World J Microbiol Biotechnol (2010) 26:1053–1057
DOI 10.1007/s11274-009-0269-y

ORIGINAL PAPER

Chelators enhanced biocide inhibition of planktonic sulfate-reducing bacterial growth

J. Wen · K. Zhao · T. Gu · I. Raad

Received: 23 September 2009 / Accepted: 27 November 2009 / Published online: 11 December 2009

Abstract Biocides are currently the primary mitigation method to control sulfate-reducing bacteria (SRB) in biofouling, reservoir souring and microbiologically influenced corrosion. Increasingly restrictive environmental regulations and safety concerns on biocide uses demand more efficient dosing of biocides. Chelators have been known to enhance antibiotics because of their properties such as increasing the permeability of the outer cell membrane of Gram-negative bacteria. Two readily biodegradable chelators, ethylenediaminedisuccinate (EDDS) and *N*-(2-hydroxyethyl)iminodiacetic acid (HEIDA) disodium salts that are touted as potential replacements of ethylenediaminetetraacetic acid (EDTA), were evaluated as potential biocide enhancers for glutaraldehyde and tetrakis hydroxymethyl phosphonium sulfate (THPS) in their inhibition of planktonic SRB growth. *Desulfovibrio vulgaris* ATCC 7757 and *Desulfovibrio desulfuricans* ATCC 14563 were grown in modified ATCC 1249 medium and in enriched artificial seawater, respectively. Laboratory tests in 100 ml anaerobic vials showed that EDDS or HEIDA alone did not inhibit SRB growth. However, when EDDS or HEIDA was combined with glutaraldehyde or THPS, each of them enhanced the biocide inhibition of planktonic SRB growth.

Keywords *Desulfovibrio desulfuricans* · Chelators · EDDS · HEIDA · Biocide · Glutaraldehyde · THPS

J. Wen · K. Zhao · 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

Sulfate reducing bacteria (SRB) are able to reduce sulfate to sulfide. The hydrogen sulfide gas generated by SRB is the primary cause for reservoir souring in the oil and gas industry (Vance and Thrasher 2005). SRB is often the culprit for microbiologically influenced corrosion (MIC) (Flemming 1996; Videla 1996). Metals such as cast iron, copper, aluminum, steel and stainless steel can be attacked by SRB, causing serious problems in systems such as cooling water systems and oil and gas pipelines (Videla 1996; Poulton et al. 1995). Fernance et al. (2007) reported a number of failures in fire sprinkler systems possibly due to MIC caused by SRB. In wastewater systems, the hydrogen sulfide generated by SRB and the subsequent oxidization product of sulfuric acid are highly corrosive to steel and concrete (Tator 2003). MIC also caused pollution concerns for potable water installations (Wagner and Chamberlain 1997). In cooling water systems, heat transfer efficiency and water flow rate were reduced by the presence of biofilms (Poulton et al. 1995). Due to increased practice of water flooding to enhance well pressures, reservoir souring due to SRB is becoming more common in the oil and gas industry (Hubert and Voordouw 2007).

Biocides are widely used to control SRB growth. To avoid unintended chemical corrosion caused by biocides, non-oxidizing biocides are favored in industries such as pulp and paper industry and the oil and gas industry (Laopaiboon et al. 2006). Glutaraldehyde and tetrakis hydroxymethyl phosphonium sulfate (THPS) are among the most popular because they are broad-spectrum and biodegradable biocides. Glutaraldehyde functions as a cross-linking agent to disable cell wall's amino groups (Denyer 1995; Russell 2002; Greene et al. 2006; Laopaiboon et al. 2006). THPS with the biologically active phosphine acting as a reducing

agent disrupts the disulphide bonds in cell proteins and enzymes (Ballantyne and Jordan 2004).

With heightened concerns over the environment in recent years, the large-scale applications of biocides are becoming increasingly more restrictive and costly. A more effective and environmental benign biocide treatment is desired. Denyer (1995) reviewed several methods that enhance biocide applications including utilization of cell permeabilizing agents, modification of the chemical structure of a biocide for better delivery, and combination of biocides with complementary mechanisms of action.

