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Action of glutaraldehyde and nitrite against sulfate-reducing bacterial biofilms

LR Gardner[a] and PS Stewart

Center for Biofilm Engineering and Department of Chemical Engineering, Montana State University-Bozeman, Bozeman, MT 59717-3980, USA

A continuous flow reactor system was developed to evaluate the efficacy of antimicrobial treatments against sulfate-reducing bacterial biofilms. An annular reactor operating at a nominal dilution rate of 0.5 h^{-1} was fed one-tenth strength Postgate C medium diluted in 1.5% NaCl and was inoculated with a mixed culture enriched from oilfield-produced water on the same medium. Thin biofilms developed in this reactor after 2 days of operation. The activity of these biofilms resulted in approximately 50 mg S l^{-1} of sulfide at steady state prior to biocide treatment. Biocide efficacy was quantified by recording the time required for sulfide production to recover following an antimicrobial treatment. In a control experiment in which pure water was applied, the time required to reach 10 mg S l^{-1} sulfide after the treatment was 1.7±1.2 h, whereas the time to reach this level of sulfide after a pulse dose of 500 mg l^{-1} glutaraldehyde was delayed to 61±11 h. Nitrite treatment suppressed sulfide production as long as the nitrite concentration remained above 15 mg N l^{-1}. Sulfide production recovered more rapidly after nitrite treatment than it did after glutaraldehyde treatment.
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Introduction

Sulfate-reducing bacteria are well known to petroleum microbiologists because the hydrogen sulfide they generate induces corrosion of metal pipes and equipment, interferes with downstream processing, and poses a health and safety risk to workers [10,14,16,17]. Particularly when seawater is injected into a reservoir in a secondary recovery process, the conditions are ripe for the activity of sulfate-reducing bacteria [1,2,4,20]. In such waterflood operations, high concentrations of sulfate, the availability of organic compounds such as fatty acids that can serve as electron donors, low oxygen tensions, and warm temperatures combine to promote the growth of sulfate-reducing microorganisms. Biocides are regularly applied in oil and gas production facilities to control the problems associated with sulfate-reducing bacteria [3,5,7–9,26,27]. The ability to control these bacteria is compromised by the fact that they grow in protected biofilms on the surface of pipes or in the reservoir formation itself.

The purpose of the work reported in this article was to develop a continuous flow biofilm reactor system suitable for use in evaluating the efficacy of antimicrobial treatments for the control of sulfate-reducing bacterial biofilms. The testing approach was demonstrated with the application of glutaraldehyde, a widely used oilfield biocide, or sodium nitrite, a putative inhibitor of SRB metabolism.

Correspondence: Dr Philip S Stewart, Center for Biofilm Engineering and Department of Chemical Engineering, Montana State University-Bozeman, Bozeman, MT 59717-3980, USA
[a]Current address: IBM, 11400 Burnet Road, Austin, TX 78758, USA.
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Materials and methods

Microorganisms and medium

Samples of produced water from the Chevron Lost Hills oilfield in California were used as the source of a microbial consortium for both planktonic and biofilm studies. Produced water was shipped to Montana in 1-l glass jars with oil added to the top of the containers to minimize aeration. A 1-ml volume of produced water was added to 30 ml of Postgate C medium (see below) and incubated at 35°C. When these cultures became turbid, they were subcultured into fresh Postgate C. Frozen stocks of this culture were prepared in 20% glycerol when the culture was in late exponential phase. Stocks were stored at −70°C.

A modified Postgate C medium [18] with a 1.5% salinity level was used. The base medium was prepared using the Hungate method [13,15] with the reducing agents sodium sulfide and sodium dithionite. Full-strength medium provided a total sulfate concentration of 1024 mg S l^{-1} with lactate serving as the primary carbon substrate. One-tenth strength medium was used in biofilm experiments by diluting full-strength Postgate C medium with 1.5% NaCl. The medium pH ranged from 7.2 to 7.3. A blanket of scrubbed nitrogen was used to maintain a positive pressure during cooling of autoclaved medium. The scrubbed nitrogen consisted of high-purity nitrogen that was passed through a 400°C oven containing a reducing column composed of copper wire. Batch and biofilm cultures were maintained at 36°C.

