Effect of periphytic *Escherichia coli* and lake water bacterial population on the biofilm establishment of a Shiga toxin producing *Escherichia coli* O157:H7 strain

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The undersigned certify that they have read, and recommended to the Graduate Studies Committee for acceptance, a thesis entitled "Effect of periphytic *Escherichia coli* and lake water bacterial population on the biofilm establishment of a Shiga toxin producing *Escherichia coli* O157:H7 strain" submitted by Tallie MacDonald in partial fulfilment of the requirement for the degree of Master of Science.

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Effect of periphytic *Escherichia coli* and lake water bacterial population on the biofilm establishment of a Shiga toxin producing *Escherichia coli* O157:H7 strain

Abstract of thesis

Biofilm studies of *Escherichia coli* (*E. coli*) have typically focused on O157:H7 in defined, laboratory medium. While there is value in such studies, they offer little in the way of explaining the behaviour and interactions of this group of bacteria with other environmental bacteria under biofilm conditions. Furthermore, with evidence mounting to support the persistence of naturalized populations of *E. coli* in the environment, a study to determine the effect of the naturalized *E. coli* and other environmental microbial populations on the biofilm development of *E. coli* O157:H7 is called for.

The biofilm developments of *E. coli* H32 strain (a pathogenic *E. coli* O157:H7 strain), *E. coli* 1A strain (isolated from a periphyton sample collected at Boulevard Lake, Thunder Bay, Ontario, Canada) and a microbial population collected from Boulevard Lake were examined using confocal scanning laser microscopy (CSLM). Biofilm formation was studied in a minimal salt medium supplemented with 0.04% glucose (MSMG). The CSLM allowed for the determination of biofilm structures. It was observed that the periphytic *E. coli* strain was able to form a thick (approximately 40 μm) structured biofilm which water channels and mushroom-like pillars were observed. The pathogenic *E. coli* strain H32, was unable to form a structured biofilm. The biofilm was scarce forming a monolayer of coverage. The lake water microbial population was able to form a structured biofilm with

lots of variations in structures from mounds to thin layers of cell coverage. The biofilm thickness was very diverse ranging from 5 to 30 μm.

In addition, the effect of the periphytic *E. coli* 1A strain and the lake water bacterial population on the biofilm establishment of the *E. coli* O157:H7 H32 strain was examined. In order to study the interactions between the two *E. coli* strains, a rifampicin resistant mutant of 1A (1A-Rif) and a green fluorescent protein gene (*gfp*) labelled and kanamycin resistant H32 mutant (H32-*gfp*) were created. These two mutant strains were used to replace the 1A and H32 strains in the mixed culture study. Three treatments were performed in the mixed culture study. The first treatment was to determine the biofilm establishment of the pathogenic H32-*gfp* strain when exposed to a pre-established periphytic *E. coli* 1A-Rif biofilm. The inoculum densities of the H32-*gfp* strain in this treatment were $1x10^7$, $1x10^6$, $1x10^5$ and $1x10^4$ CFU/mL and the biofilm cell densities of H32-*gfp* and 1A-Rif were determined by drop-plating after 48 h. At the inoculum density of $1x10^7$ CFU/mL of H32-*gfp*, the pre-established 1A-Rif biofilm helped H32-*gfp* to form more biofilm by increasing the biofilm density of H32-*gfp* by a magnitude of 1 log, when compared with the monoculture H32-*gfp* biofilm cell density in the absence of the pre-established 1A-Rif biofilm. However, at inoculum densities of $1x10^5$ and $1x10^4$ CFU/mL, the 1A-Rif biofilm decreased the ability of H32-*gfp* to form biofilm significantly ($p<0.05$). The inverse of this experiment was performed where the 1A-Rif strain was exposed to a pre-established H32-*gfp* biofilm. In this setting, the pre-established H32-*gfp* biofilm significantly decreased the ability of 1A-Rif to form biofilm ($p<0.05$) regardless the inoculum densities of 1A-Rif ranging from $1x10^7$ and $1x10^4$ CFU/mL.

The second treatment explored the biofilm forming ability of the pathogenic *E. coli* H32-*gfp* strain when it was exposed to a clean substratum simultaneously with the periphytic *E. coli* 1A-Rif strain. In this treatment, the 1A-Rif inoculum density was held at a fixed level of $1x10^7$ CFU/mL and it was co-inoculated with various concentrations of H32-*gfp* ranged at $1x10^7$, $1x10^6$, $1x10^5$ and $1x10^4$ CFU/mL. The biofilm cell densities of H32-*gfp* and 1A-Rif were determined after 48 h of growth. In contrast to the previous treatment with pre-established 1A-Rif biofilm, the reduction of H32-*gfp* inoculum density from $1x10^7$ to $1x10^4$ CFU/mL did not significantly reduce the density of H32-*gfp* biofilm cells established on the substratum and the final biofilm cell densities of H32-*gfp* were 6.86, 6.70, 6.60, and 6.66 Log CFU/cm^2 when the inoculum densities were $1x10^7$, $1x10^6$, $1x10^5$ and $1x10^4$ CFU/mL, respectively. However, when compared to the H32-*gfp* monoculture biofilm, the presence of 1A-Rif (at the co-inoculum ratio of 1:1) increased the biofilm density of H32-*gfp* by 0.4 Log ($p=0.06$). When the inoculum ratio of 1A-Rif: H32-*gfp* were 1:0.1, 1:0.01 and 1:0.001, the final biofilm densities of H32-*gfp* were not significantly changed by the presence of the 1A-Rif co-inoculum ($p=0.738$). The inverse of this experiment was performed where H32-*gfp* was inoculated at a fixed density of $1x10^7$ CFU/mL and 1A-Rif was co-inoculated at $1x10^7$, $1x10^6$, $1x10^5$ and $1x10^4$ CFU/mL. In this setting, the presence of the H32 co-inoculum significantly decrease the biofilm establishment of the 1A-Rif strain ($p<0.05$).

The third treatment was to examine the effect of a lake water microbial population (collected from Boulevard Lake) on the biofilm establishment of the pathogenic *E. coli* H32-*gfp* strain. The lake water bacterial population was able to form a mature biofilm on a sterile glass microscope coverslip immersed in the MSMG growth medium in 48 h. In this treatment, various concentrations of H32-*gfp* ($1x10^7$, $1x10^6$, $1x10^5$, $1x10^4$, $1x10^3$, $1x10^2$,

$1x10^1$ and $1x10^0$ CFU/mL) were exposed to a 48 h pre-established lake water biofilm. The biofilm cell densities established by the various H32-*gfp* inoculum levels were determined at 48 h. When compared to the H32-*gfp* monoculture biofilm, the pre-established lake water biofilm did not affect the amount of H32-*gfp* biofilm formed on the substratum at the inoculum density of $1x10^7$ CFU/mL. However, the lake water biofilm significantly decreased ($p<0.05$) the establishment of H32 biofilm at inoculum levels from $1x10^6$ to $1x10^2$ CFU/mL. Furthermore, the lake water biofilm completely inhibited the formation of H32-*gfp* biofilm at the inoculum densities of $1x10^1$ and $1x10^0$ CFU/mL.

The establishment of the H32-*gfp* strain was also examined in a co-cultured condition. In this experiment, the lake water bacterial density in the MSMG medium was adjusted to $3x10^3$ CFU/mL, which was at the same density as the natural bacterial density in the lake water samples collected from Boulevard Lake. An equal volume of the lake water bacteria-MSMG suspensions were mixed with a concentration series of H32-*gfp* to achieve final inoculum densities ranged at $1x10^7$, $1x10^6$, $1x10^5$, $1x10^4$, $1x10^3$, $1x10^2$, $1x10^1$ and $1x10^0$ CFU/mL of H32-*gfp*. The establishment of the H32-*gfp* biofilm was determined at 48 h. When compared to the H32-*gfp* monoculture biofilm, the lake water bacterial population did not significantly changed the biofilm establishment of the H32-*gfp* ($p=0.74$) at the inoculum density of $1x10^7$ CFU/mL. However, at the inoculum densities between $1x10^6$ to $1x10^1$ CFU/mL, the lake water bacterial population decreased the biofilm formation ability of H32-*gfp* significantly ($p<0.05$). Furthermore, the lake water bacterial population completely inhibited the biofilm formation of H32-*gfp* at an inoculum density of $1x10^0$ CFU/mL. Based on this study, the associated implications of *E. coli* O157:H7 establishing in the environment is a cause for concern. The risks associated for public health are far-reaching and must be taken seriously. .

Chapter 1. Literature review on *Escherichia coli* and its ability to establish biofilm in the presence of other *Escherichia coli* strains and environmental bacteria

1.1. *Escherichia coli*

A diverse bacterial species that consists of mostly non-pathogenic strains, *Escherichia coli (E. coli) is* a Gram-negative rod shaped (bacilli) bacterium. The length ranges from 2.0 - 6.0 µm and the cells are1.1 - 1.5 µm wide. However, the bacterium can acquire a spherical shape under carbon starvation (Percival, et al., 2014).

Many *E. coli* strains are capsulated and these capsules are composed of acidic polysaccharides. Mucoid strains of *E. coli* produce extracellular mucus consisting of polysaccharides composed of certain K antigen or with a common acid polysaccharide made of colonic acid (Jimenez, et al., 2012). *E. coli* contains fimbriae with various composition and antigen specificity. As these fimbriae are hydrophobic, they provide host or organ specific adhesion properties. Most *E. coli* strains are non-pathogenic. However, some groups can cause severe diarrheal disease, occasionally with fatal outcome. *E. coli* is of fecal origin and almost exclusively found in the digestive tract of warm blooded animals, including humans. As a consequence, detection of *E. coli* in potable water is used as an indicator of human or animal fecal contamination and is referred to as the coliform index (Percival, et al., 2014).

E. coli was first identified in 1885 and named *Bacterium coli commune*, by Dr. Theodor Escherich, a German paediatrician. He identified the bacterium through studies

of the intestinal flora of infants. Later the bacterium was found to have pathogenic properties involving extraintestinal infection. There are six major types of diarrheagenic *E. coli* and also extraintestinal pathogenic *E. coli* strains that are linked with urinary tract infections and neonatal meningitis. Each type combines some form of initial attachment to the host cell with subsequent adverse effects, either through the elaboration of a toxin or direct action. The diarrheagenic *E. coli* types include: the enterohaemorrhagic *E. coli* (EHEC) which is associated with watery diarrhea, hemorrhagic colitis and hemolytic uremic syndrome; the enterotoxigenic *E. coli* (ETEC) which is associated with watery diarrhea in children and travelers diarrhea; the enteroinvasive *E. coli* (EIEC) which is associated with watery diarrhea, inflammatory colitis and dysentery; the enteropathogenic *E. coli* (EPEC) which is associated with infant and childhood diarrhea; the enteroaggregative *E. coli* (EAEC) which is linked to persistent diarrhea in children and adults; and the diffuse adherent *E. coli* (DAEC) which is known to cause childhood diarrhea. Each specific type causes diarrheal disease through different mechanisms and each disease presents with different clinical symptoms (Percival, et al., 2014; Johnson, 2011).

The EHEC are the main cause of food and water borne illness and express numerous virulence factors. The most prevalent genes that EHEC exhibit are shiga toxins, *stx1* and *stx2,* which have the potential to cause hemorrhagic colitis, hemolytic uremic syndrome and even death.

EPEC stains are capable of adhering to the surface of mucosal cells and causing changes to the microvilli and structural rearrangement of the host cells (Rappelli, et al., 2001; Watterworth, 2003).The EPEC strains contain a plasmid denoted as EPEC

adherence factor (*eaf*). The *eaf* plasmid encodes an EPEC virulence factor named bundle-forming pili. EPEC infections are the result of bacterial invasion of the host cell and the resulting impact on the signal transduction system (Tobe, et al., 1999).

ETEC strains produce a heat labile enterotoxin (Elt) and/or a heat stable enterotoxin (Est).They are the most common etiological agent of traveler's diarrhea. Heat labile enterotoxin involves the activation of the enzyme adenylate cyclase that causes an increase in intracellular cyclic AMP. The increased cAMP levels disrupt the activity of the sodium chloride transporters of the host cell which results in an ion imbalance. The imbalance causes water loss by the cell resulting in diarrhea (Salyers & Whitt, 1994). The heat stable enterotoxins are low weight polypeptides that are able to alter the movement of fluid and electrolytes across the intestinal epithelium. This starts a cascade involving the intracellular accumulation of cyclic GMP and results in the secretion of chloride ions into the intestinal lumen (Salyers & Whitt, 1994).

EIEC resemble *Shigella* in respect to pathogenicity and most virulence genes are encoded on large virulence plasmids almost identical to those of *Shigella* spp. (Rappelli, et al., 2001). EIEC cause food associated diarrhea and dysentery. EIEC are capable of penetrating and multiplying within colonic epithelia cells. It has been determined that only those harbouring a large plasmid termed the invasiveness plasmid (*ial*) display the invasive phenotype and that this plasmid encodes the necessary products for invasiveness (Rappelli, et al., 2001).

UPEC are the main cause of extraintestinal infections such as urinary tract infections. UPEC's virulence strategies are to invade the bladder epithelial cells (Salyers & Whitt, 2002) . There are many virulence factors with UPEC strains. Major factors

include iron acquisition, where UPEC use siderophore-based acquisition systems. Iron is important in cellular respiration (Madigan, et al., 2009) . There are some strains of UPEC that secrete the alpha-hemolysin (HylA) which can lyse red blood cells. The HlyA toxin can create pores on the host cell membrane (Salyers & Whitt, 2002). The final set of UPEC virulence genes that were examined were the *papA* and *papC* genes. Their functions are involved with the formation of the P fimbria. The fimbria mediates the first steps of the adhesion process of the UPEC to the epithelial cells of the host and causes pyelonephritis (Madigan, et al., 2009) .

1.2. *Escherichia* coli O157:H7

One of the most prominent and well-studied *E. coli* serotypes includes enterohaemorrhagic *E. coli* O157:H7. Members of the O157 serogroup have the common somatic cell surface O antigen, whilst the flagellar H antigen is used to define the specific serotype. *E. coli* O157:H7 is considered one of the most problematic and pathogenic serotypes.

Enterohemorrhagic *E. coli* O157:H7 is a rapidly emerging bacterial pathogen. It can produce a Shiga toxin which has dramatic effects on the kidney, leading to hemolytic uremic syndrome (HUS). *E. coli* O157:H7 can be classified as either enterohaemorrhagic *E. coli* (EHEC) or Shiga toxin (STEC) (Rigsbee, et al., 1997). Recent outbreaks have suggested that inadequately cooked beef is the most common cause of EHEC infections. The bacteria can be isolated from cattle, however only a portion of cattle possess the bacteria. It also has been documented that geese and deer have the potential to be sources of fecal pollution and lead to the transmission of EHEC in the environment

(Somarelli, et al., 2007). Other outbreaks of EHEC have been linked to the transmission of EHEC into water bodies. Runoff water from grazing fields can transport the bacteria into local watersheds and into drinking water supplies as seen in 1990 in Missouri, where over 200 illnesses were reported due to the contamination of drinking water by EHEC (Rigsbee, et al., 1997). In 2000, a well in Walkerton, Ontario was contaminated with cattle manure from a nearby farm and was not properly treated. This resulted in more than 2,300 people experiencing gastroenteritis, 65 were hospitalized, 27 developed haemolytic uraemic syndrome, and 7 deaths occurred (Hrudey, et al., 2003). Runoff waters from farmlands can potentially enter into recreational waters.

From 2007 to 2008, there were 134 reported bacterial waterborne outbreaks in recreational waters in 38 states in the U.S and Puerto Rico. During those outbreaks, 13,966 bacterial infection cases resulted. The National Resources Defense Council conducted an epidemiological study and found that approximately 10% of beachgoers report getting sick after swimming in the Great Lakes. Sixty two percent of the beach closings/advisories in the U.S. were due to unknown sources of contamination in 2008. Also in 2008, Wisconsin had 578 closing or advisory notices for beaches, California had 1003, and Illinois had 396. Almost all of these cases were due to high levels of bacterial indicators, such as *E. coli* and Enterococci (Dorfman & Rosselot, 2009).

In the article, *On Notice for a Drinking Water Crisis in Canada*, Emma Lui discuses that both Canada and First Nation communities, there were 1,838 reported drinking water advisories (DWA) as of January 2015. A drinking water advisory is a preventative measure put in place to protect public health from drinking water that could be contaminated. Boil water advisories are recommend to the public, to bring their water to a

roaring boil for a minimum of 1 minute before drinking and using for other purposes. It is imposed when disease causing bacteria, viruses or parasites is found in the drinking water (Canada, 2015). As of January 2015, there were 169 DWA in 126 First Nation communities (Canada, 2015). The drinking water advisories in Indigenous communities are reported as follows: Ontario has the highest DWA reporting with 79 cases, followed by British Columbia with 35, Saskatchewan 24, Alberta 17, the Atlantic 7 and Quebec with 2 DWA (Lui, 2015). Most DWA in Indigenous communities are boil water advisories. Reasons for these DWA included, unacceptable microbiological quality, inadequate disinfection or disinfectant residuals, operation of system would compromise public health, significant deterioration in source water quality, unacceptable turbidity or particle count and equipment malfunction during treatment or distribution. Some of the oldest advisories date back as far as 1995 in Neskantaga First Nation in Ontario. Shoal Lake First Nation No. 40, outside of the city of Kenora in Ontario, has had a boil water advisory in place for more than 17 years (Lui, 2015). In Alberta, the 42 DWA were caused by negative pressure leading to stagnant water, that creates the risk of bacterial growth and pathogen infiltration, ground water under direct influences of surface water and the total coliform bacterial were listed as the cause for most of the advisories.

Another possibility is that *E. coli* could end up in estuaries where they could concentrate in shellfish. The FDA reported that the survivability of EHEC strains in the environment is similar to that of non-O157:H7 strains, causing alarm as a potential public health threat (Rigsbee, et al., 1997). Not only has the contamination of drinking water and reactional water posed a risk to human health, there is potential for irrigation water to be contaminated and cause problems.

Outbreaks of human infection associated with consumption of raw fruits and vegetables and unpasteurized fruit juices have occurred with increased frequency (Beuchat, 2002). Contamination of raw produce with pathogenic and non-pathogenic microorganisms can occur at any points of time. Including pre-harvest contamination sources include soil, manure, human and farm animal faeces and irrigation water. These factors influence the composition of indigenous microbial flora as well as the survival of and growth of human pathogens. The association between contaminated fresh vegetables and food-borne disease has led to increasing concerns about contamination with faecal pathogenic bacteria. Manure used as a fertilizer or soil amendment, as well as contaminated irrigation water represent a potential source of pathogens to contaminate fruits and vegetables. *E. coli* O157:H7 and *Samonella* are carried by animals and shed in their feces. Non-composted or improperly composted manure used on the farm, or manure that enters surface waters, may contain these pathogens and subsequently contaminate produce (Beuchat, 2002). Irrigation of vegetables and fruits with contaminated water increases the presence of pathogenic bacteria (Ibenyassine, et al., 2006). Possible reasons for higher number of outbreaks or infections can be due to the use of animal manure rather than chemical fertilizer, as well as untreated sewage or irrigation water containing pathogens can contribute to the risk of human illness associated with pathogenic microorganisms (Beuchat, 2002). All types of produce have the potential to harbour pathogens, but *Shigella* spp., *Salmonella*, entertoxigenic and enteroheorrhagic *E. coli*, *Campylobacter* spp., *Listeria monocytogenes, Yersinia enterocolitica, Bacillus cereus*, *Clostridium botulinum*, viruses and parasites are of great health concern. Fruits and vegetables can become contaminated with pathogenic microorganisms while growing in fields, orchards, vineyards, or greenhouses or during

harvesting, post-harvesting, processing, distribution and preparation on food service (Beuchat, 2002). Microbial aggregates that may harbour bacteria, yeasts and molds within an exopolysaccharide matrix have been observed on plant surfaces and these structures are referred to as a biofilm (Beuchat, 2002).

1.3. Biofilms

Microbiologists define biofilms as an assemblage of cells irreversibly attached to a surface and enclosed in a primarily polysaccharide matrix (Donlan, 2002). Although it is now readily apparent that many bacteria spend large portions of their lives dwelling in these surface-associated communities, modern research involving biofilms has been late in getting started. Antonie van Leeuwenhoek first described biofilms using a simple light microscope in the early 18th century, but it wasn't until the middle of the 20th century that any significant progress was made concerning the physiology of these surface-associated cells (Donlan, 2002).

