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Chemical and microbiological changes in laboratory incubations of nitrate amendment ''sour'' produced waters from three western Canadian oil fields

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Nitrate addition to oil field waters stops the biogenic formation of sulfide because the activities of nitrate-reducing bacteria (NRB) suppress the activities of sulfate-reducing bacteria (SRB). In general, there are two types of NRB — the heterotrophic NRB and the chemolithotrophic NRB. Within the latter group are the nitrate-reducing, sulfide-oxidizing bacteria (NR-SOB). To date, no study has specifically addressed the roles of these different NRB in controlling sulfide concentrations in oil field produced waters. This study used different culture media to selectively enumerate heterotrophic NRB and NR-SOB by most probable number (MPN) methods. Produced waters from three sulfide-containing western Canadian oil fields were amended with nitrate as an electron acceptor, but no exogenous electron donor was added to the serum bottle microcosms. Changes in the chemical and microbiological characteristics of the produced waters were monitored during incubation at 21°C. In less than 4 days, the sulfide was removed from the waters from two of the oil fields (designated P and C), whereas nearly 27 days were required for sulfide removal from the water from the third oil field (designated N). Nitrate addition stimulated large increases in the number of the heterotrophic NRB and NR-SOB in the waters from oil fields P and C, but only the NR-SOB were stimulated in the water from oil field N. These data suggest that stimulation of the heterotrophic NRB is required for rapid removal of sulfide from oil field-produced waters.
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Keywords: nitrate; nitrate-reducing bacteria; petroleum; sulfate; sulfate-reducing bacteria; sulfide

Introduction

Sulfate-reducing bacteria (SRB) and their activities in oil fields have been studied for many years. At one time, they were shown to be capable of releasing bitumen from oil sands as well as conventional oil from laboratory test columns, but the detrimental effects of SRB far outweigh any positive contribution they could have in oil fields [20]. The major detrimental effect is production of H_2S, which is toxic, causes ''souring'' of oil, and induces corrosion in oil fields. Hydrogen sulfide leads to the production of iron sulfide, which precipitates and reduces oil recovery [7,20,34,36,43].

Besides SRB, oil reservoirs have diverse and active anaerobic microbial populations [33], although there is some doubt as to whether all of the microbes in an oil field are indigenous [33,45]. However, many oil fields that have been subjected to water-flooding, which repressurizes the reservoir by injecting water into the oil-bearing stratum, have many types of bacteria in an ecosystem that allows for production of H_2S [33]. The use of drilling mud and the addition of makeup water to the oil reservoir are ways in which sulfate, needed for H_2S production by SRB, is introduced into oil reservoirs [19].

Because H_2S production is a detriment to oil fields, much effort and expense have been spent to eliminate SRB. The most widely used method for H_2S control is biocide application to the oil reservoir [3,21]. Once bacterial corrosion has been established, high-concentration, long-term biocide treatment is necessary. The amount of biocide required for an oil field waterflood operation, in which produced water is separated and recycled through the oil reservoir, could be more than 100,000 l $year^{-1}$ [21]. Although biocides are useful, they are not always effective in the short- or long term [41]. An alternative method that has been considered to eliminate H_2S and control the activities of SRB in oil reservoirs is treatment with nitrate.

Studies in which nitrate was added to anaerobic wastewater [24,39], oily wastes from ships [30], and oil field-produced waters [8,37] have shown that nitrate stops the production of sulfide. Nitrate stimulates nitrate-reducing bacteria (NRB) that outcompete SRB for electron donors and produce nitrite or nitrous oxide, as illustrated below, which increases the redox potential of the environment (above -100 mV) to inhibit the strictly anaerobic SRB:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \quad (1)$$

Mixed cultures that contained NRB and the redox indicator, resazurin, turned from colorless to pink when the redox of the medium increased as a result of the accumulation of products from nitrate reduction [23,25,41]. Overall, the main advantage of nitrate reduction by NRB is the formation of endproducts that are less harmful than H_2S [56].

Two major types of NRB can be stimulated by the presence of nitrate. One is the chemoorganotrophic (heterotrophic) NRB that use organic compounds as electron donors. As is illustrated in Eqs. (2) and (3), using acetate as an electron donor, nitrate

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reduction yields more energy per mole of terminal electron acceptor than sulfate reduction [51]:

$$5CH_3CO_2^- + 8NO_3^- + 3H^+ \rightarrow 10HCO_3^- + 4N_2 + 4H_2O$$
$$\Delta G^{\circ\prime} = -495 \text{ kJ (mol } NO_3^-)^{-1} \quad (2)$$

$$CH_3CO_2^- + SO_4^= \rightarrow 2HCO_3^- + HS^-$$
$$\Delta G^{\circ\prime} = -47 \text{ kJ (mol } SO_4^{2-})^{-1} \quad (3)$$

Thus, heterotrophic NRB outcompete heterotrophic SRB for electron donors, thereby suppressing sulfide production. Oil field waters contain dissolved organic compounds including short-chain fatty acid anions like acetate, propionate, and butyrate as well as aromatic compounds such as toluene and phenols [1,17,33,36,46] that are substrates for heterotrophs.

