Biofilm Formation of Environmental and Enterohemorrhagic *Escherichia coli*

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August 31st, 2010

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Your file Votre référence
ISBN: 978-0-494-71952-7
Our file Notre référence
ISBN: 978-0-494-71952-7

ABSTRACT OF THE THESIS

Bacteria are capable of existence in two separate lifestyles: sessile and planktonic. Sessile bacteria grow in surface-associated communities enmeshed in an extracellular matrix know as biofilms. Biofilms confer upon constituent bacteria a heightened resistance to physical and chemical stress and represent the preferred mode of growth. Early studies of biofilm development were limited by the shortcomings inherent in microscopic techniques of the 1980's. Subsequent improvement however, particularly the use of confocal scanning laser microscopy, allowed for the observation of fully hydrated, living biofilms which contributed to the running theory of what constitutes biofilm structure, particularly at maturity. And while useful, the model of biofilm development and structure is based largely upon the study of *Pseudomonas aeruginosa*- an obvious shortcoming when one endeavors to study biofilm formation in other bacteria. Indeed, studies have examined biofilm development of *E. coli* but focus on either O157:H7, a pathogenic isolate responsible for causing gastrointestinal disease in humans, as well as K-12, a standard laboratory strain and are almost always carried out in defined laboratory media. Additionally, growing evidence indicates that *E. coli* are capable of existence in aqueous environments beyond the protective mammalian host. Thus, an examination of biofilm formation of not only clinical, but of environmental isolates of *E. coli*, was undertaken here.

Enterohemorrhagic serogroups O157, O111 and O26 as well as environmental biofilm strains collected from three Northwestern Ontario water bodies were screened for their respective abilities to form biofilm in optimal laboratory medium. DNA-fingerprinting was then used in an effort to identify genetic diversity of the strains employed. Subsequently, one representative each from the O157 and environmental biofilm groups of *E. coli*, along with K-12, were selected for microscopic examination of biofilm development in optimal lab medium as well as filter-sterilized sewage-contaminated and uncontaminated lake water. Planktonic development was similarly monitored. When screened for biofilm formation in optimal laboratory medium, it was obvious that the environmental biofilm *E. coli* were significantly better formers than the others. Moreover, the O157 and O26 strains were nearly incapable of forming biofilm. It was further discovered that DNA fingerprint was not predictive of biofilm forming ability. Microscopic examination of the three representative *E. coil* selected revealed vastly differing biofilm phenotypes between organisms in the same medium as well as for the same organism in different media. The only bacterium to reach maturity was the environmental isolate, and then, only when grown in laboratory medium. Also noteworthy was the semi-mature biofilm formed by H32 in the sewage contaminated lake water. This presents public health concerns as it indicates that pathogenic *E. coli* have the capacity to adhere

to abiotic surfaces, potentially in waters that see public use. In rich laboratory medium, planktonic and biofilm trends were very similar. However, in the poor environmental media, plankotnic cell densities fell over time while biofilm populations remained constant suggesting that indeed, the formation of biofilm provides a protective measure for bacteria when faced with poor environmental conditions. In addition to being the first to examine biofilm structure and development of pathogenic *E. coli* alongside natural varieties isolated from temperate water bodies, this research provides a useful foundation for the further study of biofilm formation of *E. coli* in temperate water bodies.

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CHAPTER 1 - LITERATURE REVIEW

1.1.1 Introduction

Escherichia coli O157:H7 is a Gram-negative, facultatively-anaerobic, rod-shaped microorganism which is typically 1-2 microns in length (Trun and Trempy 2003). It was first identified as the causative agent of a previously undocumented gastrointestinal disease which caused illness in 47 patients in Oregon, U.S.A. and Michigan, U.S.A. in 1982. Those who fell ill initially experienced intense abdominal cramping and watery diarrhea. This was followed by profusely bloody diarrhea with some patients also experiencing low-grade fever. Treatment of the disease with antimicrobials proved ineffective and patients typically experienced symptoms for three to seven days (Riley *et al.* 1983). The immunocompromised, very young and very old are at the greatest risk for mortality when infected with *E. coli* O157:H7 (Trun and Trempy 2003).

As a member of the gut microflora of most healthy warm-blooded organisms, the vast majority of *E. coli* are non-pathogenic to humans (Leclerc *et al.* 2001). Indeed, most individuals will play host to seven or eight strains of *E. coli* at any point in their life with no ill effect (Bettelheim, 1997). Nevertheless, because of its typical association with fecal pathogens, and its inability to persist for long periods once shed from the host, *Escherichia coli* is used as an indicator organism of fecal contamination when assessing drinking and recreational water

quality (Leclerc *et al.* 2002).

1.1.2 Biofilms

Escherichia coli can exist in either a free-living, planktonic phase, or in a sessile community attached to a substrate, known as a biofilm. Biofilms are complex, highly organized, communities of bacteria enclosed in an exopolysaccharide matrix adhered to a surface (Costerton *et al.* 1995). These surface-associated communities were recognized as early as 1933 (Henrici 1933) and represent the preferred method of growth for bacteria in aqueous environments (Geesey *et al.* 1977, Lappin-Scott and Costerton 1995). Aggregation on a surface provides bacteria with a facility for improved cell-cell signaling as well as the benefit of nutrients which are likely concentrated there (Baty *et al.* 2000). Unfortunately, the understanding of biofilm development, historically, has been based on the process as performed by *Pseudomonas aeruginosa* (O'Toole *et al.* 2000). Thus, the model for the temporal organization of the discreet steps involved in biofilm formation is based upon *P. aeruginosa*. Typically, biofilm formation involves five sequential steps (Costerton *et al.* 1995; Costerton *et al.* 1987):

1) Transitory contact; initial association with a surface which yields reversible attachment by some cells.

2) Cells from stage one develop an irreversible attachment with the surface.

Attachment is facilitated by the production of the exopolysaccharide matrix.

3) Cells form small aggregates known as microcolonies, dependent upon their position within the biofilm.

4) Mature biofilms are evident in the fourth stage: complex aggregations of mushroom-like structures.

5) Cells begin to detach from the biofilm, either individually or en-masse. The latter is known as sloughing. Detachment provides a means of dispersal, enabling the spread of the bacteria to other favourable surfaces.

1.1.3 Biofilm structure

Of central importance in a discussion of biofilm structure is the extra cellular matrix (ECM). Composed of polysaccharides, proteins, nucleic acids - all secreted by the cells - and other captured substances, the ECM plays an essential role in the architecture and maturation of *E. coli* K-12 biofilms (Danese *et al.* 2000). Within the ECM, mushroom-shaped aggregates of cells grow upward from the substrate and are separated by water channels which provide a means of importing and exporting nutrients and wastes respectively (Lawrence *et al.* 1991; Davey *et al.* 2003). These channels are essential for ensuring a consistent supply of nutrients to microcolonies embedded within a biofilm (De Beer *et al.* 1994; Massol-Deya *et al.* 1995), as diffusion alone would be insufficient (Massol-Deya *et al.* 1995). Incidentally, De Beer *et al.* (1994) noted that these channels

represent a primary link between form and function in biofilms. Interestingly, exopolysaccharide production is shown to serve different purposes in various organisms. In *E. coli* K-12, EPS production is triggered by cellular attachment to a surface (Prigent-Combaret *et al.* 1999) and is thus unnecessary in the process of transitory contact and initial attachment (Danese *et al.* 2000). In contrast, *Vibrio cholerae* (Watnick and Kolter 1999) and *Staphylococcus epidermidis* (McKenny *et al.* 1998) must necessarily produce EPS as a means of primary attachment to a surface.

Frequently, the cells located in the center of mature biofilms will show reduced metabolic activity relative to those proximal to the biofilm's surface (Sternberg, 1999). However, metabolic activity can be restored to these cells by supplying them with a more readily useable carbon source (Sternberg, 1999).

1.1.4 Increased robustness in biofilm cells

Indicative of the protective role they play, biofilms develop in environments where nutrients are growth-limiting, irrespective of the high energy required in their synthesis (Dewanti and Wong 1996). This is suggestive of the advantages growth as a biofilm confers upon microorganisms. Indeed, compared to planktonic counterparts, the bacteria in biofilms show increased tolerance for insult including dessication (Costerton *et al.* 1987), antibiotic treatment (Nickel *et al.* 1985a; Nickel *et al.* 1985b; Hoyle and Costerton 1991),

exposure to biocides such as detergent and chlorine (De Beer *et al.* 1994b; Hoyle and Costerton 1991; Lisle *et al.* 1998) and defense of the host (Jensen *et al.* 1990). Nutrient limitation and changes in growth rate which often accompany the transition to a sessile lifestyle can have profound effects on the fatty-acid and phospholipid components of the cellular envelope (Glibert and Brown 1978). This in turn, can alter the targets of various antimicrobials (Gilbert and Brown 1978). Therefore, changes in growth rate and nutrient availability are indirectly responsible for the altered susceptibility of microorganisms in biofilms. In the case of *E. coli,* the activity of polymycin B is significantly diminished in carbon and nitrogen poor environments (Wright and Gilbert 1987). Furthermore, slow-growing cultures of *E. coli* show no susceptibility towards the antibiotics cefonicid and ceftriaxone (Cozens *et al.* 1986).

1.1.5 Escherichia coli biofilms

Our understanding of the development and architecture of *E. coli* biofilms is limited compared to that of *P. aeruginosa* (Wood *et al.* 2006). What is known about biofilm formation by *E. coli* originates mostly from the study of the non-pathogenic, K-12 laboratory strain of the organism (Reisner *et al.* 2006). The study of mature *E. coli* biofilm structure using K-12 is problematic as some strains do not form mature biofilms in the absence of supplied conjugative plasmids which are necessary for the mediation of cellular interactions (Ghigo 2001; Reisner *et al.*

2003). Additionally, genetic differences among strains makes applying results obtained through the study of K-12 to other strains risky (Resnier et al 2006). Nevertheless, Pratt and Kolter (1998) and Wood *et al.* (2006), have successfully demonstrated the ability of certain K-12 strains to form mature biofilms (Reisner *et al.* 2003).

1.1.6 Biofilm formation by pathogenic E. coli

The growth of *E. coli* O157:H7 biofilms in vitro was first recognized by Dewanti and Wong (1995). They discovered that biofilm development among the pathogenic isolates tested was best in nutrient-poor medium. *Escherichia coli* O157:H7 was grown in Trypticase Soy Broth (TSB), a 1/5th dilution of TSB, BactoPeptone and minimal salt medium with 0.04% glucose (MSM). Examination of biofilms grown in MSM, revealed shorter cells and a thicker, extracellular matrix, relative to those in other media tested.

Sheike *et al.* (2001) have shown the importance of aggregative adherence fimbriae (AAF) in the mediation of cell-cell adhesion in enteroaggragative *Escherichia coli* (EAEC). It was determined that mutants deficient in the production of AAF lost the ability to colonize glass and polystyrene. The limitations of the experiment must be considered however, note the authors. In the intestine, EAEC from biofilms which colonize the surface of the mucosa and are, in large part, formed of mucus secreted by the host. The assay employed

stains in large part, the mucus which makes up biofilms in vivo. As host secretions are unavailable in in-vitro experiment, results of staining may not have been entirely representative of reality.

Many studies highlight the importance of curli in the development of biofilms by pathogenic strains of *E. coli*. Ryu et al. (2004) demonstrated the importance of curli in biofilm formation on stainless steel chips. Cookson et al. (2002) also showed the importance of curli in the attachment of pathogenic *E. coli* to an abiotic surface and, furthermore, showed that curli were also important in biofilm maturation.

Reisner *et al.* (2006), conducted a study in an attempt to determine whether pathogenic *E. coli* showed an enhanced ability to form biofilms compared with non-domesticated strains collected from human stool. The in vitro biofilm formation of *E. coli* isolated from 105 healthy and 68 diarrhea afflicted children, as well as from 90 bacteremic patients and 102 males suffering from ascending urinary tract infections was examined. Biofilm formation was also compared to that of pathogenic laboratory strains as well as to K-12. Natural *E. coli* isolates showed diverse biofilm-forming ability in the four media tested (Luria-Bertani, minimal medium supplemented with glucose (ABTG) or casamino acids (ABTCAA), diluted porcine mucus and urine). Biofilm formation was not dependent upon strain of origin, but rather, on growth medium. Moreover, a test

strain's biofilm development in one medium was not predictive of its ability to form biofilms in another. Lastly, the authors concluded that pathogenic *E. coli* did not exhibit improved biofilm formation in vitro relative to non-pathogenic isolates.

1.1.7 Escherichia coli in the environment

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 and 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; Rangel et al 2005; Olsen *et al.* 2002). 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).

Beginning in the 1980's, persistent populations of *E. coli* were identified in both tropical and temperate environments. In a study conducted by Carillo *et al.* (1985), all sites sampled along the Mayemes River, Puerto Rico, showed persistent populations of *E. coli*. Particularly interesting, the site furthest upstream from the only sewage input point source on the river, assumed to be pristine and free of any fecal contamination, showed levels of *E. coli* second only

to samples taken immediately downstream from the sewage inflow. That is to say, sites downstream from the sewage input showed densities of *E. coli* significantly lower than those found in an uncontaminated site. A second examination of the sites conducted by Lopez-Torres *et al.* (1987) yielded similar results. Furthermore, examinations of rivers in Hawaii (Hardina and Fujioka 1991) and Guam (Fujioka *et al.* 1999), showed the presence of populations of *E. coli* in water which consistently exceeded the United States Environmental Protection Agency standards for recreational water quality of 126 CFU/ 100 mL water. The conclusions in each study were similar: natural, self-sustaining populations of *E. coli* likely exist in tropical watersheds making *E. coli* an unsuitable indicator of fecal contamination in those environments.

The presence of natural *E. coli* populations, however, is not limited to tropical environments. Persistent populations of *E. coli* have also been identified both in aquatic and terrestrial habitats in temperate regions. *Escherichia coli* have been shown to persist for extended period in forest soils in Great Lakes watersheds (Byappanahalli *et al.* 2006; Ishii *et al.* 2006). Not only were the bacteria examined in these two studies (Byappanahalli *et al.* 2006; Ishii *et al.* 2006) capable of survival in temperate conditions, they were able to re-colonize sterile soil added to their habitat. Beach sand of Lakes Michigan (Whitman and Nevers 2003) and Superior (Ishii *et al.* 2007) are also home to long-lasting communities of

E. coli.

Periphytic algal communities in Lake Superior have also been shown to support populations of *E. coli* which persist throughout the summer, and some which are even capable of over-wintering in the lake (Ksoll *et al.* 2007).

1.1.8 Naturalized populations of Escherichia coli

Ksoll *et al.* (2007) isolated several unique strains of *E. coli* from epilithic periphyton for which matches could not be found in the Duluth *E. coli* DNA Fingerprint Library- a comprehensive collection of DNA fingerprints of *E. coli* of various sources known to occur in the Duluth area. As the organisms were found to occur uniquely in the periphyton without an identifiable external source, it was concluded that they had become naturalized and could be considered autochthonous members of periphytic communities. Further the authors demonstrated uniqueness among strains isolated from periphytic algal communities both before and after winter. The presence of *E. coli* in periphyton prior to, and following, the winter is strong evidence for the likelihood of naturalized, autochthonous environmental strains.

Ksoll *et al.* (2007) commented that differential growth and survival in the environment can be sufficient impetus for the creation of novel environmental strains. Indeed, the idea of unique environmental strains is supported by research undertaken by McLellan (2004). It was determined that *E. coli* sampled

at a particular riverine site bore more similarity to isolates found at other sites, or which were collected at other times, than to isolates collected from known sources (eg: gulls) on a particular site. It has also been demonstrated by Ishii *et al.* 2006 that some strains collected from riparian soils in a Lake Superior watershed have unique horizontal fluorophore enhance rep-pcr (HFERP) fingerprints and may have developed as naturalized environmental strains unique to certain conditions and locations.

1.1.9 Temporal variation of Escherichia coli in the environment

Temporal variation of persistent environmental populations of *E. coli* has been examined with mixed results. Based on a lack of correlations between substrate moisture content and soil and air temperatures, Byappanahalli *et al.* (2006) concluded that season plays no part in the densities of *E. coli* found in a forest soil. In contrast, Whitman and Nevers (2003) showed a moderate to strong correlation between air and water temperatures and *E. coli* cell densities in foreshore and submerged sands of a Lake Michigan beach. Koiralaa *et al.* 2008 showed temporal variation of fecal coliforms in stream sediment: both short term and long term. The authors found that levels were highest in the summer and lowest in the winter. The soils of a Lake Superior watershed examined By Ishii *et al.* (2006) revealed a three order-of-magnitude difference in cell densities from midsummer to midwinter. *Escherichia coli* cell densities in the soils examined

from June to October were as high as 3 X 10^3 colony forming units per gram of soil (CFU/g soil). Lowest cell densities were observed in midwinter and were less than 1 CFU/g soil. Examination of periphytic algal communities in Lake Superior also showed seasonal variation in *E. coli* cell densities. Fecal coliform densities in the periphyton sampled increased from 100 - 10 000-fold seven weeks after initial sampling at three sites. *E. coli* represented approximately 40% of total fecal coliforms sampled, depending on site. Counts then began a slow and steady decline as the summer progressed toward fall (Ksoll *et al.* 2007).

