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Enhanced Mitigation of Pipeline Biocorrosion Using A Mixture of D-Amino Acids with A Biocide - DTPH56-13-H-CAAP08

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On

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by

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Preface

Pipeline corrosion is a serious threat to pipeline integrity and safety. Corrosion can lead to leaks of pipeline fluids that harm the environment and cause explosion harzards. Financial losses can be very severe from delivery interruptions and repairs. Biocorrosion, also known as Microbiologically Influenced Corrosion (MIC), amounts to 20% of all corrosion losses. In the 2006 Alaska pipeline leak due to a ¼" pinhole, MIC was a primary suspect. The leak led to a major spike in world oil prices. Production at Prudhoe Bay was disrupted for serveral months and the financial losses reached many millions of dollars including 20 million dollars in government fines.

Biofilms cause MIC and thus MIC mitigation is about biofilm treatment. Sessile cells in biofilms are notoriously far more difficult to treat than planktonic cells because biofilms employ various defense mechanisms, including diffusional limitation, lowered metabolic rate to reduce intake, formation of persistent cells, upregulation of resistance genes, efflux pumps, etc. Biofilms usually require ten times or higher biocide concentrations to treat than planktonic cells. Recently, D-amino acids were found to be biocide enhancers in biofilm mitigation. All bacterial cell walls contain D-alanine terminus in their peptidoglycan molecules. Its substitution by other D-amino acids such as D-tyrosine is suspected to trigger the dispersal of bacterial biofilms such as those of *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. It was discovered by our group that the biofilm dispersal signalling is not effective for recalcitrant biofilms such as the corrosive *Desulfovibrio vulgaris* (a sulfate reducing bacterium) biofilm formed on a carbon steel surface. A biocidal stress in the form of a biocide such as 50 ppm (w/w) of tetrakis hydroxymethyl phosphonium sulfate (THPS) is required to "convince" the biofilm to disperse. D-amino acids are naturally occurring. They are found in animals and humans. A significant fraction of L-amino acids is converted to D-amino acids when food products are heat processed. Laboratory experiments have found that D-tyrosine, D-leucine, D-tryptophan, and D-methionine are effective biocide enhancers. They are effective at ppm dosages when combined with THPS in the prevention of biofilm establishment and removal of established biofilms using pure-stain *D. vulgaris* biofilm as a model.

This project investigated various D-amino acids as biocide enhancers to mitigate biofilms that cause MIC. Experimental tasks were finished to evaluate these as well as additional D-amino acids in combination with THPS (one of the most popular biocides) to treat field biofilm consortia. Field biofilms were used to validate the new environmentally friendly biocide enhancer technology.

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List of Abbreviations

APB	Acid Producing Bacteria
CLSM	Confocal Laser Scanning Microscope
D-ala	D-alanine
D-asn	D-asparagine
D-asp	D-aspartic acid
D-glu	D-glutamic acid
D-his	D-histidine
D-leu	D-leucine
D-met	D-methionine
D-phe	D-phenylalanine
D-ser	D-serine
D-thr	D-threonine
D-trp	D-tryptophan
D-tyr	D-tyrosine
D-val	D-valine
EDDS	Ethylenediamine-N,N'-disuccinic Acid
EDTA	Ethylenediaminetetraacetic Acid
GHB	General Heterotrophic Bacteria
L-tyr	L-tyrosine
MIC	Microbiologically Influenced Corrosion
MPN	Most Probable Number
m-RNA	Messenger RNA
NRB	Nitrate Reducing Bacteria
PBS	Phosphate Buffered Saline

SEM	Scanning Electron Microscope
SRB	Sulfate Reducing Bacteria
THPS	Tetrakis Hydroxymethyl Phosphonium Sulfate
TRB	Thiosulfate Reducing Bacteria

1 Introduction

Biocorrosion, also known as microbiologically influenced corrosion (MIC), is a major problem in the oil and gas industry, as well as other industries such as water utilities. Even stainless steel is not immune to MIC. MIC accounts for 20% of all corrosion of metals and building materials according to Flemming [1]. Walsh et al. estimated MIC damages at $30 - 50 billion per year in the US alone [2]. The well-known 2006 Alaska pipeline leak was caused by a ¼" pinhole that was likely to the result of MIC [3]. The leak caused a spike in world oil prices. MIC is becoming more and more important because infrastructures are aging and enhanced oil recovery is practiced more often than ever. When injected water is pumped out the reservoir with oil, oil wetting of the pipe wall may switch to water wetting if there is a large fraction of water. Water-wetting increases the MIC threat greatly compared with oil-wetting and intermittent water-wetting because water allows a much larger variety of microbes to flourish on pipe walls. Due to depleting reserves, previously unproductive reservoirs are still in production by increasing well pressure that is done using water or CO_2 injection (also known as flooding). Most often, seawater is used due to scarcity of fresh water. Seawater contains nutrients for microbial growth. It also contains bacteria such as sulfate reducing bacteria (SRB) and other organisms. Bacteria may already be in reservoirs since geological times [4]. Gas pipelines are not immune to MIC either because trace amount of moisture is unavoidable due to condensation. Gas pipeline leaks and explosions have been blamed on corrosion in numerous reports. MIC was a suspect in some of those cases [5–7].

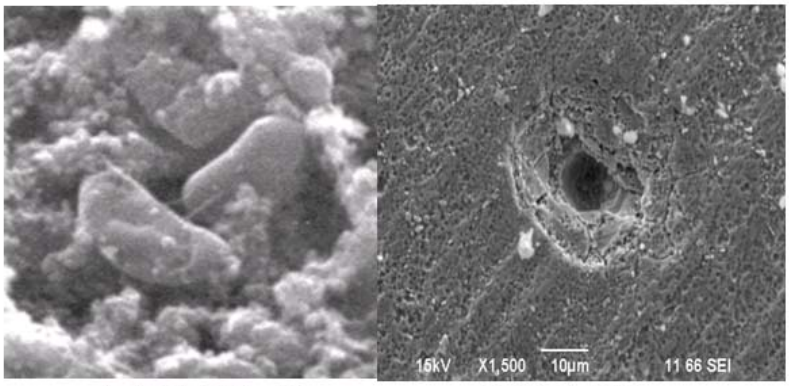

Figure 1-1. An SRB biofilm and an MIC pit found underneath the biofilm under scanning electron microscope (SEM).

1.1 MIC mechanisms

Biofilms cause MIC (Figure 1-1). They also prevent corrosion inhibitors from reaching the metal surface [8]. MIC causes pitting corrosion rather than uniform corrosion. MIC has been classified into three basic categories by Gu and Xu recently [9]. Type I MIC is caused by SRB, nitrate reducing bacteria (NRB) and methanogens. These microbes use anaerobic respiration in their metabolism. They utilize the extracellular electrons released by insoluble elemental iron (Fe^0) oxidation for reduction of an oxidant such as sulfate in the cytoplasm.

Anodic: $Fe \rightarrow Fe^{2+} + 2e^-$ (Iron oxidation) $-E^{o\prime} = +447$ mV (1)

Cathodic: $SO_4^{2-} + 9H^+ + 8e^- \rightarrow HS^- + 4H_2O$ $E^{o\prime} = -217$ mV (2)

The reduction potential ($E^{o\prime}$) of Fe^{2+}/Fe^0 is equal to −447 mV and $E^{o\prime}$= −217 mV for SO_4^{2-}/HS^- [10]. $E^{o\prime}$ is measured at 25°C, pH 7, 1 M for solutes (1 bar for gases) except H^+. It uses the standard hydrogen potential as its reference. The cell potential for the redox reaction combining the two reactions above is +230 mV. This positive value corresponds to the Gibbs free energy change $\Delta G^{o\prime}$= −178 kJ/mol sulfate for the redox reaction. This negative $\Delta G^{o\prime}$ value means the redox reaction generates energy and thus the corrosion process is thermodynamically favourable under the conditions defined for $E^{o\prime}$. The actual conditions may differ. However, the Gibbs free energy change will remain negative for this non-borderline case. Despite the thermodynamic driving force, due to a high activation energy for Reaction (2), biofilm catalysis is needed. Xu and Gu [11] showed that starting with the same mature *Desulfovibrio vulgaris* biofilms grown in a full-strength culture medium, subsequent starvation of carbon source made the biofilms more corrosive. This was because that due to a lack of organic carbon, the sessile SRB cells switched to Fe^0 as an electron donor (fuel molecule).

In Type II MIC, corrosive metabolites secreted by microbes such as acid producing bacteria (APB) are responsible. Fermentative microbes often produce acids such as volatile fatty acids (formic acid, acetic acid, etc.) that serve as proton reservoirs. Proton reduction can replace Reaction (2) to absorb the electrons released by iron oxidation in Reaction (1), if the proton concentration is sufficiently high (i.e., pH sufficiently low). Type III MIC is also known as biodegradation. Some biofilms can secrete enzyme to degrade polymers such as polyurethanes and plasticizers and utilize the degradation products as organic carbon and energy sources.

All three types of MIC are due to biofilms because biofilms either directly attack metals in Type I MIC, in which planktonic cells are in capable, or they secrete locally high concentrations of corrosive compounds. Sessile cell concentrations in a biofilm can be two-order of magnitude higher than planktonic cells. Thus, biofilm treatment is the key to MIC mitigation.

1.2 Current MIC mitigation methods

Currently, "scrub and spray" is still the basic approach in the pipeline industry. Pigs are used to scrub the internal pipeline surfaces and to apply biocides in pigging runs. Pigs are usually driven by pressure. Earlier pigs made the oink sound while moving inside pipelines, thus giving the name to the devices. Figure 1-2 shows a pig inside a pipeline. A "smart" pig can be used to detect corrosion and spray biocides. Two pigs are sometimes used to squeeze a biocide liquid plug in the middle while traveling downstream. This maintains a high biocide concentration in the plug, but it limits the biocide exposure time. Pipeline pigging can be very expensive due to downtime, labor, equipment and chemical costs. Because pipelines cannot be kept sterile, repeated applications are required. A more effective biocide treatment can prolong the time gap between treatments. It should be noted that some older pipelines are not piggable due to sharp turns in their designs. This makes their maintenance even more challenging.

Figure 1-2. A pig inside a pipeline.

Because the treatment chemicals will be discharged after use, environmental regulations require that only biodegradable chemicals can be used in pipelines. Due to this reason, compounded by cost and also efficacy factors, the two dominant biocides used in oil and gas pipelines in the past few decades remain to be tetrakis hydroxymethyl phosphonium sulfate (THPS) and glutaraldehyde. They are broad-spectum biocides. Both are environmentally friendly and relatively safe for operators in field operations including offshore platforms. Unfortuately, like antibiotics, repeated use of the same biocides promote microbial resistance. This causes dosage escalations and eventually these biocides may become ineffective for some fields. Although occasionally some new biocides are introduced into the market, no blockbuster biocides that can replace THPS or glutaraldehyde are expected any time soon. One strategy is to enhance existing biocides.

A biocide is often used in a biocide cocktail. For example, a biocide is sometimes mixed with a surfactant [12] to deliver it to a pipe wall surface more effectively. Two or more biocides may be mixed together if they are compatible, but this is often inconvenient in applications. A relatively new invention is to use chelators to chelate the calcium and potassium ions in the cell wall [13]. Chelators such as ethylenediaminetetraacetic acid (EDTA) and its more biodegradable substitute ethylenediamine-N,N'-disuccinic acid (EDDS) are found to be biocide enhancers. They make cell walls more permeable to a biocide [14,15]. However, a minimum of 1000 ppm (w/w) EDTA or EDDS is typically required [16] because the pipeline fluid environment often contains scales that consume some chelators. This dosage exceeds the 500 ppm THPS or glutaraldehyde dosage which is considered a high end concentration.

Biofilms employ several defence mechanisms to counter harsh environmental conditions such as antimicrobial/biocide attacks. They include diffusional barriers to prevent biocide penetration, lowered metabolic rates to reduce antimicrobial intake, formation of persistent cells to rebuild the biofilm when the environmental conditions improve, upregulation of antimicrobial resistant genes and efflux pumps, etc. [17]. It is commonly acknowledged that ten times (10X) or higher antimicrobial/biocide concentrations are usually needed to treat sessile cells compared with that needed for treating planktonic cells [18]. Concentrations as high as 1,000X have been reported [19]. When biofilms are dispersed, they are much easier to treat. A novel strategy to treat medically important biofilms was discovered by a group of medical researchers [20]. They found that D-amino acids can disperse biofilms, converting sessile cells in a biofilm community into individual planktonic cells. They proposed that biocides can be used more effectively after using D-amino acids' dispersing of biofilms. However, our research found that D-amino acids alone failed to disperse SRB biofilms on carbon steels because they are far more tenacious that the medically signficiant biofilms they tested. This work used a combination of D-amino acids and biocides for treating tough corrosive biofims inside pipelines.

1.3 D-amino acids are novel biocide enhancers: Rational, methods and existing data

All proteins synthesized right after messenger RNA (m-RNA) translation consist of only L-amino acids. D-amino acids are enantiomers of L-amino acids as shown in Figure 1-3. Abiotic organic synthesis in a chemical reactor produces a 50:50 mixture of D- and L-amino acids. Most L-amino acids are nowadays produced using biosynthesis through fermentation to produce only the desired L-amino acid form. D-amino acids occur in peptides via two different mechanisms: (1) posttranslational conversion of L- to D-amino acids in the peptides originally synthesized (primarily in eukaryotic ribosomes), and (2) peptide synthesis through nonribosomal peptide synthetases, independent of m-RNA. The

second method is used frequently in bacteria [21]. With the advances in analytical methods, especially high performance liquid chromatography, and growing interests in the biological functions D-amino acids, researchers have discovered that D-amino acids are far more prevalent than previously thought. D-amino acids are distributed widely in nature. In fact, D-amino acids are not only found in microorganisms, but also in animals and even humans [22]. Depending on the age and environmental conditions, biological materials such as silk, bone, shells and teeth have an increasing D/L ratio for each amino acid due intrinsic first-order racemization reaction. This property has been used to date archaeological objects [23].

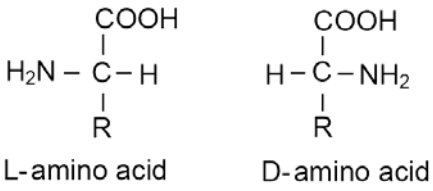

Figure 1-3. Structures of L- and D-amino acids.

It is well known that all bacterial cell walls contain peptidoglycan molecules. As seen in Figure 1-4, peptidoglycan is a polymer of β-(1,4)-linked N-acetylglucosamine and N-acetylmuramic acid. All the lactyl groups in N-acetylmuramic acid are substituted with stem peptides consisting of four alternating D- and L-amino acids [24]. Peptidoglycan molecules in a cell wall maintain the bacterial cell's shape, strength and resistance to the high osmotic pressure of its protoplast [21]. Gram-positive bacilli and Gram-negative bacteria possess meso-diaminopimelic acid as the third amino acid (Figure 1-4a), while most other Gram-positive bacteria possess L-lysine as the third amino acid (Figure 1-4b) [24]. In both cases, D-alanine (D-ala) is the terminal amino acid of the peptide chain. Peptidoglycan synthesis has been used as a key target in many antibiotics. D-amino acids at high concentrations have been used to alter peptidoglycan synthesis in order to inhibit bacterial growth [21]. Thus, it is not surprising that D-amino acids are found in some antibiotic peptides [25]. In fact, D-amino acids are commercially available to the pharmaceutical industry for drug synthesis.

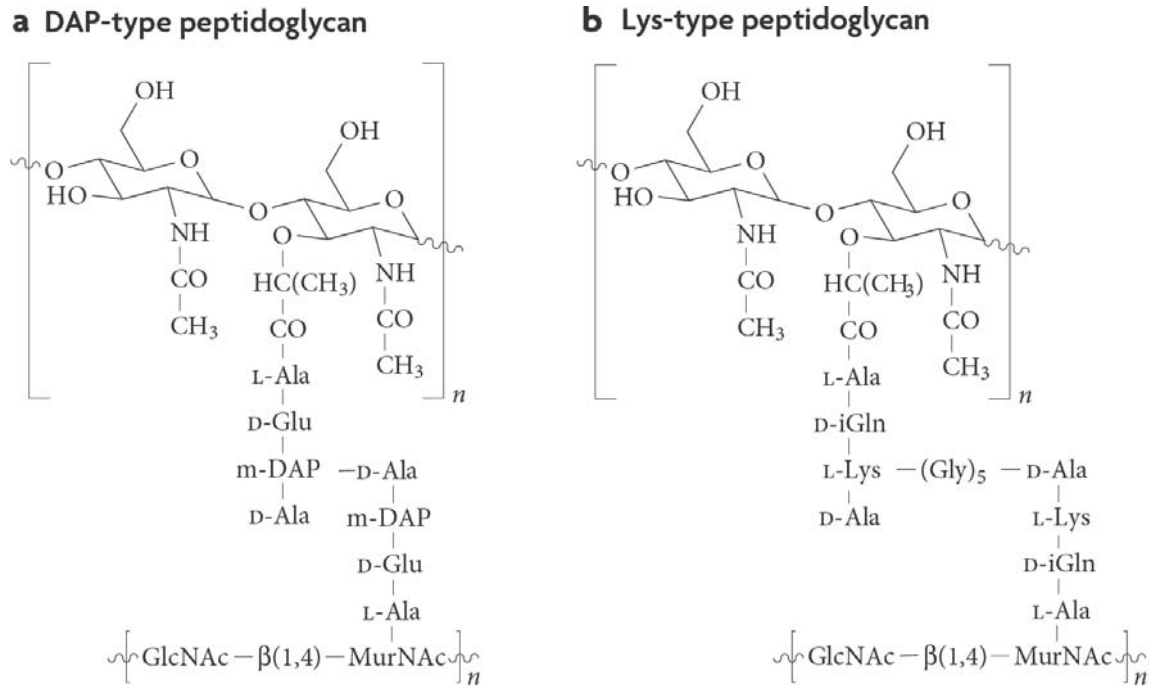

Figure 1-4. Two types of peptidoglycan in bacterial cell walls [24].

In 2009, Lam et al. [26] found that D-amino acids play a key role in peptidoglycan synthesis and speculated that the synthesis of D-amino acids may be a common strategy for bacteria to adapt to environmental conditions. Cava et al. [21] speculated that in times of nutritional limitation and other cellular stresses, bacteria release extracellular D-amino acids that signal to the biofilm community to regulate peptidoglycan amount, composition and strength. Recently, Kolodkin-Gal et al. [20] discovered that some D-amino acids (D-tyrosine (D-tyr), D-methionine (D-met), D-tryptophan (D-trp) and D-leucine (D-leu)) dispersed bacterial biofilms at very low concentrations (μM to mM) for *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. They also prevented biofilm formation. Kolodkin-Gal et al. hypothesized that these D-amino acids can substitute the D-ala terminus in peptidoglycan molecules, thus sending a biofilm dispersal signal. This hypothesis was supported by their experimental data showing that adding a high concentration of D-ala in a D-tyr solution rendered the D-tyr treatment ineffective. They proposed a strategy of biofilm mitigation by applying D-amino acids first to convert sessile cells to planktonic cells and subsequently applying an antimicrobial for an easier kill.

Pure D-tyr can be isolated from a D/L-tyr mixture produced from organic synthesis using selective degradation of L-tyr by microbes [27]. It can also be produced from hydroxyphenylpyruvate using enzymes [28]. D-tyr exists in many food products such as casein, soybean, wheat gluten and fish due to the conversion of L- to D-tyr during

alkaline or heat treatment of food products [29]. L-tyr has an isoelectric point of 5.6. Between pH 3 and 8, L-tyr has a low solubility ≤ 3 mM [30]. Its enantiomer, D-tyr has similar physical properties. D-tyr stands out among various D-amino acids as having a particularly low solubility at pH 7. In biofilm dispersal tests, its required minimum concentration for efficacy is particularly low (≤ 1 ppm by mass) [13,16], probably by nature's design. In order to make a concentrated stock solution in lab tests, it is necessary to adjust pH to a far more acidic or alkaline pH.

Table 1-1. D-tyr and THPS for the prevention and removal of *D. vulgaris* biofilm [13]

Treatment	Sessile cell count in batch test (cells cm^{-2})*	Sessile cell count after 1-h treatment (cells cm^{-2})**	Sessile cell count after 3-h treatment (cells cm^{-2})**
No treatment chemicals (control)	$\geq 10^7$	$\geq 10^6$	$\geq 10^6$
100 ppm D-tyr	$\geq 10^6$	$\geq 10^5$	$\geq 10^5$
50 ppm THPS	$\geq 10^4$	$\geq 10^4$	$\geq 10^3$
50 ppm THPS + 1 ppm D-tyr	<10	<10	<10
100 ppm THPS	$\geq 10^2$	<10	<10

*Sessile cell count on carbon steel coupon surface in 37°C ATCC 1249 medium for 7 days in biofilm prevention test.

**Sessile cell count on carbon steel coupon surface with established mature SRB biofilm after 1-hour treatment and 3-hour treatment in an anaerobic chamber at room temperature, respectively.

Table 1-1 and Figure 1-5 show the results of D-tyr + THPS for biofilm prevention [17]. D-tyr alone only achieved 1-log reduction (90% removal) of SRB sessile cells on C1018 coupons compared with untreated control indicating that D-tyr alone did not adequately trigger SRB biofilm dispersion. When treated with 100 ppm THPS, a 5-log reduction (99.999% removal) of SRB sessile cells was achieved compared with untreated control. The higher log reduction is very desirable because it means a much longer treatment time for the cells to rebound. Compared with the 3-log reduction obtained by 50 ppm THPS without D-tyr, the binary combination of 50 ppm THPS and 1 ppm D-tyr achieved a 6-log reduction compared with untreated control, resulting in undetectable sessile cells. For the established biofilm removal test data shown in Columns 3 and 4 in Table 1-1, similar results were obtained in both 1-hour and 3-hour tests. The binary combination of 50 ppm THPS + 1 ppm D-tyr achieved the same biofilm eradication effect as 100 ppm THPS without D-tyr (5-log reduction). This means 1 ppm D-tyr successfully halved the THPS

dosage. The data suggest that D-tyr strongly enhanced THPS in SRB biofilm prevention and removal tests. Figure 1-5C indicates that D-tyr alone even at a high concentration of 100 ppm was insufficient for *D. vulgaris* biofilm dispersal. Apparently, this SRB biofilm was more recalcitrant than those biofilms tested by Kolodkin-Gal et al. [20]. A biocide stress was needed to "convince" the *D. vulgaris* biofilm to disperse.

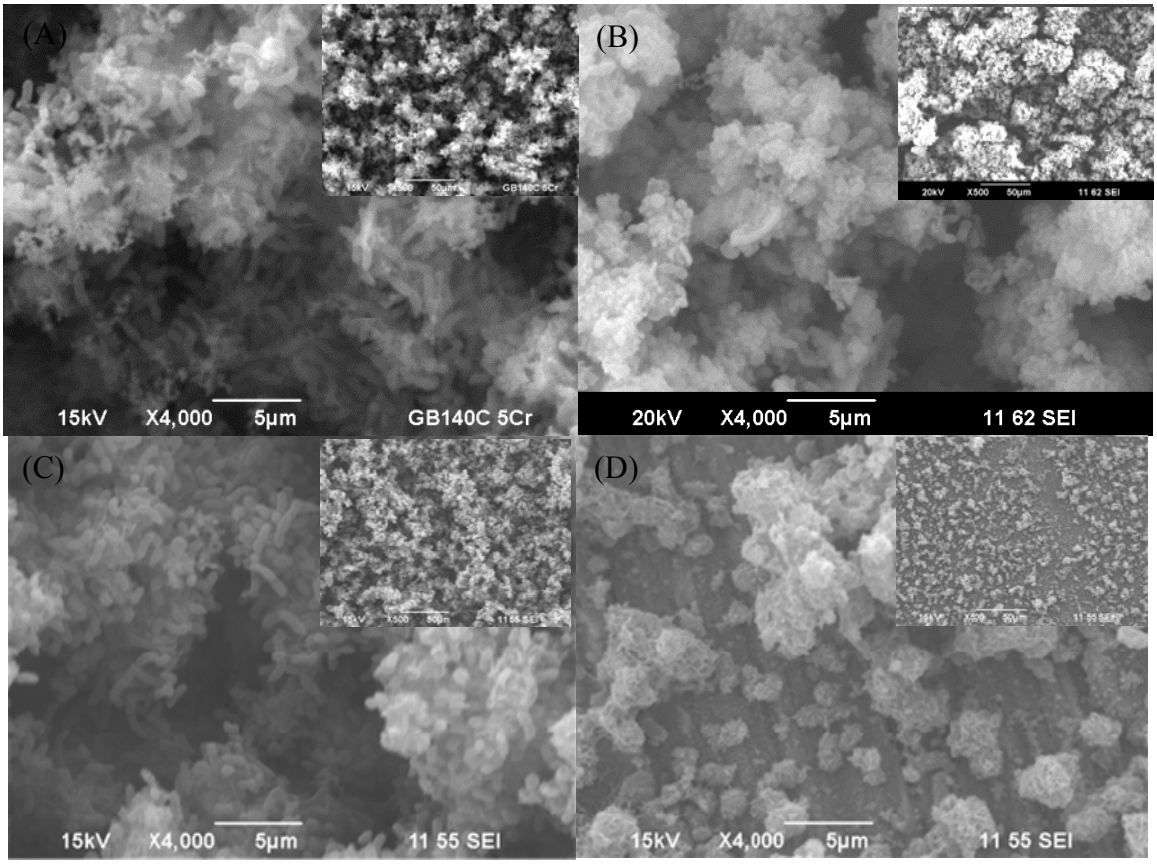

Figure 1-5. SEM images for 7-day coupons from 37°C *D. vulgaris* cultures in ATCC 1249 medium in the biofilm prevention test: (A) control coupon with no treatment chemicals added to the culture medium, (B) 100 ppm THPS, (C) 100 ppm D-tyr, and (D) 50 ppm THPS + 1 ppm D-tyr. (Scale bars for the small inserted images are 50 µm) [17].

