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Control of biogenic H_2S production with nitrite and molybdate

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The effects of the metabolic inhibitors, sodium nitrite and ammonium molybdate, on production of H_2S by a pure culture of the sulfate-reducing bacterium (SRB) *Desulfovibrio* sp. strain Lac6 and a consortium of SRB, enriched from produced water of a Canadian oil field, were investigated. Addition of 0.1 mM nitrite or 0.024 mM molybdate at the start of growth prevented the production of H_2S by strain Lac6. With exponentially growing cultures, higher levels of inhibitors, 0.25 mM nitrite or 0.095 mM molybdate, were required to suppress the production of H_2S. Simultaneous addition of nitrite and molybdate had a synergistic effect: at time 0, 0.05 mM nitrite and 0.01 mM molybdate, whereas during the exponential phase, 0.1 mM nitrite and 0.047 mM molybdate were sufficient to stop H_2S production. With an exponentially growing consortium of SRB, enriched from produced water of the Coleville oil field, much higher levels of inhibitors, 4 mM nitrite or 0.47 mM molybdate, were needed to stop the production of H_2S. The addition of these inhibitors had no effect on the composition of the microbial community, as shown by reverse sample genome probing. The results indicate that the efficiency of inhibitors in containment of SRB depends on the composition and metabolic state of the microbial community. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 350–355.

Keywords: souring; sulfate-reducing bacteria; oil reservoir; molybdate; nitrite; reverse sample genome probing

Introduction

Biogenic production of H_2S in oil reservoirs subjected to water flooding (souring) is a serious concern for the oil industry, since it decreases the quality of gas and oil and increases the cost of production. Sulfate-reducing bacteria (SRB) are believed to be major players in souring of oil fields. These organisms use a variety of organic compounds, including components of the oil, as electron donor for reduction of sulfate to sulfide [7]. The hydrogen sulfide produced is a serious concern because of its toxicity and corrosiveness. It also forms insoluble sulfides that plug oil-bearing strata and stabilize undesirable oil–water emulsions [3]. Souring increases the sulfur content of the oil, thereby decreasing its value. The problems associated with souring imply a need for control of H_2S production and its release into the environment. One of the most common approaches for mitigation of souring is to contain the activity of SRB by treatment of injected water with biocides such as glutaraldehyde and cocodiamine. Resistance of SRB to high levels of cocodiamine biocides (500 mg/l) was recently demonstrated [11]. The biogenic production of H_2S can be controlled by addition of nitrite and/or molybdate (two metabolic inhibitors of SRB) and the inhibitory action of these compounds is synergistic [6,8,10]. Although the inhibitory effects of nitrite and molybdate on SRB activity have been known for years, the dependence of the minimum required level of inhibitor on the composition of the microbial community or on the metabolic state of the SRB is not known and was investigated in the present work.

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Materials and methods

Microorganisms and media

Pure culture of the SRB *Desulfovibrio* sp. strain Lac6, isolated from western Canadian oil fields [18], was used in this study. Strain Lac6 was maintained on saline Postgate's medium C [9,15]. Coleville synthetic brine, developed by Gevertz *et al.* [4], was modified and used to study the biogenic production of H_2S [15]. All medium components were combined and the pH was adjusted to 7.0, using either 2 M HCl or 2 M NaOH. Medium was dispensed in 100-ml aliquots in 120-ml serum bottles. The bottles were sealed, flushed with mixed gas (85% N_2, 5% H_2, 10% CO_2 by volume) and autoclaved. A culture of strain Lac6 was used as the inoculum (3% v/v). The cultures were maintained at 30°C and were subcultured weekly. To simulate an SRB population, which could prevail in an oil reservoir, modified synthetic brine was inoculated with Coleville-produced water (5% v/v).

Field sample

The produced water sample (4 l) was obtained from the Coleville oil field, located near Kindersley, Saskatchewan, Canada. In this field, oil is produced by water flooding. Upon arrival, the sample was stored in a Coy anaerobic chamber equilibrated with mixed gas.

