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A SEMI-CONTINUOUS SYSTEM FOR MONITORING MICROBIALLY INFLUENCED CORROSION

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A SEMI-CONTINUOUS SYSTEM FOR MONITORING MICROBIALLY INFLUENCED CORROSION

A THESIS APPROVED FOR THE
DEPARTMENT OF MICROBIOLOGY AND PLANT BIOLOGY

BY

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I would like to dedicate this work to my family for their unwavering support and constant encouragement in pursuing my dreams.

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Abstract

Microbially Influenced Corrosion (MIC), also known as biocorrosion, has significant impacts on the environment and economy. Typical systems to study biocorrosion are either dynamic (once-through flow) or static (serum bottle incubations). Dynamic systems can be materials, cost and personnel intensive, while static systems quickly become nutrient limiting and exhibit long incubations. A semi-continuous biocorrosion cell was developed to address these issues. Low carbon shim steel was used as a test surface. Initial results revealed that 50 ppm glutaraldehyde (GLT), a common oil field biocide, in an abiotic cell (24.5×10^{-3} mm/y) was 3.6 times more corrosive than a biocorrosion cell inoculated with a sulfate-reducing bacteria (SRB) enrichment (6.73×10^{-3} mm/y). The SRB inoculated cell treated with GLT reduced the corrosion rate from 6.73×10^{-3} mm/y to 3.68×10^{-3} mm/y. It was hypothesized that a biocide-surfactant combination would enhance a biocide's activity, lowering corrosion in a semi-continuous biocorrosion cell. The biocide and surfactant were GLT (30 ppm) and Tween 80 (TW80; 100 ppm). MIC increased in the presence of a non-inhibitory concentration of GLT (23.4×10^{-3} mm/y), compared to the untreated +SRB condition (8.29×10^{-3} mm/y). The non-ionic surfactant alone reduced MIC (4.57×10^{-3} mm/y) and even more in combination with GLT (3.69×10^{-3} mm/y). Approximately half of the 16S rDNA sequences in the biofilm on the test surface were identified as belonging to the genera *Desulfovibrio* and *Desulfomicrobium.* The utility of a semi-continuous system for MIC studies and biocide testing was demonstrated. The concept of regular partial medium replacement is applicable to different corrosion cell

and corrosion coupon geometries. Biocide-surfactant combinations may have the potential to reduce the concentration of biocide used in the field. Additionally, Acid-Producing Bacteria (APB) are important across many industries ranging from food microbiology to the oil and gas industry. In oil and gas fields, APB are cultured using a standard phenol red medium outlined by the American Petroleum Institute and NACE. In this study, enumerating APB from oilfield produced water was non-optimal using the medium outlined in the literature. To address this issue, a semi-defined medium for enumerating APB was developed, resulting in higher recoveries compared to a standard phenol red medium (e.g., 1.1×10^4 APB/cm^2 vs < 0.4 APB/cm^2).

Chapter 1: A Semi-Continuous System for Studying MIC

ABSTRACT

Microbially Influenced Corrosion (MIC), also known as biocorrosion, has significant impacts on the environment and economy. Typical systems to study biocorrosion are either dynamic (once-through flow) or static (serum bottle incubations). Dynamic systems can be materials, cost and personnel intensive, while static systems quickly become nutrient limiting and exhibit long incubations. A semi-continuous biocorrosion cell was developed to address these issues. Low carbon shim steel was used as a test surface. Initial results revealed that 50 ppm glutaraldehyde (GLT), a common oil field biocide, in an abiotic cell (24.5 x 10^{-3} mm/y) was 3.6 times more corrosive than a biocorrosion cell inoculated with a sulfate-reducing bacteria (SRB) enrichment (6.73 x 10^{-3} mm/y). The SRB inoculated cell treated with GLT reduced the corrosion rate from 6.73 x 10^{-3} mm/y to 3.68 x 10^{-3} mm/y. It was hypothesized that a biocide-surfactant combination would enhance a biocide's activity, lowering corrosion in a semi-continuous biocorrosion cell. The biocide and surfactant were GLT (30 ppm) and Tween 80 (TW80; 100 ppm). MIC increased in the presence of a non-inhibitory concentration of GLT (23.4 x 10^{-3} mm/y), compared to the untreated +SRB condition (8.29 x 10^{-3} mm/y). The non-ionic surfactant alone reduced MIC (4.57 x 10^{-3} mm/y) and even more in combination with GLT (3.69 x 10^{-3} mm/y). Approximately half of the 16S rDNA sequences in the biofilm on the test surface were identified as belonging to the genera *Desulfovibrio* and *Desulfomicrobium*. The utility of a semi-continuous system for MIC studies and biocide testing was demonstrated. The concept of regular partial medium replacement is applicable to different corrosion cell

and corrosion coupon geometries. Biocide-surfactant combinations may have the potential to reduce the concentration of biocide used in the field.

INTRODUCTION

Corrosion is a global, complex process influenced by a multitude of factors. The economic impact of corrosion accounts for 3% of the United States' gross domestic product (Koch et al., 2001). Corrosion affects many industries and municipalities, such as oil and gas, shipping and transportation, water systems and architecture (Kip and van Veen, 2015; Koch et al., 2001). This introduction focuses on corrosion in the oil and gas industry. The total cost of corrosion in the oil and gas industry is estimated at $1.37 billion annually (Simmons, 2008). Microorganisms are a leading cause of corrosion in the oil and gas industry, impacting a majority of oil infrastructure, including pipelines, storage tanks and separators (Duncan et al., 2009; Javaherdashti, 2017). The cost of microbially influenced corrosion (MIC) has been speculated ranging from 20% (Booth, 1964) to 50% (Flemming, 1996) of the total corrosion cost. Corrosion can lead to pipeline and storage tank failures, which result in a release of crude oil into the environment, significantly impacting environmental ecosystems, the economy and water supplies (Koch et al., 2001; Schmitt, 2009). In 2006 in Prudhoe Bay, Alaska, 212, 252 gallons of crude oil leaked onto the tundra (Meggert and Giguere, 2008). The final report stated that the above ground pipeline failure was a result of MIC (Fineburg, 2006). MIC prevention and mitigation protocols were not followed, resulting in this environmental disaster (Fineburg, 2006). The total cost of corrosion accounts for production shutdown, environmental damage, inventory loss and infrastructure repair and replacement (Kennard and McNulty, 1993).

