

Characterization of Sediment, Sewage and O157 *E. coli*:

**A comparison of their virulence genes, biofilm forming capacities, antibiotic resistance
and level of reactive oxygen species (ROS)**

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Abstract of Thesis

Even though sediment *Escherichia coli* is a common source of contamination in freshwater environments, their virulence and physiological characteristics are not well studied. The goal of this study was to compare the virulence genes, biofilm forming capacity, antibiotic resistance and reactive oxygen species (ROS) level of sediment *E. coli* isolated from three freshwater lakes (Lake Simcoe, Georgian Bay, and Boulevard Lake) with no exposure to sewage effluent to sewage *E. coli* from two sewage treatment plants (Orillia and Thunder Bay Ontario) and a collection of known O157 *E. coli*. Multiplex PCR analysis on nine virulence genes (*hlyA*, *iroN*, *papA*, *hs*, *hl*, *ial*, *bfpA*, *stx1*, and *stx2*) revealed that no sediment, and very few (3%) sewage *E. coli* contained diarrheagenic and shiga toxin genes. However, 12.5% of sediment and 42% of sewage isolates contained one or more uropathogenic genes. Interestingly, only the *iroN* gene was detected in the sediment isolates. The biofilm assays determined that sediment *E. coli* were significantly better biofilm formers ($p < 0.001$) than the sewage and O157 *E. coli*, and the sediment *E. coli* was able to form 2 and 3.5 times as much biofilm as the sewage and O157 *E. coli*, respectively. The antibiotic resistances of the isolates to eight antibiotics were determined using the Kirby-Bauer disk diffusion method and the antibiotic resistance patterns of the *E. coli* samples illustrated that the sediment, sewage and O157 isolates belonged to three distinctive populations. Furthermore, there was an overall significant difference between the three sample groups ($p < 0.05$), where sediment was the most susceptible and sewage was the most resistant to the antibiotics tested. It was also determined that the level of ROS in biofilm *E. coli* cells was significantly lower than their planktonic counterparts ($p < 0.001$). A negative correlation ($p = 0.066$) was observed when comparing the isolates' biofilm forming capacities

with their intrinsic level of ROS, whereby isolates with higher tolerance to oxidative stress (i.e. higher amounts of cellular ROS) were associated with lower biofilm forming capability. Furthermore, isolates with higher resistance to antibiotics in their planktonic state also showed lower biofilm forming capacity.

It has been previously determined that the *rpoS* gene, a crucial regulatory gene in biofilm phase bacteria, is able to improve the survival of *E. coli* by optimizing the size of the biofilm matrix. The role of stationary phase sigma factor (RpoS) of an *E. coli* O157:H7 H32 strain in the biofilm phase was examined by comparing the biofilm formation capacity, reactive oxygen species (ROS) level, and antibiotic resistance of the wildtype H32 to its *rpoS* mutant. The mutant strain formed significantly more biofilm ($p < 0.05$) than the wildtype strain with the mutant strain forming twice as much biofilm as the wildtype strain. To investigate the level of ROS in the two *E. coli* strains, a DCF-DA assay was conducted and revealed a significant difference between the exponential growth phase and biofilm state of the wildtype H32 strain with biofilm cells illustrating over 100,000 times lower ROS levels ($p < 0.05$) than exponential phase cells (values of 4.04×10^{-5} ROS/ μg protein $\pm 2.67 \times 10^{-5}$ and 4.057 ± 0.251 ROS/ μg protein respectively). Within the biofilm phase, the mutant cells illustrated significantly higher ROS levels than the wildtype cells ($p < 0.05$) with values of 0.373 ± 0.250 ROS/ μg protein and $4.04 \times 10^{-5} \pm 2.67 \times 10^{-5}$ ROS/ μg protein respectively, supporting the idea that the *rpoS* gene is an essential regulatory gene for lowering the ROS level within bacteria. The levels of antibiotic resistance of the wildtype and mutant *E. coli* biofilm cells were determined for eight antibiotics using the Kirby-Bauer disk diffusion method. When compared with the wildtype H32 strain, the *rpoS* mutant strain showed an overall lower resistance across the eight antibiotics tested in the

biofilm state ($p < 0.05$). With the support of the ROS data, the decreased antibiotic resistance of the H32 mutant in biofilm state further illustrates the role of *rpoS* on increasing antibiotic resistance of biofilm cells through the reduction of cellular ROS level.

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Chapter 1:

Literature Review:

**Virulence Factors, Biofilm Forming Capacity, Antibiotic Resistance,
and Oxidative Stress of *Escherichia coli***

1.1 Introduction

Escherichia coli (*E. coli*) has been studied for many years and is used as a model type strain to examine the physiology, biochemistry and the genetics of gram-negative bacteria. There are many subtypes and strains of *E. coli* that can be found in a wide variety of environments including humans, water, periphyton, sediment, and animals (Quero et al., 2015). Very few studies have examined the differences between the various sources of *E. coli* and compared them on a large scale. In this context, this study aims to examine the *E. coli* from sediment in fresh water lakes in Northwestern and Southern Ontario and compare them to the *E. coli* found in sewage and a known collection of O157 pathogenic *E. coli*. The characteristics of the *E. coli* to be studied include their virulence genes, biofilm forming capacity, antibiotic resistance, and intrinsic levels of reactive oxygen species (ROS). It is important to study each of these characteristics as each can be attributed to either bacterial survival or disease-causing abilities. Being capable of surviving in stressful conditions is essential for *E. coli* in the natural environment. Both biofilm forming capabilities and the tolerance to oxidative stress have been linked to overall survival capabilities (Jakubowski and Bartosz. 2000). Bacteria, such as *E. coli*, are of major concern to humans due to their ability to cause diseases such as urinary tract infections or diarrheal diseases. The ability to cause disease is only possible for *E. coli* due to the possession of virulence genes. Without these genes, the bacteria would be of minimal health threat to the human population. Disease causing *E. coli* are even more of a health concern if they show resistance to antibiotics. With the number of antibiotic resistant bacteria growing each year, antibiotic resistance is a prominent area of microbiology research today. By examining all of these factors in the sewage and sediment *E. coli*, a more comprehensive

understanding of this bacteria living within the sediment can be established. Without this investigation, connections between each of these innate abilities within *E. coli* populations cannot be fully understood.

1.2 *Escherichia coli*

Belonging to the family Enterobacteriaceae, *E. coli* are gram-negative rod shaped, aerobic bacteria that can normally be found in the gut flora of mammals (Beloin et al., 2008; Holt, 1993; Kuhnert et al., 2000; Merckx-Jacques et al., 2013; Percival et al., 2014). Most *E. coli* are harmless, however, there are some strains that can be harmful and cause diseases in humans (Merckx-Jacques et al., 2013). In the 1890's, *E. coli* became a biological indicator for water safety, and since then it has been used as part of the recreational and drinking water regulations (Edberg et al., 2000). In fresh water, *E. coli* remains to be one of the most reliable indicators of fecal contamination thus far as both human and cattle remain to be primary reservoirs of pathogenic *E. coli* groups (Health Canada, 2012). In the Guidelines for Canadian Recreational Water Quality, bacteria must be present in concentrations of less than 100 CFU/100 mL for recreational waters (Health Canada, 2012). Drinking water guidelines in Canada are stringent with bacteria concentrations required to be nonexistent at a concentration of 0 CFU/ 100 mL as this would indicate fecal contamination, and the possible presence of enteric pathogens (Health Canada, 2017).

Many *E. coli* are transmitted through the fecal-oral route, but other vectors can lead to water contamination (Centre for Food Security and Public Safety, 2009). If humans were to drink this contaminated water, they stand the risk of being infected and developing *E. coli*

related diseases. Contamination of the public water supply with potentially pathogenic *E. coli* can be detected by normal water testing procedures (Merkx-Jacques et al., 2013). However, if water treatment is insufficient or if animal feces containing these pathogenic bacteria enter fresh water, the pathogen may not be removed effectively (Merkx-Jacques et al., 2013). It is important for scientists to be able to detect and identify each of the *E. coli* virotypes in order to evaluate the possible risk of *E. coli* contamination from within the water (Merkx-Jacques et al., 2013). A virotype is defined by Merkx-Jacques et al. (2013) to be the potential for pathogenicity, which is based on the results of genotyping. These virotypes can be isolated from various environments and placed into various categories allowing for the determination of the risk to human health (Merkx-Jacques et al., 2013). Each of the known virotypes tested currently is associated with a specific disease. In order to reduce the amount of potentially harmful *E. coli* in lakes and rivers used by humans, livestock should be kept away from water supplies, and watershed from the farms that house livestock should be actively monitored (Meays et al., 2004). Monitoring of this bacterium requires extremely sensitive detection methodology to determine the presence of pathogenic *E. coli* in the environment. The methodology must be precise due to the fact that there are low frequencies of the pathogenic bacteria living alongside many other non-pathogenic organisms. These low frequencies of pathogenic bacteria are a cause for concern given that Ivnitski et al. (1999) found that the infectious dose for enterohemorrhagic *E. coli* can be as low as 10 cells. With such a small dose of the pathogen required to cause disease, the identification of these harmful genes within the environment needs to be determined. The similarities of the strains living within the water and within the human gut are not widely known, and this is why *E. coli* in environmental samples

needs to be studied. It needs to be determined if the new environmental strains are pathogenic and potentially harmful to the human population.

Researchers have found that *E. coli* can persist in the environment such as in the sand, algae, sediment, and fresh water periphyton (Quero et al., 2015). It has been determined that *E. coli* are present in higher densities in sediment than in the general water column (Badgley et al., 2010). Sediment *E. coli* can detach from the sediment particles with the movement of water and be released. *E. coli* released from the sediment or periphyton may artificially inflate the bacterial counts and indicate fecal contamination where there is none (Moreira et al., 2012). In the fresh water lakes and beaches in Northwestern Ontario, there is a natural *E. coli* population. A study conducted by Ksoll et al. (2007) examined the *E. coli* from sand on the beaches of Lake Superior. They found that naturalized *E. coli* populations were highest in August (Ksoll et al., 2007). Exploration into the *E. coli* that establish in the sediment has not been widely undertaken. A study was conducted by Burton et al. (1987) where it showed that *E. coli* could survive longer in sediment than *Salmonella Newport* but not as long as *Pseudomonas aeruginosa* or *Klebsiella pneumoniae*. Luna et al (2010) tested *E. coli* isolated from marine sediment and determined that between 65-90% of those isolates belonged to the phylogroups known to cause extraintestinal diseases. There are few studies however, that have built upon these findings. While it is understood that *E. coli* living in the secondary habitats in the environment display different characteristics from those isolated from humans, little is known regarding the naturalized *E. coli* living in freshwater sediments. It is not known if the freshwater sediment *E. coli* contain genes that would allow them to cause disease.

1.3 Pathogenicity

Environmental water samples are currently monitored only for *E. coli* counts, as per Health Canada guidelines (Health Canada, 2017). However, these samples could be analyzed for different virulent factors and pathotypes that allow for the pathogenicity of the environmental *E. coli* to be detected in order to promote a healthier water system for humans around the world.

Pathogenic *E. coli* are responsible for various types of clinical infections and can be separated into two main pathotypes: either diarrheagenic *E. coli* that cause diarrheal diseases, or extraintestinal pathogenic *E. coli* that cause urinary tract infections, sepsis, and meningitis (Caprioli et al., 2005; Reisner and Krogfelt, 2006). There are six pathotypes of diarrhea *E. coli* including Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) (Kuhnert et al., 2000; Van Elsas et al., 2012). Urinary tract infections (Extraintestinal infections) are caused by the strains of Uropathogenic *E. coli* (UPEC) that are responsible for 70-90% of the estimated 150 million urinary tract infections diagnosed worldwide each year (Pitout, 2012).

Genetic determinants (virulence genes or virulence factors) render the *E. coli* as pathogenic for both humans and animals (Van Elsas et al., 2011). Many of the virulence genes are located on the mobile elements such as plasmids, phages, and transposons (Van Elsas et al., 2012). These mobile regions are important as they allow the virulence factors to be transmitted horizontally between strains. Virulence factors included adhesions, host cell surface modifying factors, invasins, capsule, toxins, and secretion systems (Van Elsas et al.,

2012). The pathogenicity of *E. coli* can be thought of as multi-factorial whereby a large number of the virulence genes contribute to the pathotype (Kuhnert et al., 2000). There are specific virulence factors that can be used to separate the organisms into different pathotypes as different virulence factors contribute to the pathogenicity of *E. coli* (Chapman et al., 2006; Van Elsas et al., 2012). Generally, strains that belong to the same pathotype have the same virulence factors involved in how they cause diseases. In order to detect these virulence factors and if they are present within the *E. coli* living in the environment, one must detect the specific virulence genes in order to determine the pathotype of the microorganism. The most common method for detecting these virulence genes is through PCR analysis. This method allows researchers to detect pathotype specific virulence genes to screen *E. coli* for their pathogenicity. However, possession of virulence genes may not necessarily translate into causing disease; the bacteria must acquire the correct combination of these genes in order to cause disease within a host. A study conducted by Albalawi (2016) investigated method development of multiplex PCR using nine pair of primers for specific virulence genes from five major pathotypes of *E. coli*. This study was conducted using over 300 isolates from sewage, geese, periphyton, and lake water samples collected in Thunder Bay, Ontario. Their findings illustrated that the isolates from the environment, whether that be from periphyton or lake water, contained minimal amounts of virulence genes, and that the major contributor to the *E. coli* in the lake was coming from periphyton (Albalawi, 2016; Moreira et al., 2012). These findings illustrate the potentially low risk of disease that the *E. coli* belonging to the natural environment have on the human population, however, further study into the *E. coli* that live in

the freshwater sediment is needed to further understand the health risk of this group of bacteria.

1.4 Biofilms

Appearing in the fossil record as early as 3.25 billion years ago, filamentous biofilms have been an integral part of prokaryotic life allowing them to survive in diverse environments (Hall-Stoodley et al., 2004). First described by Anton van Leeuwenhoek, biofilms were discovered when he took plaque off of his teeth and examined the complexity of the microbial community up close (Donlan and Costerton, 2002). These biofilm cells are very different from the rapidly growing planktonic cells because changes in the bacteria's physiology occur when the bacteria form biofilms. Biofilms have been defined as organized bacterial communities inside of an extracellular polymeric matrix that can adhere to each other, biotic and abiotic surfaces (Costerton, 1995). It is believed that these complex structures evolved as an adaptive behaviour in order to survive hostile environments (Beloin et al., 2008; Macia et al., 2014). This discovery has led to a tremendous amount of research on these unique structures to determine why they are so different and how the bacteria can alter their physiology.

The structure of microbial biofilms is elaborate and intricate in that bacteria are able to produce an "open system of microcolonies and water channels" while producing exopolysaccharides to enclose this matrix structure (Van Acker et al., 2014). This structure allows for different bacteria to work and live in harmony allowing each other to survive in different conditions (Costerton, 1995). Within the biofilm, microcolonies are created to better suit each bacteria's needs; for instance, bacteria can stabilize each other metabolically in order

for the colony to be maintained (Donlan and Costerton, 2002). Biofilm structures are formed through several steps (Hall-Stoodley et al., 2004;). First, the cells must be able to attach to the surface (through surface proteins [gram positive bacteria] or through pili and flagella [gram negative bacteria]) (Beloin et al., 2008; Macia et al., 2014). Next, the cells will form reversible attachment to the surface. This reversible attachment will become irreversible and strengthened by various fimbriae, including pili. The bacteria will then be able to use quorum sensing to be able to modulate biofilm formation, produce an extracellular matrix and proliferate. The matrix is made up largely of water (97%) in addition to exopolysaccharides, with smaller amounts of DNA, proteins, and other compounds from the host environment (Zhang et al., 1998). The last stage in biofilm formation is where some of the bacteria exit the biofilm and begin to colonize and form more biofilms on the surrounding surfaces. This entire process is only possible because of cell-to-cell signaling where quorum sensing can help regulate cell density and with the help of signaling molecules, the transcription of certain biofilm genes. These biofilm populations have different genes expressed within different regions of the biofilm based upon the characteristics of those bacteria and the environment surrounding them (Costerton et al., 1999; Van Acker et al., 2014). The ability to express different genes within the same structure and respond together to environmental stresses has allowed bacteria to survive in some hostile environments. One reason for surviving such hostile environments is because the biofilm structure itself allows nutrients to circulate in an otherwise nutrient low environment.

It is important to study biofilms because they impact our lives every day. Biofilms can be a source of the infection and can also protect bacteria from antibiotic treatments (Pratt and

Kotler, 1998). Biofilm organisms can cause blockage of pipes and fouling on machine surfaces leading to downtime in industry. One area not fully examined is the biofilms within sediments. Quero et al. (2015) discussed how marine sediments were able to act as reservoirs for *E. coli* outside of the mammalian host which could give rise to potentially pathogenic *E. coli* within the environment. The need for further investigation into this new reservoir for *E. coli* cannot come soon enough as researchers aim to combat *E. coli* infections that are now showing high frequencies of being resistant to antibiotics.

1.5 Antibiotic Resistance

1.5.1 Antibiotic Mechanisms

Since the age of tuberculosis and the bubonic plague, scientists have been at battle with pathogenic microorganisms in order to cure humans and animals from infections (Tenover, 2006). Since their development, antibiotics have allowed for humans to survive infections and diseases that would not have been possible decades before. Now, there is a growing concern regarding the level of increased resistance to these trusted antibiotics, which means humans are now racing to find alternative mechanisms to dismantle harsh microbial infections. In order to understand resistance, one must first understand how antibiotics work and their various mechanisms of action.

Antibiotics are largely separated into two groups: those that induce bacterial cell death (bactericidal) and those that inhibit cell growth (bacteriostatic) (Kohanski et al., 2010). Bactericidal antibiotics can cause various effects including the prevention of DNA replication through gyrase inhibitors, cause damage to the cell membrane whereby it loses its integrity,

and in inhibit protein synthesis whereby the ribosome binding is affected. Bacteriostatic antibiotics can inhibit DNA synthesis, RNA synthesis, cell wall synthesis or protein synthesis all inhibiting the bacteria from growing. It is important to note that at low concentrations, bactericidal antibiotics can act bacteriostatically and only turn lethal when present at high concentrations (Cloete, 2003). Likewise, very high concentrations of bacteriostatic drugs can exhibit bactericidal effects on bacteria (Kohanski et al., 2010).

Antibiotics can affect bacteria through many avenues including: disruption of bacterial membrane structures (polymixins), inhibition of cell wall synthesis (beta-lactams and glycopeptides), inhibition of metabolic pathways (sulfonilamides), inhibition of protein synthesis (aminoglycosides, macrolides, and tetracyclines to name a few), and the inhibition of nucleic acid synthesis (rifampin and fluoroquinolones). Antibiotics can inhibit protein synthesis do so by binding to either the 30S or 50S subunit of the ribosome in order to inhibit the synthesis of proteins required for growth. Antibiotics that can inhibit DNA synthesis do so by causing breaks in the DNA strand leading to a lethal outcome. If an antibiotic affects the bacterial cell membrane in terms of permeability, it can cause leakage of the bacterial contents slowly causing cell death (Gallant et al., 2005). Antibiotics that can inhibit the bacterial cell wall synthesis, such as penicillin, work by interfering with the enzymes that are needed to construct the peptidoglycan layer. Antibiotics can target a metabolic pathway, such as folic acid synthesis, whereby the antibiotic will compete to bind with para-aminobenzoic acid (PABA) which will stop the bacteria from forming folate, in turn causing cell death (Tenover, 2006).

1.5.2 Antibiotic Resistance Mechanisms

Some bacteria are intrinsically resistant to one or more than one class of antibiotics, and others acquire the resistance through mutation or through acquisition of resistance gene(s) from other bacteria (Tenover, 2006). The main challenge with antibiotic resistance in bacteria is that it is occurring at an accelerating rate while the ability for the world to combat this issue is rapidly declining. Antibiotic resistance mechanisms of bacteria have surged in the past three decades due to more and more antibiotics being prescribed. The target bacteria are able to adapt and exchange genetic material that confers an advantage, allowing them to survive (Neu, 1992).

Some mechanisms of bacterial resistance to antibiotics include the production of specific enzymes, the expression of efflux pumps, through gene acquisition, and through mutations (Gallant et al., 2005). The ability to produce specific enzymes, such as β -lactamases, allow the bacteria to hydrolyze the ring structure of penicillin and cephalosporin thereby destroying the antibiotics before it can elicit an effect in the cell. Another mechanism of resistance is through efflux pumps (Mah and O'Toole, 2001). These pumps are able to move antibiotics out of the cells before they reach their target, thereby decreasing the effect of the drug on the cells (Spoering and Lewis, 2001). The expression of efflux pumps also allows for the bacteria to be able to reduce the drug permeability of the outer membrane. The third mechanism of resistance is through the acquisition of genes that allow for bacteria to change their cell walls where the binding site of the antibiotic is no longer recognizable. Lastly, bacteria can also have mutations that would alter the target sites of the antibiotics.

Resistance mechanisms have been and are currently being investigated by researchers to hopefully be able to combat the growing problem of antibiotic resistance. The majority of antibiotics will target proliferating cells, not cells that are slowly respiring. This slow rate of respiration may contribute to how cells within the biofilm (cells under the stringent response) are able to display higher tolerance to antibiotics (Nguyen et al., 2011; Van Acker et al., 2014). With more than 60-80% of bacterial infections currently being treated involve some level of biofilm formation, it reiterates how widespread biofilms are in our lives within the developed world (Fux et al., 2005).

1.5.3 Antibiotic Resistance of Biofilm Cells

There is a growing concern regarding biofilms and human health as biofilm infections are difficult to be treated. It is important to further study the complex mechanisms of biofilms since many of the antibacterial agents currently used are designed to target planktonic cells (Costerton, 1995). These planktonic cells are physiologically different from biofilm cells, and antibacterial agents need to be designed specifically for biofilm bacteria in order to penetrate and kill the bacteria within the biofilm (Lewis, 2001).

Biofilms have a very important characteristic that they have shown increasing tolerance to antimicrobial agents (Cloete, 2003; Kirby et al., 2012). There are several mechanisms that explain the increase of antibiotic resistance of the biofilm cells. The first mechanism is thought to be from the exopolymeric matrix itself and it provides a barrier to reduce diffusion of polar and charged antimicrobial agents. The second mechanism is thought to be through the channels that circulate nutrients throughout the biofilm. These channels arise from the

arrangement of organisms within the biofilm based on their nutritional and growth needs [i.e. the outer layer allows for organisms that are aerobic and metabolically active, whereas the inner layers allow for reduced growth rates of the bacteria]. The variation in the conditions may play a role in which antibiotics can actually penetrate the biofilm. Since fluoroquinolones are not active under anaerobic conditions, so it is possible that they will not reach the inner layers of the biofilm. The third idea is related to the persister phenomenon. Persister cells have been described as the cells that remain dormant when bacteria are under high levels of stress or in the stationary phase (Lewis, 2001). These cells can occur in biofilms because they are protected by the other cells in the outer biofilm layers. This protection allows the cells to gradually build up tolerance to antibiotics. Many cells within the biofilm are also slow growing and enter stationary phase, which means that they may be undetected by antibiotics that target rapidly growing and respiring cells. Another antibiotic resistance mechanism can be caused by gene transfer. This mechanism is based upon bacteria having the ability to horizontally transfer antibiotic resistance genes that will allow them to survive treatment of antibiotics when they are in the biofilm community. This biofilm community facilitates the transfer of antibiotic resistance genes more easily than in planktonic bacteria. Each of these resistance mechanisms can facilitate biofilm cells surviving antibiotic treatment and make them more resistant to various stresses in the environment.

The relationship between antibiotic resistance and biofilm formation has been studied in *Acinetobacter baumannii* by Qi et al. (2016). They found that isolates with higher levels of antibiotic resistance tended to be weaker biofilm formers but the exact genetic mechanism for this phenomenon is not yet known. Gallant et al. (2005) studied the genetic link between

biofilm formation and antimicrobial resistance in *P. aeruginosa* and *E. coli*. They showed that when the bacteria increase their resistance to β -lactam antibiotics by acquiring the TEM-1 β -lactamase gene, they also showed a decrease in biofilm forming capacity. This can be explained by the fact that the β -lactamase also causes defects in motility, adherence, and biofilm formation of the bacteria. Gallant et al. (2005) concluded that in order for bacteria to acquire the resistance of certain antibiotics, it may come at the expense of other protective phenotypes such as the ability for form biofilms. However, the question on how weak biofilm formers are able to achieve high levels of resistance to antibiotics still remains and further investigations are needed to explain this phenomenon.

1.6 Bacterial Response to Oxidative Stress

1.6.1 Reactive Oxygen Species and their Regulation

Oxidative stress is caused by excess reactive oxygen species (ROS, such as superoxide anions, hydrogen peroxide, and hydroxyl radicals) in cells that can cause damage to DNA, RNA, protein, and lipids (Farr and Kogoma 1991; Tamarit, 1998; Vatansever et al., 2013). It is important to note that these ROS molecules are normal byproducts of cellular respiration in aerobic organisms. The problem arises when these active molecules are overproduced and not neutralized by antioxidant enzymes produced by the cell, which leads to oxidative stress and eventually cell death (Poole, 2012; Storz and Imlay, 1999).

There are three major antioxidant enzymes that are important for cells to combat the ROS: superoxide dismutase, catalase, and glutathione peroxidase (Wang et al., 2012; Volodymyr, 2011). Superoxide dismutases (SODs) convert superoxide anion into hydrogen

peroxide and oxygen (Schellhorn and Hassan, 1988). These SODs have at least one first transition series metal (Fe, Mn, or Cu) at the active site. Catalase catalyzes hydrogen peroxide to form neutral products oxygen and water. Lastly, glutathione peroxidase catalyzes the destruction of hydrogen and hydrogen peroxides by using glutathione as an electron donor. During periods of oxidative stress, *E. coli* have different regulators, OxyR and SoxRS, that can undergo conformational changes in order to control the expression of cognate genes (Chiang and Schellhorn, 2012). These two regulators, OxyR and SoxRS, allow for different superoxide dismutases and catalase genes to be expressed in oxidative stress conditions. *E. coli* have two of these antioxidant enzymes produced within their cells: superoxide dismutase and catalases. These two enzymes are known to eradicate superoxide and hydrogen peroxide (Dwyer et al. 2009;). The hydroxyl radical generated in oxidative stress is not removed by these two enzymes which presents large problems for the bacterial cells.

