THE CHARACTERIZATION OF BIOFILM ATTACHMENT TO METAL INTERFACES:
EFFECTS OF SUBSTRATUM PROPERTIES

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by
Marcel D Mendes

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COMMITTEE MEMBERSHIP

TITLE: The Characterization of Biofilm Attachment to Metal Interfaces: Effects of Substratum Properties

AUTHOR: Marcel D Mendes

DATE SUBMITTED: June 2012

COMMITTEE CHAIR: Dr. Daniel W Walsh, Professor

COMMITTEE MEMBER: Dr. Lanny Griffin, Department Chair

COMMITTEE MEMBER: Dr. Robert Crockett, Professor

ABSTRACT

The Characterization of Biofilm Attachment to Metal Interfaces: Effects of Substratum Properties

By

Marcel D Mendes

Bacteria are among the most abundant microorganisms on earth, and can be found in essentially every environment. When a clean surface is exposed to media containing planktonic bacteria, the bacterial cells will attach to the surface and aggregate to form what is known as a biofilm. Biofilms have been shown to negatively affect many industries including medical, industrial, and food science applications. While biofilms have been well characterized from a microbiology perspective, there has been much less research from a materials science standpoint. It is hypothesized that the material properties of the substratum (such as the micro-structure) have a significant impact on biofilm growth. To research this hypothesis, protocol was established in order to produce, analyze, and study biofilms in a static exposure system. Though simple, the static bioreactor was proved to be adequate for inducing microbial attachment to processed samples. Methodologies for analyzing the established biofilms were presented, and an experimental procedure was proposed that enables the correlation of material properties to microbial growth on the substratum. The experimental procedure utilized Design of Experiments in a three factor, two level study that identified the interaction of Material Composition, Surface Conditions, and the Effect of Welding on microbial growth. In a trial iteration of the experiment, samples of 303 and 304 Stainless Steel were mounted in Bakelite and processed. Some samples were sanded to 600 grit sand paper, while others were polished to 1 μ m. The samples were exposed to biologically active natural water and imaged with scanning electron microscopy. Preliminary results were presented, and limitations of the study were identified.

Keywords: Biofilms, Microbial Attachment, Substrate Properties, Materials Science

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CHAPTER 1

Introduction

Bacteria are among the most abundant microorganisms on earth, and can be found in essentially every environment. Bacteria are able to colonize in a diverse range of conditions due to their well developed survival mechanisms that enable them to create their own micro-environment. Planktonic bacterial cells, or cells that are free floating in a bulk fluid, will aggregate in a sessile community known as a *biofilm*. Biofilms are structured groups of bacterial cells that adhere to a surface in order to establish a protected consortium where they can multiply and divide. Biofilms negatively affect many industries, and the study of biofilms may provide new insight in methods of control. Biofilms have been well characterized from a microbiology perspective; however there has been much less insight from a materials science standpoint. More focus should be made on engineering materials that can resist biofilm growth via mechanical properties.

Bacterial Cell Biology

There are many types of bacteria that can be found in biofilms; the type is dependent on the extent of the cell growth and the rate of the cell growth. The extent of bacterial growth is explained through the stoichiometry and energetics of the overall growth process, while the rate of bacterial growth is characterized by the available nutrient concentration. A biofilm is composed of two major components: the microorganisms (bacteria), and the matrix of extracellular polymer substance (EPS) that houses them. In a biofilm, there may be as many as 10^{16} cells/ m^{-3} , which is much higher than that normally found in a populated suspension of fluid. Bacteria in suspension congregate to form biofilms in order to establish a microenvironment that houses and protects the concentrated group of cells (Characklis and Marshall 1990).

Biofilms may contain a variety of organisms such as viruses, eukaryotes, and prokaryotes- though bacteria are the dominant organism that forms a biofilm. Bacteria are equally as diverse, though they are all prokaryotes. Bacteria do not contain a nucleus, and the DNA is a single circular molecule inside cell.

Bacteria respire through the plasma membrane, and some bacterial types utilize photosynthesis. Bacteria have been classified in their own kingdom, the Procaryotae, which is composed of four divisions based on cell structure, respiration type (aerobic or anaerobic), and the use photosynthesis. There are many physiological subgroups that classify bacteria even further. Anoxygenic photobacteria photosynthesize under anaerobic conditions and do not evolve oxygen as a byproduct, whereas Oxygenic photobacteria photosynthesize under aerobic conditions and do produce oxygen as a reaction product.

Chemolithotrophic bacteria are not photosynthetic; they oxidize inorganic compounds as an energy source and harvest carbon from CO₂ to synthesize organic compounds. All Heterotrophic bacteria require organic carbon substrates for energy production, though there can be Aerobic Heterotrophs and Anaerobic Heterotrophs. Aerobic Heterotrophs require the presence of oxygen, and utilize glycolysis and the Krebs cycle. Anaerobic Heterotrophs do not require oxygen to produce ATP, instead they utilize fermentation and denitrification to produce methane (Characklis and Marshall 1990).

Bacteria are unicellular organisms that produce rigid cell walls which protect the underlying plasma membrane and intracellular components such as DNA. The plasma membrane is composed of phospholipids and proteins that is around 8 nm thick. Each phospholipid contains a region that is hydrophilic and a region that is hydrophobic; oriented in a double layered structure called the phospholipid bilayer. This membrane acts as a selectively permeable barrier to transport solutes and nutrients into and out of the cell; ensuring that all energy related reactions take place within the cell cytoplasm. The cytoplasm is composed of DNA, ribosomes, and reaction enzymes. Outside the plasma membrane, the cell wall protects the cell and maintains structural integrity and is composed of a thick layer of peptidoglycans (Characklis and Marshall 1990). Bacteria are characterized by two types of cell walls that are found, based on a “gram stain” that stains the cell wall blue. Gram-positive bacteria (which

stain blue) contain a very thick cell wall, mostly of peptidoglycans. Gram-negative bacteria (which stain red) have a thinner cell wall, with a much smaller layer of peptidoglycans. Gram-negative bacteria also have an outer membrane composed of phospholipids, lipopolysaccharides, and proteins (See Figure 1).

Figure 1- Cell walls of Gram-negative and Gram-positive bacteria.
Image adapted from Betsy et al. 2005.

This outer membrane acts as an extra permeable barrier to further filter substances in and out of the cell (Wicken 1984). In both types of bacteria, beyond the cell wall is the Extracellular Polymeric Substance (EPS). This EPS will either attach to the cell wall, in which case it is referred to as a capsule, or it may depart from the cell wall and freely move between cells, when it is referred to as a slime. The EPS is typically a “mass of tangled polymeric fibers” that reach out from the cell wall. It is hypothesized that these fibers are responsible for adhering to material surfaces. It is also thought that these fibers will attach to the fibers of other bacterial cells, creating flocs in suspensions and films on surfaces. There are also surface appendages that extend from the bacterial cell including flagella (used for movement in liquids), sex pili (used for cell communication and conjugation), fimbriae (which also act as adhesive structures during attachment), and extensions of the cell wall called prosthecae (Characklis and Marshall 1990). All of these functions and components allow bacterial cells to survive and thrive in a variety of environments.

The Biofilm Process

Bacteria are present as planktonic microbes in the bulk fluid of almost every system –from a living body, to an industrial pipeline, to a food processing plant. The course of successive steps in which bacteria moves out of the bulk fluid and accumulate on a surface to create a biofilm is known as the biofilm process. There are four main processes that occur: (1) attraction, (2) adhesion, (3) growth and division, and (4) dispersal . The first process is characterized by reversible attachment of bacteria to the surface (or substratum), the second process is considered irreversible attachment (adsorption) to the substratum, the third process is characterized by accumulation and growth of the bacterial cells to create a biofilm (See Figure 2 and Figure 3), and the fourth process is marked by the detachment of a group of cells from the biofilm into the bulk fluid to spread and populate elsewhere (Characklis and Marshall 1990).

Figure 2 Scanning electron micrograph depicting a developed biofilm (A), the substratum (B), and an attached cell (C). Adapted from Donlan 2002.

These four processes are further broken down into 8 events: (1) organic molecules from the bulk fluid attach to the substratum and condition the surface, (2) planktonic microbial cells that are present in the bulk fluid are attracted to the substratum, (3) some cells that reach the substratum reversibly adhere (also called *adsorb*), (4) some cells that are reversibly adhered *desorb* (unattach) the surface due to fluid sheer stress or other chemical/biological factors, (5) a portion of the cells that remain on the surface will become irreversibly adhered, (6) the permanently adsorbed cells will grow and divide to create a biofilm, using nutrients available in the bulk fluid, (7) more cells will attach to the adsorbed cells, expanding the biofilm, (8) sections of the biofilm will detach and reenter the bulk fluid for dispersal elsewhere. It is important to note that *attachment* refers to the adhering of matter to the biofilm while *adsorption* is adherence of matter to the substratum; *detachment* is the loss of matter from the biofilm where as *desorption* is the loss of matter from the substratum. Each step is governed by physical, chemical, and biological processes, and it is important to understand the specifics of each process in order to take a preventative approach in controlling the negative effects of biofilms.

Figure 3- The Biofilm Formation Process.
Image adapted from Rendueles and Ghigo (2012).

When a clean surface (such as an implant) is exposed to the bulk fluid in a system (blood), it is coated with organic molecules, proteins, and charged ions that are transported out of the bulk fluid and

attached to the surface of the substratum. This can happen within seconds of exposure, and results in a “conditioned substratum.” These organic molecules that are attracted to the surface are typically polysaccharides or glycoproteins; the protein molecules in the bulk fluid are constantly exchanged with those adhered to the surface. Bacterial attraction (process one) is dependent on the resulting surface properties of the conditioned substratum (charge, surface tension, “sticking efficiency,” etc.). It has been shown that hydrophobic proteins adhere better to high free energy surfaces (such as stainless steel), whereas fatty acids will adhere better to metal surfaces that have been cleaned with solvents and to hydrophobic polymeric materials (which are factors that will influence bacterial cell attachment) (Verran and Jones 2000).

Adsorption of bacterial cells to the conditioned substratum can be either passive or active. Passive adsorption is governed, not by the bacteria, but by the bulk fluid dynamics, gravitational forces (sedimentation) and Brownian diffusion (Characklis and Marshall 1990). Active adsorption occurs when the bacterial cell actively facilitates the adherence to the substrate and is governed by the cell surface features such as pili, flagella, EPS capsules, and adhesin proteins (Kumar and Anand 1998). Once the cell has been attracted to the substratum, it temporarily adheres to the conditioned surface. This temporary adsorption is reversible and is typically long range interactions such as van der Waals forces, hydrophobic interactions, steric interactions, and double layer electrostatic forces. The initial adherence is said to be reversible because the bacterial cells are still influenced by Brownian motion and can be easily removed from the surface by low shear stresses (such as fluid flow). These interactions generally have a low heat of adsorption per chemical bond of around 20-50 kJ/ mol⁻¹ (Characklis and Marshall 1990). It has been shown that microorganisms such as bacteria adhere much quicker to non-polar and hydrophobic surfaces, and much slower to hydrophilic surfaces (Donlan 2002).

If the bacterial cell remains adhered, and is not removed by external forces, the cell irreversibly adsorbs to the surface (Process Two) by anchoring the cellular surface appendages (pili, flagella, and adhesion proteins) to the conditioned substratum. The time it takes the cell to irreversibly attach to the

surface is referred to as the “critical residence time,” and is usually between 20 minutes and 4 hours (Chmielewski and Frank 2003). In 1998, Pratt was able to show that the bacterial cells and the conditioned substratum are often both negatively charged, so the bacterial cell and the surface are usually not in direct contact (explaining the reversible attachment). The bacterial cell anchors its surface appendages through stronger short range forces such as covalent bonds, hydrogen bonds, ion-dipole interactions, dipole-induced dipole (Debye) interactions, and dipole-dipole interactions to create the irreversible adsorption (Characklis and Marshall 1990). These interactions have a much higher heat of adsorption per chemical bond, between 40-400 kJ/ mol⁻¹. Often, the extracellular polymeric substances (EPS) plays a large factor in the adhesive interactions. This is because the polymers can absorb to the surface by creating many bonds along the polymer chain (though they are all weak, a large number of them can create a firm hold). The bacteria, which are often negatively charged, are able to adhere to a negatively charged surface through the polymer chains of the EPS which will contain positively charged regions. After anchoring, is very difficult to remove the bacterial cell and requires either high sheer forces (such as physical scrubbing) or chemical breakdown using detergents, surfactants, or sanitizers (Characklis and Marshall 1990). The cells that are adhered to the surface will more easily capture other particles that are suspended in the bulk fluid because the cells are generally rougher than the substratum and typically have a readily available EPS to stick to. This accumulation of particles and other cells is called attachment, and differs from the initial adsorption of the cells to the substratum.

Process Three is characterized by the growth and division of the bacteria as the collection of cells becomes a biofilm. This will be shown in the significant increase in the metabolic activity of the cells as the biofilm begins to colonize. The metabolic processes are categorized into four fundamental stages: growth, product formation, maintenance, and death (lysis). The growth of the cells in the biofilm involves absorbing nutrients from the bulk fluid and circulating them within the biofilm. The biofilm will then begin to form products such as another EPS layer, known as the slime. This layer serves to protect the biofilm by concentrating nutrients, preventing access of biocides, and providing structure to the biofilm. It

has been shown through confocal laser scanning microscopy that the cells will group into regions and push outwards from the substratum to form mushroom-like towers. It has been hypothesized that this takes place in order to create water channels that increase fluid flow in the biofilm- which will help replenish nutrients and aid in waste removal (Wimpenny, Manz, and Szewzyk 2000). Once developed, there is little required maintenance in the biofilm. The biofilm may form regions of anaerobic respiration, conserving energy and decreasing the amount of nutrients needed deeper in the film. Some of the cells that are compromised or simply too aged will lyse and die. The total growth and division process of the biofilm is a function (cumulative total) of the growth of the cells, the products formed, the amount of maintenance needed, and the number of cell deaths (Characklis and Marshall 1990).

The fourth and final process of biofilm formation is marked by dispersion. This occurs when cells (or other biofilm matter such as EPS) are released from the biofilm into the bulk fluid (see Figure 4). Just as attachment was the addition of matter to the biofilm, detachment is the removal of matter from the biofilm (recall that removal of matter from the substrate would be desorption). This is hypothesized to have a large impact on cell turnover within the biofilm as new planktonic cells are accumulated and old cells within the biofilm are released.

Figure 4- Events of biofilm formation. Adapted from Costerson 2007.

