Interaction of *Pseudomonas putida* and *Listeria monocytogenes* in Mixed Culture Biofilms

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Chapter 2: *Pseudomonas putida* increases growth of *Listeria monocytogenes* in mixed culture flow cell biofilms

Abstract

Listeria monocytogenes is a foodborne pathogen that causes problems in many food processing plants because it produces biofilms and thus is difficult to control by regular cleaning and sanitizing procedures. It has been stated that the growth of *L. monocytogenes* is enhanced in mixed culture biofilms, but little information is available that provides a mechanistic explanation for this. Mixed culture biofilms with *Pseudomonas putida* (labeled with green fluorescent protein) and *L. monocytogenes* EGD were examined to determine whether one organism would enhance growth of the other. Mono and mixed culture biofilms were grown on glass cover slips, in M9 1x minimal salt medium supplemented with 1mM glucose at 22°C for 24 hours using a flow cell. Images were taken using a scanning electron microscope and with a confocal scanning laser microscope, after staining *Listeria* cells with a Texas Red-X conjugate of wheat germ agglutinin. Confocal images captured at inlet, middle and outlet of the flow cell were analyzed with the novel biofilm program PHLIP for total biovolume and mean thickness. At the inlet, almost all biofilms produced highest total biovolume and mean thickness; this was significant for *P. putida* ($P < 0.05$). In mixed culture biofilms at the inlet of the flow cell, biovolume contributed by *L. monocytogenes* cells, though not statistically significant, was higher than in monoculture *L. monocytogenes* biofilms (532% increase). In contrast, in mixed biofilms in the middle section and at the outlet, biovolume contributed by *P. putida* cells was much lower than in monoculture *P. putida* biofilms, with reductions of 183 % (middle) and 793% (outlet). To explain these findings, attachment and detachment were measured using cell concentration changes in effluent within mixed and monoculture flow cell biofilms of *L. monocytogenes* and *P. putida*. Although findings were inconclusive, confocal images of *P. putida* effluent suggest an increase in detachment. Dissolved oxygen was measured in planktonic cultures and glass wool biofilms using the same conditions as flow cell biofilms, as well as in mature biofilms suspended in PBS in order to measure dissolved oxygen without the influence of planktonic cells and the impact of media. Compared to *P. putida*, oxygen depletion from *L. monocytogenes* was much less severe. Dissolved oxygen began at approximately 8 mg/ml for all planktonic cultures but within hours declined for all cultures. Dissolved oxygen within the *L. monocytogenes* monoculture only dropped to 6.20 mg/ml, however

continued to decline reaching 2.40 mg/ml and 2.62 mg/ml for *P. putida* and mixed culture biofilms respectively. When we measured dissolved oxygen in the glass wool biofilms we found much less dissolved oxygen depletion than in the planktonic study, nevertheless, trends in dissolved oxygen levels were similar to planktonic cells. Similar trends to those found in biofilms formed in flow cells in relation to cell concentrations were also found within biofilms formed on glass wool.

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Chapter 1: Literature Review

1.1. *Listeria monocytogenes*

1.1.1. Introduction

Listeria monocytogenes is a psychrotrophic Gram-positive pathogen regularly involved in outbreaks of food-borne disease in which milk, cheese, meat, and vegetables are often the sources of infection (Chavant et al. 2001). Early studies, such as those by Weis and Seeliger (1975) showed that *L. monocytogenes* is an organism very ubiquitous and that humans and animals are exposed to this organism frequently in the natural environment. Evidence showed that high incidence of *Listeria* can often be found in plants and soil samples, as well as in the intestinal tract of animals, allowing *L. monocytogenes* to further be spread in the environment.

Perhaps one of the most problematic characteristics of *L. monocytogenes* was shown when Glass and Doyle (1989) demonstrated that *L. monocytogenes* can grow on a variety of processed meat products at refrigeration temperature (4.4°C), however, the rate of growth appeared to depend on the type and pH of the product. These results indicate the importance of preventing contamination of food products with *L. monocytogenes* because refrigeration may not be enough to keep the food safe. This is why *L. monocytogenes* is such a problem in food processing plants, and unfortunately there is much opportunity for *L. monocytogenes* to grow in these plants. When animals are slaughtered, microbial flora remains on the surface of the carcasses, and despite the decontamination processes carried out (scorching the skin or using hot water, organic acid, or phosphate solutions), part of the microflora survives. These microorganisms come into contact with the surfaces of equipment in cutting halls and other processing areas. *L. monocytogenes* can detach from the carcass and then contaminate products placed on the equipment. Conveyor belts are among the surfaces which, coming into contact with food, can remain heavily contaminated even after hygiene operations (Midelet and Carpentier 2002)

Another problematic scenario is that *L. monocytogenes* forms biofilms on numerous surfaces, generally in association with other microorganisms such as *Pseudomonas spp* (Sasahara and Zottola 1993). Biofilms are an important source of contamination for materials and foods coming into contact with them. Several reports have shown that *L. monocytogenes* in biofilms, compared to planktonic cells, is more resistant to environmental changes and cleaning or disinfection treatments. (Chavant et al. 2001). These biofilms allow organisms to survive for prolonged periods, depending on the amount and nature of residual soil, temperature, and relative humidity. Areas that are more prone to biofilm development include dead ends, joints, valves, and gaskets. In addition, equipment surfaces can be corroded with age. Pits and cracks may develop in which soil and bacteria can collect (Wong 1998).

1.1.2. Listeriosis

L. monocytogenes causes an invasive disease called listeriosis that most often manifests as meningitis or meningoencephalitis in nonpregnant, generally immunocompromised adults or infection of the fetus in pregnant women. (Gahan and Hill 2005). The organism has the ability to cross three significant barriers in humans, namely the intestinal barrier, the blood–brain barrier and the fetoplacental barrier. (Lecuit 2005) Thus, *L. monocytogenes* is readily isolated from clinical specimens obtained from normally sterile sites such as blood, cerebrospinal fluid, and amniotic fluid. (Schuchat et al. 1991).

The clinical course of infection usually begins about 20 hours after the ingestion of heavily contaminated food in cases of gastroenteritis, while the incubation period for the invasive illness is generally much longer, around 20 to 30 days. Similar incubation periods have been reported in animals for both gastroenteric and invasive disease (Vazquez-Boland et al. 2001). Transmission of *L. monocytogenes* after ingestion of food first requires penetration of the organism through the intestine (Schuchat et al. 1991). Several *L. monocytogenes* virulence factors mediating the key steps in cell infection have been identified. These include: internalin (also called InlA) and InlB (another member of the internalin multigene family, characterised by the presence of leucine-rich repeats),

which are responsible for internalization of *L. monocytogenes* in intestinal epithelial cells (Lecuit 2005). Quantitative electron microscopic studies show that *L. monocytogenes* moves from phagocytic vacuoles to the cytoplasm, where replication is improved by more favorable growth conditions. The ability to disrupt vacuolar membranes is considered important in the virulence of *L. monocytogenes*. (Schuchat et al. 1991). Nevertheless, before *L. monocytogenes* begins its food-borne infection, it comes across a number of hurdles that make up the hosts physico-chemical defence system. In order to quickly colonize the host gastrointestinal tract prior to invasion, the pathogen must survive acid conditions within the stomach as well as elevated osmolarity and the presence of bile salts within the small intestine. (Gahan and Hill 2005)

L. monocytogenes can be found frequently in the environment, and many studies have shown that human exposure to *L. monocytogenes* is not uncommon, yet invasive listeriosis occurs rarely (Schuchat et al. 1991). However, the disease still remains a concern because of its overall mortality rate of up to 30% (Lecuit 2005). Factors that may influence whether invasive disease will occur include the virulence of the infecting organism, size of the inoculum, and the susceptibility of the host (Schuchat et al. 1991). Illness has been reported in apparently immunocompetent individuals after ingestion of 10^6 to 10^9 cells, and it is probable that smaller populations will cause illness in immunocompromised persons (Lammerding et al. 1992). Generally speaking however, little is known about the infective dose of *L. monocytogenes*; though, animal studies and epidemiological findings indicate that certain populations are at much greater risk than healthy individuals (Baloga and Harlander 1991). These populations that are more at risk include people who are immunosuppressed and the elderly, due to absent or weakened cellular immunity. More frequent use of immunosuppressive medications for the treatment of various conditions and in organ transplantation has increased the immunosuppressed population and brought increased attention to listeriosis. Also, with the epidemic of acquired immunodeficiency syndrome (AIDS) and the rapid expansion of the population at substantial risk for listeriosis, this disease may be more frequently diagnosed (Schuchat et al. 1991). However, though human immunodeficiency virus (HIV) infection is a significant risk factor for listeriosis, AIDS is the underlying predisposing condition in only 5 to 20% of listeriosis cases in nonpregnant adults.

Furthermore, though it has been estimated that the risk of contracting listeriosis is 300 to 1,000 times higher for AIDS patients than for the general population, listeriosis remains a relatively rare AIDS-associated infection (Vazquez-Boland et al. 2001).

Another high-risk population includes pregnant women. Women infected with *L. monocytogenes* during pregnancy may develop mild, flulike symptoms or remain asymptomatic. However, infection of the fetus can result in abortion or birth of an infant with neonatal listeriosis, a condition known as granulomatosis infantiseptica that is characterized by the appearance of scattered granulomatous microabscesses and high mortality rates (Vazquez-Boland et al. 2001). Listeriosis may develop at any time during pregnancy, although most infections are detected in the third trimester. Infections occurring earlier in pregnancy may not be recognized if cultures are not obtained, and failure to culture the products of conception when spontaneous abortion or stillbirth occurs complicates the problem of estimating the proportion of fetal loss that may be attributable to listeriosis (Schuchat et al. 1991).

1.1.3. Listeria monocytogenes Serotypes

Serological typing by methods such as enzyme-linked immunosorbent assays (ELISA) is based on antibodies that specifically react with somatic or ''O'' antigens and flagellar or ''H'' antigens of *Listeria* species. *L. monocytogenes* is grouped into serotypes according to which O or H antigens are displayed. Although, serological confirmation is not required for identification of *L. monocytogenes*, serology is often used to determine the frequency of specific serotypes in epidemiological studies and for tracking environmental contamination (Gasanov et al 2004). Numerous molecular subtyping techniques such as Random Amplified Polymorphic DNA (RAPD) and Restriction Endonuclease Analysis (REA) have identified two major phylogenetic divisions within the species which are based on evolutionary lineage (Mereghetti et al 2002). Division I consists of serotypes 1/2b, 3b, 4b, 4d, and 4e, and Division II consists of serotypes 1/2a, 1/2c, 3a, and 3c. A third division consisting of less common serotypes 4a and 4c has also been identified (Borucki and Call 2003). Some argue that although serotyping has defined 16 serotypes of *L. monocytogenes*, this is of limited value in epidemiology

because over 90% of listeriosis is caused by pathogenic *Listeria* spp. belonging to a small number of serotypes (1/2a, 1/2b, or 4b, Baloga and Harlander 1991). In a study by Lammerding et al. (1992) the course of peroral listeric infection of pregnant immunocomprimised mice was monitored. *L. monocytogenes*, serotype 4b, 1/2a, and 1/2b colonized the gastrointestinal tract, translocated to the livers and spleens of mice by day 1 postinoculation, and multiplied in these tissues until day 4. Infection of the placental tissues occurred by days 3 and 4 and was followed by infection of the fetuses. Livers and spleens showed a cellular immune response. *L. monocytogenes*, serotype 3a, did not colonize the gastrointestinal tract, nor was it isolated from any internal tissue. Rarely, outbreaks have been caused by non 4b serotypes, a serotype 3a strain outbreak was linked to contaminated butter in Finland, and a serotype-1/2a outbreak of gastrointestinal listeriosis was linked to sliced turkey in the United States (Gray et al. 2004). It does not appear though, that serotypes isolated from food always correspond to those in clinical cases. Gilot et al. (1996) serotyped 832 *L. monocytogenes* strains collected in Belgium, over the period 1990 to 1992. Of these strains, 95 were isolated in the sporadic cases of listeriosis, and 737 were isolated from food. Most of the strains were typed as 1/2a (46.2%), 4b (21.8%), 1/2c (16.6%), and 1/2b (12.7%). Few strains belonged to serotypes 3a (1.3%), 3b (1.0%), 4c (0.2%), 3c (0.1%), and 4a (0.1%). The distribution of serotypes in the *L. monocytogenes* population isolated from food was different from that obtained with the population of strains of human origin. Strains isolated from cerebrospinal fluid, blood, and amniotic fluid in human clinical patients with listeriosis belonged to four different serotypes: 4b (45.2%), 1/2a (32.6%), 1/2b (19.0%), and 1/2c (3.2%). Whereas 45.2% of the strains isolated from human clinical patients belonged to serotype 4b and 3.2% belonged to serotype 1/2c, only 19.5% of the strains isolated from all food were serotyped as 4b, while 12.3% were typed as 1/2c.

There has also been a number of studies conducted that attempted to divide the serotypes even further. Serotype 4b strains are highly represented among clinical isolates, accounting for a large fraction of sporadic listeriosis in humans. Furthermore, most of the recent major epidemics involved serotype 4b strains (Zheng and Kathariou 1995) Thus, Ericsson et al. (1995), using the restriction enzyme AluI, obtained four different cleavage profiles of chromosomal DNA. Strains of serotype 4b were divided

into two groups. Again, because outbreaks of human listeriosis are often caused by serotype 4b strains, typing methods have been focused on and developed especially for strains belonging to this serotype. However, *L. monocytogenes* serogroup 1/2 strains seem to have become increasingly prevalent in human cases. Serogroup 1/2 strains are also often isolated during routine food examinations (Unnerstad et al.1999)

1.1.4. Listeria monocytogenes in Vegetation

One of the earliest large outbreaks of listeriosis occurred when perinatal listeriosis occurred in 1.3% of births at the maternity hospital in Halifax, Nova Scotia. Between March and September 1981, there were 7 adult and 34 perinatal cases of listeriosis, including 5 spontaneous abortions and 4 stillbirths. The case fatality rate among liveborn infants was 27%. In contrast to most patients with sporadic disease, nonpregnant adult patients with listeriosis in this outbreak had no evidence of an underlying immunosuppressive condition. The source of contamination was from coleslaw eaten prior to their illness. Coleslaw from a refrigerator of one patient and two unopened packages from the manufacturer grew *L. monocytogenes* serotype 4b. The cabbage came from a farmer whose sheep had died of listeriosis the year before the outbreak. The farmer used composted and raw sheep manure to fertilize his cabbage fields, which may have led to contamination of the crop. Cabbage was stored over the winter and spring in a large shed; growth of *L. monocytogenes* was probably enhanced by prolonged cold storage. (Schuchat et al. 1991). Today contaminated vegetables and cabbage are still a concern. Hough et al. (2002) have developed a quantitative real-time polymerase chain reaction detection method for *L. monocytogenes*. The reproducibility of the system showed that log differences in *L. monocytogenes* numbers added to cabbage could be reliably distinguished. The system allowed quantitative results to be obtained within 8 hours and was relatively inexpensive, showing good potential for routine analytical use. DNA fingerprinting methods including Random Amplified Polymorphic DNA (RAPD) and Restriction Endonuclease Analysis (REA) were used by Aguado et al. (2004) to examine ready-to-eat vegetables in a processing plant over 23 months. Out of 906 samples, the incidence of *Listeria* contamination was only 1.2%, discovering only 11

strains predominantly from tomatoes and green beans. Due to this low incidence rate it was difficult to track a route of contamination. Even though there was a low incidence of *L. monocytogenes,* presence of non-pathogenic *Listeria* species found in greater numbers was used as a warning indicator for poor working conditions that could lead to contamination.

Lin et al. (1996) examined occurrence *of L. monocytogenes*, at 4 supermarkets, 14 fast food chain restaurants, and 13 family restaurants. They homogenized salad samples and incubated them in trypticase soy broth for 6 hours, then in specific selective enrichment media before plating onto sets of selective agar plates. *L. monocytogenes* was only found in one of 63 samples. They concluded that the quality of vegetable salads in supermarkets, family restaurants and fast food chain restaurants was safe. More recently, Cho et al. (2004) isolated *Listeria spp* from 402 naturally contaminated domestic ready-to-eat (RTE) vegetable samples and examined the susceptibility to 12 antibiotics. From these, 17 samples (4.2%) were found to be contaminated with *Listeria* species. Among the 17 *Listeria spp.* isolates, only 2 (11.8%) were *L. monocytogenes* and 15 (88.2%) *L. innocua.* Antibiotic susceptibility test showed that the *Listeria spp.* isolates were very susceptible to the antibiotics tested, except for nalidixic acid.

Contamination of vegetables by *L. monocytogenes* is also a concern for livestock that are fed silage which is made from vegetation. Abortion outbreaks in sheep and goats have been linked with the consumption of silaged oranges and artichokes. This is most likely due to the high moisture content of silage that produces an excellent environment not only for mould, but also *L. monocytogenes* (Caro et al. 1990). A more recent report from Wagner et al. (2005) described an outbreak in a flock of 55 sheep which were fed grass silage contaminated with *Listeria.*

1.1.5. Listeria monocytogenes in Milk

A major outbreak occurred in Massachusetts and Connecticut during July and August 1983, where pasteurized whole or 2% milk was implicated epidemiologically as the vehicle of transmission of *L. monocytogenes* (Hayes et al. 1986). An inspection of the plant in which the milk was processed uncovered no evidence of improper pasteurization

(Doyle et al 1987). Most infections occurred in nonpregnant adults, all 49 of whom had immunosuppressive conditions. In addition, there were seven perinatal cases. The overall case fatality rate was 29%. Serotype 4b accounted for 32 of the 40 isolates that could be tested. (Schuchat et al.1991). Results of a study on the outbreak indicated that *L. monocytogenes* can survive the minimum high-temperature, short-time treatment (71.7°C, 15 s) required by the U.S. Food and Drug Administration for pasteurizing milk. (Doyle et al 1987). Borucki et al. (2004) performed a study in order to determine whether the dairy farm serves as a major reservoir for *L. monocytogenes*. They used pulsed-field gel electrophoresis (PFGE) to compare profiles of *L. monocytogenes* isolated from U.S. Pacific Northwest bulk milk, the environment of the dairy farm, and bovines to isolates of human sporadic disease cases. Thirty-one percent of strains from bulk milk had matching profiles, indicating the same strain was found region wide. When they compared farm associated strains to the human sporadic strains, there was a match in 23.3% of the strains indicating that dairy farms can indeed act as a reservoir for *L. monocytogenes*. However a study by Frye and Donnelly (2005) indicates that there is a low prevalence of *L. monocytogenes* in pasteurized milk produced in the U.S. From the 5519 samples they collected over 5 weeks from pasteurized whole milk, nonfat milk, and chocolate milk, they found that only one sample was positive for *L. monocytogenes* out of the 1846 nonfat milk samples (0.05%), which was 0.018% of all 5519 samples.