1.1.10 Sources of planktonic bacteria E. coli in the environment

Escherichia coli sampled in water is frequently assumed to originate from some point source and to exist only ephemerally (Leclerc *et al.* 2001). However, there is much evidence to suggest that detectable contributions to these counts originate from non-point sources: algae, sand and sediment. *Escherichia coli* often associates with aquatic algae and with decaying aquatic vegetation and it has been suggested that this association often leads to the inoculation of the surrounding waters (Ishii *et al.* 2006b; Weiskel *et al.* 1996; Whitman and Nevers, 2003; Byappanahalli *et al.* 2003). The contribution of such *E. coli* to surrounding wetland areas is reliant mostly upon physical forces generated by the movement of water, although some cells do detach in the absence of wave action (Ksoll *et al.* 2007). Sand has also been shown capable of harbouring reproducing populations

of *E. coli* (Alm *et al.* 2003; Whitman and Nevers 2003; Anderson *et al.* 2005; Alm *et al.* 2006; Byappanahalli *et al.* 2006b). It has been shown that *E. coli* have the capacity to colonize and reproduce in beach sand once introduced into the environment (Whitman and Nevers 2003; Alm *et al.* 2006). In a study conducted by Whitman and Nevers (2003), the authors indicated that the majority of *E. coli* samples collected from water at a Lake Michigan swimming beach originated from beach sand. Release of *E. coli* from sand on beaches in Lake Superior examined by Ishii *et al.* (2007) is mainly via mechanical disturbance of wave action. In the same study, it was shown that densities of naturalized populations of *E. coli* were highest in August. The authors proposed that it was therefore likely these naturalized strains of *E. coli* could be main contributors to counts in the water column for the month of August.

The release of bacteria from sediments has been previously examined in lakes (Ishii *et al.* 2007), reservoirs (Grimes 1975; Grimes 1980) and streams (Matson *et al.* 1978; Sherer *et al.* 1992: Crabill *et al.* 1999). Many studies examining fecal coliform and *E. coli* survival in sediment have noted significantly higher numbers of the organisms in sediment than in the water column. Alm *et al.* showed that mean summer abundance of *E. coli* was 3-38 times higher in the top 20 cm of sediment than it was in the overlying water. Matson *et al.* (1978) also show a high sediment/water abundance ratio for fecal coliforms. Typically,

mechanical action and disturbance is cited as the cause for resuspension of living fecal coliforms, *E. coli* in particular, from sediment (Grimes 1975; Matson *et al.* 1978; Grimes 1980 and Sherer *et al.* 1992). Crabill *et al.* (1999) have indicated that storm surges and recreational activity are responsible for resuspending sediment-borne microorganisms in streams. Laliberte and Grimes (1982) comment that *E. coli* counts taken from water samples are more a representation of bacterial abundance in disturbed sediment than a true representation of bacterial abundance in the water. Further, Burton *et al.* (1987) suggest that planktonic bacterial sampling is inadequate. They cite the fact that grab-bag collection of water provides a snapshot of the transient planktonic bacteria present in the water, and could be greatly improved by also considering those contained within sediment. Thus, there is a consensus that bacteria, other than those present in planktonic phase, should be considered when assessing recreational waters for safety.

1.1.11 An example of the relationship between biofilm and planktonic E. coli in the environment

Epilithic periphyton is a ubiquitous form of algae that develops on many diverse submerged surfaces: rocks, sand, vegetation, steel and concrete. It is composed of diatoms, green and blue-green algae, bacteria protozoa and fungi (Ksoll *et al.* 2007). One goal in a study performed by Ksoll *et al.* (1997) was to examine colonization and survival of *E. coli* in periphyton communities using a

microcosm experiment and to estimate the contribution of periphyton to the water column. When performing the microcosm experiment, it was found that two days after inoculation, the number of *E. coli* attached to periphyton covered rocks was 200-fold higher than on rocks which had been scrubbed and sterilized prior to inoculation. This indicates that the attachment of the strain of *E. coli* used in the experiment benefited from the presence of periphyton. Notable, however, is the strain of *E. coli* used in the microcosm experiment: NRR27. The strain was a neomycin-, rifampin-, and nalidixic acid resistant mutant and was created from a non-pathogenic periphyton strain collected from Lake Superior. Thus, the microcosm experiment does not investigate the capability of shiga-toxin producing *E. coli* to attach to periphyton on rocks. Rather, the study provides a baseline for future investigation into planktonic and biofilm dynamics, particularly of *E. coli* collected from the environment, but also of pathogenic varieties.

1.2 THESIS OBJECTIVES

The first objective of this thesis was to compare biofilm formation of several strains of pathogenic and environmental biofilm *E. coli* in optimal medium and to subsequently identify genetic similarity between them. This was accomplished by:

i) Collecting biofilm samples from three water bodies in Northwestern Ontario and isolating *E. coli* contained therein

ii) Using a high throughput crystal-violet microplate assay to quantify biofilm formation in a total of 46 strains of *E. coli*

iii) Extracting DNA from all 46 organisms, amplifying the 16SrDNA region via rep PCR and processing it using fingerprint alignment software to inform on genetic relatedness

The second objective of this thesis was to examine biofilm development of three strains of *E. coli*: H32, 1A and K-12 microscopically in optimal laboratory medium as well as relatively nutrient-poor environmentally relevant media. This was accomplished by:

i) Growing biofilms of the organisms of interest on glass cover slips in MSM-0.04% glucose, filter sterilized lake water and a filter sterilized 1:1 mixture of lake water and untreated sewage.

ii) Staining the glass coverslips with attached biofilms to create slides of living fully hydrated biofilms.

iii) Observing biofilms using confocal scanning laser microscopy (CSLM) and analyzing images for biovolume, average thickness and percent substratum coverage using Phobia Laser Imaging Processor

iv) Monitoring plankotnic development of the same organisms over the course of the experiment.

CHAPTER 2 – SCREENING FOR BIOFILM FORMATION IN OPTIMAL MEDIUM

ABSTRACT

Though *Escherichia coli* is a gut microorganism of warm-blooded animals, thriving in the conditions found therein, there is considerable evidence to suggest that the bacterium may be able to survive for long periods or even become naturalized in, temperate aquatic environments. Here, *E. coli* were collected from epilithic periphyton, also known as biofilm, in three water bodies in Northwestern Ontario over the summer and fall of 2008. A crystal violet assay was used to assess the biofilm forming ability of the environmental isolates as well as members of three enterohemorrhagic *E. coli* serogroups O157, O26 and O111 in laboratory medium. The environmental biofilm isolates produced significantly more biofilm than any of the enterohemorrhagic groups ($p<0.001$) with the O157 and O26 groups forming almost none. *E. coli* O111 developed biofilm intermediate to those formed by the environmental, O157 and O26 groups. In addition to showing environmental biofilm isolates of *E. coli* to be more proficient biofilm formers than *E. coli* O157, O26 and O111, this study is the first to demonstrate the persistence of *E. coli* biofilms in inland water bodies of Northwestern Ontario.

2.0 INTRODUCTION

Architecturally and physiologically complex, biofilms are accepted as the dominant bacterial lifestyle in most systems (Stoodely et al., 2004). Organisms show an increased tolerance to a variety of stresses when grown in association with a surface relative to their planktonic brethren (Stoodely et al., 2004). Although microorganisms are capable of independent, free-living existence in their environment, but proliferation is vastly improved through interaction and the formation of communities on a surface (Caldwell et al., 1997). Though *Pseudomonas aeruginosa* is perhaps the most well-studied biofilm forming microbe (Costerton et al., 1999; Wimpenny et al., 2000), *Escherichia coli* also receives its share of attention. Much investigation has been devoted to understanding the role of *E. coli* biofilms in pathogenesis. Uropathogenic *E. coli* biofilms have been implicated as the causative agents in bilary tract infection, urinary catheter cystitis and urinary tract infection (Costerton et al., 1999). Biofilms of *E. coli* O157:H7, an enterohemorrhagic strain, have also been shown to develop in the environs of contaminated slaughtering house equipment (Skandamis et al., 2009). Subsequent contact of meats prepared within the confines of the slaughtering house creates a vector whereby disease-causing organisms may be consumed by humans (Kusumaningrum et al., 2003).

Academically, our understanding of the structure and development in biofilms of *E. coli* is based largely upon the study of *E. coli* K-12 (Van Houdt and Michiels, 2005). This is particularly interesting given that biofilm development in *E. coli* has consistently been shown to be strain dependent (Yeh and Chen, 2004; Reisner et al., 2006). Thus, it behooves the researcher to examine individual strains of *E. coli* to assess their biofilm-forming character rather than relying upon an extrapolation of results from the study of other strains.

Escherichia coli is an endemic bacterial species of the mammalian gut, particularly the colon (Smith, 1965; Tancrede, 1992). Humans will acquire the organism during or shortly after birth, and support a population composed of 2-3 resident strains and 3-4 transient strains throughout life (Sears et al., 1949, Bettelheim, 1997). This endemism, coupled with the bacterium's low tolerance for conditions disparate from those in the mammalian gut, contributed to its selection as an indicator of fecal contamination in natural environments (Jimenez et al., 1989; Tallon et al., 2005). Nonetheless, mounting evidence suggests that it may not be the reliable indicator once assumed. The organism has frequently been isolated from various tropical and, interestingly, temperate environments independent of recorded fecal contamination (Carillo et al., 1985, Solo-Gabrielle et al., 2000; Byappanahalli et al., 2006; Whitman et al., 2007) . Carillo et al. (1985) collected the organism from the those areas of the Mayemes River falling within

the boundaries of the protected Luquillo Experimental Forest, well up-stream of any anthropogenic activity including agriculture and municipal sewage input. None of the organisms isolated therein were known to cause disease in humans.

While it is understood how pathogenic *E. coli* in the planktonic condition may behave once introduced into the environment (Henis et al., 1989; Bretfar and Hoffel, 1992), little is know about the behaviour of said organisms in the sessile condition. Biofilms of commensal, non-pathogenic *E. coli* growing naturally have been recorded. Periphytic communities of non-pathogenic *E. coli* have been isolated repeatedly from the waters of Northern Lake Superior near Duluth, Minnesota (Ksoll et al., 2007). Among these were several *E. coli* strains of undefined origin, thus deemed naturalized, self-sustaining members of the periphytic community. We suspect that *E. coli* capable of self-sufficient existence in such biofilms are endowed for a sessile lifestyle subject to the rigors of environmental conditions. Pathogenic *E. coli* on the other hand, suited to the more consistently moderate environment of the mammalian guts, may not possess the resources and/or genetic traits that would allow them to develop sessile communities when faced with the harsh conditions encountered in temperate water bodies. Thus, a comparison of environmental and pathogenic serogroups will be undertaken here to identify trends exhibited by both groups.

The purposes of this study were three-fold: (1) to collect biofilm *E. coli* from three water bodies in Northwestern Ontario, (2) to determine optimal laboratory conditions to examine differences in biofilm formation and finally, (3) to use the optimal condition to examine differences in biofilm-forming capacity of the collected environmental biofilm *E. coli* as well as representatives of enterohemorrhagic serogroups O157, O111 and O26.

2.1 MATERIALS AND METHODS

2.1.1 Location and nature of sampling sites

Biofilm samples were taken at three sites from each of three water bodies in the Thunder Bay District of Northwestern Ontario. Boulevard Lake, Thunder Bay, Ontario is a widening of the Current River and is frequented by swimmers throughout the summer. Sites one and two were located about 50 m North and South, respectively of the public docks on the western side of the lake. The third site was located immediately west of the dam at the south end of the lake. Chippewa Park, Thunder Bay, Ontario, is a bay on Lake Superior and is also a popular local swimming destination. The three sites sampled were approximately 100 m, 175 m and 225 m west of the beach on the western shore of the bay. Billy Lake, Dorion, Ontario, is a small, undeveloped boreal lake. The three sites sampled were approximately 0 m, 5 m and 10 m south of the access

point on the eastern side of the lake. Samples were collected from all three sites on June 12, July 20, September 23 and October 26 and 27 of 2008.

2.1.2 Collection of environmental biofilm E. coli

At each sampling site indicated above, a submerged rock, with an uppermost surface about 30-40 cm below water's surface was selected for sampling. Rocks were removed from the water by hand and placed on shore with the surface to be sampled facing up. Prior to removal from the water, rocks were gently shaken ten times to remove sediment from the surface to be sampled. All rocks were handled with latex examination gloves (Fisherbrand) disinfected with 85% ethanol.

A sterile, square rubber template delineating an area of 10 cm x 10 cm was placed on the rock and used to quantify the substratum surface to be sampled. Material was scraped from within the area marked by the template 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 Na_2HPO_4, KH_2PO_4, 1L distilled H_2O, pH adjusted to 7.4) in a sterile 125 mL Nalgene bottle. All samples were transported to the laboratory in a cooler packed with ice. In all cases sample processing was initiated within 24 hours of collection.

2.1.3 Water chemistry

In addition to the collection of bacterial isolates, water was sampled from each water body examined in this study as follows: A sterile 500 mL 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.

2.1.4 Isolation of biofilm E. coli

Five mL of the biofilm sample suspended in PBS were drawn through a sterile 47-mm mixed cellulose ester filter (Fisherbrand water testing membrane filter, pore size 0.45 um, Thermo Fisher Scientific, Whitby, ON, Canada) using a sterile pneumatic pump funnel filtration apparatus. Subsequently, the funnel was rinsed with 5 mL sterile PBS to remove any organisms remaining on the wall of the filter funnel onto the filter. Using a 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 hours 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, Calagry, Alberta, Canada) for

isolated colonies. These plates were then incubated at 37°C for 22 hours. 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-Proskauer, Citrate) test series to provide additional confirmation of identity. Isolates positively identified as *E. coli* were assigned an alpha-numeric catalogue number for identification and were maintained in Trypticase Soy Broth (TSB; Becton, Dickinson and Company, Sparks, MD, USA) supplemented with a 25% (v/v) solution of glycerol at -80°C.

2.1.5 Enumeration of heterotrophic bacteria of biofilm samples

In addition to *E. coli*, heterotrophic background bacteria were enumerated and collected from biofilm samples. A 10X dilution series was created from the suspended biofilm sample in sterile PBS buffer. A 200-µL portion from each dilution was spread-plated onto R2A agar (Beckton Dickson and Co., Sparks, MD) using a flame sterilized glass spreading rod. This was done in triplicate. Plates were incubated overnight at 30°C in an incubator. The number of colonies growing on each plate was counted and recorded. Randomly selected colonies were selected and preserved in frozen culture, as described above, for future use.

2.1.6 16S RNA gene sequencing to confirm E. coli identification

2.1.6a DNA extraction

Extraction of genomic DNA was performed using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA). An overnight culture was created by inoculating the bacterium of interest in 30 mL of Luria-Bertani broth and incubating overnight at 37°C and 150 rpm. One mL of the overnight culture was centrifuged at 13 000 x *g* in a Micromax table top centrifuge (Thermo Fisher Scientific) for two minutes to create a pellet. The pellet was resuspended in a Nuclei Lysis Solution (supplied with kit), incubated in an 80°C water bath (Isotemp 205, Fisherbrand) for five minutes and then allowed to cool to room temperature. Three μL of RNase solution (supplied with kit) was then mixed into the cell lysate solution and subsequently incubated in a 37°C water bath (Isotemp 205, Fisherbrand) for 45 minutes. After cooling to room-temperature, the cell lysate solution was combined with 200 μL of Protein Precipitation Solution (supplied with kit) and vortexed vigorously for 20 seconds. The solution was then chilled on ice for five minutes and centrifuged at 13 000 g for three minutes. The DNA-containing supernatant was collected and combined with 600 μL room temperature isopropanol. DNA was precipitated by centrifuging the solution at 13 000 g for two minutes. After pouring off the supernatant, the DNA

pellet was washed with 600 μL of room temperature 70% ethanol and then allowed to air dry on clean, absorbent paper towel.

2.1.6b PCR

A 50 μL reaction mix designed for the amplification of the 341-907 nucleotide region of the 16S RNA gene (i.e. rDNA) was prepared as follows: 29 μL of nuclease free ddH_2O, 5 μL 10X buffer, 5 μL dNTP mix (0.2 mM for each kind of nucleotide), 1 μL primer 1 (10 μM), 1 μL of primer 2 (10 μM), 1 μL of *Taq* DNA polymerase (1 unit/μL), 3 μL of 25 mM $MgCl_2$, and 5 μL (about 0.1 μg) of template DNA. The primers employed were 16S 341-F (primer 1), 5'-CCTACGGGAGGCAGCAG-3', and 16S 907-R (primer 2), 5'-CCCTCAATTCCTTTGAGTTT-3'. All other PCR reagents used herein were procured from Fermentas International (Burlington, ON, Canada). The 16s rDNA fragment was amplified using a Sprint PCR thermocycler (Thermo Fisher Scientific, Waltham, MA, USA) set to produce the following conditions: an initial denaturation cycle at 95°C for 3 minutes, 35 cycles of denaturation at 95°C for one minute, annealing at 50°C for one minute, and extension at 72°C for one minute; followed by a final extension at 72°C for ten minutes and a final indefinite hold at 4°C.

Forty-five μL of PCR product with 8.5 μL of 6X loading dye were loaded into the wells of a thick 1% (w/v) agarose gel and run for 45 minutes at 120V. The

agarose gel was prepared as follows: 1 g agarose gel (Fisher Scientific; Fairlawn, NJ, USA), 0.2 μg/mL (final concentration) ethidium bromide (Bio Rad Laboratories, Hercules, CA, USA), 100 mL TBE buffer. Gel fragments of approximately 1 g were excised from the gel and purified using the Fermentas Pure Extreme DNA Extraction kit (Fermentas, Burlington, ON, Canada) as follows: The band of interest was excised from the gel and combined with 0.5 mL of TBE Conversion Buffer and 4.5 mL Binding Solution (each supplied with kit) and incubated in a 55°C water bath (Isotemp 205, Fisherbrand Scientific) for five minutes. Subsequently, 6 μL of resuspended silica powder suspension (supplied with kit) was added. The mixture was then incubated for five minutes in a 55°C water bath (Isotemp 205, Fisherbrand Scientific) and vortexed at two minute intervals followed by centrifugation at 13 000 x g for five minutes to form a pellet. The supernatant was discarded and the pellet washed three times with ice-cold Wash Solution (supplied with kit). After the third wash the pellet was air-dried for 15 minutes. Using sterile ddH_2O, the pellet was resuspended to elute DNA contained therein. The solution was incubated in a 55°C water bath (Isotemp 205, Fisherbrand Scientific) for five minutes and spun at 13 000 x g for thirty seconds. The DNA-containing supernatant was collected and centrifuged again at 13 000 x g for an additional thirty seconds. Following this final centrifugation, the DNA concentrations of the remaining supernatant were

verified by comparing to a 1 Kbp DNA ladder standard on a 1% (w/v) agarose gel run at 120 V for forty-five minutes.