Ethylenediaminetetraacetic acid (EDTA) is a widely used chelator in many industries. In recent years, it has been used to treat contamination of medical devices such as catheters (Banin et al. 2006). Raad et al. (2003, 2007) found that EDTA combined with minocycline is very effective in eradicating biofilms on catheter surfaces. Raad and Sherertz (2001) further patented the idea of using chelators as biocide enhancers. EDTA is generally considered slowly biodegradable and there is a concern that it may accumulate in the environment after it is discharged (European Commission 2004). Ethylenediaminedisuccinate (EDDS) is a popular alternative to EDTA due to its biodegradability (Schowanek et al. 1997). Claimed as a readily biodegradable chelator, the degradation or mineralization (the process of conversion of organic substance to carbon dioxide) half-life of [S,S]-EDDS in soil is 2–3 days and 6.3 days in unacclimated river water based on a die-away test (Jaworska et al. 1999). *N*-(2-Hydroxyethyl)iminodiacetic acid (HEIDA) disodium salts have an OECD (Organization for Economic Co-operation and Development) 306 value of 89% in 28 days and it reaches passing level (60%) within 20 days (Dow Chemical Co. technical report XUS40855.01 2007). This work evaluated two green chelators, EDDS and HEIDA, for their abilities to enhance the inhibition of planktonic SRB growth in combination with either glutaraldehyde or THPS.

Materials and methods

Two SRB strains were used in this work: *Desulfovibrio vulgaris* ATCC 7757 and *D. desulfuricans* subsp. *aestuarii* ATCC 14563. Among them, the ATCC 14563 strain is a marine strain of SRB that favors salty water. The ATCC 1249 medium was used for growing the ATCC 7757 SRB seed culture. The growth medium was then modified by lowering ferrous concentration to 25 ppm in subsequent cultures. The ATCC 1250 medium was used for culturing ATCC 14563 SRB. An enriched artificial seawater (ASW) was made with the following components for each liter of water: Instant Ocean® (Aquarium Systems, Inc., Mentor, OH, USA) synthetic sea salt mix 36 g, $Fe(NH_4)_2(SO_4)_2$ 125 mg, sodium lactate (60 wt% syrup) 4.5 ml and yeast extract 1 g. The enriched ASW was used to grow ATCC 14563 SRB.

EDTA disodium salt (Fisher Scientific, Fair lawn, NJ, USA) was used in this work. EDDS (Octaquest® E30, trisodium salt of EDDS) was obtained from Octel Performance Chemicals (now Innospec in Ellesmere Port, Cheshire, UK). HEIDA was purchased from Sigma–Aldrich (St. Louis, MO, USA). It was neutralized with sodium hydroxide to obtain a disodium salt before use. SRB growth was carried out in 100 ml anaerobic vials. Each culture medium was autoclaved at 121°C for 20 min. After sterilization, the medium was sparged with filtered nitrogen for about 45 min to remove dissolved oxygen. The pH of the modified ATCC 1249 medium was adjusted to 7.0 before inoculation. The medium was then transferred to an anaerobic chamber with a nitrogen environment where inoculation took place. A 50 ml culture medium was added to each vial followed by addition of a specified amount of a biocide and/or a biocide enhancer. After the vials were inoculated with a 2–4 day-old SRB seed culture, they were sealed and then incubated at 37°C for up to 13 days. During the incubation period, samples were periodically withdrawn using sterile syringes from the vials to monitor planktonic SRB growth. Motile planktonic SRB cells in the samples were counted under an optical microscope at 400× magnification using a hemocytometer counting chamber.

Results and discussion

In this work, both the modified ATCC 1249 medium and the enriched ASW were used for SRB growth. Atkinson and Bingman (1996) compared several commercial synthetic sea salts including the sea salt mix used in this work with typical ocean water. The enriched ASW was used in this work because without the enrichment, SRB growth would be too slow for laboratory testing. Glutaraldehyde and THPS are known to interact with such a culture medium, and in this kind of culture medium biocidal effects become biostatic effects (Gardner and Stewart 2002). If the biocide concentration is not high, planktonic bacterial growth will overcome biocide inhibition and take off. The time duration of the inhibition (i.e., suppression or delay in attaining stationary phase) of planktonic bacterial growth is often used to compare the efficacies of biocides in the treatment of planktonic bacteria (von Rege and Sand 1998; Gardner and Stewart 2002; de Savaria and de Mele 2005).