1.1.6. Listeria monocytogenes in Cheese

Outbreaks of *L. monocytogenes* are not exclusive to North America and continue to occur worldwide. Until 2001, food-borne outbreaks were not recognized in Japan although sporadic cases of neonatal listeriosis had been reported. In February 2001, Makino et al. (2001) found that *L. monocytogenes* serotype 1/2b was isolated from a washed-type cheese during routine *Listeria* monitoring of 123 domestic cheeses. Further samples from products and the environments at the plant that produced the contaminated cheese were examined for and *L. monocytogenes* serotype 1/2b was detected in 15 cheese

samples, Studies with people who had consumed cheese from the plant revealed 86 persons who had been infected with *L. monocytogenes*. Thirty-eight of those people had developed clinical symptoms of gastroenteritis or the common cold type symptoms after the consumption of cheese. Isolates from those patients exhibited identical serotype, pathogenicity for mice and HeLa cells, DNA fingerprinting patterns and PCR amplification patterns. From the epidemiological and genetic evidence, it was concluded that the outbreak was caused by cheese.

Da Silva et al. (1998) examined the incidence of *L. monocytogenes* in a variety of cheeses from Rio de Janeiro supermarkets and street markets. Samples went through an enrichment procedure, followed by plating and serotyping. They found that 10.68% of the cheeses were contaminated with *L. monocytogenes*, while 12.62% were contaminated with *Listeria innocua*, which is often a sign of *L. monocytogenes* contamination. Of the *L. monocytogenes* strains found 59.90% were serotype 1/2a, 27.54% were serotype 1/2b, and 12.56% were serotype 4b. Even though many of the strains were serotypes found in many cases of listeriosis, and the incidence was fairly high, no major outbreaks associated with cheese have occurred in Brazil. A similar study by Wagner et al. (2006) examined 2615 samples of cheese from Austria over a 3 year period. Out of 181 cheese factories, 50 (27.6%) had isolates of *L. monocytogenes* which accounted for a total of 182 isolates, however none we linked to any oubreaks. From 14 of the factories they were able to recover *L. monocytogenes* four or more times, and deemed these heavily contaminated. Five of the 182 isolates, could not be serotyped; of the remaining 177, 49.7% were serotype 1/2b, 26.6% were serotype 1/2a, and 22% were serotype 4b. Using pulsed-field gel electrophoresis they found that the 4b serotypes had a noticeably high genetic diversity.

1.1.7. Listeria monocytogenes in Meats

Meat is perhaps the most common source of *L. monocytogenes* in foods. Bohaychuk et al. (2006) examined 800 samples of raw ground beef, raw chicken legs, raw pork chops, processed turkey breast, roast beef, fermented sausage, chicken wieners,

and beef wieners from four supermarket chains in Edmonton, (Alberta, Canada). They found that *L. monocytogenes* was isolated from all meat and poultry product types except for roast beef. The majority of samples contained *L. monocytogenes* that belonged to serotypes 1/2a and 1/2b. Raw pork chops had the highest diversity of serotypes and were the only product type that contained a sample with *L. monocytogenes* serotype 4b. Overall *L. monocytogenes* was found in 52% of raw ground beef, 34% of raw chicken legs, 24% of raw pork chops, 4% of fermented sausages, 3% of processed turkey breast, 5% of beef wieners, and 3% of chicken wieners. An earlier study in Belgium by Coillie et al. (2004) collected ready to eat fish and meat products from four local supermarkets over a seven month period. In total, 138 fish products and 77 meat products, were examined and *L. monocytogenes* was isolated from 23.9% of the fish and 14.3% of the meat samples. Strains were serotyped by PCR. Most strains (89%) tested belonged to the serotype 1/2a. Four strains were classified as 1/2b, one as 1/2c, and one as 4b. In the period in which the strains were isolated from the food products, the serological distribution of the 30 clinical cases observed in Belgium was 1/2a (43.3%), 1/2b (13.3%), 1/2c (3.3%), and 4b (40%).

1.2. Biofilms

1.2.1. Introduction

According to Parsek and Fuqua (2004), the study of microbial biofilms has been gaining in popularity, and over time more attention is being paid to biofilms. Though this ultimately provides increasing amount of knowledge in the field, it makes research increasingly difficult as well. Researchers wishing to conduct biofilm research or compare their results with those of other laboratories will realize that there are few standardized systems or protocols for studying biofilms. Nonetheless, increasing progress is being made.

Dolan (2002) defines biofilms as an assemblage of microbial cells that is irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in a

matrix of primarily polysaccharide material. This matrix, more commonly known as the EPS (extracellular polymeric substances), is one of the most distinctive features of biofilms compared to planktonic populations, but characterizing the composition of the EPS matrix can be difficult. The composition of the EPS matrix varies depending on the organisms present and environmental conditions (Parsek and Fuqua 2004). In general however, it is mostly composed of a mixture of components that include polysaccharides, protein, nucleic acids, and other substances. The best studied of these components are the polysaccharides also termed exopolysaccharide. Most bacteria are able to produce polysaccharides, either as wall polysaccharides (capsules) or as extracellular secretions released into the surrounding environment (Davey and O'Toole 2000). Some of these polysaccharides are neutral or polyanionic, as in the case of Gram-negative bacteria. The presence or uronic acids (such as D-glucuronic, D-galacturonic, and mannuronic acids) or ketal-linked pyruvate confers the anionic property. This property is important because it allows association of divalent cations such as calcium and magnesium, which have been shown to cross link with the polymer strands and provide greater binding force in a developed biofilm. In the case of some Gram-positive bacteria, the chemical composition of the exopolysaccharides may be quite different and may be primarily cationic (Dolan 2002). EPS has also been reported to provide protection from a variety of environmental stresses, such as UV radiation, pH shifts, osmotic shock, and desiccation (Davey and O'Toole 2000) as well as antimicrobial and sanitizing agents (Borucki et al. 2003). With its many properties, and the fact that EPS may account for as much as 50% to 90% of the total organic carbon of biofilms, it is evident that it truly is an integral part of the biofilm (Dolan 2002).

Though EPS help facilitate initial attachment, full scale colonization occurs only when multiple organisms are attached. When different species have attached, colonization patterns will differ depending on the species, some organisms will join the developing biofilm, whiles others will colonize other areas of the substrate (Poulsen 1999). What ultimately dictates where and how many species of bacteria grow and colonize is communication between cells called quorum sensing. Quorum sensing is controlled by autoinducers, which are signaling molecules realeased by bacteria. At certain concentrations, these activate or repress certain genes, which will reflect on how

the community will behave (De Kievit and Iglewski 2000). Gram-negative bacteria have been found to use N-acyl homoserine lactones as autoinducers, these will then bind to a transcriptional activator and expression of target genes will begin (Schripsema et al. 1996). In contrast, Gram-positive bacteria, use peptide signal molecules, which will interact with histidine kinase (Kleerebezem et al 1997).

1.2.2. Listeria monocytogenes Biofilms

Biofilms of *L. monocytogenes* have been a major concern especially in food processing environments. In these environments biofilms can be difficult to control since they can form where water is plentiful and cleaning is not performed properly. The common sites for *L. monocytogenes* isolation from processing plants are filling or packaging equipment, floor drains, walls, cooling pipes, conveyors, collators used for assembling product before packaging, racks for transporting products, hand tools or gloves, and inside freezers (Tompkin et al. 1999).

Some arguments have been put forth as to whether *L. monocytogenes* forms a classic biofilm or if observed strain differences in biofilm formation assessed by staining with crystal violet merely reflect differential adherence to the substrate (Kalmokoff et al. 2001.) However, *L. monocytogenes* is not known to produce capsules, and therefore it was hypothesized by Borucki et al. (2003) that if they could stain the cells with a carbohydrate-binding dye, they could measure actual EPS production. They developed a microtiter plate assay using ruthenium red, which is a carbohydrate binding dye. It was found that all *L. monocytogenes* tested in the study clearly stained by this method, which is consistent with production of biofilm. Accordingly, many researchers have focused their attention on biofilm formation and adhesion of *L. monocytogenes*.

Djordjevic et al. (2002) examined 31 *L. monocytogenes* strains for biofilm formation using PVC microtiter plates; at the same time their intention was to try to standardize the experiment. They found that cellular growth rates and final cell density did not correlate with biofilm formation after 40 hours at 32°C, indicating that differences in biofilm formation were due to factors other than the ability to grow in modified Welshimer's broth. The study also showed a difference in biofilm formation between

phylogenetic divisions of *L. monocytogenes*. The majority of Division I strains had significantly greater biofilm formation than Division II and Division III strains. In addition, *L. monocytogenes* biofilm formation on PVC was analyzed by quantitative epifluorescence microscopy and results were compared to the PVC microtiter plate biofilm assay. Similar trends in biofilm formation were observed using the microscopic and the microtiter plate assays, indicating that the PVC microtiter plate assay may be used as a rapid, simple method to assess biofilm production.

Borucki et al. (2003) used a similar technique when they assayed 80 *L. monocytogenes* strains for biofilm formation. Significant differences were evident between phylogenetic divisions of *L. monocytogenes*, but in contrast to the report by Djordjevic et al. (2002), Division II strains were significantly better biofilm formers than Division I strains. These conflicting conclusions might arise due to differences in methodology, samples sizes, and specific strains used in the analyses. Variation in biofilm formation at the level of serotypes was also detected. However, although. Serotypes 3a, 1/2c, and 1/2a had the highest average intensity values, the range of absorption values was too high and the differences between serotypes were not statistically significant. It was also found that persistent strains, those that cause a prolonged contamination were significantly better biofilm formers than were strains obtained sporadically (nonpersistent), from bulk milk tanks. In the same study SEM was used to examine a high biofilm former (strain M39503A) and a low biofilm former (strain M35584A) grown on stainless steel and PVC. The high-biofilm-forming strain was originally classified as a persistent strain from bulk milk samples, and it clearly produced a dense, three-dimensional composite of cells with well-distributed channels and pores on both stainless steel and PVC. Conversely, the low biofilm-forming strain (nonpersistent from bulk milk) produced only sparse aggregates of cells on stainless steel and predominantly single attached cells on PVC.

Another study that examined a number of strains was done by Chae and Schraft (2000). They studied adhesion and biofilm growth at 37°C on glass slides for 13 strains of *L. monocytogenes*, and they also compared both planktonic growth and biofilm formation. It was determined that after 3 h of incubation, bacterial cells for all 13 *L. monocytogenes* strains were found attached to glass slides and that all strains formed

biofilms during the subsequent 24 hour incubation period. The number of attached bacteria ranged between 10^2 and 10^4 cfu/cm^2, and plate counts of 24 hour biofilms were between 10^3 and 10^5 cfu/cm^2. For five strains of *L. monocytogenes* (Murray, 7148, 7163, 5015-3, and 23074) the biofilms were grown over 4 days, swabbed and enumerated. It was found that there was no significant difference between Murray and 7163, and between 23074 and 5015-3, after 1 day of biofilm formation. However, at days 2, 3, and 4 of biofilm growth, there were significant differences in the mean cell numbers among the five strains of *L. monocytogenes*, except between Murray and 7163 (P=0.4) on day 2, and between 23074 and 7148 (P=0.11) on day 3. The cell counts in biofilms formed by all five strains increased for the first 2 days reaching 10^5 cfu/cm^2, except for *L. monocytogenes* 7148, which had a cell density of 10^4 cfu/cm^2. The study also showed that growth behavior of *L. monocytogenes* strains in planktonic may be different from the growth behavior within the biofilms. For example, Chae and Schraft (2000) found that although *L. monocytogenes* 7148 had the lowest relative attachment and biofilm growth, this strain had the same maximum growth rate as 5105-3. In addition, although *L monocytogenes* Murray and 7163 had the same initial ability to attach to the substratum, and the same biofilm growth, planktonic maximum growth rates and generation times for those strains were significantly different.

Not all studies have included a broad spectrum of strains. Another study by Chae and Schraft (2001) focused on two of the strains from the previous study, *L. monocytogenes* Murray and 7148, and analyzed the relationship between culturable and viable-but-non-culturable (VBNC) cells, or cells that can still cause disease but will not normally grow on media. Total cell numbers obtained for swabbed biofilms were compared to the number of cells detected within *in situ* biofilms under confocal scanning laser microscopy (CSLM). It was observed that on day 2, the number of cells enumerated *in situ* was identical to cell numbers counted after swabbing. In older biofilms however, cell counts obtained *in situ* were found to be higher than the counts for swabbed biofilms. This difference was attributed to the formation of VBNC in biofilms.

Knowing that *L. monocytogenes* is a problem in food processing plants, a study by Wong (1998) examined biofilms on materials closer to those that would be found at these processing plants. These materials included stainless steel and buna-n rubber.

Wong (1998) found that *L. monocytogenes* could survive for prolonged time periods on stainless steel and buna-n rubber and, under favorable conditions, even multiply on stainless steel. Temperature, relative humidity, soil, and surface type affected the behavior of surface-associated *L. monocytogenes*. In addition, the nature of the attachment surface affected the efficacy of sanitizers.

A similar study was performed by Chavant et al. (2002), who examined the surface adhesion and biofilm formation on hydrophilic stainless steel and hydrophobic polytetrafluoroethylene (PTFE) *by L. monocytogenes* LO28 under different conditions. The colonization of the two surfaces, monitored by scanning electron microscopy, epifluorescence microscopy, and cell enumeration by plate counting, showed that the strain had a strong capacity to colonize both surfaces irrespective of the incubation temperature. However, biofilm formation was faster on the hydrophilic substratum. After 5 days at 37 or 20°C, the biofilm structure was composed of aggregates with a threedimensional shape, but significant detachment took place on PTFE at 37°C. At 8°C, only a bacterial monolayer was visible on stainless steel, while no growth was observed on PTFE. Similarly Beresford et al. (2001) examined attachment of *L. monocytogenes* (strain 10403S) to materials in food processing environments. Specifically they studied several varieties of stainless steel, aluminum, polycarbonate, as well as several varieties of plastics, and rubber and examined both short term and long term attachment. The authors reported that, with the exception of polypropylene, the type of material may not be as important as much as the length of time in which the material comes in contact with *L. monocytogenes*. They found that the amount of attached cells did not vary between materials for short term, nor long term, and that there was a significant increase in cells after the 2 hour period independent of material type. Though many studies have been conducted that examined *L. monocytogenes* biofilms, it is important to realize that most biofilms are not monocultures.

1.2.3. Multispecies Biofilms

Except for of certain types of infections, most biofilms consist of multiple species of both eukaryotic and prokaryotic organisms. There are obvious consequences to different organisms in a biofilm as they are present in high density and close proximity. The potential for interspecies communication, competition, and cooperation is high. How different species perceive and respond to one another is a key feature of any multispecies system. Although this fact is widely appreciated, most of the biofilm research being conducted in the field today involves pure-culture systems. This is not surprising, trying to study the complexity present in simple, pure-culture biofilms is a challenge on its own (Parsek and Fuqua 2004). Nevertheless, some studies have already been reported in the field of mixed culture biofilms.

Localized growth and detachment events at the microscopic scale were studied by Stoodley et al. (2001). They used image analysis to quantify the size distribution and detachment rate for discrete biofilm cell clusters. The four species biofilm, composed of Gram-negative environmental isolates including *Pseudomonas aeruginosa* (ATTC 700829), *Pseudomonas fluorescens* (ATTC 700830), *Klebsiella pneumoniae* (ATTC 700831), and *Stenotrophomonas maltophilia.* The experiment was also broadened to include an undefined biofilm developed from a tap water inoculum to determine if similar detachment patterns might occur in "natural" biofilms. To support results from direct observation of the biofilm, they microscopically quantified the distribution of particulate biomass in the filtered effluent from the tap water biofilm. In both biofilms there was a steady detachment of cell clusters over the 16 -19 hour monitoring periods. The mixed culture biofilm produced an estimated detachment population as high as 3.6×10^9 CFU/min, representing a significant transfer of biomass from the biofilm to the liquid phase. When they examined the effluent from the tap water biofilm, similar results were observed.

Other studies have examined whether different microorganisms in a multi-species biofilm have had an impact on each other. Cowan et al. (2000) examined the response of a dual species biofilm to two compounds that are commonly found in mixed organic chemical waste. The biofilm consisted of *P. putida* DMP1, a p-cresol-degrading

organism, and *Pseudomonas sp.* strain GJ1, a 2-chloroethanol (2-CE)-degrading organism. The morphology, species distribution, and EPS production of biofilms grown under increasingly inhibitory toxicant concentrations were examined using scanning confocal laser microscopy. The planktonic growth and toxicity data obtained for the two pseudomonads suggested that GJ1 had a limited chance of survival in the presence of p-cresol. It was also evident that a single strain could not effectively remediate a mixed waste stream containing 2-CE and p-cresol. GJ1 benefited from the close interactions with DMP1 in a dual-species biofilm. In the presence of p-cresol, GJ1 was present in the greatest proportion when it was paired with DMP1 in a biofilm. The fact that GJ1 was almost eliminated from single-species or GJ1-ATCC 33456 dual-species biofilms at a p-cresol concentration of 1.9 mM implies that it benefited from being coupled with DMP1, in addition to the nonspecific resistance to toxicants that is associated with biofilms. GJ1 depended on DMP1 to mineralize p-cresol and thus detoxify the medium in its vicinity.

Similarly Norwood and Gilmour (2000), examined whether certain species in a biofilm have an effect on the other microorganisms. This study involved the mixed culture of *L. monocytogenes, Pseudomonas fragi* and *Staphylococcus xylosus* to steady state using a chemostat. The steady-state output of a chemostat was then transferred to the constant-depth film fermenter (CDFF) and the growth of these three organisms into a multispecies biofilm was achieved. This model was then used to determine the effect of increasing levels of sodium hypochlorite on the three microorganisms. The steady-state biofilms in the CDFF were exposed to increasing strengths of sodium hypochlorite; 200, 500 and 1000 p.p.m. free chlorine, but a substantial two-log cycle drop in bacterial numbers was only achieved at 1000 p.p.m. free chlorine. In planktonic culture all three organisms were completely eliminated when exposed to 10 p.p.m. free chlorine for a 30 second period. This study clearly illustrates the increased resistance of biofilms compared to planktonic cells. Mathematicians and physicists have used this type of data to model biofilm formation.

1.2.4. Biofilm Modeling

Several mathematical models of biofilm have been reported in the literature. Kreft et al. (2001) describe an example of an individual-based model (IbM) of biofilms. This fully quantitative IbM, called BacSim, was the first spatially explicit IbM of bacterial growth. BacSim essentially consists of two parts: one deals with the simulation of the growth and behavior of individual bacteria as autonomous agents; the other deals with the simulation of substrate and product diffusion and reaction. Since biofilm growth is usually a much slower process than diffusion of substrate into the biofilm, the diffusion process can be simulated assuming the growth process to be frozen, and the growth process can be simulated assuming the diffusion process to be in a pseudo-steady state.

