicillin (µg/mL)		**Cefotaxime (µg/mL)**			**Streptomycin (µg/mL)**		**Oxytetracycline (µg/mL)**		
	512[a]	>1024	256	512	>1024	512	>1024	128	512	>1024
E. coli	2.88	97.12		50.96	49.04	8.65	91.35	0.96	11.54	87.5
***Salmonella* spp.**	11.43	88.57	0.95	38.1	60.95	12.38	87.62		11.43	88.57

[a]represents the highest concentration tested for that particular trial in which there was growth

4.3. Acid Tolerance

Acid tolerance was tested using synthetic gastric juice as a means to determine whether bacteria would be able to survive in the selective pressures of a host (*i.e.* stomach acid). All isolates were grown overnight (~ 16h) either in biofilms, or in planktonic cultures and then diluted and incubated in synthetic gastric juice after which they were plated and checked for survival. Each isolate was tested in triplicate (an example of the raw data can be found in Table A4 of Appendix B). Figure 4.8 summarizes acid tolerance data for *E. coli* (panel A) and *Salmonella* spp. (panel B) based on location with and without the formation of biofilms. *E. coli* isolates from Scugog River, Gull River and Nunavut were more acid tolerant when they formed biofilms. Isolates from Grand River and Grand Bend were equally tolerant as planktonic and sessile cultures. *E. coli* isolates from the Coboconk

Lagoon were more tolerant without the formation of biofilms (100% compared to 8.3%). Most *Salmonella* spp. isolates were equally acid tolerant with and without the formation of biofilms. An exception to this trend was noted with isolates from Gull River and Omeemee where they were more tolerant as biofilms (100% compared to 25% and 0% without biofilm formation, respectively), while Fleming isolates were more tolerant as planktonic cultures (100% as opposed to 78% with biofilm formation). Acid tolerance for the total amount of *E. coli* and *Salmonella* spp. was similar for biofilm forming isolates, but *Salmonella* spp. isolates were more tolerant as planktonic cultures compared to planktonic *E. coli* (see Table 4.7.).

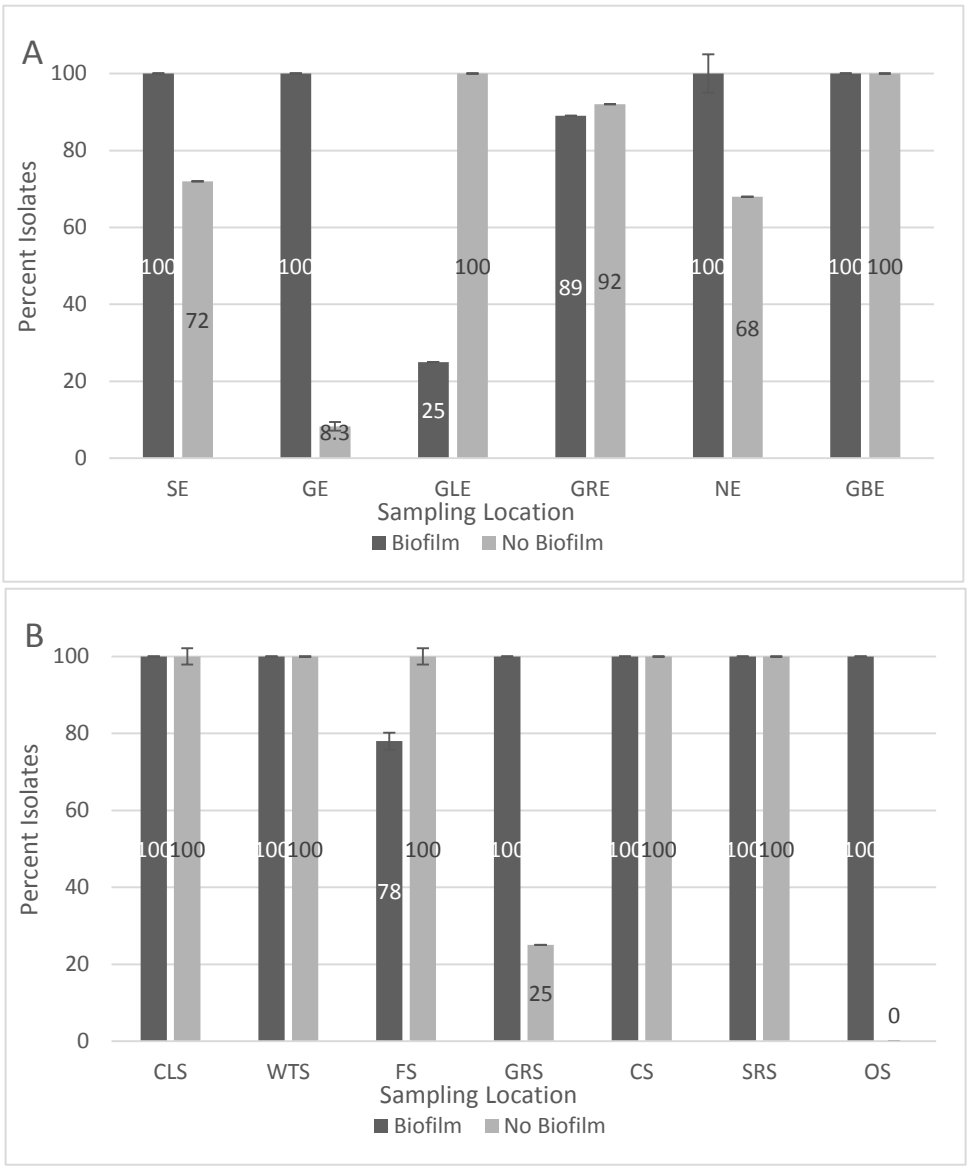


Figure 4.11. Acid tolerance of *E. coli* isolates *Salmonella* spp. isolates based on sampling locations. Panel A represents *E. coli* and panel B represents *Salmonella* spp. The specific locations were SE= Scugog River *E. coli*, GE= Gull River *E. coli*, GLE*= Coboconk Lagoon *E. coli*, GRE= Grand River *E. coli*, NE*= Nunavut *E. coli*, GBE= Grand Bend *E. coli*, CLS= Clair Lake *Salmonella* spp., WTS*= Water Treatment Center *Salmonella* spp., FS*= Fleming *Salmonella* spp., GRS= Gull River *Salmonella* spp., CS*= Coboconk Lagoon *Salmonella* spp., SRS= Scugog River *Salmonella* spp., OS*= Omeemee *Salmonella* spp.. Each isolate had three replicates.

Table 4.7. Summary Table of *E. coli* and *Salmonella* spp. Acid Tolerance.

	Acid Tolerance (%)	
	E. coli	*Salmonella* spp.
Biofilm	88.9	86.8
No Biofilm	75.9	90

Acid tolerance data was further summarized in Figure 4.12 in order to analyze the effects of predominantly host and predominantly non-host locations of origin on the ability of isolates to survive in acidic environments. Predominantly host *E. coli* and *Salmonella* spp. were more acid-tolerant as planktonic cultures (83.9% compared to 70% with biofilm formation for *E. coli* and 88.7% as opposed to 82.5% with biofilm formation for *Salmonella* spp.), while predominantly non-host isolates were more tolerant with the formation of biofilms (97.6% as opposed to 71.8% without biofilm formation for *E. coli* and 100% compared to 93.6% for *Salmonella* spp. isolates). This was also evident when looking at the total host and total non-host percentage of tolerance.

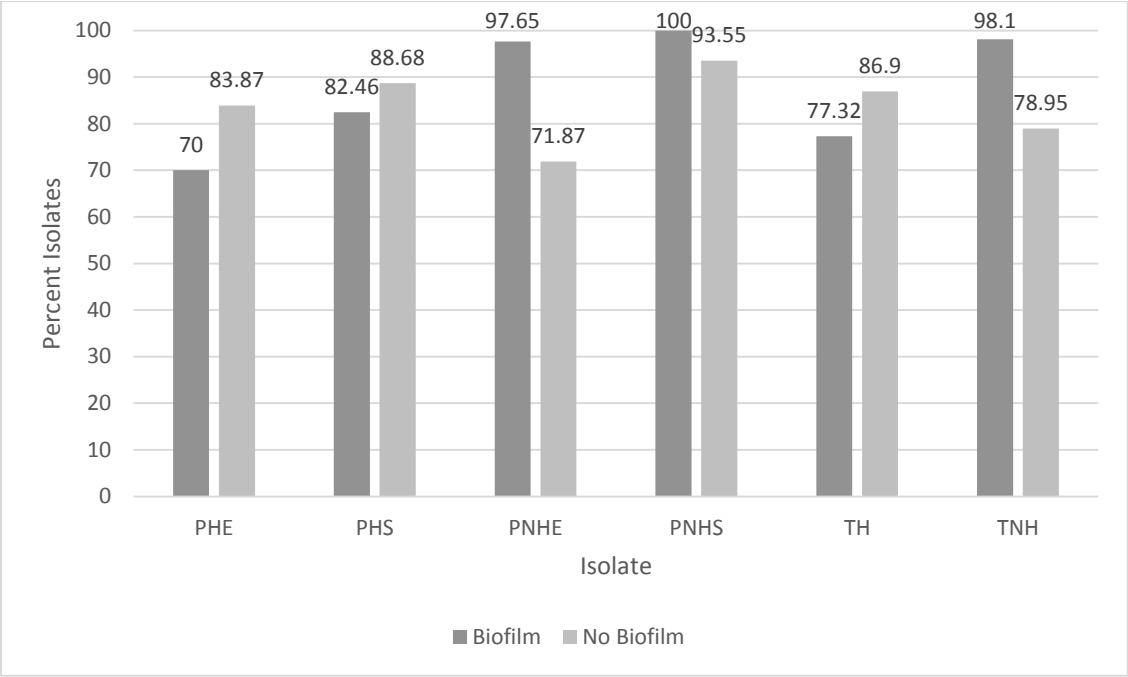


Figure 4.12. Acid tolerance of predominantly host and predominantly non-host isolates. The subdivisions were PHE= predominantly host *E. coli*, PHS= predominantly host *Salmonella* spp., PNHE= predominantly non-host *E. coli*, PNHS= predominantly non-host *Salmonella* spp., TH= total host, TNH= total non-host. Each isolate had three replicates.

4.4. Curli and Cellulose Expression

The presence of curli and cellulose was detected using congo red and calcoflour plates. Each isolate was inoculated on the plates and incubated at four different temperatures, after which observations were made on the morphology and fluorescence (where appropriate) of the isolates. Each isolate was tested in triplicate (an example of the raw data can be found in Table A5 of Appendix B). Expression of curli and cellulose by *Salmonella* spp. can be seen in Figure 4.13. At 37°C, 71% of isolates from Fleming (FS*) expressed both curli and cellulose, and 71% of Water Treatment Center (WTC*) isolates expressed cellulose. *Salmonella* spp. isolates from Omeeme (OS*), Scugog River (SRS) and Coboconk Lagoon (LS*) expressed curli at 37°C and 28°C. Clair Lake isolates changed expression throughout the different temperatures tested. At 37°C, 17.8% of the Clair Lake isolates expressed cellulose and 10.7% expressed curli, while at 28°C 35.7% of the Clair Lake isolates expressed both curli and cellulose, and 14.3% expressed only curli, and only cellulose. At 23°C, 42.5% of these isolates expressed both curli and cellulose, followed by 35% and 10°C. Isolates from Omeemee, Scugog River, Coboconk Lagoon and Gull River did not express curli or cellulose at 23°C and at 10°C.