Efficacies of biocide enhancers in modified ATCC 1249 medium

The ATCC 7757 SRB strain was used in this section. Figures 1, 2 and 3 show the enhancements of glutaraldehyde

and THPS by EDDS and HEIDA. EDDS or HEIDA alone without a biocide did not inhibit planktonic SRB growth even at a high concentration as indicated by the 0 ppm biocide curves in Figs. 1 and 2a. With 20 ppm THPS and 0 ppm chelator, the SRB cell number started to take off and quickly reached the stationary phase following a take-off delay of 2 days after inoculation as seen in Fig. 1. When 20 ppm THPS was combined with EDDS, the delay was extended to 4.5 days after inoculation. Although 500 ppm and 2,000 ppm of EDDS did not show much difference on the enhancement of THPS, the SRB cell number for the treatment using 20 ppm THPS plus 2,000 ppm EDDS gradually increased. Its time to attain the stationary phase was 3.4 days longer than the one treated with 500 ppm EDDS. When the THPS concentration was increased to 30 ppm, with the help of EDDS, the SRB cell number remained low 10 days after inoculation when the test stopped, while 30 ppm of THPS alone lost its inhibition of SRB growth.

HEIDA showed similar enhancement of THPS inhibition on planktonic SRB growth in Fig. 2b. Figure 2b shows that the SRB cell number in the vial took off 8 days after inoculation and then quickly reached the stationary phase when the vial was treated with 30 ppm THPS with 0 ppm HEIDA, while in another vial 30 ppm THPS combined with HEIDA continued to suppress SRB growth up to 10 days after inoculation when the test ended. Figure 3 shows that EDDS enhanced glutaraldehyde just like it did for THPS in Fig. 1. Three days after inoculation, with 30 ppm glutaraldehyde the SRB cell number started to take off and then reached the stationary phase quickly. However, when combined with EDDS, SRB growth take-off was delayed further. With 500 ppm EDDS and 30 ppm glutaraldehyde, the delay time for SRB growth take-off was about 7 days after inoculation. When EDDS dosage was increased to 1,000 ppm, the SRB cell count was kept at a low level of 4.0×10^5 cells/ml 12 days after inoculation when the experiment was terminated. When the glutaraldehyde concentration was increased to 50 ppm, SRB growth was inhibited regardless of EDDS concentration during the lab experiments.

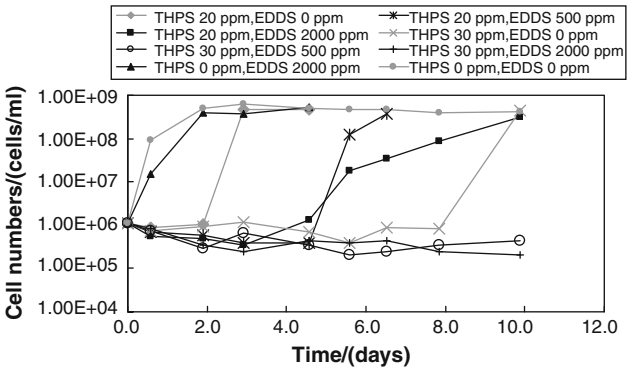

Fig. 1 EDDS enhancement of THPS in modified ATCC 1249 medium. (Initial cell concentration was 1.14×10^6 cells/ml. Final pH range 6.8–7.1)