Effects of nitrite and/or molybdate on biogenic H_2S production

Modified synthetic brine containing different concentrations of sodium nitrite, ranging from 0.05 to 2 mM (2.3–92 ppm nitrite ion), and/or of ammonium molybdate tetrahydrate $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, in the range 0.001–0.19 mM (1–200 ppm molybdate ion), was used to study the effects of the inhibitors on biogenic production of H_2S. Serum bottles containing these media were inoculated with 3 ml of strain Lac6 culture, grown in saline Postgate's medium C. The cultures were incubated at 30°C. In

another experiment, different quantities of sodium nitrite (final concentration: 0.05–1 mM) and/or of ammonium molybdate (final concentration: 0.001–0.19 mM) were added to a set of exponentially growing strain Lac6 cultures. The concentrations of sulfate and sulfide were monitored during the course of both experiments.

A similar study was conducted with a consortium of SRB enriched from Coleville-produced water. Nitrite (0.025–2 mM) and/or molybdate (0.001–0.19 mM) was added to modified synthetic brine. The serum bottles were then inoculated with Coleville-produced water (5% v/v). In another set of experiments, nitrite (2–20 mM) and/or molybdate (0.19–2.85 mM) was added to a set of serum bottles containing an exponentially growing consortium of SRB in modified synthetic brine. The concentrations of sulfate and sulfide were monitored. Compositions of some emerged communities were determined by reverse sample genome probing at the end of the experiments.

Analysis of microbial community composition

The composition of the microbial communities was analyzed by reverse sample genome probing. DNA was isolated from mixed cultures as described previously [13]. The concentration of purified DNA was determined fluorimetrically [16]. Purified DNA (100 ng) was mixed with 2.5 ng of bacteriophage λ DNA and labeled with the random hexamer procedure, using [α-^{32}P] dCTP [16]. The denatured, labeled probe was hybridized with the oil field master filter for 16 h under stringent conditions at 68°C [17]. Following washing and drying, the filters were exposed to BAS-III type imaging plates, which were scanned with a BAS 1000 Bioimage analyzer. The measured hybridization intensities were used to calculate the fraction of each microorganism in the microbial community. The master filter contained the denatured chromosomal DNA of oil field bacteria standards, including 30 SRB and 16 heterotrophs [11,15], and two sulfide-oxidizing bacteria [4,15]. Standards are defined as bacteria with genomes that have no or limited cross-hybridization [14]. A batch of 200 master filters had been prepared in previous work [12] by spotting denatured chromosomal DNA of standards in known amount (usually 100 ng) at known locations on 8×7 cm Hybond-N grids. Denatured bacteriophage λ DNA (5, 10, 20, 30, 50, 100, 200 and 400 ng) was also included on the filters. Following