In *E. coli*, superoxide dismutase and catalase are two forms of enzymes that can remove oxidative stress molecules. There are three forms of superoxide dismutases present: MnSOD, Manganese containing SOD (encoded by *sodA*); FeSOD, Iron containing SOD (encoded by *sodB*); and CuZnSOD, Copper and Zinc containing SOD (encoded by *sodC*). These SODs will convert superoxide anions into hydrogen peroxide molecules that can be further broken down through another enzyme present in the bacteria, catalase. *E. coli* also have two different catalases that breakdown hydrogen peroxide into water and oxygen molecules (Tanaka et al., 1997). These two catalases are hydroperoxidase I (HPI) and hydroperoxidase II (HP II) which are encoded by *katG* and *katE* respectively. The *katE* gene is the main form of catalase in stationary phase in *E. coli* and other gamma-proteobacteria. This is controlled by the RpoS regulon and is important

for bacteria to resist oxidative stress under stationary phase (Baez and Shiloach, 2013). The OxyR regulon is inversely correlated with intracellular hydrogen peroxide levels, and deletion of the *katG* gene shows an increase of hydrogen peroxide levels in exponential phase. Overall, these two catalases allow for hydrogen peroxide-induced oxidative stress to be combatted within the *E. coli* cells. Both of the enzymes, SOD and catalase, allow for *E. coli* to combat oxidative stress. It is important for these enzymes and other gene regulators to be further investigated in order to understand the impact of the ROS on the survival of the bacteria in environmental conditions.

1.6.2 Antibiotic Resistance and Oxidative Stress

How antibiotics eventually come to kill the bacterial cell is a widely examined phenomenon today. Most antibiotics aim to kill microbes through interaction with specific cellular targets (Wang et al., 2010). Kohanski et al. (2007) explained that oxidative stress may play a role in a secondary killing mechanism of bactericidal antibiotics. Bactericidal antibiotics, which attack bacteria through their specific cellular targets, have also been found to stimulate the production of ROS in bacteria that can lead to cell death (Kohanski et al., 2007). The same ROS production was not found at low concentration of bacteriostatic drugs, which merely aim to inhibit bacterial growth. With bactericidal antibiotics, a cell will undergo drug induced stress. This stress will increase its level of aerobic respiration thereby generating excess superoxide anion, hydrogen peroxide and hydroxyl radical molecules (Wang et al., 2010). Along with increased aerobic respiration, the destabilization of iron-sulfur clusters in dehydratase enzymes occurs providing the perfect opportunity for the iron molecule to be released as a ferrous ion

that will eventually generate hydroxyl radicals through the Fenton reaction. During this process, the dismutation of hydrogen peroxide will generate additional hydroxyl radicals that are toxic to the cells (Wang and Zhao, 2010; Van Acker et al., 2014). This rise in the level of hydroxyl radicals will in turn lower the number of cells that survive antibiotic treatment (Wang et al., 2010). By altering the central metabolism of the cell, bactericidal antibiotics in this way can cause drug-induced killing through respiration and iron metabolism (Dwyer et al., 2014; Van Acker et al., 2014; Vatansever et al., 2013).

It has been hypothesized that antibiotic tolerance of some pathogenic bacteria has been the result of altering the oxidative stress response and defense genes within these organisms (Dwyer et al., 2014). Dwyer et al. (2014) has indicated that bactericidal antibiotics are capable of *“altering cellular respiration and inducing lethal levels of intracellular hydrogen peroxide”*. Dwyer et al. (2014) goes on to say that the toxicity from ROS is generated as an effect of the antibiotic reacting with the cell in the first place. The mechanism of antibiotic induced cellular death through oxidative stress proposed in 2007, has led to greater understanding of how these bactericidal antibiotic compounds interact with the bacterial cell (Van Acker et al., 2014). There are very few studies on determining the cellular level of ROS and its effect on antibiotic resistance. If the theory with bactericidal antibiotics is correct, then cells with higher levels of oxidative stress tolerance should be more resistant to antibiotics. This increased tolerance to oxidative stress has not been shown or correlated to antibiotic resistance. This study will aim to fill in some of these gaps whereby *E. coli* isolates from sediment, sewage, and O157 groups will be studied in order to determine the intrinsic level of oxidative stress (i.e. cellular ROS concentration) in and antibiotic resistance of the bacteria in both the planktonic and biofilm

state. With a greater understanding into how the bacteria behave in the different phases of growth, some of the questions regarding intrinsic ROS will be able to be answered.

1.7 The Stationary Phase Sigma Factor

E. coli have an important stress regulator, the stationary phase sigma factor (σ^S or RpoS), that is a subunit of the RNA polymerase encoded by the *rpoS* gene (Hengge-Aronis and Storz, 2011). As bacteria enter stationary phase or under specific stress conditions, the RpoS regulon achieves maximum levels (Battesti et al., 2011; Schellhorn, 2014). The RpoS allows the cell to navigate through stimuli such as environmental changes and stress adaptations in order to survive. This 38 kDa protein controls the function of many genes in the stationary phase that can respond to oxidative stress (Baez and Shiloach, 2013). With many known functions, and some still left unknown, *E. coli* bacteria use this regulon to increase their tolerance to acids in the stomach of mammals and survive the harsh environment. RpoS functions by interacting with the core RNA polymerase to control the expression of specific stress tolerance genes. In *E. coli*, the RpoS regulon controls approximately 10% of the bacteria's genome and in periods of stress or stationary growth for *E. coli*, there are over 140 genes that are up-regulated by RpoS (Sheldon et al., 2012). Mutations of *rpoS* in *E. coli* have been shown to cause sensitivities to stresses including starvation, pH, heat, and oxidative stress of the bacteria.

Battesti et al. (2011) described the induction of *rpoS* as a response to unfavorable conditions or starvation that requires the cell to sense the stresses (Poole, 2012). One of these sensing responses is known as the stringent response (Battesti et al., 2011; Chang et al., 2002; Poole, 2012; Zuo et al., 2013). The bacteria will elicit the stringent response when it is

deprived of amino acids. This nutritional stress causes an increased expression of survival genes that will allow the bacteria to capitalize the use of scarce nutrients through transcriptional switching (Chang et al., 2002; Poole, 2012; Zuo et al., 2013). During this response, a signaling molecule called alarmone or (p)ppGpp, (guanosine 5'-(tri)diphosphate 3'-diphosphate) will be produced to improve the RpoS activity and prevent its degradation. The RpoS will then activate the production of stress tolerance proteins that increase survival of the bacteria in stressful environments.

One gene that has been attributed to bacteria biofilm formation during periods of stress is the *rpoS* (Adams and McLean, 1999; Sheldon et al., 2012). This gene allows RNA polymerase to bind to the promoter of the *rpoS*-dependent genes to allow for their transcription to occur. This gene has been found to be expressed differently in biofilm and planktonic states of bacteria (Donlan and Costerton, 2002). The *rpoS* gene has been shown to affect biofilm formation of bacteria and has essentially no effect on planktonic cell growth (Mah and O'Toole, 2001).

Battesti et al. (2012) reviewed the effect of RpoS in relation to oxidative stress. They found that the RpoS response seems to be only able to respond to oxidative stress when a cell is in stationary phase. Stationary phase cells are described by Battesti et al. (2012) to be resistant to oxidative damage by hydrogen peroxide due to RpoS dependent gene *katE*, which encodes for the catalase enzyme that breaks down hydrogen peroxide to oxygen and water. Sheldon et al. (2012) proposed that the *rpoS* gene will regulate bacteria to form biofilm at an optimal size so that nutrients can reach the bacteria. Therefore, RpoS is essential for the survival of biofilm bacteria. Although the RpoS has been related to the reduction of ROS in

stationary growth phase of bacteria, it is not clear if it has the same function in biofilm cells.

We propose that not only does the RpoS optimize the size of biofilm matrix, but it also reduces the concentration of ROS in the biofilm cells and hence increase the antibiotic resistance of the bacteria in biofilm phase.

1.8 Thesis Objectives

Pianetti et al. (2004) has shown that *E. coli* in sediment can be released into the water column during periods of high water disturbance. However, little is known about the properties of freshwater sediment *E. coli* including their virulence genes, biofilm forming capacity, antibiotic resistance and cellular ROS concentration. Furthermore, the role of *rpoS* on the biofilm forming capacity, ROS and antibiotic resistance of shiga toxin producing O157:H7 *E. coli* is unknown. Therefore, the objectives of this study are:

1. To determine and compare the virulence genes (a total of nine virulence genes representing both uropathogenic and diarrheagenic *E. coli*) possessed by the sediment, sewage, and O157 *E. coli*.
2. To determine and compare the biofilm forming capacity of the three groups of *E. coli*.
3. To determine and compare the antibiotic resistance of the three groups of *E. coli* to eight common antibiotics in both the planktonic and biofilm states.
4. To determine and compare the intrinsic level of ROS of the three groups of *E. coli* in both the planktonic and biofilm states.
5. To examine the correlations between biofilm forming capacity and antibiotic resistance, and the biofilm forming capacity and ROS of the *E. coli* samples.

6. To determine the biofilm forming capacity of the *E. coli* O157:H7 strain H32 and its *rpoS* mutant.
7. To investigate the level of ROS contained within the wildtype and mutant strains in both the exponential and biofilm phases.
8. To assess the effect of the *rpoS* gene on the bacterium's antibiotic resistance to eight common antibiotics in both the exponential and biofilm phases.

1.9 References

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Chapter 2.

**Determining the Virulence Genes, Biofilm Forming Capacity,
Antibiotic Resistance, and Level of Reactive Oxygen Species
of Freshwater Sediment, Sewage, and O157 *E. coli***

2.0 Abstract

Even though sediment *Escherichia coli* is a common source of contamination in freshwater environments, their virulence and physiological characteristics are not well studied. The goal of this study was to compare the virulence genes, biofilm forming capacity, antibiotic resistance and reactive oxygen species (ROS) of sediment *E. coli* isolated from three freshwater lakes (Lake Simcoe, Georgian Bay, and Boulevard Lake) with no exposure to sewage effluent to sewage *E. coli* from two sewage treatment plants (Orillia and Thunder Bay Ontario) and a collection of known O157 *E. coli*. Multiplex PCR analysis on nine virulence genes (*hlyA*, *iroN*, *papA*, *hs*, *hl*, *ial*, *bfpA*, *stx1*, and *stx2*) revealed that no sediment, and very few (3%) sewage *E. coli* contained diarrheagenic and shiga toxin genes. However, 12.5% of sediment and 42% of sewage isolates contained one or more uropathogenic genes. Interestingly, only the *iroN* gene was detected in the sediment isolates. The biofilm assays determined that sediment *E. coli* were significantly better biofilm formers ($p < 0.001$) than the sewage and O157 *E. coli*, and on average the sediment *E. coli* was able to form 2 and 3.5 times as much biofilm as the sewage and O157 *E. coli*, respectively. The antibiotic resistances of the isolates to eight antibiotics were determined using the Kirby-Bauer disk diffusion method and the antibiotic resistance patterns of the *E. coli* samples illustrated that the sediment, sewage and O157 isolates belonged to three distinctive populations. Furthermore, there was an overall significant difference between the three sample groups ($p < 0.05$), where sediment was the most susceptible and sewage was the most resistant to the antibiotics tested. It was also determined that the level of ROS in biofilm *E. coli* cells was significantly lower than their planktonic counterparts ($p < 0.001$). A negative correlation ($p = 0.066$) was observed when comparing the isolates' biofilm forming capacities

with their intrinsic level of ROS, whereby isolates with higher tolerance to oxidative stress (i.e. higher amount of cellular ROS) were associated with a lower biofilm forming capability. Furthermore, isolates with higher resistance to antibiotics in their planktonic state also showed lower biofilm forming capacity.

2.1 Introduction

E. coli have been able to persist outside of the mammalian host and establish in secondary environments including natural freshwater sediments (Ksoll et al., 2007; Quero et al., 2015; Sherer et al., 1992). In the environment, bacteria are able to form biofilms on the interface of the sediment and the water allowing for increased survival (Sherer et al., 1992). *E. coli* survival has been shown to increase in the sediment due to the existence of fine soil particles, nutrients, and high organic matter contents and these bacteria are present in higher concentrations than in the water column (Badgley et al., 2010; Sherer et al., 1992). Biofilms are complex microbial communities enclosed in an exopolymeric matrix (Costerton, 1995; Lewis, 2001; Van Acker et al., 2014). This matrix structure allows bacteria to adapt to various environmental conditions and survive various stresses (Costerton, 1995). Many studies have demonstrated that *E. coli* in sediments were able to live considerably longer than when they were in the water column (Burton et al., 1987, Hood et al., 1982, Sherer et al., 1992). While it is known that some *E. coli* strains can survive and establish themselves in the sediment, other physiological characteristics of this group of bacteria have not been fully studied (Luna et al., 2010; Quero et al., 2015; Sherer et al., 1992).

With *E. coli* contamination in fresh water being an important concern for health reasons, it is essential to know the sources of contamination of this group of bacteria. Sewage is a major source of *E. coli* contaminations of fresh water environments. Sixty percent of this sewage *E. coli* has been shown by Anastasi et al. (2010) to contain one or more uropathogenic or diarrheagenic virulence genes. Furthermore, sewage effluents may also carry other human pathogens that increases the health risk of this contaminated water (Merckx-Jacques et al., 2013). However, a secondary source of contamination can be from *E. coli* residing in the sediment and other environmental sources such as periphyton (Moreira et al., 2012). To determine the potential health risk caused by this group of *E. coli*, it is important to understand the pathogenicity and physiology of these naturalized sediment *E. coli*. To date, little is known about the characteristics of the sediment *E. coli*.

Characteristics such as virulence gene possession, biofilm forming capabilities, antibiotic resistance, and the intrinsic level of reactive oxygen species (ROS) can all contribute to the viability of *E. coli* in the environment. Although every *E. coli* virulence gene has an important role for the bacteria to cause diseases to humans, most pathogenic strains require a combination of several virulence genes to express their pathogenicity (Chapman et al., 2006). Furthermore, some of these virulence genes, such as the iron sequestering gene, can be useful for the bacteria to survive and establish in the environment (Neilands, 1995). Luna et al. (2010) investigated this naturalized sediment *E. coli* and noted high percentages of virulence genes contained within the sediment *E. coli* of marine and coastal waters. The high frequency of virulence genes within the sediment could indicate that the sediment *E. coli* is becoming a

reservoir for pathogenic bacteria. However, little is known about the pathogenicity of naturalized *E. coli* in fresh water sediments with no exposure to sewage contamination. A strong capacity of forming biofilm is essential for *E. coli* to survive and establish in the environment because biofilm cells express stress tolerance responses that allow the bacteria to persist in stressful conditions. Reisner et al. (2006) showed that human *E. coli* isolates exhibited a wide range of biofilm-forming competence. However, it has also been shown that *E. coli* populations established in fresh water periphyton and on macroalgae have exhibited high biofilm forming capacity (Moreira et al., 2012; Quero et al., 2015), inferring that the ability of forming biofilm is an important factor for the bacteria to survive and establish in the environment. Currently, little is known about the biofilm forming capacity of sediment *E. coli*. An understanding of these bacteria's ability to form biofilm will provide us valuable information about their persistence in the environment and risks to human health.

Antibiotic resistance has become a major health concern as antibiotics are prescribed more and more each year. Now, bacteria that were once sensitive to antibiotics are increasingly difficult to be treated. With more than 25 million courses antibiotics prescribed each year in Canada, the effects of these antibiotics once they leave our bodies and enter the environment is a growing concern (Canadian Institute for Health Information, 2017). Most antibiotic compounds are not metabolized fully by our bodies or broken down at wastewater treatment plants, and because of that they are a source of antibiotic contamination in the fresh water environments (Kummerer, 2009; Watkinson et al., 2007). This increase in the concentration of antibiotics that enter the natural water system presents a health concern

where there is a risk of bacteria within the environment becoming resistant to these antibiotics (Watkinson et al., 2007). Therefore, it is important to determine the antibiotic resistance patterns of the freshwater sediment *E. coli*.

Besides the conventional cellular target-related antibiotic killing mechanisms, a new model has been proposed in which bactericidal antibiotics have secondary pathway that induces ROS production in bacterial cells and inflicts damages to the bacteria (Dwyer et al., 2014). This secondary mechanism occurs through interaction with a bacteria's TCA cycle and electron transport chain to overproduce ROS such as superoxide, hydrogen peroxide, and hydroxyl radicals. When these ROS molecules are produced at a much faster rate than what occurs in respiration, the bacteria will go into oxidative stress (Dwyer et al., 2014). Eventually, this increase in oxidative stress will be overwhelming for the bacteria and will cause cell death (Dwyer et al., 2014). With this new secondary mechanism of antibiotic killing with bactericidal antibiotics being increasingly studied, the need for understanding into how bacteria respond to oxidative stress is critical. Currently, little is known about the cellular levels of ROS in environmental *E. coli*. By determining the intrinsic cellular ROS levels of these bacteria, it will provide important information for our understandings on the bacteria's ability to survive oxidative stress and exposure to antibiotics.

In this study, we examined the virulence genes, biofilm forming capacities, antibiotic resistance patterns, and cellular ROS levels of *E. coli* isolated from sediment, sewage, and a collection of shiga toxin producing *E. coli* O157. Sediment and sewage samples were obtained

from both Northern and Southern Ontario to allow for a better geographic representation of *E. coli* isolates. The objectives of this study were:

1. To determine the virulence genes (a total of nine virulence genes representing both uropathogenic and diarrheagenic *E. coli*) possessed by the sediment, sewage, and O157 *E. coli*.
2. To determine the biofilm forming capacity of the three groups of *E. coli*.
3. To determine the antibiotic resistance of the three groups of *E. coli* to eight common antibiotics in both the planktonic and biofilm states.
4. To determine the intrinsic level of ROS of the three groups of *E. coli* in both the planktonic and biofilm states
5. To examine the correlations between biofilm forming capacity and antibiotic resistance, and the biofilm forming capacity and ROS of the *E. coli* samples.

2.2 Materials and Methods

2.2.1 Bacterial Strains Used

Known uropathogenic, diarrheagenic and shiga toxin-producing *E. coli* strains were used in this study as positive controls for the detection of virulence genes in sediment and sewage *E. coli* through the use of multiplex PCR method. ATCC 25922 *E. coli* strain obtained from Cedarlane Corporation (Burlington, Ontario) was used as positive control for uropathogenic virulence genes. Positive controls of diarrheagenic virulence genes were detected in Enterotoxigenic *E. coli* (ETEC) strains ETEC 505 and ETEC 07, an Enteroinvasive *E. coli* (EIEC) strain 0136, and an Enteropathogenic (EPEC) strain 055 provided by Dr. B. Ciebin, Ontario

Ministry of Health, Etobicoke, Ontario. Shiga toxin I and II genes in Hemorrhagic *E. coli* were detected using a shiga toxin-producing *E. coli* positive control strain EC 920004 provided by Dr. C. Gyles at the University of Guelph, Guelph, ON.

The uropathogenic *E. coli* genes examined in this study were *iroN*, *hylA*, and *papA*. The diarrheagenic *E. coli* genes examined were *hs*, *hl*, *ial*, and *bfpA*, in addition to hemorrhagic genes *stx1* and *stx2*. The *iroN* gene allows bacteria to acquire iron through siderophore based acquisition systems. *HylA* is the gene associated with alpha-hemolysin that can destroy red blood cells and the *papA* gene is involved in P fimbrial adhesion formation. While the *hs* and *hl* genes code for heat stable and heat labile enterotoxin genes belonging to Enterotoxigenic *E. coli*. The *ial* gene, belonging to Enteroinvasive *E. coli* was detected as it has been associated with diarrhea and dysentery. The bundle forming pili gene, *bfpA*, is essential for attaching to the host cell, was also included in this investigation from Enteropathogenic *E. coli*. The isolates were also tested for the presence of Enterohemorrhagic *E. coli* genes shiga toxin I and II genes (*stx1* and *stx2*) that have been known to cause hemorrhagic colitis and hemolytic uremic syndrome.

Eight additional samples belonging to the O157 serogroup were also provided by Dr. C. Gyles (Guelph, ON) and used to compare with *E. coli* isolated from the sediment and sewage for their biofilm forming capacities, antibiotic resistance, and level of intrinsic ROS. Strains EC920004, EC920026, and EC920037 were isolated from bovine, whereas strains EC970112, EC961020, EC961085, B0962889, and B0965105 were isolated from human sources.

2.2.2 Sample Collection and *E. coli* Isolation

2.2.2a Sediment Samples

In this study, sediment samples were collected from locations in both Southern and Northwestern Ontario. The Southern Ontario samples were taken from four locations in southern Ontario, two from around Lake Simcoe [44° 23'21.6780"N, 79° 41'25.1916"W] and two from the southeastern shore of Georgian Bay [44°50'30"N 79°59'21"W]. The Northwestern Ontario samples were collected from around Boulevard Lake in Thunder Bay, Ontario [48.4604° N, 89.1989° W]. The sample sites can be visualized on the map displayed in Image 2.1. The water temperatures at the sampling sites were ranged between 18-25°C. Subsamples of the sediments collected were sent to the Lakehead University Environmental Laboratory where they were chemically analyzed, and results were shown in Table 2.1.

Lake Simcoe and Georgian Bay sediment samples were collected on September 9 and 10 of 2016 using a stainless-steel sediment corer rinsed with 70% ethanol and sterile double distilled water, which was then submerged in the sediment to a depth of approximately 6 inches to collect the sample. The sediment was placed into a sterile 50ml Eppendorf centrifuge tubes. The sediment corer was rinsed with 70% ethanol and sterile double distilled water, and additional samples were taken and placed into sterile centrifuge tubes. A total of three separate samples were taken from each sampling site. These samples were then placed in a cooler with ice packs and shipped to Lakehead University in Thunder Bay overnight where the samples were processed within 24 hours. Samples were collected from Holland River in the Township of Innisfil [44° 9'45.83"N 79°31'15.77"W], Kettle Lake, Awenda Park [44°50'40.59"N

79°58'22.58"W], Farlain Lake Township [44°49'46.13"N 79°58'26.07"W], and Ben's Ditch [44°35'30.59"N 79°25'15.29"W].

The Boulevard Lake sediment samples were collected on August 2, 2017, approximately 1-2 meters away from the shoreline, either just off the beach area or off the river embankment. A sediment corer was pushed into the sediment of the lake by hand and the core sample was capped and placed in a cooler. Three replicate samples were collected at the location and the process was repeated at three other locations around Boulevard Lake. The sediment samples were transported to the Lakehead University Microbiology Laboratory for processing immediately.

Upon arrival at the Thunder Bay campus, all samples were immediately processed as follows. After a sub-sample was sent for chemical analysis, the rest of the sample was used for isolation of *E. coli*. In sterile 100 mL milk dilution bottles, 10 g of sediment was shaken with 90 mL of 0.85% sterile saline for 5 minutes to release bound bacteria from the sediment. After letting settle for 45 minutes, 10mL of the supernatant was filter through a sterile 0.45 µm membrane filter (Thermo Fisher Scientific, Whitby, ON). The filter was then placed onto a Differential Coliform Agar plate (DC Agar, Oxoid Limited, Basingstoke, England) with sterile forceps. The DC agar plate was incubated at 37°C in an Isotemp 205 Incubator (Thermo Fisher Scientific). After 24 hours, blue colonies were retrieved individually with sterile toothpicks from the filter, streaked onto new DC agar plates and incubated for 24 hours at 37°C. A blue colony from this new DC agar plate was chosen and re-streaked again onto an additional DC agar plate to obtain a pure culture of the isolate. The isolates were confirmed to be *E. coli* through the Indole, Methyl Red, Voges-Proskauer, and Citrate utilization (IMViC) test whereby the bacteria

was indole and methyl red positive, and the Vogues-Proskauer and Citrate tests were negative. The bacteria were then sub-cultured onto a Trypticase Soy Agar plate (TSA, containing 30 g Trypticase Soy Broth (TSB) and 15 g Granulated Agar (Becton Dickinson and Company, New Jersey, USA) where it was incubated for 24 hours at 37°C. A single colony was then cultured in TSB and stored in a 25% glycerol solution (v/v final concentration) at -80°C for long-term storage.

2.2.2b Sewage Samples

Thunder Bay sewage *E. coli* was previously isolated from untreated sewage collected from the Thunder Bay Waste Water Treatment Plant (Thunder Bay, ON) in 2010 and 2011 (from July to September). Untreated sewage samples were also obtained from the City of Orillia Wastewater Treatment Centre (Orillia, ON) in September 2017. *E. coli* bacteria were isolated using the same procedure described in the sediment *E. coli* isolation process with slight modifications. Samples were diluted 1000X in sterile milk dilution bottles in a two-step procedure, where 10 ml of raw sewage sample was shaken with 90 mL of 0.85% sterile saline for 5 minutes to release bound bacteria. After it settled for 45 minutes, 1 mL of the 10X dilution was added to 99 ml of 0.85% saline and shaken for an additional 5 minutes. After it settled for an additional 45 minutes, 1 mL of the supernatant was filter through a sterile 0.45 µm membrane filter (Thermo Fisher Scientific). The filter was then placed onto a Differential Coliform Agar plate (Oxoid Limited) with sterile forceps. The DC agar plate was incubated at 37°C in a Isotemp 205 Incubator (Thermo Fisher Scientific). After 24 hours, the *E. coli* samples

were isolated using the same procedure for the sediment samples and stored in a 25% glycerol solution (v/v final concentration) for long term storage.

