D-Tryptophan as a Biocide Enhancer for *Desulfovibrio vulgaris* Biofilm Mitigation and Biocorrosion of Carbon Steel by Nitrate-Reducing *Pseudomonas aeruginosa*

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This thesis titled
D-Tryptophan as a Biocide Enhancer for *Desulfovibrio vulgaris* Biofilm Mitigation and
Biocorrosion of Carbon Steel by Nitrate-Reducing *Pseudomonas aeruginosa*

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ABSTRACT

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D-Tryptophan as a Biocide Enhancer for *Desulfovibrio vulgaris* Biofilm Mitigation and Biocorrosion of Carbon Steel by Nitrate-Reducing *Pseudomonas aeruginosa*

Director of Thesis: Tingyue Gu

There have been many reported failures due to biocorrosion, also known as microbiologically influenced corrosion (MIC), throughout the years. Additional research is needed in this area focusing on mechanisms and mitigation. Treatments for MIC rely primarily on biocides and pigging. Biocide enhancers are an excellent way to improve upon already existing biocides. D-amino acids as biocide enhancers for tetrakis (hydroxymethyl) phosphonium sulfate (THPS) have shown promise in recent years. In this work, D-tryptophan was combined with THPS at various concentrations to determine its efficacy as a biocide enhancer against sulfate-reducing bacteria (SRB) *Desulfovibrio vulgaris* biofilms. It was determined that D-tryptophan could be an effective biocide enhancer for THPS at high concentrations.

Though not as well studied as SRB, nitrate-reducing bacteria (NRB) can also cause MIC failures. Additional research is needed to further understanding and mechanistic study in this area. Anaerobic experiments were conducted with *Pseudomonas aeruginosa* grown as an NRB. Three different strains were tested under the same conditions to determine if there was any significant difference in their MIC. It was determined that there were no significant differences in the MIC caused by the three strains.
To my parents, Dennis T. and Susan R. Lindenberger.

This work would have never been possible without all their love and support.
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CHAPTER 1: INTRODUCTION

Corrosion is the deterioration of a material (often a metal) due to a reaction with its environment. The reaction is often electrochemical in nature, especially pertaining to metals. This electrochemical process occurs because of a coupled oxidation-reduction (redox) reaction. For a redox reaction to take place, there needs to be both an anodic and a cathodic reaction. When corrosion of a metal takes place, the anodic reaction is oxidized which causes dissolution of the metal. The cathodic reaction is the reduction of whatever environmental factor (an oxidant) is causing the corrosion to occur. These reactions coupled together allow for the transfer of electrons, and thus corrosion, to take place.

A 2002 2-year study estimated annual direct corrosion costs of $276 billion (approximately 3.1% of the Gross Domestic Product (GDP)) in the United States (Koch et al., 2002). This same study also estimated the annual indirect corrosion costs to be equal to that of the direct corrosion costs, for a staggering total estimate of about 6% of the U.S. GDP. From that same study, it was also estimated that direct corrosion costs of $3.7 and $1.4 billion could be attributed to petroleum refining and oil and gas exploration and production, respectively.

There are many different forms of corrosion, with one of particular interest being biocorrosion, also known as microbiologically influenced corrosion (MIC). Of all the damage caused by corrosion of metals and building materials, it is estimated that MIC is responsible for about 20% (Flemming, 1996). MIC is the corrosion of a material caused by the presence of microorganisms. Microorganisms can be responsible for corrosion in
primarily two different ways: the direct utilization of electrons from a metal surface and the corrosion caused by the presence of their metabolites secreted as a byproduct of their metabolism. Only a few layers of microorganisms in close proximity to the metal surface are capable of utilizing electrons directly from the metal surface. The metabolites produced by some microorganisms may reduce the local pH and cause corrosion in the same manner as had the metabolites been present there by any other means. Not all microorganisms are capable of causing MIC and some may only cause it under certain conditions (Gu, 2012a).

Only microorganisms in biofilm may cause MIC. Microorganisms in biofilm, or sessile cells, are communities of cells attached to a surface held together by exopolymeric substances (Costerton, 2007). Some cells in direct contact with a metal surface can utilize electrons directly from the metal surface when there is a limited availability of carbon source (Xu and Gu, 2014). Free floating or planktonic cells do not have the ability to cause MIC because there is no way to transfer electrons into the cell from the metal as electrons cannot “swim” (Zhang et al., 2015). The microorganisms that cause MIC due to their metabolites induce MIC in biofilm when their metabolites create a local pH drop at the metal surface which accelerates the corrosion process (Gu, 2012a, 2012b).

The first mention that microorganisms might be responsible for corrosion was proposed by Garrett in 1891 (Crookes, 1891). Since then, there has been a great deal of supporting evidence for MIC including many reported failures in various industries and a great deal of supporting research. The first mechanism to describe MIC by SRB was not developed until von Wolzogen Kühr and van der Vlugt introduced their classic cathodic
depolarization theory (CDT) (1934). This theory was able to describe MIC on a basis of electrochemistry. CDT has been widely debated by many researchers for many years since. The Biocatalytic Cathodic Sulfate Reduction (BCSR) theory proposed by Gu et al. (2009) was the first theory that could also account for pitting corrosion due to MIC which, due to its complicated nature, had previously been too difficult to model. BCSR theory is a mechanistic model based on electrochemical kinetics and mass transfer.

There have been numerous problems attributed to MIC throughout the years. This was facilitated by the more common practice of secondary oil recovery. Secondary oil recovery uses water injection wells to inject water into an oil reservoir to increase the pressure to force more oil out of the production well. The primary concern with this is that the water used for this process is often whatever water is available, in many cases seawater. This water may contain many different microorganisms which could potentially contribute to corrosion. Additional chemicals may be used to treat certain kinds of bacteria, but some (e.g., nitrate) may actually help other bacteria thrive (Gieg et al., 2011).

The two primary ways to mitigate MIC are biocide treatment and pigging (Gieg et al., 2011). Biocides, in general, are chemicals used to kill or limit the growth of living organisms. Biocides have their limitations as they can cause environmental concerns and high financial costs. This is especially problematic as higher dosages are needed to overcome microbial resistance to biocides (Gieg et al., 2011). Pigging uses “pigs,” devices which are propelled through pipelines by fluid flow, to remove debris and aid in the inspection of the interior of the pipeline (Tiratsoo, 1992). Pigs have their limitations
as well as they cannot move through pipes with various diameters, sharp bends, etc. (Tiratsoo, 2013). Additional research is needed in this area to enhance mitigation efforts.

One area of research to aid in MIC mitigation efforts is biocide enhancers. Biocide enhancers use some other additive(s) to enhance the efficacy of current biocides. One group of biocide enhancers worth particular mention are D-amino acids. This technology is based on research that demonstrated that some D-amino acids can trigger a dispersal signal in the cell wall (Kolodkin-Gal et al., 2010). This, when paired with certain biocides (e.g., THPS, glutaraldehyde), has shown an enhanced mitigation effect especially for a recalcitrant SRB biofilms such as *Desulfovibrio vulgaris* (Xu, Wen, Fu, et al., 2012; Xu, Li, et al., 2012).
CHAPTER 2: LITERATURE REVIEW

2.1 Microbiologically influenced corrosion

The first mention of corrosion that resulted from the presence of microorganisms was proposed by Garrett in 1891. Garrett concluded that the corrosion of lead in water could only be attributed to the presence of metabolites in the water produced by bacteria under anaerobic conditions (Crookes, 1891). This research was particularly important at the time as lead pipes were more commonly used for water pipelines and the dissolution of lead pipes from corrosion could result in lead poisoning.

Another early investigation into corrosion influenced by the presence of microorganisms was described by Gaines (1910). In this study, steel conduits were found corroded due to exposure to unusually high sulfur content in the soil. This high sulfur content was attributed to the presence of bacteria. Gaines mentions that the corrosion of steel pipes in the soil could be caused by the microbes directly, or indirectly when the microbes create acidic conditions (1910). This is one of the first early accounts of corrosion attributed to SRB.

Throughout the years many problems have been reported with MIC in various industries. Some of these major industries include the oil and gas industry, fire protection systems, wastewater treatment facilities, copper potable water systems and heat exchangers, nuclear and hydroelectric power plants, and the pulp and paper industry (Gu et al., 2014). While most of these issues go on unnoticed by the general public, a few incidents have received significant media attention.
A couple of the most widely publicized incidents in the United States occurred in Prudhoe Bay, Alaska (Jacobson, 2007) and Carlsbad, NM (NTSB Report, 2003). In 2006, a serious leak from a transit pipeline at the Prudhoe Bay oil field in Alaska resulted in a loss of 200,000 gallons of crude oil and the eventual shutdown of the pipeline for repairs. The incident initially resulted from a 0.25-in. by 0.5-in. hole that was discovered in the bottom of the pipeline which was later concluded could only be attributed to MIC. This incident resulted in significant financial repercussions (Jacobson, 2007). The other incident was the rupture of a natural gas pipeline near Carlsbad, NM in 2000 which resulted in the death of 12 people camping under the bridge that supported the pipeline and damaged two nearby steel suspension bridges. Damages were estimated around $1 billion (NTSB Report, 2003).

2.2 Biofilms

Biofilms can cause a number of serious problems in industry including MIC and biofouling. MIC is caused by the presence of microorganisms in biofilm on a surface. Biofouling is the buildup of microorganisms in biofilm on a surface, which can cause obstructions of the fluid flow in pipelines, heat exchangers, etc. Biofilms are composed of communities of microorganisms attached to a surface and usually form an organic matrix. Most biofilms in nature are comprised of many different microorganisms that work together to form complex communities. A biofilm comprised of many different microorganisms is known as a consortium.

The cells living in biofilm are known as sessile cells, while free floating cells are known as planktonic cells (Costerton, 2007). Sessile cells are held in place by pili and
exopolymeric substances (EPS), which consists of polysaccharides, proteins, and extracellular DNA. The microorganisms are also able to organize themselves to form tunnels for the movement of nutrients and wastes in and out of the biofilm (Costerton, 2007). Even though the microorganisms can organize themselves in this manner, in biofilm they can still act as a barrier between the metal surface and the surrounding bulk solution. This can cause nutrient and pH gradients. When the microorganisms cannot get the nutrients they need from the bulk, they settle for whatever else is available in their local surroundings. Microorganisms prefer to get their energy from a carbon source because they can produce more energy, but when their carbon source is exhausted they will continue to survive by the utilization of electrons from a metal surface (Gu, 2012b).

The development of a biofilm was described by Stoodley et al. through a series of five stages (2002). The first stage was the initial attachment of the cells to a surface. The second stage was characterized by the adhesion of the cells to the surface due to the production of EPS. The early stages of biofilm architectural development is the third stage. The fourth stage is characterized by the biofilm architectural development achieving maturation. The fifth and final stage is the dispersal of cells from the biofilm back to their planktonic state (Stoodley et al., 2002).

Microorganisms in biofilm are much more resistant to biocides and antibiotics than planktonic cells. Individual bacterial cells naturally have several defense mechanisms that help protect them from antibiotics, including efflux pumps, which pump the antibiotic out of the cell to prevent the accumulation of a lethal dosage in the cell, enzyme modification, which causes antibiotic deactivation, and mutation of the target
structure, which lowers the binding affinity of the antibiotic (Stewart and Costerton, 2001; Walsh, 2000). Biofilms, however, provide additional protection to the sessile cells within them, which cannot be accounted for by the aforementioned defense mechanisms (Stewart and Costerton, 2001). There appears to be no one factor to explain the extra protection afforded to the cells in biofilm, but some possible explanations include a diffusion barrier created by the biofilm matrix, which limits the penetration of an antibiotic into the biofilm, the additional antibiotic resistance exhibited by cells during stages of slow or no growth and the heterogeneity exhibited in cell growth due to variations in a localized environment within the biofilm (Mah and O’Toole, 2001). All of these factors help account for the observed tenacity of microorganisms in biofilm.