Oilfield conditions typically select for anaerobic microorganisms since environment within oil and gas infrastructure is mostly anaerobic. A microbial community in the oil

field infrastructure can include *Clostridia*, *Deltaproteobacteria*, *Thermatogae* and *Synergistia* (Duncan et al., 2009; Duncan et al., 2014; Liang et al., 2014). Sulfate-reducing bacteria (SRB), thiosulfate-reducing bacteria (TRB), acid-producing bacteria (APB) and iron-reducing bacteria (IRB) contribute to MIC (Enning and Garrelfs, 2014; Javaherdashti, 2017). Most MIC studies focus on SRB and their role in iron corrosion, oil field souring and reservoir plugging (Beech and Sunner, 2007; Cord-Ruwisch et al., 1987; Enning et al., 2012). Sulfate-reducing microorganisms belong to two archaeal and five bacterial phylogenetic lineages (Muyzer and Stams, 2008). The most common bacterial phylogenetic lineage is the Deltaproteobacteria followed by the *Clostridia* and *Thermodesulfobacteria* (Muyzer and Stams, 2008). Other sulfidogenic bacteria regularly detected in the oil field include *Dethiosulfovibrio* and *Garciella,* TRB which belong to the class *Clostridia* (Magot et al., 1997; Miranda-Tello et al., 2003). SRB alone are not as corrosive as they are in consortium with other microorganisms (Booth et al., 1964). Mirobial communities are common in corrosion samples compared to single isolate populations. Vigneron et al. (2016) performed a molecular analysis on three biofilms collected from steel pipes exhibiting increased corrosion rates in the field. The authors found that most of the biofilm sequences consisted of *Desulfovibrio* species. However, other microorganisms such as *Pseudomonas, Pelobacter* and *Geotoga* were also detected. It is important to note the importance of a mixed microbial population in a biofilm and its impact on iron corrosion.

MIC is corrosion facilitated by the presence of microorganisms making kinetically unfavorable corrosive reactions favorable (Enning and Garrelfs, 2014). Corrosion of iron is an electrochemical process consisting of spatially separated anodic and cathodic

reactions (Beech and Gaylard et al., 1999; Enning and Garrelfs, 2014). SRB reduce sulfate to sulfide via anaerobic respiration. They utilize the fermentation products, such as lactate, acetate and H_2, of other microorganisms as electron donors (Beech and Gaylarde, 1999; Muyzer and Stams, 2008). In anoxic conditions, SRB can use electrons directly from the metal, sulfate, and organic matter to create corrosive species, such as hydrogen sulfide and hydrogen (Enning and Garrelfs, 2014). Some SRB with hydrogenases can remove cathodic hydrogen from the metal resulting in an increased corrosion rate at the anodic end, also known as cathodic depolarization, and produce hydrogen by the releasing excess electrons via hydrogenase enzyme (Enning and Garrelfs, 2014; Muyzer and Stams, 2008). The sulfide and hydrogen produced can cause stress cracking corrosion which is an abiotic process indirectly facilitated by SRB (Enning and Garrelfs, 2014). Corrosion can also be driven by acid reacting with the metal. For MIC to occur in a system, the following must be present: corroding microorganisms, an electron donor and acceptor, water and a susceptible surface (Javaherdashti, 2017). Microbes adhere to metal at the metal-water interface forming heterogeneous biofilms which enhance the rate of oxidation of the iron by influencing the chemistry surrounding the metals involved in oil infrastructure (Enning and Garrelfs, 2014; Hamilton, 1998; Javaherdashti, 2017, Lee and Newman, 2003). Rate of corrosion depends on the availability of nutrients in the system.

These microorganisms get into the oil facilities by techniques such as water flooding, secondary oil recovery, hydraulic fracturing, utilizing drilling muds and leaving wells stagnant (Fink, 2012). Many of these processes inject water from ponds and aquifer brine thereby also injecting these microbes. Additionally, many drilling

muds containing carbohydrates as a main ingredient providing an adequate food sources for bacteria (Fink, 2012). Sulfate and thiosulfate, common electron acceptors utilized by sulfidogenic bacteria, enter oil and gas systems either via the contaminated water or because they are major components of oilfield chemicals. Barium sulfate is a weighting agent used in drilling muds, and chromium sulfate and aluminum sulfate are common crosslinking agents (Fink, 2012). Sodium thiosulfate is a common gel thickening agent (Lukach and Zapico, 1995). In addition, carbonates and acetates are used as boundary lubricants (Fink, 2012). Awareness of the compounds and chemicals being used in the field is important as they can exacerbate biocorrosion in the presence of microbiology.

Preventing microbiology from becoming prevalent in oil and gas systems is done via a variety of methods. Preventing, mitigating and treating MIC in the oil and gas industry is achieved by mechanical, electrical and chemical processes. Coatings, special paints and polymers are used to prevent corrosion in addition to ensuring that there are no metal deformities or crevices the bacteria can stick to and cause problems. Mechanically, MIC is mitigated through a process called pigging. In this process, a PIG (Pipeline Inspection Gauge) is used to scrape out the inside of pipelines removing any biofilm or paraffin build up. Electrical processes include anodic or cathodic protection (Javaherdashti, 2017). This is a technique used to prevent bacteria, therefore biofilms, from adhering to the metal infrastructure. Chemical treatments of oil infrastructure include corrosion inhibitors or biocides (Javaherdashti, 2017). There are multiple classes of biocides and every class functions differently due to their different chemical structures. Commonly used biocides include GLT, benzalkonium chloride (BAC), cocodiamine, quaternary amine compounds (quat) and

tetrakis(hydroxymethyyl)phosphonium sulfate (THPS; Javaherdashti, 2017; Xue and Voordouw, 2015). Determining which biocide is best for the situation depends on multiple factors including pH and salinity of environment, temperature, sensitivities to light or other chemicals present in the system as well as toxicity and safety.

Glutaraldehyde (GLT) is a common biocide used in the oil and gas industry (Kahrilas et al., 2014). GLT is an advantageous biocide due to its broad spectrum of activity, its solubility, stability at a wide range of pH and salinities, and its biodegradability (Javaherdashti, 2017; Leung, 2001). GLT is used individually and in combination with other compounds such as quats and nitrites (Greene et al., 2006). The literature states that glutaraldehyde functions by cross-linking amino acids and proteins (Gorman et al., 1980). A preliminary biocorrosion study, using a low carbon steel test surface, revealed that 50 ppm GLT in an abiotic corrosion assay was 3.6 times more corrosive than those inoculated with a SRB enrichment (Appendix I). Biocides are generally not thought to cause corrosion but recent studies have focused on the effect biocides have on MIC and corrosion. One study found that most of the biocides tested promoted corrosion in the presence of bacteria despite their ability to inhibit microbial activity (Harris et al., 2010). The biocides that were most effective at lowering viable cell counts also had the highest average pitting rate (Harris et al., 2010).