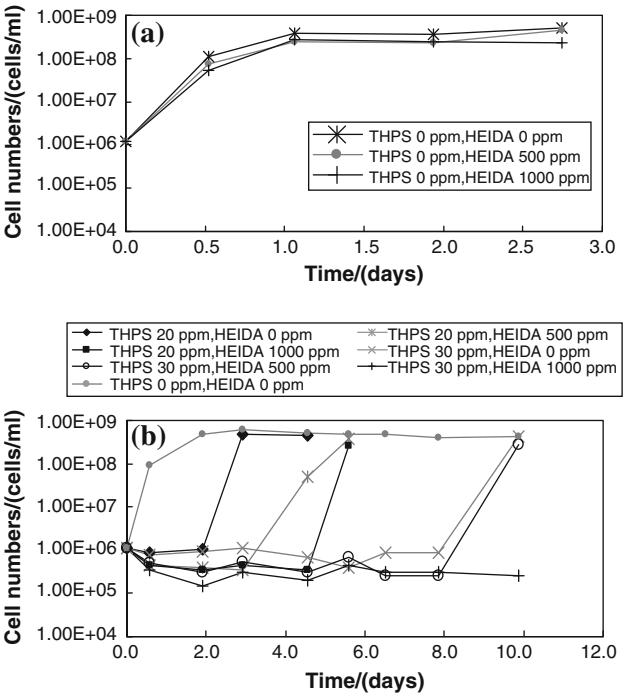

Fig. 2 HEIDA enhancement of THPS in modified ATCC 1249 medium: **a** Initial cell concentration was 1.21×10^6 cells/ml. Final pH range 7.0–7.1; **b** Initial cell concentration was 1.14×10^6 cells/ml. Final pH range 6.4–7.0

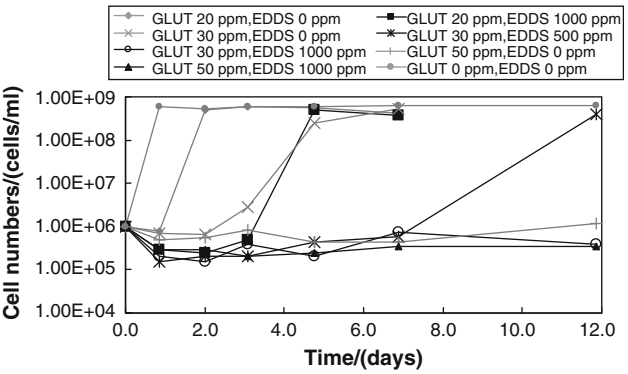

Fig. 3 EDDS enhancement of glutaraldehyde in modified ATCC 1249 medium. (Initial cell concentration was 9.70×10^5 cells/ml. Final pH range 6.7–7.3)

Efficacies of biocide enhancers in enriched ASW

The ATCC 14563 marine SRB strain was used in this section. Initial SRB cell concentration after inoculation was 1.42×10^6 cells/ml. The final pH range was found to be 6.95–7.4 for EDTA test and 6.45–7.1 for EDDS tests. Figures 4 and 5 show that both EDTA and EDDS enhanced

glutaraldehyde in inhibiting planktonic SRB growth in enriched ASW, while EDTA or EDDS alone did not show any significant inhibition.

Compared to the results involving the ATCC 7757 SRB grown in modified ATCC 1249 medium, a higher concentration of EDDS was needed to enhance glutaraldehyde in the enriched ASW. This was likely because salt ions in the salty medium interacted with the chelator, thus reducing its availability. It was found that 500 ppm chelators (EDTA or EDDS) did not provide significant enhancement for the inhibition of planktonic SRB growth by glutaraldehyde (data not shown). When the chelator concentration was increased to 1,000 ppm EDTA or 2,000 ppm EDDS and combined with 30 ppm glutaraldehyde, the SRB growth did not take off up to 13 days after inoculation when the test ended as shown in Figs. 4 and 5. Figures 4 and 5 indicate that the treatment of 20 ppm of glutaraldehyde combined with 1,000 ppm of EDTA or 2,000 ppm of EDDS achieved similar efficacy to the treatment of 30 ppm glutaraldehyde alone.

In the modified ATCC 1249 medium and the enriched ASW, EDTA, EDDS and HEIDA enhanced THPS and glutaraldehyde in their inhibition of planktonic SRB growth. Considering the different mechanisms of action against bacteria for THPS and glutaraldehyde, it is likely that the enhancement by chelators may be because chelators can target the outer cell membrane by removing some Mg^{2+} and Ca^{2+} ions, thus increasing the permeability of the outer membrane for Gram-negative cells such as SRB as mentioned by Vaara (1992).