Henrici first observed in 1933 that bacteria preferentially associate with any surface introduced to a culture (Ghannoum & O'Tool, 2004). Heukelekian and Heller (1940) followed this with the observation that the addition of a surface to a culture significantly increased bacterial growth and activity. In 1969, Jones *et al.* (1969) took advantage of the relatively new scanning and transmission electron microscopy technologies to analyze the matrix that surrounds biofilm cells. They concluded that the matrix was primarily composed of polysaccharides, a finding that was corroborated by Costerton *et al.'s* (1978) study involving mutants that could not produce extracellular polysaccharides (EPS). By 1978, the concept of reversible and irreversible attachment

had been introduced by Marshall (Ghannoum & O'Tool, 2004), so all the pieces were in place to synthesize a rudimentary definition of a biofilm. From 1980 onwards, researchers took this newfound understanding of microbial communities and applied it to various other disciplines, including medicine and food processing. More recently, the incorporation of molecular methods has allowed for an even greater understanding of how bacteria communicate with one another to develop the complex architecture of a mature biofilm.

Biofilms contain a large number of cells and quite importantly these populations are phenotypically heterogeneous. Bacteria can adhere to and grow on nearly every surface. As the bacteria grow they become encased in a self-produced extracellular matrix, the bacterial cell will then cluster together and form what is known as a biofilm. Biofilms can range from a few layers of cells to macroscopic structures such as stromatolites and microbial mats. Biofilms that grow in natural settings often result in mutualistic symbioses. The ability of bacteria to adhere to, and form biofilms on almost every surface gives these bacteria an advantage in every aspect. In clinical settings, biofilms can form on medical devices and often result in problematic chronic infections. In addition to this problem, bacteria can form biofilms on biotic surfaces such as skin and teeth. In an industrial setting, biofilm forming bacteria can foul equipment thereby causing many costly issues and time delays (Vlamakis & Kolter, 2011).

Biofilms provide several benefits to cells that inhabit in the structure, including increased resistance to antimicrobials, protection against protozoan grazing and host defences. For instance, the extracellular matrices can serve as a diffusion barrier to small molecules (Vlamakis & Kolter, 2011). Biofilms trap nutrients for microbial growth

and help prevent the detachment of cells from surfaces exposed in flowing systems. Biofilms typically contain several porous layers and the cells in each layer can be examined with a scanning laser confocal microscope. Biofilms may contain one or more species of microorganisms. Biofilms are very functional microbial communities. A biofilm that forms around a tooth surface will contain several hundred different phylotypes bacteria which include both the *Bacteria* and *Archaea* domains (Madigan, et al., 2009).

While the type of microorganisms and cell density play a large role in determining biofilm formation, external conditions can also significantly influence whether or not microbes will form mature biofilms. Biofilm formation is initiated by an interaction between planktonic cells and a substratum through two attachment processes, the reversible attachment and irreversible attachment (Sauer, 2003). These two attachment steps are mediated by surface proteins used for motility (*e.g.* flagella) and attachment (*e.g.* LapA in *Pseudomonas fluorescens*). This attachment may not occur, however, if the substratum does not possess the proper physical characteristics for particular bacteria. Fletcher and Loeb (1979) demonstrated differential biofilm formation in *Pseudomonas* strains exposed to surfaces with varying charge and hydrophobicity. *Pseudomonas aeruginosa* has also been shown to adhere more readily to positively charged and hydrophobic surfaces (Li & Logan, 2004). Other external environmental factors have been shown to mediate biofilm formation. These include the presence of conditioning films, solution chemistry, and fluid dynamics (Rijnaarts, et al., 1993; Li & Logan, 2004).

Conditioning films are formed when a substratum adsorbs proteins or other organic molecules that are present in the fluid environment (Murga, et al., 2001). This phenomenon has been studied extensively in the colonization of indwelling medical

devices by bacteria. Indwelling medical devices, such as central venous catheters, readily adsorb blood plasma proteins such as fibronectin and fibrinogen. These proteins have been demonstrated to enhance the attachment of Gram-positive organisms such as *Staphylococcus aureus* or inhibit the attachment of Gram-negative bacteria such as *Escherichia coli* (Esperson, et al., 1990; Abraham, et al., 1983). Conditioning films found in the environment, however, seem to be a prerequisite of environmental biofilm formation (Siboni, et al., 2007). The adsorption of polysaccharides, proteins, lipids, and humic acids from seawater onto a polyurethane surface has been demonstrated to increase the oxygen and nitrogen content on the substratum surface, as well as increase the overall substratum roughness, allowing for a greater surface area for bacteria to attach (Bakker, et al., 2004). Additionally Siboni et al, (2007) have demonstrated that the total organic carbon content of the conditioning film decreased with bacterial colonization. This suggests that bacteria use the conditioning film as a food source.

The solution chemistry of the surrounding fluid can also play a large role in determining aquatic biofilm formation. Oliviera et al, (1994) have demonstrated that *Pseudomonas fluorescens* exhibits optimal biofilm growth at a neutral pH. While this may be due in part to a neutral pH representing optimal growth conditions, a low pH has been shown to prevent interactions between metallic ions and polymeric organic substances, such as those used by *Pseudomonas spp.* for attachment (Ferris, et al., 1989). Conversely, a low pH has been shown to increase the adhesion of exopolymers to carboxylated latex (Xu, et al., 2005). Additionally, low ionic strength solutions were shown to increase the ability of exopolymers to adhere to surfaces (Xu, et al., 2005). These conflicting results serve to illustrate the complexity of microbe-surface interactions.

The temperature of the fluid medium also plays a significant role in the development of biofilms. Temperatures at approximately 22 °C have been demonstrated to promote peak biofilm development of some *E. coli* strains (Moreira, et al., 2011). However, cold-water conditions, less than 15°C, have been shown to inhibit biofilm growth (Donlan, et al., 1994).

Fluid dynamics can also affect biofilm formation. In moving water systems, fluid dynamics largely dictate whether or not a bacterium will be transported from the bulk liquid to the liquid-surface interface (Rijnaarts, et al., 1993). Rinjaarts *et al.* (1993) studied the deposition of bacteria in static and dynamic systems. In static systems, it was found that the rate of diffusion and level of bacterial motility governed the deposition of bacteria from the bulk liquid onto the substratum. Bacterial deposition in dynamic systems, conversely, is governed by convective diffusion. High linear volumetric flow rates result in an increased deposition of bacteria on the surface until the point where shear forces begin to disturb attached cells (Rijnaarts, et al., 1993).

Although external conditions demonstrate a significant role in controlling the formation of environmental biofilms, there are a myriad of other factors that can affect the attachment and subsequent growth of microbial communities on surfaces. These factors include substrate type, flow rate and type (turbulent or laminar) of surrounding environmental conditions and temperature. It is very important to take these extenuating conditions into account when conducting experiments *in vitro*. Although the genetic components for biofilm formation may be present, the bacteria might be prevented from establishing a biofilm due to adverse environmental conditions.

1.4. Biofilm life cycle

Biofilm and planktonic cells differ significantly in their physiology, gene expression pattern and morphology. Bacteria are able to switch between the planktonic and biofilm life styles. Planktonic bacteria are free floating single-cell bacteria in aquatic environments; whereas a biofilm is defined as a sessile microbial community characterized by adhesion to a solid surface enclosed in a matrix of exopolymeric substances, proteins and DNA. The bacteria growing in biofilms are less sensitive to treatments of antimicrobial agents as compared to planktonic cells. The extracellular matrix can reduce the penetration of antibiotics into the biofilms and the matrix-embedded bacterial cells. Together with the dormant metabolic state of the biofilm cells, they are more resistant to antibiotics than their planktonic counterparts (Madigan, et al., 2009).Transition of the planktonic cells to biofilm growth is regulated by a variety of environmental and physiological cues, such as bacterial cell density, nutrient availability and cellular stress (Landini, et al., 2010).

The formation of biofilm is initiated with attachment of a cell to a surface which then starts to express some biofilm-specific genes. The genes encode proteins that synthesize intercellular signalling molecules and initiate biofilm matrix formation. Once committed to biofilm formation, a previously swimming cell loses its flagella and becomes non-motile. The switch from planktonic to biofilm growth is triggered by the synthesis of cyclic dimeric guanosine (c-di-GMP), a derivate of the nucleotide guanosine triphosphate. C-di-GMP is made by a series of proteins associated with membrane integrated sensory proteins that in some way detect an opportunity for surface associated growth (Madigan, et al., 2009).

The development of the biofilm starts with a reversible attachment process between the planktonic bacteria and a substratum. In the second stage of the biofilm forming process, some of the bacteria become irreversible attached to the submerged surface. The third stage of biofilm formation is the formation of microcolonies from individual attached cells. The fourth stage is the maturation of the biofilm, where the mature biofilm develops structures such as pillars and water channels. Quorum sensing is a regulatory process dependent on bacterial cell density and is typically involved in the activation of genes related to biofilm maturation and maintenance. The quorum sensing process is activated at high bacterial cell densities. It will then induce the micro-colonies to transform into mature biofilm structures where local cell concentrations can be more than tenfold higher than their planktonic counterparts. The fifth and final step in biofilm development cycle is biofilm dispersal, where there is breakdown of the exopolymeric substance matrix and release of bacterial cells out into the environment to allow for new biofilm formation to occur (Landini, et al., 2010; Madigan, et al., 2009).

1.5. Robustness of biofilms

Biofilms plague both medical and industrial surfaces and are difficult to treat with common antimicrobial strategies. Cells residing within biofilms are often tolerant to antimicrobial agents at concentrations thousands of times higher than what is necessary to eradicate the same cells growing in planktonic phase. This recalcitrance is likely tied to a combination of physical and physiological factors (Zuroff, et al., 2010).

The role of biofilms in enhancing the survival and growth of pathogenic organisms and thus serving as a reservoir for disease is of upmost importance. Biofilms can act either as

a temporary or permeant refuge for pathogenic bacteria. Being a member in a biofilm community increases the capacity of pathogenic microorganisms to persist. Thus, pathogens in environmental biofilms are a concern even though they may not increase their biomass (Flanders & Yildiz, 2004).

Biofilms can act as reservoirs of pathogenic organisms due to growth benefits and the protection from conventional means for controlling bacterial growth. A biofilm offers resistance to antimicrobial compounds through poor antibiotic penetration due to extracellular polymeric substances, slow cell growth and adaptive stress responses (Flanders & Yildiz, 2004).

The presence of pathogenic bacteria in biofilms allows for the persistence, survival, and growth of pathogens and enhances the risk to human health. The presence and survival of biofilms in drinking water and on food sources is of great concern. The direct ingestion of pathogens from these sources can cause detrimental impact to human health. The World Health Organization estimates that 20% of the world's population lacks access to safe drinking water, which accounts for 200 million cases of diarrhea and 2.1 million deaths caused by diarrheal illness each year (Flanders & Yildiz, 2004). In Canada, Public Health Ontario published their dissertation on Reportable Disease Trends in Ontario which states that the annual incidence rates of shiga toxin-producing *E. coli* (STEC) increased in 2011 after four years of consistent decline (Ontario Agency for Health Protection and Promotion (Public Health, 2011). In 2011, the incidence rate of STEC was highest among persons under the age of ten years. STEC cases occur throughout the year, but most cases occur from May to October. STEC accounted for two percent of all reported cases of enteric diseases reported in Ontario in 2011. In 2011, 232

cases were reported for an incidence rate of 1.73 cases per 100,000 people. In 2011, the number of reported cases increased by 52% in comparison to 2010 which was 153 cases. The most frequently reported risk factors associated with STEC contamination were animal contact 45% (73/163), consumption of raw unwashed fruits of vegetables or unpasteurized juice at 26% (43/163) and recreational water contact at 23% (37/163), consumption of raw/ undercooked ground beef 14.7% (24/163) (Ontario Agency for Health Protection and Promotion (Public Health, 2011).

1.6. *Escherichia coli* in the environment

E. coil O157:H7 has been recognized in both sporadic cases and outbreaks as an important cause of hemorrhagic colitis and hemolytic uremic syndrome. Epidemiologic investigations revealed that cattle, especially young animals, are important reservoirs of this pathogen. *E.coli* of O157:H7 infections have been linked to drinking water and reactional waters. Several recent outbreaks associated with drinking reservoirs, well water and swimming in recreational lakes raise concerns about waterborne illness caused by this pathogen. In 1982 in Michigan and Oregon, *E. coli* O157:H7 was first recognized as a pathogen during an outbreak investigation of hemorrhagic colitis (Rangel, et al., 2005). *E. coli* O157:H7 was first isolated from surface water in a reservoir in 1986. In the summer of 1991, the infection of 21 children with *E. coli* O157:H7 was associated with swimming in a fecally contaminated reactional lake in Oregon. A large waterborne outbreak of O157:H7 occurred during the winter of 1991 in Cabool, Missouri, where residents obtained apparently contaminated drinking water from an un-disinfected

groundwater source. The outbreak resulted in 243 documented cases of diarrhea, including 32 hospitalizations and four deaths (Wang & Doyle, 1998).

Although contraction of the bacterium is typically associated with the ingestion of contaminated foods such as undercooked ground beef or vegetable products (Doyle, 1991; Mead & Griffin, 1998), there have been many instances of individuals becoming ill after swimming in contaminated streams and lakes or as a result of contaminated municipal water distribution systems (Ackman, et al., 1997). From 1982 until 2002, there were thirty-one waterborne outbreaks of *E. coli* O157:H7 in the United States. Of the thirty-one, twenty-one were associated with swimming in contaminated recreational water (Rangel, et al., 2005).

The source of *E. coli* is the gastrointestinal tract of animals, including humans. Cattle have been identified as a main reservoir and other animals also excrete the bacterium in their faeces, although there is little evidence of animal illness. Farmed and wild animals grazing in water catchment areas are a potential source of fecal contamination and therefore waterborne infections. Other sources include accidental ingress of raw sewage into water distribution systems (Chalmers, et al., 2000).

E. coli survives for long periods of time in bovine faeces, depending mainly on temperature, moisture content and water activity (Wang, et al., 1996). At 5°C, culturable organisms were detected for up to 70 days and at 37°C for up to 49 days, signifying that the *E. coli* need not be excreted directly into water sources, but can survive in cattle excrement. Survival has also been reported for 130 days in soil cores containing rooted grass (Maule, 1999). This allows for *E. coli* to enter water sources in runoff from agricultural land, either on the soil surface, through the soil matrix or through drainage

systems (Chalmers, et al., 2000). Once the *E. coli* is in the water, it is capable of surviving for many days particularly at cold temperatures. Studies of municipal water, reservoir water and recreational lake water have shown greater survival at 8°C compared with 15° and 25°C (Wang & Doyle, 1998).

In water treatment facilities, Ontario water is subjected to vigorous treatment and water quality testing. The processes that make potable water safe, palatable, clear, colourless, odourless, reasonably soft and noncorrosive are outlined in the Safe Drinking Water Systems, Ontario Regulation 170/03 (Ontario, 2014). Ontario's microbiological standard for recreational water is 100 CFU/100 mL *E. coli* and for potable drinking water 0 CFU/100mL. Disinfection occurs in the last stage of water treatment. Chlorination and ozone are the most common disinfectants used. Both the pathogenic and non-pathogenic *E. coli* are susceptible to chlorination. They should not be present in correctly treated and protected water mains. However, in experimental nutrient-limited conditions, *E. coli* O157 can develop a resistance to a low level of chlorine concentration (Chalmers, et al., 2000). The presence of fecal indictor microbes, such as *E. coli*, in water is indicative of fecal contamination and water treatment failure.

1.7. Thesis Objectives

Fecal contamination of Lake Superior and other water sources around the world is a threat to human health and the ecosystem. The goal of our study is to determine if periphytic *E. coli* and the periphytic community will increase or decrease the establishment of a highly pathogenic *E. coli* O157:H7 strain. The first objective of this study is to characterize the biofilm forming ability and the 3-D biofilm structure of a

periphytic *E. coli* 1A strain isolated from Boulevard Lake, Thunder Bay, Ontario and a shiga-like toxin producing *E. coli* O157:H7 H32 strain. The second objective is to determine the effect of the periphytic *E. coli* 1A on the biofilm establishment of the *E. coli* O157:H7. The third objective is to determine the effect of a lake water bacterial population on the biofilm establishment of the *E. coli* O157:H7.

Chapter 2. Effect of periphytic *Escherichia coli* and lake water bacterial population on the biofilm establishment of a Shiga toxin producing *Escherichia coli* O157:H7 strain

2.1. Introduction

Over the years research has shown that much of microbiological life occurs in biofilms. Waterborne pathogens are not an exception as congregation and integration into biofilms can offer considerable advantages. Biofilms provide shelter and protection for pathogenic bacteria and allow different species to come into close contact, and this allows communication and transfer of genetic materials (Nocker, et al., 2014). Many pathogens can actively form biofilms by themselves or attach to existing biofilms, referred as primary or secondary colonization, respectively (Szewzyk, et al., 2000). Biofilm formation is not only a transition from the free–floating to the sessile state, but surface attachment is typically accompanied by a change in cellular physiology. Biofilm formation characteristics of bacteria are influenced by the physiology of the bacteria and the biological, physicochemical and hydrodynamic conditions of the environment. An important trigger for biofilm formation is the exposure to environmental stresses such as nutrient depletion, organism density, temperature changes and other biotic and abiotic factors. The transition into the biofilm state is often considered to be a protective reaction used to overcome stresses (Nocker, et al., 2014).

Periphyton is a biofilm community of diatoms, green algae, cyanobacteria, bacteria, protozoa and viruses. These biofilms are attached to most natural and artificial submerged surfaces and are therefore often profuse in rivers and lakes. Epilithic

periphyton attached to rocks may offer habitat for population of fecal coliforms and *E. coli* in near shore aquatic environments (Ksoll, et al., 2007). In many cases, bacteria have been shown to gain a fitness advantage when residing in a mixed-species versus a single species biofilm. Bacteria living in a two species community were demonstrated to undergo mutations which improved productivity and stability compared to the parent community (Klayman, et al., 2009). Only bacteria and algae have the ability to actively form biofilm by attaching to surfaces with the secretion of exopolysaccharides. The biofilm matrix offers refuge for other organisms. The attachment and intra- and inter-species communication can lead to the formation of complex microbial communities that host a wide spectrum of microorganisms, including pathogens (Nocker, et al., 2014). Biofilms allow pathogenic bacteria to withstand environmental challenges, increase persistence, survival and growth, and enhance the risk to human health relative to their planktonic counterparts (Flanders & Yildiz, 2004; Sheldon, et al., 2012). In nature, bacteria are able to form single species biofilms or to coexist in multispecies communities and form mixed-culture biofilms on a wide variety of solid surfaces. Detached biofilms can become a continuous source of cross-contamination (Wang, et al., 2012).

Of all the pathogenic *Escherichia coli* strains, *Escherichia coli* O157:H7 is particularly interesting because it is a major cause of hemorrhagic colitis and hemolytic uremic syndrome (HUS). This is a consequence of the presence and expression of Shiga-toxin genes. Furthermore, this pathogen has a very low infective dose of less than 100 cells (Ngwa, et al., 2013). Shiga-toxin producing *E. coli* (STEC) strains of various serotypes are important pathogens responsible for numerous outbreaks, with symptoms ranging from bloody diarrhea to more severe diseases. Of the many STEC serotypes,

O157:H7 is the most frequently identified serotype that causes food-borne outbreaks worldwide (Wang, et al., 2012). STEC O157:H7 has been documented to survive for extended periods in water and has been linked to outbreaks associated with drinking water. As seen in Walkerton, Ontario in May 2000, this serotype infected 2500 individuals and with 7 fatalities (Ngwa, et al., 2013). *E. coli* O157:H7 is a pathogen in both food and water, and has the ability to form biofilm as an individual culture or form biofilm with a companion (Wang, et al., 2012). Biofilms in drinking water distribution systems and groundwater aquifers have been shown to harbour pathogens (Nocker, et al., 2014). Therefore, it is imperative to study how pathogenic *E. coli* becomes established in natural fresh waters and in periphyton.

In this study, we examined biofilm formation of a pathogenic strain of *E. coli,* a periphytic strain of *E. coli* and a lake water bacterial suspension using confocal laser microscopy and plate counting methods. This thesis will address how effectively *Escherichia coli* O157:H7 can associate with a periphytic *E. coli* biofilm and with a mixed-culture biofilm composed of a bacterial population recovered from lake water samples of Boulevard Lake, Thunder Bay, Ontario, Canada. The objectives of this study were to:

(1) Characterize the biofilm structures of a periphytic *E. coli* strain (1A), a rifampicin resistant 1A strain (1A-Rif), an *E. coli* O157:H7 strain (H32) and a *gfp*-labelled kanamycin resistant H32 strain (H32-*gfp);*

(2) Determine the effect of the periphytic *E. coli* 1A strain on the biofilm establishment of the *E. coli* O157:H7 H32 strain;

(3) Determine the effect of a lake water bacterial population on the biofilm establishment of the *E. coli* O157:H7 H32 strain.

