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Control of microbial souring by nitrate, nitrite or glutaraldehyde injection in a sandstone column

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Microbial souring (production of hydrogen sulfide by sulfate-reducing bacteria, SRB) in crushed Berea sandstone columns with oil field-produced water consortia incubated at 60°C was inhibited by the addition of nitrate (NO_3^-) or nitrite (NO_2^-). Added nitrate (as nitrogen) at a concentration of 0.71 mM resulted in the production of 0.57–0.71 mM nitrite by the native microbial population present during souring and suppressed sulfate reduction to below detection limits. Nitrate added at 0.36 mM did not inhibit active souring but was enough to maintain inhibition if the column had been previously treated with 0.71 mM or greater. Continuous addition of 0.71–0.86 mM nitrite also completely inhibited souring in the column. Pulses of nitrite were more effective than the same amount of nitrite added continuously. Nitrite was more effective at inhibiting souring than was glutaraldehyde, and SRB recovery was delayed longer with nitrite than with glutaraldehyde. It was hypothesized that glutaraldehyde killed SRB while nitrite provided a long-term inhibition without cell death. Removal of nitrate after as long as 3 months of continuous addition allowed SRB in a biofilm to return to their previous level of activity. Inhibition was achieved with much lower levels of nitrate and nitrite, and at higher temperatures, than noted by other researchers.

Keywords: souring; nitrate; nitrite; glutaraldehyde; inhibition

Introduction

Souring, the production of hydrogen sulfide (H_2S) in oil reservoirs which have been subjected to water flooding, costs oil companies billions of dollars every year [17]. The process is believed to be mediated by sulfate-reducing bacteria (SRB) present in the reservoirs [8,9]. These bacteria are strict anaerobes that may accumulate in biofilms on reservoir rock and reduce sulfate (SO_4^{2-}) to hydrogen sulfide. Major carbon and energy sources used by SRB in reservoirs are believed to be short-chain organic acids. Recent studies have found that hydrocarbons in petroleum may also serve as donors for sulfate reduction (Jackson and McInerney, unpublished results; [26]).

The reservoir-souring process is influenced by water injection, the oil-bearing formation, biotransformation (souring), and phase separation. Produced water separated from the formation mixture is recycled in some applications for water injection of the oil reservoirs. Problems associated with H_2S production in oil reservoirs include H_2S toxicity, corrosion, and reservoir plugging by SRB biomass and precipitated metal sulfides, both of which retard secondary oil recovery. In addition, H_2S production significantly raises the sulfur content of the produced natural gas, where pipeline standards limit the H_2S concentration to several ppm. The problem becomes more serious when sea water, which contains a high sulfate concentration, is used to inject oil reservoirs [27]. Sea water is used in many areas because of its proximity and availability.

Several methods have been investigated for mitigating souring. Biocides such as glutaraldehyde inhibit SRB [28]. Glutaraldehyde (glutaric dialdehyde) is a highly toxic, corrosive chemical often added in slugs or pulses to injection water to inhibit souring. In test systems studied by Ruseska *et al* [28], 1.0 mM glutaraldehyde was somewhat effective in lowering H_2S production by sessile organisms (an undefined mixed population from an oilfield), as SRB counts were reduced by about 70% after 20 days of daily treatment. Grab and Theis [12] found that 1.0 mM glutaraldehyde was very effective against both a planktonic pure culture of *Desulfovibrio desulfuricans* and against sessile organisms (a three-log reduction in bacterial numbers). Higher concentrations (2.2 mM) almost completely killed a mixed-population biofilm from the North Slope. H_2S had no effect on glutaraldehyde efficacy. In a field study, 1.0 mM glutaraldehyde was very effective in reducing SRB cell counts [12]. Eagar *et al* [11] found that similar concentrations of glutaraldehyde (1.0–2.0 mM) were needed to kill cells of *Pseudomonas fluorescens* in biofilms.

However, application of biocides has several drawbacks. The concentration of a biocide found to be inhibitory in laboratory studies may be insufficient in the field, where strains other than those used in the laboratory are present. Chemical components in reservoirs may scavenge biocides through reaction or sorption, making application of biocides in large waterflooding projects expensive. In addition, SRB form biofilms which protect the organism from the action of the biocide. Elimination of sulfate (by membrane separation, for example) from the injection water is another possibility for controlling SRB [2]; however, it is expensive.

The use of nitrate (NO_3^-) to control noxious odors caused by SRB in waste water treatment was recognized as early as 1929 [1]. Nitrate treatment was believed to have only a transient effect on sulfide production, since any organic

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matter remaining after nitrate was depleted could still be used to reduce sulfate to sulfide. However, long-term inhibition of sulfide production by nitrate addition, probably due to changes in the redox potential by the production of nitrite or nitrous oxide, has been observed (Jackson and McInerney, unpublished results; [16]). The relative importance of the individual nitrogen oxides in creating and maintaining oxidized conditions, thereby inhibiting SRB growth, has not been studied. Another method for microbial control of souring is reoxidation of sulfide to sulfate, using nitrate as the electron acceptor, by a sulfide-resistant strain of *Thiobacillus denitrificans*, strain F [19,20]. In experiments using sandstone cores where SRB and *T. denitrificans* strain F were both present, no H_2S was detected and no black precipitate (iron sulfide) was formed. The above studies were all done at mesophilic temperatures (30–40°C). Nitrate treatment to control souring in thermophilic reservoirs such as those in the North Sea or the North Slope of Alaska has not been reported.

Nitrate reduction comprises a series of reactions which may form one of several products, eg nitrite (NO_2^-), nitrogen gas (N_2) or ammonia (NH_3). Reduction of nitrate to any of these products has a much more favorable Gibbs free energy than does sulfate reduction [18]. Thus, if both denitrifying bacteria (DNB) and sulfate-reducing bacteria (SRB) are present in a consortium, the DNB should dominate. If the two types of bacteria utilize a common substrate such as *n*-butyrate or acetate, the DNB should outcompete the SRB if both nitrate and sulfate are present in excess, which could limit or eliminate souring activity. Competitive removal of short-chain organic acids by DNB reduced the H_2S concentration in an experimental porous media system [14]. Some sulfate reducers can also reduce nitrate, usually completely to ammonia [31]. Several researchers [10,16] have proposed that observed microbial metabolism of nitrate in experimental systems was due to SRB, not DNB. In either case, activity on nitrate would limit the production of H_2S, which was the goal of this research.