Planktonic experiments

Planktonic batch experiments were performed in 50-ml vials containing 30 ml of Postgate C medium. Prior to filling them with medium, vials were purged of oxygen by alternately applying vacuum and then scrubbed nitrogen gas. To study batch growth, a vial was inoculated with 1 ml of frozen culture and sulfate and

sulfide concentrations were subsequently measured every 2 h. The culture was grown for about 30 h until the bacteria were in late exponential or early stationary phase. A 1-ml aliquot was transferred to a sterile, oxygen-free, and medium-free environment of 100 ml of 1.5% NaCl in a 250-ml bottle. The solution was incubated for 6.5 h. This time was analogous to the nutrient medium flush time in the biofilm experiments. At 6.0 h, the bottles were sampled for sulfide and sulfate. Upon completion of this "flush" stage, a 1-ml aliquot was transferred to 100-ml batch vials containing 60 ml of sterile, anoxic 1.5% NaCl amended with glutaraldehyde. These vials were incubated at 35°C for a period of 7 h. At the end of the 7-h contact period, the suspensions were centrifuged at 12,000 g and 4°C for 10 min and the supernatant containing glutaraldehyde was discarded. Immediately after centrifuging the suspension and decanting the supernatant, a sterile and anoxic volume of 30 ml of Postgate C medium was added to resuspend the pellet. The tube was vortexed for 2 min. These suspensions were then transferred to sterile and oxygen-free vials. The ability of the planktonic sulfate-reducing bacteria to recover from the biocidal activity was quantified by sampling and analyzing the levels of sulfate and biogenic sulfide as they incubated at 35°C. Untreated controls were performed in which cultures were put through this protocol without glutaraldehyde present.

Biofilm reactor

A continuous flow annular reactor (Model 920; BioSurface Technologies, Bozeman, MT) was used to grow biofilms and evaluate biocide action. The reactor had a rotating inner cylinder and stationary outer cylinder. Temperature control was maintained *via* a water jacket connected to a recirculating water bath. The inner cylinder held 20 removable polycarbonate slides on the outer wall. Each slide had an area of approximately 22 cm^2. Reactor operating parameters are listed in Table 1.

The mixing characteristics of the annular reactor were experimentally investigated by injecting a slug dose of red food coloring into the reactor and monitoring absorbance at 500 nm in the reactor effluent with an in-line spectrophotometer. Natural log of absorbance *versus* time data were regressed to determine the dilution rate (the negative of the slope of this line).

The reactor was cleaned and the reactor and process lines were assembled and disinfected by pumping a 20 mg l^{-1} sodium hypochlorite solution for 10 min followed by a 2-h rinse with sterile ultrapure water. The reactor was filled with one-tenth strength Postgate C medium. This medium was achieved by simultaneous feeding to the reactor 0.79 ml min^{-1} Postgate C medium and 7.13 ml min^{-1} 1.5% NaCl. The reactor was inoculated by injecting half of a 1.5-ml frozen stock culture into the reactor and letting the reactor operate in batch mode for 2 h. Continuous flow was then initiated to support the proliferation of aerobic organisms. The remaining 0.75-ml aliquot of frozen culture was used to inoculate a 30-ml vial containing Postgate C medium and this was allowed to grow for approximately 14 h. At this point, the reactor was returned to batch mode, inoculated with this 30-ml dose of sulfate-reducing organisms, and maintained in batch mode for an additional 48 h. Continuous flow was reinitiated at a total flow rate of 7.9 ml min^{-1}.