2.2. Materials and Methods

2.2.1. Bacterial strains and growth media

For this study, all organisms were cultured in 50 mL of sterile Trypticase Soy Broth (TSB; Becton, Dickinson and Company, Sparks, MD, USA) at 37°C with 200 RPMs of orbital shaking. Stock cultures of all *E. coli* were maintained in TSB, supplemented with a 50% (v/v) solution of glycerol at -80°C and the final concentration of glycerol was 25% (v/v). The *E. coli* were recovered from frozen stock then streaked to produce isolated colonies on Trypticase Soy agar (TSA) and followed by overnight incubation at 37°C.

The wild-type *E. coli* O157:H7 strain employed in this study was obtained from Dr. C. Gyles at the University of Guelph (Guelph, ON, Canada). Strain identification number EC9620004 was used, corresponding to *E. coli* strain H32 (Gyles, et al., 1998). This strain possesses both the *stx1* and *stx 2* genes that encode for the shiga toxin I and II, respectively (Table 1). It also contains the *eae* gene for intimin, the virulence factor responsible for mediating the attachment of *E. coli* to colonic epithelial cells (Gyles, et al., 1998).

H32-*gfp* represents a genetically modified strain of H32, where a green fluorescent protein and kanamycin resistance genetic cassette (*gfp*-kan) has been inserted into the chromosome of the bacterial strain by a Tn5 transposon system (Sheldon, et al., 2012).

The periphytic *E. coli* 1A strain was isolated from a rock adjacent to the main beach of Boulevard Lake (48°27'34"N and 89°12'26"W) in Thunder Bay, Ontario, Canada by Moreira et al. (2011) using the following protocol:. A submerged rock, with an uppermost surface about 30-40 cm below water's surface, was selected for sampling. Periphytic material was scraped from within a marked area of 10 x 10 cm using a sterile spatula and suspended in 50 mL of sterile phosphate buffered saline (PBS, 8.00 g NaCl, 0.20 g KCl, 1.44 g Na2HPO4, 0.24 g KH2PO4, 1L distilled H_2O, pH adjusted to 7.4) in a sterile 125 mL Nalgene bottle. The isolation of the *E. coli* from the periphyton sample was carried out as follows; 5 mL of the periphyton suspension were drawn through a sterile 47-mm mixed cellulose ester filter (Fisherbrand water testing membrane filter, pore size 0.45 µm, Thermo Fisher Scientific, Whitby, ON, Canada) using a pneumatic pump funnel filtration apparatus. Using sterilized forceps (Fisherbrand), the filter was placed face up on Membrane Fecal Coliform Agar (mFC: Becton, Dickson and Company, Spark, MD, USA). The plate and filter were incubated at 44.5°C for 22 h in an Innova incubator (New Brunswick Scientific, Edison, NJ, USA). Blue colonies growing on the filter were selected and streaked on CHROMagar *E. coli* agar (Dalynn Biologicals, Calgary, AB, Canada) for isolated colonies. These plates were then incubated at 37°C for 22 h. Blue colonies appearing on CHROMagar *E. coli* agar plates were presumptively identified as *E. coli*. Additionally, blue colonies were subjected to the IMVic (Indole, Methyl Red, Vogues-Proskaure, Citrate) test series to provide additional confirmation of identity. The 1A strain was one of the periphytic *E. coli* isolates which formed a large amount of biofilm in a polystyrene 96-well plate-crystal violet assay (Moreira, et al., 2011). The 1A isolate was maintained in TSB with 25% (v/v) of glycerol at -80°C.

In order to study the interactions between the H32 strain and the 1A strain, a spontaneous rifampicin resistant mutant of 1A was isolated. The isolation protocol is described as follows: The wild type 1A *E. coli* strain was inoculated in a sterile TSB tube and then incubated for 24 h at 37°C with shaking at 50 RPMs. After the growth, 200 µL of the 1A culture was spread-plated onto a 50 mg rifampicin/L Tryptic Soy Agar (TSA) plate. The plate was incubated for 24 h at 37°C. After growth, cells from a single colony were streaked onto another 50 ppm rifampicin TSA plate. The plate was incubated for 24 h at 37°C. To ensure the stability of the rifampicin resistance phenotype, cells from a single colony were then spread onto a regular TSA plate and grown under the same conditions as above. The spreading was followed by an additional streaking of a single colony onto another 50 ppm rifampicin TSA plate. This process was repeated twice to ensure maximal stability of the rifampicin resistance. The rifampicin resistant strain (1A Rif) was maintained on 50 ppm rifampicin TSA plates for short term storage. For long term storage, the 1A-Rif strain was maintained in Tryptic Soy Broth (TSB) with a 25% glycerol final concentration and stored at -80°C.

All bacterial strains were cultured in TSB at 37°C, unless otherwise noted. The wild type H32 and 1A strains were grown and maintained without antibiotics. The antibiotic resistant mutants were cultured as follows: H32-*gfp* was cultured in growth media containing 50 µg/mL of kanamycin and 1A-Rif was grown with 50 µg/mL of rifampin. Stock cultures were prepared for each strain and stored at -80°C. Each of the bacterial strains were recovered prior to each experiment by streaking for the isolated colonies on TSA and then plating on TSA with the appropriate antibiotic.

2.2.2. DNA extraction and PCR detection of the virulence genes of the *E. coli* strains

E. coli strains 1A, 1A-Rif, H32, H32-*gfp* were streaked from -80 °C stock cultures on to fresh Tryptic Soy Agar (TSA) plates. Each strain was inoculated into a test tube (Borosilicate Fisher Scientific) with 5 mL of sterile Tryptic Soy Broth (TSB). The inoculated cultures were then incubated and shaken at 37°C and at 200 RPMs for 24 h.

To extract the DNA from the cells, XS lysis buffer (1% w/v potassium ethyl xanthogenate, 100 mol l^{-1} Tris–HCl, 20 mmol l^{-1} EDTA, 1% w/v SDS, and 800 mmol l^{-1} ammonium acetate) was used as described by Yang (2013). Firstly, 1000 µL of each culture was added to a 1.5 mL sterilized tube (Fisher Scientific) and centrifuged for 5 minutes at 18,800xg. The supernatant was then decanted off, leaving a pellet of cells. A second 1000 µL of sample was then added and the procedural steps were repeated. Thirdly, 800 µL of XS lysis buffer and 2 µL of RNase (10µg/mL) were added to the cell pellet to disrupt membranes, denature proteins and break down RNA of the bacterial cells. The sample was vortexed until the pellet was resuspended, then placed in a floating tray and incubated for 1 h in a 37°C water bath. After incubation, the samples were relocated to a 70°C water bath for a 1 h incubation period to completely lyse the cells. The samples were then mixed by inverting the tubes for 1 minute then placing the samples in ice for 30 minutes to precipitate the cell debris from the cell suspension. The samples were centrifuged for 10 minutes at 18800 x g to separate the denatured proteins and fragments of cell debris. For each strain, 750 µL of supernatant was transferred to a new sterilized 1.5 mL tube (Fisher Scientific) and 750 µL of isopropyl alcohol was added to precipitate the DNA. The samples were then cooled in a -80°C freezer for 1 h then

transferred to -30°C for overnight precipitation. The samples were removed from the -30°C freezer and centrifuged for 10 minutes at 18,800 x g. The supernatant was decanted off and the DNA pellet was then washed with 70 % ethanol twice. The DNA samples were then placed in a biosafety cabinet for 1h and allowed to air dry. Each tube received 100 µL of UV treated sterilized double distilled water and was vortexed until the pellet was dissolved. The DNA was examined with a 1% Agarose gel (50mL 1XTAE, 0.5g Agarose, and 5 µL Ethidium Bromide). To load the gel, 2 µL of 6 x loading dye (Fermentas.) and 10 µL of DNA sample were mixed and loaded into the well of the gel. The electrophoresis parameters included 100 V for 30 minutes in 1x Tris-acetate EDTA buffer (TAE, 242g Tris base, 100 mL of 0.5M EDTA, 57.1 mL glacial acetic acid, adjust pH to 8.2) and visualization was accomplished with a Syngene Chemi Genius Bio Imaging System with Gene snap program (Fisher Scientific).

The virulence genes in the *E. coli* strains were tested by PCR assays to confirm that *E. coli* H32 and H32-*gfp* were EHEC and that 1A and 1A-Rif were not. To confirm the absence of pathogenicity factors of other *E. coli pathotypes,* the following genes were tested. The 1A, 1A-Rif, H32, H32-*gfp* strains were tested for the presence of the shiga toxin I gene (*stx*1), shiga toxin II gene (*stx*2), the most prevalent genes that EHEC exhibit are shiga toxins, *stx*1 and *stx*2, which has the potential to cause hemorrhagic colitis, hemolytic uremic syndrome and even death.

The heat stable enterotoxin gene (*est*), heat labile enterotoxin gene (*elt*), is the most common etiological agent of traveler's diarrhea invasiveness plasmid loci gene (*ial*) is associated diarrhea and dysentery, adherence factor gene (*eaf*) results in the infections of bacterial invasion of the host cell and the resulting impact on signal transduction

system. UPEC are the main cause of extraintestinal infections such as urinary tract infections. The virulence genes of UPEC include iron acquisition gene (*iroN*) used in siderophore-based acquisition systems, alpha-hemolysin gene (*hylA*) which can lyse red blood cells, and P fimbrial adhesion genes (*papA* and *papC*) which are involved with the formation of the P fimbria. The fimbria mediates the first steps of the adhesion process of the UPEC to the epithelial cells of the host and causes pyelonephritis (Madigan, et al., 2009) .

The *E. coli* ETEC 505 strain was the positive control for the *est* gene, ETEC 07 for the *elt*, ETEC 326 for *est* and *elt*, EIEC 0164 and 0136 for *ial*, EPEC 2348 and 055 for *eaf*, and ATCC 25922 for *iroN, hylA, papA* and *papC*. The *E. coli* JM 109 strain was used as a negative control for the PCR assay. The PCR was conducted in a MJ Mini Thermal Cycler (BioRad). One µL of genomic DNA (approximately 10 ng DNA) was added to each PCR reaction mixture containing 0.2 $mmol^{-1}$ of each dNTP, 2.5 $mmol^{-1}$ $MgCl_2$, 1 × PCR buffer, 1.0 U of *Taq* polymerase and 0.3 μmol^{-1} of each primer. The protocol consisted of an initial denaturation at 95°C for 1 minute, followed by 34 cycles, each consisting of stages at 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The PCR products were analyzed by electrophoresis in 1% agarose gel containing TAE buffer (40 $mmol^{-1}$ Tris-HCl, 20 $mmol^{-1}$ acetic acid and 1 $mmol^{-1}$ EDTA) and ethidium bromide (1 μg^{-1}).

2.2.3. Growth curves of planktonic *E. coli* strains

Individual *E. coli* colonies were inoculated into 250 mL Erlenmeyer flasks (Fisher Scientific), each containing 50 mL of sterile TSB. The flasks containing *E.coli* 1A-Rif and

H32-*gfp* were supplemented with 50 µL of rifampicin (50 mg/mL) and 50 µL of kanamycin (50 mg/mL) into the broth respectively and the wild type strains were inoculated into 50 mL of TSB without antibiotic. The samples were incubated at 37°C overnight with orbital shaking at 150 RPMs. After growth, the cells were washed with sterile phosphate buffer saline (PBS), dissolved salts comprising of 8.0 g NaCl, 0.20 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 in 800 mL ddH_2O adjusted to a pH of 7.4 with 1M HCl and then adjusted to a final volume of 1000 mL by ddH_2O. To wash each *E.coli* strain, 15 mL of the bacterial culture was added to a sterile disposable 50 mL tube (Fisher Scientific) and centrifuged for 5 minutes at 2683 x g (Sorvall RT1 Centrifuge, Thermo Scientific). After separation the supernatant was removed and the pellet was resuspended in 10 mL PBS, this process was repeated three more times. After the final wash, the supernatant was decanted. The cells were resuspended in either 15 mL TSB or Minimal Salt Medium with 0.04% glucose (MSMG, 1.249 mM KH_2PO_4, 3.73 mM K_2HPO_4, 0.4 mM $MgSO_4$, 0.02 mM $FeSO_4$, and 1.4 mM NH_4Cl). A new sterile 250 mL Erlenmeyer flask, containing 47.5 mL sterile growth media, was inoculated using the 2.5 mL sample to an OD_{600nm} of approximately 0.05.

2.2.4. Confocal Imaging for biofilm growth

For these experiments, the biofilms were grown on 22 x 22 mm glass coverslips (Fisher Scientific Canada, Ottawa, ON, Canada) previously cleaned as follow by the following procedure: 30 minutes immersion in 15.7 M nitric acid, three subsequent washes in sterile ddH_2O, a rinse in absolute methanol and finally autoclaved for 20 minutes. The coverslips were placed in sterile 6-well cell culture plates (Costar, Corning Inc.) with 5 mL of approximately $1x10^7$CFU/mL cell samples in MSMG. Every 24 h the

spent media was removed gently by aspiration and replaced with 5 mL of sterile fresh media. The *E.coli* biofilms were grown for 48 h at 22°C with orbital shaking at 25 RPMs. The coverslips were then removed from the wells carefully, using sterile forceps and rinsed in two beakers of 100 mL of sterile ddH_2O, and stained by flooding them for 10 minutes with 200 µL of SYTO 9 (diluted to 5 µL/mL in sterile ddH_2O). All strains used for the experiment, including the *E. coli* H32-*gfp* were stained with SYTO 9 (Molecular Probes, Eugene, OR, USA) to ensure comparable fluorescence. SYTO 9 is a green fluorescent nucleic acid stain, which stains both the wild type strains and the antibiotic resistant strains, whereas the *gfp*-labelled strain is visible without the SYTO 9 stain. After staining, the un-adhered cells and excess dye were removed by submerging the coverslips twice in sterile ddH_2O.

The coverslip was then placed face-down over the concavity of a concave microscope slide filled with 200 µL of sterile ddH_2O to prevent drying. Excess sterile ddH_20 was blotted from the margins of the cover slip using a clean paper towel. The margins of the cover slip were sealed using nail polish and the exposed side of the coverslip was cleaned and decontaminated by wetting a cotton swab with 10 % sodium hypochlorite and gently swabbing the surface of the coverslip. The wash procedure was repeated with ddH_2O, 85 % ethanol, and ddH_2O again, using a new cotton swab for each application. Slides were placed in darkness at 4°C to prevent photo-bleaching of the SYTO 9 and to preserve the biofilms.

An Olympus FluoView™ FV300 confocal scanning laser microscope (CSLM), equipped with a 60 x PlanApo NA 1.4 oil immersion objective lens and a 10 mW, 488 nm argon laser (Olympus Corporation, Tokyo, Japan) was utilized to visualize the biofilms.

Detection of SYTO 9 fluorescence was performed using a DM570 dichroic mirror and band pass emission from 510 to 530 nm, using a FVX-BA 510-530 filter set, due to the maximal emission of SYTO 9 occurring at 510 nm (Molecular Probes, Eugene, OR, USA). To reduce photo-bleaching from occurring, the laser intensity was set to 1% for all image capturing. Each coverslip had 5 fields of view; the fields of view were randomly moved over the coverslip to allow for a non-bias representation of the biofilm.

2.2.5. Establishment of monoculture biofilms with various inoculum densities

After careful examination between the wild types, 1A, H32 and the antibiotic resistant mutant strains 1A-Rif, H32-*gfp* of *E. coli,* it was observed that there was no difference between the wild type strains and their respective antibiotic resistant mutants (Figures 1 - 6). Therefore, the mutant strains were used in the remainder of the study. For the biofilm experiments, the *E. coli* strains (1A-Rif, H32-*gfp*) were freshly streaked out onto TSA plates with the respective antibiotics (50 µg/mL of kanamycin, 50 µg/mL of rifampin). The *E. coli* strains were grown overnight at 37°C. The isolated colonies were then inoculated into 50 mL of sterile TSB with the appropriated antibiotics in 125 mL Erlenmeyer flasks (Fisher Scientific). These cultures were incubated at 37°C and shaken at 150 RPMs for 24 h. The *E. coli* cultures were then divided 20 mL each distributed into two sterile 50 mL tubes (Fisher Scientific). Samples were centrifuged for 5 minutes at 13584 x g at 4°C. The supernatant was decanted and 15 mL of sterile phosphate buffered saline (PBS) was added to the pellet and vortexed. The cells were washed with sterile PBS and repeated 3 times as described in section 2.2.3. After the final wash, cells

were resuspended in sterile MSMG to an optical density (OD_{600nm}) of 1 ± 0.05 (about $1x10^9$ CFUs/mL). The cell suspension was further diluted approximately 100 X in MSMG to achieve a final cell density of $1x10^7$ CFU/ mL. Several different inoculant density treatments were performed. The monocultures were initially inoculated in the 6-well culture plates with decreasing densities, $1x10^7$, $1x10^6$, $1x10^5$ and $1x10^4$ CFU/mL. These concentrations allowed for the development and understanding of how the biofilms will behave under the experimental design of testing monocultures of 1A-Rif and H32-*gfp* to pre-established treatments and co-culture treatments. The four different *E.coli* inocula were transferred via a 25 mL sterile, disposable, serological pipette (Fisher Scientific) into 6-well cell culture cluster plate (Costar, Corning NY.). A final volume of 5mL was added to each well in triplicate. As stated before, the inoculum culture was incubated at 22°C, to simulate the temperature of the lake water in the summer months. A gentle agitation on an orbital shaker was performed at 25 RPM. This speed is analogous to the gentle lapping of waves on the shoreline. The biofilms were allowed to grow and mature over a set length of specified time (monocultures harvested every 24 h for 4 days, pre-established treatments after 48 h and 96 h due to the two set of growing bacteria at different times, and co-culture treatments after 48 h), with aspiration of old media every 24 h, while replenishing with new sterile MSMG media. The biofilms developed and matured over time and harvesting of the biofilms was essential to determine the biofilm density and any changes in that occurred in the biofilm.

2.2.6. Harvesting of the biofilms

Removal of the biofilms from the coverslips was facilitated by immersing the coverslips sequentially in 2 beakers of 100 mL of 0.85% sterile saline (8.5 g NaCl dissolved 1 liter of ddH_2O) to remove planktonic cells and debris not related to the biofilm. After rinsing coverslips were placed on top of a sterile piece of paper towel in a sterile petri dish (Fisher Scientific). Sterile cotton applicators (Fisher Scientific Canada, Ottawa, ON, Canada) were utilized to scrub the sampling area a defined number of times. The first cotton applicator was drawn over the surface of the coverslip 30 times in a unidirectional manner. The second cotton applicator was immersed in a sterile PBS solution, and then used to streak 90° to the original direction, 30 times. The third cotton applicator was used dry and was streaked 180° to the original direction, 30 times, and was used to go over the perimeter of the cover slide. The cotton tip applicators were deposited into a sterile test tube (15 x 150 mm borosilicate glass culture tube, Fisher Scientific) containing 5 mL of sterile PBS. The test tube containing 5 mL of PBS and three cotton tips was immediately vortexed vigorously for 1 minute to dislodge the cells, and 1 mL of sample was removed to perform a 10 X dilution series in sterile PBS. From each dilution, 5 drops of 5 µL were sampled and drop-plated onto their respective antibiotic TSA plate. Prior to counting colonies and calculating biofilm cell density, the plates were incubated for 24 h at 37°C. When examining the time course experiment, the biofilms were harvested every 24 h. Subsequently, for all co-culture experiments, the biofilms were harvested every 48 h, and for all pre-established experiments, the biofilms were harvested at 48 h and 96 h.

2.2.7. Monoculture time course experiment

The monoculture experiments are important to examine and model the behaviour of each strain of *E. coli* and to determine the characteristics needed to study the bacterial dynamics in biofilm formation. The *E.coli* 1A-Rif and H32-*gfp* were prepared as previously described. Inoculum densities of approximately $1x10^7$, $1x10^6$, $1x10^5$, $1x10^4$ CFU/mL, were added to the 6-well cell culture cluster plates. The time course experiment was performed over 96 h. Therefore, four sets of coverslips were inoculated at time zero. At 24 h intervals, biofilms of one set were harvested and enumerated as described above. Fresh MSMG was added to the remaining biofilms at each 24 h interval. A drop plate assay was performed on each of the samples to determine their biofilm cell densities.

2.2.8. Pre-established Biofilms

In the environment, pre-established biofilms are attached to most natural or artificial surfaces and are therefore abundant in rivers and lakes (Ksoll, et al., 2007). The *E. coli* cultures were prepared as described above. The inoculum densities of 1A-Rif and H32-*gfp* were varied depending on the experiment performed. The inoculant density of the pre-established *E.coli* was at $1x10^7$ CFU/mL for this experiment, with decreasing inoculant concentrations of the competitor *E. coli* strain. The pre-established 1A-Rif biofilm was grown for 48 h incubated at 22°C with orbital shaking at 25 RPMs, with the media changed at the 24 h mark, as described above. After 48 h, three 1A-Rif biofilm samples were harvested and their biofilm cell densities were determined, thereby elucidating how the 1A-Rif biofilm behaves without a competitor growing in its presence.