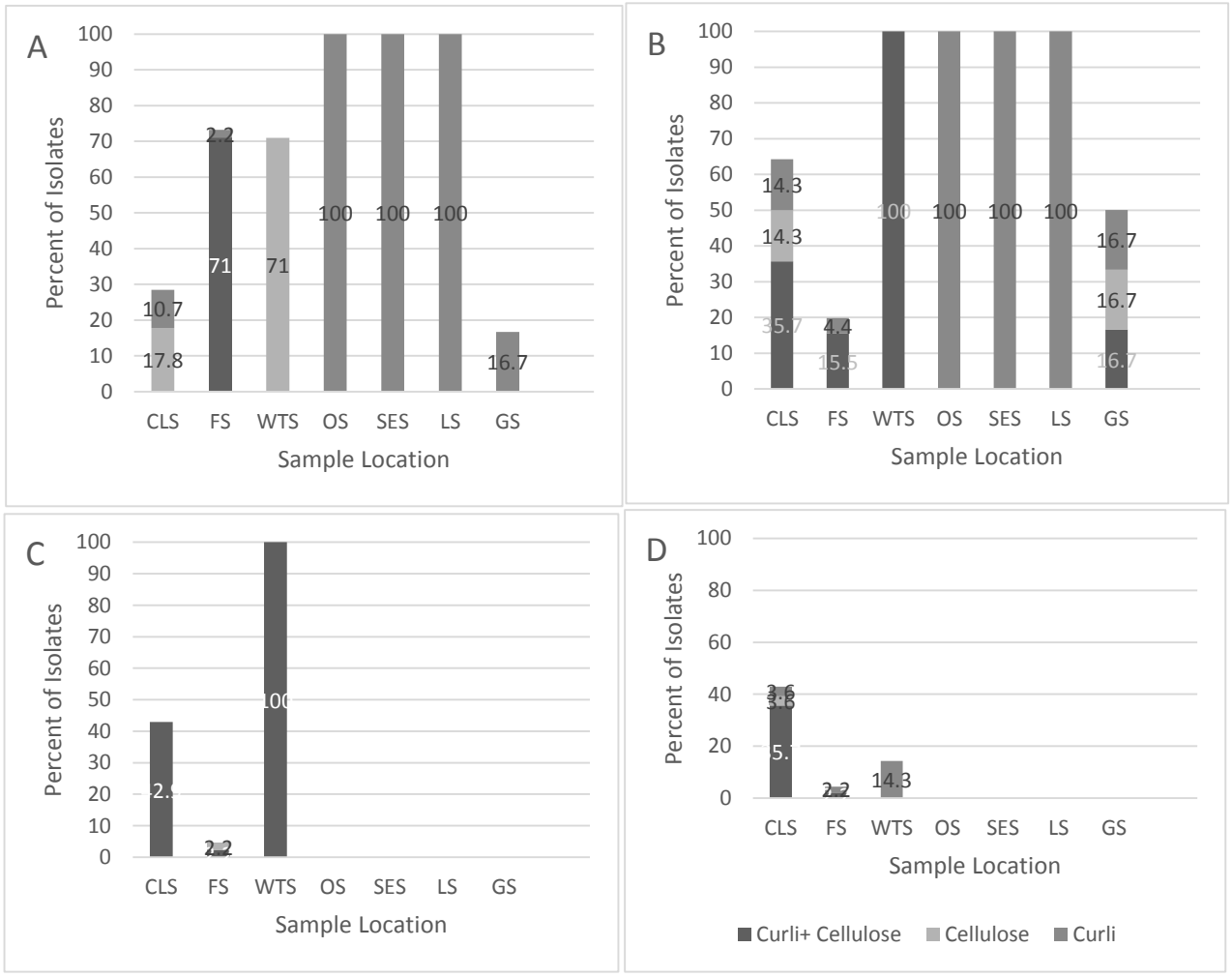


Figure 4.13. Percent expression of curli and cellulose by *Salmonella* spp. isolates. The temperatures tested were A) 37°C, B)28°C, C)23°C and D)10°C. The legend depicts the shading corresponding with curli, cellulose or curli and cellulose expression. Isolates were divided based on location where CLS= Clair Lake, FS*= Fleming, WTS*= Wastewater Treatment Plant, GRS= Gull River, LS*= Coboconk Lagoon, SRS= Scugog River, OS*= Omeemee Water Pollution Treatment Pond. Each isolate was tested in triplicate.

As opposed to *Salmonella* spp., *E. coli* isolates from all locations were able to express curli and cellulose components at all the temperatures tested (see Figure 4.14). At 37°C, 100% of isolates from Gull River (GE) and Nunavut (NE*) expressed either curli and cellulose, or only curli. All isolates from Gull River, also expressed curli and cellulose components at 28°C. *E. coli* isolates from Scugog River (SGE) exhibited the highest proportion of component expression at 10°C (60%), while Gull River isolates expressed the most components at 28°C (100%) and 37°C (100%) and both locations had the highest component expression at 23°C (70%).

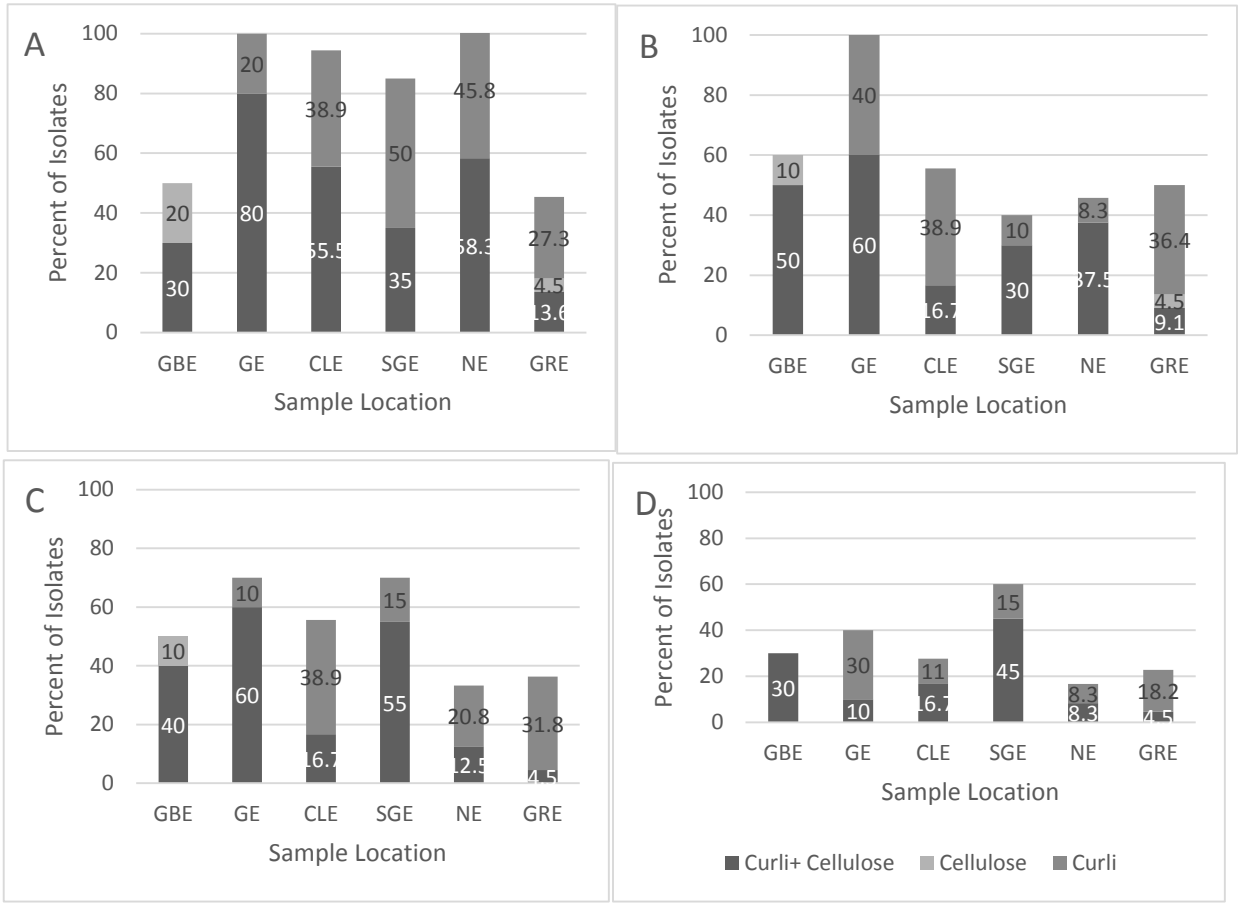


Figure 4.14. Percent expression of curli and cellulose by *E. coli* isolates. The temperatures tested were A) 37°C, B) 28°C, C) 23°C and D) 10°C. The legend depicts the shading corresponding with curli, cellulose or curli and cellulose expression. The locations of isolations are as follows: GBE= Grand Bend, GE= Gull River, CLE*= Coboconk Lagoon, SGE= Scugog River, NE*= Nunavut, GRE= Grand River. Each isolate was tested in triplicate.

In general, isolates expressed either both curli and cellulose or just curli, very few expressed only cellulose (>10%). A summary of the overall curli and cellulose expression by *E. coli* and *Salmonella* spp. isolates can be found in Table 4.8. The most cellulose-only expression was observed at 37°C (2.9% by *E. coli* and 9.9% by *Salmonella* spp.) and the least cellulose expression was noted at 10°C (0% for *E. coli* and 1% by *Salmonella* spp..) The most component expression was observed at 37°C (79.8% of *E. coli* isolates and 61.4% of *Salmonella* spp. isolates), and the least was observed at 10°C (31.7% of *E. coli* isolates and 14.8% of *Salmonella* spp.. At all temperatures, *E. coli* isolates exhibited a higher percentage of component expression compared to *Salmonella* spp. isolates.

Table 4.8. Summary of Total *E. coli* and *Salmonella* spp. Curli and Cellulose Expression in Percentages.

	Growth Temperature			
	37°C	28°C	23°C	10°C
E. coli	Percent expression (%)			
Curli and Cellulose	43.3	29.8	26.9	18.3
Cellulose	2.9	1.92	0.96	0
Curli	33.6	22.1	22.1	13.5
Total[a]	79.79	53.8	50	31.7
Salmonella spp.	Percent expression (%)			
Curli and Cellulose	31.7	24.8	19.8	10.9
Cellulose	9.9	5	1	1
Curli	19.8	21.8	0	3
Total	61.4	51.5	20.8	14.8

[a]Total represents the total amount of isolates expressing components

4.5. Serotyped Isolates

Salmonella spp. isolates from the Water Treatment Center (WTC*), Fleming (FS*) and Clair Lake (CLS) were sent for serotyping, The Fleming isolates were unsuccessfully serotyped. Results for the Water Treatment Center Isolates and Clair Lake isolates can be found in Table 4.9. *Salmonella* spp. isolates from WTC* were different serotypes, with the exception of two that were designated as *Salmonella* Heidleberg. The Clair Lake Group consisted of seven Typhimurium serotypes, six Braenderup, three Hartford, two I:Rough-O:e,h:e, n,z15, seven I:4,5, 12:b:-, one I:Rough-O:e,h:- and two I:Rough-O:y:e,n,x. The table also summarizes results for biofilm formation, acid tolerance antibiotic resistance and morphotype at 37°C. Most of the isolates were weak biofilm formers, with the exception of the Typhimurium serotype. Six out of eight Isolates from the WTC expressed cellulose, two out of seven isolates serotyped as I:4,5,12:b:- expressed curli, and three expressed cellulose. One out of the two isolates serotyped as I:Rough-O:-:e,n,x expressed cellulose. The greatest level of antibiotic resistance was observed in isolates from WTC, with all but one being resistant to multiple antibiotics. In contrast, only two of the Clair Lake isolates showed antibiotic resistance by the disk diffusion method. MBECs of >1024µg/Ml were observed in three Braenderup serotypes, two Typhimurium serotypes, both I:Rough-O:e,h:e, n,z15 serotypes and two I:4,5,12:b:- serotypes. *Salmonella* Typhimurium and one

Salmonella I:4,5,12:b:- isolate were able to produce moderate biofilms without the presence of curli fimbriae and cellulose components.

Table 4.9. Summary of Serotyped *Salmonella* spp. Isolates.