Table 1 Annular reactor parameters

Parameter	Value	Units
Inner drum rotation rate	150	rpm
Nominal dilution rate	0.5	h^{-1}
Postgate C medium flow rate	47.4	ml h^{-1}
1.5% NaCl flow rate	428	ml h^{-1}
Operating temperature	36	°C
Volume	1.0	l
Wetted surface area	0.26	m^2
Nitrogen sparge flow rate	370–520	ml h^{-1}

Biocide treatment and sampling

Because glutaraldehyde was discovered to react with yeast extract, a component of the Postgate C medium, the flow of Postgate C medium flow was turned off for 6.5 h in advance of the biocide dose. The 1.5% NaCl dilution water continued to flow and displaced any residual medium from the reactor. A 1-ml dose of 50% glutaraldehyde was injected through the influent line to the 1-l reactor volume. A 1-ml sample from the bulk liquid was immediately taken for glutaraldehyde analysis. Sampling continued every 15 min for the next 7 h. Samples were filtered through a 0.2-μm pore size cellulose acetate filter then analyzed for glutaraldehyde by gas chromatography. The flow of nutrient medium was reinstated at the completion of the 7-h biocide treatment. Sulfate and sulfide concentrations were monitored to follow the recovery of the sulfate-reducing bacteria.

Other studies were performed to investigate the effectiveness of nitrite as an inhibitor of sulfate reduction. A 24-h continuous dose of approximately 110 mg N l^{-1} of sodium nitrite was delivered to the reactor. The nitrite solution was prepared anoxically in 1.5% NaCl and this amended salt solution replaced the usual 1.5% NaCl dilution water flow. The flow of the nutrient medium was maintained in the reactor. Nitrite levels were sampled from both the influent and bulk fluid ports with the latter at a frequency of every 30 min. Sulfide and sulfate concentrations were also obtained at these intervals. At the end of the 24-h period, the nitrite/NaCl carboy was taken off-line and replaced with an anoxic carboy of 1.5% NaCl. Sampling continued as the nitrite was displaced from the reactor. Sulfide and sulfate levels were continually monitored to investigate any lasting suppression of sulfide generation by the sulfate-reducing bacteria.

Aqueous phase sampling and analytical methods

Aqueous samples from the rotating annular reactor were anoxically retrieved from the bulk liquid phase through a septum sampling port. These samples as well as samples from the influent stream were split and analyzed for sulfate and sulfide. Sulfide concentrations were determined by a modified methylene blue method [6] while sulfate levels were measured by ion chromatography. Aliquots to be analyzed for sulfide were immediately fixed in 1% zinc acetate. Aliquots to be analyzed for sulfate were processed through a Dionex OnGuard-Ag cartridge (Sunnyvale, CA) followed by a 0.2-μm filter to remove excess chloride. Glutaraldehyde was determined by gas chromatography using a glass packed column in an 185°C oven with flame ionization detection. Nitrite was determined using a commercial colorimetric kit (Hach, Loveland, CO).

Biofilm sampling and analytical methods

Polycarbonate slides were removed from the reactor for measurement of biofilm areal carbon density and biofilm thickness. Biofilm-covered slides were removed through the threaded coupon port on top of the annular reactor. Sampling was performed by

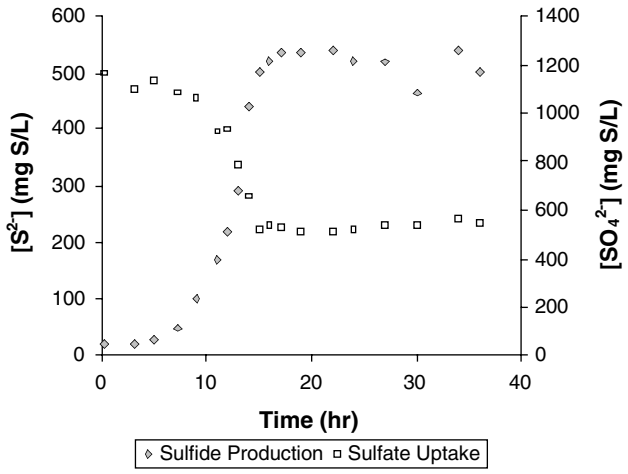

Figure 1 Sulfide and sulfate concentrations during a batch planktonic experiment.