Here the efficacies of nitrate and nitrite addition to control microbial souring in a crushed sandstone column at 60°C were studied. This entailed measuring the effects on H_2S production of adding and then removing nitrate or nitrite from this system. An experiment was also performed to determine the effectiveness of glutaraldehyde compared to nitrite in lowering H_2S concentrations.

Materials and methods

Reactor system

The anaerobic upflow porous media reactor system used was a packed bed (50 × 5.5 cm) column made of polycarbonate and equipped with sampling ports (Figure 1) located at regular intervals on one side of the column (10, 20, 30 and 40 cm from the inlet), while two ports were located on the opposite side of the column at 5 and 15 cm from the inlet. The column inlet and outlet were sampled directly through the heavy-wall butyl rubber tubing (Cole-Parmer, Masterflex neoprene tubing, Vernon Hills, IL, USA) used to minimize oxygen diffusion. Porous media were contained in the reactor by brass gauge mesh over each end of the column. Flow was dispersed by a funnel at each end

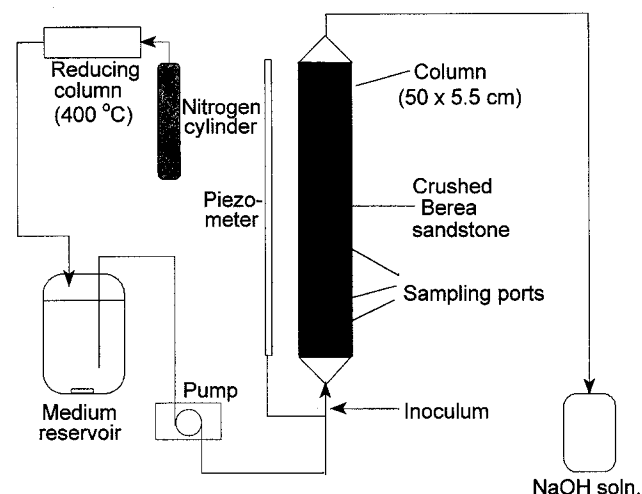


Figure 1 Schematic diagram of the anaerobic upflow crushed sandstone column.

of the reactor column. Aqueous medium was slowly purged with N_2, from which traces of O_2 had been removed in a column containing copper turnings at 400°C. The medium flask was kept sterile with 0.22-μm bacterial air vents (Gelman, Ann Arbor, MI, USA) at the gas inlet and outlet. A glass flow break separated the medium flask from the column inlet. Volatile H_2S in the effluent was trapped in a 0.1 M NaOH solution.

Crushed Berea sandstone, a standard material used by many oil companies for *in situ* investigation of oil recovery, was used as the porous medium [30]. Solid Berea sandstone was obtained from the Idaho National Engineering Lab (Idaho Falls, ID, USA) and pulverized with a grinding wheel. The crushed sandstone contained particles ranging from 35 to 300 μm in diameter with over 50% of the particles residing in the 220-μm regime. This provided a column diameter to grain diameter ratio of greater than 180, which is sufficient to ensure that the radial velocity variations due to wall effects were negligible [29]. Sand particles were pretreated by heating them at 400–500°C for 4–5 h to remove organic material and bound water, and autoclaved before packing them in a column. The sand pretreatment procedure, reactor sterilization, column packing procedure and porosity determination were described previously [5]. The porosity of each sand bed was 0.50 ± 0.02.

Nitrate, when added, was included in the medium as sodium nitrate. Nitrite was added from a stock solution through a Masterflex peristaltic pump. The solution was prepared by adding potassium nitrite to nanopure water in a 100-ml stoppered vessel, then autoclaving it. A 24-h timer (Fisher Scientific, cat. No. 06-662-12, Pittsburgh, PA, USA) was used to control the nitrite addition rate by adding a solution of 86 mM for a specified number of minutes at 4-h intervals. Two schemes of nitrite addition with the peristaltic pump were used—pulse addition and continuous addition. Pulse addition of nitrite was achieved by operating the nitrite pump for 5 min (at a rate of 11 ml h^{-1}) at 4-h intervals. This resulted in a time-averaged concentration of 0.71 mM over the 4-h time period but a pulse of 34 mM over the first 5 min at the column inlet. Continuous addition

of nitrite (0.71, 0.86 and 1.0 mM) was achieved by inserting a 250-ml flask, for the mixing of the nitrite stock solution and the anaerobic medium, directly ahead of the column inlet. This system, which provided a mixing time of almost 10 h, avoided the problem of nitrite reacting with the medium over extended periods of time, yet provided enough residence time to dampen the nitrite pulse. Nitrite concentrations using this continuous addition method were very consistent over time throughout the column, with no observable pulse at the first sampling port located 5 cm up the column (data not shown). Continuous addition was used in the experiments which involved glutaraldehyde, as glutaraldehyde (50% w/w, Aldrich, Milwaukee, WI, USA) was thawed and added directly to the medium supplying the column. When nitrate was added, the same levels of sulfate and organic acids were maintained in the medium.

Bacterial consortia and cultivation

Separate samples of produced water from the Ninian North Sea oil field and the Kuparuk North Slope oil field were used as the microbial consortia; the water was concentrated (100×) on-site, flash-frozen and stored at −70°C. Previous experiments indicated the frozen cultures maintained the same microbial activities as on-site produced waters before freezing. Cultures used to inoculate the porous medium reactors were prepared by inoculating 1 ml of frozen culture into a 50-ml vial (sealed and filled with purified N_2 gas in the head space) containing 30 ml of medium inside an anaerobic chamber (model No. 12356, Coy Laboratory Products, Grass Lake, MI, USA). The vials were incubated at 60°C for 7–14 days. One vial was used to inoculate the porous medium reactor at the inlet end.