This detachment of cells from the biofilm can be caused by erosion, sloughing, and abrasion (Characklis and Marshall 1990). Erosion of the biofilm is a result of the fluid dynamics and flow of the bulk fluid as it moves over the biofilm. Fluid shear stresses at the interface of the biofilm will cause portions of the biofilm to shear off into the bulk fluid. This can also be caused by increasing biofilm thickness; as the biofilm protrudes farther into the bulk fluid it will have more surface area causing resistance to the flow, and thus will experience more shear force. Sloughing of the biofilm refers to a “rapid, massive loss” of matter (Howell and Atkinson 1976). Sloughing is caused by an external stimulus that is not typically a natural occurrence in the local environment, such as an instant extreme load on the substratum or the introduction of a substance that inhibits bacterial attachment. External stimuli, such as chemical treatments, have been used as a method for biofilm control as they cause large detachment of biofilm matter. These can include oxidizing biocides (such as chlorine), surfactants (like detergents), and

nonoxidizing biocides (such as glutaraldehyde). These chemicals all work in different ways to break off portions of the biofilm or prevent further attachment of cells by inducing sloughing. The last type of detachment is caused by abrasion. This is characterized by the loss of matter due to mechanical or physical contact, such as the scrubbing of a tooth brush on dental biofilms or the contact of backwash sand in a water filter. It is hypothesized that dispersion by erosion is a survival mechanism because the bacteria use the flow of the bulk fluid to spread to new locations while maintaining turnover of older cells (Characklis and Marshall 1990).

Microbiology of Biofilm Formation

Biofilms have been studied extensively from a microbiological perspective. There are a number of studies that work to explain the biological functions of the bacteria at each step of formation. This is best detailed in a paper by Fey and Olson (2010), where the authors describe the biology of each step in the formation process of *Staphylococcus Epidermidis*. The attachment of *S. epidermidis* is most dependent upon specific proteins (such as AtlE and Aae). These adherence proteins regulate the attachment of the bacteria to surfaces by non-covalently binding to proteins that are already attached to the surface (within minutes of biomaterial implantation, the surface of the implant is coated with human serum proteins such as collagen, fibronectin, fibrinogen, and vitronectin). During the initial bacterial attachment to surfaces, bacteria also release extracellular DNA (eDNA). This gene expression functions to produce adherence factors that have been labeled “Microbial Surface Components Recognizing Adhesive Matrix Molecules” (MSCRAMMs). Fey and Olson continue to identify the specific bacterial adherence protein that is used for attachment to each surface protein, as well as each MMSCRAMM that is produced by the eDNA (Fey and Olson 2010). This list is not directly relevant, though it is important to understand that the microbiology of the attachment phase is extensive and complex.

Once the bacteria have attached to the surface, molecules such as polysaccharide intercellular adhesion (PIA) are synthesized to enable the accumulation of more bacteria. This molecule is a homoglycan that is synthesized by the *ica* operon gene (composed of four genes, *ica A- ica D*). It is a

polar molecule, which aids in its processing of the *ica* gene. The four *ica* genes cause PIA to attach to the cell surface, aid in surface colonization, and assist in “immune system evasion.” Fey and Olson explain that PIA also determines the three dimensional structure of the biofilm. If there is low sheer stress (or fluid flow) over the surface, biofilms will develop “tower formations” as they reach out away from the biomaterial. When the tower is sheared off, it helps to disperse the bacteria to colonize elsewhere. In environments with high sheer stress, such as in a catheter lumen, towers are not formed and focus is made on protecting the underlying bacteria. Once the bacteria have accumulated on the surface, a maturation phase to culture the bacteria occurs followed by a dispersal phase to spread the bacteria to new locations. Fey and Olson identify each protein and gene expressed to enable cellular communication, culture growth, and nutrient transport. The main concept is that the biofilm is able to create a microenvironment that hosts the perfect conditions for growth. PH levels, ATP production, phenotypes expression, and areas of anaerobic/ aerobic conditions are all able to be controlled (Fey and Olson 2010).

The microbiological approach has been extensively studied and well characterized, providing much insight to the inner workings of the biofilm. It has identified important genes and specific proteins that enable key events to occur. However, the microbiology based approach does little to address the material surface that is being colonized. From an engineering standpoint, it is more important to understand how the surface properties of the material affect bacterial attachment and biofilm growth. More focus in industry should be put on characterizing surface properties that decrease probability of infection, which can be used as a crucial tool in device design.

Extracellular Polymeric Substance (EPS)

Extracellular polymeric substance is a natural structure that is secreted by bacterial cells (as described above). When bacteria have accumulated to form a biofilm, the EPS that normally resides on the outside of the cells will congregate and form a matrix around the biofilm. During early research of biofilms it was thought that the EPS was made up of polysaccharides, and so was named “extracellular polysaccharides” (or exopolysaccharides). It was then discovered that the EPS actually contained

proteins, nucleic acids, and lipids as well (See Figure 5). Because it represented more of a polymeric material, it was renamed to “exopolymers,” and is now more appropriately referred to as “extracellular polymeric substance.” Extracellular polymeric substances are repeating organic molecules that are made of similar amino acids. The polymeric properties come from the repeating structure of differing units, and contain organic substituents (such as succinyl, pyruvyl, and acetyl groups) or inorganic substituents (such as sulfate). Proteins within the EPS are substituted with fatty acids, forming lipoproteins, or glycosylated with oligosaccharides, forming glycoproteins. These can all be used as energy storage for consumption by the biofilm (Wingender, Neu, and Flemming 1999).

Figure 5- Components of EPS between bacterial cells.
Adapted from Wingender, Neu, and Flemming 1999.

The EPS is typically secreted by living cells (such as bacteria) and is transported across the cellular membrane to reside in the surrounding matrix. There are many mechanisms through which the EPS may be secreted, such as: during the growth phase due to eDNA, through the release of cellular components like lipopolysaccharides through the outer membrane, through accumulation of the products of cell lysis (cell walls and membranes), or through the accumulation of EPS that was produced at another

location and carried by the bulk fluid. Regardless of how the EPS is produced by the cell, it exists either as a loosely attached capsule or is detached from the cell to create a surrounding matrix known as the slime (Wingender, Neu, and Flemming 1999).

This slime (EPS that is spread throughout the biofilm) is very important to the biofilm community, as it maintains the structural and functional integrity of the biofilms. The EPS acts as a glue network that keeps the bacterial cells together, providing important structural support and mechanical stability through the non-covalent bonding of the polysaccharide chains (Mayer et al. 1999). It also functions to keep the biofilm anchored to the surface of the substratum so that it is not swept away by the bulk fluid, allowing the bacterial cells to accumulate and colonize the surface. The cross linking of the polymer chains allow for the biofilm to produce the three-dimensional structures that aid in nutrient transport (the mushroom towers that protrude from the biofilm). This is a result of the protein network in the matrix cross linking polysaccharides to produce the water channels and tower structures that are found in developed biofilms (Higgins and Novak 1997). It also functions to retain water inside the biofilm community, creating a microenvironment that is conducive to bacterial growth. This is especially important when the biofilm is established in dryer locations that lack readily available water (Or, Phutane, and Dechesne 2007). The EPS also acts as a protective barrier for the underlying bacterial cells by preventing harmful “intruders” from entering the biofilm (see Figure 6). These “intruders” may include the human complement system and antibody response (during infection), biocides such as antibiotics or disinfectants, or another type of chemical or cell that would pose threat to the living bacteria population.

Figure 6- The biofilm EPS and surrounding components. Adapted from Eberl 2006.

The EPS is also responsible for some cell to cell recognition between bacteria, the retention of water in the biofilm, the adsorption of organic compounds to be used for nutrients, the accumulation of enzymes secreted by the cells, the enzymatic digestion of exogenous molecules (also for nutrients), and the release of cells (detachment) by EPS degradation (Wingender, Neu and Flemming 1999). The fact that the EPS can play such an important role in preventing the human immune system, antibiotics, or other biocides from eradicating the bacteria make it a topic of high interest in current medicine. It is important to understand the structure and function of the EPS, especially for applications in medical biology (such as device related infections). Though the EPS is not directly affected by the material properties of the substratum, it is very useful in understanding and predicting how the biofilm will colonize and populate the surface of the material.

Quorum Sensing

Bacteria are able to thrive as a biofilm community due to their ability to communicate between cells. This sophisticated cell-to-cell communication is a very important survival mechanism that enables the cells to act collectively as a group, and has been termed quorum sensing (QS). Quorum sensing is defined as “the regulation of gene expression in response to fluctuations in cell-population density” (Miller and Bassler 2001). Gene expression in bacteria has been shown to regulate events such as biofilm relocation (for nutrient supply), virulence, symbiosis, conjugation (transfer of DNA), competence (taking

up DNA), and, recently, formation. This is done through a signal molecule known as the autoinducer (AI) and a transcriptional activator known as the R-protein. There are many signaling cascade pathways through which this communication takes place, and each is specific to the type of bacteria (species) in the biofilm.

Quorum sensing occurs in both Gram-negative bacteria and Gram-positive bacteria, though Gram-negative is more commonly studied due to its typical AI/R-protein components. Gram-positive bacteria communicate through processed oligo-peptides, whereas Gram-negative bacteria utilize acylated homoserine lactones (AHLs) (See Figure 7). The autoinducer in quorum sensing is a small molecule that is generated by the cell to diffuse throughout the biofilm. When the concentration of the AI in the biofilm reaches a critical level, the AI is then able to bind (and activate) its R-protein, which then induces the transcription of the corresponding target gene (Reference Cell density image). If a single bacterial cell is releasing AIs into the biofilm, the concentration will be too low to be detected by surrounding cells; in order for the AIs to activate the R-proteins, there must be many cells releasing the same AI to reach the critical concentration (thus the name *quorum* sensing) (Wilson and Devine 2003).

Figure 7- Cell density and AI Concentration. Adapted from Wilson and Devine 2003.

Quorum sensing was discovered in the bacterial species *Vibrio fischeri*, which produce light emissions at high cell populations (due to the large levels of AI). Quorum sensing in *V.fischeri* is

regulated by two R-proteins (regulatory proteins), LuxI and LuxR (See Figure 8) . These R-proteins, when activated by high concentrations of AIs, induce gene transcription (five genes- *luxCDABE*) that produces the proteins and enzymes required to emit light. This is an example of a simple cell to cell communication that produces a response in the overall biofilm; this LuxI and LuxR pathway is used by over 25 different bacterial species (Engebrecht, Nealson, and Silverman 1983).

Figure 8- Gram-negative QS (left) and Gram-positive QS (right). Adapted from Wilson and Devine 2003.

There are other quorum sensing cascade pathways that are more complex than 2 simple R-proteins. *Pseudomonas aeruginosa* is the most extensively studied with respect to quorum sensing, as it is a very serious human pathogen with a relatively complex cascade pathway. *P. aeruginosa* utilizes two complete cellular pathways, *las* and *rhl*. The Las system consists of LasR, a transcriptional activator, and LasI, a synthase enzyme, to produce a specific homoserine lactone (3O-C₁₂-HSL). The rhl system utilizes a transcription activator (RhlR) and enzyme (RhlI) to produce a different homoserine lactone, C₄-HSL (See Figure 9).

Figure 9- LasI/LasR-RhlI/RhlR- QS in bacteria. Adapted from Wilson and Devine 2003.

Together, these systems regulate (through activation, regulation, and negative feedback) the expression of virulence factors that determine the degree of pathogenicity for *P. aeruginosa* (Wilson and Devine 2003). It has also been revealed that the Las system is directly responsible for the three dimensional structure of *P. aeruginosa*. The 3O-C₁₂-HSL signal molecule is responsible for coordinating the cells in the biofilm to build the distinct mushroom-like structures that facilitate water channels and nutrient exchange both in and out of the biofilm (Davies et al. 1998). It has also been shown that the Las signal molecule plays a significant role in biofilm initiation (attachment of cells) and development (through communication between cells); the removal of the Las signal molecule significantly inhibited the initiation and formation of *P. aeruginosa* (de Kievit et al. 2001). These are all examples of how different quorum sensing pathways elicit specific responses by the biofilm community. It is important to understand how quorum sensing is utilized by biofilms; this exemplifies that bacteria is more efficient when established in a cooperative community than when existing as a planktonic organism (See Figure 10). Quorum sensing is also an important area of study- if the communication pathway between cells could be interrupted, the success of the biofilm may be severely hindered.

Figure 10- Importance of Quorum Sensing. Adapted from Romeo 2008.

Multispecies Biofilms

The majority of biofilm research done today utilizes monopopulation biofilm models- studying biofilm cultures that contain only one species of bacterium. This is beneficial in understanding the events of formation and microbiology of biofilms, but it may not be directly relevant to preventing biofilms in industry. In reality, biofilms often survive as multispecies units and can contain several distinct species of bacteria. Multispecies biofilms can be referred to as a consortia (many units sharing resources as one) or a coaggregation (collecting many into a single mass), and are characterized by “genetically distinct” bacteria adhering to one another. The attachment between different species is regulated by the adhesion protein of one bacterium type and the corresponding saccharide receptor on the other type (Stoodley, Sauer, Davies, & Costerton 2002). There are hundreds of different adhesin proteins and saccharide receptors, though only the concept of attachment is directly relevant. In this way, bacteria are limited to which types of different bacteria species they can adhere to (though more than 1000 strains are able to co-aggregate).

A general model of the biofilm formation process was previously described (attraction, adhesion, growth and division, dispersion). Multispecies populations are very similar to this process, with some important differences. The first bacterial species, called primary (or early) colonizers, adhere to the

conditioned substratum and begin to multiply. As this develops into a young biofilm, the microenvironment in the community becomes favorable for bacteria. Secondary (or late) colonizers then attach to the primary colonizers (through the adhesion protein/ receptor saccharide process), and the biofilm develops into a multispecies consortia (See Figure 11, a & b). There are two methods for which the secondary colonizers join the biofilm: (1) single bacterial cells in the bulk fluid (planktonic cells) attract and adhere to the genetically different cells in the established biofilm (accumulating cell by cell) or (2) the planktonic cells in the bulk fluid first aggregate into a floating mass (Seen in Figure 11, c), which then aggregates to the established biofilm as one unit (Rickard 2003). In either case, the secondary colonizers adhere to the primary colonizers through specific adhesion/ receptor pairing, and the late cells develop a subset of bacterial species in the biofilm. As each species divides and grows individually, they are clustered together into mosaics (Seen in Figure 11, d) (Molin, Haagensen, Barken, & Sternberg 2000). It is believed that the secondary species will add to the EPS of the biofilm, providing further strength and protection to the community.

Figure 11- Formation of multispecies biofilms (a) Primary Colonizers (B) Microcolonies (C) Secondary Colonizers (D) Mature Multi-Species Biofilm. Adapted from Rickard 2003.

Mathematical Modeling

There have been many attempts to describe the conceptual understanding of biofilms in mathematical terms. This can be useful in attempting to understand or predict what the system is doing or how the processes of the system work. These models vary greatly, from simple calculations describing the biofilm as a whole to complicated algorithms that model the three-dimensional structure. Current mathematical models are typically only useful in a specific area, such as mass balance equations for the system (transport) or growth and rate equations (kinetics) of the cells, because encompassing every aspect of the biofilm into one model is severely impractical. These models may be very useful in predicting the behavior of a process, though they still may contain assumptions that are over simplified. Because of this, mathematical models should only be used as a *tool* for predictions and should not be expected to hold true during research.