2.2.3 Detection of Virulence Genes in *E. coli* isolates

2.2.3a DNA Extraction and Purification

A total of 149 *E. coli* isolates from sediment (n=72), sewage (n=66) and O157 (n=11) were screened in the detection of virulence genes. *E. coli* from an isolated colony was transferred into 5mL of sterile TSB and incubated at 37°C in an Innova 4430 Incubator Shaker (New Brunswick Scientific, Midland, ON) at 150 rpm overnight. One mL of the *E. coli* culture was placed into a sterile 1.5 mL Eppendorf tube and centrifuged in an Eppendorf Centrifuge 5430 (Thermo Fisher Scientific) for 5 minutes at 18,800 x g. The supernatant was removed from the sample and an additional 1 mL of the *E. coli* culture was added into the tube. The Eppendorf tube was centrifuged again at 18,800 x g for 5 minutes. The supernatant was removed, and the cells were re-suspended in 800 µL of XS buffer solution (1%w/v potassium ethyl xanthogenate, 100 mM Tris-HCl, 20 mM EDTA, 1% w/v SDS, and 800 mM ammonium acetate) as described by Yang (2013). Two µL of RNase (10mg/ml) (Fermentas, Burlington, ON) was added to the sample and the tube was vortexed to ensure adequate mixing before being incubated at 37°C in an Isotemp 205 water bath (Fisher Scientific) for one hour. The sample was then transferred to a 70°C Isotemp water bath for an additional hour. The sample was placed on ice for 30 minutes and centrifuged for 10 minutes at 18,800 x g, after which, 750 µL of the supernatant was transferred to a new sterile Eppendorf tube where 750 µL of 100% isopropyl alcohol was added to precipitate the DNA. The samples were then cooled in a -30°C

Freezer (ThermoForma, Fisher Scientific) for 12-20 hours. After cooling, the DNA was pelleted by centrifuging the samples at 18,800 x g for 10 minutes. The supernatant was discarded, and the DNA was washed twice with 750 μ L of 70% ethanol. Next, the samples were left to air dry in a Biosafety Cabinet (BSC) for approximately 30 minutes. The DNA samples were then dissolved in 100 μ L of sterile UV treated double distilled water.

A 1% agarose gel was prepared to detect the DNA samples through gel electrophoresis. Half of a gram of Low EEO Multipurpose Agarose (Thermo Fisher Scientific) was placed into a 125 mL Erlenmeyer flask where 50 mL of 1X TAE buffer (242 g Tris base, 100 mL of 0.5 M EDTA, 57.1 mL glacial acetic acid, adjusted to pH of 8.2) was added and the agarose suspension was heated in a Dual Wave Microwave System (General Electric) for 1.5 minutes. Five μ L of ethidium bromide solution (10 mg/mL, BioRad) was added to the gel-solution and mixed thoroughly before it was poured into the gel tray. A gel comb with the appropriate number of wells was added to the top of the gel solution where the gel was left to solidify for 20 minutes. The gel tray was then placed into the electrophoresis chamber (BioRad) and submerged with 1X TAE buffer before the comb was gently removed. The gel was loaded with 3 μ L of the ready to use GeneRuler 1kb Plus DNA ladder 0.1 μ g/ μ L (Fermentas) in the first and last wells. On a parafilm strip, 2 μ L of 6X Loading DNA Dye (Thermo Fisher Scientific) was placed on the parafilm where 10 μ L of the extracted DNA sample was added. The loading dye and sample were mixed before being added into the corresponding well. The gel was run at 100 V for 30 minutes using a BioRad gel electrophoresis power supply apparatus (BioRad, Mississauga, ON). The DNA bands in the agarose gel were imaged using the Syngene Chemi Genius Bio Imaging System with Gene Snap program (Synoptics Group).

2.2.3b Singleplex and Multiplex PCR Assays

The virulence of the *E. coli* samples isolated from the sediment, sewage and O157 groups were compared by detecting the presence of specific *E. coli* virulence genes using the polymerase chain reactions (PCR). Singleplex and multiplex PCR were performed on the positive controls to optimize the reaction conditions. Nine biomarkers were targeted by the PCR assays in this study: *hs* (heat stable toxin gene), *hl* (heat labile toxin gene), *ial* (invasion associated loci gene), *bfpA* (bundle-forming pili gene), *stx1* (shiga toxin 1 gene), *stx2* (shiga toxin 2 gene), *iroN_{E. coli}* (catechol siderophore receptor gene), *hlyA* (hemolysin gene) and *papA* (pyelonephritis-associated pili gene) (Table 2.2).

Singleplex PCR reaction was prepared by including the following ingredients: 31 μL of UV treated autoclaved double distilled water, 5 μL of 2 mM dNTPs mix (Fermentas), 5 μL of 25 mM MgCl_2 (Fermentas), 5 μL of 10X Taq Polymerase Buffer (Fermentas), and 1 μL of Taq DNA Polymerase (1U/ μL , Fermentas). Next, 1 μL of each of the appropriate forward and reverse primers was added (reaching a final concentration of 0.2 μM , Table 2.2) to the reaction tube before 1 μL of the template DNA was added (approximately 150 ηg of DNA) to yield a total PCR reaction volume of 50 μL .

The PCR tubes were then gently vortex with a Vortex-Genie 2 (Scientific Industries, Bohemia, New York, USA) to mix the ingredients. All the PCR tubes were then placed into the MJ Mini Thermocycler (BioRad). The PCR reaction involved the following steps: the samples were initially heated at 95°C for 5 minutes. The PCR cycling then began at 95°C for 1 minute to denature the DNA, 55°C for 1 minute for annealing, 72°C for 1 minute for extension. After 35 cycles, the samples were kept at 72°C for 10 minutes and left at 4°C until the samples were

retrieved from the PCR machine. The PCR samples were analyzed by agarose gel electrophoresis and visualized with the Syngene Chemi Genius Bio Imaging System as described previously (section 2.2.3a).

Multiplex PCR reactions were performed to detect the presence of nine virulence genes in three reactions. Unlike in the singleplex PCR, each multiplex reaction contained three sets of primers. The PCR primers were put into groups based on their amplicon size and interactions to ensure that nonspecific amplification was eliminated while also ensuring that the three amplicons in each grouping were well separated (Table 2.3). Group 1 included the heat stable toxin (*hs*), the heat labile toxin (*hl*), and the catechol siderophore receptor (*iroN_{E. coli}*) genes. Group 2 included the invasion associated loci (*ial*), the bundle-forming pili (*bfpA*), and the hemolysin (*hylA*) genes. Group 3 included the shiga toxin 1 (*stx1*), the shiga toxin 2 (*stx2*), and the pyelonephritis-associated pili (*papA*) genes. Positive controls were used as outlined in Table 2.2. All three primer pairs were added with 1 μ L each in both the forward and reverse direction (each primer had a final concentration of 0.2 μ M). Each multiplex PCR reaction tube contained the following reagents: 27 μ L of UV treated autoclaved double distilled water, 5 μ L of 2 mM dNTP mix, (Fermentas), 5 μ L of 25 mM MgCl₂ (Fermentas), 5 μ L of 10X Taq Polymerase buffer (Fermentas), and 1 μ L of Taq DNA Polymerase (1 U/ μ L, Fermentas). The template DNA added at a volume of 1 μ L (approximately 150 ng) to give a total reaction volume of 50 μ L. The multiplex PCR program and cycles were performed as previously described in the singleplex PCR assay and the PCR products were visualized with a 1% agarose gel electrophoresis as described previously.

2.2.4 Biofilm Forming Capacity

The biofilm forming capacities of the sediment, sewage and O157 *E. coli* were compared. To initiate biofilm growth within a 96-well flat bottomed polystyrene microplate (Costar, Corning, New York, NY), *E. coli* inoculum was prepared as described by Moreira et al. (2011) with minor modifications. Eighty two of the 149 *E. coli* isolates in the multiplex PCR assay (47 sediment, 24 sewage, and 11 O157 isolates) were grown on TSA plates. Three replications were performed for each isolate where the inoculum was placed into 30 mL of sterile TSB in a 125 mL Erlenmeyer flask and incubated overnight at 37°C with shaking at 150 rpm. Ten mL of each overnight culture was withdrawn and transferred into a sterile 15 mL disposable centrifuge tube. The centrifuge tubes were centrifuged at 2050 x g at 4°C for 10 minutes. The supernatant was removed, and the cells were washed by re-suspending the pellet with 10 mL of sterile phosphate buffer saline (PBS) (8.0 g NaCl, 0.20 g KCl, 1.44 g Na₂HPO₄, and 0.24 g KH₂PO₄ per liter of double distilled water adjusted to a pH of 7.4 with 1 M HCl). The cells were centrifuged again, and the washing process was repeated for a total of three times. The washed cells were re-suspended with 10 mL of sterile double distilled water and vortexed to ensure full dislodgement of the pellet. Two mL of the cell suspension was transferred to a new tube with 5 mL of sterile double distilled water. The optical density was adjusted with a NovaSpec spectrophotometer (Biochrom LTD, Cambridge, UK) to an OD₆₀₀ of 1.00 ± 0.05 which was approximately 1x10⁹ CFU. Ten µl of the OD₆₀₀ 1.00 culture was transferred into a sterile 96-well flat bottomed polystyrene plate (Costar). Four separate wells for each culture were included in the experiment. All three sample replicates were included in the same row of the microplate. One hundred and ninety µl of Minimal Salt Medium with 0.04% glucose (MSMG)

(1.249 mM KH₂PO₄, 3.73 mM K₂HPO₄, 0.4 mM MgSO₄, 0.02 mM FeSO₄, and 1.4 mM NH₄Cl) was transferred to each well to give a final total volume of 200 µl. The last row of the plate was left blank to be used in determining the background staining of crystal violet in the biofilm assay. The lid was placed on the 96-well plate and parafilm was used to seal the lid to prevent evaporation during incubation. The 96-well plate was then placed inside of a covered Rubbermaid bin with damp paper towel. The plastic bin was then incubated for 48 hours at 22°C with gently shaking at 25 rpm. The 96 well plate was removed from the bin and the planktonic cells were emptied from the wells. Three separate washings of the plate were performed with sterile double distilled water before the plate was air dried inside the BSC for 30 minutes. The biofilm cells were stained with 150 µl of a 0.1% crystal violet solution for 10-15 minutes. Excess crystal violet solution was removed from the plate and the plate was washed three times with sterile double-distilled water before left to dry in the BSC for 30 minutes. To release the crystal violet from the biofilm cells, 200 µl of a de-staining solution made up of 80% acetone and 20% ethanol (v/v) was added to each well of the plate and left for 10 minutes. One hundred and fifty µl of the de-staining solution was transferred into a new 96-well plate for absorbance measurement at 595 nm using a Fluostar Optima automated plate reader (BMG Labtech, Offenburg, Germany). The background staining of the crystal violet was subtracted from each measurement to quantify the amount of biofilm formed by each sample. The average of the three independent replicates was used to represent the biofilm forming capacity for each of the sediment, sewage and O157 *E. coli* isolates.

2.2.5 Antibiotic Resistance Testing

2.2.5a Antibiotic Resistance Testing under CLSI Conditions

The same 82 *E. coli* isolates that were screened for their biofilm forming capacities were used again for the antibiotic resistance testing (47 sediment, 24 sewage, and 11 O157 isolates). Eight antibiotics were chosen based on numerous factors including their presence in the environment, frequency in human prescriptions filled, and to ensure variation in the antibiotic target on the bacteria (Kohanski et al., 2010; Kümmerer, 2009; Kümmerer, 2003; Public Health Agency of Canada, 2016). The eight antibiotics used in this experiment were ampicillin, colistin, gentamycin, erythromycin, vancomycin, sulfanilamide, tetracycline, and ciprofloxacin (Sigma Aldrich Canada, Oakville, ON). Sterile filter paper disks (6mm in diameter) were prepared in the lab from Whatman Filter paper #3 (General Electric Healthcare, Mississauga, ON) that was cut using a standard 1-hole punch and autoclaved for 15 minutes. To comply with the Clinical Laboratory Standards Institute (CLSI) standard for antibiotic testing with the Kirby-Bauer method, Mueller Hinton agar (Oxoid) plates were used. Antibiotic solutions were freshly prepared on the day of the experiments and antibiotic powders were weighed out using an analytical balance and placed into sterile 50 ml tubes (Thermo Fisher Scientific). The appropriate solvent was added to the antibiotic powder (Table 2.4) and mixed using a vortex shaker until the antibiotic was dissolved. Using a 10 ml sterile syringe, the antibiotic solution was filter sterilized with a sterile 0.2 µm polycarbonate hydrophilic membrane filter (EMD Millipore, Etobioke, Ontario) before being placed into a sterile 15 ml centrifuge tube (Thermo Fisher Scientific). Ten µL of antibiotic solution was added to each individual filter paper disk to achieve the desired amount of antibiotic per disk (Table 2.4). The disks were left to dry in the

BSC for 2 hours. Blank disks (i.e. disks without antibiotics) were prepared using just the solvent specific to antibiotic of interest. These blanks acted as controls to ensure that the solvent of the antibiotic and the disk itself did not affect the zone of inhibition.

The *E. coli* samples cultured in TSB overnight at 37°C were washed with sterile PBS as previously described, except, the optical density of the final cell suspension was adjusted to an OD₆₀₀ of 0.10 ± 0.05 which is equitable to the McFarlane standard for turbidity of 0.5. Two hundred µL of each of the OD₆₀₀ of 0.10 cultures were spread evenly on the Mueller-Hinton Agar plates and left to dry for approximately 30 minutes in a BSC before the antibiotic disks were placed onto the agar. Six replications of each antibiotic were performed on each *E. coli* sample, in addition to a blank disk to act as the control. The disks were gently pushed onto the agar to ensure proper contact before incubation. The sample plates were incubated for 18 hours in an Isotemp Incubator (Thermo Fisher Scientific) at 37°C. After which diameters of the inhibition zones were measured.

2.2.5b Modified Antibiotic Resistance Testing under Optimal Biofilm Growth Conditions

A subsample of 21 isolates used in the standard CLSI antibiotic resistance testing was used in the following experiments where there were 10 sediment, 8 sewage, and 3 O157 isolates. To examine the antibiotic resistance of the *E. coli* isolates under optimal biofilm forming conditions, adjustments to the standard CLSI protocol were implemented. The incubation temperature was adjusted to 22°C instead of 37°C to resemble the optimal temperature for biofilm formation (Moreira et al, 2011). The cultures were grown in MSMG broth to resemble the biofilm assay protocol established by Moreira (2011). Lastly, Mueller

Hinton agar was substituted by agar plates made with MSMG in order to keep the media conditions comparable to biofilm growth.

Planktonic cultures were prepared in 30 mL of sterile MSMG incubated overnight at 22°C with shaking at 150rpm (Moreira et al, 2011). One mL of each overnight culture was transferred into fresh sterile MSMG, vortexed and incubated for an additional 6 hours at 22°C with shaking at 150 rpm. This allowed for the bacterial culture to be in the exponential phase of growth, rather than in stationary phase. When the optical density (at 600nm) reached approximately 0.2 ± 0.05 , the cells were harvested and washed with sterile PBS twice as described previously. The planktonic cells were then re-suspended to an optical density of 0.2 ± 0.05 with sterile double distilled water. Two hundred μL of planktonic culture was transferred onto an MSMG plate and spread evenly on a plate and left to dry for 1 hour in the BSC. Antibiotic disks were then placed onto the respective plates as described previously and plates were incubated at 22°C for 18 hours. After incubation, zones of inhibition were measured and recorded.

In order to determine the antibiotic resistance of biofilm cells exposed to biofilm conditions, poloxamer 407 (Sigma Aldrich) plates were used. The protocol to prepare these biofilm plates was adopted from Yamada et al. (2011) with modifications. Poloxamer 407 was incorporated into MSMG media at a 30% concentration. The poloxamer-MSMG growth medium was refrigerated at 4°C for 48 hours in order for the poloxamer to fully dissolve into the solution. The poloxamer growth solution was autoclaved for 20 minutes and returned to the refrigerator for 48 hours to allow the medium to liquefy. The liquid poloxamer growth medium was poured into sterile petri plates inside the BSC in volumes of approximately 30 mL.

The poloxamer-MSMG plates were solidified and maintained at room temperature until the experiment.

To determine the antibiotic resistance of the *E. coli* biofilm cells on MSMG-poloxamer plates, biofilm cell suspensions were prepared as described by Magajna and Schraft (2015). *E. coli* samples were grown in MSMG broth as described for the planktonic cultures, except eliminating the additional transfer of culture and the 6 hours of additional incubation. Ten mL of each overnight culture was transferred into a sterile 15 mL disposable centrifuge tube. The cells were washed three times with sterile PBS and re-suspended with sterile double distilled water to an OD_{600nm} of about 0.05. One mL of this cell suspension was then transferred into 20 mL of MSMG in a sterile 250 mL Pyrex glass bottle containing 0.1 g of glass fibre filters (pore size 0.7 µm, Whatman GF/F) and incubated for 48 hours at 22°C with shaking at 25 rpm. To harvest the biofilm cells from the glass fibre filters, the planktonic cells were removed aseptically and discarded using a sterile glass pipette. The filters were washed three times inside the glass bottles by adding 10 mL of sterile PBS and swirling gently before removing the liquid from the bottle. The filter disks were transferred aseptically using sterile forceps to a sterile 100 mL glass bottle containing 5g of sterile glass beads (450-600 µm, Sigma Aldrich). Eight mL of sterile double distilled water was then added to the glass bottle, and the sample was vortexed vigorously for 3 minutes. The biofilm cell samples were then transferred into individual sterile stomacher bags to remove excess glass fibres and the biofilm cell samples were collected in sterile 15 mL tubes. The biofilm cell samples were then adjusted to an OD₆₀₀ of 0.2 ± 0.05 with sterile double distilled water and kept on ice until ready to use. Antibiotic disks were prepared as described previously. Two-hundred-µL of biofilm culture was

transferred onto an MSMG-30% poloxamer plate and left to dry for 1 hour. Antibiotic disks were then placed onto respective plates as described previously and the plates were incubated at 22°C for 18 hours. After incubation, zones of inhibition were measured and recorded.

2.2.6 Measuring Intrinsic ROS of *E. coli*

The same group of 21 isolates (10 sediment, 8 sewage, and 3 O157 isolates) used in the modified antibiotic resistance testing was used in the intrinsic level of reactive oxygen species (ROS) assay. The intrinsic levels of ROS of planktonic and biofilm *E. coli* cells from the sediment, sewage and O157 samples were examined by DCF-DA (2',7'-di-chlorofluoresceine diacetate). The planktonic and biofilm *E. coli* cells were prepared using the same procedures described in the modified antibiotic resistance testing protocol whereby both planktonic and biofilm cells were grown at 22°C and in MSMG with biofilm cells being grown on glass fibre filters. A 1000 µM DCF-DA solution was prepared and 10 µL portions of the solution were added to sterile 15mL tubes containing 1980 µL of either the planktonic or biofilm *E. coli* cell samples suspended in sterile double distilled water. The cell samples were incubated in the dark at 25°C for 30 minutes to allow the DCF-DA to interact with the cells. In a dark room, 100 µL of each sample was then transferred into a Costar 96-well flat-bottomed plate (Corning). Each sample was loaded into six individual wells where the fluorescence was measured at 570 nm excitation/ 585nm emission using a BMG Labtech FLUOrstar OPTIMA plate reader. The average of the six fluorescence measurements was determined for each sample where the fluorescence was proportional to the amount of ROS in the sample.

One mL aliquots of the planktonic and biofilm *E. coli* cultures were collected, and their protein concentrations were determined. Protein concentrations of the cell samples were determined by the B-PER Bacterial Protein Extraction Reagent (Thermo Fisher Scientific) and Coomassie (Bradford) Protein Assay kit (Thermo Fisher Scientific). One mL of each cell suspension was washed and pelleted in a sterile 1.5 mL Eppendorf tube. One mL of the B-Per Reagent was added to the cell pellet and vortexed until the pellet was homogenized before it was incubated at room temperature for 15 minutes. The lysate was then centrifuged for 5 minutes at 15,000 x g to remove the cell debris and the protein concentration of sample was determined by the Bradford assay. The Coomassie (Bradford) Protein Assay (Thermo Fisher Scientific) was performed as outlined in the manufacturer's manual for the microplate protocol. Protein standards were prepared using BSA at concentrations of 0, 2.5, 5, 10, 15, 20 and 25 µg/mL. Each *E. coli* protein extract (150 µL) was added to the microplate in triplicate where 150µL of the Coomassie (Bradford) Reagent was added to each well containing samples or standards. The plate was allowed to incubate at room temperature for 10 minutes. The absorbance of each well was measured at 595nm using a Fluostar Optima automated absorbance plate reader (BMG Labtech, Offenburg, Germany). A standard curve was constructed using the BSA standards and the protein concentrations of the *E. coli* samples were determined by comparing their absorbance to the BSA standard calibration curve.

2.2.7 Statistical Analysis

Chi-Square Analysis (StatsToDo.com) was performed to compare the percentage of virulence genes between the three groups of *E. coli* (sediment, sewage, and O157) in this study.

Other statistical analyses were performed by the SigmaPlot 12 Software integrated with SigmaStat (Systat Software Inc., San Jose, USA) unless otherwise stated. One-way analysis of variance (ANOVA) was performed in various sections of this study: biofilm forming capacities, antibiotic resistance, and ROS concentrations. ANOVAs were carried out in three independent samples and further replications of each sample varied from 3-6 depending on individual experiments. The biofilm forming capacities of the three sample sources were compared using the average values of three independent replicates samples for each *E. coli* tested. The standard CLSI antibiotic resistance of the three groups of *E. coli* were compared and analyzed by the one-way ANOVA test. The comparisons between the zone of inhibition for each sample source in the planktonic and biofilm state was also performed with one-way ANOVAs using three replications for each sample and each antibiotic tested. Lastly, the comparison between the intrinsic level of ROS in the planktonic and biofilm cells were determined by one-way ANOVA using six experimental replications. The patterns of antibiotic resistance of the three *E. coli* groups were compared using discriminant function analysis in the SPSS statistic program (IBM, Markham, ON) using the six zones of inhibitions measured for each sample for all eight antibiotics under standard CLSI conditions. Linear regression was performed (SigmaPlot12) to determine the correlations between: (1) the ROS in a planktonic cell and Δ ROS from planktonic to biofilm phase; (2) the antibiotic resistance and biofilm forming capacity; and (3) the relationship between the amount of ROS and biofilm forming capacity.

2.3. Results

2.3.1 Virulence Gene Detection

A total of 149 *E. coli* isolates were screened using multiplex-PCR assays for nine-different virulence markers representing the five types of pathogenic *E. coli* (EHEC, ETEC, EPEC, EIEC, and UPEC) examined in this study. Positive controls of the virulence genes were used to optimize the reaction conditions of the multiplex PCR assay (Figure 2.1). The presence of these virulence genes in the positive controls were detected through both singleplex and multiplex PCR reactions. Figure 2.1 exhibits the results of the three primer groups in both singleplex and multiplex PCR reactions. Figure 2.1(A) illustrates the singleplex PCR detection of the *hs* gene of ETEC 505 (lane 1), *hl* gene of ETEC 07 (lane 2), and *iroN*_{*E. coli*} gene of UPEC 25922 (lane 3) with amplicons of 170, 322, and 665 base pairs (bp) respectively. Multiplex PCR reactions in Figure 1(A) can be found in lanes 4 through 6 (*hs*, *hl*, and *iroN*_{*E. coli*} respectively) whereby only the target gene is amplified in each positive control strain, regardless of the presence of the other two pairs of multiplex primers. Lane 7 of Figure 2.1(A) illustrates the detection of all three virulence genes simultaneously in one multiplex PCR reaction with no non-specific amplification. Similar to Figure 2.1(A), Figure 2.1(B) illustrates specific amplification of *ial* (650 bps), *bfpA* (324 bps), and *hlyA* (1000 bps) for both singleplex and multiplex PCR reactions. Additionally, Figure 2.1(C) illustrates the amplification of *stx1* (150 bps), *stx2* (255 bps), and *papA* (720 bps) through both PCR reactions.

Seventy-two sediment (27 from Lake Simcoe, 23 from Georgian Bay, and 22 from Boulevard Lake), 66 sewage (57 from Thunder Bay and 9 from Orillia), and 11 O157 *E. coli* isolates were examined by the multiplex PCR assay. Overall, Chi-Square analysis revealed an

overall significant difference between the three groups in terms of both of their overall uropathogenic ($\chi^2= 20.44$, $p<0.0001$) and diarrheagenic gene possession ($\chi^2= 124.65$, $p<0.0001$) (Table 2.5 and Table 2.6) with the sewage isolates contained significantly more uropathogenic virulence genes than sediment samples ($p<0.001$).

Most of the sediment samples were negative for uropathogenic, diarrheagenic and hemorrhagic genes (Table 2.5 and Table 2.6). The only virulence gene detected in sediment *E. coli* isolates was *iroN* (the iron sequestering gene), which was positive to 12.5% of the samples (Table 2.5). Neither the diarrheagenic or hemorrhagic *E. coli* genes were detected in these isolates. Forty-two percent of the sewage samples were positive for uropathogenic genes, consisting either of *iroN*, *hylA*, *papA* or combinations of the three (Table 2.5). Nine percent of the sewage isolates contained the *iroN* gene, 5% contained the *hylA* gene, and 12% contained the *papA* gene. Four percent of sewage isolates contained both the *iroN* and *hylA* gene, 5% contained the *iroN* and *papA* gene, while 4% contained all three *iroN*, *hylA*, and *papA* genes. In terms of diarrheagenic and hemorrhagic gene possession, only one sewage isolate contained the *ial* gene, while another isolate contained the *stx1* gene (Table 2.6). The O157 *E. coli* strains were used in this study as positive controls for the hemorrhagic genes. None of the O157 isolates contained the uropathogenic or diarrheagenic genes examined in this study. While 73% of the strains contained only the *stx1* gene and 27% contained both the *stx1* and *stx2* genes (Table 2.6).