A high-salinity anaerobic medium suitable for recovery and growth of marine SRB [25] was used for the batch cultures and columns (Table 1). Short-chain organic acids were used as the carbon sources since they were the major carbon sources found in produced water [3,8]. A concentration of 200 mg L^{-1} (varying slightly with each preparation) for each of the organic acids was used in the medium. Therefore, influent concentrations were 4.35 mM of formic acid, 3.33 mM of acetic acid, 2.70 mM of propionic acid, and 2.27 mM each of *n*-butyric and isobutyric acids. The medium sulfate concentration was in excess at 9.4 mM (based on sulfur, S) to avoid sulfate limitation. The medium was purged with N_2 and reduced with small amounts of $Na_2S_2O_4$ (0.34 mM as S) and $Na_2S{\cdot}9\,H_2O$ (1.5 mM as S initially, then lowered to 0.5 mM). The pH of the medium was 6.5–7.0.

The reactors were incubated at 60°C. The flow rate of the medium was maintained at 650 ml day^{-1}, which equals an average pore velocity of 55 cm day^{-1}. A similar flow regime is used by oil companies for water injection (Burger ED, personal communication, Environmental Biocontrol International). The hydraulic residence time in each column was 20 h.

Table 1 Medium composition (per liter)

Carbon and energy sources	
formic acid	200 mg
acetic acid	200 mg
propionic acid	200 mg
isobutyric acid	200 mg
n-butyric acid	200 mg
Base medium	
Na_2SO_4	1.35 g
NaCl	20 g
KCl	0.3 g
NH_4Cl	0.3 g
$MgCl_2{\cdot}6H_2O$	3 g
KH_2PO_4	0.2 g
$CaCl_2{\cdot}2H_2O$	0.15 g
Trace elements [25]	1 ml
Vitamin solution [25]	1 ml
$NaHCO_3$ solution [25]	30 ml
Selenite (Na_2SeO_3) solution [25]	1 ml
Dithionite ($Na_2S_2O_4$) solution [25]	1 ml
Sulfide ($Na_2S{\cdot}9H_2O$) solution [25]	3 ml

Analytical methods

Aqueous hydrogen sulfide was analyzed by the methylene blue method [7]. Because of the high sample dilutions used, the detection limit for H_2S was approximately 0.15 mM as sulfur. Sulfate and nitrate were measured using a Dionex ion chromatograph (model Al-450; Dionex Co, San Francisco, CA, USA) with a pulse electrochemical detector (model DX 300) using a Dionex Ionpac AS4A-SC column (2 mm). Both hydrogen sulfide and sulfate measurements were expressed in terms of sulfur (S). Organic acids were measured using the same ion chromatograph with a Dionex Ionpac AS10 column (4 mm). Nitrite was analyzed spectrophotometrically using a test kit (Hach Co, cat. No. 20596, Loveland, CO, USA) at 546 nm. Both nitrite and nitrate were expressed in terms of nitrogen (N). Cell numbers in suspension were determined by acridine orange direct counts [15]. At the conclusion of the nitrate addition experiment, the column was dismantled and biomass was measured by another method: sectioning the sandstone packing into lengths, drying at 100°C, then ashing at 400°C. It was determined earlier in an identical system [6] that both the carbon remaining in the aqueous medium and the cells in suspension were negligible compared to the rest of the biomass, so this should be an accurate method for determining attached biomass (biofilm).

Eh measurement

Oxidation-reduction potential (ORP or Eh) was measured by microelectrodes (Microelectrodes, Inc, Londonderry, NH, USA) connected to a pH-Eh meter (Cole-Parmer Chemcadet). The Eh microelectrode (Ag/AgCl) and reference microelectrode were encased in separate 18-gauge needles for insertion through the thick-walled butyl rubber stoppers at each sampling port in the column. The two microelectrodes were taped together for ease in handling and to minimize the distance between them. The microelectrodes were rinsed in ethanol to maintain sterile conditions, inserted through the stopper into the column, allowed to reach steady state (up to 2 h), then rinsed and inserted into the next stopper. All Eh readings were taken on the same day while the column was at steady state. The microelectrodes were calibrated using pH 4 and pH 7 solutions saturated with quinhydrone at 25°C.

Results

Effect of nitrate

The effects of nitrate addition on actively souring microbial biofilms were demonstrated in experiments utilizing two different microbial consortia, operated side-by-side in identical reactor systems. The time course of souring with the North Slope consortium, and the substrate and product profiles along the column, were described previously [6]. Results described here are from new experiments, so the North Slope souring data shown are slightly different from those presented elsewhere. Souring data from the North Sea consortium were similar to the North Slope results and, as will be shown, both consortia were similarly affected by the addition of nitrate. In the North Sea column, 3.6 mM nitrate was included in the aqueous medium approximately 1 week after H_2S concentrations reached steady state, or about 3 months after the initial inoculation of the reactor. One week after nitrate addition, H_2S concentrations remained at the same high level, so the column was reinoculated with actively growing cells from a batch culture in a vial containing the original North Sea consortium. About 10 days after this inoculation, a decrease in nitrate in the column was first noted. Within 3 more days, essentially all the nitrate was consumed, a stoichiometrically equivalent amount of nitrite was detected in the column (Figure 2), and the H_2S concentrations throughout the column decreased from greater than 3.0 mM to below the detection limit of 0.15 mM. Similarly, nitrate utilization and inhibition of souring was seen in the North Slope column after reinoculation of the column with freshly obtained North Slope-produced water. Subsequently, the time course of the inhibition of souring was similar to that seen in the North Sea column: once nitrate disappearance and nitrite appearance were noted, disappearance of H_2S from the column followed in nearly plug-flow fashion.