The most useful, basic models for research include conservation of mass for the system. Conservation of mass is accounted for by defining how much mass is accumulating in the system. Accumulation is equal to the net flow in and out, added to the amount produced inside the system, subtracted by the amount of mass consumed in the system. This is defined as seen below (in both descriptive and mathematical forms):

$$\left(\begin{array}{c} \text{Net rate of} \\ \text{accumulation} \\ \text{of mass} \\ \text{of component} \\ \text{in the system} \end{array} \right) = \left(\begin{array}{c} \text{Mass flow} \\ \text{of the} \\ \text{component} \\ \text{into} \\ \text{the system} \end{array} \right) - \left(\begin{array}{c} \text{Mass flow} \\ \text{of the} \\ \text{component} \\ \text{out of} \\ \text{the system} \end{array} \right) + \left(\begin{array}{c} \text{Rate of} \\ \text{production} \\ \text{of the} \\ \text{component by} \\ \text{transformations} \end{array} \right) - \left(\begin{array}{c} \text{Rate of} \\ \text{consumption} \\ \text{of the} \\ \text{component by} \\ \text{transformations} \end{array} \right)$$

$$\frac{\partial C}{\partial t} = -\frac{\partial j_x}{\partial x} - \frac{\partial j_y}{\partial y} - \frac{\partial j_z}{\partial z} + r$$

In this model, the change in concentration (δC) with respect to the change in time (δt) is related to the overall change in mass flow (in-out) plus the overall rate (production- consumption). The mass is modeled as change in flux (δj) in every direction (x,y, and z) and the overall rate is simply described by

(r). Through some assumptions and algebra, this basic rule of transport can be adjusted to fit a general model of biofilms:

$$\frac{d(V_B C_B)}{dt} = F_{in} - F_{ef} + F_F + F_B$$

The net accumulation in the system becomes a product of the volume of the bulk fluid (V_B) and the concentration in the bulk fluid (C_B), which are related to the influent and effluent component mass flow rates (F_{in} and F_{ef}) and the overall transformation flow rates in the bulk fluid and the biofilm (F_F and F_B) (Eberl et al. 2006). Each specific step will not be analyzed as the model is only for example purposes and is not the focus of this paper. There are other models that focus on specific processes, such as stoichiometry, rate, growth, maintenance, dispersal, and structural development.

Relevance of Biofilm Research

Bacteria are among the most abundant microorganism on earth, thus biofilms can be found almost anywhere. Because biofilms are quick to establish and difficult to remove, they are commonly found in a variety of industries. If the biofilms presence is undesirable or causes damage, it is said to be biofouling. In a small number of applications biofilms are not considered to be negative or harmful, but in the vast majority of industries biofilms are a severe problem that causes much damage. Biofilms have been reported in industries such as food processing, dairy processing, industrial water, gas, and oil lines, wastewater treatment plants, and medical applications (including dental, orthopedic, cardiac, etc.). In all of these examples, biofouling occurs in the form of bacterial and pathogen contamination of food, decreased flow through pipes, reduction in heat transmission, corrosion of metal pipes and surfaces, contamination of the bulk fluid flowing through the pipe, and severe infection or medical device failure. Biofilms impact these industries with large costs due to prevention and maintenance, and pose serious health threats to consumers and patients. Biofilm research is applicable to all of these industries;

understanding and characterizing how bacteria form biofilms can lead to new technologies for preventing the negative side effects of biofouling.

Food Processing

Bacterial contamination is a common problem in the food processing industry, including dairy processing plants, raw meat and poultry processing centers, and food canneries. These food processing facilities typically include cold wet environments made of stainless steel and plastics, which are factors that promote biofilm growth. If these surfaces are not properly cleaned and maintained, biofilms can lead directly to food borne illnesses by contaminating the products as they pass over the biofilm infected areas. Bacteria such as Salmonella, Staphylococcus aureus, multiple Pseudomonas and Listeria monocytogens have been known to contaminate raw meats and poultry as they are prepared in these facilities. These bacteria have been observed to harbor on surfaces that collect condensation such as conveyor belts, floor drains, storage tanks, and hand trucks. In dairy processing, bacteria (*Listeria*, *Staphylococcus*, *Escherichia coli*, etc.) has been discovered on wooden shelves of cheese ripening rooms and on the metal surfaces of buffer tanks, pasteurization lines, silos, and raw milk lines (Sharma 2003). Surfaces that contain irregularities such as crevices, pits, valves, and gaskets are at a higher risk for contamination. In processing facilities that involve raw meat, biofilms have been found on hotdog case strippers, rollers (conveyor belts), packaging equipment, slicer blades, and other pieces of equipment that are challenging to clean and are wet for long periods of time, resulting in food that poses serious health risks to consumers. Pathogen-containing biofilms have even been found in vegetable processing plants; bacteria are often brought into the facility in the soil that is attached to the vegetables (such as broccoli and spinach) (Chmielewski and Frank 2003). Chemical cleaning and sanitizing is a crucial procedure in preventing food borne illnesses, and is often not sufficient to preventing contaminations in the food processing industry.

Industrial

Industrial applications are also significantly affected by biofouling. This can occur in cooling water systems, storage tanks, drinking water and wastewater treatment plants (reverse osmosis membranes), ship hulls, porous media used in oil-bearing lines, ion exchangers, and drinking water delivery systems. Biofouling in industrial applications are typically less health-risk associated and more economically inhibiting. In pipelines and filters, there is significant energy loss due to an increase in the friction of the bulk fluid. Biofilms also increase the heat transfer resistance, which has an effect on heat exchangers like those used in a power plant condenser. There is also a significant increase in cost for replacing equipment that is prematurely compromised by these effects. If equipment is down for repair, there are costs associated with the stop of production (in oil production, it would be equal to the amount of oil not being produced during downtime). Quality control issues can also be associated with biofouling of heat exchange systems. For example, the thickness of rolled steel may vary outside of the specification limits if the steam system used by the plant is contaminated with biofilms (Characklis and Marshall 1990). Biofilms have a significant economic impact on the industrial sector; the monetary impact is estimated to be in the hundreds of billions of dollars in the United States.

Corrosion

Microbial corrosion also plays a significant role in the biofouling of metal surfaces. There are many mechanisms for corrosion, including pitting and crevice corrosion. Corrosion is caused by an electrochemical process that includes an oxidation (anode) and a reduction (cathode) reaction. These occur together in an electrochemical cell that contains each half cell (one anode and one cathode) as well as a path for electron flow to occur and a separate path for ion flow to occur, much like a battery (See Figure 12) (Characklis and Marshall 1990). The corrosion reaction can be a result of biofilms in many ways; the oxygen (cathodic reactant) at the substratum surface below a biofilm will be consumed, the mass transport resistance (of the corrosion products and reactants) near the surface will be increased,

corrosive substances (acids) can be generated by biofilms, or the metabolic reactions within the biofilm can serve as the cathodic reactants of corrosion.

Figure 12- - Basic Corrosion Cell. Adapted from Lewandowski and Beyenal 2008.

Figure 13- Pit corrosion due to oxygen depletion under biofilm.
Adapted from Lewandowski and Beyenal 2008.

In Figure 13, the electrochemistry of pitting corrosion facilitated by biofilm growth on the surface of the substratum is exemplified. The localized area is depleted of oxygen under the biofilm, so the anodic reaction will be the oxidation of the metal while the cathodic reaction will be the reduction of oxygen. Biofilms may also produce corrosive substances that degrade the surface of the substratum, such as acids. These also function to alter the localized pH around the biofilm and further corrode the metal (Lewandowski and Beyenal 2008). Corrosion can be severely deleterious to metals used in any type of

application. Corroded pipelines may have a serious economical impact due to replacement times and materials cost. They also may contaminate the fluid flowing through those pipes, such as drinking water, milk, oil, fuel, food products, etc. Corrosion due to biofilms is common on prominent features within the pipe system such as sharp turns, gaskets, and valves. Biofilm corrosion of gaskets and valves may pose serious safety issues due to leaking or the inability to control fluid flow.

Medical Industry

Biofilm growth also has severely negative effects on the medical industry, and has been shown to contaminate dental tissues and implants, catheters, heart valves, pacing systems, orthopedic implants, and human integument. Biofilm contamination on these medical devices leads to infection of the surrounding tissues, and can pose a very serious health risk to the patient. Infections have been classified into three categories: superficial immediate infections, deep immediate infections, and late infections. Superficial immediate infections occur on the surface of the surgery site, and are associated with the bacteria that dwell on the skin (such as the infection of a burn dressing). Because the infection site is accessible, they are typically easily treated with antibiotics or surface disinfectants. More serious infections are those that occur deep in the tissue at the implant site. An immediate deep infection is associated with bacteria introduced at the site during the procedure, and can be from bacteria on the skin of the patient, hands and medical tools of the surgeons, or on the implant itself. A late infection is one that develops in the deep tissue of the implant site months to years after the surgery. This is attributed to planktonic bacteria in the blood, such as bacteria that dispersed into the bloodstream from another biofilm infection somewhere else in the body (Temenoff and Mikos 2008). Deep infections, whether immediate or late, pose the greatest health risk to patients as they are very difficult to treat and typically spread through important organs such as muscle and bone.

Prosthetic Implant Infection (known as PII) is a deep infection example that is caused by biofilm growth on the surface of the implant. This is a rising medical problem, as the use of plates, screws, intramedullary rods (IM nails), and artificial joints have become increasingly popular. In 2005, there were

over 500,000 hip and knee replacements alone. Though infection occurs in a minority of patients, it has a high cost of treatment as well as a high rate of mortality. The cost of treating a single implant infection can be as large as \$50,000, and may result in removal of the implant (Brady et al. 2008). The majority of infections are immediate, and occur within 3 months of implantation of the device. Often, external bacteria are directly introduced into the patient during the surgery. Once introduced, the surgical wound will include compromised soft tissue as well as clotted blood and provide an ideal environment for bacteria to colonize. Through MSCRAMMs, bacteria are able to bind to a variety of proteins in the host, such as fibrinogen, fibronectin, collagen, and elastin (Brady et al. 2008). The infection will cause increased temperature and swelling in the local tissues; this may destroy surrounding leukocytes, increase bone and blood pressure, decrease the partial pressure of oxygen in the blood, and decrease the pH of the environment. All of these factors will serve to decrease local circulation and increase the spread of the infection. In bone implants, the infection spreads through the Haversian canal system and into the cortex bone, causing the tissue periosteum to lift and separate from the surface of the bone. This will destroy the local capillaries, and can lead to the death of large portions of cortical and cancellous bone.

The current gold standard of infection diagnosis requires culture and identification of the bacterial species- available only through tissue biopsies. Once identified, drugs such as antibiotics may be administered, though they may not be very effective in treating the established infection. Biofilms have many mechanisms which enable them to resist antibiotic and host immune responses. Some bacterial strains have developed to resist certain types of antibiotics- such as methicillin-resistant *S. aureus* (MRSA). Other drugs such as vancomycin and rifampin must be considered, though they are often less effective. In either case, antibiotics must be administered for at least 4-6 weeks and up to 6 months for an infected knee replacement (Brady et al. 2008).

Bacteria also have many mechanisms in which to avoid the immune response of the host. The polysaccharide capsule of each cell (EPS) function to prevent the immune cells (such as macrophages) from phagocytosing the bacteria. Antibodies and complement proteins are able to attach to the EPS,

though the thick polysaccharide layer prevents them from interacting with the receptors of the immune cells. This can lead to a proinflammatory response, causing more damage to the surrounding tissue. Apart from the biofilm community, the planktonic bacteria may also invade the eukaryotic cells of the host such as osteoblasts, fibroblasts, and eukaryotic cells (Wilson and Devine 2003).

Bacterial biofilms have also been observed to colonize on intravascular catheters. There are many associated repercussions, though the most serious medical condition is catheter-related bloodstream infections, or CR-BSI. In the United States, there are over 5 million intravascular catheters inserted each year, and more than 200,000 result in blood-stream infections, costing between \$296 million and \$2.3 billion (Mermel 2000). While the chances of developing CR-BRI are low, the mortality rate for established infections are estimated to be as low as 10% and as high as 25%. The risks of CR-BRI are proportional to the duration of the catheter placement: the site of the catheter insertion, the anatomic location of the vein used (jugular, subclavian), the technique of insertion (surgical vs. non-surgical), the type of material the catheter is made of, the number of the catheter lumens, and the environment under which the catheter is inserted (emergency insertion vs scheduled and prepared insertion) all have significant effects on increasing the risk of infection. The mechanisms of infection development and avoidance of the host immune response in CR-BRI are very similar to those of prosthetic implants; however in CR-BRI the bacteria are dispersed directly into the blood stream. Bacterial biofilms have been observed on other types of catheters as well, including urinary tract catheter and dialysis catheters, though the effects of the infection are less serious than CR-BRI (Wilson and Devine 2003).

Bacterial biofilms have also been observed to colonize on living tissue, such as the human integument. It has been revealed that bacteria, such as *S. epidermidis*, are able to grow between the squamous skin cells of humans. It was observed that in dry areas the bacterial cells reside at least 5 cells deep, and 15-20 skin cells deep in wet tissues (such as injured areas and surgical sites). The bacterial cells were found to be on the surface of the skin as well as structurally integrated deep with the squamous skin cells. This provides some insight as to how deep implant infections may occur; after sterile preparation of

the skin (surgery protocol), only the superficial bacterial cells were killed while the deeper integrated cells remained unharmed. Bacterial infection of the human integument has very serious medical repercussions, such as in the infection of the human female reproductive system. Bacterial establishment on the fallopian tubes causes scarring that can lead to infertility, as well as leading to infection in the ovaries and the peritoneal cavity. Bacterial infections may be introduced through sexual activity or even intrauterine contraceptive devices. The uterine tract, the biliary system, the digestive system, and the pulmonary system have all been shown to establish bacterial infections, and all pose serious health risks to the patient (Costerton 2007).

Biofilms also have been shown to have significant negative effects in the dental industry. Biofilms exist as dental plaque on the surfaces of human teeth and gums. Using nutrients from saliva and food, bacterial biofilms are able to survive and multiply and can cause much damage in the mouth of the host. Immediately after brushing, bacteria that are left in the mouth (tongue, gums, cheeks, etc.) begin to repopulate on the teeth. Inside the biofilm an area of anaerobic respiration occurs close to the substratum, fermenting sugars and producing acids as byproducts. This has been shown to demineralize the enamel surface of the teeth, which leads to the development of caries (cavities). If not properly cleaned and maintained, biofilm plaque can cause an extreme amount of damage to the natural teeth of the host (Stoodley, Wegel, Gieske, deBeer, & Ohle 2008).

Current Methods of Biofilm Prevention

There are three main methods used to control biofilm growth on surfaces: (1) physical (mechanical) removal of bacteria from the surface, (2) chemical application (antibiotics, biocides, antiseptics, etc.) to kill the living bacteria, and (3) modifying the surface properties of the substratum to prevent bacterial attachment. Often, physical scrubbing of the colonized surface is difficult because the biofouled surface may not be easily accessible (such as inside pipes, food processing equipment, or on medical implants). Chemical applications have been shown to have limited effect due to the natural structure of biofilms, and often the environmental impact of harsh chemicals may be a limiting factor.

Focus in industry has turned to the design of the material, utilizing materials science and engineering to produce surfaces that resist biofilm growth by inhibiting bacterial attachment. Surface modification approaches usually change the surface chemical composition (morphology), surface topography (roughness), hydrophilic/ hydrophobic nature, surface energy, and polarity (Vladkova 2009). All of these modification techniques function to prevent or hinder biofilms by creating a surface that bacteria cannot initially attach to; though some are not relevant in the medical device industry (such as thickness of coatings, polymer chemistry, and slippage) as these affect large scale biofouling (such as the attachment of barnacles to ocean vessels).