The second type of NRB is the chemolithotrophic NRB. Among these are *Thiobacillus denitrificans* and the nitrate-reducing, sulfide-oxidizing bacteria (NR-SOB). If these bacteria are present in the oil field, they will gain energy by oxidizing reduced inorganic sulfur compounds. They are also capable of forming products from nitrate reduction that will raise the redox potential of the environment. As a result, the NR-SOB not only remove sulfide, but also suppress sulfide formation by the SRB [22].

Although several laboratory [8,18,50] and field [14,27,49] studies have demonstrated that nitrate amendment to oil field waters increases the number of NRB and controls sulfide production, the relative roles of the heterotrophic NRB and chemolithotrophic NRB have not been established. In part, this is because the formulations of the media used for their enumeration have not been selective for these two individual types of NRB. That is, culture media used by different research groups often contained nutrients that allowed the growth of both heterotrophic and chemolithotrophic NRB. For example, the medium used by Davidova *et al* [8] contained thiosulfate, an electron donor for some chemolithotrophic NRB, and yeast extract, a potential electron donor for heterotrophic NRB. Similarly, the medium used by Telang *et al* [50] for the enumeration of NR-SOB contained acetate, which would allow the growth of some heterotrophic NRB.

Molecular biology techniques have also been used to monitor changes in oil field microbial communities. For example, two species of NR-SOB were isolated from an oil field water [15]; then the DNA from these isolates was used for reverse sample genome probing (RSGP) [49]. The monitoring method is very specific, and without a DNA standard of a particular heterotrophic nitrate-reducing bacterium, the method is insensitive to the presence of that bacterium in oil field waters.

Eckford and Fedorak [10] used three different types of culture media to enumerate different nutritional types of NRB using most probable number (MPN) methods. The media used for the chemolithotrophic NRB were free of organic components to prevent the growth of heterotrophic NRB, and the medium used for heterotrophic NRB was free of reduced inorganic sulfur species to prevent the growth of chemolithotrophic NRB.

Because no previous study had specifically enumerated the different nutritional types of NRB in nitrate-amended "sour" oil field waters, we used three media formulations [10] to determine which types of planktonic NRB were stimulated in laboratory microcosms. Produced waters from three oil fields were used, and the chemical and bacterial changes in the microcosms were followed over time. In two cases, both the NR-SOB and heterotrophic NRB increased in number and sulfide was removed quickly. In the third case, only the NR-SOB increased in number and the sulfide was removed slowly.

Materials and methods

Produced waters for nitrate amendment

Produced water samples from three oil fields were collected in sterile, anaerobic serum bottles. Some characteristics of these souring oil fields and the samples are summarized in Table 1. Although oil fields P and N are from the same formation, they are from different pools that are about 5 km apart and they are not connected. Further details on sampling and other oil field parameters were reported previously [10]. Oil field P was not being treated with biocides, but oil fields N and C were receiving biocides. The operators at oil field N turned off the biocide feed 1 week prior to sampling to minimize the effects of the biocide on this study, but the operators of oil field C did not stop the biocide feed during sample collection.

The samples were transported on ice to the laboratory and stored at 4°C until the serum bottle microcosms were established to test the effects of nitrate amendment. Samples with elevated sulfide concentrations were chosen for the serum bottle microcosm studies.

Nitrate amendment tests

The nitrate amendment test was designed to observe the chemical and bacterial changes over 38 days following the addition of 10 mM nitrate to oil field waters. For each produced water studied, changes in the nitrate-amended samples were compared to changes in an unamended sample and a sterile control. Microcosms were established within 2 days of obtaining the produced water samples, so MPN values determined on the original water samples were considered the time zero counts in the serum bottle microcosms.