Samples were sequenced at the Mobix Laboratory (McMaster University, Hamilton, ON, Canada). Isolates were identified by comparison to homologous sequences of known identity using the Basic Local Alignment Search Tool (BLAST) available from the National Center for Biotechnology Information (NCBI, Zhang et al., 2000).

2.1.7 Bacterial strains

Escherichia coli 0157:H7 and H-, O111:H8 and H-, and O26: H11 and H- strains used in this study were obtained from C. Gyles at the University of Guelph (Guelph, ON, Canada). Environmental isolates of *E. coli* were isolated and identified in this study as indicated above. Eleven representatives of O157 (four of bovine and seven of human origin), seven representatives of O111 (four of bovine and three of human origin), eleven representatives of O26 (five of bovine and six of human origin) and seventeen environmental biofilm *E. coli* isolates were examined in this study. For a comprehensive list of organisms used for this study see (Table 2).

For this study, all organisms were cultured in 35 mL sterile Luria-Bertani (Miller) broth (LB; Becton, Dickinson and Company, Sparks, MD, USA) at 37°C with 150 rpm. Stock cultures of all *E. coli* were maintained in Trypticase Soy

Broth (TSB; Becton, Dickinson and Company, Sparks, MD, USA) supplemented with a 25% (v/v) solution of glycerol at -80°C. *E. coli* were recovered from frozen stock by streaking for isolated colonies on Luria-Bertani agar (LBA) and incubating overnight at 37°C.

2.1.8 Biofilm quantification

Biofilm inocula were created by streaking frozen stock onto Luria-Bertani agar (LBA, Becton, Dickinson and Company, Sparks, MD, USA) for isolated colonies. Individual colonies were then sub-cultured onto new LBA plates. An isolated colony was selected from the sub-culture plate and used to inoculate 30 mL of Luria-Bertani broth in a 125 mL Erlenmyer flask. The flask was incubated overnight at 37°C with shaking at 150 rpm. Ten mL of overnight culture was withdrawn from the flask using a sterile disposable 10 mL glass pipette (Costar, Corning, NY, USA) and transferred to a 15 mL disposable centrifuge tube (Fisherbrand, Thermo Fisher Scientific,). The cells in the tube were washed three times as follows: centrifugation at 2050 x *g*, disposal of the supernatant, addition of 10 mL of sterile PBS followed by vortexing for 30 seconds. After the final wash step, cells were resuspended to an optical density ($OD_{@\ 600nm}$) of 1 ± 0.05 (about $1x10^9$ CFUs/mL). The OD1 cells were serially diluted and five 5 µL drops of each dilution was drop plated onto LBA to confirm cell densities. The biofilm inoculum was created by combining 10 µL of the OD1 cell

suspension with 190 µL of the medium of interest: MSM-0.04% glucose (MSM-glu) or 1 X TSB. The experiment was repeated in both media. MSM-glu was suggested as an appropriate medium for the culture of *E. coli* biofilms by Dewanti and Wong (1995) while TSB was selected as a comparatively rich laboratory medium. Eight replicate wells in a 96-well, flat-bottomed polystyrene microplate (Costar, Corning, NY, USA) were used for each isolate/or strain (200 µl inoculum/well). Inoculated plates were then incubated at the desired temperature: (the experiment was repeated at 22°C and 37°C) for 48 hours with 25 rpm in an Innova 4430 incubator (New Brunswick Scientific, Edison NJ, USA). Incubation temperatures were chosen according to their ecological and biological relevance. Throughout the summer months, temperatures of the water bodies examined hovered near 22°C. And while a temperature as high as 37°C was never recorded, it represents the optimal growth temperature for *E. coli*, thus the basis for the selection of each temperature. After incubation, plates were removed from the incubator and wells washed by rinsing three times with sterile double distilled water. Rinsate was removed from wells by gently shaking over a waste container and blotting on paper towel. Plates were then allowed to air dry for 30 minutes in a Labguard level 2 biosafety cabinet (NuAire, Minneapolis, MN, USA). After drying, replicate wells were stained with 200 µL of 0.1% (w/v) crystal violet solution (Harleco, Gibbstow, NJ, USA) for ten minutes. Residual

crystal violet was removed from the microplates by shaking over a waste container and rinsing wells three times with sterile double distilled water. Plates were then allowed to air dry for thirty minutes in the Labguard level 2 biosafety cabinet. The stain was re-solubilized by exposure to 200 μL of a 20% acetone and 80% ethanol solution for ten minutes. One hundred and fifty μL of re-solubilized stain was then transferred to the corresponding well of a replicate microplate. Absorbance readings of the re-solubilized stain were taken at 600 nm using a Fluostar Optima, automated absorbance plate reader (BMG Labtech, Offenberg, Germany). Absorbance readings were also taken of eight blank wells per microplate, treated as above but without inoculum, to be used as a control. The average absorbance of the 8 blank wells was subtracted from the absorbance reading of each treatment well. This was a means of eliminating background absorbance created by the plate. With the blank absorbance subtracted, the average of each eight treatment-well group was taken as the absorbance reading for that bacterium under the given conditions. This average was used to compare biofilm development between bacteria under the four conditions tested. As a means of comparing biofilm formation among serogroups, averages of all eight treatment-well averages of the bacteria within a serogoup were calculated.

2.1.9 Statistical analyses

A two-way analysis of variance was used to analyze the differences in environmental biofilm *E. coli* cell densities over time. For the purpose of identifying an optimal medium-temperature combination a treatment group was considered to be the mean absorbance reading of eight replicate wells per strain of *E. coli* with each of four combinations of incubation temperature and growth medium as the factors. A Kruskall-Wallis one-way analysis was used to compare the treatment groups, followed by a Nemenyi's multiple comparison procedure. Biofilm forming capacity of environmental and enterohemorrhagic *E. coli* under optimized conditions was evaluated using a one-way analysis of variance. All statistical analyses were performed using Sigmaplot and Sigmastat software (SPSS, IBM, Chicago, Illinois).

2.2 RESULTS

2.2.1 Collection and isolation of E. coli from the environment

E. coli were collected from all three target water bodies in Northwestern Ontario. *E. coli* strains 1A, 1B, and 1D were isolated from a rock adjacent to the dam and 1G from a rock upstream from the public beach on Boulevard Lake. Strains 2A, 2B, 2C, 2D and 2E were collected from a submerged log, 2L and 2S from a rock, each South of the access point on Billy Lake. Strains 3B, 3C, 3D, 3E, 3F, and 3J were each isolated from a common rock east of the public beach in

Chippewa Park. Physical and chemical characteristics including water temperature, pH, and nutrient concentrations of each water body examined are listed in Appendicies 1.2 and 1.3, respectively.

The sites were each sampled once per month, from June to October with the exception of August, wherein no sampling took place. No trends in cell density, with respect to temporal variation were evident. However, *E. coli* were consistently present at detectable levels in each water body studied (Figure 1).

Using CHROMagar *E. coli* agar (Dalynn Biologicals), *E. coli* were presumptively identified as *E. coli*. Identification was corroborated using the IMViC test series (IMViC; Indole, Methyl Red, Vogues-Proskauer, Citrate tests) (Bergey's, look up). All strains examined showed a 99% similarity with *E. coli* K-12 (substrain MG1655) with the exception of 2F, which bore a 98% similarity to the former (Table 1) (Blattner *et al.*, 1997). A similarity of greater than 97% between a known bacterium and one which is unknown allows identification of the latter to the level of species (Woo *et al.* 2009).

2.2.2 Heterotrophic bacterial enumeration

In addition to *E. coli*, heterotrophic background bacteria from biofilms were also isolated and enumerated. Heterotrophic bacteria were present in densities from roughly 10^7CFU/100 cm^2 to 10^9 CFU/100 cm^2 (Figure 2). Thus, in biofilm, *E. coli* represented less than one percent of the total bacterial population.

2.2.3 Medium selection for biofilm screening

Four medium-temperature combinations were examined in order to establish an optimum set of conditions under which to examine biofilm formation. Biofilm formation of all 46 strains of *E. coli* was examined at 22°C and 37°C in a Minimal-Salt-Medium supplemented with 0.04% glucose (MSM-glu) and 1X trypticase soya broth. Biofilm formation by the O157 and O26 serogroups showed no difference in any of the four medium-temperature combinations examined, (H32, 2-Way ANOVA, n=10, df= 9, p=0.403) and (O26, 2-Way ANOVA, n=11, df-10, p=0.097), respectively (Figure 3). Members of the O111 serogroup formed more biofilm in MSM-glu at 22°C than in MSM-glu at 37°C ($p<0.001$) or 1X TSB at 37°C ($p<0.001$), however, no difference was observed when compared to 1X TSB at 22°C. The environmental *E. coli* showed biofilm formation significantly higher in MSM-glu at 22°C than 1X TSB at 37°C ($p<0.001$) and MSM-glu at 37°C ($p<0.001$), though comparison to biofilm formed in 1X TSB at 22°C showed no difference (Figure 3). Thus, MSM-glu and 22°C were chosen as the optimal conditions used to screen for biofilm formation.

2.2.4 Biofilm formation of environmental and enterohemorrhagic E. coli under optimized conditions

Under optimized condition in MSM-0.04%glu at 22°C, environmental isolates of *E. coli* were the strongest biofilm formers and formed significantly

more biofilm than the 0157 and O26 serogroups, $p<0.001$ and $p<0.001$ respectively, with the latter two forming almost none (Figure 3). 1G, an environmental isolate, formed much less biofilm than other members of the environmental group (Figure 4) and was also displaced from other isolates collected from Boulevard Lake based on DNA fingerprint analysis (Figure 5). Little difference in biofilm forming character was observed between the groups O157 and O26, $p=0.997$. Showing a less distinct tendency toward a particular trend in biofilm formation, O111 demonstrated an intermediate tendency to form biofilm: higher than either O157 or O26, $p<0.001$, but significantly less than the environmentals, $p=0.022$.

2.2.5 REP-PCR

In general, the REP-PCR DNA fingerprints (Figure 5) of *E. coli* strains within a serogoup clustered more closely together than those between serogroups and often showed similarity greater than 82%. However, some exceptions to this trend were noted. The fingerprints of *E. coli* H40, a strain of O111, fell within cluster II, otherwise dominated by members of the O26 serogroup. Additionally, two members of the O157 serogroup; H19 and H32, also fell within the realm of cluster II and were particularly distant from cluster V, which included the remaining 9 strains of O157. Further, *E. coli* collected from one environmental site also tended to cluster closely, though again, with

exceptions. *E. coli* isolates from Site 1, all fell within the same cluster except for 1G which was included in cluster I (otherwise dominated by *E. coli* O111) and showed more similarity with members of the O111 serogroup (~ 82% similarity) than with its environmentally isolated brethren (77% similarity). Moreover, 1G formed significantly less biofilm than other members of the environmental group ($p<0.001$)(Figure 4). Site 2 *E. coli* isolates shared cluster III with about 90% similarity. *E. coli* from the third collection site are represented by cluster IV save for 3E which was included in cluster III. Worth noting is that all environmental isolates are distinct. This is to say there were no bacteria replicated in the study.

2.3 DISCUSSION

E. coli was collected over a period of five months from three water bodies in Northwestern Ontario. Cell densities of *E. coli* growing in biofilm were not different between sites ($p=0.795$) nor did they vary significantly from one sampling event to the next ($p=0.282$). Nonetheless, *E. coli* was present on all sites throughout the summer and fall suggesting that it is capable of persisting throughout the seasons where water temperatures fluctuated between 5°C and 23°C (Appendix 1.1). And while no trend in temporal variation was apparent in the data, this does not necessarily indicate that none exists. Sampling earlier and later in the year may have helped to clarify a temporal trend in bacterial densities. Ksoll et al. (2007) observed that beginning in the spring, the density of

E. coli in biofilm growing on rocks along the shore of Lake Superior increased until mid-late summer and then gradually decreased until freeze-up. The high variability exhibited by the temporal data herein is likely reflective of the largely differing cell densities exhibited from one rock to another at each sampling site

Escherichia coli has been isolated from myriad different natural tropical and temperate ecosystems: farms, lakes, streams, forest soil, beach sand, and epilithic periphyton (Carillo et all, 1985; Whitman and Nevers, 2003; Whitman et al. 2006; Whitman et al. 2003; Ksoll et al, 2007). Its discovery as a component of epilithic periphyton, also known as biofilms, represents a departure from the all-so-common focus inherent in the literature: the isolation of the organism in its free-living state. Moreover, it demonstrates agreement with the theory that the majority of microorganisms in a population will preferentially exist in the sessile state (Costerton et al., 1995). With reports of existence of naturalized *E. coli* biofilm populations in a temperate water body documented (Byapanhalli et al., 2006; Ksoll et al., 2007), it becomes important to study the behaviour of both environmental and pathogenic varieties of the bacterium.

Here environmental strains of *E. coli* collected from biofilms growing in northern Lake Superior and two other water bodies in the vicinity of Thunder Bay, Ontario as well as members of three enterohemorrhagic serogroups of *E. coli* were examined for biofilm formation. This was done with a goal of elucidating

whether enterohemorrhagic strains of *E. coli*, known to cause disease in humans, could become established in much the same way as their environmental counterparts. The ability of enterohemorrhagic *E. coli* to establish natural, sessile communities once shed in the environment represents an obvious risk to public health and merits investigation. In this study, it was observed that *E. coli* isolated from the environment were far more adept at forming biofilm than any of the pathogenic *E. coli* examined. This suggests, perhaps, that the environmental biofilm isolates are endowed for an existence beyond their primary human or animal hosts. In other words, these microorganisms have become a member of the naturally occurring microflora adept at coping with the rigors encountered beyond the limits of said host. Based on repeated isolation, and an absence of similarity with a source library Ksoll et al. (2007), concluded that *E. coli* collected from periphyton communities of northern Lake Superior had become naturalized and self-sustaining. This was not the first instance of the organism being isolated from an environment without a link to a discernible source of input. Repetitive extragenic palindromic polymerase chain reaction was used by Byappanhalli et al. in 2006 to show that a consortium of *E. coli* sampled from forest soils draining a Lake Michigan watershed were genetically distinct from any likely animal sources in the region, such as ducks, geese, terns and deer. Lopez-Torres et al. (1987) acknowledged the likelihood of *E. coli* becoming part of

the autochthonous microflora of the Mayemes River in Puerto Rico. The investigators found that *E. coli* were capable of persisting for long periods in the river ecosystem all-the-while remaining physiologically active. More importantly, fecal contamination was completely absent within those areas wherein *E. coli* had been collected indicating that the *E. coli* present must have been naturalized. Thus, it is possible that the *E. coli* collected from biofilms in Boulevard Lake, Chippewa Park and Billy Lake from spring until fall 2008 may be naturalized members of the local microfauna.

REP-PCR fingerprinting was performed on each *E. coli* strain/isolate screened herein for biofilm formation. The *E. coli* isolates collected from the environment were all distinct from one another, thus eliminating any suspicion that observed similarities in biofilm formation among environmental biofilm *E. coli* were a result of duplicates of the same organism. Strains within serogroups usually clustered together though there were exceptions. Most notable were the separation of 1G (environmental), H32 (O157), and H19 (O157) from the clusters formed by their respective serogroups. Interestingly, when screened under optimal conditions, 1G proved to be an outlier forming significantly less biofilm than any other member of the environmental biofilm *E. coli* ($p<0.001$). In contrast, H32 and H19, with fingerprints largely different from other representatives of the O157 serogroup, showed biofilm formation characteristic of 0157

membership. Furthermore, H32 and 1A, poor and proficient biofilm formers, respectively, showed only a 15% difference in REP-PCR fingerprint- a stronger similarity than was observed between individual members of the environmental *E. coli* which were isolated at a common collection site. Thus, it is difficult to draw conclusions regarding the link between REP-PCR fingerprint and biofilm-forming proficiency of the broad range of *E. coli* examined.

Revealed by this study is the fact that *E. coli* are constitutively present in biofilms during the summer and fall months in the three Northwestern Ontario water bodies examined. Though *E. coli* has been shown to exist as a member of large bacterial biofilm consortium in Lake Superior (Ksoll et al., 2007), this is the first record of the bacteria's presence in biofilms in smaller, inland lakes. Moreover, this study is the first to consider and compare the biofilm forming competency of *E. coli* isolated from the environment alongside enterohemorrhagic varieties, namely O157, O26 and O111, under optimal laboratory conditions. Differences in biofilm synthesizing capabilities of the environmental and enterohemorrhagic *E. coli* screened were significant thereby creating opportunities for future mechanistic studies focusing on the elucidation of the physiological traits responsible for the phenotypes observed.

Figure Legend

Figure 1. Mean densities and standard deviations of three measurements of densities of *E. coli* in biofilms sampled From Boulevard Lake, Chippewa Park (Lake Superior) and Billy Lake from spring to fall 2008.

Figure 2. Mean densities and standard deviations of three measurements of densities of background bacterial isolated from biofilms collected from three water bodies in Northwestern Ontario from spring to fall 2008.

Figure 3. Average absorbance readings (relative biomass) and standard deviations of environmental isolates of *E. coli* as well as *E. coli* O111, O157 and O26 under four medium-temperature combinations. Environmental strains are significantly more efficient formers of biofilm than the O157 and O26 serogroups in MSM-0.04% Glu at 22°C (1-way ANOVA).

