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ORIGINAL PAPER

A green triple biocide cocktail consisting of a biocide, EDDS and methanol for the mitigation of planktonic and sessile sulfate-reducing bacteria

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Abstract Sulfate-reducing bacteria (SRB) cause souring and their biofilms are often the culprit in Microbiologically Influenced Corrosion (MIC). The two most common green biocides for SRB treatment are tetrakis-hydroxymethylphosphonium sulfate (THPS) and glutaraldehyde. It is unlikely that there will be another equally effective green biocide in the market any time soon. This means more effective biocide treatment probably will rely on biocide cocktails. In this work a triple biocide cocktail consisting of glutaraldehyde or THPS, ethylenediaminedisuccinate (EDDS) and methanol was used to treat planktonic SRB and to remove established SRB biofilms. *Desulfovibrio vulgaris* (ATCC 7757), a corrosive SRB was used as an example in the tests. Laboratory results indicated that with the addition of 10–15% (v/v) methanol to the glutaraldehyde and EDDS double combination, mitigation of planktonic SRB growth in ATCC 1249 medium and a diluted medium turned from inhibition to a kill effect while the chelator dosage was cut from 2,000 to 1,000 ppm. Biofilm removal was achieved when 50 ppm glutaraldehyde combined with 15% methanol and 1,000 ppm EDDS was used. THPS showed similar effects when it was used to replace glutaraldehyde in the triple biocide cocktail to treat planktonic SRB.

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Introduction

Microbial contamination is a threat to many industries, such as the food industry, water utilities, nuclear power plant and the oil and gas industry (Carpentier and Cerf 1993; Javaherdashti 1999; Diosi et al. 2003; Meyer 2003). Microbes often form a biofilm on a solid surface. It consists of bacteria and extracellular polymeric substances (EPS) that hold them together. A biofilm offers the microbes protection against fluid shear, harmful pH swings and biostatic and biocidal chemicals. Usually ten times or higher biocide concentrations are needed to eradicate biofilms compared to the dosages needed to treat planktonic microbes (Meyer 2003). Corrosive biofilms, often Sulfate Reducing Bacteria (SRB) biofilms, on internal pipeline surfaces result in the so-called Microbiologically Influenced Corrosion (MIC), which was a primary suspect in the March 2006 Alaska pipeline leak (Jacobson 2007). Pigging and biocides are widely used to mitigate MIC in the oil and gas industry (Videla 1996). Due to more restrictive environmental regulations and increasing costs, more effective biocide treatment with lower dosages is desired.

Raad and Sherertz (2001) patented the use of a lock solution consisting of ethylenediaminetetraacetic acid (EDTA) which is a chelator and an antibiotic to eradicate biofilms on catheters for cancer patients. Raad et al. (2007) further improved the cocktail treatment by adding 25% (v/v) ethanol. The new triple combination is highly effective in removing biofilms on catheters after 15 min of exposure. Our previous work (Wen et al. 2009; 2010) demonstrated that chelators also enhanced biocide

treatment against planktonic and sessile SRB and reduced biocide dosages considerably in the removal of established SRB biofilms. In addition to EDTA which is considered slowly biodegradable, ethylenediaminedisuccinate (EDDS) that is readily biodegradable (Schowanek et al. 1997) was also found to be as effective as EDTA (Wen et al. 2009; 2010). In this work, a triple biocide combination consisting of glutaraldehyde or tetrakis-hydroxymethylphosphonium sulfate (THPS), and methanol was used to treat planktonic SRB and to prevent SRB biofilm establishment.

Materials and methods

Desulfovibrio vulgaris ATCC 7757 was grown in ATCC 1249 medium and two other modified culture media. Experiments using 120 ml anaerobic vials followed the procedures described by Wen et al. (2009, 2010) with some differences. To reduce the interactions between the nutrients in the culture medium, a 1/4 strength diluted medium was also used to evaluate the biocide cocktail performance. It contained only four ingredients in the ATCC 1249 medium, namely $MgSO_4$, lactate, yeast extract, and Fe^{2+} at concentrations 1/4 of those in the full ATCC 1249 medium. EDDS (Octaquest® E30, a trisodium salt of EDDS) was provided by Octel Performance Chemicals (now Innospec in Ellesmere Port, Cheshire, UK). Methanol and glutaraldehyde were purchased from Fisher Scientific (Pittsburgh, PA, USA), and THPS from Sigma–Aldrich (St. Louis, MO, USA).