2.3.2 Biofilm Forming Capacity

To compare biofilm forming capabilities between the sediment, sewage and O157 *E. coli* samples, a total of 82 isolates (a subsample from the original 149 samples examined in the multiplex PCR assay) were tested under the optimal biofilm forming condition at 22°C for 48 hours in MSMG. This group of 82 samples included 47 sediment samples (20 from Lake Simcoe, 15 from Georgian Bay, and 12 from Boulevard Lake), 24 sewage samples (9 from Orillia and 15 from Thunder Bay), and 11 O157 isolates. This reduction in number of samples allowed for a more manageable sample size that included representation of all sample locations.

Sediment *E. coli* samples displayed the highest biofilm forming capabilities amongst the three groups of *E. coli* (Figure 2.2). Sediment samples had an average absorbance of 0.700 ± 0.293 , about twice the biofilm forming capacity of sewage samples with an average absorbance of 0.354 ± 0.197 . In addition, the sediment *E. coli* on average formed 3.5 times as much biofilm as the O157 samples with an average absorbance of 0.210 ± 0.022 .

The three sample types, sediment, sewage, and O157, illustrated an overall significant difference from each other when comparing their biofilm forming capabilities using Tukey's One-Way ANOVA ($p < 0.001$). Comparisons between sediment and sewage, as well as between sediment and O157 illustrated significant differences between sample groups ($p < 0.001$). There was no significant difference between the sewage and O157 samples ($p = 0.253$).

2.3.3 Antibiotic Resistance

2.3.3a Antibiotic Resistance at Standard Conditions (37°C Using Mueller-Hinton Agar Plates)

To use a more manageable number of samples when testing for antibiotic resistance, 49 isolates were selected from the three groups of *E. coli* tested in the biofilm assay. The isolates were chosen covering the full range of biofilm forming capacities from each sample group. The isolates selected included 19 sediment isolates (9 from Lake Simcoe, 6 from Georgian Bay and 4 from Boulevard Lake), 19 sewage isolates (4 from Orillia and 15 from Thunder Bay) as well as the 11 O157 isolates.

In accordance with the Clinical Laboratory Standards Institute (CLSI) 2013 guidelines for antibiotic resistance testing, all samples were screened for their antibiotic resistance using Mueller-Hinton plates and an incubation temperature of 37°C. A canonical plot was constructed through discriminant function analysis to compare the antibiotic resistance patterns (zone of inhibition distances) of the sediment, sewage and O157 *E. coli* (Figure 2.3). The canonical plot illustrates a clear separation between the three sample groups indicating that the antibiotic resistance patterns of the *E. coli* between these groups are very different from each other but within the same group, the isolates are very similar and clustered together. The discriminant function analysis used a two-function model showing significant group differences ($\chi^2=187.61$ and $p<0.001$) between the three groups of *E. coli* with function 1 and 2 represented 96.5 and 3.5% of the variables respectively. Classification from the discriminant function analysis showed that overall 98.0% of original grouped cases were correctly classified in their source groups (Table 2.7) where 100% of the sediment, 100% of the sewage, and 91.7% of the O157 isolates were correctly classified into their respective groups.

The sewage samples had a significantly higher level of resistance than both the sediment and O157 *E. coli* isolates (p-values of <0.001 and <0.001 respectively). The mean zone of inhibition for O157 was the greatest at 20.18 mm \pm 0.285, indicating that it was the least resistant sample group. The sediment had a mean zone of inhibition of 18.94 mm \pm 0.217, while the sewage was the most resistant with a mean zone of inhibition of 18.49 mm \pm 0.217 (Figure 2.4).

Each isolate was classified as either resistant, intermediate, or susceptible to the eight antibiotics according to the CLSI (2013) standard. The total number of isolates that were classified as either resistant or intermediate were tabulated and compared statistically using chi-square analysis (Table 2.8). Overall, the sewage *E. coli* samples illustrated higher levels of resistance than the sediment to the antibiotics examined. Four of the eight antibiotics tested (colistin, gentamycin, tetracycline, and ciprofloxacin) were highly effective on the *E. coli* with no resistant strains found for either the sediment or sewage samples. Two antibiotics, vancomycin and erythromycin, were not effective for either the sediment or sewage samples with 100% of the isolates being resistant. One antimicrobial, sulfanilamide, was found to be ineffective on sewage *E. coli* with 100% of sewage isolates being resistant, however only 4% of sediment were resistant to this antibiotic. Ampicillin was 100% effective for the sediment *E. coli* however 32% of the sewage isolates were found to be resistant. The variations in the levels of resistance in sample type for ampicillin and sulfanilamide were found to be significant upon Chi Square analysis ($\chi^2 = 8.05$ with $p=0.018$ and $\chi^2= 34.26$ with $p<0.001$ respectively). All other antibiotics had no significant differences between the three groups based on their CLSI classification ($p>0.05$). Overall, the sewage sample group illustrated the greatest level of resistance to the

eight antibiotics examined (based on the diameter of their average zone of inhibition) in this experiment while the O157 group illustrated the highest degree of susceptibility.

2.3.3b Antibiotic Resistance in Biofilm Growth Conditions

To compare the antibiotic resistances of the planktonic and biofilm cells, a subgroup of 21 isolates was chosen from the original 49 samples. These isolates covered the full range of antibiotic resistance and biofilm forming capabilities in each sample group. The subgroup of samples contained 10 sediment (4 from Lake Simcoe, 2 from Georgian Bay and 4 from Boulevard Lake), 8 sewage (4 from Orillia and 4 from Thunder Bay) and 3 O157 isolates. A one-way ANOVA was performed to determine if the antibiotic resistance of the *E. coli* isolates changed when the experiment was conducted in MSMG at 22°C rather than at the CLSI conditions. The results demonstrated that the overall antibiotic resistance of the *E. coli* grown under the optimal biofilm condition was significantly different from those grown under standard CLSI conditions ($p < 0.001$). Furthermore, all antibiotics except ciprofloxacin ($p = 0.726$), illustrated a significant difference between the two growth conditions ($p < 0.05$) (Table 2.9). This new regimen using MSMG agar media at 22°C was used to compare the antibiotic resistances between planktonic and biofilm cells using the Kirby-Bauer disk diffusion method. Planktonic cell resistance was conducted on MSMG agar plates, whereas biofilm cell antibiotic resistance was conducted using plates made up of 30% poloxamer 407 in MSMG (ensuring the cells were in the biofilm state). Overall, biofilm cells were found to be significantly more resistant than their planktonic counterparts ($p < 0.001$) (Figure 2.5). The overall inhibition zone of the planktonic cells was 1.7 times of the biofilm cells.

Between the three groups of planktonic *E. coli*, two-way ANOVA revealed that the overall antibiotic resistance of the sediment isolates was significantly lower than that of the O157 samples ($p=0.039$) but was not significantly different from the sewage isolates. Whereas with biofilm cells, there was no significant differences between the three groups of samples (Figure 2.6). There was however, a significant difference between planktonic and biofilm cells within each sediment, sewage and O157 sample group ($p<0.001$, $p<0.001$, and $p=0.009$ respectively) (Figure 2.6).

Statistical analysis was also conducted for each antibiotic to compare the resistance between the planktonic and biofilm cells. Two-way ANOVA revealed that all antibiotics except for gentamycin and erythromycin (p -values of 0.056 and 0.171 respectively) had significant differences observed between the two growth phases (Figure 2.7). Five of the eight antibiotics (ampicillin, vancomycin, tetracycline, ciprofloxacin, and sulfanilamide) exhibited greater levels of antibiotic resistance in the biofilm state ($p<0.05$). One antibiotic, colistin, exhibited an opposite effect, whereby biofilm cells were less resistant than planktonic cells. This opposite effect can be explained by the fact that colistin targets non-respiring cells (i.e. biofilm cells) rather than metabolically active cells (i.e. planktonic cells) (Pamp et al., 2008).

2.3.4 Reactive Oxygen Species

The intrinsic levels of ROS of the planktonic and biofilm cells of the three groups of *E. coli* were determined using the DCF-DA fluorescence assay. A total of 21 samples consisting of 10 sediment (4 from Lake Simcoe, 2 from Georgian Bay and 4 from Boulevard Lake), 8 sewage (4 from Orillia and 4 from Thunder Bay) and 3 O157 isolates were analyzed in this study.

The overall ROS levels of the biofilm samples were significantly lower than that of the planktonic samples ($p < 0.001$). On average, the ROS levels of planktonic cells were 424% larger than the biofilm cells (Figure 2.8). Furthermore, the ROS levels of the biofilm cells were significantly lower than the planktonic cells for the sediment and sewage samples with both p -values being < 0.001 (Figure 2.9). However, because of the large deviation and small sample size of the O157 isolates used, there was no significant difference between the planktonic and biofilm cells within this group ($p = 0.166$). It is important to note that regardless of sample source, the ROS level within the planktonic cells was not significantly different from each other ($p = 0.426$) and similar results were observed in the biofilm cells ($p = 0.233$), indicating that sample source did not affect the intrinsic ROS level of the *E. coli* cells.

A significant correlation ($p < 0.001$, $R^2 = 0.9557$) was observed between the intrinsic level of planktonic ROS and the overall change of ROS (Figure 2.10). The greater the amount of ROS in the planktonic cells, the greater the decrease in ROS was observed when the cells were transition into the biofilm state. This indicates that despite the varying levels of ROS in planktonic *E. coli* samples, the ROS level within biofilm cells drops to a similar minimal level regardless of the source or strains of the *E. coli*.

2.3.5 Correlations of Biofilm Forming Capacity versus Antibiotic Resistance and ROS

Linear regression analysis showed a negative correlation between the biofilm forming capacity of the *E. coli* isolates and their resistance to antibiotics, indicating that isolates with greater ability in forming biofilm tend to be less resistance to antibiotics. Figure 2.11 illustrates this trend across each of the eight antibiotics. This correlation was found to be significant, for

gentamycin ($p < 0.001$), tetracycline ($p = 0.026$), sulfanilamide ($p < 0.001$) and vancomycin ($p = 0.050$). Similar trends were observed for ampicillin, colistin and ciprofloxacin while the correlations were not significant (p -values of 0.681, 0.800, and 0.072 respectively). Since 19 of the 21 isolates were 100% resistant to erythromycin, the regression analysis between the biofilm forming capacity of the bacteria and their antibiotic resistance was not reliable. A negative correlation ($p = 0.066$) was also observed between the biofilm forming capacities of the isolates and their intrinsic ROS levels of the planktonic cells (Figure 2.12). This illustrated that planktonic isolates with higher intrinsic ROS (i.e. higher resistance to oxidative stress) showed lower ability to form biofilm.

2.4 Discussion

While most *E. coli* are harmless, some strains can cause intestinal or extra-intestinal diseases (Health Canada, 2012). *E. coli* have been found to survive and establish in various environments including the periphyton, soil, sand, and algae (Byappanahalli and Fujioka 1998; Ishii et al. 2006; Kon et al. 2007; Ksoll et al. 2007; Whitman et al. 2003), however, the sediment has only recently been studied as a reservoir for *E. coli* (Luna et al., 2010; Quero et al., 2015). High densities of *E. coli* in the environment have been correlated with increased gastrointestinal symptoms amongst freshwater swimmers (Alm et al., 2006). However, water testing for contamination is based on the presence of *E. coli* in the water column, not in the sediment (Luna et al., 2010). This lack of sediment analysis presents a problem since *E. coli* have been found to accumulate in the sediment and have the potential of being re-suspended into the water column if disturbed (Alm et al., 2006; Le Fevre and Lewis, 2003). The populations of *E. coli* in the sediment have not been widely studied, thus this investigation aims to examine *E. coli* isolated from sediment and to compare them to those isolated from sewage and a collection of shiga toxin producing *E. coli* O157. A total of nine virulence genes were examined in this study belonging to the uropathogenic, diarrheagenic, and hemorrhagic *E. coli* groups. These genes enable *E. coli* to become pathogenic towards its host and elicit an infection.

In this study, none of the sediment isolates tested were found to contain genes belonging to either the diarrheagenic or hemorrhagic genes groups (Table 2.5 and Table 2.6). A relatively low percentage (12.5%) of *E. coli* from the sediment was found to contain the *iroN* gene that belongs to the uropathogenic genes group. This *iroN* gene has clinical importance for

uropathogenic *E. coli* as it allows them to elicit infections (Anastasi et al., 2012; Wiles et al., 2008). It causes this infection by forming an iron siderophore-based acquisition system that gives the bacteria the ability to use iron found in the host (Anastasi et al., 2012; Wiles et al., 2008). The *iroN* gene has also been shown by Neilands (1995) to offer bacteria the ability to sequester iron from the environment, where the amount of iron available to the bacteria is low. The possession of this gene within sediment *E. coli* populations does not necessarily translate into negative effects on human health as it may have been acquired for better survival in the environment. Anastasi et al. (2012) found similar results to this experiment when they examined sediment *E. coli* and found that environmental samples harboured only the *hylA* or *iroN_{E.coli}* genes. With an understanding that the *iroN* gene may be present in bacteria for survival in the environment, it makes sense why the sediment *E. coli* were found to have this virulence gene. Future monitoring will need to be conducted to determine if other known virulence genes become established in the sediment *E. coli*.

There are minimal studies examining *E. coli* established within the sediment. Luna et al. (2010), one of the most notable studies on sediment *E. coli*, found that there was a large genetic diversity within sediment *E. coli* populations and concluded that marine sediments do not select for specific genotypes. This genetic heterogeneity between sediment *E. coli* could be caused by fecal contamination from waste waters, the geographical and climate of the sediment, and from horizontal gene transfer that is facilitated by the sediment bacterial community (Luna et al., 2010). If an area where the sediment *E. coli* is collected from is frequently contaminated, then this would artificially alter the amount of virulence genes possessed by these bacteria. Likewise, the temperature and geographical region that the

sediment exists in also plays a role in *E. coli* distribution. Luna et al. (2010) analyzed sediments from marine waters for virulence genes using multiplex PCR to reveal that between 65-90% of sediment isolates tested contained at least one virulence gene from *E. coli* that have the potential to cause disease in humans (their study tested 11 extra-intestinal virulence factors including *pap*, *sfa/foc*, *afa*, *eaeA*, *ibeA*, *traT*, *hlyA*, *stx1*, *stx2*, *aer*, and *fyuA*). While Luna et al. (2010) revealed that the areas where their samples were collected have the possibility of being contamination with sewage, these findings illustrate how sediment is a potential reservoir for pathogenic bacteria. These bacteria could be released from the sediment by either weather/wave conditions (Fevre and Lewis, 2003), or from animal traffic (Sherer et al., 1992). The two Southern Ontario locations used in this study of sediment *E. coli* (Georgian Bay and Lake Simcoe), were determined to have minimal urban influence with less than or equal to 10% urban cover (N. Fligg., 2018). Boulevard Lake in Northwestern Ontario also has minimal exposure to sewage contamination. This low level of contamination from sewage may explain the observation from this study where very low percentages of the sediment *E. coli* were found to contain virulence genes. With the only virulence gene found in the sediment being the *iroN* gene, this may infer that the composition of virulence genes in a sediment *E. coli* population could be an indicator for the quality of the environment in which they reside, rather than to human infection. Further studies into the environmental properties of sediment bacteria and their need to be included in water quality testing should be examined in order to more accurately determine the level of water contamination.

In contrast to sediment, sewage has been more widely studied due to the high frequency of fecal contamination in the environment. Overall, the sewage *E. coli* isolates were

found to contain significantly more virulence genes than both the sediment and O157 samples for both uropathogenic and diarrheagenic genes. In this study, almost half (42%) of the sewage samples were positive for uropathogenic genes *ironN*, *hlyA*, *papA*, or combinations of the three (Table 2.5). There were only 1.5% of the sewage isolates that contained the *ial* gene, and only 1.5% contained the *stx1* gene. Mokracka et al. (2011) used multiplex PCR to detect virulence genes (*eae*, *bfpA*, *ST[hs]*, *LT[hI]*, *ipaH*, *stx1* and *stx2*) in municipal waste water and found that 50.5% of the *E. coli* isolates were positive for these virulence genes. Diarrheagenic pathotypes made up 21% of all isolates tested and 17.4% of the isolates had three or more virulence genes in their study (Mokracka et al., 2011). Sabaté et al. (2008) also analyzed human wastewater and found that it contained a high level of pathogenicity islands. Despite the fact that all sewage studies show a high levels of *E. coli* with virulence genes, the frequency and composition of the various virulence genes ranges widely for each study. This range in sewage virulence frequency indicates that the populations feeding into each sewage treatment plant are not uniform.

E. coli have evolved to survive and establish in non-enteric habitats due to a high degree of versatility and genetic diversity (Quero et al., 2015; Touchon et al., 2009). One of these factors includes forming biofilms as it allows bacteria to survive and establish in an environment where there are fluctuating harsh conditions (Hall-Stoodley et al., 2004). The ability of bacteria to form biofilms has been studied extensively by scientists. However, there has been little direct comparisons on how an isolates' ability to form biofilms is related to its' establishment within the environment. Even less information is known on how the ability of bacteria to form biofilms is related to their resistance to antibiotics and tolerance to oxidative stress.

The biofilm forming capacity was found to be significantly different amongst the three groups of samples (Figure 2.2). Comparisons between the three groups illustrated that sediment samples were the best biofilm formers. This makes sense for the bacteria, as they would have to survive harsh, non-optimal conditions in the sediment environment (including nutrient limitation and stress) (Hall-Stoodley et al., 2004). Sewage *E. coli* illustrated heterogeneity between isolates, with some being moderate biofilm formers and others being poor biofilm formers, however, the general overall finding was that sewage isolates were poorer biofilm formers than sediment *E. coli*. Similar observations were shown by Resner et al. (2006). By examining over 300 *E. coli* isolates retrieved from healthy and unhealthy individuals, they found that the isolates illustrated varying biofilm forming capabilities. The findings in Resner et al. (2006) and in this study illustrate how *E. coli* from mammalian sources are not uniform and that under different conditions, biofilm forming capabilities may be more advantageous than in others. The sewage isolates' biofilm forming capabilities differ greatly from the pathogenic O157 isolates, which exhibited low biofilm forming capabilities across all samples tested with minimal variation amongst the samples. This lack of variation amongst the O157 samples may be a reflection of the relatively short evolutionary history of this serotype of shiga toxin producing *E. coli* (Percival et al., 2014).

With the large number of antibiotics being prescribed today, it has created a problem for health professionals where bacteria that could once be treated with these antibiotics have developed resistance, making bacterial infections more and more difficult to treat. Antibiotics for this experiment were chosen based on numerous factors including how frequently they were prescribed, their presence in the environment, their level of effectiveness, and their

target on the bacteria. Ciprofloxacin, tetracycline, erythromycin, and sulfanilamide have all been commonly prescribed and found in the environment (Kummerer, K., 2003; Public Health Agency of Canada, 2016). Antibiotics such as ampicillin, vancomycin, and gentamycin are also commonly used antibiotics but have shown resistance in either clinical or environmental settings (Boles and Singh, 2008; Kummerer, 2003; Sáenz et al., 2004). The last antibiotic used in this study was colistin, a polymyxin antibiotic that is currently being used as a last resort antibiotic due to its effectiveness against multidrug resistant Gram-negative bacteria (Falagas and Kasiakou, 2006; Li et al., 2006; Pamp et al., 2008). Although some antibiotics used in this study (namely erythromycin and vancomycin) are known to be ineffective against *E. coli*, the degrees of response to these antibiotics (i.e. diameters of the zone of inhibition) varied for the different *E. coli* strains. The antibiotic resistance patterns across the eight antibiotics tested illustrated clear differences between the sediment, sewage, and O157 *E. coli* groups (Figure 2.3 and Table 2.7). This agrees with our findings in the biofilm and virulence gene experiments where the three groups of *E. coli* were physiologically distinct. With established sediment *E. coli* populations not being previously examined in detail (Ishii et al., 2005), this study offers new information into how resistant these bacteria intrinsically are, and how they are different from the *E. coli* in sewage. The most resistant group was found to be sewage *E. coli*, while the sediment was the least resistant group (Figure 2.4) (Table 2.8). Sediment *E. coli* are not known to be exposed to antibiotic contamination as commonly as sewage *E. coli*. This range in exposure to antibiotics may explain the lower levels of antibiotic resistance found within the sediment *E. coli* in this study. However, with the continuous rise of antibiotic contamination in the aquatic environment, the risk of multiple antibiotic resistant bacteria will only increase.

The Kirby-Bauer disk diffusion method for antibiotic resistance of planktonic cells has been well documented. However, recent studies have shown that this method, with modifications, can also be used to study the resistance of biofilm cells. Gilbert et al. (1998) assessed biofilm cells' response to biocide treatment using 30% hydrogels composed of poloxamer F127, a non-toxic, di-block copolymer of polyoxyethylene and polyoxypropylene. They compared the protein expression of planktonic, biofilm, and poloxamer grown cultures using SDS-PAGE to determine the characteristics of the poloxamer grown cells. They found that poloxamer cultures exhibited a general protein pattern and phenotype similar to the biofilm cells, thus confirming the hydrogel method as a simple and reliable assay to produce and study biofilm cells. Yamada et al. (2011) furthered the use of hydrogels for biofilm antibiotic resistance testing by using 30% poloxamer 407 incorporated into Mueller Hinton media to create biofilm growth plate media. A protocol similar to Yamada et al. (2011) was employed in this study to accurately compare the antibiotic resistance patterns of both planktonic and biofilm cells using the Kirby-Bauer disk diffusion method.

Antibiotic resistance testing comparing the planktonic and biofilm cultures in this study illustrated clear differences, with the biofilm cells being significantly more resistant to antibiotics than their planktonic counterparts ($p < 0.001$) (Figure 2.7). This increased level of resistance to antibiotics has been attributed to phenotypical differences amongst planktonic and biofilm cells including the exopolymeric matrix and reduced cellular activity. When biofilm cells are enclosed in the exopolymeric matrix, it makes it difficult for polar and charged antibiotics to reach the bacteria in order to elicit its effect (Cloete, 2003; Kirby et al., 2012). Cells in a biofilm also enter stationary phase, where they can reduce their metabolism and

respiration levels to become virtually undetected by antibiotics seeking out actively respiring bacteria (Lewis et al., 2001). Colistin is an example of an antibiotic that is more effective on cells with low metabolic activity and less effective on cells with higher levels of metabolic activity (Pamp et al., 2008). The trend observed between planktonic and biofilm cultures with colistin confirmed the findings of Pamp et al. (2008) where the planktonic cells were significantly more resistant to colistin ($p < 0.05$) than their biofilm counterparts. Two antibiotics, gentamycin and erythromycin, were not found to be significantly different between the two stages and there is not a complete consensus for this similarity.

This study compares the cellular levels of ROS in *E. coli* obtained from different sources (sediment, sewage, and O157). The findings concur with other studies (Jakubowski and Bartosz 2000; Jakubowski et al. 2000) in that significantly lower levels of ROS were found in biofilm cells than planktonic cells regardless of their sources ($p < 0.001$) (Figure 2.9). Jakubowski et al. (2000) explained that biofilm cells have higher protection against oxidative stress because they have reduced metabolic process, which can lead to lower concentrations of free radicals generated within the cells. Biofilm cells have been found to be very different from their planktonic counterparts in a variety of ways, one of which involves their stress responses. As indicated previously in the literature review, the RpoS (stationary phase sigma factor) is responsible for initiating a range of stress responses in the bacterial cell once it enters a biofilm state (Schellhorn, 2014). It is not until the cell enters a biofilm state where the RpoS sigma factor is produced and can elicit a stress tolerance effect (Battesti et al., 2011; Schellhorn, 2014; Mah and O'Toole, 2001). These responses include an increase in the number of stress response proteins produced by the bacteria, such as catalase, that can reduce the levels of ROS and

hence reduce oxidative stress of the bacteria (Jakubowski and Walkowiak, 2015). This concurs with the findings in this study where the concentration of ROS in planktonic cells was significantly higher than those in biofilm cells ($p < 0.001$) (Figure 2.8). While the level of ROS in planktonic cells varies between the isolates, the overall differences between the three groups were not significant (Figure 2.9). Furthermore, the biofilm cells of all of the isolates had similar levels of ROS and no significant differences were observed between these sample groups.

For cells capable of forming strong biofilms, Penterman et al. (2014) proposed that there are fitness trade-off mechanisms that the bacteria will have to undergo. Therefore, when certain genetic adaptations are evolved in bacteria for a specific growth and/or environmental condition, it will reduce the fitness of the bacteria in other conditions (Futuyma and Moreno, 1988). For example, a trade-off mechanism has been shown to be between biofilm capabilities and antibiotic resistance by Gallant et al. (2005) and Qi et al. (2016). The findings of these studies indicated that bacteria must balance the ability of either forming biofilms or being resistant to antibiotics. Qi et al. (2016) examined over 200 isolates of *Acinetobacter baumannii* for this relationship between antibiotic resistance and biofilm forming capacity and concluded that bacteria with increased antibiotic resistance formed weaker biofilms. Gallant et al. (2005) compared the ability of β -lactamase positive bacteria to form biofilms with and without the presence of β -lactam antibiotics. The results indicated that an increase in resistance to antibiotics came at the expense of forming weaker biofilms (Gallant et al., 2005). The authors suggested that the peptidoglycan required for the cell to adhere to the surface and form biofilms may be altered by β -lactamases (Gallant et al., 2005). While the relationship between antibiotic resistance and biofilm forming capacity has only recently been proposed, no study

has examined this relationship across a large group of *E. coli* with a variety of antibiotics. This investigation revealed a negative correlation between the antibiotic resistance in planktonic cells and biofilm forming capacity of 21 *E. coli* isolates (10 from sediment, 8 from sewage, and 3 from the O157 group) (Figure 2.11). This correlation was significant for five antibiotics, namely, gentamycin, tetracycline, sulfanilamide, erythromycin, and vancomycin (90% confidence) that expands upon the theory that bacteria must trade-off survival mechanisms. In agreement with the fitness trade-off theory, the *E. coli* isolates with strong abilities to form biofilms illustrated lower levels of resistance to a wide variety of antibiotics.

