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Monitoring of microbial souring in chemically treated, produced-water biofilm systems using molecular techniques

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Abstract The identification of bacteria in oil production facilities has previously been based on culture techniques. However, cultivation of bacteria from these often-extreme environments can lead to errors in identifying the microbial community members. In this study, molecular techniques including fluorescence in situ hybridization, PCR, denaturing gradient gel electrophoresis, and sequencing were used to track changes in bacterial biofilm populations treated with nitrate, nitrite, or nitrate + molybdate as agents for the control of sulfide production. Results indicated that nitrite and nitrate + molybdate reduced sulfide production, while nitrate alone had no effect on sulfide generation. No long-term effect on sulfide production was observed. Initial sulfate-reducing bacterial numbers were not influenced by the chemical treatments, although a significant increase in sulfate-reducing bacteria was observed after termination of the treatments. Molecular analysis showed a diverse bacterial population, but no major shifts in the population due to treatment effects were observed.

Keywords Oil souring · Chemical treatment · Fluorescence in situ hybridization · Polymerase chain reaction · Denaturing gradient gel electrophoresis · Sulfate-reducing bacteria

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Introduction

Oil-bearing formations that are subject to seawater injection during secondary oil recovery are often the sites of significant hydrogen sulfide production, caused by the activity of sulfate reducing bacteria (SRB) [9]. The microbiological origin of sulfide in oil fields has been recognized for more than 75 years [4] and is known to reduce the quality of oil and gas and to cause problems such as plugging of reservoirs, corrosion of metal equipment, and toxicity to workers [20].

Non-oxidizing biocides such as glutaraldehyde [12, 29], diamines [28], and tetrakishydroxymethylphosphonium sulfate [16] have been used to reduce microbial activity in oil production facilities. The efficacy of these treatments is typically dependent on reservoir conditions, such as temperature, permeability, and water chemistry, and cases of resistance to non-oxidizing biocides following prolonged use have been reported [12]. In addition, these biocides may pose hazards for both workers and the environment, in some cases necessitating alternative control measures.

The use of nitrate for sulfide control was introduced in the sewage treatment industry in the early 1900s [6]. Laboratory and full-scale investigations confirmed the ability of nitrate to control SRB activity in oil-field systems [16, 29]. Although the exact mechanism of control has not been determined, it is postulated that the effect may involve competition for electron donors by nitrate-reducing bacteria (NRB) and/or direct inhibition of SRB by nitrite produced as a result of nitrate reduction by NRB [10]. Interestingly, combined treatment of SRB with nitrate and molybdate shows a synergistic effect [20, 30]. Other research suggests that the use of nitrite alone as an alternative to nitrate may promote higher reactivity and more rapid scavenging of sulfide [26]. Field applications of nitrite have generally been successful, but inconsistent results at locations with high levels of dissolved iron have prompted further studies [25, 26]. Field results indicate that sulfide production

from oil and gas wells may be inhibited for an extended period (less than 6 months) following nitrite treatment. This effect is thought to result from iron sulfide dissolution, which then creates a sulfide sink in the subsurface and eventually leads to re-precipitation of produced sulfide as iron sulfide.

In the past, culture-based methods have been used as the primary means of bacterial identification and enumeration in oil fields. Cultivation of bacteria from extreme environments is difficult and may lead to incorrect conclusions regarding the diversity and metabolic activity of the microbial consortium and, thus, to improper design of control strategies [32]. In other complex environments, molecular techniques such as PCR, denaturing gradient gel electrophoresis (DGGE) [1, 31], fluorescence in situ hybridization (FISH) [2], and microautoradiography [22] are widely used for identifying bacteria and assessing their activity and physiology.

In this study, three souring control strategies were investigated in reactors containing a mixed population enriched from oil-field produced water. The control strategies were based on additions of: (1) nitrite, (2) nitrate, or (3) nitrate + molybdate. The objective of this investigation was to assess the impact of the treatments on SRB activity and sulfide production, using a number of molecular approaches involving PCR, DGGE, 16S rDNA sequencing, and FISH.