Sulfate concentrations measured in both columns

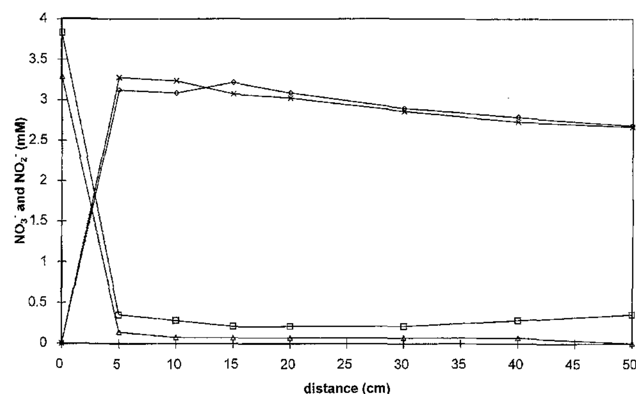


Figure 2 Nitrate (NO_3^-) and nitrite (NO_2^-) concentrations in the North Sea and North Slope columns after addition of 3.6 mM nitrate. Data points are averages from 3 consecutive days of sampling with replicates (two samples per day), taken when the columns were at steady state. Standard errors are not shown because they were very small (≤0.14 mM). Column distances 0 cm and 50 cm indicate the inlet and outlet of the columns, respectively, with sampling ports also located at 5, 10, 15, 20, 30 and 40 cm. Symbols: □, NO_3^- concentration, North Sea column; ◇, NO_2^- concentration, North Sea column; △, NO_3^- concentration, North Slope column; X, NO_2^- concentration, North Slope column.

(Table 2) corroborated the H_2S data and the hypothesis that souring was completely inhibited. Approximately 6.25 mM sulfate was consumed under souring conditions in each column at steady state, with 3.9 mM H_2S produced in the North Sea column and 5.6 mM H_2S produced in the North Slope column. After nitrate addition, however, there was no significant change in sulfate concentration throughout either column ($P = 0.05$), even though sulfate was still available at high concentrations (Table 2). In the North Sea column, the lower H_2S level (3.9 mM H_2S produced versus 6.25 mM sulfate consumed) was probably due to the column not being at steady state yet with respect to iron sulfide precipitation. A small amount of iron was contained in the aqueous medium and iron was also present in the crushed sandstone. Hydrogen sulfide reacts rapidly with iron to form a black iron sulfide precipitate, which could be seen as it migrated upward through the column. At the time these data were taken, some produced H_2S was probably still reacting with iron at the end of the North Sea column and was removed from the aqueous phase.

Organic carbon consumption in each column (Table 3) is consistent with the sulfate and sulfide data. Before nitrate addition, formate and *n*-butyrate were the preferred substrates for North Sea SRB, and *n*-butyrate was also completely consumed in the North Slope column. The net production of acetate in both cases was due, it is believed, to incomplete oxidation of propionate and *n*-butyrate, and the lack of acetate utilization by SRB. Both phenomena were seen in single-substrate batch experiments [22]. After nitrate addition, small amounts of acetate, isobutyrate, *n*-butyrate and formate were consumed in the North Sea column, and acetate and formate were consumed in the North Slope column. It is believed these were the electron donors for nitrate reduction. The other carbon sources were not significantly consumed ($P = 0.05$) after nitrate addition. A smaller amount of total substrate was consumed after nitrate addition for two reasons: a lower level of electron acceptor consumed (3.6 mM NO_3^- *vs* 6.25 mM SO_4^{2-}) and a lower requirement of electrons. Sulfate reduction requires eight electrons per mole whereas nitrate reduction to nitrite consumes only two electrons per mole.

Nitrate reduction occurred primarily in the first 5 cm of the column and stopped at nitrite (Figure 2). In both columns, the concentration of nitrate decreased from approxi-

Table 2 The effect of nitrate on sulfate concentrations (mM as S) in crushed Berea sandstone columns inoculated with North Sea and North Slope consortia

Distance from column inlet (cm)	North Sea		North Slope	
	Before NO_3^-	With NO_3^-	Before NO_3^-	With NO_3^-
0	8.71 ± 0.22	9.06 ± 0.22	7.56 ± 0.31	9.81 ± 0.19
5	4.22 ± 0.31	8.97 ± 0.22	1.47 ± 0.06	9.63 ± 0.22
10	4.09 ± 0.19	8.94 ± 0.16	1.41 ± 0.03	9.88 ± 0.22
15	4.25 ± 0.41	8.81 ± 0.16	1.13 ± 0.03	9.63 ± 0.28
20	3.50 ± 0.19	9.00 ± 0.13	1.13 ± 0.03	9.66 ± 0.13
30	2.44 ± 0.13	8.84 ± 0.09	0.91 ± 0.03	9.72 ± 0.06
40	1.75 ± 0.19	8.84 ± 0.16	1.38 ± 0.22	9.59 ± 0.09
50	2.31 ± 0.16	8.94 ± 0.16	1.94 ± 0.13	9.53 ± 0.13

Table 3 The effect of nitrate on organic acid concentrations as steady state in the columns

Column	Position	Nitrate present	Organic acid concentration (mM)				
			Formate	Acetate	Propionate	Isobutyrate	*n*-Butyrate
North Sea	influent	no	5.04 ± 0.02	4.18 ± 0.23	2.38 ± 0.01	2.07 ± 0.01	1.99 ± 0.01
North Sea	effluent	no	0	6.83 ± 0.12	0.50 ± 0.04	0.82 ± 0.03	0
North Sea	influent	yes	4.39 ± 0.04	3.63 ± 0.03	2.97 ± 0.03	2.63 ± 0.05	2.32 ± 0.02
North Sea	effluent	yes	4.15 ± 0.04	3.15 ± 0.03	3.07 ± 0.09	2.16 ± 0.08	2.20 ± 0.03
North Slope	influent	no	5.17 ± 0.11	3.72 ± 0.08	2.62 ± 0.07	2.19 ± 0.06	1.85 ± 0.06
North Slope	effluent	no	0.52 ± 0.07	7.78 ± 0.23	0.42 ± 0.01	0.76 ± 0.06	0
North Slope	influent	yes	4.13 ± 0.02	2.73 ± 0.08	2.12 ± 0.04	1.60 ± 0.01	1.67 ± 0.03
North Slope	effluent	yes	3.93 ± 0.02	2.45 ± 0.02	2.14 ± 0.01	1.67 ± 0.01	1.58 ± 0.03

mately 3.6 mM in the influent to about 0.36 mM by the first sampling port. In the North Sea column, about 80% of the nitrate was recovered as nitrite at the first port, but 100% of the nitrate remained as nitrite in the North Slope column. In both columns, no significant change in nitrate concentration was seen after 5 cm but nitrite concentrations steadily decreased throughout the column. Other possible products of nitrate reduction, such as nitrous oxide, nitrogen gas and ammonia, were not determined.