disinfecting this area with ethanol while flooding the port with a nitrogen blanket. The nutrient medium and dilution water flows were ceased with the influent and effluent lines clamped off. Once a coupon was removed from the reactor environment, it was immediately replaced with a new one. Coupon selection throughout the different phases of an experiment was based upon a sequence determined by a random number generator before beginning the experiment.

Extracted slides were rinsed gently in 1.5% NaCl for 5 min, then scraped into a 100-ml beaker and rinsed with a total of 10 ml of 1.5% NaCl. The contents of the beaker were processed with a tissue homogenizer for 1 min. Total organic carbon was determined using a Dohrman DC-80 Total Organic Carbon analyzer (Tekmar, Cincinnati, OH).

Biofilm thickness was quantified by microscopic examination of frozen biofilm cross sections [11,28]. Frozen sections 5 μm thick were deposited on glass microscope slides, stained with 1 μg ml^{-1} 4′,6-diamidino-2-phenylindole, and examined using a Nikon Eclipse E800 microscope with epifluorescence illumination. Digital images were captured using a 40× oil immersion objective in conjunction with an Optronics Engineering CCD camera. The images were converted to a TIF file format and imported into *ImageTool* software (http://ddsdx.uthscsa.edu/dig/itdesc.html). Twenty-one regions were randomly selected for measurement of biofilm thickness along the length of a sectioned sample.

Analysis of biocide penetration

The extent of biocide penetration into the biofilm was analyzed by calculating a dimensionless observable modulus comparing the rates of biocide reaction and diffusion. This modulus and the basis for its calculation have been presented elsewhere [24]. The overall reaction rate of the biocide with the biofilm was calculated from a material balance around the reactor using data on the biocide concentration decay during and immediately following biocide dosing. The reaction rate thus calculated reflects the loss of concentration of the antimicrobial agent in solution in excess of the loss due to dilution. The values of the diffusion coefficients of glutaraldehyde and nitrite in the biofilm were taken as 3.9×10^{-6} and 7.8×10^{-6} cm^2 s^{-1}, respectively. These values reflect estimates of the aqueous diffusion coefficients of the two solutes [12,25] adjusted for the presence of the biofilm [23].

Results

Postgate C-enriched cultures exhibited primarily Gram-negative staining. Most of the microorganisms were motile, curved rods. A typical progression of sulfate and sulfide concentrations during a batch planktonic experiment is shown in Figure 1. The maximum specific growth rate of bacteria in Postgate C medium at 35°C, as derived from the sulfide *versus* time curve, was 0.28 ± 0.02 h^{-1}. The yield of sulfide on sulfate in planktonic batch growth was 0.86 ± 0.04 g sulfide–sulfur g sulfate–sulfur^{-1}.

Glutaraldehyde at a concentration of 50 mg l^{-1} retarded recovery of sulfide production in batch vials (Table 2). The mean time to reach a concentration of 10 mg S l^{-1} sulfide after biocide treatment was 47 ± 4 h in the untreated control, whereas it was 143 h for the 50 mg l^{-1} glutaraldehyde treatment. This difference in recovery time was statistically significant ($P=0.002$). When 100 mg l^{-1} glutaraldehyde was applied, no recovery of sulfide production was detected over the course of posttreatment observation (385 h). These data show that glutaraldehyde, at a concentration of 100 mg l^{-1} and a contact time of 7 h, completely suppressed the activity of sulfate-reducing bacteria in batch cultures.

The biofilm annular reactor operated at a nominal dilution rate (influent flow rate divided by reactor fluid volume) of 0.47 h^{-1}. Duplicate tracer studies indicated a dilution rate of 0.45 ± 0.01 h^{-1} and confirmed that the reactor fluid was well mixed.

