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ORIGINAL PAPER

D-amino acids for the enhancement of a binary biocide cocktail consisting of THPS and EDDS against an SRB biofilm

D. Xu · J. Wen · W. Fu · T. Gu · I. Raad

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Abstract Biofilms of sulfate reducing bacteria (SRB) are often responsible for Microbiologically Influenced Corrosion (MIC) that is a major problem in the oil and gas industry as well as water utilities and other industries. This work was inspired by recent reports that some D-amino acids may be useful in the control of microbial biofilms. A D-amino acid mixture with equimolar D-tyrosine, D-methionine, D-tryptophan and D-leucine was tested in this work for their enhancement of a biocide cocktail containing tetrakis (hydroxymethyl) phosphonium sulfate (THPS) and ethylenediamine-*N*,*N*'-disuccinic acid (EDDS). *Desulfovibrio vulgaris* (ATCC 7757) was cultured in ATCC 1249 medium. Its biofilm was grown on C1018 carbon steel coupons. Experimental results indicated that the triple biocide cocktail consisting of 30 ppm THPS, 500 ppm EDDS and 6.6 ppm D-amino acid mixture (with equimolar D-tyrosine, D-methionine, D-tryptophan and D-leucine) was far more effective than THPS and EDDS alone and their binary combination. The triple biocide cocktail effectively prevented SRB biofilm establishment and removed the established SRB biofilm. The D-amino acid mixture alone did not show significant effects in the two tasks even at 660 ppm.

Keywords D-amino acids · Biocide cocktail · THPS · SRB · Biofilm

D. Xu · J. Wen · W. Fu · T. Gu (✉)
Department of Chemical and Biomolecular Engineering,
Ohio University, Athens, OH 45701, USA
e-mail: gu@ohio.edu

I. Raad
Department of Infectious Diseases, Infection Control,
and Employee Health, The University of Texas
M. D. Anderson Cancer Center, Houston, TX 77230, USA

Introduction

Biofilm, a community of microorganisms, can attach on non-biological or biological surfaces (Hall-Stoodly et al. 2004). Bacteria embedded in the biofilm produce a extracellular polymeric substances (EPS) which offer them protection against the changing environmental conditions such as pH, fluid shear stress and antimicrobial agents (Hall-Stoodly et al. 2004; Zuo 2007). Biofilms cause problems in many fields, such as hospitals, water utilities, the food industry, and the oil and gas industry (Ganesh-Kumar and Anand 1998; Hall-Stoodly et al. 2004; Hu et al. 2005; Little and Lee 2007). Sulfate reducing bacteria (SRB), which form a corrosive biofilm, can cause Microbiologically Influenced Corrosion (MIC) (Videla 1996). Pitting corrosion caused by SRB biofilms was blamed for some pipeline failures (Abedi et al. 2007). MIC due to SRB biofilms was considered a primary suspect in the Alaska pipeline leak in the spring of 2006 (Jacobson 2007).

To mitigate biofilms and MIC, scrubbing including pigging and biocide/biostat dosing are often used (Videla 1996, 2002). Sessile cells in a biofilm are protected by the biofilm. A much higher, often 10 times higher, biocide dosage is needed to eradicate a biofilm compared with the dosage needed for planktonic cells (Videla 1996). Repeated applications are often required to control biofilms (Vance and Thrasher 2005). Tetrakis (hydroxymethyl) phosphonium sulfate (THPS) and glutaraldehyde are the two most popular green biocides in the oil and gas industry. The recommended THPS dose by Dow Chemicals (2009) is 93–350 ppm for 2–6 h in shock treatment, or 14–100 ppm for a continuous dose of its AQUCAR THPS 75 formulation containing 76.5% (w/w) THPS for oil and gas pipelines. Biocide efficacy for SRB treatment is often measured in log scale reduction of cell numbers (Keasler et al. 2010;

Dow Chemicals 2009; Anonymous 2005; Cowan 2005) instead of using numbers such as 99 or 99.9% kill rate.

Large-scale biocide applications, such as those in oil and gas fields or pipelines are becoming very expensive with the increasingly restrictive environmental regulations. More effective biocide dosing is desired. Biocide enhancers are helpful in cutting down biocide dosages while improving their efficacies. Raad et al. (2003) demonstrated that ethylenediaminetetraacetic acid (EDTA) enhanced microbial biofilm removal on catheters by antibiotics. They also reported that 25% (v/v) ethanol accelerated the biofilm treatment greatly when it was combined with minocycline and EDTA (Raad et al. 2007). Inspired by this discovery, Wen et al. (2011) found that a triple combination of 50 ppm glutaraldehyde, 1,000 ppm EDDS (ethylenediamine-*N*,*N*'-disuccinic acid) and 15% (v/v) methanol was efficacious in the removal of established SRB biofilms on mild steel coupons. EDDS, a readily biogradable chelator, was believed to weaken the cell wall and thus enhanced glutaraldehyde's biocidal effect.