For the rest of the 48 h 1A-Rif biofilm samples, the competitor H32-*gfp E. coli* was introduced, ranging in inoculum density from $1x10^7$, $1x10^6$, $1x10^5$, and $1x10^4$ CFU/ mL. Samples with the newly introduced *E.coli* were incubated for 48 h and the medium was exchanged at 24 h as described above. Three independent biological replicates were used in each treatment. The samples were then incubated at 22°C for 48 h with agitation at 25 RPMs. The mixed culture biofilms were harvested and enumerated after the second 48 h incubation period as described above. Harvesting of biofilms followed the same procedure described above. The pre-established treatment contained both H32-*gfp* and 1A-Rif cultures of biofilm. The biofilm cell densities of the two *E. coli* stains were determined by a drop-plating method using both the kanamycin and rifampin TSA plates. The drop plate method was described as above.

In contrast to the above treatment where 1A-Rif was able to form a pre-established biofilm, another set of treatments in this experiment was to investigate the effect of a pre-formed H32-*gfp* biofilm and monitor the effects on the establishment of a 1A-Rif biofilm. The H32-*gfp* cell density was inoculated at $1x10^7$CFU/mL, incubated at 22°C with orbital shaking at 25 RPMs the medium was exchanged as described above. The 48 h biofilm exposed to 1A-Rif with inoculant densities of $1x10^7$, $1x10^6$, $1x10^5$ and $1x10^4$ CFU/ml. The biofilms were grown for 48 h, as described above. The pre-established biofilms had both H32-*gfp* and 1A-Rif cultures. The biofilm cell densities of the two *E. coli* stains were enumerated and were determined by a drop-plating assay using both the respected kanamycin or rifampin TSA plates. The drop plate method was described as above.

For each experiment samples with monoculture biofilms were grown as control. Monocultures were harvested and enumerated at 48 h and at 96 h. The 48 h and 96 h biofilm determined how the *E. coli* strains grew without the competitor.

2.2.9. Co-cultures experiment

In this part of the study, establishment of H32-*gfp* biofilm and 1A-Rif biofilm were determined when a substratum was exposed to the two *E. coli* strains simultaneously. Cultures of the two *E. coli* inocula were prepared as described above. In one set of treatments, the *E.coli* 1A-Rif inoculant density was held at $1x10^7$ CFU/mL and the concentrations of the H32-*gfp* co-inoculant were set as $1x10^7$, $1x10^6$, $1x10^5$ and $1x10^4$ CFU/ml. Three replicates of each inoculant ratio were utilized. All samples were incubated at 22°C for 48 h with shaking at 25 RPM. The MSMG media was changed after 24 h by pipetting out the spent MSMG medium from the edges of the wells and adding 5 mL of fresh sterile MSMG. After 48 h the biofilms were removed and washed as described above. Harvesting and enumeration of biofilms was conducted utilizing the procedure outlined above. The samples were enumerated on both the kanamycin and rifampin TSA plates due to the mixed culture samples containing both H32-*gfp* and 1A-Rif.

The experiment was conducted again with inverse parameters, where H32-*gfp* was inoculated with ~ $1x10^7$ CFU/mL and 1A-Rif was inoculated with densities ranging from $1x10^7$, $1x10^6$, $1x10^5$, $1x10^4$ CFU/mL. Media was exchanged after 24 h, as outlined above. After 48 h the biofilms were removed and washed as described above. Harvesting and enumeration of biofilms was conducted utilizing the procedure outlined above. The

samples were enumerated on both the kanamycin and rifampin TSA plates due to the mixed culture samples containing both H32-*gfp* and 1A-Rif.

Monocultures, of H32-*gfp* and 1A-Rif were prepared, harvested and enumerated as described above. Each inoculum density was used to compare the co-culture results and check for changes between the monoculture treatment and the co-culture treatment.

2.2.10. Lake water biofilms

Water samples were taken from one location at Boulevard Lake (48°27′34″N and 89°12′26″W), adjacent to the main beach, in Thunder Bay, Ontario, Canada. Three sterile 1 L Nalgene bottles were used at the site to collect water approximately one foot under the surface. The Nalgene bottle was opened immediately prior to its immersion in the water. The bottle was thrust downward into the water upside down (mouth down) and inverted once fully submerged. After being allowed to fill completely, the bottle was capped, while still submerged, and transported to the Lakehead University Water Testing Facility for analysis of water chemistry. The lake water sample was tested for planktonic bacterial cell density. Three 1 mL aliquots were taken and serially diluted and 100 µL portions were spread-plated on to R2A agar plates (Becton, Dickson and Company, MD, USA). The plates were incubated at 30°C for 7 days with plate examined every 24 h. The chemical and biochemical properties of the unfiltered lake water were analyzed by the Lakehead University Centre for Analytical Service (LUCAS) and are reported as follows (in mg/liter): 9.7dissolved organic carbon, 3.51 Chloride,0.032 Nitrate, 0.038 Total Aluminum, 0.016 Total Barium, 9.654 Total Calcium, 0.002 Total Copper, 0.375 Total Iron, 0.55 Total Potassium, 2.88 Total Magnesium, 0.0188 Total Manganese, 2.81 Total

Sodium, 0.86 Total Sulfur, 0.019 Total Strontium, 0.001 Total Zinc, 2.06 Sulphate (SO_4^-), The levels of nitrite, arsenic, beryllium, cadmium, cobalt, chromium, molybdenum, nickel, phosphorus, lead, vanadium and phosphate (PO_4^-) were below the detection limits (<0.005 mg/liter, with the exception of PO_4^{3-} -P, which had a detection limit of 0.025 mg/liter) The water pH and temperature were 7.5 and 7.8°C, respectively, at the time of collection.

2.2.11. Monitoring growth of mixed culture biofilms formed by planktonic lake water bacteria

The following procedure was used to harvest lake water bacteria and transfer them to MSMG medium for biofilm growth. One hundred and sixty mL of lake water was drawn through 8 sterile 25 mm Isopore hydrophilic polycarbonate membranes with a 0.2 µm pore size (EMD Milipore water testing membrane filter, pore size 0.2 µm; Thermo Fisher Scientific, Whitby, ON, Canada) using 25 mm vacuum filter (Merck Millipore) and a sterile pneumatic pump funnel filtration apparatus (model SA55NXGTE-4870 Fisher Scientific, St. Louis MO. USA.) allowed for even vacuum and collection of bacterial cells from the lake water. The filters were individually placed in a sterile 50 mL disposable centrifuge tubes (Fisher Scientific) with 20 mL of sterile MSMG vortexed for 120 seconds three times with five sterile 6 mm glass beads (Fisher Scientific, Germany). The lake water bacterial suspension samples were transferred into a sterile 250 mL beaker where the pooled samples were mixed. The filters were removed from the centrifuge tubes and placed on to R2A plates for conformation that all the bacterial cells had been removed from the filters. The R2A plates were incubated at 30°C for 4 days and no bacterial

growth was observed on the filters. The pooled lake water bacterial suspension was further examined to determine the planktonic cell counts; three 100 µL samples were spread plated onto R2A plates and incubated for 4 days to determine the density.

The pooled lake water bacterial suspension sample was transferred into the 6-well cell culture plates with sterile coverslips as biofilm substrata. A time course experiment to determine the growth dynamics of the lake water bacterial biofilm on the coverslip substratum was monitored over 6 days. The spent MSMG medium was replaced with fresh sterile MSMG every 24 h as described previously. The harvest of the biofilms from the coverslips was performed every 24 h. The biofilm samples were serially diluted and 100 µL spread plated on to R2A Agar. The plates were incubated at 30°C for 48 h and the CFUs were counted to obtain cell density.

2.2.12. Pre-established lake water biofilms with H32-*gfp*

Lake water bacterial samples were prepared as described earlier and the lake water biofilm was grown for 48 h. Fresh H32-*gfp* culture was streaked onto TSA kanamycin plates and incubated at 37°C overnight. An isolated colony of H32-*gfp* was inoculated into 50 mL of TSB kanamycin at 37°C and 200 RPMs. The H32-*gfp* culture was washed and prepared in the same manner as described before, with inoculum densities adjusted to $1x10^7$, $1x10^6$, $1x10^5$, $1x10^4$, $1x10^3$, $1x10^2$, $1x10^1$ and $1x10^0$ CFU/mL. The H32-*gfp* inoculums were added to the pre-established lake water biofilm, 5 mL of each H32-*gfp* sample at various inoculum densities was added into each well. The cell culture plates were incubated at 22°C with orbital shaking at 25 RPMs. A control sample of the 48 and 96 h lake water biofilm was harvested to establish the cell density for the

lake water biofilm; harvesting and serial dilutions were performed in the same procedure as mentioned.

Monocultures of each inoculum density of H32-*gfp* were made for comparison of the treatments allowing to observer changes of how H32-*gfp* biofilm formation will respond to the lake water biofilm. The H32-*gfp E. coli* were grown for 48 h, with a media change after 24 h from the initial inoculation. At the 48 h mark, the biofilms were harvested. The harvesting follows the same protocol as mentioned.

2.2.13. Co-culture lake water with H32-*gfp*

Fresh H32-*gfp* was streaked onto TSA kanamycin plates and incubated at 37°C overnight. An isolated colony of H32-*gfp* was inoculated into 50 mL of TSB kanamycin at 37°C and 200 RPMs. H32-*gfp* cultures was washed and prepared in the same manner as described before.

Utilizing a sterile pneumatic pump funnel filtration apparatus, 160 mL of lake water was filtered through a sterile 25 mm Isopore hydrophilic polycarbonate membrane (EMD Milipore, Thermo Fisher Scientific, Whitby, ON, Canada). The bacteria on the filter were transferred to 160 mL of sterile MSMG medium as described earlier.

To perform the co-culture treatments of exposing the cover slip substratum to a mixture of the lake water bacteria and H32-*gfp E. coli* simultaneously, 2.5 mL of the lake water bacterial suspension was added to each well. In addition, 2.5 mL-portion of H32-*gfp* samples were mixed into the lake water bacterial suspension in the wells to achieve final inoculant concentrations of $1x10^7$, $1x10^6$, $1x10^5$, $1x10^4$, $1x10^3$, $1x10^2$, $1x10^1$ and $1x10^0$

CFU/mL. Monocultures of H32-*gfp* with decreasing inoculum densities were set up as described earlier as experimental controls for the experiment.

The 6-well cell culture plates were incubated at 22°C with agitation at 25 RPMs. The MSMG growth medium was changed every 24 h as described before. The co-culture lake water bacteria+H32-*gfp* biofilm was harvested at 48 h. The harvesting follows the same procedure as discussed previously. The H32-*gfp* biofilm cell density was determined by drop-plating on TSA kanamycin plates and incubated at 37°C for 24 h. Lake water bacterial density in the biofilm was determined by spread-plating onto R2A plates and incubated at 30°C for 72 h. Colony forming units were recorded and used to determine the biofilm cell densities.

2.2.14. Statistical analysis

All experiments were performed in triplicate with three independent biological replicates. The normalized data were analyzed and graphed in Microsoft Excel 2010 (Redmond, Washington, Computer Software) using one-way analyses of variance. In addition, normalized data were analyzed in SigmaStat (SPSS Inc., Chicago, IL.) using one-way analyses of variance (ANOVAs).

2.3. Results

2.3.1. PCR detection of pathogenic genes

To determine the pathogenicity of the periphytic strains (1A and1A-Rif) and the O157:H7 strains (H32 and H32-*gfp*), they were tested for the presence of ten different genetic markers which represent five pathogenic types of *E. coli* (i.e. EHEC, EPEC, ETEC, EIEC and UPEC). Several *E. coli* strains were used as positive and negative controls for the PCR tests (Table 1). 1A and 1A-Rif were negative for all markers. Therefore, they are likely to be non-pathogenic. H32 and H32-*gfp* tested positive for *stx1* and *stx2* genes and negative for the rest of the pathogenic genes (Table 1).

Table 1 Characterization of pathogenic genes of periphytic *E. coli* 1A wild type and mutant 1A-Rif, *E. coli* H32 wild type and mutant H32-*gfp*, (-) sign is absent genes, (+) is present genes.

	stx1	***stx2***	***est***	***elt***	***ial***	***eaf***	***iroN E.coli***	***hlyA***	***papA***	***papC***
1A	-	-	-	-	-	-	-	-	-	-
1A-Rif.	-	-	-	-	-	-	-	-	-	-
H32	+	+	-	-	-	-	-	-	-	-
H32-*gfp*	+	+	-	-	-	-	-	-	-	-
EHEC 920004	+	+	-	-	-	-	-	-	-	-
UPEC 25922	-	-	-	-	-	-	+	+	+	+
EIEC 0164 & 0136	-	-	-	-	+	-	-	-	-	-
EPEC 2348 & 055	-	-	-	-	-	+	-	-	-	-
ETEC 505	-	-	+	-	-	-	-	-	-	-
ETEC 07	-	-	-	+	-	-	-	-	-	-
ETEC K326	-	-	+	+	-	-	-	-	-	-
EC JM 109	-	-	-	-	-	-	-	-	-	-

[1]

[1] Only four diarrheagenic virotypes will be examined: enterohemorrhagic (EHEC- *stx1*, *stx2*), enteropathogenic (EPEC- *eaf*), enterortoxigenic (ETEC *est*, *elt*), enteroinvasive (EIEC- *ial*), one extraintestinal *E. coli;* uropathogenic (UPEC- *iroN E. coli*, *hylA*, *papA*, *papC*).

2.3.2. Planktonic growth curves

In order to compare biofilm development between the four strains of *E. coli,* it is important to understand the growth abilities in planktonic conditions. Planktonic growth curves of the four *E. coli* strains were determined in two different growth media, the nutrient rich TSB and nutrient limited MSMG. The planktonic growth curves of all the wild type strains and mutant strains were similar in TSB. In the TSB growth medium, H32 and H32-*gfp* were able to grow faster between 60 to 120 minutes in comparison to1A and 1A-Rif. In addition, all four bacterial strains were starting to reach a plateau in 165 minutes (Panel A). The MSMG media is designed for oligotrophic bacteria and to imitate the low carbon nutrient conditions of the lake water. The four strains grew slowly in the MSMG medium, reaching a plateau in 300 minutes (Panel B). The OD of the first *E. coli* cultures data point was measured at time zero for the MSMG experiments. During the first 120 minutes the OD was measured every 60 minutes due to the insignificant changes observed over shorter increments of time. This phenomenon is likely a result of the low carbon nutrient content of the media. After 120 minutes, two additional data points were recorded with 90 minutes apart. The final data points were recorded after 1260, 1320, and 1680 minutes in the following day. The experiment was performed in triplicate with three independent replicates.

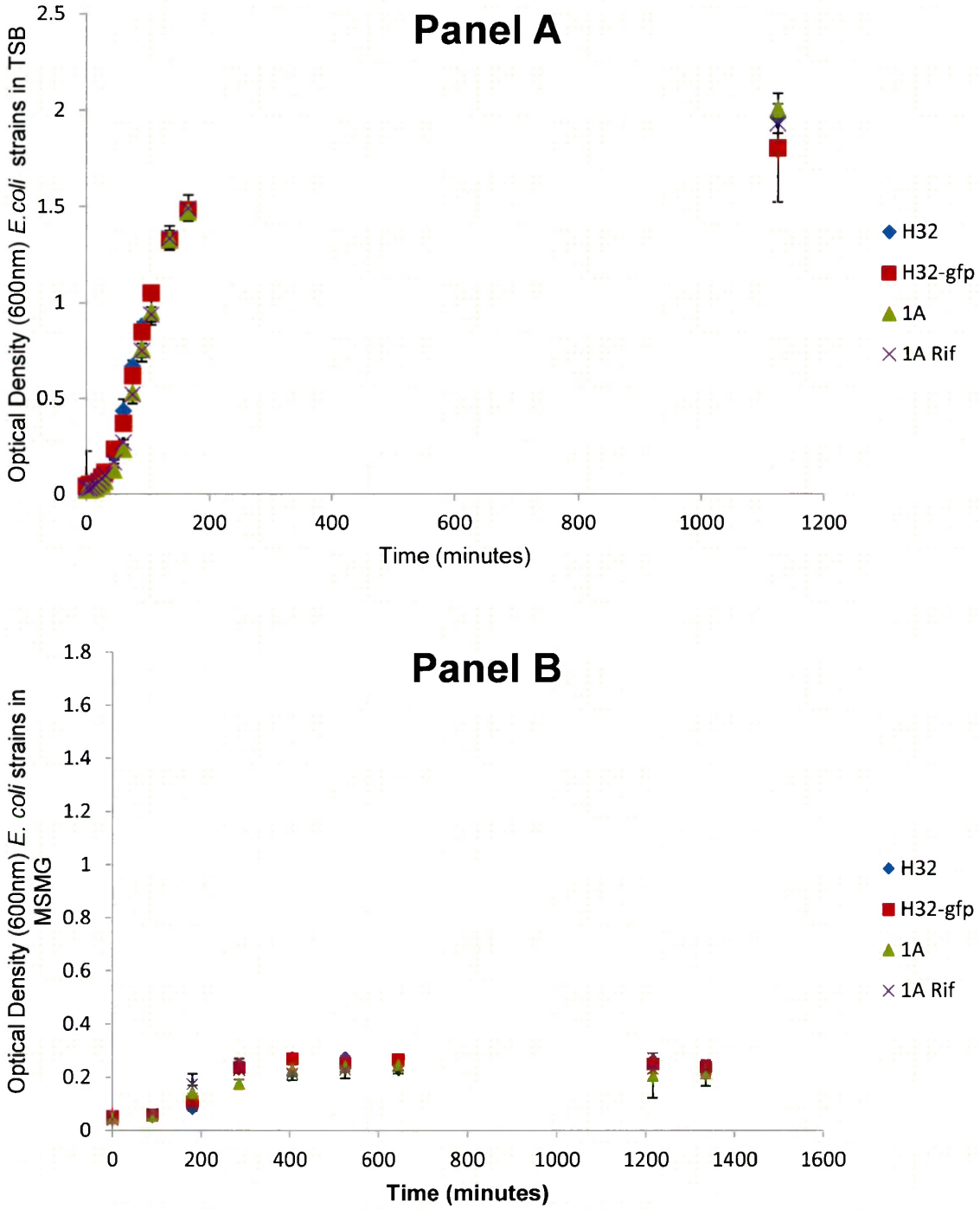

Figure 1 Planktonic growth curves

Panel A, Planktonic growth curves of *E. coli* H32 (◆), H32-*gfp* (■), and *E. coli* 1A (▲), 1A-Rif (X) in nutrient rich media (TSB). Panel B, Planktonic growth curves *E. coli* H32 (◆), H32-*gfp* (■), and *E. coli* 1A (▲), 1A-Rif (X) in MSMG.

2.3.3. Biofilm formation characterization and analysis

The periphytic *E. coli* 1A strain formed notably more biofilm than the shiga toxin producing *E. coli* O157:H7 H32 strain (Figure 2, Panel A). Figure 2 panel A reveals that 1A forms uniform coverage over the cover slip. The cross section displays mushroom- and pillar-like appendages and water channels (Figure 3 and Figure 4). The thickness of the 1A biofilm varies depending on the location sampled but can be as much as 40 µm (Figure 2, Panel A). Panel B of Figure 2 displays the H32 biofilm which is notably less able to cover the surface of the coverslip. The cross-section of the biofilm displays how sparse the cells are in comparison to the biofilm of the periphytic *E. coli* 1A. The *E. coli* 1A strain had definitive structures within its biofilm, such as pillars, mushroom-like appendages and water channels (Figure 3 and Figure 4). The biofilm of the pathogenic *E. coli* H32 strain was devoid of all observable structures and water channels. It only formed a monolayer on the coverslip. These images of the 1A strain and the H32 strain confirm the differences in the ability of the two strains to form biofilm.