There are many mechanisms by which bacteria attach to surfaces (the adhesion mechanism is usually dependent on the bacterial strain type), so designing a material that universally prevents attachment is complicated. However, it has been shown that the hydrodynamic conditions of the surface are a strong determining factor in attachment. Ikada et al. predicted through the use of the DLVO theory that adhesion (or surface energy) approaches zero when the surface has a water contact angle of 0° or 90° (meaning the surface is super hydrophilic or super hydrophobic). This was partially confirmed in an experiment that showed bovine serum albumin (BSA) attachment to a polymer surface was significantly decreased when the surface approached these conditions. Though surface appendages of the bacterial cell play an important role in later biofilm development, it has been shown that the thermodynamic properties are more important in initial attachment (Ikada, Suzuki, & Tamada 1984). The thermodynamic theory of Derjaguin, Landau, Verwey, and Overbeek (DLVO theory, which characterizes colloids at solid-liquid interfaces) has been used many times to explain the adhesion of bacteria to surfaces; taking into account DLVO forces (electrostatic forces, van der Waals interactions, etc.) polar interactions, ion bridging, and steric interactions, are very important in developing “non-sticky” surfaces (Derjaguin 1955).

Surface free energy and critical surface tension (wettability) have been extensively studied with respect to decreasing adherence, and for many years optimal conditions remained in debate. In 2003, the “Baier curve” was presented and accepted as the best relationship between adhesion and surface energy.

This established that the lowest surface energy did not result in the lowest relative adhesion, as was the belief for many years and the source for the debate (Anderson et al. 2003). It was shown that the lowest relative adhesion occurred at a surface free energy of 20-30 mN/m (See Figure 14).

Figure 14- The Baier Curve. Adapted from Anderson et al. 2003.

Fracture mechanics can also be applied to the surface adhesion of the material. Griffith fracture mechanics can be considered in modeling the force required to break an adhesive from an elastomer substrate. This equation can be modified to directly relate this “pull off force” of a substrate to the material properties, such as critical surface tension and Elastic Modulus. Elastic modulus is easily engineered in a material, and current antifouling elastomers in industry have an elastic modulus from 1.4 to 3 MPa (Vladkova 2009).

Manufacturing and Welding

Welding is perhaps the most prominent method used in metal manufacturing, more common than other forms of metal bonding such as fasteners and rivets. Welds are found in almost every metallic device used in the medical, industrial, and food processing industries. Every industrial or food processing system most likely contains some form of a weld- whether it is in a pipeline, holding tank, or piece of machinery. Medical devices such as stents, pacemakers, and orthopedic implants (bone plate, hip implant,

etc.) are also often held together with welds. All of these surfaces are also fluid contacting surfaces and are susceptible to microbial growth and biofilm development.

While there are many different types of welding processes, the thermal cycles experienced by the material are all very similar. Extreme thermal gradients can exist over a very short range; near the arc the gradient can reach several $1000^{\circ}\text{K}/\text{mm}$, which results in violent forces that act on the substratum (refer to Figure 15). Note that the right side of the image shows the large thermal gradient with respect to the distance from the arc, disregard the left side of the image). The extreme thermal process associated with welding has very serious implications on the resulting microstructure of the material. It can alter the surface texture around the weld, create segregations and phase boundaries between granular regions, alter the grain size in regions near the weld (cause grain growth or even refinement and recrystallization), form precipitates near the weld, oxidize the surface of the welded region, and create large residual stresses due to the expansion and contraction as the material changes phases (Williams 1991).

Figure 15- Temperature gradient ($^{\circ}\text{K}$) with respect to distance (mm). Adapted from Murphy et al. 2010.

During the welding process, the electrode can either act as the Cathode or the Anode, in which case electrons flow either from the electrode to the base metal or from the base metal to the electrode.

Electrons are responsible for more than 80% of the energy transfer during the welding process, as well as the large temperatures found near the electrode. The severe temperatures are enough to melt the base metal (and filler material, if present) into what is called the weld pool (refer to Figure 16). In order to solidify the weld pool, the latent heat of fusion must be dissipated from the solid-liquid interface. Solidification Theory attempts to explain and predict the behavior of this phase change from the liquidus to the solidus in the material. As the pool cools, Epitaxial Nucleation occurs at the solid liquid interface- often followed by Competitive Growth amongst the grains. For a detailed discussion of Solidification Theory (including Epitaxial Nucleation and Competitive Growth), please refer to Appendix A.

Figure 16- The liquid weld pool near an arc. Adapted from Walsh 2012.

The molten weld pool is not the only region to be thermodynamically influenced. As the heat dissipates from the weld pool during solidification, it heats up the surrounding base metal. The region around the weld pool that increases in temperature is known as the Heat Affected Zone, or HAZ. The temperature gradient from the weld pool to the base metal produces regions that contain very distinct microstructures that directly alter the material's mechanical properties. The HAZ not only affects the physical and chemical properties surrounding the weld, but also has a significant impact on the bioactivity of the surface. For a detailed discussion of the HAZ and the resulting microstructure and material properties, please refer to Appendix B.

CHAPTER 2

Objectives

The objective of this thesis is to establish a protocol to produce, analyze, and study biofilms grown in a static exposure system. Deliverables will include (1) proof that a static system bioreactor will produce microbes attached to a substratum, resulting in a biofilm, (2) a specific protocol for exposing, fixing, and preparing the developed biofilm for imaging, (3) methodology for analyzing biofilm growth, and (4) an experimental procedure that will enable the correlation of material properties to microbial attachment and biofilm growth.

CHAPTER 3

Methods

The following protocol will cover the methods used for (1) bioreactor set up, (2) sample preparation and characterization (pre- exposure), (3) sample exposure, (4) bacterial fixation and chemistry, (5) sample characterization and imaging (post exposure), and (6) biofilm analysis. For a list of the materials used in the study, please refer to Appendix E.

Bioreactor Set Up

1. A 500ml beaker, or equivalent glassware, was used as a static bioreactor. The beaker and all associated tools were disinfected with 70% IPA to sanitize the system. All components of the system were sprayed and wiped clean with a paper towel.
2. The system was assembled and prepared for the experiment by laying out all the appropriate components and ensuring they fit in the proper places. This includes placing rubber stoppers (or equivalent glassware lid), preparing suspending wires, and readying the fixing chemicals.

Sample Preparation and Characterization (Pre Exposure)

1. The sample was sized to 1cm x 2cm using a horizontal band saw (chop saw).
2. The sample was then mounted in Bakelite using a Buehler SimpliMet 2 Mounting Press.
 - a. For specific mounting protocol, refer to Appendix C- Specimen Mounting Procedure.
3. Using the 250, 400, and 600 grit sand papers, the samples were sanded to remove any major scratches or inclusions.
4. The sanded samples were then imaged to characterize the rough surface.
5. The samples were then polished using the 6 μ m and 1 μ m polishing wheels (and appropriate diamond polishing suspension).

- a. Some samples were polished only with the courser wheel, while others were brought all the way down to the finishing wheel in order to produce samples with different surface properties.
6. After polishing, the sample was then prepared for imaging by an Electrochemical Etch (with 10% oxalic acid) at 4 volts and 2 amps for approximately 15-20 seconds.
7. The sample was then imaged with the optical light microscope to identify the microstructure of the surface.
 - a. On the non-welded samples, the bulk material was imaged for surface properties and the presence of inclusions. On the welded samples, the images were concentrated on the bulk material, the HAZ, and the welded region.
 - b. Several images were taken on each sample in order to well characterize the surface.
8. The samples were re-polished to remove the damage done by the etching procedure before exposing.
9. The sample was then sanitized with 70% IPA to remove any microbes present on the surface. This ensures that the biofilms that develop are those that have attached to the surface from the bulk fluid.

Sample Exposure

1. Biologically active natural water was collected from a local pond (stream by campus) for use as the bulk fluid (media).
2. Approximately 400 ml of media was added to the static bioreactor, or until the sample was completely submerged.
3. The samples were placed at the bottom of the bioreactor, with the mounted portion exposed to the media. Smaller samples (or those not mounted in Bakelite), should be suspended in the media using nylon monofilament (fishing line).

4. The bioreactor was capped off and sealed with a rubber stopper, parafilm, or appropriate glassware lid in order to create an anaerobic environment by sealing off the exposure chamber from the atmosphere.
5. Samples were left in the static bioreactor for a period of 24 and 48 hours.
6. Immediately upon removal, the samples were submerged for three minutes in each of the following: 3.7% Formaldehyde, pure Ethanol, and pure Acetone.
 - a. For specific fixing protocol, refer to Appendix D- Microbial Fixing Procedure.

Sample Characterization & Imaging (post exposure)

1. Samples can then be imaged without further processing if an “Environmental” SEM is used, such as a Hitachi TM-1000. If other imaged systems are to be used, the samples should be sputter coated with palladium- gold in order to produce the best images.
 - a. For specific sputter coating protocol, refer to APPENDIX E- Sputter Coating Procedure.
2. Using the Hitachi TM-1000, the samples were then imaged in several locations on the exposed surface.
3. The images were recorded in order to analyze the microbial activity and the biofilm growth on the surface of the substratum.

Biofilm Analysis

1. Microbial attachment was observed with Scanning Electron Microscopy.
2. Potential correlations were made by visual inspection.

CHAPTER 4

Results

The primary goal of this thesis was to establish a protocol to culture, analyze, and study biofilms grown in a static exposure system. Four specific Deliverables were defined in order to achieve this goal; analyzing each deliverable individually will exemplify how the overall goal of this thesis was achieved.

Deliverable (1)

As outlined in the Objectives, the first deliverable of this thesis is to prove that a static system bioreactor will produce microbes attached to a substratum, resulting in a biofilm. Following the protocol previously described in the methods section, a 303 Stainless Steel sample was longitudinally mounted, polished to 1 μ m, and characterized via optical light microscopy. The sample was then exposed to biologically active natural water for a period of 48 hours. The sample was fixed and imaged with a Hitachi TM-1000 Tabletop Scanning Electron Microscope.

Figure 17- 303 SS longitudinally mounted and polished pre-exposure (left) and after 48 hour exposure to biologically active natural water (right).

Comparing the images in Figure 17, there was clearly microbial attachment to the substratum in the static system bioreactor. The large dark mass in the center of the image on the right is most likely a

developed colony of microbes (biofilm), with the lightly colored spots being individual microbes. The cloudy regions in the dark mass are thought to be the mushroom towers forming from the EPS. The image on the left shows the longitudinal sulfide inclusions that are present in the 303 SS. Looking closely at the image on the right, we can see these same longitudinal inclusions beneath the colony of microbes. The smaller dark spots around the colony were not expected and were present on the surface of every exposed sample. After research, it is believed that a thin film developed and covered the entire surface of the sample due to the long exposure time. It is hypothesized that the chemicals used in fixing desiccated this film and caused it to contract into smaller clumps, creating the “EPS footprint” that appears. This EPS footprint was visible on every exposed sample, and ranged in severity with respect to the substrate properties as well as the exposure time.

Deliverable (2)

The second Deliverable was to develop specific protocol for characterizing, exposing, and preparing a sample for microbial attachment. The methods section outlines the specific steps of the protocol, and supplementary Standard Operating Procedures can be found in Appendices C-E. These are provided in case future work is carried out by individuals who are unfamiliar with the machines and chemicals required. The full protocol, including the supplemental information, is listed below:

- **Bioreactor Set Up**
 - Use a 500ml beaker, or equivalent glassware, as a static bioreactor. Disinfect the beaker and all associated tools with 70% IPA to sanitize the system. Spray and wipe clean all components of the system with a paper towel.
 - Assemble the system and prepare for the experiment by laying out all the appropriate components and ensuring they fit in the proper places. This includes placing rubber stoppers (or equivalent glassware lid), preparing suspending wires, and readying the fixing chemicals.
- **Sample Preparation and Characterization (Pre Exposure)**
 - Size the sample to 1cm x 2cm using a chop saw (horizontal band saw).
 - Mount the sample in Bakelite using a Buehler SimpliMet 2 Mounting Press.
 - Specimen Mounting Procedure
 - Clean specimens to remove cutting and handling residues.
 - Remove debris from the mold assembly.
 - Apply a thin coat of mold release compound to the mold assembly.
 - Raise mold ram to up into position.

- Center the specimen on the ram.
 - Lower the ram assembly approximately 3 inches.
 - Pour a predetermined amount of resin into mold.
 - Clean and remove any excess resin from around the mold assembly threads.
 - Lock the mold assembly cover.
 - Slowly raise the ram into the up position.
 - Apply and maintain the recommended heat and pressure for the specified period of time, approximately 10-15 minutes.
 - Apply the cooling cover and let sit for 2-3 minutes.
 - Remove the mounted specimen and let cool to room temperature before handling.
 - Clean the mold and ram assembly.
- Using the 250, 400, and 600 grit sand papers, sand the samples to remove any major scratches or inclusions.
- Image the sanded samples with the optical light microscope to characterize the rough surface.
- Polish the samples using the 6 μ m and 1 μ m polishing wheels (and appropriate diamond polishing suspension)
 - Polish one set of samples with the 6 μ m polishing wheel and another set down to the 1 μ m polishing wheel.
- After polishing, prepare the sample was for imaging by an Electrochemical Etch (with 10% oxalic acid) at 4 volts and 2 amps for approximately 15-20 seconds.
- Image the sample with the optical light microscope to identify the microstructure of the surface.
 - On the non-welded samples, image the bulk material for surface properties and the presence of inclusions. On the welded samples, images should be concentrated on the bulk material, the HAZ, and the welded region.
 - Take several images on each sample in order to well characterize the surface
- Re-polish the samples to remove the damage done by the etching procedure before exposing.
- Sanitize the sample with 70% IPA to remove any microbes present on the surface. This ensures that the biofilms that develop are those that have attached to the surface from the bulk fluid.
- Sample Exposure
 - Collect biologically active natural water (or other media).
 - Add approximately 400 ml of media to the static bioreactor, or until the sample is completely submerged.
 - Place the samples at the bottom of the bioreactor, with the mounted portion exposed to the media. Smaller samples (or those not mounted in Bakelite), should be suspended in the media using nylon monofilament (fishing line).
 - Cap off the bioreactor and seal with a rubber stopper, parafilm, or appropriate glassware lid in order to create an anaerobic environment.
 - Leave samples in the static bioreactor for the designated period of time.
 - Immediately upon removal, submerge the samples for three minutes in each of the following: 3.7% Formaldehyde, pure Ethanol, and pure Acetone
 - Microbial Fixing Procedure

- Ensure proper ventilation in the fixing area, a Fume Hood is recommended. Ensure proper lab safety equipment is worn (closed toed shoes, long pants, latex or polyethylene gloves, and protective eyewear).
- Prepare all solutions. Ensure that all solutions are pure and that all glassware is clean
 - 3.7 % Formaldehyde 200 mL
 - Pure Ethanol 200 mL
 - Pure Acetone 200 mL
- Use tongs to remove the exposed samples from the static bioreactor. Do not touch the surface that is to be examined
 - Remove and place one sample at a time in order to ensure accuracy
- Place the sample in the 3.7% Formaldehyde solution for approximately 3 minutes.
- Remove the sample from the Formaldehyde and place it in the Pure Ethanol for another 3 minutes.
- Remove the sample from the Ethanol and place it in the Pure Acetone for a final 3 minutes.
- Remove the sample from the Acetone and place it on a clean, covered surface to prevent contamination (such as a cleaned Tupperware container).
- Sample Characterization & Imaging (post exposure)
 - Samples can then be imaged without further processing if an “Environmental” SEM is used, such as a Hitachi TM-1000. If other imaged systems are to be used, the samples should be sputter coated with palladium- gold in order to produce the best images.
 - Sputter coating procedure
 - Hand contact with the samples should be minimized, and personal protective equipment such as latex or polyethylene gloves should be worn. The samples should be gripped only on the edges of the Bakelite.
 - Place the sample in the vacuum chamber.
 - Ensure that the sealing gaskets are clean prior to replacement of the chamber
 - Replace the vacuum chamber and check that the vent valve is shut.
 - Turn the power switch on and draw a vacuum of 10^{-1} mbar.
 - Open the leak valve and allow the vacuum to stabilize at 2 mbar.
 - Use the test switch to check the current. The value should stabilize at 15-18 mA. If not, adjust the leak valve to the proper setting.
 - Set the timer switch to the desired setting, usually about 30 to 45 seconds for a mounted specimen.
 - Press the start switch and wait for the time to elapse.
 - Break the vacuum with the vent valve and remove the sample from the vacuum chamber, using clean polyethylene gloves.
 - Return the chamber to the proper position and turn off the power switch.
 - Using the Hitachi TM-1000 (or other SEM), image the samples in several locations on the exposed surface.
 - Record the images in order to analyze the microbial activity and biofilm growth on the surface of the substratum.
- Biofilm Analysis
 - Determine which method of analysis is to be used, and record results.