The binary combination of 50 ppm THPS and 100 ppm D-met performed better in MIC pitting mitigation compared with 50 ppm THPS alone and 500 ppm D-met alone (Figures 1-6(A-C)). The binary biocide cocktail also had the lowest normalized weight loss of the corrosion coupons compared with 50 ppm THPS treatment and 500 ppm D-met treatment (Figure 1-6D). Both D-tyr + THPS and D-met + THPS lab tests indicated that a biocide stress was required for D-amino acids in SRB biofilm dispersal.

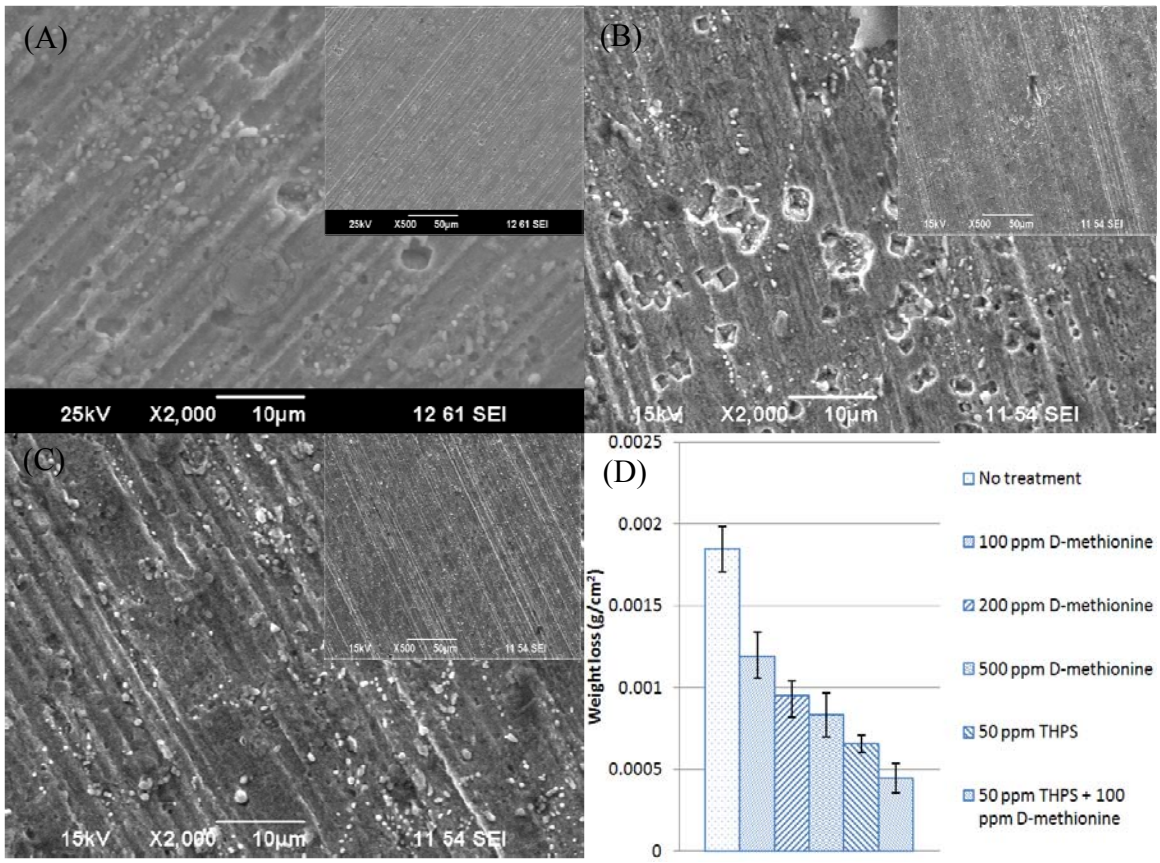

Figure 1-6. SEM images of coupon surfaces after *D. vulgaris* biofilm removal for coupons obtained after 7 days of incubation at 37°C from ATCC 1249 medium with the addition of: (A) 50 ppm THPS, (B) 500 ppm D-met, (C) 50 ppm THPS + 100 ppm D-met, respectively, accompanied by normalized weight loss data shown in (D). (Scale bars for the small inserted images are 50 µm) [31].

Xu et al. found that a 6.6 ppm D-amino acid mixture consisting of equimolar D-tyr, D-met, D-trp and D-leu considerably enhanced the 30 ppm THPS + 500 ppm EDDS binary combination in both biofilm prevention and biofilm removal tests [32]. They showed that the triple combination of 30 ppm THPS + 500 ppm EDDS + 6.6 ppm D-amino acid mixture achieved 5-log and 4-log reductions compared with the untreated control in biofilm prevention and biofilm removal tests, respectively. Although superior biocide enhancement results have been achieved in laboratory tests using pure-strain SRB biofilms, the efficacy of the enhanced biocide system remains to be seen in the treatment of field biofilm consortia.

2 Background

2.1 Problem statement

MIC has been a major problem in the field operations of many industries. It is usually caused by biofilm consortia rather than a pure-strain biofilm. The process of MIC caused by biofilm consortia is more complicated than that by a pure-strain biofilm, such as the *D. vulgaris* biofilm. Moreover, various coexisting species in the biofilm community protect each other from the external hazardous factors such as biocide stress and pH swing. Thus, biofilm consortia are more difficult to mitigate than pure-strain biofilms. Because biofilms are the primary cause of MIC, the mitigation of MIC is about biofilm treatment. Current field biofilm mitigation methods have limitations. A green and novel method to mitigate field biofilm consortia is urgently needed.

2.2 Project scope

The primary objective of this project was to develop a new environmentally friendly biocide enhancer system to mitigate field biofilm consortia effectively and economically. Between the two most popular biocides (glutaraldehyde and THPS), THPS was chosen because glutaraldehyde is a cross-linking agent (one of its main antimicrobial mechanisms) that reacts with D-amino acids, rendering them ineffective. The whole project was centered on increasing the efficacy of THPS in the mitigation of field biofilm consortia. The evaluation of the enhancement by D-amino acids in the THPS treatment of field biofilm consotia isolated was conducted. In this project, four tasks were completed.

Task 1. Testing D-tyr and D-met against field biofilm consortia

Two different biofilm consortia collected from two different oil and gas fields were used to evaluate the efficacy of THPS + D-amino acid biocide combination. SRB, APB and general heterotrophic bacteria (GHB) were assayed using microbiological and imaging methods. Both biofilm prevention and biofilm removal tests were conducted. Test conditions are listed in Table 2-1. Results were compared with previously obtained data for the pure-strain *D. vulgaris* biofilm.

Table 2-1. Test conditions for mitigation of biofilm consortia

Biofilms	Two field biofilm consortia
Biocide cocktail	THPS + D-tyr, THPS + D-met
Temperature	37°C for prevention test and 25°C for removal test
Test duration	7 days for biofilm prevention test, 3 hours for biofilm removal test
Coupon	C1018 carbon steel

Task 2. Screening additional D-amino acids

So far, only D-tyr, D-leu, D-trp, and D-met data have been evaluated and published. Additional D-amino acids may also be suitable biocide enhancers. In this task, more inexpensive D-amino acids were screened for potential field applications. Test conditions are listed in Table 2-2.

Table 2-2. Test conditions for additional D-amino acids

Bacteria	*D. vulgaris* (ATCC 7757)
Biocide cocktail	THPS + D-amino acid
Temperature	37°C for prevention test and 25°C for removal test
Test duration	7 days for biofilm prevention; 3 hours for biofilm removal
Coupon	C1018 carbon steel

Task 3. Using D-amino acid mixtures as biocide enhancers

A mixture of several D-amino acids might be better against field biofilm consortia because different bacteria may be susceptible to different D-amino acids. Several combinations were tested. They consisted of different D-amino acids selected among D-tyr, D-leu, D-trp, D-met, and the promising candidates from the newly tested amino acids from Task 2. Table 2-3 shows test conditions.

Table 2-3. Test conditions for THPS + mixtures of D-amino acids

Biofilms	Two biofilm consortia
Biocide cocktail	THPS + D-amino acids (various choices and dosages)
Temperature	37°C for prevention test and 25°C for removal test
Test duration	7 days for biofilm prevention; 3 hours for biofilm removal
Coupon	C1018 carbon steel

Task 4. Field testing

We collaborated with a large polymer manufacturing company in the US (name withheld by request) to treat the biofilms from their cooling towers. C1018 carbon steel coupons were placed in the cooling system to allow biofilms to grow first. They were then used for enhanced biocide treatment testing. Table 2-4 shows the test conditions.

Table 2-4. Test conditions

Biocide cocktail	Bleach + D-amino acids
Temperature	25°C
Test duration	4 hours for biofilm removal
Coupon	C1018 carbon steel

3 Testing D-tyrosine and D-methionine with THPS against field biofilm consortia

3.1 Introduction

D-met and D-tyr were effective biocide enhancers for THPS against *D. vulgaris* as described in the Introduction section. In order to figure out whether they are able to enhance THPS in the mitigation of field biofilm consortia, D-met and D-tyr were tested with 50 ppm THPS against them in the biofilm prevention test and the biofilm removal test. Two field biofilm consortia were collected from two different places in an oil and gas field operated by a major oil company (name withheld by request).

3.2 Experimental methods

3.2.1 Biofilm consortia, culture medium, and chemicals

Two biofilm consortia labeled as Consortium I and Consortium II were isolated from two field water samples collected from an oil and gas field using the ATCC 1249 medium (an SRB culture medium). The composition of the medium is listed in Table 3-1. L-cysteine with a final concentration of 100 ppm was added to the medium to perform as an oxygen scavenger. Since the two biofilm consortia were cultured in an anaerobic environment, the prevention of oxygen leak was quite significant. All the chemicals used in this project were purchased from Fisher Scientific (Pittsburgh, PA, USA). The original field water sample was stored in 1.5 ml centrifugal tubes and frozen in a −20^{o}C freezer. Each seed culture was used for one month before switch backing to the frozen seed culture to prevent the subsequent cultures being different from the original.

Table 3-1. Composition of ATCC 1249 medium

	Chemicals	Amount
Component I	$MgSO_4 \cdot 7H_2O$	4.1 g
	Sodium citrate	5.0 g
	$CaSO_4$	1.0 g
	NH_4Cl	1.0 g
	Distilled water	400.0 ml
Component II	K_2HPO_4	0.5 g
	Distilled water	200.0 ml
Component III	Sodium lactate	4.5 ml
	Yeast extract	1.0 g
	Distilled water	400.0 ml
Component IV	5% (w/w) $Fe(NH_4)_2(SO_4)_2$	20 ml

3.2.2 Substratum for biofilm growth

Coin shaped C1018 (UNS G10180) carbon steel coupons were used for biofilm growth. The composition of C1018 carbon steel is listed in Table 3-2. Coupons were painted with Teflon except the top surface with a surface area of 1.12 cm^2, which was exposed to the medium solution. The top surface of coupons was sequentially polished with #180, 400, and 600 grit sandpapers and washed with pure isopropanol. Afterwards, coupons were dried under UV light for 20 minutes.

Table 3-2. Composition of C1018 carbon steel

Element	C	Mn	P	S	Si	Fe
Amount (wt%)	0.14-0.20	0.60-0.90	0.04	0.05	0.15-0.30	98.81-99.26

3.2.3 Biofilm prevention and biofilm removal tests

Two tests were done to evaluate the efficacy of D-amino acid + THPS combinations. In the biofilm prevention test, a D-amino acid solution, the culture medium, chemical solutions, and tools used in manipulation were sterilized in an autoclave for 20 minutes. All solutions were sparged with the filter-sterilized nitrogen gas for 45 minutes to remove solved oxygen to achieve a strict anaerobic condition. Mixing and inoculation were done in an anaerobic glovebox, which was sparged with the filter-sterilized nitrogen gas for 45 minutes with the help of a vacuum pump. In the manipulation, 3 duplicate coupons were put into 100 ml culture medium with 1 ml biofilm consortium seed culture and treatment chemicals. The vials used in this project were 125 ml anaerobic vials. The test matrix is shown in Table 3-3. Afterwards, vials were put into an incubator at 37^oC for 7 days. After 7 days, coupons were taken out for cell counting and biofilm observation.

The biofilm removal test was carried out to simulate a pipeline biocide dosing that places a biocide solution between two traveling pigs. In the biofilm removal test, coupons were incubated without treatment chemicals following the same procedure as in the biofilm prevention test. Coupons covered with mature biofilm consortia were harvested after 3 days. Then they were taken out and washed three times, 15 seconds each time, using a phosphate buffered saline (PBS) buffer solution with pH adjusted to 7.4. The purpose was to remove the planktonic cells and residual medium. Afterwards, three coupons were put into a Petri dish containing 50 ml PBS solution with treatment chemicals for 3 hours, simulating an exposure time of 3 hours in a slow moving pigging run. The operation was done in the anaerobic chamber. The test matrix is shown in Table 3-4.

Table 3-3. Conditions for biofilm prevention test

Biofilm	Consortium I and Consortium II
Culture medium	ATCC 1249 medium
Treatment method	D-amino acids + 50 ppm THPS
Temperature	37^{o}C
Treatment duration	7 days
Coupon	C1018 carbon steel

Table 3-4. Conditions for biofilm removal test

Biofilm	Consortium I and Consortium II
Culture medium	ATCC 1249 medium (to grow biofilms first)
Treatment method	D-amino acids + 50 ppm THPS
Temperature	25^{o}C
Test duration	3 hours in Petri dishes
Coupon	C1018 carbon steel

3.2.4 Biofilm analyses

The sessile cells on coupon surfaces were counted using the Sani-Check SRB test kit (Biosan Laboratories, Inc., Warren, MI, USA). The biofilm on a coupon was scrapped off using a small brush that came with the test kit. Then the brush with sessile cells was rinsed within 10 ml sterilized distilled water. Afterwards, the brush and the coupon were vortexed with the 10 ml PBS for 15 seconds to make sure no sessile cells attached on the coupon surface. After vortexing, the brush was inserted in a small tube with a solid medium in it. Mineral oil was dropped on the top of the solid medium in the test kit's vial to form an oxygen barrier. The tube was incubated at 37^{o}C. The sessile cell concentration was logarithmically related to the number of days it tool for the black color (iron sulfide generated by SRB growth) showed up. Another most probably number (MPN) test kit was also used to count the SRB sessile cells. It confirmed the results obtained using the Sani-Check SRB test kit. This vendor also supplied GHB and APB test kits. The detailed data obtained using Biotechnology Solutions (BTS) (Houston, TX) test kits are in Appendix section 9.1.

The biofilm morphology was checked using SEM (Model JSM-6390 SEM, JEOL, Japan). The coupons for SEM observation were immersed into 4% (w/w) glutaraldehyde solution for 2 hours. During this process, the sessile cells were killed and "fixed" onto the coupon surface. The next step was dehydration. Coupons were dehydrated with 25%, 50%, 75%, and 100% (v/v) isopropanol sequentially, 5 minutes at each concentration. Then, a critical point dryer was used to dehydrate with supercritical carbon dioxide. It would remove the water from biofilms completely. After dehydration, coupons were coated with a thin layer of palladium to provide conductivity for SEM imaging.

3.3 Results and discussion

3.3.1 Field biofilm consortia

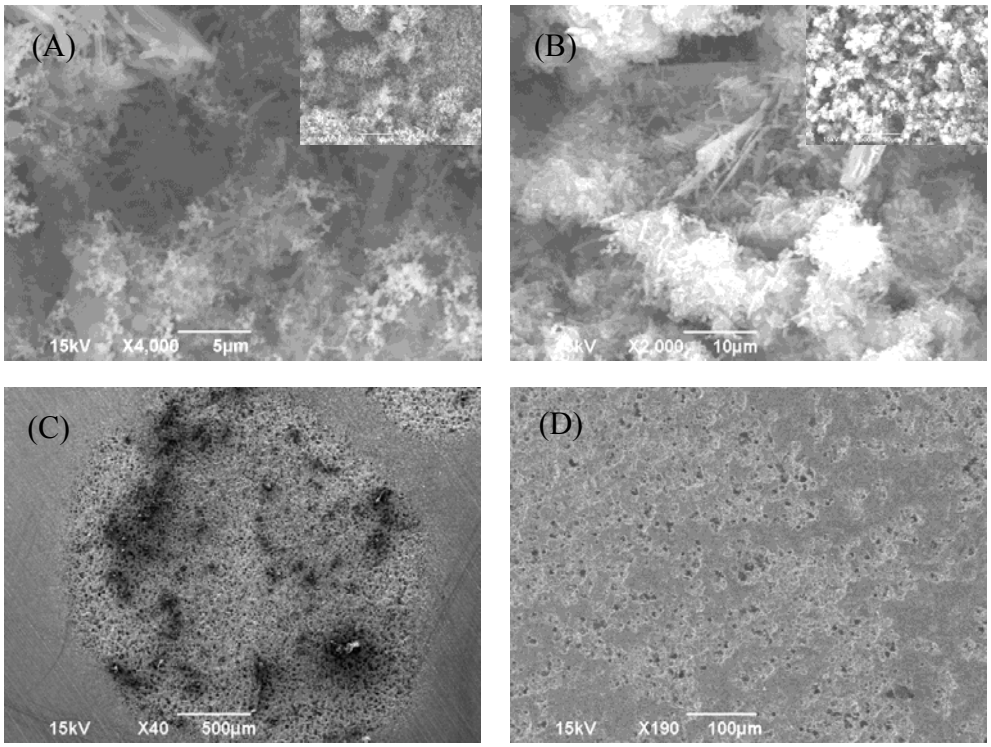

Figure 3-1. Biofilm images and bare coupon surface images after biofilm removal for coupons in the ATCC 1249 medium after 10 days of incubation (without biocide treatment) at 37^{o}C: (A) biofilm Consortium I, (B) biofilm Consortium II, (C) coupon surface after removal of biofilm Consortium I, and (D) coupon surface after removal of biofilm Consortium II.

Two biofilm consortia were cultured in the ATCC 1249 medium in 120 ml anaerobic vials without treatment chemicals at 37°C for 10 days. After a 10-day incubation period, the planktonic cell concentration in the medium was 10^9 cells/ml. Figure 3-1 shows that both Consortium I and Consortium II formed robust biofilms on C1018 coupons. Various cell shapes indicate that there were multiple microbial species in both biofilm consortia. Underneath the biofilm consortia were corrosion pits. These pits indicate that Consortia I and II caused severe MIC. The specific weight losses obtained on coupons were 0.0047 g/cm^2 and 0.0058 g/cm^2 for Consortia I and II, respectively. Both weight loss values were larger than the 0.0018 g/cm^2 caused by the pure-strain *D. vulgaris* biofilm at the same test condition.

The metagenomics analysis of the biofilm consortia was done by Ecolyse, Inc. (College Station, TX). The sample preparation, shipping, and the detailed report are attached in Appendix 9.2. All species in Consortia I and II are shown in Table 3-5. In the consortia, there were NRB, thiosulfate reducing bacteria (TRB), Sulfidogen, and Biodegradation (HC). An NRB medium was used to isolate NRB from the field collected sample solution. However, no bacterial growth was observed in the medium vials. The detailed data are attached as Appendix 9.3. Sulfidogens reduce sulfur and release sulfide as a product. Abbreviated from the word biodegradation, Biodeg microbes utilize matters that are usually decomposed by most bacteria. HC simply means hydrocarbon (degrading microbes). Biofilm Consortium I includes all 6 species and biofilm Consortium II only has 4 of all identified species, except *Sallmonella enterica* and *Soehngenia sp*. *Bacillus sp* has been studied in MIC research for decades [33]. Xu et al. [34] reported MIC of C1018 carbon steel caused by *B. licheniformis*, a *Bacillus sp*. However, some *Bacillus sp* are not corrosive at all. A number of *Bacillus* strains even have the ability to inhibit corrosion [35]. Most *Bacteroides sp* are found in mammalian gastrointestinal flora [36]. They secret organic acids, which are possible to cause metabolite MIC or Type II MIC [37]. *Garciella sp* and *Soehngenia sp* have been isolated from the oil and gas field [34-36]. *Garciella sp* can reduce thiosulfate and produce hydrogen sulfide, which is corrosive [39]. Although *Salmonella sp* bacteria are found in the natural environment including animals. *Soehngenia sp* have been isolated from oil and gas fields. *Salmonella sp* can reduce tetrathionate and sulfate, and *Soehngenia sp* microbes are capable of using sulfate and thiosulfate as electron acceptors [40,41]. Both *Salmonella sp* and *Soehngenia sp* produce hydrogen sulfide, and may be related to MIC. *Tepidibacter sp* can grow in oilfield conditions or oil polluted soils. No clear metabolism is known for *Tepidibacter sp* to cause MIC [42,43]. Although most of the identified species are related to MIC, many of them have not been intensively investigated in MIC research.

Table 3-5. Metabolic assignments of dominant bacterial species (percentage)

Species	Consortium I	Consortium II	Trait
Bacillus sp	<1	6.0	Biosurfactant producing; Varies
Bacterioides sp	2.9	1.4	Fermenting bacteria
Garciella sp	27.7	78.8	NRB; Sulfidogen; TRB
Salmonella enterica	61.5	0	SRB; TRB
Soehngenia sp	4.1	0	Fermenting bacteria
Tepidibacter sp	2.7	13.6	Biodeg (HC)

3.3.2 D-tyrosine as a biocide enhancer for THPS

In Tables 3-6 and 3-7, 1 ppm D-tyr did not promote the biocidal effect of 50 ppm THPS in both biofilm prevention and removal tests for the mitigation of Consortium I because the biofilm was recalcitrant. The cocktail only achieved 2 log SRB sessile cell reduction compared with untreated control. In the mitigation of pure-strain *D. vulgaris* biofilm, 1 ppm D-tyr was sufficient to enhance 50 ppm THPS, achieving more than 5 log reduction compared with untreated control in SRB sessile cell count [17]. When 50 ppm TPHS was combined with 50 ppm D-tyr, the combination achieved 4 log reduction of the SRB sessile cell count in biofilm prevention and removal tests compared with the untreated control. Similarly, 1 ppm D-tyr did not promote the biocidal effect of 50 ppm THPS in the mitigation of Consortium II because it was also a recalcitrant biofilm. Tables 3-8 and 3-9 show that 1 ppm D-tyr was not enough to promote 50 ppm THPS to obtain more than 3 log reduction in the SRB sessile cell count. The combination of 50 ppm THPS + 50 ppm D-tyr showed 4 log reduction of the SRB sessile cell count in both biofilm prevention and removal tests compared with the untreated control. In Tables 3-6 and 3-7, the symbol ≥ is used by the MPN method as a convention.

Although in the mitigation of biofilm consortia, 50 ppm D-tyr promoted 50 ppm THPS achieving a higher SRB sessile cell reduction, the biocidal effect was not as good as that in the mitigation of the *D. vulgaris* biofilm. Figures 3-2 and 3-3 show that in the mitigation of *D. vulgaris* biofilm, no sessile cells was found while in the mitigation of the two biofilm consortia sessile cells were easily found on coupon surfaces in both biofilm prevention and removal tests. However, visible sessile cells in Figures 3-2 and 3-3 were fewer than those in Figure 3-1 (untreated control).

Table 3-6. SRB sessile cell count at the end of 7-day biofilm prevention test against Consortium I

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^7$
100 ppm THPS	$\geq 10^6$
50 ppm THPS + 1 ppm D-tyr	$\geq 10^5$
50 ppm THPS + 50 ppm D-tyr	$\geq 10^3$

Table 3-7. SRB sessile cell count after 3-hour treatment of biofilm removal against Consortium I

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^7$
100 ppm THPS	$\geq 10^5$
50 ppm THPS + 1 ppm D-tyr	$\geq 10^5$
50 ppm THPS + 50 ppm D-tyr	$\geq 10^3$

Table 3-8. SRB sessile cell count at the end of 7-day biofilm prevention test against Consortium II

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^7$
100 ppm THPS	$\geq 10^6$
50 ppm THPS + 1 ppm D-tyr	$\geq 10^5$
50 ppm THPS + 50 ppm D-tyr	$\geq 10^3$

Table 3-9. SRB sessile cell count after 3-hour treatment of biofilm removal against Consortium II

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^7$
100 ppm THPS	$\geq 10^6$
50 ppm THPS + 1 ppm D-tyr	$\geq 10^5$
50 ppm THPS + 50 ppm D-tyr	$\geq 10^3$

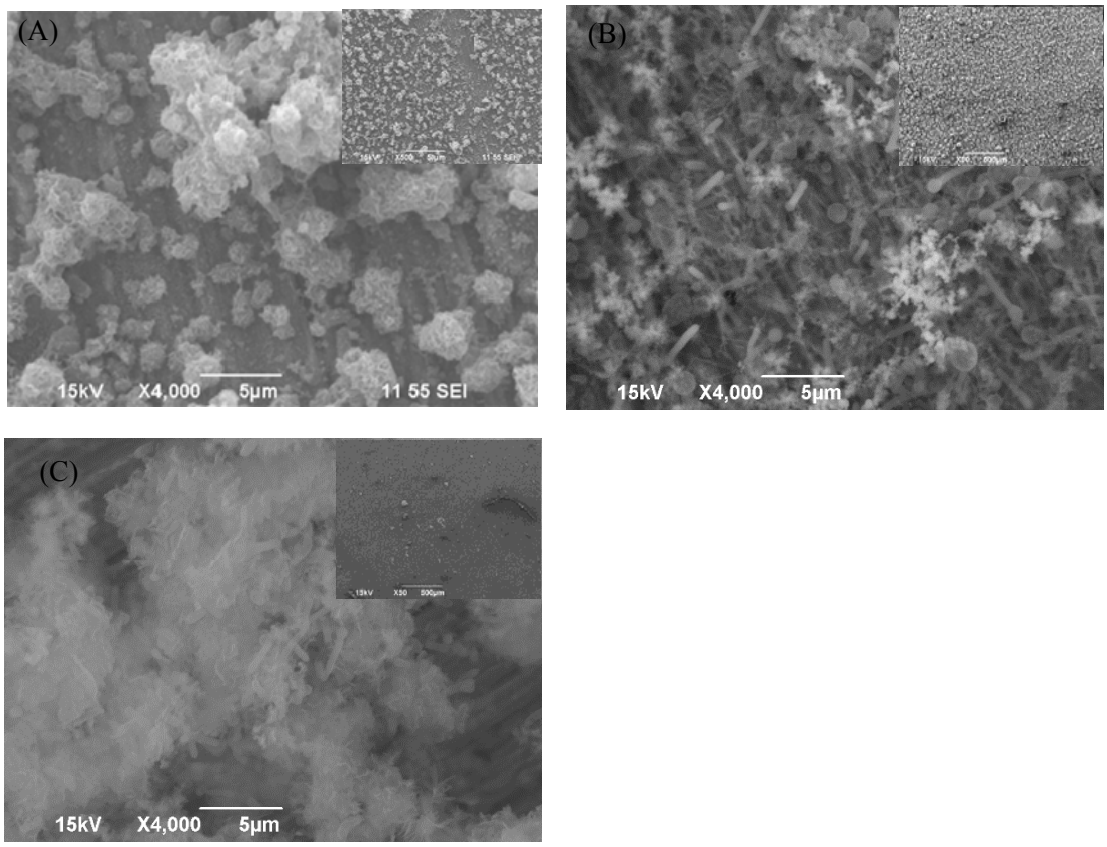

Figure 3-2. Images of biofilms on coupons after 7-day incubation in the biofilm prevention test in ATCC 1249 medium: (A) *D. vulgaris* biofilm treated with 50 ppm THPS and 1 ppm D-tyr [17], (B) biofilm Consortium I treated with 50 ppm THPS and 50 ppm D-tyr, and (C) biofilm Consortium II treated with 50 ppm THPS and 50 ppm D-tyr. (The scale bar in the inserted small image is 50 µm.)

