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A novel peptide at a very low concentration enhanced biocide treatment of corrosive biofilms

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ABSTRACT

Microbiologically influenced corrosion (MIC) is caused by problematic biofilms in many industries, especially the oil and gas industry. In this work, a novel peptide (labeled as “Peptide A”) was used to enhance tetrakis hydroxymethyl phosphonium sulfate (THPS) biocide to treat the corrosive sulfate reducing bacterium (SRB) *Desulfovibrio vulgaris* and a tough field biofilm consortium (labeled as “Consortium II”) on C1018 carbon steel coupons. This peptide was synthesized based on the active sequence derived from an anti-biofilm protein produced by a sea anemone. Only 10 nM Peptide A or 18 ppb (w/w) was used in the biofilm prevention test and 10 – 100 nM was tested in the biofilm removal test. The cocktail of 50 ppm THPS + 10 nM Peptide A achieved 2 extra log reduction of SRB sessile cell count in the 7-day biofilm prevention test compared with 50 ppm THPS treatment alone. In the 3-hour biofilm removal test that started with mature biofilms, the combination of 50 ppm THPS + 100 nM Peptide A achieved 2 extra log reduction compared with 50 ppm THPS treatment alone. Peptide A alone showed no log reduction in the mitigation of biofilm Consortium II. However, in the *D. vulgaris* biofilm removal test, 10 nM Peptide A achieved 1-log reduction and 100 nM achieved 2 logs.

Key words: biocide, peptide, biofilm, microbiologically influenced corrosion

INTRODUCTION

Microbiologically influenced corrosion (MIC) is a major problem in many industries, such as the oil and gas industry.[1] MIC accounts for 20 – 40% of all corrosion damages. Billions of dollars are lost because of MIC every year in the United States.[2] Sulfate reducing bacteria (SRB) are often the culprit in MIC, but sometimes acid producing bacteria and other microbes are also involved. Pipeline systems are usually anaerobic because corrosive oxygen is removed. SRB can easily grow in an anaerobic environment where sulfate is available. Crude oil contains SRB[3] and seawater injection can also bring SRB[4] that cause corrosion and reservoir souring.[5] Even in an aerobic environment, SRB can grow underneath an aerobic biofilm that provides an anaerobic local environment.

Most microbes live in biofilm communities in nature. A biofilm is a barrier to limit the penetration of biocides.[6] Microbes in biofilms can slow down metabolic rates to lower the intake of biocides.[7] Biofilms can also produce persister cells[8] and use efflux pumps[9] to defend against biocidal attacks. A field biofilm consortium contains different types of microbes that offer synergy against biocides. Thus, sessile cells in a biofilm need a much higher biocide concentration to treat than planktonic cells.[10] However, continued use of a biocide will promote resistant microbes and eventually lead to dosage escalation over time. Unfortunately, only a few biocides such as tetrakis hydroxymethyl phosphonium sulfate (THPS) and glutaraldehyde are used available for large-scale field applications due to considerations for broad-spectrum efficacy, environmental restrictions, operational safety and cost. One strategy to overcome biocide dosage escalation is to enhance the existing biocides. D-amino acids are naturally occurring. They are non-biocidal, but they were found to disperse relatively weak biofilms.[11] However, for recalcitrant industrial biofilms, a biocide stress is needed for them to work. It was reported that D-tyrosine (D-tyr) and D-methionine (D-met) individually enhanced THPS against the *Desulfovibrio vulgaris* biofilm.[10, 13] A mixture of D-amino acids was found to enhance THPS[14] and two other biocides[15] in the mitigation of a field biofilm consortium containing SRB and other microbes. However, glutaraldehyde is not compatible with D-amino acids because of its ability to crosslink amino acids.[16]

Some peptides were also reported as signal molecules that trigger biofilm disassembly.[17] The anti-biofilm Peptide 1018 at low concentrations that did not inhibit the growth of planktonic cells was found to disperse several biofilms.[18] Some other non-biocidal peptides were also reported as natural biofilm dispersal agents.[19, 20]Data from biofilm prevention and detachment tests showed an effective dosage at the nano molar (nM) level was able to achieve 50% – 90% reduction of microbial attachment. In the oil and gas industry, a high kill or biofilm removal rate is desired because it means the time for the next treatment will be prolonged.

In this work, a peptide (labeled as “Peptide A”) was used as the biocide enhancer in combination with THPS to treat a corrosive SRB and a tough field biofilm consortium. This peptide was synthesized based on the active sequence derived from a protein produced by a sea anemone for protection against biofilms. This kind of proteins were said to prevent marine-aquatic plants and animals from the attachment of harmful biofilms in the marine environment.[19] Ten nM Peptide A was used in the biofilm prevention test and 10 – 100 nM were used in the biofilm removal test.

EXPERIMENTAL PROCEDURE

In this work, *D. vulgaris* (ATCC[†] 7757) and a field biofilm consortium (labeled as “Consortium II”)[15] were used to test the efficacy of the cocktail of Peptide A and THPS. The ATCC 1249 culture medium was used to culture *D. vulgaris* and Consortium II. The culture medium, 125 ml anaerobic vials, vial caps, pipette tips, and tweezers were sterilized in an autoclave for 20 minutes at 121^{o}C. Peptide A provided was first dissolved in dimethyl sulfoxide (DMSO) to make a stock solution with a concentration of 10 mg/ml. The stock solution was diluted with sterilized deionized water in tests. Liquid solutions were

[†]Trade name

sparged with filtered N_2 for 45 minutes to remove dissolved O_2 before incubation. C1018 carbon steel coupons had a top surface area of 1 cm^2. The coupon surfaces were coated with Teflon paint except the top surface. Coupons were polished with 180, 400, and 600 grit sandpapers sequentially. They were then cleaned with isopropanol and dried under UV light for 20 minutes. All chemicals used in this study were purchased from Fisher Scientific (Pittsburgh, PA, USA) or Sigma-Aldrich (St Louis, MO, USA).