Figure 4. A comparison of biofilm formation of the various strains of environmental and enterohemorrhagic *E. coli* examined organized by serogroup. Each triangle represents on an individual isolate of the indicated group. Star indicates the outlier 1G.

Figure 5. Percent similarity of aligned REP-PCR fingerprints of all representatives of environmental and enterohemorrhagic *E. coli* examined in this study.

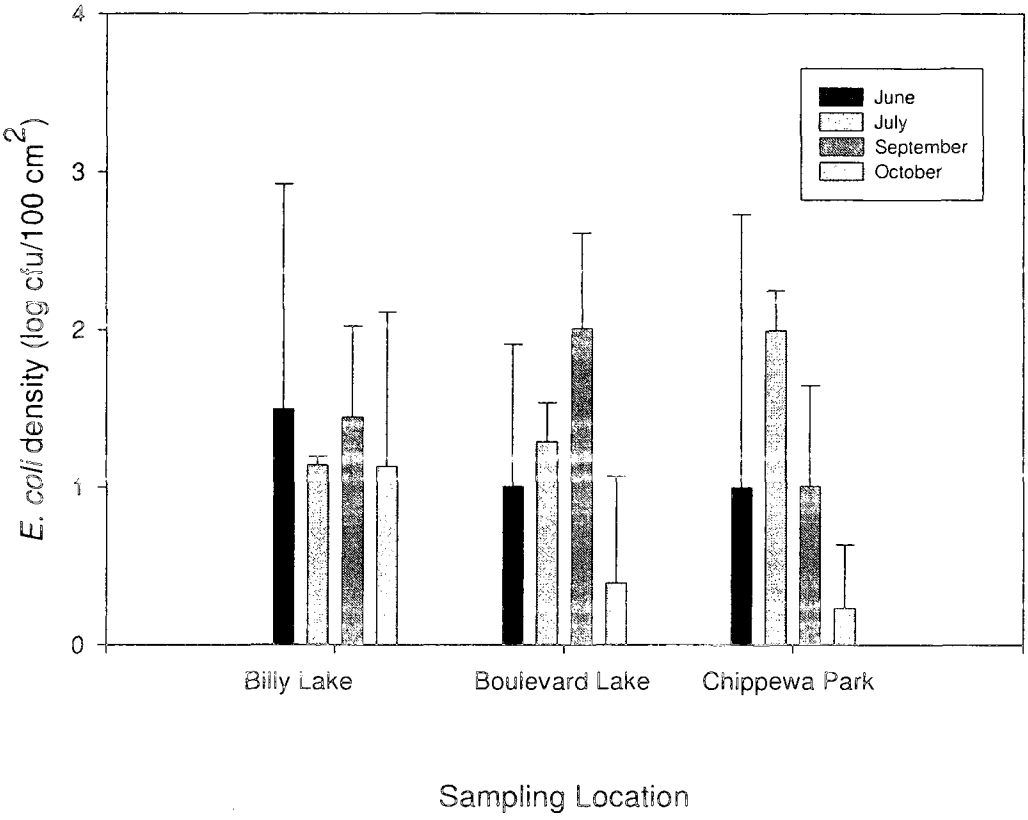
4
3
2
1
0
E. coli density (log cfu/100 cm2)
June
July
September
October
Billy Lake
Boulevard Lake
Chippewa Park
Sampling Location

Figure 1

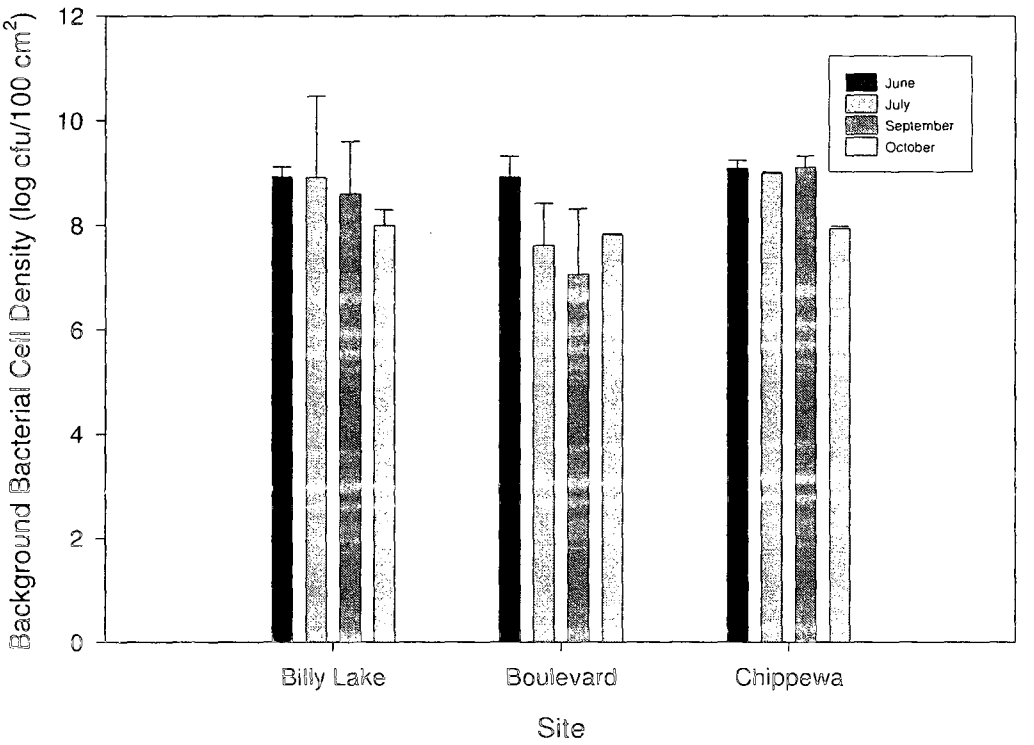
Background Bacterial Cell Density (log cfu/100 cm2)
12
10
8
6
4
2
0
June
July
September
October
Billy Lake
Boulevard
Chippewa
Site

Figure 2

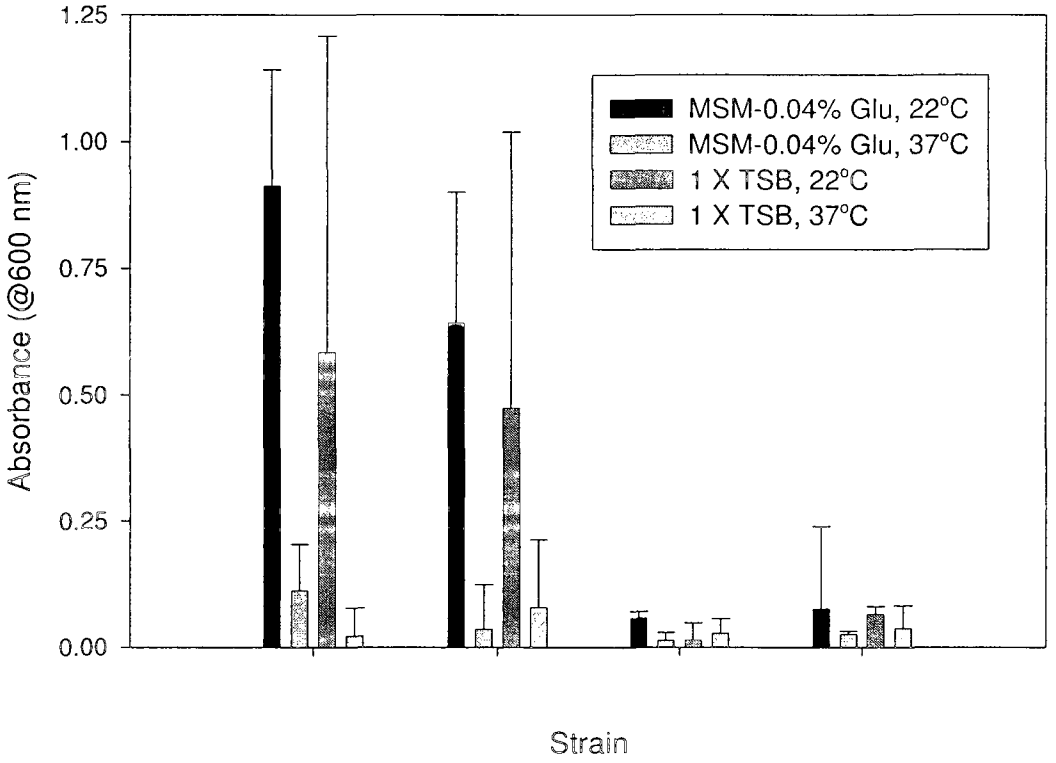
1.25
1.00
0.75
0.50
0.25
0.00
Absorbance (@600 nm)
MSM-0.04% Glu, 22°C
MSM-0.04% Glu, 37°C
1 X TSB, 22°C
1 X TSB, 37°C
Strain

Figure 3

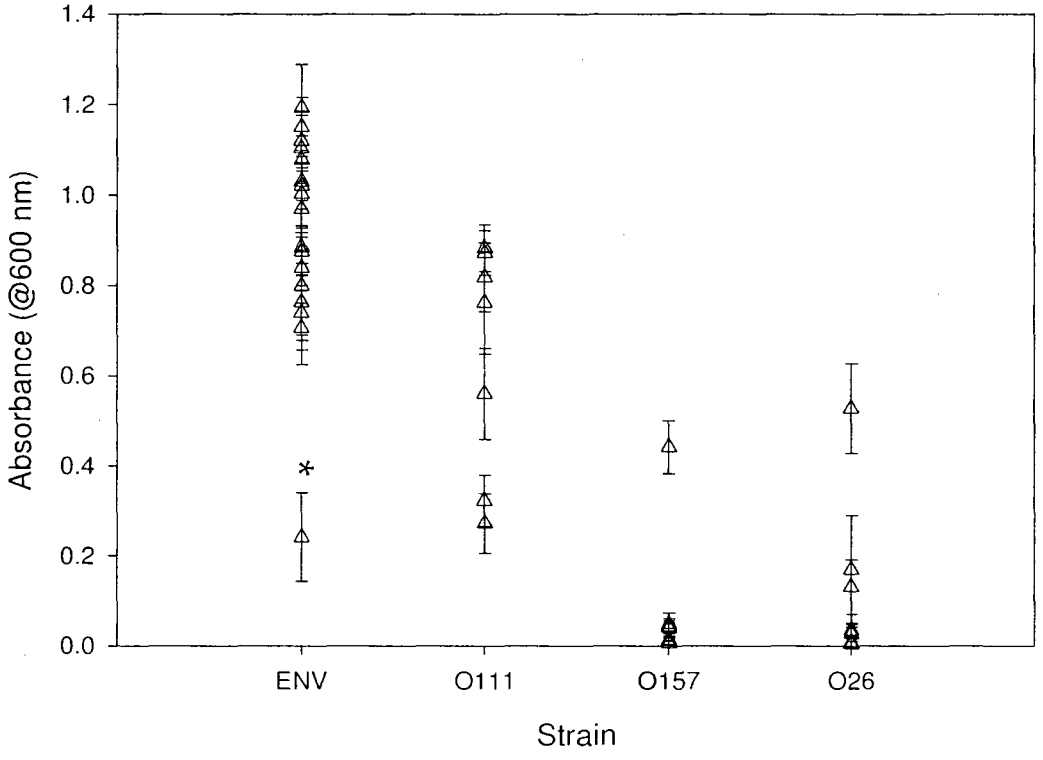
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Absorbance (@600 nm)
*
ENV
O111
O157
O26
Strain

Figure 4

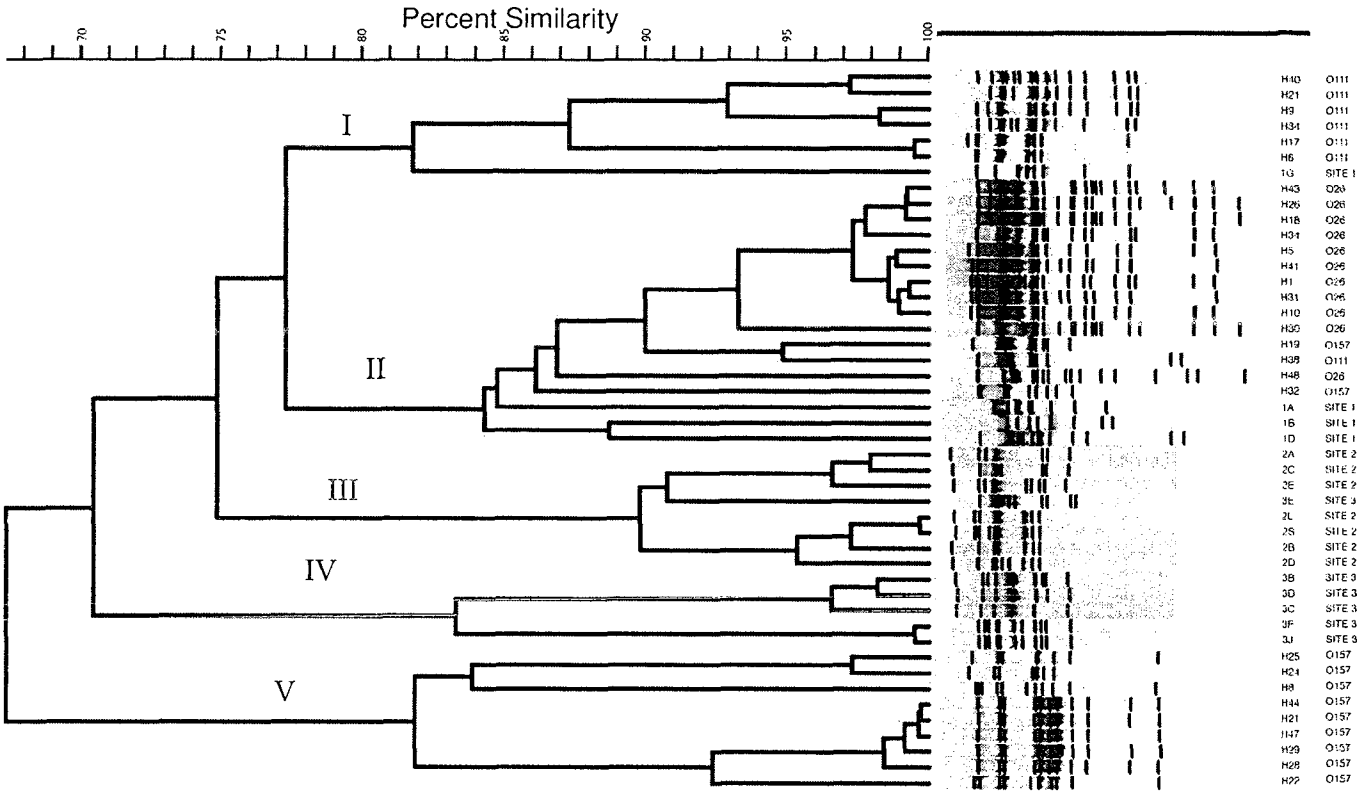

Figure 5

Table 1. Percent similarity of 16S rRNA gene of collected environmental *E. coli* isolates to that of standard *E. coli* K-12 (substrain MG1655).

Strain	Collection Location	% Similarity (K-12)	No. Bases Used in Alignment
1A	Boulevard Lake	99%	502
1D	Boulevard Lake	99%	530
1G	Boulevard Lake	99%	536
2A	Billy Lake	99%	388
2F	Billy Lake	98%	354
2L	Billy Lake	99%	535
2S	Billy Lake	99%	529
3A	Chippewa Park	99%	535
3F	Chippewa Park	99%	532
3J	Chippewa Park	99%	307

Table 2. Origin, serotype and strain of the enterohemorrhagic *E. coli* used in this study.

Strain	Serotype	Origin
H32	O157: H7	Bovine
H47	O157: H-	Human
H29	O157: H7	Human
H21	O157: H7	Human
H44	O157: H7	Bovine
H28	O157: H-	Bovine
H19	O157: H-	Human
H22	O157: H7	Human
H25	O157: H-	Human
H24	O157: H-	Human
H8	O157: H7	Bovine
H17	O111: H-	Bovine
H6	O111: H-	Bovine
H34	O111: H-	Human
H40	O111: H-	Bovine
H12	O111: H-	Human
H38	O111: H-	Human
H9	O111: H8	Bovine
H35	O26: H11	Human
H43	O26: H11	Bovine
H18	O26: H-	Human
H26	O26: H-	Bovine
H48	O26: H11	Human
H10	O26: H11	Human
H1	O26: H11	Bovine
H5	O26: H	Human
H31	O26: H-	Bovine
H4	O26: H-	Human
H30	O26: H11	Bovine

CHAPTER 3 – ANALYSIS OF ENVIRONMENTAL AND ENTEROHEMORRHAGIC *E. coli* BIOFILMS USING CONFOCAL SCANNING LASER MICROSCOPY

ABSTRACT

Bioiflm studies of *Escherichia coli* have typically focused either on O157:H7 or the standard K-12 strain in defined, laboratory medium. While there is value in such studies, they offer little in the way of explaining the behaviour of the bacterium in natural conditions. Furthermore, with evidence mounting to support the likelihood of persistent, naturalized populations of *E. coli*, a study of biofilm development in natural conditions is called for. Here, the biofilm development of three strains of *E. coli* H32 (O157:H7), K-12 (standard laboratory strain) and 1A (collected from a biofilm in a Northwestern Ontario lake) was examined using confocal scanning laser microscopy. Biofilm formation was studied in minimal salt medium supplemented with 0.04% glucose (MSM-glu), filter sterilized lake water as well as in a 1:1 mixture of filter sterilized lake water and untreated sewage (50-50). Planktonic survival of the bactetria was also monitored throughout the course of the experiment. The environmental isolate 1A was the only strain capable of developing mature biofilm. Mature 1A biofilms formed only in MSM-glu. In 50-50 H32 was able to form biofilms which were

structured but which did not reach maturity. Interestingly, in lake water and 50-50, biofilm formation of all three strains was positive while planktonic cell densities continually decreased over time. Opposing trends in biofilm and planktonic development in reduced media indicate that biofilm formation is a survival strategy under conditions of stress. Lastly, H32's ability to form semi-mature biofilm in 50-50 suggests the likelihood of its establishment if introduced into a water body contaminated with sewage- not an unlikely event. The associated implications for public health are far-reaching and must be taken seriously.