Biocide combinations have been of interest in recent years. These "synergistic" combinations have been documented as being effective at lowering bacterial concentrations in the field. Some studies indicated the presence of a surfactant helped enhance the inhibitory activity of biocides (Gopi et al., 2000). Xu et al. (2012) tested the effects of D-tyrosine and THPS had on *Desulfovibrio vulgaris* biofilm and determined

that 100 ppm D-tyrosine alone and 50 ppm THPS alone had no impact on biofilm development. However, 50 ppm THPS in combination with 1 ppm D-tyrosine prevented the establishment of a *D. vulgaris* biofilm and sessile bacteria were undetectable (Xu et al., 2012). Another study determined the effect of Triton CF 54 and Triton DF 12, two non-ionic surfactants, on isothiazolone biocides (Williams, 2008). Williams (2008) determined that the biocides were more effective at decreasing viable cell counts in combination with surfactant. Synergistic combinations of biocides have been described in water treatment and the oil and gas industry (Haack and Greenly, 1991; Ludensky et al., 1998). Some Triton surfactants are listed by DOW as oil field chemicals used in drilling fluids, acidizing and stimulation, drilling cutting and well cleaning, and enhanced oil recovery (https://www.dow.com/surfactants/applications/other.htm). Common synergistic biocides are GLT/Quat combinations. The literature describes quaternary amines as cationic surfactants (Xue and Voordouw, 2015). Surfactants are compounds used to reduce interfacial and surface tension, typically as emulsifiers, detergents and dispersants commonly used in food, pharmaceutical, environmental, research, cosmetic, and oil and gas industries (Jayashree and Vasudevan, 2007; Torres et al., 2012). In the oil and gas industry, surfactants are used as surface protectants, corrosion inhibitors, oil dispersants and to enhance oil recovery (Abdallah and El-Etre, 2003; Hegazy et al., 2012; Javaherdashti 2017; Place et al., 2016; Youssef et al., 2009). Tween 80 (TW80), also known as Polysorbate 80, is the surfactant studied in this experiment. TW80 is a main component of the oil dispersant Corexit, used in the Gulf of Mexico after the Deepwater Horizon oil spill (Place et al., 2016). The Food and Drug Administration (FDA) approved TW80 as a solubilizing and dispersing agent in pickles

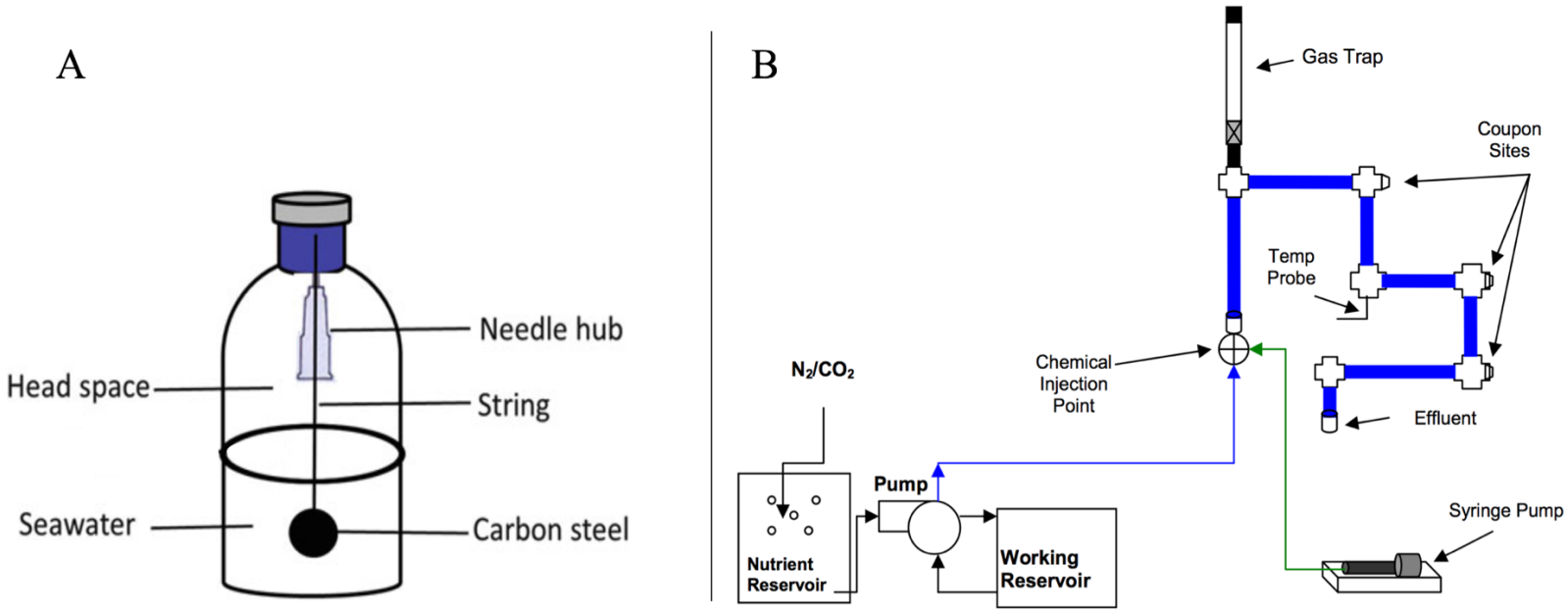

Figure 1. A) Diagram of a serum bottle incubation (Liang et al., 2015) B) Schematic of a once-through flow cell (Harris et al., 2010)

and vitamins, and as an emulsifier in ice cream and sherbet. TW80 has also been documented to reduce end-product inhibition of *Rhodococcus erythropolis* during desulfurization (Feng et al., 2006). TW80 is ideal for use in the oil and gas because it does not contribute to galvanic corrosion due to its ion neutrality, is biodegradable and deemed environmentally safe (Banin et al., 2006; Food and Drug Administration, 2017).

Two types of systems, dynamic and static, are commonly used for studying MIC in a laboratory setting (Harris et al., 2010; Liang et al., 2015; Figure 1). An example of a dynamic system is the once-through flow cell (Duncan et al., 2014; Harris et al., 2010; Stipanicev et al., 2013; Tanner, 1985). Dynamic systems allow for a more accurate modeling of pipeline corrosion. Due to the constant flow of nutrients and bacteria in brine, dynamic systems can be materials and personnel intensive. This system can examine multiple test surfaces simultaneously and is constantly flowing with medium and inoculum with a typical incubation time of 30 days. One study examined the two corrosion inhibitors (inhibitor A and inhibitor B) for their impact on corrosion and the microbial community of corrosion cells (Duncan et al., 2014). Overall, it was concluded that pitting rates decreased in the presence of both corrosion inhibitors although pit morphology varied based on the inhibitor (Duncan et al., 2014). Also, corrosion inhibitors differentially impacted microbial populations (Duncan et al., 2014). A serum bottle incubation is an example of a static system (Liang et al., 2015). All components are initially added into the bottle with incubations ranging from 90 days to over 300 days (Cote et al., 2013; Liang et al., 2015). Long incubations can lead to nutrient depletion which can greatly reduce bacterial activity, including MIC, within a system.