Eberl and Efindiev (2003) have presented a more sophisticated mathematical model. Their model does not simply model biofilm growth, but is used to study the disinfection of microbial biofilms by antibiotics. The model they propose is a fully continuous biofilm formation mechanism that had been introduced as a mono-species/mono-substrate prototype model and was subsequently applied to a biofilm system with two species. The system describes antibiotic disinfection of biofilms, where in addition to viable biomass, inert biomass must be taken into account. According to Eberl and Efindiev (2003), this simple system has the advantage that it was extensively studied experimentally and numerically. The model itself is based on a system of diffusion-reaction equations that comprises degeneracy as well as fast diffusion. Consequently, since the overall mechanisms leading to mixing or separation of several species in a biofilm are not yet fully understood, no general multi-species biofilm model can be formulated at present.

Unlike other mathematical models, De Jong et al. (2002) have presented a three-tiered model that has been shown to predict food product contamination and thus optimizing and improving food production chains. They used process models that describe production chains as model reactors, kinetic models that can predict transformation of food components and contaminants, and lastly predictive kinetic models that that estimate production costs. Using their model in a case study of dairy

heat treatment, they were able to reduce overall annual production costs, as well as impacting energy consumption that could further reduce costs.

1.3 Study Objectives

There is a large amount of information concerning different serotypes of *L. monocytogenes*, and their prevalence in cases of listeriosis. However, there needs to be more investigation into mixed culture biofilms for this bacterium in order to get an understanding of how the actual biofilms found in food processing plants function with respect to *L. monocytogenes*. Thus, this study evaluated whether *P. putida*, a microorganism commonly associated with *L. monocytogenes*, enhances the growth of *L. monocytogenes* in biofilms, and in turn whether *L. monocytogenes* has any affect on *P. putida*. The secondary goal of this study is to attain data on attachment and detachment as well as the role of oxygen in *L. monocytogenes* and *P. putida* mixed culture biofilms. Oxygen was chosen because it may be limited in closed systems like flow cells and glass jars. Furthermore, experiments were conducted in high nutrient concentrations, and therefore nutrients are unlikely to be limiting factors of growth in my experimental systems. There appears to be difficulty modeling mixed species biofilms, and this study may provide information and experimental tools to further investigation in this field.

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Chapter 2: Effect of *Pseudomonas putida* on growth of *Listeria monocytogenes* in mixed culture flow cell biofilms

2.1. Introduction

Listeria monocytogenes is a Gram-positive microorganism, widely distributed in the environment and commonly resistant to environmental stress. It is associated with plant material, alive or dead, water, and soil (Notermans et al., 1998). This organism causes severe nonenteric disease (meningitis, septicemia) in immunocompromised individuals and abortion in pregnant women (Djordjevic et al., 2002). Food has been shown to be the primary mode of transmission of *L. monocytogenes*, which has been implicated in numerous food-borne disease outbreaks (Farber and Peterkin, 1991). These foods include soft cheeses, fish products, vegetables, milk, paté and other cooked meat products (Norwood and Gilmour, 2000). It is believed that contamination of these products by *L. monocytogenes* occurs during post-process procedures rather than through the organism surviving the processing itself (McLauchlin 1987).

Food processors have always relied on physical and chemical methods to eliminate microorganisms from surfaces. However, research in this area has indicated that adherent microorganisms in biofilms may be much more resistant to sanitizing compounds than planktonic cells (Norwood and Gilmour, 2000). In these food-processing environments biofilms can be difficult to control since they can form where water is plentiful and cleaning is not performed properly. The common sites for *L. monocytogenes* isolation from processing plants are filling or packaging equipment, floor drains, walls, cooling pipes, conveyors, collators used for assembling product for packaging, racks for transporting products, hand tools or gloves, and freezers (Tompkin et al. 1999). These sites may harbour microbial biofilms.

Several methods are used to study biofilms, most common are microscopy techniques (light, laser-scanning confocal, transmission electron, and scanning electron microscopy), which allow to observe microbial colonization directly (Djordjevic et al. 2002). Scanning electron microscopy (SEM) is an effective way to examine biofilm

topology at a high magnification, however artifacts such as microstructure distortion may result from SEM sample preparation (Surman et al. 1996). Novel staining protocols, such as those developed by Priester et al. (2007), may alleviate the problem of these artifacts. However, the use of SEM along with other forms of microscopy can be used to detect any artifacts present, while providing complementary information of several biofilm parameters (Cortizo et al. 2003). Epifluorescence microscopy data can lead to underestimates of biofilm levels, since thickness may not be measured properly depending on the technique, and lead to overestimates of the areas covered by cells, if some extracellular polymer is stained. Despite these concerns, direct microscopy is important for obtaining reliable results when studying the bacteria attached on surfaces (Djordjevic et al. 2002). The advantage of confocal microscopy, is that it also lets the viability of the biofilm to be visualized, and is an effective means to examine biofilm-substrate interactions (Surman et al. 1996).

Most biofilms consist of multiple species, which include prokaryotic and in some cases eukaryotic organisms. When different organisms in a biofilm are present in high density and close proximity, the potential for interspecies communication, competition, and cooperation is high. How different species perceive and respond to one another is a key feature of any multispecies system. Although this fact is widely appreciated, most of biofilm research conducted today involves pure-culture systems. (Parsek and Fuqua 2004). Never the less, much work has already been done in the field of mixed culture biofilms, including those of *L. monocytogenes.*

Flow cells have been an effective way to study these biofilms (Sternberg et al. 1999), as they produce controlled environments that can be used to monitor biofilm growth with respect to different parameters. Biofilms will form in protected areas under high flow conditions, compared to even growth under low flow (Stoodley et al. 2005). Within these flowing systems colonies will behave differently depending on nutrient flow, and age of the biofilm (Sternberg et al. 1999).

It has been stated that the growth of *L. monocytogenes* is enhanced in mixed culture biofilms. Carpentier and Chassaing (2004) found that four non-*Listeria* strains they tested increased the population of *L. monocytogenes* when grown in mixed culture biofilms.

A study by Bremer et al. (2001) demonstrated that *L. monocytogenes* attachment and survival increased in the presence of *Flavobacterium* species. Hassan et al. (2004) and Sasahara and Zottola (1993) found that certain strains of *Pseudomonas* species increase the amount of *L. monocytogenes* that will colonize a substrate, but little information is available to provide a mechanistic explanation for this. Thus, this study proposes to look at whether *P. putida*, a microorganism commonly associated with *L. monocytogenes*, enhances the growth of *L. monocytogenes* in biofilms, and in turn whether *L. monocytogenes* has any affect on *P. putida*. We propose to take a different approach compared to previous studies that mostly looked at cell counts, and instead examine different parameters of the biofilm (biovolume, and mean thickness), as well as examining different areas of the biofilm (inlet, middle, outlet) within a flow cell.

2.2. Methods and Materials

2.2.1. Microorganisms and culture conditions

L. monocytogenes EGD (ATCC 4428) and *P. putida* GFP9 were used in the various experiments. Stock cultures of both microorganisms, were maintained at –80 °C in 50% glycerol. *P. putida* GFP9 was originally isolated from a biofilm in a milk processing line and genetically labeled using a Mini-Tn5-luxAB plasmid to produce a green fluorescence (Chumkhunthod et al. 1998). Both *L. monocytogenes* and *P. putida* were grown on trypticase soy agar (TSA; Difco Laboratories, Michigan, USA) for 24 hours, *L. monocytogenes* at 37°C and *P. putida* at 30°C. The TSA plates and cultures were then stored at 4°C for up to one week. For each experiment, a fresh culture was grown on TSA plates for 24 hours, one isolated colony was then inoculated into 50ml of trypticase soy broth (TSB; Difco Laboratories) and incubated at 37°C for *L. monocytogenes* and 30°C for *P. putida*. After 24 hours, cultures were washed by centrifuging twice at 3100 xg in phosphate-buffered-saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) for 10 minutes, at 4°C. Using a NovaSpec light spectrophotometer (Biochrom LTD, Cambridge, UK) both cultures were

diluted with PBS to an OD_{600} of 0.324 ± 0.005 , this standardized the cultures to ~10^8 CFU/ml.

2.2.2. Preparation of glass cover slips, and flow cell

Glass cover slips (2.5cm x 6.0cm and 2.5cm x 2.5 cm Fisherfinest) were washed in 500ml of 2% RBS Detergent Concentrate solution (RBS; Pierce, Rockford, IL). The cover slips were placed in the solution for 10 minutes in a 50°C water bath. Next, the cover slips were washed with 500ml of distilled tap water and placed back into the 50°C water bath for 25 minutes. Lastly, the cover slips were rinsed by agitating in 500ml of distilled water for 5 intervals of 1 minute. After drying, a 2.5cm x 2.5cm cover slip was placed into a FC81 flow cell (BioSurface Technologies Corporation, Bozeman, MT) beneath the 2.5cm x 6.0cm cover slip. This was necessary because the flow cell required a 2.5cm x 6.0cm cover slip, however, only the 2.5cm x 2.5cm cover slip would fit into the critical point dryer. Only the 2.5 x 6.0cm cover slips were used when growing biofilms for confocal scanning laser microscopy. For the flow cell a Masterflex.L/S digital economy drive (model # 7524-50), cartridge pump (model # 7519-15), and cartridge (model # 7519-65) were used. Flow cell and tubing were autoclaved and cooled to room temperature prior to each experiment to ensure that all components of the equipment were sterile.

2.2.3. Biofilm growth

For each experiment, three biofilms were grown at 22°C, a *L. monocytogenes* monoculture, a *P. putida* monoculture and a combination of equal part *L. monocytogenes* and equal part *P. putida*. Approximately 3 ml of standardized cultures were inoculated into the flow cell with a 10 ml syringe and left in the flow cell for 1 hour to allow cells to adhere to the glass cover slip. After adhesion M9 1x minimal salt medium (Difco

Laboratories; Becton, Dickinson, and company, Sparks, MD) with 1mM glucose was pumped through the flow cell at a rate of 3.3 ml/hr for a 24-hour period.

2.2.4. Scanning Electron Microscope (SEM) preparation and imaging

At the end of the 24-hour growth period the 2.5 x 2.5 cm glass cover slip was removed from the flow cell and placed into a petri dish containing 2.5% glutaraldehyde and left at 4°C for 24-hours. Following this step, the cover slip was run through an ethanol series. Starting with 5% ethanol, the cover slip was moved every 15 mins through 9 increasing concentrations until reaching 100% ethanol, where it was placed into a Sorvall critical point drier (Newtown, CT) for critical point drying. Upon dehydration, the cover slip was coated with gold using an Ernest F. Fullen gold target sputter coater. For each biofilm, 5 images were taken at 250x, 500x, 1000x, 2500x, and 5000x magnification using a Jeol 5900 LV scanning electron microscope.

2.2.5. Confocal Scanning Laser Microscopy (CSLM) Preparation, Imaging, and Analysis

After 24 hours of biofilm growth, 4% paraformaldyhyde was pumped through the flow cell, and was left for 1 hour to kill the bacterial cells of the biofilm. PBS was then pumped through the flow cell for 1 hour to wash the paraformaldyhyde out. Then, the biofilm was stained by pumping 4 ml of 10μg/ml propidium iodide (PI) for the first set of biofilms, and 5μg/ml of Texas red wheat germ agglutin for the second set of biofilms (*L. monocytogenes* monoculture, and mix only) into the flow cell, and allowing 30 minutes for the biofilm to take up the stain. Then, PBS was pumped at a rate of 3.3 ml/hr for 1 hour to wash the stain from the flow cell.

Without removing the slide from the flow cell, images of the biofilms were taken using an Olympus FV300 confocal scanning laser microscope (CSLM). Images were acquired with a 60x PlanApo NA 1.4 oil immersion lens, using sequential scanning. Argon (10 mW, force air cooled, blue 488nm) and HeNe Green(1mW, 543nm) lasers

were used with FVX-BA 510-530 band pass emission. Three images were taken of the inlet, 5 in the middle, and 3 at the outlet of the flow cell. For the first set of biofilms, PI was used to visualize all 3 biofilms. For the second set of biofilms, images were taken of *P. putida* within the biofilm by means of the green fluorescence protein (GFP) for *P. putida* and by means of the wheat germ agglutinin for *L. monocytogenes*. These images were analyzed for total biovolume, and mean thickness using the biofilm image analysis program PHLIP (Phlip: The new Laser Scanning Microscope Image Processing Package) (Mueller et al., 2004).

For each biofilm and parameter, a one-way analysis of variance with an alpha value of 0.05 was used to examine whether there was any difference between the biofilms at the inlet, middle and outlet. A one-way analysis of variance was also used on the first set of biofilm data to determine whether *L. monocytogenes EGD* and *P. putida* monoculture biofilms varied from each other, as well as the mixed culture biofilm at the inlet, middle and outlet. Using the second set of biofilm data t-tests were used to determine whether the bacteria in the monoculture biofilms differed from their counterparts within a mixed culture biofilm.

2.3. Results

The exact counts for bacterial cultures prior to inoculation into the flow cell were 7.89 ± 0.29 log CFU/ml for *L. monocytogenes*, and 8.22 ± 0.18 log CFU/ml for *P. putida*. These were confirmed by plate counting on TSA plates incubated for 37°C and 30°C respectively.

Images of biofilms used for SEM analysis can be seen in Figures 2.1 – 2.3. Two sets of biofilms were grown in triplicate, each stained by a different method and then visualized by confocal scanning laser microscopy, and analyzed by PHLIP. Images from each set can be seen in Figures 2.4 and 2.5. The first set was stained exclusively with propidium iodide, the second was stained with Texas red wheat germ agglutin to visualize *L. monocytogenes*, and a GFP signal was used to visualize *P. putida*.

Biovolume and mean thickness was analyzed at the inlet, middle and outlet of the flow cell for the *L. monocytogenes* monoculture biofilm, the *P. putida* monoculture, and the mixed culture biofilm for both sets (Figure 2.6). *L. monocytogenes* produced the lowest biovolume of all the biofilms. This was seen in both data sets producing only 150 - 228 μm^3 per 0.0006 cm^2 for data set 1, and 284 - 772 μm^3 per 0.0006 cm^2 for data set 2 (Figure 2.6A and 2.6C). This was much less compared to the *P. putida* and the mixed culture biofilms, which produced biovolume ranging from 3578 – 18 228 μm^3 per 0.0006 cm^2 and 2011 – 23 783 μm^3 per 0.0006 cm^2 respectively between both data sets (Figure 2.6A and 2.6C). Though not as pronounced, the same trend was seen for mean thickness. *L. monocytogenes* had a mean thickness of 3.7 - 4.4 μm, for data set 1, and 2.4 – 3.7 μm for data set 2 at the inlet, middle and outlet respectively (Figure 2.6B and 2.6D). Mean thickness for the *P. putida* and the mixed culture biofilms ranged from 7.6 – 26.2 μm and 4.8 – 24.9 μm respectively between both data sets (Figure 2.6B and 2.6D).

To further visualize the biofilm structures, a total of 15 biofilm images were produced using the SEM (Figures 2.1 – 2.3). The images were used as a 3 dimensional visual indicator of the characteristics and differences between the monoculture *L. monocytogenes EGD* and *P. putida* biofilms, as well as the mixed culture *L. monocytogenes/P. putida* biofilm. It is evident that the *L. monocytogenes* produced the thinnest biofilm relative to the other 2 biofilms, while *P. putida* produced the thickest (Table 2.1.). This can also be seen in both sets of confocal images, where *L. monocytogenes* produced a monolayer while *P. putida* produced round structures (Figures 2.4A and 2.4B). It seems however, that a mixture of the two species produced a biofilm of intermediate thickness (Table 2.1.). Looking at EPS production, the *L. monocytogenes EGD* biofilm was the only one of the three where there was no EPS directly observed in any of the images (Table 2.1.) No EPS could be discerned in the confocal images. When examining biovolume, and substratum coverage, the mixed culture biofilm scored the highest in both categories compared to the two monoculture biofilm, *L. monocytogenes EGD* was moderate in both categories, while *P. putida* had moderate biovolume, but lacked in substratum coverage (Table 2.1). When visually inspecting the confocal images, the mixed culture biofilm at the inlet and the *P. putida*

biofilm appeared to produce the most biovolume and substratum coverage (Figures 2.4 and 2.5).

With respect to biovolume, at different locations in the flow cell, statistically significant variation between all three biofilm types was observed in the middle of the flow cell for data set 1, where PI signal was used. In contrast, at the inlet and outlet, the biovolume of the three biofilm types was not different (Figure 2.6A). Similar trends were observed for thickness (Figure 2.6B). Interestingly, this trend was not apparent for the data set 2 biofilms (Figures 2.6C and 2.6D). We proceeded to perform t-tests in order to determine significance of this apparent difference in total biovolume and mean thickness between data sets (Tables 2.2 and 2.3). For almost all biofilms, the biovolume calculated from the confocal images was higher for data set 2, where biofilms were stained with Texas red WGA and the total biovolume was determined by adding the signal of GFP and WGA. This difference between data sets was significant for the *L. monocytogenes* monoculture biofilm in the middle of the flow cell and for *P. putida* biofilms in the middle and at the outlet of the flow cell (Table 2.2). Similar results were observed with respect to biofilm thickness for the *P. putida* monoculture biofilms, and mixed culture biofilms, but this was only significant for *P. putida* monoculture biofilms (Table 2.3). Since only limited information could be extracted from data set 1, due to the inability to visualize both microorganisms individually, further analysis was carried out for data set 2 only.

Data in figures 2.6A and 2.6C show a trend where biovolume was higher at the inlet than the middle and the outlet for both *P. putida* monoculture and mixed culture biofilms. Although these differences were not statistically significant, the biovolume at the inlet, in the middle, and at the outlet of the flow cell were investigated in more detail. Individual biovolume of *L. monocytogenes* and *P. putida* for mixed and monoculture biofilms were compared for the three locations (Figure 2.7). No difference in biovolume for *L. monocytogenes* monoculture biofilms was observed. *L. monocytogenes* biovolume in the mixed culture biofilm and *P. putida* biovolume in the *P. putida* monoculture biofilm were higher at the inlet than in the middle and at the outlet of the flow cell, although this was not statistically significant. Clearly, biovolume of *P. putida* growing in

a mixed culture biofilm was significantly higher at inlet than in the middle and at the outlet of the flow cell (P = 0.006).

Lastly, also using the second set of biofilm data, t-tests were performed to determine whether total biovolume and mean thickness of *L. monocytogenes* and *P. putida* biofilms differed between mixed and monocultures. Biovolume and thickness of *L. monocytogenes* were higher at the inlet for mixed culture biofilms, although this was statistically not significant. (Figures 2.8A and 2.8B)

In contrast, total biovolume (P = 0.002) and mean thickness (P = <0.001) of *P. putida* were significantly lower at the outlet of the flow cell in mixed culture biofilms, compared to monoculture biofilms (Figures 2.8D and 2.8C).