Materials and methods

Reactor operation

Four suspended coupon reactors (Biosurface Technologies, Bozeman, Mont.), each consisting of a glass chamber filled with 300 ml of liquid solution, were operated at a flow rate of 60 ml h^{-1} (hydraulic retention time of approximately 5 h) at room temperature (23°C). The reactors were equipped with seven polypropylene rods, each with three mild steel coupons. Before operation the reactors were washed with detergent, rinsed with distilled water, and autoclaved. Coupons were degreased with 90% ethanol and rinsed with acetone before insertion. To begin operation, the reactors were filled with sterile medium and run with continuous flow for 1 day under anaerobic conditions. Different enrichment cultures, including a general aerobic bacterial (GAB) enrichment, a SRB enrichment, a denitrifying bacterial (DNB) enrichment, and a nitrate-reducing, sulfur-oxidizing bacterial (NR-SOB) enrichment and originating from production water from Saudi ARAMCO (Dhahran, Saudi Arabia), were used as inocula. The reactors were continuously fed with a modified Postgate C medium [24] containing 0.5 g l^{-1} KH_2PO_4, 1.0 g l^{-1} NH_4Cl, 2.25 g l^{-1} Na_2SO_4, 0.06 g l^{-1} $CaCl_2{\cdot}6H_2O$, 0.06 g l^{-1} $MgSO_4{\cdot}7H_2O$, 0.03 g l^{-1} $FeSO_4{\cdot}7H_2O$, 0.3 g l^{-1} $C_6H_5Na_3O_7{\cdot}2H_2O$, 20 g l^{-1} NaCl, 0.12 g l^{-1} acetic acid, 0.05 g l^{-1} propionic acid, 0.02 g l^{-1} butyric acid, and 3.0 g l^{-1} pyruvate. The salinity of the medium was approximately 2% and the pH was adjusted to 7.0–7.5 with 12 N sodium hydroxide.

The nitrite, nitrate, and nitrate + molybdate treatments were started 41 days after inoculation, when mature and active SRB communities were established. Reactor 1 (R1) was used as a control and received only normal medium with no amendment, whereas reactor 2 (R2) received 100 mg l^{-1} NO_2–N added to the normal medium as $NaNO_2$, reactor 3 (R3) received 100 mg l^{-1} NO_3^-–N as $NaNO_3$, and reactor 4 (R4) received 100 mg l^{-1} NO_3^-–N as $NaNO_3$ and 35 mg l^{-1} molybdate as $Na_2MoO_4{\cdot}2H_2O$. All treatments were applied for 6 days, representing the standard duration used in field treatment procedures within the petroleum industry.

Enrichment cultures

The reactors were inoculated with 15 ml of each enrichment culture, all of which were grown at pH 7.0–7.5 with a salt concentration of 2%. GAB were enriched on an aerobic marine broth standard medium (ref. 2216; Difco). SRB were enriched using a modified Postgate B medium [24]. DNB were cultivated in an anaerobic medium consisting of 5.0 g l^{-1} peptone, 3.0 g l^{-1} beef extract, 1.0 g l^{-1} KNO_3, and 20 g l^{-1} NaCl. Finally, NR-SOB were cultivated in a complex anaerobic medium consisting of 0.25 mg l^{-1} H_2SO_4, 1.14 mg l^{-1} $MnSO_4{\cdot}7H_2O$, 0.25 mg l^{-1} $ZnSO_4{\cdot}7H_2O$, 0.25 mg l^{-1} H_3BO_3, 0.0125 mg l^{-1} $CuSO_4{\cdot}2H_2O$, 0.0125 mg l^{-1} $NaMoO_4{\cdot}2H_2O$, 0.0225 mg l^{-1} $CoCl_2{\cdot}6H_2O$, 0.1 g l^{-1} nitrilotriacetic acid, 0.29 mg l^{-1} $FeCl_3$, 0.06 g l^{-1} $CaSO_4{\cdot}2H_2O$, 0.78 g l^{-1} $MgSO_4{\cdot}7H_2O$, 0.07 g l^{-1} Na_2HPO_4, 0.063 g l^{-1} KH_2PO_4, 0.02 g l^{-1} NH_4Cl, 0.24 g l^{-1} $CaCl_2{\cdot}2H_2O$, 0.68 g l^{-1} $MgSO_4{\cdot}7H_2O$, 0.13 g l^{-1} $(NH_4)_2SO_4$, 1.0 g l^{-1} KNO_3, 0.68 g l^{-1} sodium acetate, 1.0 mg l^{-1} resazurin, 1.9 g l^{-1} $NaHCO_3$, 20 g l^{-1} NaCl, and 0.2 g l^{-1} $Na_2S{\cdot}9H_2O$.