The protocol listed above is an effective way to prepare and characterize the substratum, expose the sample to media containing planktonic bacteria, and fix the attached microbes for imaging without damaging the sample. Each step adds important information to the resulting data, and aids in gathering insight for the analysis. Once the skills to process and prepare the raw samples were developed, it was relatively easy to carry out the protocol consistently sample to sample. The most challenging stages were obtaining quality images with the Hitachi Tabletop SEM and determining how to analyze the acquired images.

Deliverable (3)

In order to analyze the post-exposure SEM images, two methodologies are proposed: a Statistical Approach and an Indexed Approach. In the statistical approach, several random images of the sample are taken in order to identify the surface features and microstructure. Each image should be a unique field of view that does not overlap another image, spread out across the surface of the sample. If enough images are taken, the overall properties of the sample surface can be determined. Once the sample is exposed, the same number of random images again containing unique fields of view should be recorded. These images can then be quantified for the amount of microbial attachment (such as a count of microbial cells per area, or a ratio of the surface area of established biofilms to the surface area in the field of view). The pre-exposure images will yield information about the surface of the sample, while the post-exposure images will provide insight to the amount of bacterial growth. There can then be a correlation made between the surface properties of the material and the microbial attachment to that surface.

The indexed approach can be used to identify specific areas of interest (such as around inclusions) that are present on the surface of the sample. This is done by mapping the surface of the sample with respect to an origin, which should be established at an easily identifiable edge or corner. From this origin, specific x and y locations of inclusions or surface geometries can be recorded so that the exact same location can be imaged before exposure and again after exposure. Like the statistical

approach, this will give insight to relating the surface properties of the sample to the microbial attachment around those properties.

While the SEM provided adequate images of the exposed sample, there are other imaging techniques that may also be effective. Confocal Laser Scanning Microscopy (CLSM) or Fluorescence Microscopy may provide additional information that an SEM cannot. It has been discovered that some cells will auto-fluoresce when excited by a specific UV wavelength. Research has been carried out to identify the wavelengths that specific strains fluoresce under. A study by Bao et al. used differing wavelengths of fluorescence microscopy to identify strains of bacteria and respective cell density in a culture (Bao et al. 2008). This could be incorporated to the methods to analyze the developed biofilms by determining which strains are in the biofilm as well as the cell density of the population. If staining were also to be incorporated, information about protein or gene expression can be recorded in the images. A Live/ Dead stain (such as Trypan Blue) will not only provide information about where the microbes attach but also which microbes are thriving in which areas. Cell Staining will provide great insight to the bacterial activity of the culture and may serve as a bridge between the current biological research and the new engineering approach that is proposed.

Deliverable (4)

The fourth objective was to develop an experiment that relates material properties to microbial growth. This experiment should take into account the affects of each material property in relation to bacterial attachment. Design of Experiments from The Engineering Statistics e-Handbook was used in order to create an experiment that is well planned and maximizes the information available. Design of Experiments is the method of determining the objectives of the study and developing process factors to test for those objectives. This is most commonly represented by the “Black Box Model.”

The Black Box Model can be used in order to determine which specific Inputs (or controlled factors) are to be used in order to characterize the output. A Black Box is used to represent the process (or

experiment), where inputs are turned into results “magically” by the process inside the box; the specific steps of the process are not what is important, so they are grouped into one step inside the box. A statistical design of experiments utilizing this method is an efficient way to develop an experiment in which the obtained data is easy to analyze and produces valid conclusions. The experiment that is proposed is a type of Screening experiment, where the factors that matter the most (of the many possible ones) are chosen in order to determine which factor has the greatest response. The experiment contains three factors each at two levels, noted by 2^3 . A study of three factors each at two levels will yield 2^3 or 8 total test combinations (runs needed). In order to be statistically sound, the complete experiment (all 8 runs) would need to be replicated at least 3 times. In design of experiments, a matrix is built in order to notate each experiment. In each experiment, each factor will have a “high” and “low” setting (denoted by +1 and -1) where that factor is either ramped up or scaled down. If all possible combinations of high and low settings for each factor are incorporated into the experiment, then it is termed a Full Factorial experiment. A full factorial 2^3 experimental matrix would then be represented as seen in Table 1.

TABLE 1: A 2^3 two-level, full factorial design table

run	X1	X2	X3
1	-1	-1	-1
2	1	-1	-1
3	-1	1	-1
4	1	1	-1
5	-1	-1	1
6	1	-1	1
7	-1	1	1
8	1	1	1

This experiment can also be represented graphically. A two level design with 3 factors creates a cube, with each direction correlating to a specific factor. This graphical representation can be seen in Figure 18. Note that the numbers above each sphere correlate to the run number in the design matrix of Table 1. An analysis matrix is then created to relate the interaction of individual factors to the overall results. The design also enables the experimenter to determine the two-element and three-element interactions in the system. An example of the full factorial 2 level, 3 factor analysis matrix is shown in Table 2 (NIST/SEMATECH 2012).

Figure 18- A 2^3 two-level, full factorial design; factors X1, X2, X3.
Adapted from NIST/ SEMATECH 2012.

TABLE 2: Analysis Matrix for a 2^3 Experiment

<i>I</i>	<i>X1</i>	<i>X2</i>	<i>X1*X2</i>	<i>X3</i>	<i>X1*X3</i>	<i>X2*X3</i>	<i>X1*X2*X3</i>
1	-1	-1	+1	-1	+1	+1	-1
+1	+1	-1	-1	-1	-1	+1	+1
+1	-1	+1	-1	-1	+1	-1	+1
+1	+1	+1	+1	-1	-1	-1	-1
+1	-1	-1	+1	+1	-1	-1	+1
+1	+1	-1	-1	+1	+1	-1	-1
+1	-1	+1	-1	+1	-1	+1	-1
+1	+1	+1	+1	+1	+1	+1	+1

The three factors proposed for the experiment are Material Composition, Surface Conditions, and Welding, and the outcome measured is the degree of microbial attachment. The major difference in Material Composition is the sulfur content of the material. This greatly affects the number and character of sulfide inclusions in samples of 303 Stainless Steel and 304 Stainless Steel. We are interested in the difference in 303 and 304 SS because 303 SS contains large amount of inclusions in the material, which may have significant affects on the microbial attachment. Measurable parameters may include inclusion size, shape, and distribution. Sample Surface conditions will be tested by exposing samples sanded down to 600 grit and samples polished to 1 μ m (again for 303 and 304 Stainless Steels). The Effect of Welding will also be studied: samples of both 303 SS and 304 SS will be exposed “as welded” (with the weld bead intact on the surface), with the weld sanded down to 600 grit paper, and with the weld polished off to 1 μ m. This will provide information about how to process welded materials in industry in order to minimize microbial attachment. Following the Design of Experiments previously described, this study can be carried out efficiently to produce results that are relate each factor to microbial attachment in a valid manner.

Trial Iteration of Proposed Experiment

In order to prove the effectiveness of the proposed experiment in Objective (4), a single experimental iteration was performed. 303 and 304 Stainless Steel rods were sectioned from a 3/8 inch diameter rod and mounted in Bakelite. A set of samples were mounted both transverse and longitudinally, in order to analyze the changing material properties. 304 SS samples previously mounted and welded with a Gas Tungsten Arc Weld (GTAW) at 7.5 mm/s were obtained (both a surface weldment and a weldment cross section). Following the protocol developed in Objective (2), all samples were sanded, imaged, polished, imaged, etched, and imaged (using optical light microscopy) in order to obtain detailed information about the varying material properties. The samples were then exposed to biologically active natural water for either 24 or 48 hours in a static bioreactor. Following the fixation protocol provided in

Appendix B, the samples were then fixed and prepared for imaging using the Hitachi TM-1000 Tabletop Environmental Scanning Electron Microscope. The results of the single iteration are presented below.

Pre Characterization Results- Optical Light Microscopy

Samples Sanded to 600 Grit Paper:

Samples of both 303 and 304 SS were mounted and sanded from 250 grit paper to 600 grit paper. These samples were then imaged using an optical microscope (see Figures 19 and 20). In Figure 19, the large dark regions are the sulfide inclusions that are present in the 303 Stainless Steel. The large lines that travel across the image are scratches resulting from the sanding process. As the sand paper gets finer, the scratches get smaller. It is important to note that Figure 20, a 304 SS mounted and sanded to 600 grit paper, does not contain the sulfide inclusions that are present in the 303 SS. Both Figure 19 and Figure 20 are at the same magnification (500x) and thus have the same scale.

Figure 19- 303 SS 600 grit at 500x. Note the large sulfide inclusions.

Figure 20- SS 600 grit at 500x. Note the lack of any sulfide inclusions.

Samples Polished to 1 μ m:

Samples of both 303 and 304 SS were mounted and sanded from 250 grit paper to 600 grit paper. Then, the samples were polished on diamond polishing wheels from 6 μ m to 1 μ m. In Figure 21 (polished 303 SS), the small abundant dots are the sulfide inclusions that are present in the material. Figure 22 (also polished 303 SS) shows the similar sulfide inclusions with a large, atypical inclusion in the center of the image. Comparing these images to Figure 23 (polished 304 SS), we see that there are much fewer inclusions in the 304 SS, and that the diamond polishing produces a surface that is very smooth and lacks many surface features. In this image, there is a group of two dark spots that may be sulfide inclusions. This was a very atypical image of the 304 SS; the rest of the 304 SS polished images looked like the smooth base material around the inclusions.

Figure 21- 303 SS 1μm diamond polished 200x. Note the abundance of inclusions.

Figure 22- 303 SS 1μm diamond polished 200x. Note the abundance of inclusions, and the large inclusion in the middle.

Figure 23- 304 SS 1μm diamond polished at 500x. Note the very few inclusions, though some were still present.

Samples Etched After Polishing:

After polishing, samples of both 303 SS and 304 SS were etched in 10% oxalic acid in order to show the grain boundaries of the sample. In Figure 24 (the 303 SS), the grain boundaries are most easily identified near the upper portion of the image (though it is still difficult). During the etching process, boundaries on the surface are corroded in order to more easily identify them. While this enhances the grain boundaries, it may also cause pitting (or pit corrosion) on the surface of the sample. In Figure 25, the grain boundaries are easily visible and there are lots of black dots on the surface. While these may appear to be sulfide inclusions, knowing that this is a 304 SS, they are most likely pits that have developed on the surface as a result of the etching process (and will be removed after re-polishing).

Figure 24- 303 SS 1 μ m diamond polished and etched at 500x. Note the visibility of the microstructure and grain boundaries.

Figure 25- 304 SS 1 μ m diamond polished and etched at 200x. Note the visibility of the microstructure and grain boundaries.

Longitudinally Mounted Samples

In order to get a better look at the sulfide inclusions present in the samples, a different perspective was required. This was obtained by mounting both 303 and 304 SS longitudinally in the Bakelite and polishing the samples down to provide a cross section of the sulfide inclusions. In Figure 26, the inclusions appear as long strings that run through the 303 SS sample rather than as tiny dots. This provides a more comprehensive look at the structure of the material. Figure 27 shows a polished cross section of 304 SS also mounted longitudinally. Note the small number of sulfide inclusions in the 304 SS compared to the 303 SS. In the Longitudinal 304 SS, a small string of inclusions is visible in the lower left hand corner. The large spot in the upper right is most likely an aluminum oxide impurity. The aluminum oxide solidifies at a higher temperature than the sulfur inclusion- so when the material is rolled, the sulfide inclusion will elongate whereas the solidified aluminum oxide impurity will remain spherical. The aluminum oxide impurities were very rare in the 304 SS Longitudinal (this was the only image captured that contained one). Figure 28 was a more typical image of the 304 SS, containing very few sulfide inclusions and no alumina oxide impurities.

Figure 26- 303 SS Longitudinally mounted, 1 μ m diamond polished at 500x. Note the long strung out sulfide inclusions

Figure 27- 304 SS Longitudinal 1 μ m diamond polished 500x. Note the large alumino silicate oxide in the upper right, and the small inclusion string in the lower left.

Figure 28- 304 SS Longitudinal 1 μ m diamond polished 500x. A typical image of the 304 SS, lacking any inclusions or oxides.

Welded Samples

In order to test for the effect of welding on microbial attachment, welded (Gas Tungsten Arc Weld- GTAW) samples of 304 SS were prepared. The samples were previously mounted and sanded, and then polished to 1 μm . The samples were etched in order to show the grain boundaries and the microstructure resulting from the welding process. Figure 29 shows the fusion zone on the left and the unaffected base material on the right. The small band in the center is the Heat Affected Zone (HAZ). On the right edge of the HAZ band, melt back along the grain boundaries into the unaffected base material is visible (the dark lines that string away from the HAZ, tracing the grain boundaries). This image exemplifies the very different microstructure that results from the welding process. The changes in the structure of the material are on the same order as the microbes ($< 10 \mu\text{m}$), and thus are hypothesized to have significant impacts on amount and location of microbial attachment. Figure 30 is the same image as Figure 29, focused to enhance the grain boundaries in the fusion zone. Figure 31 is another 304 SS GTAW polished and etched. The fusion zone in this image is on the right, while the unaffected base material is on the left. This image shows the native grain boundaries of the unaffected base metal (on the right), and how the weld refines the crystals (or grain) in the fusion zone.

Figure 29- 304 SS weldment polished and etched 200x. Note the changing microstructure of the different zones. Left to right: fusion zone, HAZ, base material. Meltback is visible along the grain boundaries.