2.3 Microorganisms responsible for MIC

There are several groups of microorganisms responsible for MIC including sulfate-reducing bacteria (SRB), nitrate-reducing bacteria (NRB), acid producing bacteria (APB), and methane-producing bacteria (MPB) or methanogens. SRB are anaerobic microorganisms that reduce sulfate to sulfide to obtain energy for their metabolism (Muyzer and Stams, 2008). NRB, as facultative anaerobes, can utilize nitrate as their terminal electron acceptor in place of oxygen under anaerobic conditions for their respiratory metabolism (Meyer, 1994). APB can cause a local pH drop due to their production of large quantities of acids (organic or inorganic) as byproducts of their metabolism (Beech and Gaylarde, 1999). MPB are anaerobic bacteria that obtain their energy through the reduction of carbon dioxide to methane (Barker, 1936).
2.3.1 Sulfate-reducing bacteria

In 1895, *Spirillum desulfuricans*, the first SRB was isolated and described by Beijerinck. Beijerinck described the black colored colonies when grown with iron salts and the ability of the bacteria to reduce sulfates to produce hydrogen sulfide. He was also surprised to discover that they were obligate anaerobes, but this proved useful during isolation. He classified this bacteria as a spirillum, but noted that it was quite different compared with other known spirilla and would likely be renamed (Beijerinck, 1895). This strain is now known as *Desulfovibrio desulfuricans*.

As obligate anaerobes, SRB are prokaryotic microorganisms that can utilize sulfate as the terminal electron acceptor for their energy metabolism by way of dissimilatory sulfate reduction in the absence of molecular oxygen (Thauer et al., 2007). Dissimilatory sulfate reduction is a respiratory process in which SRB reduce sulfate to sulfide to produce compounds necessary for growth and energy (Akagi, 1995).

Many SRB are known to contain hydrogenase enzymes (hydrogenase-positive bacteria). Hydrogenases, located primarily in the periplasm, are responsible for the uptake of hydrogen which acts as an electron donor in the reduction of sulfate (Ljungdahl et al., 2003). It is believed that hydrogenase enzymes in SRB may be responsible for initiating MIC by the removal of cathodic hydrogen from the steel surface (Bryant et al., 1991).

There are five known phylogenetic lineages of SRB which are mesophilic δ-proteobacteria, thermophilic Gram-negative bacteria, Gram-positive bacteria,
Euryarchaeota, and Archaeoglobus. The genera Desulfovibrio is classified as a mesophilic δ-proteobacteria (Thauer et al., 2007).

Despite their classification as strict anaerobes, it was later shown that some SRB could survive in the presence of oxygen (Cypionka, 2000), and in some cases are even capable of aerobic respiration (Dannenberg et al., 1992; Lemos et al., 2001). Many SRB have the ability to reduce oxygen to water (Cypionka, 2000). Dannenburg et al. found that even though some SRB were capable of aerobic respiration, it generally did not support bacterial growth (1992).

It has also been shown that SRB can utilize a variety of alternative terminal electron acceptors for anaerobic respiration. In addition to sulfate, some SRB have been shown to use elemental sulfur, nitrate, fumarate, dimethylsulfoxide, manganese(IV) and iron(III) (Thauer et al., 2007) and even uranium(VI) (Lovley and Phillips, 1992) as terminal electron acceptors.

The ability of some SRB to utilize nitrate as a terminal electron acceptor is of particular note especially in the case in which nitrate is used as an inhibitor for reservoir souring caused by SRB (Gieg et al., 2011). It has been shown that some SRB are capable of dissimilatory nitrate or nitrite reduction to ammonia (Widdel and Pfennig, 1982). This reduction process is known as nitrate or nitrite ammonification (Dalsgaard and Bak, 1994; Seitz and Cypionka, 1986). Seitz and Cypionka found that a higher growth rate could be observed for some SRB utilizing nitrate over sulfate as their terminal electron acceptor (1986). In other instances, it was found that the nitrate ammonification process was hindered by the presence of sulfate or sulfide (Dalsgaard and Bak, 1994).
2.3.2 Nitrate-reducing bacteria

Nitrate-reducing bacteria (NRB) are a group of facultative anaerobes that, though they prefer oxygen, in its absence, can utilize nitrate as their terminal electron acceptor for respiration (Meyer, 1994). NRB can reduce nitrate by either denitrification or dissimilatory nitrate reduction to ammonium (DNRA). In denitrification, nitrate or nitrite is reduced to nitric oxide or nitrous oxide which is then eventually reduced to nitrogen gas (N\textsubscript{2}). The main enzymes that catalyze this process are nitrate reductase, nitrite reductase and nitrous oxide reductase (Meyer, 1994). In DNRA, the nitrate or nitrite is reduced to ammonium (NH\textsubscript{4}+) (Su et al., 2012).

2.4 Mechanisms and classifications of MIC

2.4.1 Cathodic depolarization theory

The first classic theory to describe MIC by SRB was introduced by von Wolzogen Kühr and van der Vlugt in 1934. In their work they describe the biocorrosion process by sulfate reducers as a bioelectrochemical process in which the sulfate reduction acts as a depolarizer and the iron is dissolved into solution as the anode (von Wolzogen Kühr and van der Vlugt, 1934). This theory became known as the cathodic depolarization theory (CDT). The following reactions were used by Gu and Xu (2010) to describe CDT:

Anodic reaction (iron dissolution): \[4\text{Fe} \rightarrow 4\text{Fe}^{2+} + 8\text{e}^-\] (2.1)

Dissociation of water: \[8\text{H}_2\text{O} \rightarrow 8\text{H}^+ + 8\text{OH}^-\] (2.2)

Cathodic reaction: \[8\text{H}^+ + 8\text{e}^- \rightarrow 8\text{H}_{\text{ads}}\] (2.3)

Cathodic depolarization of hydrogenase: \[8\text{H}_{\text{ads}} (\rightarrow 4\text{H}_2) \rightarrow 8\text{H}^+ + 8\text{e}^-\] (2.4)

Sulfate reduction by SRB: \[\text{SO}_4^{2-} + 8\text{H}^+ + 8\text{e}^- \rightarrow \text{S}^{2-} + 4\text{H}_2\text{O}\] (2.5)
Corrosion product formation: \[ \text{Fe}^{2+} + \text{S}^{2-} \rightarrow \text{FeS} \] (2.6)

Corrosion product formation: \[ 3\text{Fe}^{2+} + 6\text{OH}^- \rightarrow 3\text{Fe(OH)}_2 \] (2.7)

Overall reaction: \[ 4\text{Fe} + \text{SO}_4^{2-} + 4\text{H}_2\text{O} \rightarrow 3\text{Fe(OH)}_2 + \text{FeS} + 2\text{OH}^- \] (2.8)

“Cathodic polarization” occurs from the cathodic reaction in which hydrogen atoms produced in Reaction 2.3 adsorb onto the cathode. The corrosion process cannot continue until the adsorbed hydrogen (\( \text{H}_{\text{ads}} \)) is desorbed from the cathode by one or both of the following rate-limiting reactions:

Chemical desorption: \[ 2\text{H}_{\text{ads}} \rightarrow \text{H}_2 \] (2.9)

Electrochemical desorption: \[ \text{H}_{\text{ads}} + \text{H}^+ + \text{e}^- \rightarrow \text{H}_2 \] (2.10)

The cathodic depolarization (Reaction 2.4) occurs when the hydrogenase enzyme produced by the SRB catalyzes the conversion of \( \text{H}_{\text{ads}} \) to \( \text{H}_2 \) and then to \( \text{H}^+ \) by reducing the activation energy needed for the reaction to proceed (Ljungdahl et al., 2003). It is also believed at neutral pH the sulfide would primarily be in the form of \( \text{HS}^- \) (Hardy, 1983).

Since the introduction of the classic CDT theory in 1934, many researchers have set out to validate it. While many early researchers agreed with the CDT (Enning and Garrelfs, 2014), even some of them did not believe it could account for all the observed bacterial corrosion (Starkey, 1947). Research conducted by Spruit and Wanklyn did not observe any significant cathodic depolarization and the removal of hydrogen by the bacteria could not account for the observed sulfate reduction (1951; Wanklyn and Spruit, 1952). Booth and Tiller conducted a series of experiments with several different strains of SRB to determine the effect of hydrogenase activity in relation to the CDT. Their results were supportive of CDT but only for hydrogenase positive SRB (Booth and Tiller, 1962,
1960; Tiller and Booth, 1962). Iverson also found results supportive of CDT with the use of benzyl viologen as an alternative electron acceptor to eliminate the production of hydrogen sulfide, but once again it was noted that it could not account for all MIC observed in nature (Iverson, 1966). Research conducted by Hardy supported CDT, but the observed cathodic polarization was transitory. He concluded that while cathodic depolarization occurs, it could not be considered the dominant mechanism (Hardy, 1983).

2.4.2 Additional MIC theories

Since the initial introduction and many validation attempts of CDT, several additional theories have since been proposed. Some of these theories include King’s Mechanism (Iron Sulfides), a Volatile Phosphorus Compound, Anodic Depolarization, Fe-Binding Exopolymers, Sulfide and Hydrogen-Induced Stress Corrosion Cracking (SCC), Sulfides, and Romero Mechanism (Three Stages Mechanism) (Beech and Gaylarde, 1999; Kakooei et al., 2012). These theories attempt to account for the shortcomings and gaps in CDT and subsequent validation attempts.

King’s Mechanism proposed that the iron sulfide that forms on the metal surface, resulting from the bacterially produced sulfide ions reacting with the ferrous ions, is actually where the cathodic reaction takes place. The iron sulfide initially forms a protective layer on the iron surface but its subsequent breakdown resulted in high corrosion rates. The high observed corrosion rates were observed and no further sulfide layers were formed as the result of the galvanic cell that formed between the iron and the iron sulfide that was initiated by the formation of a sufficient iron sulfide layer (King and Miller, 1971).
Iverson proposed a theory suggesting that the observed corrosion was due to the production of a highly reactive phosphorus compound by the SRB as part of the phosphorus cycle and it was this compound that was responsible for reacting with iron to form iron phosphide (Fe₃P) and thus causing the corrosion of the metal (Iverson, 2001; Iverson and Olson, 1983).

Anodic depolarization was another theory that was discussed by several researchers (Crolet, 1992; Obuekwe et al., 1981; Wanklyn and Spruit, 1952). As described by researchers Wang and Liang, the anodic depolarization of sulfide causes an acceleration of the anodic dissolution of iron by SRB. The S²⁻ that is a metabolite produced by the SRB will react with the ferrous ions from the steel to produce iron sulfide which will further increase the corrosion of the metal (Wang and Liang, 2007).

An EPS mediated mechanism of metal accumulation is based on the ability of EPS to complex with metal ions. The EPS produced by SRB can bind with metal ions, including iron, which accelerates the corrosion process. The presence of EPS can lead to localized corrosion due to creation of a localized chemical gradient (Beech, 2004; Beech and Cheung, 1995).

The presence of SRB can lead to environments highly favorable to cause hydrogen embrittlement and corrosion-fatigue cracking in metals due to their production of hydrogen sulfide. Hydrogen embrittlement is the result of the permeation of hydrogen atoms into the lattice structure of a metal which can cause corrosion-fatigue cracking and lead to premature failure (Edyvean et al., 1997).
Little et al. investigated the effects of biomineralization on MIC. The dissolution of the biomineral layer could lead to an increase in the corrosion. It was also demonstrated that the sulfides that formed on the metal surface due to the presence of the bacteria would accelerate the corrosion rate by shifting the corrosion potential in a more negative direction (Little et al., 1998).

The Romero Mechanism, or Three Stages Mechanism, is characterized by three separate stages. The first stage involved the adsorption of the bacterial cells and iron sulfide to the metal surface where a low, more general corrosion was observed. The second stage was characterized by the formation of a more dense iron sulfide film along with EPS which provided further protection of the metal surface from general corrosion, but the start of microbial corrosion was observed. The third and final stage involved a more severe and localized microbial corrosion attack of the metal due to a localized pH drop caused by the microbial corrosion of the steel in the presence of HS⁻ (de Romero, 2005).

2.4.3 Biocatalytic Cathodic Sulfate Reduction theory

More recently, a new theory called the Biocatalytic Cathodic Sulfate Reduction (BCSR) theory was proposed by Gu et al. (2009). This theory introduced a new mechanistic model based on electrochemical kinetics and mass transfer to describe MIC by SRB. Initially, SRB form a dense biofilm on the steel surface. This dense biofilm may eventually cause a mass transfer barrier to form limiting the availability of a carbon source to the cells, particularly those closest to the steel surface. The SRB must turn to some other alternative energy source for survival. In the case of SRB biofilm on steel, the
bacteria turn to steel as an alternative source of electrons for maintenance energy. Elemental iron is oxidized outside the cell and electrons are brought into the cytoplasm of the cell via the electron-transport chain. Once in the cell, sulfate reduction takes place in the cytoplasm via the adenosine phosphosulphate (adenylylsulphate) (APS) pathway,

\[
\text{SO}_4^{2-} \rightarrow \text{APS} \rightarrow \text{HSO}_3^- \text{ (bisulfate)} \rightarrow \text{HS}^- \text{ (bisulfide)}
\]  
(2.11)

The path from sulfate to bisulfide is catalyzed by the enzymes ATP sulfurylase, APS reductase and bisulfate reductase (Ljungdahl et al., 2003; Thauer et al., 2007).