Biofilm prevention and removal tests were used to evaluate the efficacy of the cocktail of Peptide A and THPS. In the biofilm prevention test, at least 3 coupons, 100 ml culture medium, treatment chemicals, 100 ppm L-cysteine and 1 ml biofilm seed culture were put into each vial. L-cysteine was used to mitigate any possible oxygen ingress. The initial planktonic cell count in each vial was 10^6 cells/ml immediately after inoculation. The vials were sealed and incubated at 37^{o}C. After 7 days, coupons were retrieved for SRB sessile cell enumeration and biofilm observation. The test matrix of the biofilm prevention test is shown in Table 1. In the biofilm removal test, biofilms were first grown on 1018 carbon steel coupons without any treatment chemicals for 3 days to reach biofilm maturity. After that, coupons were retrieved and washed to rinse off planktonic cells with a pH 7.4 phosphate buffered saline (PBS) solution. Then two coupons were submerged in 50 ml pH 7.4 deoxygenated PBS solution with treatment chemicals in a Petri dish in an anaerobic chamber at 25^{o}C. After the treatment, coupons were taken out for SRB sessile cell enumeration and biofilm observation. The test matrix of the biofilm removal test is shown in Table 2.

Table 1
Test matrix of biofilm prevention test

Biofilm	*D. vulgaris* and Consortium II
Culture medium	ATCC 1249 medium with and without treatment chemicals
Treatment method	THPS, Peptide A, Peptide A + THPS
Concentration	50 ppm THPS, 10 nM Peptide A, 50 ppm THPS 10 + 10 nM Peptide A
Temperature	37 ^{o}C
Incubation duration	7 days
Coupon	C1018 carbon steel

Table 2
Test matrix of established biofilm removal test

Biofilm	*D. vulgaris* or Consortium II pre-grown on coupons
Culture medium	ATCC 1249 medium (to grow biofilms first)
Growth time	3 days to reach maturity
Treatment method	THPS, Peptide A, Peptide A + THPS
Concentration	10 nM and 100 nM Peptide A, 50 ppm THPS + 10 nM or 100 nM Peptide A
Treatment time	3 hours (*D. vulgaris*), and 3 - 4.5 hours (Consortium II) in Petri dishes followed by PBS wash to remove planktonic cells and residual biocides
Temperature	25 ^{o}C
Coupon	C1018 carbon steel

An assay kit (Sani-Check Product #100 from Biosan Laboratories†, Warren, MI, USA) was used to determine the numbers of sessile SRB cells. The procedure was reported previously.[10] The time it took to show the black (FeS) color reflects the SRB cell concentration based on the vendor's calibration. The coupon preparation details for scanning electron microscope (SEM) (Model JSM-6390, JEOL†, Tokyo, Japan) observation were reported elsewhere.[10] The coupon surface was coated with a palladium film to provide conductivity before SEM observation. A confocal laser scanning microscope (CLSM) (Model LSM 510, Carl Zeiss†, Jena, Germany) was used to observe live and dead cells in a biofilm. To do this, biofilms was stained using the Live/Dead BacLight† Bacterial Viability Kit L7012 (Life Technologies†, Grand Island, NY, USA). In CLSM images, green dots indicate live cells and red dots indicate dead cells.

RESULTS

Figure 1 shows the SRB sessile cell counts of *D. vulgaris* biofilm and biofilm Consortium II after the 7-day biofilm prevention test using Peptide A to enhance THPS. In the 7-day *D. vulgaris* biofilm prevention test, the SRB sessile cell count of the no treatment control was 10^6 cells/cm^2. Fifty ppm THPS reduced the sessile cell concentration from 10^6 cells/cm^2 to 10^4 cells/cm^2. Without THPS, 10 nM peptide A did not reduce the SRB sessile cell concentration for both biofilms. However, 10 nM Peptide A enhanced 50 ppm THPS by achieving an extra 2-log reduction of sessile SRB cells. In the 7-day biofilm Consortium II prevention test, Peptide A alone did not reduce the SRB sessile cell concentration. When 50 ppm THPS treatment alone was used, it achieved only 1-log sessile cell count reduction. The combination of 50 ppm THPS + 10 nM Peptide A achieve 2 extra log reduction compared with 50 ppm THPS treatment alone.