Sampling

Samples for microbiological analysis were taken before starting the treatments (day 41), immediately after the treatments were terminated (day 47), and 24 days after termination of treatments (day 71). Samples for chemical analysis from both the influent and effluent were taken once daily before and after the treatments and twice daily during treatment; and they were stored at −18°C until analysis. Biofilm samples from mild steel coupons were removed by scraping with cell scrapers (Orange Scientific, Braine–l'Alleud, Belgium) and added to 3 ml of sterile-filtered water (0.2 μm). Concurrently, samples for PCR were scraped into sterile tubes and stored at −18°C.

Chemical analysis of samples

Sulfate and nitrate were analyzed using a DX500 ion chromatograph (Dionex Corp., Sunnyvale, Calif.) equipped with an Anion–IonPac AS4A-SC column and conductivity detector. The column eluent was a mixture of 1.8 mM sodium carbonate and 1.7 mM sodium bicarbonate. Samples were pre-filtered using On Guard–Ag filters (Dionex Corp.). Hydrogen sulfide was measured spectrophotometrically according to previously published methods [8]. Nitrite was measured with a nitrite kit (HACH 2240-00; HACH, Loveland, Colo.). An inductively coupled plasma emission spectrometer (Accuris model; Fison Instrument, Dearborn, Mich.) was used for the analysis of total iron and molybdate [13].

Total number of bacteria and FISH

The total number of bacteria in the biofilm on the mild steel coupons was determined using the general bacterial stain 4′,6-diamino-2-phenylindole (DAPI); and evaluation was performed by epifluorescence microscopy, using a Nikon E800 microscope. FISH was performed with fluorescently labeled, 16S rRNA-targeted oligonucleotide probes (Thermo Electron, Ulm, Germany) on homogenized biofilm samples, using methods described by Amann [3]. Samples were fixed in fresh 4% paraformaldehyde/PBS or 50% ethanol and then washed three times in filtered (0.2 μm) distilled water. Subsamples were stained with DAPI (1 mg l^{-1}) for 30 min, immobilized on slides (Marienfeld, Bad Mergentheim, Germany), and hybridized in triplicate according to Amann [3]. The probes tested are listed in Table 1. A Zeiss epi-fluorescence microscope with filter sets 09 and 10 was used for the FISH analysis (Zeiss, Jena, Germany). Cy3-labeled probes were used for enumeration of specific types of bacteria [7], while a Fluos-labeled EUB338 probe was applied simultaneously as a positive internal control. The total number of DAPI-stained bacteria in the range 20–200 was counted for each microscopic field; and in the same fields the number of Cy3-labeled cells was determined for the specific bacterial group of interest (Table 1) . Other details about the probes and the hybridization conditions can be found in probeBase [18]. The positive controls applied were a number of pure cultures (*Escherichia coli*, *Desulfovibrio desulfuricans*), enrichment cultures from oil-field systems (SRB), and a number of activated sludge samples where the examined genus/species were known to be present.

PCR, DGGE, and sequencing

Nucleic acid extraction and purification was performed using a FastDNA Spin kit for soil (Invitrogen Corp., Carlsbad, Calif.), according to the manufacturer's instructions. The universal primer set 341F/907R [19] was used for PCR. DGGE was performed with the same primer set, with a GC-rich clamp added to the 5′ end of the 341F primer [11]. PCR was conducted using 25 μl of AccuPrime Supermix II (Invitrogen Corp.), 0.02 nmol of each primer, 1–2 μl of DNA template, and 21–22 μl of nuclease-free water, to total a 50-μl reaction volume. For PCR without the GC clamp, an initial denaturation step at 94°C for 3 min was used, followed by 30 cycles of denaturation at 94°C for 45 s, primer annealing at 59°C for 1 min, and primer extension at 70°C for 1.5 min. A final extension step at 70°C for 7 min was used, followed by a final holding step at 4°C. All denaturation steps for PCR with GC-clamp were conducted at 96°C. The PCR products of the correct length were confirmed by electrophoresis, using a 1.5% agarose gel.