Isolate		Biofilm Formation (37°C)				Acid Tolerance (+/-)		Antibiotic Resistance	MBEC >1024µg/mL			
WTC		Serotype	Salt	No Salt	Morphotype	Biofilm	No Biofilm	Disk Diffusion	AMP	CTX	STREP	OXY
	WTCR5	I:6, 7:r:-	weak	weak	pdar/saw	+	+	CTX, STREP	√	√	√	√
	WTCT4	Heidleberg	weak	weak	saw	+	+	ATM, CIP, STREP	√	√	√	√
	WTCT27	Heidleberg	weak	weak	saw	+	+	STREP	√	√	√	√
	WTCR9	Infantis	weak	weak	pdar/saw	+	+	ATM, STREP	√		√	√
	WTCR22	Thompson	weak	weak	pdar/saw	+	+	ATM, STREP	√	√	√	√
	WTCR20	Monschaui	weak	weak	pdar/saw	+	+	CTX, OXY, SXT	√	√	√	√
	WTCR30	Schwarzengrund	weak	weak	pdar/saw	+	+	ATM, CTX, STREP	√	√	√	√
	WTCR6	Stanley	weak	weak	pdar/saw	+	+	CTX, OXY, SXT	√	√	√	√
CL												
N=7	**SCS7**	Typhimurium	weak	moderate	saw	+	+		√		√	√
	SCS8	Typhimurium	moderate	moderate	saw	+	+		/	/	/	/
	SCS1	Typhimurium	weak	moderate	saw	+	+		√	√	√	√
	SCS4	Typhimurium	moderate	moderate	saw	+	+		/	/	/	/
	SCI11	Typhimurium	weak	weak	saw	+	+		√		√	√
	SCS3	Typhimurium	weak	moderate	saw	+	+		√	√	√	
	SCS6	Typhimurium	weak	moderate	saw	+	+				√	√
	SCS9	Braenderup	weak	weak	saw	+	+		/	/	/	/
N=6	**SCO4**	Braenderup	weak	weak	saw	+	+					
	SCO6	Braenderup	weak	weak	saw	+	+					
	SCS5	Braenderup	weak	weak	saw	+	+		√			√
	SCO5	Braenderup	weak	weak	saw	+	+		√	√	√	√
	SCO2	Braenderup	weak	weak	saw	+	+		√	√	√	√
N=3	**SCI8**	Hartford	weak	weak	saw	+	+					
	SCI1	Hartford	weak	weak	saw	+	+			√		√
	SCI7	Hartford	weak	weak	saw	+	+			√		√
N=2	**SCO7**	I:Rough-O:e,h:e, n	weak	weak	saw	+	+					
	SCS2	I:Rough-O:e,h:e, n	weak	weak	bdar	+	+					
N=7	**SCO1**	I:4,5, 12:b:-	moderate	moderate	saw	+	+					
	SCI4	I:4,5,12:b:-	weak	weak	saw	+	+					
	SCI9	I:4,5,12:b:-	weak	weak	saw/pdar	+	+		√	√	√	√
	SCI12	I:4,5,12:b:-	weak	weak	saw/bdar	+	+		√	√	√	√
	SCO3	I:4,5,12:b:-	weak	weak	saw/bdar	+	+	AMC	√	√	√	
	SCI3	I:4,5,12:b:-	weak	weak	pdar	+	+		√	√	√	√
	SCI5	I:4,5,12:b:-	weak	weak	pdar	+	+		√	√	√	√
N=1	**SCI2**	I:Rough-O:e,h:-	weak	weak	pdar	+	+		√	√	√	√
N=2	**SCI10**	I:Rough-O:y:e,n,x	weak	weak	saw	+	+		√	√	√	√
	SCI6	I:Rough-O:-:e,n,x	weak	weak	saw/pdar	+	+	ATM	√		√	√

” / ” data not available

5. Discussion

Biofilm-forming bacteria are an ongoing concern as they are able to attach themselves to food, food packaging, water pipes and medical devices (Mah and O'Toole, 2001; Fux *et al*., 2004*;* Kumar, 1998). Since biofilms are so versatile, and are a growing health concern (Licking, 1999), it is important to study and understand the conditions under which bacteria are able to produce biofilms. In this study, *E. coli* and *Salmonella* spp. were extracted from watershed locations, storm water ponds, wetlands and water treatment centers designed for the treatment of human waste. Isolates were subsequently classified as predominantly host and predominantly non-host depending on the location of extraction. Isolates from areas which treated, or were in direct contact with human waste were considered predominantly host. In order to understand their ability to persist, isolates were tested for biofilm forming capabilities under host and non-host mimicking conditions, ability to withstand stressors such as antibiotic treatment and acidic environments, as well as their expression of curli and cellulose biofilm components and the overall relationship to antibiotic resistance expression.

5.1. Serotyped Isolates

Salmonella spp. isolates that were extracted from the Water Treatment Center, Fleming and Clair Lake were sent for serotyping. Not all isolates were able to be serotyped due to time constraints because a few sampling events were scheduled after the original samples were sent for serotyping. The most frequently observed serotypes in the sample group were *S.* Typhimurium, *S.* Braenderup, *S.* Hartford and *S.* Heidleberg, which are serotypes that often cause symptoms of salmonellosis, including fever, nausea, vomiting, abdominal cramps and diarrhea (CDC, 2013). Other serotypes found were *S.* Infantis, *S*. Thompson, *S.* Monschaui, *S.* Stanley and *S*. Scwarzengrund (Table 4.9). There were also 4 other serotypes named based on their serotype profile: I:Rough-O:e,h:e, n,z15, I:4,5, 12:b:-, I:Rough-O:e,h:- and I:Rough-O:y:e,n,x. The most variety in serotypes was found in isolates from the Water Treatment Plant.

Isolates from the Fleming location were not successfully serotyped. This is most likely due to the isolates being stored in the -80°C freezer for prolonged periods of time, between experiments. When they entered their dormant state, their physiology and antigen expression could have changed from what it originally was in the environment. Once they were taken out of the freezer, they were no longer exposed to the stressors they faced in the environment and may not have expressed the same antigens they did before, making them hard to serotype.

It is important to note that although some isolates were the same serotype, they responded differently. For example, there were seven isolates which were *S.* Typhimurium yet their biofilm formation varied between weak and moderate, they all did not express curli fimbriae or cellulose, and there were some differences in their MBECs towards different antibiotics. A previous study conducted by Romling *et al.* (2003) which studied cellulose and curli expression of *S.* Typhimurium and *S.* Enteriditis from human and animal sources found that approximately 90% of isolates expressed the components at 37°C. Curli and cellulose were not expressed by the *S.* Typhimurium isolates in the present study which could be because they have been in the non-host environment for prolonged periods of time which may have altered the expression profiles from what they might have been immediately after recently leaving a host. The isolates may have become more accustomed to biofilm formation and component expression in non-host conditions, as it was observed that *S.* Typhimurium isolates had some curli and cellulose expression at lower temperatures (data not shown). Although the isolates used in this study were isolated from environmental locations, they may have the ability to colonize within a host if given the opportunity, as evidenced by the isolation of serotypes such as *S.* Typhimurium from Clair Lake, which can cause salmonellosis upon ingestion (CDC, 2013). Therefore, it is important to study environmental isolates and gain a greater understanding of their ability to survive in host and non-host conditions.

5.2. Biofilm Formation

The degree of biofilm formation was categorized as previously described by Stepanovic *et al.* (2000), as no, weak, moderate and strong biofilm formers. Biofilm experiments were replicated 3 times under each condition. Each isolate was inoculated into 4 wells, and each experiment was repeated 3 times, meaning isolates could have 12 values for the biofilm formation under each condition. Some isolates, however, had 8 or 4 values due to improper growth in certain experiments, and loss of data, but overall this experimental setup was successful in providing data on the degrees of biofilm formation.

Overall, a large proportion of isolates were able to form biofilms. At 10°C, 89.9% of isolates formed biofilms, 98.3% at 23°C, 96.7% at 28°C and 98.8% at 37°C (Table 4.2). This data suggests that the preferred temperatures for the formation of biofilms are 23°C and 37°C. The most biofilms qualified as "strong" were observed at 23°C. This is consistent with previous research which, using *Salmonella enterica* serovar Enteriditis tested at 5°C, 20°C and 37°C, found that 20°C was the optimum biofilm forming temperature for *Salmonella* (Giaouris *et al.,* 2005). The study conducted by Giaouris and colleagues did not use a large sample size like the present study, yet the same trend of strong biofilm formation at ~20°C was observed. There are limited studies for *E. coli* using the same temperature range and media used in this project. A previous study conducted by Uhlich and colleagues (2013) using 73 *E. coli* strains (from seven serogroups) retrieved

from research institutions, tested biofilm formation at 25°C, 30°C and 37°C in YESCA (0.5g/L yeast extract; 10g/L Casamino acids) nutrient broth. The study found that strains were able to form biofilms at 25°C and 30°C, but little biofilm formation was observed at 37°C. In addition to biofilm formation, Uhlich and colleagues (2013) also tested the congo red binding affinity of their isolates (testing curli and cellulose presence), and found that even though congo red binding affinity was high at 37°C (in particular for O157:H7 isolates), biofilm production was higher at 25°C and 30°C. These findings are consistent with the data from the present study where the highest proportion of strong and moderate biofilm formation for *E. coli* was observed at 23°C and 28°C (79.6% and 73.8% in no salt, respectively), and most component expression was observed at 37°C (79.8%) (see Figure 4.1 for biofilm formation and Table 4.8 for component expression).

Overall, *Salmonella* spp. were able to form more biofilms in comparison to *E. coli* (Figure 4.1). At all temperatures, there was a higher proportion (24.3% vs. 1.9% at 10°C, 5.6% vs. 0% at 23°C and 28°C, 2.8% vs. 0% at 37°C, in media with no salt) of *E. coli* isolates unable to form biofilms. *Salmonella* spp. exhibited not only more biofilm formers, but also higher levels of moderate and strong biofilms. The biofilm formation capabilities of *E. coli* and *Salmonella* spp. should also be compared to biofilm component data (found in Table 4.8), because it has been found that isolates which express curli and cellulose biofilm components are able to form biofilms (Romling, 1998). It was noted that a higher proportion of *E. coli* isolates (79.8% at 37°C) were able to express curli fimbriae and cellulose components in comparison to *Salmonella* spp. isolates (61.4% at 37°C),

indicating that biofilm formation was not directly related to the expression of components (similar to data found by Uhlich *et al.,* 2003). This could mean that many *Salmonella* spp. isolates used other components to aid in the formation of their biofilm, for example the O-antigen capsule (Crawford, 2008).

It should be noted that a shortcoming of the biofilm assay that was employed in this thesis (and routinely by others) may be missing some forms of biofilms, and therefore, underestimating the level of biofilm formation. The assay used to analyze biofilm formation only accounts for cells that are adhered to the surface of the wells. After incubation, the 96-well plates with cultures were flipped upside down and washed out in tubs of water, in order to dispose of all unattached cells and media components that may increase background staining (O'Toole, 2011). This means that any biofilms not attached to the surface (*e.g.* pellicles formed at the air liquid interface or cells clumped together in suspension as flocculates) are not captured in downstream measurements because they would be discarded during this initial step. Exopolysaccharide-only biofilms in particular, may not be captured properly because they tend to form diffuse biofilms that do not anchor firmly to the surface (Solano *et al.,* 1998). This phenomenon could also be organism/strain dependent. For example, if *E. coli* isolates formed more biofilms at the air liquid interface, while *Salmonella* spp. formed more biofilms that attached to sides and bottoms of the wells, then due to the design of the experiment, *E. coli* would show up as a weaker or no-biofilm former. Thus, there may have been more biofilms than were recorded using the biofilm assay approach outlined in this thesis. Recognizing this concern, the present study also

employed the plate morphology (congo red and calcofluor-based plates) assay to help gain a broader understanding of biofilm formation on semi-solid media and to complement the routinely used liquid-based growth of the biofilm assay. For future studies with the liquid-based biofilm assay, it may also be helpful to take notes/pictures of the various biofilms directly following incubation, looking for pellicles or aggregation of cells that are not attached throughout the well prior to removing the growth culture. Regardless of these concerns and recommendations, the biofilm assay technique is still the most widely used method and currently offers the best way to compare biofilms across the literature.

Data of the isolates from the biofilm assay were divided based on location of extraction and observed for differences in biofilm formation. Overall, biofilm formation patterns for predominantly host isolates and predominantly non-host isolates were very similar to each other throughout all temperatures tested. *E. coli* isolates from certain non-host locations, such as Grand Bend and Gull River, were able to form biofilms at all temperatures (Figure 4.2, Figure 4.3). Isolates from the Gull River produced the most biofilms qualified as "strong" at 10°C compared to any other temperature. This could be because isolates from this location have been in the environment for a prolonged period of time and are more accustomed to environmental temperatures. As expected, *E. coli* isolates from predominantly host locations such as Nunavut and Coboconk Lagoon, were able to form more biofilms at 37°C (host temperature) than other temperatures tested, and highest amount of biofilm production was observed at 28°C (which is consistent with previous findings of biofilm formation at <30°C) (Olsen *et al.,* 1989; Olsen *et al.,* 1993; White-

Ziegler *et al.*, 2008). *E. coli* and *Salmonella* spp. isolates were generally able to form higher amounts of biofilm in media with no salt (Figure 4.2.- Figure 4.5.). Media with salt and no salt was used as a means to compare biofilm formation in conditions that might be encountered in a host or a non-host environment. In a non-host environment, such as in lakes or rivers, bacteria would encounter areas of low/ no salt, while within a host, bacteria would be more likely to be in a salt environment. Previous studies have not compared *E. coli* and *Salmonella* spp. biofilm growth in liquid media with and without salt. It has been found, using mouse virulent *Salmonella* spp. strains, that curli fibres grow best on agar plates with no salt compared to a high salt environment (Romling *et al.*, 1998). If this is also true for other protein biofilm components, it could explain why biofilm formation is more prominent in media without salt.