2.4. Discussion

SEM analysis was used to give qualitative insight into growth characteristics and morphology of both monoculture and mixed culture biofilms of *L. monocytogenes EGD* and *P. putida* grown at 22°C. Information from SEM was also compared to the confocal microscopy data. Several notable characteristics were evident from the SEM images. Firstly, biofilms of *L. monocytogenes EGD* and *P. putida* had very different architecture which had a direct impact on substratum coverage and thickness. The *L. monocytogenes EGD* biofilm seemed to entirely consist of a single cell layer, but as noted in Table 2.1, covered a larger surface area than *P. putida*. This single layer of *L. monocytogenes* biofilms was also described by Carpentier and Chassaing (2004). This could indicate a competitive strategy of listeriae to cover more surface area or simply the organism's inability to produce thicker biofilms due to a limiting factor. Consequently, the thicker biofilm produced by *P. putida,* which was described by Cowan et al. (2000) as tower- or mushroom-shaped, might indicate, an opposite strategy, where the bacteria establish a thicker biofilm before competing for space. Thus, a positive relationship may exist for these two species, for example, the growth of *L. monocytogenes EGD* could be enhanced because *P. putida* protects *L. monocytogenes EGD*. Also, the lack of visible EPS in the *L. monocytogenes EGD* biofilm and a presence of EPS in the *P. putida* biofilm might

indicate that the EPS plays an important role in growth of a thick biofilm (Carpentier and Chassaing 2004)

Though there was no visible EPS in the SEM images of *L. monocytogenes EGD* biofilms, the substratum with this biofilm seems to be decorated, possibly by *L. monocytogenes* EPS (Figure 2.1E). It is also important to note that the lack of visible EPS may be the result of preparation of the *L. monocytogenes EGD* cover slip during the biofilm drying process required for SEM. A study done by Borucki et al. (2003) confirms the presence of EPS in *Listeria* biofilms. They used a crystal violet microtiter assay to differentiate strong and weak biofilm forming strains of *L. monocytogenes*. They found that all strains tested positive for extracellular polysaccharides, with the highest biofilm formers producing noticeably more EPS. This however is not conclusive either, as it may be a difference in conditions and strains that may be causing the lack of visible EPS as well.

This ambiguity illustrates the limitation of SEM imaging. Because it is mostly a qualitative process, it is difficult to extract quantitative information from SEM images. Another difficulty is the process itself. Though the SEM preparation was carefully carried out, it must be accepted that when the cover slip with the biofilm is removed from the flow cell, the biofilm could be disturbed. Similarly, as noted by Priester et al. (2007) the preparation and drying steps may have an effect as well, more notably on the EPS. However this does not mean the SEM images are irrelevant. Contrary, the SEM images are useful as a means to get a preliminary idea of the biofilm structures expected. The SEM images are helpful in this particular experiment because it was possible to reinforce the results observed from some of the CLSM data.

In all cases the biofilm produced by *L. monocytogenes EGD* had much lower total biovolume, and to a lesser extent, lower mean thickness than that of *P. putida* and mixed culture biofilms (Figure 2.6). This trend of *Pseudomonas* has been also reported in a study for the inactivation of *L. monocytogenes/Pseudomonas* biofilms by peracid sanitizers. Fatemi and Frank (1999) noted that *Pseudomonas* predominated in initial biofilm populations, similar to our 24 hour biofilms. We also reported significant variation in biovolume and mean thickness that was mostly seen in data set 1. This reinforces the theme of edge effects noted by Sekar et al. (1998), in which there is

variation in the colonization of microorganisms at edges of test substrata. These variations could be masking the trends found for the middle of the biofilm in the flow cell.

In this study, three main areas of the biofilms were examined with a focus on total biovolume, those found at the inlet, middle, and outlet of the flow cell. The results of the one-way analysis of variance for inlet, middle, and outlet within biofilms showed that some of the biofilms did vary significantly depending on location. There appeared to be more variation when examining *P. putida* in the mixed biofilm (Figure 2.7). Also, it was evident as shown in Figure 2.6 that in almost all cases of the mono and mixed culture biofilms, there was greater total biovolume at the inlet, when compared to the middle and outlet, even in those biofilms where the analysis of variance reported no significant difference. In a proposed mathematical model of *L. monocytogenes* and *P. putida* biofilms, Eberl and Schraft (2005) suggested that there is more growth at the inlet of the flow cell due to the high amount of available oxygen for *P. putida*, however the biofilm decreases at the middle and outlet in mixed culture due to *L. monocytogenes* using up available oxygen at a faster rate despite being a facultative anaerobe.

Before concluding on the effect of *P. putida* on *L. monocytogenes EGD* biofilms, it is important to note that it was not possible to view *L. monocytogenes EGD* exclusively in the mixed culture biofilm in data set one (Figure 2.4). This was due to the fact that there was cross detection between the GFP and PI fluorescent signals. The problem of cross detection, or unwanted stain binding between stains, is not uncommon. A similar problem occurred with fluorescent antibodies binding to *C. testeroni*, in a study by Carpentier and Chassaing (2004). Due to this problem, the images of their mixed biofilm could not be taken in an identical spot when observing emission from the GFP and PI. Because *P. putida* was already labeled with a green fluorescence protein, the original objective was to stain the entire biofilm and view the GFP and propidium iodide emission signal separately. However, this was in fact only possible in data set two (Figure 2.5) by using the WGA instead of propidium iodide. Though this method allowed for a differentiation between the two species, the procedure was not always flawless and at times the two emission spectra would overlap causing incomplete differentiation between fluorescent signals, often referred to as cross talk. This problem was not visually apparent

when using the confocal microscope. However, it was problematic during image analysis that would pick up slight cross talk in certain images due to the sensitivity of the PHLIP program. This was overcome by subtracting *P. putida* confocal images from *L. monocytogenes* images using Image Pro (Media Cybernetics Inc., Bethesda, MD, USA) image analysis software. Since *P. putida* is already labeled with a green fluorescent protein, the next logical step would be to label *L. monocytogenes* with another fluorescent protein in order to facilitate further study more efficiently and with greater analytical precision.

T-tests showed that *P. putida* had significantly less total biovolume at the outlet, and significantly less mean thickness at the middle and outlet when in mixed culture than in monoculture biofilms, again likely due to the oxygen theory proposed by Eberl and Schraft (2005)(Figure 2.8). The lack of significant increase of *L. monocytogenes* biofilms when grown with *P. putida* is similar to results reported by Carpentier and Chassaing (2004). They found that 4 out of 29 microorganisms examined had a positive effect on *L. monocytogenes* settlement, however this increased *L. monocytogenes* population by no more than 1 log CFU/cm^2. Interestingly, none of the 4 microorganisms was a *Pseudomonas* species; organisms that did have a positive effect included *Kocuria varians, Staphylococcus capitis, Stenotrophomonas maltophilia*, and *Comamonas testosteroni*. In fact, their results indicate that of the 6 *P. putida* strains that they tested, 5 had a negative effect on the growth of *L. monocytogenes* biofilms, while one had no effect. However, there were other studies reporting results similar to mine. Hassan et al. (2004) found that *L. monocytogenes* attached in significantly greater numbers (> 3-log difference) to surfaces with preexisting *P. putida* biofilms than to *Pseudomonas*-free surfaces. It was also shown that *L. monocytogenes* survived better in the presence of *P. putida* with no added nutrients for 35 days, with numbers of survivors in the range of 3 to 4 log CFU/cm^2 in the presence of *P. putida* and less than 2.9 log CFU/cm^2 in pure culture. Sasahara and Zottola (1993) also found that in pure cultures, attachment of *L. monocytogenes* to glass cover slips was sparse, while *P. fragi* accumulated on glass coverslips as a confluent layer of cells. When *L. monocytogenes* was grown in mixed culture with *P. fragi*, an exopolymer-producing microorganism, attachment and microcolony formation by *L. monocytogenes* was enhanced. This may indicate that it is

in fact the EPS enhancing the variable parameters of the growth of *L. monocytogenes EGD*, however Carpentier and Chassaing (2004) could not find a link between EPS production by pure-culture non-*Listeria* biofilms and their ability to affect *L. monocytogenes* biofilm population size. They do suggest though, that it may be the nature of the EPS rather than the quantity that could affect the *L. monocytogenes* population.

The investigation into both growth enhancement of *L. monocytogenes* by *P. putida* in mixed culture biofilms and the examination of growth change at different locations within flow cell biofilms was successful. Our studies suggests a decrease in both total biovolume and mean thickness, for *P. putida* when grown with *L. monocytogenes* and that biofilm growth is not uniform across the substratum. Although mechanistic explanations were looked at in this study, further experiments focused on different parameters are necessary. Examination of oxygen availability could explain the loss of *P. putida* growth and adhesion experiments could further evaluate how the growth of *L. monocytogenes* is affected by *P. putida* in mixed culture biofilms.

Table 2.1. Visual comparison of monoculture *L. monocytogenes EGD*, *P. putida* and mixed culture *L. monocytogenes EGD/ P. putida* biofilms based on SEM images.

Biofilm	Biovolume	Substratum Coverage	Thickness	EPS
L. monocytogenes EGD	Moderate	Moderate	Low	Absent
P. putida	Moderate	Low	High	Visible
Mixed *L. monocytogenes EGD/P. putida*	High	High	Moderate	Visible

Table 2.2. T-test of total biovolume (μm^3) for biofilms between data set 1 and 2.

Biofilm	Area	Mean Set 1 (PI Signal)	Mean Set 2 (GFP and WGA Signal)	P-value
L. monocytogenes EGD	Inlet	199.10 ± 130.79	772.25 ± 552.28	P = 0.155
	Middle	150.39 ± 64.39	590.88 ± 382.21	P = 0.035
	Outlet	228.45 ± 103.07	283.67 ± 21.40	P = 0.415
P. putida	Inlet	15941.71 ± 11598.62	18287.88 ± 8414.51	P = 0.791
	Middle	3757.67(median)	11858.31(median)	P = 0.008*
	Outlet	4664.41 ± 3124.75	14329.87 ± 2859.82	P = 0.017
Mixed *L. monocytogenes EGD/P. putida*	Inlet	12778.22 ± 9981.12	23798.31 ± 9485.40	P = 0.238
	Middle	5357.38 ± 1161.59	6147.62 ± 4869.02	P = 0.733
	Outlet	2473.32 ± 817.78	2010.96 ± 1002.04	P = 0.569

*Based on Mann-Whitney Rank Sum Test (data did not meet prerequisites for t-test)

Table 2.3. T-test of mean thickness (μm) for biofilms between data set 1 and 2.

Biofilm	Area	Mean Set 1 (PI Signal)	Mean Set 2(GFP and WGA Signal)	P-value
L. monocytogenes EGD	Inlet	4.44 ± 1.29	2.44 ± 0.62	P = 0.073
	Middle	3.89 ± 1.72	3.50 ± 0.39	P = 0.628
	Outlet	3.73 ± 0.36	3.71 ± 0.23	P = 0.937
P. putida	Inlet	12.66 ± 10.33	21.25 ± 10.46	P = 0.369
	Middle	7.59 ± 1.50	22.51 ± 2.11	P = <0.001
	Outlet	9.40 ± 5.23	26.17 ± 0.98	P = 0.005
Mixed *L. monocytogenes EGD/P. putida*	Inlet	12.89 ± 8.241	24.89 ± 8.60	P = 0.156
	Middle	10.42 ± 2.05	13.21 ± 7.07	P = 0.421
	Outlet	8.89 ± 5.23	4.85 ± 0.98	P = 0.258

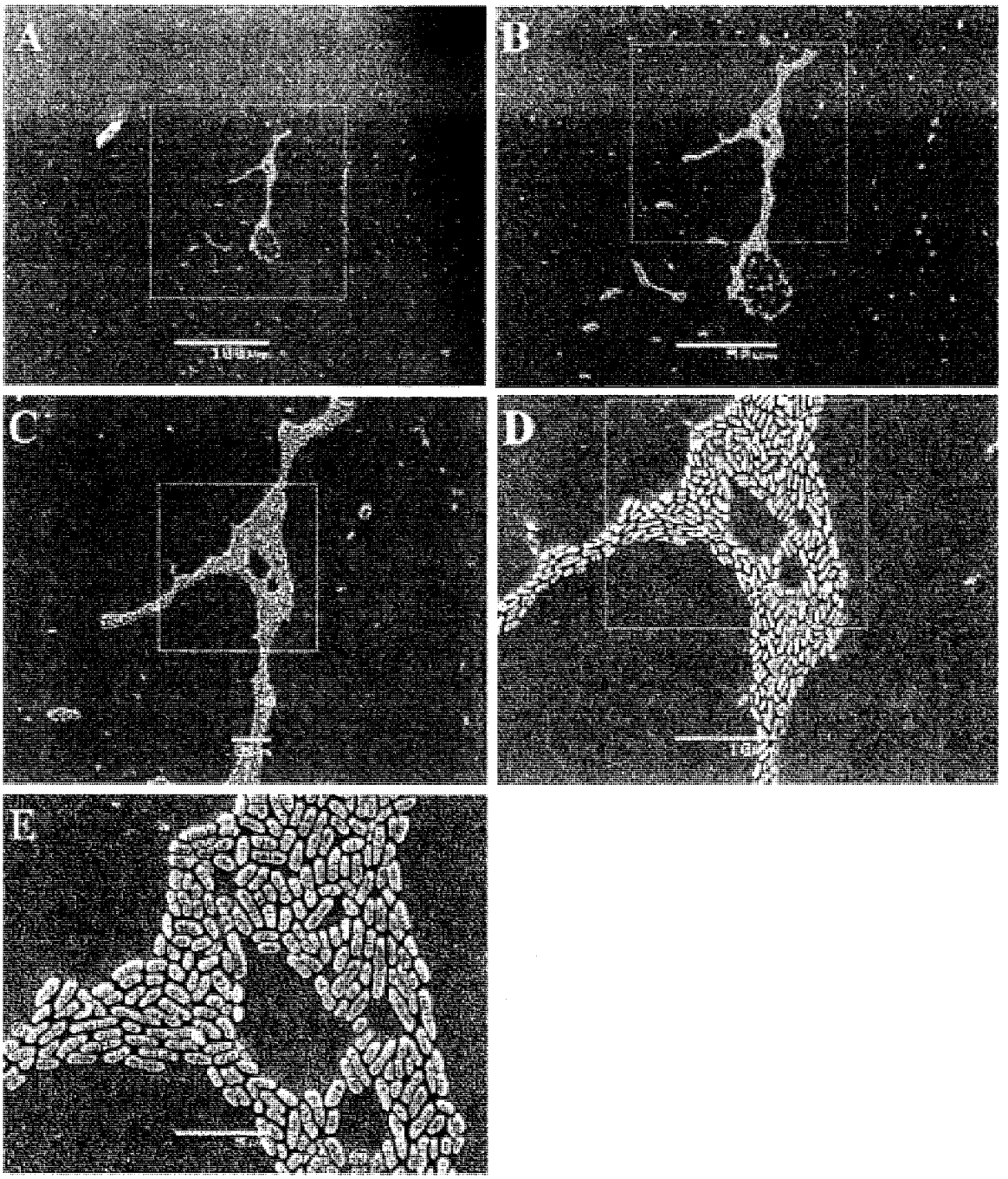

Figure 2.1. SEM images of *L. monocytogenes EGD* biofilm at (A) 250x, (B) 500x, (C) 1000x, (D) 2500x, (E) 5000x magnification. Biofilms were grown on glass at 22°C for 24 hours.

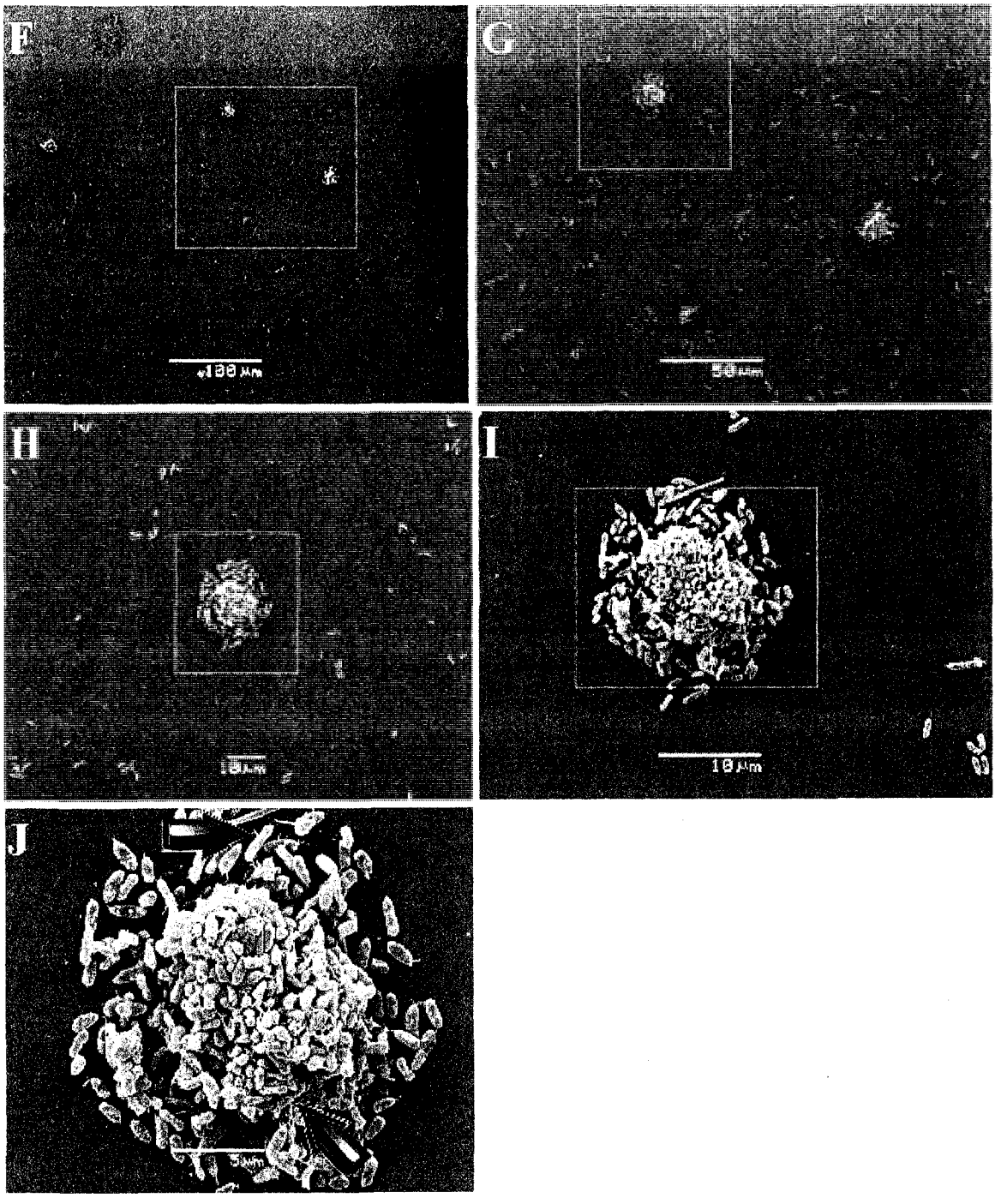

Figure 2.2. SEM images of *P. putida* biofilm at (F) 250x, (G) 500x, (H) 1000x, (I) 2500x, (J) 5000x magnification. Biofilms were grown on glass at 22°C for 24 hours. Arrows indicate EPS.