DGGE was performed using a DCode DGGE system (Bio-Rad Laboratories, Hercules, Calif.). Acrylamide gels (8%) with a denaturant gradient from 40% to 70% were loaded with PCR products mixed with 2× loading dye. The gels were run at 60°C and 60 V for approximately 16 h, then stained with SYBR Green I (Molecular Probes, Eugene, Ore.) for 20 min prior to imaging, using a FluorChem 8800 imaging system (Alpha Innotech, San Leandro, Calif.). Bands in the DGGE gels were afterwards purified for sequence analysis by removing the individual bands for immediate re-amplification with the primer set 341F/907R. Final purification was achieved using a QIAquick gel extraction kit (Qiagen, Valencia, Calif.).

Table 1 The 16S rRNA-targeted oligonucleotide probes which were applied to biofilm samples from mild steel coupons [18]

Probe	Sequence (5′ → 3′)	Target organism
EUB338	Mix of the probes EUB338, EUB338II and EUB338III	Bacteria
cgrid EUB338	GCTGCCTCCCGTAGGAGT	Bacteria
EUB II338	GCAGCCACCCGTAGGTGT	Planctomycetes/*Pirellula* spp
EUB III338	GCTGCCACCCGTAGGTGT	Verrucomicrobia
SRB385	CGGCGTCGCTGCGTCAGG	Deltaproteobacteria
SRB385Db	CGGCGTTGCTGCGTCAGG	Desulfobacteriaceae
DSV698	GTTCCTCCAGATATCTACGG	*Desulfovibrio* spp
NIT3	CCTGTGCTCCATGCTCCG	*Nitrobacter* spp
AT1458	GAATCTCACCGTGGTAAGCGC	*Azoarcus–Thauera* cluster
NSV443	CCGTGACCGTTTCGTTCCG	*Nitrosospira* spp
SPH120	GGGCAGATTCCCACGCGT	*Sphingomonas* spp
NONEUB	ACTCCTACGGGAGGCAGC	Nonsense probe

One forward and one reverse sequencing reaction, respectively, were performed for each band, using the BigDye terminator kit ver. 3.1 (PE Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions. Sequencing of purified DNA was performed on an ABI Prism 310 genetic analyzer (PE Applied Biosystems). In some cases, the reactions were supplemented with 5% dimethyl sulfoxide to reduce potential detrimental effects caused by secondary structures. The 16S rDNA sequences were compiled and aligned using the automatic nucleic acid aligner in the ARB software package (http://www.arb-home.de) and alignments were refined manually. Checks for chimeric sequences were conducted using the CHECK_CHIMERA program of the Ribosomal Database Project (http://rdp.cme.msu.edu) and the program BELLEROPHON [14]. Only unambiguously aligned sequences were used for the calculation of trees by distance matrix, parsimony, and maximum likelihood approaches, using default settings in the ARB software.

Results

Sulfate and sulfide

The effect of the treatments on SRB activity was determined by measuring the concentrations of sulfate and sulfide in the effluent. The treatments were initiated when a high level of sulfide was measured in the effluent (average 19 mg l^{-1}). This corresponded with the formation of a thick black biofilm on the coupons. Prior to treatment, the concentration of sulfate in the effluent had averaged 515 mg l^{-1} (Table 2). This concentration increased in the reactors receiving nitrite (R2) and nitrate + molybdate (R4), to a maximum of approximately 550 mg l^{-1}, while sulfate dropped in the control (R1) and the nitrate-only reactor (R3), suggesting the suppression of SRB activity as a result of the nitrite and nitrate + molybdate treatments. Conversely, sulfide production was significantly higher in R1 and R3 compared with R2 and R4 during dosing (Table 2). After termination of the treatments the sulfide levels in R1 and R3 remained significantly higher than in R2 and R4. These results corresponded with the variations in sulfate concentration observed in the effluent.