Serum bottles (158 ml) were flushed with N_2, sealed, and then sterilized by autoclaving. To each bottle, 100 ml of produced water sample was transferred aseptically and anaerobically from the sample collection bottles returned from the oil field. Care was taken to avoid adding oil to the microcosms so that the only source of electron donor was dissolved compounds present in the produced waters. No other potential electron donor was added to the microcosms, and all experiments were done without phosphate supplementation. However, no special precautions were taken to

Table 1 Some characteristics of the three western Canadian oil fields that were sampled for this study

Characteristic	P[a]	C	N
Nearest town	Stettler	Coleville	Stettler
Oil-bearing formation	Glauconitic	Bakken	Glauconitic[b]
Field depth (m)	1300	810	1400
Production started in	1994	1951	1992
Water flooding started in	1994	1958	1994
Average water cut (%)	95	95	55
Sampling dates	December 2000	July 2001	May 2001
Source of sample for nitrate amendment	Preinjection site	Free water knockout	Water storage tanks
Sulfide (mM)	0.78	2.7	0.94
Sulfate (mM)	5.9	0.56	4.4

[a]This sample was denoted "Pa3" by Eckford and Fedorak [10].
[b]Also referred to as the Upper Mannville formation [8].

use phosphate-free glassware, and the phosphorus content of the produced waters was not determined. The nitrate-amended and unamended microcosms were prepared in triplicate. For each test, two sterile controls were made from oil field water samples that had been autoclaved on two occasions for 30 min at 121°C and 15 psi.

A 1 M solution of potassium nitrate was prepared using boiled, distilled, deionized water. The solution was sparged with N_2, sealed in a serum bottle, autoclaved, and stored until needed. After the oil field waters were added to the serum bottles, 1 ml of the 1 M potassium nitrate solution was added to the three serum bottles that were designated nitrate-amended. Immediately after the amended, unamended, and sterile controls were prepared, time zero samples were removed for chemical analyses. The serum bottle microcosms were incubated in the dark at room temperature (approximately 21°C) and, at various times, samples were removed for chemical analyses and microbial counts.

Samples were removed from the serum bottle microcosms every few days for chemical analyses until the sulfide was depleted, and then samples for chemical analyses were removed only when samples were taken for the MPN procedures. The MPN analyses were done repeatedly from one of the replicate serum bottle microcosms (chosen at random). For oil field sample P, MPN determinations were done on samples taken from the microcosms at the time of inoculation and after 38 days of incubation. For oil field samples C and N, samples were withdrawn on days 0, 7, 14, 21, 28, and 38 for MPN determinations. Due to the large number of tubes of media required for the MPN methods, the three-tube MPN procedure was done on samples removed from the nitrate-amended and unamended microcosms, but only the 10^{-1} dilution from the sterile control was inoculated into the four different media to ensure sterility.

Microbial counting methods and analytical methods

The three-tube MPN procedure was done using heterotrophic NRB medium for heterotrophic NRB [9], S8 medium for thiosulfate-oxidizing NRB [9], modified CSB medium for NR-SOB [10], and modified Butlin's medium for SRB [10]. The appropriate amount of NaCl was added to each medium to match the chloride concentration in each produced water sample [10]. Briefly, the heterotrophic NRB medium contained nutrient broth as the electron donor, the S8 medium contained thiosulfate as the electron donor, and the modified CSB medium contained sulfide as the electron donor and was devoid of acetate. Modified Butlin's medium for SRB contained lactate as the electron donor. The inoculated tubes were incubated for 30 days at room temperature in the dark. The MPN tubes were scored positive for growth of (a) heterotrophic NRB if nitrate was consumed and nitrous oxide was produced in the heterotrophic NRB medium [9,12]; (b) thiosulfate-oxidizing NRB if nitrate was consumed or nitrite formed in the S8 medium [9]; and (c) SRB if the iron nails in the medium turned black from the formation of FeS [11]. Growth of NR-SOB in the modified CSB medium was scored by two methods based on color changes in the medium. This medium contained resazurin, a redox indicator, which was oxidized from colorless to pink by the formation of nitrous oxide by the NRB [25]. Scoring the MPN tubes on the basis of the appearance of the pink color is referred to as method A. In some instances, after 30 days of incubation, the medium in lower dilution MPN tubes was pink, and the medium in some of the next higher dilutions was yellow. This was most evident in the enumerations of samples from oil field N. With extended incubation (up to 5 months), the medium in most of the tubes that were yellow turned pink. Scoring the MPN tubes positive on the basis of the appearance of either the pink or yellow color after 30 days of incubation is referred to as method B. The MPN results were compared by the statistical method of Cochran [6].

With the exception of the first sample enumerated for NR-SOB (the time zero sample from oil field P), all of the inoculated tubes of modified CSB medium were incubated in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) to ensure that O_2 from air could not cause the redox indicator to oxidize. *Arcobacter* sp. strain FWKO B and *Thiomicrospira* sp. strain CVO, which are known NR-SOB [15], were obtained from Dr. G. Voordouw's laboratory (University of Calgary) and these were used as positive controls for the modified CSB medium.