Bacteria use biofilms to attach to host surfaces and avoid detection by the host immune response (Parsek and Singh, 2003). In this study, all *Salmonella* spp. isolates were able to form biofilms at host temperatures (*i.e.* 37°C), but low amounts of biofilm (weak) were mainly produced by these bacteria. This may suggest that this amount is enough to efficiently accomplish colonization and persistence within a host. The highest amount of biofilm (moderate, strong) was observed at 23°C. While biofilm formation can be beneficial for bacteria within a host, a stronger biofilm might be needed to protect the bacteria from environmental factors such as desiccation, or UV radiation (Olson *et al.*, 2002; Fux *et al.*, 2005). Bacteria may be creating stronger biofilms in the presence of non-host temperatures in order to protect themselves from non-host environmental factors,

while creating biofilms of less thickness, just enough to mask their antigens at 37°C. In the environment, a larger biofilm would be more beneficial to the bacterial cells because it would give them a larger surface area that would allow for the trapping of food, nutrients and moisture.

Data of biofilm formation based on predominantly host and predominantly non-host locations were compared in order to analyze whether being in the non-host environment for short (predominantly host) or prolonged periods of time (predominantly non-host) had an effect on bacterial biofilm formation. Predominantly host and non-host *Salmonella* spp. had the highest percentage of moderate biofilms at 37°C in media with no salt (27% and 44%, respectively). Overall, at 37°C, predominantly host and predominantly non-host isolates had similar patterns of biofilm formation. This could mean, that even though the isolates are from different sources, they could behave in similar ways when encountering a host. For example, as seen in the *Salmonella* spp. serotyping section, isolates from a predominantly non-host location (Clair Lake) were serotypes associated with salmonellosis (Typhimurium, Braenderup, Hartford). Although isolates were subdivided into predominantly host and predominantly non-host groups, these isolates are all essentially environmental isolates and can be expected to behave in similar ways under certain circumstances.

One of the trends observed throughout the biofilm experiments, was that biofilm formation was lowest at 10°C. At this temperature, most isolates exhibited low biofilm formation

with the exception of predominantly host *Salmonella* spp. which formed ~60% moderate biofilms, indicating that these isolates are able to successfully establish themselves within a host environment and outside of the host. At lower temperatures, bacterial growth slows (Rahm, 2015), which likely also slows the production of biofilm components resulting in most isolates to be weak biofilm formers at lower temperatures (*ie.*10°C). The exception was predominantly host *Salmonella* spp., which were able to form moderate biofilms at low temperatures, likely due to the expression of alternate biofilm components such as colonic acid.

5.3. Antibiotic Resistance

Due to the rampant use of antibiotics (including human, animal and agriculture treatments) researchers have noted a worrying increase in inadvertent antibiotic exposure to other microorganisms present in the environment (Furuya *et al.,* 2006). Thus, it was suspected that antimicrobial resistance would be observed among our environmental isolates of *E. coli* and *Salmonella* spp. and may also be associated with biofilm formation. Antibiotic resistance of these isolates was tested using a disk diffusion assay (CLSI, 2007) and the MBEC assay (Ceri *et al.,* 1998), as previously described. The disk diffusion assay is standardized and useful for profiling resistance patterns, but the assay only tests the growth/resistance of bacteria on a solid surface (which is not necessarily a good representative environment of *in vivo* conditions where bacteria are immersed in solution).

The MBEC assay was originally designed by Ceri and colleagues (1998) to capture bacteria in the air-liquid-surface environment using what was then called the Calgary Biofilm Device. The device is a microtiter plate without wells, and pegs on the lid, which allows for biofilm formation of the same isolate throughout the plate on each peg (*i.e.* mimic of the liquid-surface interface). The lid is then transferred to a 96-well plate with varying antibiotic concentrations in each well. The biofilm is then removed from the pegs and plated for viability. The assay was adapted for the use of microtiter plates. Because of the large sample size in this study, the use of the Calgary Biofilm Device would be costly. Biofilms were grown in microtiter plates, after which the wells were washed out and filled with antibiotic-containing media. Following incubation the wells were washed out again and the biofilm was scraped from the wells and then plated for viability. While different from the Calgary Biofilm Device, this adaptation was a close mimic of their original technique. The modified version used in this study proved to be successful as it generated reproducible results. The method was also more cost effective, particularly for this study which used a large number of isolates, because multiple isolates could be inoculated into a microtiter plate (as opposed to one isolate per plate for the Calgary Biofilm Device).

The MBEC assay was employed as a complimentary technique to view antibiotic resistance of biofilm-embedded isolates. Although bacteria are able to form biofilms on solid surfaces, liquid media provides an environment more closely related to the location of isolation (rivers, watersheds) and to host environments, where bacteria would be submerged in liquid. Within a biofilm, bacteria can be protected by the exopolysaccharides

in the biofilm inhibiting the entry of antimicrobial compounds, either through the thickness of the biofilm or by causing compounds to bind to the matrix (Costerton *et al.,* 1995; Lewis, 2001; Mah and O'Toole, 2001). Additionally, bacteria embedded in a biofilm have a lower metabolic activity which slows their uptake of antimicrobial compounds, making the antibiotic ineffective (Fux *et al.,* 2004). Isolates were indeed better protected from antibiotics within the biofilm, as most had a MBEC >1024 µg/mL (Table 4.6). Eradication concentrations were tested up to 1024 µg/mL as previously suggested from MBEC assays (Ceri *et al.,* 1997, 1999; Sepandj *et al.,* 2004). This concentration was selected as the highest concentration tested because it is above the highest serum concentration, and higher concentrations would not be clinically relevant (Qu *et al.*, 2010).

The modified MBEC and disk-diffusion assays were conducted using antibiotics chosen based on suggested groupings by CLSI (2007). Eight antibiotics were used for the disk diffusion assay, while four of these were chosen for subsequent testing using the MBEC assay since the sample size was large for this type of assay (which was also only being tested for the first time in the adapted form described above). The four antibiotics chosen were from four different subclasses (Penicillin, Cephalopsorins, Tetracyclines and Aminoglycosides) to encompass a good selection of both bacteriostatic and bactericidal modes of action (see Table 3.3). While this strategy proved to be a good initial survey of the effects of common antibiotics, the research would benefit from future studies that employ a wider panel of antibiotics, in order to gain a better understanding of resistance patterns that were uncovered herein.

The antibiotics tested in the MBEC assay, Streptomycin, Ampicillin and Cefotaxime are bactericidal while Oxytetracycline is bacteriostatic. Bactericidal antibiotics are able to kill the bacteria while bacteriostatic antibiotics inhibit the growth of additional bacteria (Pankey and Sabath, 2004). Most isolates (88%) were able to survive concentrations of 1024 µg/mL in Oxytetracycline (a bacteriostatic agent), and there was very little difference between the treatment with bacteriostatic and bacteriocidal antibiotics. Although it could be expected that when isolates are treated with bacteriostatic antibiotics, cells might be able to grow again once re-inoculated into antibiotic-free medium, since the cells are not killed but kept in the stationary phase of growth, this is not the case. There is often little difference between the treatment of infections using bacteriostatic and bactericidal antimicrobials (Pankey and Sabath, 2004), which was also evidenced in the findings of this study.

Data generated through the use of the disk diffusion assay showed that *E. coli* isolates exhibited multiple resistances to antibiotics (Table 4.3). Isolates from the Coboconk Lagoon were resistant to each antibiotic tested, while Nunavut isolates were resistant to 87% (7/8) antibiotics tested (Table 4.3). Similar results were found by Reinthaler *et al.* (2003), where *E. coli* sampled from three areas of wastewater treatment, treating municipal sewage as well as hospital sewage, showed high occurrences of antibiotic resistance. In addition to the previously mentioned locations, a similar pattern of resistance was observed in Grand River isolates, which showed some resistance to all antibiotics tested as well as isolates from Scugog River and Gull River which exhibited resistance to 6/8 antibiotics tested. This is inconsistent with a previous study of antibiotic resistance of South Carolina

urban watersheds, where only 15% of *E. coli* isolates showed resistance to antibiotics (Webster *et al.*, 2004). The difference in resistance could be due to an increase of antibiotic use over the years, resulting in a higher occurrence of antibiotic resistance, or due to the general differences in the urban watersheds. It is possible, that the South Carolina urban watersheds were less impacted by antibiotics than the watersheds sampled in this study. For example, the Grand River spans from Dufferin County to Port Maitland on Lake Erie and is impacted by municipal and agricultural activities which could result in the runoff of antimicrobial containing compounds.

Similar to the high antibiotic resistance rates of the previously mentioned *E. coli* isolation locations, Grand Bend *E. coli* isolates were resistant to 6/8 antibiotics tested. Grand Bend isolates were sampled on a day with warnings of high *E. coli* levels. The samples were taken approximately 3 meters from shore, which has been found to be an area of high bacterial counts (Crowe, 2015). The great lakes are impacted by humans, urban runoff, agriculture and waterfowl (Crowe, 2015), so bacteria could have entered the Great Lakes from an area impacted by antibiotic usage, which could have encouraged the bacteria to form antibiotic resistance mechanisms. For example, bacteria entering the lake through a water pipe could have been part of a surface-attached biofilm, increasing the chances of encountering other antibiotic resistant microorganisms and exchanging resistance genes, resulting in multiple antibiotic resistance. Aside from the potential of antimicrobial exposure, environmental pressures may have altered the drug susceptibilities in bacteria. Previous studies have found that stress responses caused by environmental stressors may

indirectly alter antimicrobial susceptibilities by regulating DNA repair systems (Hastings *et al.,* 2004; Poole, 2012). Antimicrobial resistance could also be stress-dependent; for example, McMahon *et al.* (2007) found that *S. enterica* and *E. coli* antimicrobial resistance increased when subjected to pH (5.0 and 4.0) and salt stresses (NaCl concentrations of 4.5% and 12%). Although antimicrobial susceptibility returned back to previously tested levels for *S. enterica* after removing the stressors, *E. coli* sustained antimicrobial resistance. This suggests that the pressures of stressors could permanently alter antimicrobial resistance in bacteria. This could indicate that environmental isolates that are exposed to environmental stressors (*e.g*. UV radiation, temperature fluctuation) for prolonged periods of time (*i.e*. isolates from predominantly non-host locations, such as Grand River and Grand Bend) have gained antimicrobial resistance as a result of other survival mechanisms.

When comparing antibiotic resistance data it was observed that *Salmonella* spp. were more susceptible to antibiotics than *E. coli* isolates (Table 4.4). The lowest instance of antibiotic resistance was observed in isolates from Clair Lake. With only 3.6% of isolates showing resistance to 2/8 antibiotics. A previous study by Morrison (2013, unpublished) also tested antibiotic resistance of *Salmonella* spp. from this storm water retention pond. Samples were taken during a dredging, which allowed for the opportunity to study microorganisms that were surviving in deposited materials sheltered from environmental factors, such as UV or temperature fluctuations, as well as after reconstruction. *Salmonella* spp. isolates from the dredged material had a high incidence of multiple antibiotic resistance, while post-dredging, the multiple antibiotic resistance decreased to 70% and 0 (on separate occasions).

Samples for the present study were collected over a year after the Morrison study. The incidence of antibiotic resistance decreased over time post-dredging during the previous study (Morrison, 2013), and levels continued to stay low in the samples collected for the current study.