Panel A **Panel B**

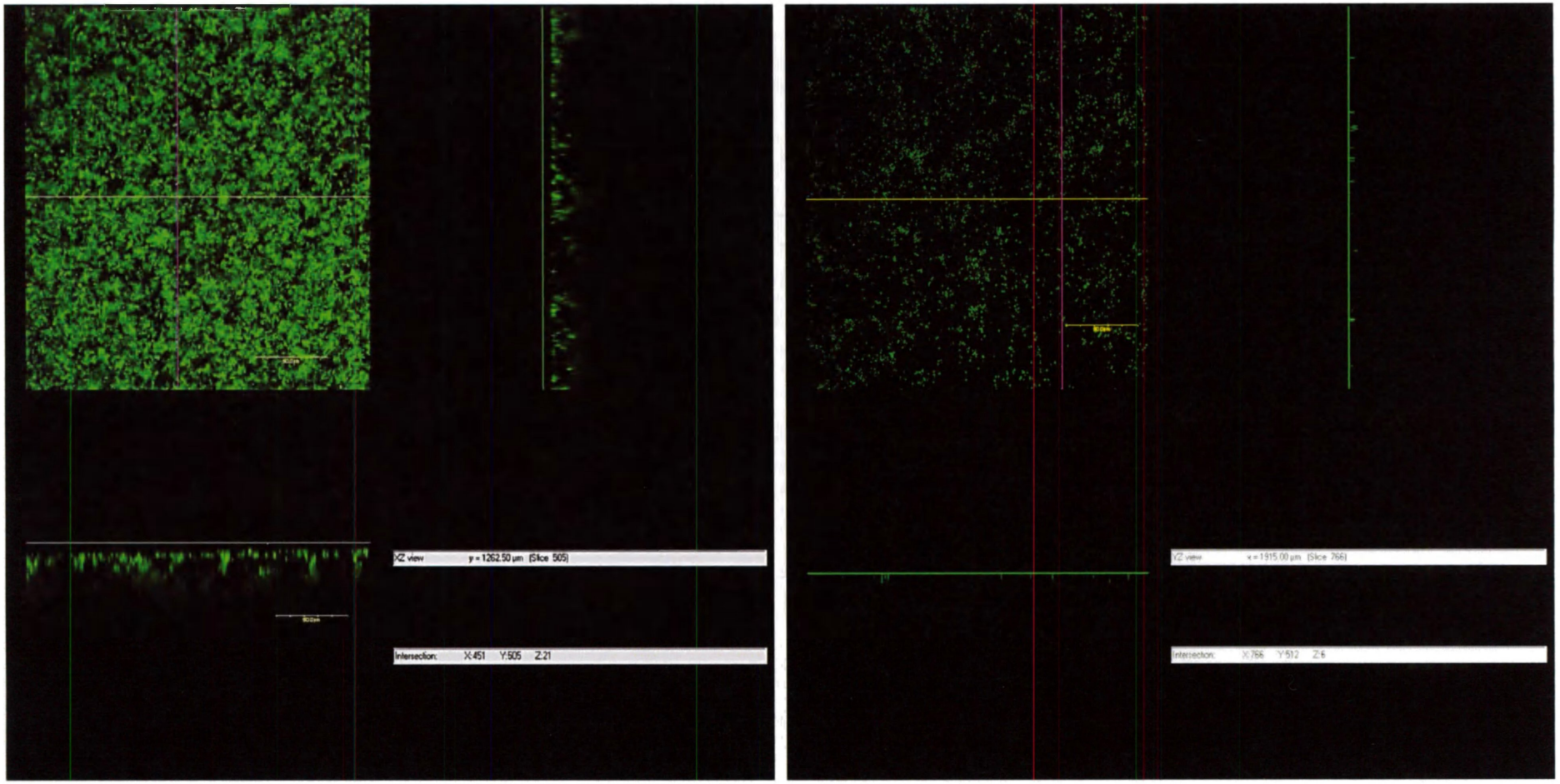

Figure 2 Biofilm formation of the wild type *E. coli strains*

The wild type strains of the periphytic 1A and pathogenic H32 *E. coli* biofilms were grown for 48 h and stained with SYTO 9. Panel A displays the periphytic 1A *E. coli* strain with the total biofilm and with a cross section of the biofilm. Panel B displays the pathogenic H32 *E. coli* strain with the total biofilm and with a cross section of the biofilm.

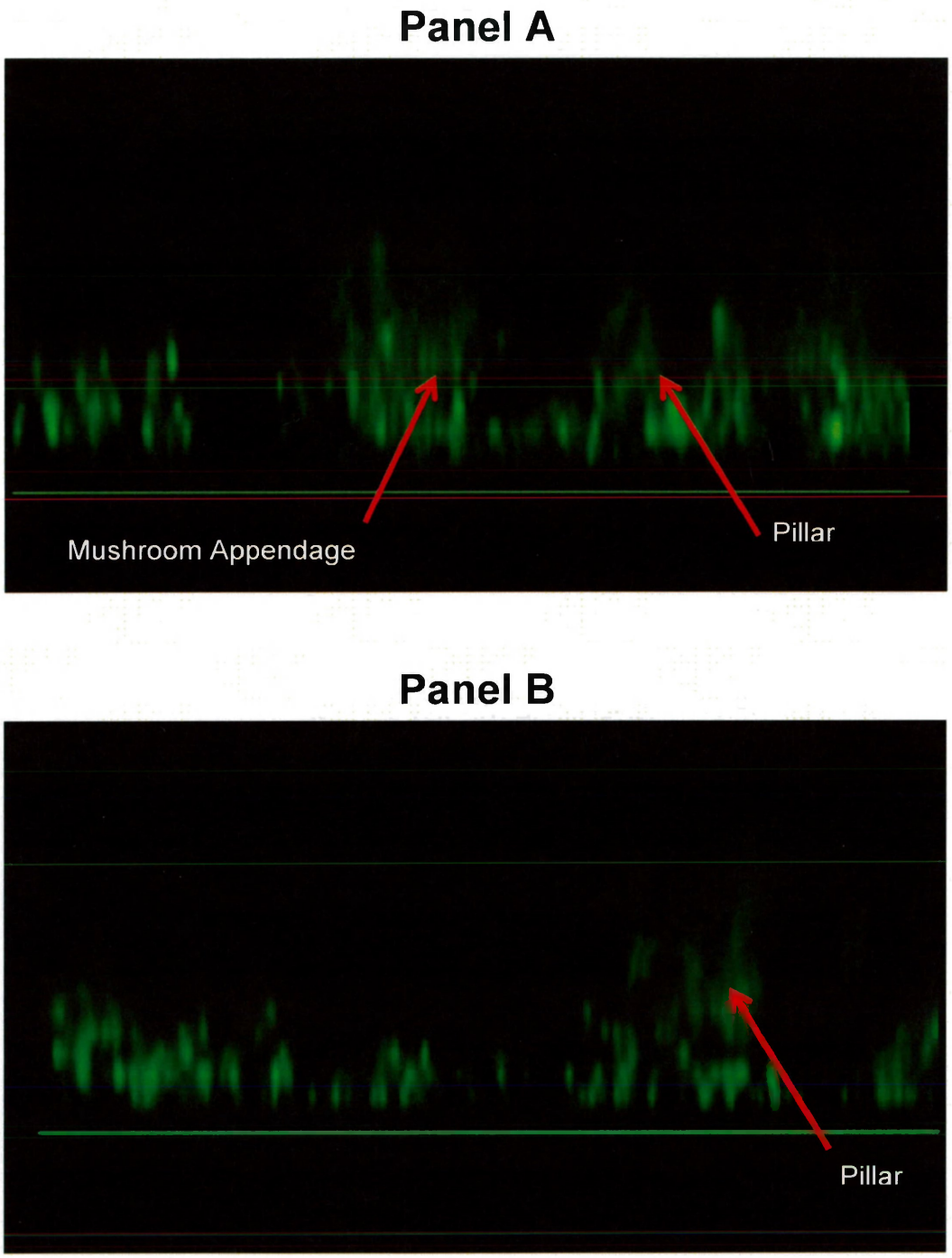

Figure 3 Cross-section of the periphytic 1A *E. coli* 48 h biofilm stained with SYTO 9.

Panel A displays a mushroom like appendage and pillar. Panel B displays a pillar like appendage.

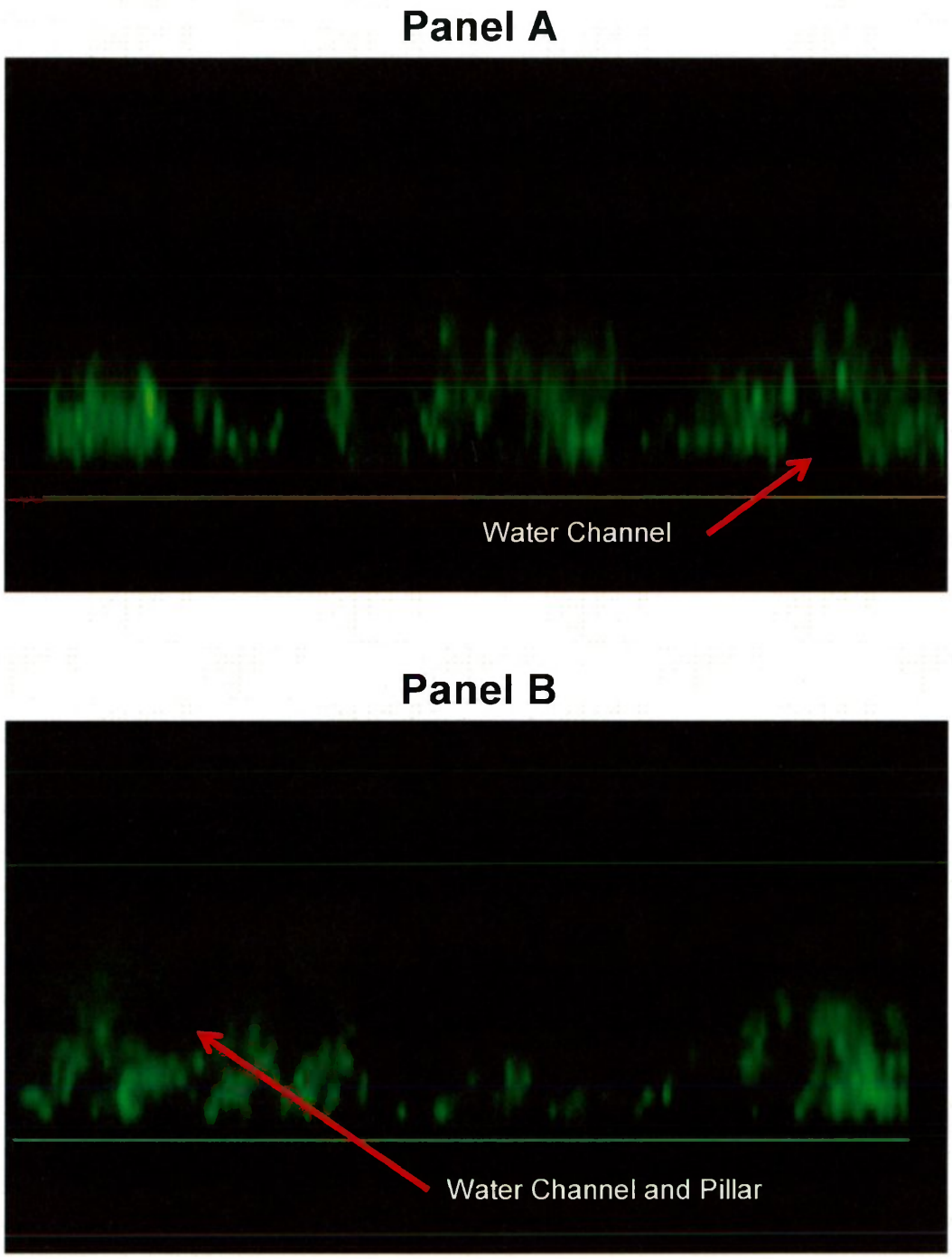

Figure 4 Cross-section of the periphytic 1A *E. coli* 48 h biofilm stained with SYTO 9.

Panel C and D display water channels, the black void in the biofilm of 1A. The cells are stained green, black arears contain no cells

2.3.3.1. Biofilm of wild type and mutant *E. coli* strains

To confirm that the biofilm capabilities of the mutant strains were similar to that of their parent wild type strains, 1A to 1A-Rif and H32 to H32-*gfp,* the biofilms of the wild type strains were compared to their respective antibiotic resistant mutant strains (Figure 5 and Figure 6).

Both 1A and 1A-Rif formed extensive biofilm coverage on the coverslip substratum (Figure 5). The top view showed that both strains were able to monopolize the coverslip surface area. Within 48 h of growth, the bottom panels display that the thickness of the biofilm was ranged from 30-40 µm. The images of the 1A and 1A-Rif biofilm confirm that there are no observable changes on the ability to form biofilm between the wild type strain and the mutant strain.

Figure 6 displays H32 and H32-*gfp* biofilms with an initial inoculum density of $1x10^7$ CFU/mL. Both strains display similar abilities to form biofilm with sparse coverage of the coverslip. Panel A displays H32 and there is scarce coverage on the cover slip and the side view shows that the H32 forms a monolayer. Panel B displays H32-*gfp*. This strain develops a similar biofilm as its wild type parent with sparse coverage on the cover slip. There is a monolayer formed over the surface of the coverslip. After 48 h of growth both strains have comparable biofilm coverage and thickness on the coverslip.

Both strains of *E. coli*, the wild type and mutant of the periphytic *E. coli* 1A, display the same biofilm-forming capabilities and able to form copious amounts of biofilm, as observed in Figure 5. For the pathogenic strain H32, both the wild type and the

H32-*gfp* mutant formed small amount of biofilms and the insertion of the *gfp* -kanamycin resistant gene cassette did not change the biofilm forming characteristic of the H32 strain, demonstrated in Figure 6. Therefore, the mutant strains of *E. coli* were used in the mixed culture study and the H32-*gfp* was used to examine the interactions between the pathogenic *E. coli* O157:H7 strain and the lake water bacteria.

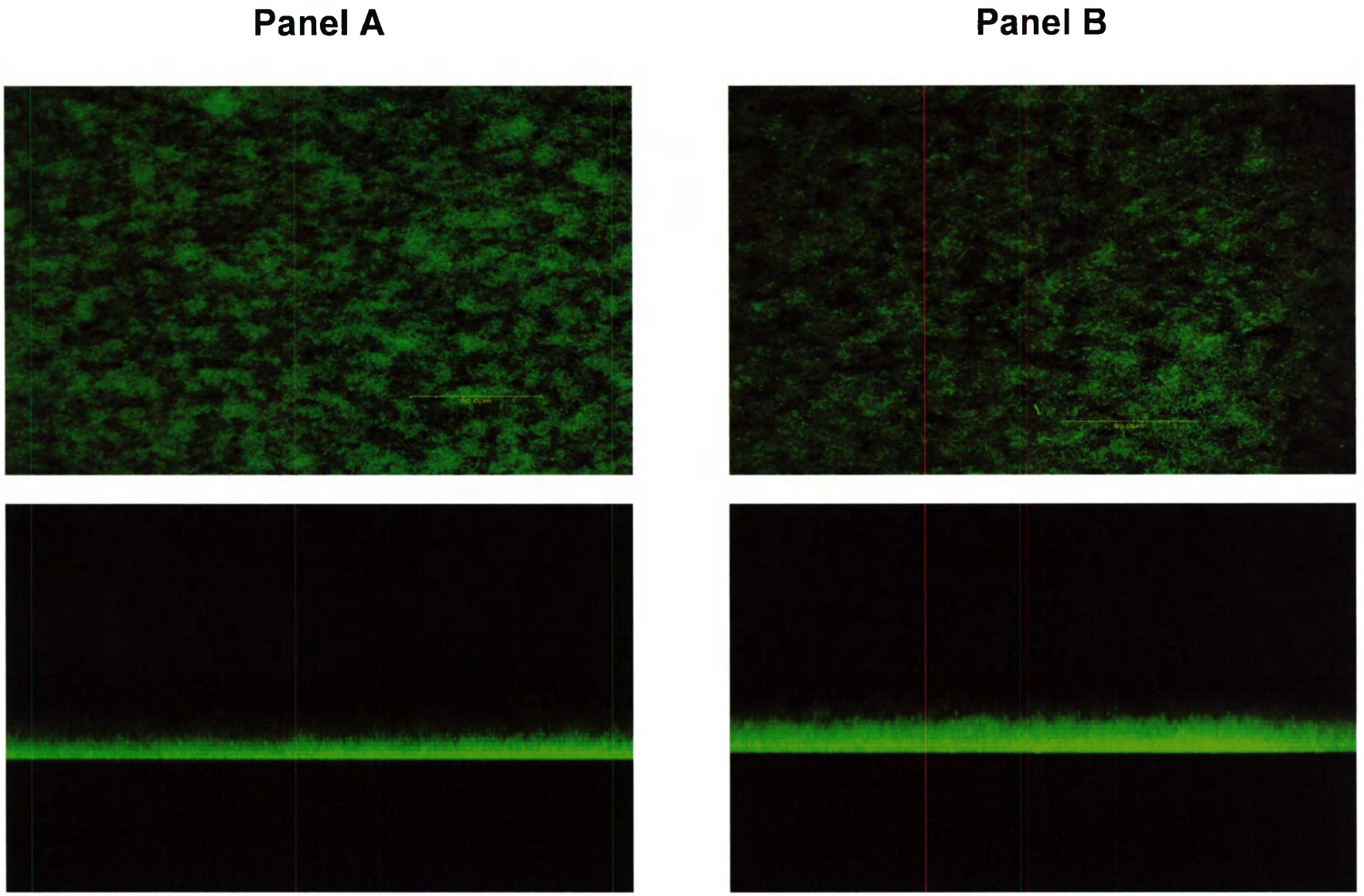

Figure 5 1A and 1A-Rif biofilms grown in MSMG.

Panel A, displays the periphytic *E. coli* 1A stained with SYTO 9 top image is the total biofilm, and the bottom image is an edge view of the biofilm. Panel B, depicts 1A-Rif, the top panel displaying the total coverage of the biofilm, the bottom image shows side view, elucidating the thickness of the biofilm.

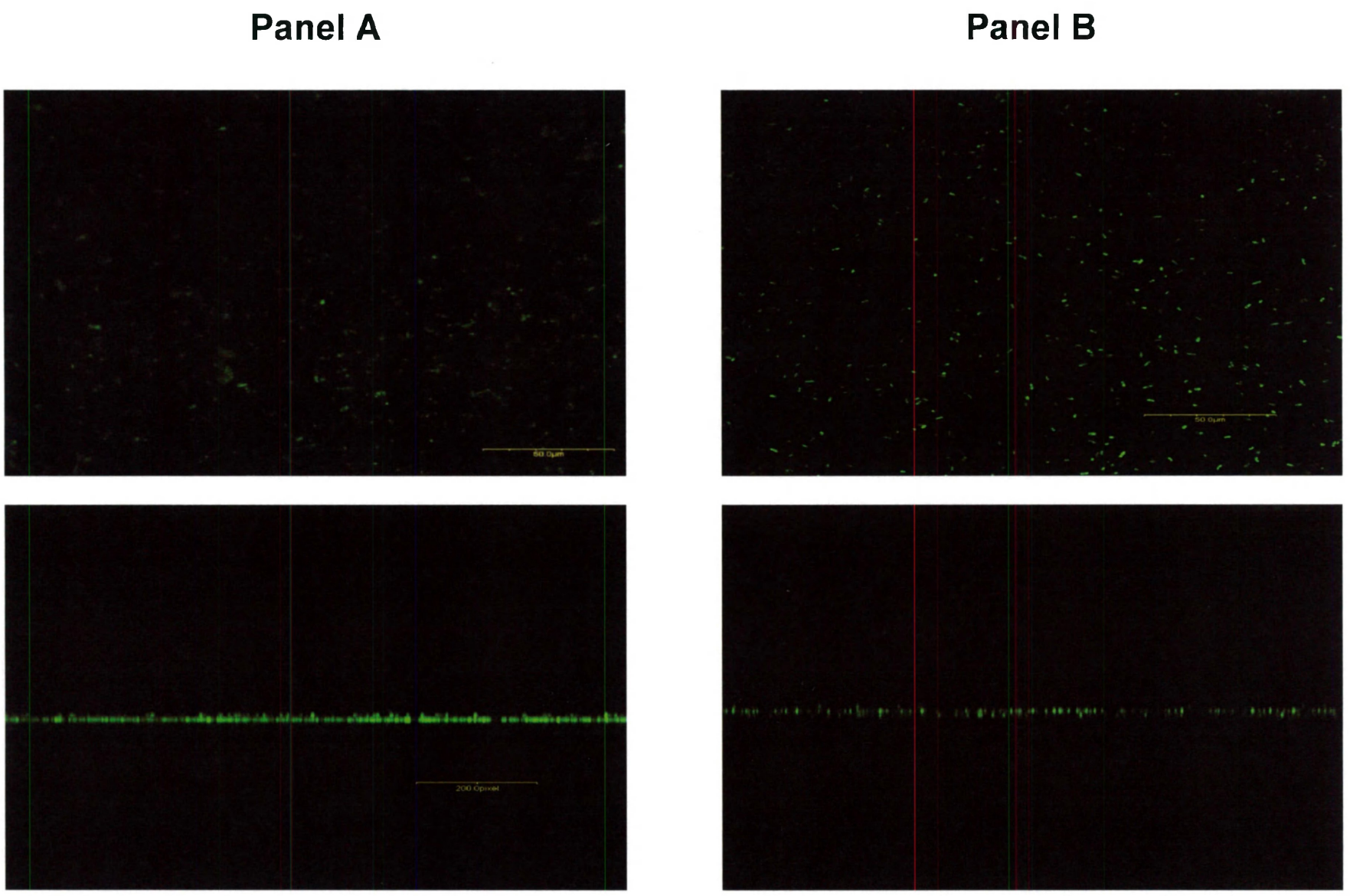

Figure 6 H32 and H32-*gfp* biofilms grown in MSMG.