Figure 30- 304 SS Weldment polished and etched at 200x. Same image as Figure 29, focused to enhance the grain boundaries in the fusion zone.

Figure 31- 304 SS weldment polished and etched 200x. Left to right: base material, HAZ, fusion zone. Note the grain boundaries in the base material.

Because the weld transmits heat not only on the surface of the sample but also down into the material (in the z direction), a cross section of the weld was taken to once again provide a different perspective. Figures 32 and 33 provide information as to how the heat transmits into the material. In these images, the weld was run over the upper portion of the sample and transmitted heat in a semi circle below it. These images are at different portions along the semi circle; In both images, the fusion zone (in the upper portion) and the unaffected base material (in the lower portion) are separated by the curved HAZ band. These images are very similar to the surface images (Figures 29-31) in that the base material has a microstructure that is obviously larger than the HAZ or the fusion zone.

Figure 32- Cross section of weld on 304 SS polished and etched at 200x.

Figure 33- Cross section weldment on 304 SS polished and etched at 10x.

On the 304 SS surface welded sample, a region of recrystallization and competitive growth was visible inside the fusion zone. This is shown in Figure 34; competitive grain growth is visible and would have developed behind the weld pool as it solidified.

Figure 34- Fusion zone of a weldment on 304 SS at 20x.

Post Exposure Results- Scanning Electron Microscope:

Coarse Samples (Sanded to 600 Grit):

The samples of 303 and 304 SS that were sanded to 600 grit paper (referred to as “coarse samples”) were exposed to biologically active natural water in the static bioreactor for 48 hours. The samples were then fixed and subsequently imaged with the Hitachi Environmental SEM. In Figure 35, the large dark regions are most likely areas of developed biofilms. The smaller dark spots that are visible make up the “EPS footprint” that remains of the desiccated surface film. Figure 36 is a magnification of the upper dark region in Figure 35. The lines from the sand paper that were visible in the coarse pre-exposure characterizations (Figures 19 and 20) are still visible in this image. Figures 37 and 38 are typical images of the 304 SS coarse samples after 48 hours of exposure. It is important to note that there are fewer dark regions than on the 303 SS image (Figure 35). Figure 39 locates a developed microbial community and Figure 40 is a greater magnification on this region, where the individual microbial cells are visible inside the dark region.

Figure 35- Coarse 303 SS exposed for 48 hours. Note the abundant large dark regions of microbial growth as well as the smaller EPS footprint.

Figure 36- Coarse 303 SS exposed for 48 hours. A magnification of the large dark region- most likely a developing biofilm. Note the lines visible from 600 grit paper on the underlying substratum.

Figure 37- Coarse 304 SS exposed for 48 hours. Note the significant decrease in microbial activity compared to the coarse 303 SS.

Figure 38- Coarse 304 SS exposed for 48 hours. A magnified image showing two or more small regions of microbial activity.

Figure 39- Coarse 304 SS exposed for 48 hours. Another possible region of microbial activity.

Figure 40- Coarse 304 SS exposed for 48 hours. A closer image of the community of microbes as seen in Figure 39. Note the scratches from the 600 grit sand paper on the underlying substratum.

Polished Samples (to 1 μ m):

Samples of 303 SS and 304 SS were polished to 1 μ m and also exposed for 48 hours. The samples were fixed and imaged in the same fashion as the coarse samples. The initial observation is that there are fewer dark regions on the 303 SS polished sample (Figure 41) than on the 303 SS coarse sample (compare to Figure 35); only 1 large dark spot is visible on the polished sample as compared to 4 or more on the coarse sample (recall that the dark regions are indicative of microbial activity). Figures 42 and 43 magnifies the polished 303 SS sample to show the dark regions with greater detail- Figure 43 is most likely a developing microbial community. It is again apparent that the 304 SS sample has less biofilm development than the 303 SS sample (both polished and exposed similarly). This is shown in Figure 44, which is a typical image of the polished 304 SS sample that is largely void of any large dark regions, indicating the reduced amount of microbial activity on the surface. Figure 45 reinforces the idea of reduced microbial activity as there are almost no groups of microbes (dark regions) in the field of view. Small groups of organisms were found spread out on the 304 SS polished as seen in Figures 46 and 47. However, these groups of cells appear to aggregate around artifacts on the surface, rather than setting down on the bulk material. The artifacts seen in Figures 46 and 47 appear as long string like structures and may have been on the surface before exposure (a result of poor cleaning), or were introduced to the surface from the biologically active natural water (such as a type of algae). Also visible on these images are what appear to be larger, unidentified eukaryotic organisms other than bacteria, also a result of the natural water. Two of these larger organisms are visible near the center of Figure 46, and eight or more are visible on Figure 47 (because the groups of cells in Figure 46 and 47 appear to be the result of many factors other than bacterial attachment and biofilm development (surface artifacts, larger organisms, etc.), these two images should not be considered when quantifying microbial attachment to the polished 304 SS).

Figure 41- 303 SS Polished to 1μm and exposed for 48 hours. Note the single large dark region of microbial activity.

Figure 42- 303 SS Polished to 1μm and exposed for 48 hours. A greater magnification of the dark region in the previous image.

Figure 43- 303 SS Polished to 1 μ m and exposed for 48 hours. Likely a developing biofilm community.

Figure 44- 304 SS Polished to 1 μ m and exposed for 48 hours. Note the lack of any large dark regions, areas which are indicative of microbial activity.

Figure 45- 304 SS Polished to 1μm and exposed for 48 hours. Note the lack of any large dark region of microbial activity.

Figure 46- 304 SS Polished to 1μm and exposed for 48 hours. Note the presence of long string-like surface artifacts and the unidentified larger organisms.

Figure 47- 304 SS Polished to 1 μ m and exposed for 48 hours. Note the presence of surface artifacts and the many unidentified larger organisms.

Longitudinal Samples

Samples of 303 SS and 304 SS longitudinally mounted and polished were also cleaned and exposed to the same media. The polished, longitudinally mounted 303 SS was exposed for a period of 48 hours, while the polished, longitudinally mounted 304 SS was exposed for a period of 24 hours. Figure 48 depicts a community of microbes among the EPS footprint of the longitudinal 303 SS sample; Figure 49 is a greater magnification of the same region. Looking closely at Figure 49, the long sulfide inclusions that are present in the longitudinal 303 SS can be seen in the underlying base material. The collection of microbes (dark mass in the center of the image) appears to be a well developed biofilm. The cloudy regions of the dark mass are remnants of thicker film regions, or the “mushroom-like” towers protruding out from the EPS, and the smaller white dots may be the individual microbes. Figure 50 depicts another region where microbial cells have attached to the surface of a longitudinal 303 SS. Another surface artifact is visible in the lower region of the image (the long cylindrical structure). Unlike Figures 46 and

47, the group of cells appear to be attached to the substratum, separated from the surface artifact (they are not attached *to* the artifact as in the previous images). While Figure 51 shows yet another group of microbes on the longitudinal 303 SS, it better displays the long sulfide inclusions (upper left portion of the image, see arrow) that are present in the longitudinal 303 SS.

Figure 48- 303 SS Polished to 1 μ m and Longitudinally mounted, exposed for 48 hours. A single large community of possible microbes.

Figure 49- 303 SS Polished to 1 μ m and Longitudinally mounted, exposed for 48 hours. A magnified view of the microbial community in the previous image. Note the sulfide inclusions visible on the underlying microstructure.

Figure 50- 303 SS Polished to 1 μ m and Longitudinally mounted, exposed for 48 hours. Note the surface artifact on the lower right, and the microbial community that is attached separately from the artifact.

Figure 51- 303 SS Polished to 1 μ m and Longitudinally mounted, exposed for 48 hours. Note the long sulfide inclusions present in the longitudinal 303 SS, most easily seen in the upper left portion of the image (arrow).

The polished, longitudinally mounted 304 SS was exposed in the same media, though it was removed and fixed after only 24 hours. Figure 52 is an image of the surface taken at 600x. The immediate difference between the samples exposed for 48 hours and this image is the reduction in the EPS footprint and the significant lack of microbial groups. It appears that the short time frame limited the amount of microbial development that could occur, and it is clear that the film causing the EPS footprint did not have enough time to develop over the whole surface. It is important to recall, though, that the 304 SS appeared to be less attractive in the other samples. The proliferation on the 303 SS may be caused, in part, by the quick reactivity of the material, and more data is required if conclusions are to be made. This image shows that the microbes were still in the attachment phase, and did not have enough time to develop into a biofilm. Magnifying one of the lighter (almost white) regions, it is easy to see that there are no longitudinal sulfide inclusions in the 304 SS as in the 303 SS; this is visible in Figure 53. The small white dots in this image may be individual microbes attaching to the surface. Figure 53 is at 1200x, and the length marker indicates that these dots appear to be on the same order as individual microbes (μ m).

Figure 54 again shows the reduced EPS footprint and the lack of large dark regions that are visible on the longitudinal 304 SS. This may be due to the fact that in the 48 hour samples the microbial communities had time to develop and produce the EPS that covers the biofilm, so when these areas were imaged the EPS rendered the region dark. If this EPS layer was to be stripped away, the plethora of microbes might be visible underneath (and should appear as light colored cells). In these images (Figures 52-54), the EPS was not given time to develop, and so the individual microbes may be visible. Figure 55 displays a very small surface artifact that was present, and what appears to be microbes attaching to this artifact. Note that this image is at 3000x, and the lightly colored dots may be individual microbes.

Figure 52- 304 SS Polished to 1 μm and Longitudinally mounted, exposed for 24 hours. Note the decreased EPS footprint and the lack of any large dark regions indicative of microbial activity.

Figure 53- Longitudinally mounted 304 SS Polished to 1 μ m and exposed for 24 hours. Possible microbes attaching to the substratum.

Figure 54- 304 SS Polished to 1 μ m and Longitudinally mounted, exposed for 24 hours. Another view of the surface; reduced EPS footprint and lack of dark regions of microbial activity.

Figure 55- 304 SS Polished to 1μm and Longitudinally mounted, exposed for 24 hours. Possible microbes attaching to a surface artifact.

Welded Samples

The polished 304 SS sample with the surface GTAW weld was cleaned and exposed for a period of 48 hours. Upon imaging, it was nearly impossible to locate the welded region. Because the sample was re-polished after etching, the fusion zone and HAZ could not be located upon initial inspection. Further, if the regions were visible upon inspection, the extensive EPS footprint that developed on the sample covered the underlying microstructure and increased the difficulty of locating the regions of interest (fusion zone, HAZ, base material, etc.). Figure 56 exemplifies how difficult it is to locate the weld region of the sample. In this image, a very large surface artifact, though interesting, inhibits the view of the underlying microstructure. In this trial iteration the welded region could not be located.

Figure 56- 304 SS Weldment Polished to $1\mu\text{m}$ and exposed for 48 hours. It is impossible to determine the underlying microstructure of the material, and thus the regions of interest (welded region, HAZ, base material) were unable to be located and studied.

CHAPTER 5

Discussion

Deliverable (1):

The first deliverable of this thesis was to prove that a static system bioreactor would generate microbes attached to a substratum, producing a biofilm. Based on the images presented in the results, most notably Figure 17, this goal was achieved. The images show that there clearly was microbial attachment to the surface of the samples, though the individual microbes were not identified or characterized. Other static bioreactor systems have been previously described, such as microtiter well plates for the characterization of bacterial strains as detailed by Stepanovic et al.; however, these flat-bottomed wells are much too small to incorporate metal samples (Stepanovic et al. 2007). This is why a static system large enough to house samples that were mounted in Bakelite needed to be tested. This initial proof of concept was important to be established if future work is to be carried out relating microbial attachment to processed surfaces. Though the static system worked well for the scope of this project, new systems simulate environments that are more relevant to those found in industry should be developed. A system that incorporates more factors (such as fluid flow) would be useful in many applications, such as the industrial, medical, and food processing industries.

Deliverable (2):

The second deliverable (and primary goal) of this project was to develop specific protocol for characterizing, exposing, and fixing the samples. While the protocol was generally successful and relatively easy to follow, some steps seemed to have limitations associated with them. An analysis of each section in the protocol is provided below:

Bioreactor Set Up

For this study, the static system bioreactor was used in order to maintain simplicity and establish initial proof of concept. The static system provides a simple environment where planktonic bacteria are

exposed to the substrate surface, without the added complications of more advanced bioreactors. A flow loop bioreactor that incorporates fluid flow not only increases the resources needed to perform the experiment (such as an external pump, media reservoir, and power source), it increases the number of factors needed to take into consideration during characterization and analysis (including fluid flow velocities and profiles around surface geometries). In order to initially establish and prove the effectiveness of these methods, a static bioreactor was sufficient for exposing the samples.

The static bioreactor used in this experiment was a simple glass Tupperware equivalent to a 500 ml beaker. The bioreactor first needed to be sanitized and cleaned of existing bacteria and debris. This was done by cleaning the surfaces with 70% Isopropyl Alcohol (IPA). Sterilization of the bioreactor can be performed, though it is not necessary for this project. In typical cell culture, sterilization to the Sterility Assurance Level (a 1-in-10⁶ chance that the surface is not sterile, even if the packaging is left undisturbed) is needed in order to be absolutely sure that the eukaryotic cells are not contaminated with prokaryotic bacteria (the bacterial cells readily grow in all environments, whereas it is much harder to maintain and culture complex cells). This method is necessary when studying monocultures of bacteria so that other strains do not contaminate the population. Monoculture biofilms are often studied from a biological standpoint in order to characterize strain-specific properties such as gene expression; however, biofilm monocultures are rarely found in nature as they typically always exists in a consortium. This thesis is focused on culturing biofilms that are relevant to real world applications where many species of bacteria exist, so sanitation with 70% IPA is adequate to remove excessive material present on the surface.

Sample Characterization (Pre-Exposure)

Utilizing the methods of material science, protocol was developed to easily characterize the properties of the material. These steps were clear and concise, while providing enough data to sufficiently characterize the material. Improvements could be added to this section, though they are not necessary and would only provide additional information. Examples may include (1) alternative etching methods in order to enhance the visible grain boundaries, (2) X-Ray Diffraction in order to provide data about the

crystal structure, chemical composition, or physical properties of the substratum, and (3) Atomic Force Microscopy to identify the nanoscale morphology of the surface.

Sample Exposure

In industry, biofilms are observed in a wide variety of media including oil, water, and blood. For this study, the type of fluid used in exposure was not the main interest. While biologically active natural water (pond water) is not directly relevant to the medical industry, the important factor is that the fluid contains planktonic bacteria that are able to attach to the surface of the substrate. It is important, however, to note that the samples should be exposed to the same media if they are to be compared during analysis. Stream water collected at different locations or times may contain different concentrations of planktonic bacteria, which may affect the amount of bacterial attachment. If differing media sources are to be used, they should be characterized for the level of bacterial contamination before sample exposure. Now that the static bioreactor and research protocol have been established, an experiment evaluating exposure in various media types is possible and should be pursued.