The sequence of events in operation and biocide treatment of the biofilm annular reactors is complicated and requires explanation

Table 2 Recovery times for sulfide production after biocide treatment

Treatment	Biofilm or planktonic	Number of experiments	Time to reach 10 mg S l^{-1} sulfide (h)	Time to reach 90% of steady-state sulfide (h)
Water	P	3	47±4	64±6
50 mg l^{-1} glutaraldehyde	P	1	143	169
100 mg l^{-1} glutaraldehyde	P	1	>385	>385
Water	B	3	1.7±1.2	7.3±2.1
500 mg l^{-1} glutaraldehyde, first dose	B	3	62±8	73±8
500 mg l^{-1} glutaraldehyde, second dose	B	2	59±18	72±13
110 mg l^{-1} nitrite, first dose	B	1	19	32
110 mg l^{-1} nitrite, second dose	B	1	5	24

Recovery time was measured beginning at the time when fresh nutrients were supplied to batch planktonic vials or the time when nutrient amendment was reinitiated in annular reactor experiments.

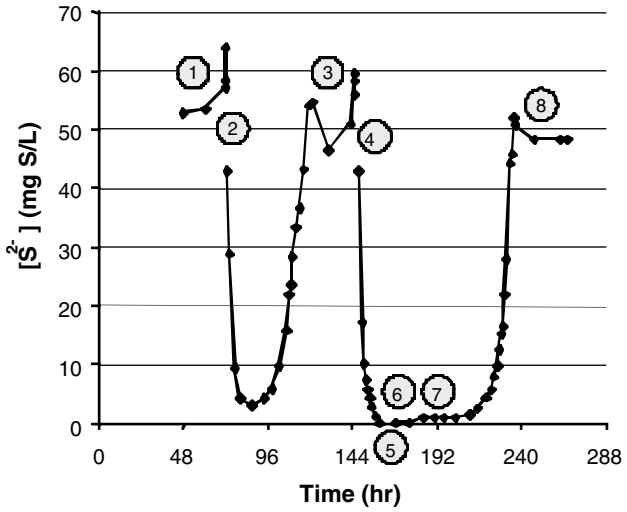

Figure 2 Sequence of events during a biofilm experiment. The reactor was operated in batch mode (1) for approximately 48 h. The sulfide concentration plateaued at approximately 50 mg S l^{-1} in this phase of operation. Flow to the reactor was initiated (2), causing sulfide to wash out of the reactor. As biofilm activity increased, the sulfide concentration in the reactor recovered, stabilizing (3) at approximately 50 mg S l^{-1}. Prior to dosing the biocide, the medium was flushed from the reactor (4) to eliminate reaction between glutaraldehyde and components of the medium. The biocide was applied as a pulse injection of concentrated agent (5) and a 7-h contact period was allowed (6) before resuming the flow of nutrients to the reactor (7). Sulfate was replenished and sulfide levels monitored during the recovery period. The concentration of sulfide eventually returned to a steady-state level of approximately 50 mg S l^{-1} (8).

(see Figure 2). After 48 h of batch culture, the sulfide concentration in the reactor was approximately 50 mg S l^{-1}. The reactor was then placed in continuous flow mode, which washed out most of the sulfide. As the biofilm grew, the sulfide concentration in the reactor rose and stabilized at a concentration of 50–60 mg S l^{-1}. Prior to