The oil field water samples and samples taken from the serum bottle microcosms were analyzed for sulfide concentration using a kit purchased from CHEMetrics (Calverton, VA). An alkaline sodium nitroprusside spot test [13] was used to detect sulfide in the MPN cultures. Chloride, sulfate, and nitrate concentrations were determined by ion chromatography [9], and nitrite was determined by a colorimetric method [5].

Results

Each of the produced water samples used for this study had elevated sulfide concentrations and sulfate available for SRB (Table 1) — conditions that are characteristic of souring oil fields. In addition, each sample contained heterotrophic NRB, NR-SOB, and SRB (Table 2). No thiosulfate-reducing NRB were detected in any of these samples. Work with the produced water P was done while the method for enumerating NR-SOB was first being implemented in our laboratory. After 30 days of incubation, some MPN cultures turned pink, but scoring these tubes as positive did not yield utilizable MPN indices. Thus, the medium in each tube was tested

Table 2 Summary of bacterial numbers, nitrate reduction rates, and depletion of sulfide in nitrate-amended microcosms with produced waters from three different oil fields

Parameter	Oil field		
	P	C	N
Initial counts (MPN ml^{-1})			
Heterotrophic NRB	7.5	4.3×10^{2}	2.3×10^{4}
NR-SOB	1.5×10^{3}[a]	2.1×10^{5}[b,c]	9.3×10^{1}[b] 2.3×10^{4}[c]
SRB	2.3×10^{3}	9.3×10^{2}	2.3×10^{3}
Maximum counts (MPN ml^{-1})			
Heterotrophic NRB	4.3×10^{5}	9.3×10^{6}	4.3×10^{4}
NR-SOB	4.3×10^{6}	9.3×10^{7}	4.3×10^{4}[b] 2.3×10^{7}[c]
Maximum increase in			
Heterotrophic NRB MPN	57,000-fold	22,000-fold	0-fold[d]
NR-SOB MPN	2,900-fold[a]	440-fold[b,c]	460-fold[b] 1,000-fold[c]
Initial nitrate reduction rate (mM day^{-1})	0.68	1.4	0.4
Sulfide depleted by day	<4	3	27

[a]MPN value from nitrite analysis.
[b]MPN value determined by method A.
[c]MPN value determined by method B.
[d]No statistical increase in MPN ($P<0.05$).

for nitrite, and those tubes that contained nitrite were scored positive. The nitrite analyses were used as the basis for MPN results given for the initial NR-SOB MPN count in sample P in Tables 2 and 3. With additional experience enumerating NR-SOB in other samples, we found that MPN values based on the appearance of nitrite were essentially the same as those determined by method A (resazurin turning pink) [10].

Nitrate amendment in produced water from oil field P

The sample used for nitrate amendment studies was a "comingled" water from a preinjection site. It was a mixture of produced water and source water taken just before reinjection into the oil reservoir. The sample had a sulfide concentration of 0.78 mM (Table 1), and the initial number of NR-SOB was greater than the number of heterotrophic NRB (Table 2). In the nitrate-amended microcosms, no sulfide was detected on day 4 (Figure 1a). Nitrate dropped from 10 mM at time zero to below detection by day 14 (Figure 1a), and the rate of nitrate loss taken from the linear portion of the nitrate loss curve was 0.68 mM day^{-1}. Nitrite was detected transiently in the nitrate amendment microcosm (Figure 1a), indicating that nitrate reduction had occurred. The sulfate concentration increased by 0.8 mM in the amended sample while the sulfide decreased by 0.78 mM, consistent with sulfide oxidation.

In contrast, the unamended microcosms showed a near stoichiometric reduction of sulfate to sulfide with a loss of 5.8 mM sulfate and gain of 5.6 mM sulfide (Figure 1b). Neither nitrate nor nitrite was detected in these unamended microcosms. The sterile control showed no change in sulfate or sulfide concentrations, and no nitrate or nitrite was detected over the 38-day testing period (Figure 1c). Sterile controls for the other two oil fields showed the same pattern as Figure 1c; thus, data from the other sterile controls are not presented. Samples taken from the sterile controls yielded no growth in any of the MPN tubes.

Data plotted in Figure 1 are the means obtained from triplicate microcosms, and in most cases, the error bars were smaller than the plotted symbol, indicating excellent reproducibility among the replicates. These data clearly show that nitrate amendment stopped sulfate reduction and contributed to the removal of sulfide originally in the produced water.