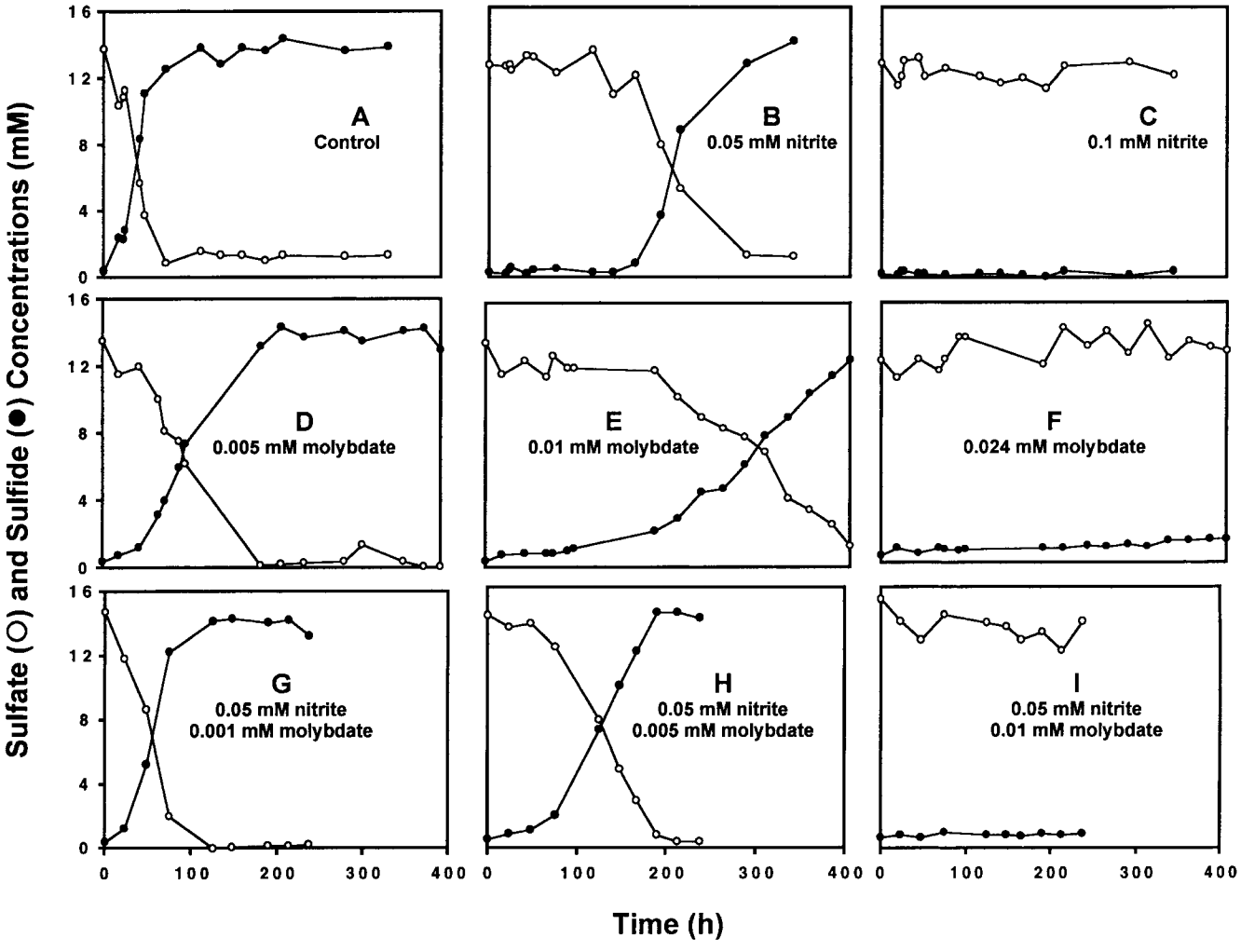

Figure 1 Effects of addition of nitrite and/or molybdate at time 0 on production of H_2S by *Desulfovibrio* sp. strain Lac6. (●) Sulfide concentration; (○) sulfate concentration.

covalent linkage of the DNA on the filter, the filters were stored at −20°C. These were then used for analysis of microbial communities.

Chemical analyses

The concentration of sulfide was determined using a colorimetric method [2]. To 0.1 ml of the sample, 0.9 ml of an acidic copper sulfate solution was added. The absorbance of the resulting mixture was measured at 480 nm. A calibration curve, prepared using standard solutions of sulfide, was used to calculate the concentration of sulfide in the sample. A turbidimetric method was used to measure the concentration of sulfate [1]. To 0.1 ml of a centrifuged sample, 0.9 ml of a conditioning agent (0.85 ml glycerol, 0.5 ml concentrated HCl, 1.3 g NaCl, 1.7 ml ethanol and 1 l deionized water) and an excess amount of finely ground $BaCl_2$ were added. These were mixed and the absorbance of the mixture was measured at 420 nm. The concentration of sulfate was calculated from a calibration curve.