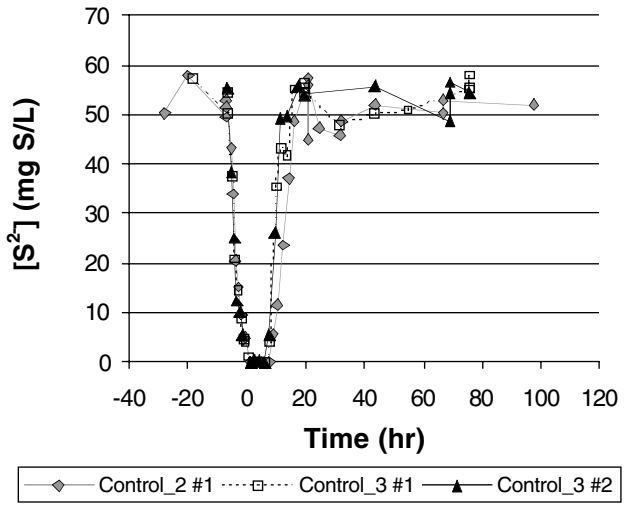

Figure 3 Sulfide concentrations in annular reactor containing sulfate-reducing bacterial biofilm when treated with water. Three runs of this untreated control are shown. Time zero denotes the pulse injection of water. Time −6.5 h corresponds to the time nutrient amendment ceased and time +7 h corresponds to the time nutrient amendment was reinitiated. Sulfide production resumed quickly once nutrients were restored to the system.

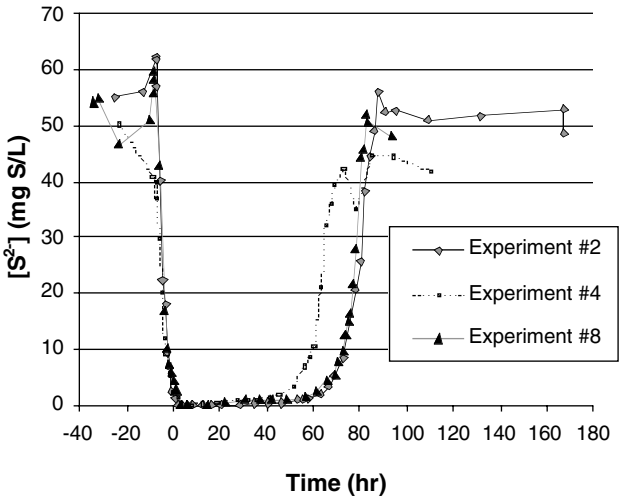

Figure 4 Sulfide concentrations in annular reactor containing sulfate-reducing bacterial biofilm when treated with a single dose of 500 mg l^{-1} glutaraldehyde. Three runs are shown. Time zero denotes the pulse injection of biocide. Time −6.5 h corresponds to the time nutrient amendment ceased and time +7 h corresponds to the time nutrient amendment was reinitiated.

glutaraldehyde treatment, the Postgate C medium was flushed from the reactor. The purpose of this was to preclude reaction of the glutaraldehyde with the yeast extract contained in Postgate C. The flush was accomplished by turning off the flow of Postgate C medium while maintaining the flow of 1.5% NaCl solution. The effect of this flush was to once again drop the sulfide concentration in the reactor to essentially zero. After 6.5 h, sulfide could not be detected and the biocide slug dose was administered. The nutrient flow remained at zero for the next 7 h to allow for contact of glutaraldehyde with the biofilm. Glutaraldehyde was washed out of the reactor during this time. After the 7-h contact period, the nutrient flow was reinstated. Sulfide production eventually