A process for fixing the attached microbes was also developed. The 3.7% Formaldehyde solution was used in order to stop and kill the bacteria without lysing the cell wall. This ended all metabolic processes in order to prevent further proliferation- which allowed for a point-in-time look at the development of the biofilm. A time study is centered on the ability to analyze the microbial development at specific points in time, so the punctuality of this step is critical. Once the biofilm is fixed on the substrate, it is necessary to dry the specimen in order to capture quality SEM images. The first dip in pure Ethanol acts as an initial dehydrating process, followed by a final dehydration in the pure Acetone. These steps combined produced a fixed and dried biofilm that is ready for SEM imaging. While this fixing procedure worked well, the development of the EPS footprint was distracting in many of the images and limited the view of the underlying microstructure. Other methods of fixing and drying have been described and should be further investigated, such as Bouin's reagent (Stepanovic et al. 2007) or drying the sample with UV light before exposure (Sreekumari, Nandakumar, & Kikuchi 2001).

Sample Characterization & Imaging (Post-Exposure)

The Hitachi TM-1000 tabletop environmental SEM used for this project worked well and provided satisfactory images, but was difficult to focus at the higher magnifications. This may be due to the limited pressure vacuum of the machine, or the lack of conductivity of the samples. This machine does not require sputter coating to capture an image, though it may help increase the quality of the pictures. A larger SEM that operates at a higher vacuum may also aid in the quality of the images. While this machine was adequate for the scope of this project, more advanced imaging systems could be pursued.

Deliverable (3):

The third deliverable was to research and propose methodologies to analyze the extent and distribution of microbial attachment. The statistical approach was utilized for the trial iteration of the developed experiment (Deliverable 4), but there were significant limitations to this analysis method. First, the images are analyzed manually and are subject to human error and cognitive biases. Second, the images were not clear enough to provide a count of individual microbial cells per area. It was difficult to tell what was a microbial cell and what was not, and almost impossible to identify specific bacterial cells. It may be more efficient to calculate the total area of attached microbes versus the total area in the field of view (such as with Image J or other such program), yet this also has limitations. The presence of the EPS footprint makes it difficult to separate a microbial community from the bare substratum. In order to address these limitations, a computer program is proposed that measures the total area of the “dark spots” (regions of microbial activity) compared to the total area of the field of view (the substratum surface area). Only calculating spots that are of a “critical radius” (as specified by the user) will ensure that the program takes into account only the areas large enough to not be part of the EPS footprint. This will better standardize the data gathered from the Statistical Approach and provide more valid conclusions.

An approach based on indexing (which maps locations on the sample surface, as previously described) is also warranted. Being able to locate specific inclusions or surface geometries will greatly

increase the quality of the data gathered. If the samples were indexed before exposure to identify specific areas of interest, they can be analyzed using the same index system to image growth at these designated areas. For example, if there is a certain inclusion or geometry on the surface of the sample that is deemed to be of interest, this can be located after exposure to see if there is a more or less biofilm growth correlated with this point. This also would be useful when studying the exposed welded samples. If the fusion zone, HAZ, and base material were all indexed prior to exposure, the regions could be easily located for analysis later. New imaging techniques as well as staining methods were identified in order to increase the amount of information that can be gathered for analysis. The limitations of the tabletop SEM have been identified, and it would be pertinent to pursue these alternatives for future studies.

When analyzing the samples, it is important to note that the microbial attachment is not the same in all regions of a given sample. Depending on the location of the field of view, different results may be acquired. It is also not the same between two samples, as no two samples were prepared or processed exactly the same. This is why many iterations/replications should be carried out in order to statistically capture the representative population.

Deliverable (4):

The fourth and final deliverable of the project was to develop an experiment that correlated material properties to microbial attachment on the substratum. Based on statistical Design of Experiments, this can be carried out to establish relations between the pertinent factors (Material Composition, Surface Conditions, and Welding's Effect). A trial iteration was carried out in order to test the effectiveness of the developed protocol as well as the practicality of the experiment.

The trial iteration provided very good insight to the effectiveness of the protocol that was developed in the methods. It also seemed to make some preliminary correlations between the studied factors and the overall microbial activity on the surface of the samples. This experiment had only one replication, and future work would be to replicate or triplicate the trials in order for the experiment to be

statistically sound. However, the present work can be used to make preliminary assumptions and guide future effort. Based on the results, there was more microbial attachment and biofilm development on the samples of 303 SS than the samples of 304 SS (best exemplified between Figures 35 and 38). The hypothesis is that the inclusions are the main reasoning for this correlation. The inclusions are the sites where the processes of corrosion begin to occur. This creates anodic and cathodic reactions, which produce Hydrogen in many cases. There are many bacterial strains that require hydrogen in order to thrive, so they typically flock to these areas around the inclusions. It is important to note that Hydrogen is only one example- there are bacteria that seek other elements such as iron or sulfates, which may also be released around corroding inclusions. It also appeared that the samples sanded to 600 grit sand paper had more microbial attachment and biofilm development than the samples that were polished to 1μ , most easily visible in Figures 41 and 45. While this may be intuitive, it is important information to further validate because it is a simple design tool that can be used for device processing in industry.

Because the welded regions could not be tracked after polishing, the welded samples were impossible to analyze in current work. Thus, no data was presented on the effect of welding and the need for sample indexing was solidified. Previous studies have shown it possible by identifying each region and correlating the degree of microbial attachment. It is known that welding alters the microstructure of the material on the scale of the individual microbes (such as the grain boundary size).

Sreekumari et al. showed that the changing regions of the weldment do significantly affect the amount of microbial attachment. In order to test these factors, 304 L Stainless Steel was welded by Gas Metal Arc Welding at 3mm/s, 36 V, and 300 A in an argon shielding gas. The samples were then etched in order to identify grain boundaries and metal regions associated with the welding. The materials were machined to separate the weld region, the HAZ, and the base metal and each portion was mounted in resin. The materials were then polished to $3\mu\text{m}$ and the surface degreased with acetone and sterilized with 70% ethanol. The samples were then sterilized and dried with UV for 10 minutes. A culture of *Pseudomonas* sp. was used for bacterial exposure, and the bacterial density of the medium was recorded

intermittently by plating on nutrient agar. Sets of each regions of welded material were exposed to both the bacterial media as well as sterile distilled water. The flasks were incubated and shaken at 28°C and 90 rpm. The coupons were exposed for periods of 4, 8, 12, 24, 96, 144, and 192 hours and three flasks were kept 16 days in order to observe corrosion.

It was shown that the area of bacterial attachment increased with time on all three regions of the material. The base metal showed the lowest area of attachment, and the welded region showed the greatest. However, by the 192 hour, the HAZ region showed approximately the same area of attachment as the welded region. In the corrosion studies, 16 days of exposure did not produce pit corrosion in the unaffected base metal or the HAZ region, though there was pitting observed in the welded region. It was also shown that initial bacterial attachment occurred on or near the grain boundaries. The sizes of the grains were measured to be the smallest in the weld region (10um) and largest in the base metal region (27um). This proved an inverse relationship to bacterial area of attachment. Differences in the composition of the metal at the grain boundaries was also shown, though there was no correlation made between the material composition and bacterial attachment (Sreekumari et al. 2001).

This study offers many improvements that could be implemented to the protocol in order to accommodate the Effect of Welding factor. Most notably, the regions were cut away from each other after etching so that each region could be exposed and studied individually. Microbial attachment to regions of welded samples have also been shown without cutting the materials. Figure 57 shows the proliferation of Biomass on the HAZ region of a weld. In this image, the welded (fusion) region is on the left, the HAZ is the band in the middle, and the base material is on the right. The sample was exposed for a period of time (not specified) and the biomass fixed. The film was desiccated, revealing the remnants attached to the HAZ region (Walsh 2012). These methods should be considered if the proposed experiment is to be carried out in the future.

Figure 57: Proliferation of Biomass in the HAZ. Adapted from Walsh 2012.

Future Work

Several improvements are critical to the successful evolution of this project. First, the media used in the system for the trial iteration was not ideal. The media contained artifacts that disrupted the natural microbial growth on the surface of the samples. While this may be found naturally occurring in industry, it limits the studies ability to correlate the design factors to the results. Other medias should be considered, such as those with known concentrations of planktonic bacteria or those relevant to industry applications (such as oil, gas, milk, processed food, or even FBS (fetal bovine serum) to simulate blood). Sreekumari et al. monitored the media by periodically measuring the planktonic bacterial concentration. Also, the environment of the bioreactor could be improved to better relate to real world applications. An incubator could be added to culture the microbes at specific temperatures (such as those found in the body). Fluorescent microscopy should also be research; auto-fluorescing cells may provide information about specific strains present in the culture.

Second, the static bioreactor was useful as a proof of concept, but has limited real world applications. There are many industries which utilize bioreactors- such as tissue and cell culture in stem cell research and waste water treatment in environmental engineering. While very complex bioreactors

have been designed for use in these fields, some simple additions may produce large improvements to the static system. The first addition proposed would be incorporating a Spin Bar to the flask to induce fluid flow, as seen in Figure 58. This would be very easy to do, and would provide control over the speed of the fluid currents by increasing/ decreasing the bar's spin velocity.

Figure 58- A Spin Bar inducing fluid flow in the system.

The next bioreactor design would include the incorporation of a flow loop and an external pump to induce directional flow in the system. A thesis by Sara Leifer has previously outlined, in detail, how to build a flow loop system that could work well for biofilm research. Figure 59 is an image that shows the final design developed in her thesis. This flow loop system was used for developing an in vitro blood vessel mimic and incorporates flow in both the luminal and transluminal directions, which is needed to condition and seed stem cells onto the tissue scaffold. This bioreactor was made from a plastic kitchen Tupperware, and connections were drilled into the sides in order to attach the flow tubes. A section of the flow tube is incorporated through an external pulsatile pump in order to create flow in the system, and a media reservoir was added in order to keep the media levels high and prevent air bubbles travelling through the system. Also, there are several valves and stop cocks used to control the flow through the bioreactor (Leifer 2008).

Figure 59- A Blood Vessel Mimic Bioreactor System designed by Sara Leifer. A system such as this could be modified and used in biofilm research to incorporate flow in the system. Adapted from Leifer 2008.

This system can be used to seed and culture Human Umbilical Vein Endothelial Cells (HUVECs), which are very complex eukaryotic cells. These cells require a very specific media, temperature, and environment in order to flourish, as well as an aseptic system that is free from bacterial contamination. In cell culture, bacterial contamination is very difficult to prevent; typically all experiments are done inside an aseptic laminar flow hood. Because this type of system has proven to work for eukaryotic cells, it should also be adequate for culturing prokaryotic cells (such as bacteria). Bacterial cells do not require such a controlled environment, as they can survive virtually everywhere. A flow loop system such as this could be modified to house bacterial biofilms and serve as an exposure system that incorporates flow of the bulk fluid.

A simple flow loop system was created based on the design of the BVM (blood vessel mimic) bioreactor described above. The luminal/ transluminal ports were eliminated and simplified into a single inlet and a single outlet. A single plastic tube was connected to each side in order to create a system that has a single loop and a single exposure chamber. The plastic tubing can be connected to a peristaltic

pump in order to provide flow through the system, and thus expose the prepared substrate to a flowing bulk fluid.

While this design incorporates fluid flow into the system, analysis of the sample is “system invasive;” the sample is destroyed during the fixing procedure and must be reprocessed before it is exposed again. An ideal bioreactor would be one that allows for sample analysis without interrupting or disturbing the system. While some analysis methods of biofilm research do require sample destruction (such as Staining), important information can still be acquired through simple imaging. Imaging can give insight to quantifying the number of adherent cells, and further development of the consortia can be characterized through the use of real time video surveillance. A system that allows external imaging or real time video to take place without disrupting or contaminating the bioreactor would serve as a tool for advanced biofilm characterization and research, and has been previously described (Palmer 1999). The system includes an external pump, a media/ waste reservoir, and a microscopy flow cell that allows for real time imaging of the developing biofilm without disrupting or contaminating the system. A system such as this would be ideal for studying microbial attachment.

Figure 60- A microscopy flow cell that allows for real time study of biofilms. Adapted from Palmer 1999.

REFERENCES

- Anderson C, Atlar M, Callow M, Candries M, Milne A, Townsin RL (2003) The development of fouling-release coatings for seagoing vessels. *J Marine Design* B4:11–23
- Bao, Ning, Balamurugan Jagadeesan, Arun K. Bhunia, Yuan Yao, and Chang Lu. "Quantification of Bacterial Cells Based on Autofluorescence on a Microfluidic Platform." *Journal of Chromatography A* 1181.1-2 (2008): 153-58. Print.
- Betsy, Dr. Tom and Keogh, Jim. 2005. *Microbiology Demystified* . McGraw-Hill Publishing. Print.
- Brady, Rebecca A, Calhoun, Jason H, Leid, Jeff G, & Shirtliff, Mark E. "Infections of Orthopaedic Implants and Devices." Ed. Shirtliff Me & Leid Jg. *SciencesNew York* (2008) : 15-55.
- Characklis, William G., and Kevin C. Marshall. *Biofilms*. New York: Wiley, 1990. Print.
- Chmielewski, R. and Frank, J. (2003), Biofilm Formation and Control in Food Processing Facilities. *Comprehensive Reviews in Food Science and Food Safety*, 2: 22–32. \
- Costerton, J. W. 2007. *The Biofilm Primer*. Springer, in press.
- Davies, D. G., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costerton, J. W. and Greenberg, E. P. (1998). The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science*, 280, 295–298.
- deKievit T R, Gillis R, Marx S, Brown C, & Iglewski B.H.(2001).Quorum-sensing genes in *Pseudomonas aeruginosa* biofilms: their role and expression patterns. *Applied and EnvironmentalMicrobiology*, 67, 1865–1873.
- Derjaguin BV. Theory of the heterocoagulation, interaction and adhesion of dissimilar particles in solutions of electrolytes . *Discuss Faraday Soc* 18 : (1955) 85–8.
- Donlan, Rodney M. "Biofilm Formation: A Clinically Relevant Microbiological Process." *Clinical Infectious Diseases* 33.8 (2001): 1387-392. Print.
- Donlan, R.M. Biofilms: Microbial life on surfaces. *Emerging Infect. Dis.*(2002), 8, 881-890.
- Eberl H, Morgenroth E, Noguera DR, Picioreanu C, Rittman B, van Loosdrecht, MCM . Mathematical modeling of biofilms. *Scientific and Technical Report No. 18*. IWA Publishing, 2006.
- Engebrecht J, Nealson K, Silverman M. 1983. Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri*. *Cell* 32:773–81
- Fey, Paul D., and Michael E. Olson. "Current Concepts in Biofilm Formation of." *Future Microbiology* 5.6 (2010): 917-33. Print.
- Higgins, M.J., and Novak, J.T. Characterization of exocellular protein and its role in bioflocculation. *Journal of Environmental Engineering*, 123 (1997), pp. 479–485