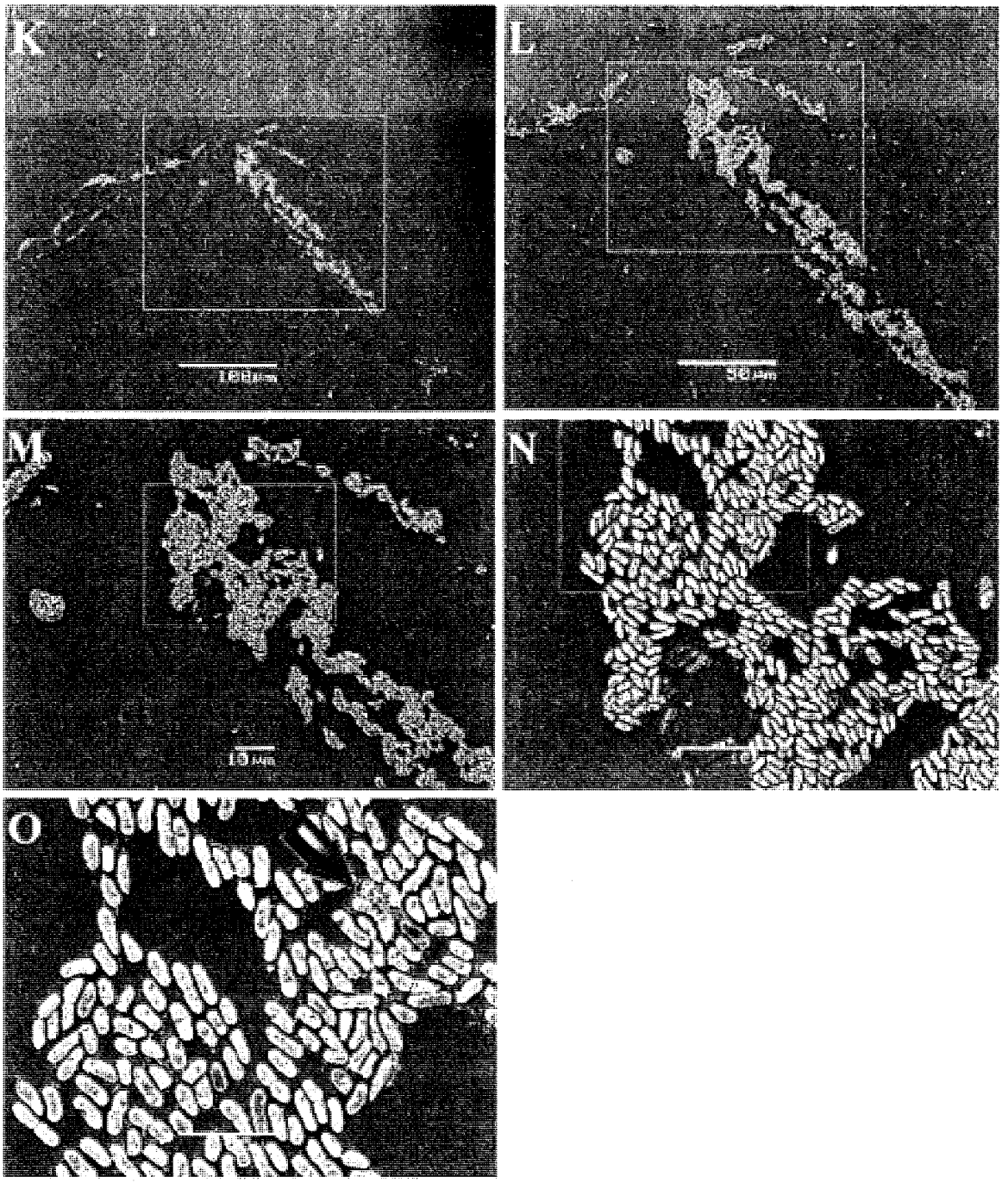

Figure 2.3. SEM images of *L. monocytogenes EGD/ P. putida* mixed culture biofilm at biofilm (K) 250x, (L) 500x, (M) 1000x, (N) 2500x, (O) 5000x magnification. Biofilms were grown on glass at 22°C for 24 hours. Arrows indicate EPS.

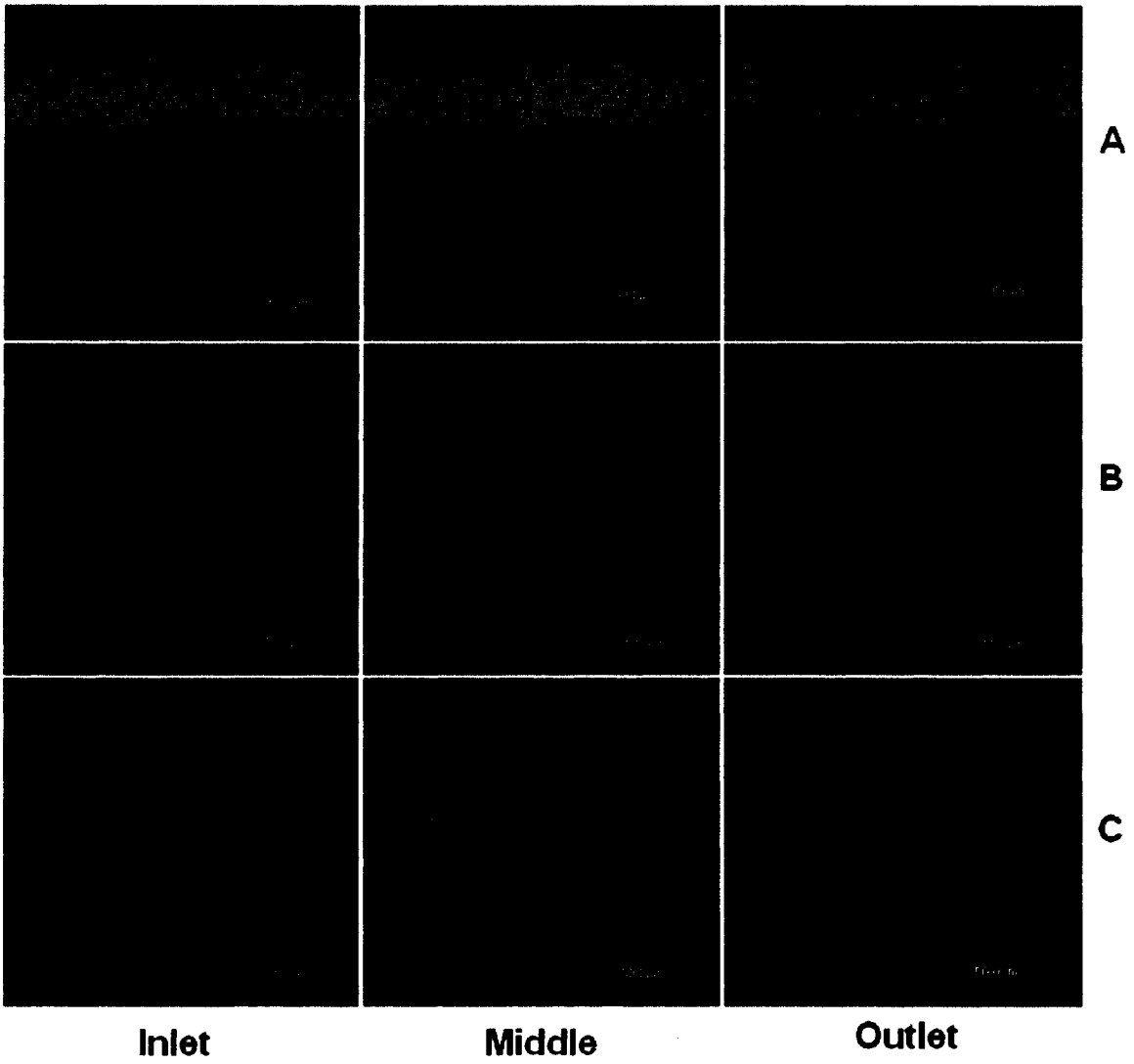

Figure 2.4. Data set 1 confocal images of *L. monocytogenes EGD* (A; red PI signal), *P. putida* (B; red PI signal), and mix biofilms (C; red PI signal) at inlet, middle, and outlet. Magnification 600x.

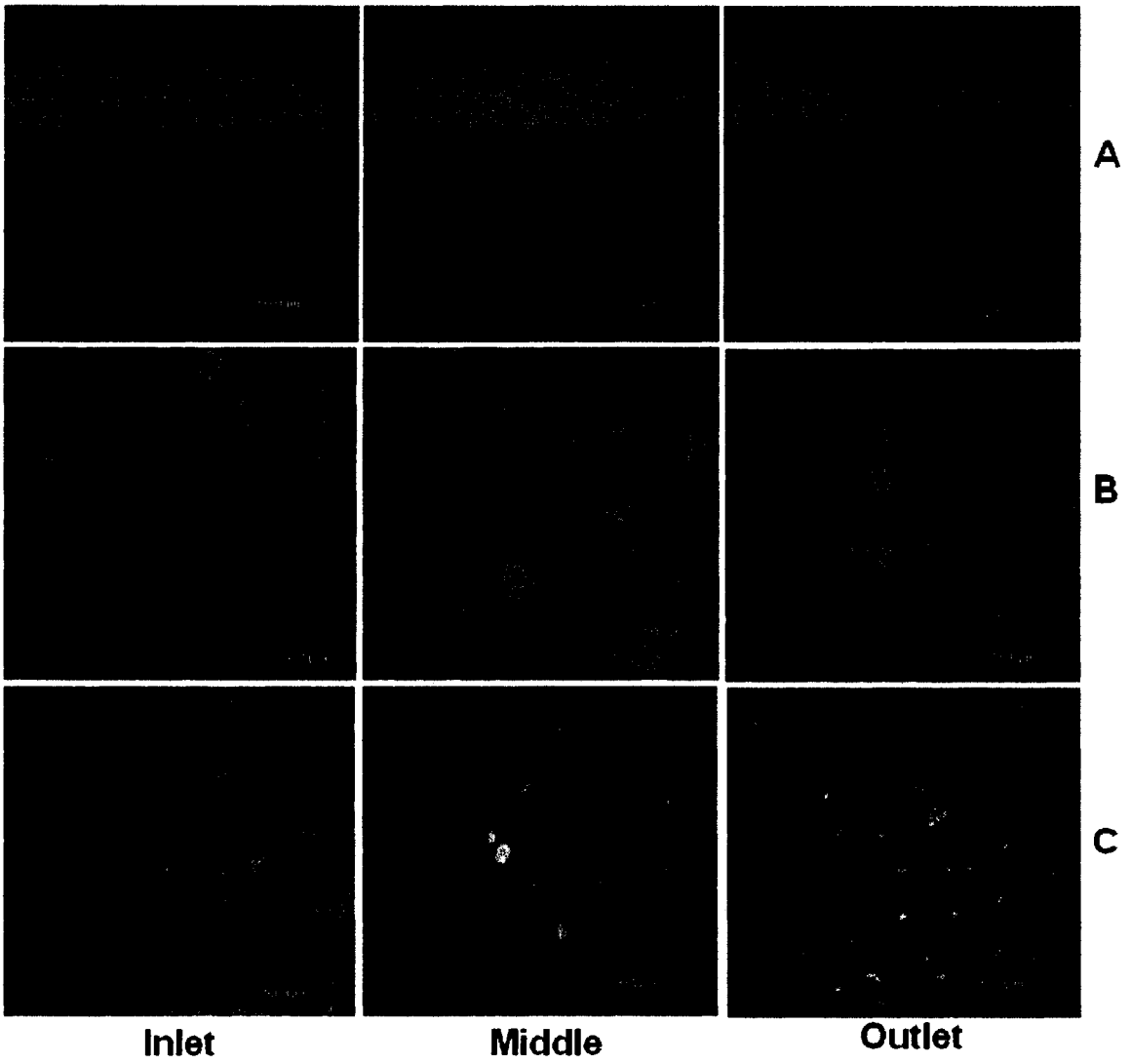

Figure 2.5. Data set 2 confocal images of *L. monocytogenes EGD* (A; red WGA signal), *P. putida* (B; green GFP signal), and mix biofilms (C; red/green WGA and GFP signal) at inlet, middle, and outlet. Magnification 600x.

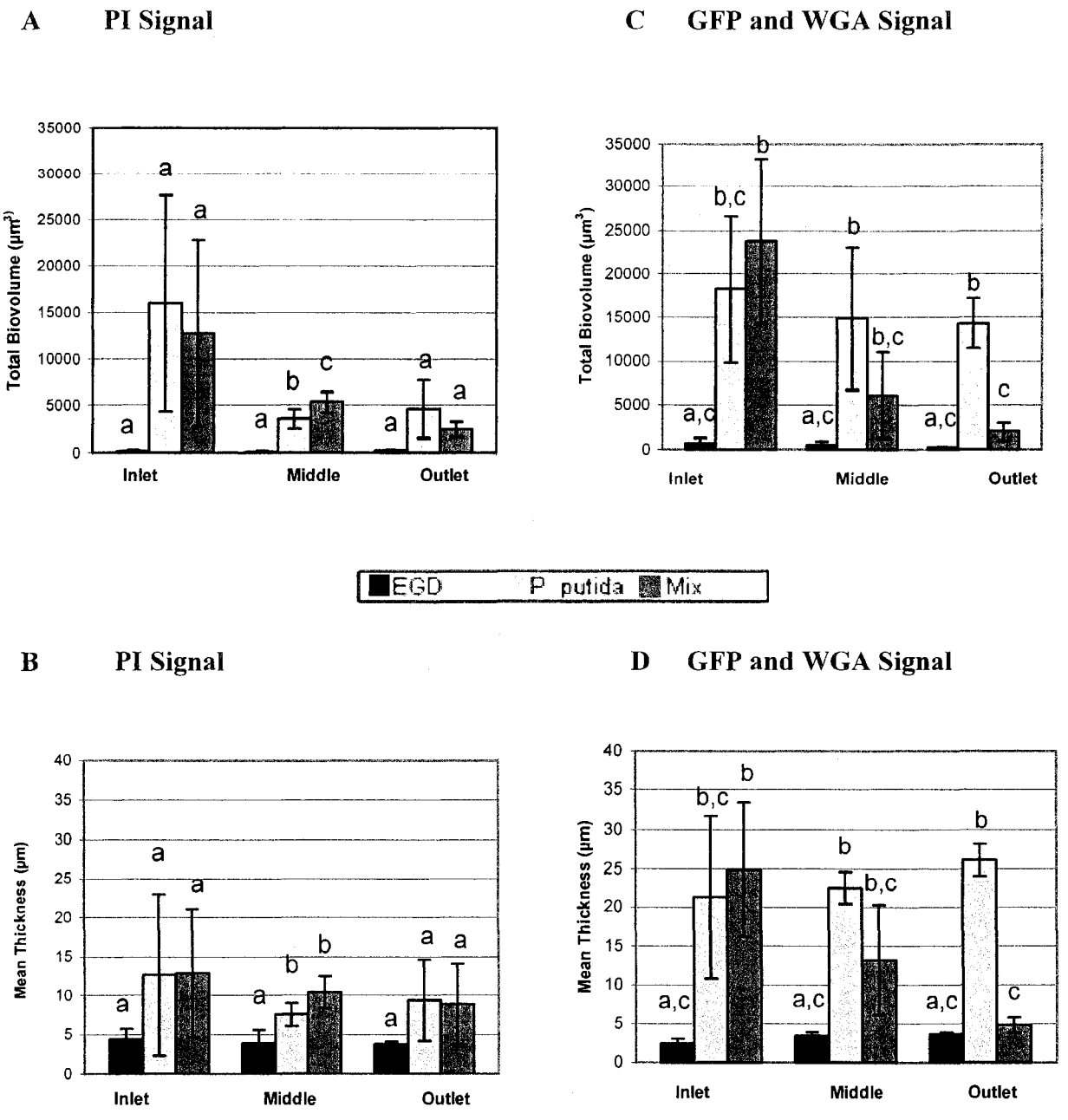

Figure 2.6. Comparison of total biovolume (μm^3) (A, C) and mean thickness (µm)(B, D) contributed by both monoculture and mixed culture *L. monocytogenes EGD* and *P. putida* biofilms comparing inlet, middle and outlet between each biofilm. Graphs A and B are from data set 1 (PI Signal), while graphs C and D are from data set 2 (WGA and GFP signal). For each sampling location bars with different letters indicate significant difference in biovolume or mean thickness between biofilms ($p < 0.05$).

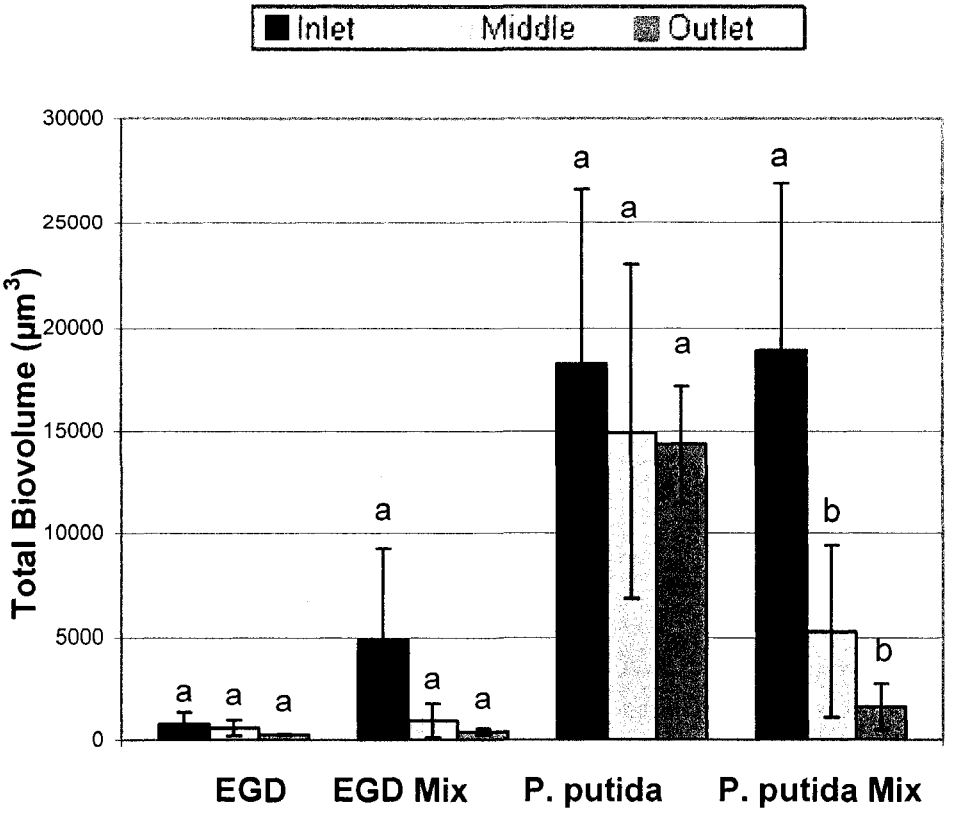

Figure 2.7. Comparison of total biovolume (μm^3) contributed by both monoculture and mixed culture *L. monocytogenes EGD* and *P. putida* data set 2 biofilms. Inlet, middle and outlet within each biofilm are being compared. For each biofilm bars with different letters indicate significant difference in total biovolume between biofilms ($p < 0.05$). EGD represents *L. monocytogenes* EGD stained with Texas Red WGA. EGD Mix represents *L. monocytogenes* EGD within the mixed culture biofilm stained with Texas Red WGA. *P. putida* represents *P. putida* visualized by its GFP signal. *P. putida* Mix represents *P. putida* within the mixed culture biofilm visualized by its GFP signal.