Recently, D-amino acids were found to play very important roles in regulating and disassembling of microbial biofilms. Lam et al. (2009) found that D-amino acids secreted by bacteria themselves probably controlled the cell wall building and modified the assembly of cell wall. Kolodkin-Gal et al. (2010) reported that a combination of four D-amino acids (D-tyrosine, D-methionine, D-tryptophan and D-leucine) at very low concentration dissembled the established bacteria biofilm of *Bacillus subtilis* and also prevented the biofilm formation of *B. subtilis, Staphylococcus aureus* and *Pseudomonas aeruginosa*. They tested various D-amino acid combinations and found that a mixture of the four D-amino acids was particularly potent. The purpose of this work was to evaluate a combination of the D-amino acid mixture for its enhancement of the THPS prevention of the establishment of an SRB biofilm and removal of an established SRB biofilm on a mild steel coupon surface. EDDS was also used in the biocide cocktail.

Materials and methods

Bacterium, culture media and chemicals and coupon for biofilm growth

Desulfovibrio vulgaris (ATCC 7757) was cultured in ATCC 1249 medium. D-tyrosine, D-methionine, D-tryptophan and D-leucine and THPS were purchased from (Sigma–Aldrich, St. Louis, MO, USA). Based on the molecular weights of the four D-amino acids, 1 mM equimolar mixture of four D-amino acids was found equivalent to 660 ppm D-amino acid mixture concentration. EDDS (Octaquest®E30, a trisodium salt of EDDS) was kindly provided by Octel Performance Chemicals (now Innospec in Ellesmere Port, Cheshire, UK). Sani-Check® Product #100 SRB test kit (Biosan Laboratories, Warren, MI, USA) was used to enumerate sessile SRB cells after they were removed from a coupon surface. SRB culture media were sterilized at 121°C and were deoxygenated by sparging filtered N_2 for 1 h before use. All manipulations were done in a glovebox filled with N_2 which provided an anaerobic environment. 125 ml anaerobic vials and disk shaped C1018 carbon steel coupons were used in the tests. The coupons were coated with Teflon except the top surface that had a surface area of 1.12 cm^2. This surface was exposed to the culture medium in the vial. After polishing with 200, 400 and 600 grit sand papers sequentially, the coupons were rinsed in isopropanol, and then sterilized under UV light for 15 min before use.

Tests for preventing biofilm establishment

One ml of a 3-day old SRB seed culture and 3 coupons were added into each 125 ml vial containing 100 ml culture medium. Right after inoculation, SRB cell concentration in each vial was roughly 10^6 cells/ml. After adding treatment chemicals, the vials were sealed in the glovebox and then incubated at 37°C. After 7 days, coupons were taken out. The biofilm on the coupon surface was observed under SEM (Scanning Electron Microscope, Model JSM-6390, JEOL, Tokyo, Japan). The procedure for coupon preparation for SEM observation of biofilms was described by Wen et al. (2009). SEM images were selected for the spot with most sessile SRB cells on each coupon.

To obtain the average sessile cell density on the coupon surface, a duplicate coupon was used. The biofilm was removed using the brush-like dipstick of the SRB kit. The dipstick was then placed in a 50 ml conical plastic test tube containing 10 ml sterile deoxygenated distilled water. The test tube was vortexed for 30 s to distribute the cells evenly in the liquid. At this time, the dipstick was able to sample the volumetric cell concentration in the liquid as required by the test kit. The dipstick was taken out using sterilized tweezers and placed into the SRB test vial containing a solid SRB medium. The incubation time needed for the black color to appear in the medium corresponded to a certain SRB concentration based on vendor's calibrations. The volumetric SRB concentration was subsequently converted to a surface area based sessile cell count based on the coupon surface area and the liquid volume (10 ml) used for sampling.