The negative correlation between the biofilm forming capability and ROS level of the planktonic *E. coli* samples (Figure 2.12) also supports the fitness trade-off theory. This overall trend was found to have a 90% confidence level ($p=0.066$) across the samples tested. For those *E. coli* isolates that exhibited lower levels of cellular ROS (i.e. lower tolerance to oxidative stress), they also showed strong biofilm forming capabilities, indicating that better biofilm formers trade-off their ability to tolerate higher levels of oxidative stress. This is the first study to observe this correlation across a large group of *E. coli* samples, however, further investigation is required to understand the cellular mechanisms that connect the biofilm forming capacity and oxidative tolerance of the bacteria.

Oxidative stress has been linked to antibiotics by Kohanski et al. (2007) who proposed that bactericidal antibiotics, regardless of their specific drug-targeted killing mechanisms, could stimulate the production of ROS in bacteria, including hydroxyl radicals, superoxide anions and hydrogen peroxide to cause cellular damages. Bactericidal antibiotics are able to alter the central metabolism of the cell by stimulating the oxidation of NADH through the electron

transport chain (Van Acker and Coenyn, 2017). The TCA cycle will also produce reducing molecules that will induce the formation of both hydrogen peroxide and superoxide (Van Acker and Coenyn, 2017). Superoxide molecules will damage the iron-sulphur clusters in cellular proteins that will react with hydrogen peroxide in the Fenton reaction to generate hydroxyl radicals. These radicals will then cause damage to DNA, lipids, and proteins causing ROS mediated cell death (Van Acker and Coenyn, 2017). Although antibiotics can induce the production of ROS and cause oxidative stress to bacteria, there are some defense systems in place within bacterial cells to mitigate the effect of oxidative stress leading to a greater degree of antibiotic resistance. These defence systems include the production of three major antioxidant enzymes: superoxide dismutase, catalase, and glutathione peroxidase (Volodymyr, 2011; Wang et al., 2012). Superoxide dismutases (SODs) convert the superoxide anion into hydrogen peroxide and oxygen (Schellhorn and Hassan, 1988). Catalase catalyzes the dismutation of hydrogen peroxide to form neutral products oxygen and water. Lastly, glutathione peroxidase catalyzes the destruction of hydrogen and hydrogen peroxide by using glutathione as an electron donor. In a previous study, Sheldon et al. (2012) illustrated that the *rpoS* gene, a crucial regulatory gene in biofilm phase bacteria, is able to improve the survival of *E. coli* in a biofilm by optimizing the size of the biofilm matrix. In this study, it was found that biofilm cells illustrate lower ROS concentrations and higher levels of resistance to antibiotics. The stationary phase sigma factor protein, RpoS, is responsible for turning on the *katE* gene which will allow for catalase enzymes to be formed (Vijayakumar et al., 2004). Since the RpoS regulon is a protein produced by the *rpoS* gene, it is possible that this gene may play a role in lowering the ROS level within biofilm cells and increasing their antibiotic resistance, and hence,

increasing their survival. Further investigations will be required to confirm the role of *rpoS* on the regulation of oxidative stress, antibiotic resistance, and survival of bacteria under the biofilm conditions.

2.5 References

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2.6 Tables

Table 2.1. Comparison of chemical content from the three locations at which sediment samples were collected in Northwestern and Southern Ontario.

Location Lake	Northwestern Ontario		Southern Ontario		Units
	Boulevard Lake	Lake Simcoe	Average	Georgian Bay	
% Moisture Content	17.97	57.71		83.74	%
Calcium	64.43	38.01		6.78	mg/L
Potassium	3.91	1.70		0.59	mg/L
Magnesium	23.9	6.37		1.19	mg/L
Sodium	170.61	7.70		0.62	mg/L
1:1 H2O:Soil Ratio	330.3	365.33		263.00	us/cm
Total Recoverable Aluminum	4763.92	4392.21		3208.13	ug/g
Total Recoverable Arsenic	<DL	<DL		18.54	ug/g
Total Recoverable Barium	40.79	32.94		99.83	ug/g
Total Recoverable Beryllium	<DL	<DL		<DL	ug/g
Total Recoverable Calcium	2200.78	55026.82		34711.80	ug/g
Total Recoverable Cadmium	<DL	<DL		0.78	ug/g
Total Recoverable Cobalt	6.57	2.96		3.32	ug/g
Total Recoverable Chromium	15.67	64.40		57.63	ug/g
Total Recoverable Copper	18.55	178.26		28.14	ug/g
Total Recoverable Iron	18645.38	7094.20		11888.10	ug/g
Total Recoverable Potassium	398.7	614.78		392.88	ug/g
Total Recoverable Magnesium	3628.9	4115.08		2687.80	ug/g
Total Recoverable Manganese	308.58	288.93		153.08	ug/g
Total Recoverable Molybdenum	<DL	<DL		<DL	ug/g
Total Recoverable Sodium	228.31	358.99		137.80	ug/g
Total Recoverable Nickel	16.61	20.24		15.49	ug/g
Total Recoverable Phosphorus	374.09	848.64		630.85	ug/g
Total Recoverable Lead	<DL	198.74		26.58	ug/g
Total Recoverable Sulfur	64.62	2495.37		14291.50	ug/g
Total Recoverable Selenium	<DL	<DL		<DL	ug/g
Total Recoverable Strontium	4.77	81.92		48.03	ug/g
Total Recoverable Titanium	335.72	596.05		299.12	ug/g
Total Recoverable Vanadium	55.45	11.44		82.03	ug/g
Total Recoverable Zinc	35.12	29.73		133.08	ug/g
pH 1:1 water to soil ratio	7.12	7.06		6.95	unit
Dissolved Organic Carbon	12.95	24.97		36.90	mg/L
Chloride (IC)	228.68	9.67		1.50	mg/L
Nitrite NO ₂ -N (IC)	<DL	<DL		<DL	mg/L
Nitrate NO ₃ -N [IC]	0.04	<DL		<DL	mg/L
Phosphate (PO ₄ -P) by IC	<DL	<DL		<DL	mg/L
Sulphate (SO ₄) [IC]	19.82	14.00		54.27	mg/L

<DL indicates below detectable limit

Table 2.2. Virotypes and strains used as positive controls to detect common uropathogenic, hemorrhagic and diarrheagenic *E. coli* genes with their respective primers.

Primer Name	Primer Symbol	Virotypes (Strain ID) Used as Positive Control
Heat stable toxin	<i>hs</i>	EPEC (EPEC 505)
Heat labile toxin	<i>hl</i>	EPEC (EPEC 07)
Catechol siderophore receptor	<i>iroN_{E. coli}</i>	UPEC (UPEC 25922)
Invasion associated loci	<i>ial</i>	EPEC (EPEC 0136)
Bundle forming pili	<i>bfpA</i>	EPEC (EPEC 055)
Hemolysin	<i>hlyA</i>	UPEC (UPEC 25922)
Shiga toxin 1	<i>stx1</i>	EPEC (EPEC 920004)
Shiga toxin 2	<i>stx2</i>	EPEC (EPEC 920004)
Pyelonephritis-associated pili	<i>papA</i>	UPEC (UPEC 25922)

Table 2.3. Primer sequences used in virulence gene detection study.

Part of set	Gene	Primer Sequence	Amplicon Size (bp)	Reference
1	<i>hs</i>	F:5'-TCTTTCCCCTCTTTTAGTCAGTC-3' R:5'-CCAGCACAGGCAGGATTAC-3'	170	Rappelli et al., 2001
1	<i>hl</i>	F:5'-TCTCTATGTGCACACGGAGC-3' R:5'-CCATACTGATTGCCGCAAT-3'	322	Rappelli et al., 2001
1	<i>ironE. coli</i>	F:5'-AAGTCAAAGCAGGGGTTGCCCCG-3' R:5'-GACGCCGACATTAAGACGCAG-3'	665	Chapman et al., 2006
2	<i>ial</i>	F:5'-GGTATGATGATGATGAGTCCA-3' R:5'-GGAGGCCAACAAATTATTCC-3'	650	López-Saucedo et al., 2003
2	<i>bfpA</i>	F:5'-GCCGCTTTATCCAACCTGGTA-3' R:5'-TGCTGGACCTACATTTAATTCC-3'	324	López-Saucedo et al., 2003
2	<i>hlyA</i>	F:5'-CATCTCTGGTTGGTGCACCGGTA-3' R:5'-AACTTGTCGGCACGCGTGGTC-3'	1000	Chapman et al., 2006
3	<i>stx1</i>	F:5'-CTGGATTTA ATGTTCGCATAGTG-3' R:5'-AGAACGCCCACTGAGATCATC-3'	150	López-Saucedo et al., 2003
3	<i>stx2</i>	F:5'-GGCACTGTCTGA AACTGCTCC-3' R:5'-TCG CCAGTTATCTGA CATTCTG-3'	255	López-Saucedo et al., 2003
3	<i>papA</i>	F:5'-ATGGCAGTGGTGTCTTTTGGTG-3' R:5'-CGTCCCACCATACGTGCTCTTC-3'	720	Chapman et al., 2006

Table 2.4. Antibiotics and solvents used in order to prepare each type of disk with the desired amount of antibiotic.

Antibiotic	Solvent	Concentration of antibiotic in solvent (µg/ml)	Amount of antibiotic* in each disk (µg)
Ampicillin	Water	1000	10
Colistin	Water	1000	10
Gentamycin	Water	1000	10
Erythromycin	2M HCl	1500	15
Vancomycin	Water	3000	30
Tetracycline	95% Ethanol	3000	30
Ciprofloxacin	0.1M HCl	500	5
Sulfanilamide	0.5M HCl	500	5

*10µl of each antibiotic solution was added to each disk.

Table 2.5. Detection of uropathogenic genes found in 72 sediment samples, 66 sewage, and 11 O157E. coli samples through the use of multiplex PCR.

Source	Uropathogenic <i>E. coli</i> genes						Total number of isolates containing one or more virulence gene
	Only <i>iroN</i>	Only <i>hlyA</i>	Only <i>papA</i>	Only <i>iroN</i> & <i>hlyA</i>	Only <i>iroN</i> & <i>papA</i>	<i>iroN</i> & <i>hlyA</i> & <i>papA</i>	
Sediment	9	0	0	0	0	0	9 ¹
72 isolates	(12.5%)	(0%)	(0%)	(0%)	(0%)	(0%)	(12.5%)
Sewage	5	4	12	2	3	2	28 ^{1,2}
66 isolates	(9%)	(5%)	(21%)	(4%)	(5%)	(4%)	(42%)
O157	0	0	0	0	0	0	0 ^{1,2}
11 isolates	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)

¹indicates significant difference with p<0.05

²indicates significant difference with p<0.001

Table 2.6. Detection of diarrheagenic genes found in 72 sediment, 66 sewage, and 11 O157E. coli samples through the use of multiplex PCR.

Source	Diarrheagenic <i>E. coli</i> genes							Total number of isolates containing one or more virulence gene
	Common Diarrheagenic <i>E. coli</i> genes				Hemorrhagic <i>E. coli</i> Genes			
	Only <i>hs</i>	Only <i>hl</i>	Only <i>ial</i>	Only <i>bfpA</i>	Only <i>stx1</i>	Only <i>stx2</i>	<i>stx1</i> & <i>stx2</i>	
Sediment	0	0	0	0	0	0	0	0 ¹
72 isolates	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
Sewage	0	0	1	0	1	0	0	2 ^{1,2}
66 isolates	(0%)	(0%)	(1.5%)	(0%)	(1.5%)	(0%)	(0%)	(3%)
O157	0	0	0	0	8	3	3	11 ²
11 isolates	(0%)	(0%)	(0%)	(0%)	(73%)	(27%)	(27%)	(100%)

¹indicates significant difference with p<0.05

²indicates significant difference with p<0.001

Table 2.7. Classification results from discriminant function analysis performed on samples from sediment (n=19), sewage (n=19) and O157 (n=11) Classification from canonical plot showed that overall 98.0% of original grouped cases were correctly classified.

		Predicted Group Membership				
		Sample Type	Sediment	Sewage	O157	Total
Original	Count (%)	Sediment	19 (100.0%)	0 (0%)	0 (0%)	19 (100.0%)
		Sewage	0 (0%)	19 (100.0%)	0 (0%)	19 (100.0%)
		O157	1 (9.1%)	0 (0%)	10 (90.9%)	11 (100.0%)

Table 2.8. Comparison of the percentage of strains classified as resistant or intermediate to the eight antibiotics tested in each of the three sample groups (sediment (n=19), sewage (n=19), and O157 (n=11)). Overall, the sewage illustrates higher levels of resistance than the sediment. Four of the eight antibiotics tested were found to be effective at eradicating the *E. coli* with no resistant strains found for either the sediment or sewage samples. Two antibiotics were not effective for either the sediment or sewage samples with 100% of the isolates being resistant. One antimicrobial, sulfanilamide, was found to be ineffective for sewage *E. coli* with 100% of sewage resistant, however only 4% of sediment were resistant to it.

Percentage of Strains that are Resistant/Intermediate Resistant ¹			
Antibiotic	Sediment	Sewage	O157
Ampicillin	0%	32%	9%
Colistin	0%	0%	0%
Gentamycin	0%	0%	0%
Vancomycin	100%	100%	91%
Tetracycline	0%	0%	0%
Ciprofloxacin	0%	0%	0%
Sulfanilamide	4%	100%	0%
Erythromycin	100%	100%	100%

¹ According to CLSI (2013) Antibiotic Resistance Classification

Table 2.9. Comparison between the two bacteria growth temperatures and their outcomes on antibiotic susceptibility (zone of inhibition) illustrates overall significant differences for each antibiotic except for ciprofloxacin.

Antibiotic	CLSI Condition	Biofilm Condition	p-value
	(37°C and Mueller-Hinton Agar Plates)	(22°C and MSMG Agar Plates)	
Mean Zone of Inhibition (mm)			
Ampicillin	28.4	21.9	<0.001
Colistin	9.7	15.5	<0.001
Gentamycin	11.8	19.6	<0.001
Vancomycin	21.0	11.0	<0.001
Ciprofloxacin	31.8	32.2	0.726
Tetracycline	24.9	22.3	0.031
Sulfanilamide	14.2	17.1	0.016
Erythromycin	6.2	9.6	0.006

2.7 Images & Figures



Image 2.1. Locations from which sediment samples were collected for this study. The green and blue locations indicate Georgian Bay and Lake Simcoe in Southern Ontario respectively. Whereas the red location indicates Boulevard Lake in Northern Ontario.

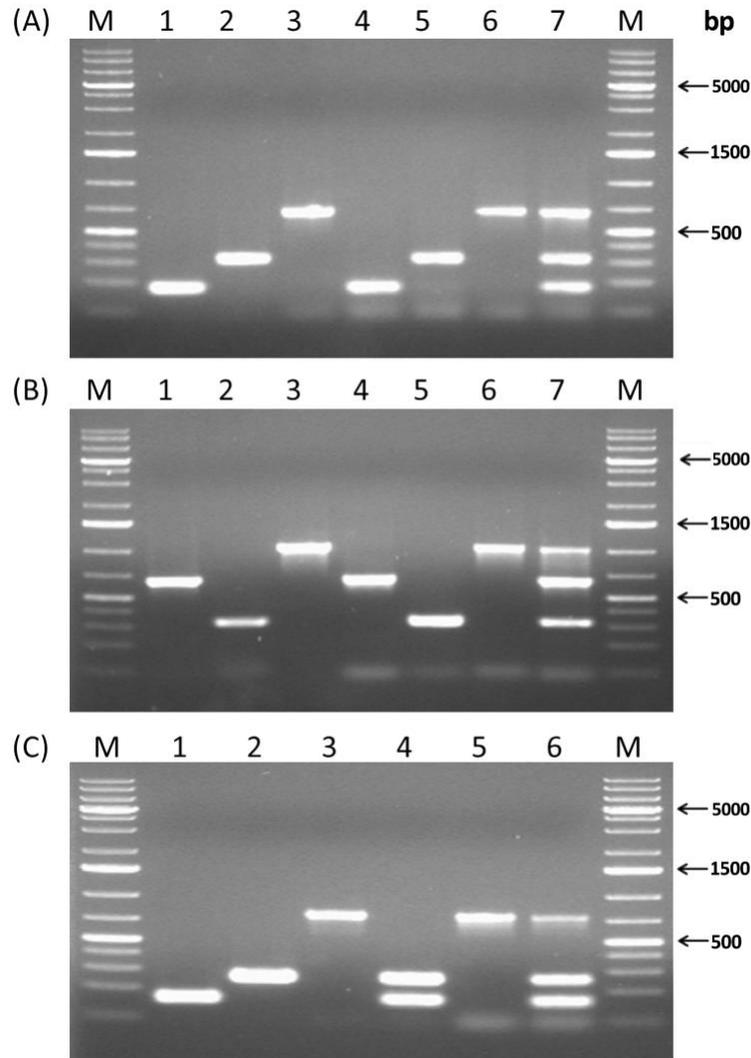


Figure 2.1. Detection of virulence genes using gel electrophoresis (1% agarose gel). electrophoresis products of positive control strains of *E. coli* that underwent singleplex (sPCR) and multiplex PCR (mPCR). Lanes M, GeneRuler 1kb DNA Ladder Plus. (A) ETEC 505 exhibiting *hs* (lane 1), ETEC O7 exhibiting *hl* (lane 2), and UPEC 25922 exhibiting *iron_{E. coli}* (lane 3) via sPCR, and the same results via mPCR (lanes 4-6). The three strains combined also exhibited the three virulence genes via mPCR (lane 7). (B) EIEC O136 exhibiting *ial* (lane 1), EPEC O55 exhibiting *bfpA* (lane 2), and UPEC 25922 exhibiting *hlyA* (lane 3) via sPCR, and the same results via mPCR (lanes 4-6). The three strains combined also exhibited the three virulence genes via mPCR (lane 7). (C) EHEC 920004 exhibiting *stx1* (lane 1), *stx2* (lane 2), and UPEC 25922 exhibiting *papA* (lane 3) via sPCR. EHEC 920004 exhibiting both *stx1* and *stx2* via mPCR (lane 4), and UPEC 25922 exhibiting *papA* via mPCR (lane 5). The two strains combined also exhibited the three virulence genes via mPCR (lane 6).

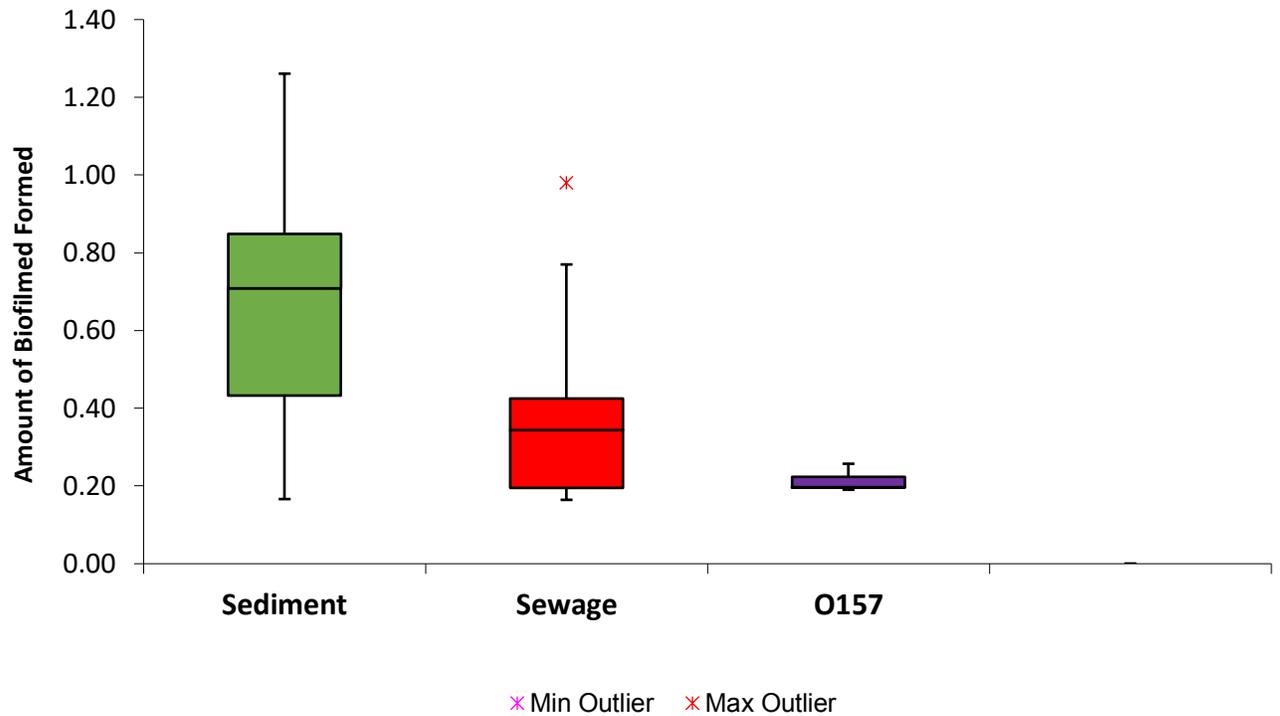


Figure 2.2. Boxplot of the overall biofilm forming capacity between sediment (n=47) and sewage (n=24) samples (obtained from Thunder Bay and Orillia ON) with known pathogenic group O157 (n=11) illustrating the significant difference between each sample group overall $p < 0.001$. Each measurement is the average result of three separate replicates for each sample type.

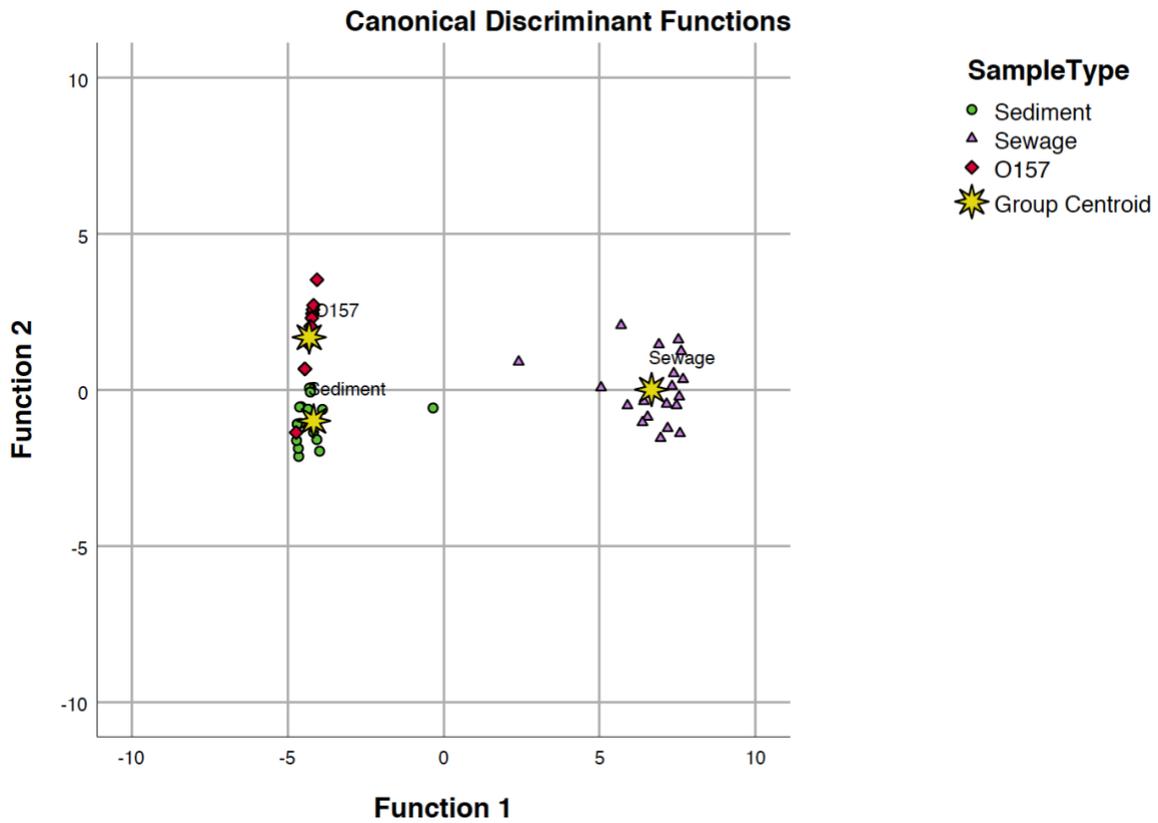


Figure 2.3. Canonical plot of the observed antibiotic resistance patterns for all eight antibiotics tested across all samples illustrated clear separation between the three sample groups: sediment (n=19), sewage (n=19) and O157 (n=11) with 98% of the original cases classified correctly.