While dilute areas such as watershed and lake locations were expected to have a low incidence of antibiotic exposure, areas of concentrated sources such as wastewater treatment were presumed to have higher antibiotic exposure (*i.e.* from human treatment). Therefore, it was hypothesized that *Salmonella* spp. isolates from the Wastewater Treatment Center and the Center for Alternative Waste Water Treatment (Fleming), Coboconk Lagoon and Omeeme would show high levels of antibiotic resistance. *Salmonella* spp. collected from the Center for Alternative Waste Water Treatment (Fleming) were previously studied by Morrison (2013, unpublished) and tested for antibiotic resistance. It was previously found that antimicrobial resistance profiles varied throughout the seasons, with the highest resistance occurring during a winter sampling event and some resistance during summer and late-summer sampling events. The Alternative Waste Water Treatment (Fleming) isolates used in this study are from the late-summer sampling event and continued to exhibit some resistance that paralleled results of new isolates from other wastewater treatment locations, specifically Coboconk Lagoon and Omeemee. Together these isolates were resistant to 50% (4/8) of antibiotics tested, with the greatest level of resistance towards Aztreonam, Amoxicillin/Clauvulanic acid and Ampicillin. *Salmonella* spp. isolates from the Wastewater Treatment Center displayed even

more widespread antibiotic resistance profiles (*i.e.*, showing resistance to 6/8 antibiotics tested). Since wastewater treatment locations were expected to have a higher incidence of antibiotic resistance compared to watershed locations, due to the influx of human-impacted water that may be inherently carrying a higher number of antibiotic resistant microorganisms, the Coboconk Lagoon and Omeemee results were lower than presumed. In fact, the resistance patterns of these isolates were more similar to *Salmonella* spp. from the watershed location, Scugog River (predominantly non-host labelled environment). Samples from the Scugog River were taken from the biofilms on the roots of the FTW's (Floating Treatment Wetlands), as well as from the water surrounding the FTW's. Biofilms often form on the surface of the root system, resulting in a high bacterial load. *Salmonella* spp. may have gained resistance through the transfer of resistance genes within the concentrated biofilms on the root system. Conversely, if samples were taken from a biofilm within the areas of wastewater treatment, it is expected that the incidence of antibiotic resistance would be higher due to exposure to possible antimicrobial containing human-impacted water.

Overall, isolates from predominantly host locations exhibited more resistance to each antibiotic when compared to predominantly non-host isolates (Table 4.5). The exception to this trend is the resistance to Amoxicillin/Clavulanic Acid which was the highest among predominantly non-host isolates. Amoxicillin/ Clavulanic Acid is widely used in veterinary medicine (Liberato *et al.*, 2011); if livestock are treated with this antibiotic, agricultural runoff may impact surrounding waters, resulting in bacterial exposure and increased

resistance to the antimicrobial. While all other groups had some resistance (3.3%- 82%) to each antibiotic, predominantly non-host *Salmonella* spp. showed resistance to only 50% of antibiotics tested (4/8). A higher prevalence in antibiotic resistance for predominantly host isolates was expected, as they would have been more likely to encounter antibiotics from human treatment, or antibiotic resistant genes from other microorganisms.

In addition to location-based antibiotic resistance patterns, total resistance to specific antibiotics should also be noted. Among all of the isolates, the most widespread resistance was to Ampicillin, followed by, Amoxicillin/ Clavulanic Acid, Streptomycin, Aztreonam, Cefotaxime, and Ciprofloxacin. The most susceptibility was observed towards Trimethoprim-Sulfamethoxazole and Oxytetracycline (Table 4.5). Sulfamethoxazole/Trimethoprim and Ampicillin were used in this study because they are often used in medicine and veterinary medicine for ear infections and urinary tract infections of which 80% are caused by *E. coli* (Jancel and Dudas, 2002), as well as upper respiratory infections, and gastrointestinal infections (Drugs, 2015; Drugs, 2015b). Urinary tract infections are the most frequently occurring infections in the United States, resulting in approximately eight million office visits per year (Orenstein, 1999; Patton *et al.*, 1991). Ampicillin is often used for urinary tract infections but antibiotics such as Trimethoprim-Sulfamethoxazole and fluoroquinones (*i.e.* Ciprofloxacin) are the preferred treatment for these infections because they minimally disrupt the microflora of the body (Jancel and Dudas, 2002). The use of antibiotics for urinary tract infections have resulted in an increased resistance to β-lactams such as Ampicillin, Cephalosporins,

Sulfamethoxazole/Trimethoprim, with the greatest level of resistance observed towards Ampicillin and first generation Cephalosporins (Jancel and Dudas, 2002). This study is consistent with this published literature, since the combined data on all the isolates revealed resistance to Ampicillin. Resistance to Trimethoprim-Sulfamethoxazole was lower than the resistance described in a clinical study conducted by Gupta and colleagues (1999) (6.3% in this study compared to 18%). The isolates in the Gupta and colleagues study (1999) were from 150 clinical patients, which may have been previously treated with Trimethoprim- Sulfamethoxazole, while this study focused on environmental isolates which may not have been as readily exposed to the antibiotic. Antimicrobial resistance of environmental *E. coli* and *Salmonella* spp. isolated from urban and rural streams was previously studied by Thomas in 2011. Similar to this study, resistance to Trimethoprim-Sulfamethoxazole and Ciprofloxacin was found to be very low (>1%) (6.3% and 5.3% resistance respectively in this study) while over 44% of isolates were resistant to Ampicillin and Streptomycin (63.4% and 29.6% respectively, in this study). Antimicrobial resistance patterns exhibited by environmental isolates in this study showed greater resemblance towards previous studies using environmental isolates (Thomas, 2011) as opposed to clinical isolates (Gupta *et al.*, 1999).

A previous study from 1999 found a large increase in resistance to Ampicillin and Tetracycline from 18% to 78% and 53% to 89%, respectively between 1994 and 1999 (Gallardo *et al.*, 1999). Although some Oxytetracycline resistance was observed, it was not as high as the Tetracycline resistance in the Gallardo study. Oxytetracycline was one

of the broad spectrum Tetracyclines which was widely used in the States after their approval in 1957 (LiverTox, 2015). Due to an increase in antibiotic resistance many tetracyclines discontinued to be used, or are only used for veterinary medicine. Today, Oxytetracycline is used mainly for acne treatment and for the treatment of infections caused by *Chlamydia* (LiverTox, 2015). Due to the overall decreased use of Tetracyclines, the isolates from this study may not have had exposure to the antibiotic to the same degree as other antibiotics, resulting in a decreased occurrence of the mechanisms needed to survive in the presence of the antibiotic, possibly indicating that if selective pressures of the antibiotic are removed, resistance could decrease, or return to a manageable state.

Overall, 97% of *E. coli* isolates and 88.6% of *Salmonella* spp. isolates had MBEC >1024 µg/mL to Ampicillin, indicating that this antibiotic may not be successful in treating biofilm infections. *Salmonella* spp. had a greater resistance to Cefotaxime with 61% exhibiting MBECs >1024 µg/mL compared to 49% of *E. coli.* Resistance towards Streptomycin and Oxytetracycline was similar for both *E. coli* and *Salmonella* spp. with approximately 88%-91% of isolates having a MBEC >1024 µg/mL. The resistance is most likely due to the possible enhanced formation of biofilms in liquid medium, since both *E. coli* and *Salmonella* spp. isolates were able to produce biofilms in liquid culture to a certain degree. When cultures on agar plates were tested for antibiotic resistance it was found that 29.6% of isolates were resistant to Streptomycin and 2.9% were resistant to Oxytetracycline (Table 4.5), which is a considerably larger difference than the resistance seen in liquid-based biofilm forming isolates. Previous studies using three lab strains of *E.*

coli, *P. aeruginosa*, and *S. aureus*, when testing minimum inhibitory concentrations, compared to minimum biofilm eradication concentrations, found that the presence of biofilms can increase bacterial antibiotic resistance by up to 10-fold compared to planktonic cultures (Ceri *et al.*,1999). Although the sample size was smaller in the previous study, MBECs of >1024 µg/mL were also observed towards certain antibiotics tested (Ceri *et al.*, 1999).

Using the MBEC assay, it was observed that *E. coli* isolates were most sensitive to Cefotaxime and most resistant to Ampicillin (Figure 4.8). For the purpose of this study, "sensitivity" is referred to MBECs >1024 µg/mL (*e.g.* 512 µg/mL or 256 µg/mL), although these are still high antibiotic concentrations. *E. coli* isolates from Grand River and Grand Bend were the most sensitive to Cefotaxime. Grand River also had some isolates with MBECs of 512 µg/mL towards Streptomycin and Oxytetracycline. Similar patterns were also observed with *Salmonella* spp. (Figure 4.9) which also showed the highest level of sensitivity to Cefotaxime, but equally resistant to Ampicillin, Streptomycin and Oxytetracycline. Collectively, 92.7% of isolates displayed MBECs >1024 µg/mL towards Ampicillin, 54.8% towards Cefotaxime, 90% towards Streptomycin and 86% towards Oxytetracycline. Resistance was expected as 98.8% of isolates were able to produce biofilms to a certain degree at 37°C (most were weak biofilms, with some moderate and strong biofilm formation). When comparing resistance of predominantly host to predominantly non-host isolates it was observed that there was a larger prevalence of MBECs of 512 µg/mL in the predominantly non-host groups (Figure 4.10). In total, a

higher proportion of predominantly host isolates were able to produce biofilms compared to predominantly non-host isolates (90% vs 81%) (Figure 4.6, Figure 4.7.). These results support the idea that the increased resistance to antibiotics could be due to the larger number of isolates which were able to form biofilms (Figure 4.6, Figure 4.7) which, as described previously can often increase the antibiotic resistance of bacteria by inhibiting antimicrobial entry. Resistance can also be due to the presence of other resistance mechanisms. Bacteria could achieve antibiotic resistance through the spread of resistance genes such as β-lactamase or through a generalized stress response (Fux *et al.*, 2004). As noted earlier, isolates from predominantly host locations were more resistant to the antibiotics used in the disk-diffusion assay than the isolates for predominantly non-host locations, meaning that they could have resistance mechanisms helping the planktonic and sessile cultures cope with antimicrobial substances.

5.4. Acid Tolerance

Bacteria with pathogenic potential are often encountered in food and water supplies and upon ingestion, they may have the ability to colonize within the gastrointestinal tract and cause illness consistent with food poisoning, often causing symptoms of diarrhea, fever, nausea and vomiting. In order for the bacteria to reach the gastrointestinal tract, it must first pass through the stomach, and survive the acidic environment. This phase of the

research tested whether isolates from the various sampling locations, if ingested, would be able to survive the acidic environment of the stomach, similar to a study performed previously by Xia and colleagues (2009) using *Salmonella* spp. isolated from various food sources. Planktonic enriched cultures and biofilm enriched cultures in liquid medium were incubated in synthetic gastric juice and tested for acid tolerance. It was hypothesized that by allowing isolates to form biofilms, they would be able to better survive acidic conditions.

The experiment was carried out as originally described by Beumer *et al.* (1992) and Xia *et al.* (2009). Alterations were made to also accommodate testing using biofilms, where rather than transferring a bacterial culture to tubes with synthetic gastric juice, the gastric juice was transferred to a tube containing a formed biofilm (similar to the antibiotic treatment assays employed), ensuring that the attached cells were fully submerged in the gastric juice. Both versions of the experiment were repeated in triplicate. The results of these experiments indicated that acid tolerance did not greatly increase with the formation of biofilms. *E. coli* isolates were 88.9% tolerant to the synthetic gastric juice with the formation of biofilm, while 75.9% were tolerant without the formation of biofilm (Figure 4.11.). *Salmonella* spp. isolates were consistently tolerant with 86.8% displaying tolerance with biofilm formation and 90% without biofilm formation.

An examination of acid tolerance levels compared to source isolation was also conducted. *E. coli* isolates from Scugog River, Gull River and Nunavut proved to be more acid tolerant

with the formation of biofilms which could indicate that isolates from these locations relied more on the protection of biofilms rather than acid tolerance pathways (*e.g.* acid-induced oxidative system). Isolates from Grand River and Grand Bend were equally tolerant in conditions that promoted biofilm growth and conditions that promoted planktonic growth. Isolates from Coboconk Lagoon were more tolerant as planktonic cultures (100% vs 25%). The *E. coli* isolates from the Lagoon could have been using an acid tolerance pathway to survive as planktonic cultures, and turned off this pathway when in a biofilm. A previous study with *E. coli*, using random insertion mutagenesis with a transposon carrying a promoterless *lacZ* gene discovered that 38% of genes were differentially expressed when the cells were in a biofilm, compared to planktonic cells (Prigent-Combaret *et al.*, 1999). If cells are differentially expressing genes with the formation of biofilms, then acid tolerance pathways might also be affected. Additionally, microorganisms within a biofilm often have a low metabolic activity (Fux *et al.*, 2004), which could have prevented the isolates from implementing their acid tolerance pathway, leaving them unprotected in the event that the synthetic gastric juice penetrated the biofilm.