The BCSR theory, which is based on biocatalysis and bioenergetics, can be represented by the following reactions (Fu, 2013; Xu, Li, Song, et al., 2013):

Anodic: \(4\text{Fe} \rightarrow 4\text{Fe}^{2+} + 8\text{e}^- \text{ (Iron oxidation)} \) \hspace{1cm} (2.12a)

\[
E_{(\text{Fe}^{2+/\text{Fe}^0})} = -0.447 + \frac{RT}{2F} \cdot \ln[\text{Fe}^{2+}] \text{ (vs. SHE)}
\]  
(2.12b)

Cathodic: \(\text{SO}_4^{2-} + 9\text{H}^+ + 8\text{e}^- \rightarrow \text{HS}^- + 4\text{H}_2\text{O} \text{ (BCSR)} \) \hspace{1cm} (2.13a)

\[
E_{(\text{SO}_4^{2-/\text{HS}^-})} = 0.252 - \frac{2.591RT}{F} \cdot \ln\left[\frac{\text{SO}_4^{2-}}{\text{HS}^-}\right] + \frac{RT}{8F} \cdot \ln\left[\frac{[\text{SO}_4^{2-}]}{[\text{HS}^-]}\right] \text{ (vs. SHE)}
\]  
(2.13b)

The anodic reaction (Reaction 2.12a) represents the dissolution of iron, while the cathodic reaction (Reaction 2.13a) represents the reduction of sulfate by biocatalysis. The two reactions are paired to form a redox couple catalyzed within an SRB cell. In the case of an available organic carbon source the following anodic reaction represented by the oxidation of lactate would be coupled with sulfate reduction in Reaction 2.13a for anaerobic respiration:

Anodic: \(\text{CH}_3\text{CHOHCOO}^- + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + \text{CO}_2 + 4\text{H}^+ + 4\text{e}^- \) \hspace{1cm} (2.14a)
The equilibrium potential \( E_e \) can be calculated in volts by the Nernst equations represented in (2.12b), (2.13b) and (2.14b) which use the Standard Hydrogen Electrode (SHE) as a reference. The Nernst equations use \( R \) as the universal gas constant, \( F \) as the Faraday constant, \( p_{CO_2} \) as the partial pressure of \( CO_2 \), and \( T \) as the temperature. The equilibrium potential can be used to calculate the redox potential for each half reaction, which can be used to calculate the change in Gibbs free energy by the following reaction:

\[
\Delta G = -nF \Delta E_{\text{cell}}
\]

(2.15)

The \( n \) is the number of electrons, \( F \) is the Faraday constant and \( E_{\text{cell}} \) is the cell potential at 1M solutes, 1 bar gases, pH 7.0 and 25°C. The change in Gibbs free energy will determine if a reaction is thermodynamically favorable and thus proceed forward. A thermodynamically favorable reaction is indicated by a negative change in Gibbs free energy which is the result of a positive change in cell potential.

Under standard conditions of pH 7.0, 25°C, 1M concentrations of sulfate and HS\(^-\), the redox potential \( (E^{o'}) \) for the sulfate/HS\(^-\) couple is -214 mV (vs. SHE), but would be closer to -200 mV under physiological conditions due to lower actual concentration values of sulfate and HS\(^-\). The redox potential for the Fe\(^{2+}\)/Fe\(^0\) couple is -447 mV (vs. SHE), which in the dissolution of iron would be +447 mV. The combination of these two redox reactions together would result in a cell potential of \( \Delta E^{o'}_{\text{cell}} = +233 \) mV, which would correspond to a change in Gibbs free energy \( \Delta G^{o'} = -180 \) kJ/mol as \( n \) equals 8, indicating a thermodynamically favorable reaction. This reaction only proceeds due to
biocatalysis by SRB and cannot proceed without it due to a high activation energy (Thauer et al., 2007; Xu, Li, Song, et al., 2013; Xu and Gu, 2011).

When organic carbon is available, in this case represented by lactate, the redox potential for the CO₂ + acetate/lactate couple is -430 mV (vs. SHE), which would be +430 mV for the oxidation of lactate. This redox couple is only slightly less negative than that of the oxidation of iron, which would actually make it slightly more thermodynamically favorable than lactate. The SRB would actually choose the lactate over the iron as lactate provides a source of organic carbon for growth and energy, while iron only provides electrons for maintenance energy (Thauer et al., 2007; Xu, Li, Song, et al., 2013; Xu and Gu, 2011).

2.4.4 Biocatalytic Cathodic Nitrate Reduction theory

The BCSR theory can also be used analogously for NRB and called the Biocatalytic Cathodic Nitrate Reduction (BCNR) theory (Xu, Li, Song, et al., 2013). In the case of NRB, nitrate (or nitrite) can be reduced to either nitrogen gas (N₂) by denitrification (Ghafari et al., 2008) or to ammonium (NH₄⁺) by dissimilatory nitrate reduction to ammonium (DNRA) (Su et al., 2012). Either of the following cathodic reactions which could be paired with the anodic dissolution of iron in Reaction 2.12a (Fu, 2013; Xu, Li, Song, et al., 2013):

Cathodic: 2NO₃⁻ + 10e⁻ + 12H⁺ → N₂ + 6H₂O  \hspace{1cm} (2.16a)

\[
E_{(2NO_3^-/N_2)} = 1.246 - \frac{2.764RT}{F} \cdot \text{pH} + \frac{RT}{10F} \cdot \ln \left( \frac{[\text{NO}_3^-]^2}{p_{N_2}} \right) \hspace{1cm} \text{(vs. SHE)} \hspace{1cm} (2.16b)
\]

Cathodic: NO₃⁻ + 8e⁻ + 10H⁺ → NH₄⁺ + 3H₂O  \hspace{1cm} (2.17a)
\[ E_{(\text{NO}_3/\text{NH}_4^+)} = 0.875 - \frac{2.879RT}{F} \text{pH} + \frac{RT}{8F} \cdot \ln \frac{[\text{NO}_3^-]}{[\text{NH}_4^+]} \text{ (vs. SHE)} \]  

(2.17b)

The partial pressure of \( N_2 \) (bar) is represented by \( p_{N_2} \) in (2.16b). The redox potential for the \( 2\text{NO}_3^-/N_2 \) couple is +749 mV (vs. SHE) and for the \( \text{NO}_3^-/\text{NH}_4^+ \) couple is +358 mV (vs. SHE). The oxidation of iron paired with the reduction of nitrate to nitrogen gas (denitrification) would yield a cell potential \( E_{\text{cell}}^{\circ} = +1196 \text{ mV} \), which would result in a change of Gibbs free energy \( \Delta G_{\text{cell}}^{\circ} = -577 \text{ kJ/mol} \) with \( n \) equal to 10. The oxidation of iron paired with the reduction of nitrate to ammonium (DNRA) would yield a cell potential \( E_{\text{cell}}^{\circ} = +805 \text{ mV} \), which would result in a change of Gibbs free energy \( \Delta G_{\text{cell}}^{\circ} = -621 \text{ kJ/mol} \) with \( n \) equal to 8. When paired with iron oxidation, both reactions result in very negative changes in Gibbs free energy indicating that both reactions are highly thermodynamically favorable (Thauer et al., 2007; Xu, Li, Song, et al., 2013).

2.4.5 Classifications of MIC

Three primary classifications of MIC based on their mechanisms were proposed by Gu (2012a). Anaerobic microorganisms that are electrogenic or use hydrogen (\( H_2 \)) as an electron carrier can use non-noble metals as an electron donor for the reduction of an oxidant by biocatalysis for their respiration metabolism are classified as Type I MIC. Type I MIC follows BCSR theory but in a more general sense in which the oxidant could instead be nitrate/nitrite or \( \text{CO}_2 \), for example, and the microorganisms could include more than just bacteria. Gu further states that Type I MIC could be divided into two distinct categories based off microbial fuel cell research: direct electron transport (DET) and mediated electron transport (MET) (2012a). In DET, electrons are transported by the
direct contact of electron transport proteins or pili with a conductive surface. MET utilizes redox active electron mediators to transfer the electrons from the conductive surface into the cytoplasm. These electron mediators are known as electron shuttles. Type II MIC is caused by the extracellular oxidation of a metal surface caused by corrosive metabolites (oxidants) secreted by anaerobic microorganisms through fermentation. This type of MIC is often due to the presence of large amounts of organic acids. Type III MIC, also known as biodegradation, is a non-electrochemical corrosion in which microorganisms use extracellular organic substances (e.g., polyurethane) as an organic carbon source for energy.

2.5 Mitigation of MIC

2.5.1 Current methods

The methods predominately used in the mitigation of MIC in industry are chemical and mechanical methods (Gieg et al., 2011). The primary choice in chemical methods is the use of biocides. Biocides are chemicals used to kill or suppress the growth of living organisms and can act by several different mechanisms depending on the biocide (Bartlett and Kramer, 2011). Two popular choices in biocides are tetrakis (hydroxymethyl) phosphonium sulfate (THPS) and glutaraldehyde. THPS is a fast acting biocide that is a quaternary phosphonium salt which works by damaging the cell membrane (Downward et al., 1997; Zhao et al., 2009). THPS is biodegradable and less environmentally hazardous than many other biocides. This biocide is particularly favorable due to its efficacy and cost (Downward et al., 1997). Glutaraldehyde is an
aldehyde-based electrophilic biocide and works by cross-linking proteins (Bartlett and Kramer, 2011).

The use of biocides is not without its limitations. Biocides are not as effective on sessile cells and require repeated application to maintain efficacy. Frequent use of biocides can also lead to the development of resistance and as a result higher dosages are necessary to remain effective. Biocides are also toxic and can cause serious environmental concerns. All of this, along with the significant financial costs, can lead to serious problems that accompany the use of biocides (Gieg et al., 2011).

The primary choice for mechanical methods of MIC mitigation in pipelines is pigging. “Pigs” are devices propelled through the pipeline by the moving fluid in the pipeline. They were originally named for the squealing noise that resulted from the rakes attached to the pig scraping against the interior walls of the pipeline. Among its several uses, pigging can be used for the removal of buildup from the pipe walls, detecting corrosion and pitting, and corrosion inhibition (Tiratsoo, 1992).

The use of pigging, however, also has its limitations in many pipelines. There are many “unpiggable” pipelines which are classified as such due to the design of the pipelines which make pigging difficult, if not impossible. Some of these difficulties include small diameters, multi-diameters, tight bends, low flow, and pipeline accessibility. While advancements have been made to solve many of these problems, more improvements are needed (Tiratsoo, 2013).
2.5.2 Biocide enhancers

One area that shows promise is the idea of biocide enhancers. Biocide enhancers are additives that work synergistically with already existing biocides to increase the mitigation effects of biocides. Raad et al. (2003) found that combining the chelator ethylenediaminetetraacetic acid (EDTA) with a low dose of the antimicrobial monocycline worked better as a biofilm inhibitor on central venous catheters (CVCs) than other antimicrobials alone. They also found the addition of 25% (v/v) ethanol further increased the efficacy of the monocycline-EDTA cocktail (Raad et al., 2007).

Wen et al. (2009) found that the efficacy of the biocide glutaraldehyde could be enhanced with addition of the biodegradable chelator ethylenediaminesuccinic acid (EDDS) against an SRB biofilm on carbon steel. They also demonstrated that combining both EDDS and methanol with biocides glutaraldehyde or THPS also enhanced the efficacy of the biocide (Wen et al., 2012; Xu, Wen, Gu, et al., 2012).

The use of D-amino acids as biocides enhancers has already shown some promise. Kolodkin-Gal et al. demonstrated that D-tyrosine, D-methionine, D-tryptophan and D-leucine could be used either individually or as a mixture to prevent biofilm formation and signal biofilm dispersal (2010). They also suggested that the dispersal of the biofilm was triggered by the substitution of the terminal D-alanine in peptidoglycan in the cell wall with a different D-amino acid. This substitution could be prevented by the addition of additional D-alanine (Kolodkin-Gal et al., 2010).