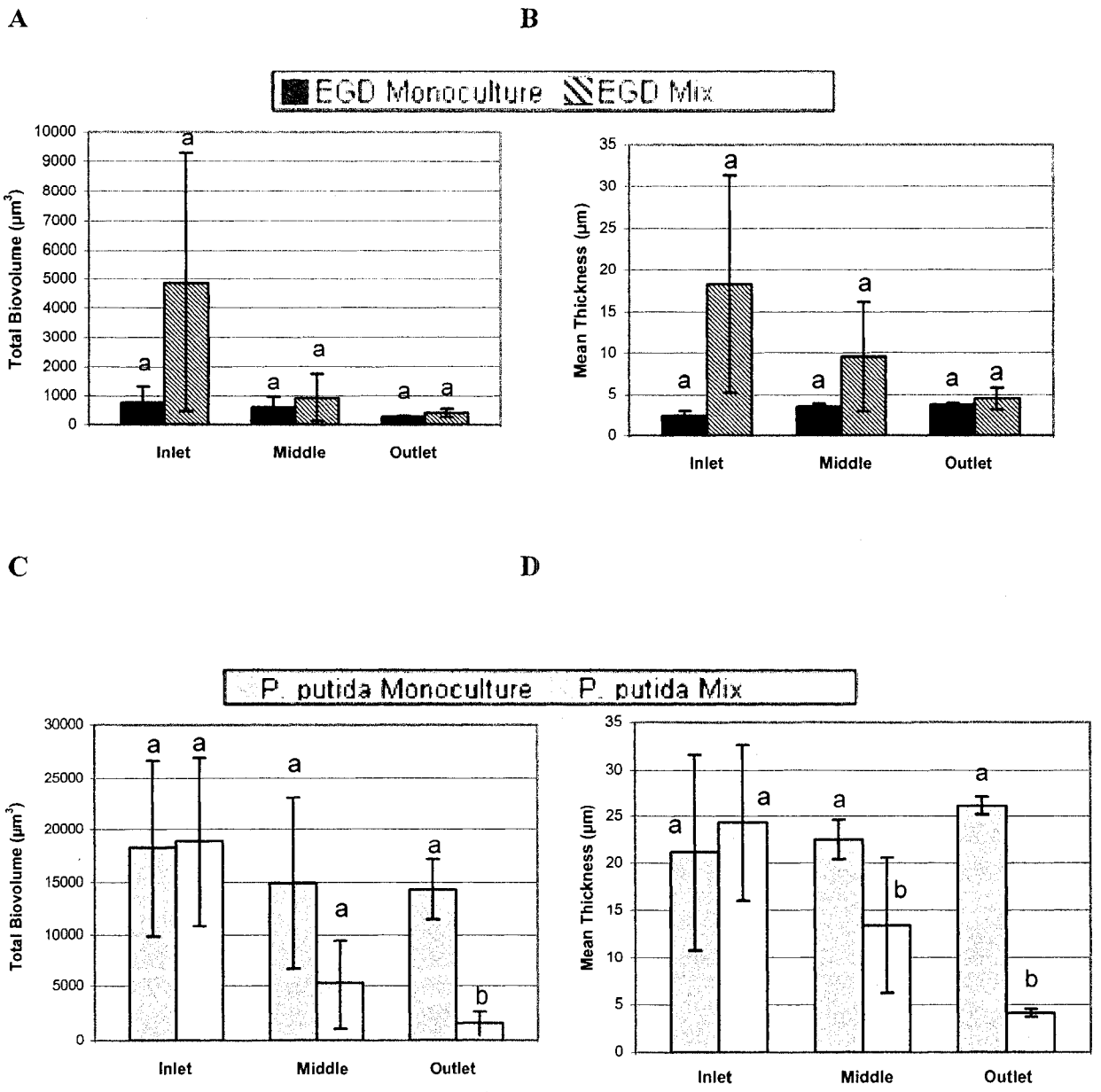

Figure 2.8. Data set 2 (WGA and GFP signal) image analysis of total biovolume (μm^3) (A, C) and mean thickness (µm) (B, D) between monoculture and mixed culture *L. monocytogenes EGD* (A, B) and *P. putida* (C, D) at inlet, middle, and outlet. For each sampling location bars with different letters indicate significant difference in total biovolume and mean thickness between biofilms ($p < 0.05$).

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Chapter 3: Effect of dissolved oxygen on growth of *Pseudomonas putida* and *Listeria monocytogenes* in mixed culture flow cell biofilms

3.1 Introduction

In the food industry where biofilms in processing plants are problematic and can lead to infection outbreaks, microbial attachment is a major concern. In our previous study (Chapter 2) we found that total biovolume and mean thickness of the food borne pathogen *Listeria monocytogenes* was not significantly increased in mixed culture biofilms with *Pseudomonas putida,* however the question of whether attachment is increased still remains to be answered. Studies such as those by Sasahara and Zottola (1993), which indicated an increase in attachment of *L. monocytogenes* serotype 3a in mixed culture biofilms with *Pseudomonas fragi* leads us to believe that increased attachment is possible in *L. monocytogenes* and *P. putida* biofilms. In the case of *L. monocytogenes,* many studies have been done with respect to attachment. Vatanyoopaisarn et al. (1999) showed that flagella play a major role in initial attachment. They found that mutants that lacked flagella had identical growth patterns as wild-type *L. monocytogenes* that was grown at 37°C, a temperature at which flagella are absent. Studies by Lemon et al. (2007), who looked at *L. monocytogenes* with both absent flagella and paralyzed flagella further emphasized the importance of flagella on biofilm attachment for *L. monocytogenes.* Both mutant types were defective in attachment and biofilm formation. According to Tresse et al. (2006) changes in pH can influence attachment of *L. monocytogenes* by affecting the regulation of flagellin genes. At pH 5 flagellin was downregulated for all strains they examined. Norwood and Gilmour (2001) found that attachment for *L. monocytogenes* is also dependent on temperature, stating that 18°C was the optimal temperature for attachment of both *L. monocytogenes* Scott A, and a persistent strain from a milk processing plant, in mixed and monoculture biofilms. Because different microorganisms can change their surrounding environment, such changes could lead to increases in attachment.

However, not only attachment plays a role in biofilm growth, detachment can be equally important and could possibly explain the loss of total biovolume and mean thickness we found in *P. putida* when grown in mixed culture with *L. monocytogenes.* For example, Stoodley et al. (2001) examined mixed culture biofilms of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens, Klebsiella pneumoniae*, and *Stenotrophomonas maltophilia.* When they monitored detachment, they found that there was steady detachment that produced up to 9 log CFU/min in the flow cell effluent and that clusters had a diameter as large as 500μm. Their data indicate that detachment can account for a large biomass loss in biofilms. They indicate that this could also be a concern when liquid samples are being examined for public health reasons, because of the massive size of possible aggregates.

For our study of mixed culture biofilms of *L. monocytogenes* and *P. putida* we postulated that available oxygen may be a limiting factor in *P. putida* biofilm growth. This led us to further investigate the impact of oxygen depletion as another possible explanation for the loss of total biovolume and mean thickness of *P. putida.* However, unlike microbial attachment, there has been little work done on the impact of oxygen on biofilms of *L. monocytogenes.* However, several studies have concentrated on *Pseudomonas* species in flowing systems. Sabra et al. (2003) found that *Pseudomonas aeruginosa* PAO1 expressed different lipopolysaccarides depending on the amount of dissolved oxygen present, becoming more adherent at higher dissolved oxygen concentrations. Similarly, Jin et al. (2006) described a higher rate of biofilm detachment for waste water treatment inoculum (microorganisms not reported) in lower dissolved oxygen.

The goal of this study is to first monitor attachment and detachment by measuring cell concentration changes in effluent within mixed and monoculture flow cell biofilms of *L. monocytogenes* and *P. putida* to determine whether *P. putida* increases attachment of *L. monocytogenes*, and whether the decrease in total biovolume, and mean thickness for *P. putida* is a result of detachment. Secondly, we want to measure dissolved oxygen and cell concentration in both planktonic cultures, and glass wool biofilms of mixed and monoculture *L. monocytogenes* and *P. putida* in order to determine whether the decline in growth or increased detachment is a result of oxygen loss.

3.2. Methods and Materials

3.2.1. Microorganisms and culture conditions

L. monocytogenes EGD (ATCC 4428) and *P. putida* GFP9 were used in the various experiments. Both microorganisms were originally taken from frozen stock that was maintained at –80 °C. *P. putida* was originally isolated from a biofilm in a milk processing line and genetically labeled using a Mini-Tn5-luxAB plasmid to produce a green fluorescence (Chumkhunthod et al. 1998). Both *L. monocytogenes* and *P. putida* were grown on trypticase soy agar (TSA; Difco Laboratories, Michigan, USA) for 24 hours, *L. monocytogenes* at 37°C and *P. putida* at 30°C. The TSA plates and cultures were stored at 4°C for up to one week. For each experiment, a fresh culture was grown on TSA plates for 24 hours, one isolated colony was then inoculated into 50ml of trypticase soy broth (TSB; Difco Laboratories) and incubated at 37°C for *L. monocytogenes* and 30°C for *P. putida*. After 24 hours, cultures were washed by centrifuging twice at 3100x g in phosphate-buffered-saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) for 10 minutes, at 4°C. Using a light spectrophotometer both cultures were diluted with PBS to an OD_{600} of 0.324 ± 0.005, this standardized the cultures to ~10^8 CFU/ml. The exact counts were 7.66 ± 0.50 log CFU/ml for *L. monocytogenes*, and 7.62 ± 0.67 log CFU/ml for *P. putida*, confirmed by plate counting on TSA plates incubated for 37°C and 30°C respectively.

3.2.2. Preparation of glass cover slips, and flow cell

Glass cover slips (2.5cm x 6.0cm Fisherfinest) were washed in 500ml of 2% RBS Detergent Concentrate solution (RBS; Pierce, Rockford, IL). The cover slips were placed in the solution for 10 minutes in a 50°C water bath. Next, the cover slips were washed with 500ml of distilled tap water and placed back into the 50°C water bath for 25 minutes. Lastly, the cover slips were rinsed by agitating in 500ml of distilled water for 5 intervals of 1 minute. After drying, a 2.5cm x 6.0cm cover slip was placed into a FC81 flow cell (BioSurface Technologies Corporation, Bozeman, MT) For the flow cell a Masterflex.L/S digital economy drive (model # 7524-50), cartridge pump (model # 7519-

15), and cartridge (model # 7519-65) were used. Flow cell and tubing were autoclaved and cooled to room temperature prior to each experiment to ensure that all components of the equipment were sterile.

3.2.3. Flow Cell Biofilm Effluent Measurements

For each experiment three biofilms were grown on a glass substratum (2.5cm x 6.0cm Fisherfinest glass cover slips) in a flow cell at 22°C, a *L. monocytogenes* monoculture, a *P. putida* monoculture and a combination of equal part *L. monocytogenes* and equal part *P. putida*. Approximately 3 ml of standardized cultures were inoculated into the flow cell with a 10 ml syringe and left in the flow cell for 1 hour to allow bacteria to adhere to the glass cover slip. After adhesion M9 1x minimal salt medium (Difco Laboratories; Becton, Dickinson, and company, Sparks, MD) supplemented with 1mM glucose was pumped through the flow cell at a rate of 3.3 ml/hr for a 24-hour period. Effluent samples were taken after a 0-3, 3-6, and 21-24 hour period. Samples were enumerated by plate counting in triplicate on TSA plates incubated at 37°C for *L. monocytogenes* and 30°C for *P. putida*. Colonies from the mixed culture biofilm were differentiated using UV light to distinguish the fluorescent *P. putida* from the non fluorescent *L. monocytogenes*.

At each time period slides were made from two 1 mL samples of *P. putida* mixed and monoculture biofilm effluent using 0.22 μm Millipore membrane filters and a Millipore vacuum filter (Millipore Corporation; Billerica, MA, USA). Five random images for a total of 10 images per time period were taken for each slide using an Olympus FV300 confocal scanning laser microscope (CSLM). Images were acquired with a 60x PlanApo NA 1.4 oil immersion lens, using an Argon (10 mW, force air cooled, blue 488nm) laser with FVX-BA 510-530 band pass emission. Images were analyzed for clumping using Image Pro (Media Cybernetics Inc., Bethesda, MD, USA) image analysis software.

3.2.4. Glass Wool Biofilms

Glass wool biofilms using 0.008 mm diameter Pyrex fiber glass (Corning Inc.; Corning NY, USA), were grown for *L. monocytogenes, P. putida*, and a combination of both in equal parts. The first set of biofilms was grown in smaller containers, and was used to determine cell concentrations from the glass wool biofilms. Next, glass wool biofilms were required for measuring dissolved oxygen (DO) from growing biofilms within media in similar conditions found in our flow cell biofilms. Larger containers were used when growing the second set of biofilms in order to properly take dissolved oxygen measurements. Lastly, mature 24 hour old biofilms had their media removed, and replaced with PBS. These biofilms were used in order to measure dissolved oxygen without the influence of planktonic cells and the impact of media.

3.2.4.1. Glass Wool Biofilms for Determining Cell Concentration

For the first set of biofilms 10 ml capped jars for each time period were filled with 0.1g of glass wool, 18 ml of M9 1x minimal salt medium with 1mM glucose, and autoclaved. These were inoculated with 2ml of standardized culture for a final volume of 20 ml and a concentration of ~10^7 CFU/ml. Jars were incubated at 22°C for 24 hours, and biofilm cells were harvested at 0, 3, 8 and 24 hours. Harvesting was done by removing supernatant waste and rinsing the biofilm three times using 25 ml of PBS. Addition of 10 ml of PBS and 7.5 g of 425 – 600 μm glass beads (Sigma – Aldrich Inc.; St. Louis, MO, USA), followed by 2 minutes of vortexing dislodged the biofilm cells from the glass wool. These were enumerated by plate counting on TSA plates incubated at 37°C for *L. monocytogenes* and 30°C for *P. putida*. UV light was used to distinguish green fluorescent *P. putida* colonies from the non fluorescent *L. monocytogenes* colonies.

3.2.4.2. Glass Wool Biofilms for Determining Dissolved Oxygen in Media

The second set of glass wool biofilms was prepared for dissolved oxygen measurements in media. Sealed jars of 500 mL volume for each time period were filled with 1 g of glass wool, 198 ml of M9 1x minimal salt medium with 1mM glucose and autoclaved. These jars were then inoculated with 2 ml of standardized culture for a final volume of 200 ml and a concentration of ~10^6 CFU/ml. Dissolved oxygen measurements were taken at 0, 2, 4, 6 and 24 hours as described below (Section 3.2.6.).

3.2.4.3. Glass Wool Biofilms for Determining Dissolved Oxygen in PBS

Lastly, glass wool biofilms were prepared for dissolved oxygen measurements in PBS. These biofilms were grown to examine dissolved oxygen from a mature biofilm no longer growing and without the impact of planktonic cells. Biofilms were first prepared in media and grown for 24 hours. Sealed jars of 500 mL volume for each time period were filled with 1 g of glass wool, 198 ml of M9 1x minimal salt medium with 1mM glucose and autoclaved. These jars were then inoculated with 2 ml of standardized culture for a final volume of 200 ml and a concentration of ~10^6 CFU/ml. After 24 hours of growth, biofilms were washed three times with 25 ml of PBS and media was replaced with 200 ml of PBS. Dissolved oxygen measurements were taken at 0, 2, 4, 6 and 24 hours as described below (Section 3.2.7.).

3.2.5. Dissolved Oxygen Measurements for Planktonic Cells

Dissolved oxygen in the solution of planktonic *L. monocytogenes, P. putida*, and a combination of equal part *L. monocytogenes* and equal part *P. putida* was measured over a 24 hour time period. Fifty ml centrifuge tubes for each time period were filled with M9 1x minimal salt medium with 1mM glucose and inoculated with washed and diluted culture for a final volume of 40 ml and a concentration of ~10^7 CFU/ml. This allowed tubes to be discarded after measurements were taken, and thus avoided contamination and aeration from the oxygen probe. The tubes were incubated at 22°C for 24 hours. A

separate set of tubes were used for determining cell concentration, and more readings of cell concentration were taken than dissolved oxygen measurements. Samples were taken at 0, 2, 4, 6, 8, 10 and 24 hours and enumerated by plate counting on TSA plates incubated at 37°C for *L. monocytogenes* and 30°C for *P. putida*. UV light was used to distinguish colonies from the mixed culture by using GFP in *P. putida*. Dissolved oxygen measurements were taken using a standardized oxygen probe at 0, 2, 4, 6 and 24 hours.

3.2.6. Dissolved Oxygen Measurements for Glass Wool Biofilms

Dissolved oxygen was measured in *L. monocytogenes, P. putida*, and a combination of equal part *L. monocytogenes* and equal part *P. putida* glass wool biofilms over a 24 hour period. Dissolved oxygen was measured using a standardized oxygen probe at 0, 2, 4, 6 and 24 hours. After measurements were taken at one time period, bottles for that time period were discarded. The next reading was taken from fresh bottles containing the glass wool biofilm growing for the desired amount of time.