The culture media, vials and seals were sterilized using an autoclave at 121°C before use. The medium, methanol and THPS stock solutions were then deoxygenated by purging filtered nitrogen for more than 45 min. Inoculation was carried out in an anaerobic chamber filled with nitrogen. 50 ml medium for planktonic SRB growth or 100 ml medium with 3 carbon steel coupons were distributed into each vial, and it was followed by the addition of treatment chemicals. A 2–3 day-old SRB seed culture was used to inoculate the vials with an initial SRB concentration of around 1×10^6 cells/ml right after inoculation unless specified otherwise. For sessile SRB growth, a disk-shaped coupon of carbon steel C1018 with a surface area of 1.12 cm^2 was used as the substratum. It was coated with Teflon except the top surface. Each coupon was polished using sand papers with 200, 400 and 600 grits successively before use. Two coupons were placed in each vial as duplicates.

To evaluate the performance of biocide cocktail treatment, planktonic SRB growth was monitored by counting the motile SRB cells using a hemocytometer under an optical microscope. To obtain the planktonic SRB cell count data points 1 ml of bulk fluid was withdrawn from an anaerobic vial and the SRB cells in the sample were counted two or three times and the results were averaged for each data point. Scanning electron microscopy (SEM) was used to visualize sessile SRB cells. The coupon preparation procedure for SEM examination was described by Wen et al. (2009).

Results and discussion

In this work, ethanol was replaced by methanol, because of the lower price of the latter alcohol (James et al. 1996) and it is already used as an anti-freeze to prevent hydrate formation in the oil and gas industry (Ng and Robinson 1985; Kelland 2006). Methanol naturally occurs in various biological processes and it can be biodegraded quickly. It is unlikely to accumulate in the environment, whether in ground and surface water, or in soil (Malcolm Pirnie 1999). It should be pointed out that some bacteria including SRB are known to utilize methanol as an electron donor directly or indirectly (Liamleam and Annachhatre 2007), but at a higher concentration methanol inhibits their growth (Snedecor and Cooney 1974). According to Braun and Stolp (1985), *D. vulgaris* does not utilize methanol. Experiments were designed to test the enhancement of the SRB mitigation using glutaraldehyde or THPS combined with EDDS by methanol.

Mitigation of planktonic SRB

Figure 1 shows that without a biocide, up to 1,000 ppm (mass) EDDS plus up to 15% methanol introduced upon inoculation to the full medium did not prevent planktonic SRB cell counts from taking off. Only SRB growth inhibition was observed. With 500 ppm EDDS plus 15% methanol or 1,000 ppm EDDS plus 10% methanol the stationary-phase SRB cell counts were about ten times lower, as compared to the control without additions. With 30 ppm glutaraldehyde, the mitigation of planktonic SRB growth in the full medium improved considerably. Figure 2 shows that 30 ppm glutaraldehyde combined with 1,000 ppm EDDS and 10% methanol caused an almost continuous decline of the planktonic SEB cell count over the one-week test period with a ten times reduction of the initial SRB concentration. The data in Fig. 2 also indicate that EDDS and methanol dosages should be higher than 500 ppm and 10%, respectively to achieve the kill effect in the full medium.

It is known that the organic carbons (reducers) in the culture medium can reduce the biocide efficacy (von Rege and Sand 1998) and the ions in the medium can consume the chelator thus requiring a higher dosage of EDDS. This suggests that in a diluted medium, lower biocide and EDDS