Tests for removal of an established biofilm

Biofilms were first pre-cultured for 7-days on coupons in the ATCC 1249 medium. The coupons covered with biofilms were harvested and rinsed with sterile deoxygenated

distilled water to remove any planktonic cells. Three duplicate coupons covered with biofilms were placed in fresh ATCC 1249 medium in each anaerobic vial. After adding treatment chemicals, the vials were sealed and then incubated at 37°C for 7 days. Coupons were then taken out and analyzed just like the coupons used in the prevention of biofilm establishment.

Results and discussion

Prevention of SRB biofilm establishment

Figure 1 shows that coupons treated with 6.6 ppm D-amino acid mixture, 30 ppm THPS + 6.6 ppm D-amino acid mixture, 660 ppm D-amino acid mixture, 30 ppm THPS + 660 ppm D-amino acid mixture, 100 ppm THPS, respectively all had numerous sessile cells (kidney bean shaped) visible on the coupon surfaces. Kolodkin-Gal et al. (2010) suggested that the D-tyrosine, D-methionine, D-tryptophan and D-leucine may be signal molecules that trigger biofilm disassembly and a synergistic effect can be achieved by combining the four D-amino acids together to form a more potent mixture than individual D-amino acids. They tested various equimolar mixture concentrations including 2.5, 5 and 15 nM (for each D-amino acid in the mixture) and found 2.5 nM to be the minimum inhibitory concentration. Figure 2a shows that when 6.6 ppb D-amino acid mixture (equivalent to 10 nM for each D-amino acid in the mixture) was added to the 30 ppm THPS + 500 ppm binary biocide cocktail, the sessile cell amount was reduced considerably compared with Fig. 1d. However, some sessile cells are still seen in Fig. 1d. When the D-amino acid concentration was increased to 6.6 ppm, sessile cells were hard to detect (Fig. 2b), indicating successful prevention of SRB biofilm establishment on the coupon surface by the triple biocide cocktail containing 30 ppm THPS + 500 ppm EDDS + 6.6 ppm D-amino acid mixture.

The sessile cell count results obtained from the SRB test kit in Table 1 were consistent with the SEM images in Figs. 1 and 2. It shows that without treatment, the sessile cell count on the coupon surface was 10^8 cells/cm^2. When treated with 30 ppm THPS, the sessile cell count decreased to 10^6 cells/cm^2. Combining 30 ppm THPS with 6.6 ppm D-amino acid mixture yielded no enhancement. When 30 ppm THPS was combined with 500 ppm EDDS, the sessile cell count decreased to 10^5 cells/cm^2, showing enhancement by EDDS. When the triple biocide cocktail containing 30 ppm THPS, 500 ppm EDDS and 6.6 ppm D-amino acid mixture, the sessile cell count dropped to 10^3 cells/cm^2, indicating a 5 log reduction compared with the sessile cell count without treatment.

Removal of established SRB biofilm

Figures 3a and b show that the treatment with 660 ppm D-amino acid mixture and 250 ppm THPS, respectively, sessile cells were numerous on the coupon surfaces. When the biofilm was treated with 30 ppm THPS combined with a 660 ppm D-amino acid mixture, or 30 ppm THPS and 500 ppm EDDS, much fewer sessile cells were observed. When a triple biocide cocktail acid containing 30 ppm THPS, 500 ppm EDDS and 6.6 ppb D-amino acid mixture was used, only a few sessile cells were observed as shown in Fig. 4a. Increasing the D-amino acid concentration from 6.6 ppb to 6.6 ppm resulted in almost complete disappearance of sessile cells from the coupon surface (Fig. 4b).

The sessile cell counts in Table 2 are consistent with the SEM observations. The table shows that the triple biocide cocktail containing 30 ppm THPS + 500 ppm EDDS + 6.6 ppm D-amino acid mixture was far more effective than 30 ppm THPS, 30 ppm THPS + 6.6 ppm D-amino acid mixture, and 30 ppm THPS + 500 ppm EDDS, resulting in a 4 log reduction in the sessile cell concentration. The sessile cell concentration in the control (no treatment) in Table 2 was 10 times lower than that of the control in Table 1. The coupon in Table 2 was taken out from a 7-day old culture and then placed in fresh medium for an additional 7 days. It was observed that some sessile cells started to disperse and became planktonic cells. The biofilm probably prefer a lower sessile cell density in the new anaerobic vial (Romeo 2006) and this probably caused the lower sessile cell count for the control in Table 2.