3.2.7. Dissolved Oxygen Measurements for 24 Hour Glass Wool Biofilms in PBS

Dissolved oxygen was measured in *L. monocytogenes, P. putida*, and a combination of equal part *L. monocytogenes* and equal part *P. putida* glass wool biofilms over a period 24 hours. These biofilms were previously grown for 24 hours, and media was replaced with PBS for the duration of the dissolved oxygen testing period. Dissolved oxygen was measured using a standardized oxygen probe at 0, 2, 4, 6 and 24. After measurements were taken at one time period, bottles for that time period were discarded. The next reading was taken from fresh bottles containing the glass wool biofilm growing for the desired amount of time.

3.3. Results

3.3.1. Effluent Measurements and Clumping Analysis

Cultures were enumerated before inoculation into the flow cell. This value is represented as time zero in Figures 3.1-A and 3.1-B. Over the 24 hour period of biofilm growth, bacterial cells leaving the flow cell gradually decreased or maintained constant cell concentration for both *L. monocytogenes* (Figure 3.1 – A) and *P. putida* (Figure 3.1 – B) in mixed and monoculture. In all cases, there were no significant differences in effluent cell concentration between mixed and monoculture biofilms except at 0 hours for *P. putida* ($p = 0.008$).

Examples of the effluent slide images for *P. putida* monoculture and mixed culture biofilm can be seen in Figures 3.2 and 3.3 respectively. In Figure 3.2 we discovered that clusters were already present in the inoculum, and after 3 hours the majority was washed out in the effluent. From 3 – 6 hours there were significantly less clusters ($P < 0.001$), and clusters represent a significantly smaller percentage when compared to single cells ($P < 0.005$) (Figures 3.4A and 3.4B); this corresponds to what we observed in Figure 3.2. Though the number of clusters significantly increased in the 21 - 24 hour sampling period when compared to the 3 – 6 hour sampling period ($P < 0.005$), the size (area) of the clusters significantly decreased ($P < 0.001$) from the inoculum and 0 – 3 sampling period (Figure 3.4C). In Figure 3.3, *P. putida* in mixed culture behaved similar to *P. putida* in monoculture within the inoculum, and at the 0 – 3 sampling period with respect to the amount of clusters. However, unlike the monoculture, large amounts of clusters are found in the effluent throughout all sampling periods, although still maintaining a smaller area in the 3 – 6, and 21 – 24 sampling periods (Figure 3.3).

3.3.2. Planktonic Dissolved Oxygen Measurements in Media

Dissolved oxygen (DO) and cell concentration were measured at various time points for planktonic cultures in M9 1x minimal salt medium with 1mM glucose. For all planktonic cultures dissolved oxygen levels at time zero were approximately 8 mg/ml (Figure 3.5C). Within the first 6 hours dissolved oxygen levels dropped for all cultures. Dissolved oxygen within the *L. monocytogenes* monoculture dropped to 6.20 mg/ml (Figure 3.5C). In contrast, dissolved oxygen dropped to 2.40 mg/ml and 2.62 mg/ml for *P. putida* monoculture and mixed cultures respectively (Figure 3.5C). Over the next 18 hours oxygen within the *P. putida* and the mixed culture continued to gradually drop reaching 1.26 mg/ml and 1.34 mg/ml respectively (Figure 3.5C). Over the same 18 hours dissolved oxygen within the *L. monocytogenes* planktonic culture increased to reach 7.84 mg/ml (Figure 3.5C).

Over the 24 hour period, cell concentration for *L. monocytogenes* counts in both mixed and monoculture dropped by approximately one $\log_{10}$ CFU/mL, while the *P. putida* counts in mixed and monocultures remained constant despite some fluctuation (Figure 3.5A).

3.3.3. Biofilm Dissolved Oxygen Measurements in Media

When we examined dissolved oxygen within media of glass wool *L. monocytogenes* biofilms, we found an increase from 6.88 mg/ml to 8.91 mg/ml (Figure 3.5D). In the case of the *P. putida* biofilms, dissolved oxygen levels dropped within the media from 6.77 mg/ml to 4.69 mg/ml (Figure 3.5D). Lastly looking at the mixed culture biofilms, dissolved oxygen followed a similar pattern to that of monoculture *P. putida.* Dissolved oxygen started at 6.74 mg/ml, climbed to 7.93 mg/ml within the first 2 hours, but ultimately dropped to 4.73 mg/ml (Figure 3.5D)

When we examined cell concentration in similar glass wool biofilms, we found that there was little effect to *L. monocytogenes* in both mixed and monoculture. Cell concentration did not vary between 3 and 8 hours, but increased nearly one half $\log_{10}$

CFU/mL after the full 24 hour period (Figure 3.5B). As for *L. monocytogenes* within the mixed culture glass wool biofilm, there was a slight, but gradual increase in concentration (Figure 3.5B). Cell concentration in the *P. putida* glass wool biofilm gradually decreased by approximately one quarter $\log_{10}$ CFU/mL after the 24 hour period (Figure 3.5B). *P. putida* in the mixed biofilm had much lower cell concentrations after the first 3 hours (approximately one $\log_{10}$ CFU/mL difference). *P. putida* counts recovered over remaining 24 hour period to reach concentrations similar to the monoculture biofilm (Figure 3.5B).

3.3.4. Biofilm Dissolved Oxygen Measurements in PBS

Comparable to the *L. monocytogenes* biofilm in media, when we measured dissolved oxygen of the mature 24 hour *L. monocytogenes* biofilm in PBS, dissolved oxygen rose from 8.02 mg/ml to 8.58mg/ml (Figure 3.6). In the mature 24 hour *P. putida* biofilm in PBS, dissolved oxygen levels decreased from 7.59 mg/ml to 7.36 mg/ml after some fluctuation (Figure 3.6). Similarly, dissolved oxygen went from 6.63 mg/ml to 6.69 mg/ml in the mature 24 hour mixed culture biofilm maintained in PBS (Figure 3.6).

3.3. Discussion

Effluent measurements were used as a simple method to detect changes in both attachment and detachment in flow cell biofilms. If attachment to the glass surface was a key factor in the changes we see in mixed culture biofilms of *L. monocytogenes* and *P. putida*, then we would expect a lower cell concentration in the effluent, most likely in the early stages of growth. Conversely, we would expect an increase in cell concentration washed out from mixed culture biofilms occurring most likely at later stages of growth when the biofilm became thicker, if detachment was a key factor. From our measurements (Figure 3.1) there was no major change throughout the 24 hour period. This was unlike a similar study by Sasahara and Zottola (1993) who looked at mixed biofilms of *L. monocytogenes* serotype 3a and *Pseudomonas fragi.* Biofilms were grown on glass cover slips in flow cells, and directly monitored with phase contrast microscopy.

When grown together, *L. monocytogenes* exhibited an increase in attachment. Sasahara and Zottola (1993) concluded that the high exopolymer producing nature of *P. fragi* was creating a favorable environment affecting characteristics such as hydrophobicity, surface change and flagellar movement.

Looking at the oxygen levels from planktonic *L. monocytogenes* and *P. putida* culture, there is no difference between oxygen depletion between *P. putida* in mixed and monoculture (Figure 3.5C). Compared to *P. putida,* oxygen depletion from *L. monocytogenes* was much less severe. Such high oxygen depletion by *Pseudomonads* was also reported by Sabra et al. (2002) when describing the environment *of Pseudomonas aeruginosa* PAO1. *P. aeruginosa* was grown at several dissolved oxygen saturation levels in a bioreactor. Dissolved oxygen levels dropped after a short period (6 – 8 hours after inoculation), even at high aeration rates. Sabra et al. (2002) suggested that it was not due to oxygen consumption by *P. aeruginosa*, but that *P. aeruginosa* was restricting oxygen transfer from the gas phase to the liquid phase. However no mechanistic explanation was offered for this hypothesis. In the first 2 hours of our experiment, dissolved oxygen levels for *P. putida* were half of those from *L. monocytogenes*. After the 6 hour period oxygen in the *L. monocytogenes* culture ceased to decline, while dissolved oxygen in *P. putida* cultures continued to drop. Because *L. monocytogenes* is a facultative anaerobe it was expected to yield a greater cell concentration compared to the aerobic *P. putida* in low oxygen conditions, but this was not seen in the planktonic studies after 24 hours of growth (Figure 3.5A). Studies of various *Pseudomonas* species such as that of Alvarev-Ortega and Hardwood (2007) suggest that microaerobic pathways exist allowing these bacteria to persist through low oxygen conditions may be a possible explanation.

When we measured dissolved oxygen in the glass wool biofilms we found much less dissolved oxygen depletion than for planktonic cells. There are several factors that we attribute to this. Firstly, because of the larger volumes required, the cell concentration was one $\log_{10}$ CFU/ml lower than in the planktonic study. This reduced the impact of the bacteria on their environment. Secondly when looking at dissolved oxygen levels of *L. monocytogenes* in media and after media replacement with PBS we see that oxygen levels rise above initial values (Figure 3.6). Compared to the planktonic study that only

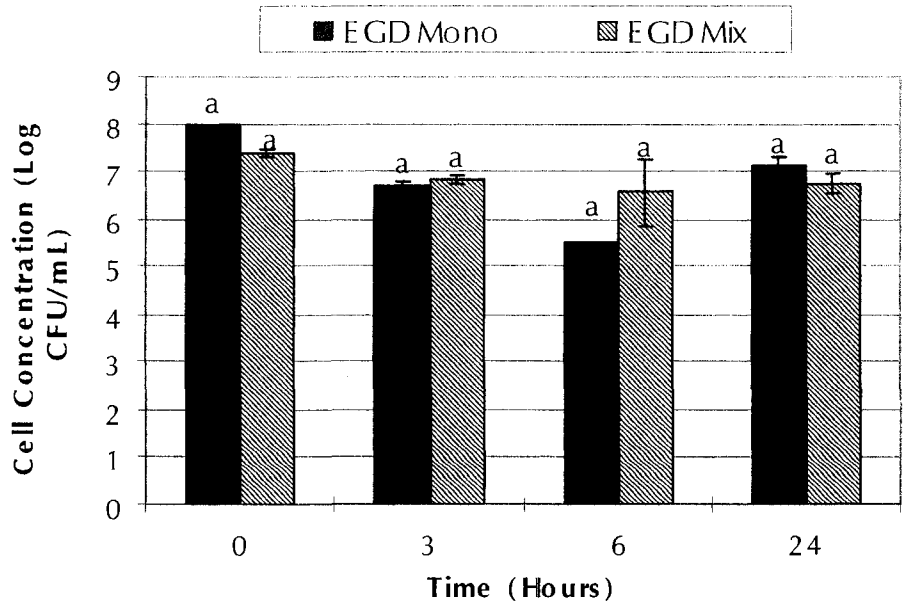
had 10 ml of headspace in the tubes for oxygen, headspace in the bottles used for the biofilm study was 300 ml. Since agitation was also used for biofilm growth, it is possible that our media and PBS were aerated during the experiment. Our goal was to evaluate how much oxygen was consumed by cells within the biofilm, thus we wanted to remove any planktonic cells within the media. We did this by replacing media with PBS and taking our measurements; however this replacing of media may have created another inadverted means of aeration.

Nonetheless, despite aeration, trends in dissolved oxygen levels are similar for biofilm cells and planktonic cells. Dissolved oxygen in media and PBS with *L. monocytogenes* does not drop, compared to the dissolved oxygen in media of the *P. putida* and mixed culture biofilm that declines by approximately 2mg/ml in PBS, and by approximately 4 mg/ml in broth (Figure 3.6). Also, the dissolved oxygen in the planktonic culture and the dissolved oxygen of the *P. putida* and mixed culture biofilms in the media and PBS follow the same trend (Figure 3.6). Unlike the planktonic culture results, and the flow cell results described in Chapter 2, cell concentrations of the glass wool biofilms appear to follow their own trends, which reinforce the idea that different approaches to biofilm growth will yield different results (Figure 3.5B). At the 3 hour period, cell concentration of *P. putida* within the mixed culture biofilm coincides with the steep loss of dissolved oxygen found in both the biofilm and more so the planktonic measurements, which is more accurate due to the lack of aeration effects (Figure 3.5C and 3.5D). Compared to the cell concentration in monoculture biofilms, this was the only variation in growth for *P. putida*, while growth of *L. monocytogenes* only varied slightly, with monoculture *L. monocytogenes* yielding a larger cell concentration after 24 hours. Our findings reflect those of Hansen et al. (2007). They looked at a *P. putida* rough variant, and wild type *P. putida* grown in mixed culture biofilms with an *Acinetobacter* species. They found that unlike the rough variant, the wild type *P. putida* had difficulty entering close structural associations with the *Acinetobacter* species even though it relied on *Acinetobacter* for nutrients. They discovered that there was a large loss in dissolved oxygen in the mixed culture biofilm, that lead to a decline in *P. putida* growth, and even detachment occurring within minutes. By experimentally increasing dissolved oxygen

levels, wild type *P. putida* was able to recover growth within an hour. Their results showed that for the wild type *P. putida* dissolved oxygen was a limiting nutrient factor.

Our study of both attachment and detachment in mixed and monoculture biofilms of *L. monocytogenes* and *P. putida* was inconclusive. Our cluster analysis could not be completed due to technical difficulties, and it is difficult to conclude whether the lack of significant attachment and detachment over time was accurate, or whether our methods were not precise enough to detect this change Further study is required that can detect attachment and detachment such as confocal time lapse imaging. Conversely our examination into the impact of oxygen levels for biofilm growth in mixed and monoculture biofilms of *P. putida* and *L. monocytogenes* was successful. Although further studies could be carried out that measure dissolved oxygen of the mature 24 hour biofilms when placed back into media, nonetheless, our findings suggest that the loss of growth in both total biovolume, and mean thickness of *P. putida* in mixed biofilms with *L. monocytogenes* is limited by the amount of dissolved oxygen.

A



B

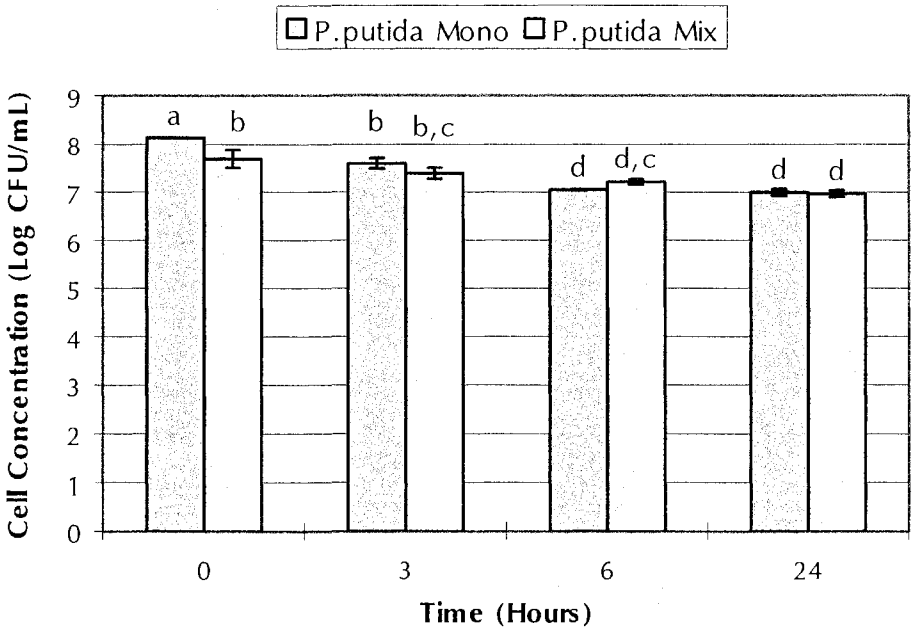


Figure 3.1. Cell concentration (Log CFU/mL) in flow cell effluent of *L. monocytogenes EGD* and *P. putida* mixed and monoculture biofilms. Time 0 represents inoculum. For each type (monoculture or mixed) biofilm bars with different letters indicate significant difference in cell concentration ($p < 0.05$).

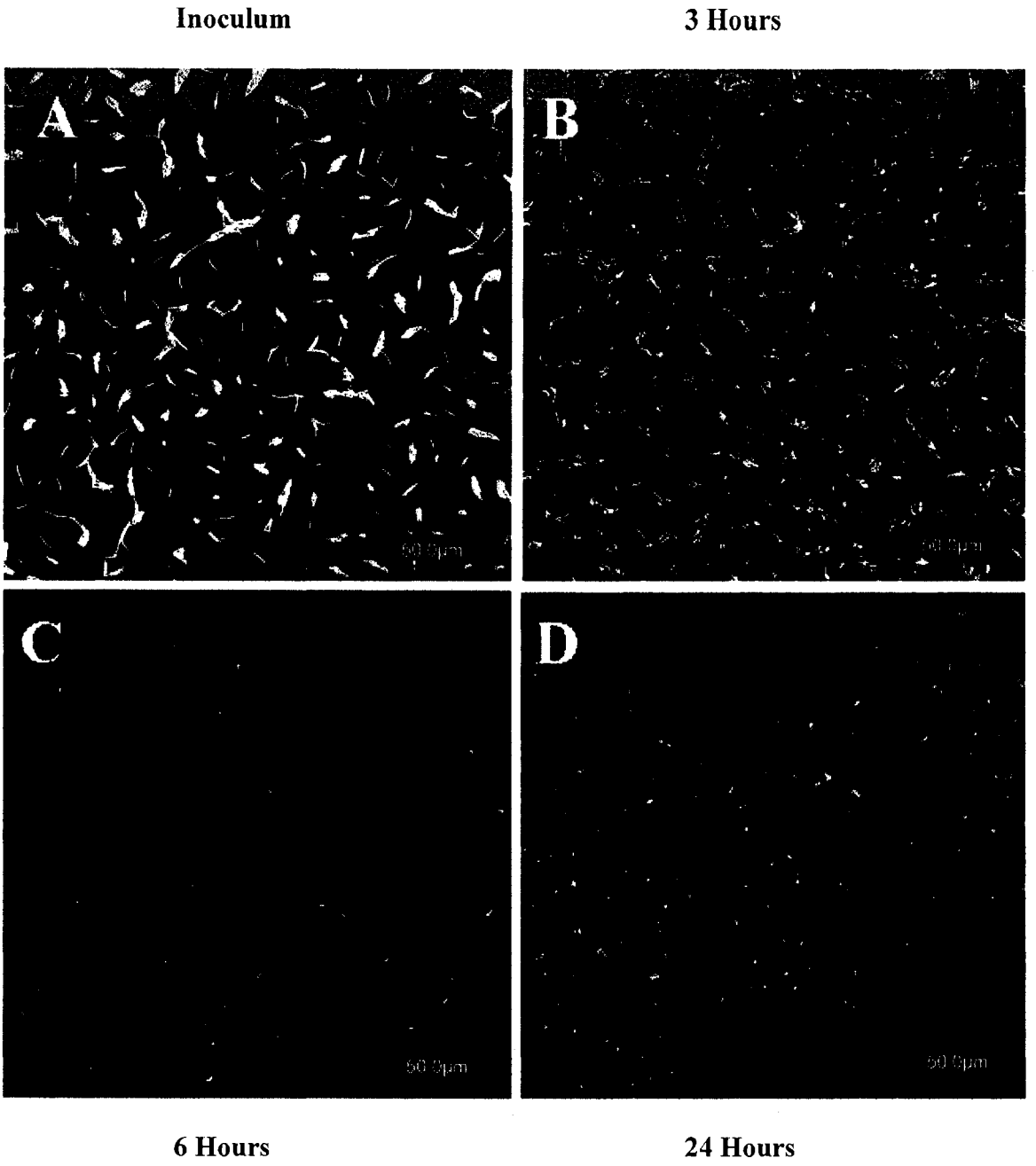


Figure 3.2. Confocal fluorescent images of monoculture biofilm for GFP-labeled *P. putida* inoculum (A), and biofilm effluent at 3 hour (B), 6 hour (C), 24 hour (D) sampling periods. Magnification 600x.