Nitrate level required for inhibition

About 3.6 mM NO_3^-, which was biologically converted to 2.8–3.6 mM NO_2^-, completely inhibited souring when added to actively souring columns (Table 2). This scenario was maintained for over 2 months while this level of nitrate was continuously fed to both columns. When the nitrate level in the North Sea column was lowered to 0.36 mM, souring was completely inhibited for another 20 days. However, when nitrate was entirely removed from the influent medium, SRB activity resumed, as indicated by the large quantities of H_2S present in the aqueous phase (Figure 3) and by the upward progression of black precipitate in the column (the sandstone had gradually returned to its natural brown color while souring had been suppressed for 3 months). When 0.36 mM nitrate was again added to the medium, nitrate was reduced to nitrite but souring was not inhibited, as the H_2S concentration remained above 3.0 mM. However, when the influent nitrate level was increased to 0.71 mM, souring was again suppressed Figure 3).

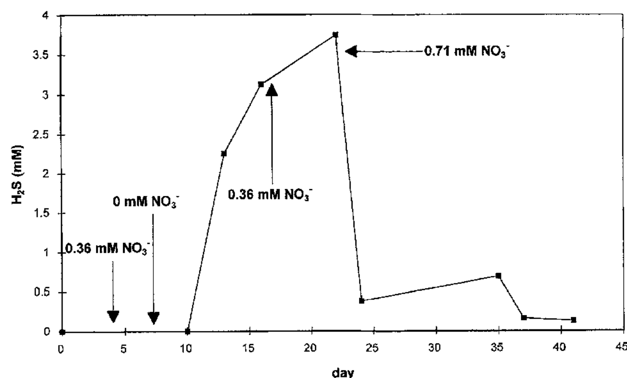


Figure 3 H_2S concentrations in the North Sea column (distance = 5 cm) with different nitrate concentrations in the medium.

This last scenario was repeated by entirely removing the nitrate, then adding 0.36 mM, and then 0.71 mM nitrate to the medium (data not shown). The same results were seen: souring returned in the absence of nitrate, it was not inhibited by 0.36 mM nitrate, but was completely inhibited by 0.71 mM and inhibition was maintained with 0.36 mM. Since about 80% of the nitrate added was recovered as nitrite in the North Sea column, this suggests approximately 0.57 mM NO_2^- was needed to completely inhibit souring, and about 0.29 mM NO_2^- maintained this inhibition.

Biomass

Total cell counts were performed at each sampling port in the columns at steady state under both sulfate-reducing and nitrate-reducing conditions. More cells were generally detected in the aqueous phase (approx 10^7 cells ml^{-1}) withdrawn from sampling ports within the column than from the effluent, probably due to some biofilm detachment when sampling within the column (data not shown). After nitrate addition, fewer cells (approximately three-fold) were detected in the North Sea column. In the North Slope column, about the same number of cells were seen before and after nitrate addition.

Dry biomass values from the bottom to the top of the column were determined in 12 sections; they ranged in no discernible pattern from 6 to 12 mg per g sand. This amount was approximately 50% higher than previously seen [6]. When coupled with the surface area available in the column, as estimated based on the average media particle size, this biomass equals approximately a 4.0 μm-thick biofilm. It has already been shown that the amount of suspended biomass was negligible compared to the attached biomass in an identical system [6].

Chemical/physical phenomena

Black iron sulfide precipitate formed in the column when H_2S was produced by SRB. During initial growth of SRB in the column, this iron sulfide precipitate proceeded upwards through the column at a rate of about 0.75 cm day^{-1}. As the precipitate reached each sampling port, H_2S was detected in the aqueous phase. No H_2S was detected ahead of the front. After nitrate was added and souring was suppressed, iron sulfide precipitate gradually disappeared and the sand returned to its original brown color. When nitrate was removed from the North Sea column after a period of 3 months, the crushed sand was sufficiently light-colored to

see the precipitate begin moving up the column after 6 days. This time the velocity of the front was much faster, about 8 cm day^{-1}. One possible reason for this order-of-magnitude increase is that less iron was available in the column to react with H_2S. Another reason is that an SRB biofilm was now more evenly distributed throughout the column, so that H_2S could be generated throughout the column after the inhibitor was removed instead of only at the inlet.

The redox potential (Eh) of the system changed dramatically during and after souring. During active souring, the Eh of the anaerobic medium decreased from about −100 mV to below −400 mV within both columns. However, after nitrate addition the Eh of the North Sea column increased to above zero. The lowest Eh (about −100 mV) was observed in the first 5 cm of the column, shortly after the reduced medium entered.

Nitrite addition

The *in situ* production of as little as 0.71 mM NO_2^- by nitrate-reducing bacteria completely inhibited H_2S production. For the North Sea column, a pulse of nitrite (34 mM for 5 min every 4 h, an average of only 0.71 mM over 4 h) achieved the same degree of souring suppression as the *in situ* production of nitrite. Sulfate concentrations did not change between the inlet and outlet of the column with pulsed nitrite addition (Figure 4). Organic acid concentrations also did not change. However, when 0.71 mM NO_2^- was added continuously, souring activity was not completely suppressed: as much as 0.47 mM H_2S was seen in the column (data not shown). While this is 90% less H_2S than seen during active souring without nitrite, it is more than that seen during the pulse addition, which injected the same amount of nitrite on a 4-h basis. The H_2S concentration decreased throughout the column in this experiment, as did the nitrite concentration (Figure 5). Organic acid profiles during continuous addition of 0.71 mM NO_2^- showed a dramatic decrease in substrate consumption compared to the actively souring case (data not shown). *n*-Butyrate and formate were still the preferred substrates for SRB during nitrite addition but at much lower consumption rates than during active souring.