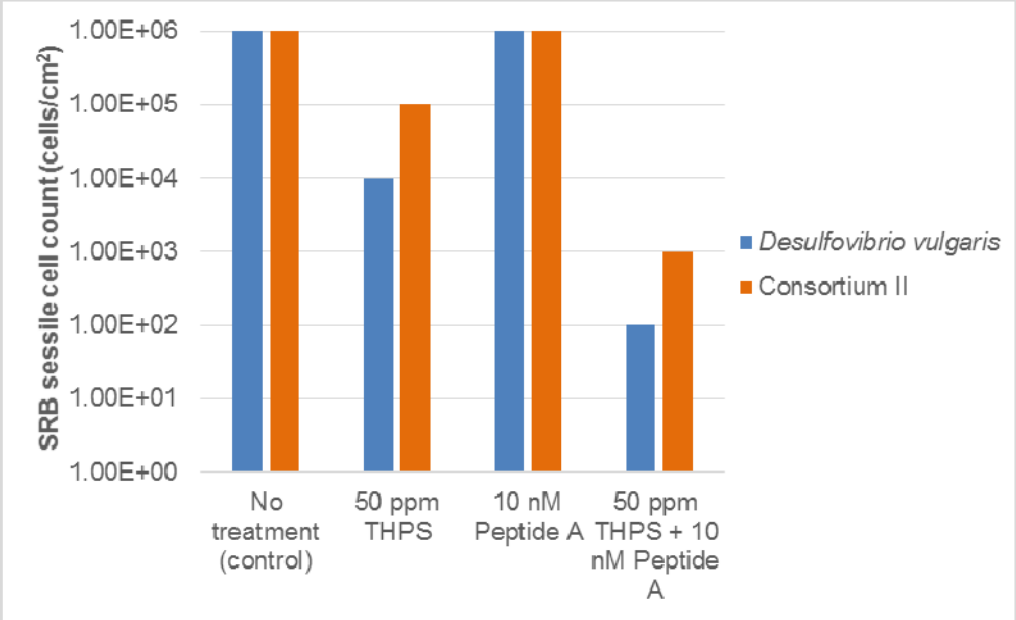

Figure 1. SRB sessile cell counts after 7-day biofilm prevention tests.

Figure 2 shows SEM images of *D. vulgaris* biofilms after the 7-day biofilm prevention test. Sessile cells were abundant on the control coupon (Figure 2A), Peptide A alone treated coupon (Figure 2C), and 50 ppm THPS alone treated coupon (Figure 2B). However, with the cocktail of 50 ppm THPS + 10 nM Peptide A (Figure 2D), the number of sessile cells was much less on the coupon surface. Similar

results were found in the SEM images of biofilms after 7-day biofilm Consortium II prevention test as shown in Figure 3. This is consistent with the SRB enumeration results in Figure 1.

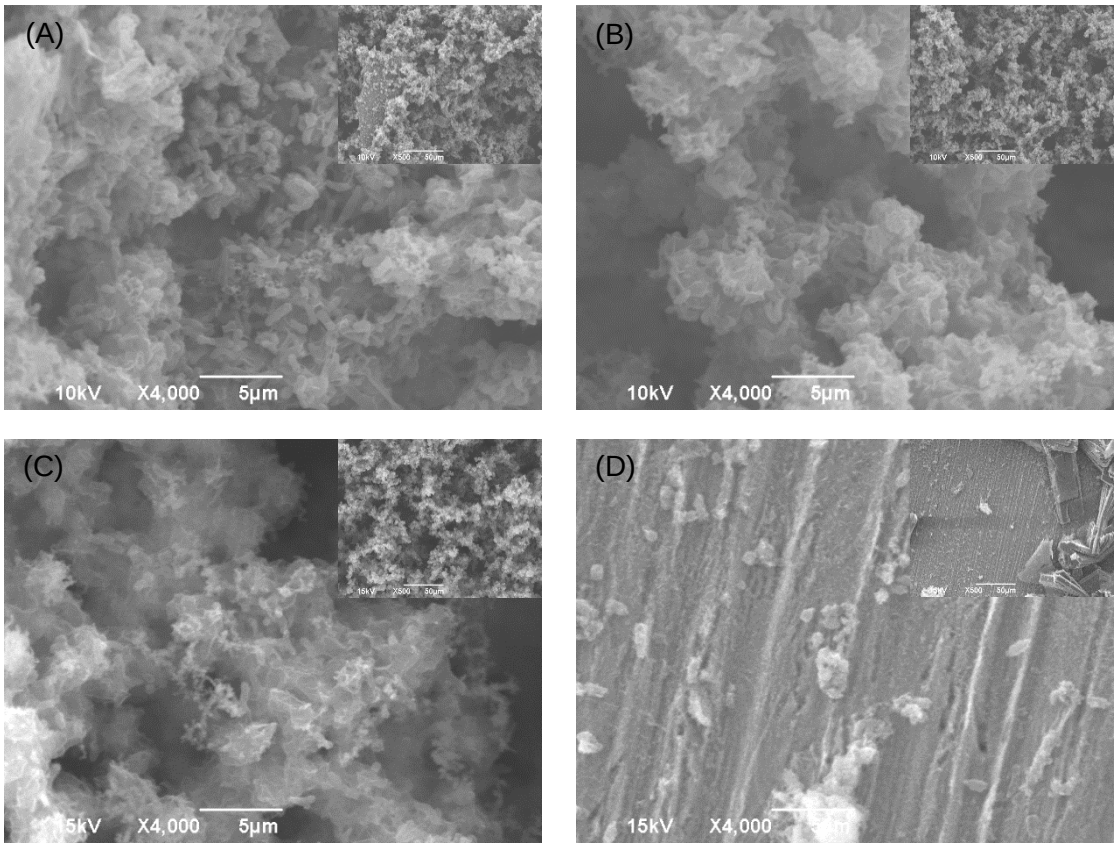

Figure 2. Images of biofilms after 7-day incubation in the *D. vulgaris* biofilm prevention test: (A) no treatment chemical (control), (B) 50 pm THPS, (C) 10 nM Peptide A, and (D) 50 ppm THPS + 10 nM Peptide A. (The scale bar in the inserted small image is 50 µm.)