Leiman et al. (2013), however, suggested that biofilm dispersal was not triggered in the peptidoglycan, but through the disruption of protein synthesis. They demonstrated
that they could inhibit the biofilm dispersal triggered by a specific D-amino acid by introducing a proportional amount of its L-amino acid enantiomer. Their results also suggested that the incorporation of D-amino acids showed some inhibition of growth and could even cause growth defects (Leiman et al., 2013).

A high concentration of an equimolar mixture of D-tyrosine, D-methionine, D-tryptophan and D-leucine could not successfully prevent or remove an established SRB biofilm on carbon steel (Xu, Wen, Fu, et al., 2012). It was found, however, that a lower concentration (6.6 ppm) of this equimolar mixture when combined with EDDS (500 ppm) and THPS (30 ppm), demonstrated an enhanced efficacy against an SRB biofilm when compared to the binary mixture alone. It was believed that the SRB biofilm was more recalcitrant than those tested by Kolodkin-Gal et al. (2010). Xu, Li, et al. (2012) found that 50 ppm THPS + 1 ppm D-tyrosine was just as efficacious as 100 ppm THPS alone in the prevention and removal of an established SRB biofilm. Xu, Li, and Gu (2013) also found that 50 ppm THPS + 100 ppm D-methionine was more efficacious than 100 ppm THPS alone in the prevention and removal of an established biofilm. They also tested D-tyrosine, D-methionine and the equimolar mixture of D-tyrosine, D-methionine, D-tryptophan and D-leucine combined with THPS against a field biofilm consortium and found much higher concentrations were required to achieve a similar efficacy as those against a *D. vulgaris* biofilm alone (Li et al., 2015).
CHAPTER 3: D-TRYPTOPHAN AS A BIOCIDE ENHANCER

3.1 Introduction

The mitigation of MIC can be a difficult task to undertake as the detection and treatment of the underlying cause is not without its serious shortcomings. The primary methods of MIC mitigation treatment are by chemical and mechanical methods for biofilm removal (Gieg et al., 2011). The oil and gas industry, which is the primary focus of this investigation, deals with many problems associated with MIC and biofouling, especially in pipelines. Their primary method of chemical treatment is the use of biocides. The biofilm can be mechanically removed by manual scraping from the inside of the pipeline by the use of pigging.

There are many different materials used in pipelines, with carbon steels being the most commonly used. Steels are a common choice largely due to their mechanical properties and cost. The primary components in steels are iron and carbon, with iron making up the largest percentage of the steel. Mild carbon steels, for example, contain 0.45-0.50 percent carbon. Additional components found in small percentages in carbon steels include manganese, silicon, phosphorus and sulfur. Steel alloys may contain components such as nickel, chromium, molybdenum and vanadium. These components can greatly influence mechanical properties and costs of the steel alloys (Judge, 1945). An example of inexpensive mild carbon steel that is used in the oil and gas industry in pipelines is C1018 (UNS G10180). The chemical composition of C1018 steel can be found in Table 3.1 (“AISI 1018 mild/low carbon steel,” 2014).
The use of chemicals to treat biofilm causing microorganisms comes with a number of drawbacks. First of all, biocides are toxic to the environment. Some biocides are far more harmful than others, but all biocides are designed to kill. The challenge is finding a balance to effectively kill the undesired microorganisms without killing off other species in the process. Additional problems with biocides include the development of resistance and the significant costs (Gieg et al., 2011).

While there are many different biocides available, a common choice in the oil and gas industry is THPS. THPS is a quaternary phosphonium salt that is a fast acting, biodegradable, and relatively environmentally friendly biocide that works by damaging the cell membrane (Downward et al., 1997; Zhao et al., 2009). THPS also rapidly oxidizes to trishydroxymethylphosphine oxide (THPO) in the environment which is considered even less toxic than THPS (Downward et al., 1997).

One area that has seen some promise in the mitigation of MIC is the development of biocide enhancers for preexisting biocides. Raad et al. (2007, 2003) enhanced the antimicrobial monocycline initially with just EDTA and then with EDTA and ethanol to reduce the biofilm formation on CVCs. Wen et al. (2009) found that the biocide glutaraldehyde could be enhanced with EDDS and Wen et al. (2012) and Xu, Wen, Gu, et al. (2012) found that both glutaraldehyde and THPS could both be enhanced by EDDS and methanol against SRB biofilms on carbon steel. Another group of biocide enhancers that have shown some promise are the use of D-amino acids. Xu et al. found that both D-tyrosine and D-methionine when combined with a low concentration of THPS could effectively prevent biofilm formation and cause the dispersal of an established SRB
biofilm on carbon steel (Xu, Li, et al., 2012; Xu, Li, and Gu, 2013). Their group also found that D-amino acids were required in higher concentrations to achieve a similar efficacy against a field consortium biofilm (Li et al., 2015).

Amino acids are the building blocks of proteins, energy metabolites, and in some cases serve as important nutrients. There are 20 “standard” amino acids that are responsible for the makeup of all proteins. These α-amino acids have a carboxylic acid group (−COOH), a primary amino group (−NH₂) and a side chain (−R group) around the α-carbon. Amino acids have two different nonsuperimposable enantiomeric forms—the L- and D- form. Proteins are only composed of the L- form (Voet and Voet, 2004). When produced commercially, amino acids are often formed in a racemic mixture of both the L- and D- forms.

D-tryptophan is one D-amino acid that has not been tested alone as a biocide enhancer for THPS. Tryptophan is a nonpolar amino acid with a bulky indole group attached to the α-carbon (Voet and Voet, 2004). The L- form has a strong bitter taste, while the D- form has a sweet flavor that is 40-times as sweet as sucrose (Bender, 1982). The D- form can be used as a sweetener and is distinguished by its long lasting bitter aftertaste (Schiffman et al., 1979).

3.2 Research objectives

The research objectives were to determine if D-tryptophan could be used as a biocide enhancer for THPS in (1) the prevention of the establishment of a *D. vulgaris* biofilm on C1018 carbon steel test coupons in ATCC 1249 medium and (2) the removal
of a 3-day established *D. vulgaris* biofilm on C1018 carbon steel test coupons in ATCC 1249 medium.

3.3 Materials and methods

3.3.1 Bacteria and culture medium

*Desulfovibrio vulgaris* (ATCC 7757) was the strain used in both the biofilm prevention and removal tests in which D-tryptophan was tested as a biocide enhancer for THPS. The *D. vulgaris* bacteria were cultured in ATCC 1249 medium, in which the components can be found in Table 3.2. The ATCC 1249 medium was prepared and autoclave sterilized at 121°C and 15 psi for 20 min. After the medium was allowed to cool, it was sparged with filter-sterilized nitrogen for 1 h and 100 ppm (w/w) L-cysteine was added as an oxygen scavenger. One milliliter of the *D. vulgaris* seed culture was added to 50 ml of the medium with L-cysteine in 125 ml anaerobic vials (Catalog No. 223748, Wheaton Industries Inc., Millville, NJ, USA) and sealed with aluminum seals with rubber septa. The seed cultures were incubated in a shaker at 37°C. The initial cell concentration of an inoculated vial was approximately $10^6$ cells/ml.

3.3.2 Substratum for biofilm growth

Square C1018 (UNS G10180) carbon steel test coupons approximately 11.1 mm x 11.1 mm were used for both the biofilm prevention and biofilm removal tests in which D-tryptophan was tested as a biofilm enhancer of THPS. The composition of C1018 carbon steel can be found in Table 3.1. The test coupons were coated with a blue Teflon coating on all but the top surface. The test coupons used for weight loss were left uncoated as a significant amount of the coating sometimes fell off during the experiment. The test
coupons were successively polished with 180, 400, and 600 grit aluminum oxide sandpapers until smooth before each experiment. Pure isopropanol was used to sterilize the test coupon surface and air dried. The test coupons were weighed when used for weight loss measurements. All test coupons were sterilized for at least 15 min under UV light in a biosafety hood.

3.3.3 D-tryptophan biofilm prevention test experimental setup

D-tryptophan biofilm prevention tests were conducted by adding 100 ml ATCC 1249 medium with 100 ppm (w/w) L-cysteine to 125 ml anaerobic vials containing three prepared test coupons. The anaerobic vials and the preparation of the medium were the same as those described in 3.3.1. The tested treatment and the bacteria were added to the anaerobic vials. Table 3.3 shows the test matrix used for these experiments. All experimental manipulations involving bacteria were conducted in an anaerobic glove box filled with nitrogen. The vials were sealed with aluminum seals with rubber septa and incubated at 37°C for 7 days. After 7 days, the vials were opened, the bulk pH of the medium was measured, and the test coupons were removed for examination. The test coupons were also weighed both immediately after their preparation for the experiment and after the biofilm removal after the experiment to determine their weight loss.

3.3.4 D-tryptophan biofilm removal test experimental setup

D-tryptophan biofilm removal tests were conducted by first establishing a biofilm on the test coupon surface for 3 days. This was done in the same manner as described in 3.3.3, but only ATCC 1249 medium with L-cysteine, the test coupons and the bacteria were added to each vial. After 3 days, the vials were opened and rinsed in phosphate
buffered saline (PBS) solution. The components of PBS solution are shown in Table 3.4. The PBS solution had been autoclave sterilized for 20 min at 121°C and 15 psi and then sparged with filtered nitrogen for 1 h. The test coupons were then soaked for 1 h in a weighing boat with 50 ml PBS solution with the tested treatment of THPS and D-tryptophan. These experimental manipulations were carried out in a nitrogen-filled anaerobic glove box.

3.3.5 Enumeration of sessile cells

After a 7-day biofilm prevention test or 1-h biofilm removal test, the test coupons were removed from the anaerobic vials and rinsed in distilled water to remove any planktonic bacteria. The enumeration of sessile cells was determined by an SRB test kit (Sani-Check Product #100, Warren, Michigan, USA). Ten milliliters (autoclave sterilized and 1-h nitrogen sparged) PBS solution and two coupons from each vial were added to a glass Petri dish. A sample applicator included with the SRB test kit was used to remove the biofilm from the test coupon. The sample applicator, the test coupons and the PBS solution from the Petri dish were vortexed together for 15 sec in a 50 ml centrifuge tube and the sample applicator was inserted in a tube of solid medium included in the SRB test kit. The sessile cell count was determined by how many days it took for the solid medium in the tube to turn black based on the vendor’s calibration.

3.3.6 Analysis of biofilm and test coupon surface

After a 7-day prevention test or 1-h removal test, the test coupons were removed from the vials or weighing boats, respectively, and rinsed with distilled water to remove any loose cells. The test coupons used for biofilm analysis were soaked in 4% w/w
glutaraldehyde for 2 h. The test coupons were then soaked in 25%, 50%, 75%, and 100% isopropanol sequentially for 5-10 min for each concentration. The test coupons were then dried in a critical point dryer (CPD) to remove the moisture from the biofilm. The test coupons were then coated with palladium using a sputter coater (Hummer 6.2, Anatech, USA) to ensure the biofilm was conductive to be imaged using a scanning electron microscope (SEM). The biofilm on the test coupons was observed using an SEM (Model JSM-6390, JEOL, Japan). The corrosion of the test coupon was observed with SEM after the biofilm and corrosion products were removed using Clark’s solution (ASTM G1-03 solution for corrosion specimen preparation) (G01 Committee, 2003). The surface of several test coupons from each test condition were scanned with an infinite focus microscope (IFM) (Model G4, Alicona, Austria) using surface profilometry to measure the pit depth of several pits to determine the deepest pits.

3.3 Results and discussion

3.3.1 D-tryptophan biofilm prevention test

The efficacy of D-tryptophan as a biocide enhancer for THPS for the mitigation of SRB biofilm *D. vulgaris* on C1018 carbon steel test coupons was evaluated through a series of experiments. Initially, lower concentrations of D-tryptophan were tested to determine if they could successfully prevent the establishment of a *D. vulgaris* biofilm on the carbon steel test coupon. Figure 3.1 shows the SEM images of 50 ppm THPS + 1 ppm D-tryptophan, 50 ppm THPS + 50 ppm D-tryptophan, 50 ppm THPS + 100 ppm D-tryptophan, and 50 ppm THPS + 300 ppm D-tryptophan. All four images show widespread biofilm and many cells on the test coupon surface. No improvement in cell
reduction can be seen in these images. The average bulk pH values for the same concentrations can be seen in Figure 3.2 along with 50 ppm THPS alone, SRB alone, and the abiotic control. The pH values of the treatments including THPS in combination with D-tryptophan only showed a slight but statistically insignificant decrease, for the small sample size in this work, when compared with the abiotic control and the SRB alone. The sessile cell count data can be seen in Table 3.5. This also agrees with what was seen in the SEM images that 50 ppm THPS in combination with up to 300 ppm D-tryptophan had little to no effect as a biocide enhancer.