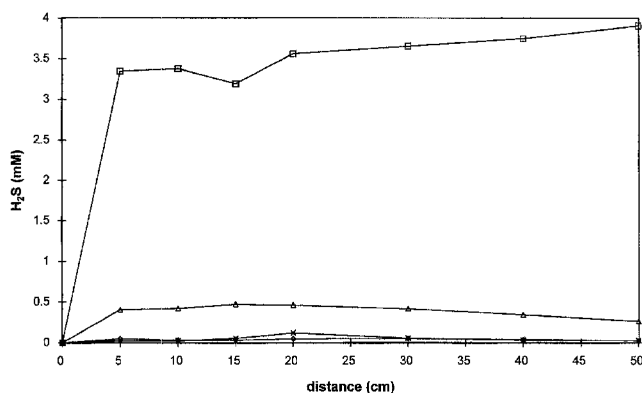


Figure 4 H_2S concentration profiles in the North Sea column after nitrite addition to the influent. Data points shown are averages from 3 consecutive days of sampling with replicates (two samples per day), taken when the column was at steady state. Standard errors are not shown because they were very small (≤0.03 mM). Column distances 0 cm and 50 cm indicate the inlet and outlet of the column, respectively, with sampling ports also located at 5, 10, 15, 20, 30 and 40 cm. Symbols □, without NO_2^-; ◇, pulse of 34 mM for 5 min every 4 h; △, continuous addition of 0.71 mM; X, continuous addition of 0.86 mM.

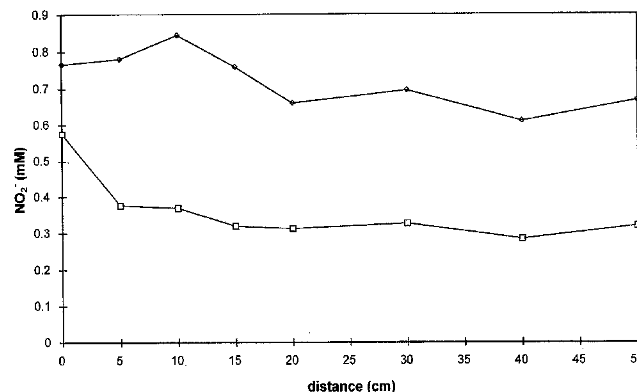


Figure 5 Nitrite concentration profiles in the North Sea column after addition of nitrite to the influent, taken at steady state. Symbols: □, continuous addition of 0.71 mM; ◇, continuous addition of 0.86 mM.

When continous addition of NO_2^- was raised to 0.86 mM in the influent, the H_2S concentration was lowered to less than 0.15 mM. Nitrite concentrations remained above 0.64 mM throughout the column (Figure 5). When continuous addition of NO_2^- was raised to 1.0 mM, H_2S concentrations throughout the column were less than 0.06 mM (data not shown), the same result observed with the pulse injection of nitrite. At nitrite addition rates of 0.86 mM and 1.0 mM, organic acid concentrations were not measured.

The anaerobic medium used in the column contained small concentrations of reducing agents and had an Eh of about −100 mV. The Eh of the souring system dropped to below −400 mV in regions where high concentrations of H_2S were present, as Eh is negatively correlated (r^2 value = 0.98) with H_2S concentration. The Eh was about 100 mV higher when souring was inhibited by nitrite.

Glutaraldehyde addition

In a preliminary experiment, glutaraldehyde was added to the souring column at a level of only 0.1 mM, with the idea that it may be more effective than seen in other studies due to the known thin biofilm in this system [6]. However, after 5 days, H_2S production was not inhibited anywhere in the column (data not shown). Glutaraldehyde concentration could not be measured with a colorimetric method [13] due to interferences from the medium, but it was included in the medium at a concentration of 0.1 mM. Subsequently, the influent glutaraldehyde concentration was increased to 1.0 mM. An immediate effect on H_2S production was seen at the first sampling port, while a delayed, more gradual inhibition effect was seen at the last sampling port (Figure 6). Glutaraldehyde was added to the column for 2 weeks, then the medium was replaced with fresh, glutaraldehyde-free medium (also without nitrite). At the second sampling port, H_2S production immediately resumed at a rate equal to the initial SRB growth in the column (Figure 7). After 2 weeks, the column had recovered to approximately the same souring steady state seen before glutaraldehyde addition, with 4.4 mM H_2S at the first sampling port.

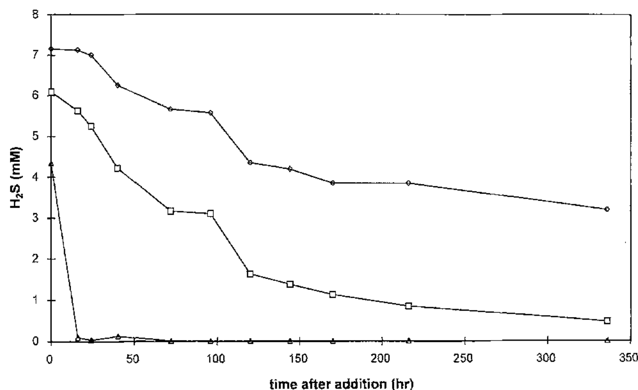


Figure 6 H_2S concentrations in the North Slope column after addition of 1.0 mM glutaraldehyde or 3.6 mM nitrite to the influent. Symbols: □, with glutaraldehyde (distance = 5 cm); ◇, with glutaraldehyde (distance = 40 cm); △, with nitrite (distance = 5 cm).