Results

Effects of inhibitors on production of H_2S by Desulfovibrio sp. strain Lac6

The effects of addition of nitrite and/or molybdate at time 0 on production of H_2S by *Desulfovibrio* Lac6 are shown in Figure 1. In the presence of 0.05 mM nitrite, an extended lag phase in the activity of Lac6 was seen after which H_2S was produced at a relatively slow rate. Complete reduction of sulfate in this case was achieved after 350 h, compared with 100 h in the absence of nitrite. With higher concentrations of nitrite (0.1–2 mM), H_2S was not detected in any of the cultures, even after long periods of incubation. Addition of molybdate had a similar effect. At the lower range of applied concentrations ($\leq$0.01 mM), the presence of molybdate led to a longer lag phase and a slower rate of H_2S production. Concentrations of molybdate above 0.01 mM completely suppressed the activity of strain Lac6. Simultaneous addition of inhibitors had a synergistic effect. In the combined form, lower levels of each inhibitor were

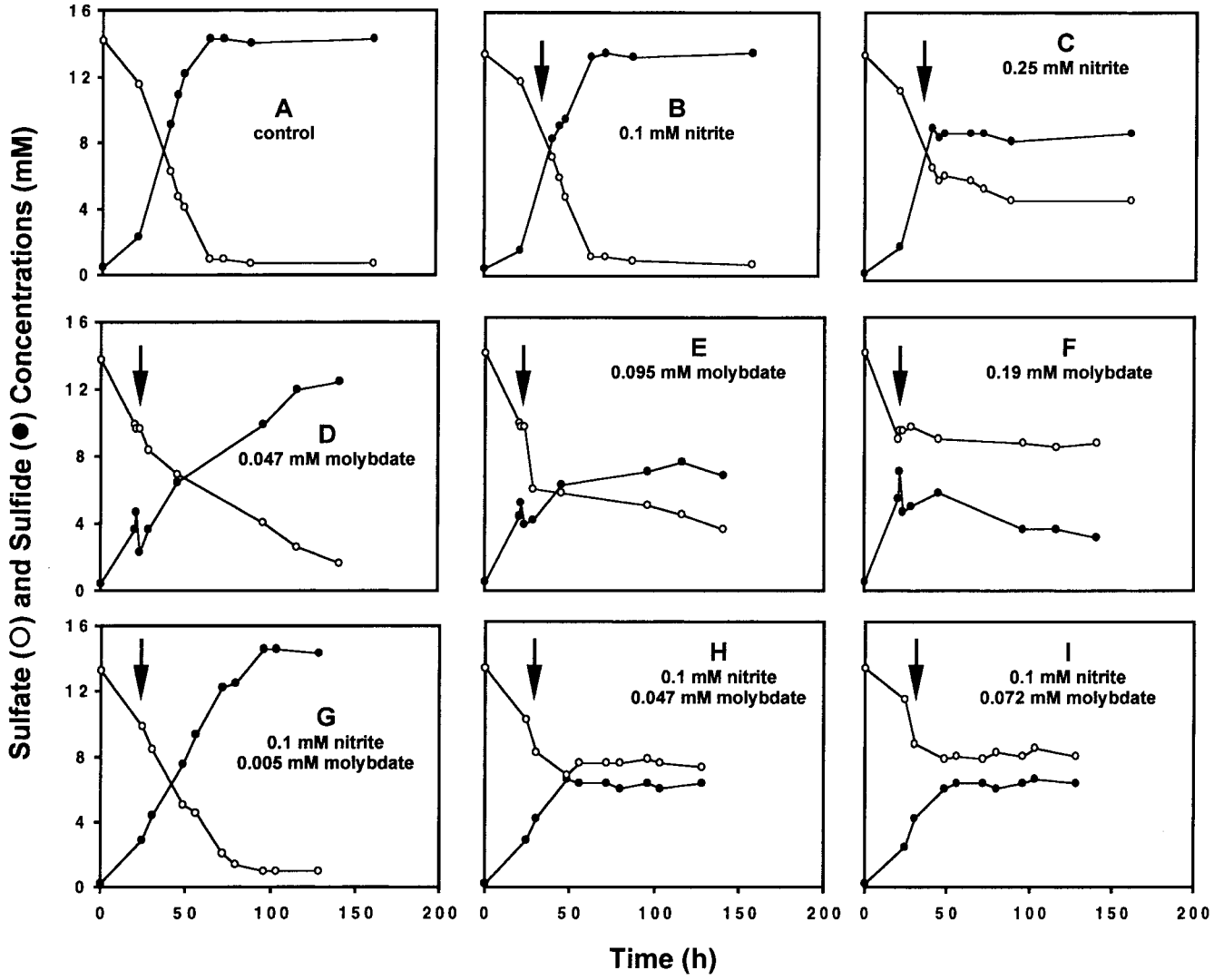

Figure 2 Effects of addition of nitrite and/or molybdate on production of H_2S by an exponentially growing culture of *Desulfovibrio* sp. strain Lac6. (●) Sulfide concentration; (○) sulfate concentration. Additions were made at the indicated time (↓).

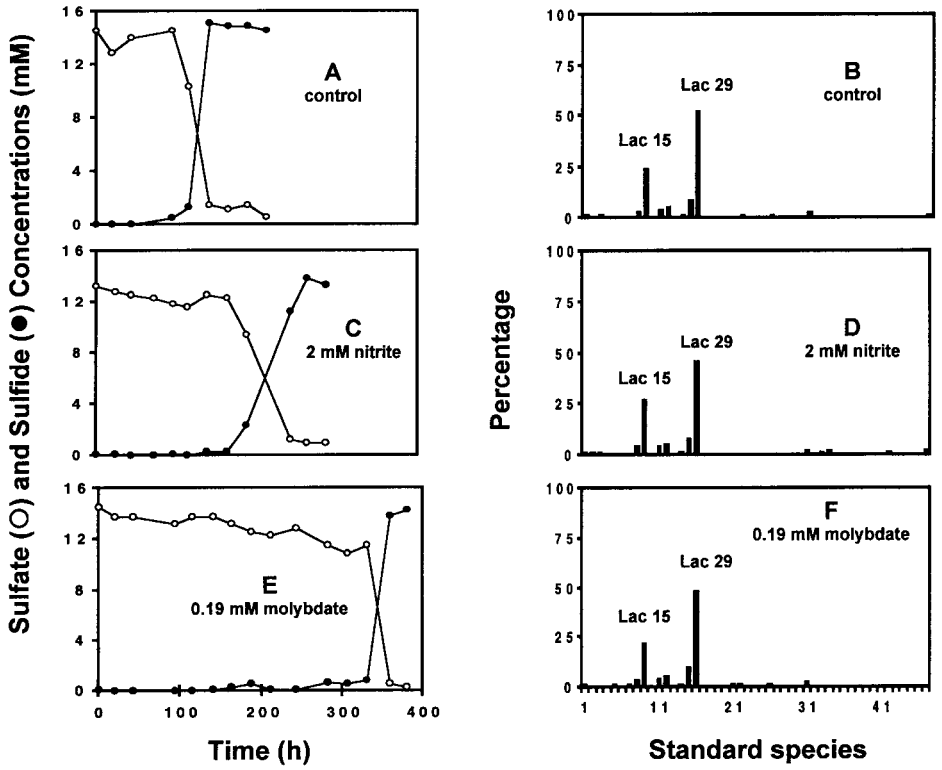

Figure 3 (A, C, E) Effects of addition of nitrite or molybdate at time 0 on production of H_2S by a consortium of bacteria enriched from Coleville-produced water. (●) Sulfide concentration; (○) sulfate concentration. (B, D, F) Composition of the emerged communities.

required to prohibit production of H_2S (0.05 mM nitrite and 0.01 mM molybdate).