3.0 INTRODUCTION

The earliest record of microscopic observation of biofilms dates back to the time of Antonie van Leeuwenhoek. Using one of his primitive microscopes he was able to observe the "animalculi" present in the dental plaque he had scraped from his own teeth (Slavkin, 1997). Fast forward 200 years to the 1970's. Fully hydrated biofilms could be observed using light microscopy adding to the momentum of research into sessile community structure (Geesy et al., 1977). The scanning electron microscope allowed a detailed view of the surface of biofilms and the matricies enclosing them (Geesy et al., 1977). Nonetheless, the understanding of biofilm structure was hindered by limitations inherent in each visualization technique. Light microscopy was deemed useful only for the

observation of thin biofilms as resolution was poor, and haziness was the norm when thicker biofilms were observed (Caldwell et al., 1986; Lawrence et al., 1989). Thus, the technique was employed only for the examination of biofilms in the earliest stages of development (Caldwell et al., 1986; Lawrence et al., 1989). Sophisticated as it was, the scanning electron microscope too was ill-suited to the study of complex sessile communities. Sample preparation necessitated the sacrifice and total dehydration of organisms to be observed and thus did not permit the viewing of living microbes (Kinner et al., 1983). The shortcomings inherent in each technique contributed to the (fundamentally flawed) running model of the time: that biofilms were more-or-less homogeneous aggregations of cells embedded randomly and evenly in a self-synthesized exopolysaccharide matrix (Costerton et al., 1987).

Enter the confocal scanning laser microscope (CSLM). Here was a tool that allowed the visualization of living, fully hydrated biofilms (Lawrence et al., 1991). Furthermore, samples at any developmental stage could be observed, thus allowing researchers the ability to record biofilm formation over time. Moreover, the tool could be interfaced with cameras to record images under the objective, but also with software capable of assessing the cellular and non-cellular areas within the biofilm matrix (Lawrence et al., 1991). Naturally, its application to the study of biofilms led to abandonment of the model portraying biofilms as

homogeneous and random in favour of one depicting a far more complex and organized system (Caldwell et al., 1992 and Lawrence et al., 1991).

Biofilm formation typically proceeds via a series of discreet steps; i. reversible attachment, ii. irreversible attachment, iii. development of micro-colonies, iv. maturation and v. detachment which culminate in the synthesis of an architecturally complex entity (Stoodley et al., 2002). Once mature, a biofilm resembles clusters of mushrooms with broad heads supported on much narrower stalks (Costerton et al., 1995). Quite elegant, this architecture is the result of a reorganization of constituent cells toward the periphery of the biofilm (Davies et al., 1998) which also creates voids between the stalks. These spaces, dubbed water channels, allow the influx and efflux of nutrients and wastes respectively (Costerton et al., 1995) and answered the question of how microorganisms embedded deep within biofilm matrix could survive (Lawrence et al., 1991).

Studies examining the development and structure of biofilms have typically focused on *Pseudomonas aeruginosa* (Costerton et al., 1999; Wimpenny et al., 2000). Indeed much of our understanding of the architecture of a mature biofilm is derived from studies of this bacterial species (Costerton et al., 1999; Wimpenny et al., 2000). Still, *E. coli* remains an important model organism in the study of biofilms and has been studied (Pratt and Kolter, 1998; Geneveaux et al,

1999; Danese et al., 2000a, Prigent-Combaret et al., 2000). Using CSLM, Reisner et al. (2003) showed that K-12, a model strain of *E. coli,* could produce a significant amount of mature biofilm given the correct conditions. In 2006, Reisner's group examined how environment and genome influence the ability of *E. coli* to form biofilm in various laboratory media, simultaneously comparing biofilm formation in the urinary tract infection-causing *E. coli* and and K-12 strains. Dewanti and Wong (1995) examined the effect of culture conditions on the development of *E. coli* O157:H7 biofilms and showed that biofilm formation was best in low nutrient media. Common to all of the above mentioned studies was the use of artificial laboratory media when assessing biofilm development. In an effort to understand bacterial behaviour and ecology, natural aquatic ecosystems must be examined.

Studies have been undertaken to examine the survival of *E. coli* in freshwater systems. However, most focus on an inoculum introduced into, and monitored in, sediment (Laliberte and Grimes, 1982; Burton Jr. et al., 1986). Microcosm experiments have examined how *E. coli* might behave when introduced into freshwater ecosystems, but also focus on the establishment in sediment (Brettar and Hoffle, 1991). Persistent planktonic populations are unlikely as protozoan grazing has been shown to quickly reduce the density of bacteria shed into a freshwater water body (Gurijala and Alexander, 1990). The

evaluation of biofilm formation and architecture by *E. coli* O157:H7 has yet to be made in natural media including lake water and sewage contaminated lake water.

In this study, scanning confocal laser microscopy was used to examine biofilm formation of three strains of *Escherichia coli*: H32 (O157:H7), K-12 (MG1655), and 1A, a collected environmental biofilm strain. Biofilm development of these *E. coli* strains was compared in MSM-glu, filter sterilized lake water and 50-50 with the goal of identifying developmental and structural differences among the three strains in laboratory and simulated environmental conditions. This research is a useful first step in understanding the biofilm forming capacities of environmental and O157:H7 *E. coli*.

3.1 MATERIALS AND METHODS

3.1.1 Media

In this study, the sessile and free-living lifestyles of *E. coli* 1A, H32 and K-12 *E. coli* were examined in three aqueous media: MSM-0.04%glu (MSM-glu; 1.249 mM KH_2PO_4, 3.73 mM K_2HPO_4, 0.4 mM $MgSO_4$. $7H_2O$, 0.02mM $FeSO_4.7H_2O$, 1.4 mM NH_4Cl, 0.04% glucose (w/v)) , filter-sterilized lake water, and a 1:1 mixture of filter-sterilized lake water and untreated sewage which shall henceforth be referred to as 50-50. MSM-glu is an artificial growth medium that provides an optimal amount of nutrients to the developing bacteria. Filter-

sterilized lake water and 50-50 were chosen to mimic the nutrient levels of natural and sewage-contaminated water bodies, respectively. Lake water was collected from Boulevard Lake, Thunder Bay, ON. Untreated sewage was collected from the Thunder Bay Waste Water Treatment facility (Thunder Bay, ON)

3.1.2 Planktonic growth and survival of E. coli 1A, H32 and K-12

In addition to examining biofilm formation over time, planktonic growth of *E. coli* H32, 1A and K-12 were examined. To create the starting inoculum, an isolated colony was used to inoculate 35 mL of Luria-Bertani broth (Difco) in a 150 mL Erlenmeyer flask. The flask was incubated overnight at 37°C with shaking at 150 rpm. Ten mL of the overnight culture was withdrawn from the flask using a sterile disposable glass pipetter (Costar, NJ, USA) and transferred to a sterile 15 mL disposable centrifuge tube (Fisher Scientific). The tube was then spun at 2050 x *g* for ten minutes at 4°C to pellet the cells. The supernatant in the tube was discarded and replaced with 10 mL ddH_2O. The tube was then vortexed to resuspend the cells. This cell washing was repeated three times. Following the third wash step, the cells were resuspended to and $OD_{@600\ nm}$ of 1 (about 10^9 CFU/mL) in the medium being considered: either MSM-glu, sterilized lake water or 50-50. The OD1 cell suspension was then diluted using the same medium until a concentration of between 10^6-10^7 CFU/mL was reached. Thirty

five mL of the diluted cell suspension was incubated in an Erlenmeyer flask at 22°C with shaking at 150 rpm for 96 hours. At 24, 48 and 96 hours 0.5 mL of the cell suspension was withdrawn and serially diluted in sterile ddH_2O. Serial dilutions were drop plated (3 replicates of five 5-μL drops per dilution) onto Luria-Bertani agar (Difco) and the plates incubated overnight at 37°C. The following day, colonies were counted and recorded.

Ten mL of an overnight culture was transferred to a 15 mL Fisher Brand centrifuge tube (Fisher Brand, USA) and centrifuged at 2050 *x g* for 10 minutes at 4°C. The supernatant was decanted and the pellet resuspended by vortexing in 10 mL of sterile ddH_2O. This cell washing procedure was repeated 3 times. Following the third wash step, the cells were resuspended to an OD_{600nm} of 1 (about 10^9 CFU/mL) in the medium being considered: either MSM-glu, filter-sterilized lake water or filter-sterilized 50-50. OD_{600nm} 1 cell suspensions were then diluted 10^2 times in the same medium yielding a cell density of about 10^7 CFU/mL. Concentration was verified by drop plating five 5-μL drops of the serially diluted 10^7 CFU/mL cell suspension onto Luria Bertani agar (LBA, Difco). Subsequently, 5 mL of the 10^7 CFU/mL inoculum was transferred to a well in a 6-well flat bottomed polystyrene microplate (Costar, Corning, NY, USA). Sterile, acid washed 22 mm X 22 mm premium glass coverslips (Fisher Scientific,,USA) were used as a substratum for biofilm growth and were placed in the bottom of

the well after the addition of inoculum. Cover slips were prepared as follows: soaking in 70% nitric acid (v/v) (Fisher Scientific) for 10 minutes, rinsing 3 times in fresh, sterile ddH_2O, and rinsing with methanol (Fisher Scientific). Cover slips were allowed to air dry on clean paper towel. Cover slips were sterilized by autoclaving at 121°C and 15 psi for 25 minutes. With the coverslip immersed in the inoculum, the lid was applied and the plate was placed inside a large Ziploc bag (Ziploc, USA) with moist paper towel to limit evaporation of media and incubated at 22°C and 25 rpm. Media was removed every 24 hours by gentle aspiration and replaced with 5 mL fresh sterile medium. Three coverslips per strain, per medium were prepared.

3.1.3 Confocal Scanning Laser Microscopy and image analysis

Slides were prepared as follows: cover slips with attached biofilms were removed from the media using sterile forceps and placed face-up on the inverted lid of the microplate. Two hundred μL of SYTO-9 stain was pipetted onto the cover slip and left for 5 minutes. Using sterile forceps, the cover slip was then washed in three separate vessels containing 200 mL sterile, ddH_2O. The coverslip was then placed face-down over the concavity of a concave microscope slide to which 200 uL of sterile, ddH_2O had been added. Excess sterile ddH_20 was blotted from the margins of the cover slip using clean paper towel. The margins

of the cover slip were then sealed using nail polish. Slides were placed in darkness at 4°C.

Biofilm observation was accomplished using an Olympus Fluoview FV300 confocal scanning laser microscope in conjunction with Fluoview 4.0 software (Olympus Corp. Japan). The CSLM was set up as follows: Objective lens- 60x PlanApo NA 1.4 oil immersion objective lens and a 10mW, 488 nm argon laser (Olympus Corp. Japan). Detection of SYTO-9 fluorescence was achieved using a DM570 dichroic mirror and band pass emission from 510-530 nm using an FVX-BA 510-530 filter set. Three replicate slides per bacterium per condition and time were created. Five vertical image stacks (z-stacks) per slide were analyzed using Phobia Laser Imaging Processor (PHLIP, (Xavier *et al.* 2003)) (run using MatLab 7.0.4 (The MathWorks 2005). Parameters analyzed included biovolume, average thickness and percent substratum coverage.

3.1.4 Plate counting to assess biofilm cell density

Quantification of biofilm cells was accomplished by washing the biofilm samples (on coverslips) once by dipping into 200 mL of sterile double distilled water. Subsequently, biofilm cells were removed from the entire surface of the cover slip by scraping using a sterile cotton swab. The slide was scraped 15 times in a vertical direction with one swab, and then 15 times horizontally with a new sterile swab. After scraping, swabs were submerged in a 15 mL centrifuge tube

(Fisher Brand) containing 3 mL sterile, room temperature PBS. The tube was vortexed on medium speed for 1 minute to release biofilm cells from the fibers of the cotton swab. The liquid contents of the tube were then serially diluted in PBS and drop plating was performed onto TSA. The plates were incubated for 24 hours at 37°C prior to counting colonies and calculating biofilm cell density.

3.1.5 Statistical analyses

One way analysis of variance was used to compare means of measured data collected from all three *E. coli* strains at a single time during the experiment, as well as the changes exhibited by one bacterium over the four time points (0, 1, 2, and 4 days). Two-way analysis of variance was used to compare the means of measured parameters of all three *E. coli* throughout an experiment. Data in figure 1, 2, 3, 4 and 5 are presented using a mean value of measurements ± SD. All statistical analyses were performed using Sigmaplot and Sigmastat software (SPSS Inc. 1997; SPSS Inc. 2000).

3.2 RESULTS

3.2.1 Planktonic development/survival

In MSM-glu 1A and H32 planktonic cell densities climbed quickly between 0 and 48 hours, after which growth leveled off. Growth of K-12 lagged behind 1A and H32 and did not begin to increase until after 24 hours. While all three *E. coli* eventually reached a common cell density of about 2.0 $X10^8$ CFU/mL

at 96 hours ($p=0.241$), K-12 showed a continuous increase between 24 and 96 hours, rather than reaching the plateau observed in the growth of the two other bacteria. Ultimately, the growth curves for 1A and H32 were not significantly different, $p= 0.870$, however each was different from that of K-12 ($p<0.001$ for both) (Figure 1A).

In lake water (Figure 1B), once again, 1A and H32 shared similar survivorship curves, $p=0.244$. Beginning at a cell density of about 5×10^7 CFU/mL, 1A and H32 each fell to a final cell density of about 9×10^4 CFU/mL. Though K-12, 1A and H32 shared similar cell densities at 0 ($p=0.608$) and 96 ($p=0.869$) hours, K-12 showed the most dramatic decrease in density between 0 and 24 hours ($p<0.001$), while 1A and H32 showed only a slight decrease between 0 and 24 hours, but one far more exaggerated between 24 and 48 hours. Thus, in lake water, once again the survival of K-12 is different from those of 1A and H32. Moreover, while in MSM-glu all three bacteria grew about two orders of magnitude, in lake water cell densities for all three fell about two orders of magnitude.

In 50-50 (Figure 1C), all three bacteria shared similar cell densities of about 5×10^6 CFU/mL at 0 hours ($p=0.082$) and about 3×10^6 at 24 hours ($p=0.721$) after which each bacterium exhibited separate trends in growth/survival. At 48 hours, H32 increased to 1.2×10^7 CFU/mL ($p<0.001$), 1A dropped to a density of about

1.3 X 10^6 CFU/mL ($p = 0.029$) while K-12 showed no change ($p=1.00$). Between 48 and 96 hours, 1A cell densities continued to fall while those of H32 and K-12 remained constant. Thus, in 50-50, 1A and H32 showed negative and positive growth, respectively with K-12 intermediate to each.

3.2.2 Biofilm cell plate counting

After only 24 hours of growth in MSM-glu (Figure 2A), 1A and H32 biofilms apparently reached a point of saturation beyond which no increase in cell density was possible. Therein, 1A and H32 showed densities of about 1.8 X 10^8 CFU/cm^2 and 6.3 X 10^7 CFU/cm^2, respectively. Neither showed any change thereafter, ($p=1.000$ for both). K-12 showed lower cell counts than 1A ($p<0.001$) or H32 ($p<0.001$) after 24 hours and exhibited subsequent growth until 48 hours ($p<0.001$) at which point a plateau was reached at about 1.9 X 10^7 CFU/cm^2. Thus at 48 hours, cells in K-12 biofilms had presumably grown to saturation as was observed in the case of 1A and H32 at 24 hours. Further, it should be noted that while the development of the three bacteria in biofilms followed a similar trend, the curves for all three bacteria were significantly different. That of 1A was higher than H32 ($p<0.001$) whose curve itself was significantly higher than that of K-12 ($p<0.001$).

In lake water (Figure 2B), as was the case in MSM-glu, 1A and H32 reached their maximum biofilm cell densities of about 1.14 X 10^6 CFU/cm^2 and

3.93 X 10^6 CFU/cm^2 after only 24 hours and showed no change thereafter ($p=0.942$) and ($p=0.574$). And while the biofilm cell count of K-12 was no different from that of 1A or H32 at 24 hours, it began, and continued, to fall until 96 hours wherein its density was about 6.27 x 10^4 CFU/cm^2- significantly lower than either 1A ($p<0.001$) and H32 ($p<0.001$). Again, as in MSM-glu, the cell density curves for 1A and H32 shared a similar trend, though that of the former exceeded the latter's ($p<0.001$). However, in this case, K-12 cell counts fell continuously beginning at 24 hours and finishing with a final cell density about two orders of magnitude lower than 1A and H32.

When grown in 50-50 (Figure 2C), no difference was observed between the growth curves of 1A, H32 or K-12, $p=0.405$. Upon reaching a cell count of about 2.5 X 10^6 CFU/cm^2, the biofilm cell densities of 1A ($p=0.201$), H32 ($p=0.054$) and K-12 ($p=0.723$) showed no change over.

3.2.3 Biofilm biovolume

In MSM-glu, the biovolumes of 1A biofilms were consistently higher than those of H32 ($p<0.001$) and K-12 ($p<0.001$), which were themselves, similar ($p=1.000$) (Figure 3A). The biovolume of 1A increased from 24 to 48 hours and subsequently leveled off. Interestingly, this was dissimilar to the trend observed in 1A biofilm cell counts in MSM-glu which showed no increase after 24 hours. In the cases of H32 and K-12 however, similar trends in biofilm cell counts and

biovolume development over time were noted, with the biovolumes of H32 and K-12 remaining unchanged over time ($p=0.159$) and ($p=0.864$). Compared at 24 hours, the biovolume measurement of 1A was 37 and 88 times more voluminous than those of H32 ($p=0.003$) and K-12 ($p=0.007$), respectively. The differences only became more exaggerated with time, though after 96 hours, biovolume readings were only 17 times higher for 1A than for H32 $p=0.002$.