Total-iron, nitrite, nitrate, and molybdate

An increase in the total iron concentration in the effluent was observed as a result of the nitrite treatment (R2), from 9 mg l^{-1} before treatment to 20 mg l^{-1} during treatment, while treatment with nitrate (R3) and nitrate + molybdate (R4) did not alter the iron concentration remarkably (Table 2). After termination of the treatments, total iron concentrations increased to 10–20 mg l^{-1} for all reactors, which was approximately twice the level measured before dosing. The concentration of total iron in the effluent was lower for the nitrate + molybdate (R4) treatment than for the nitrite (R2) treatment and was about the same level as the nitrate (R3) treatment, suggesting that molybdate did not influence the solubilization of iron. The observed effluent nitrite concentration (R2) ranged over 10–25 mg l^{-1} during treatment and was below detection by the last 3 days of dosing (data not shown). After dosing for 2 h, the effluent nitrate concentrations for R3 and R4 ranged over 33–43 mg l^{-1} . However, after 22 h of dosing, nitrate could not be detected in either reactor. A similar observation was made for molybdate, where the concentrations decreased approximately 60% during dosing, suggesting either adsorption or consumption of the molybdate (data not shown).

Total number of bacteria

The total number of biofilm bacteria prior to treatment ranged from 5×10^8 to 3×10^9 cells cm^{-2} (Fig. 1). During the treatment period, the numbers increased in the control reactor, while the treatment with nitrite showed a decrease. The bacterial numbers remained almost constant for the nitrate (R3) and nitrate + molybdate (R4) treatments. During the time-period in which application of standard medium to all reactors (days 47–71) was resumed, the cell numbers decreased slightly in the control reactor (R1), decreased more dramatically in R3 and R4, and remained stable in R2.

Fluorescence in situ hybridization

Quantitative FISH was also applied to evaluate the impact of the treatments on bacterial composition of the

Table 2 Concentrations of sulfate, sulfide, and total iron measured in the effluent before, during, and after treatment with nitrite, nitrate, or nitrate + molybdate (±95% confidence interval is indicated)

Reactor	Sulfate (mg l^{-1})			Sulfide (mg l^{-1})			Total iron (mg l^{-1})		
	Before	During	After	Before	During	After	Before	During	After
Control		467 ± 0.8	437 ± 0.8		44 ± 0.2	36 ± 0.2		9 ± 0.2	10 ± 0.2
Nitrite	515 ± 1.4	567 ± 0.8	523 ± 1.3	19 ± 0.6	6 ± 0.1	26 ± 0.2	9 ± 0.2	20 ± 0.2	15 ± 0.3
Nitrate		468 ± 0.9	441 ± 0.6		52 ± 0.3	58 ± 0.3		8 ± 8	19 ± 0.2
Nitrate + molybdate		541 ± 0.6	540 ± 0.8		15 ± 0.1	26 ± 0.2		11 ± 0.1	15 ± 0.3

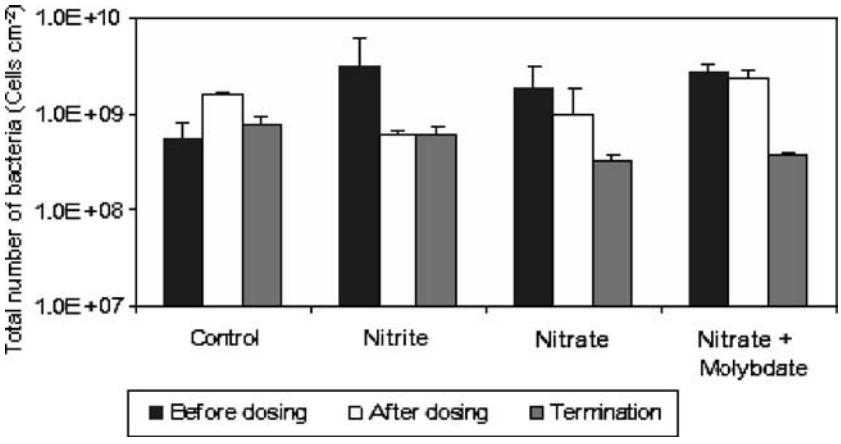

Fig. 1 The total number of bacteria determined with the general fluorescent stain DAPI in biofilms from mild steel coupons. The error bars represent 95% confidence intervals