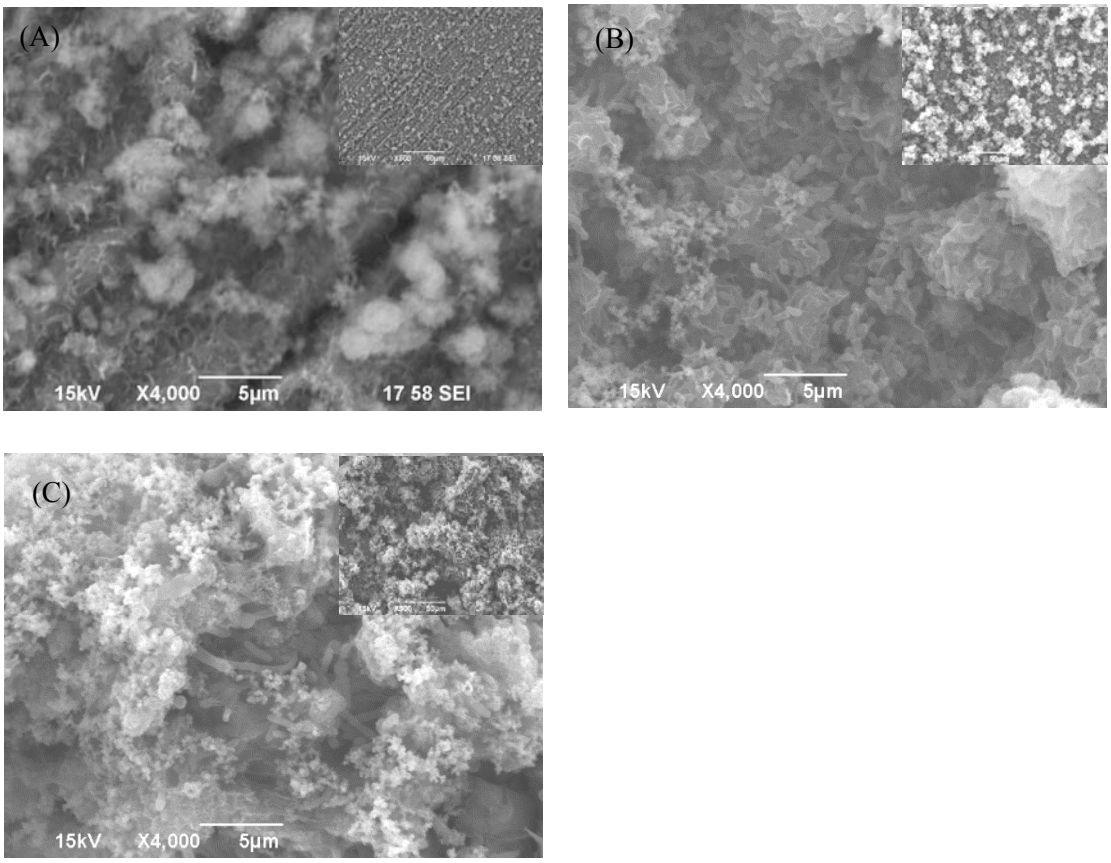

Figure 3-3. Images of biofilms on coupons after 3-hour treatment in the biofilm removal test: (A) *D. vulgaris* biofilm treated with 50 ppm THPS and 1 ppm D-tyr [17], (B) biofilm Consortium I treated with 50 ppm THPS and 50 ppm D-tyr, and (C) biofilm Consortium II treated with 50 ppm THPS and 50 ppm D-tyr. (The scale bar in the inserted small image is 50 µm.)

3.3.3 D-methionine as a biocide enhancer for THPS

Similar to the results of D-tyr tests, 100 ppm D-met did not enhance 50 ppm THPS in the mitigation of both biofilm consortia either. In the mitigation of the *D. vulgaris* biofilm, 100 ppm D-met showed enhanced efficacy for 50 ppm THPS and the combination achieved more than 5 log reduction in the sessile cell count compared with the untreated control [31]. This is not surprising, because biofilm consortia are typically far more recalcitrant than pure-strain biofilms. In the mitigation of the two biofilm consortia, 100 ppm D-met was not sufficient to promote 50 ppm THPS in biofilm prevention test (Tables 3-10 and 3-12) and biofilm removal test (Tables 3-11 and 3-13). The combination of 50 ppm THPS and 200 ppm D-met achieved 4 log reduction of the SRB sessile cell count compared with the untreated control in the biofilm prevention and removal tests

against both Consortia I and II. In Figures 3-4 and 3-5, sessile cells were not noticeable after the same treatment for *D. vulgaris* while in the mitigation of both consortia sessile cells were found easily. Nevertheless, they were much fewer than those in Figure 3-1.

It was not a surprise that individual D-amino acids with a lower concentration did not promote the biocidal effect of 50 ppm THPS in mitigation of biofilm consortia. Higher concentrations of individual D-amino acids enhanced 50 ppm THPS, but the enhancement was less than that achieved in the mitigation of the *D. vulgaris* biofilm with a lower concentration. Sessile cells in a biofilm consortium have synergistic effects that defend the community against biocide attacks. The two field biofilm consortia were collected from field locations with persistent biofilm problems. It was possible that for the two more recalcitrant field biofilm consortia, 50 ppm THPS was not a sufficient biocide stress. Both THPS and D-amino acid concentrations need to be increased if better treatment outcome is desired.

Table 3-10. SRB sessile cell count at the end of 7-day biofilm prevention test against Consortium I

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^7$
100 ppm THPS	$\geq 10^6$
50 ppm THPS + 100 ppm D-met	$\geq 10^6$
50 ppm THPS + 200 ppm D-met	$\geq 10^3$

Table 3-11. SRB sessile cell count after 3-hour biofilm removal test against Consortium I

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^7$
100 ppm THPS	$\geq 10^5$
50 ppm THPS + 100 ppm D-met	$\geq 10^6$
50 ppm THPS + 200 ppm D-met	$\geq 10^3$

Table 3-12. SRB sessile cell count at the end of 7-day biofilm prevention test against Consortium II

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^7$
100 ppm THPS	$\geq 10^6$
50 ppm THPS + 100 ppm D-met	$\geq 10^6$
50 ppm THPS + 200 ppm D-met	$\geq 10^3$

Table 3-13. SRB sessile cell count after 3-hour biofilm test against Consortium II

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^7$
100 ppm THPS	$\geq 10^6$
50 ppm THPS + 100 ppm D-met	$\geq 10^6$
50 ppm THPS + 200 ppm D-met	$\geq 10^3$

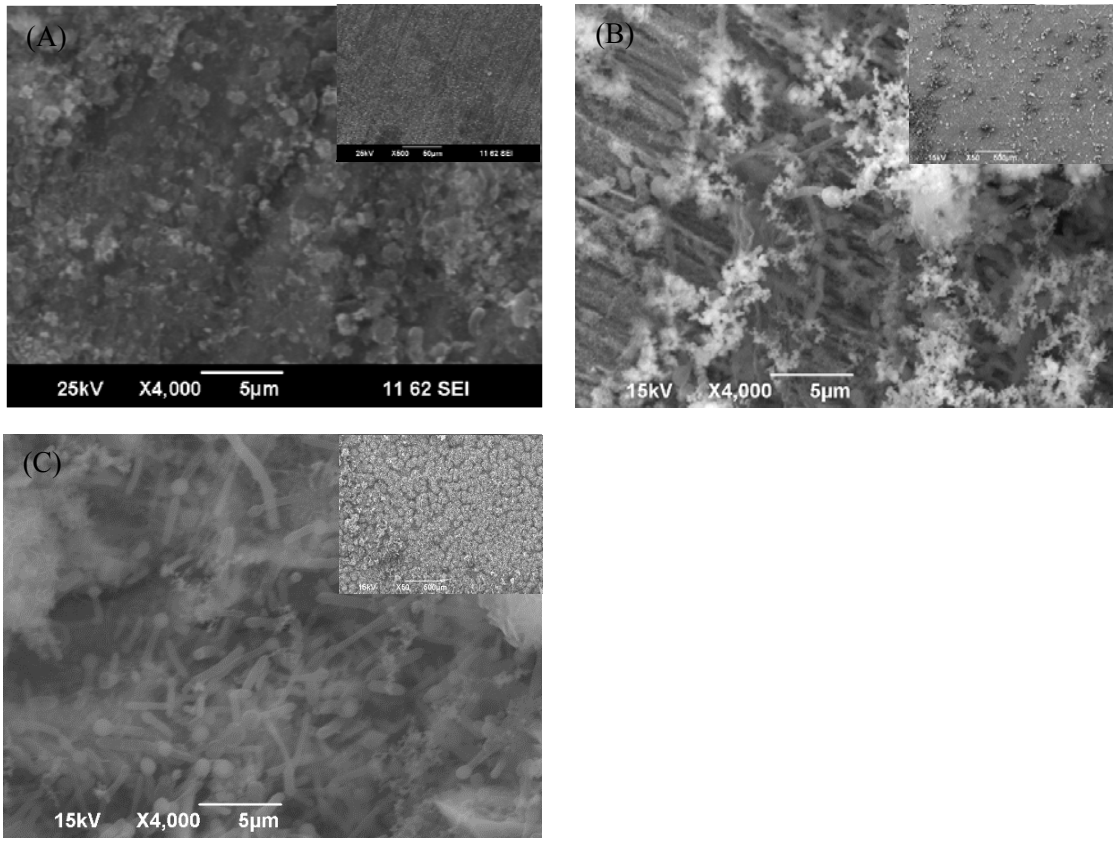

Figure 3-4. Images of biofilms on coupons after 7-day incubation in the biofilm prevention test in the ATCC 1249 medium: (A) *D. vulgaris* biofilm treated with 50 ppm THPS + 100 ppm D-met [31], (B) biofilm Consortium I treated with 50 ppm THPS + 200 ppm D-met, and (C) biofilm Consortium II treated with 50 ppm THPS + 200 ppm D-met. (The scale bar in the inserted small image is 50 µm.)

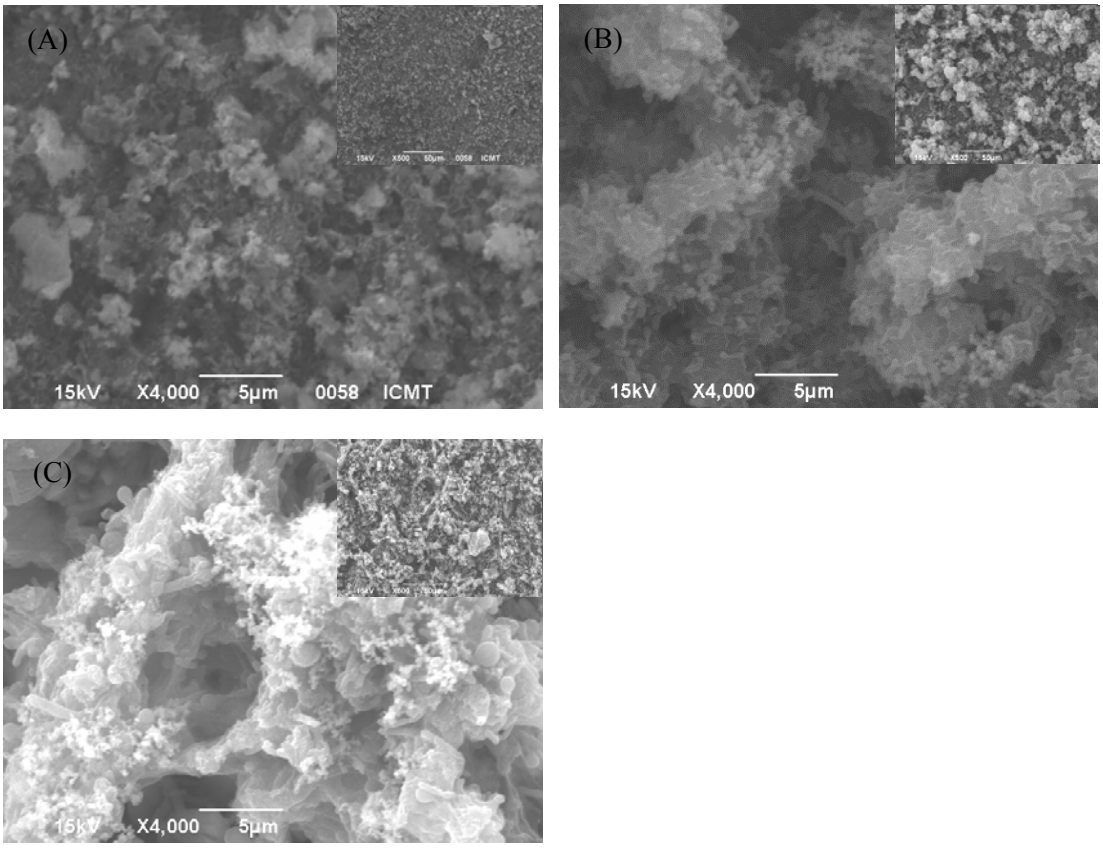

Figure 3-5. Images of biofilms on coupons after 3-hour treatment in the biofilm removal test: (A) *D. vulgaris* biofilm treated with 50 ppm THPS + 100 ppm D-met [31], (B) biofilm Consortium I treated with 50 ppm THPS + 200 ppm D-met, and (C) biofilm Consortium II treated with 50 ppm THPS + 200 ppm D-met. (The scale bar in the inserted small image is 50 µm.)

3.4 Summary

In summary, D-met and D-tyr individually did not enhance the efficacy of 50 ppm THPS in the mitigation of the two field biofilm consortia. In the mitigation of the pure-strain *D. vulgaris* biofilms, 100 ppm D-met or 1 ppm D-tyr combined with 50 ppm THPS remarkably improved the efficacy of 50 ppm THPS. One possible reason is that multiple D-amino acids are necessary due to the microbial diversity in the biofilm consortia. It is likely that different D-amino acids may be prone to different D-amino acids. Therefore, a mixture of D-amino acids is likely needed in the mitigation of biofilm consortia.

4 Screening additional D-amino acids

4.1 Introduction

It is possible that D-amino acids other than D-met and D-tyr trigger biofilm disassembly. Thus, it is desirable to screen all inexpensive D-amino acids as biocide enhancers. In this section, eight D-amino acids were tested to enhance 50 ppm THPS in the mitigation of the *D. vulgaris* biofilm. They were D-valine (D-val), D-phenylalanine (D-phe), D-threonine (D-thr), D-serine (D-ser), D-asparagine (D-asn), D-glutamic acid (D-glu), D-histidine (D-his), and D-aspartic acid (D-asp). All of them were purchased from Fisher Scientific.

4.2 Experimental methods

The biofilm prevention and the biofilm removal tests were performed to evaluate if these eight D-amino acids could enhance the efficacy of 50 ppm THPS. The procedure of both tests and the biofilm analyses were described in Section 3.2.

4.3 Results and discussion

D-ser and D-thr were tested to mitigate the *D. vulgaris* biofilm and two field biofilm consortia used in Task 1. Both biofilm prevention and removal tests were performed. The test condition and procedures were the same as the description in the Section 3.2. The results are summarized as follows.

Table 4-1. Sessile cell count at the end of 7-day biofilm prevention test against *D. vulgaris*

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^7$
100 ppm THPS	$\geq 10^4$
500 ppm D-ser	$\geq 10^6$
50 ppm THPS + 500 ppm D-ser	$\geq 10^2$

In Tables 4-1 and 4-2, 500 ppm D-ser enhanced the efficacy of 50 ppm THPS against the *D. vulgaris* biofilm in both prevention and removal tests. The combination of 500 ppm D-ser + 50 ppm THPS achieved 5 log reduction of the sessile cell count compared with the untreated control in the biofilm prevention test and 4 log reduction in the biofilm removal test. In Figure 4-1, no sessile cell was found on the coupon surface in the biofilm prevention test and only a few sessile cells were found in the biofilm removal test.

Table 4-2. Sessile cell count after 3-hour treatment of biofilm removal against *D. vulgaris*

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^7$
100 ppm THPS	$\geq 10^4$
500 ppm D-ser	$\geq 10^5$
50 ppm THPS + 500 ppm D-ser	$\geq 10^3$

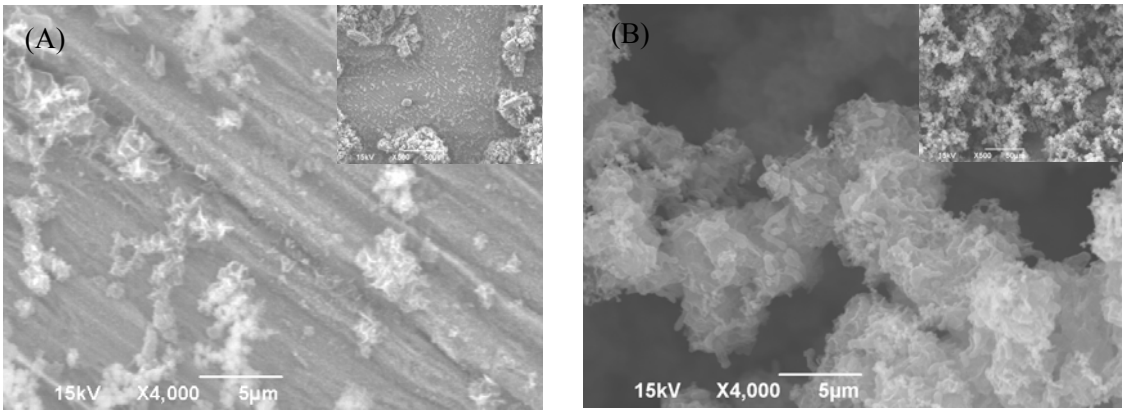

Figure 4-1. Images of biofilm on coupons after: (A) 7-day incubation in the biofilm prevention test in the ATCC 1249 medium containing 50 ppm THPS and 500 ppm D-ser, and (B) 3-hour treatment in a Petri dish containing PBS buffer and 50 ppm THPS and 500 ppm D-ser. (The scale bar in the inserted small image is 50 μm.)

The result of D-thr as a biocide enhancer of THPS in the mitigation of *D. vulgaris* biofilm was similar to that of D-ser. In Tables 4-3 and 4-4, 200 ppm D-thr enhanced the efficacy of 50 ppm THPS against the *D. vulgaris* biofilm in both the prevention and removal tests. The combination of 200 ppm D-thr + 50 ppm THPS achieved 4 log reduction of the sessile cell count compared with the untreated control in both the prevention and removal tests. Figure 4-2 shows that sessile cells were hard to find on the coupon surface.

Table 4-3. Sessile cell count at the end of 7-day biofilm prevention test against *D. vulgaris*

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^7$
100 ppm THPS	$\geq 10^6$
500 ppm D-thr	$\geq 10^6$
50 ppm THPS + 200 ppm D-thr	$\geq 10^3$

Table 4-4. Sessile cell count after 3-hour treatment of biofilm removal against *D. vulgaris*

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^7$
100 ppm THPS	$\geq 10^5$
500 ppm D-thr	$\geq 10^6$
50 ppm THPS + 200 ppm D-thr	$\geq 10^3$

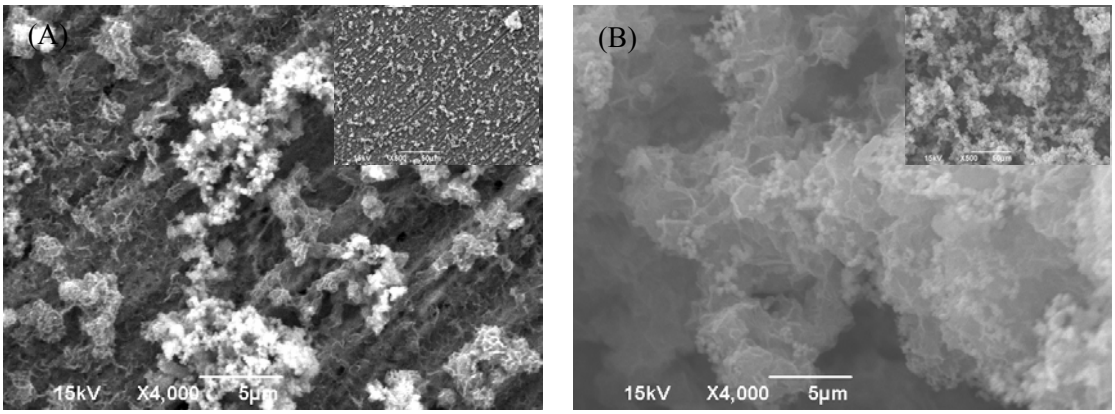

Figure 4-2. Images of biofilm on coupons after: (A) 7-day incubation in the biofilm prevention test in the ATCC 1249 medium containing 50 ppm THPS + 200 ppm D-thr, and (B) 3-hour treatment in a Petri dish containing PBS buffer and 50 ppm THPS + 200 ppm D-thr. (The scale bar in the inserted small image is 50 μm.)

D-his and D-asp were tested to enhance 50 ppm THPS in the mitigation of *D. vulgaris* biofilm. Table 4-5 shows that both D-his and D-asp individually alone did not enhance the efficacy of 50 ppm THPS in the biofilm prevention test.

Table 4-5. Sessile cell count at the end of 7-day biofilm prevention test against *D. vulgaris*

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^5$
50 ppm THPS	$\geq 10^4$
50 ppm THPS + 1000 ppm D-his	$\geq 10^4$
50 ppm THPS + 1000 ppm D-asp	$\geq 10^5$

D-phe was tested as a biocide enhancer of THPS. It was found that 100 ppm D-phe did not enhance the efficacy of 50 ppm THPS. A new test using a high concentration of D-phe was done. The result shows 500 ppm D-phe + 50 ppm THPS achieved 4 log reduction of sessile cell count while 50 ppm THPS alone achieved 1 log reduction of

sessile cell count in biofilm prevention test compared with the untreated control (Table 4-6).

Table 4-6. Sessile cell count at the end of 7-day biofilm prevention test against *D. vulgaris*

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^5$
50 ppm THPS	$\geq 10^4$
1000 ppm D-phe	$\geq 10^5$
50 ppm THPS + 500 ppm D-phe	<10

D-phe was tested to enhance 50 ppm THPS in the biofilm removal test in the mitigation of the *D. vulgaris* biofilm. The result shows that 500 ppm D-phe + 50 ppm THPS achieved 4 log reduction of sessile cell count compared with the untreated control in the removal test (Table 4-7). In Figure 4-3, sessile cells were fewer on the coupon surface after 3-hour treatment with 500 ppm D-phe + 50 ppm THPS than on the control coupon surface (untreated control) and on the coupon surface after 3-hour treatment with 50 ppm THPS. In conclusion, D-phe is an effective enhancer for THPS in the mitigation of *D. vulgaris*, although the minimum concentration of D-phe is rather high.

Table 4-7. Sessile cell count at the end of 3-hour biofilm removal test against *D. vulgaris*

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^6$
50 ppm THPS	$\geq 10^3$
1000 ppm D-phe	$\geq 10^6$
50 ppm THPS + 500 ppm D-phe	$\geq 10^2$

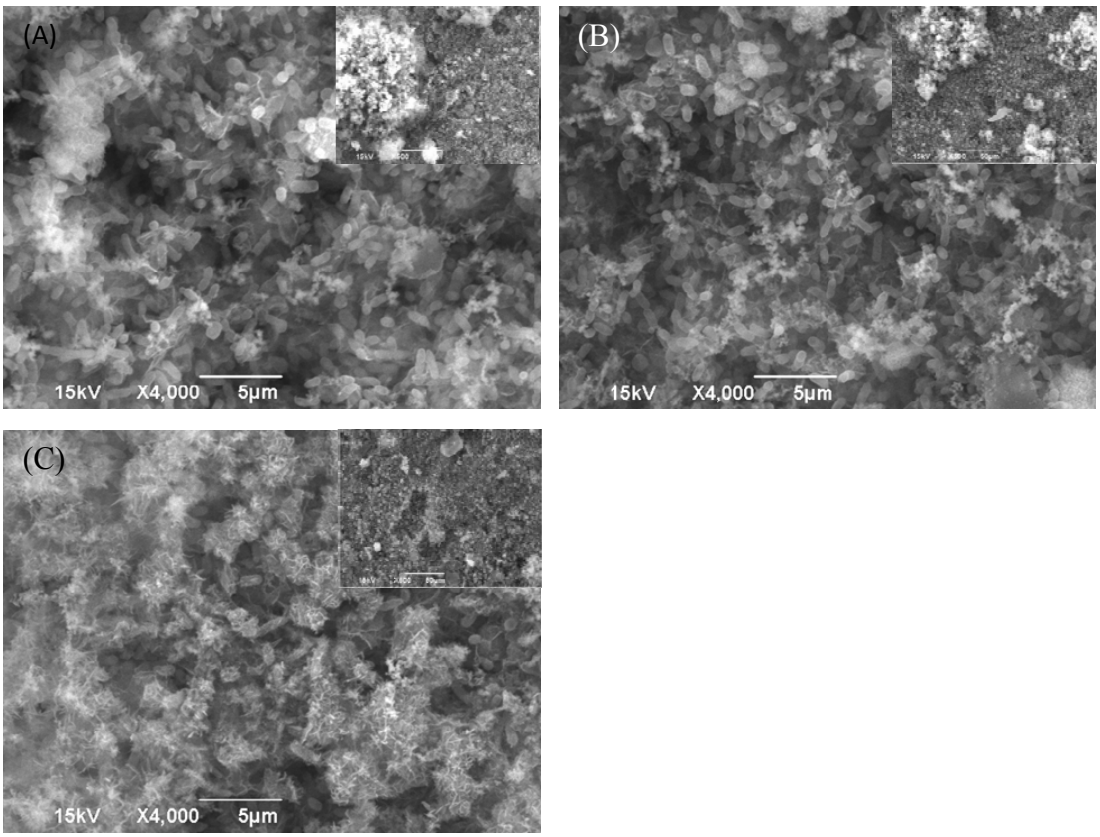

Figure 4-3. Images of biofilms after 3-hour treatment in a Petri dish containing PBS buffer and: (A) no treatment chemicals (control), (B) 50 ppm THPS, and (C) 50 ppm THPS + 500 ppm D-phe in the biofilm removal test. (The scale bar in the inserted small images is 50 µm.)