Contrary to biovolume observations made in MSM-glu, in lake water (Figure 3B), 1A showed no change in biovolume from day one to day four ($p=0.131$). Nonetheless, these findings agree with those from the biofilm plate counting assay which showed densities of 1A cells remained static over time. Conversely, the biovolumes of both H32 and K-12 remained stable between 24 and 48 hours, but increased thereafter, $p<0.001$ and $p=0.01$, respectively. Moreover, the biofilm cell count for K-12 in lake water fell between 48 and 96 hours- a marked contrast to the increase in biovolume observed here. Also noteworthy was that by 96 hours, K-12 had formed more biovolume than either 1A ($p=0.003$) or H32 ($p<0.001$), thus making it the best biofilm former, in terms of biovolume, in lake water (Figure 6B).

In 50-50 (Figure 3C), a medium intended to replicate a nutrient regime similar to one which may be found in a contaminated water body, biovolume formation over time is the same for 1A and H32, $p=0.096$. However, 1A and H32

each form more biovolume over time than K-12, $p<0.001$ and $p<0.001$, respectively. Moreover, 1A and H32 reached biovolume saturation after only 24 hours, neither showing any change over time, $p=0.057$ and $p=0.129$, respectively. This pattern was mirrored by the lack of change in biofilm cell count the two bacteria exhibited in 50-50. The same cannot be said for K-12 however, whose biovolume increased slightly after 48 hours, $p=0.021$, but whose biofilm cell count showed no change over time.

3.2.4 *Average biofilm thickness*

Following from biovolume measurements in MSM-glu, 1A forms biofilms significantly thicker than H32 and K-12 over time, $p<0.001$ and $p<0.001$ (Figure 4A). Moreover, while the biovolume of 1A increases in MSM-glu over time, average thickness does not follow. 1A, like H32 and K-12, exhibits no change in average thickness over time. Nonetheless, by 96 hours, 1A biofilms are 3.6 and 2.3 times thicker than H32 ($p<0.001$) and K-12 ($p=0.002$) biofilms, respectively (Figure 6A).

In lake water, average thickness is comparatively low (Figure 4B). Though 1A develops significantly thicker biofilms than H32 ($p<0.001$) and K-12 ($p<0.001$) over time, by 96 hours its thickness fell to a point where it is no longer different from the other two (Figure 6B). Noteworthy here is that average thicknesses of H32 and K-12 were in the order of less than 1 to 2 μm for H32 and

K-12 in lake water, while in MSM-glu they were between 2 and 3 μm. In contrast, the average thickness of 1A in MSM-glu was considerably higher than in lake water with values as high as 6.25 μm in MSM-glu, but only as high as 1.5 in lake water. Thus a heightened sensitivity to nutrients is observed in the case of 1A.

Interestingly, in 50-50 H32 seems to hold an advantage in terms of its ability to form biofilms which were, on average, thicker than those of 1A ($P=0.007$) and K-12 ($p<0.001$) (Figure 4C). Though none of the three organisms showed a change in mean thickness over time, H32 was certainly advantaged in its ability to form biofilms, thicker on average, than the other two *E. coli* examined (Figure 6C).

3.2.5 Percent substratum coverage by biofilms

Percent substratum coverage was highest for all three organisms in MSM-glu with 1A showing a maximum coverage of nearly 65%. In 50-50, percent substratum coverage did not exceed 7 percent while in lake water values no higher than 1 percent were observed for any of the isolated tested. In MSM-glu, coverage was highest for 1A ($p<0.001$), which was not surprising given accompanying measurements of biovolume. Substratum coverage of 1A peaked at 48 hours at about 65 percent, but then fell to 35 percent by 96 hours. H32 coverage in MSM-glu continued to climb until 96 hours when it reached nearly

20 percent whileK-12 coverage, remained unchanged over time (p=0.903), hovering around two percent.

3.3 DISCUSSION

Given the previously-identified physiological differences between the sessile and free-living lifestyles of a bacterium, when examining biofilm formation it is also valuable to consider planktonic development. Thus, all three *E. coli* studied here were evaluated for both planktonic, and biofilm development in each medium employed. Two themes became obvious over the course of the study. First, in MSM-glu, a relatively rich medium when compared to the other two employed, all three bacteria in the planktonic condition grew slowly to exceed initial inocula concentrations by about two orders of magnitude by four days. In contrast, the majority of biofilm growth took place between 0 and 1 days, for the same bacteria. Noteworthy is that while planktonic growth curves for the three were similar, 1A clearly held an edge when it came to biofilm formation with a biofilm roughly 40-90 times more voluminous than the others in the first 48 hours. The second trend observed was that in reduced media (lake water and 50-50) planktonic and biofilm development showed opposite trends. While in each medium planktonic cell populations continued to decline after inoculation, biofilm populations grew and remained stable after one day. A notable exception was the development of H32 in 50-50. While its non-

pathogenic planktonic counterparts were subject the post-inoculation decline mentioned above, H32 grew after inoculation. Irrespective of the developmental differences in the planktonic condition, the data support a fundamental truth: biofilm formation is a survival mechanism which allows bacteria to survive in conditions unforgiving to planktonic growth. In a 2005 study, Ryu and Beuchat showed the protection conferred upon biofilms of *E. coli* O157:H7 grown on stainless steel chips when faced with chlorine disinfection- one not enjoyed by planktonic analogs. Similarly, Uhlich et al. (2006) identified increased resistance to hydrogen peroxide and ammonium sanitizer in biofilms of *E. coli* O157:H7 relative to planktonic cells. It is difficult to provide an explanation for why planktonic *E. coli* cell densities fell in lake water and 50-50. Whether it was the result of nutrient deficiency or some secondary metabolite production of non-target bacteria in the lake water or untreated sewage prior to filtration, our data suggest biofilm development allowed these *E. coli* to persist where the planktonic contingent could not.

Biofilms are propagated in three separate ways: the redistribution of cells along surface of the substratum (Korber et al., 1995), the recruitment of cells from the bulk fluid (Tolker-Nielson et al., 2000), and through division of cells within the biofilm (Heydorn et al., 2007). It is likely that the heterogeneous structure of 1A biofilms observed in MSM-glu, and of H32 in 50-50 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 mushroom shaped structures observed. Further, as medium was changed every 24 hours post-inoculation, the recruitment of cells from the overlying medium too would have been unlikely to play a significant role in biofilm growth. This leaves the division of existing biofilm cells as the most plausible explanation for the mode of development of the 1A and H32 biofilms observed in MSM-glu (Figure 6A) and 50-50 (Figure 6H), respectively. Conversely, flagellar mediated motility may have played a role in the construction of the small clusters of 1A, K-12 and H32 cells observed in lake water (Figures 6D, 6E, 6F).

As a biofilm thickens, cells deep within the matrix begin to lose activity owed to the reduced access to nutrient available in the bulk fluid (Sternberg et al., 1999). As a means of circumventing the increasingly limited access to nutrients, a growing biofilm will adopt a three-dimensional structure typified by mushroom or tulip shaped projections away from the substratum (Costerton et al., 1999). By erecting pillars into the bulk fluid and simultaneously creating water channels which permeate the biofilm matrix, access to nutrients is improved for cells embedded therein (de Beer et al., 1994). The structures of 1A biofilms grown in MSM-glu are consistent with the model of a mature biofilm, as defined for the well-characterized biofilms of *Pseudomonas aeruginosa* (Costerton

et al., 1999). In 50-50, H32 developed a biofilm which lacked full differentiation and thickness to be similarly termed mature. No other examples of *E. coli* biofilms reaching structural maturity were observed-owed likely to a lack of nutrients.

Bacterial biofilms growing in oligotrophic environments, such as those present in the abyssal depths of the oceans must contend with the low nutrient availability inherent in those environments (Kjelleberg, 1993). What biofilms exist in these circumstances are often poorly differentiated and metabolically reserved. However, the sudden release of volcanic gasses from a deeps sea vent or "black smoker", initiates a flurry of growth leading to the development of mature biofilms. The same can likely be said of the environmental biofilm *E. coli* collected for this study. Though only capable of forming small colonies a few cells thick on cover glass surface in lake water, 1A developed thick, heterogeneous biofilms when grown in MSM supplemented with a readily useable carbon source: glucose. These findings underpin the importance of the examination of biofilm development in both rich and natural media in this study. For example, casual observation of H32's ability to form biofilm in lake water would lead the researcher to conclude that in a natural setting, the bacterium is incapable of the formation of mature biofilms. However, a sudden introduction of nutrients- mimicked using 50-50 (50-50 has about 50 percent more available

carbon than lake water, See Appendix 1.3) – allowed H32 to develop biofilms that were much thicker than those which it formed in lake water.

The records of the ability of *E. coli* O157:H7 to from biofilm are contradictory. The bacterium's capacity to from sessile communities in a flow cell culture supplied with TSB was examined by Klayman et al. (2009). The inoculated bacteria were incapable of fulfilling even the earliest stage of biofilm development: reversible attachment. Conversely, Dewanti and Wong (1995) showed that *E. coli* O157:H7 could form biofilm, roughly 10^5CFU/cm^2 after two days, when grown in minimal salt medium amended with 0.04% glucose. The results of this study agree with those of the Dewanti group with respect to the ability of *E. coli* O157:H7 to form biofilm. However, the evidence of biofilm formation presented by the Dewanti group in MSM-glu was limited to plate counting and electron microscopy. Measurements of biovolume, average thickness, and percent substratum coverage were not taken. In contrast, here, CSLM was used to examine each of these parameters and to allow the observation of living, fully-hydrated biofilms. Further, this research sought to study biofilm development using and environmentally relevant medium, namely, 50-50 which was conceived to mimic conditions which could be encountered by *E. coli* O157:H7 in a water body recently contaminated with untreated sewage. After 96 hours of growth in MSM-glu (Figure 6B) H32 formed

modest biofilm. However, in 50-50, the bacterium was capable of forming thicker, more well-differentiated biofilm(Figure 6H). This highlights, once again, the importance of medium in biofilm formation.

Indeed, there is agreement in the literature that the biofilm formation of *E. coli* is largely dependent upon growth medium (Dewanti and Wong, 1995; Naves et al., 2008). However, central to a discussion of the impact of nutrient availability/medium-appropriateness on the development of a biofilm, is the mechanism that affects the differences in biofilm phenotypes. Danese et al (2000) as well as Watnick et al. (2001) have demonstrated the importance of colanic acid production on the maturation process of *E. coli* and *Vibrio cholerae* biofilms, respectively. Further, evidence also exists for the link between medium composition and a bacterium's ability to synthesize colanic acid in it. When Hetzner et al. (2001) and Davies et al. (1998) studied the biofilm formation of the same non-mucoid strain of *Pseudomonas* sp., Hetzner's group found a thin, poorly differentiated biofilm while Davies' group found one heterogeneous and with distinct mushroom-shaped clusters. Hetzner attributed the different phenotypes to a difference in nutrient availability of the media employed in each study. Similarly, while in MSM-glu 1A formed thick heterogeneous biofilm (Figure 6A), but one that was thin and relatively undifferentiated in 50-50 (Figure 6G) (the opposite response was observed for H32), the respective phenotypes

could be attributed to the varying abilities of each to synthesize colanic acid in separate media. Thus, an organism need not necessarily be a universally good producer of colanic acid, and thus, biofilm, in all media. Though, given the appropriate conditions, it may be capable of attaining maturity.

As disparity existed between planktonic and biovolume data, so too was there an unexpected difference between biofilm plate count, and biovolume, data. The quantity of biofilm measured in this experiment proved dependent upon the method of examination used. In MSM-glu, biovolume measurements indicated 1A produced significantly more biofilm than H32 or K-12. Plate counting revealed that while the number of cells differed between the three *E. coli*, the discrepancies were not nearly as exaggerated as would be expected from measurements of biovolume. Thus, plate counting was not as predictive of biofilm formation as CSLM. This is consistent with the results of Naves *et al.* (2008) and Larsson *et al.* (2008). When examining biofilms of *E. coli*, Larsson *et al.* (2008) reported a total loss of cells after 70 hours using plate counting. 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) analysis of the same populations showed that in fact, after 96 hours, *E. coli* were still present at a concentration of nearly 10^6 CFU/ cm^2. These data present alternate evidence for *E. coli's* potential loss of culturability when grown in biofilm.

In this study, MSM-glu was the only medium to support plankotnic and biofilm growth, rather than survival. In sterile lake water and 50-50, relatively nutrient-poor compared to MSM-glu, planktonic densities generally decreased over time, while those biofilm remained constant over time. This highlights the fact that biofilms represent a survival mechanism for *E. coli* when faced with difficult conditions. Moreover, H32 formed biofilm particularly well in 50-50. This ability to from biofilm is troublesome as it indicates the high likelihood of *E. coli* O157:H7 becoming established in the environment given appropriate conditions. Structurally, these biofilms were not as well developed as those of 1A in MSM-glu, but nonetheless showed differentiation which may indicate an intermediate step in the progression towards maturity. The latter formed biofilms which were thick and heterogeneous, in other words, mature-complete with characteristic tulip shaped structures. Other than the two bacteria/condition combinations mentioned, no other thick, differentiated biofilms were observed. Additionally, a large discrepancy was observed between measurements of biovolume and biofilm cell plate counts suggesting that, cells of biofilms examined lost their culturability shortly after inoculation. Thus, this study has contributed not only to the understanding of biofilm development as a protective and preferred bacterial lifestyle, but has also spoken to the physiological

response of *E. coli* to differing natural environmental conditions- information which is crucial to the safe and effective management of recreational water.

FIGURE LEGEND

Figure 1. Means and standard deviations of nine measurements of the survival of planktonic *E. coli* 1A, H32 and K-12 after 24, 48 and 96 hours incubation at 37°C with 150 rpm in: (A) MSM-glu, (B) Sterilized lake water (C) 1:1 mixture of sterilized lake water and sterilized untreated sewage (50-50).

Figure 2. Means and standard deviations of nine measurements of biofilm cell densities of *E. coli* 1A, H32 and K-12 in (A) MSM-Glu, (B) sterile lake water and (C) a sterile 1:1 mixture of lake water and untreated sewage (50-50) .

Figure 3. Means and standard deviations of 15 measurements of biovolume of *E. coli* 1A, H32 and K-12 biofilms after 24, 48 and 96 hours of incubation at 22°C with 25 rpm in (A) MSM-glu (B) sterilized lake water (C) 1:1 mixture of sterilized lake water and sterilized untreated sewage (50-50) calculated using PHLIP. Biovolume analysis was performed on the biofilm covering an area of 55 338 μm^2.

Figure 4. Means and standard deviations of 15 measurements of average thickness of *E. coli* 1A, H32 and K-12 biofilms after 24, 48 and 96 incubation at 22°C with 25 rpm in (A) MSM-glu (B) sterilized lake water (C) 1:1 mixture of sterilized lake water and sterilized untreated sewage (50-50) calculated using

PHLIP. The analysis of average thickness was performed on the biofilm covering an area of 55 338 μm^2.

Figure 5. Means and standard deviations of 15 measurements of percent substratum coverage of *E. coli* 1A, H32 and K-12 biofilms after 24, 48 and 96 hours incubation at 22°C and 25 rpm in (A) MSM-glu. (B) sterilized lake water (C) 1:1 mixture of sterilized lake water and sterilized untreated sewage (50-50) calculated using PHLIP. The analysis of percent substratum coverage was performed on the biofilm covering an area of 55 338 μm^2.

Figure 6. Confocal images of hydrated *E. coli* (A) 1A, (B) H32 and (C) K-12 in MSM-glu; (D) 1A, (E) H32 and (F) K-12 in filter-sterilized lake water; (G) 1A, (H) H32, and (I) K-12 in filter sterilized 50-50. The upper image is a bird's eye view of the biofilm showing substratum coverage while the lower image is a profile showing thickness.