THPS can damage the cell outer membrane and also inhibits the lactate dehydrogenase enzyme that is critical in SRB metabolism (Downward et al. 2000). In this work, both EDDS and the D-amino acids enhanced THPS successfully in the prevention and removal of an SRB biofilm with only 30 ppm THPS in the triple biocide cocktail. The experimental data indicated that for the SRB biofilm system in this work, the D-amino acid mixture alone was ineffective even at a very high concentration. The SRB biofilm was a thin but dense biofilm that adhered to the coupon surface tenaciously. It was obviously far more difficult to treat than the *B. subtilis, S. aureus* and *P. aeruginosa* biofilms that were successfully treated by Kolodkin-Gal et al. (2010) using only ppb level of the D-amino acid mixture alone. The SRB biofilm in this work seemed to largely ignore the biofilm dispersal signaling by the D-amino acid mixture when the mixture was used alone. However, in the presence of the THPS biocide stress (enhanced by EDDS), the D-amino acid mixture was able to "convince" the SRB biofilm to disperse. This outcome was a result of synergy among the biocide cocktail components.

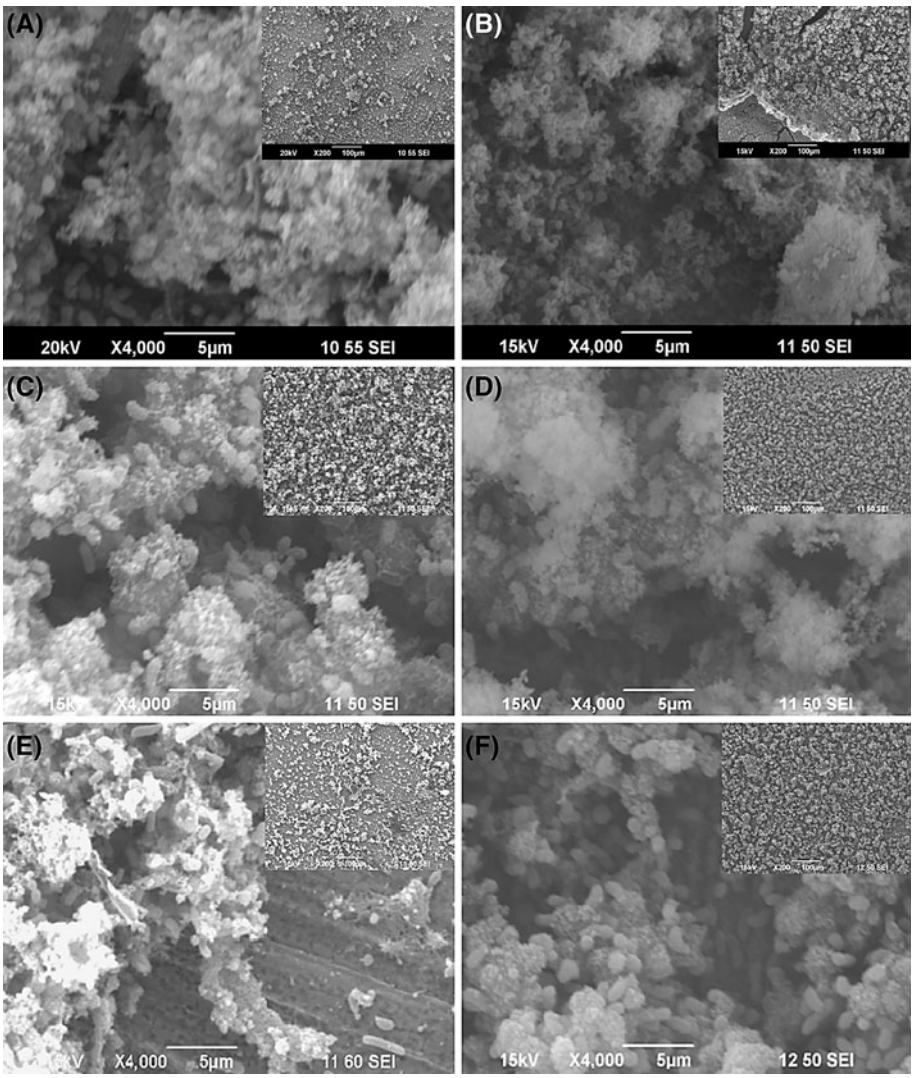

Fig. 1 SEM images for 7-day coupons in ATCC 1249 medium with **a** 6.6 ppm D-amino acid mixture, **b** 30 ppm THPS + 6.6 ppm D-amino acid mixture, **c** 660 ppm D-amino acid mixture, **d** 30 ppm THPS + 660 ppm D-amino acid mixture, **e** 100 ppm THPS treatment, **f** 30 ppm THPS + 500 ppm EDDS. *Scale bars* for the small inserted images are 100 μm