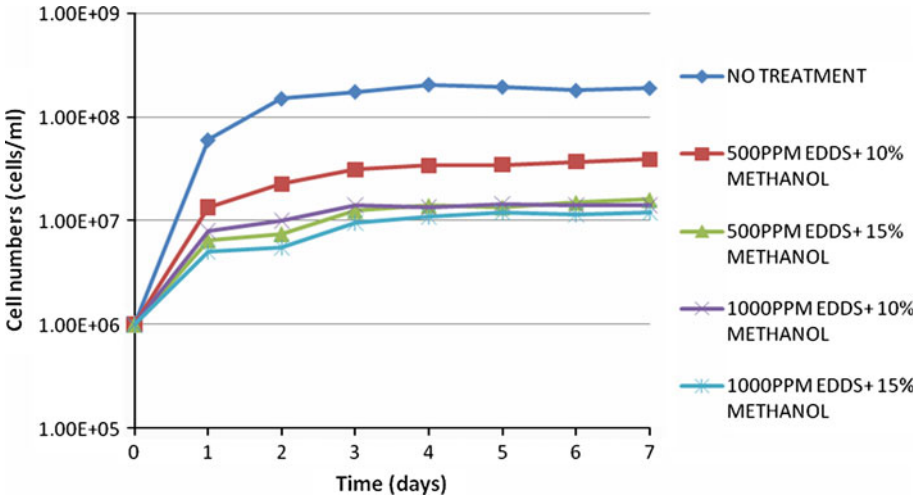

Fig. 1 Effects of EDDS and methanol on planktonic SRB growth in ATCC 1249 medium

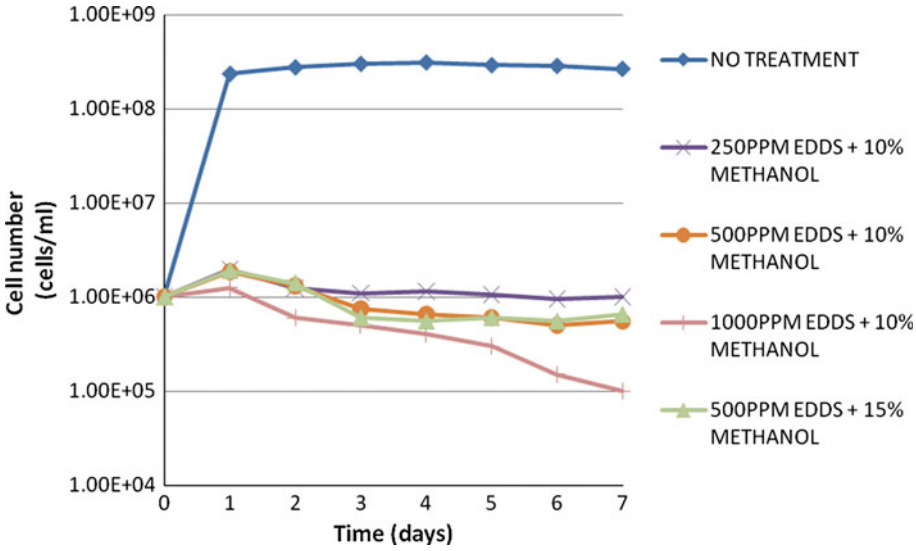

Fig. 2 Effects of the triple biocide cocktail treatment of EDDS and methanol plus 30 ppm glutaraldehyde on planktonic SRB growth in ATCC 1249 medium

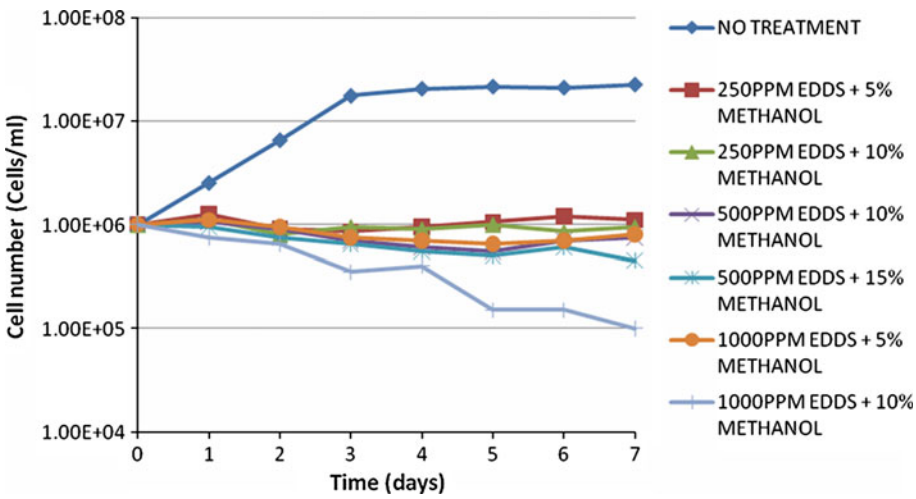

Fig. 3 Effects of the triple biocide cocktail treatment of EDDS and methanol plus 30 ppm glutaraldehyde on planktonic SRB growth in 1/4 strength diluted medium