When comparing acid tolerance levels of *Salmonella* spp. to the locations of isolation, it was noted that isolates from Clair Lake, the Water Treatment Center, Coboconk Lagoon and Scugog River were 100% tolerant with and without the formation of biofilms, isolates from Fleming were more tolerant without biofilm formation (100% vs 78%) and isolates from Gull River were more tolerant with the formation of biofilms (100% vs 25%). *Salmonella* spp. acid tolerance has previously been studied by Xia *et al.* (2009) using 16

strains isolated from various food sources (spices, meat and cheese). Out of the total number of strains, 60% of them were found to be resistant using the same assay and it was deduced that the rest of the isolates were defective in the acid-tolerance pathway (Xia *et al.*, 2009). Acid tolerance of *Salmonella* spp. (in planktonic state) observed in the present study was higher than the tolerance observed by Xia and colleagues (2009) (90% vs 60%), which indicates that environmental isolates were able to use acid-tolerance pathways to survive in acidic environments, which may be of particular concern if these isolates encountered a host.

Acid tolerance was also compared between predominantly host isolates and predominantly non-host isolates. It was noted that predominantly host *E. coli* and *Salmonella* spp. were slightly more acid tolerant as planktonic cultures (84% vs 70% and 88% vs 82%, respectively) (Figure 4.12). Predominantly non-host isolates were more tolerant to the synthetic gastric juice if a biofilm was present; 98% vs 72% for *E. coli* and 100% vs 94% for *Salmonella* spp.. The observed pattern could be due to isolates from the predominantly host environment being previously exposed to a mammalian host, and already being selected for through the pressures of the low pH environment. These isolates may have had acid tolerance mechanisms while isolates from predominantly non-host environments, having not been exposed as recently to acidic environments, may have down regulated acid tolerance mechanisms thereby relying on their biofilms to protect them from the acidic environment.

5.5. Curli and Cellulose Expression

It has previously been noted that *E. coli* and *Salmonella* spp. form biofilms composed of curli fimbriae and cellulose (Branda *et al.*, 2005; Zogaj *et al.*, 2001; Romling *et al.*, 2003). Both *E. coli* and *Salmonella* spp., can produce biofilms using other components, for example Ag43, type I pili, or colanic acid, but curli fimbriae and cellulose were found to be expressed by both groups of microorganisms, under varying conditions. Since previous studies have noted the presence of curli and cellulose in many *E. coli* and *Salmonella* spp. biofilms, it was hypothesized that biofilm forming isolates would also express curli fimbriae and cellulose components. Expression of components was hypothesized to change with the change of temperature, just as biofilm formation varied with temperature.

The presence of *E. coli* and *Salmonella* spp. biofilm components was detected using congo red and calcoflour agar. The agar plates used for this assay contained no salt, making the results most comparable to biofilm formation results in media with no salt, due to the similarity in growth conditions. Overall, expression of curli and cellulose components was most prevalent at 37°C and weakest at 10°C. At host temperature, 79.8% of *E. coli* isolates expressed either curli fimbriae, or cellulose or both components, which decreased with temperature to 31.7% at 10°C (Table 4.8). The same pattern was seen with *Salmonella* spp. isolates which had 61.4% component expression at 37°C, and 14.8% at 10°C. Comparably, the previous biofilm assays employed in this study revealed that all *Salmonella* spp. isolates

and 97.2% of *E. coli* isolates were able to form biofilms at 37°C (Figure 4.1), indicating that these isolates must also use other components for the formation of biofilms (*e.g.* colanic acid, type I pili). In conditions of no salt, 100% of *Salmonella* spp. isolates were able to form biofilms at 37°C, 28°C and 23°C, and 98% were able to form biofilms at 10°C. Biofilm formation for *E. coli* isolates decreased as the temperature decreased, 97% were able to form biofilms at 37°C, 94.4% at 28°C and 23°C, and 75.7% at 10°C. Expression of curli fimbriae and cellulose also decreased with temperature (79.8% at 37°C compared to 31.7% at 10°C for *E. coli* isolates and 61.4% compared to 14.8% at 10°C for *Salmonella* spp. isolates), but there was still a higher proportion of biofilm formers when compared specifically to curli and cellulose expression. Additionally, most of the moderate and strong biofilms were observed at 23°C and 28°C indicating that strong biofilm formation is not necessarily due to the presence of curli and cellulose components. Alternatively, the moderate and strong degrees of biofilm formation could essentially be the same as weak biofilms, but with added layers of bacteria and other biofilm components. Future studies could explore this by monitoring expression of alternate biofilm components (*e.g.* type I pili, colanic acid) in parallel to biofilm formation at temperatures which promote strong and moderate biofilm formation (23°C and 28°C).

Biofilm component expression was examined based on source of isolation. Certain locations, such as Omeeme, Scugog River and Coboconk Lagoon *Salmonella* spp. expressed the same component at different temperatures (curli fimbriae at 37°C and 28°C), while isolates from most locations changed expression throughout the variety of

temperatures tested. For example, isolates from Clair Lake expressed only curli and only cellulose at 37°C, all morphotypes at 28°C, both curli and cellulose at 23°C, and all morphotypes again at 10°C. Expression of components has not previously been studied at lower temperatures (23°C, 10°C) but it has been found, using *E. coli* and *Salmonella* spp. isolated from animals and humans, that expression of components can vary between 37°C and 28°C (Bokranz *et al.,* 2005; Romling *et al.,* 2003), which is consistent with findings in the present study where alternating component exposure by environmental isolates was observed throughout the tested temperatures (10°C to 37°C) (Figure 4.13, Figure 4.14).

Upon evaluating component expression by all *E. coli* isolates, it was found that *E. coli* expressed the curli only morphotype, as well as the curli and cellulose morphotype at all temperatures tested. There have not been many studies testing the effects of temperature on *E. coli* curli and cellulose expression, but a previous study by Saldana *et al.* (2009) tested curli expression of 20 Enterohemorrhagic and Enteropathogenic *E. coli* strains at 37°C. Expression was tested to determine whether the pathogens would be able to attach and colonize at host temperatures. It was found that 80% of their strains were able to produce curli at host temperature. This is consistent with the present study in which 76.9% of the 105 *E. coli* isolates tested were able to express curli at host temperature, providing similar results to the Enterohemorrhagic and Enteropathogenic strains and proving that these trends hold true to a wider scale with environmental isolates that are likely not as highly pathogenic. Another study, using seven *E. coli* clinical isolates, found that urinary tract *E. coli* isolates were capable of producing curli and cellulose at 28°C but not 37°C,

while commensal *E. coli* isolates were able to express the components at both temperatures (Weiss-Muszkat *et al.,* 2010). Isolates used in this experiment were able to express curli and cellulose components at both 28°C and 37°C (Figure 4.14) making them more similar to the commensal isolates in the Weiss-Muszkat study. Environmental *E. coli* profiled in this study exhibited similar biofilm component expression characteristics to *E. coli* isolates previously studied from host locations (Saldana *et al.,* 2009; Weiss-Muszkat *et al.,* 2010), which are known to be able to colonize within a host, indicating that environmental isolates may be able to express components to colonize within a host.

A pattern that was observed throughout the *E. coli* and *Salmonella* spp. isolates, was the low expression of the cellulose-only morphotype (2.88% and 9.9% at 37°C for *E. coli* and *Salmonella* spp., respectively). This was also observed in the results of previous studies, where *Salmonella* spp. most commonly expressed curli and cellulose (Romling *et al.,* 1998; 1999; 2003). In these studies, mutants were created to express either curli or cellulose. The cellulose-only morphotype may not be often expressed, because biofilm production is generally enhanced by the presence of an anchoring protein. Cellulose biofilms have been shown to provide tensile strength and protection to the community, but without the expression of proteins such as curli, the biofilm may be hindered in attaching to surfaces (Solano *et al.,* 1998). Bacteria may be selecting against the cellulose-only morphotype, because cells may not be well-protected if they are unable to attach to a surface. The small amount of isolates exhibiting the cellulose-only morphotype, could have also been

expressing alternate proteins that aid in attachment, not recognized by the congo red and coomassie blue dye, therefore allowing them to form biofilms at the air liquid interface.

6. Summary and Conclusions

The overall objective of this study was to better elucidate the association between host and non-host biofilms. In order to examine that, *E. coli* and *Salmonella* spp. isolates were tested for their ability to form biofilms under non-host conditions, and their capability to survive certain stressors such as antibiotics and acidic environments following the formation of biofilms. The isolates were also subdivided into two groups, predominantly host and predominantly non-host. The predominantly host isolates were extracted from areas of wastewater treatment and could have recently been in a host environment, while predominantly non-host isolates were isolated from watersheds and storm water ponds and were expected to be more accustomed to environmental fluctuations.

The first hypothesis of the study was:

"*More environmental isolates of E. coli and Salmonella* spp. *from predominantly host locations will form biofilms than isolates from non-host locations*."

The first hypothesis was tested by isolating *E. coli* and *Salmonella* spp. from predominantly host and predominantly non-host environmental sources, and identifying biofilm formers. Biofilm formation was examined using a microtiter biofilm assay, where isolates were inoculated into media with and without salt (to mimic host and non-host environments, respectively) and incubated at 10°C, 23°C, 28°C and 37°C. This was the first study to examine biofilm formation in both a low (10°C) and warm (37°C) temperature range. Overall, it was observed that most isolates were able to form biofilms at low and warm temperatures. The largest proportion of biofilm formation was observed at 37°C, in media with no salt. It had previously been noted that curli fibres prefer to grow in media with no salt (Romling *et al.,* 1998). If expression of biofilm components is more prominent in media with no salt, then biofilm formation may also be observed in higher proportion under similar conditions, as evidenced by biofilm formation in this study. For example, 100% of *Salmonella* spp. isolates formed biofilms (73.8% of which were categorized as moderate or strong biofilms) in media with no salt at 28°C, while in media with salt, 96% of isolates formed biofilms (58.2% of which qualified as moderate or strong). It was found that the highest proportion of strong biofilms were formed at 28°C and 23°C, which could be due to bacteria requiring a thicker biofilm for protection from environmental factors, while

biofilms classified as weak would be sufficient for protection within a host (*i.e.* just enough biofilm to mask bacterial antigens from host immune response). When comparing biofilm formation between predominantly host isolates and predominantly non-host isolates, it was noted that the patterns of biofilm formation were similar through all the temperatures tested. Overall, the first hypothesis was not supported: Environmental isolates of *E. coli* and *Salmonella* spp. from predominantly host locations did not exhibit more biofilm formation that isolates from predominantly non-host locations, biofilm formation patterns were similar.

Hypothesis 2:

"*Biofilm forming isolates will be able to better survive in various environmental, non-host conditions and host-like conditions."*

The second hypothesis was tested by exposing isolates to various temperatures in media with salt and no salt (as summarized above), antibiotic testing and acid tolerance testing. It was found that *E. coli* and *Salmonella* spp. isolates were able to form biofilms under host and non-host conditions. Isolates were tested for their ability to survive antimicrobial treatment and synthetic gastric juice, which are both conditions that bacteria might encounter within a host. It was found that isolates were more resistant to antibiotics when biofilms were formed in liquid cultures, as opposed to isolates grown on agar plates, as evidenced by the majority of isolates exhibiting MBECs >1024 µg/mL. It has previously

been noted that the presence of biofilms can increase an organism's antibiotic resistance by 10-fold (Ceri *et al.,* 1999). Biofilms were hypothesized to increase isolates' abilities to survive in synthetic gastric juice. This was the first study to test acid tolerance in conditions that encouraged biofilm formation for gram negative bacteria. This present study found that isolates from the predominantly non-host locations, the presence of biofilms increased the microorganisms' resistance to the acidic environment (98.1% compared to 79% without biofilm formation). Predominantly host isolates were better able to survive in the synthetic gastric juice in cultures that promoted planktonic growth (86.9% compared to 77.3% with biofilm formation). This was suspected to be due to the predominantly host isolates being more recently exposed to a host environment, and as such, have had to use their acid-tolerance pathways, while the predominantly non-host isolates had grown accustomed to the non-host environment and relied on the general protection of the biofilm. Biofilms were able to protect the microorganisms from antibiotics and synthetic gastric juice, when acid tolerance pathways were not employed. Overall, it can be concluded that the formation of biofilms aided in the survival of isolates under various host and non-host conditions.