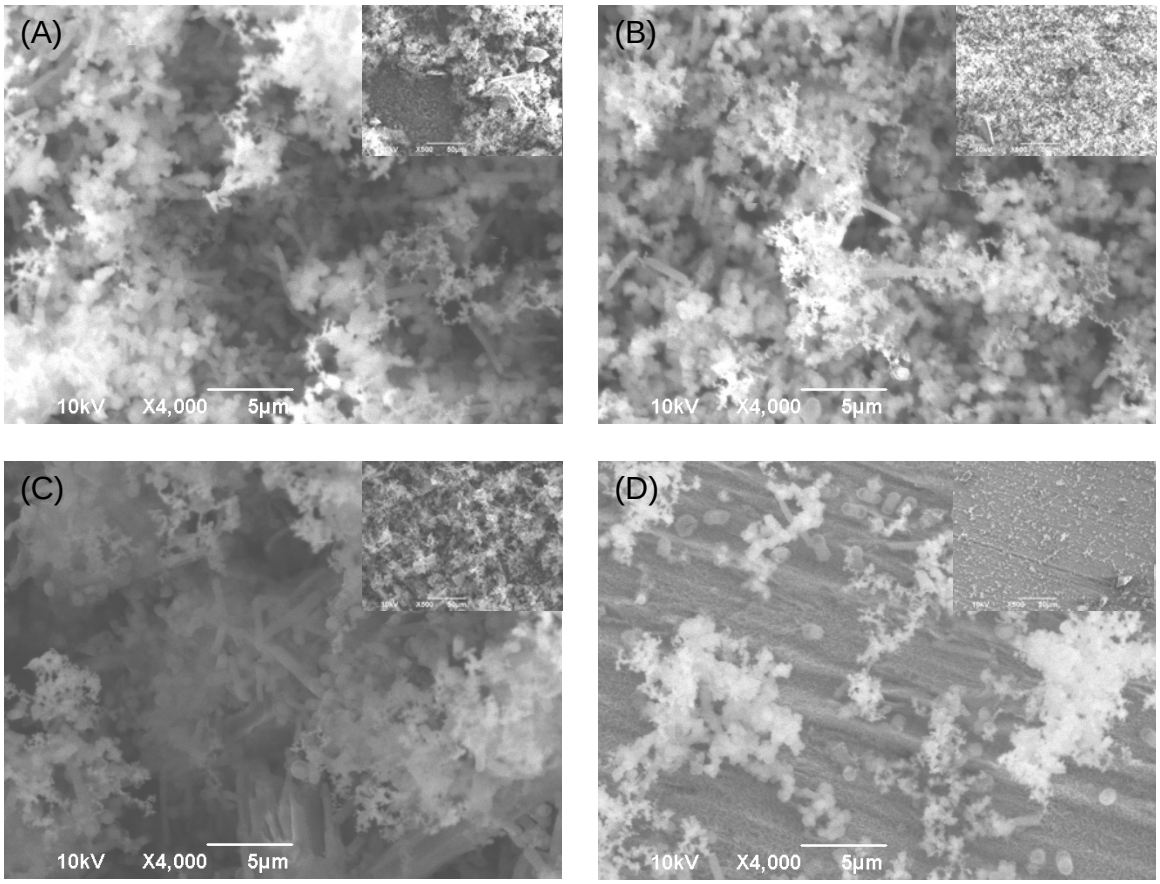

Figure 3. SEM images of sessile cells on C1018 after 7-day biofilm Consortium II prevention test: (A) no treatment (control), (B) 50 ppm THPS, (C) 10 nM Peptide A, and (D) 50 ppm THPS + 10 nM Peptide A. (The scale bar in the inserted small image is 50 μm.)

SEM shows different cell morphology, but it cannot indicate live and dead cells. CLSM can be used to observe live and dead sessile cells in biofilms. In the 7-day biofilm Consortium II prevention test, the sessile cells on untreated (control) (Figures 4A) and Peptide A treated (Figures 4C) coupons were all live cells. For the 50 ppm THPS alone treated coupon in Figures 4B, the number of dead cells increased, but live cells still dominated. After the treatment with 50 ppm THPS + 10 nM peptide, dead cells in Figures 4D far outnumbered live cells.