There were populations of heterotrophic NRB and NR-SOB in this water (Table 3). On day 38, samples were taken from the nitrate-amended and the unamended microcosms. There was a marked increase in both NRB populations over the 38-day incubation (Table 3), with increases in heterotrophic NRB and NR-SOB of about 57,000- and 2,900-fold, respectively (Table 2). These increases were consistent with the depletion of nitrate from the microcosms (Figure 1a). The unamended microcosm showed a slight increase in the heterotrophic NRB population ($P<0.05$) and no increase in the NR-SOB population ($P<0.05$) (Table 3). The SRB population showed a slight increase ($P<0.05$) in the amended microcosm and no change in the unamended sample in 38 days. Jack and Westlake [21] also observed an increase in the number of SRB after 1.6 mM nitrate was added to a field test facility. In their study, the number of SRB increased 100-fold over 30 days.

Table 3 Bacterial counts in the produced water sample from oil field P

Bacterial types	Initial counts (MPN ml^{-1})	Counts after 38 days incubation (MPN ml^{-1})	
		Nitrate-amended	Unamended
Heterotrophic NRB	7.5	4.3×10^{5}	9.3×10^{1}
NR-SOB	1.5×10^{3}[a]	4.3×10^{6}[b]	3.9×10^{3}[b]
SRB	2.3×10^{3}	2.3×10^{4}	2.3×10^{3}

[a]MPN value from nitrite analysis.
[b]MPN value determined by method A.

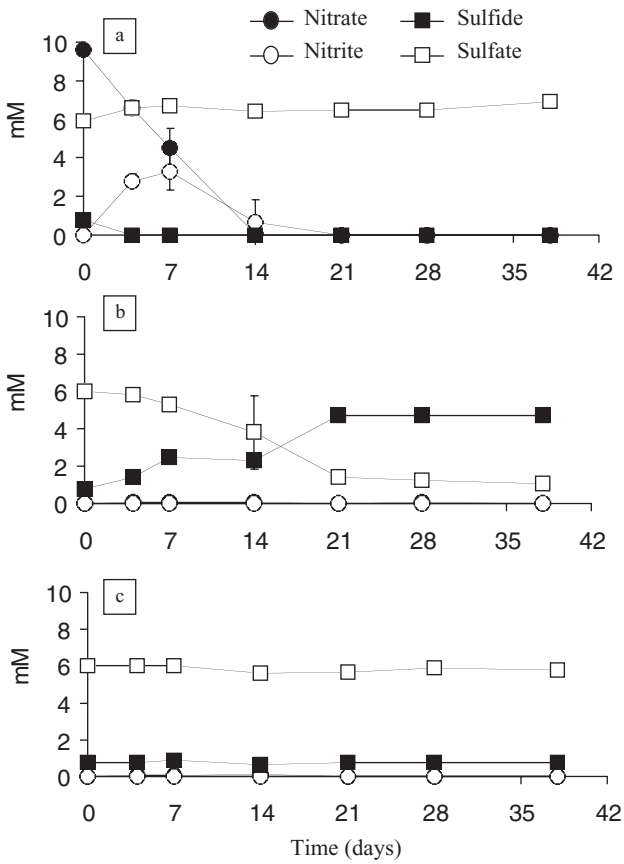

Figure 1 Chemical analyses of microcosms that contained produced water from oilfield P: nitrate-amended (a), unamended (b), sterile control (c). The plotted values are means of three replicates. The error bars, which are often smaller than the size of the symbols, show 1 SD.

Nitrate amendment in produced water from oil field C

We chose to study a produced water sample from the free water knockout at oil field C because several other studies have described NR-SOB from these waters [15,27,49,50], and the oil field has a severe souring problem. The chemical analyses showed that there were some differences among the triplicate nitrate-amended microcosms over the incubation period. The results shown in Figure 2a are from the microcosm that was sampled for bacterial enumerations. Initially, this microcosm contained 2.7 mM sulfide, which increased to 3.1 mM by day 1 and then dropped below detection by day 3 in the nitrate-amended microcosm (Figure 2a). The rate of nitrate consumption over the first 3 days was 1.4 mM day^{-1}. The nitrate concentration then remained at about 5–6 mM for the rest of the incubation. The sulfate concentration increased noticeably over the first 14 days of incubation, with a total increase of 3.5 mM by day 38, closely matching the 3.1 mM decrease in sulfide. The nitrite concentration was at a maximum of 1.8 mM on day 3 and then gradually decreased to 0.2 mM by day 38.