D-asn was tested to enhance 50 ppm THPS in the biofilm prevention test of the mitigation of the *D. vulgaris* biofilm. The sessile cell concentration on the untreated control coupon surface was 10^6 cells/cm^2 in Table 4-8. Fifty ppm THPS achieved 3 log reduction of sessile cell count and 500 ppm D-asn did not reduce the sessile cell concentration compared with the untreated control. It was found that 500 ppm D-asn + 50 ppm THPS achieved 4 log reduction of sessile cell count in the biofilm prevention test compared with the untreated control.

Table 4-8. Sessile cell count at the end of 3-hour biofilm removal test against *D. vulgaris*

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^6$
50 ppm THPS	$\geq 10^3$
500 ppm D-asn	$\geq 10^6$
50 ppm THPS + 500 ppm D-asn	$\geq 10^2$

4.4 Summary

D-val, D-phe, D-thr, D-ser, and D-asn enhanced the biocidal effect of 50 ppm THPS in the *D. vulgaris* biofilm prevention test. In Table 4-9, 50 ppm THPS achieved 2 log reduction of the sessile cell count compared with the untreated control. The 5 D-amino acids increased the efficacy of 50 ppm THPS by at least 2 log. On the coupon surface after the treatment with 50 ppm THPS + 500 ppm D-phe, sessile cells were undetectable. However, D-glu, D-his, and D-asp did not show enhancement for 50 ppm THPS. In the biofilm removal test, the 5 D-amino acids individually showed enhancement as well (Table 4-10).

Table 4-9. Sessile cell count of 7-day biofilm prevention test against *D. vulgaris*

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^6$
50 ppm THPS	$\geq 10^4$
50 ppm THPS + 500 ppm D-val	$\geq 10^2$
50 ppm THPS + 500 ppm D-phe	<10
50 ppm THPS + 200 ppm D-thr	$\geq 10^3$
50 ppm THPS + 500 ppm D-ser	$\geq 10^2$
50 ppm THPS + 500 ppm D-asn	$\geq 10^2$
50 ppm THPS + 1000 ppm D-glu	$\geq 10^4$
50 ppm THPS + 1000 ppm D-his	$\geq 10^4$
50 ppm THPS + 1000 ppm D-asp	$\geq 10^5$

Table 4-10. Sessile cell count of 3-hour treatment of biofilm removal against *D. vulgaris*

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^7$
50 ppm THPS	$\geq 10^4$
50 ppm THPS + 500 ppm D-phe	$\geq 10^2$
50 ppm THPS + 200 ppm D-thr	$\geq 10^3$
50 ppm THPS + 500 ppm D-ser	$\geq 10^3$
50 ppm THPS + 500 ppm D-asn	$\geq 10^2$
50 ppm THPS + 500 ppm D-val	$\geq 10^3$

So far, a total 12 commercially available D-amino acids have been tested as biocide enhancers to enhance THPS biocide. The rest of D-amino acids are far more expensive and thus not selected at this time. It was found that 9 of the 12 D-amino acids enhanced the efficacy of 50 ppm THPS in the mitigation of the *D. vulgaris* biofilm. D-glu, D-asp,

and D-his did not enhance the efficacy of 50 ppm THPS. D-glu and D-asp have similar side chains, the former being one carbon longer. The list of the 12 D-amino acids and the effective concentrations are shown in Table 4-11.

Table 4-11. Sessile cell count at the end of 3-hour biofilm removal test against *D. vulgaris*

D-amino acid	Effective concentration (ppm)	D-amino acid	Effective concentration (ppm)
D-tyr	1	D-thr	500
D-met	100	D-ser	500
D-trp	1500	D-asn	500
D-leu	1500	D-glu	not effective
D-val	500	D-his	not effective
D-phe	500	D-asp	not effective

5 Using D-amino acid mixtures as biocide enhancers

5.1 Introduction

D-amino acids are promising biocide enhancers for 50 ppm THPS in the mitigation of the *D. vulgaris* biofilm. However, individual D-amino acids failed to enhance or less effectively enhanced 50 ppm THPS in the mitigation of two field biofilm consortia. Rather than a single D-amino acid, a D-amino acid mixture is likely required because different bacteria may be susceptible to different D-amino acids. A total 4 D-amino acid mixtures were tested to enhance 50 ppm THPS in the mitigation of two field biofilm consortia, which were used in Task 1. The investigation of which D-amino acid played an important role in the mixtures was also conducted.

5.2 Experimental methods

In this task, several D-amino acid mixtures (Table 5-1) were used to enhance THPS. Confocal laser scanning microscope (CLSM) (LSM 710, ZESIS, Germany) was used to observe cells in a biofilm on a coupon surface. The dye was Live/Dead® BacLightTM Bacterial Viability Kits L7012 purchased from Life Technologies (Grand Island, NY, USA). It has two components, SYTO 9 and propidium iodide. Coupons were soaked in the dye mixture of these two components with a ratio of 1:1 (v/v) for 15 minutes. SYTO 9 labeled all population in biofilm and propidium iodide only stained cells with damaged membranes (dead cells). The living cells appeared as green dots and the dead cells red dots. After 15 minutes of incubation, coupons were taken out and adhered to a microscope slide. Ten µl distilled water was dropped on the coupon surface to make the coverslip stick to the surface. Afterwards, an 18 mm square coverslip was placed on the coupon surface.

Table 5-1. D-amino acid combinations used to enhance 50 ppm THPS

Mixture	D-amino acids			
D4-1	D-tyr	D-met	D-trp	D-leu
D4-2	D-phe	D-thr	D-ser	D-val
D4-3	D-glu	D-asn	D-asp	D-his
D8-1	D-tyr	D-met	D-trp	D-leu
	D-val	D-phe	D-thr	D-ser
D8-2	D-val	D-phe	D-thr	D-ser
	D-glu	D-asn	D-asp	D-his
D12-1	D-tyr	D-met	D-trp	D-leu
	D-val	D-phe	D-thr	D-ser
	D-glu	D-asn	D-asp	D-his

5.3 Results and discussion

5.3.1 Equimolar mixture of D-methionine, D-tyrosine, D-leucine and D-tryptophan

An equimolar mixture of D-met, D-tyr, D-leu, and D-trp was tested as a biocide enhancer to enhance the efficacy of 50 ppm THPS in the mitigation of two field biofilm consortia tested in Task 1 and Task 2. The composition of the D-amino acid mixture is shown in Table 5-2.

Table 5-2. Composition of D-amino acid in the mixture

D-amino acids	Amount in 50 ppm D-amino acids mixture
D-met	11 ppm
D-tyr	13.5 ppm
D-leu	10 ppm
D-trp	15.5 ppm

Tables 5-3 and 5-4 show that the D-amino acid mixture enhanced THPS mitigation of Consortium I. In biofilm prevention test, 50 ppm D4-1 + 50 ppm THPS achieved 4 log reduction of the SRB sessile count compared with the untreated control. In the biofilm removal test, 30 ppm D4-1 + 50 ppm THPS achieved 4 log reduction of the SRB sessile cell count as well compared with the untreated control. Figure 5-1 shows a visual confirmation that the sessile cell count decreased markedly in both the biofilm prevention and removal tests compared with Figure 5-1A.

Table 5-3. SRB sessile cell count at the end of 7-day biofilm prevention test against Consortium I

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^7$
100 ppm THPS	$\geq 10^6$
50 ppm D4-1	$\geq 10^5$
50 ppm THPS + 50 ppm D4-1	$\geq 10^3$

Table 5-4. SRB sessile cell count after 3-hour treatment of biofilm removal against Consortium I

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^7$
100 ppm THPS	$\geq 10^5$
50 ppm D4-1	$\geq 10^5$
50 ppm THPS + 30 ppm D4-1	$\geq 10^3$

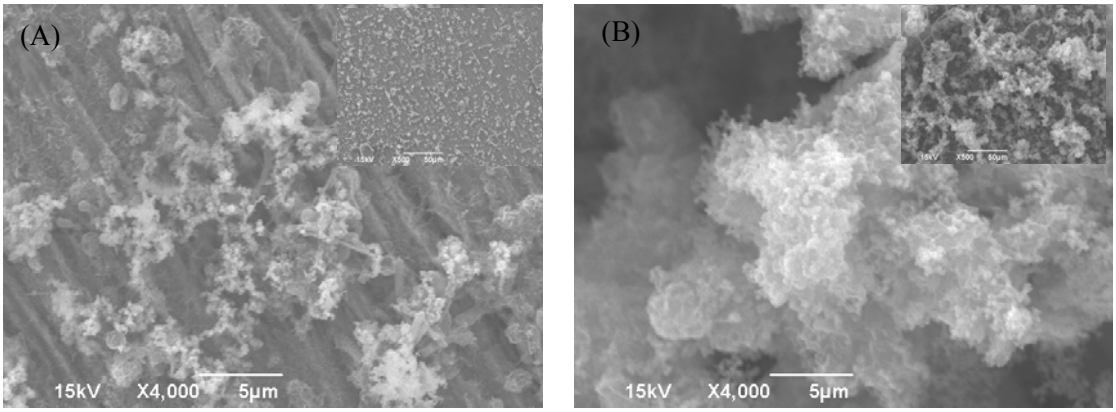

Figure 5-1. Images of biofilm Consortium I on coupons after: (A) 7-day incubation in the biofilm prevention test in the ATCC 1249 medium containing 50 ppm THPS + 50 ppm D4-1, and (B) 3-hour treatment in a Petri dish containing C1018 carbon steel coupons, 50 ml PBS buffer with 50 ppm THPS + 30 ppm D4-1. (The scale bar in the inserted small image is 50 µm.)

In the mitigation of Consortium II, Tables 5-5 and 5-6 show that the D-amino acid mixture enhanced THPS in both the biofilm prevention and biofilm removal tests. The cocktail of 50 ppm D-amino acid mixture + 50 ppm THPS achieved 3 log reduction of the SRB sessile cell count in both tests compared with the untreated control. In Figure 5-2, sessile cells were fewer than those in Figure 3-1, SEM images of Consortia I and II without treatment chemicals, for both the biofilm prevention and removal tests. They were more numerous than those in Figure 5-1. The mitigation of Consortium II required a higher concentration of D-amino acids than the mitigation of Consortium I. It shows that Consortium II was more recalcitrant than Consortium I.

Table 5-5. SRB sessile cell count at the end of 7-day biofilm prevention test against Consortium II

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^7$
100 ppm THPS	$\geq 10^6$
50 ppm D4-1	$\geq 10^6$
50 ppm THPS + 50 ppm D4-1	$\geq 10^4$

Table 5-6. SRB sessile cell count after 3-hour treatment of biofilm removal against Consortium II

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^7$
100 ppm THPS	$\geq 10^6$
50 ppm D4-1	$\geq 10^6$
50 ppm THPS + 50 ppm D4-1	$\geq 10^4$

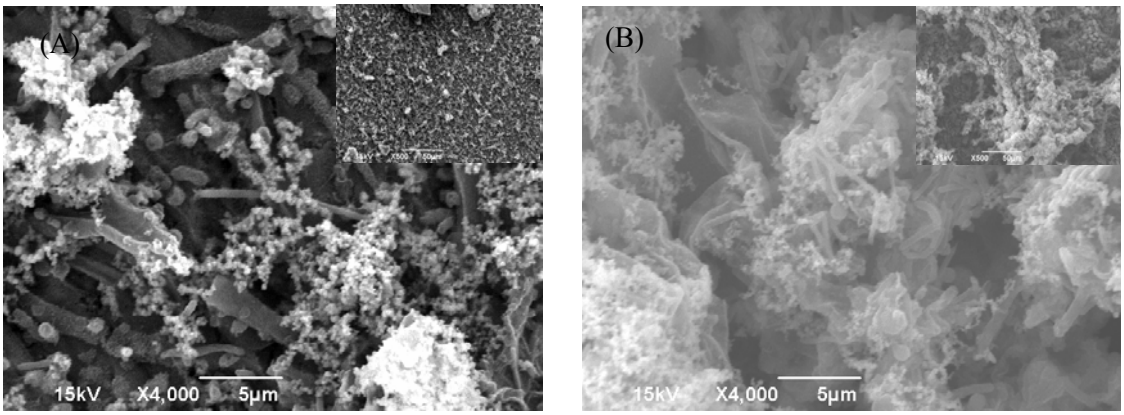

Figure 5-2. Images of biofilm Consortium II on coupons after: (A) 7-day incubation in the biofilm prevention test in the ATCC 1249 medium containing 50 ppm THPS + 50 ppm D4-1, and (B) 3-hour treatment in a Petri dish containing C1018 carbon steel coupons, 50 ml PBS buffer with 50 ppm THPS + 50 ppm D4-1. (The scale bar in the inserted small image is 50 µm.)

5.3.2 Equimolar mixture of D-histidine, D-asparagine, D-aspartic acid, and D-glutamic acid

An equimolar mixture of D-his, D-asn, D-asp, and D-glu (D4-3) was tested to enhance 50 ppm THPS in the mitigation of the two biofilm consortia. Table 5-7 shows that the combination of 50 ppm THPS + 25 ppm D4-3 achieved 1 log reduction of SRB sessile cell count in the biofilm prevention test compared with the untreated control. The treatment of 50 ppm THPS + 50 ppm and 100 ppm D4-3 both achieved 2 log reduction of SRB sessile cell count compared with the untreated control. In the mitigation of biofilm Consortium II, the treatment of 50 ppm THPS + 25 ppm, 50 ppm, and 100 ppm D4-3 all achieved 1 log reduction of SRB sessile cell count in the biofilm prevention test compared with the untreated control. Fifty ppm THPS treatment alone achieved no reduction of SRB sessile cell count in the prevention test against the biofilm consortia. Sessile cells in biofilm consortia I and II could be easily found on carbon steel coupon surfaces after 7-day incubation in the biofilm prevention test in Figure 5-3. In Table 5-8,

treatments of 50 ppm THPS + 25 ppm, 50 ppm, and 100 ppm D4-3 did not reduce the sessile cell count of both biofilm consortia in the biofilm removal test. Fifty ppm THPS treatment alone achieved no reduction of sessile cell count in the removal test against the biofilm consortia as well compared with the untreated control. This means D4-3 did not enhance 50 ppm THPS.

Table 5-7. SRB sessile cell count at the end of 7-day biofilm prevention test against biofilm consortia I and II

Strain	Treatment	Sessile cell count (cells/cm^2)
Consortium I	No treatment chemicals (control)	$\geq 10^6$
	200 ppm D4-3	$\geq 10^6$
	50 ppm THPS	$\geq 10^6$
	50 ppm THPS + 25 ppm D4-3	$\geq 10^5$
	50 ppm THPS + 50 ppm D4-3	$\geq 10^4$
	50 ppm THPS + 100 ppm D4-3	$\geq 10^4$
Consortium II	No treatment chemicals (control)	$\geq 10^6$
	200 ppm D4-3	$\geq 10^6$
	50 ppm THPS	$\geq 10^6$
	50 ppm THPS + 25 ppm D4-3	$\geq 10^5$
	50 ppm THPS + 50 ppm D4-3	$\geq 10^5$
	50 ppm THPS + 100 ppm D4-3	$\geq 10^5$

It was found that 50 ppm equimolar mixture of D-his, D-asn, D-asp, and D-glu did not enhance the efficacy of 50 ppm THPS by much in the mitigation of the two biofilm consortia. The combination of 50 ppm mixture + 50 ppm THPS achieved 1 or 2 log reduction of SRB sessile cell count in the prevention test and no reduction of SRB sessile cell count in the removal test compared with the untreated controls. In Section 5.3.1, the combination of 50 ppm equimolar mixture of D-tyr, D-met, D-leu, and D-trp + 50 ppm THPS achieved 4 log reduction of SRB sessile cell count in the prevention test and 3 log reduction of SRB sessile cell count in the removal test against the same consortia compared with the untreated control.

Table 5-8. SRB sessile cell count after 3-hour treatment of biofilm removal against biofilm consortia I and II

Strain	Treatment	Sessile cell count (cells/cm^2)
Consortium I	No treatment chemicals (control)	$\geq 10^6$
	200 ppm D4-3	$\geq 10^6$
	50 ppm THPS	$\geq 10^6$
	50 ppm THPS + 25 ppm D4-3	$\geq 10^6$
	50 ppm THPS + 50 ppm D4-3	$\geq 10^6$
	50 ppm THPS + 100 ppm D4-3	$\geq 10^6$
Consortium II	No treatment chemicals (control)	$\geq 10^6$
	200 ppm D4-3	$\geq 10^6$
	50 ppm THPS	$\geq 10^6$
	50 ppm THPS + 25 ppm D4-3	$\geq 10^6$
	50 ppm THPS + 50 ppm D4-3	$\geq 10^6$
	50 ppm THPS + 100 ppm D4-3	$\geq 10^6$

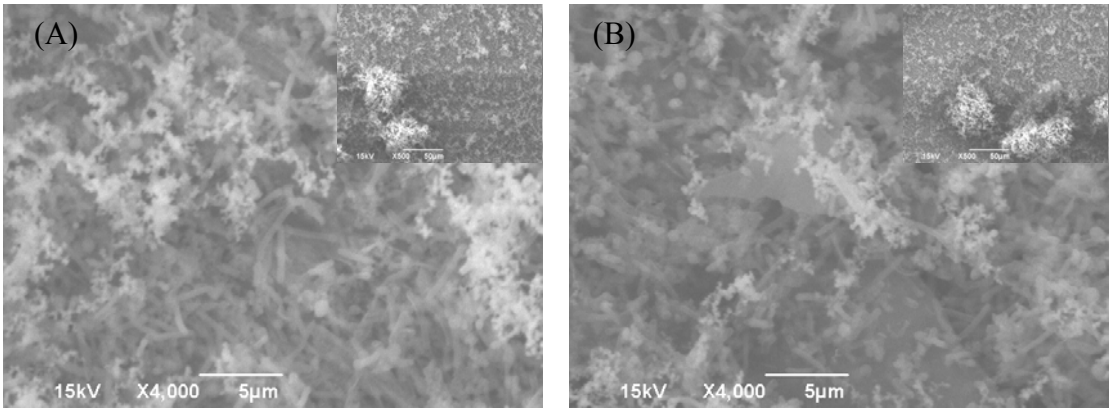

Figure 5-3. SEM images of biofilms on carbon coupons after 7-day incubation in the biofilm prevention test in ATCC 1249 medium: (A) biofilm Consortium I treated with 50 ppm THPS and 100 ppm D4-3, and (B) biofilm Consortium II treated with 50 ppm THPS and 100 ppm D4-3. (The scale bar in the inserted small image is 50 µm.)

5.3.3 Equal mass fraction mixture with eight D-amino acids

A D-amino acid mixture was prepared of eight D-amino acids with equal mass fractions. They are D-tyr, D-met, D-trp, D-leu, D-val, D-phe, D-thr, and D-ser. Each of them was an effective biocide enhancer for THPS in the mitigation of *D. vulgaris* on carbon steel. Due to the solubility of this D-amino acid mixture (D8-1), the concentration of the stock solution was 8,000 ppm instead of 10,000 ppm as other two mixtures tested in previous

works. The D-mix was tested with 50 ppm THPS in the mitigation of biofilm Consortia I and II.

In the mitigation of Consortium I, the cocktail of 50 ppm THPS + 30 ppm D8-1 achieved 5 log reduction of SRB sessile cell count compared with the untreated control, while 50 ppm THPS treatment alone achieved 2 log reduction compared with the untreated control (Table 5-9). It was noticeable that 30 ppm D8-1 enhanced 50 ppm THPS achieving 3 extra log reduction of SRB sessile cell count compared with the 50 ppm THPS control. In Table 5-10, the cocktail of 50 ppm THPS + 30 ppm D8-1 achieved 4 log reduction of SRB sessile cell count in the removal test compared with the untreated control, while 50 ppm THPS alone achieved 2 log reduction of SRB sessile cell count compared with the untreated control, which is the same as in the biofilm prevention test. SEM images in Figure 5-4 confirmed the SRB sessile cell count data. Sessile cells were hard to find on the coupon surfaces after the treatment of 50 ppm THPS + 30 ppm D8-1 in both biofilm prevention and removal tests. In conclusion, 30 ppm D8-1 enhanced 50 ppm THPS in both biofilm prevention and removal tests in the mitigation of biofilm Consortium I.

Table 5-9. SRB sessile cell count in Consortium I after 7-day biofilm prevention test

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals(control)	$>10^7$
50 ppm THPS	$>10^5$
100 ppm D8-1	$>10^6$
50 ppm THPS + 30 ppm D8-1	$>10^2$

Table 5-10. SRB sessile cell count in Consortium I after 3-hour treatment in a Petri dish in the biofilm removal test

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$>10^7$
50 ppm THPS	$>10^5$
100 ppm D8-1	$>10^6$
50 ppm THPS + 30 ppm D8-1	$>10^3$

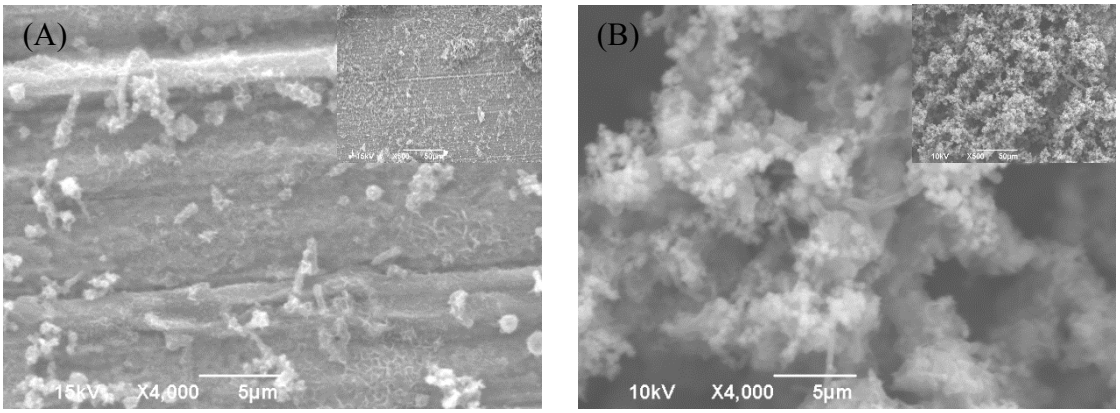

Figure 5-4. Images of biofilm Consortium I after: (A) 7-day incubation in the biofilm prevention test in ATCC 1249 medium containing 50 ppm THPS + 30 ppm D8-1 and (B) 3-hour treatment in a Petri dish containing PBS buffer and 50 ppm THPS + 30 ppm D8-1 in the biofilm removal test. (The scale bar in the inserted small images is 50 µm.)

The SRB sessile cell count data in the mitigation of biofilm Consortium II are shown in Tables 5-11 and 5-12. The treatment of 50 ppm THPS achieved 2 log reduction of the SRB cell count, while 30 ppm D8-1 + 50 ppm THPS achieved 5 log, indicating 3 log enhancement (Table 5-11). When 50 ppm D8-1 was used to enhance 50 ppm THPS, SRB sessile cells on the coupon surface were undetectable. In the biofilm removal test, 50 ppm THPS + 30 ppm D8-1 achieved 4 log reduction of the SRB sessile cell count which was 2 log more than that achieved by 50 ppm THPS treatment alone compared with untreated control in Table 5-12. In Figure 5-5, sessile cells are hard to find after the treatments of 50 ppm THPS + 30 ppm D8-1 in both biofilm prevention and biofilm removal tests. Therefore, D8-1 is considered an effective biocide enhancer for THPS in the mitigation of Consortium II.

Table 5-11. SRB sessile cell count in Consortium II after 7-day biofilm prevention test

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$>10^7$
50 ppm THPS	$>10^5$
100 ppm D8-1	$>10^6$
50 ppm THPS + 30 ppm D8-1	$>10^2$
50 ppm THPS + 50 ppm D8-1	<10

Table 5-12. SRB sessile cell count in Consortium II after 3 hour treatment in a Petri dish in the biofilm removal test

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$>10^7$
50 ppm THPS	$>10^5$
100 ppm D8-1	$>10^7$
50 ppm THPS + 30 ppm D8-1	$>10^3$

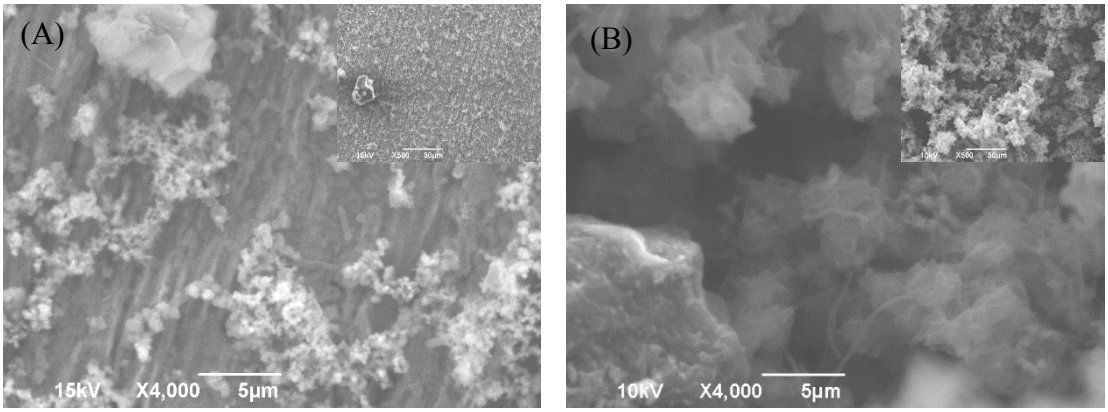

Figure 5-5. Images of biofilm Consortium II after: (A) 7-day incubation in the biofilm prevention test in ATCC 1249 medium containing 50 ppm THPS + 30 ppm D8-1 and (B) 3-hour treatment in a Petri dish containing PBS buffer and 50 ppm THPS + 30 ppm D8-1 in the biofilm removal test. (The scale bar in the inserted small images is 50 µm.)