With these results, the concentration of D-tryptophan was increased further. An image of the anaerobic vials after a 7-day biofilm prevention test can be seen in Figure 3.3. This image shows a reduction in the visible black color produced by the SRB can be seen at much higher concentrations, with noticeable reductions at 50 ppm THPS + 1000 ppm D-tryptophan and 50 ppm THPS + 1500 ppm D-tryptophan. This can also be seen with the naked eye on test coupons removed from the vials in Figure 3.4, with the most visible reduction in the black color on the test coupon treated with 50 ppm THPS + 1500 ppm D-tryptophan. The average bulk pH values of the treatments tested with higher D-tryptophan concentrations can be found in Figure 3.5. The pH values show a general decreasing trend with increasing D-tryptophan concentration in combination with 50 ppm THPS. This decrease in pH value in general is very small and the pH value of 50 ppm THPS + 1500 ppm D-tryptophan is similar to that of 100 ppm THPS alone. There is a statistically significant decrease in the pH values between SRB only (no treatment) and
50 ppm THPS + 1500 ppm D-tryptophan. The pH value of 1500 ppm D-tryptophan alone is also comparable to SRB only (no treatment).

Figure 3.6 shows an SEM image of the *D. vulgaris* biofilm on the carbon steel test coupon surface without any treatment. In this image, many cells can be seen along with the rest of the biofilm. An SEM image of the *D. vulgaris* biofilm on the carbon steel test coupon surface treated with 1500 ppm D-tryptophan alone can be seen in Figure 3.7. Not much difference can be seen in the biofilm and number of cells between Figures 3.6 and 3.7. This is a good indication that 1500 ppm D-tryptophan alone did not have much effect in the reduction of the number of sessile cells attached to the carbon steel test coupon surface in the 7-day biofilm prevention test. Figures 3.8 and 3.9 are the *D. vulgaris* biofilm on the carbon steel test coupon after a 7-day biofilm prevention test with 50 ppm THPS and 100 ppm THPS treatment, respectively. Figure 3.8 still shows many cells along with the biofilm. Very little difference can be seen between Figures 3.6 and 3.8 which indicates that a treatment of 50 ppm THPS alone is not enough for a noticeable reduction of the *D. vulgaris* biofilm. Figure 3.9, however, shows a significant reduction of biofilm and cells on the carbon steel test coupon surface when compared to Figure 3.6, with no treatment, and Figure 3.8, treated with 50 ppm THPS alone. Polish lines are even visible on the carbon steel test coupon surface. This is a good indication that 100 ppm THPS alone is a strong enough dosage to significantly reduce the establishment of a *D. vulgaris* biofilm in ATCC 1249 medium in a 7-day biofilm prevention test.

Figure 3.10 shows an SEM image of the *D. vulgaris* biofilm treated with 50 ppm THPS + 500 ppm D-tryptophan on the carbon steel test coupon after a 7-day biofilm
prevention test. The image shows very little reduction in the number of cells on the carbon steel test coupon surface when compared with no treatment in Figure 3.6. The most noticeable reduction in the biofilm, however slight, can be seen in primarily in the inserted images at 50X magnification. Figure 3.11, which shows the biofilm on the carbon steel test coupon surface treated with 50 ppm THPS + 1000 ppm D-tryptophan, starts to definitely show a reduction in the biofilm on the carbon steel test coupon surface. This, once again, is most noticeable in the inserted image at 50X magnification. The reduction can be seen both when compared with Figure 3.6 with no treatment and Figure 3.10 with 50 ppm THPS + 500 ppm D-tryptophan. There are still lots of cells visible on the carbon steel test coupon surface at 4000X magnification. The dosage was increased to 50 ppm THPS + 1500 ppm D-tryptophan which can be seen in Figure 3.12. This image shows a significant reduction in the number of cells found on the carbon steel test coupon surface when compared to Figure 3.6 with no treatment, and Figures 3.10 and 3.11 with increasing dosages of D-tryptophan in combination with THPS. Even some of the polish lines can be seen in Figure 3.12, indicating the bare surface is visible under the biofilm that formed on the carbon steel test coupon surface. The increasing reduction of biofilm visible in the SEM images of the test coupon surface is a good indication that the increased concentration of D-tryptophan acted as a biocide enhancer for THPS.

The efficacy of D-tryptophan as a biocide enhancer for THPS can also be supported by the sessile cell count data after a 7-day biofilm prevention test found in Table 3.6. This data shows that there is no significant reduction in sessile cell count for both 50 ppm THPS alone and 50 ppm THPS + 500 ppm D-tryptophan treatments with
only a 1-log reduction in sessile cells. This is supportive of little to no change seen between Figure 3.6 and Figures 3.8 and 3.10. Increasing the dosage to 50 ppm THPS + 1000 ppm D-tryptophan only increases to a 2-log reduction in cells compared to no treatment. While this is indicative of an improvement in efficacy of the increased concentration of D-tryptophan, a more significant reduction in cells is still desired. A 50 ppm THPS + 1500 ppm D-tryptophan treatment results in a 4-log reduction in cells when compared to no treatment. This is a significant reduction in sessile cells and is comparable to the efficacy observed by 100 ppm THPS alone. A treatment of 1500 ppm D-tryptophan alone demonstrated only a 2-log reduction in sessile cells. This indicates that D-tryptophan alone is not very effective at reducing the number of sessile cells attached to the carbon steel test coupon surface. Table 3.6 indicates that neither 50 ppm THPS alone nor 1500 ppm D-tryptophan alone are all that effective at reducing the sessile cell counts, but when used in combination a 4-log reduction can be achieved.

The data from the SEM images and the sessile cell counts are further supported by the average normalized weight loss, also known as specific weight loss, and general corrosion rate seen in Figure 3.13. Figure 3.13 shows the decrease in (A) weight loss, and thus (B) the corrosion rate, observed with increasing dosage of D-tryptophan in combination with THPS. The weight loss that resulted from a 50 ppm THPS + 1500 ppm D-tryptophan treatment is comparable to that of 100 ppm THPS alone, which is supportive of the lack of cells observed in Figures 3.12 and 3.9 and the sessile cell count data in Table 3.6. Both of these treatments exhibited weight loss values similar to that of the abiotic control, which is also a strong indication that both treatments were quite
The weight loss that resulted from 1500 ppm D-tryptophan alone was similar to that of 50 ppm THPS alone and 50 ppm THPS + 500 ppm D-tryptophan, which also supports the results observed in the SEM images and the sessile cell count data. The reduction in weight loss when compared to no treatment (SRB only) indicates that there was some effect by these treatments, but not nearly as effective as 50 ppm THPS + 1500 ppm D-tryptophan or 100 ppm THPS alone, which both demonstrated a statistically significant decrease compared to no treatment. The weight loss from the 50 ppm THPS + 1000 ppm D-tryptophan treated coupons exhibited a weight loss between that of those treated with 50 ppm THPS + 500 ppm D-tryptophan and 50 ppm THPS + 1500 ppm D-tryptophan which supports the previous data that increasing the concentration of D-tryptophan in combination with THPS will demonstrate a further reduction in the biofilm on the carbon steel test coupon surface and thus a reduction in the corrosion.

An SEM image of the carbon steel test coupon surface for the abiotic control can be seen in Figure 3.14. This figure shows a clean surface with distinct polish lines, indicating the ATCC 1249 medium alone is not corrosive. In Figure 3.15 (A), on the other hand, many pits can be seen on the carbon steel test coupon surface with no treatment (SRB only), with a clearer image of a pit in Figure 3.15 (B). Many pits can also be seen in Figure 3.16 (A), which is an image of the carbon steel test coupon surface treated with 50 ppm THPS alone, and Figure 3.16 (B) is a clearer image of a pit from the same test coupon. It does not appear that 50 ppm THPS alone had much effect on the mitigation of pitting corrosion on the carbon steel test coupon surface as the pits still appear similar in size and number as that in Figure 3.15 with no treatment (SRB only).
Figure 3.17 shows the carbon steel test coupon surface treated with 50 ppm THPS + 500 ppm D-tryptophan. Figure 3.17 (A) shows smaller pits than those seen in Figure 3.16 indicating that the addition of 500 ppm D-tryptophan to 50 ppm THPS show some enhancement in the mitigation of MIC. This is supportive of what was observed in the slight reduction of the observed weight loss in Figure 3.13. The pit observed in Figure 3.17 (B) appears to be slightly smaller in size, also supporting these results. Figure 3.18 shows the carbon steel test coupon surface treated with 50 ppm THPS + 1000 ppm D-tryptophan. Figure 3.18 (A) shows pits fewer in number and smaller in size than those observed in Figures 3.16 (A) or 3.17 (A) indicating a further reduction in the pitting corrosion with increasing concentration of D-tryptophan in combination with the same concentration of THPS. The closer image of the pits in Figure 3.18 (B) also appear to be smaller both in width and depth than the pit seen in Figure 3.17 (B). This further supports the reduction in weight loss observed in Figure 3.13, the reduction of the biofilm visible in inserted image in Figure 3.11 and the decrease in sessile cells in Table 3.6. Figure 3.19 shows the carbon steel test coupon surface treated with 50 ppm THPS + 1500 ppm D-tryptophan and only a few small, shallow pits can be observed. The pit shown in Figure 3.19 (B) shows only small, shallow pitting even when compared to that in Figure 3.18 (B) treated with 50 ppm THPS + 1000 ppm D-tryptophan. This observation of such a significant reduction in the pitting corrosion compared to no treatment (SRB only), 50 ppm THPS alone or even 50 ppm THPS + 500 ppm D-tryptophan supports the other data that the higher dosage of D-tryptophan acts as a biocide enhancer for THPS. The image of the carbon steel test coupon surface seen in Figure 3.20 was treated with 1500 ppm D-
tryptophan alone. In Figure 3.20 (A) many pits can be seen on the carbon steel surface. While there still appear to be a large number of pits seen on the carbon steel test coupon surface (Figure 3.20 (A)), the pits do not appear to be as deep (Figure 3.20 (B)) as those seen Figure 3.15 (B) with no treatment (SRB only), indicating that there was some mitigation effect by the high dosage of D-tryptophan alone. These results support those seen previously in Figure 3.13 and Table 3.6. The carbon steel test coupon surface with 100 ppm THPS treatment can be seen in Figure 3.21. Very few, mostly small pits can be seen on the surface in Figure 3.21 (A) and the pit seen in (B), though small, is larger than the rest. The pitting seen in this figure is much smaller than that in Figure 3.16, with the 50 ppm THPS treatment only, indicating that the increase in concentration shows a reduction in the pitting corrosion on the carbon steel test coupon surface. The pitting corrosion seen in Figure 3.21 (A) is similar to that seen in Figure 3.19 (A) which provides additional evidence that the combination of 50 ppm THPS + 1500 ppm D-tryptophan produces results similar to those of a higher dosage of THPS.

Sample pit depths observed on the carbon steel test coupons by IFM surface scans for SRB, 50 ppm THPS and 50 ppm THPS + 1500 ppm D-tryptophan are shown in Figure 3.22. While the pits in general are not all that deep, a slight difference can be observed, especially with the 50 ppm THPS + 1500 ppm D-tryptophan treatment, with a 1 µm reduction in pit depth. These observations support those seen in the SEM images of the pits on the test coupon surfaces in Figures 3.14-3.21 and the reduction in weight loss in Figure 3.13 (A). Figure 3.23 (A) shows the average pit depth observed on the carbon steel test coupons by IFM surface scans taken from the five deepest pits observed on
three coupons for each treatment. As seen in Figure 3.22, very little difference in average pit depth can be observed between any of the treatments, but in general there is a decreasing trend in the average pit depth. There is a statistically significant decrease (p-value = 0.03) between the pit depths without treatment and those treated with 50 ppm THPS + 1500 ppm D-tryptophan. The average pitting corrosion rate can be seen in Figure 3.23 (B). The corrosion rate calculated from weight loss in Figure 3.13 (B) is higher than that calculated from the pit depth indicating that the corrosion was not localized corrosion.