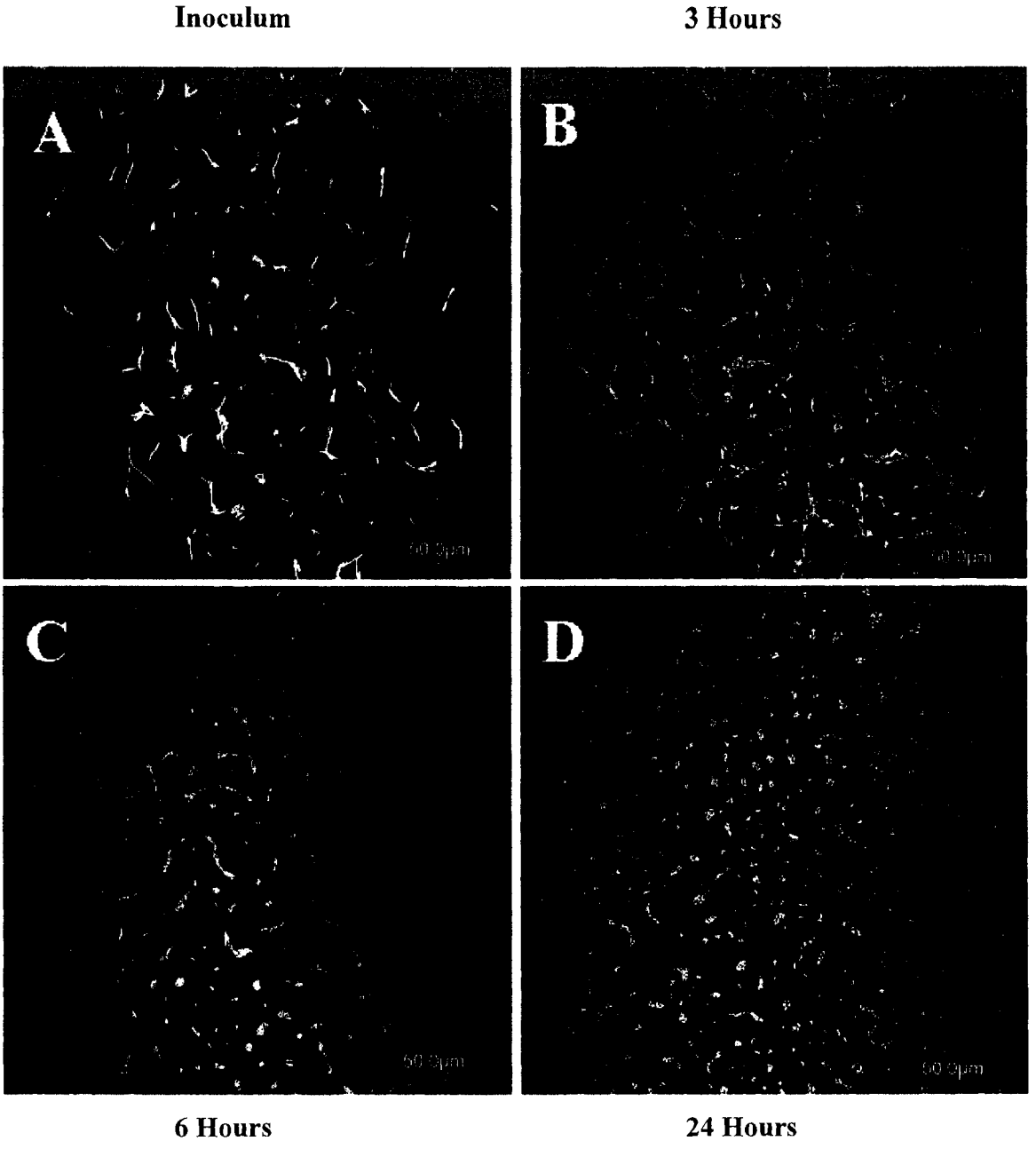
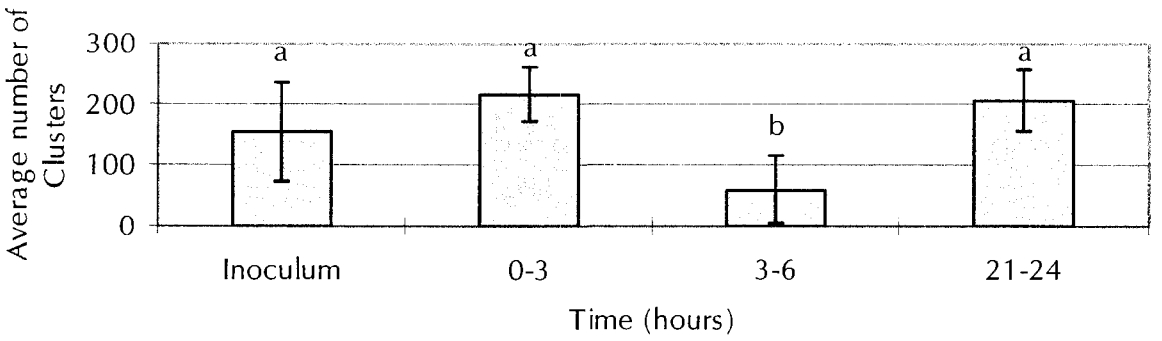
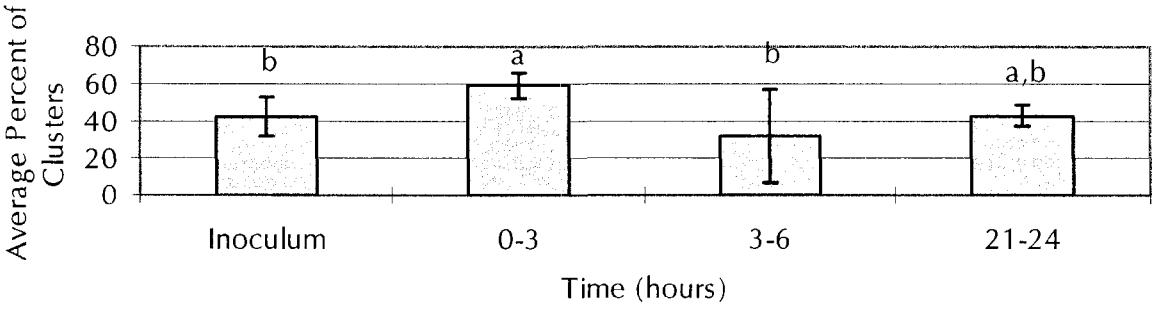


Figure 3.3. Confocal fluorescent images of mixed biofilm for GFP-labeled *P. putida* inoculum (A), and biofilm effluent at 3 hour (B), 6 hour (C), 24 hour (D) sampling periods. Magnification 600x.

A



B



C

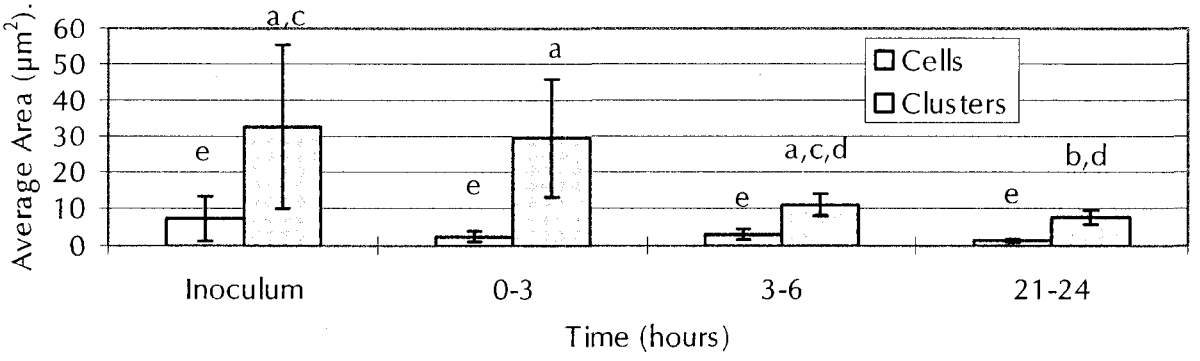


Figure 3.4. Average number of clusters (A), average percentage of clusters (B), and average area of bacterial clusters and cells (C) in monoculture *P. putida* biofilm effluent. For each sampling period bars with different letters indicate significant difference within biofilm effluent. ($P < 0.05$).

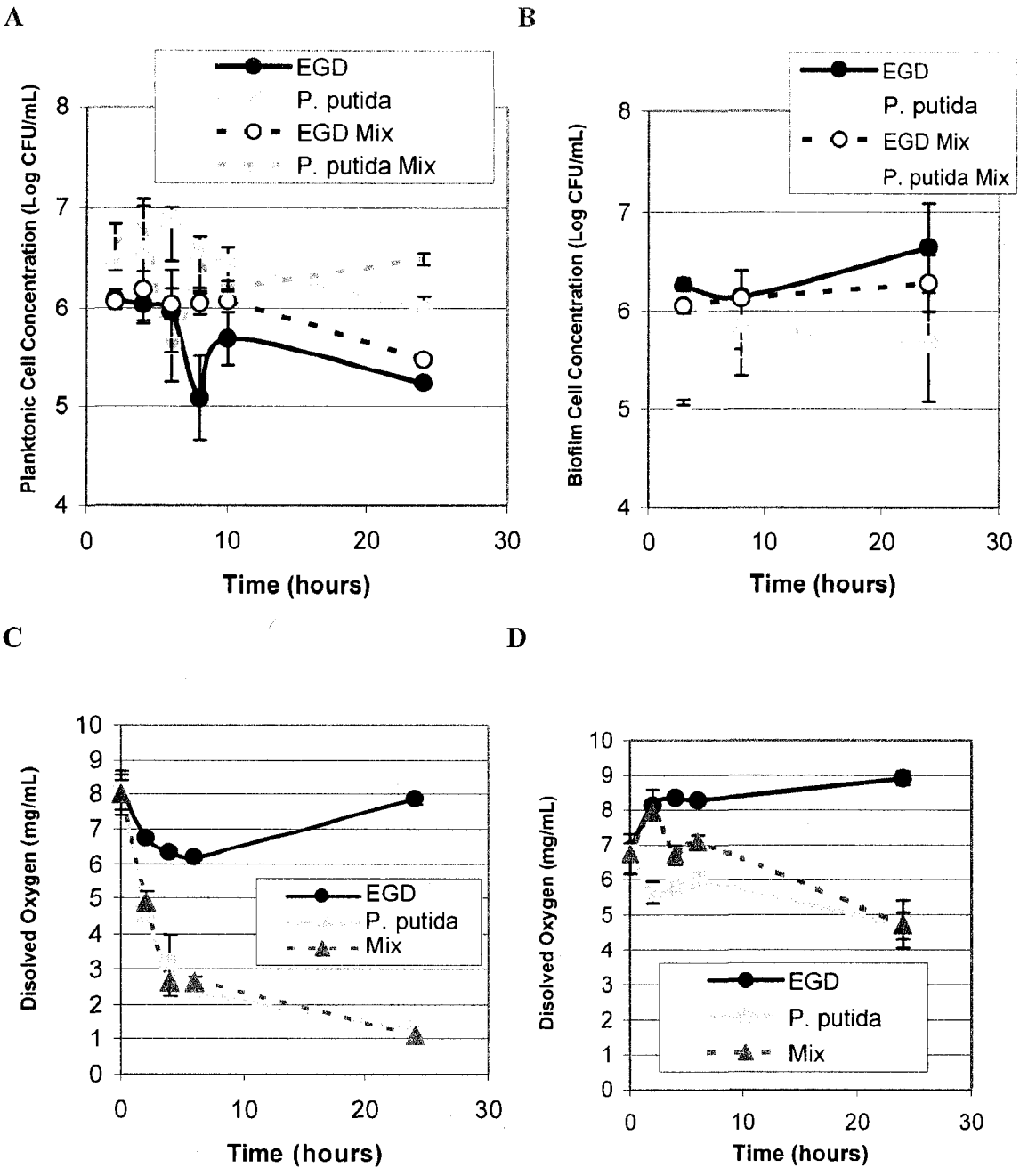


Figure 3.5. Dissolved oxygen (mg/mL) (C, D) and cell concentration (Log CFU/mL) (A, B) from mixed and monoculture cell suspensions (A, C) and biofilms (B, D) of *L. monocytogenes EGD* and *P. putida.*

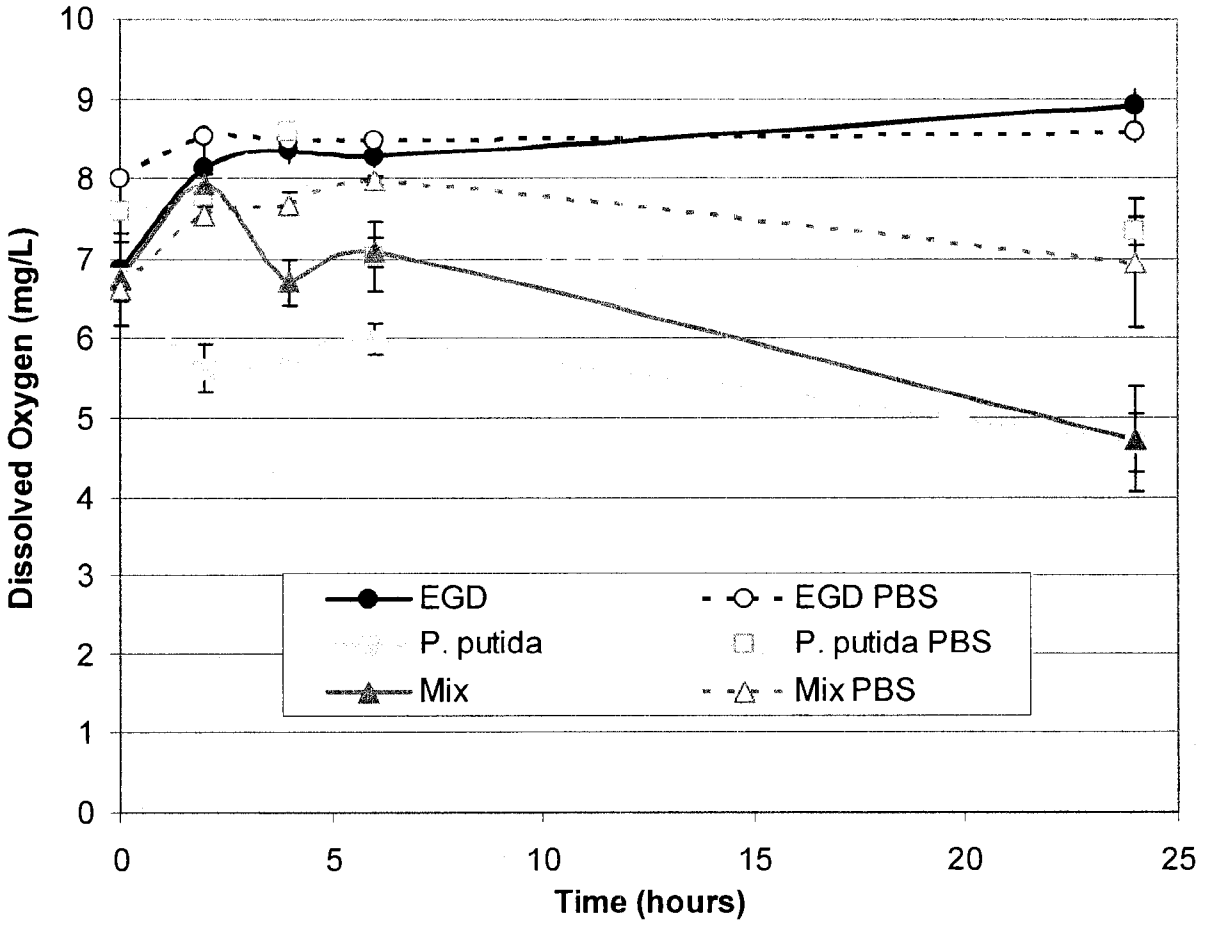


Figure 3.6. Dissolved oxygen (mg/mL) from *L. monocytogenes EGD* and *P. putida* mixed and monoculture biofilms in media and PBS. Solid symbols represent biofilm in media, while open symbols represent 24 hour mature biofilm in PBS.

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Chapter 4: Summary and Conclusions

In this study we evaluated whether biofilm growth of *Listeria monocytogenes* would be enhanced when grown with *Pseudomonas putida*, a microorganism commonly associated with *L. monocytogenes*, and also whether *L. monocytogenes* has any effect on *P. putida*. We also examined attachment and detachment as well as the role of oxygen in *L. monocytogenes* and *P. putida* mixed culture biofilms.

In chapter 2 we examined whether total biovolume, and mean thickness of mixed culture biofilms was affected and whether there was any difference in the biofilm at the inlet, middle, and outlet within the flow cell. Our findings indicate no significant enhancement of growth in total biovolume, and mean thickness for *L. monocytogenes*, but instead a loss of total biovolume, and mean thickness in *P. putida.* We also discovered that although not always significant, there was more growth at the inlet than middle and outlet in all biofilms.

In chapter 3 we attempted to further examine the change in growth of *L. monocytogenes* and *P. putida* in mixed culture biofilms by examining attachment and detachment using cell concentration from flow cell effluent, and measuring dissolved oxygen in both planktonic cultures, and glass wool biofilms. We found no significant change in cell concentrations in the flow cell effluents and concluded that no change in attachment, or detachment had occurred. However, more sensitive studies, such as real-time microscopy studies, are required in order to confirm these findings. When we examined the impact of dissolved oxygen on biofilm growth in mixed and monoculture biofilms we concluded that the loss of growth in both total biovolume, and mean thickness of *P. putida* is related to the amount of dissolved oxygen. Studies on specific compounds found in *L. monocytogenes* and *P. putida* EPS and how they affect growth of each microorganism could offer more explanations to the growth decline of *P. putida* in mixed culture biofilms and provide more information into growth enhancement reported by other studies for *L. monocytogenes.*