The results of addition of nitrite and/or molybdate to an exponentially growing culture of strain Lac6 (20–50 h after

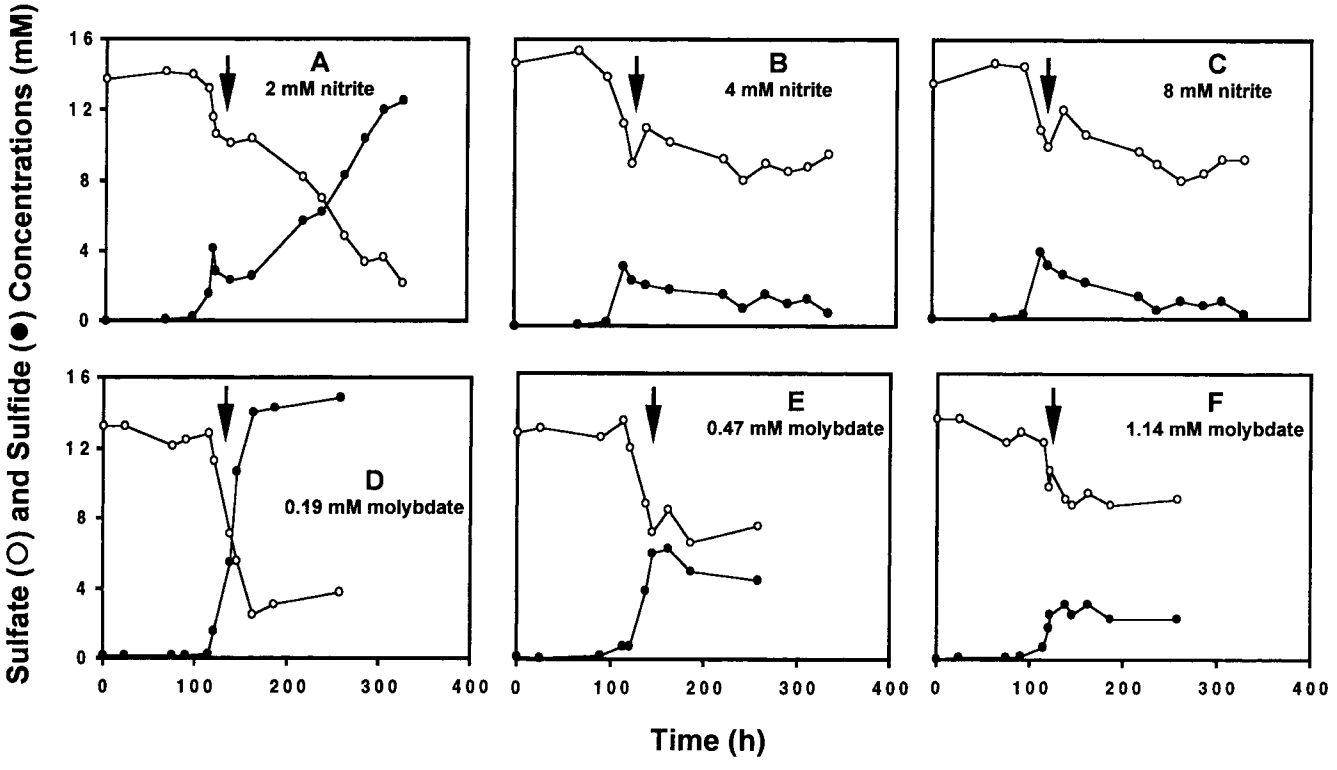

Figure 4 Effects of addition of nitrite or molybdate on production of H_2S by an exponentially growing consortium of SRB enriched from Coleville-produced water. (●) Sulfide concentration; (○) sulfate concentration. Additions were made at the indicated times (↓).