The results for the D-tryptophan biofilm prevention test indicate that D-tryptophan could, at high concentration (1500 ppm), effectively be used as a biocide enhancer for THPS in the prevention of a D. vulgaris biofilm on carbon steel. D-tryptophan was not, however, as effective as a biocide enhancer for THPS as either D-tyrosine or D-methionine, which were just as effective as D-tryptophan at much lower concentrations (1 ppm and 100 ppm, respectively) (Xu, Li, and Gu, 2013; Xu, Li, et al., 2012). Kolodkin-Gal et al. (2010) included it in their quaternary mixture of D-amino acids they found triggered biofilm dispersal at a much lower concentration than the individual D-amino acids alone. This indicates that though D-tryptophan alone may require a higher concentration to be an effective biocide enhancer for THPS, it may work synergistically at a lower concentration with other D-amino acids.

3.3.2 D-tryptophan biofilm removal test

After it was determined that a high concentration of D-tryptophan in combination with THPS was effective at reducing the number of SRB sessile cells and pitting
corrosion on carbon steel, a biofilm removal test was conducted to determine if D-tryptophan could be an effective biocide enhancer in the removal of an established *D. vulgaris* biofilm. The carbon steel test coupon surface with a 3-day established biofilm with no treatment, soaked for 1 h in only PBS solution can be seen in Figure 3.24. This image shows a well developed biofilm with lots of sessile cells, indicating that 3 days was long enough to establish a mature biofilm and the PBS solution alone did not appear to have a negative effect on the biofilm. Figure 3.25 shows the carbon steel test coupon surface with a 3-day established biofilm with a 1-h 50 ppm THPS + 1500 ppm D-tryptophan treatment in PBS solution. The image shows a well developed biofilm with lots of sessile cells, which is basically no different than that with no treatment in Figure 3.24. The carbon steel test coupon surface with a 3-day established biofilm with a 1-h 50 ppm THPS + 2000 ppm D-tryptophan treatment in PBS solution can be seen in Figure 3.26. This image also shows a well developed biofilm with lots of sessile cells appearing essentially the same as Figures 3.24 and 3.25. Figure 3.27 shows the carbon steel test coupon with a 3-day established biofilm with a 1-h 2000 ppm D-tryptophan treatment alone in PBS solution. This image appears as Figures 3.24-3.26 with a well developed biofilm and lots of sessile cells. The carbon steel test coupon surface with a 3-day established biofilm with a 1-h 100 ppm treatment in PBS solution can be seen with a well developed biofilm and lots of sessile cells in Figure 3.28. Once again the image of this treatment did not appear any different than any of the others for the 1-h biofilm removal test.
The sessile cell count data for the 1-h biofilm removal test can be seen in Table 3.7. The data here indicates some differences in sessile cell counts that could not be seen in the SEM images. One possible explanation for this is that the SEM images can only show the shape of what is attached to the test coupon surface and cannot indicate whether the cells are alive or dead. A 1-h treatment of 50 ppm THPS + 1500 ppm D-tryptophan or 50 ppm THPS + 2000 ppm D-tryptophan in PBS solution only show at 1- to 2-log reduction in sessile cell counts which is a slight reduction, but not nearly comparable to the 4-log reduction the 50 ppm THPS + 1500 ppm D-tryptophan treatment exhibited in the biofilm prevention test. A 3-log reduction can be seen in Table 3.7 from a 1-h treatment of 2000 ppm D-tryptophan alone which is slightly better than when in combination with 50 ppm THPS. A 1-h treatment of 100 ppm THPS alone in PBS solution demonstrated the most significant reduction in sessile cells of any of the treatments with a 6-log reduction. The SRB test kits after 5-days incubation for each of the five conditions tested for the 1-h biofilm removal test can be seen in Figure 3.29. As seen in this picture, the solid medium in the test kit from the test coupons treated with 100 ppm THPS never turn black, so despite the appearance of sessile cells in the SEM images the cells were most likely not viable cells.

There are several possible reasons that the results for D-tryptophan as a biocide enhancer for THPS were much better in the biofilm prevention test versus the biofilm removal test. First of all, the treatment in the biofilm prevention test was added along with the bacteria, which would help prevent the establishment of a biofilm in the first place. The exposure to the various treatments in the biofilm prevention test was also
considerably longer, allowing more time for the treatment to take effect. Also, the 1-h shock treatment in the biofilm removal test was a very short test on an already established biofilm, meaning it had a tougher task with a shorter completion time. It seemed like the higher dosage of THPS was very effective in that short period of time, but that 1 h may not have been enough time to really see more significant biofilm dispersal effects from D-tryptophan.

3.4 Summary

D-tryptophan was tested and determined to be an effective biocide enhancer for THPS at high concentrations against *D. vulgaris* biofilm on carbon steel in biofilm prevention. D-tryptophan required a much higher concentration to be as effective of biocide enhancer as D-tyrosine or D-methionine. Combining D-tryptophan with pH adjusted THPS showed very little influence on the pH value. D-tryptophan alone and in combination with THPS only showed a slight effect in the removal of an established *D. vulgaris* biofilm in a 1-h biofilm removal test.
Table 3.1.

<table>
<thead>
<tr>
<th>Component</th>
<th>Chemical composition of C1018 carbon steel in wt%</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0.14-0.20</td>
</tr>
<tr>
<td>Mn</td>
<td>0.60-0.90</td>
</tr>
<tr>
<td>P</td>
<td>0.04</td>
</tr>
<tr>
<td>S</td>
<td>0.05</td>
</tr>
<tr>
<td>Si</td>
<td>0.15-0.30</td>
</tr>
<tr>
<td>Fe</td>
<td>98.81-99.26</td>
</tr>
</tbody>
</table>

Table 3.2.

<table>
<thead>
<tr>
<th>Component</th>
<th>Composition of ATCC 1249 Broth</th>
</tr>
</thead>
</table>
| Component I | MgSO₄  2.0 g  
Sodium citrate  5.0 g  
CaSO₄  1.0 g  
NH₄Cl  1.0 g  
Distilled water  400.0 ml |
| Component II | K₂HPO₄  0.5 g  
Distilled water  200 ml |
| Component III | Sodium lactate  3.5 g  
Yeast extract  1.0 g  
Distilled water  400 ml |
| Component IV | Fe(NH₄)₂(SO₄)₂  Filter-sterilize 5% (w/w) ferrous ammonium sulfate. Add 0.1 ml of this solution to 5.0 ml of medium prior to inoculation. |
Table 3.3.

Test matrix for biocide enhancer biofilm prevention test

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Conditions</th>
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</thead>
<tbody>
<tr>
<td>SRB strain</td>
<td><em>Desulfovibrio vulgaris</em> (ATCC 7757)</td>
</tr>
<tr>
<td>Temperature</td>
<td>37°C</td>
</tr>
<tr>
<td>Culture medium</td>
<td>ATCC 1249 Broth</td>
</tr>
<tr>
<td>Oxygen scavenger</td>
<td>100 ppm (w/w) L-cysteine</td>
</tr>
<tr>
<td>Initial pH</td>
<td>7.0±0.1</td>
</tr>
<tr>
<td>Biocide</td>
<td>50 ppm THPS (adjusted to pH 7)</td>
</tr>
<tr>
<td>Biocide enhancer</td>
<td>0, 500, 1000, 1500 ppm D-tryptophan</td>
</tr>
<tr>
<td>Test duration</td>
<td>7 days</td>
</tr>
<tr>
<td>Test material</td>
<td>C1018 carbon steel</td>
</tr>
</tbody>
</table>

Table 3.4.

Components of phosphate buffered saline (PBS) solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
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<tbody>
<tr>
<td>NaCl</td>
<td>8.0 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>1.44 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.24 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

*pH adjusted to 7.4*
Table 3.5.
Sessile cell count from carbon steel test coupons after 7-day biofilm prevention test

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sessile cell count (cells/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment (control)</td>
<td>≥10⁶</td>
</tr>
<tr>
<td>500 ppm D-tryptophan</td>
<td>≥10⁵</td>
</tr>
<tr>
<td>50 ppm THPS</td>
<td>≥10⁵</td>
</tr>
<tr>
<td>50 ppm THPS + 1 ppm D-tryptophan</td>
<td>≥10⁵</td>
</tr>
<tr>
<td>50 ppm THPS + 50 ppm D-tryptophan</td>
<td>≥10⁵</td>
</tr>
<tr>
<td>50 ppm THPS + 100 ppm D-tryptophan</td>
<td>≥10⁵</td>
</tr>
<tr>
<td>50 ppm THPS + 300 ppm D-tryptophan</td>
<td>≥10⁵</td>
</tr>
</tbody>
</table>

Table 3.6.
Sessile cell count from carbon steel test coupons after 7-day biofilm prevention test

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sessile cell count (cells/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment (control)</td>
<td>≥10⁶</td>
</tr>
<tr>
<td>1500 ppm D-tryptophan</td>
<td>≥10⁴</td>
</tr>
<tr>
<td>50 ppm THPS</td>
<td>≥10⁵</td>
</tr>
<tr>
<td>50 ppm THPS + 500 ppm D-tryptophan</td>
<td>≥10⁵</td>
</tr>
<tr>
<td>50 ppm THPS + 1000 ppm D-tryptophan</td>
<td>≥10⁴</td>
</tr>
<tr>
<td>50 ppm THPS + 1500 ppm D-tryptophan</td>
<td>≥10³</td>
</tr>
<tr>
<td>100 ppm THPS</td>
<td>≥10²</td>
</tr>
</tbody>
</table>
Table 3.7.

Sessile cell count from carbon steel test coupons after 1-h biofilm removal test

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sessile cell count (cells/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment (control)</td>
<td>$\geq 10^{6}$</td>
</tr>
<tr>
<td>50 ppm THPS + 1500 ppm D-tryptophan</td>
<td>$\geq 10^{4}$</td>
</tr>
<tr>
<td>50 ppm THPS + 2000 ppm D-tryptophan</td>
<td>$\geq 10^{5}$</td>
</tr>
<tr>
<td>2000 ppm D-tryptophan</td>
<td>$\geq 10^{3}$</td>
</tr>
<tr>
<td>100 ppm THPS</td>
<td>$&lt; 10$</td>
</tr>
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</table>
Figure 3.1. SEM images of biofilms on test coupons after 7-day biofilm prevention test: (A) 50 ppm THPS + 1 ppm D-tryptophan, (B) 50 ppm THPS + 50 ppm D-tryptophan, (C) 50 ppm THPS + 100 ppm D-tryptophan, (D) 50 ppm THPS + 300 ppm D-tryptophan. Scale bar for inserted images is 500 µm.
Figure 3.2. Average bulk pH values for lower tested concentrations of D-tryptophan biofilm prevention test with *D. vulgaris*. Error bars represent standard deviation.

Figure 3.3. Anaerobic vials with test coupons after 7-day biofilm prevention test. (1) No treatment (SRB only), (2) 50 ppm THPS, (3) 50 ppm THPS + 500 ppm D-tryptophan, (4) 50 ppm THPS + 1000 ppm D-tryptophan, (5) 50 ppm THPS + 1500 ppm D-tryptophan.
Figure 3.4. Test coupons after removal from anaerobic vials after 7-day biofilm prevention test. (1) No treatment (SRB only), (2) 50 ppm THPS, (3) 50 ppm THPS + 500 ppm D-tryptophan, (4) 50 ppm THPS + 1000 ppm D-tryptophan, (5) 50 ppm THPS + 1500 ppm D-tryptophan.

Figure 3.5. Average bulk pH values for higher tested concentrations of D-tryptophan biofilm prevention test with *D. vulgaris*. Error bars represent standard deviation.
Figure 3.6. SEM images of biofilm on test coupons after 7-day biofilm prevention test in ATCC 1249 medium with *D. vulgaris* with no treatment. Scale bar for inserted image is 500 µm.

Figure 3.7. SEM images of biofilm on test coupons after 7-day biofilm prevention test in ATCC 1249 medium with *D. vulgaris* treated with 1500 ppm D-tryptophan. Scale bar for inserted image is 500 µm.
Figure 3.8. SEM images of biofilm on test coupons after 7-day biofilm prevention test in ATCC 1249 medium with *D. vulgaris* treated with 50 ppm THPS. Scale bar for inserted image is 500 µm.