Images in Figure 5-6 are CLSM images of biofilm Consortium II in the biofilm prevention and removal tests. Figure 5-6A shows that a large amount of sessile cells, both living and dead cells, were detected after 7-day incubation with 50 ppm THPS in the biofilm prevention test. Combined with 30 ppm D8-1, the efficacy of 50 ppm THPS was enhanced since fewer sessile cells were detected and most of the detected cells were dead in Figure 5-6B. A similar trend was found in the removal test as well. In Figure 5-6C, after 3-hour treatment with 50 ppm THPS, a great number of living cells (green dots) were detected accompanied by a small fraction of dead cells (red dots). In Figure 5-6D, most detected cells appear as dead cells. The treatment of 50 ppm THPS + 30 ppm D8-1 (Figure 5-6D) had a better biocidal effect than 50 ppm THPS treatment alone (Figure 5-6C) in the removal test. Therefore, the CLSM images in Figures 5-6 strongly suggest that D8-1 enhanced the efficacy of 50 ppm THPS in the mitigation of field biofilm Consortium II.

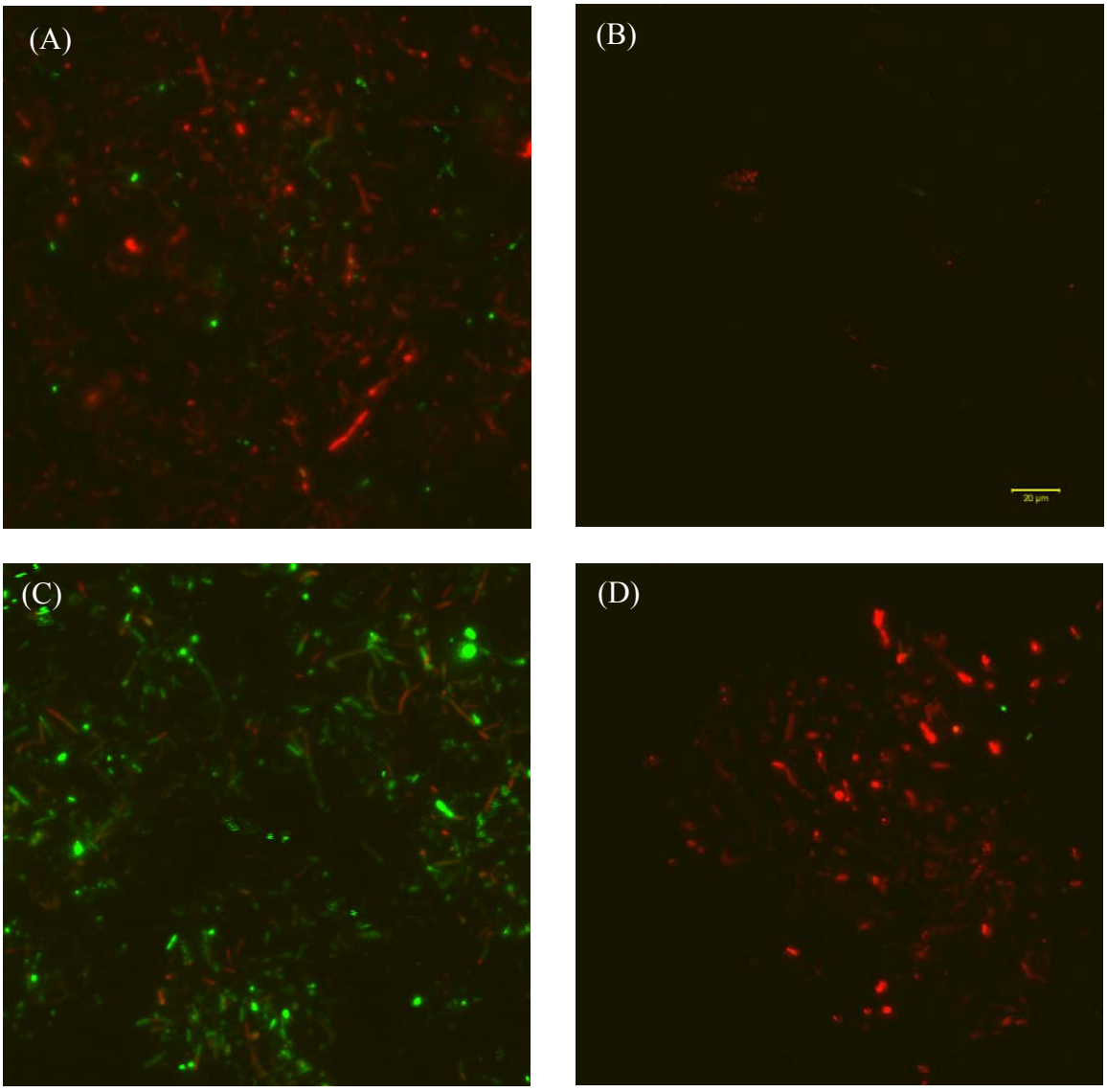

Figure 5-6. CLSM images of biofilm Consortium II after: 7-day incubation in the biofilm prevention test in ATCC 1249 medium containing (A) 50 ppm THPS and (B) 50 ppm THPS + 30 ppm D8-1; 3-hour treatment in a Petri dish containing PBS buffer containing (C) 50 ppm THPS and (D) 50 ppm THPS + 30 ppm D8-1 in the biofilm removal test.

5.3.4 Equimolar mixture of D-tyrosine and D-methionine

The equimolar mixture of D-tyr and D-met was tested with THPS in the biofilm prevention test against Consortium II. The sessile cell concentration was 10^6 cells/cm^2 in Table 5-13. Tests using 50 ppm THPS, 50 ppm equimolar mixture of D-tyr and D-met, and 50 ppm THPS + 50 ppm equimolar mixture of D-tyr and D-met all did not reduce the SRB sessile cell counts. In Figure 5-7, abundant sessile cells were found on the coupon

surfaces in these three treatments. There was no visual difference between the biofilm morphology on the untreated control coupon surface and the coupon surfaces with these three treatments. The results suggested that the equimolar mixture of D-tyr and D-met was not an effective biocide enhancer in lab tests. Thus, there was no surprise that an equimolar mixture of D-tyr and D-met did not enhance 250 ppm THPS in the mitigation of their field biofilm consortium in a test conducted by an industrial collaborator. A D-amino acid mixture including more types of D-amino acids was necessary in the field tests.

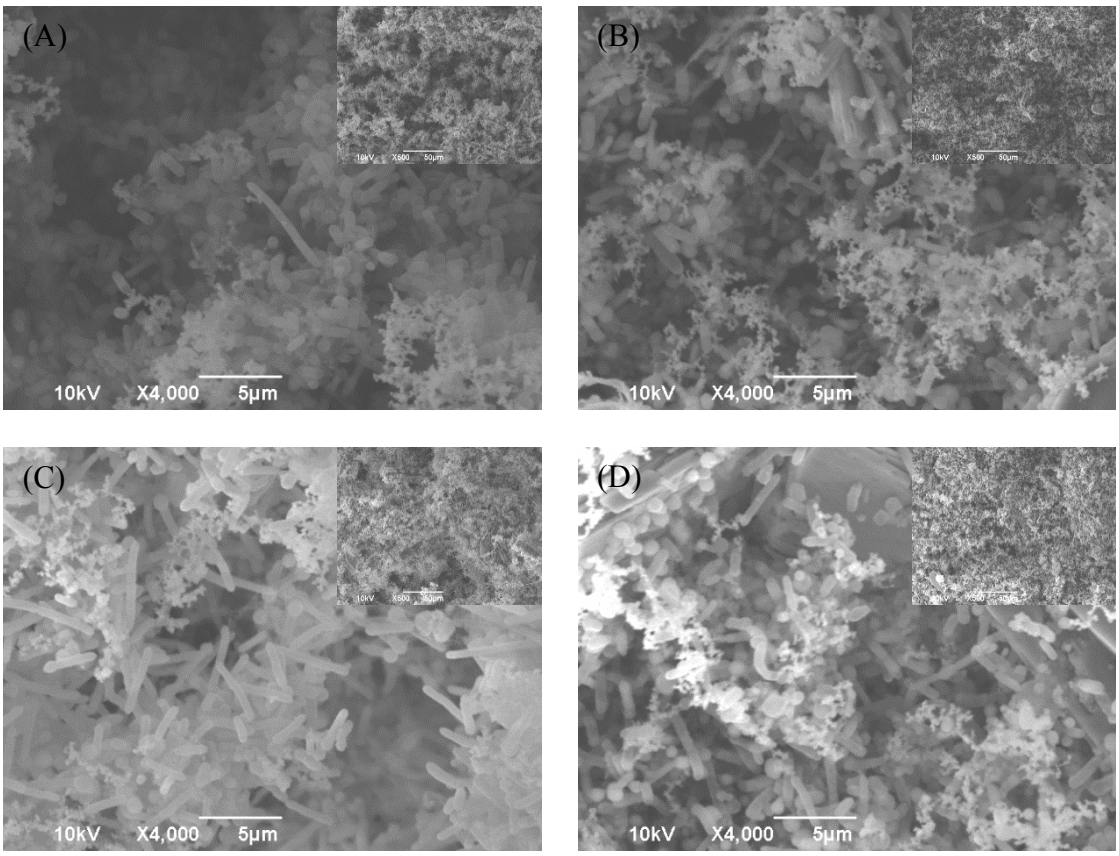

Figure 5-7. Images of biofilms after 7-day incubation in the biofilm Consortium II prevention test in the ATCC 1249 medium containing: (A) no treatment chemicals (control), (B) 50 pm THPS, (C) 50 ppm equimolar mixture of D-tyr and D-met, and (D) 50 ppm THPS + 50 ppm equimolar mixture of D-tyr and D-met. (The scale bar in the inserted small image is 50 µm.)

Table 5-13. SRB sessile cell count at the end of 3-hour biofilm removal test in Petri dishes against Consortium II

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^6$
50 ppm THPS	$\geq 10^6$
50 ppm equimolar mixture of D-tyr and D-met	$\geq 10^6$
50 ppm THPS + 50 ppm equimolar mixture of D-tyr and D-met	$\geq 10^6$

5.3.5 Investigation of the importance of each D-amino acid in a D-amino acid mixture to enhance 50 ppm THPS against Consortium II

Table 5-14. SRB sessile cell count after 7-day biofilm prevention test using 50 ppm THPS

Mixture	Concentration (ppm)	Sessile cell reduction* (log reduction)
D4-1	50	3
D4-2	25	2
D4-3	50 100	1 1
D8-1	50	5
D8-2	50	1
D12-1	50	4

*For biofilm Consortium II. The SRB sessile cell reduction for the treatment of 50 ppm THPS without D-amino acids was 1 log reduction compared with the untreated control.

Table 5-15. D-amino acid combinations used to enhance 50 ppm THPS

Mixture	D-amino acids				Concentration (ppm)
D4-1	D-tyr	D-met	D-try	D-leu	50
D8-1	D-tyr D-val	D-met D-phe	D-try D-thr	D-leu D-ser	50
D6-1	D-tyr D-ser	D-met D-thr	D-trp	D-leu	50
D5-1	D-tyr D-ser	D-met	D-trp	D-leu	50
D5-2	D-tyr D-thr	D-met	D-trp	D-leu	50

Table 5-16. Conditions for optimizing the D-amino acid mixture in the biofilm prevention test

Biofilm	Consortium II
Culture medium	ATCC 1249
Treatment method	THPS, D-amino acid mixtures (equal mass), THPS + D-amino acid mixtures
Temperature	37°C
Incubation duration	7 days
Coupon	C1018 carbon steel

The biofilm prevention test was used to optimize the D-amino acid mixture with 50 ppm THPS for biofilm mitigation. The test conditions are shown in Table 5-16. To optimize the equal-mass mixture consisting of D-met, D-tyr, D-trp, D-leu, D-val, D-phe, D-ser, and D-thr (D8-1) for the enhancement of 50 ppm THPS in the mitigation of biofilm Consortium II, the combination of D-met, D-tyr, D-trp, and D-leu (D4-1) in Table 5-15 was first considered. Any one of the D-amino acids used alone and any binary D-amino acid combination did not show enhancement of 50 ppm THPS against the field biofilm. Table 5-17 shows SRB sessile cell counts after treatments using 50 ppm THPS + D4-1 minus one D-amino acid at a time. Although they all achieved the same cell count reduction, the weight loss data in Figure 5-8 show that they led to higher weight losses than the complete D4-1 mixture without dropping one D-amino acid. CLSM images indicated that these combinations (D4-1 minus D-trp, D-tyr, D-met, or D-leu one at a time in Figure 5-9(E-H)) led to more living cells (green dots) after the 7-day biofilm prevention test than the D4-1 enhanced treatment (Figure 5-9D). Thus, D-met, D-tyr, D-trp, and D-leu are all considered essential in the combination.

In Section 5.3.3, it was shown that D8-1 achieved the best enhancement. Tests were done by dropping each of the 4 extra (compared with D4-1) D-amino acids (D-val, D-phe, D-ser, and D-thr) in D8-1 one at a time, the SRB sessile cell counts are shown in Table 5-18. Dropping D-val or D-phe didn't affect the SRB sessile cell count, while dropping D-ser or D-thr led to 1-log SRB sessile cell count increase. The results were confirmed by the weight loss data in Figure 5-10 that shows dropping D-ser or D-thr led to higher weight losses. This was corroborated by the CLSM images in Figure 5-11. Figure 5-11 shows that the treatment without D-ser or D-thr in D8-1 led to more living cells. Thus, further optimization should be based on these six D-amino acids (D-met, D-tyr, D-trp, D-leu, D-ser, and D-thr).

Since the D4-1 mixture achieved better enhancement for 50 ppm THPS than the mixture of D-val, D-phe, D-ser, and D-thr, the four D-amino acids in D4-1 are deemed all important and should be included in a D-amino acid mixture. Three equal mass combinations among the six D-amino acids (D-met, D-tyr, D-trp, D-leu, D-ser, and D-thr) shown in Table 5-15 as D6-1, D5-1 and D5-2 were tested to enhance 50 ppm THPS against the field biofilm Consortium II. The SRB sessile cell counts in Table 5-19 indicate that D6-1 didn't achieve better results than D5-1 and D5-2. Figure 5-12 also shows the treatments of 50 ppm THPS + 50 ppm D5-1 and 50 ppm THPS + D5-2 led to smaller weight losses than the cocktail of 50 ppm THPS + 50 ppm D6-1. The CLSM images in Figure 5-13 confirm the results. Figure 5-13 shows that the treatment with D5-1 or D5-2 in combination with 50 ppm THPS resulted in much fewer living cells than the treatment with D6-1 in combination with 50 ppm THPS against the field biofilm Consortium II in the 7-day biofilm prevention test.

Table 5-17. SRB sessile cell counts after biofilm prevention test using D-amino acids in D4-1 to enhance 50 ppm THPS against the field biofilm Consortium II

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^7$
50 ppm THPS	$\geq 10^5$
50 ppm D4-1	$\geq 10^6$
50 ppm THPS + 50 ppm D4-1	$\geq 10^4$
50 ppm THPS + 38 ppm D4-1 minus D-trp	$\geq 10^4$
50 ppm THPS + 38 ppm D4-1 minus D-tyr	$\geq 10^4$
50 ppm THPS + 38 ppm D4-1 minus D-met	$\geq 10^4$
50 ppm THPS + 38 ppm D4-1 minus D-leu	$\geq 10^4$

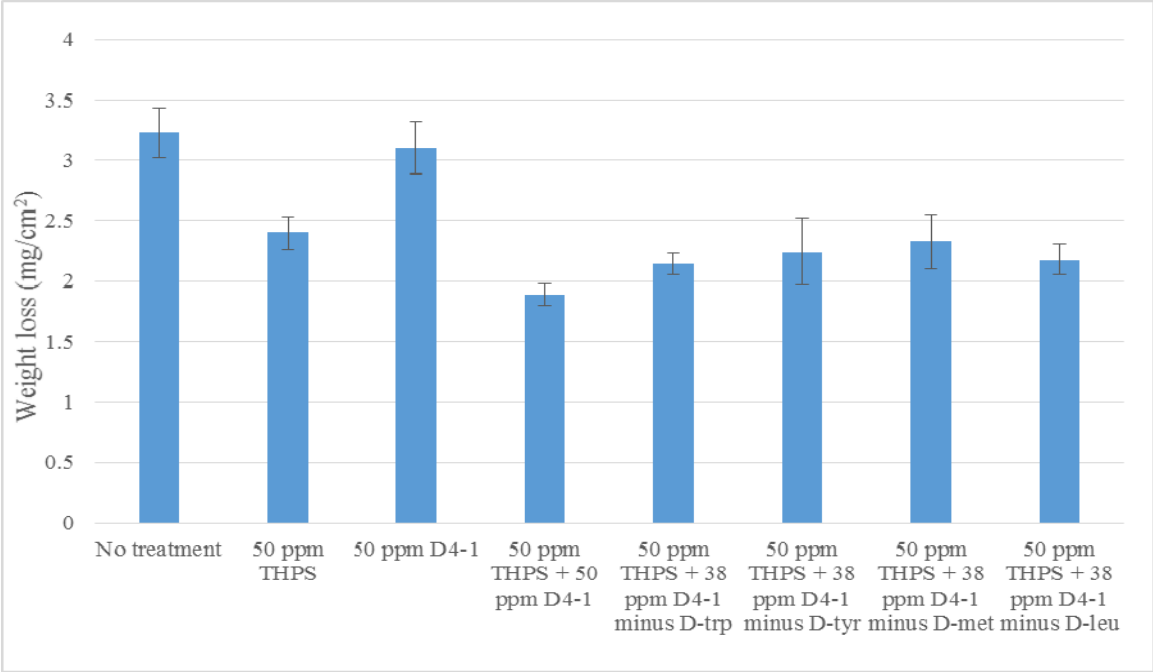

Figure 5-8. Coupon weight loss data after biofilm prevention test using D-amino acids in D4-1 to enhance 50 ppm THPS against the field biofilm Consortium II.

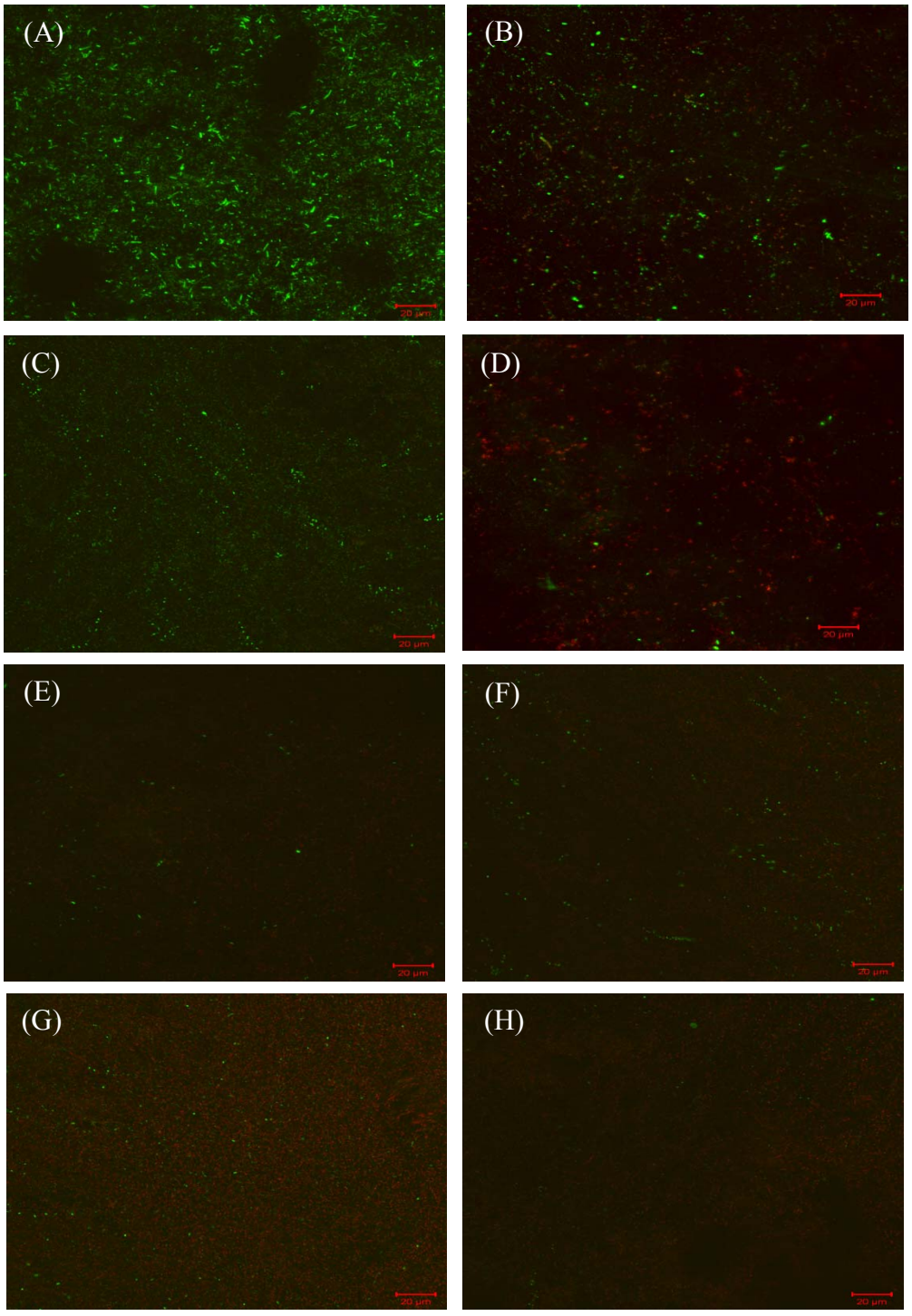

Figure 5-9. CLSM images of biofilms after 7-day incubation in the biofilm Consortium II prevention test: (A) no treatment chemicals (control), (B) 50 ppm THPS, (C) 50 ppm D4-1, (D) 50 ppm THPS + 50 ppm D4-1, (E) 50 ppm THPS + 38 ppm D4-1 minus D-trp, (F) 50 ppm THPS + 38 ppm D4-1 minus D-tyr, (G) 50 ppm THPS + 38 ppm D4-1 minus D-met, and (H) 50 ppm THPS + 38 ppm D4-1 minus D-leu.

Table 5-18. SRB sessile cell counts after 7-day biofilm prevention test using D-amino acids in the D8-1 to enhance 50 ppm THPS against the field biofilm Consortium II

Treatment	Sessile cell count (cells/cm^2)
50 ppm D8-1	$\geq 10^6$
50 ppm THPS + 50 ppm D8-1	$\geq 10^3$
50 ppm THPS + 44 ppm D8-1 minus D-ser	$\geq 10^4$
50 ppm THPS + 44 ppm D8-1 minus D-val	$\geq 10^3$
50 ppm THPS + 44 ppm D8-1 minus D-phe	$\geq 10^3$
50 ppm THPS + 44 ppm D8-1 minus D-thr	$\geq 10^4$

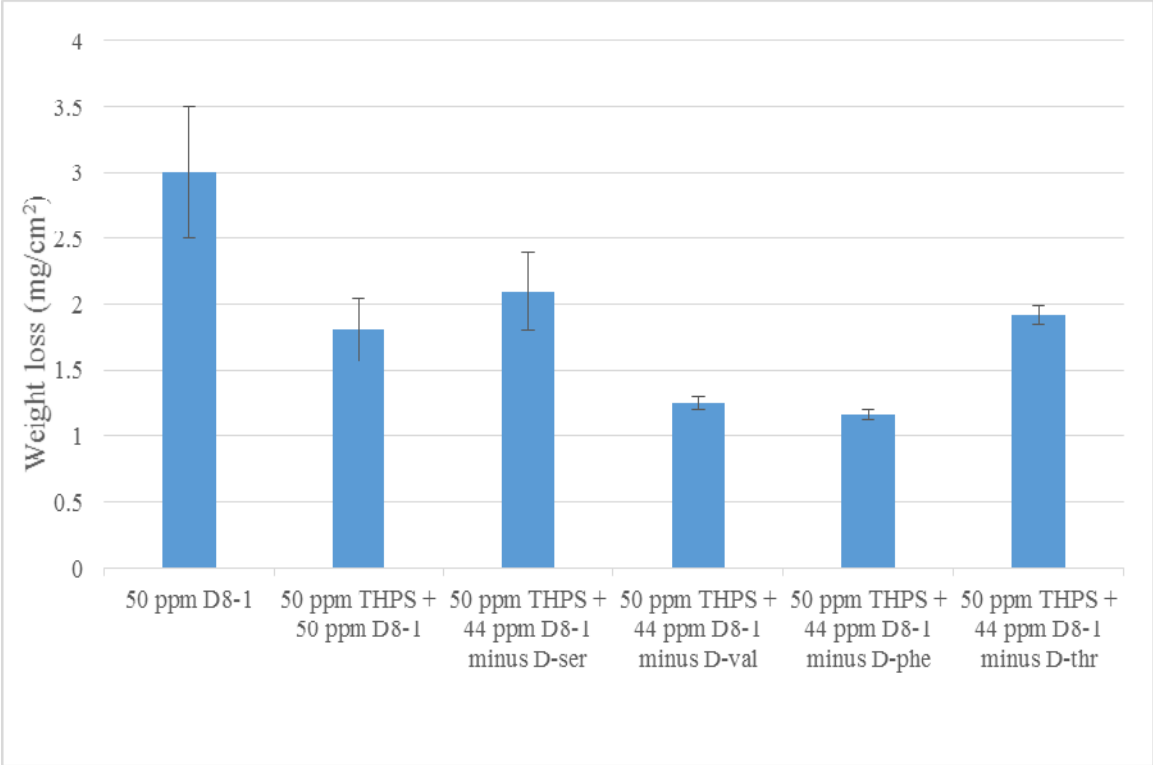

Figure 5-10. Weight loss data after 7-day biofilm prevention test using D-amino acids in the D8-1 to enhance 50 ppm THPS against the field biofilm Consortium II.