Raad et al. (2003, 2007) successfully used up to 30 mg/ml of EDTA combined with ethanol and minocycline to eradicate microbial organisms embedded in biofilms on catheter surfaces in a patented medical application (Raad and Sherertz 2001). EDDS and HEIDA are both touted as readily biodegradable chelators for the replacement of EDTA in its numerous industrial applications. In this study, EDDS and

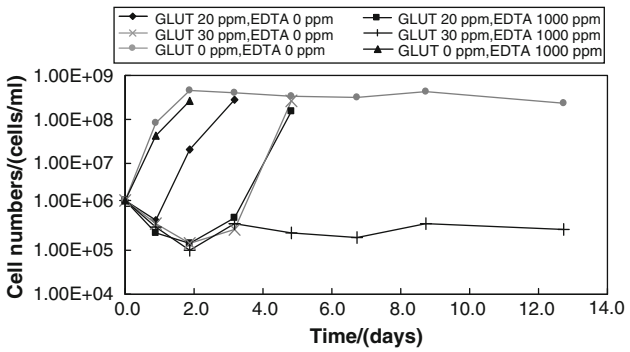

Fig. 4 EDTA enhancement of glutaraldehyde in enriched ASW. (Initial cell concentration after inoculation was 1.42×10^6 cells/ml. EDTA solution pH adjusted to 7 before being added to the broth. Final pH range 6.95–7.4)

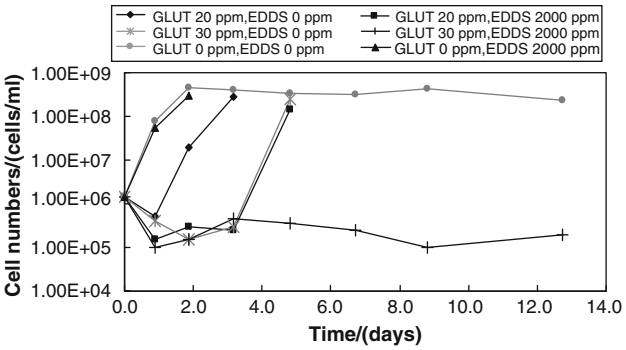

Fig. 5 EDDS enhancement of glutaraldehyde in enriched ASW. (Initial cell concentration after inoculation was 1.42×10^6 cells/ml. Final pH range 6.45–7.1)

HEIDA were found to enhance THPS and glutaraldehyde inhibition of planktonic SRB growth. It is possible that field applications in a liquid with a very low ionic strength probably requires a much lower concentration because the chelators are not consumed by cations such as Ca^{2+} and Na^{+} in the liquid. It is also reasonable to believe that with a biocide concentration higher than the low concentration tested in this work, the requirement for chelator could be less.

Conclusions

Experimental data using two lab strains of SRB demonstrated that EDTA, EDDS and HEIDA enhanced glutaraldehyde and THPS in their inhibition of planktonic SRB growth. EDDS and HEIDA appeared similarly effective as EDTA. In the enriched ASW, a higher concentration of chelator was needed.

Acknowledgments This work was supported by a grant from Enhanced Corrosion Prevention, LLC, and a seed grant from the M. D. Anderson Cancer Center.

References

Atkinson M, Bingman C (1996) The composition of several synthetic seawater mixes. J Aquaric Aquat Sci 8:39–43

Ballantyne B, Jordan SL (2004) Biocides. In: Marrs TC, Ballantyne B (eds) Pesticide toxicology and international regulation. Wiley, Chichester, pp 384–385

Banin E, Brady KM, Greenberg EP (2006) Chelator-induced dispersal and killing of *Pseudomonas aeruginosa* cells in a biofilm. App Environ Microbial 72:2064–2069. doi:10.1128/AEM.72.3.2064-2069.2006

De Savaria SG, de Mele MFL (2005) Enhancement of glutaraldehyde biocidal efficacy by the application of an electric field. Effect on sessile cells and on cells released by the biofilm. World J Microbiol Biotechnol 21:1077–1081. doi:10.1007/s11274-004-8288-1

Denyer SP (1995) Mechanisms of action of antibacterial biocides. Int Biodeter Biodegr 36:227–245. doi:10.1016/0964-8305(96)00015-7

Dow Chemical Co., XUS40855.01 (2007) Developmental chelating agent. Available via http://www.prochem.ch/html/forum/XUS40855.01%20Data%20Sheet.pdf (a technical report). Accessed 01 Feb 2009

European Commission (2004) Edetic acid (EDTA) (CAS No: 60-00-4): European Union Risk Assessment Report vol 49. European Chemicals Bureau, Luxembourg. Available via http://ecb.jrc.ec.europa.eu/documents/Existing-Chemicals/RISK_ASSESSMENT/REPORT/edtareport061.pdf. Accessed 01 Feb 2009