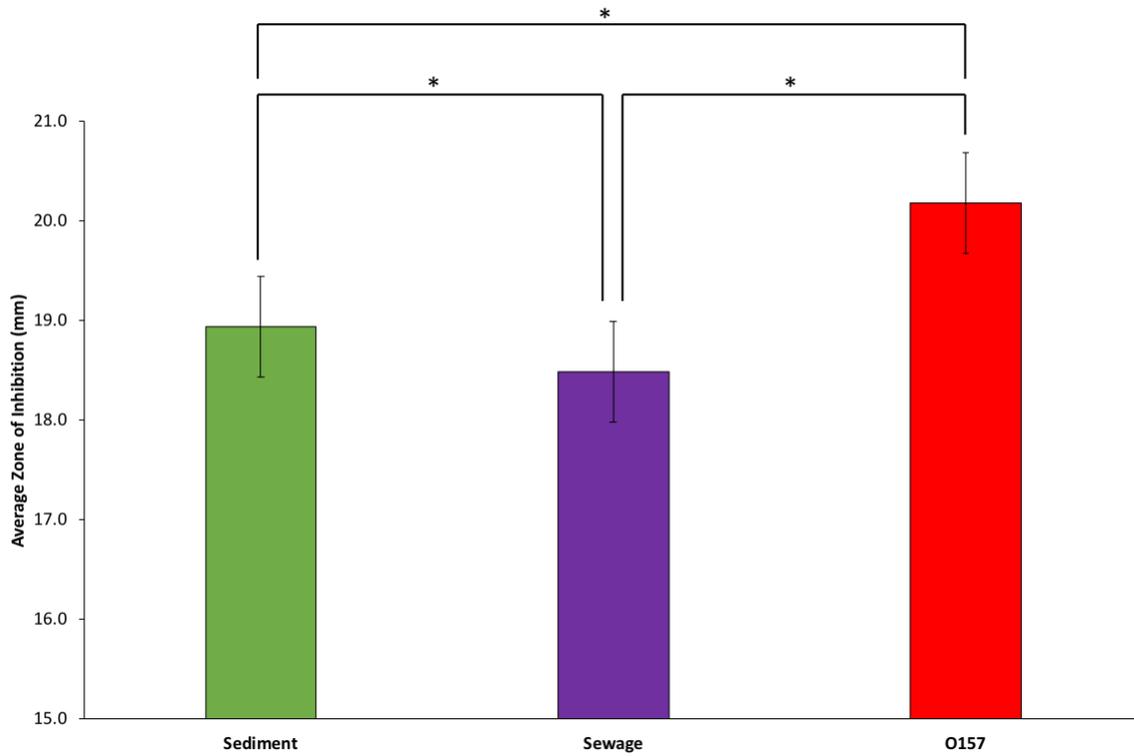


Figure 2.4. Comparison of the zone of inhibition to the eight antibiotics tested in each of the three sample groups (sediment (n=19), sewage (n=19), and O157 (n=11)). The resistance of the three sample sources are significantly different from one another overall ($p < 0.05$). The most resistant source is sewage with a mean zone of inhibition of 18.488mm, followed by sediment with a mean of 18.943mm, and the most susceptible source was O157 with a mean of 20.182mm.

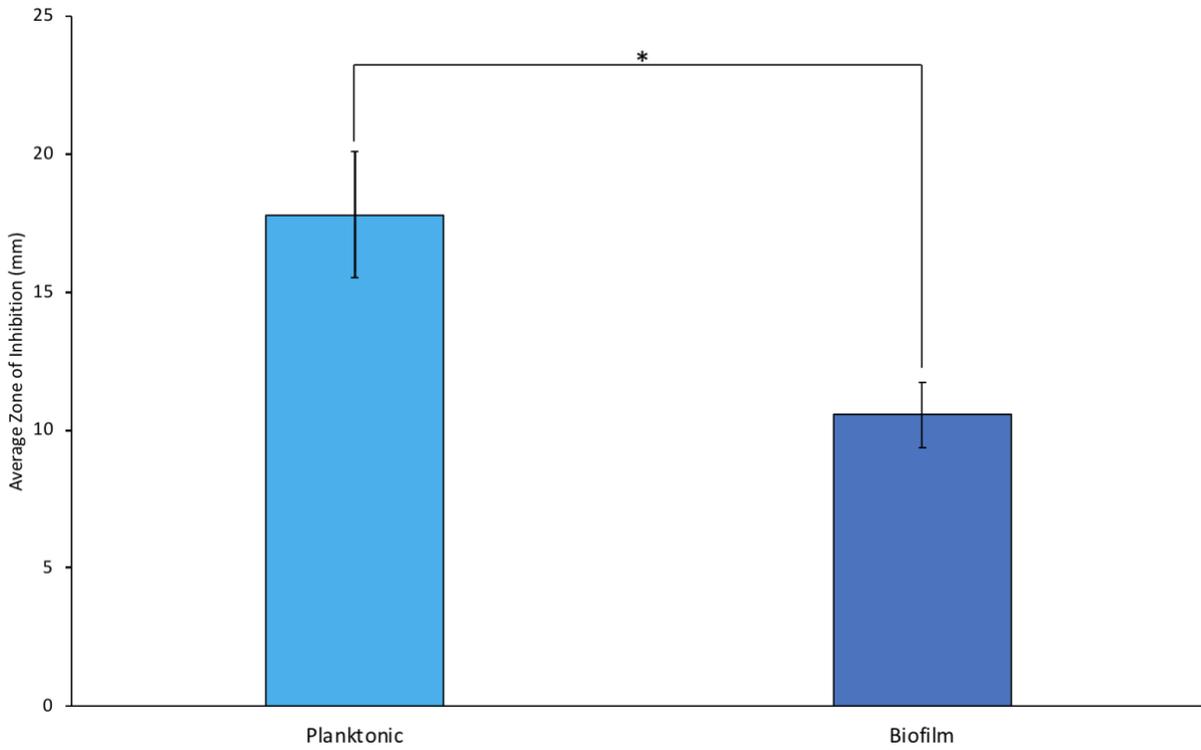


Figure 2.5. Comparison of mean overall zone of inhibition for all eight antibiotics tested in both the planktonic and biofilm states across all 21 samples. Statistical comparison illustrates a significant difference between sample source and sample state ($p < 0.001$). *indicates $p < 0.001$

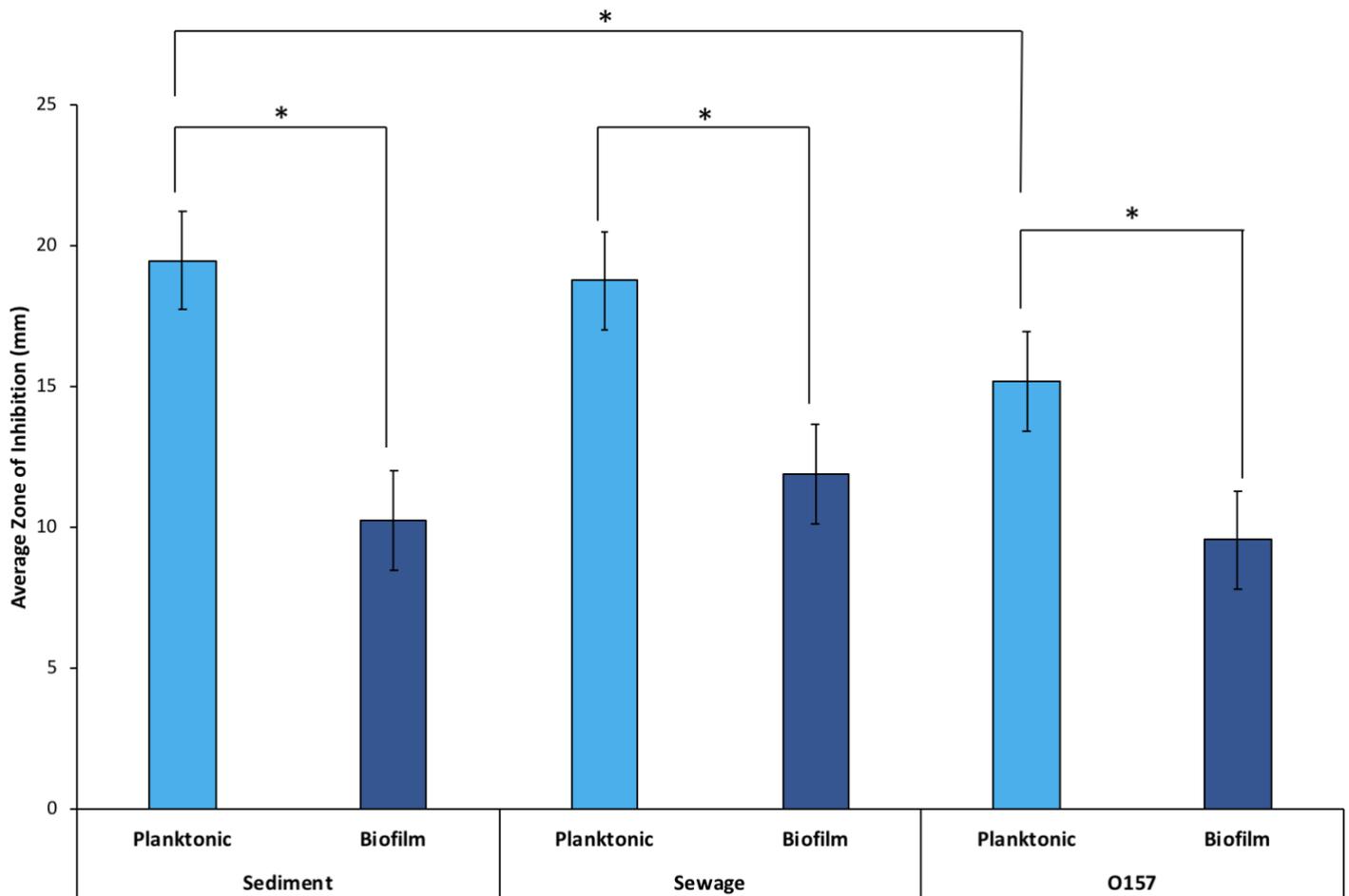


Figure 2.6. Comparison of mean zone of inhibition for each sample source (sediment (n=10), sewage (n=8), and O157 (n=3)) for all eight antibiotics tested in both the planktonic and biofilm states. Statistical comparison illustrates a significant difference overall between sample source and sample state ($p < 0.001$). * indicates $p < 0.05$

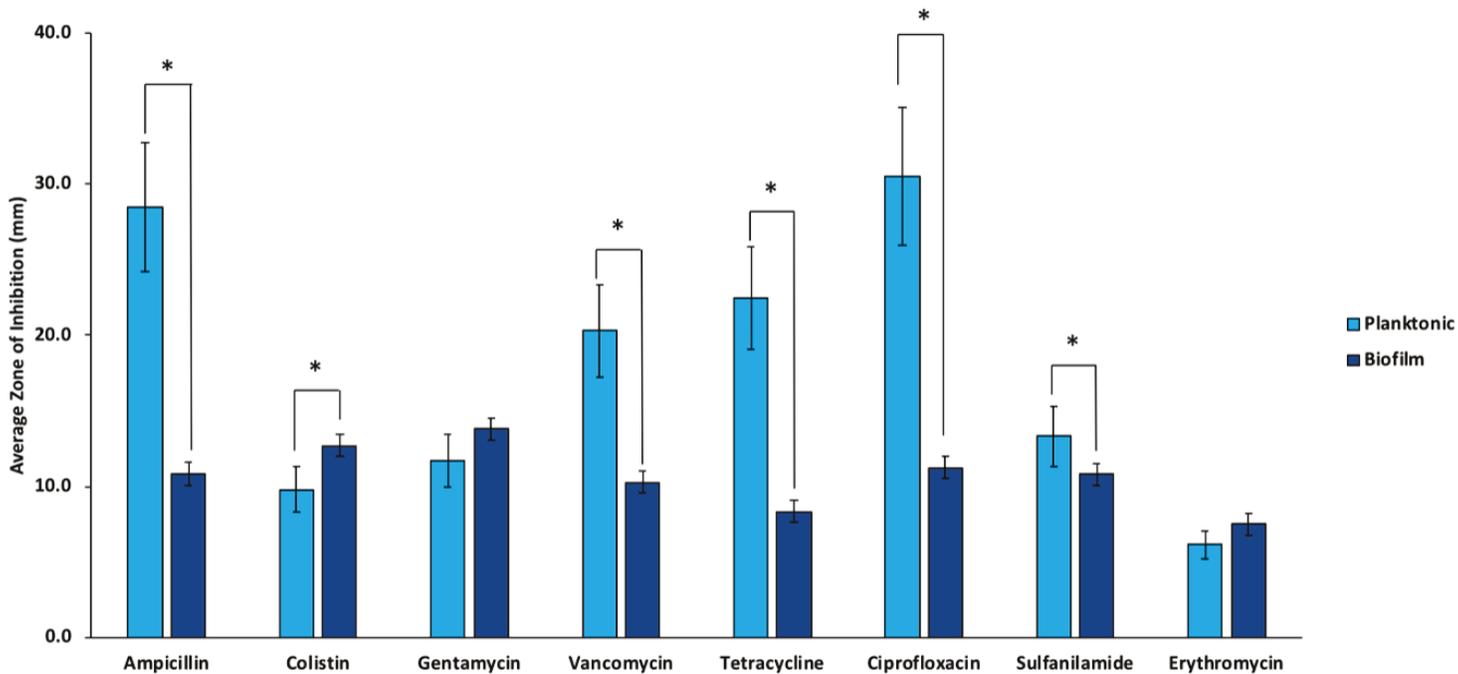


Figure 2.7. Comparison of mean zone of inhibition across all sample groups (sediment (n=10), sewage (n=8), and O157 (n=3)) for each of the eight antibiotics tested in both the planktonic and biofilm states. The resistance is inversely proportional to the size of the zone of inhibition. Statistical comparison illustrates an overall significant difference between antibiotics and sample state ($p < 0.001$). *indicates $p < 0.05$

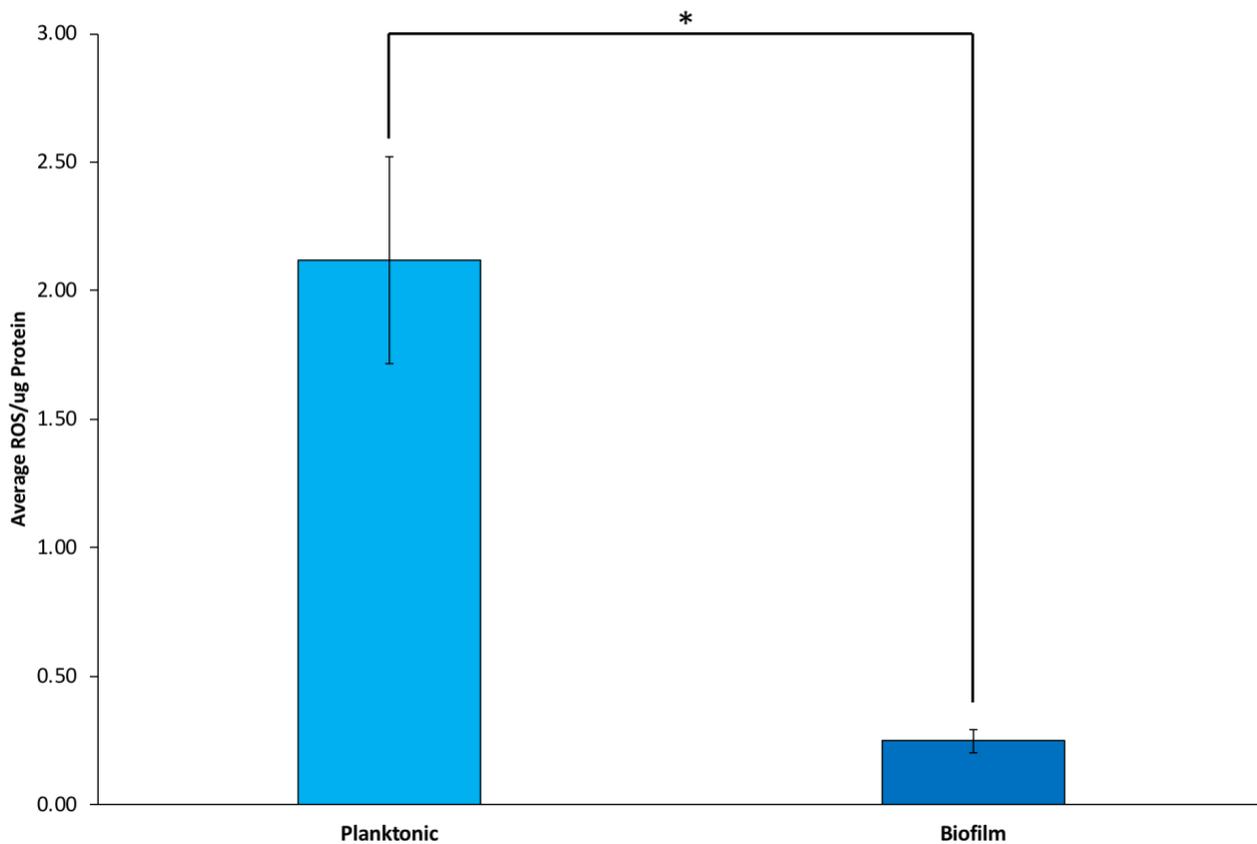


Figure 2.8. Comparison of mean ROS/ μg protein for all sample groups used in this experiment, sediment ($n=10$), sewage ($n=8$), and O157 ($n=3$), in both the planktonic and biofilm state. Significant differences between the two states ($p<0.001$) were found with biofilm cells having significantly lower amounts of ROS than their planktonic counterparts. * indicates $p<0.001$

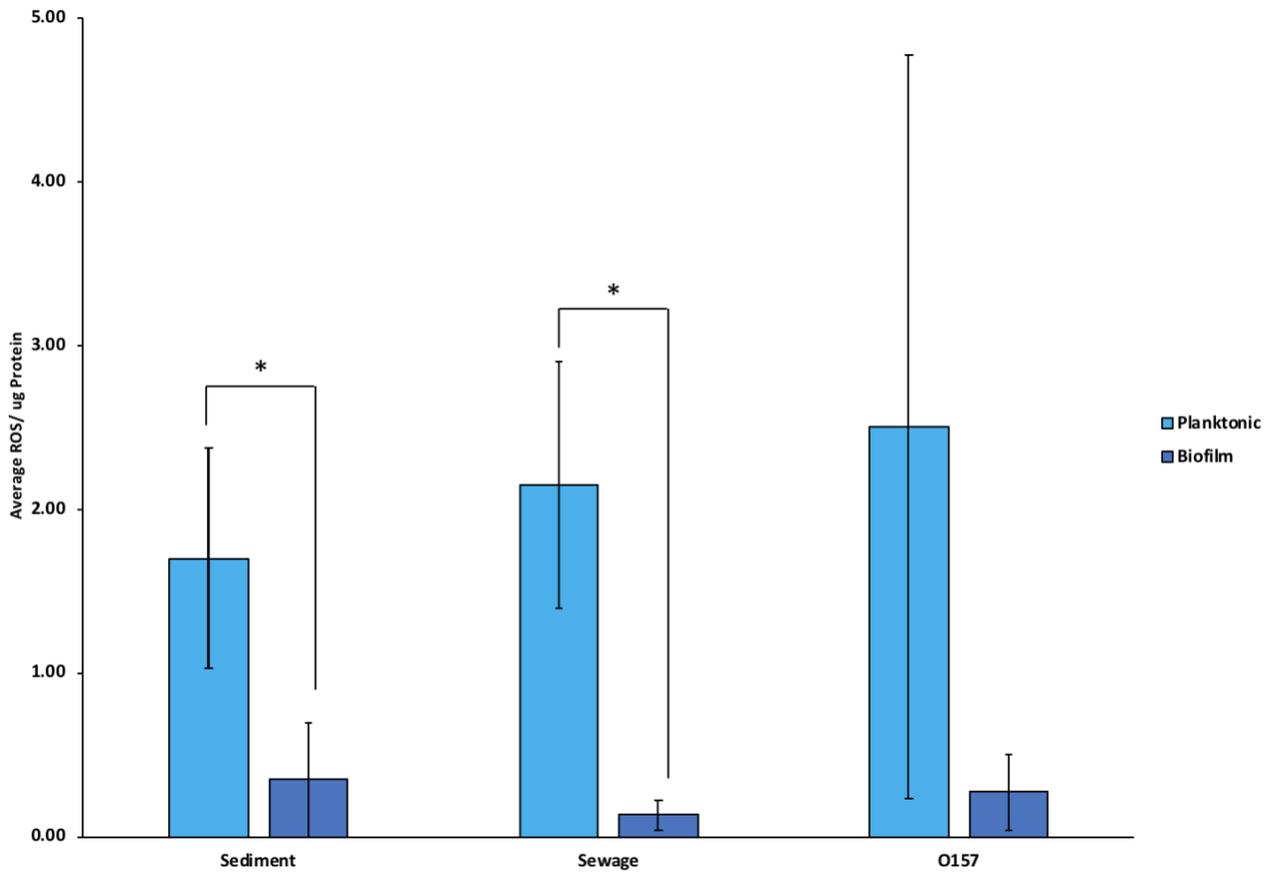


Figure 2.9. Comparison of mean ROS/ μg protein for each of the sample groups used in this experiment, sediment (n=10), sewage (n=8), and O157 (n=3), in both the planktonic and biofilm state. Significant differences between the two states ($p < 0.001$) were found for both sediment and sewage samples where biofilm cells have significantly lower ROS. * indicates $p < 0.001$

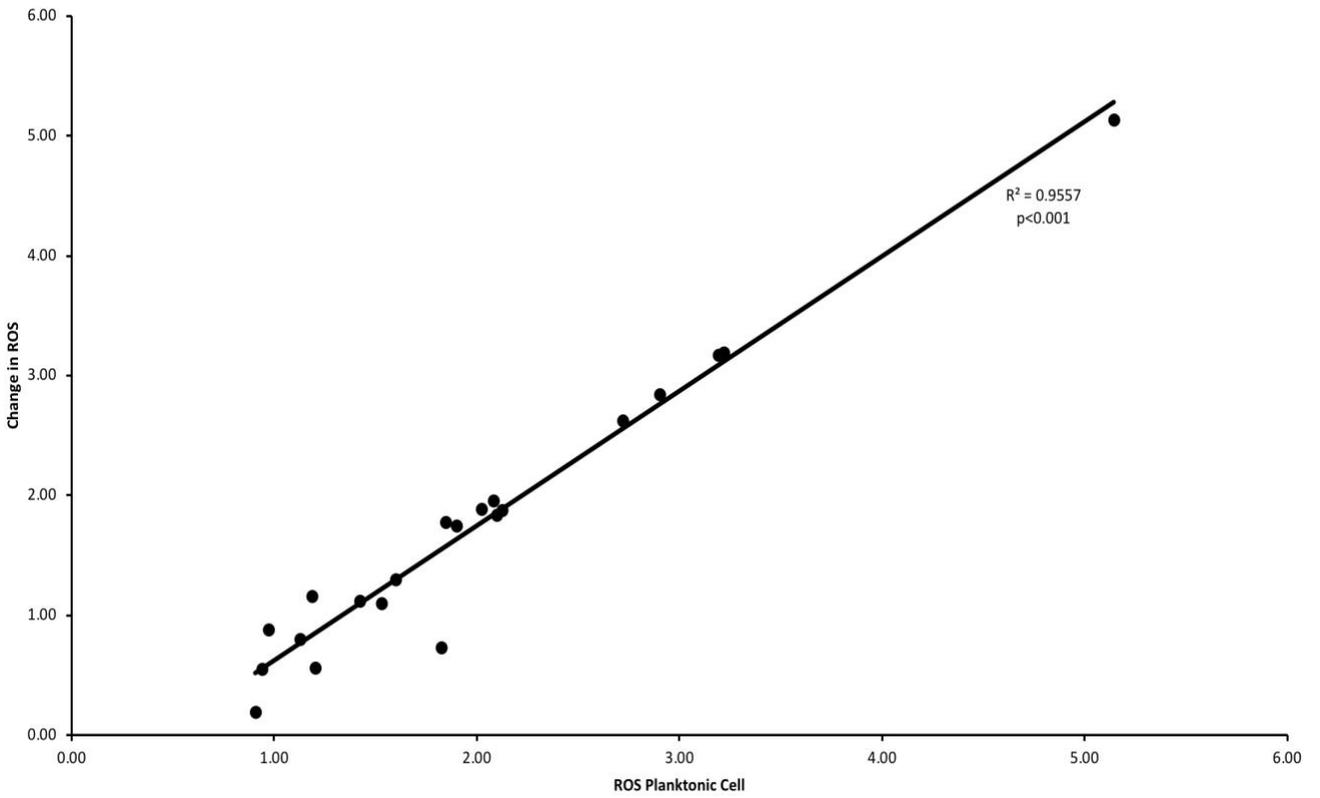


Figure 2.10. Trend observed between the amount of reactive oxygen species (ROS) in the planktonic cell and overall change in ROS from planktonic to biofilm state for each sediment (n=10), sewage (n=8), and O157 (n=3) samples was found to be significant ($p < 0.001$).