Table 1 Effects of simultaneous addition of high concentrations of nitrite and molybdate on production of H_2S by an exponentially growing consortium of SRB enriched from Coleville-oil-field-produced water

Nitrite (mM)	Molybdate (mM)				
	0.095	0.19	0.28	0.38	0.47
0.5	no significant effect	no significant effect	no significant effect	immediate suppression	immediate suppression
1.0	no significant effect	no significant effect	no significant effect	immediate suppression	immediate suppression
1.5	no significant effect	inhibition	immediate suppression	immediate suppression	immediate suppression
2.0	immediate suppression	immediate suppression	immediate suppression	immediate suppression	immediate suppression
3.0	immediate suppression	immediate suppression	immediate suppression	immediate suppression	immediate suppression

The term "inhibition" was used when addition of inhibitors decreased the rate of H_2S production.
The term "immediate suppression" was used when addition of inhibitors stopped the production of H_2S.

inoculation) are presented in Figure 2. Nitrite at concentrations of 0.05 and 0.1 mM did not have a significant effect on H_2S production. With 0.05 mM nitrite, H_2S production continued with a rate similar to that observed before addition of nitrite, while with 0.1 mM nitrite, a decreased rate was seen. Addition of nitrite at concentrations of 0.25 mM and higher resulted in an immediate halt in bacterial activity. Molybdate at concentrations ranging from 0.001 to 0.01 mM did not affect the production of H_2S. With 0.024 and 0.047 mM molybdate, a decrease in the production rate of H_2S was observed, but the bacteria were still able to reduce the sulfate completely. Addition of molybdate at concentrations of 0.095 mM and higher immediately stopped the H_2S production. Combination of nitrite and molybdate at high concentrations imposed a very strong inhibition effect on the activity of strain Lac6. Synergy of these two inhibitors was again observed. For instance, combination of 0.1 mM nitrite and 0.047 mM molybdate was enough to stop H_2S production, while the levels required for each inhibitor individually were 0.25 and 0.095 mM, respectively.

Studies with a consortium of SRB enriched from Coleville-produced water

Addition of nitrite at concentrations as high as 2 mM only extended the lag phase of H_2S production by a consortium of bacteria from Coleville-produced water. The rate of H_2S production during the exponential phase of bacterial growth was similar in the presence of 0.025–1 mM nitrite, but was slower with 2 mM of nitrite (Figure 3C). Addition of nitrite did not have a significant effect on the composition of the emerged microbial community. In all cases, *Desulfovibrio* sp. strains Lac15 and Lac29 were the dominant components of the community, similar to that observed in the absence of nitrite (Figure 3, B and D). Initial addition of molybdate had similar effects. Increased concentrations of molybdate led to a longer lag phase, but molybdate even at 0.19 mM did not contain the activity of SRB (Figure 3E). The compositions of communities that emerged in the presence of different molybdate concentrations were similar to one another, with *Desulfovibrio* sp. strains Lac15 and Lac29 being the dominant components (Figure 3F). The initial addition of both nitrite and molybdate, even at a combination of 2 mM nitrite and 0.19 mM molybdate, did not have a significant effect on production of H_2S by the SRB consortium. Apparently, higher concentrations of nitrite and molybdate were needed to suppress the activity of SRB in the cultures inoculated with produced water. The composition of the emerged microbial communities was not influenced by the presence of nitrite and molybdate, with *Desulfovibrio* sp. strains Lac15 and Lac29 being the prominent components (not shown).

Figure 4 summarizes the results of addition of nitrite or molybdate at higher concentrations to an exponentially growing consortium of SRB enriched from Coleville-produced water. With 2 mM nitrite, H_2S production proceeded with a rate slightly slower than that observed before addition of nitrite. Nitrite at concentrations of 4 mM and higher strongly suppressed the activity of SRB. Due to reaction of nitrite and sulfide, and perhaps activity of nitrite-dependent, sulfide-oxidizing bacteria, a decrease in the concentration of sulfide was observed in these cultures during the rest of the experiment. Similarly, addition of molybdate at concentrations of 0.47 mM and higher inhibited H_2S production. Following the addition of molybdate, the production of H_2S stopped and the sulfide concentration remained constant.