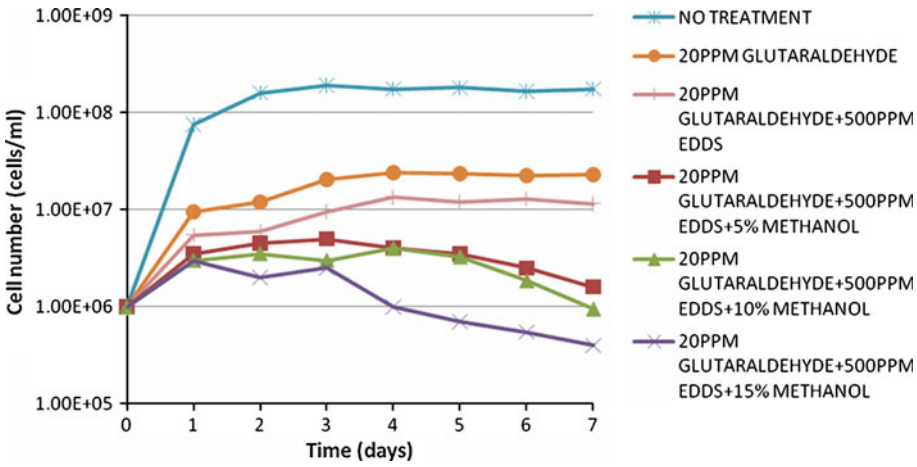

Fig. 4 Effects of the triple biocide cocktail treatment of EDDS and methanol plus 20 ppm glutaraldehyde on planktonic SRB growth in 1/4 strength diluted medium

concentrations may be used. It is reasonable that for less nutritious medium, the amount of EDDS and/or methanol can be decreased as shown in Fig. 3. In the 1/4 strength medium, SRB cell numbers at stationary phase were about ten times lower than in the full medium. 250 ppm EDDS and 5% methanol with 30 pm glutaraldehyde inhibited SRB growth (Fig. 3); a similar effect was observed in undiluted medium with 250 ppm EDDS and 10% methanol with 30 pm glutaraldehyde (Fig. 2). Figure 4 shows the effects of the triple biocide cocktail treatment of EDDS and methanol in the presence of 20 ppm glutaraldehyde on SRB growth in 1/4 strength diluted medium. As methanol concentration was increased from 10% in Fig. 3 to 15%, while the glutaraldehyde concentration was reduced to 20 ppm when combined with 500 ppm EDDS to kill SRB in Fig. 4, the planktonic SRB cell numbers first increased slightly, but it started to decline after 3 days and reached a level that was lower than the initial value.

Methanol and EDDS also enhanced THPS treatment of SRB with similar results. It was observed that 30 ppm THPS + 1,000 ppm EDDS + 10% methanol achieved the kill effect in the 1/4 strength medium.

Treatment of established SRB biofilms

Wen et al. (2009) found that 30 ppm glutaraldehyde combined with 2,000 ppm EDDS successfully removed an established SRB biofilm pre-grown on a carbon steel coupon in the ATCC 1250 medium (i.e., modified Barr's medium for sulfate reducers with 2.5% NaCl) modified by using 25 ppm of Fe^{2+} and then exposed to the fresh medium mixed with glutaraldehyde and EDDS. Without EDDS, even 500 ppm glutaraldehyde did not completely remove the biofilm at the same culture conditions (Wen et al. 2009). In this work, the new triple biocide combination was used. Figure 5a shows that when treated with 50 ppm glutaraldehyde and 15% methanol, sessile cells were still present in the biofilms. When a triple combination consisting of 15% methanol + 50 ppm glutaraldehyde + 1,000 ppm EDDS was used, the established