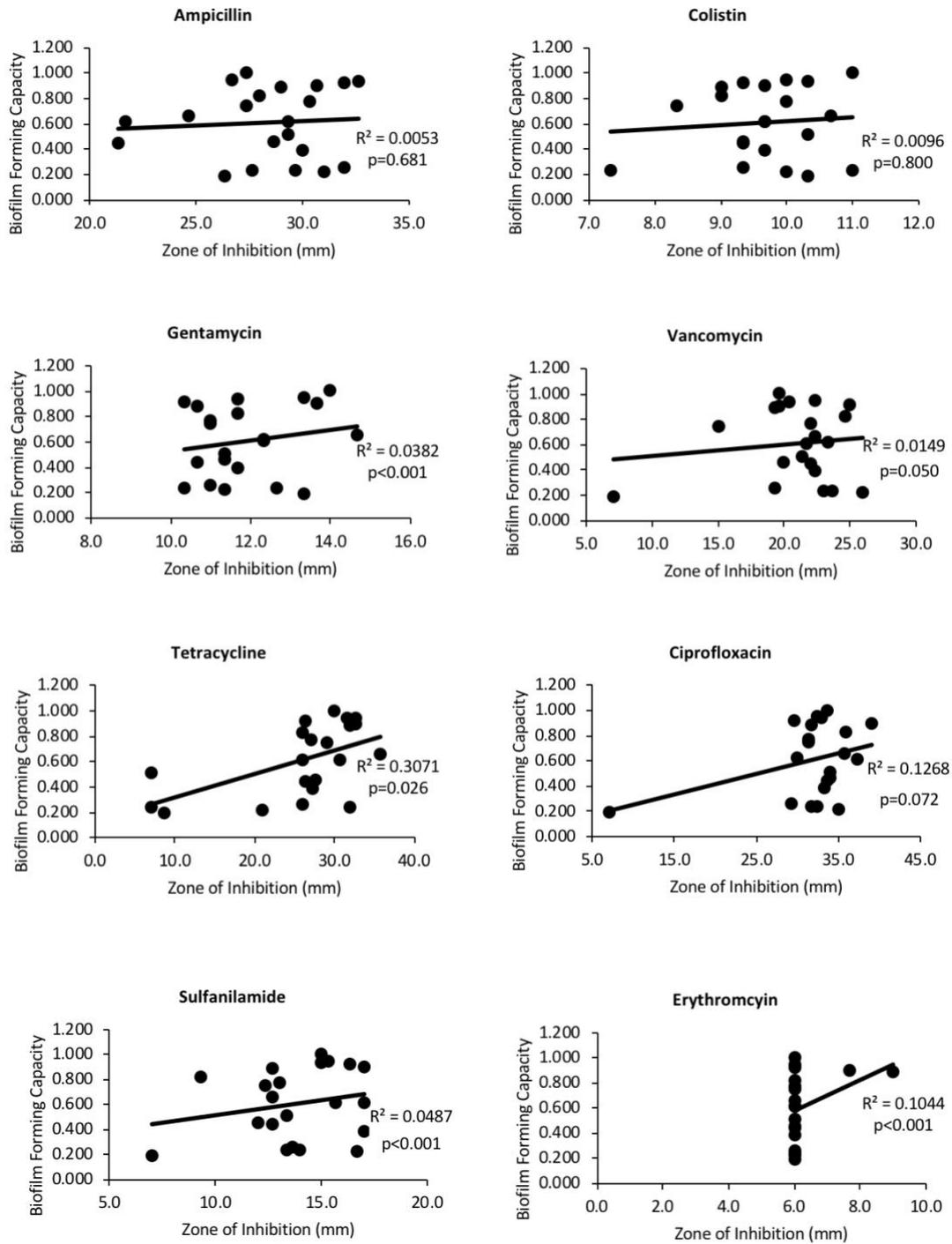


Figure 2.11. Trends observed between the antibiotic resistance in the planktonic cell (zone of inhibition) and biofilm forming capacity for each of the sediment (n=10), sewage (n=8), and O157 (n=3) samples across each of the antibiotics. (Plot illustrates the average of six separate zone of inhibition measurements)

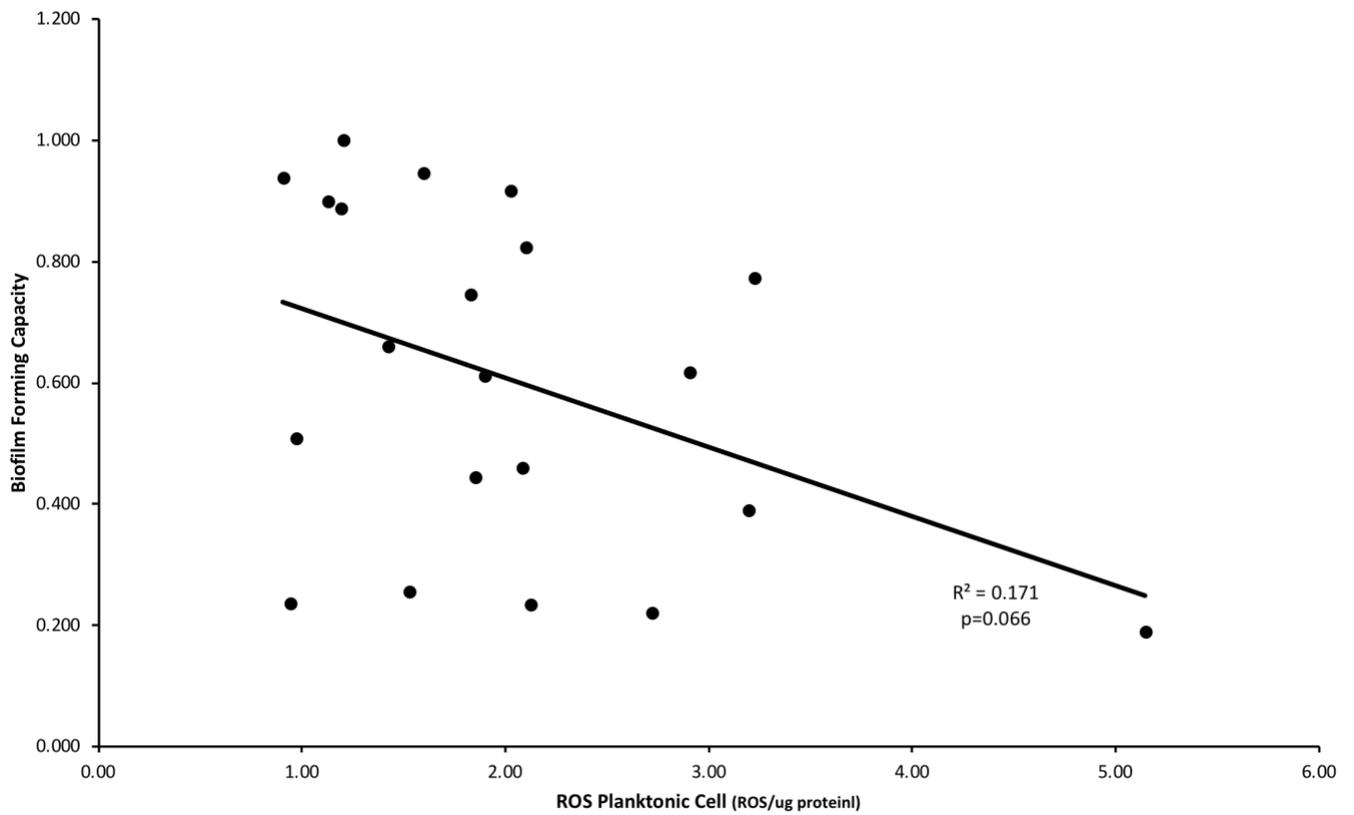


Figure 2.12. Trend observed between the amount of reactive oxygen species (ROS) in the planktonic cell and biofilm forming capacity for each of the sediment (n=10), sewage (n=8), and O157 (n=3) samples.

Chapter 3.

Examining the Role of *rpoS* gene on Biofilm Forming Capacity, the Concentration of Reactive Oxygen Species, and Antibiotic Resistance of an *E. coli* O157:H7 in the Biofilm State

3.0 Abstract

The role of stationary phase sigma factor (RpoS) of an *E. coli* O157:H7 H32 strain in the biofilm phase was examined by comparing the biofilm formation capacity, reactive oxygen species (ROS), and antibiotic resistance of the wildtype H32 to its *rpoS* mutant. The mutant strain formed significantly more biofilm ($p < 0.05$) than the wildtype strain with the mutant strain forming twice as much biofilm as the wildtype strain. To investigate the level of ROS in the two *E. coli* strains, a DCF-DA assay was conducted and revealed a significant difference between the exponential growth phase and biofilm state of the wildtype H32 strain with biofilm cells illustrating over 100,000 times lower ROS levels ($p < 0.05$) than exponential phase cells (values of 4.04×10^{-5} ROS/ μg protein $\pm 2.67 \times 10^{-5}$ and 4.057 ± 0.251 ROS/ μg protein respectively). Within the biofilm phase, the mutant cells illustrated significantly more ROS than the wildtype cells ($p < 0.05$) with values of 0.373 ± 0.250 ROS/ μg protein and $4.04 \times 10^{-5} \pm 2.67 \times 10^{-5}$ ROS/ μg protein respectively, supporting the idea that the *rpoS* gene is an essential regulatory gene for lowering the ROS level within bacteria. The levels of antibiotic resistance of the wildtype and mutant *E. coli* biofilm cells were determined for eight antibiotics using the Kirby-Bauer disk diffusion method. When compared with the wildtype H32 strain, the *rpoS* mutant strain showed an overall lower resistance across the eight antibiotics tested in the biofilm state ($p < 0.05$). With the support of the ROS data, the decreased antibiotic resistance of the H32 mutant in biofilm state further illustrates the role of *rpoS* on increasing antibiotic resistance of biofilm cells through the reduction of cellular ROS concentration.

3.1 Introduction

Bacteria have various mechanisms to combat stressful environments whether that be through entering a stationary state, forming biofilms, or by using stress response regulators to control the stress level of the bacteria. One gene that has been shown to affect all three of these mechanisms is the *rpoS* gene, the stationary phase sigma factor gene. This gene will produce the RpoS stationary phase sigma factor (Kohanski et al., 2007). With this sigma factor, general stress responses can be regulated to combat various stressful situations that the bacteria may encounter while in the biofilm phase.

The *rpoS* gene has been shown to affect biofilm formation whereby *E. coli* cells with this gene mutated were able to form more biofilm than the wildtype strain (Sheldon et al., 2012). While this enhanced biofilm formation was observed for the mutant strain, the biofilm cell survival ability decreased, indicating that the *rpoS* gene is essential for optimal biofilm cell survival (Sheldon et al., 2012). When there is an excessive increase in the amount of the biofilm matrix produced, the nutrients from the environment are not able to circulate through the biofilm thereby limiting the amount of nutrients these biofilm cells receive. While the biofilm effects of the *rpoS* gene have been somewhat previously studied, few studies have examined this gene and its role in oxidative stress or antibiotic resistance.

Oxidative stress can be defined as the overproduction of reactive oxygen species (ROS), namely superoxide, hydrogen peroxide, and hydroxyl radicals, within the cell that can eventually lead to cell death (Vatansever et al., 2013). Normally *E. coli* have mechanisms that can combat these ROS molecules, since they are normal by-products of respiration. However, under oxidative stress conditions, the bacteria cannot react to the overproduction of these

molecules, and the cell can eventually die. To combat this stress, bacteria have evolved to possess survival mechanisms to combat these ROS molecules at stationary phase and biofilm state. One of these mechanisms is through the RpoS, stationary phase sigma factor. Controlled by the *rpoS* gene, the RpoS sigma factor is a 38 kDa protein that can control over 200 genes, with many of these genes able to respond to oxidative stress (Gambino and Cappitelli, 2016). One gene that plays a role in an *E. coli*'s response to oxidative stress in the biofilm phase is the *katE* gene (Vijayakumar et al., 2004). The *katE* gene is responsible for the major form of catalase in the biofilm phase (Vijayakumar et al., 2004). Catalase will form neutral products of oxygen and water from the ROS molecule hydrogen peroxide to combat oxidative stress. This gene and the production of catalase is essential for *E. coli* to be able to withstand periods of oxidative stress. The *rpoS* gene has been examined in relation to oxidative stress by Loewew and Triggs (1984) where they observed that the absence of the *rpoS* gene has also been found to make bacteria more susceptible to the lethal effects of exogenous hydrogen peroxide (Loewew and Triggs, 1984). Nguyen et al. (2011) examined the link between the role of stringent response genes (*relA* and *spoT*) and the increase of antibiotic resistance of *Pseudomonas aeruginosa* during nutrient limited growth conditions. They illustrated that the stringent response mediated antibiotic resistance was caused by the reduction of ROS within the *P. aeruginosa* cells under starvation conditions. With the known effect that the *rpoS* gene plays an important role in reducing the oxidative stress in *E. coli* cells under biofilm conditions, we propose that *rpoS* is also partly responsible for the increase of antibiotic resistance of the biofilm cells.

It has recently been determined that a secondary antibiotic killing mechanism is at play in bacteria whereby bactericidal antibiotics stimulate oxidative stress within the bacterial cell (Kohanski et al., 2007). With bactericidal antibiotics, the proposed mechanism for this secondary effect has to do with producing high levels of ROS through an increase activity of the TCA cycle and the electron transport chain. While the mechanism is not fully understood, control of oxidative stress may be related to a bacteria's ability with withstand antibiotics. In Chapter 2, we illustrated that *E. coli* cells possess significantly less reactive oxygen species in the biofilm phase than in the actively growing planktonic cells. This study also supported the notion that biofilm cells are generally more resistant to antibiotics than planktonic cells. Therefore, the effects of the *rpoS* gene's ability to control oxidative stress in relation to antibiotic resistance needs to be further examined.

In this study, we examined the characteristics of a shiga toxin producing *E. coli* O157: H7 strain H32, and a *rpoS* mutant of this strain that was previously engineered by Sheldon et al. (2012). This examination will develop a better understanding of the role of the *rpoS* gene and its influence on the *E. coli*'s ability to form biofilms, combat oxidative stress, and be resistant to antibiotics. The objectives of this study were:

1. To determine the biofilm forming capacity of both the *E. coli* O157:H7 strain H32 and its *rpoS* mutant.
2. To investigate the level of reactive oxygen species (ROS) contained within the wildtype and mutant strains in both the exponential and biofilm phases.
3. To assess the effect of the *rpoS* gene on the bacterium's antibiotic resistance to eight common antibiotics in both the exponential and biofilm phases.

3.2. Materials and Methods

3.2.1 Bacterial Strains

The shiga toxin producing O157:H7 strain H32 used in this study was provided by Dr. C. Gyles at the University of Guelph (Guelph, ON, Canada). H32 is a bovine isolate and possesses genes for both Shiga-like toxin I and II (Watterworth et al., 2006). It also contains the *eae* gene for intimin, the virulence factor responsible for mediating the attachment of *E. coli* to colonic epithelial cells. A *rpoS* mutant of this strain was engineered by Sheldon et al. (2012) in which a gentamycin resistance gene (*accC1*) was inserted in the middle of the *rpoS* of the wildtype H32 strain. The *rpoS* mutant strain was also labelled with a *gfp* (green fluorescent protein gene) genetic marker by a Tn5 transposon system, allowing the *rpoS* mutant to be monitored in environmental samples. Sheldon et al. (2012) also showed that the addition of the *gfp* marker does not affect the physiology of the *rpoS* mutant. The two bacterial strains were transferred from frozen stock cultures onto Trypticase Soy Agar plates (TSA, containing 30 g Trypticase Soy Broth (TSB) and 15 g Granulated Agar (Becton Dickinson and Company, New Jersey, USA)) and the plates were incubated for 24 hours at 37°C.

3.2.2 Biofilm Forming Capacity

The biofilm forming capacities of the H32 wildtype and H32 *rpoS* mutant strains were compared using the same method as described previously in this thesis (section 2.2.4). Biofilm growth experiments were performed within a 96-well flat bottomed polystyrene microplate (Costar, Corning, New York, NY). Overnight cultures were washed three times with 10 mL of sterile phosphate buffer saline (PBS) (8.0 g NaCl, 0.20 g KCl, 1.44 g Na₂HPO₄, and 0.24 g KH₂PO₄

in 1 L double distilled water adjusted to a pH of 7.4). The optical density of the cell suspension was adjusted with a NovaSpec spectrophotometer (Biochrom LTD, Cambridge, UK) to an OD₆₀₀ of 1.00 ± 0.05 with sterile double distilled water. Ten µl of the OD₆₀₀ 1.00 culture was transferred into a sterile 96-well flat bottomed polystyrene plate (Costar) where four separate wells were inoculated for each culture and 190 µl of Minimal Salt Medium with 0.04% glucose (MSMG) (1.249 mM KH₂PO₄, 3.73 mM K₂HPO₄, 0.4 mM MgSO₄, 0.02 mM FeSO₄, and 1.4 mM NH₄Cl) was transferred to each well and was then incubated for 48 hours at 22°C with gently shaking at 25 rpm. The 96-well plate was washed three times with sterile double distilled water before being air dried for 30 minutes. The biofilm cells were stained with 150 µl of a 0.1% crystal violet solution for 10-15 minutes. Excess crystal violet solution was removed from the plate and the plate was washed three times with sterile double-distilled water and left to dry for an additional 30 minutes. Two-hundred µl of a de-staining solution made up of 80% acetone and 20% ethanol was added to each well for 10 minutes to release the crystal violet from the cells. One hundred and fifty µl of the de-staining solution was transferred into a new plate for absorbance measurement at 595 nm using a Fluostar Optima automated plate reader (BMG Labtech, Offenburg, Germany). The average of the three independent replicates was used to represent the biofilm forming capacity for each *E. coli* strain.

3.2.3 Measuring Cellular ROS of Exponential and Biofilm Phase *E. coli* Cells

The cellular levels of ROS of exponential phase and biofilm *E. coli* cells from the two strains were examined by DCF-DA (2',7'-di-chlorofluoresceine diacetate). The exponential and biofilm *E. coli* cells were prepared whereby both exponential and biofilm cells were grown at

22°C and in MSMG with biofilm cells being grown on glass fibre filters. Exponential cultures were prepared from overnight cultures and transferred into MSMG media for an additional 6 hours to reach the exponential phase of growth. Upon reaching an $OD_{600nm} = 0.2 \pm 0.05$, the cells were washed with sterile PBS twice as described previously and re-suspended in sterile double distilled water. Biofilm cultures were prepared from overnight cultures washed three times with sterile PBS and re-suspended with sterile double distilled water to an OD_{600nm} of about 0.05. One mL of this cell suspension was then transferred into 20 mL of MSMG in a sterile glass bottle containing 0.1 g of glass fibre filters (pore size 0.7 μm , Whatman GF/F) and incubated for 48 hours at 22°C with shaking at 25 rpm. To harvest the biofilm cells from the filters, the filters were washed three times with sterile PBS before the filter disks were transferred to a sterile glass bottle containing 5g of sterile glass beads (450-600 μm , Sigma Aldrich). Eight mL of sterile double distilled water was then added to the glass bottle, and the sample was vortexed vigorously for 3 minutes. The biofilm cell samples were then transferred into individual sterile stomacher bags to remove excess glass fibres and the biofilm cell samples were collected in sterile 15 mL tubes. The biofilm cell samples were then adjusted to an OD_{600} of 0.2 ± 0.05 with sterile double distilled water and kept on ice until ready to use. A 1 mL subsample of each exponential and biofilm cell suspension was collected to be used for determining protein concentrations.

A 1000 μM DCF-DA solution was prepared and 10 μL portions of the solution were added 1980 μL of either the exponential or biofilm *E. coli* cell samples suspended in sterile double distilled water. The cell samples were incubated in the dark at 25°C for 30 minutes to allow the DCF-DA to interact with the cells. One hundred μL of each sample was transferred

into a Costar 96-well flat-bottomed plate (Corning). Each sample was loaded into six wells where the fluorescence was measured at 570nm excitation/ 585nm emission using a BMG Labtech FLUOrstar OPTIMA plate reader. The average of the six measurements was used as the proportional to the amount of ROS in the sample.

One mL subsample of the exponential and biofilm *E. coli* strains was used for determining their protein concentrations with the B-PER Bacterial Protein Extraction Reagent (Thermo Fisher Scientific) and Coomassie (Bradford) Protein Assay kit (Thermo Fisher Scientific). Each cell suspension was washed and pelleted where 1 mL of the B-Per Reagent was added to the pellet and vortexed until homogenized before being incubated at room temperature for 15 minutes. The lysate was then centrifuged for 5 minutes at 15,000 x g and the protein concentration of sample was determined by the Bradford assay. The Coomassie (Bradford) Protein Assay (Thermo Fisher Scientific) was performed as outlined in the manufacturer's manual for the microplate protocol.

3.2.4 Comparing Antibiotic Resistance of Exponential and Biofilm Phase *E. coli* Cells

The antibiotic resistance of H32 and its *rpoS* mutant in both the log phase and biofilm state were determined by a modified Kirby-Bauer antibiotic disc method. The eight antibiotics used in this experiment were ampicillin, colistin, gentamycin, erythromycin, vancomycin, sulfanilamide, tetracycline, and ciprofloxacin (Sigma Aldrich Canada, Oakville, ON), and the antibiotic discs of these antibiotics were prepared as described in Section 2.2.5a of Chapter 2 of this thesis.

To prepare the exponential phase bacterial cultures, the two bacterial strains were grown in MSMG broth and incubated at 22°C overnight. One mL of each overnight culture was transferred into fresh sterile MSMG growth medium and incubated for an additional 6 hours at 22°C to achieve an exponential growth phase. The exponential phase cells were then harvested and spread on MSMG plates to determine their resistance to the eight antibiotics as previously described in Section 2.2.5b of Chapter 2 of this thesis. Antibiotic discs were placed on the MSMG plates with the log phase bacteria and the plates were incubated at 22°C for 18 hours. After incubation, zones of inhibition were measured and recorded.

In order to determine the antibiotic resistance of biofilm cells exposed to biofilm condition, MSMG media was incorporated with 30% poloxamer 407 (Sigma Aldrich) to create biofilm culture plates as described previously in Section 2.2.5b. Biofilm cells were prepared from overnight cultures whereby 10 mL of each culture was washed three times with sterile PBS and then re-suspended to an OD_{600nm} of about 0.05. One mL of this cell suspension was transferred into 20 mL of MSMG in a sterile 250 mL glass bottle containing 0.1 g of glass fibre filters (pore size 0.7 µm, Whatman GF/F) and incubated for 48 hours at 22°C with shaking at 25 rpm. To harvest the biofilm cells from the filters, the planktonic cells were removed, the filters were washed three times with 10 mL of sterile PBS, and then the filters were transferred into a sterile 100 mL glass bottle containing 5g of sterile glass beads (450-600 µm, Sigma Aldrich). Eight mL of sterile double distilled water was added to the bottle, and the sample was vortexed vigorously for 3 minutes. The biofilm cell samples were then transferred into sterile stomacher bags to remove the loose fibres from the biofilm cell suspensions. The concentration of biofilm cell samples were adjusted to an OD₆₀₀ of 0.2 ± 0.05 , and samples were transferred

onto MSMG-30% poloxamer plates as described in Section 2.2.5b. Antibiotic disks were then placed onto respective plates as described previously and the plates were incubated at 22°C for 18 hours. After incubation, zones of inhibition were measured and recorded.

3.2.5 Statistical Analysis

Statistical analyses were performed using the SigmaPlot 12 Software integrated with SigmaStat (Systat Software Inc., San Jose, USA) unless otherwise stated. One-way analysis of variance (ANOVA) and T-test were performed in various sections of this study: biofilm forming capacities, ROS concentrations, and antibiotic resistance, and ROS concentrations. The biofilm forming capacities of the two strains were compared by T-test using the average values of three independent replicates each *E. coli* strain tested. The comparison between the intrinsic level of ROS in the exponential and biofilm cells were determined by one-way ANOVA using six experimental replications. Lastly, the comparisons between the zone of inhibition for each strain in the exponential and biofilm state was performed with one-way ANOVAs using three replications for each strain and each antibiotic tested.

3.3 Results & Discussion

When a bacterial cell is in the biofilm state, almost 50% of the biofilm-related genes are controlled by RpoS, the stationary phase sigma factor, which activates responses to various stresses including oxidative stress (Gambino and Cappitelli, 2016). Gambino and Cappitelli (2016) indicated that the RpoS regulon plays an essential role in biofilm development since it controls many of the genes that will induce biofilm growth under nutrient limited conditions. In

this study, the *rpoS* gene was examined to determine its effects on biofilm formation, antibiotic resistance, and its impact on the level of oxidative stress of a shiga toxin producing O157:H7 *E. coli* strain, H32. The role of the *rpoS* gene on biofilm formation, antibiotic resistance and ROS in the biofilm phase of the *E. coli* strain was examined by comparing the wildtype H32 to its *rpoS*-knockout mutant.

To compare biofilm forming capabilities between the wildtype and the *rpoS*-knockout H32 mutant strains, a standard crystal violet staining method of biofilm quantification previously optimized by Moreira et al. (2012) was employed. A significant difference ($p < 0.05$) was observed in terms of their biofilm forming capabilities where the mutant strain was able to form twice as much biofilm as the wildtype strain with average absorbance's of 0.3270 ± 0.0022 and 0.1570 ± 0.0076 respectively (Figure 3.1). The *rpoS* gene has been attributed to bacterial biofilm formation during periods of stress (Adams and McLean, 1999; Donlan and Costerton, 2002; Sheldon et al., 2012). This study confirmed the results reported by Sheldon et al. (2012) whereby, under nutrient limited conditions, the *rpoS* deficient strain was able to form significantly more biofilm than the wildtype H32 strain. It is important to note that Sheldon et al. (2012) reported that this effect of the *rpoS* gene is only found when the bacteria are under nutritional stress, not under high nutrient availability. However, Sheldon et al. (2012) also indicated that the greater amount of biofilm formed by the *rpoS*-mutant strain did not translate into increased survival of these cells. They suggested that this reduced survival might be explained by the lack of stress controls from the RpoS regulon that would in turn, lead to the bacteria becoming overwhelmed from stress. This leads to the conclusion that while the *rpoS*-mutant strain may form more biofilm, this higher amount of biofilm may not be optimal for cell

survival. This decreased survival may be due to the limited ability for nutrients to circulate through the thick matrix, thereby limiting the nutrients available to the bacterial cells, and in turn putting the cells in a stressed state. While Sheldon et al. (2012) illustrated that the *rpoS* gene plays an important role in optimizing biofilm formation, other roles of the regulon, such as the control of ROS and antibiotic resistance of the biofilm cells, have not been examined. Battesti et al. (2012) previously determined that the RpoS regulon is only able to respond to oxidative stress when a cell is in stationary phase (Adams and McLean, 1999). The *rpoS* gene plays an essential role in regulating oxidative stress levels in the biofilm cells by turning on their catalase production, and this theory was examined to determine if the mutant cells would display higher levels of stress. The evidence was clear in our results that the level of ROS in mutant biofilm cells was significantly higher ($p < 0.05$) than the wildtype biofilm cells with concentrations of 0.373 ± 0.13 ROS/ μg protein and $0.454.04 \times 10^{-5} \pm 0.01$ ROS/ μg protein, respectively (Figure 3.2). In the biofilm state, it was evident that the *rpoS* gene was active and hence the biofilm H32 cells illustrated significantly lower levels of ROS ($p < 0.05$) than the log phase H32 planktonic cells. Despite expecting a similar ROS level for the H32 wildtype and *rpoS* mutant samples in the log phase, the average ROS concentration in the mutant cells was 20% lower than that of the wildtype cells in the planktonic log phase. This may be due to the fact that some of the *rpoS* mutant cells that may have reached their stationary phase. Those cells without a functional *rpoS* gene to reduce the stress condition would then be killed, and this could cause an overall decrease on the ROS/protein concentration in the *rpoS* mutant samples. The *rpoS* gene allows *E. coli* to produce the stationary phase sigma factor, which controls the *katE* gene that allows the cell to produce catalase, which can degrade the

ROS molecule hydrogen peroxide into water and oxygen (Vijayakumar et al., 2004). Without the *rpoS* gene, and without catalase, the *E. coli* mutant illustrated an increase in ROS molecules contained within the biofilm cells. The significant increase of ROS in the H32 *rpoS* mutant may be another explanation of the observation of Sheldon et al. (2012) that the *rpoS* mutant decreased its viability and survival in biofilm phase despite forming larger biofilm on a substratum.