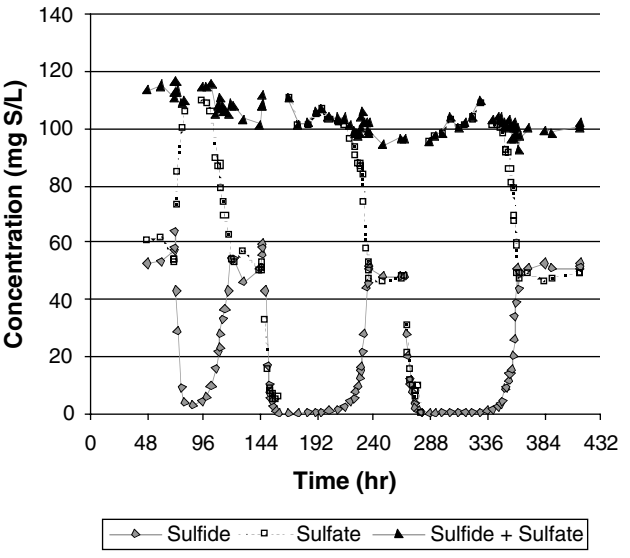

Figure 5 Sulfide concentrations in annular reactor containing sulfate-reducing bacterial biofilm when treated with a repeated dose of 500 mg l^{-1} glutaraldehyde. The first biocide dose was applied at 153 h and the second dose was applied at 273 h.

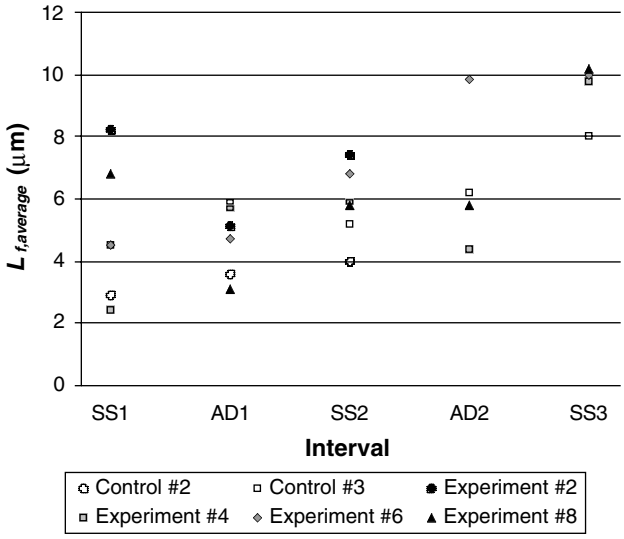

Figure 6 Progression of average biofilm thickness, $L_{f,average}$, during annular reactor experiments. The acronyms indicate intervals: SS1, steady state 1, prior to first biocide dose; AD1, after first biocide dose; SS2, steady state 2, after first biocide dose; AD2, after second biocide dose; SS3, steady state 3, after second biocide dose.

recovered; the recovery time was quantified by two parameters (Table 2).

In untreated control experiments, water was dosed instead of glutaraldehyde, but the sequence of events was otherwise identical to that outlined above and explained in Figure 2. Sulfide production recovered rapidly after this procedure (Figure 3). The time to reach 10 mg S l^{-1} sulfide posttreatment was 1.7±1.2 h and the time to reach 90% of the previous steady-state sulfide concentration was 7.3±2.1 h. When glutaraldehyde was applied at a nominal initial dose concentration of 500 mg l^{-1}, the recovery of sulfide production was delayed (Figure 4). The time to reach 10 mg S l^{-1} sulfide posttreatment was 61±11 h and the time to reach 90% of the previous steady-state sulfide concentration was 73±9 h. These recovery times represent significant retardation compared to the respective untreated control values ($P=3\times10^{-4}$ and $P=2\times10^{-5}$, respectively). Two experiments were performed in which repeated glutaraldehyde treatment was applied (Figure 5). The second dose of glutaraldehyde had a similar effect as the first dose (Table 2).

Nitrite treatment suppressed sulfide production as long as the nitrite concentration remained above about 15 mg N l^{-1}. Sulfide production recovered more rapidly after nitrite treatment than it did after glutaraldehyde treatment. For example, the time to reach 90% of the pretreatment steady state sulfide concentration was 28 h for nitrite treatment, whereas it was 73 h for glutaraldehyde treatment.