Chemical analyses of samples from the other two nitrate-amended microcosms that contained produced water from oil field

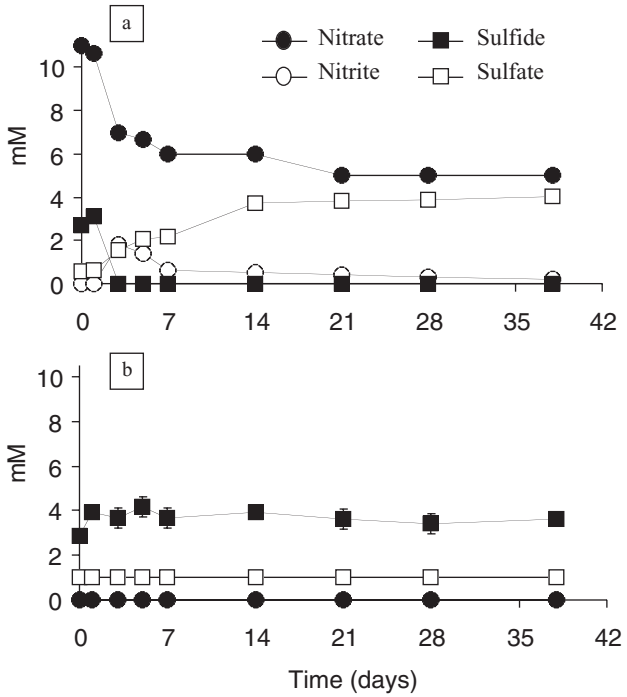

Figure 2 Chemical analyses of a microcosm that contained produced water from oilfield C: nitrate-amended (a), unamended (b). See text for discussion of the results of the other two replicate microcosms.

C showed a slight increase in sulfide over the first few days of incubation, and then a complete loss of sulfide as observed in Figure 2a. In one of these microcosms, the sulfate concentration remained stable at 0.6 mM during the 38-day period, and after a drop in nitrate concentration during the first 8 days of incubation, the nitrate concentration remained at 9 mM for the duration of the incubation. No nitrite was detected in this microcosm. In the third microcosm, there was a rapid decrease in nitrate over the first 3 days, followed by a gradual decrease in nitrate resulting in a final concentration of 4.5 mM on day 38. This was accompanied by an increase in nitrite concentration to 4 mM by day 38. Sulfate accumulated in this microcosm, as was observed in Figure 2a, with the final concentration being 3 mM. The reasons for the discrepancies among these triplet microcosms are unknown, and these were the only three microcosms in the entire study that showed this high variability. The sample from oil field C was the only sample collected while biocide was being injected into the produced water, and the presence of the biocide may have influenced the results. Nonetheless, all three nitrate-amended microcosms demonstrated nitrate consumption and sulfide removal.

There was little variability among the three unamended microcosms, so chemical parameters in Figure 2b are the means of the triplicate microcosms. The sulfide increased to 4 mM by day 5, and sulfate remained fairly steady at 0.68–0.54 mM throughout the testing period. Neither nitrate nor nitrite was detected in the microcosms.

Bacterial enumerations were done on samples from one nitrate-amended and one unamended microcosm at intervals of 7–10 days. The MPN results are shown in Figure 3. Initially, the number of NR-SOB (2.1×10^5 ml^{-1}) was much greater than the number of heterotrophic NRB (4.3×10^2 ml^{-1}) (Table 2). There was no increase in the number of heterotrophic NRB (Figure 3a) or NR-SOB (Figure 3b) in the unamended microcosm that contained produced water from oil field C. In contrast, there was a rapid increase in the number of heterotrophic NRB and NR-SOB by day 7 in the amended microcosm (Figure 3a and b). The number of heterotrophic NRB and NR-SOB increased 22,000- and 440-fold, respectively. These proliferations occurred during the time when nitrate consumption was the most rapid, and sulfide was depleted from the microcosm (Figure 2a). On day 7, the number of heterotrophic NRB and NR-SOB was 9.3×10^6 and 9.3×10^7 ml^{-1}, respectively. Over the remainder of the incubation, the heterotrophic NRB number remained high, whereas the NR-SOB number dropped to near their original count (Figure 3a and b). The SRB number did not change in the amended microcosm and showed a slight increase in the unamended microcosm with a maximum on day 7 (Figure 3c).

Nitrate amendment in produced water from oil field N

The water sample for oil field N was taken from the outlet of the storage tanks, just before reinjection into the reservoir. The initial sulfide concentration in these microcosms was 0.94 mM (Figure 4a). Nitrate consumption was observed over the first 13 days, and the utilization rate was 0.4 mM day^{-1}. Unlike the results from the other two oil field waters, sulfide persisted until after day 20, when it decreased to below detection on day 27 (Figure 4a). The sulfate concentration remained stable between 4 and 5 mM, and nitrite was only detected on one occasion (day 3).