One study used a serum bottle incubation to determine corrosion rates of pigging samples. This study showed the abiotic control as having the highest corrosion rate of 0.95 mm/y compared to the biotic sample 1 and sample 5 with corrosion rates of 0.18 mm/y and 0.06 mm/y (Cote et al., 2013). The authors explained this unexpected result as being a result of organic compounds in the medium reacting with the corrosion test surface. A semi-continuous system for studying MIC was developed to address the issues addressed above, such as high background corrosion values, nutrient depletion and cost of materials, equipment and personnel. In Tanner et al. (1985), greater than 500 L of media per individual test loop was used in a four-week incubation. Typically, eight loops were run simultaneously. In Harris et al. (2010), the starting volume in the working reservoir (bioreactor - $10,000; Figure 1B) was 20 L of inoculated medium, which was constantly being replenished to prevent nutrient depletion and flushed with N_2/CO_2 to maintain an anoxic environment. However, in a semi-continuous system each test cell would require less than 200 ml of media and the replacement of gas tanks multiple times would not be necessary. If the Harris et al. (2010) study was replicated in triplicate, only 5 L of media would be used for the entirely of the experiment.

After corrosion studies, like the ones described in the previous paragraph, are conducted, the metal is analyzed both quantitatively and qualitatively. The easiest analysis indicating corrosion is weight loss. The weight loss of the test surface is determined after the biofilm is scraped off the surface and corrosion rates are calculated. Other quantitative measurements include profilometry, linearized polar resistance and electrochemical impedance spectroscopy (Cote et al., 2013, Liang et al., 2017; Mansfield and Little, 1991). Qualitative analyses to measure corrosion include scanning

electron microscopy and visual observations of metal deformities (Beech, 2004). Many of these surface analyses are specialized, expensive and require training. The cost of profilometer is $60,000. A goal of this research is to develop a method for studying corrosion in which the analyses focus solely on weight loss and observation to reduce the cost of analysis.

Purposes of this experiment were to determine the utility of a semi-continuous system to study MIC and to determine whether the presence of a surfactant enhanced biocide activity. It was hypothesized that, in a semi-continuous biocorrosion test cell, TW80 would enhance the biocidal activity, resulting in reduced MIC.

MATERIALS AND METHODS

Inoculum and Bacterial Enumeration

SRB were enriched in 50 ml of SRB medium (Tanner 1989; Tanner 1996) in 125 ml serum seal bottles containing 1 g of powdered iron, prepared anaerobically under N_2 gas (Balch et al., 1976). Enrichments were inoculated with 0.5 ml of sediment collected from the Duck Pond located at the University of Oklahoma in Norman, OK. Enrichments were incubated for two weeks at 38˚C.

Most probable number technique (3 tube; Banwart, 1981) was used to determine planktonic and biofilm counts for SRB and General Heterotrophic Bacteria (GHB). MPN medium was SRB medium and the GHB MPN medium was 1/3 strength Tryptic Soy Broth (no. 211825, Becton Dickinson and Co., Sparks, MD). Medium was prepared anaerobically in Hungate type tubes (no. 2047-16125, Bellco Glass, Inc., Vineland, NJ). Enumerations were incubated for 28 days before a final count was taken.

A Semi-Continuous Biocorrosion Testing System

Low carbon steel shim (no. 16130, Precision Brand Products, Inc., Downers Grove, IL) was used as the test surface. Test surfaces were handled on clean aluminum foil, using forceps and gloves, and cut into appropriate size (10 cm x 1.5 cm). The test surface was rinsed and cleaned with acetone twice and dried for 10 minutes in a drying oven at 100˚C. Initial weights were recorded and test surfaces were placed inside of a 25 mm Pyrex® screw cap tubes (no. S76112G, Fisher Science Education) and taken into an anaerobic chamber (Coy Laboratories Products, Inc., Grass Lake, MI). Tubes were sealed with a no. 2 stopper (no. 14-30D, Fisher Science Education) and removed from the chamber. Stoppers were cut flush with the tube top and secured with screw caps containing pre-drilled holes. Corrosion cells were gas exchanged under N_2 (Balch et al., 1976) and sterilized.

The medium used in the corrosion cells was a modified SRB medium (Tanner, 1996) designed to promote biofilm formation (/liter): 50 ml SRB feeding mineral solution; 5 ml vitamin solution (Tanner, 2007); 3 ml trace metal solution (Tanner, 2007); 1 g TES; 1.5 ml 60% sodium lactate; 0.5 g yeast extract (no. 212750, Becton Dickinson and Co., Sparks, MD); pH 7.3-7.5. SRB feeding mineral solution contained (/liter): 90 g NaCl; 10 g Na_2SO_4; 5 g $(NH_4)_2SO_4$; 4 g $MgSO_4 \cdot 7H_2O$; 6 g KH_2PO_4; 0.5 g $CaCl_2 \cdot 2H_2O$. Forty ml of sterile SRB feeding medium was aseptically added to each corrosion cell. One ml of inoculum from the SRB enrichment was added to each biotic corrosion cell. Glass syringes were used (no. 5292, 5293 and 5294, Becton Dickinson and Co., Rutherford, NJ) to add medium, biocide and inoculum. Corrosion cells were

incubated at 30˚C for seven weeks. Five ml was removed from each biocorrosion cell daily and replaced with 5 ml of SRB feeding medium.

Treatments began one week after the start of incubation, during which biofilms developed in the inoculated biocorrosion cells. The biocide used was 30 ppm GLT and the surfactant used was 100 ppm TW80. Treatment conditions with and without SRB inoculum included: untreated; +GLT; +TW80; + GLT+TW80. Treatments were performed in triplicate. Biocide and surfactant amendments adjusted to 30 and 100 ppm final concentrations, respectively, at each feeding.

Final Processing of Corrosion Cells and Corrosion Evaluation

Corrosion cells were disassembled inside of a Coy chamber after final planktonic counts were taken after seven weeks. The test surface was suspended in 20 ml of sterile, anoxic 1% NaCl. Biofilm was mechanically removed from the test surface into a sterile reservoir (no. 13-681-502, Fisher Science Education) using a sterile plastic spatula. Biofilm recovery from the low carbon steel surface was easier compared to recovery from a carbon steel surface (data not shown). The 1% NaCl with biofilm were collected into a sterile serum seal tubes (no. 2048-00150, Bellco Glass, Inc., Vineland, NJ) containing 6 mm Pyrex beads (no. 11-312010D, Fisher Science Education). The tubes were then sealed with a sterile butyl rubber stopper (no. CLS-4209-14, Chemglass Life Sciences, Vineland, NJ), removed from the chamber and crimp-sealed (no. 06-406-14B, Fisher Science Education).