Panel A displays the pathogenic *E. coli* O157:H7, H32 stained with SYTO 9 the top image is of the total I biofilm, and the bottom panel is of the side view biofilm.. Panel B, depicts H32-*gfp*, displaying the total coverage of the biofilm, the bottom image shows side view, exposing the thickness of the biofilm

2.3.4. Confocal images of unstained and stained H32-*gfp* treatments

Figure 7 shows the effect of 1A-Rif (with both pre-established treatment and co-culture treatment at an inoculant 1A-Rif density of $1x10^7$ CFU/mL) on the biofilm establishment of the H32-*gfp* (inoculated at $1x10^7$ CFU/mL) using fluorescent microscopy. The top panels of A, B and C represent images of H32-*gfp* without SYTO 9 staining. The top panels of B and C were mixed culture treatments with 1A-Rif in pre-established and co-culture treatments respectively. Both the top panels show notably more H32-*gfp* biofilm than the monoculture H32-*gfp* biofilm (top panel of A). The lower three panels represent samples stained with SYTO 9. The upper and lower panels of Figure 7, Panel A are very similar showing that the *gfp*-labelled H32-*gfp* cells emitted sufficient fluorescence to be detected without the help of the SYTO 9 stain. The lower panels of Figure 7, Panel B and Figure 7, Panel C show substantially more biofilms than their respective top panels because both 1A-Rif and H32-*gfp* biofilm cells are revealed in the two lower panels. However, only H32-*gfp* biofilm cells are visible in the top panels.

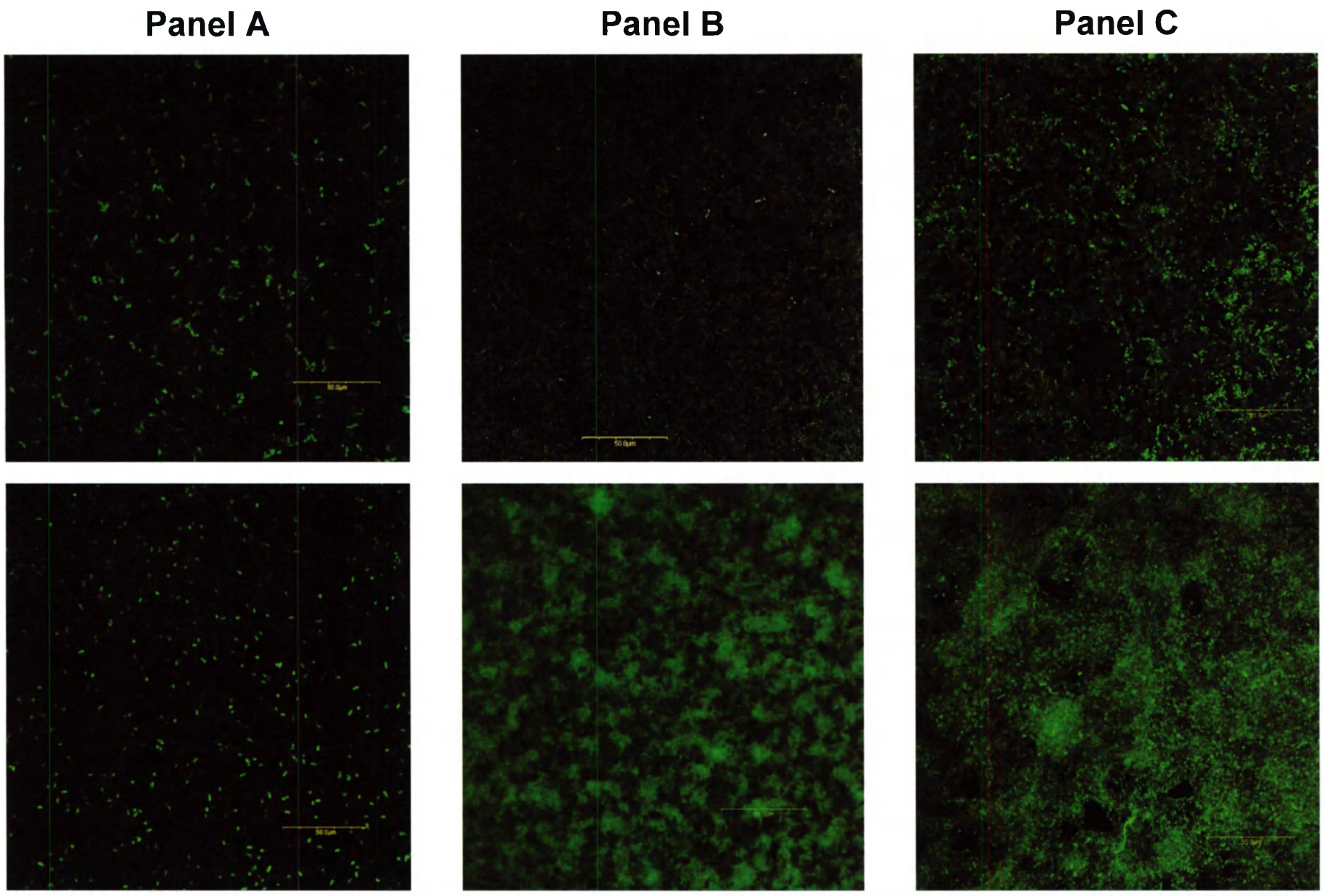

Figure 7 H32-*gfp* biofilms top panels unstained and bottom panels are stained with SYTO 9 all inoculums were a 1:1 ratio of 1 x10^7 CFU/mL.

Panel A monoculture of H32-*gfp* top panel is unstained and the bottom panel is stained bottom, Panel B displays, a pre-established 1A-Rif biofilm with the inoculation of H32-*gfp* the top panel is unstained and bottom is stained, Panel C, Co-culture of 1A-Rif and H32-*gfp* where the top panel is unstained and the bottom panel is stained. All inoculum densities are kept at 1 x10^7 CFU/mL. All bottom panels stained with SYTO9 dye.

2.3.5. Biofilm growth curves of 1A-Rif and H32-*gfp*

The growth curves of biofilm provide vital information about how quickly the bacteria colonize a given area and reach a saturation point. The experiment (Figure 8, Panel A) shows how 1A-Rif behaves. Inoculum density and maximum saturation of the biofilm are related, when inoculated with a higher inoculum this results with more biofilm being formed as compared to a lower inoculum which forms less biofilm. For each inoculum concentration, the biofilm reaches its maximum saturation within 24 h and the biofilms remain at that level for the 4 days of the experiment.

Therefore it was observed that at the higher inoculum density of $1x10^7$ CFU/mL, it reaches saturation quickly. As the inoculum density decreased tenfold each time, there was an inability of 1A-Rif to reach its maximum biofilm density, revealing that the amount of biofilm formed by 1A-Rif was dependent on the initial inoculum concentration of 1A-Rif. 1A-Rif was not very efficient in initiating the colonization of the substratum. The amount of biofilm formed by $1x10^7$, $1x10^6$, $1x10^5$, $1x10^4$ CFU/mL of inoculum were 8.16, 7.62, 6.44 and 5.54 Log CFU/cm^2, respectively after 24 h.

However, the growth dynamics of the H32-*gfp* biofilm was very different from that of the 1A-Rif biofilm (Figure 8, Panel B). The biofilm forming capacity of this *E. coli* strain was not dependent on its initial inoculum density. Regardless of the inoculant density ($1x10^4$ to $1x10^7$ CFU/mL) of H32-*gfp*, the same biofilm density was reached within 24 h. The tenfold dilution of H32-*gfp* did not have an effect on the ability to form biofilm. The amount of biofilm formed from $1x10^7$, $1x10^6$, $1x10^5$, $1x10^4$ CFU/mL of inoculum was 6.90, 6.67, 6.64 and 6.53 Log CFU/cm^2, respectively after 24 h.

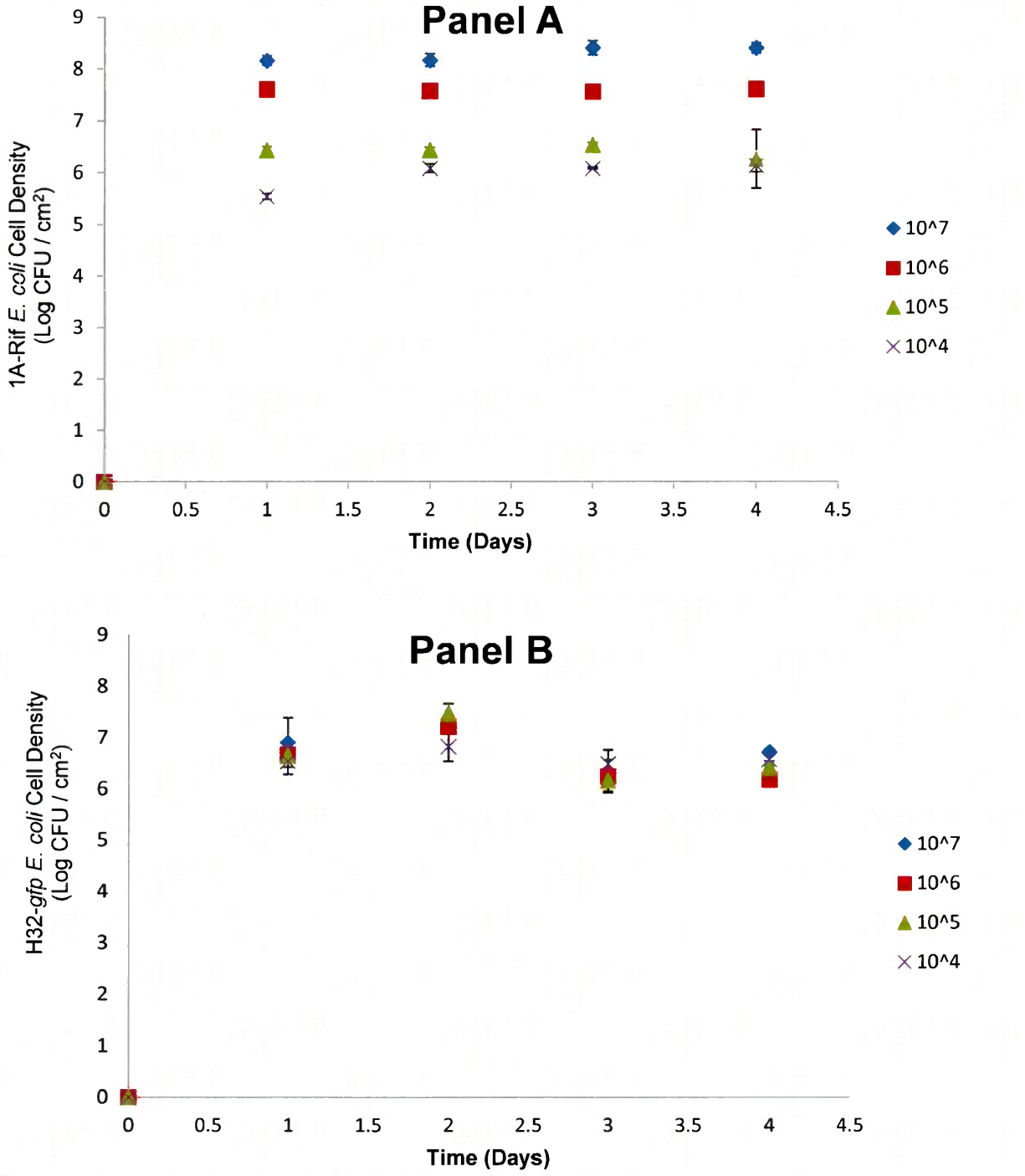

Figure 8 1A-Rif and H32-*gfp* biofilm time curves.

Panel A, displays the growth curves of *E. coli* 1A-Rif with decreasing inoculate densities, Panel B, displays the growth curves of H32-*gfp* with decreasing inoculate densities. The *E. coli* strains were grown over a 4 day period, with orbital shaking at 25 RPMs and incubation temperature of 22°C. The experiment used the inoculum densities of 10^7, 10^6, 10^5 and 10^4 CFU/mL to initate biofilm formation.

2.3.6. Biofilm quantification

2.3.6.1. Effect of 1A-Rif on the establishment of H32-*gfp* biofilm

To quantify the effect of 1A-Rif on the biofilm forming capability of H32-*gfp*, the mutant strains of *E. coli* 1A-Rif and H32-*gfp* were studied in three conditions. As mentioned before in the methods section, conditions include monocultures, co-culture and pre-established treatments. The purpose of each treatment is to develop an understanding of how H32-*gfp* will grow and form biofilm under the prescribed conditions.

The first treatment (i.e. Figure 9, Panel A, 1st column) was to study how H32-*gfp* - grew as a monoculture, with decreasing inoculum densities. The inoculum densities were $1x10^7$, $1x10^6$, $1x10^5$ and $1x10^4$ CFU/mL, cultured in the same fashion as stated before. After 48 h of growth, the biofilm cell densities of the H32-*gfp* formed by inoculum densities of $1x10^7$, $1x10^6$, $1x10^5$ and $1x10^4$ CFU/mL were 6.47, 6.64, 6.46 and 6.25 Log CFU/cm^2, respectably (Figure 9 Panel A).

The second treatment (i.e. Figure 9, Panel A, 2nd column) was conducted with a pre-established 1A-Rif biofilm starting with an initial inoculum density of $1x10^7$CFU/mL. This treatment is analogous to an environmental setting where there is an established biofilm community growing and thriving. Then a source of contamination is introduced into the environment and allows for the *E. coli* pathogen to take hold in a pre-established biofilm.

The second column represents the attachment of the H32-*gfp* on the substratum that was pre-established by the 1A-Rif biofilm (Figure 9, Panel A). In comparison to the biofilm cell density of the 48 h monoculture H32-*gfp* biofilm, the 48 h H32-*gfp* that was

inoculated onto the pre-established 1A-Rif biofilm illustrates novel findings in regards to inoculum density. The first set of columns represents inoculum density of $1x10^7$ CFU/mL. At this inoculant density, the pre-established 1A-Rif biofilm increased the biofilm establishment of H32-*gfp* significantly ($p<0.05$, p value was 0.007) from 6.47 Log CFU/cm^2 (in monoculture biofilm condition) to 7.24 Log CFU/cm^2 (in pre-established 1A-Rif condition). When the inoculum density of H32-*gfp* was $1x10^6$ CFU/mL, the biofilm densities of H32-*gfp* were 6.64 Log CFU/cm^2 and 6.83 Log CFU/cm^2 under mono-culture and pre-established 1A-Rif conditions, respectively. Under this inoculant density, the pre-established 1A-Rif biofilm did not affect the establishment of the H32-*gfp* significantly ($p>0.05$ and p value was 0.223). At the inoculum density of $1x10^5$ CFU/mL; the H32-*gfp* monoculture biofilm density was 6.64 Log CFU/cm^2. In the presence of the pre-established 1A-Rif biofilm, it reduced the H32-*gfp* biofilm density significantly to 5.74 Log CFU/cm^2. When the inoculant density of H32-*gfp* was lowered to $1x10^4$ CFU/mL, the pre-established 1A-Rif suppressed the attachment and growth of the pathogenic *E. coli* even more to 4.94 Log CFU/cm^2. Pre-established 1A-Rif biofilm hindered the development of the H32-*gfp* biofilm when the inoculum densities of 1A-Rif if were $1x10^5$ CFU/mL or lower.

The third treatment in this experiment is co-inoculation, where both *E. coli* species were introduced into the 6 well culture plates simultaneously as described in the methods section. 1A-Rif was kept at an inoculum density of $1x10^7$CFU/mL, and H32-*gfp* was inoculated at the same time with decreasing concentrations from $1x10^7$ down to $1x10^4$ CFU/mL. This type of treatment exhibits how the two bacterial strains competed with each other for the establishment of biofilm on a new substratum.

The co-inoculation value of H32-*gfp* when compared to the monoculture of H32-*gfp* at $1x10^7$CFU/mL resulted with the biofilm cell density values at 6.87 and 6.47 Log CFU/cm^2 respectively. These values confirm that the H32-*gfp,* when co-inoculated, grew better biofilm than its monoculture without 1A-Rif at 90 % of confidence level (p=0.075). When H32-*gfp* was inoculated at $1x10^6$ CFU/mL, its biofilm cell density was 6.70 Log CFU/cm^2 in the co-culture treatment compared to the 6.64 Log CFU/cm^2 in the monoculture condition. In this instance, the biofilm cell density of H32-*gfp* was not significantly affected by 1A-Rif co-inoculant (p=0.738). Interestingly, further reduction of the inoculum ratio between the 1A-Rif and H32-*gfp* to 1:0.01 and 1:0.001 did not reduce the biofilm cell density of the H32-*gfp* significantly (p>0.05) on the substratum (Figure 9, Panel A).

Figure 9 Panel B, shows the biofilm cell densities of 1A-Rif, in this set of experiments. The data displays how 1A-Rif behaves with the presence of H32-*gfp.* 1A-Rif was inoculated at $1x10^7$ CFU/ mL in all trials, the graph displays the monoculture, pre-established and co-cultures. The growth of a 4 day monoculture of 1A-Rif biofilm compared to the 4 day pre-established 1A-Rif biofilm with H32-*gfp.* The second treatment is comparing a 2 day monoculture of 1A-Rif to the co-inoculation of 1A-Rif with H32-*gfp.*

The day 4 monoculture of 1A-Rif was 8.30 Log CFU/cm^2. The 1A-Rif biofilm cell density after exposed to $1x10^7$ H32-*gfp* was 8.13 Log CFU/cm^2 and it was not significantly different from the monoculture 1A-Rif biofilm (p=0.342). The biofilm cell densities of 1A-Rif were 8.31, 8.34, 8.34 Log CFU/cm^2 when exposed to H32-*gfp* at $1x10^6$, $1x10^5$ and $1x10^4$ CFU/mL, respectably, and they were not significantly different from the mono-culture 1A-

Rif biofilm cell density (Figure 9 Panel). These values show that the 1A-Rif pre-established 1A-Rif biofilm was not affected by the presence of H32-*gfp* strain.

In the co-culture treatment, the 48 h 1A-Rif monoculture had a cell density of 8.11 Log CFU/cm^2. The 1A-Rif biofilm cell densities when co-inoculated with 1x10^7, 1x10^6, 1x10^5 and 1x10^4 CFU/mL of H32-*gfp* were 6.51, 6.58, 6.50 and 6.55 Log CFU/cm^2, respectively (Figure 9, Panel B). This is a significant decrease in 1A-Rif's ability to form biofilm when inoculated as a co-culture with H32-*gfp* ($p < 0.05$).