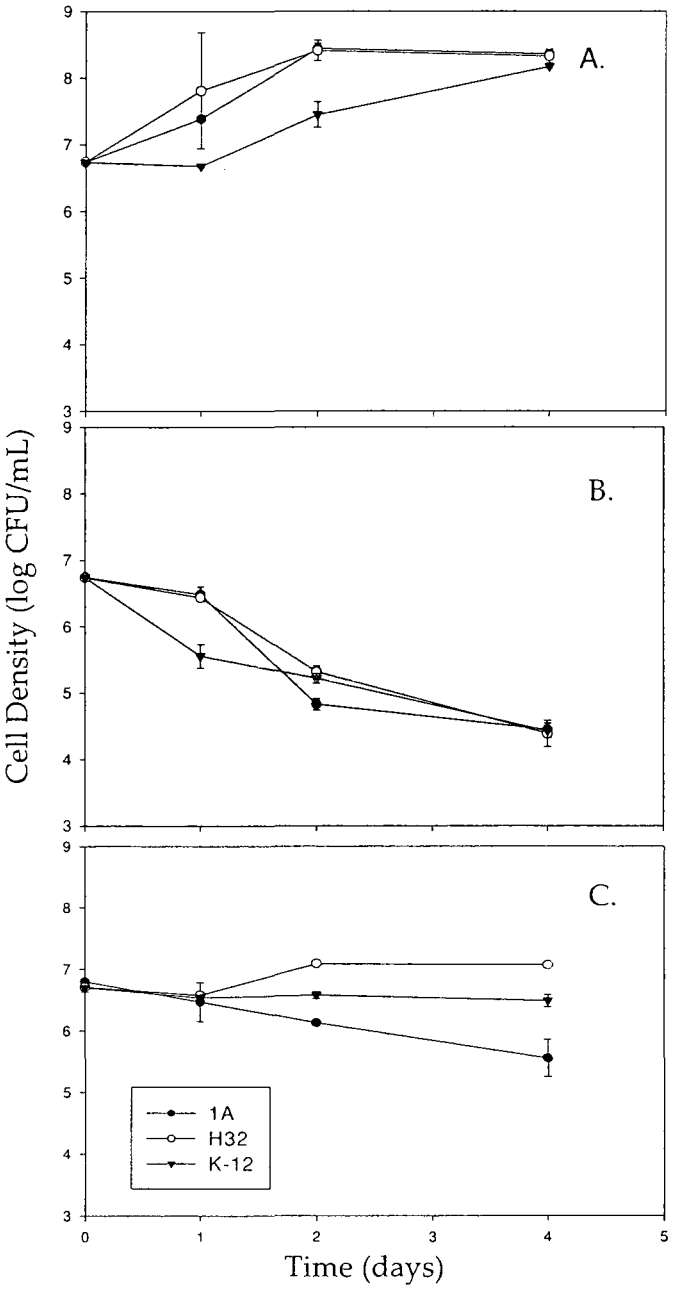

Figure 1

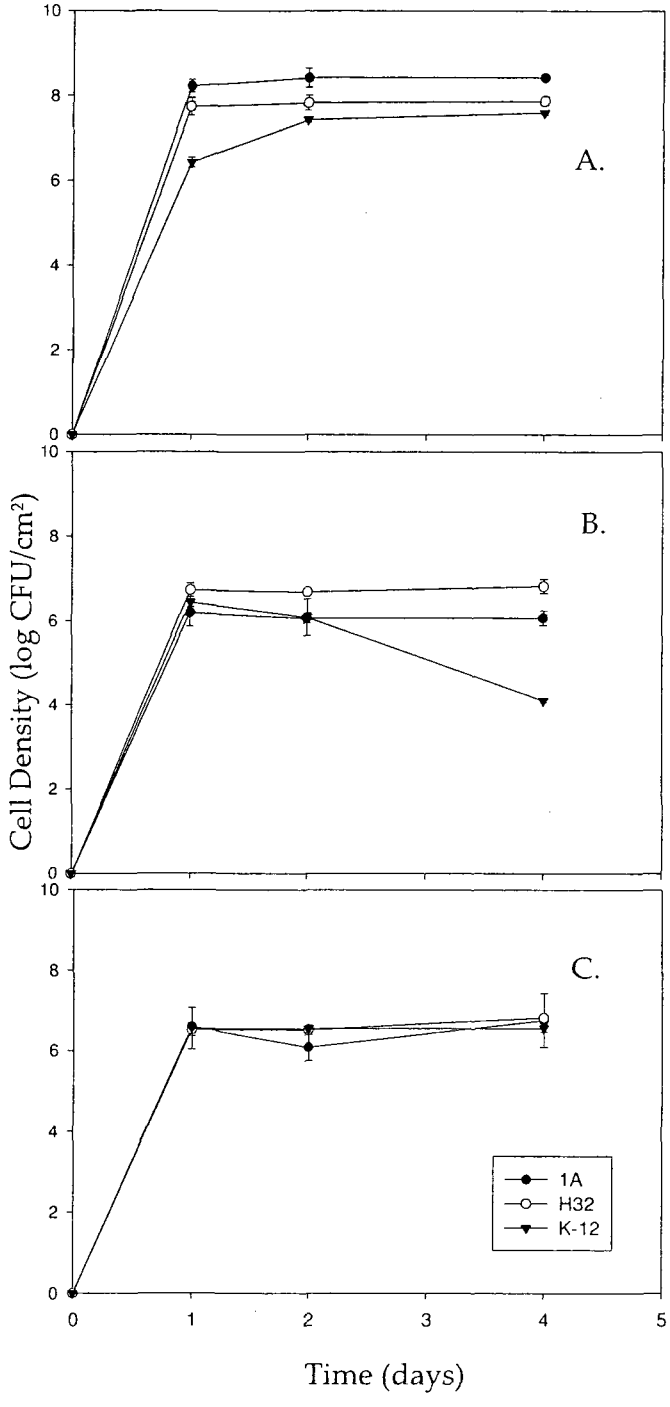

Figure 2

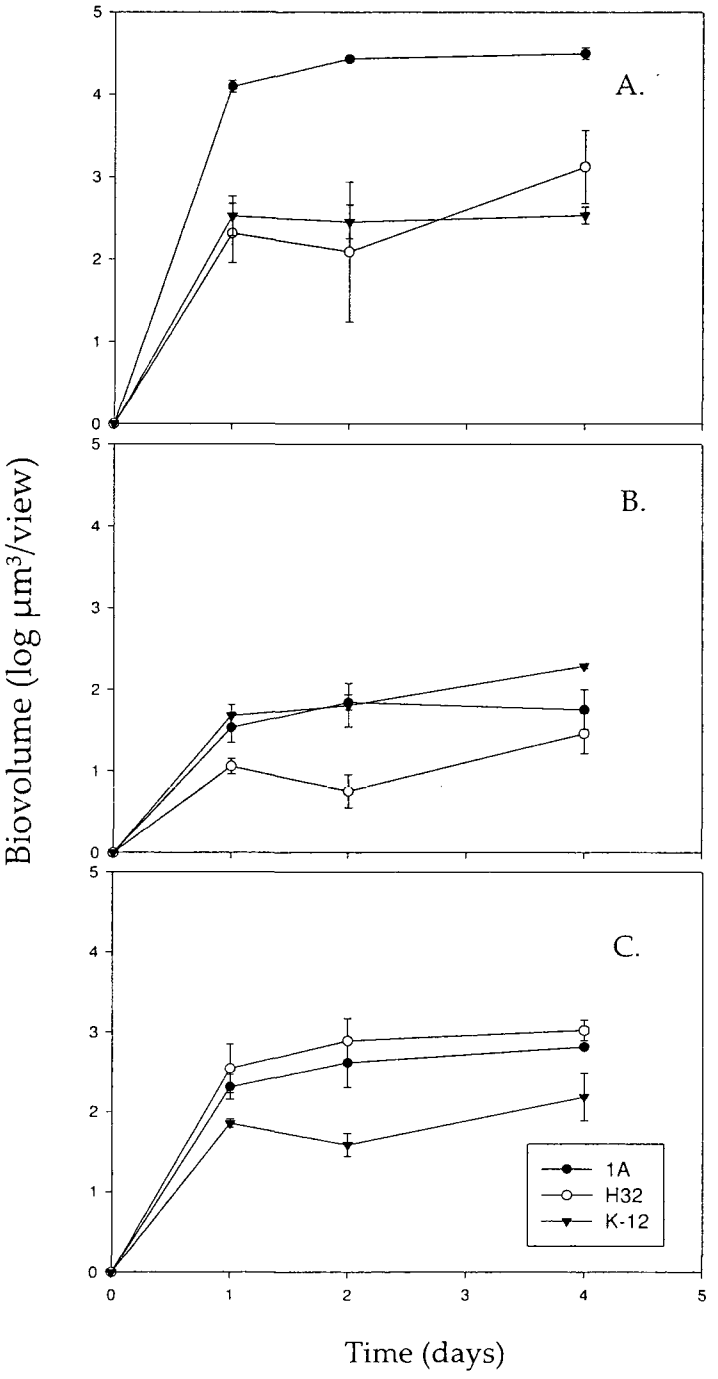

Figure 3

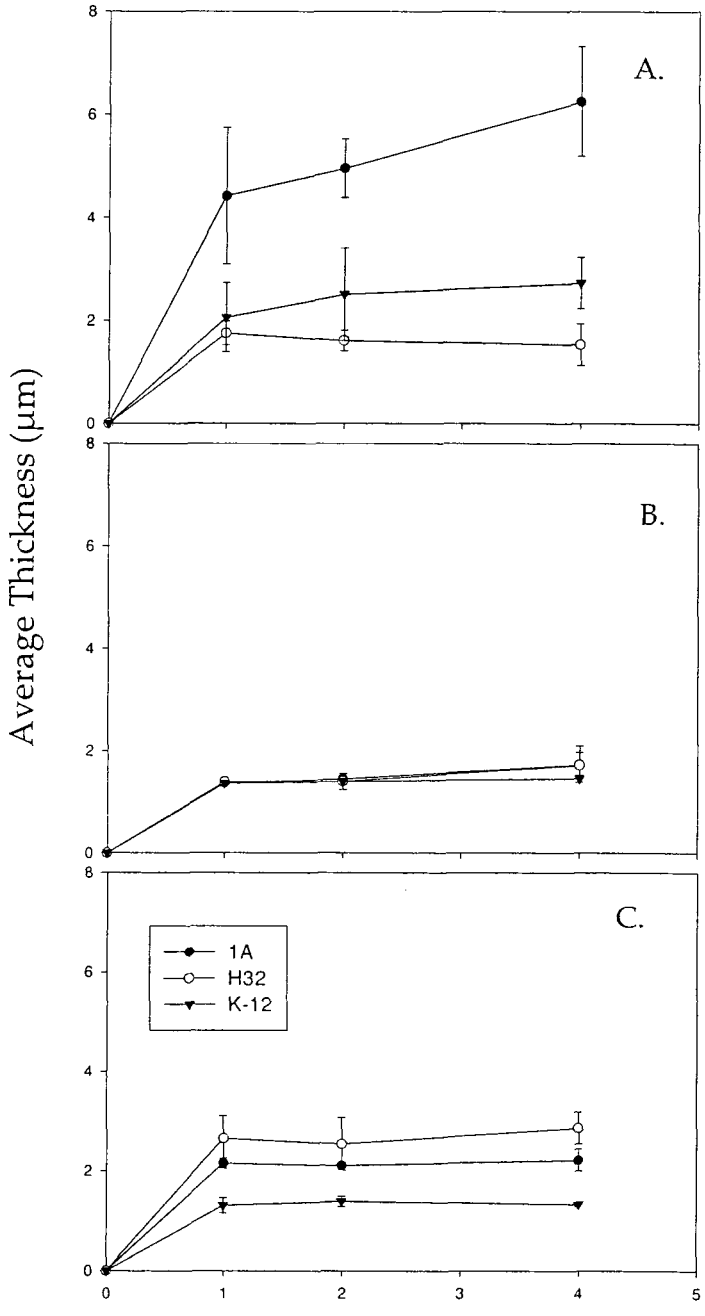

Figure 4

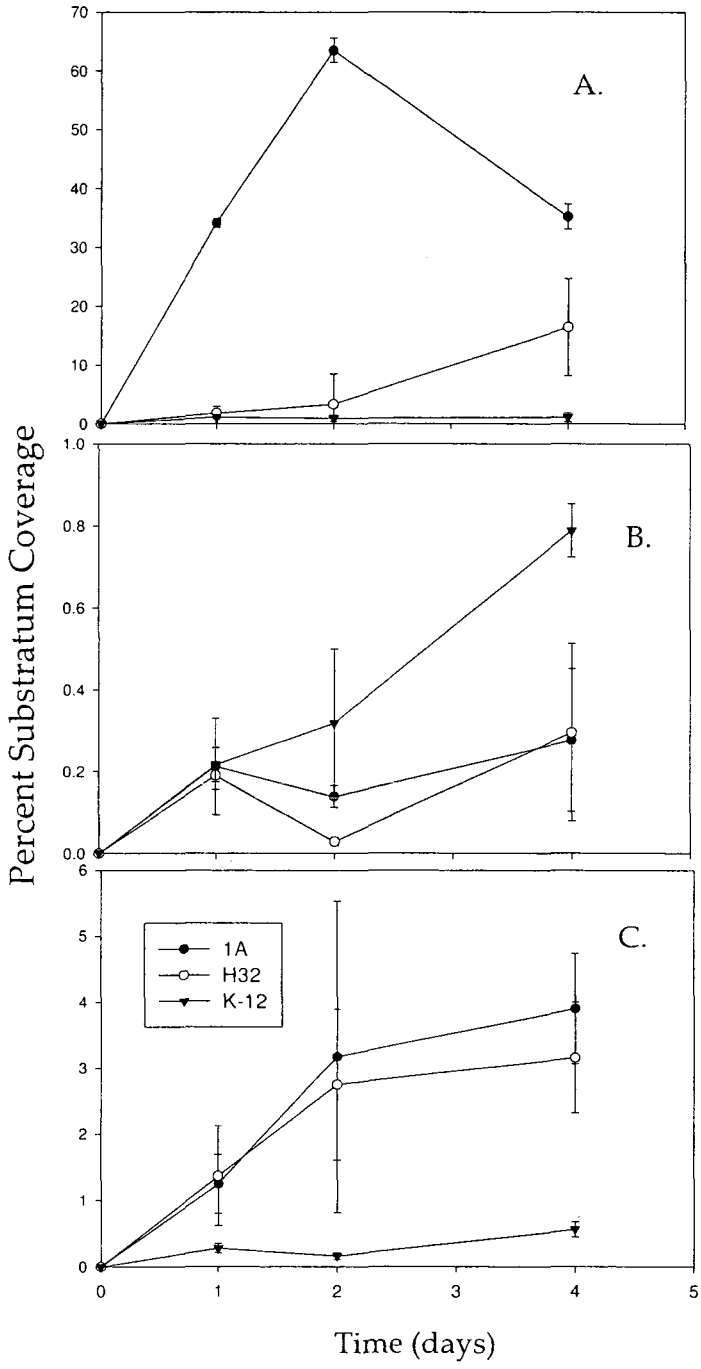

Figure 5

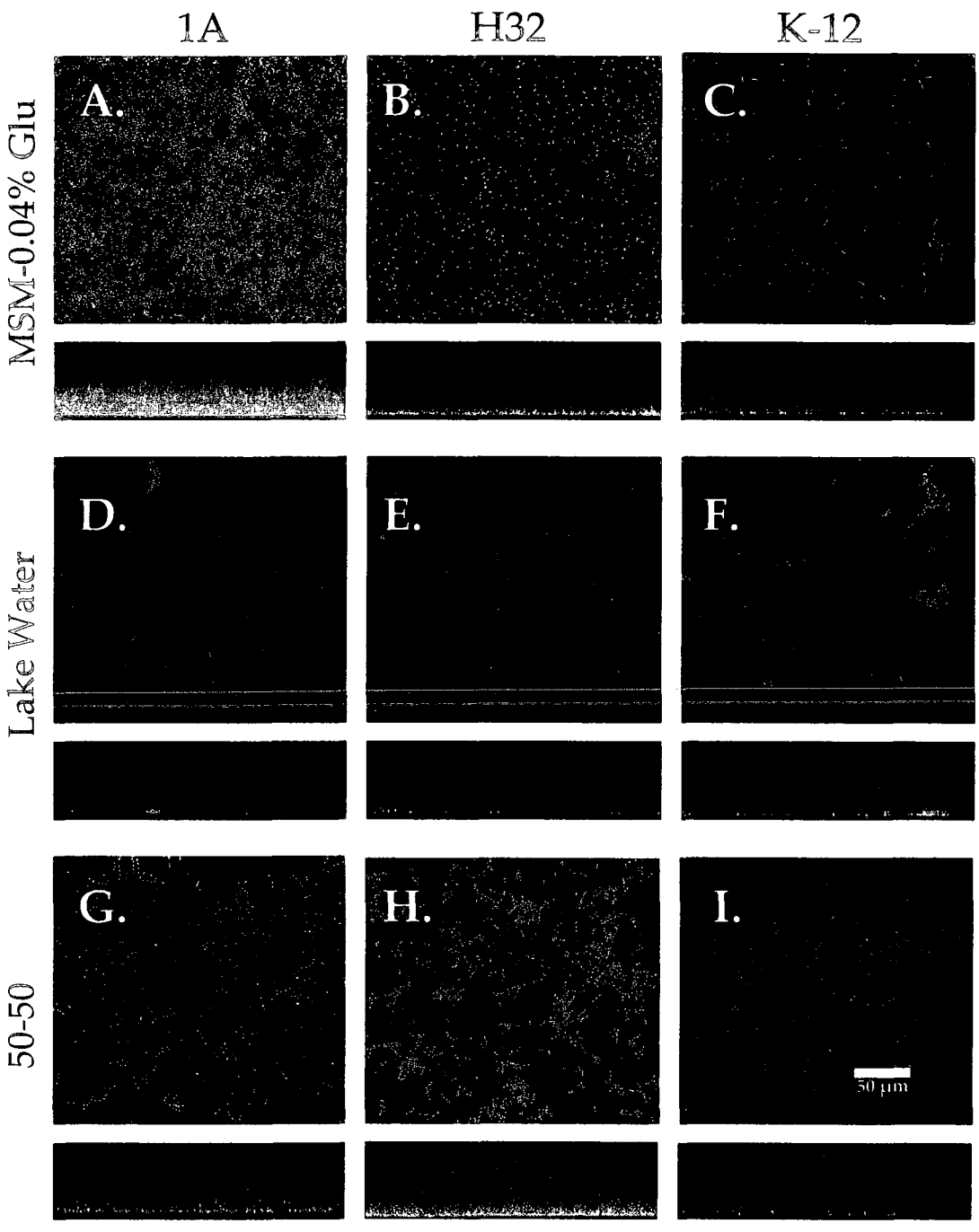

Figure 6

CHAPTER 4: CONCLUSIONS OF THE THESIS

Once assumed incapable of lengthy persistence beyond the animal host, *E. coli* has shown its aptitude to persevere in both tropical and temperate watersheds (Carillo et al., 1985; Lopez-Torres et al., 1987; Whitman and Nevers, 2003; Ksoll et al., 2007). This has brought into question its utility and reliability as an indicator organism of fecal contamination. Here, the continuous presence of persistent populations of *E. coli* growing in biofilms in three temperate water bodies was identified. This falsifies the assumption that *E. coli* found in a water body could only exist as a result of fecal contamination. Certainly if *E. coli* is present in biofilms, its detection in the overlying water may indicate the release of cells from a biofilm rather than its arrival in association with fecal material. Thus, a study aimed at identifying the likelihood of biofilms releasing constituent cells into the overlying water should be undertaken. Certainly, if biofilms are shown to release cellS into the overlying water column, a review of current water testing protocol would be called for.