Cleaning of the test surfaces included washing with 4% citric acid and gently scrubbing using a plastic scouring pad, twice, followed by a final citric acid rinse and two anoxic water rinses (ASTM International, 2011; Rajagopal and LeGall, 1989). Test surfaces were thoroughly dried with a Kimwipe before their removal from the chamber to prevent the surfaces from rusting once exposed to air. Outside of the chamber, the test surfaces were rinsed with ethanol and acetone, and allowed to sit in a drying oven for 10 min at 100˚C prior to taking final weight loss measurements. Weight loss was used to calculate general corrosion rates in millimeters per year (mm/y) following ASTM Standards (G1-03, 2011) protocol. A two-tailed, two-sample equal variance Student's t-test was performed on all conditions with significance indicated as $p < 0.05$.

Analyses

Sulfide, pH and ferrous iron levels were measured bi-weekly. A CHEMets sulfide test kit (no. K-9510, Midland, VA), pH strips (no. 1.09543.0007, colorpHast, EMD Millipore Corporation, Billerica, MA) and Eisen-Test strips (no. 1100040001, EMD Chemicals, Gibbstown, NJ) were used. Glutaraldehyde assays were performed daily using a glutaraldehyde test kit (no. 25872-00, Hach Company, Loveland, CO) to ensure GLT concentrations were at 30 ppm post feeding.

DNA was extracted from a pelleted untreated SRB biofilm (BF) sample obtained after coupon cleaning of each replicate using MoBio PowerSoil Kit (MoBio Laboratories, Carlsbad, CA) following manufacturer protocol. Primers S-D-Arch-O519-a-S-15 and S-D-Bact-0785-b-A-A18 were used for 16S rDNA amplification (Klindworth et al., 2012). All three samples were tagged with a unique barcode and sequenced on an Illumina MiSeq platform using pair-end 600V3 at Oklahoma Medical Research Foundation in Oklahoma City, OK (Hamady et al., 2008). Paired-end reads were joined together after sequencing and demultiplexed in QIIME (Quantitative Insights Into Microbial Ecology) software package (Caporaso et al., 2010). Chimeras were removed before Operational Taxonomic Units (OTUs) were assigned at 97% similarity via the USEARCH algorithm (Edgar, 2010). Taxonomic identity was determined using 16S rDNA small subunit sequences from the SILVA ribosomal RNA database (Pruesse et al., 2007).

RESULTS

Bacterial Enumeration, Sulfide, Glutaraldehyde and pH

Planktonic counts for SRB and General Heterotrophic Bacteria (GHB) were measured at time zero (T_0) and averaged 7.3 x 10^4 SRB/ml and 9.1 x 10^2 GHB/ml. Abiotic counts were below the limit of detection for both SRB and GHB (< 4 x 10^{-1} cell/ml) for T_0 and time final (T_f). In all conditions tested, SRB biofilm cell counts decreased compared to the untreated cells ($p < 0.05$; Table 1). Reductions in planktonic SRB and GHB counts were not detected in the conditions tested when compared to the untreated control, except for the planktonic GHB counts treated with the biocide-surfactant combination. Sulfide was not detected in the abiotic tubes. Higher sulfide levels were observed in the +SRB+TW80 treatment compared to the untreated cells (Table 2). The pH for all the corrosion cells started at 7.4. The pH decreased in tubes treated with +GLT and +TW80 to 7.1 and 6.5 in the biocide-surfactant combination tubes (Table 2). Abiotic tube pH was between 7.1 and 7.4 throughout the experiment. Ferrous iron levels were detected in all abiotic cells at 10 ppm. In the final week of incubation, ferrous iron was detected at 3 ppm in biotic tubes treated with GLT and not in any other conditions.

Table 1. T_f SRB and GHB biofilm and planktonic viable cell counts for all treatments [a].

Condition	T_f Planktonic Count (cells ml^{-1})		T_f Biofilm Count (cells cm^{-2})	
	SRB	GHB	SRB	GHB
Untreated	6.7×10^7	$> 1 \times 10^7$	6.9×10^7	6.9×10^6
+GLT	$> 9 \times 10^6$	$> 9 \times 10^6$	$> 7 \times 10^6$	7.0×10^6
+TW80	1.5×10^7	5.4×10^7	$6.0 \times 10^{6\,*}$	$2.7 \times 10^{6\,*}$
+GLT+TW80	2.0×10^7	$2.7 \times 10^{6\,*}$	$1.7 \times 10^{6\,*}$	$1.6 \times 10^{6\,*}$

[a] Mean values. * Indicated statistically significant values when compared to the untreated ($p < 0.05$). All tests were conducted in triplicate. GLT and TW80 concentrations were at 30 ppm and 100 ppm, respectively.

Semi-Continuous System and Corrosion Rates

In Table 2, weight loss of the +SRB test surfaces increased from an average of 18.8 mg in the untreated, inoculated controls to 70.8 mg for the +SRB+GLT (Table 2). In the presence of 100 ppm TW80, the biocorrosion coupons had an average weight loss of 13.8 mg (Table 2). Biocorrosion cells with +SRB+TW80 and +SRB+GLT+TW80 had less weight loss than the +SRB. The +SRB+GLT was 2.8 times more corrosive than the untreated +SRB. The +SRB+GLT biocorrosion test cells had the most corrosion, with a corrosion rate of 23.4×10^{-3} mm/y (Fig. 1). Within the abiotic treatments, -SRB+GLT was the most corrosive with a corrosion rate of 5.20×10^{-3} mm/y. In this study, -SRB+GLT was just as corrosive as the +SRB with statistically the same corrosion rates ($p > 0.05$). Within each condition, biotic corrosion rates were higher compared to abiotic corrosion rates ($p < 0.05$). When compared, all datasets were determined to be statistically significant from each other except when comparing the abiotic (-SRB) and biotic (+SRB) values for cells treated with TW80 and with GLT+TW80 ($p = 0.235$ and $p = 0.201$, respectively). There was no difference in corrosion rates in the biocorrosion cells treated with the surfactant or the biocide-surfactant combination.