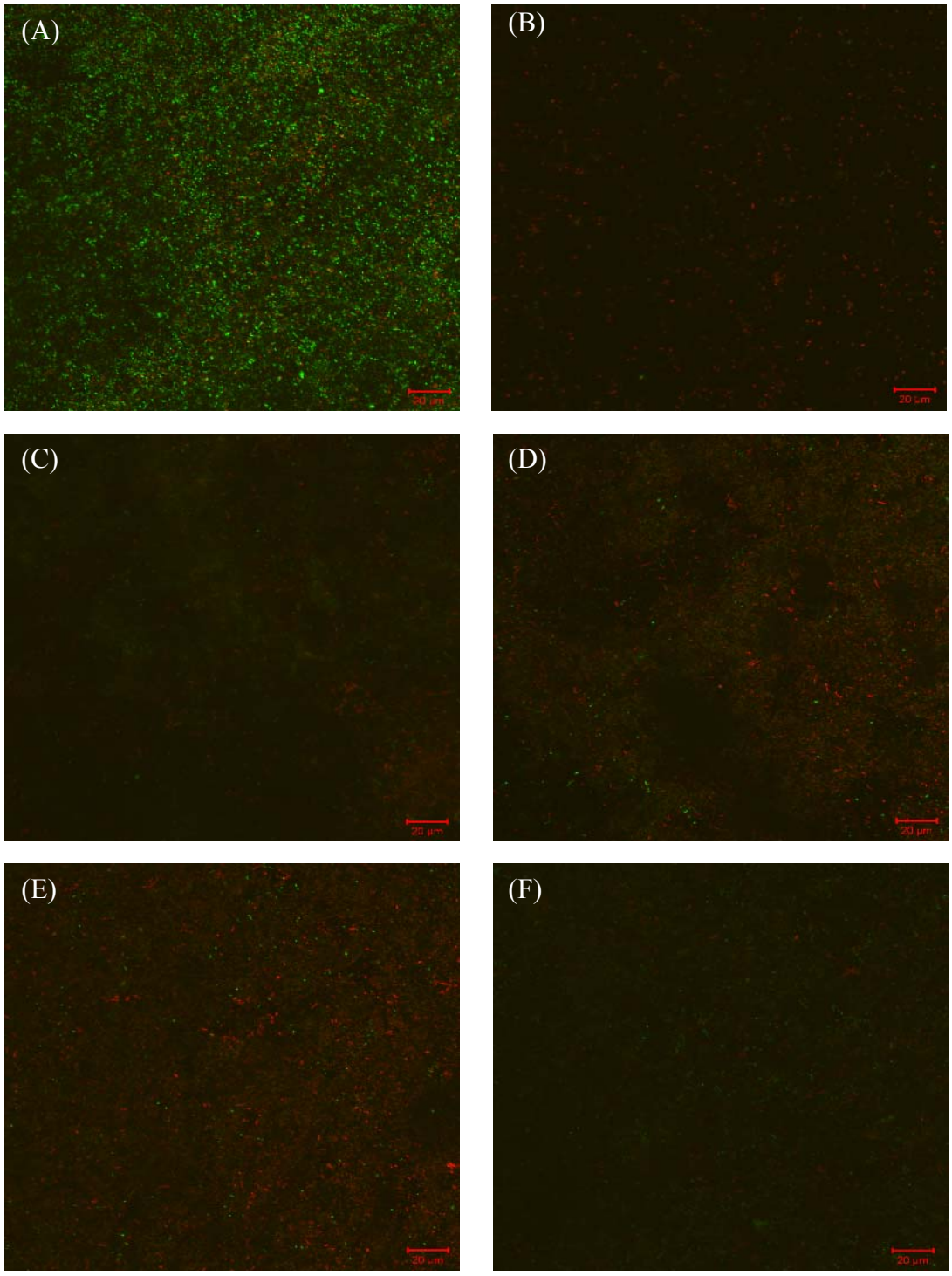

Figure 5-11. CLSM images of biofilms after 7-day incubation in the biofilm Consortium II prevention test: (A) 50 ppm D8-1, (B) 50 ppm THPS + 50 ppm D8-1, (C) 50 ppm THPS + 44 ppm D8-1 minus D-ser, (D) 50 ppm THPS + 44 ppm D8-1 minus D-val, (E) 50 ppm THPS + 44 ppm D8-1 minus D-phe, and (F) 50 ppm THPS + 44 ppm D8-1 minus D-thr.

Table 5-19. SRB sessile cell counts after 7-day biofilm prevention test using different D-mix to enhance 50 ppm THPS against the field biofilm Consortium II

Treatment	Sessile cell count (cells/cm^2)
50 ppm THPS + 50 ppm D6-1	$\geq 10^4$
50 ppm THPS + 50 ppm D5-1	$\geq 10^3$
50 ppm THPS + 50 ppm D5-2	$\geq 10^3$

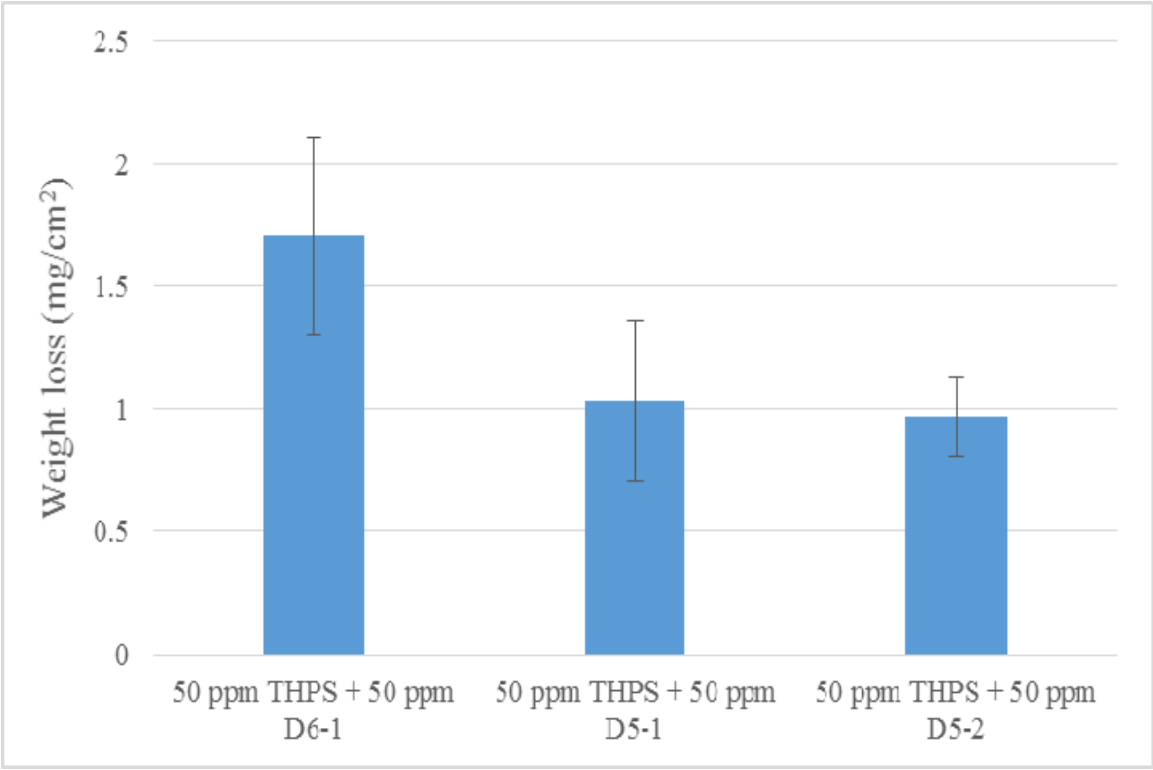

Figure 5-12. Weight loss data after 7-day biofilm prevention test using different D-amino acid mixtures to enhance 50 ppm THPS against the field biofilm Consortium II.

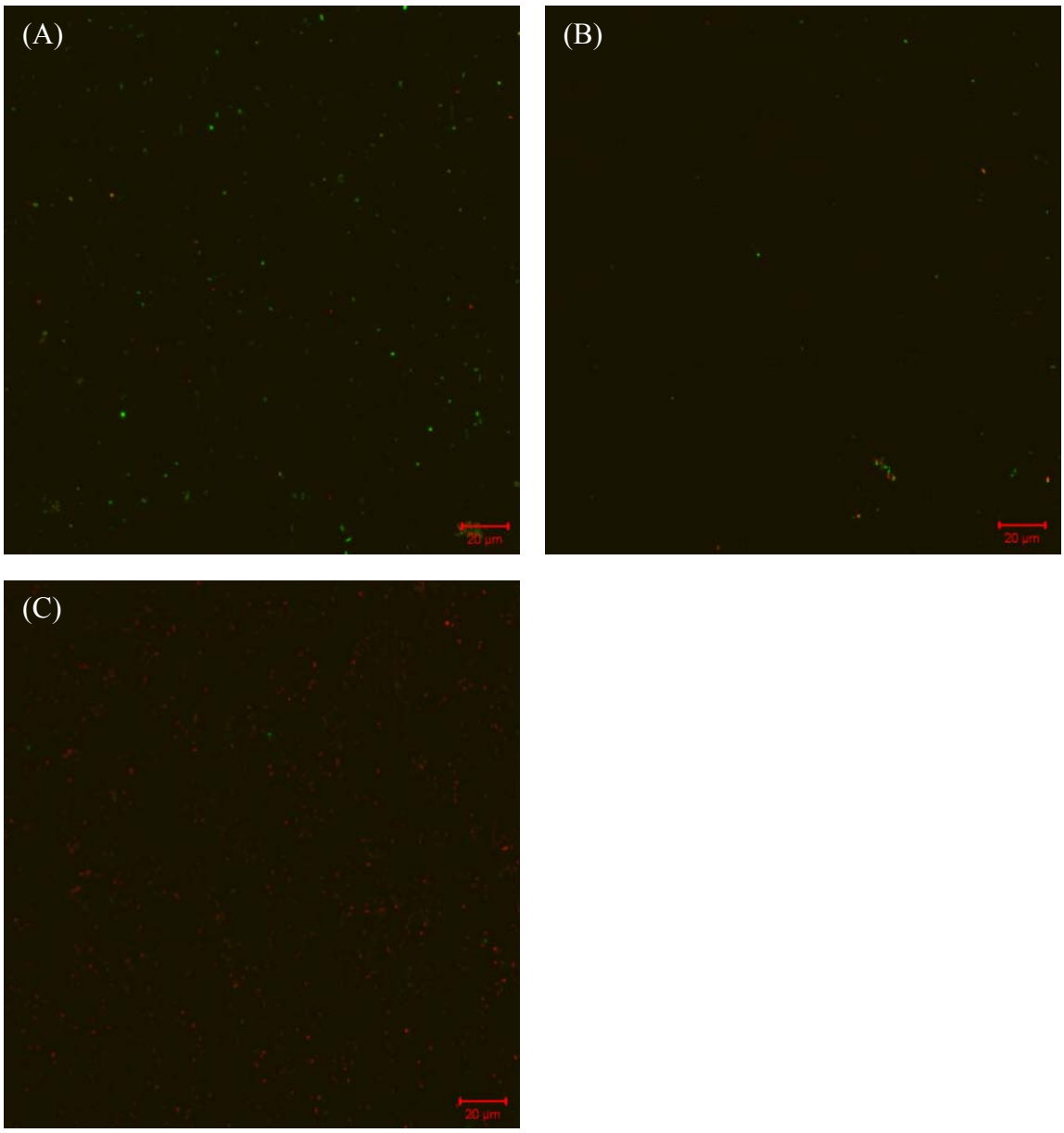

Figure 5-13. CLSM images of biofilms after 7-day biofilm Consortium II prevention test: (A) 50 ppm THPS + 50 pm D6-1, (B) 50 ppm THPS + 50 ppm D5-1, and (C) 50 ppm THPS + 50 ppm D5-2.

5.4 Summary

An industry partner tested 50 ppm equimolar mixture of D-met and D-tyr with 250 ppm THPS against a tough microbial biofilm consortium of theirs. However, no significant enhancement was achieved. Tests of using the same D-amino acid mixture as biocide enhancer and 50 ppm THPS were repeated in the lab. It was found that 50 ppm equimolar mixture of D-met and D-tyr did not enhance 50 ppm THPS against biofilm Consortium II. The 30 ppm D-amino acid mixture consisting of eight D-amino acids (D8-1) enhanced the efficacy of 50 ppm THPS against the same consortium. The result suggests that D-amino acid mixture containing more D-amino acids than D-met and D-tyr would be necessary in the mitigation of the field biofilm consortia. A total of 6 D-amino acid mixtures were tested with 50 ppm THPS in the mitigation of two field biofilm consortia (Consortia I and II). The mixture consisting of D-met, D-tyr, D-trp, D-leu, D-val, D-phe, D-ser, and D-thr achieved the best enhancement for 50 ppm THPS. The optimization of the D-amino acid mixture consisting of D-met, D-tyr, D-trp, D-leu, D-val, D-phe, D-ser, and D-thr was carried out with 50 ppm THPS in the mitigation of the field biofilm Consortium II. Based on the SRB sessile cell data, weight loss data and CLSM observation, the mixture of equal mass D-met, D-tyr, D-trp, D-leu, and D-ser (labeled as D5-1) or D-met, D-tyr, D-trp, D-leu, and D-thr (labeled as D5-2) achieved the same enhancement as the mixture of equal mass D-met, D-tyr, D-trp, D-leu, D-ser, D-val, D-phe, and D-thr (labeled as D8-1) for 50 ppm THPS.

6 Testing of field cooling tower biofilms

6.1 Introduction

A collaboration was carried with a polymer plant in the US (name withheld by request) to solve biofilm corrosion and fouling problems in their cooling towers. Unlike oil and gas pipelines that are anaerobic, cooling towers are open to the air. Thus, aerobic biofilms will grow. Underneath the aerobic biofilms, anaerobic biofilms find a locally anaerobic environment to thrive.

Chlorine is a widely used biocide for the control and removal of organisms in drinking water system and in cooling systems [44,45]. In swimming pools, chlorine is used to remove algae. It is also used to control harmful algal blooms in aquaculture farms [46]. Chlorine is an active oxidizing agent which can damage cell membranes, proteins, and nucleic acids in various organisms [47]. In the aquatic environment, Cl_2 hydrolyses rapidly (Reaction 3). Cl_2 easily dissolves in water to form the hypochlorous acid (HClO).

$$Cl_2 + H_2O \leftrightarrow HClO + HCl \qquad (3)$$

Bacteria easily grow in cooling water systems which are warm and contains some nutrients. Biofouling is a common and significant problem in the operation of cooling water systems [48,49]. It is often recommended to use low-level HClO as a biocide for treatment of cooling water systems [50]. D-amino acids were found to trigger biofilm disassembly. D-amino acids have to be compatible with biocides if used as biocide enhancers. Glutaraldehyde is a widely used biocide, which has the ability to crosslink amino acids. It was reported that neither 10 ppm D-tyr nor 100 ppm D-met could enhance 50 ppm glutaraldehyde against the *D. vulgaris* biofilm in a 7-day biofilm prevention test because of glutaraldehyde's reactivity [51].

Chlorine reacts readily with a wide variety of biomolecules including proteins, DNA, lipids, cholesterol, NADH and free thiols and disulfides [52–55]. Proteins consist of one or more polypeptides arranged in a biologically functional way. Peptides contain approximately 50 or fewer amino acids. Take amino acids into account, the rates of some of the reactions of HClO with amino acids, peptides and proteins have been determined by kinetic studies and stopped-flow methods [56,57].

The field biofilms were collected by plant operators using C1018 carbon steel coupons from a water cooling system in the polymer plant. Bleach is the biocide of choice in the cooling systems. D-amino acids were tested to enhance the efficacy of bleach.

6.2 Experimental methods

6.2.1 Abiotic chemical compatibility test

Free chlorine concentrations were measured using SenSafe Free Chlorine Water Check test strips (Industrial Test Systems, Inc., Rock Hill, SC). Figure 6-1 shows that detection levels of test strips are from 0 - 6 ppm, with a color from white to dark blue. Test matrix is shown in Table 6-1.

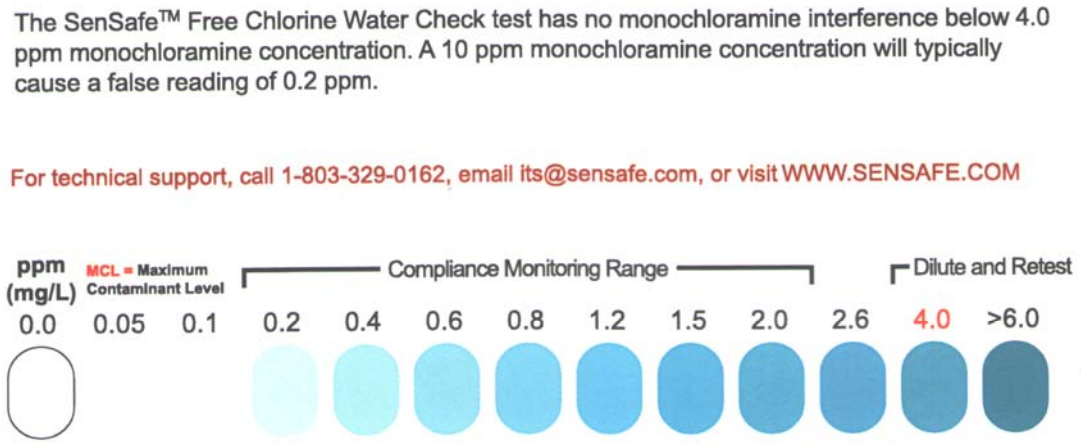

Figure 6-1. Detection levels of SenSafe free chlorine water check test strips from 0 - 6 ppm provided by Industrial Test Systems, Inc.

Table 6-1. Test matrix of abiotic chemical compatibility test

SRB strain	No microbes
Solvent	Deionized water
Treatment method	5 ppm chlorine + 100 ppm D-met or + 1 ppm D-tyr
Temperature	25°C
Treatment duration	3 h
Coupon	None

6.2.2 Compatibility test with microbes

The biofilm prevention test was done to evaluate the efficacy of chlorine and D-amino acids. In the biofilm prevention test, biofilms were incubated on C1018 coupons in the ATCC 1249 medium with treatment chemicals and 1 ml *D. vulgaris* biofilm seed culture for 7 days. Test matrix is shown in Tables 6-2.

Table 6-2. Test matrix of biofilm prevention test

SRB strain	*D. vulgaris*
Culture medium	ATCC 1249 medium
Treatment method	5 ppm chlorine + 100 ppm D-met or + 1 ppm D-tyr
Temperature	37^{o}C
Treatment duration	7 days
Coupon	C1018 carbon steel

6.2.3 Sequential treatment using bleach and a D-amino acid mixture against field biofilm samples on carbon steel coupons from the polymer plant

Table 6-3. Conditions for D4-1 enhancement of bleach in biofilm removal test

Biofilm	Consortium from a water cooling tower
Growth time	3 weeks
Treatment method	Bleach, D4-1, Bleach + D4-1
Treatment time	Control: 4 hours without treatment D4-1 alone: 2 hours with 50 ppm D4-1 + 2 hours with no treatment chemicals Biocide alone: 2 hours with bleach + 2 hours with no treatment chemicals Sequential treatment: first 2 hours with bleach + 2 hours with 50 ppm D4-1 Sequential treatment: first 2 hours with 50 ppm D4-1 + 2 hours with bleach
Temperature	25^{o}C
Coupon	C1018 carbon steel

Five 3-week old (i.e., coupons were placed in the cooling water system to allow biofilms to grow for 3 weeks) coupons from the polymer plant were tested with bleach and D4-1 (composition in Table 5-14). Since the active component NaClO in bleach can react with D-amino acids, a sequential treatment of bleach (containing 2 ppm active NaClO) and 50 ppm D4-1 was tried. The test matrix is shown in Table 6-3. After the treatment, coupons were taken out for CLSM observation and sessile cell enumeration. MPN test kits (BTS) were used to count the SRB, APB and GHB sessile cells after biocide treatment. For the detailed experimental designs, MPN test kit methods and CLSM observation, please see Appendix II.

6.3 Results and discussion

6.3.1 Compatibility tests

In the compatibility test, various D-amino acids were mixed with bleach (containing 5 ppm NaClO) in dark environment for 3 hours. Figure 6-2 shows that D-amino acids consumed all the ClO^-. Thus, the reaction happened between the ClO^- and D-amino acids rather quickly.

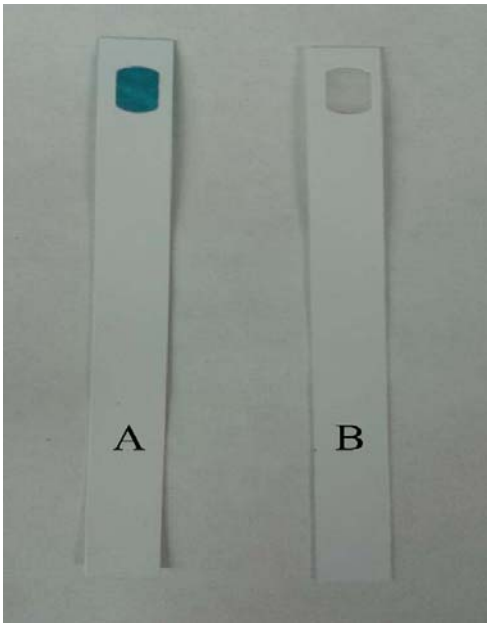

Figure 6-2. Bleach (containing 5 ppm NaClO) with 10 ppm D-amino acid in water after 3 hours: (A) bleach, and (B) bleach + 10 ppm D-amino acid.

In Table 6-4, the sessile cell concentration of the control coupon without any biocide treatment was 10^6 cell/cm^2. Five ppm chlorine treatment alone only achieved 1 log reduction compared with the untreated control. The combinations of 5 ppm chlorine + 100 ppm D-met and 5 ppm chlorine + 1 ppm D-tyr did not achieve any enhancement compared with the 5 ppm chlorine treatment. In Figure 6-3, sessile cells were easily found on the surface of the control coupon. Therefore, a sequential treatment was needed to avoid the chemical reaction between chlorine and D-amino acids.

Table 6-4. Sessile cell count after 7-day biofilm prevention test

Treatment	Sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^6$
5 ppm chlorine	$\geq 10^5$
100 ppm D-met	$\geq 10^5$
100 ppm D-tyr	$\geq 10^6$
5 ppm chlorine + 100 ppm D-met	$\geq 10^6$
5 ppm chlorine + 1 ppm D-tyr	$\geq 10^5$

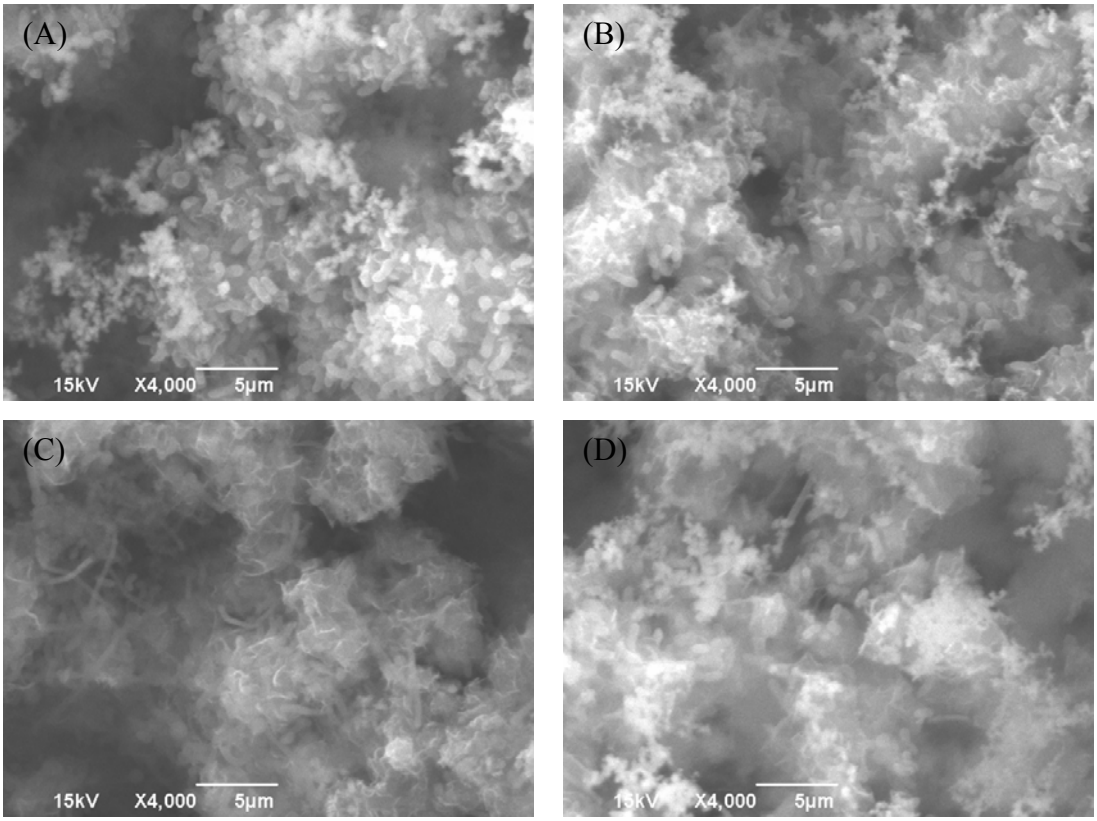

Figure 6-3. Images of biofilms after 7-day incubation in the biofilm prevention test in ATCC 1249 medium containing: (A) no treatment chemicals (control), (B) 5 ppm chlorine, (C) 5 ppm chlorine + 100 ppm D-met, and (D) 5 ppm chlorine + 1 ppm D-tyr.