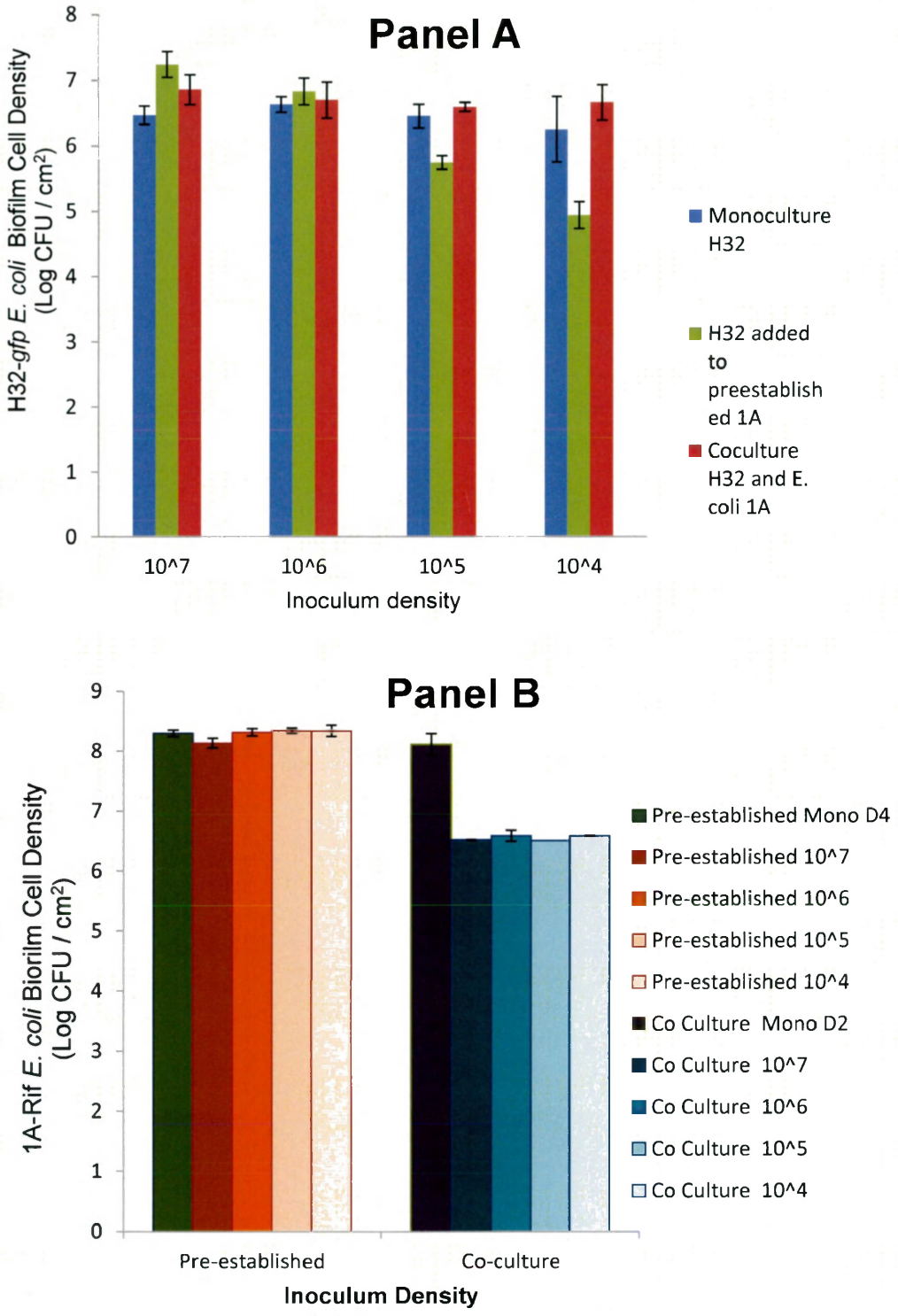

Figure 9 Biofilm cell density analysis of H32-*gfp* how 1A-Rif effects H32-*gfp* biofilm establishment

1A-Rif held at 1x10^7 CFU/mL in all trials, Panel A, displays the biofilm cell density of H32-*gfp*, as a monoculture, inoculated at 10^7, 10^6, 10^5 and 10^4 CFU/mL. The pre-established biofilm of 1A-Rif with H32-*gfp* inoculated at 10^7, 10^6, 10^5 and 10^4 CFU/mL. Co-culture biofilms were inoculated with 1A-Rif and H32-*gfp* 10^7, 10^6, 10^5 and 10^4 CFU/mL. Panel B displays the growth of 1A-Rif *E. coli* grown as 4 day monoculture with the 4 day pre-established biofilms inoculated with H32-*gfp with* 10^7, 10^6, 10^5 and 10^4 CFU/mL. Co-culture displays a 2 day monoculture of 1A-Rif with H32-*gfp* inoculated *at* 10^7, 10^6, 10^5 and 10^4 CFU/mL.

2.3.6.2. Effect of H32-*gfp* on the establishment of 1A-Rif biofilm

The previous experiments examined when 1A-Rif was inoculated at a constant inoculant density of $1x10^7$ CFU/mL as the inoculum densities of H32-*gfp* were varied in the experiments. In the following study, the H32-*gfp* inoculum was kept at a constant density of $1x10^7$ CFU/mL and the inoculant density of the 1A-Rif varied from $1x10^7$ to $1x10^4$ CFU/mL in the monoculture, pre-establishment and co-culture treatments.

The first treatment was to study how 1A-Rif grew as a monoculture, with decreasing inoculum densities. The inoculum densities of 1A-Rif were $1x10^7$, $1x10^6$, $1x10^5$ and $1x10^4$ CFU/mL, cultured in the same fashion as stated before. After 48 h of growth, the biofilms were harvested and a tenfold serial dilution was made on the samples and plated on to TSA rifampicin plates. The biofilm cell densities of 1A-Rif at inoculum densities of $1x10^7$, $1x10^6$, $1x10^5$ and $1x10^4$ CFU/mL were 8.27, 8.19, 7.06 and 6.43 Log CFU/cm^2, respectively (Figure 10, Panel A).

The second column represents the biofilm establishment of 1A-Rif in the presence of a pre-established H32-*gfp* biofilm on the substratum (Figure 10, Panel A). H32-*gfp* was grown for 48 h, and then 1A-Rif was inoculated onto the H32-*gfp* biofilms. For the pre-established H32-*gfp* treatment, the biofilm cell densities of the 1A-Rif were 7.41, 6.15, 5.43 and 4.62 Log CFU/cm^2 with decreasing inoculum densities of $1x10^7$, $1x10^6$, $1x10^5$ and $1x10^4$ CFU/mL, respectively.

The third treatment in this experiment was co-inoculation, where both *E. coli* species were introduced into the 6-well culture plates simultaneously. H32-*gfp* was kept at an inoculum density of $1x10^7$CFU/mL, and 1A-Rif was inoculated at the same time

with decreasing concentrations from $1x10^7$ down to $1x10^4$ CFU/mL. The biofilm cell density values of the monoculture 1A-Rif were 8.27, 8.19, 7.06 and 6.43 Log CFU/cm^2 when the inoculant densities were $1x10^7$, $1x10^6$, and $1x10^5$ and $1x10^4$CFU/mL, respectably. The co-inoculated values from $1x10^7$ to $1x10^4$CFU/mL were 6.66, 5.67, 4.51 and 3.32 Log CFU/cm^2. There was an overall 1.5 to 3.5 log decrease when comparing the monocultures to their respected co-culture counterpart. In this set of treatments, the H32-*gfp* strain significantly hindered the biofilm formation of 1A-Rif in both the pre-establishment and co-culture treatments ($p<0.05$) (Figure 10, Panel A).

Panel B of Figure 10 shows how the H32-*gfp* biofilm grows with the addition of 1A-Rif in the treatments. The biofilm cell density value for the 4 day monoculture biofilm of H32-*gfp* was 7.62 Log CFU/cm^2. The biofilm cell densities of the H32-*gfp* with the addition of 1A-Rif were 7.84, 7.75, 7.76 and 7.86 Log CFU/cm^2 when the inoculant densities were, $1x10^7$, $1x10^6$, $1x10^5$ and $1x10^4$ CFU/mL, respectively. There was no significant changes in the amount of biofilm being formed between the monoculture and the pre-established culture of H32-*gfp* ($p=0.013$). In the second treatment with the co-culture, the cultures were monitored for 48 h. The monoculture cell density of H32-*gfp* was 6.47 Log CFU/cm^2. The co-cultured H32-*gfp* values when inoculated with 1A-Rif with decreasing inoculum densities were 6.54, 6.81, 6.52, 6.65 Log CFU/cm^2. In this set of treatments H32-*gfp* was not significantly affected by the presence of 1A-Rif being co-cultured together ($p<0.05$).

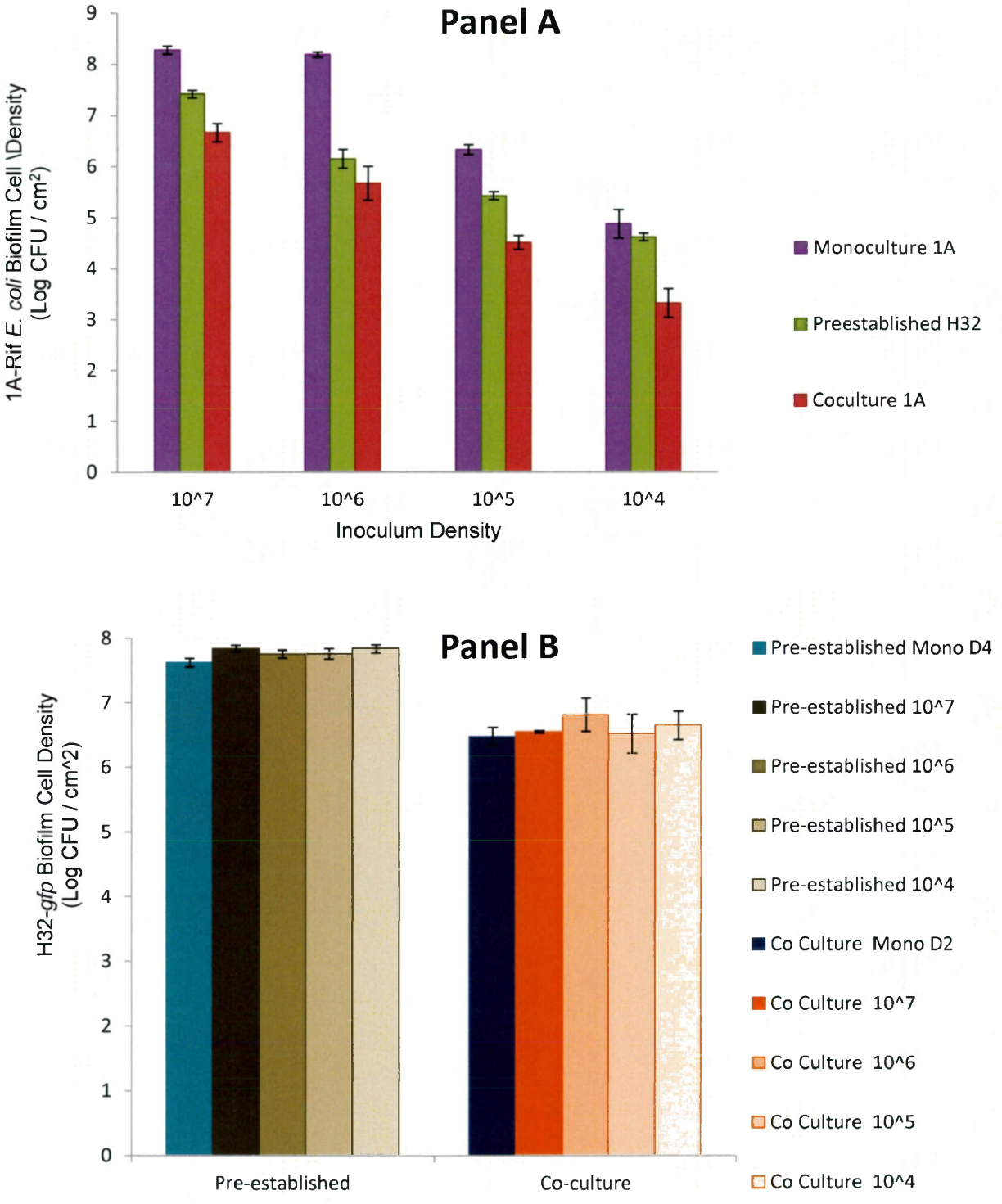

Figure 10 Biofilm cell density analysis of 1A-Rif how H32-*gfp* effects 1A-Rif biofilm establishment

H32-*gfp was* held at 1x10^7 CFU/mL in all trials, Panel A, displays the biofilm cell density of 1A-Rif, as a monoculture, inoculated at 10^7, 10^6, 10^5 and 10^4 CFU/mL. The pre-established biofilm of H32-*gfp* inoculated with 1A-Rif at 10^7, 10^6, 10^5 and 10^4 CFU/mL. Co-culture biofilms were inoculated with H32-*gfp* and 1A-Rif at 10^7, 10^6, 10^5 and 10^4 CFU/mL. Panel B displays the growth of H32-*gfp E. coli* grown as 4 day monoculture with the 4 day pre-established biofilms inoculated with1A-Rif with 10^7, 10^6, 10^5 and 10^4 CFU/mL. Co-culture displays a 2 day monoculture of H32-*gfp* with 1A-Rif inoculated *at* 10^7, 10^6, 10^5 and 10^4 CFU/mL

2.3.7. Lake water biofilms

Examining lake water has many potential anomalies due to the vastness of the mixed microbial species. Therefore, it was important to study how the lake water bacteria would behave as a biofilm. The lake water contains a mixed culture of bacteria. Therefore, it is important to study the biofilm forming abilities without H32-*gfp*. Figure 11, displays the time course used to establish the point of saturation for the biofilm. Within 24 h, there was significant biofilm growth and biofilm formation by the lake water bacteria. Within 48 h, there was complete saturation of the coverslip and as time progressed, the lake water biofilm matured and very little change occurred with the biofilm cell density over time. The most dramatic development of the biofilm occurred within 48 h. The experiment was carried out over 144 h. The biofilm cell densities increased from 5.73 to 7.54 between the 24 and 48 h time points and remained at ~7.64 Log CFU/ cm^2 for the remaining days tested.

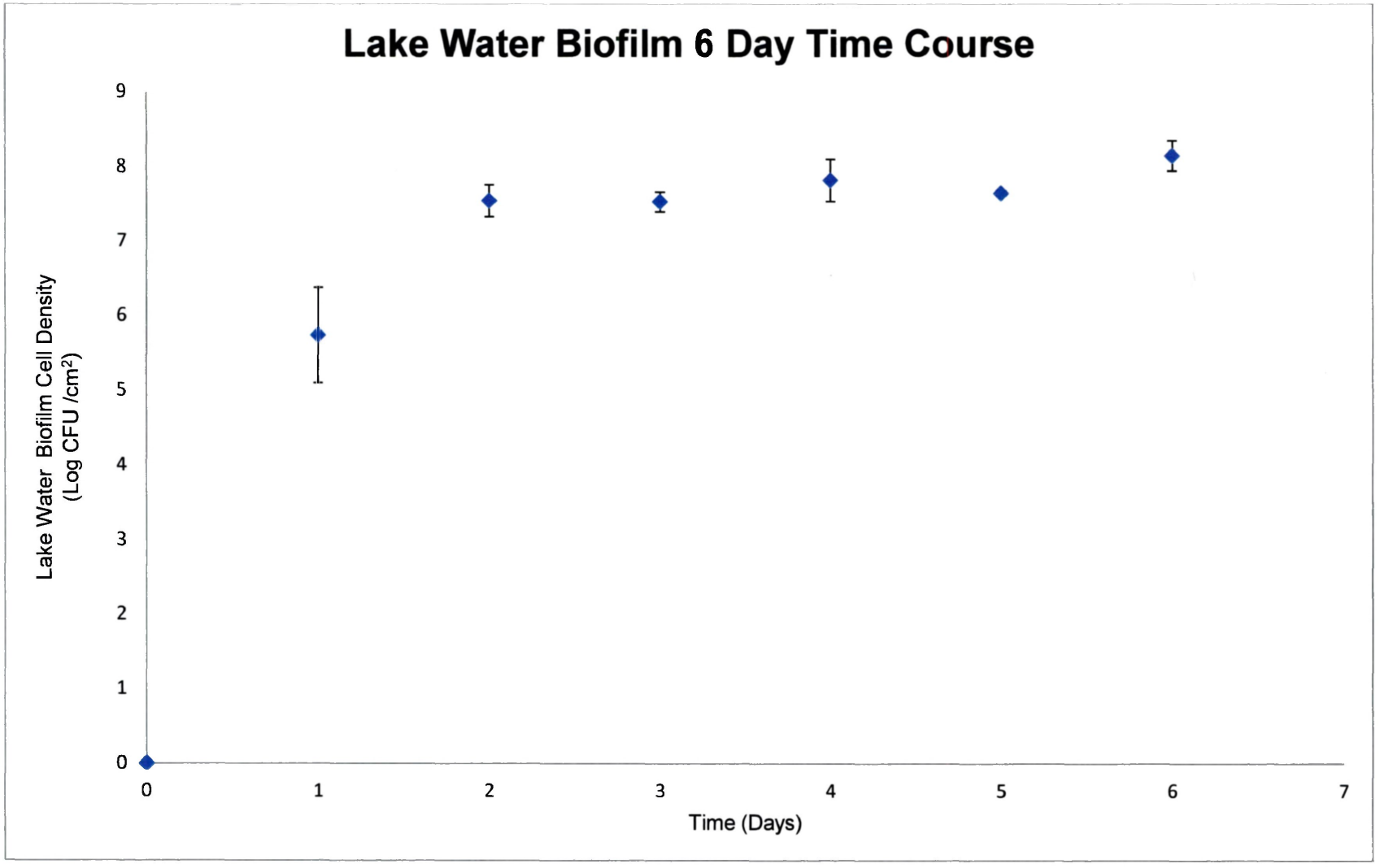

Figure 11 Time course of lake water biofilm.

Lake water bacterial biofilm suspension grown over 6 days

2.3.8. Lake water bacterial biofilm imaging

The lake water biofilm required staining in order to be visualized by fluorescence microscopy. Figure 12 is an image of a 48 h lake water biofilm stained with SYTO 9 dye. In panel A, it shows that a variety of bacteria are growing on the coverslip glass. There were different sizes and shapes of the bacteria found in this sample, ranging from rod-shape to coccoid shape bacteria. In addition, clusters of cells and dome shape biofilm structures could be found as part of the lake water biofilms. Within 48 h, there was a strong colonization of the coverslip. Panel B displays the side view of the lake water bacterial biofilm. This view presents the complexity of a mixed species bacterial community. The topography is different compared to the periphytic 1A *E. coli* biofilm which was uniformly thick throughout the biofilm whereas the lake water biofilm was irregular, with cell clusters and individual 3-D structures arise from the thin layer of biofilm formed on the surface of the substratum.

Panel A **Panel B**

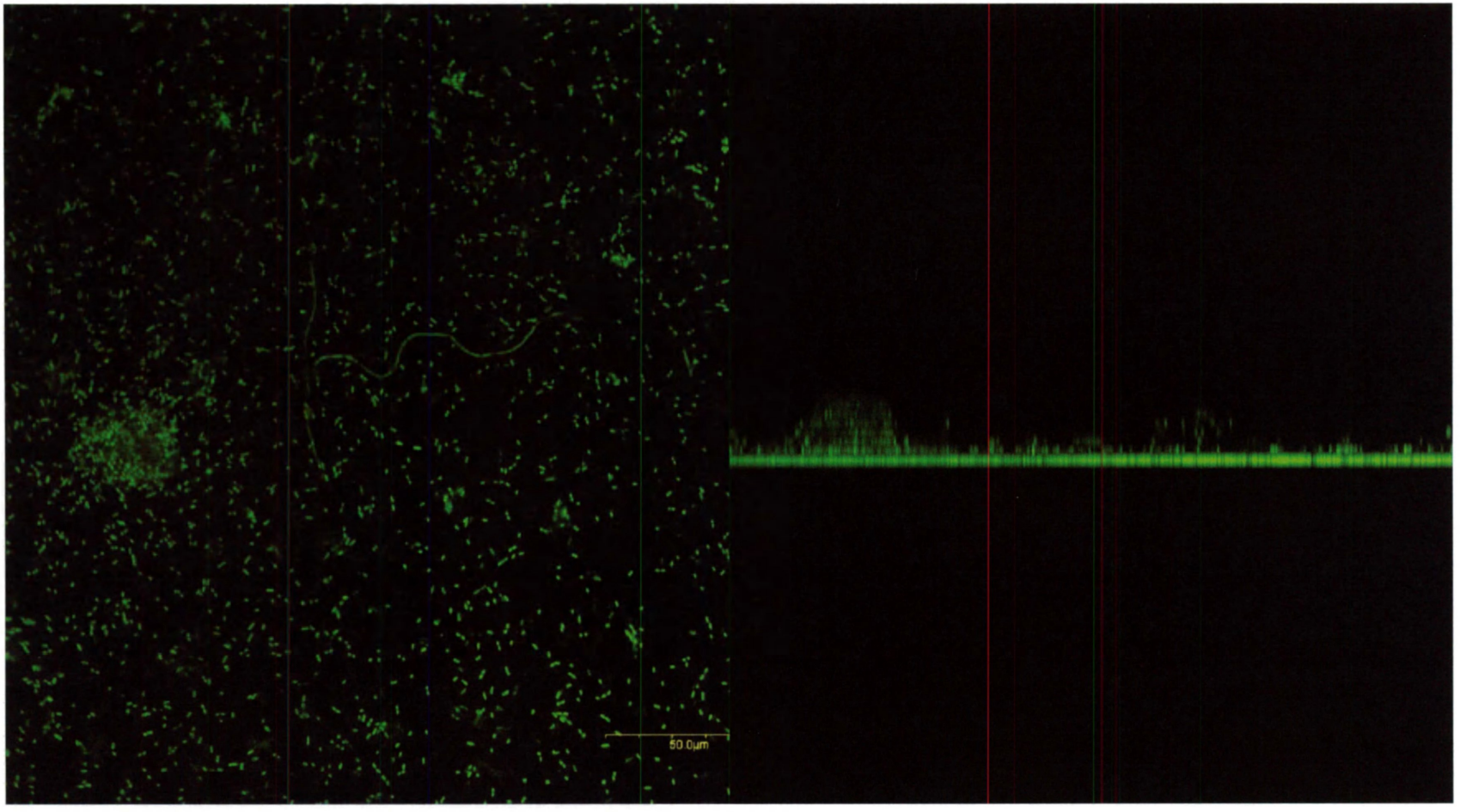

Figure 12 Lake water bacterial suspension biofilm.

The lake water bacterial suspension biofilm was grown for 48 h and stained with SYTO 9. Panel A displays the lake water suspension total biofilm Panel B, displays the cross section of the lake water biofilm.