Hypothesis 3:

"Biofilm formers will express curli fimbriae and cellulose biofilm components to various degrees that may affect the type and resilience of the biofilm formed."

The third hypothesis was tested by performing compositional analysis of biofilms with the use of congo red and calcoflour plates to detect the presence of curli fimbriae and cellulose components. Curli fimbriae and cellulose have previously been found to be expressed by *E. coli* and *Salmonella* spp. (Branda *et al.,* 2005; Zogaj *et al.,* 2001; Romling *et al.,* 2003), thus, it was hypothesized that environmental isolates from this study would express the biofilm components under various conditions. This was the first study to our knowledge, to test curli and cellulose expression at lower temperatures (10°C, 23°C). Expression of components was highest at 37°C (79.8% and 61.4% for *E. coli* and *Salmonella* spp., respectively) and lowest at 10°C (31.7% and 14.8% for *E. coli* and *Salmonella* spp., respectively), with a higher proportion of *E. coli* isolates expressing more curli and cellulose than *Salmonella* spp. isolates. Previous studies have noted the expression of components at 37°C and 28°C by *E. coli* and *Salmonella* spp. isolated from humans and animals (Branda *et al.,* 2005; Zogaj *et al.,* 2001; Romling *et al.,* 2003), but these studies did not explore the same range of temperatures tested in this study, nor component expression of environmental isolates. Overall, it was observed that while some biofilm formers (ranging from 14.8% to 79.8% throughout the temperatures) expressed curli and cellulose, biofilms were also formed without the presence of these components, indicating

that isolates are using additional components to aid with biofilm formation. The third hypothesis was partially supported because while some weak, moderate and strong biofilm formers expressed curli and cellulose components, biofilms were also formed using additional components not explored in this thesis.

It was evident from this study that environmental isolates of *E. coli* and *Salmonella* spp. are not only able to survive in non-host environments, but also in situations that mimic a host (*i.e.* acidic environments, antibiotic exposure). It was observed that isolates were able to form biofilms at host and non-host temperatures and that these biofilms (particularly liquid-culture biofilms) aided bacterial survival in the presence of antimicrobials and in some cases, synthetic gastric juice. Even without the formation of biofilms (in liquid culture), isolates exhibited antimicrobial resistance (63.4% of isolates were resistant to ampicillin). This suggests that action should be taken to prevent improper use of antibiotics in order to reduce bacterial exposure to antimicrobials. Additionally, since bacteria are able to produce biofilms, which provide antimicrobial tolerance and protection from acidic environments, at a variety of host and non-host temperatures, it is important to continue research in bacterial biofilms, geared towards finding methods of biofilm formation prevention.

The integrative nature of this thesis can be observed in part through the methodology which used microbiology and molecular techniques. Molecular techniques such as PCR were used in the isolation of *Salmonella* spp.. Microbiology techniques were routinely used to study

biofilm formation, antibiotic resistance, acid tolerance and curli and cellulose expression. Overall, *Salmonella* spp. and *E. coli* isolates were studied as individual organisms and also as part of a community (biofilms) in the presence of temperature stressors, antibiotics, and acidic environments. The data provided valuable insight on the abilities of biofilms to protect microorganisms from stressors.

In addition, this research used *E. coli* and *Salmonella* spp. environmental isolates from a variety of locations including areas where isolates were recently exposed to the non-host environment (predominantly host) and areas where isolates have been in the non-host environment for extended periods of time (predominantly non-host). This provided a great opportunity to study the effects of areas impacted by humans, agriculture, waterfowl and environmental factors on the bacteria present in those areas. Environmental isolates were collected and studied for their ability to persist in the environment, and for their potential to survive within a host, having implications in medicine, food industry and environmental studies.

Future Directions and Recommendations

- Additional biofilm studies with more thorough analysis of biofilm components should be performed using bacteria not only from environmental sources, but also from sources of host infection in order to better understand the impact that a host

vs. non-host environment can have on the biofilm forming capabilities and the type of components expressed by the bacteria resulting in biofilm formation.

- Additional experiments could include testing environmental isolates (predominantly host and predominantly non-host) for their ability to invade/attach to mammalian cells and form biofilms, in order to gain a better understanding of how the biofilm forming capabilities of the environmental isolates affect their ability to infect a host.

- Since greater than 98% of isolates tested in this study were able to form biofilms under a variety of different conditions, and biofilms have been shown to cause chronic infections, it is important to study methods of minimizing biofilm formation, for example through the use of materials that do not promote bacterial attachment, such as plasma modified biomaterials (Bazaka *et al.*, 2011).

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Appendix A

Recipes:

LB broth: 5g yeast extract, 10g Nacl, 10g tryptone, 1L dH_2O

LB broth with no salt: 5g yeast extract, 10g tryptone, 1L dH_2O

LB agar: 5g yeast extract, 10g NaCl, 10g tryptone, 1L dH_2O

LB slants: prepare LB agar and autoclave. Allow to 50°C then pour into a culture tube filling halfway. Lay tubes onto a slant board and allow to solidify.

Synthetic Gastric Juice: 8.3g protease-peptone, 3.5g glucose, 2.05g NaCl, 0.6g KH_2PO_4, O.11g $CaCl_2$, 0.37g KCl, 0.05g porcine bile, 0.1g lysozyme, 13.3mg pepsin, 1L dH_2O, pH adjusted to 3.0 with 6N HCl.

1X TAE (tris-acetate- EDTA)- prepare 1L of 50X solution and dilute accordingly, 242.5g Tris, 57 mL glacial acetic acid 100mL 0.5M EDTA, add deionized water to 1L.

Sodium-Free Phosphate Buffer- 5mL $MgCl_2$, 1.25mL KH_2PO_4, 1L MiliQ H_2O

Appendix B

Table A1. Absorbance of *E. coli* Biofilms at 10°C in LB With Salt.

**E. coli* isolates from Scugog River

	10-Nov				23-Nov				28-Nov			
Isolate*	**Replicate 1**	**Replicate 2**	**Replicate 3**	**Replicate 4**	**Replicate 1**	**Replicate 2**	**Replicate 3**	**Replicate 4**	**Replicate 1**	**Replicate 2**	**Replicate 3**	**Replicate 4**
MS1	-0.012	-0.013	-0.012	-0.013	0.016	0.015	0.011	0.01	0.121	0.083	0.11	0.13
MS2	-0.011	-0.011	-0.011	-0.013	0.073	0.125	0.083	0.05	0.08	0.121	0.138	0.024
MS3	-0.012	-0.011	-0.012	-0.01	0.01	0.01	0.008	0.007	0.123	0.054	0.055	0.044
MS4	-0.013	-0.013	-0.013	-0.012	-0.003	-0.003	-0.002	-0.004	0.072	0.069	0.066	0.055
MS5	-0.013	-0.012	-0.013	-0.011	0.018	0.022	0.004	0.127	0.051	0.046	0.045	0.034
MS6	-0.012	-0.012	-0.013	-0.012	0.137	-0.001	0.085	0.001	0.14	0.063	0.049	0.048
MS7	-0.011	-0.012	0.123	0.094	0.017	0.011	0.094	0.013	0.062	0.064	0.055	0.053
MS8	-0.004	-0.01	0	-0.011	0.527	0.153	0.562	0.414	0.049	0.05	0.05	0.052
RPL21	0.46	0.421	0.415	0.54	0.116	0.103	0.053	0.136	0.086	0.107	0.097	0.072
RPL22	0.007	0.01	0.007	0.016	0.018	0.009	0.009	0.011	0.114	0.074	0.069	0.093
RPL23	0.046	0.038	0.036	0.022	0.045	0.043	0.047	0.093	0.485	0.426	0.429	0.358
RPL24	0.028	0.02	0.006	0.021	0.037	0.045	0.045	0.044	0.056	0.047	0.042	0.044
MSPL21	0.003	0.021	0.001	0.001	0.001	0.012	0.007	0.052	0.001	0.008	0.008	0.005
MSPL22	0.002	0.002	0	0.004	0.001	0.003	0.001	0.003	0.027	0.02	0.012	0.057
MSPL23	0.004	0.003	0.002	0.002	0.064	0.045	0.026	0.025	0.364	0.495	0.105	0.172
MSPL24	0.003	0.002	0.001	0	0.076	0.101	0.021	0.003	0.039	0.038	0.034	0.019
MSPL25	0.008	0.003	0.002	0	0.153	0.216	0.205	0.146	0.348	0.27	0.404	0.358
MSPL26	0.003	0.004	0	0.003	0.175	0.144	0.175	0.046	0.159	0.219	0.031	0.109
MSPL27	0.002	0	0.002	0	0.013	0.013	0.012	-0.001	0.044	0.051	0.057	0.028
MSPL28	0.002	0.001	0	0.002	0.003	0.004	0	0.005	0.005	0.006	0.012	0.002
CR21	0.025	0.036	0.022	0.022	0.086	0.238	0.062	0.054	0.118	0.113	0.097	0.11
CR21	0.016	0.041	-0.006	0.004	0.065	0.143	0.06	0.056	0.014	0.026	0.016	0.015
CR23	1.015	0.919	0.262	0.903	0.108	0.101	0.104	0.184	0.124	0.114	0.071	0.122
CR24	0.124	0.659	0.004	0.064	0.086	0.266	0.273	0.286	0.014	0.014	0.011	0.008
CR25	0.125	0.023	0.004	0.091	0.031	0.131	0.14	0.042	0.463	0.415	0.397	0.315
CR26	0.037	0.115	0.132	0.032	0.065	0.069	0.118	0.058	0.019	0.02	0.018	0.012
CR27	0.119	0.109	0.117	0.088	0.052	0.053	0.058	0.048	-0.003	-0.004	-0.005	-0.002
CR28	0.018	0.036	0.024	0.155	0.06	0.065	0.066	0.052	0.008	0.006	0.007	0.002
CRR2	0.025	0.027	0.018	0.026	0.06	0.067	0.07	0.063	0.012	0.007	0.007	0.017
CRR3	0.006	0.003	0.005	0.002	0.058	0.205	0.052	0.152	0.131	0.115	0.084	0.093
CRR4	0.143	0.027	0.019	0.085	0.062	0.062	0.059	0.058	0.421	0.642	0.608	0.636

Table A2. Example of Antibiotic Resistance Results Using the Disk Diffusion Assay.

	CTX					AMC				
Isolate*	Trial 1	Trial 2	Trial 3	AVG	R/I/S	Trial 1	Trial 2	Trial 3	AVG	R/I/S
E20-6	26		36	31	S	19		25	22	S
E16-6	26	10	33	23	S	23	14	24	20.33333333	S
E6-5	23	11	31	21.6666667	I	21	15	22	19.33333333	S
E13-6	26	10	39	25	S	21	13	24	19.33333333	S
E19-6	29	10	30	23	S	20	16	22	19.33333333	S
E16-6	24	11	33	22.6666667	S	16	16	24	18.66666667	S
E4-5	28	12	30	23.3333333	I	20	15	21	18.66666667	S
E6-6	29	10	31	23.3333333	S	19	14	22	18.33333333	S
E18-6	26	10	33	23	S	16	15	24	18.33333333	S
E4-6	25	12	30	22.3333333	S	18	15	22	18.33333333	S
E12-6	25	10	29	21.3333333	I	18	14	21	17.66666667	S
E14-6	25	10	32	22.3333333	S	18	14	21	17.66666667	S
E9-6	8	12	30	16.6666667	I	14	14	23	17	S
E7-6	26	9	26	20.3333333	I	17	12	20	16.33333333	I
E7-5	23	10	25	19.3333333	I	17	15	14	15.33333333	I
E2-6	10	10	28	16	I	16	12	15	14.33333333	I
E15-6	26	10	30	22	S	18	14	11	14.33333333	I
E11-6	26	9	0	11.6666667	R	20	14	0	11.33333333	R
E1-6	26	11	0	12.3333333	R	18	15	0	11	R
E11-5	26	18	16	20	I	19	14	0	11	R
E3-6	25	8	0	11	R	20	12	0	10.66666667	R
E17-6	9	10	0	6.33333333	R	14	13	0	9	R
	T					S				
Isolate*	Trial 1	Trial 2	Trial 3	AVG	R/I/S	Trial 1	Trial 2	Trial 3	AVG	R/I/S
E20-6	21		22	21.5	S	14		18	16	S
E16-6	20	18	22	20	S	16	23	19	19.33333333	S
E6-5	18	19	20	19	S	13	25	16	18	S
E13-6	20	20	23	21	S	16	25	16	19	S
E19-6	21	18	19	19.3333333	S	16	24	18	19.33333333	S
E16-6	19	19	22	20	S	0	25	19	14.66666667	S
E4-5	20	18	20	19.3333333	S	15	16	18	16.33333333	S
E6-6	21	18	20	19.6666667	S	12	23	16	17	S
E18-6	18	18	22	19.3333333	S	14	25	19	19.33333333	S
E4-6	20	18	21	19.6666667	S	15	16	19	16.66666667	S
E12-6	18	19	19	18.6666667	S	15	20	19	18	S
E14-6	19	18	21	19.3333333	S	15	23	16	18	S
E9-6	18	19	21	19.3333333	S	18	25	0	14.33333333	S
E7-6	18	19	19	18.6666667	S	13	25	18	18.66666667	S
E7-5	18	16	18	17.3333333	S	15	25	16	18.66666667	S
E2-6	15	20	0	11.6666667	R	26	25	0	17	S
E15-6	21	18	20	19.6666667	S	16	25	16	19	S
E11-6	20	18	19	19	S	0	22	0	7.333333333	R
E1-6	16	18	21	18.3333333	S	14	28	13	18.33333333	S
E11-5	19	16	21	18.6666667	S	12	26	9	15.66666667	R
E3-6	18	15	0	11	R	13	19	0	10.66666667	R
E17-6	20	18	20	19.3333333	S	15	23	0	12.66666667	I