The results of simultaneous addition of nitrite and molybdate to an exponentially growing consortium of SRB (enrichment of Coleville-produced water) are summarized in Table 1. In this set of experiments, due to reaction of nitrite with sulfide and precipitation of elemental sulfur, as well as the presence of molybdate which induced a strong yellowish–brownish colour in the cultures, the accurate measurement of sulfide and sulfate concentrations was not possible. However, the analytical technique was good enough to enable us to assess the activity of SRB qualitatively. As shown in Table 1, addition of 0.5 or 1 mM nitrite, together with 0.095, 0.19 or 0.28 mM molybdate, and 1.5 mM nitrite, together with 0.095 mM molybdate, did not influence the activity of SRB. Combination of 1.5 mM nitrite and 0.19 mM molybdate inhibited, but did not stop, production of H_2S. The presence of higher concentrations of nitrite and molybdate resulted in an immediate halt in production of H_2S. The synergistic inhibition effects of nitrite and molybdate were again observed. While addition of 2 mM nitrite or 0.095 mM of molybdate alone could not prevent production of H_2S, the same quantities of each inhibitor were sufficient to suppress the activity of SRB when combined.

Discussion

SRB are implicated in sulfide build-up in enclosed working environments in the offshore oil and gas industries and are the principal causative organisms in microbially influenced corrosion of platform structures, transmission lines and general equipment. They are also the likely cause of major reservoir damage including

souring of the produced oil and gas, and plugging of the geological formation [5]. The detrimental consequence of SRB activity has caused the oil industry to invest in strategies for their containment. Oil production facilities are routinely treated with biocides. Biogenic production of H_2S is also controlled by addition of inhibitors such as nitrite and molybdate. Reinsel *et al.* [10] showed that continuous addition of 0.71–0.86 mM nitrite to columns packed with Berea sandstone, inoculated with oil-field-produced water, completely inhibited the production of H_2S and that nitrite was more effective at inhibiting souring than was glutaraldehyde. The synergism of nitrate, nitrite and molybdate in containment of SRB has been reported by Hitzman *et al.* [6]. The efficiency of such treatments and the level of required inhibitor depend on a variety of parameters. Based on the results of the present study, one of the influential factors on the efficient level of inhibitor is the concentration and metabolic state of the bacteria. For instance, addition of 0.1 mM nitrite and/or 0.024 mM molybdate was enough to prevent the activity of strain Lac6 in its early stage of growth, while with exponentially growing cells, the necessary levels of nitrite and molybdate were 0.25 and 0.095 mM, respectively. A comparison of the inhibitor level required to suppress the activity of a consortium of SRB (4 mM nitrite and/or 0.47 mM molybdate) with those needed for strain Lac6 (0.25 mM nitrite and/or 0.095 mM molybdate) revealed that the efficient level of inhibitor was also dependent on the type of bacteria and the composition of the microbial community. The reverse sample genome probing profile of the Coleville-produced water enrichments showed that the addition of either inhibitor at various concentrations had no effect on the composition of the microbial community and, in all cases, *Desulfovibrio* sp. strains Lac15 and Lac29 were the prominent components. The similarity of reverse sample genome probing patterns in the presence and absence of inhibitors indicated that the community members were not differentially resistant to the action of inhibitors. In general, the results of the present study indicate that the efficiency of an inhibitor in containment of SRB is specific for each system and environment. Such factors as type, composition and metabolic state of the microbial community influence the required level of inhibitor. In addition, it may be expected that the mode of bacterial growth and the hydrodynamic characteristics of the cells, either freely suspended cells or in biofilm, influence the efficacy of the metabolic inhibitors. In order to control the undesirable activity of SRB, each system must be assessed individually and it is not feasible to apply a universal strategy.

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