6.3.2 Field sample collection and biofilm analysis

C1018 carbon steel coupons retrieved from a water cooling system in the polymer plant after exposure for different time durations were received. Overnight shipping was used to eliminate possible sample deterioration. The chewing gum-shaped (3" x 0.5") coupons were inserted into 30 ml anaerobic vials with the cooling water. The headspace in each

vial was reduced as much as possible to minimize air exposure. MPN test kits (BTS) were used to count and detect planktonic bacteria in the water sample in the headspace of each vial. It was found that there were 10^3 cells/ml SRB, 10^2 cells/ml APB, and 10^2 cells/ml GHB in the water samples.

The biofilm on the coupon surface was observed using CLSM. Figure 6-4 shows the CLSM images of the biofilm on the coupon exposed to the field location for 1 week. There were not many living cells found on the CLSM image. In the 3D view, the biofilm was not well developed. In Figure 6-5, more living cells were found on the coupon exposed to the field for 2 weeks, and the biofilm was thicker than that on the 1-week coupon. The 3D CLSM image in Figure 6-5 shows that the thickness of the biofilm was around 20 μm. The biofilm was fluffy as observed by naked eyes. In future biocide tests, 2-week or older biofilms were used.

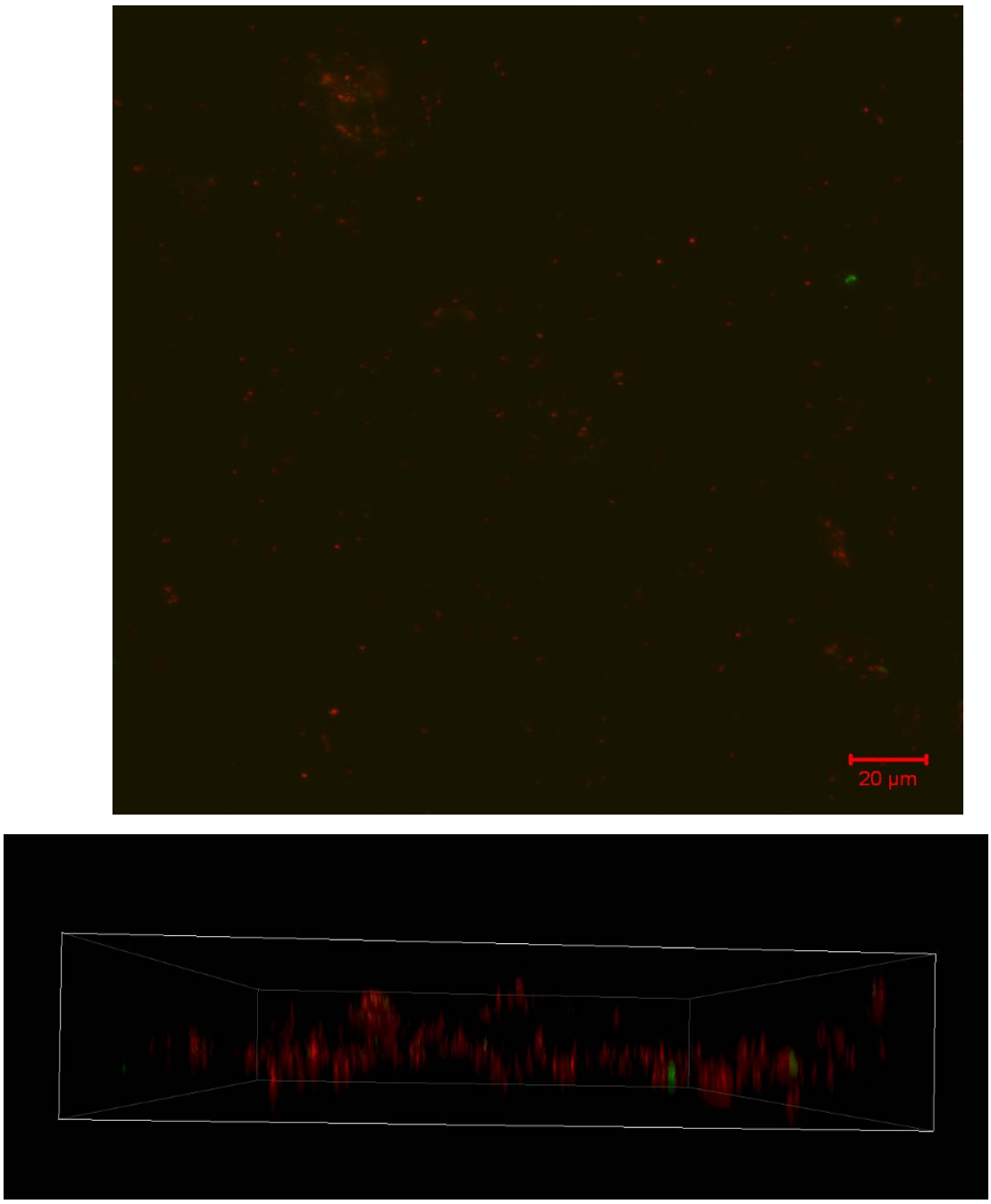

Figure 6-4. CLSM images of the 1-week biofilm from a cooling water system.

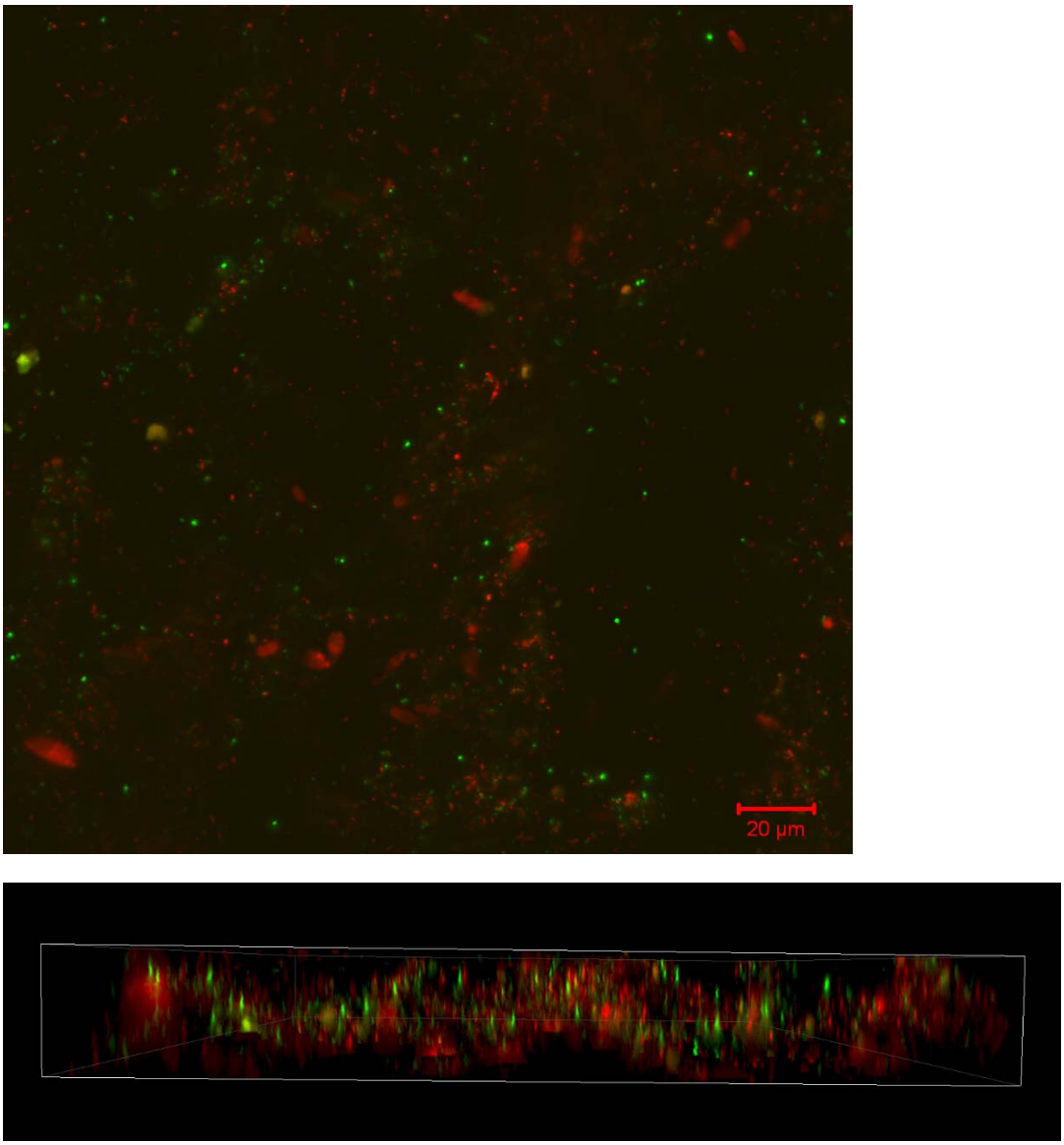

Figure 6-5. CLSM images of the 2-week field biofilm from a cooling water system.

6.3.3 Field sample sequential test using bleach and D-amino acids

Five C1018 carbon steel coupons retrieved from the polymer plant after exposure for 3 weeks were received. Overnight shipping was used to minimize possible sample deterioration. MPN test kits (BTS) were used to count and detect the sessile cells after treatments. The biofilm removal test lasted 4 hours in the 100 ml PBS solution (pH 7.4). Fifty ppm D4-1 and bleach (containing 2 ppm active NaClO) were separately applied for a duration of 2 hours. During the second 2 hours, the coupon was immersed in the PBS solution. The sequential treatment in Table 6-3 shows that the coupon first immersed in

the PBS solution containing bleach for 2 hours. After that, it was taken out and put into another PBS solution containing 50 ppm D4-1. Sessile cell count data after the biofilm removal test are shown in Table 6-5. Fifty ppm D4-1 alone achieved 1 log reduction each in APB, SRB and GHB sessile cell counts compared with the untreated control. Bleach alone achieved 1-log more in APB sessile cell reduction than 50 ppm D4-1 treatment alone. The sequential treatments both showed 1-log more reduction in the SRB sessile cell count than bleach treatment alone. Table 6-5 also shows the order (bleach first or second) in the sequential treatment method didn't matter in this work. The CLSM images in Figure 6-6 confirm the sessile cell count data. More living cells are seen in the 50 ppm D4-1 treatment alone case (Figure 6-6B), and more dead cells (red dots) are seen in Figure 6-6D and Figure 6-6E compared with bleach alone (Figure 6-6C).

Table 6-5. Sessile cell counts of field biofilm consortium from a cooling tower after 4-hour biofilm removal test

Treatment	APB sessile cell count (cells/cm^2)	SRB sessile cell count (cells/cm^2)	GHB sessile cell count (cells/cm^2)
No treatment chemicals (control)	$\geq 10^5$	$\geq 10^4$	$\geq 10^5$
50 ppm D4-1	$\geq 10^4$	$\geq 10^3$	$\geq 10^4$
Bleach	$\geq 10^3$	$\geq 10^3$	$\geq 10^4$
Bleach followed by 50 ppm D4-1	$\geq 10^3$	$\geq 10^2$	$\geq 10^4$
50 ppm D4-1 followed by bleach	$\geq 10^3$	$\geq 10^2$	$\geq 10^4$

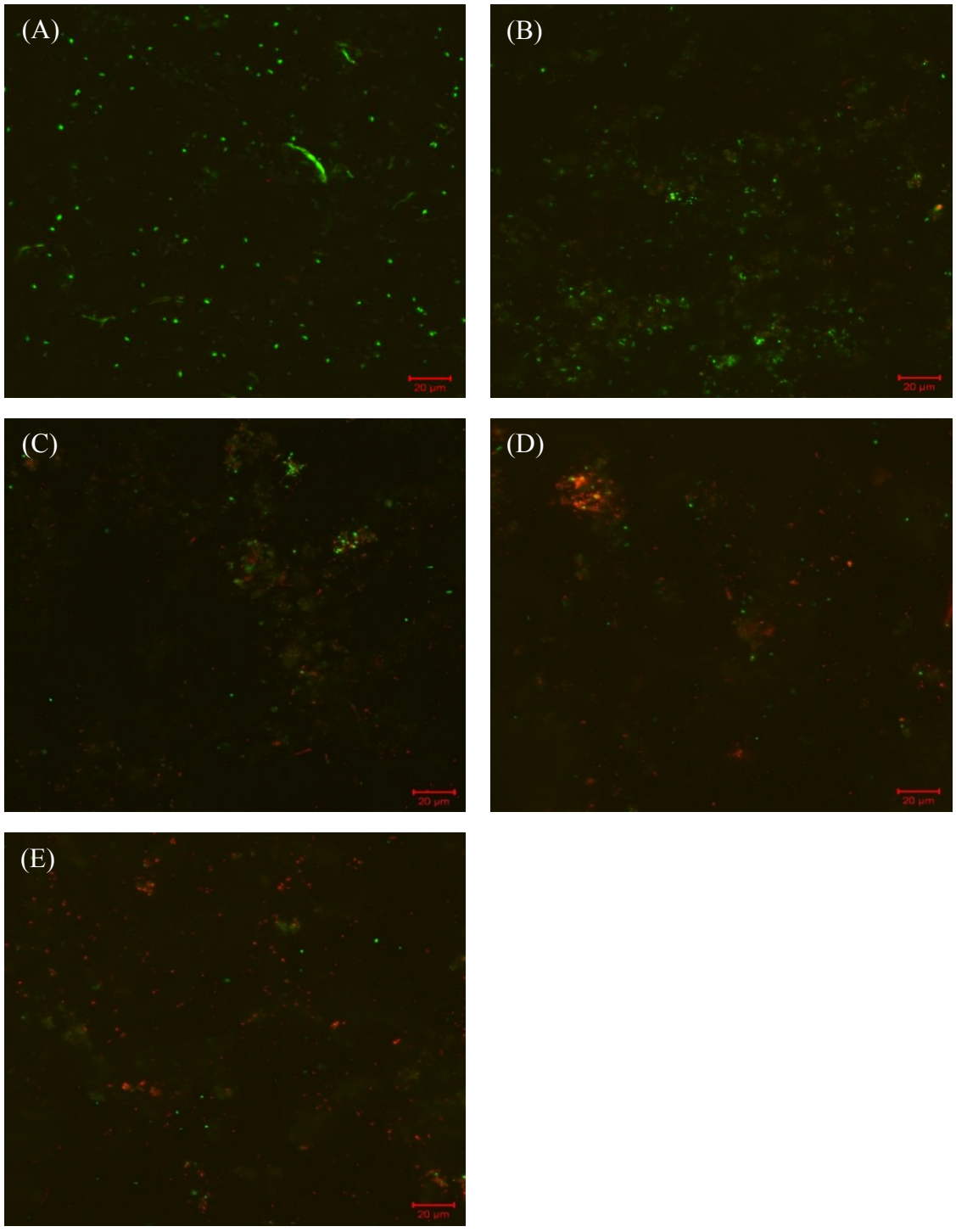

Figure 6-6. CLSM images of biofilms after 4-hour biofilm removal test: (A) no treatment chemicals (control), (B) 50 ppm D4-1 alone for 2 hours + no treatment chemicals for 2 hours, (C) bleach alone for 2 hours + no treatment chemicals for 2 hours, (D) bleach alone for 2 hours + 50 ppm D4-1 alone for 2 hours, and (E) 50 ppm D4-1 alone for 2 hours + bleach alone for 2 hours.

6.4 Summary

Literature review shows chlorine can react with amino acids, peptides and proteins. When using 5 ppm sodium hypochlorite reacted with different 10 ppm D-amino acids in water, in 3 hours 10 ppm D-amino acids can consume all the ClO^-. During the prevention test with *D. vulgaris*, the added 100 ppm D-met and 1 ppm D-tyr did not increase the efficacy of 5 ppm chlorine due to chemical incompatibility. The SEM images of the prevention test confirm this outcome. Five 3-week old coupons with biofilm from a cooling tower in a polymer plant were tested with bleach and an equimass D-amino acid mixture containing D-met, D-tyr, D-trp, and D-leu (labeled as D4-1). Since the active component NaClO in bleach can react with D-amino acids, a sequential treatment of bleach (containing 2 ppm active NaClO) followed by 50 ppm D4-1 was tried. The results showed 2-log reduction in the APB sessile cell count, 2-log reduction in the SRB sessile cell count, and 1-log reduction in the GHB sessile cell count compared with untreated control. The sequential treatment showed 1 extra log reduction in the SRB sessile cell count, but no further reduction for APB and GHB sessile cell counts compared with the bleach treatment alone.

7 Conclusions

This project successfully developed a new method to enhance the efficacy of biocides using D-amino acid mixtures in the mitigation of field biofilm consortia.

First of all, in lab tests, individual D-amino acids were found inadequate to enhance the efficacy of THPS against two tough field biofilm consortia, although in the mitigation of *D. vulgaris* biofilms, 100 ppm D-met or 1 ppm D-tyr combined with 50 ppm THPS remarkably improved the efficacy of 50 ppm THPS. An industry partner (a major company) confirmed this. They tested 50 ppm equimolar mixture of D-met and D-tyr with 250 ppm THPS against a different microbial consortium and found that no significant enhancement was achieved. The possible reason was that multiple D-amino acids are necessary due to the multi-species in the biofilm consortia. It is likely that each D-amino acid works with specific bacteria, but not all bacteria Therefore, a mixture of D-amino acids were tested in the mitigation of biofilm consortia.

Other D-amino acids were studied in the mitigation of *D. vulgaris* biofilm combined with 50 ppm THPS. D-val, D-phe, D-thr, D-ser, and D-asn were found to enhance the biocidal effect of 50 ppm THPS in the *D. vulgaris* biofilm prevention test. However, D-glu, D-his, and D-asp did not show enhancement for 50 ppm THPS. So far, 12 commercially available D-amino acids have been tested as biocide enhancers to enhance THPS. The rest of the D-amino acids are more expensive (research chemical prices). It was found that 9 of the 12 D-amino acids enhanced the efficacy of 50 ppm THPS in the mitigation of the *D. vulgaris* biofilm. D-glu, D-asp, and D-his did not enhance the efficacy of 50 ppm THPS.

The cocktails of D-amino acid mixtures and THPS were tested in the mitigation of two field biofilm consortia (Consortia I and II) from an oil field. The result showed that 30 ppm equal-mass D-amino acid mixture including D-met, D-tyr, D-trp, D-leu, D-val, D-phe, D-ser, and D-thr (labeled as D8-1) enhanced the efficacy of 50 ppm THPS against the consortia. The result suggested that D-amino acid mixtures with more components in addition to D-met and D-tyr would be necessary in the mitigation of the field biofilm consortia. The optimization of the D8-1 was carried out with 50 ppm THPS in the mitigation of the field biofilm Consortium II (tougher than Consortium I). Based on the SRB sessile cell data, weight loss data and CLSM observation, the mixture of equal-mass D-met, D-tyr, D-trp, D-leu, and D-ser (labeled as D5-1) and D-met, D-tyr, D-trp, D-leu, and D-thr (labeled as D5-2) achieved the same enhancement as D8-1 for 50 ppm THPS against the Consortium II.

Finally, a D-amino acid mixture was tested against field biofilms from a polymer plant. Coupons with biofilms were retrieved from a cooling tower. Bleach was used as the biocide in the cooling tower. The active component NaClO in bleach reacted with D-

amino acids in the compatibility tests. Therefore, a sequential treatment of bleach (containing 2 ppm active NaClO) followed by 50 ppm D4-1 was tried. The results showed 2-log reduction in the APB sessile cell count, 2-log reduction in the SRB sessile cell count, and 1-log reduction in the GHB sessile cell count compared with the untreated control. This sequential treatment showed 1 extra log reduction in the SRB sessile cell count, but no further reduction for APB and GHB sessile cell counts compared with the bleach treatment alone.

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9 Appendix

9.1 MPN method

Three culture media (Figure 9-1) were used to count sessile cell in *D. vulgaris* biofilms and two field biofilm consortia. They were Modified Postgate's B for SRB, Standard Bacterial Nutrient Broth for GHB, and Phenol Red Dextrose for APB. Biofilms were scraped off from carbon steel coupons and serially diluted in sterilized distilled water from 10 to 10^6 times. The data reading was based on the NACE Standard TM0194-2004.

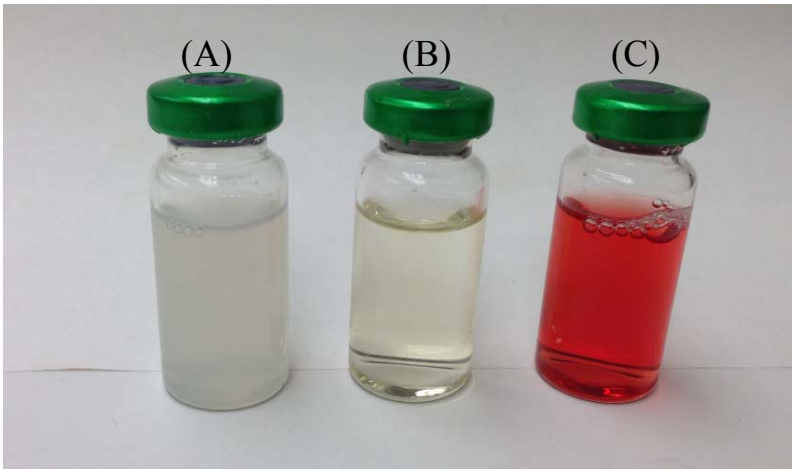

Figure 9-1. Three culture media (Biotechnology Solutions (Houston, TX)) used in this MPN method to count the sessile cells: (A) Modified Postgate's B for SRB, (B) Standard Bacterial Nutrient Broth for GHB, and (C) Phenol Red Dextrose for APB.

Figure 9-2 shows that the concentrations of SRB sessile cell in *D. vulgaris* biofilm and two biofilm consortia were 10^6 cells/cm^2. This value was consistent with the value obtained using the Biosan Sani Check SRB test kit. Figure 9-3 shows that all vials had no color indicating no GHB detected in the two field biofilm consortia. Figure 9-4 shows that the medium color turned to yellow from red which indicated the presence of APB. However, according to the instruction of Biotechnology Solutions: because there was no biomass in each vial (i.e., culture medium not turbid), it was a false positive. (Figure 9-5 shows an example from the test kit vendor of biomass presence indicated by the APB culture medium turbidity.) Therefore, no APB was detected in two field biofilm consortia. It is understandable that GHB and APB were not detected in the field biofilm consortia because they had been cultured in ATCC 1249 medium, which is designed for sulfate reducers.

The method requires a waiting time of 14 to 28 days to observe the color change and the cell growth. It is time consuming for SRB cell count compared with the Biosan Sani Check SRB test kit. Therefore, the Biosan kit were used as the primary way to count the SRB sessile cell. For new field biofilm consortia, if GHB or APB were detected, Biotechnology Solutions media were used to count GHB or APB cells.

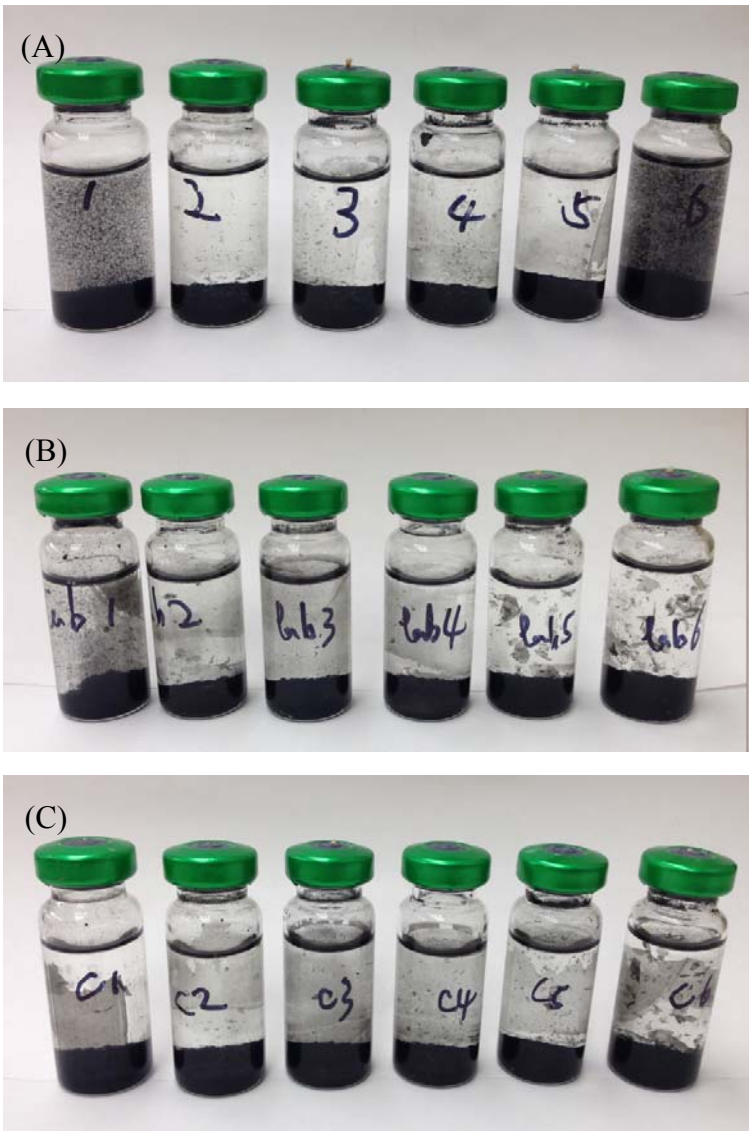

Figure 9-2.Vials of Modified Postgate's B medium used to count SRB sessile cells in *D. vulgaris* biofilm and two biofilm consortia: (A) *D. vulgaris*, (B) Consortium I, and (C) Consortium II. The numbers 1 to 6 indicate the actual dilution.