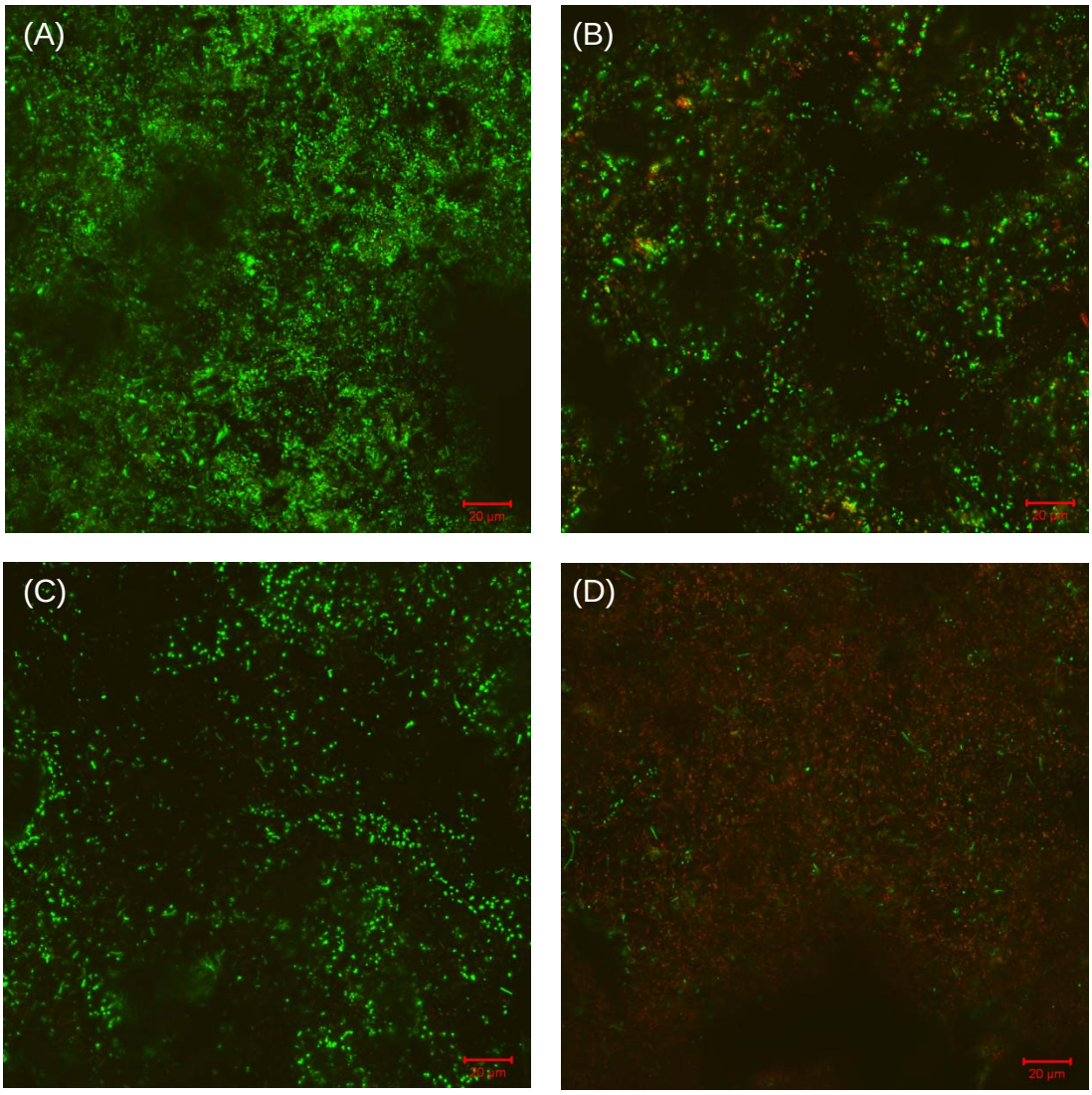

Figure 4. CLSM images of biofilms after 7-day incubation in the biofilm Consortium II prevention test: (A) no treatment (control), (B) 50 ppm THPS, (C) 10 nM Peptide A, and (D) 50 ppm THPS + 10 nM Peptide A.

Figure 5 shows the SRB sessile cell counts of the *D. vulgaris* biofilm and the biofilm Consortium II after the 3-hour biofilm removal test. In the *D. vulgaris* biofilm removal test, 10 nM Peptide A treatment alone achieved 1-log reduction while 100 nM Peptide A treatment alone achieved 2-log reduction compared with the no treatment control. Treatments of 50 ppm THPS + 10 nM peptide A and 50 ppm THPS + 100 nM peptide A achieved 1 and 2 extra log reduction respectively compared with 50 ppm THPS treatment alone case. In the biofilm Consortium II removal test, Peptide A treatment alone did not reduce any SRB sessile cell concentration. However, the combinations of 50 ppm THPS + 10 nM Peptide A and and 50 ppm THPS + 100 nM peptide A both achieved 2 extra log reduction compared with the 50 ppm THPS treatment alone. In summary, Peptide A enhanced the efficacy of 50 ppm THPS in the 3-hour biofilm removal test.