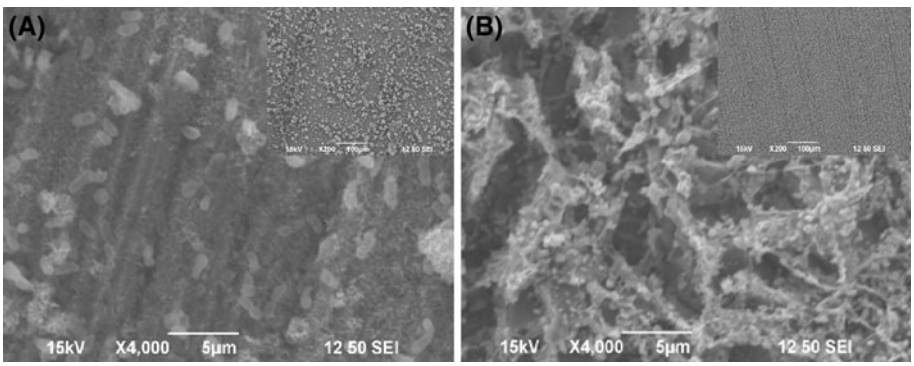

Fig. 2 SEM images for 7-day coupons in ATCC 1249 medium with **a** 30 ppm THPS + 500 ppm EDDS + 6.6 ppb D-amino acid mixture, **b** 30 ppm THPS + 500 ppm EDDS + 6.6 ppm D-amino acid mixture. *Scale bars* for the small inserted images are 100 μm. 6.6 ppb D-amino acid mixture equals to 10 nM D-amino acid mixture

Conclusion

The preliminary experimental data in this work indicated that a high concentration (660 ppm) of a D-amino acid mixture (with equimolar D-tyrosine, D-methionine, D-tryptophan and D-leucine) failed to prevent the SRB biofilm establishment or to remove a mature SRB biofilm. However, the triple biocide cocktail consisting of 30 ppm THPS, 500 ppm EDDS and 6.6 ppm D-amino acid mixture (with equimolar D-tyrosine, D-methionine, D-tryptophan and D-leucine) was far more effective than THPS and EDDS alone and their binary combination. The triple

Table 1 Sessile cell counts 7 days after using different treatment methods for the prevention of SRB biofilm establishment in ATCC 1249 medium

Treatment	Sessile cell count (cells/cm^2)
No treatment	$\geq 10^8$
30 ppm THPS	$\geq 10^6$
30 ppm THPS + 6.6 ppm D-amino acid mixture	$\geq 10^6$
30 ppm THPS + 500 ppm EDDS	$\geq 10^5$
30 ppm THPS + 500 ppm EDDS + 6.6 ppm D-amino acid mixture	$\geq 10^3$