Figure 3.9. SEM images of biofilm on test coupons after 7-day biofilm prevention test in ATCC 1249 medium with *D. vulgaris* treated with 100 ppm THPS. Scale bar for inserted image is 500 µm.
Figure 3.10. SEM images of biofilm on test coupons after 7-day biofilm prevention test in ATCC 1249 medium with *D. vulgaris* treated with 50 ppm THPS + 500 ppm D-tryptophan. Scale bar for inserted image is 500 µm.

Figure 3.11. SEM images of biofilm on test coupons after 7-day biofilm prevention test in ATCC 1249 medium with *D. vulgaris* treated with 50 ppm THPS + 1000 ppm D-tryptophan. Scale bar for inserted image is 500 µm.
Figure 3.12. SEM images of biofilm on test coupons after 7-day biofilm prevention test in ATCC 1249 medium with *D. vulgaris* treated with 50 ppm THPS + 1500 ppm D-tryptophan. Scale bar for inserted image is 500 µm.
Figure 3.13. (A) Average normalized weight loss from carbon steel test coupons after 7-day biofilm prevention test with *D. vulgaris*. (B) Average general corrosion rate calculated from weight loss data. Error bars represent standard deviation.
Figure 3.14. SEM images of the surface of a carbon steel test coupon after 7-day biofilm prevention test in ATCC 1249 medium without *D. vulgaris* (Abiotic Control) at (A) 500X and (B) 2000X magnification.
Figure 3.15. SEM images of the surface of a carbon steel test coupon after 7-day prevention test in ATCC 1249 medium with *D. vulgaris* with no treatment at (A) 500X and (B) 2000X magnification.
Figure 3.16. SEM images of the surface of a carbon steel test coupon after 7-day prevention test in ATCC 1249 medium with *D. vulgaris* treated with 50 ppm THPS at (A) 500X and (B) 2000X magnification.
Figure 3.17. SEM images of the surface of a carbon steel test coupon after 7-day prevention test in ATCC 1249 medium with *D. vulgaris* treated with 50 ppm THPS + 500 ppm D-tryptophan at (A) 500X and (B) 2000X magnification.
Figure 3.18. SEM images of the surface of a carbon steel test coupon after 7-day prevention test in ATCC 1249 medium with *D. vulgaris* treated with 50 ppm THPS + 1000 ppm D-tryptophan at (A) 500X and (B) 2000X magnification.
Figure 3.19. SEM images of the surface of a carbon steel test coupon after 7-day prevention test in ATCC 1249 medium with *D. vulgaris* treated with 50 ppm THPS + 1500 ppm D-tryptophan at (A) 500X and (B) 2000X magnification.
Figure 3.20. SEM images of the surface of a carbon steel test coupon after 7-day prevention test in ATCC 1249 medium with *D. vulgaris* treated with 1500 ppm D-tryptophan at (A) 500X and (B) 2000X magnification.
Figure 3.21. SEM images of the surface of a carbon steel test coupon after 7-day prevention test in ATCC 1249 medium with *D. vulgaris* treated with 100 ppm THPS at (A) 500X and (B) 2000X magnification.
Figure 3.22. Sample pit depth of C1018 carbon steel test coupons for SRB, 50 ppm THPS, and 50 ppm THPS + 1500 ppm D-tryptophan after 7-day prevention test from IFM surface scan.
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Figure 3.25. SEM images of the biofilm on the carbon steel test coupon after 1-h biofilm removal test treated with 50 ppm THPS + 1500 ppm D-tryptophan. Scale bar for inserted image is 500 µm.
Figure 3.26. SEM images of the biofilm on the carbon steel test coupon after 1-h biofilm removal test treated with 50 ppm THPS + 2000 ppm D-tryptophan. Scale bar for inserted image is 500 µm.

Figure 3.27. SEM images of the biofilm on the carbon steel test coupon after 1-h biofilm removal test treated with 2000 ppm D-tryptophan. Scale bar for inserted image is 500 µm.
Figure 3.28. SEM images of the biofilm on the carbon steel test coupon after 1-h biofilm removal test treated with 100 ppm THPS. Scale bar for inserted image is 500 µm.

Figure 3.29. Sani-Check test kits after 5-day incubation of the biofilm on carbon steel test coupons after 1-h removal test. (1) No treatment, (2) 50 ppm THPS + 1500 ppm D-tryptophan, (3) 50 ppm THPS + 2000 ppm D-tryptophan, (4) 2000 ppm D-tryptophan, (5) 100 ppm THPS.
CHAPTER 4: NITRATE-REDUCING *PSEUDOMONAS AERUGINOSA*

4.1 Introduction

While there has been much research and focus on MIC by SRB, very little research has been done with NRB in regards to MIC. More problems may be anticipated with NRB, especially with the use of nitrate as an inhibitor of SRB (Bødtker et al., 2008; Gieg et al., 2011). Halim et al. (2011) demonstrated the corrosivity of NRB associated with nitrate injection to mitigate SRB. They found that the addition of nitrate did indeed suppress the growth of SRB, but also could promote the growth of NRB, which was also corrosive. Xu, Li, Song, et al. (2013) also demonstrated the corrosivity of NRB in a laboratory investigation with carbon steel and found it to be even more corrosive than SRB. Their results also support the BCNR theory, which uses biocatalysis and bioenergetics to explain MIC by NRB (Xu, Li, Song, et al., 2013). More research is needed in this area of MIC by NRB to develop a further understanding into its mechanism, which is needed to improve the diagnosis and mitigation of MIC.

*Pseudomonas aeruginosa* are Gram-negative rod-shaped bacteria that metabolize by respiration. Most strains possess a single polar flagellum for motility. While they are commonly found in soil and water, they can also be found on the surface of plants and occasionally animals. *P. aeruginosa* is considered an opportunistic pathogen based on its limited ability to infect a host only when its defenses are compromised (Todar, 2014). While *P. aeruginosa* prefer to grow aerobically, in the absence of oxygen, they can use denitrification to utilize nitrate or nitrite as an alternative electron acceptor (Eschbach et
al., 2004). This discovery has led them from being classified as strict aerobes to more of a facultative anaerobe. Very little research has been done with corrosion caused by \textit{P. aeruginosa}. Morales et al. (1993) found that \textit{P. aeruginosa} increased the localized corrosion and negatively affected the passive film of stainless steel. While their tests were conducted in the absence of oxygen, the bacteria used in experiments were initially grown aerobically. Hamzah et al. (2013) investigated the corrosion of stainless steel caused by \textit{P. aeruginosa} under aerobic conditions. While they found that the presence of the bacteria increased the corrosion of the stainless steel, they failed to address the corrosion effects of oxygen under aerobic conditions.

In this research, the three different strains of \textit{P. aeruginosa} that were used for experiments were PAO1, \textit{pilT}, and \textit{bdlA}. PAO1 is a wild type of \textit{P. aeruginosa}, while \textit{pilT} and \textit{bdlA} have both been genetically altered to produce stronger biofilms. The \textit{pilT} strain has been genetically altered to have more Type IV pili, which promote surface attachment (Mukherjee et al., 2012). The \textit{bdlA} strain has been genetically altered to resist biofilm dispersion due to changes in nutrient concentrations. The term \textit{bdlA} stands for biofilm dispersion locus (Morgan et al., 2006).

4.2 Research objectives

The research objectives were first to determine if \textit{P. aeruginosa} was corrosive when grown anaerobically with KNO$_3$ as an electron acceptor (as an NRB). Next, three different strains of \textit{P. aeruginosa} were compared to determine if the genetically-modified \textit{pilT} and \textit{bdlA} strains were more corrosive than the wild-type PAO1 strain. Then the wild
type PAO1 strain was tested to determine if there were any changes to the corrosion of carbon steel without the addition of L-cysteine as an oxygen scavenger.

4.3 Materials and methods

4.3.1 Bacteria and culture medium

The three strains of *P. aeruginosa* used in this study were wild-type PAO1 and genetically-engineered *pilT* and *bdlA*. All three strains were obtained from Dr. Daniel Hassett of the University of Cincinnati. Each strain was initially cultured aerobically in autoclave sterilized Luria-Bertani (LB) medium for two days. The composition of LB medium is as follows: 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 1000 ml distilled water. The bacteria were then cultured anaerobically in the medium described in Table 4.1. This medium was prepared and autoclave sterilized for 20 min at 121°C and 15 psi and allowed to cool. The medium was then sparged with filter-sterilized nitrogen for 1 h and L-cysteine was added in the amount of 100 ppm (w/w) as an oxygen scavenger when applicable. One milliliter of each aerobic seed culture was added along with 50 ml of the prepared medium with L-cysteine (when applicable) into separate 125 ml anaerobic vials (Catalog No. 223748, Wheaton Industries Inc., Millville, NJ, USA) and sealed with aluminum seals with rubber septa. The seed cultures were incubated in a shaker at 37°C. Upon inoculation, each vial contained an initial cell concentration of approximately $10^6$ cells/ml. Additional experiments were conducted without the addition of L-cysteine as well.
4.3.2 Substratum for biofilm growth

Round C1018 (UNS G10180) carbon steel test coupons approximately 11.5 mm in diameter were used for experiments comparing the two genetically-modified strains with the wild type. Square C1018 (UNS G10180) carbon steel test coupons approximately 11.1 mm wide were used for the experiments with the wild type strain without L-cysteine. The composition of C1018 carbon steel can be found in Table 3.1. A blue Teflon coating was used to coat the test coupon surface leaving only the top surface exposed for the square test coupons used in the wild type strain experiments without L-cysteine, but the round test coupons were left uncoated leaving more surface area exposed to the test conditions. The test coupons were prepared by successively polishing with 180, 400, and 600 grit aluminum oxide sandpapers until smooth. The test coupon surface was then sterilized with pure isopropanol and air dried. The test coupons were weighed and then sterilized under UV light in a biosafety hood for at least 15 min.

4.3.3 Setup of PAO1, pilT, and bdlA experiments

The test matrix for this experiment can be found in Table 4.1. The medium described in Table 4.1 and all the other equipment used for this experiment were first sterilized in an autoclave for 20 min at 121°C at 15 psi. The medium was allowed to cool and then sparged with filter-sterilized nitrogen for 1 h. Then 100 ppm (w/w) L-cysteine was added to the medium as an oxygen scavenger. Three of the prepared carbon steel test coupons were added to each of the anaerobic vials along with 100 ml of the autoclaved and sparged medium. One milliliter of seed culture (PAO1, pilT, or bdlA) was added to each vial. The vials were sealed with aluminum seals with rubber septa and incubated at
37°C for 7 days. This same procedure was also followed for PAO1 alone without the addition of L-cysteine. After the 7 days, the anaerobic vials were opened, the test coupons were removed and the bulk pH of the medium was measured. Weight loss was also determined by weighing the test coupons both immediately after preparation for the experiment and after the biofilm removal after the experiment.

4.3.4 Analysis of biofilm and test coupon surface

After the carbon steel test coupons were removed, the same procedure was followed as described in 3.3.6 to prepare the biofilm and test coupon surface for observation by SEM and surface profilometry analysis by IFM (Model ALC13, Alicona, Austria).

4.4 Results and discussion

4.4.1 PAO1, pilT, and bdlA as NRB

The three strains of *P. aeruginosa* were tested as NRB to determine first if they were corrosive and also to determine if there was any significant difference in the corrosion they caused. The carbon steel test coupons in the anaerobic vials can be seen in Figure 4.1 for the three tested strains and the abiotic control. From this image, no change can be observed in the medium and test coupons in the abiotic control. The vials containing each of the three strains all turned a similar dark brown color that resulted from the growth of the bacteria and any corrosion that may have occurred. The biofilm can also be seen on the test coupons. This biofilm was lighter in color and did not stick to the test coupons nearly as tightly as that of *D. vulgaris*. 
The SEM image of the PAO1 biofilm on the carbon steel test coupon surface can be seen in Figure 4.2. From the figure, it can be seen that (A) the PAO1 biofilm covers the entire test coupon surface with a thick biofilm made up of primarily very long, thin cells (B). Figure 4.3 shows the pilT biofilm on the carbon steel test coupon surface, which again appears to (A) cover the entire surface in a thick biofilm and (B) have the biofilm formed of primarily very long, thin cells. The image of the bdlA biofilm on the carbon steel test coupon surface can be seen in Figure 4.4, which like PAO1 and pilT shows (A) a thick biofilm covered surface with (B) primarily long, thin cells. Very little difference can be observed between Figures 4.2, 4.3 and 4.4, indicating that there is very little difference in the morphology of the three strains when grown under these conditions.