Fernance N, Farinha PA, Javaherdashti A (2007) SRB-assisted MIC of fire sprinkler piping. Mater Performance 46(2):46–49

Flemming HC (1996) Economical and technical overview. In: Heitz E, Flemming HC, Sand W (eds) Microbially influenced corrosion of materials. Springer, Berlin, pp 5–14

Gardner LR, Stewart PS (2002) Action of glutaraldehyde and nitrite against sulfate-reducing bacterial biofilms. J Ind Microbiol Biotechnol 29:354–360. doi:10.1038/sj.jim.7000284

Greene EA, Brunelle V, Jenneman GE, Voordouw G (2006) Synergistic inhibition of microbial sulfide production by combinations of the metabolic inhibitor nitrite and biocides. App Environ Microbial 72:7897–7901. doi:10.1128/AEM.01526-06

Hubert C, Voordouw G (2007) Oil field souring control by nitrate-reducing *Sulfurospirillum* spp. that outcompete sulfate-reducing bacteria for organic electron donors. Appl Environ Microbiol 73:2644–2652. doi:10.1128/AEM.02332-06

Jaworska JS, Schowanek D, Feijtel TCJ (1999) Environmental risk assessment for trisodium [S, S]-ethylene diamine disuccinate, a biodegradable chelator used in detergent applications. Chemosphere 38:3597–3625. doi:10.1016/S0045-6535(98)00573-6

Laopaiboon L, Phukoetphim N, Laopaiboon P (2006) Effect of glutaraldehyde biocide on laboratory-scale rotating biological contactors and biocide efficacy. Electron J Biotechnol 9:358–369. doi:10.2225/vol9-issue4-fulltext-10

Poulton WIJ, Cloete TE, von Holy A (1995) Microbiological survey of open recirculating cooling water systems and their raw water supplies at twelve fossil-fired power stations. Water SA 21:357–364

Raad I, Sherertz R (2001) Chelators in combination with biocides: treatment of microbially induced biofilm and corrosion. US patent 6,267,979

Raad I, Chatzinikolaou I, Chaiban G, Hanna H, Hachem R, Dvorak T, Cook G, Costerton W (2003) In vitro and *ex vivo* activities of minocycline and EDTA against microorganisms embedded in biofilm on catheter surfaces. Antimicrob Agents Chemother 47:3580–3585. doi:10.1128/AAC.47.11.3580-3585.2003

Raad I, Hanna H, Dvorak T, Chaiban G, Hachem R (2007) Optimal antimicrobial catheter lock solution, using different combinations of minocycline, EDTA, and 25-percent ethanol, rapidly eradicates organisms embedded in biofilm. Antimicrob Agents Chemother 51:78–83. doi:10.1128/AAC.00154-06

Russell AD (2002) Antibiotic and biocide resistance in bacteria: introduction. J Appl Microbiol Suppl 92:1s–3s. doi:10.1046/j.1365-2672.92.5s1.14.x

Schowanek D, Feijtel TCJ, Perkins CM, Hartman FA, Federle TW, Larson RJ (1997) Biodegradation of [S, S], [R, R] and mixed stereoisomers of ethylene diamine disuccinic acid (EDDS), a transition metal chelator. Chemosphere 34:2375–2391. doi:10.1016/S0045-6535(97)00082-9

Tator KB (2003) Preventing hydrogen sulfide and microbiologically influenced corrosion in wastewater facilities. Mater Performance 42(7):32–37

Vaara M (1992) Agents that increase the permeability of the outer membrane. Microbiol Rev 56:395–411

Vance I, Thrasher DR (2005) Reservoir souring: mechanism and prevention. In: Ollivier B, Magot M (eds) Petroleum microbiology. ASM Press, Washington, DC, pp 123–142

Videla HA (1996) Manual of biocorrosion. CRC Press, Boca Raton

Von Rege H, Sand W (1998) Evaluation of biocide efficacy by microcalorimetric determination of microbial activity in biofilms. J Microbiol Methods 33:227–235. doi:10.1016/S0167-7012(98)00055-4

Wagner D, Chamberlain AHL (1997) Microbiologically influenced copper corrosion in potable water with emphasis on practical relevance. Biodegradation 8:177–187. doi:10.1023/A:1008206918628