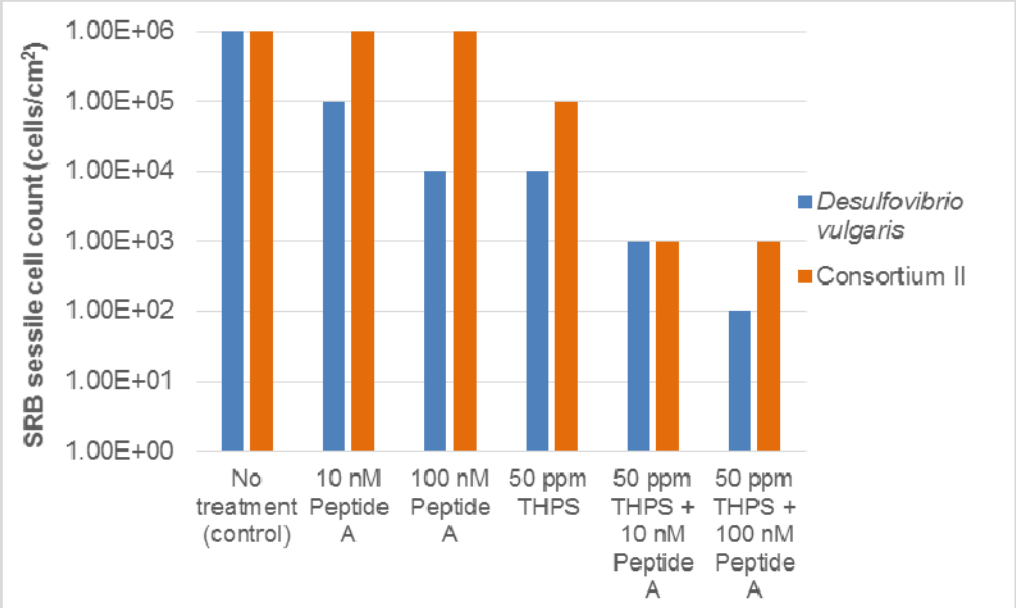

Figure 5. SRB sessile cell counts after 3-hour treatment in the biofilm removal tests.

SEM images of *D. vulgaris* biofilms after 3-hour treatment are shown in Figure 6. Sessile cells were easy to find on coupon surfaces with no treatment (control), Peptide A alone treatment, and 50ppm THPS alone treatment in the 3-hour biofilm removal test. Sessile cells were less but still noticeable on coupon surfaces in treatments of 50 ppm THPS + 10 nM peptide A and 50 ppm THPS + 100 nM peptide A. In this case, SEM images was not able to tell the differences between these treatments. CLSM was required. Figure 7 is the CLSM images of biofilm Consortium II in the 3-hour biofilm removal test. Live cells were found in large quantities on coupons for the untreated (control) coupons (Figure7A) and the coupons treated with different concentrations of Peptide A (Figures 7B and 7C). More dead sessile cells were found with the treatment of 50 ppm THPS (Figure 7D). Mostly dead sessile cells were found with the treatments of 50 ppm THPS + 10 nM and 50 ppm THPS + 100 nM Peptide A (Figures 7E and 7F). The CLSM images are generally consistent with the SRB sessile cell counts in Figure 5. If dead cells detach from the coupons surfaces, an SEM image can reflect the efficacy of a biocide treatment of a coupon surface. It also shows different cell shapes, which is helpful for mixed-culture biofilms. However, if recently killed sessile cells stay on the coupon surface, CLSM should be used.