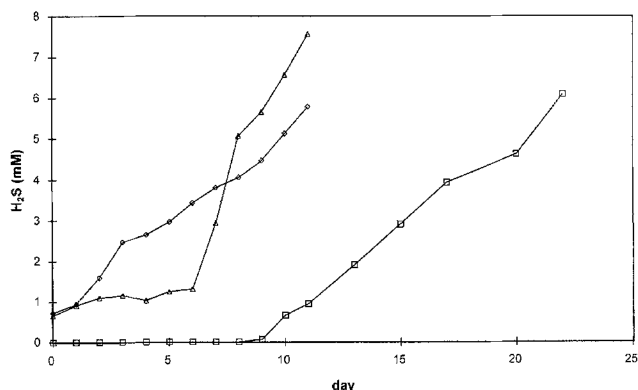


Figure 7 H_2S concentrations in the North Slope column at distance = 5 cm. Symbols: □, initial souring; ◇, after glutaraldehyde removal; △, after nitrite removal.

At this point, continuous addition of 3.57 mM nitrite was begun. At the first sampling port, H_2S production was reduced to less than 0.15 mM within 1 day, a much quicker and more effective inhibition than with glutaraldehyde (Figure 6). Nitrite addition was stopped after 3 weeks and subsequent recovery at the second sampling port was much slower compared to that observed following glutaraldehyde treatment (Figure 7). After six days of partial inhibition, H_2S production rapidly increased to the levels seen before nitrite or glutaraldehyde addition.

Discussion

This work resulted in two important findings. First, nitrate inhibited biogenic souring at thermophilic temperatures. Previous studies on nitrate control of sulfide production were with mesophilic, freshwater systems [1,14,16,19,20]. It was not known previously whether nitrate-reducing bacteria were present in thermophilic, saline oil reservoirs. This study showed that consortia enriched from produced water from the Ninian and Kuparuk oil reservoirs contained microorgansims that metabolize nitrate to nitrite. Five thermophilic denitrifying (nitrate-reducing) bacteria have been isolated from freshly collected Kuparuk-produced water (Jackson and McInerney, unpublished results). Characterization of two of these isolates showed that they reduced nitrate to nitrogen and, in the presence of reduced sulfur compounds, nitrite accumulated. The fact that these bacteria were present in produced water from two different oil fields suggests they may be widely distributed in oil reservoirs. This would make application of this technology easier since inoculation and establishment of a specific bacterial species will probably not be required.

The second important finding is that only low levels of nitrate (as little as 0.71 mM) were needed to inhibit souring, even though high concentrations of sulfate (about 9.4 mM) and organic acids (2.27–4.35 mM) were present. Jenneman *et al* [16] found that about 60 mM nitrate was required for the long-term inhibition of sulfide production in dilute sewage sludge; 20 mM nitrate only temporarily inhibited sulfide production. McInerney *et al* [19] used 40 mM nitrate to inhibit sulfide production by a biofilm growing on porous rock. The use of nitrate to abate odors caused by SRB in waste water treatment usually requires repeated treatments that result in high chemical costs. The minimum nitrate concentration needed to inhibit sulfide production in our studies was about 0.71 mM. Once sulfide production was inhibited, only about 0.36 mM nitrate was needed to prevent reinitiation of souring. Thus, the use of nitrate to control souring may not result in high chemical costs, which makes this technology much more economically attractive.

Amount required for inhibition

Sulfide production was also inhibited with low levels of nitrite. Either a pulse of 34 mM NO_2^- or continuous addition of 0.86 mM NO_2^- or greater completely inhibited H_2S production (99% or greater inhibition compared to the actively souring case without nitrite), and maintained this level of inhibition. With pulse addition, the actual nitrite concentration reaching the microorganisms in the column was most likely significantly less than 34 mM due to diffusion, mechanical dispersion and possibly reaction. The effects of disperson on nitrite concentration would be greater at distances farther up the column. However, the majority of souring activity (ie the highest gradients of H_2S production and substrate consumption) was detected at the inlet of the column in this and other studies [3]. Consequently a pulse addition, in which the concentration of inhibitory agent is highest at the beginning of the column, would be most effective. With both the North Sea and North Slope consortia, 0.57 mM NO_2^- was measured in the column after addition of 0.71 mM NO_3^- and this was sufficient to completely inhibit souring. It is unclear why approximately 50% more nitrite (0.86 mM *vs* 0.57 mM) was required when nitrite was added directly to the North Sea column instead of being biologically produced.

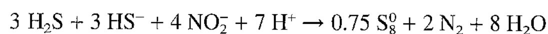
Nitrite has not been previously described as a potent inhibitor of SRB by other researchers. Postgate [24] compiled an extensive list of SRB inhibitors, including several nitro compounds, but nitrite is not one of these. US Patent No. 4 681 687 to Mouche *et al* [21] describes experiments in which alkali metal nitrites added in a concentration of 1.1 mM NO_2^--N: inhibited sulfide production in flue gas desulfurization sludges, but this patent explicitly states that 0.55 mM NO_2^--N and below was not inhibitory. We found

that 0.71 mM NO_2^- reduced but did not completely inhibit sulfide production.

Several mechanisms have been proposed to explain the cessation of biogenic H_2S production after nitrate addition. These include the oxidation of sulfide to sulfate using nitrate as the electron acceptor by chemolithotrophic bacteria such as *Thiobacillus* species [14,19,20], the ability of NRB to outcompete SRB for common electron donors [14,20], the use of nitrate by SRB [10], or the inhibition of sulfide production due to the production of intermediates of nitrate reduction such as nitrite or nitrous oxide [1,16]. Stoichiometric calculations show that 1.6 mmol of nitrate are needed for the oxidation of each mmol of sulfide. With a steady state sulfide production of 3.1–3.9 mM, this would require continuous injection of 5.3–6.4 mM nitrate, much higher than the concentration of nitrate used in this study. Significant levels of acetate, propionate and isobutyrate were detected in the effluent after souring was inhibited. Thus, the system did not become electron donor-limited, making it unlikely that the cessation of sulfide production was due to competition for common electron donors. Since the concentrations of sulfate and the different organic acids were much higher than the amount of nitrate, it is also unlikely that the cessation of sulfide production was due to the preferential use of nitrate by SRB. When both sulfate and nitrate were present, Dalsgaard and Bak [10] found that sulfate was reduced unless the sulfate concentration was low (less than 0.5 mM). The fourth possibility, the cessation of sulfide production due to inhibition of sulfate reduction by an intermediate of nitrate reduction, appears to be the most likely explanation.