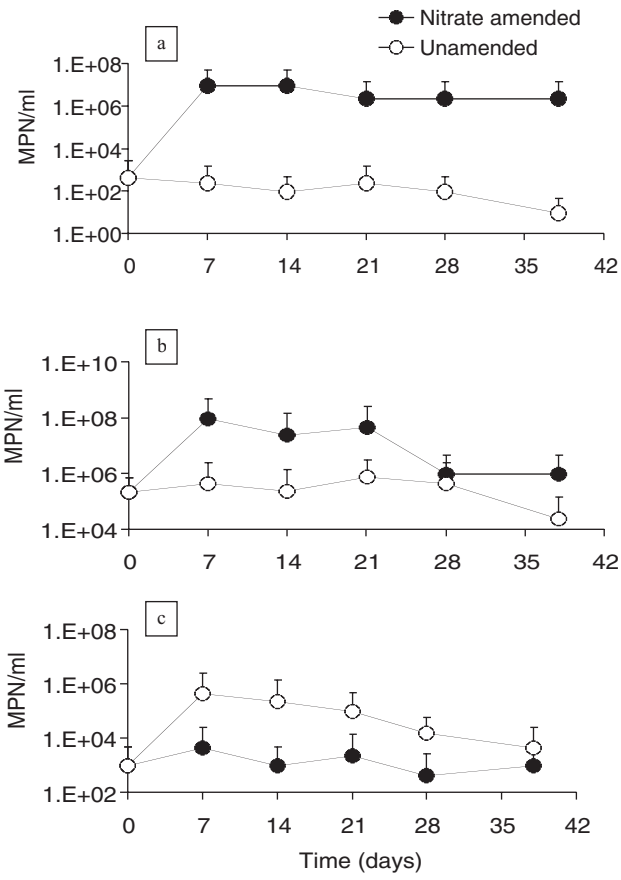

Figure 3 Heterotrophic NRB (a), NR-SOB (b), and SRB (c) counts in samples from a microcosm that contained produced water from oil field C. The NR-SOB MPN values were determined by method A, and the error bars show the 95% confidence intervals.

In the unamended microcosms, there was a rapid decrease in sulfate between days 3 and 9 (Figure 4b). This decrease of 4.4 mM sulfate was accompanied by an increase of about 5 mM sulfide. Neither nitrate nor nitrite was detected in these unamended microcosms. The results in Figure 4 again illustrate that nitrate amendment controls sulfide formation.

As shown in Table 2, the produced water from oil field N initially contained 2.3×10^4 heterotrophic NRB ml^{-1} and 93 NR-SOB ml^{-1} (based on enumeration method A). The increase in the NRB population in the nitrate-amended microcosms was much lower than was seen in the nitrate amendment tests for oil fields P and C. The number of heterotrophic NRB in the amended microcosms was the same ($P<0.05$) as those in the unamended microcosms (Figure 5a) until day 38. There was no statistically significant increase in the number of heterotrophic NRB during this incubation (Table 2), whereas the number of NR-SOB increased 460-fold ($P<0.05$, based on method A) during the first 7 days of the incubation (Figure 5b) and remained between 4.3×10^2 and 4.3×10^4 ml^{-1} over the duration of the experiment. Although the numbers of NR-SOB in the nitrate-amended microcosm were also statistically ($P<0.05$) higher than those in the unamended microcosm on days 21 and 38, the MPN values were quite similar in the two sets of microcosms (Figure 5b). There was an increase in the number of SRB during the first 7 days of incubation of the unamended microcosm (Figure 5c), during the rapid consumption of sulfate (Figure 4b). This increase was not observed in the amended microcosm (Figure 5c).

Color changes in the serum bottles and the modified CSB medium

Generally, the liquid in the unamended microcosms was colorless or black and it did not change over the incubation period. A black precipitate, which was presumably iron sulfide formed as a result of sulfide production by the SRB, was observed in the unamended microcosms at the end of the incubation period.

A transient yellow color appeared in the liquid of the nitrate-amended microcosms that contained oil field waters C or N. These liquids then turned to grey or brown as the sulfide was being removed. For example, the nitrate-amended microcosms that contained produced water from oil field N turned yellow on day 2, lost this color on day 3, and then turned yellow again on day 13, remaining yellow until day 20. The water in these microcosms then turned grey and remained grey until the end of the experiment. This transient yellow color has been observed by others who carried out studies with nitrate amendment microcosms [14,23], and the yellow color was attributed to the formation of polysulfides [23].