2.3.9. Lake water microbial biofilm with H32-*gfp*

The lake water biofilm experiments are essential to this study because the establishment of pathogenic bacteria within periphytic biofilms is a growing concern. It is important to study how this periphytic community would respond to an invasion of shiga toxin-producing *E.coli.*

The examination of the lake water biofilm and how it affected the growth of H32-*gfp* are seen in Figure 13. Panel A, shows the results for H32-*gfp*. Inoculum densities H32-*gfp* were examined from $1x10^7$ down to $1x10^0$ CFU/mL. These densities covered the vastness of the bacterial ranges that could exist in the environment.

The monocultures of H32-*gfp* at each inoculum density were able to form biofilm, displaying the versatility of this pathogen which can grow and form biofilm with extremely low inoculum densities. The biofilm growth of monoculture of H32-*gfp* was compared to growth on were a pre-established lake water biofilm and to co-inoculation of the lake water bacterial suspension with H32-*gfp.*

The lake water planktonic cell density was determined to be $3.35x10^3$ CFU/mL. The monoculture H32-*gfp* biofilm cell densities established by inoculant densities of $1x10^7$, $1x10^6$, $1x10^5$, $1x10^4$, $1x10^3$, $1x10^2$, $1x10^1$ and $1x10^0$ were 7.16, 7.23, 7.40, 7.33, 6.27, 5.39, 4.76 and 2.98 Log CFU/cm^2, respectively (Figure 13, Panel A). In the pre-established treatment, where the lake water microbial biofilm was grown for 48 h and then inoculated with different inoculum densities of *E. coli* H32-*gfp*. The H32-*gfp* cell densities from $1x10^7$ to $1x10^0$ were as follows respectably, 7.09, 6.58, 4.95, 3.58, 2.52

Log CFU/cm^2 and zero for both 1x10^1 and 1x10^0 CFU/mL (Figure 13, Panel A). The lake water biofilm had a strong impact on H32-*gfp* ability to form biofilm.

The lake water biofilm has no observable effect on the ability for H32-*gfp* to grow and establish biofilm at the higher inoculum density of 1x10^7 CFU/mL. At the inoculum density of 1x10^{6}CFU/mL and lower, the lake water microbial biofilm has a negative impact on H32-*gfp* ability to form biofilm. With the inoculum densities of 1x10^1 and 1x10^0 CFU/mL the lake water biofilm completely impeded the ability for H32-*gfp* to establish biofilm.

For the last treatment, co-inoculation, both the H32-*gfp* and the lake water bacterial suspension were added simultaneously to the 6 well culture plates. The biofilm cell density of H32-*gfp* generated by inoculant density ranging from 1x10^7 to 1x10^1 CFU/mL were 6.82, 6.53, 6.49, 6.46, 5.49, 3.93 and 3.04 Log CFU/cm^2, and for 1x10^0 CFU/mL, there was zero H32-*gfp* biofilm growth detected. At the high H32-*gfp* inoculum density of 1x10^{7}CFU/mL, there was little effect from the lake water bacterial population on the establishment of H32-*gfp*. However with the H32-*gfp* inoculum densities of 1x10^6 and lower, the lake water microbial population negatively affected the ability of H32-*gfp* to form biofilm. There were significant decreases of H32-*gfp* in the co-culture biofilms compared to the H32-*gfp* monoculture biofilms with the same respective inoculum densities. With the lowest H32-*gfp* inoculum density of 1x10^0 CFU/mL, the lake water biofilm completely inhibited H32-*gfp* ability to from biofilm.

In addition to analyzing H32-*gfp,* the lake water biofilm cell density was also examined (Figure 13, Panel B). The lake water microbial biofilm densities in the pre-

established treatment were compared to a lake water bacterial biofilm absent of H32-*gfp*. The control, four day pre-established biofilm cell density was 7.82 Log CFU/cm^2. The experimental lake water biofilm inoculated with deceasing inoculum densities of H32-*gfp* (1x10^7-1x10^0 CFU/mL) were 7.45, 7.43, 7.37, 7.44, 7.29, 7.16, 7.15 and 7.12 CFU/cm^2. The cell densities for the lake water biofilm with the addition of H32-*gfp* did not change greatly. The lake water bacterial biofilm exhibits robustness in its ability to adapt to change. This would imply that the lake water biofilm was not significantly affected by the presence of the H32-*gfp* strain ($p > 0.05$).

In the co-culture treatment, the control of lake water biofilm was grown without the addition of H32-*gfp*. The cell density of the control lake water biofilm was 7.54 Log CFU/cm^2. In comparison to the co-inoculation treatment with H32-*gfp*, the values of cell density experienced a slight decrease in the ability to form biofilm. The cell densities of the lake water biofilm when inoculated with H32-*gfp* (1x10^7-1x10^0 CFU/mL) were 7.20, 6.80, 6.77, 6.88, 6.99, 6.83, 6.79 and 6.99 Log CFU/cm^2. There was a slight decrease in the lake water biofilm cell density when H32-*gfp* was introduced. However the lake water biofilm was a stronger competitor overall compared to H32-*gfp*, as the lake water bacteria inhibited the biofilm growth of the pathogen when the inoculant density of the pathogen was ranged from 1x10^6 to 1x10^0 CFU/mL.

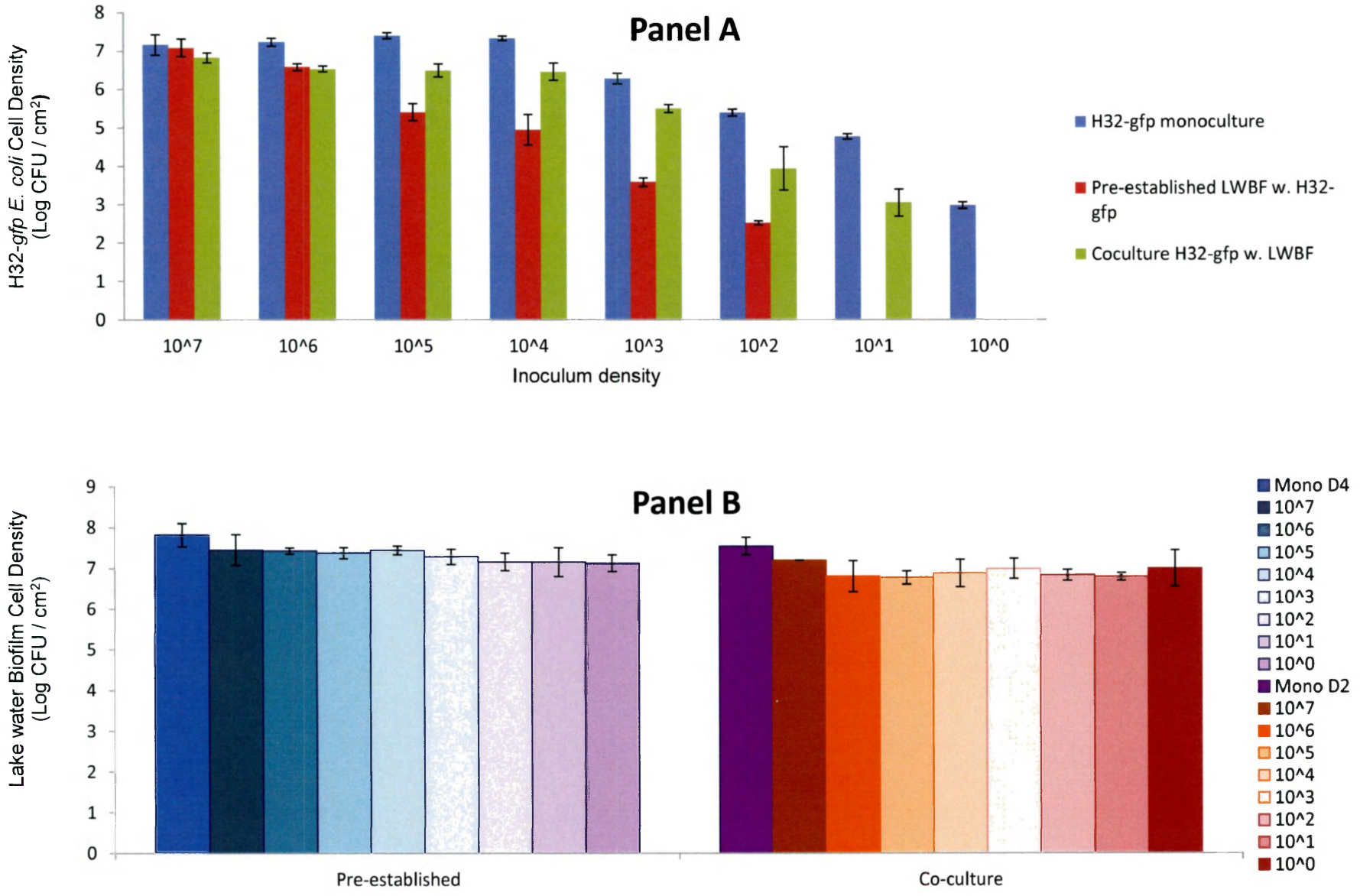

Figure 13 Biofilm cell density analysis of H32-*gfp and* how the lake water biofilm effects H32-*gfp* biofilm establishment.

Panel A, displays the biofilm cell density of H32-*gfp,* as a monoculture, inoculated at 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 CFU/mL. The pre-established biofilm of lake water with H32-*gfp* inoculated at 10^7, 10^6, 10^5,10^4, 10^3, 10^2, 10^1 and 10^0 CFU/mL. Co-culture biofilms were inoculated with lake water bacterial suspension and H32-*gfp* 10^7, 10^6, 10^5 and 10^4, 10^3, 10^2, 10^1 and 10^0 CFU/mL. Panel B displays the growth of the lake water biofilm grown as 4 day culture and with the 4 day pre-established biofilms inoculated with H32-*gfp* with 10^7, 10^6, 10^5, 10^4 10^3, 10^2, 10^1 and 10^0 CFU/mL. Co-culture displays a 2 day culture of lake water biofilm with H32-*gfp* inoculated *at* 10^7, 10^6, 10^5, 10^4 10^3, 10^2, 10^1 and 10^0 CFU/mL

2.4. Discussion

Escherichia coli have been isolated from many different tropical and temperate ecosystems such as farms, lakes, streams, forest soil, beach sand, and epilithic periphyton (Carillo, et al., 1985; Whitman & Nevers, 2003; Ksoll, et al., 2007). With the reports of existence of naturalized *E. coli* biofilm populations in a temperate water body (Byappanahalli, et al., 2006; Ksoll, et al., 2007), it becomes important to study the behaviour of both naturalized and pathogenic *E. coli* in the environment. One significant aim of this study was to determine whether an enterohemorrhagic *E. coli* O157:H7 strain, known to cause disease in humans, could become established in much the same way as the naturalized *E. coli*. The ability of enterohemorrhagic *E. coli* to become established in a sessile community represents an obvious risk to public health and merits investigation.

The biofilm-forming capabilities of *E. coli* O157:H7 strains H32, H32-*gfp*, 1A and 1A-Rif were investigated in this study. In agreement with previous findings, *E. coli* O157:H7 biofilm formation was found to be strongly influenced by the media employed in cultivation (Dewanti & Wong, 1995; Oh, et al., 2007; Reisner, et al., 2006) . All four *E. coli* strains exhibited biofilm forming ability under low nutrient conditions, in MSMG. The formation of *E. coli* O157:H7 biofilms under nutrient-limitation contrasts biofilm formation in most other Gram-negative bacteria, where low nutrient conditions typically signal a reversion to the planktonic state (O'Toole, et al., 2000)

The physiological differences between the sessile and free-living lifestyles of the four *E. coli* strains have been identified. A theme became obvious over the course of the study. Under planktonic condition, all four bacterial strains grew rapidly in TSB. In 120

minutes, their cell densities increased by about two orders of magnitude compared to their initial inoculum concentrations at OD of 0.05. In MSMG, the planktonic growth was drastically hindered and cells were unable to reach an OD of 1. The majority of the growth for all four *E. coli* strains took place between the initial inoculation to 1 day. While planktonic growth curves for the four strains were similar, it is noteworthy that 1A and 1A-Rif clearly held an edge when it came to biofilm formation, with a biofilm roughly 40 times thicker than that of H32 and H32-*gfp* in the first 48 h. Furthermore, H32 and H32-*gfp* were able to form biofilm much more quickly than the 1A strains, at lower inoculum densities (Figure 9, Figure 10 and Figure 13).

Biofilms are propagated in three separate methods: (1) the redistribution of cells along the surface of the substratum (Moreira, et al., 2011), (2) the recruitment of cells from the bulk fluid (Tolker-Nielson, et al., 2000), and (3) through division of cells within the biofilm (Heydorn, et al., 2000). It is likely that the heterogeneous structure of 1A-Rif and biofilms observed in MSMG were the result of this last mode of propagation. A simple spreading of cells along the surface would not have led to an increase in average thickness, nor would it lead to the development of mushroom shaped structures. For H32-*gfp*, it is likely that the redistribution was via the first case method because there is an absence of biofilm structures and the biofilm is sparse indicating that there is little cell division occurring.

In this study, it was observed that the 1A *E. coli* isolated from periphyton in Boulevard Lake was far more adept at forming biofilm than the pathogenic *E. coli* O157:H7 H32. This suggests that the periphytic biofilm isolate was genetically endowed for an existence beyond its primary human or animal hosts. In other words, periphytic *E.*

coli strains have become a member of the naturally occurring microflora that are adept at coping with the rigors encountered beyond the limits of their animal host (Moreira, et al., 2011). This was not the first instance of *E. coli* being isolated from an environment without a link to a discernible source of input. Repetitive extragenic palindromic polymerase chain reaction was used by Byappanhalli (2006) to show that a consortium of *E. coli* sampled from a Lake Michigan watershed were genetically distinct from any likely animal sources in the region, such as ducks, geese, terns and deer. Lopez-Torres (1987) acknowledged the likelihood of *E. coli* becoming part of the autochthonous microflora of the Mayemes River in Puerto Rico. More importantly, fecal contamination was absent within those areas wherein *E. coli* had been collected, indicating that the *E. coli* present must have been naturalized. The investigators found that *E. coli* were capable of persisting for long periods in the river ecosystem all-the-while remaining physiologically inactive. Thus, it is possible that the *E. coli* collected from biofilms in Boulevard Lake, may be naturalized members of the local microfauna (Moreira, et al., 2011).

Little is known about the structure and functions of environmental *E. coli* strains. Moreira *et al* (2011) demonstrated that the periphytic *E. coli* isolates from Boulevard Lake shared a common characteristic of being good biofilm-formers. Using a representative of the isolated periphytic *E. coli*, we further investigated the structure of this group of *E. coli,* Figure 3 and Figure 4, depict intricate biofilm architecture of 1A with the different types of 3-D structures, including water channels, mushroom- and pillar-like structures. These structures can lead to the formation of a mature periphytic biofilm. Similar biofilm structure can be found in *Pseudomonas aeruginosa* biofilm. The cells adhere to solid surfaces and remain in a dormant state until nutrients become available to permit biofilm

growth and maturation (Toutain, et al., 2004). This complex architecture is known to facilitate efficient nutrient uptake by allowing the flow to permeate into the biofilm from the bulk liquid via the water channels, thereby delivering nutrients and other essentials to deeply embedded parts of the biofilm community (Purevdorj-Gage & Stoodley, 2004). Visual characteristics of biofilms growing in diverse environments are strikingly similar, suggesting convergent biofilm survival strategies conferred in part by structural specialization (Purevdorj-Gage & Stoodley, 2004). Biofilms growing in fast moving water tend to form filamentous streamers, as seen in periphyton in rivers. In quiescent water, biofilms tend to form isotropic mushroom or pillar-like structures and these structures can be formed in a laboratory by a diverse range of microorganisms (Purevdorj-Gage & Stoodley, 2004). With regards to the 1A Rif's biofilm, these structures may help the survival of the periphytic *E. coli* in the environment.

It has been shown that *E. coli* O157:H7 can establish better biofilm with the help of other good biofilm formers (Klayman, et al., 2009; Ksoll, et al., 2007). Coexistence of multiple bacterial species, such as *Pseudomonas aeruginosa, Salmonella, Staphylococcus, Bacillus* and *E. coli* are frequently observed in food-processing plants and multi-species biofilms often demonstrate higher resistance to common sanitizers than monospecies biofilms (Wang, et al., 2012) .Yet, little effort has been spent in examining factors that can affect these interactions such as the effect of; (1) inoculant density (2) pre-establish biofilms, and (3) and co-culture biofilms. In the literature, most studies use one inoculant density or one type of experimental treatment. These types of omissions can lead to a biased conclusion in their studies. Our study which included a spectrum of inoculant densities and treatments produced the following novel findings. The pre-

established biofilm treatments, are analogous to environmental conditions. The 'first come, first takeover' hypothesis is applicable to pre-established biofilms. Only when inoculate density was high, such as $1x10^7$ CFU/mL, was there proliferation of both biofilms. As the inoculate density decreased below $1x10^6$ CFU/mL, there was suppression from the pre-established biofilm.

The co-culture treatment represents new bacteria entering a new environment simultaneously. It was reported that due to complexities of the biofilm mode of growth, multiple species can coexist despite one organism having a much higher growth rate then another. In many cases bacteria have been shown to gain a fitness advantage when residing in a mixed-species versus single species biofilm. Bacteria living in a two- species community were demonstrated to undergo mutations which improved productivity and stability compared to the parent community (Klayman, et al., 2009). For the co-inoculated biofilms, in our study, the 'fastest and more competitive win the race' analogy was observed. When both 1A-Rif and H32-*gfp* strains were inoculated at the same time, the more competitive H32-*gfp* strain established first and formed more biofilm (Figure 10, Panel A). Because the lake water bacterial sample was composed of a complex microbial community, it was more competitive than the single strain H32-*gfp* culture. Therefore, both the pre-established and co-inoculated lake water bacterial samples suppressed the establishment of the pathogenic *E. coli* O157:H7 strain at inoculant densities of $1x10^6$ and below (Figure 13, Panel A).

2.5. Conclusion

In conclusion, the biofilm forming ability of different *E. coli* strains can vary tremendously. The periphytic *E. coli* 1A formed copious amount of biofilm with pillar and water channel structures. In contrary, the O157:H7 H32 strain formed an unstructured mono-layer biofilm. The presence of 1A (either as a pre-established biofilm or as a co-inoculum culture) increased the biofilm establishment of H32 only when the inoculum density of H32 is at $1x10^7$ CFU/mL. When the inoculum density of H32 was at $1x10^6$ CFU/mL, neither a pre-established 1A biofilm nor a co-inoculum 1A culture could affect the H32 biofilm establishment. At the inoculum densities of $1x10^5$ and 10^4 CFU/mL of H32, only the pre-established 1A biofilm treatment decreased the H32 biofilm formation. In contrary, biofilm formation of H32 was not affected by the presence of 1A in the co-culture treatment. The lake water microbial population from Boulevard Lake (either as a pre-established biofilm or as a co-inoculum culture) had a greater negative effect on the biofilm establishment of H32 than the 1A treatments. The lake water microbial population significantly decreased the biofilm formation of H32 at inoculum density at $1x10^6$ CFU/mL or lower.

Therefore, the associated implications of *E. coli* O157:H7 establishing in the environment is a cause for concern. The risks associated for public health are far-reaching and must be taken seriously. This study presents novel experiments with regard to types of treatments performed such as the various inoculant densities used. The two different culture methods employed, pre-established or the co-culture treatments. The examination of the biofilm forming capacities between both strains of *E. coli* species 1A

and H32 as well as a naturalized lake water microbial population was analyzed. Presently, few peer reviewed journal articles exist that explain the behavior and interactions of *E. coli* with other environmental bacteria under biofilm conditions.

2.6. Future Work and Directions

The results of this thesis revel that the pathogenic *E. coli* strain H32 is capable of forming biofilm with periphytic *E. coli* and with a mixed community of lake water bacteria. The growth of the H32 biofilm under high inoculum densities has the ability to outgrow its competitor in either the pre-established biofilm treatments or in the co-inoculation treatments under a 24, 48 and 96 h study. The ability to form biofilm under these types of conditions can pose potential risk to natural environment if the pathogen is exposed to these conditions. Recommendations for future would to carry out long term studies of the biofilms for longer than 96 h. An analysis of an older biofilm will give insight as to how mature biofilms behave with the addition of a pathogenic bacterium.

This study focused on a few different inoculum densities combinations, and three types of treatments, monocultures, pre-established biofilms with inoculation of another species of *E. coli* and co-inoculation, the addition of both bacterial species simultaneously. Future experiments could include the analysis of different temperatures, such as colder temperatures, below 22°C and warmer temperatures above 22°C.

Novel experiments could include the analysis of the chemical compounds secreted by the biofilms, test for any stimulant and or inhibitory compounds that are secreted by the biofilm. The addition of a red fluorescent protein to the 1A-Rif *E. coli* would prove beneficial for analysis of structures of the biofilm.

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