biofilm. The proportion of Bacteria (targeted with probe EUB338) constituted 66–81% of the total number of bacteria for all sampling events (Fig. 2). It is unknown whether the remaining bacteria were dead, inactive, or impermeable to the probe or whether this result indicated the presence of a rather large number of Archaea which would not hybridize with the general bacterial probe (EUB338). The SRB population (targeted with probes SRB385, SRB385Db) constituted 31–64% of the total number of bacteria (Fig. 3). The SRB population mainly comprised Desulfovibrioaceae, while the abundance of Desulfobacteriaceae was very low (data not shown). No change in the relative abundance of SRB was observed before dosing and during treatment in any of the reactors. However, the relative abundance of SRB increased slightly after termination of the nitrite treatment (R2) and increased significantly after termination of the nitrate (R3) and nitrate + molybdate (R4) treatments. Further tests using specific probes targeting nitrate and/or nitrite-reducing bacteria, previously injected in the same oil-field and wastewater sites, did not show hybridization of these probes to the biofilm bacteria. Again, these results may indicate that the probes employed in our study did not target all of the microbes present, some of which were capable of nitrate and/or nitrite reduction.

DGGE and phylogeny

In order to more critically evaluate treatment effects on the bacterial populations in the reactors, PCR, DGGE, and 16S rDNA sequencing were conducted. The DGGE banding patterns indicated limited bacterial diversity within the microbial populations; and no major shifts in population diversity were observed in response to the various chemical treatments (data not shown). Although these results suggested that the treatments did not significantly affect the bacterial populations, it is possible that the treatment period of 6 days was too short to cause any observable changes in the populations. In order to establish a phylogenetic tree of identified organisms, a number of the bands representing the diversity of the total sample set were cut out of the DGGE gel and sequenced (Fig. 4). The numbers in Fig. 4 represent individual bands cut out of the DGGE gels and the tree reveals clusters of Firmicutes, Gammaproteobacteria, and Deltaproteobacteria (SRB) in the

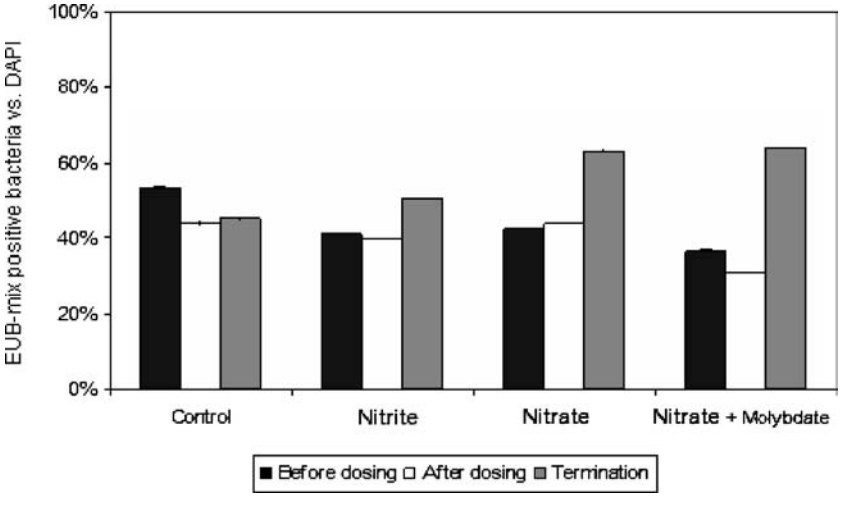

Fig. 2 The number of Bacteria determined with FISH (probe EUB338) compared with the total number of bacteria (DAPI) in biofilms from mild steel coupons. The error bars represent 95% confidence intervals