All corrosion cells inoculated with the SRB enrichment turned black after two days due to iron sulfide production. All test surfaces inoculated with SRB when removed from the corrosion cells were black, covered with biofilm and iron sulfide. After cleaning, the test surfaces were visually examined and the test surfaces from +SRB+GLT treatment were observed to have significant metal loss, including the formation of large holes with surface areas ranging from 4.0 to 4.5 cm^2 (Fig. 2). Smaller holes measured from 0.3 cm to 0.5 cm in diameter. Figure 2 shows a large hole

with approximately a 4.0 cm^2 surface area loss and a smaller hole measuring 0.5 cm in diameter. All +SRB test surfaces corroded along the edges and appeared to have lost their metallic sheen. The untreated abiotic (-SRB) test surfaces appeared to have little physical damage and maintained their metallic sheen.

Table 2. Test surface average weight loss (mg), pH and sulfide levels (ppm)

Condition	- SRB Weight loss (mg)	+ SRB Weight loss (mg)	pH	Sulfide (ppm)
Untreated	9.57 ± 1.74	18.8 ± 2.40	7.4	100
+GLT	15.7 ± 4.96	70.8 ± 3.82	7.1	30
+TW80	3.97 ± 0.42	13.8 ± 3.26	7.1	120
+GLT+TW80	4.50 ± 0.96	11.2 ± 2.70	6.5	80

- SRB is abiotic; + SRB is biotic. GLT and TW80 concentrations were at 30 ppm and 100 ppm, respectively.

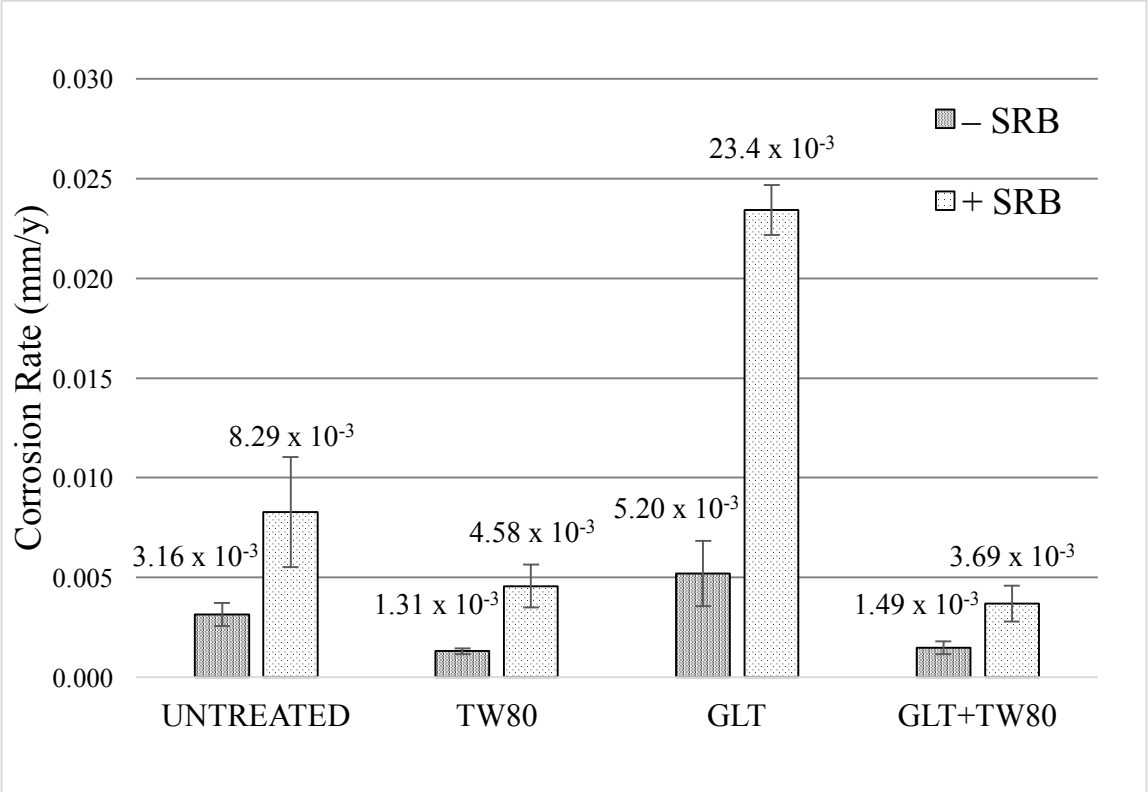

Figure 2. Average corrosion rates in millimeters per year (mm/y) for all conditions. - SRB is abiotic; + SRB is biotic. GLT and TW80 concentrations were at 30 ppm and 100 ppm, respectively.

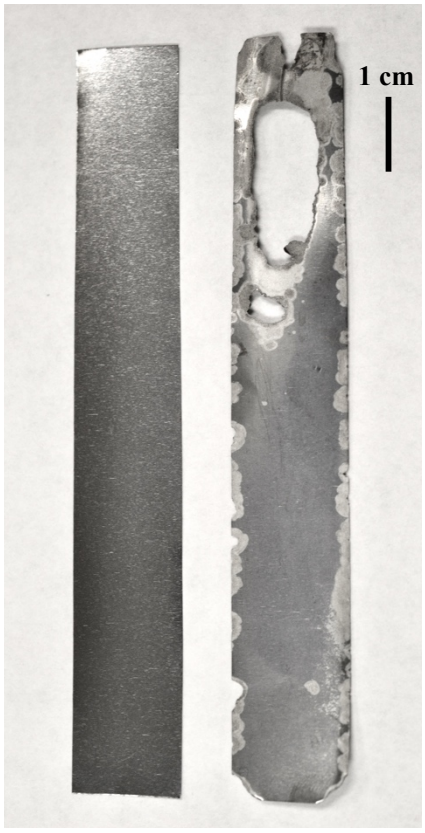

Figure 3. Low-carbon steel coupons from an abiotic untreated (–SRB) biocorrosion cell (left) and a biotic glutaraldehyde treated (+SRB+GLT) cell.

Biofilm Community Analysis

Five class level lineages were detected from three untreated SRB biofilms (BF) above 1% with Delta-proteobacteria being the most dominant (55.0% in SRB BF 1, 47.7% in SRB BF 2 and 66.1% in SRB BF 3), followed by Clostridia (13.2% in SRB BF 1, 18.3% in SRB BF 2 and 10.7% in SRB BF 3), Synergistia (13.1% in SRB BF 1, 14.7% in SRB BF 2 and 7.7% in SRB BF 3), Bacteroidia (12.6% in SRB BF 1, 13.8% in SRB BF 2 and 8.2% in SRB BF 3) and Alpha-proteobacteria (1.5% in SRB BF 1, 1.2% in SRB BF 2 and 1.4% in SRB BF 3; Table 3). The molecular analysis of the untreated SRB BF 1, 2 and 3 indicated 58, 59 and 61 operational taxonomic units (OTUs) at the genus level, respectively. Prominent genera identified in the all three SRB BF include: *Desulfovibrio* and *Desulfomicrobium* belonging to the Delta-proteobacteria class; *Parabacteroides* belonging to Bacteriodales class, and unidentified genera within the families *Peptostreptococcaceae* or *Synergistaceae*. Twenty-four OTUs at the genus level were at an abundance lower than 1% in SRB BF 1, 23 OTUs in SRB BF 2 and 24 OTUs in SRB BF 3.