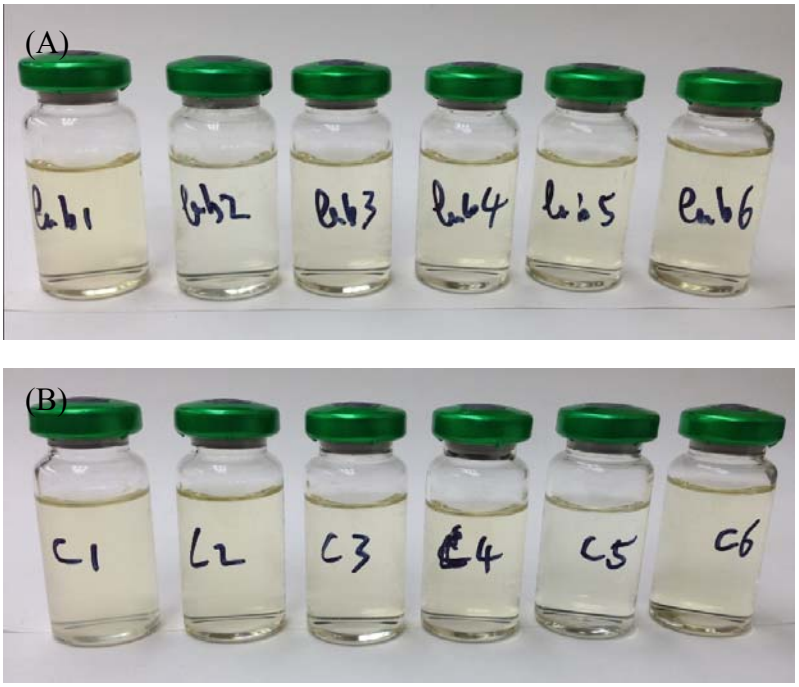

Figure 9-3. Vials of Standard Bacterial Nutrient Broth medium used to count GHB sessile cells in two biofilm consortia: (A) Consortium I and (B) Consortium II. The numbers 1 to 6 indicate the actual dilution.

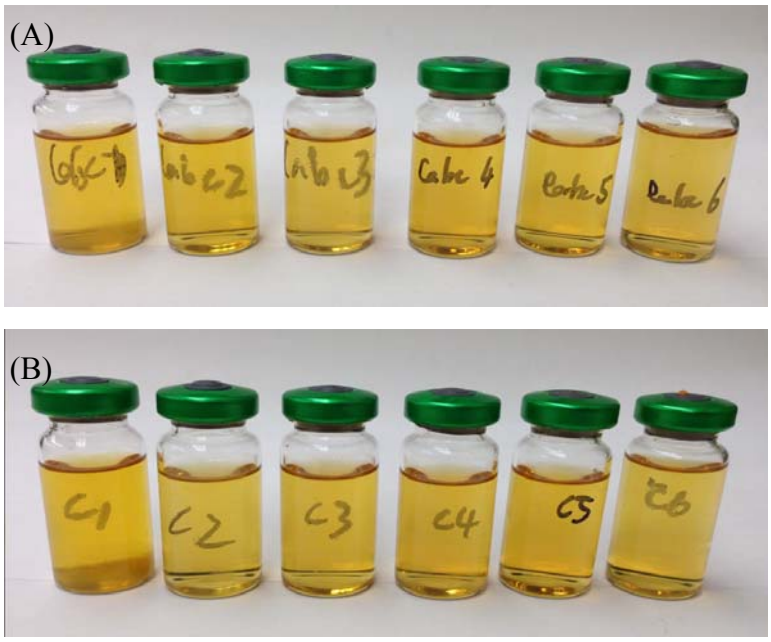

Figure 9-4. Vials of Phenol Red Dextrose medium used to count APB sessile cells in two biofilm consortia: (A) Consortium I and (B) Consortium II. The numbers 1 to 6 indicate the actual dilution.

Figure 9-5. Color change of Phenol Red Dextrose medium due to: (A) pH and (B) bioactivity. The cloudiness is noticeable in the right vial.

(Source: http://www.biotechnologysolutions.com/bacterial_media_products/phenol_red_dextrose)

9.2 Microbe identification report of two biofilm consortia used in this project

The identification of the field biofilm consortia was carried out by Ecolyse, Inc. (College Station, TX). A 200 ml seed culture medium of each consortium was shipping to Ecolyse, Inc overnight. The planktonic cell concentration in the seed culture is around 10^8 to 10^9 cells/ml. So, 200 ml seed culture medium is enough for identification according to the guideline provided by Ecolyse, Inc. The package of the culture medium was packed with ice bags. The overnight shipping eliminated the influence of cell amount deduction due to the shipping process.

Ecolyse, Inc.

MicrobeID™ Report

Project ID: OG150404

Yingchao Li

Ohio University

Monday, April 27, 2015

Ecolyse, Inc.

Sample Information

Sample Count: 2
Shipped From: Ohio University, 171 Stocker Center
Athens, Ohio 45701

Shipped Date: 4/13/15
Arrived Date: 4/14/15

2 samples were received at Ecolyse Labs on 4/13/15. These consisted of 2 liquid samples.

- Archaeal and Bacterial populations of all samples were analyzed in parallel by 16s metagenomic sequencing, using Ion PGM platform.

TABLE 1. Sample Overview

Sample	Sample Label	Ecolyse Test Requested	DNA (ng/mL)	CFU*/mL
001	C1	1. Metagenomics	49.00	1.63E+08
002	C2	1. Metagenomics	60.00	2.00E+08
*Assumes 3.3 fg/DNA per cell.				

Project Results Overview: Bacterial Diversity Analysis

Genetic-Based Diversity Analysis-Method

- Total DNA is isolated from the sample.
- Bacterial and Archaeal diversity is determined by 16s metagenomics analysis, Ion PGM.
- Following traits assigned to identified bacteria and archaea where possible:
  - **Sulfidogen**-includes all bacteria that can make sulfide or H_2S. This includes "true" SRB as well as TRB (thiosulfate-reducing bacteria) SuRB(sulfur-reducing bacteria) and peptide-fermenting bacteria (such as some Clostridia)
  - **SRB**-(sulfate-reducing bacteria) "true" SRB, utilize sulfate as respiratory electron acceptor
  - **APB**-(acid-producing bacteria) these make organic and/or inorganic acids. Not all APB result in a lowering of ambient pH.
  - **IRB**-(iron-reducing bacteria) many are strongly corrosive
  - **NRB**-(nitrate-reducing bacteria) many bacteria are nitrate reducers. Of particular relevance to the O&G industry are the NRSOB (nitrate-reducing sulfur-oxidizing bacteria) promoted by nitrate injections.
  - **Biodeg**-biodegrading bacteria. These bacteria are capable of breaking down unusual substrates such as O&G hydrocarbons, petrochemicals, cellulose, toxic chemicals etc.
- Percent of population, and number of unique microbial types (OTU) are provided as results

Genetic – Based Diversity Analysis – Overview Results

- DNA was isolated from 2 samples (Table 1).
- 12962 microorganisms were analyzed genetically.
- These were grouped into 11 different microbial types (OTU).
- 0 Archaeal OTU were present in the samples.
- Metabolic assignments were provided for 9 of the 11 OTU's identified.
- The distribution of SRB, IRB, APB, NRB, and Biodeg is provided (Table 2, Figure 1).
- A list of the most abundant bacteria (greater than 1% of the population) is provided (Table 3).
- The degree of similarity of each sample to every other sample is provided.
- A complete list of all bacteria in the samples is available upon request.

Table 2. OG150404 Summary of Bacteria and Archaea Diversity Using Genetic Analysis

Samples are highlighted by abundances: samples highlighted in yellow have >1% metabolism of interest. Samples highlighted in grey do not have this metabolism present.

Sample ID	Organisms Tested	Bacteria & Archaea OTU	Sulfidogens (TRB + SRB)	SRB	IRB	APB	NRB	Biodeg
OG150404-001 C1	6988	11	89.47% 3 otu	61.55% 1 otu	None	None	27.73 1 otu	2.68% 1 otu
OG150404-002 C2	5938	5	78.81% 1 otu	None	None	None	78.81 1 otu	13.59% 1 otu
TOTAL	***12926***	***11***	***3 otu***	***1 otu***	***None***	***None***	***1 otu***	***1 otu***

Table 3. Project OG150404 Metabolic Assignments of Dominant Bacterial Species

All bacterial species present in at least 1% of one sample are given, along with the percent abundance in that sample and a characteristic trait of relevance. Samples are highlighted by abundances: >10%, yellow; >1%, green; 0, grey. A full list of all bacteria identified in these samples is available upon request.

Species	C1	C2	Trait
Bacillus sp	<1%	6.0	Biosurfactant Producing; Varies
Bacteroides sp	2.9	1.4	Ferm
Garciella sp	27.7	78.8	NRB; Sulfidogen; TRB
Salmonella enterica	61.5	0	SRB; TRB
Soehngenia sp	4.1	0	Ferm
Tepidibacter sp	2.7	13.6	Biodeg (HC)

Trait abbreviations:
Biodeg (HC), Biodegradation of Hydrocarbons; Ferm, Fermenting Bacteria; NRB, Nitrogen-Reducing Bacteria; SRB, Sulfate-Reducing Bacteria; TRB, Thiosulfate-Reducing Bacteria.

Comparisons of Populations Between Samples

To get an overall view of a system, it is helpful to compare populations between different locations. Locations with similar bacterial populations reflect a combination of a common source of bacterial contamination along with a common physical environment.

Another way to present this information as a numerical value is to compare all samples to each other. This can be calculated as the number of microbes that are present in both samples as a function of all microbes present in both samples. Samples showed a comparison value of 0.625, meaning the samples had 62.5% of microbial taxa present in both samples.

Ecolyse, Inc.

Figure 1. Chart Showing Distribution of Select Traits Between Samples

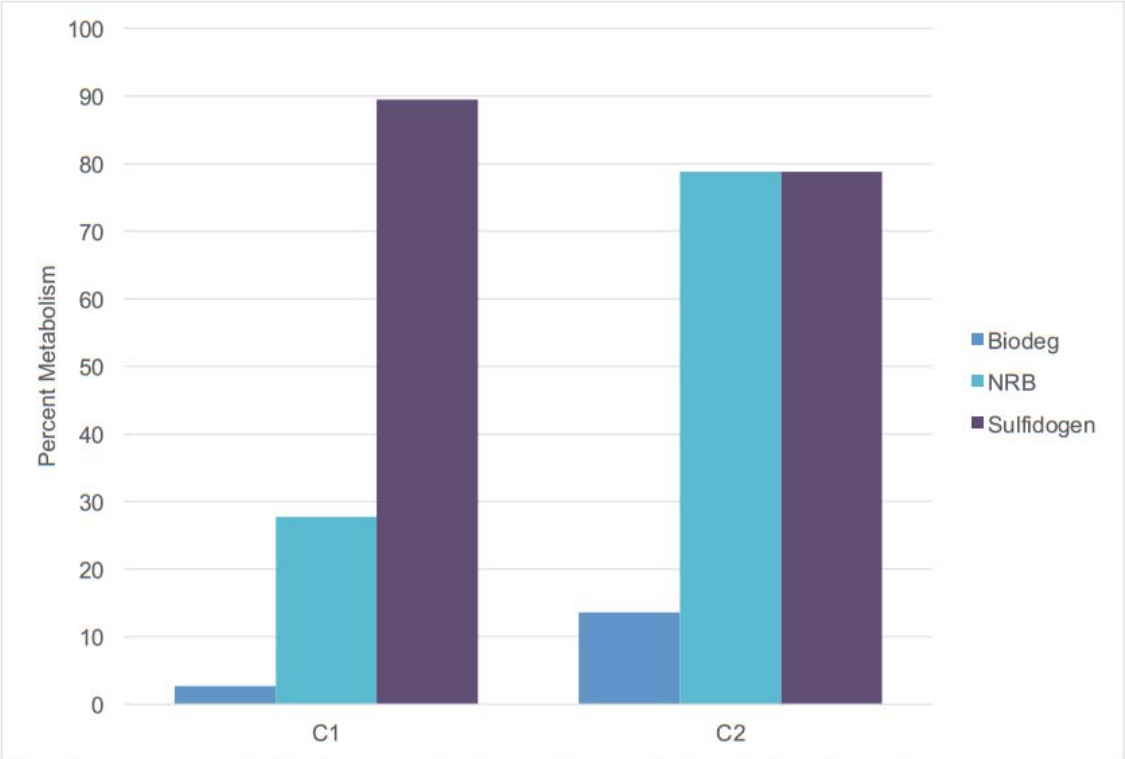

Figure 1 presents a graph with the percent abundance of key metabolic traits in each sample.

APPENDIX A. Methods

For microbial analysis, DNA was subject to bacterial tag-encoded FLX amplicon sequencing (bTEFAP) using primers 515F- GTGCCAGCMGCCGCGGTAA and
806R- TAATCTWTGGGVHCATCAGG.

Samples were amplified for pyrosequencing using a forward and reverse fusion primer. The forward primer was constructed with (5'-3') linker (CCATCTCATCCCTGCGTGTCTCCGACTCAG), an 8-10bp barcode, and the XXXXXXXX primer (see above primer). The reverse fusion primer was constructed with (5'-3') a biotin molecule, the linker (CCTATCCCCTGTGTGCCTTGGCAGTCTCAG), and the XXXXXXX primer (see above primer). Amplifications were performed in 25 ul reactions with Qiagen HotStar Taq master mix (Qiagen Inc, Valencia, California), 1ul of each 5uM primer, and 1ul of template. Reactions were performed on ABI Veriti thermocyclers (Applied Biosytems, Carlsbad, California) under the following thermal profile: 95○C for 5 min, then 35 cycles of 94○C for 30 sec, 54○C for 40 sec, 72○C for 1 min, followed by one cycle of 72○C for 10 min and 4○C hold.

Amplification products were visualized with eGels (Life Technologies, Grand Island, New York). Products were then pooled equimolar and each pool was cleaned with Diffinity RapidTip (Diffinity Genomics, West Henrietta, New York), and size selected using Agencourt AMPure XP (BeckmanCoulter, Indianapolis, Indiana). Size selected pools were then quantified and 150 ng of DNA were hybridized to OT2-400 Ion Sphere beads (Life Technologies) to create single stranded DNA following Ion PGM Protocols (Life Technologies). Single stranded DNA was diluted and used in emPCR reactions, which were performed and subsequently enriched. Sequencing following established manufacture protocols (Life Technologies).

APPENDIX B. Overview of Select Metabolic Processes

Notes on Taxonomic and Metabolic Assignment

Organisms are referred to by the identity of the most closely matched organism in the database. However, this does not indicate 100% identity. Metabolic assignments are inferred by the metabolic characteristics of the most closely related organism for which experimental data has been provided. Some metabolic groupings are overlapping and non-exclusive, e.g. many fermentative organisms generate organic acids or are capable of sulfidogenisis under some conditions. The methods utilized for sample processing and genetic analysis are described in Appendix A. An overview of select metabolisms is provided in Appendix B.

APB: Acid-Producing Bacteria

Acid-producing bacteria are of specific interest to the oilfield community as acid production directly and aggressively promotes corrosion. Several metabolic pathways result in the production of acids, including fermentation pathways that generate organic acids such as lactic acid and acetic acid, as well as those that generate inorganic acids such as sulfuric acid as a byproduct of the oxidation of inorganic sulfur compound. It should be noted that not all fermentative pathways result in acidification of the surrounding environment. The identification of bacteria as acid producing does not necessarily indicate acidification of bulk fluids.

Biodeg: Biodegradation

Some bacterial genera and species have the capacity to utilize “atypical” or “unusual” substrates as carbon sources. These bacteria are loosely referred to as Biodeg, for “Biodegradation”. The definition used here for “atypical or unusual substrates” with reference to bacterial metabolism includes compounds that most bacteria cannot utilize as a food source. Unusual compounds Biodeg organisms might be able to “eat” include disinfectants, antibiotics, xenobiotics and detergents. Some degrade long chain polymers of sugars and carbohydrates, such as those found in cell wall materials. Others are able to degrade hydrocarbons. Hydrocarbons, including alkanes, alkenes, aromatic hydrocarbons, and waxes, are found naturally in great variety in crude oil and other petroleum compounds. Due to their structural diversity, most bacteria lack the capacity to utilize petroleum hydrocarbons as food sources. Each type of hydrocarbon-degrading microorganism is likely to be capable of metabolizing a few specific types of hydrocarbons.

IRB: Iron-Reducing Bacteria, Fe(III)RB

In the absence of oxygen, many microbes can use Fe(III) as an electron acceptor, reducing it to Fe(II). Iron reduction has been observed under both acidophilic and neutrophilic conditions. Two common iron-reducing genera are Shewanella and Geobacter. In addition to IRB activity, Shewanella species produce chelators that solubilize Fe(III) oxides (Lovley et al, 2004). Shewanella are capable of growing in corrosive biofilms where they have been shown to remove the protective H2 film layer that normally protects iron surfaces from corrosion under anoxic conditions. IRB should not be confused with iron oxidizing bacteria, which are aerobes responsible for a rust brown staining and slimy growth in surface waters.

NRB: Nitrate Reducing Bacteria

NRB are able to reduce nitrates to nitrites, nitrous oxide, or nitrogen under anaerobic conditions in a process termed denitrification. Most are heterotrophic facultative anaerobic bacteria

including such common bacteria as *Paracoccus, Pseudomonas, Alcaligenes,* and *Bradyrhizobioium*. A few bacteria use such reduction processes as hydrogen acceptor reactions and hence as a source of energy; in this case the end product is ammonia. Denitrification is a normal part of nitrogen cycling and not all NRB are of concern to O&G infrastructure.

A subcategory of NRB is the **NRSOB**: Nitrate-Reducing Sulfur-Oxidizing Bacteria are a specific subgroup of NRB whose levels are increased in reservoirs following nitrate injections (Gittel et al 2009; Grigoryan et al, 2008; Hubert and Voordouw, 2007). Growth of NRSOB suppresses the activity of SRB, and thus reducing sulfidogenisis. Some Epsilonproteobacteria can also oxidize petroleum sulfur compounds and utilize nitrate as an electron acceptor for growth, and thus may be considered hydrocarbon degrading. Massive dominance of related Epsilonproteobacteria has been observed in other petroleum samples, for example in formation waters from a Canadian oil sands reservoir containing severely biodegraded oil. (Kodama, Y and Kazuya Watanabe, 2003; Hubert et al, 2011). Sulfurospirillum are nitrate-reducing, sulfur oxidizing bacteria (NRSOB) members of the class Epsilonproteobacteria and are sometimes referred to as "Campylobacter" in older publications. The way in which nitrate addition can affect the SRB population involves several pathways. First, nitrate is a thermodynamically more favorable electron acceptor than sulfate, thus NRB have a competitive advantage. To emphasize the complexity of the metabolism in oilfield samples, it should be noted that under some conditions, these bacteria are also sulfidogens capable of reducing sulfur and thus producing H_2S (Finster K et al, 1997).

Sulfidogenesis: (e.g. SRB, TRB, SuRB)
The metabolic pathways of most interest to the oilfield community are those that generate significant levels of hydrogen sulfide (H_2S). In addition to inorganic processes, biogenic processes can generate significant levels of hydrogen sulfide, primarily through the action of sulfidogenic bacteria. Bacteria that evolve hydrogen sulfide are commonly referred to as "sulfidogens". Sulfate-reducing bacteria (SRB) are particularly aggressive at sulfide production and are the group of bacteria most commonly implicated oil filed biogenic sulfide production (Barton et al, 2009). Hydrogen sulfide formation by sulfate-reducing bacteria (SRB) under strict anaerobic circumstances is a common problem in sediments, sewer systems, oil reservoirs and anaerobic effluents (Holmer & Storkholm, 2001; McComas et al., 2001). The emission of H_2S into the atmosphere of sewer systems does not only imply odor nuisances and possible health risks. It also induces the biological production of sulfuric acid in the aerobic zones, causing severe corrosion of the inner surface of concrete sewer structures (Sand, 1987; Vincke et al., 2002). Hence, preventive or curative actions are needed.

While SRB are traditionally associated with O&G system sulfide generation, sulfur- and thiosulfate- reducing bacteria (SuRB and TRB, respectively) can also generate significant levels of H_2S and contribute to corrosion and souring (Hulecki JC et al, 2009, Magot et al 1997, Agrawal et al, 2010). Compared to SRB, the TRB are harder to classify taxonomically, as they are members of bacterial genera that can include non-tSRB members. Examples of sulfidogenic TRB commonly found in oilfield samples include *Halanaerobium congolense*, as well as some *Thermoanaerobacter*, and *Spirochaeta*. Additionally, many common enteric bacteria are sulfidogenic, including *Citrobacter* and *Salmonella*.

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Thermophiles:
A thermophile is an organism that can survive and often thrives in environments having relatively high temperatures ranging between 45 and 122 °C.

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9.3 NRB isolation

An NRB medium was used to isolate NRB from the field collected sample solution. The components of the medium are listed in Table 9-1. Ten milliliters of a trace element stock solution were added into the culture medium. The components of the trace element solution are listed in Table 9-2. A total of 2 ml sample solution was added to 50 ml NRB medium in the anaerobic chamber. However, after 15 days of incubation, no significant bacterial growth (i.e., no turbidity increase) was observed as shown in Figure 9-6. No planktonic cell was found under hemocytometer. It was possible that no NRB was presented in the field sample solution. The medium used in this task is optimized for *Bacillus licheniformis*. A more general medium for NRB might be necessary to isolate NRB from this water sample.

Table 9-1. Component of NRB medium

Component	Amount
Sucrose	10 g
K_2HPO_4	13.9 g
KH_2PO_4	2.7 g
NaCl	1 g
Yeast Extract	1 g
$NaNO_3$	2.5 g
$MgSO_4$	0.25 g
Distilled Water	1 L

Table 9-2. Component of the trace element solution

Component	Amount
$MnCl_2 \cdot 4H_2O$	180 mg
$CoCl_2 \cdot 6H_2O$	270 mg
H_3BO_3	50 mg
$CuCl_2 \cdot 2H_2O$	24 mg
$NaMoO_4 \cdot 2H_2O$	23 mg
$ZnCl_2$	19 mg
Distilled Water	100 ml

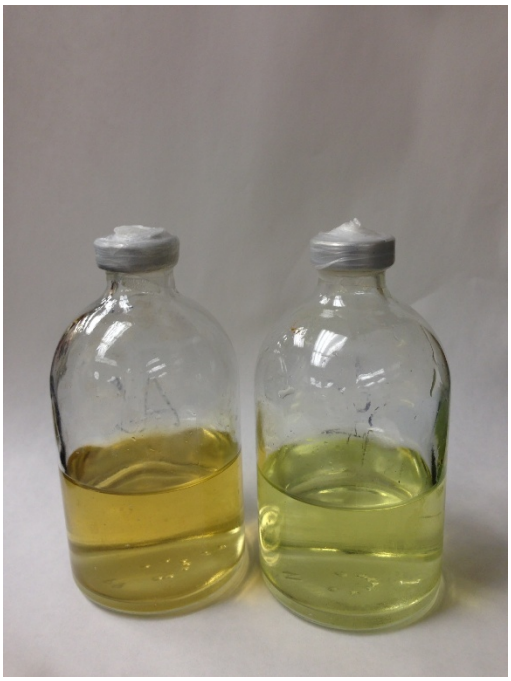

Figure 9-6. Left vial: 50 ml NRB medium and inoculated with 2 ml field sample solution incubated at 37^{o}C for 15 days; Right vial: uninoculated original culture medium before incubation.

9.4 Publications resulted from this work

The following works acknowledged the support or partial support of the PHMSA CAAP project.

SCI journal papers:

[1] D. Xu, Y. Li, F. Song, and T. Gu, "Laboratory investigation of microbiologically influenced corrosion of C1018 carbon steel by nitrate reducing bacterium *Bacillus licheniformis*," *Corros. Sci.*, vol. 77, pp. 385–390, 2013.

[2] Y. Li, R. Jia, H. H. Al-Mahamedh, D. Xu, and T. Gu, "D-amino acids enhanced biocide mitigation of field biofilm consortia in lab tests." Submitted.

[3] R. Jia, D. Yang, Y. Li, and T. Gu, "Mitigation of the *Desulfovibrio vulgaris* biofilm using alkyldimethylbenzylammonium chloride enhanced by D-amino acids." Submitted.

NACE conference papers:

[1] R. Jia, D. Yang, Y. Li, H. H. Al-Mahamedh, and T. Gu, "Enhancement of alkyldimethylbenzylammonium chloride and tributyl tetradecyl phosphonium chloride biocides using D-amino acids against a field biofilm consortium," *NACE Corrosion/2016*, Vancouver, Canada, 2016. (Accepted)

[2] Y. Li, P. Zhang, D. Xu, C. Xu, and T. Gu, "D-amino acids enhanced biocide mitigation of field biofilm consortia in lab tests," *NACE Corrosion/2015*, Dallas, USA, 2015.

[3] Y. Li, D. Xu, P. Zhang, W. Fu, and T. Gu, "D-amino acids enhanced biocide mitigation of problematic biofilms," *NACE Corrosion/2014*, San Antonio, USA, 2014.

NACE conference posters:

[1] D. Yang, "A sequential treatment designed for D-amino acids to enhance chlorine biocide treatment," *NACE Corrosion/2016*, Vancouver, Canada, 2016. (Accepted)

[2] W. Cai, "D-amino acids as biocide enhancers for the mitigation of the *Desulfovibrio vulgaris* biofilm on carbon steel: Mechanism and Efficacy," *NACE Corrosion/2015,* Dallas, USA, 2015.

[3] A. L. Lindenberger, "Mitigation of *Desulfovibrio vulgaris* biofilm and MIC of carbon steel by D-tryptophan as a biocide enhancer for THPS," *NACE Corrosion/2015,* Dallas, USA, 2015.

[4] Y. Li, "Investigation of a D-amino acid mixture as a biocide enhancer of THPS biocide in the mitigation of a field biofilm consortium," *NACE Corrosion/2015,* Dallas, USA, 2015.

[5] D. Yang, "D-phenylalanine as an enhancer of THPS for the mitigation of the *Desulfovibrio vulgaris* biofilm," *NACE Corrosion/2015,* Dallas, USA, 2015.

[6] W. Cai, "D-Leucine as an enhancer of biocide in mitigation of sulphate reducing bacterium (SRB) biofilm," *NACE Corrosion/2014*, San Antonio, USA, 2014.

[7] A. L. Lindenberger, "Comparison of two biocide enhancers for THPS mitigation of an SRB biofilm on carbon steel," *NACE Corrosion/2014*, San Antonio, USA, 2014.