**E. coli* isolates from Grand River

CTX= Cefotaxime 30µg, AMC= Amoxicillin/Clauvulanic acid 20/10 µg, T= Oxytetracycline 30 µg and S=Streptomycin 10 µg.

R=resistant, I= intermediate resistance, S= susceptible

Table A3. MBECs of a Subset of *E. coli* Isolates.

	Ampicillin			Cefotaxime			Streptomycin			Oxytetracycline		
Isolate*	256µg/mL	512µg/mL	1024µg/mL	256µg/mL	512µg/mL	1024µg/mL	256µg/mL	512µg/mL	1024µg/mL	256µg/mL	512µg/mL	1024µg/mL
GB1	+++	+++	+++	+++	+++	---	+++	+++	+++	+++	+++	+++
GB2	+++	+++	+++	+++	+++	-++	+++	+++	++-	+++	+++	+-+
GB3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
GB4	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
GB5	+++	+++	+++	+++	+++	---	+++	+++	+++	+++	+++	+++
GB6	+++	+++	+++	+++	+++	--+	+++	+++	+++	+++	+++	+++
GB7	+++	+++	+++	+++	+++	---	+++	+++	+++	+++	+++	+++
GB8	+++	+++	+++	+++	+++	---	+++	+++	++-	+++	+++	+++
GB9	+++	+++	+++	+++	+++	---	+++	+++	+++	+++	+++	--+
GB10	+++	+++	+++	+++	+++	---	+++	+++	+++	+++	+++	+++

**E. coli* isolates from the Grand Bend location

"+" indicates growth "-" indicates no growth (therefore susceptible), each symbol is one replicate

Table A4. Acid Tolerance of a Subset of *E. coli* Isolates.

	Isolate*	Trial 1	Trial 2	Trial 3	Positive Control
Scugog River	**MS1**	+	+	+	+
	MS2	+	+	+	+
	MS3	+	+	+	+
	MS4	+	+	+	+
	MS5	+	+	+	+
	MS6	+	+	+	+
	MS7	+	+	+	+
	MS8	+	+	+	+
	RPL22	+	+	+	+
	RPL23	-	-	-	+
	RPL24	-	-	-	+
	MSPL21	-	-	-	+
Gull River	**CR21**	-	-	+	+
	CR22	+	+	+	+
	CR23	-	-	-	+
	CR24	-	-	-	+
	CR25	-	-	-	+
	CR26	-	-	-	+
	CR27	-	-	-	+
	CR28	-	-	+	+
	CRR2	-	-	-	+
	CRR3	-	-	-	+
	CRR4	-	-	-	+

*Subset of isolates from Scugog River and Gull River

"+" indicates growth, therefore acid tolerance, "-" indicates no growth

The positive control is the isolate grown in LB media.

Table A5. Cellulose and Curli Expression of a Subset of *E.coli* Isolates.

	37°C						28°C					
	Trial 1		Trial 2		Trial 3		Trial 1		Trial 2		Trial 3	
Isolate*	**Congo Red**	**Calcoflour**	**Congo Red**	**Calcoflour**	**Congo Red**	**Calcoflour**	**Congo Red**	**Calcoflour**	**Congo Red**	**Calcoflour**	**Congo Red**	**Calcoflour**
RPL21	rdar	+	rdar	+	rdar	+	rdar/saw	+/-	rdar/saw	+/-	bdar	-
MSPL25	bdar	-	bdar	-	saw/bdar	-	bdar	-	bdar	-	bdar	-
MSPL27	rdar	+	bdar	-	saw	-	bdar	-	bdar	-	saw	-
RPL24	rdar	+	rdar	+	rdar/saw	-	rdar	+	rdar	+	bdar	-
RPL23	rdar	+	rdar	+	rdar	+	rdar	+	rdar	+	bdar	-
MSPL26	rdar	+	rdar	+	rdar	+	rdar	+	rdar	+	rdar	+
MSPL21	rdar	+	rdar	+	rdar/saw	+	ng		ng		ng	
MSPL23	rdar	+	rdar	+	saw/bdar	-	rdar	+	rdar	+	rdar	+
	23°C						**10°C**					
	Trial 1		Trial 2		Trial 3		Trial 1		Trial 2		Trial 3	
Isolate*	**Congo Red**	**Calcoflour**	**Congo Red**	**Calcoflour**	**Congo Red**	**Calcoflour**	**Congo Red**	**Calcoflour**	**Congo Red**	**Calcoflour**	**Congo Red**	**Calcoflour**
RPL21	bdar	-	bdar	-	bdar	-	bdar	-	bdar	-	bdar	-
MSPL25	bdar	-	bdar	-	rdar/saw	+/-	rdar/saw	+/-	rdar/saw	+/-	rdar/saw	+/-
MSPL27	bdar	-	bdar	-	ng		rdar	+	rdar	+	saw	-
RPL24	pdar/saw	+/-	pdar/saw	+/-	pdar/saw	+/-	saw	-	saw	-	saw	-
RPL23	pdar/saw	+/-	pdar/saw	+/-	pdar/saw	+/-	saw	-	saw	-	saw	-
MSPL26	rdar	+	rdar	+	rdar	+	rdar/saw	+/-	rdar/saw	+/-	rdar	+
MSPL21	saw	-	saw	-	ng		saw	-	saw	-	saw	-
MSPL23	rdar/saw	+/-	rdar/saw	+/-	rdar	+	bdar	-	bdar	-	bdar	-

*Subset of *E. coli* isolates from Scugog river

rdar represents curli and cellulose expression, pdar only cellulose, bdar only curli and saw is neither component

Table A6. CLSI 2007 Table of *Enterobacteriaceae* Antibiotic Resistance.

Organism \ Antimicrobial Agent	Ampicillin	Amoxicillin-clavulanate	Ampicillin-sulbactam	Piperacillin	Ticarcillin	Cephalosporin I: Cefazolin, Cephalothin	Cephamycins: Cefoxitin, Cefotetan	Cephalosporin II: Cefuroxime	Tetracyclines	Nitrofurantoin	Polymyxin B Colistin
Citrobacter freundii	R	R	R			R	R	R			
Citrobacter koseri	R	R	R	R	R						
Enterobacter aerogenes	R	R	R			R	R	R			
Enterobacter cloacae	R	R	R			R	R	R			
Escherichia coli	There is no intrinsic resistance to β-lactams in this organism.										
Escherichia hermannii	R				R						
Hafnia alvei	R	R	R			R	R				
Klebsiella pneumoniae	R				R						
Morganella morganii	R	R				R		R	R	R	R
Proteus mirabilis	There is no intrinsic resistance to β-lactams in this organism.								R	R	R
Proteus penneri	R					R		R	R	R	R
Proteus vulgaris	R					R		R	R	R	R
Providencia rettgeri	R	R				R			R	R	R
Providencia stuartii	R	R				R			R	R	R
Salmonella and *Shigella* spp.	There is no intrinsic resistance to β-lactams in these organisms; see Table 2A, comment (6) for reporting.										
Serratia marcescens	R	R	R			R	R	R		R	R
Yersinia enterocolitica	R	R			R	R					

(CLSI, 2007)

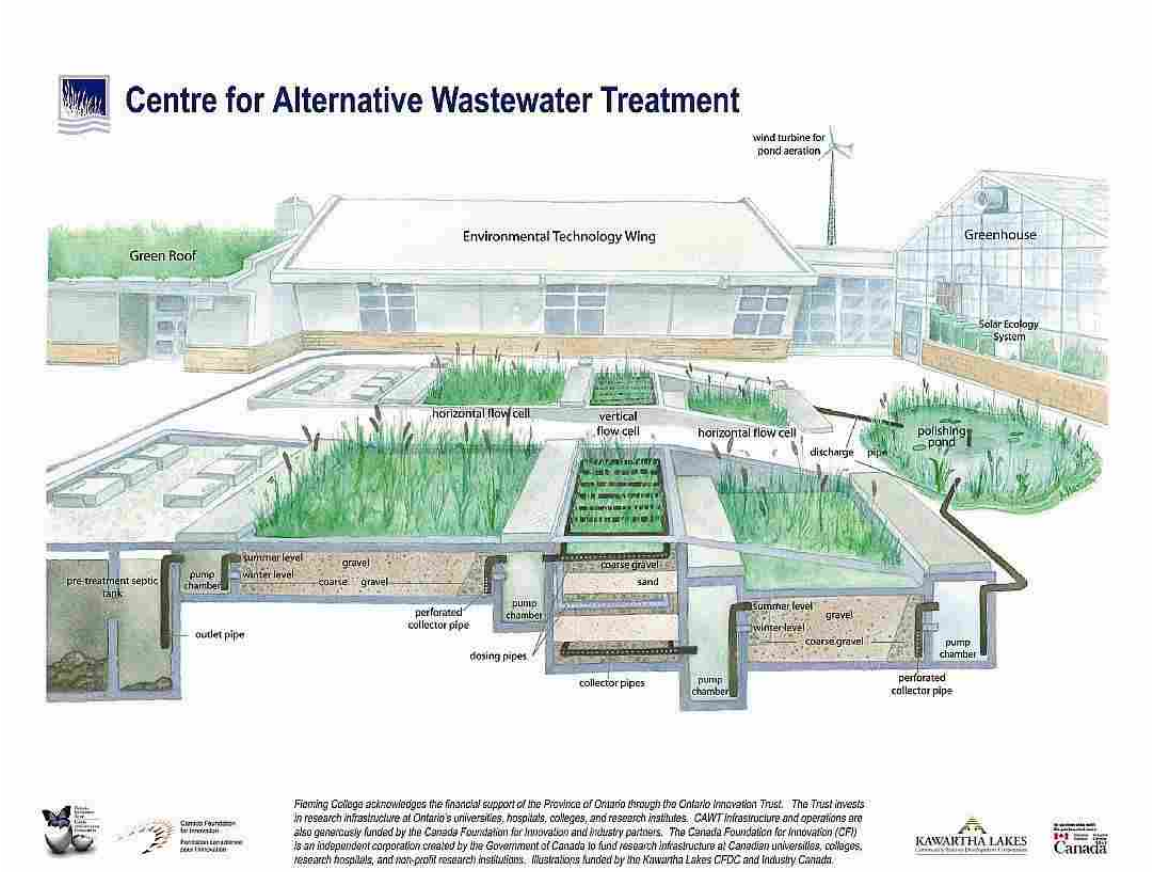

Figure A1. Schematic representation of the CAWT constructed wetland. The wetland is constructed to treat domestic waste from the Frost Campus of Fleming College and consists of three test vaults and a final polishing pond. The wastewater is then either discharged into the municipal sewer system or recycled back into the wetland (image obtained from http://www.iees.ch/EcoEng071/EcoEng071_Wootton.html)