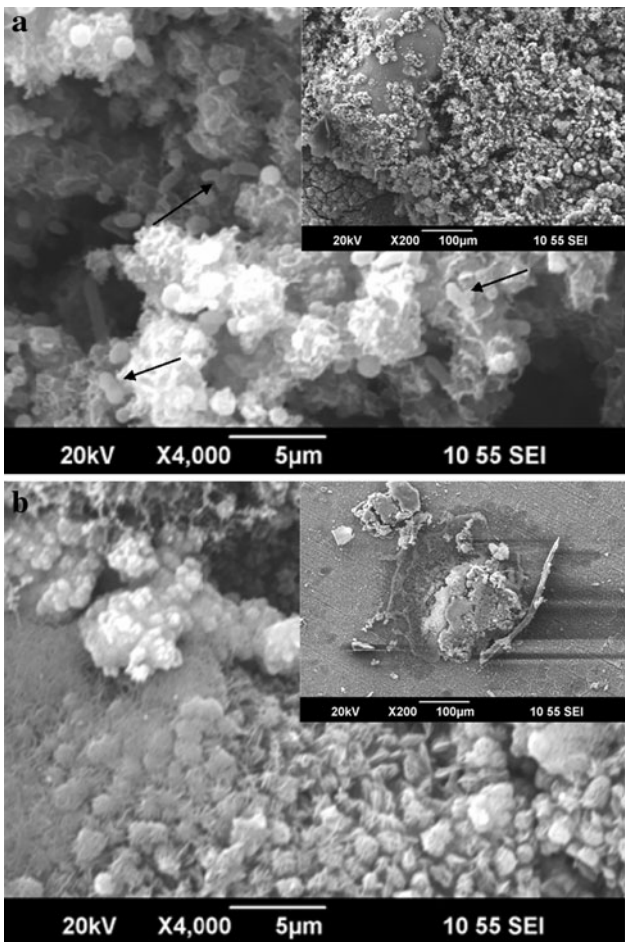

Fig. 5 Effect of 15% methanol + 50 ppm glutaraldehyde on treating an established SRB biofilm in ATCC 1249 medium with initially 25 ppm Fe^{2+} after 9 days of exposure. **a** Combined with 0 ppm EDDS, sessile SRB are visible. *Arrows* indicate SRB cells; **b** combined with 1,000 ppm EDDS, sessile SRB are hard to find

biofilm was successfully removed. Figure 5b shows that no sessile SRB cells were present 9 days after the biocide treatment. With the help of 15% methanol, the EDDS dosage was halved but 50 ppm glutaraldehyde was needed because 30 ppm was found inadequate.

The role of chelator as a biocide enhancer has been discussed by Raad et al. (2007) and Wen et al. (2009, 2010). Chelators may increase the permeability of the outer membrane for Gram-negative cells such as SRB because they can chelate Mg^{2+} and Ca^{2+} ions in that membrane. This makes the cells more susceptible to biocide attack (Hancock 1984; Varaa 1992). Alcohols such as ethanol and isopropyl alcohol are widely used as disinfectants because, as solvents, they denature proteins (Morton 1983; McDonnell and Russell 1999; Choi et al. 2001). Methanol is a similar solvent, thus it probably denatures the proteins in the outer membrane and weaken it, making a biocide more effective. Morton (1983) mentioned that methanol at above 50% can be effective as a biocide against vegetative bacteria. EDDS and methanol at relatively low dosages did not offer much biocidal effects as shown in Fig. 1. However, they enhanced glutaraldehyde and THPS considerably in the triple biocide cocktail.

Conclusions

The triple biocide cocktail containing glutaraldehyde or THPS, EDDS and methanol showed efficacies in SRB treatment. With the addition of 10–15% of methanol, mitigation of planktonic SRB growth changed from an inhibition effect to a kill effect. Experimental results also showed that 50 ppm glutaraldehyde enhanced by 15% methanol and 1,000 ppm EDDS removed an established biofilm in the modified ATCC 1250 medium.

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