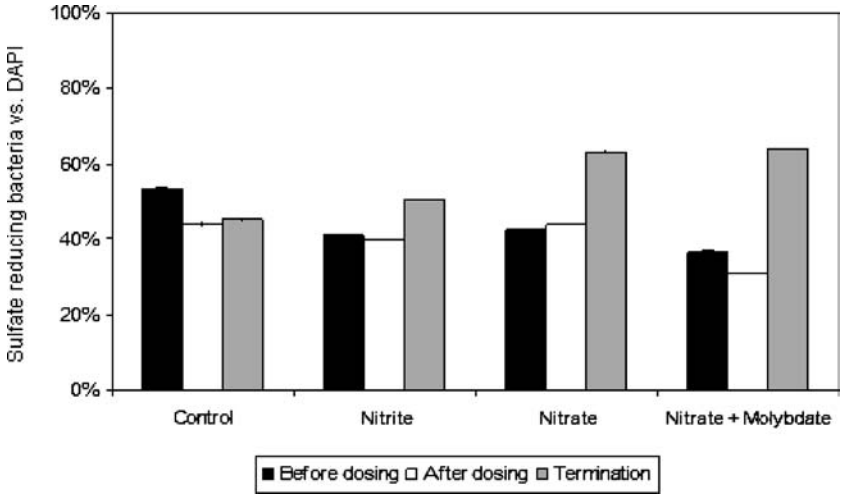

Fig. 3 The number of SRB determined with FISH compared with the total number of bacteria (DAPI) in biofilms from mild steel coupons. The error bars represent 95% confidence intervals

biofilm samples. The closest relatives were found to be members of the genera *Clostridium*, *Pseudomonas*, and *Desulfovibrio*, respectively.

Discussion

Reduced sulfide production resulted from the treatments with nitrite and nitrate + molybdate. However, complete cessation of sulfate reduction was not observed for any of the applied treatments. In addition, the nitrate-only treatment clearly had the least effect on inhibiting sulfide production. No long-term effects were observed in any of the treatments, despite the fact that total inhibition of SRB activity has been reported in studies involving oil-field production water receiving additions of either 180 mg l^{-1} nitrite or 60 mg l^{-1} molybdate [21]. Other studies have shown total inhibition of SRB activity in the treatment range of 250–1100 mg l^{-1} molybdate, depending on the sample source [27]. Also, Eckford and Fedorak [10] showed that 620 mg l^{-1} nitrate was sufficient to stop sulfide production by SRB in oil-field produced water, presumably as a result of increased NRB activity. In this study, SRB inhibition was observed at concentrations two to six times lower than those applied in the studies cited above. However, it must be emphasized that those studies showed that continuous dosing was required to completely suppress sulfide production.

A possible explanation for the differing results in the present study compared with the other studies discussed might be that the microbial populations from different sites are very different and therefore also might respond differently to similar treatments. In cases where continuous dosing is required to maintain suppression of sulfide production, it seems likely that either the present NRB population was out-competing the SRB population for electron donors or the SRB were capable of

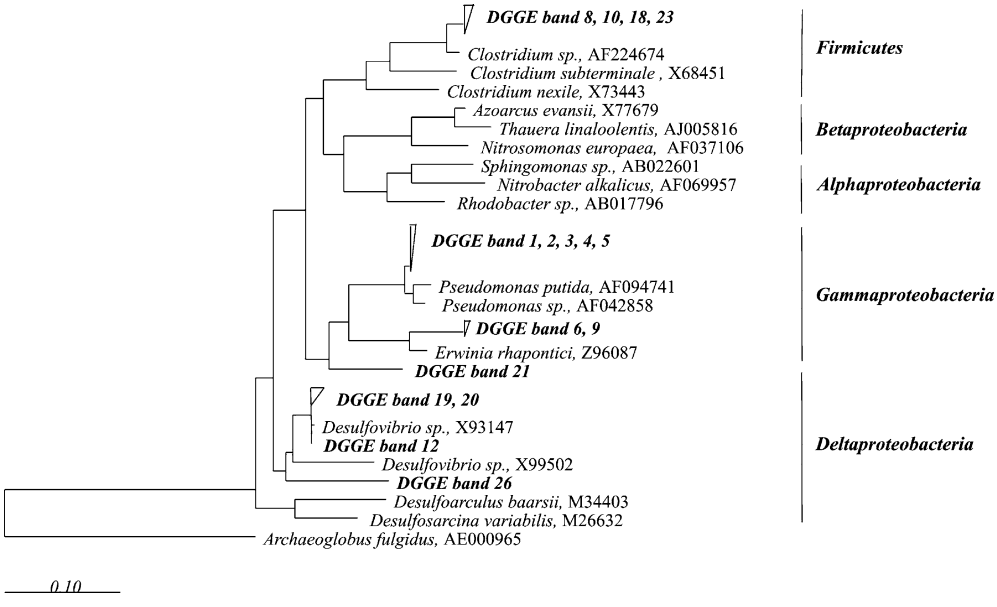

Fig. 4 Phylogenetic tree based on the sequencing of DGGE bands from the different treatments, showing the relationships between biofilm bacteria from the mild steel coupons. The bar shows 10% difference between related bacterial species