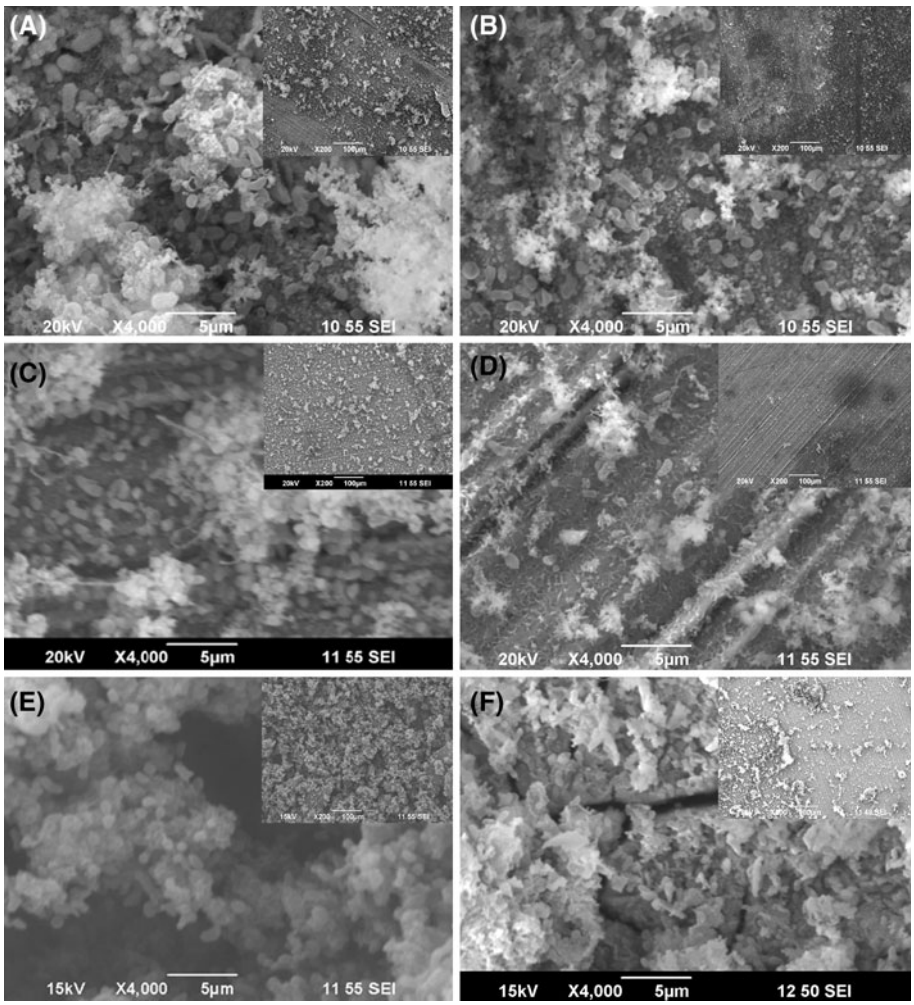

Fig. 3 SEM images for coupons in ATCC 1249 medium covered with mature biofilms for 7 days then they were treated with **a** 6.6 ppm D-amino acid mixture, **b** 30 ppm THPS + 6.6 ppm D-amino acid mixture, **c** 660 ppm D-amino acid mixture, **d** 30 ppm THPS + 660 ppm D-amino acid mixture, **e** 250 ppm THPS treatment, **f** 30 ppm THPS + 500 ppm EDDS. *Scale bars* for the small inserted images are 100 μm

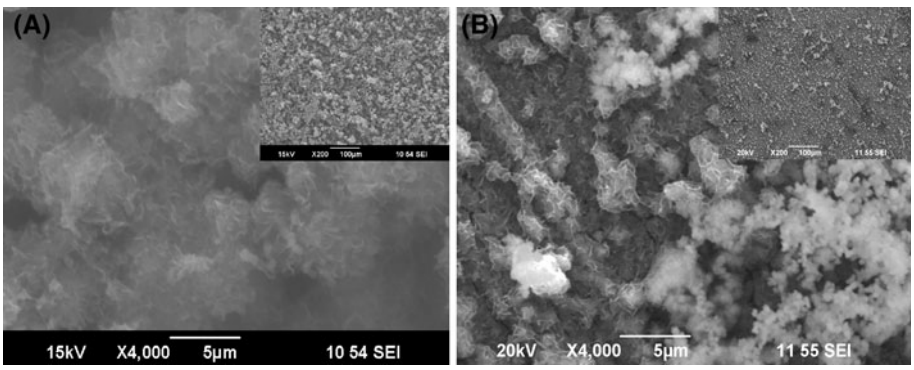

Fig. 4 SEM images for coupons in ATCC 1249 medium covered with well-established biofilms for 7 days then they were treated with **a** 30 ppm THPS + 500 ppm EDDS + 6.6 ppb D-amino acid mixture, **b** 30 ppm THPS + 500 ppm EDDS + 6.6 ppm D-amino acid mixture. *Scale bars* for the small inserted images are 100 μm

Table 2 Sessile cell counts 7 days after using different treatment methods to treat established SRB biofilms in ATCC 1249 medium

Treatment	Sessile cell count (cells/cm^2)
No treatment	$\geq 10^7$
30 ppm THPS	$\geq 10^6$
30 ppm THPS + 6.6 ppm D-amino acid mixture	$\geq 10^6$
30 ppm THPS + 500 ppm EDDS	$\geq 10^5$
30 ppm THPS + 500 ppm EDDS + 6.6 ppm D-amino acid mixture	$\geq 10^3$

biocide cocktail was quite effective in the prevention of SRB biofilm establishment and in the removal of established SRB biofilms. These three concentrations in the triple biocide cocktail may serve as guidelines for further testing (including field testing) of the biocide system. For a less recalcitrant biofilm system with a less nutritious environment, the cocktail may be much more effective even at lower D-amino acid concentrations. However, with a more recalcitrant system such as a well-established synergistic biofilm consortium, increased concentrations may be required to achieve the desired outcome.

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