With the decreased ability to combat oxidative stress, the H32 *rpoS* mutant cell may also be less able to survive antibiotic treatment under biofilm condition. When Gambino and Cappitelli (2016) studied the RpoS regulon, they suggested that ROS might be a trigger for an adaptive mechanism that would make biofilm cells more antibiotic resistant. However, they did not propose a biochemical mechanism that connects the cellular level of ROS to the antibiotic resistance of the biofilm cells. Because antibiotics can induce the production of ROS in bacterial cells and cause damages to the bacteria (Van Acker and Coenyn, 2017), we propose that the RpoS will turn on the production of catalase in the biofilm cells to reduce the oxidative stress of the ROS, and hence increase the resistance of the cells against antibiotics. Since we have demonstrated that the H32 *rpoS* mutant had significantly higher ROS in biofilm cells than the H32 wildtype strain, we also need to show that the H32 *rpoS* mutant is less resistance to antibiotics than its wildtype counterpart under biofilm condition.

To examine the effect of *rpoS* on antibiotic resistance, the antibiotic resistances of H32 were compared with its *rpoS* mutant in both biofilm and planktonic phases. In biofilm phase, the overall antibiotic resistance of the H32 was significantly greater than its *rpoS* mutant (Figure 3.3). This coincided with the observation that the ROS concentration in the H32 biofilm cells

was significantly lower than the *rpoS* mutant (Figure 3.2). At individual antibiotic levels, the *rpoS* mutant strain showed lower resistance to all eight antibiotics with various extents (Figure 3.4). For four of the eight antibiotics tested (colistin, gentamycin, vancomycin, and sulfanilamide), they were significantly more effective in killing the mutant than the wildtype strain (p-values of 0.042, <0.001, 0.013, and 0.002, respectively). For ampicillin and tetracycline, the mutant strain showed substantially less resistance than the wildtype (p-values of 0.067 and 0.078 respectively). Finally, the resistance of the mutant strain against ciprofloxacin and erythromycin was either slightly less than or similar to the wildtype. The variation of the differences between the eight antibiotics may be due to the strength and antibacterial properties of these antibiotics.

For the planktonic cells, at which most of the cells were in their exponential growth phase, the overall antibiotic resistance of the H32 was not significantly different from its *rpoS* mutant (Figure 3.5). At individual antibiotic levels, four of the eight antibiotics, including ampicillin, colistin, tetracycline and erythromycin, did not have any significant difference between the two strains (Figure 3.6). However, the other four antibiotics (gentamycin, vancomycin, ciprofloxacin and sulfanilamide) show different effect on the two strains. This may be due to the different antibacterial properties of these antibiotics. Furthermore, despite the fact that most of the planktonic cells were in the exponential growth phase, some of the wildtype H32 cells could be in stationary phase and had their *rpoS* activated.

With the wildtype H32 having a significant lower ROS concentration and greater resistance to antibiotics than its *rpoS* mutant in the biofilm phase, it supports our hypothesis that the *rpoS* gene increases the antibiotic resistance of biofilm cells by reducing the cellular

level of ROS. When the gene is present and active, the bacterial cells were better able to survive antibiotic treatment in biofilm state. This increased level of survival can be related to the alleviation of the secondary killing mechanism of bactericidal antibiotics which causes an overproduction of ROS in the bacteria. This secondary mechanism with antibiotics occurs by increasing the bacteria's TCA cycle and electron transport chain. The increase in NADPH and electron transport activity will overproduce superoxide, hydrogen peroxide, and hydroxyl radicals, and hence causing oxidative damages to the cells (Dwyer et al., 2014). Kohanski et al. (2007) also illustrated that antibiotics could use internal iron from the iron-sulfur clusters to promote the Fenton reaction to form hydroxyl radicals. With the *rpoS* gene active in the biofilm state, it turns on numerous stress protein genes including the *katE* (catalase gene) and other oxidative stress protein genes to reduce the damaging effect of the ROS caused by the secondary mechanism of antibiotics.

3.4 References

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3.5 Figures

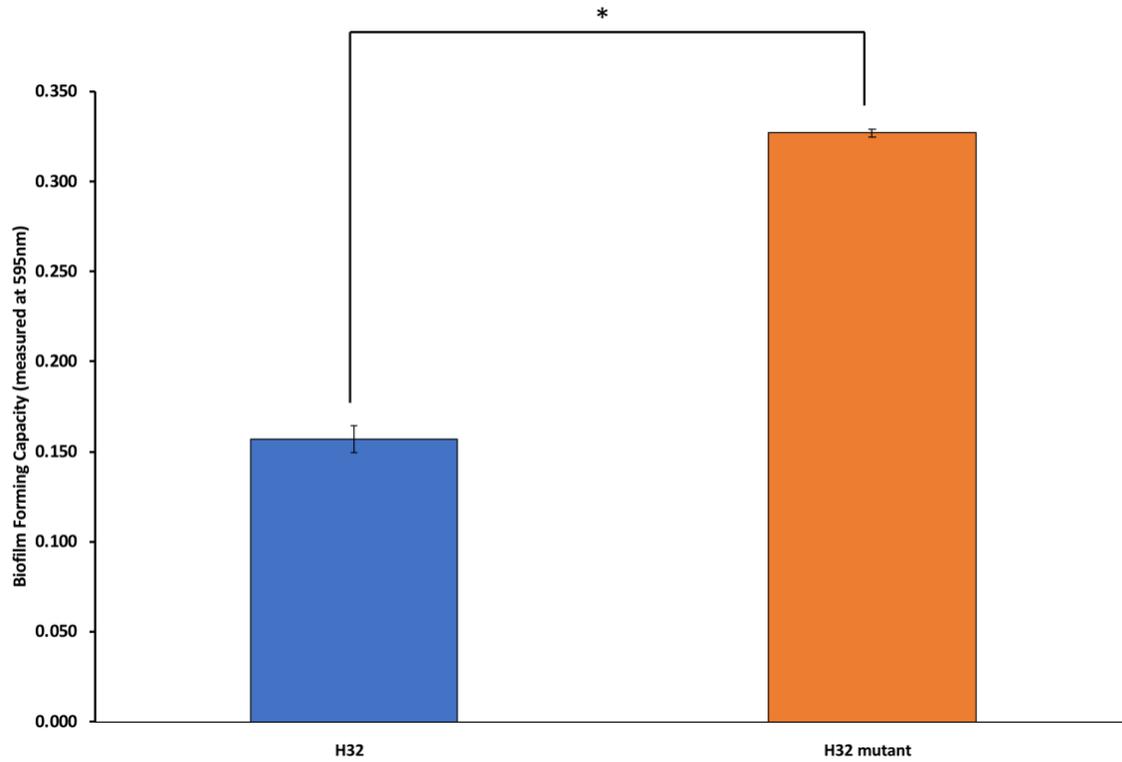


Figure 3.1. The average biofilm forming capacities of the wildtype H32 strain and the *rpoS*-deficient strain illustrated a significant difference between the two isolates ($p < 0.05$).

* indicates $p < 0.05$

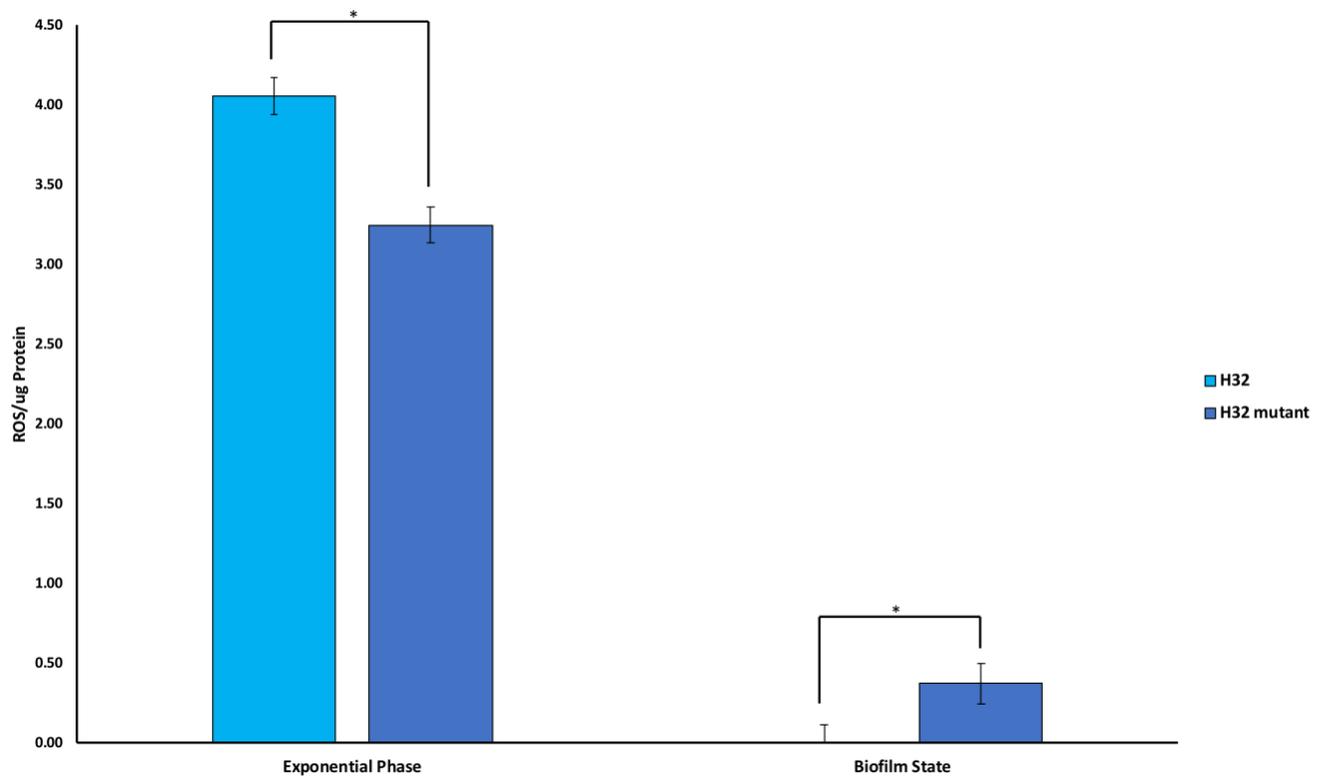


Figure 3.2. Comparison of mean ROS/ μg protein for each of the sample used in this experiment in both the exponential phase and biofilm state. Significant differences between the two states ($p < 0.05$) were found with biofilm cells having significantly lower amounts of ROS than their exponential counterparts. The mutant cell illustrated significantly more ROS in the biofilm state than the wildtype strain ($p < 0.05$). * indicates $p < 0.05$

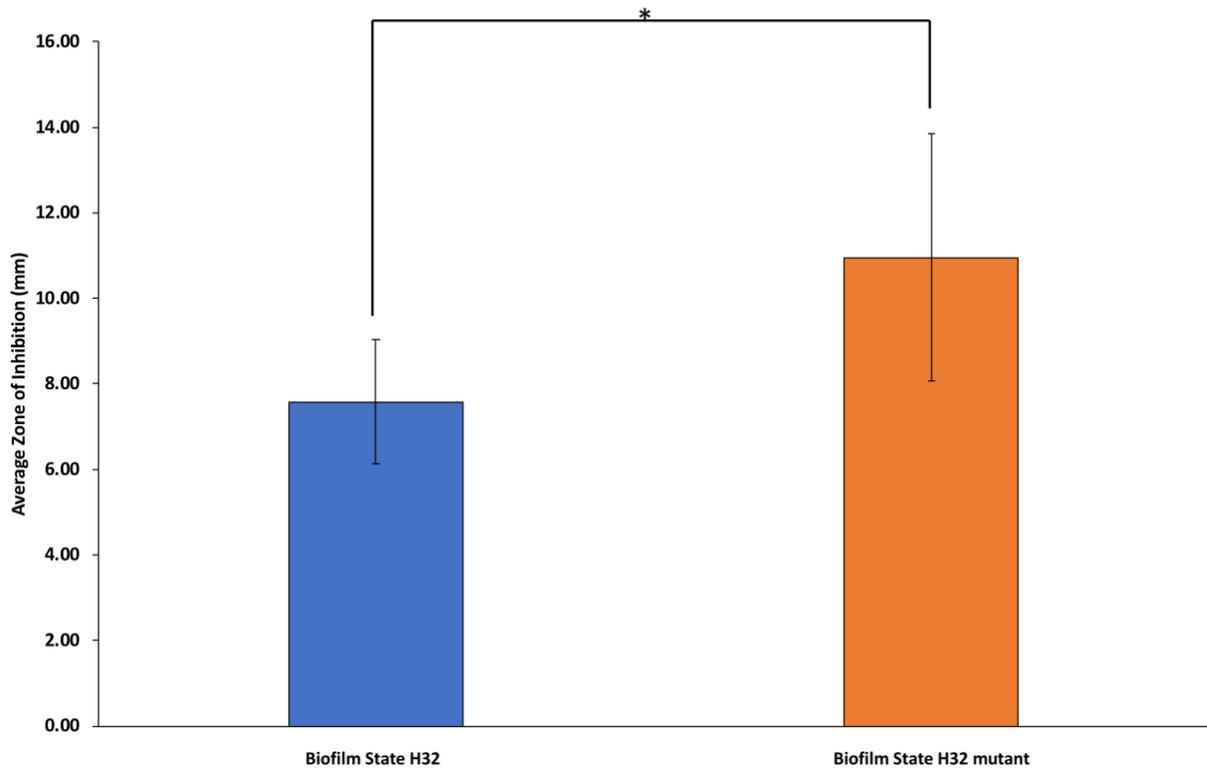


Figure 3.3. Comparison of average zone of inhibition for both the wildtype and mutant H32 strains across the eight antibiotics tested in the biofilm state. Statistical comparison illustrates a significant difference between the two isolates in the biofilm state ($p < 0.05$).

* indicates $p < 0.05$

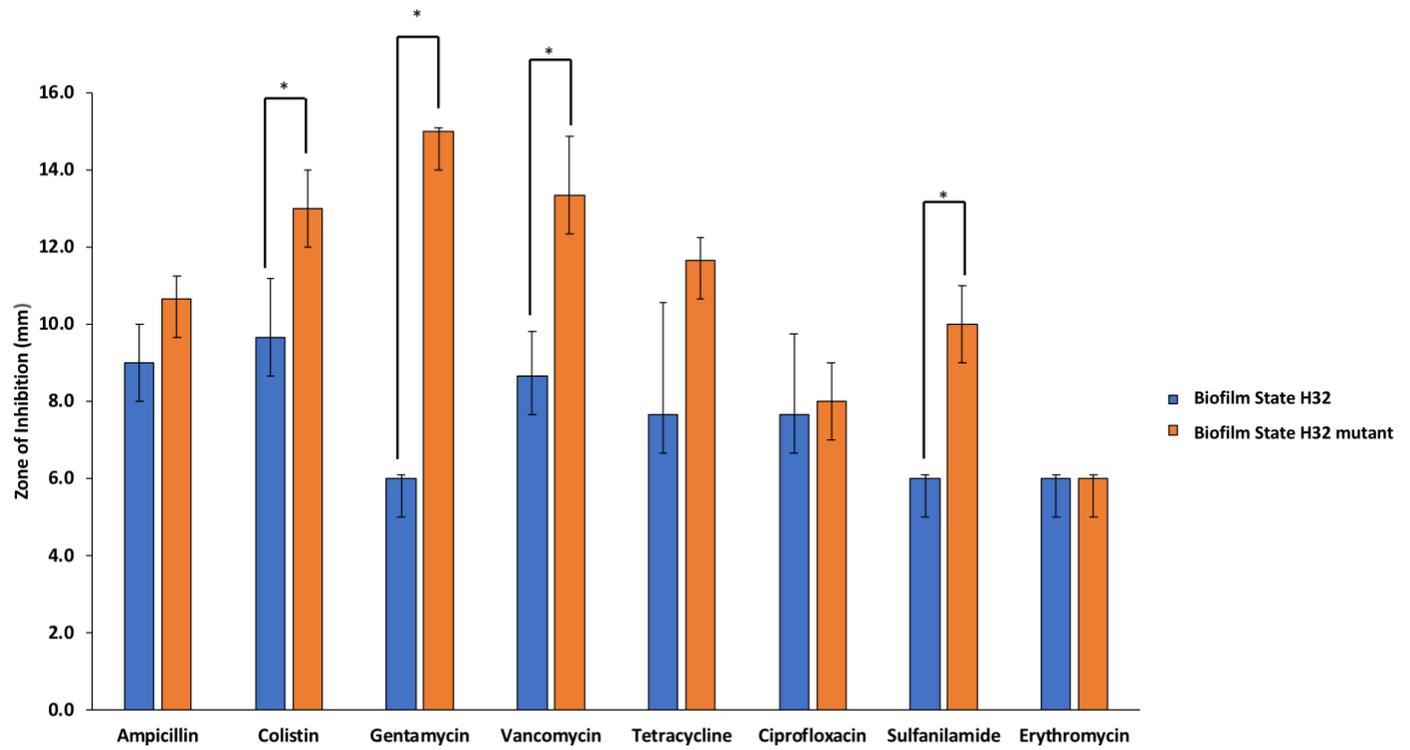


Figure 3.4. Comparison of mean zone of inhibition for both the wildtype and mutant H32 strains for each of the eight antibiotics tested in the biofilm state. Statistical comparison illustrates that in the biofilm state, the mutant H32 strain is more susceptible to the antibiotics tested (significant for four of the antibiotics tested) and is significant overall. * indicates $p < 0.05$

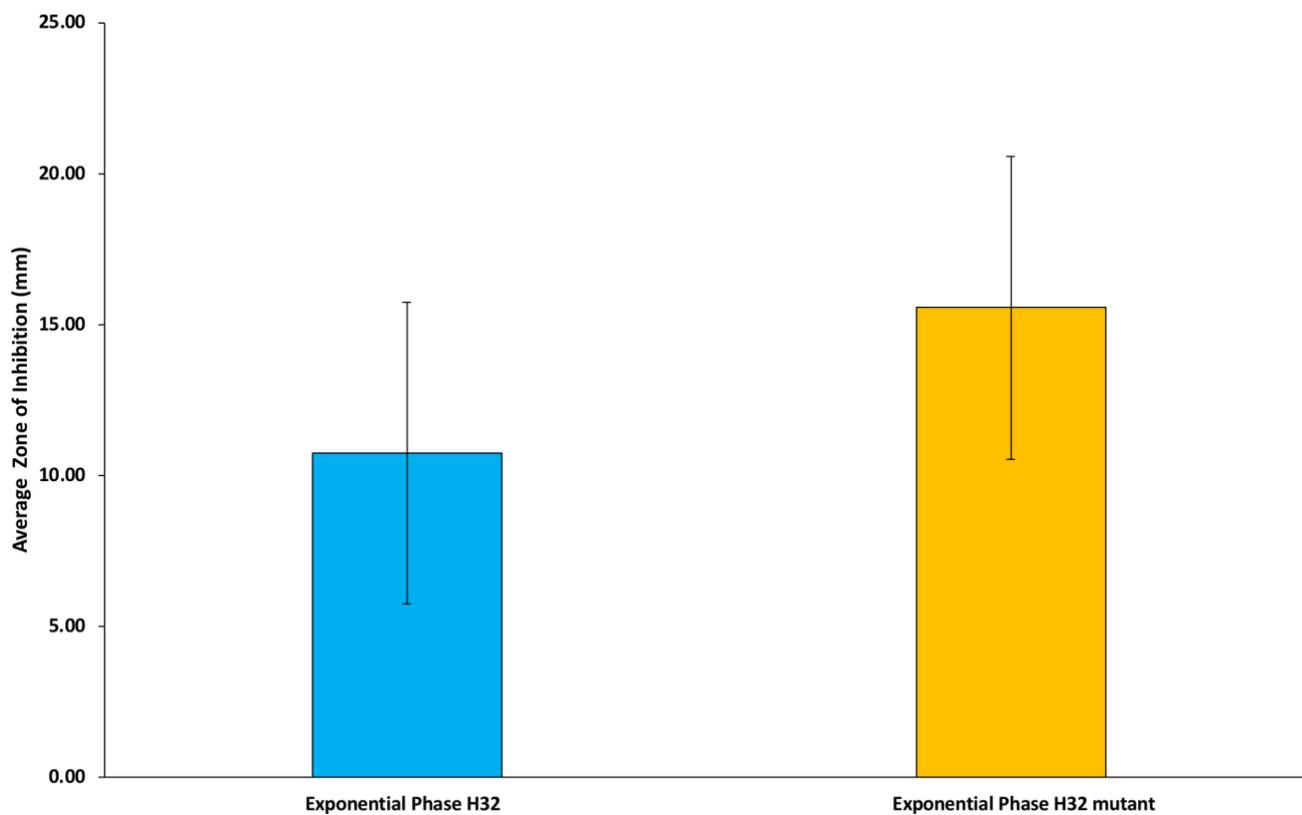


Figure 3.5. Comparison of average zone of inhibition for both the wildtype and mutant H32 strains across the eight antibiotics tested in the exponential phase. Statistical comparison illustrates that in the exponential phase, the mutant H32 strain is generally more susceptible to the antibiotics tested, however this is not statistically significant.

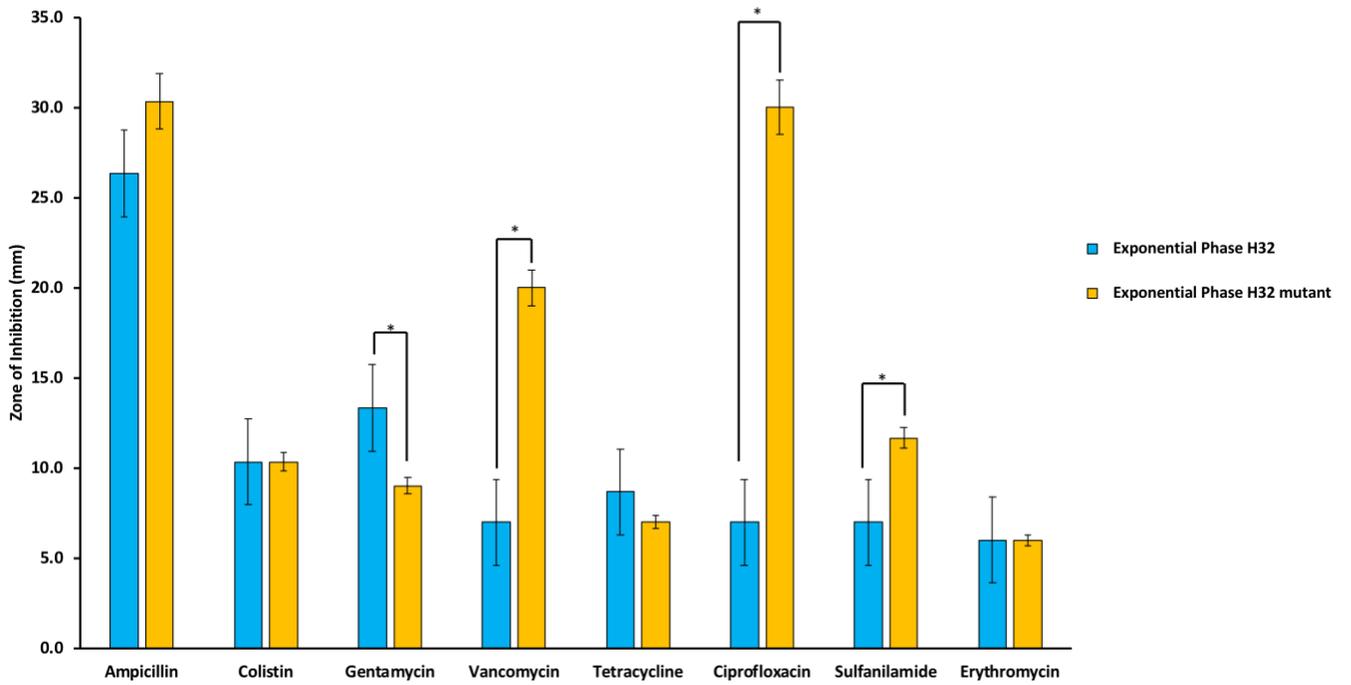


Figure 3.6. Comparison of zone of inhibition for both the wildtype and mutant H32 strains for each of the eight antibiotics tested in the exponential phase. Statistical comparison illustrates that in the exponential phase, the mutant H32 strain is generally more susceptible (significant for half of the antibiotics tested), however overall this is not statistically significant.

* indicates $p < 0.05$