A thin biofilm developed in the annular reactor at steady state prior to biocide treatment (step 3 on Figure 2). The mean thickness at the first steady state was 4.9±2.2 μm. There was not much change in the mean thickness of the biofilm following biocide treatment and subsequent periods of regrowth (Figure 6). Immediately after the first dose of glutaraldehyde, the mean biofilm thickness was 4.6±1.4 μm, which was unchanged from the pretreatment thickness. This indicates that while glutaraldehyde suppressed sulfate reduction, it did not remove the biofilm. Likewise, there was no evidence that nitrite treatment removed biofilm. The biofilm thickness immediately after nitrite treatment was 4.7±2.6 μm. Measurements of total organic carbon content of the biofilm, expressed as an areal carbon density, were noisy but

Figure 7 Corrosion of bottom plate bearing from a biofilm annular reactor. The bearing on the right came from a reactor that grew sulfate-reducing bacterial biofilms. A clean bearing is shown for comparison (left). The bearings are 2.7 cm in diameter.

also indicated little net change throughout the experimental period following the first steady state. The areal carbon density at the first steady state ranged from 27 to 187 μg C cm^{-2} with a mean value of 71±62 μg C cm^{-2}.

The extent of biocide penetration into the biofilm was evaluated by calculating an observable modulus that compares the relative rates of biocide reaction and diffusion in the biofilm. This modulus was of the order of magnitude of 10^{-3} or less for both glutaraldehyde and nitrite treatments. The physical interpretation of this result is that biocide reaction is much slower than diffusion or, more plainly put, that both agents penetrate the biofilm readily.

Most of the annular reactor is fabricated from polycarbonate and other plastic polymers. A stub shaft and bearing, both made of 316 stainless steel, also come in contact with the fluid in the reactor vessel. These components both showed evidence of corrosion after several weeks of reactor operation. Metal surfaces were pitted and blackened (Figure 7).

Discussion

Because sulfide production by sulfate-reducing bacteria is the activity that is problematic in engineered systems, we have used measures of sulfide production to assess the efficacy of biocide treatments. In particular, measures of the time required for the resumption of sulfide production after a biocide treatment appear to be repeatable and useful indicators of biocide efficacy. This represents a modest departure from the traditional emphasis on culturing bacteria or measuring parameters of the biofilm. As partial justification for this departure, consider that neither glutaraldehyde nor nitrite had much effect on biofilm thickness, but both chemicals did significantly interfere with sulfide generation. This probably reflects the fact that microbial killing and inhibition are distinct processes from that of biofilm removal.

Measurements of glutaraldehyde efficacy reported here are in rough agreement with published studies in which biofilm systems were used [5,8,9,21,27]. Our data suggest a modest degree of protection of bacteria in the biofilm state compared to free-floating cells. For example, a 7-h 50 mg l^{-1} dose of glutaraldehyde suppressed sulfide production for 143 h in planktonic cells, whereas a higher concentration dose (equivalent to 157 mg l^{-1} when time averaged over 7 h) in a biofilm reactor had less effect (sulfide production suppressed for 61 h). Reduced susceptibility in the biofilm state is well known [25]. The fact that glutaraldehyde penetrated biofilms but the bacteria were still less susceptible suggests that resistance mechanisms based on the heterogeneous physiological status of biofilm bacteria may play a role in protecting these cells from antimicrobial agents.

In earlier work with continuous flow biofilm reactors like the one used in this work, a second dose of biocide (monochloramine) was clearly less effective than the first dose [22]. No evidence of such an adaptive response could be found in the present experiments using repeated doses of glutaraldehyde or sodium nitrite.

Our data are insufficient to perform a full comparison of the relative merits of glutaraldehyde and sodium nitrite as control agents for sulfate-reducing bacterial biofilms. The data do show that glutaraldehyde has longer-lasting effects than sodium nitrite. The opposite conclusion was drawn in a comparison of these two agents in a porous media column [19]. The difference may have to do with the presence of precipitated sulfide and the capacity of the porous media to sequester sulfide. Another factor could be that glutaraldehyde was added in the absence of nutrients while nitrite was added in the presence of nutrients.

Acknowledgements

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