switching to a more energetically favorable electron acceptor, such as nitrate. A recent study by Larsen et al. [17], using the activity-based microautoradiography technique, showed that SRB were actively respiring despite continuous dosing with nitrate in the Halfdan Field in the North Sea. These results support the FISH results in this present study, where no major population changes were observed due to treatment with nitrate since some SRB can utilize nitrate as an electron acceptor.

The mechanisms of nitrite-mediated sulfide reduction are thought to involve both the chemical oxidation of iron sulfide and the suppression of SRB activity. This latter effect is thought to result from the more energetically favorable coupling of organic substrate oxidation to nitrite reduction by NRB, which allows them to effectively compete with SRB for organic electron donors [26]. Both of these proposed mechanisms are supported by the results of our study, in which we observed increased iron dissolution simultaneous with decreased consumption of sulfate during treatment with nitrite. Although NRB activity was also indicated by the consumption of nitrate in R3 and R4, NRB presence could not be confirmed using FISH. This might be due to lack of NRB activity, impermeable cells, or the use of specific probes that did not target the NRB present. The use of FISH for identifying a NRB population is a difficult task compared with enumeration of the SRB. Sulfate reduction is solely performed by the SRB that belong to fairly restricted phylogenetic lines; and the majority can be targeted with the probes applied in this study. In the case of nitrate reduction, this process can be performed by a phylogenetically diverse group of microbes, implying a more difficult task for the identification and enumeration of the NRB.

SRB were detected by FISH in the reactor receiving nitrate + molybdate (R4) after the 6 days of treatment. This continued presence of SRB under presumably adverse conditions could have been due to the short dosing period, during which the SRB were not eliminated from the microbial population, but only responded by decreased activity (i.e., sulfate reduction due to treatment with molybdate, a metabolic inhibitor of SRB, specifically blocking ATP production [23, 24]). Thus, FISH would not necessarily reflect a change in activity, since FISH targets the ribosomes. The other ''active'' constituents of the treatments may have produced similar effects.

Detection of population changes over a short period of time in a biofilm community could also be performed by the application of molecular methods involving more labile or activity-based targets (e.g., mRNA) which reflect the activity of bacteria more accurately than DNA-based techniques [5].

Although none of the treatments reduced the SRB abundance immediately following treatment, SRB abundance in all treated reactors increased within the 3 weeks between cessation of the treatments and termination of the experiment. In the nitrate and nitrate + molybdate reactors, this increase in SRB numbers was pronounced (Fig. 3). A likely reason for this observation could be that some SRB were able to use nitrate as an electron acceptor during the treatment and, therefore, were not inhibited when nitrate was switched off. Although SRB are often considered to be strict anaerobes, several recent studies have shown significant SRB activity in the presence of nitrate and under microaerophilic conditions [15]. The treatments did not cause a shift in the microbial population during treatment as predicted, although a significant reduction in sulfide production occurred. The practical importance of this result is that short-term treatments reduce sulfide production in the short term, primarily because the population continues as it was before the treatment started. Therefore, in order to obtain an on-going low production of sulfide, a continuous treatment is recommended.

In conclusion, effluent sulfide was reduced in reactors treated with nitrite and nitrate + molybdate, but no lasting sulfide inhibition was obtained. Nitrate alone did not result in sulfide inhibition at the applied concentration. The number of bacteria in the biofilm was not affected by any of the treatments, nor were SRB numbers reduced. On the contrary, SRB seemed to be stimulated in the weeks following treatment to levels in excess of pre-treatment conditions. DGGE also showed that the treatments did not alter the composition of the microbial populations in the treated reactors. This study confirms that, while nitrate/nitrite treatments can be effective sulfide-controlling agents while they are present, they are not expected to favorably alter biofilm composition following short-duration treatments. Furthermore, these data suggest the overlap of SRB and NRB populations such that, in the absence of nitrate or nitrite, the same sessile consortia may rapidly revert to sulfate reduction.

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