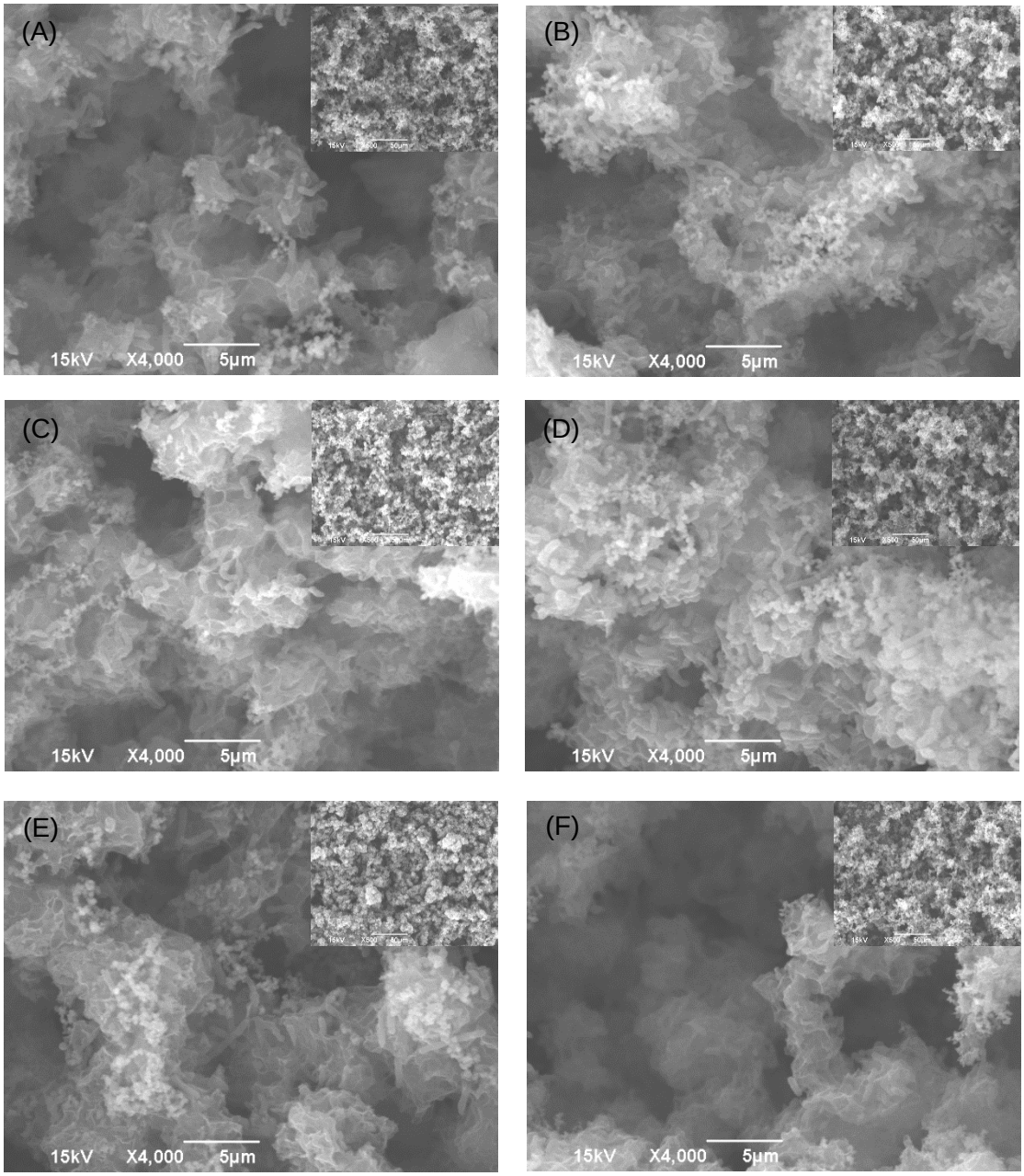

Figure 6. SEM images of *D. vulgaris* biofilms after 3-hour treatment in a Petri dish containing PBS buffer and: (A) no treatment (control), (B) 10 nM Peptide A, (C) 100 nM Peptide A, (D) 50 ppm THPS (secondary control), (E) 50 ppm THPS + 10 nM Peptide A, and (F) 50 ppm THPS + 100 nM Peptide A in the biofilm removal test. (The scale bar in the inserted small images is 50 µm.)

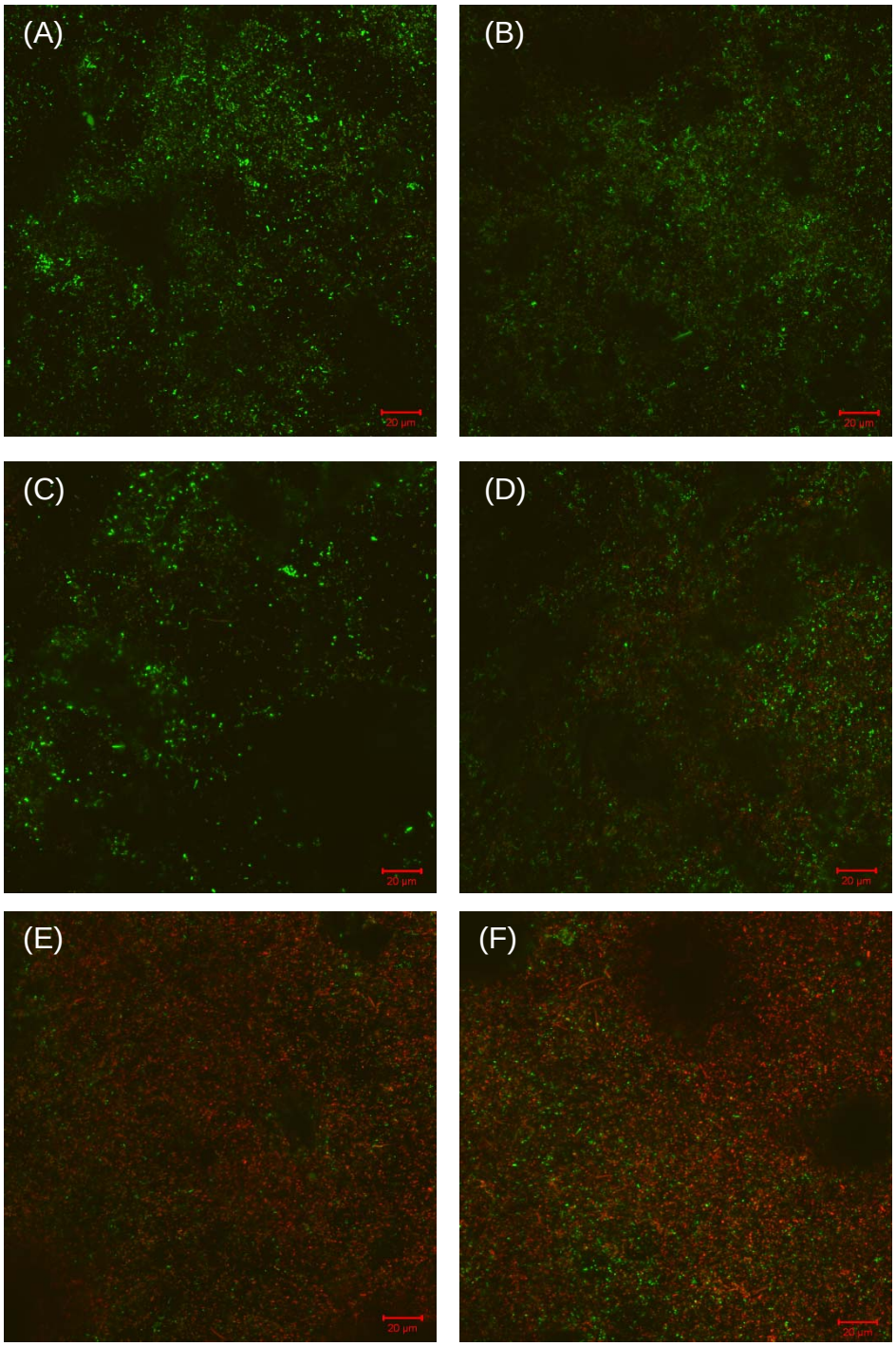

Figure 7. CLSM images of biofilm Consortium II after 3-hour treatment in a Petri dish containing PBS buffer and: (A) no treatment (control), (B) 10 nM Peptide A, (C) 100 nM Peptide A, (D) 50 ppm THPS, (E) 50 ppm THPS + 10 nM Peptide A, and (F) 50 ppm THPS + 100 nM Peptide A in the biofilm removal test.