The assumption of *E. coli's* poor persistence in the environment was also falsified in this study. Indeed, in filter-sterilized lake water inocula of *E. coli* 1A, H32 and K-12 yielded a planktonic population which fell precipitously over time alongside a sessile population which grew over the same period. Similar observations were made for 1A and K-12 in filter-sterilized 50-50. This speaks to

the general tendency of bacteria to preferentially select a sessile lifestyle but also speaks to the potential abilities of pathogenic strains of *E. coli* to form persistent biofilms in nutrient conditions found in nature. Interestingly, in 50-50 H32 was capable of positive growth in both the planktonic and sessile conditions. This suggests a heightened ability to take advantage of nutrients present in untreated sewage: unsurprising as H32 is an enteric pathogen. More importantly though, this study identifies the need for careful monitoring of a water body following accidental contamination with sewage.

While the results of this study indicate the potential for pathogenic *E. coli* to form biofilm under nutrient conditions similar to those which may be found in the environment, it did not examine the interaction which may occur between said *E. coli* and resident bacteria in the same environs. Background bacterial populations may improve or hinder the production of biofilms of the three *E. coli* examined here. Thus, there is the need to re-examine biofilm formation of the *E. coli* used in this study using non-sterile media. Without question, this would provide a more complete understanding of how biofilm may be formed by pathogenic and non-pathogenic *E. coli* in the environment.

In addition to being the first to compare biofilm formation of environmental and pathogenic isolates of *E. coli* in environmentally relevant media, this research provides a foundation upon which the understanding of *E.*

coli biofilm formation in the environment, as well as the biofilm-planktonic cell dynamic, can be furthered.

Table 1. Highlights of measurements of biovolume of *E. coli* 1A, H32 and K-12 biofilms grown in MSM-0.04% Glu using CSLM.

	MSM-0.04% Glu		
Biovolume	1A (30902 μm³/view)	>>> H32 (1318 μm³/view)	> K-12 (338 μm³/view)
Average Thickness	1A (6.25μm)	>>> H32 (1.53μm)	~ K-12 (2.73μm)
% Substratum Coverage	1A (35.21%)	>>> H32 (6.45%)	~ K-12 (1.13%)

Table 2. Highlights of measurements of biovolume, average thickness and percent substratum coverage of *E. coli* 1A, H32 and K-12 biofilms grown in filter-sterilized Lake Water using CSLM.

	Filter Sterilized Lake Water		
Biovolume	K-12 (190.54 μm³/view)	~ 1A (56.23 μm³/view)	> H32 (28.84μm³/view)
Average Thickness	1A (1.74μm)	~ H32 (1.72μm)	~ K-12 (1.45μm)
% Substratum Coverage	K-12 (0.78%)	> H32 (0.29%)	~ 1A (0.27%)

Table 3. Highlights of measurements of biovolume, average thickness and percent substratum coverage of *E. coli* 1A, H32 and K-12 biofilms grown in filter-sterilized 50-50 using CSLM.

	Filter Sterilized 50-50				
Biovolume	H32 (1047.12 μm³/view)	~	1A (645.65 μm³/view)	>>	K-12 (151.35 μm³/view)
Average Thickness	H32 (2.86μm)	~	1A (2.22μm)	>	K-12 (1.33μm)
% Substratum Coverage	1A (3.90%)	~	H32 (3.16%)	>>	K-12 (0.56%)

Table 4. Trends in planktnic and biofilm development in MSM-0.04% Glu, filter-sterilized Lake Water and filter-sterlized 50-50

	Planktonic Viable Cell Count	Biofilm Viable Cell Count
	MSM-0.04% Glu	
1A	+ve	+ve
H32	+ve	+ve
K-12	+ve	+ve
	Lake Water	
1A	-ve	+ve
H32	-ve	+ve
K-12	-ve	+ve
	50-50	
1A	-ve	+ve
H32	+ve	+ve
K-12	-ve	+ve

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APPENDIX

5.0 Environmental metadata for summer 2008 *E. coli* collections

Site	Date	Water Temp. (°C)	pH
Billy Lake	6/12/2008	10.5	7.7
Boulevard Lake	6/12/2008	15	7.1
Chippewa Park	6/12/2008	17.5	7.1
Billy Lake	7/20/2008	19	7
Boulevard Lake	7/20/2008	23	7.3
Chippewa Park	7/20/2008	21	8.3
Billy Lake	9/23/2008	17	7.3
Boulevard Lake	9/23/2008	22	7.2
Chippewa Park	9/23/2008	20	7.6
Billy Lake	10/26/2008	5.5	7.9
Boulevard Lake	10/27/2008	5.8	6.9
Chippewa Park	10/27/2008	5.2	7.2

5.1 Water chemistry analysis data of water collected from all three target water bodies during biofilm sample collections of summer/fall 2008.

	June			July		
	Billy Lake	Boulevard	Chippewa	Billy Lake	Boulevard	Chippewa
Description						
Calcium	41.28	6.51	12.98	42.37	8.34	13.58
Potassium	0.90	0.69	0.73	0.87	0.73	0.61
Magnesium	13.36	2.11	3.74	13.9	3.21	3.26
Sodium	1.44	2.16	5.00	1.5	2	2.45
Dissolved Organic Carbon	4.4	7.6	9.4	4.1	8.3	4.0
Chloride (IC)	0.84	2.42	6.24	0.78	2.38	2.60
Nitrite NO2-N (IC)	<DL	<DL	<DL	<DL	<DL	<DL
Nitrate NO3-N [IC]	<DL	0.042	0.146	<DL	0.039	0.264
Total Aluminum	0.011	0.084	0.159	0.025	0.076	0.069
Total Arsenic	0.006	<DL	<DL	0.007	<DL	<DL
Total Barium	0.275	0.015	0.016	0.306	0.023	0.010
Total Berryllium	<DL	<DL	<DL	<DL	<DL	<DL
Total Cadmium	<DL	<DL	<DL	<DL	<DL	<DL
Total Cobalt	<DL	<DL	<DL	<DL	<DL	<DL
Total Chromium	<DL	<DL	<DL	<DL	<DL	<DL
Total Copper	0.006	0.003	0.010	0.005	0.006	0.005
Total Iron	0.020	0.131	0.529	0.021	0.155	0.212
Total Manganese	0.0009	0.0019	0.0214	0.0008	0.0024	0.0121
Total Molybdenum	<DL	<DL	<DL	<DL	<DL	<DL
Total Nickel	<DL	<DL	<DL	<DL	<DL	<DL
Total Lead	<DL	<DL	<DL	<DL	<DL	<DL
Total Sulfur	1.17	1.23	2.48	1.21	1.67	1.56
Total Strontium	0.050	0.016	0.029	0.043	0.021	0.026
Total Titaniuim	<DL	<DL	<DL	<DL	<DL	<DL
Total Vanadium	<DL	<DL	<DL	<DL	<DL	<DL
Total Zinc	0.002	0.005	0.003	0.004	0.004	0.003
Phosphate (PO4-P) by IC	<DL	<DL	<DL	<DL	<DL	<DL
Sulphate (SO4) [IC]	3.18	3.09	5.99	4.29	3.31	4.25

5.1 cont'd

	September			October		
	Billy Lake	Boulevard	Chippewa	Billy Lake	Boulevard	Chippewa
Description						
Calcium	38.49	14.07	14.39	45.54	13.25	14.2
Potassium	0.91	0.72	0.66	1.03	0.69	0.63
Magnesium	14.60	4.66	3.42	15.06	4.54	3.54
Sodium	1.62	3.24	4.07	1.61	3.11	4.1
Dissolved Organic Carbon	4.1	6.0	4.0	4.1	6.3	3.8
Chloride (IC)	1.48	4.13	3.21	1.61	4.01	3.44
Nitrite NO2-N (IC)	<DL	<DL	0.027	<DL	<DL	0.045
Nitrate NO3-N [IC]	<DL	0.046	0.190	0.015	0.033	0.2
Total Aluminum	<DL	0.013	0.046	<DL	0.02	0.025
Total Arsenic	<DL	<DL	<DL	<DL	<DL	<DL
Total Barium	0.277	0.018	0.012	0.292	0.021	0.017
Total Berryllium	<DL	<DL	<DL	<DL	<DL	<DL
Total Cadmium	<DL	<DL	<DL	<DL	<DL	<DL
Total Cobalt	<DL	<DL	<DL	<DL	<DL	<DL
Total Chromium	<DL	<DL	<DL	<DL	<DL	<DL
Total Copper	0.015	0.009	0.036	0.009	0.007	0.039
Total Iron	0.023	0.194	0.103	0.016	0.113	0.127
Total Manganese	0.0002	0.0017	0.0046	0.0013	0.002	0.004
Total Molybdenum	<DL	<DL	<DL	<DL	<DL	<DL
Total Nickel	<DL	<DL	<DL	<DL	<DL	<DL
Total Lead	<DL	<DL	<DL	<DL	<DL	<DL
Total Sulfur	1.22	1.80	1.95	1.20	1.06	1.97
Total Strontium	0.051	0.028	0.026	0.053	0.026	0.022
Total Titaniuim	<DL	<DL	<DL	<DL	<DL	<DL
Total Vanadium	<DL	<DL	<DL	<DL	<DL	<DL
Total Zinc	0.003	<DL	0.004	0.026	<DL	0.003
Phosphate (PO4-P) by IC	<DL	<DL	<DL	<DL	<DL	<DL
Sulphate (SO4) [IC]	2.93	4.60	5.31	3.39	4.43	5.01

5.2 Chemical analysis of the filter-sterilized lake water and 50-50.

Description	Lake Water	50-50
Calcium	10.06	10.73
Potassium	0.51	8.15
Magnesium	3.26	7.60
Sodium	3.05	32.90
Dissolved Organic Carbon	7.6	12.4
Chloride (IC)	5.15	65.30
Nitrite NO2-N (IC)	<DL	<DL
Nitrate NO3-N [IC]	0.159	<DL
Total Aluminum	0.026	0.011
Total Arsenic	<DL	<DL
Total Barium	0.017	0.009
Total Berryllium	<DL	<DL
Total Cadmium	<DL	<DL
Total Cobalt	<DL	<DL
Total Chromium	<DL	<DL
Total Copper	0.013	0.006
Total Iron	0.152	0.108
Total Manganese	0.0111	0.0295
Total Molybdenum	<DL	<DL
Total Nickel	<DL	<DL
Total Lead	<DL	<DL
Total Sulfur	1.27	3.99
Total Strontium	0.022	0.026
Total Titaniuim	<DL	<DL
Total Vanadium	<DL	<DL
Total Zinc	0.003	0.006
Phosphate (PO4-P) by IC	<DL	0.162
Sulphate (SO4) [IC]	5.24	12.33

5.3 Aligned 16SrDNA of Environmental Biofilm *E. coli* Isolates and *E. coli* K-12 (MG1655)

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1A
Query  1       TCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATACCTTTACTCATT 60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224191  TCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATACCTTTGCTCATT  224250
Query  61      GACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG  120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224251  GACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG  224310
Query  121     GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCA  180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224311  GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCA  224370
Query  181     GATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTC  240
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224371  GATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTC  224430
Query  241     GTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACC  300
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224431  GTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACC  224490
Query  301     GGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCA  360
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224491  GGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCA  224550
Query  361     AACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCC  420
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224551  AACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCC  224610
Query  421     CTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCA  480
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224611  CTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCA  224670
Query  481     AGGTTAAAACTCAAAGGAATTG  502
               ||||||||||||||| ||||||
Sbjct  224671  AGGTTAAAACTCAAATGAATTG  224692

1D
Query  2       TGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAG  61
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224168  TGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAG  224227
Query  62      TAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTG  121
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224228  TAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTG  224287
Query  122     CCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG  181
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224288  CCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG  224347
Query  182     CACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATC  241
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224348  CACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATC  224407
Query  242     TGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAAT  301
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224408  TGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAAT  224467
Query  302     GCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCT  361
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224468  GCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCT  224527
Query  362     CAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC  421
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224528  CAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC  224587
Query  422     GATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCG  481
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224588  GATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCG  224647
Query  482     ACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAAGGAATTGACGG  530
               |||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  224648  ACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGG  224696

1G
Query  1       GCAGCC-TGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGG  59
               |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224161  GCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGG  224220
Query  60      AAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAA  119
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224221  AAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAA  224280
Query  120     CTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCG  179
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224281  CTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCG  224340
Query  180     TAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAA  239
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224341  TAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAA  224400
Query  240     CTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCG  299
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Sbjct  224401   CTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCG  224460
Query  300      GTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGAC  359
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224461   GTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGAC  224520
Query  360      TGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC  419
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224521   TGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC  224580
Query  420      CGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGT  479
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224581   CGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGT  224640
Query  480      TAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAAGGAATTGACGGG  536
                ||||||||||||||||||||||||||||||||||||||||||||| |||||||||||
Sbjct  224641   TAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGG  224697

2A
Query  1        GCAGCC-TGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGG  59
                |||||| |||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224161   GCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGG  224220
Query  60       AAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAA  119
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224221   AAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAA  224280
Query  120      CTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCG  179
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224281   CTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCG  224340
Query  180      TAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAA  239
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Sbjct  224341   TAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAA  224400
Query  240      CTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCG  299
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224401   CTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCG  224460
Query  300      GTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGAC  359
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224461   GTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGAC  224520
Query  360      TGACGCTCAGGTGCNAAAGCGTGGGGAGC  388
                |||||||||||||| ||||||||||||||
Sbjct  224521   TGACGCTCAGGTGCGAAAGCGTGGGGAGC  224549

2F
Query  1        GCAGCC-TGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTC-GCGGGGAGG  58
                |||||| ||||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct  224161   GCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGG  224220
Query  59       AAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAA  118
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224221   AAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAA  224280
Query  119      CTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCG  178
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224281   CTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCG  224340
Query  179      TAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAA  238
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224341   TAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAA  224400
Query  239      CTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCG  298
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224401   CTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCG  224460
Query  299      GTGAAATGCGTAGAGATCTGGAGGAATACCGGNGGCGAANGCGGCCCCCTGGAC  352
                |||||||||||||||||||||||||||||||| |||||| ||||||||||||||
Sbjct  224461   GTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGAC  224514

2L
Query  1        GCAGCC-TGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGG  59
                |||||| |||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224161   GCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGG  224220
Query  60       AAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAA  119
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224221   AAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAA  224280
Query  120      CTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCG  179
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224281   CTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCG  224340
Query  180      TAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAA  239
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224341   TAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAA  224400
Query  240      CTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCG  299
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224401   CTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCG  224460
Query  300      GTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGAC  359
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224461   GTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGAC  224520
Query  360      TGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC  419
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224521   TGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC  224580
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Query  420     CGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGT  479
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Sbjct  224581  CGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGT  224640
Query  480     TAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAAGGAATTGACGG  535
               |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  224641  TAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGG  224696

2S
Query  3       TGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTC-GCGGGGAGGAAGGGAG  61
               |||||||||||||||||||||||||||||||||||||||||| |||||||||||||||
Sbjct  224168  TGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAG  224227
Query  62      TAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTG  121
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224228  TAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTG  224287
Query  122     CCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG  181
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224288  CCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG  224347
Query  182     CACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATC  241
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224348  CACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATC  224407
Query  242     TGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAAT  301
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224408  TGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAAT  224467
Query  302     GCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCT  361
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224468  GCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCT  224527
Query  362     CAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC  421
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224528  CAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC  224587
Query  422     GATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCG  481
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224588  GATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCG  224647
Query  482     ACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAAGGAATTGACGG  530
               |||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  224648  ACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGG  224696

3A
Query  1       GCAGCC-TGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGG  59
               |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224161  GCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGG  224220
Query  60      AAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAA  119
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224221  AAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAA  224280
Query  120     CTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCG  179
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224281  CTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCG  224340
Query  180     TAAAGCGCACGCAGGCGGTCTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAA  239
               |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  224341  TAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAA  224400
Query  240     CTGCATCTGATACTGGCAGGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCG  299
               |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  224401  CTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCG  224460
Query  300     GTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGAC  359
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224461  GTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGAC  224520
Query  360     TGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC  419
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224521  TGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC  224580
Query  420     CGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGT  479
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224581  CGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGT  224640
Query  480     TAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAAGGAATTGACGG  535
               |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  224641  TAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGG  224696

3F
Query  1       GCC-TGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAG  59
               ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224164  GCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAG  224223
Query  60      GGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTC  119
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224224  GGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTC  224283
Query  120     CGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAA  179
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Sbjct  224284  CGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAA  224343
Query  180     AGCGCACGCAGGCGGTCTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTG  239
               |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  224344  AGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTG  224403
Query  240     CATCTGATACTGGCAGGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTG  299
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Sbjct  224404   CATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTG  224463
Query  300      AAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGA  359
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224464   AAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGA  224523
Query  360      CGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGT  419
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224524   CGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGT  224583
Query  420      AAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAA  479
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224584   AAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAA  224643
Query  480      GTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAAGGAATTGACGG  532
                |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  224644   GTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGG  224696


3J
Query  3        TGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAG  62
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224168   TGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAG  224227
Query  63       TAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTG  122
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224228   TAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTG  224287
Query  123      CCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG  182
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224288   CCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG  224347
Query  183      CACGCAGGCGGTCTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATC  242
                |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224348   CACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATC  224407
Query  243      TGATACTGGCAGGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAAT  302
                |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224408   TGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAAT  224467
Query  303      GCGTA  307
                |||||
Sbjct  224468   GCGTA  224472
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