Table 3. 16S rDNA libraries depicting relative abundance of microbes in the three untreated SRB biofilms.

	Relative Abundance - %		
Class	SRB BF 1	SRB BF 2	SRB BF 3
Deltaproteobacteria	55.0	47.7	66.1
Clostridia	13.2	18.3	10.7
Synergistia	13.1	14.7	7.7
Bacteroidia	12.6	13.8	8.2
Alphaproteobacteria	1.5	1.2	1.4
Other	- [a]	- [a]	- [a]

[a] 11 OTUs at the class level with less than 1% relative abundance.

DISCUSSION

A goal was to assess the utility of a semi-continuous system for monitoring and studying MIC. Typically, medium to high carbon steels (C1018, C1080, respectively) are used as corrosion test surfaces in corrosion studies (Abd-El-Nabey et al., 2016; Cote et al., 2013; Liang et al., 2014; Liang et al., 2017). This corrosion cell used a low carbon shim steel with a C1008/C1010 carbon rating meaning that the carbon content ranges from 0.08 to 0.1% of the steel's mass (ASTM International, 2016). Corrosion was measured absent the need for specialized equipment such as profilometry, linearized polar resistance and scanning electron microscopy, techniques sometimes used for corrosion analysis (Cote et al., 2013, Liang et al., 2017; Mansfield and Little, 1991). After seven weeks, significant destruction of test surfaces was observed on the +SRB+GLT corrosion cells. The amount of GLT utilized for this treatment was not enough to control bacterial activity in the system. GLT is used in the field at concentrations ranging from 50 ppm to 2,500 ppm (McGinley, et al. 2011; Morris and van der Kraan, 2017). Glutaraldehyde is categorized as a non-oxidizing biocide, thus expecting it to contribute minimally to corrosion (Fink, 2012). However, this research found it to exacerbate corrosion. This could be explained by GLT's electrophilic chemistry (Uhr et al., 2013).

It is recommended to only treat pipelines when SRB or bacteria counts are indicated as high or dangerous. Preventative treatment using GLT could result in adverse effects and increased corrosion rates. Additionally, when treating, it is crucial that appropriate biocide concentrations are maintained for pipelines, storage tanks and oil/water/gas separators and are not treated at lower concentrations than desired to prevent increasing

corrosion rates. A study suggested that pipelines should be treated with higher concentrations of GLT to ensure that GLT is not completed degraded before reaching the terminal end of the pipeline (McGinley et al., 2011). Pipeline flow rate would be an important factor in determining biocide concentrations. As mentioned earlier, an abiotic system with only GLT at 50 ppm was more corrosive than an untreated +SRB biocorrosion cell (Appendix I).

The presence of a surfactant reduced the overall corrosion in the system (Fig. 1). The TW80 and GLT individually and in combination reduced biofilm activity, protecting the test surface. The corrosion rates for the TW80 treatment were not statistically different ($p > 0.05$) from the +GLT+TW80 combination. The addition of a surfactant alone did not enhance the efficacy of the biocide since viable cell counts were greater than 10^6 cell/ml but possibly prevented further development of any existing biofilm. These results care similar to those found by Nielson et al. (2016). In their study, they found that the addition of 1,000 ppm TW80 prior to biofilm development prevented bacterial adhesion to abiotic surfaces. However, if the medium was amended with TW80 after biofilm development, the TW80 inhibited any further development (Nielsen et al., 2016). This may raise the presumption that surfactant addition alone can be used as an alternative method of corrosion control. The idea of using solely a surfactant as a means of controlling corrosion in place of a biocide is risky. TW80 affects microorganisms differently and is not always inhibitory. TW80 inhibited the growth of *Listeria monocytogenes* and *Pseudomonas fluorescens* but stimulated the growth of *Staphylococcus aureus* (Nielsen et al., 2016). Bacteria which possess lipase LipA, such as *Pseudomonas aeruginosa* and *S. aureus*, can cleave Tween 80 at the ester

bond into oleic acid and an alcohol, inactivating the surfactant and allowing the cleavage products to be carbon sources (Banin et al., 2006; Kerwin, 2008; Toutain-Kidd et al., 2009).

The population profile identified within the SRB biofilm enrichment used in this study are similar to population profiles observed in other anaerobic environments. As expected, the majority of bacteria present in the enrichments were SRB, *Desulfovibrio* and *Desulfomicrobium* (Fig. 3). These results are similar to that of Vigneron et al. (2016), which demonstrated that *Desulfovibrio* dominated the microbial community in biofilms found on an offshore oil production facility. Although sequences belonging to the classes Synergistia and Clostridia were not identified on a genus level, many genera within those classes have been found in oil fields (Dahle et al., 2008; Duncan et al., 2014; Orphan et al., 2000; Pham et al., 2009). Some bacteria belonging to the class Synergistia are sulfide producers, such as *Thermovirga,* isolated from an oil well in the North Sea (Dahle and Birkeland, 2006) and *Dethiosulfovibrio,* isolated from a corroding offshore oil well (Magot et al., 1997). Genera within the class Clostridia include *Thermoanaerobacter* and *Garciella*, which are common oil field isolates (Cayol et al., 1995; Miranda-Tello et al., 2003).

The concept of regular partial medium replacement is applicable to different corrosion cell and corrosion coupon geometries. Biocide-surfactant combinations may have the potential to reduce the concentration of biocide used in the field. Further studies should test biocide-surfactant combination at varying concentrations against an oilfield enrichment for MIC studies. In conclusion, this study successfully demonstrated

the utility of a semi-continuous system and documented that the presence of surfactant reduced corrosion.

Chapter 2: Optimization of Acid-Producing Bacteria Medium

ABSTRACT

Acid-Producing Bacteria (APB) are important across many industries ranging from food microbiology to the oil and gas industry. In oil and gas fields, APB are cultured using a standard phenol red medium outlined by the American Petroleum Institute and NACE. In this study, enumerating APB from oilfield produced water was non-optimal using the medium outlined in the literature. To address this issue, a semi-defined medium for enumerating APB was developed, resulting in higher recoveries compared to a standard phenol red medium (e.g., 1.1×10^4 APB/cm^2 vs < 0.4 APB/cm^2).