- Howell J. A. & Atkinson B. (1976) Sloughing of microbial film in trickling filters. *Water Res.* 10, 307-315
- Ikada Y , Suzuki M , Tamada Y (1984) Polymer surfaces possessing minimal interaction with blood components . In: Shalaby SW , Hoffman AS , Ratner BD , Horbett TA (eds.) *Polymers as biomaterials*. Plenum , New York .
- Kumar CG, Anand SK. 1998. Significance of microbial biofilms in food industry: a review. *Int. J Food Microbiol* 42:9-27.
- Leifer, Sara M. *Design and Optimization of a Blood Vessel Mimic Bioreactor System for the Evaluation of Intravascular Devices In Simple and Complex Vessel Geometries*. Thesis. California Polytechnic State University San Luis Obispo, 2008. Print.
- Lewandowski, Z, and Beyenal H. "Mechanisms of Microbially Influenced Corrosion." Ed. H C Flemming et al. *Corrosion* 1 (2008) : 35-64.
- Mayer, C., Moritz, R., Kirschner, C., Borchard, W., Maibaum, R., Wingender, J. & Flemming, H. C. The role of intermolecular interactions : studies on model systems for bacterial bio@lms. (1999).*Int J Biol Macromol* 26, 3-16.
- Mermel, L. A. (2000). Prevention of intravascular catheter-related infections. *Annals of Internal Medicine*, 132, 391–402
- Miller, Melissa B., and Bassler, Bonnie L. "Quorum Sensing in Bacteria." *Annual Review of Microbiology* 55 (2001): 165-99. Print.
- Molin, S, Haagensen, JAJ, Barken, KB & Sternberg, C. 2000, 'Microbial communities: aggregates of individuals or coordinated systems', *Community structure and co-operation in biofilms* (Eds.: D. G. Allison) Cambridge University Press, Cambridge, pp. 199-214.
- Murphy, Anthony B., Manabu Tanaka, Kentaro Yamamoto, Shinichi Tashiro, John J. Lowke, and Kostya Ostrikov. "Modelling of Arc Welding: The Importance of including the Arc Plasma in the Computational Domain." *Vacuum* (2010): n. pag. Print.
- NIST/SEMATECH e-Handbook of Statistical Methods, <http://www.itl.nist.gov/div898/handbook>, June 12, 2012.
- Or, D., Phutane, S. & Dechesne, A. Extracellular polymeric substances affecting pore-scale hydrologic conditions for bacterial activity in unsaturated soils. *Vadose Zone J.* 6, 298–305 (2007).
- Palmer, R.J., Jr. (1999). Microscopy flowcells: Perfusion chambers for real-time study of biofilms. *Methods in Enzymology* 310, 160-166
- Rendueles, O. and Ghigo, J.-M. (2012), Multi-species biofilms: how to avoid unfriendly neighbors. *FEMS Microbiology Reviews*. doi: 10.1111/j.1574-6976.2012.00328.x
- Rickard, A. "Bacterial Coaggregation: An Integral Process in the Development of Multi-species Biofilms." *Trends in Microbiology* 11.2 (2003): 94-100. Print.

Romeo, Tony. *Bacterial Biofilms*. Berlin: Springer, 2008. Print.

Sharma, Manvi. In vitro propagation of mixed species biofilms using online consortia for dairy processing lines. National Dairy Research Institute: Milchwissenschaft Vol. 58. 2003

Sreekumari, KR, K Nandakumar, and Y Kikuchi. "Bacterial Attachment to Stainless Steel Welds: Significance of Substratum Microstructure." *GBIOFOULING*, 17.4 (2001): 303-316.

Stepanović, Srdjan, Dragana Vuković, Veronika Hola, Giovanni Di Bonaventura, Slobodanka Djukić, Ivana Ćirković, and Filip Ruzicka. "Quantification of Biofilm in Microtiter Plates: Overview of Testing Conditions and Practical Recommendations for Assessment of Biofilm Production by Staphylococci." *Apmis* 115.8 (2007): 891-99. Print.

Stoodley P, Sauer K, Davies DG, & Costerton JW. Biofilms as complex differentiated communities. *Annu. Rev. Microbiol.* 56,(2002) 187–209.

Stoodley P, Wegel J, Gieske A, deBeer D, & Ohle C. "Biofilm plaque and hydrodynamic effects on mass transfer, fluoride delivery and caries." *Journal of the American Dental Association* 139.9 (2008) : 1182-1190.

Temenoff, J. S., and Mikos, Antonios G. *Biomaterials: the Intersection of Biology and Materials Science*. Upper Saddle River, NJ: Pearson/Prentice Hall, 2008. Print.

Verran J, Jones M. 2000. Problems of biofilm in the food and beverage industry. In: Walker Suramn S J, Jass J, editors. *Industrial Biofouling*. Cichester, N.Y.: John Wiley. p145-73.

Vladkova, T. "Surface Modification Approach to Control Biofouling." *Marine and Industrial Biofouling*. Ed. Hans-Curt Flemming et al. Springer, 2009. 135-163.

Walsh, Daniel W. Class Notes. Materials Engineering 440: Welding Metallurgy. 5/10/2012.

Wicken, A. J. (1984). Bacterial cell walls and surfaces. In 'Bacterial Adhesion: Mechanisms and Physiological Significance'. (Eds M. Fletcher and D. C. Savage.) (Plenum Publishing Corporation: New York.) Print.

Williams, Larry J. *Microbiologically Influenced Corrosion of Stainless Steel Weldments*. Thesis. California Polytechnic State University San Luis Obispo, 1991. Print.

Wilson, Michael, and Devine, Deirdre. *Medical Implications of Biofilms*. Cambridge, UK: Cambridge UP, 2003. Print.

Wimpenny J, Manz W, Szewzyk U. 2000. Heterogeneity in biofilms. *FEMS Microbiol Rev* 24:661-71

Wingender J, Neu TR, Flemming H-C, editors. *Microbial extracellular polymeric substances*. Berlin: Springer, 1999.

APPENDIX A

Solidification Theory

Solidification Theory is used in welding metallurgy to describe and predict how a molten weld pool will solidify from the liquidus phase to the solidus phase. In order for this to happen, the latent heat of fusion must be dissipated from the weld pool into the surroundings. As the material cools from the liquidus phase, the solid begins to form on a preexisting substrate crystal structure. The process of depositing the atoms into the solid structure of the crystal is known as Epitaxial Nucleation (which literally means, “Upon the Form”). As atoms accumulate on each crystal, grains begin to form (known as Grain Growth). The direction of this grain growth is highly dependent on the growth of the weld pool, which is controlled by the welding velocity.

Welding velocity (or the speed of the weld pool across the substrate) will produce a specific weld bead shape; high velocities will produce a teardrop-shaped weld pool, while slower velocities will produce an elliptical-shaped weld pool. This is seen in Figure A.1.

Figure A.1- Weld pool shape as a result of welding velocity.

In the images above, V is the velocity of the weld pool and R is the Growth Rate of the weld pool. R describes the speed and direction of the weld pool growth, and is always perpendicular to the edge of the weld pool. The maximum thermal gradient is also perpendicular to the edge of the weld pool, as the weld pool dissipates heat in the normal direction from the edge. R and V are related by the angle between them, ϕ , such that $R = V \cos(\phi)$.

Epitaxial Nucleation will follow the direction of the weld pool as it cools, and grains will begin to develop as the weld pool moves along. However, the grains do not all grow at the exact same direction or rate. The grains whose $\langle 100 \rangle$ direction align closest to the maximum thermal gradient (normal to the weld pool) will persist by “cutting off” the other grains. This is known as Competitive Growth. In Figure A.2, the grain whose $\langle 100 \rangle$ matches up closest to the maximum thermal gradient cuts off the other grains around it.

Figure A.2- Competitive Growth between grains based on the $\langle 100 \rangle$ direction.

Competitive Growth has important implications on the resulting microstructure that the weld leaves behind. Grain growth is related to the maximum thermal gradient, which is related to the normal

direction of the weld pool, which changes with respect to the weld pool shape. So by controlling the weld pool shape (by changing the velocity of the weld pool), the resulting grain structure can be manipulated. Figure A.3 is a diagram that relates the resulting grain structure to the shape of the weld pool (left) and images from polished, etched welded samples (right).

Figure A.3- Grain Structure resulting from weld pool shape.

Grain boundaries are typically areas of stress concentrations and are also susceptible to corrosion. If a crack were to develop along the grain boundaries, it would be very easy for it to propagate along the center of the “columnar” grain structure that is left by the high speed, teardrop weld pool, whereas the “circular” grain structure of the slower, elliptical weld pool would be much more difficult to fracture. This is why Solidification Theory (Epitaxial Nucleation and Competitive Grain Growth) is important in Welding Metallurgy.

The above information was adapted from:

Walsh, Daniel W. Class Notes. Materials Engineering 440: Welding Metallurgy. 5/10/2012.

Kou, Sindo. *Welding Metallurgy*. Hoboken, NJ: Wiley-Interscience, 2003. Print.

APPENDIX B

Heat Affected Zone (HAZ)

As the weld pool solidifies the latent heat of fusion is dissipated, heating up the surrounding metal. As the metal is heated, very interesting changes occur in the microstructure near the weld pool. This area is known as the Heat Affected Zone (HAZ). Regions closer to the weld pool will reach higher temperatures, which decrease farther away from the weld pool; the region that does not see an increase in temperature is labeled the “Unaffected Base Metal.” In a low alloy steel material, there are nine regions in the heat affected zone:

1. Composite Zone
2. Unmixed Zone
3. Partially Melted Region (also known as the Partially Melted Zone- “PMZ”)
4. Grain Coarsened Region
5. Grain Refined Region
6. Partially Refined Region
7. Spheroidized Region
8. Strain Aged Region
9. Unaffected Base Metal

Figure B.1 relates each of the above regions to the corresponding location on a phase diagram for a low alloy steel.

Figure B.1- The nine HAZ regions of a low alloy steel in relation to the corresponding phase diagram.

Each region produces a very different microstructure of the grains, which directly affects the resulting material properties. Each of these areas have individual material properties associated with them. Every material will undergo changes in the region near the weld pool; the HAZ is not limited to low alloy steel. For example, Figure B.2 shows the heat affected zone in an annealed brass material. This figure offers a pictorial representation of the grain structure in each region, the temperature associated with each region, and the resulting Tensile Strength and Hardness. Though this image is for annealed brass, it is representative of the behavior of most metals.

Figure B.2- HAZ in an annealed brass material.

Moving from right to left in Figure B.2, the first region is the Unaffected Base Metal which naturally has small grains and a tensile strength and hardness that is characteristic of the metal type (whatever properties the metal started out with). The next region in the image is the Grain Coarsened Region; the increase in temperature increases the grain size, which decreases the strength and hardness. At the tail of this region is the Partially Melted Zone. In this region, some of the material is melted due to the high temperatures. The liquid will wick the surface of the grains, pinning the grain size and preventing further grain growth. This slightly increases the strength due to the wicked grain boundaries. At the edge of this region exists the Fusion Zone (also referred to as the Welded Region or the Composite Zone). This is where Epitaxial Nucleation and Competitive Growth occur. There is a sharp increase in the strength and hardness in this area because there are subgrain boundaries developing inside the grains, which block dislocation motion.

The Heat Affected Zone is very complicated and has significant impacts on the resulting microstructure and material properties of the metal. The changes in temperature alter the microstructure by affecting the grains in the material. These changes are on the same scale as bacterial cells, and thus are believed to have significant impacts on microbial attachment. The process of welding redistributes material and concentrates solutes at the edges of grain boundaries. This change in chemistry at the grain boundaries is also believed to have an effect on the behavior and attachment of microbes to the material.

The above information was adapted from:

Walsh, Daniel W. Class Notes. Materials Engineering 440: Welding Metallurgy. 5/10/2012.

Kou, Sindo. *Welding Metallurgy*. Hoboken, NJ: Wiley-Interscience, 2003. Print.

APPENDIX C

Specimen Mounting Procedure

1. Clean specimens to remove cutting and handling residues.
2. Remove debris from the mold assembly.
3. Apply a thin coat of mold release compound to the mold assembly.
4. Raise mold ram to up into position.
5. Center the specimen on the ram.
6. Lower the ram assembly approximately 3 inches.
7. Pour a predetermined amount of resin into mold.
8. Clean and remove any excess resin from around the mold assembly threads.
9. Lock the mold assembly cover.
10. Slowly raise the ram into the up position.
11. Apply and maintain the recommended heat and pressure for the specified period of time,
approximately 10-15 minutes.
12. Apply the cooling cover and let sit for 2-3 minutes.
13. Remove the mounted specimen and let cool to room temperature before handling.
14. Clean the mold and ram assembly.

APPENDIX D

Microbial Fixing Procedure*

Ensure proper ventilation in the fixing area, a Fume Hood is recommended. Ensure proper lab safety equipment is worn (closed toed shoes, long pants, latex or polyethylene gloves, and protective eyewear).

1. Prepare all solutions. Ensure that all solutions are pure and that all glassware is clean
 - a. 3.7 % Formaldehyde 200 mL
 - b. Pure Ethanol 200 mL
 - c. Pure Acetone 200 mL
2. Use tongs to remove the exposed samples from the static bioreactor. Do not touch the surface that is to be examined
 - a. Remove and place one sample at a time in order to ensure accuracy
3. Place the sample in the 3.7% Formaldehyde solution for approximately 3 minutes.
4. Remove the sample from the Formaldehyde and place it in the Pure Ethanol for another 3 minutes.
5. Remove the sample from the Ethanol and place it in the Pure Acetone for a final 3 minutes.
6. Remove the sample from the Acetone and place it on a clean, covered surface to prevent contamination (such as a cleaned Tupperware container).

The sample is now ready for analysis.

*Procedures adapted from Williams 1998

APPENDIX E

Sputter Coating Procedure*

Sputter coating the samples permits viewing the microbes with the scanning electron microscope. Prior to coating the samples should be fixed according to the fixing procedure. Hand contact with the samples should be minimized, and personal protective equipment such as latex or polyethylene gloves should be worn. The samples should be gripped only on the edges of the Bakelite.

1. Place the sample in the vacuum chamber.
 - a. Ensure that the sealing gaskets are clean prior to replacement of the chamber
2. Replace the vacuum chamber and check that the vent valve is shut.
3. Turn the power switch on and draw a vacuum of 10^{-1} mbar.
4. Open the leak valve and allow the vacuum to stabilize at 2 mbar.
5. Use the test switch to check the current. The value should stabilize at 15-18 mA. If not, adjust the leak valve to the proper setting.
6. Set the timer switch to the desired setting, usually about 30 to 45 seconds for a mounted specimen.
7. Press the start switch and wait for the time to elapse.
8. Break the vacuum with the vent valve and remove the sample from the vacuum chamber, using clean polyethylene gloves.
9. Return the chamber to the proper position and turn off the power switch.

*Procedures adapted from Williams 1998

APPENDIX F

List of Materials Used

The following is a list of materials that were used in the study.

1. Samples
 - a. 303 Stainless Steel
 - b. 304 Stainless Steel
2. Bakelite
3. Flask/ 500 ml beaker
4. Biologically Active Natural Water
5. Rubber Stopper/ Parafilm/ Glassware lid
6. Sanitizer 70% IPA
7. Fixing Chemicals
 - a. 3.7% Formaldehyde
 - b. Pure Ethanol
 - c. Pure Acetone
8. Personal Protective Equipment
 - a. Tongs (to lift samples from media/ chemicals)
 - b. Lab Glasses/ Goggles
 - c. Latex/ Polyethylene Gloves
9. Machines
 - a. Horizontal Band Saw
 - b. Bakelite Mounting Press
 - c. Polishing Wheels and Sanding Stations
 - d. Scanning Electron Microscope
 - e. Optical Light Microscope