Jenneman *et al* [16] found that high levels of nitrous oxide (0.1–2 mM) were produced following the addition of nitrate to sewage sludge, and postulated that the inhibition of sulfate reduction was due to the cytotoxic effect of nitrous oxide on SRB, or to the increase in Eh following nitrous oxide production. The Eh in the North Sea column did increase after nitrate addition but it is doubtful whether this was due to nitrous oxide production. At the first sampling location, nearly all of the nitrate was reduced to nitrite. Also, we have found addition of nitrite to the North Sea column inhibited sulfide production. Mueller [22] found the addition of as little as 0.36 mM nitrite to batch cultures of SRB increased the Eh from −390 mV to −30 mV. The addition of 0.71 mM NO_2^- inhibited sulfide production by 90% and raised the Eh by approximately 100 mV throughout the column, from about −450 to −350 mV. However, the Eh is still much lower than the level where SRB activity would be inhibited [24].

A possible explanation for the inhibition of souring and alteration of the Eh after nitrite addition is the proposed reaction of nitrite with H_2S:



$$3\,H_2S + 3\,HS^- + 4\,NO_2^- + 7\,H^+ \rightarrow 0.75\,S_8^0 + 2\,N_2 + 8\,H_2O$$

Although the simultaneous disappearance of H_2S and NO_2^- and formation of a yellow precipitate in the column after nitrite addition may indicate that the above reaction was occurring, the oxidation of sulfide by nitrite could not be duplicated in batch experiments under conditions identical to the column (60°C, high salinity-produced water, pH 7 and 9). Therefore the slow disappearance of NO_2^- in the column was probably not due to this reaction. With a steady-state sulfide production of 3.1–3.9 mM, much higher amounts of nitrite would be needed to completely inhibit sulfide production by this chemical reaction. Finally, the fact that sulfate levels between the inlet and outlet ends of the column remained unchanged indicate that this reaction cannot explain the inhibition of souring by nitrite.

Nitrite has long been known to inhibit a wide variety of microorganisms, leading to its widespread use in the food industry [23]. It is difficult to determine the mechanism by which nitrite inhibits microorganisms or spore outgrowth since the nitrite ion is capable of a variety of reactions. Several mechanisms have been described [23] including: 1) formation of an inhibitory substance during heating; 2) reaction with thiols to form nitrosothiols, which interfere with key metabolic steps; 3) oxidation or reduction of key enzymes, enzyme cofactors, nucleic acids or membrane components; and 4) reaction with cellular iron.

It is believed that low concentrations of nitrate are much more effective at inhibiting H_2S production in the present system than in previously studied systems because the inhibitory agent is nitrite. In other systems, the effect of nitrate was to either: a) outcompete sulfate as an electron acceptor for the same carbon source; b) oxidize H_2S to sulfate; or c) produce nitrous oxide as an inhibitor. The novel aspect of the present system is that low levels of nitrite, added directly or microbially produced from nitrate, successfully inhibit H_2S production.

Comparison of nitrite to glutaraldehyde

H_2S production recovered quickly after removal of glutaraldehyde, increasing at a rate equal to that after the column was initially inoculated. This suggests that glutaraldehyde did not have any long-term inhibitory effects but instead lowered the SRB population. After glutaraldehyde removal, SRB apparently multiplied at their original growth rate and simultaneously produced H_2S. In fact, previous studies of biofilm regrowth after disinfection with another biocide, chlorine, showed growth rates doubled or tripled after removal of the chlorine, even with repeated applications [4]. In the present study, the H_2S concentration began to increase 6 days after nitrite was removed from the column. Once H_2S production began, it increased much more rapidly than either the initial growth curve or the glutaraldehyde regrowth curve. These two phenomena suggest SRB were not killed by nitrite but were still inhibited after nitrite was removed, and the original number of cells were present and able to produce H_2S once the inhibitory effect stopped.

Our studies were conducted to simulate the *in situ* conditions of the Ninian and Kuparuk oil reservoirs as accurately as possible, with the exception of pressure. The original water in the formation had low levels of sulfate and high concentrations of several organic acids [3,8], and sea water was injected into the formations to improve oil recovery. It was at this time that sulfide production began [3,8]. The temperature of reservoirs that have been water-flooded is about 60°C. Concentrations of the organic acids in the North Slope-produced water after souring began were 10.3 mM acetate, 1.0 mM propionate, 0.08 mM *n*-butyrate,

0.01 mM isobutyrate and no formate. This is very similar to the effluent organic acid concentrations of the columns under souring conditions. Since souring must occur deep within the reservoir where sulfate-rich injection water mixes with organic acid-rich formation water, mitigation technologies that rely on the use of biocide treatments are difficult and expensive to deliver a sufficient concentration of biocide to the active zone of souring, since the enormous surface area of reservoir rock provides many sites where sorption of a biocide can occur. Nitrate and nitrite are very mobile chemicals in subsurface environments and do not adsorb to most porous materials. Thus, transport of the chemical should not be a limiting factor. Reduction of nitrate to nitrite will likely occur in the zone where souring occurs, since this is where the electron donors needed for nitrate reduction will be present. This would result in production of nitrite at the site where it is most needed. Nearly identical results were obtained when nitrate was used to control souring by microbial consortia obtained from two different oil fields. This shows that the process is reproducible and should be effective in many oil reservoirs.

Acknowledgements

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