CONCLUSIONS

The results in this work demonstrated that Peptide A at a very low concentration (10 nM or 18 ppb) enhanced 50 ppm THPS in the biofilm prevention test against a pure-strain SRB biofilm and a field

biofilm consortium by achieving 2 extra log reduction of SRB sessile cells compared with using THPS alone. One hundred nM Peptide A (180 ppb) enhanced 50 ppm THPS in the 3-hour biofilm removal test for both biofilms with 2 extra log reduction. Further investigations are needed to see whether Peptide A can enhance THPS against other biofilms and whether it can enhance other biocides.

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REFERENCES

1. R.H. Gaines, “Bacterial activity as a corrosive influence in the soil,” *J. Ind. Eng. Chem.* 2 (1910): pp. 128–130.

2. D. Walsh, D. Pope, M. Danford, T. Huff, “The effect of microstructure on microbiologically influenced corrosion,” *JOM* 45 (1993): pp. 22–30.

3. Y. Tanaka, M. Sogabe, K. Okumura, and R. Kurane, “A highly selective direct method of detecting sulphate-reducing bacteria in crude oil,” *Lett. Appl. Microbiol.* 35 (2002): pp. 242–246.

4. T. Thorstenson, E. Sunde, G. Bodtker, B.L. Lillebo, T. Torsvik, J. Beeder, “Biocide replacement by nitrate in sea water injection systems,” Corrosion/2002, paper no. 02033 (Denver, Colorado: NACE, 2002).

5. B. Anandkumar, R.P. George, S. Maruthamuthu, N. Parvathavarthini, U.K. Mudali, “Corrosion characteristics of sulfate-reducing bacteria (SRB) and the role of molecular biology in SRB studies: an overview,” *Corros. Rev.* 34 (2016): pp. 41–63.

6. P.S. Stewart, M.J. Franklin, “Physiological heterogeneity in biofilms,” *Nat. Rev. Microbiol.* 6 (2008): pp. 199–210.

7. E. Tuomanen, D.T. Durack, A. Tomasz, “Antibiotic tolerance among clinical isolates of bacteria,” *Antimicrob. Agents Chemother.* 30 (1986): pp. 521–527.

8. T.-F. C. Mah, G.A. O’Toole, “Mechanisms of biofilm resistance to antimicrobial agents,” *Trends Microbiol.* 9 (2001): pp. 34–39.

9. C. Walsh, “Molecular mechanisms that confer antibacterial drug resistance,” *Nature* 406 (2000): pp. 775–781.

10. D. Xu, Y. Li, T. Gu, “A synergistic d-tyrosine and tetrakis hydroxymethyl phosphonium sulfate biocide combination for the mitigation of an SRB biofilm,” *World J. Microb. Biot.* 28 (2012): pp. 3067–3074.

11. I. Kolodkin-Gal, D. Romero, S. Cao, J. Clardy, R. Kolter, R. Losick, “D-amino acids trigger biofilm disassembly,” *Science* 328 (2010): pp. 627–629.

12. S.A. Leiman, J.M. May, M.D. Lebar, D. Kahne, R. Kolter, R. Losick, “D-amino acids indirectly inhibit biofilm formation in *Bacillus subtilis* by interfering with protein synthesis,” *J. Bacteriol.* 195 (2013): pp. 5391–5395.

13. D. Xu, Y. Li, T. Gu, “D-methionine as a biofilm dispersal signaling molecule enhanced tetrakis hydroxymethyl phosphonium sulfate mitigation of *Desulfovibrio vulgaris* biofilm and biocorrosion pitting,” *Mater. Corros.* 65 (2014): pp. 837–845.

14. Y. Li, R. Jia, H.H. Al-Mahamedh, D. Xu, T. Gu, “Enhanced biocide mitigation of field biofilm consortia by a mixture of D-amino acids,” *Front. Microbiol.* 7 (2016): pp. 896–909.

15. R. Jia, D. Yang, Y. Li, H.H. Al-Mahamedh, T. Gu, “Enhancement of alkyldimethylbenzylammonium chloride and tributyl tetradecyl phosphonium chloride biocides using D-amino acids against a field biofilm consortium,” Corrosion/2016, paper no. 7279 (Vancouver, BC, Canada: NACE, 2016).

16. Y. Li, D. Xu, P. Zhang, W. Fu, T. Gu, “D-amino acids enhanced biocide mitigation of problematic biofilms,” Corrosion/2014, paper no. 3877 (San Antonio, TX: NACE, 2014).

17. D. McDougald, S.A. Rice, N. Barraud, P.D. Steinberg, S. Kjelleberg, “Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal,” *Nat. Rev. Microbiol.* 10 (2011): pp. 39-50.

18. C. de la Fuente-Núñez, F. Reffuveille, E.F. Haney, S.K. Straus, R.E.W. Hancock, “Broad-spectrum anti-biofilm peptide that targets a cellular stress response,” *PLoS Pathog.* 10 (2014): e1004152.

19. A. Zlotkin, inventor, Hutchison Biofilm Solutions Limited, “Dispersion and detachment of cell aggregates,” U.S. Patent No. 9,284,351, 15-Mar-2016.

20. A. Zlotkin, inventor, Hutchison Biofilm Solutions Limited, “Peptides and compositions for prevention of cell adhesion and methods of using same,” U.S. Patent No. 9,045,550, 02-Jun-2015.