The carbon steel test coupon surface can be seen in Figure 4.5 for the abiotic control. The surface of the abiotic control test coupon still has distinct polish lines which is an indication that the medium itself is not corrosive. Figure 4.6 shows the carbon steel coupon surface after removal of the PAO1 biofilm. This image shows a widespread general corrosion. This general corrosion can also be seen on the carbon steel test coupons after removal of the pilT biofilm (Figure 4.7) and after the removal of the bdlA biofilm (Figure 4.8). The different tested strains of P. aeruginosa seemed to show very little difference in the corrosion they caused to carbon steel. The observed corrosion seemed more general and along grain boundaries than that seen in Chapter 3 by D. vulgaris which was primarily in the form of pitting corrosion.

The bulk pH values of the medium measured after 7 days for the abiotic control and the three tested strains can be seen in Figure 4.9. The abiotic control remained
essentially unchanged at neutral pH 7, while the pH value with the three tested strains increased by about 1 pH unit to around pH 8. While the pH did increase, it did not increase that drastically from neutral pH. Also, the pH of the medium did not become acidic, so it was not due to acidic conditions that the corrosion of the carbon steel occurred.

Figure 4.10 is the (A) average normalized weight loss and the (B) general corrosion rate calculated from the weight loss data for the abiotic control, PAO1, pilT and \textit{bdlA}. There was essentially a negligible weight loss observed from the abiotic control test coupons supporting that the medium alone was not corrosive. A large weight loss was observed from all three of the tested \textit{P. aeruginosa} strains which led to an average corrosion rate of approximately 0.5 mm/yr. This is more than twice of that observed from \textit{D. vulgaris} alone. The higher weight loss was not surprising, however, as there was a great deal of general corrosion observed in the SEM images in Figures 4.5, 4.6 and 4.7 which would lead to a higher weight loss. There are also rather large standard deviations seen for each of the three strains. These wide ranges of weight loss values were not observed so much within each anaerobic vial, but more from experiment to experiment. It appeared that growth of the bacteria greatly influenced the weight loss of the test coupons in which the better the bacteria were able to grow the higher the observed weight loss. A single factor ANOVA test was done to determine if there was any significant difference in the weight loss between the three strains. As can be seen from the single factor ANOVA test in Table 4.2, the F value was smaller than the critical F value which
indicates that statistically the variance in weight loss between the three different strains was insignificant.

SEM images of a pit observed from each of the three tested strains can be seen in Figure 4.11. The pits do not appear that much different from each other with them all appearing to act along the grain boundaries. The image of the pit for pilT is a bigger pit at a smaller magnification than the other two, but smaller pits like those of the other two strains appear in this bigger pit. The deepest pit for each strain as determined by IFM surface scans can be seen in Figure 4.12. The pits are all of a similar depth which supports that there is essentially no difference in the corrosion of carbon steel between the three strains when grown anaerobically under these conditions as NRB. The pit depths observed in Figure 4.12 correspond to a pitting corrosion rate of 0.68 mm/yr for PAO1, 0.51 mm/yr for pilT, and 0.47 mm/yr for bdlA. The pitting corrosion rates were not significantly larger than those calculated by weight loss in Figure 4.10 (B) indicating the observed corrosion was not considered localized corrosion.

4.4.2 PAO1 without L-cysteine

The SEM image of the PAO1 biofilm on the carbon steel test coupon can be seen in Figure 4.13. The biofilm in Figure 4.13 (A) does not appear quite as thick as the PAO1 biofilm in Figure 4.2 (A). At higher magnification, the biofilm looks quite a bit different from that of PAO1 with L-cysteine (Figure 4.2 (B)). The cells are still similar in diameter, but appear much shorter in length and with more EPS visible around the cells. According to Yoon et al. (2011), the elongation of the P. aeruginosa cells is a normal response when grown under anaerobic conditions by nitrate respiration. The elongation of
the cells is due to the cells not dividing properly and assists in biofilm formation. While in both cases PAO1 was grown under anaerobic conditions, the addition of L-cysteine eliminated any trace amounts of oxygen from the beginning of the experiment which would have allowed for more cell elongation to occur. Without the addition of L-cysteine as an oxygen scavenger, it may have allowed trace amounts of oxygen to be left in the medium which would have been utilized by the bacteria first before switching over to nitrate respiration. This could have resulted in less elongation of the cells. An EDX of the PAO1 biofilm can be seen in Figure 4.14 in which the primary component of the biofilm is iron, and being that there were only traces of iron in the medium, the majority of that iron must have come from the carbon steel test coupons.

The average bulk pH values for PAO1 without L-cysteine compared to those of the previous data with L-cysteine can be seen in Figure 4.15. Once again the pH values for the abiotic control remained neutral at around pH 7. The pH value increased some, but not quite as much as with the addition of L-cysteine. There is a statistically significant difference between the average pH values with and without the addition of L-cysteine. It is likely that the addition of L-cysteine caused the bacteria to produce additional metabolites which further increased the pH value than without the presence of L-cysteine.

Figure 4.16 shows the (A) average normalized weight loss and (B) general corrosion rate calculated from the weight loss data for PAO1 without L-cysteine compared to the previous PAO1 weight loss data with L-cysteine. A slight, though statistically insignificant, increase can be seen in the average normalized weight loss of the abiotic control without the addition of L-cysteine compared to that with the addition
of L-cysteine. This slight increase was to be expected as L-cysteine was added as an oxygen scavenger and the trace amounts of oxygen would not have been eliminated without it and would lead to an increase in the weight loss, and thus the corrosion rate. A similar, though statistically insignificant, increase was also seen for the average normalized weight loss of that of PAO1 without the addition of L-cysteine compared with that with the addition of L-cysteine. Unlike the abiotic control though, the bacteria would use the oxygen present and then switch over to nitrate reduction.

4.4 Summary

The wild type and the two genetically-engineered strains of P. aeruginosa were grown as NRB and tested to determine if there were any differences in the corrosion observed between them. It was determined that no significant difference was observed in the corrosion of the carbon steel and the morphology of the cells in biofilm. Further tests were conducted to determine the effects of the addition of L-cysteine as an oxygen scavenger on the wild type PAO1. It was found that the weight loss and corrosion changed very little, but the morphology of the cells changed significantly with the addition of L-cysteine resulting in much longer cells.
Table 4.1.

Test matrix for comparison of *P. aeruginosa* strains

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Conditions</th>
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<tr>
<td><em>P. aeruginosa</em> strain</td>
<td>PAO1, <em>pilT</em>, or <em>bdlA</em></td>
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<tr>
<td>Temperature</td>
<td>37°C</td>
</tr>
<tr>
<td>Culture medium</td>
<td>Tryptone 10 g</td>
</tr>
<tr>
<td></td>
<td>Yeast extract 5 g</td>
</tr>
<tr>
<td></td>
<td>NaCl 5 g</td>
</tr>
<tr>
<td></td>
<td>KNO₃ 10 g</td>
</tr>
<tr>
<td></td>
<td>Distilled water 1000 ml</td>
</tr>
<tr>
<td>Oxygen scavenger</td>
<td>100 ppm (w/w) L-cysteine</td>
</tr>
<tr>
<td>Initial pH</td>
<td>7.0±0.1</td>
</tr>
<tr>
<td>Test duration</td>
<td>7 days</td>
</tr>
<tr>
<td>Test material</td>
<td>C1018 carbon steel</td>
</tr>
</tbody>
</table>

Table 4.2.

ANOVA: Single Factor-Comparing variance in weight loss of PAO1, *pilT*, and *bdlA*

SUMMARY

<table>
<thead>
<tr>
<th>Groups</th>
<th>Count</th>
<th>Sum</th>
<th>Average</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO1</td>
<td>64</td>
<td>0.542653</td>
<td>0.008479</td>
<td>2.46E-05</td>
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<tr>
<td><em>pilT</em></td>
<td>64</td>
<td>0.570044</td>
<td>0.008907</td>
<td>2.35E-05</td>
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<tr>
<td><em>bdlA</em></td>
<td>64</td>
<td>0.469794</td>
<td>0.007341</td>
<td>1.39E-05</td>
</tr>
</tbody>
</table>

ANOVA

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
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<td>Between Groups</td>
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<td>4.2E-05</td>
<td>2.028216</td>
<td>0.134424</td>
<td>3.043722</td>
</tr>
<tr>
<td>Within Groups</td>
<td>0.003909</td>
<td>189</td>
<td>2.07E-05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.003993</td>
<td>191</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
Figure 4.1. Test coupons of Control, PAO1, pilT, and bdlA in anaerobic vials after 7-day corrosion test.
Figure 4.2. SEM images of the PAO1 biofilm on the carbon steel test coupon surface after a 7-day corrosion test at (A) 100X and (B) 4000X magnification.
Figure 4.3. SEM images of the pilT biofilm on the carbon steel test coupon surface after a 7-day corrosion test at (A) 100X and (B) 4000X magnification.
Figure 4.4. SEM images of the bdlA biofilm on the carbon steel test coupon surface after a 7-day corrosion test at (A) 100X and (B) 4000X magnification.
Figure 4.5. SEM images of the carbon steel surface of the test coupon after 7-day corrosion test with only medium (Control) at (A) 100X and (B) 2000X magnification.
Figure 4.6. SEM images of the carbon steel surface of the test coupon after 7-day corrosion test with PAO1 at (A) 100X and (B) 2000X magnification.
Figure 4.7. SEM images of the carbon steel surface of the test coupon after 7-day corrosion test with *pilT* at (A) 100X and (B) 2000X magnification.
Figure 4.8. SEM images of the carbon steel surface of the test coupon after 7-day corrosion test with \textit{bdla} at (A) 100X and (B) 2000X magnification.
Figure 4.9. Average pH values of the bulk medium after 7 days. Error bars represent standard deviation.

Figure 4.10. (A) Average normalized weight loss of C1018 carbon steel test coupons after 7 days. (B) Average general corrosion rate calculated from weight loss data. Error bars represent standard deviation.
Figure 4.11. SEM images of pits on the carbon steel test coupon surface of (A) PAO1, (B) pilT, and (C) bdlA after 7-day corrosion test. Scale bar of inserted images is 100μm.
Figure 4.12. Sample pit depth of C1018 carbon steel test coupons for PAO1, pilT, and bdlA after 7-day corrosion test from IFM surface scan.
Figure 4.13. SEM images of the PAO1 biofilm on a carbon steel test coupon without addition of L-cysteine after a 7-day corrosion test at (A) 100X and (B) 4000X magnification.
Figure 4.14. EDX of the PAO1 biofilm on a carbon steel test coupon without addition of L-cysteine after a 7-day corrosion test.
Figure 4.15. Average pH values of the bulk medium after 7 days. (Control without L-cysteine from 10-day test.) Error bars represent standard deviation.
Figure 4.16. (A) Average normalized weight loss of C1018 carbon steel test coupons after 7-day corrosion test with and without L-cysteine. (Control without L-cysteine from 10-day test.) (B) Average general corrosion rate calculated from weight loss data. Error bars represent standard deviation.
CHAPTER 5: CONCLUSION

The first part of this research was conducted to determine if D-tryptophan could be used as a biocide enhancer for THPS. D-tyrosine and D-methionine have already demonstrated a high effectiveness as biocide enhancers of THPS at low concentrations. It was found that D-tryptophan could be used as a biocide enhancer for THPS at higher concentrations in the prevention of a *D. vulgaris* biofilm. D-tryptophan did not seem to be an effective biocide enhancer for THPS on an established *D. vulgaris* biofilm with a short exposure time.

The second part of this research was conducted to determine if there were any differences in the corrosion of three different strains (one wild type and two genetically-engineered) of *P. aeruginosa* grown as NRB. Additional testing was also done to determine if the addition of L-cysteine as an oxygen scavenger had any effect on the corrosion observed by the wild type. It was found that there was no significant difference between the three different strains in the corrosion that was observed on carbon steel. The removal of L-cysteine did not seem to impact the corrosion rate much, but there was a significant difference in the appearance of the cells on the carbon steel surface.
REFERENCES


Fu, W., 2013. Investigation of Type II of microbiologically influenced corrosion (MIC) mechanism and mitigation of MIC using novel green biocide cocktails.