INTRODUCTION

Acid-producing bacteria (APB) are fermentative microbes found in oil field infrastructure, anaerobic digesters, soil, sediment, human microbiota and fermented foods. APB can cause corrosion, in addition to facilitating SRB corrosion (Soracco et al, 1988), thereby the enumeration and cultivation of APB from a system is important for detecting potential MIC problems (Javaherdati, 2017). APB have been isolated from oil and gas fields (Dahle et al., 2008; Duncan et al., 2014; Orphan et al., 2000; Pham et al., 2009). Duncan et al. (2014) determined that environmental communities from oil field samples in an experimental flow loop system shifted from being dominated by Gamma-proteobacteria to Clostridia, Beta-Proteobacteria and Gamma-Proteobacteria in the presence of corrosion inhibitors. As mentioned in Chapter 1, corrosion inhibitors are commonly used in oil and gas fields and increasing evidence has documented their impact on shifting microbial communities towards APB rich populations (Duncan et al., 2014). An increase in pitting rates have been attributed to APB under low sulfate conditions (Gu, 2012). Gu (2012) concluded that with techniques such as water flooding becoming more prevalent, the threat for failures in water-injection pipelines and water-wetted oil transport pipelines increases as a result of MIC pitting caused by APB. Field monitoring tests for enumerating and detecting these bacteria are routine and important as they can bring light to corrosion problems. The accuracy of these methods is important.

Some goals of a prior study, which led to this work, included enriching for, enumerating and isolating APB from produced water samples from oil fields in south Texas. Molecular surveys were performed on all samples and indicated the presence of

APB in most of the samples. APB enrichments using a standard phenol red medium were established for samples determined to be positive for APB via molecular analysis. These initial APB enrichments were unsuccessful. This led to the optimization of the medium and the development of a semi-defined APB medium. APB were enriched and isolated from these oil field samples using this semi-defined medium. *Clostridium sporogenes* and *Garciella* sp. were two APB isolated from the production water.

A semi-defined medium (RST-APB) including a mineral solution, vitamin solution and trace metal solution was developed for improved enumeration of APB. Common APB media contain peptone and beef extract as major medium components (American Petroleum Institute, 1982; NACE Standard, 2014). The undefined medium components were reduced in the semi-defined APB medium developed here.

MATERIALS AND METHODS

The most probable number (MPN) technique (Banwart, 1981) was used to enumerate APB from six samples. Two samples (Duck Pond (Norman, OK) and oil/water/gas separator A) were tested under low NaCl conditions (0.1% NaCl), and four of the samples (oil/water/gas separator B, biofilm mixed culture, planktonic mixed culture and *Garciella* sp.) were tested at 4% NaCl (Table 4). Standard phenol red medium (RP-38) contained (/liter): 0.5 g beef extract; 2 g peptone; 5 g glucose; 18 mg phenol red, pH 7.0 (American Petroleum Institute, 1982; NACE Standard, 2014). RST-APB medium was made with (/liter): 10 ml mineral solution (Tanner, 2007); 10 ml vitamin solution (Tanner, 2007); 5 ml trace metal solution (Tanner, 2007); 5 g glucose; 2 g yeast extract; 18 mg phenol red; pH 7.0. MPNs were incubated for 28 days at 30˚C.

RESULTS

Viable cell counts using the APB-RST medium were greater than or equal to counts using the RP-38 phenol red medium. Viable cell counts were below the limit of detection with counts $< 4 \times 10^{-1}$ cells/ml in three of the six samples using a standard phenol red medium (Table 4). In those same samples, APB were recovered using the semi-defined RST-APB medium. APB from a planktonic mixed culture were recovered above the limit of detection at $> 1 \times 10^{7}$ cell/ml using RST-APB medium, whereas only 2.3×10^{1} cells/ml were recovered using a standard phenol red medium (Table 4; Figure 4).

Table 4. Samples tested, with salinity (% - w/v), and viable cell counts.

Sample	NaCl	Viable Cell Count (cells/ml) RST-APB	RP-38
Duck Pond water	0.1%	4.6×10^{4}	4.6×10^{4}
Oil/water/gas separator A	0.1%	1.5×10^{3}	4.6×10^{2}
Oil/water/gas separator B	4.0%	2.3×10^{1}	$< 4 \times 10^{-1}$
Biofilm mixed culture [a,b]	4.0%	3.2×10^{4}	$< 4 \times 10^{-1}$
Planktonic mixed culture [a]	4.0%	$> 1 \times 10^{7}$	2.3×10^{1}
Garciella sp.	4.0%	4.6×10^{5}	$< 3 \times 10^{-1}$

[a] Mixed culture contained *Garciella sp.*, *Desulfovibrio alaskensis* and *Staphylococcus epidermidis*

[b] Viable cell count in cells/cm^2

Figure 4. APB media comparisons. On the left is the standard phenol red medium and on the right, is the RST-APB medium. Red indicates no growth. Yellow or clear indicates growth.

DISCUSSION

APB can impact many industries, such as food microbiology and the oil and gas industry, therefore an optimized medium for recovering APB was developed. Most MIC may be attributed to SRB, but preliminary biocorrosion screenings indicated that an APB oil field isolate was more corrosive (9.2×10^{-3} mm/y; 3.8×10^{-3} mm/y) than a SRB oil field isolate (1.1×10^{-3} mm/y; Appendix II). In the biofilm community analysis described in Chapter 1, 10.7% to 18.3% of the sequences in the untreated SRB biofilms were identified as belonging to *Clostridia* and many genera within the class are classified as APB. This semi-defined medium generally recovered higher counts than the APB medium that is commonly used (Table 4). Use of a standard phenol red medium may result in false negatives in APB enrichment and enumeration. In this study, false negatives were observed in APB enumerations indicated as being below the limit of detection ($< 4 \times 10^{-1}$ cell/ml; Table 4) when using the RP-38 standard medium. The semi-defined medium described reduced the possibility of false negatives. In conclusion, this study successfully documented the optimization of APB recovery using a semi-defined medium.

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Appendix A

Corrosion rates of SRB treated with 50 ppm GLT in a 5 week preliminary study

Condition	Corrosion Rate (mm/y)
-SRB	0.18×10^{-3}
-SRB+GLT	24.5×10^{-3}
+SRB	6.73×10^{-3}
+SRB+GLT	3.69×10^{-3}

- SRB is abiotic; + SRB is biotic

Appendix B

Corrosion rates of single strains APB and SRB isolated from produced water.

Isolate	Corrosion Rate (mm/y)
Clostridium sporogenes (APB)	9.2×10^{-3}
Garciella sp. (TRB/APB)	3.8×10^{-3}
Desulfovibrio alaskensis (SRB[a])	1.1×10^{-3}
Desulfovibrio alaskensis G20 (SRB[b])	9.7×10^{-4}

[a] SRB isolated from a bulk separator in the field.
[b] SRB lab strain