As expected from the literature [26,50], many of the inoculated tubes of modified CSB used for the MPN determinations turned pink as the resazurin was oxidized by nitrous oxide produced by the NR-SOB [25,26,50]. However, some of the culture medium turned yellow. For example, after 30 days of incubation, many culture tubes of modified CSB medium inoculated with dilutions of the produced water from oil field N were pink. Several tubes of medium at the higher 10-fold dilutions of the sample were yellow.

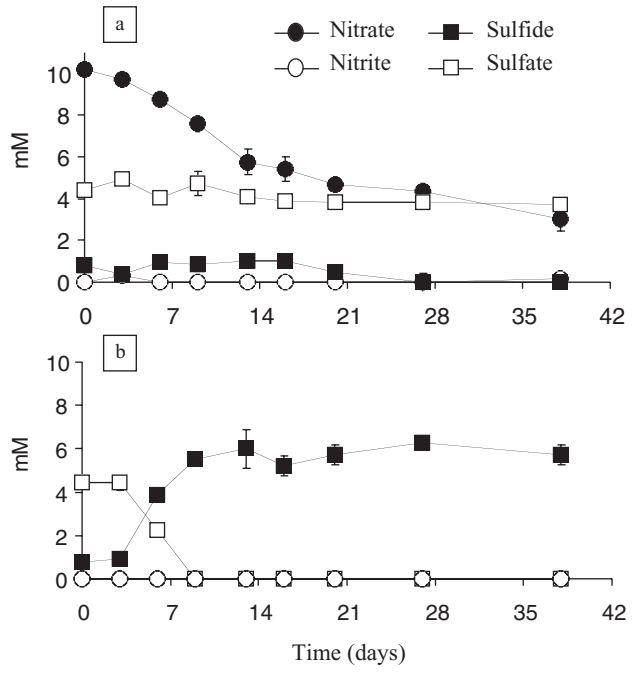

Figure 4 Chemical analyses of microcosms that contained produced water from oilfield N: nitrate-amended (a), unamended (b). The plotted values are means of three replicates. The error bars, which are often smaller than the size of the symbols, show 1 SD.

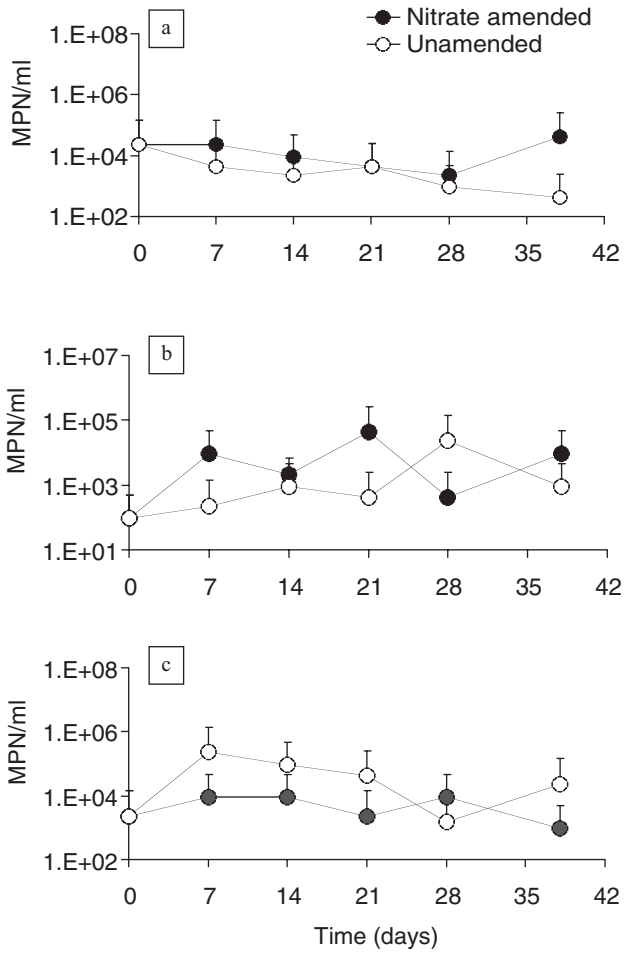

Figure 5 Heterotrophic NRB (a), NR-SOB (b), and SRB (c) counts in samples from a microcosm that contained produced water from oil field N. The NR-SOB MPN values were determined by method A, and the error bars show the 95% confidence intervals.

These tubes were incubated longer to see if the cultures would turn pink. By 2 months, many of the MPN tubes that were yellow had turned pink. At 5 months, the majority of the tubes with yellow medium had turned pink. These cultures were then tested for sulfide consumption. The medium that turned from yellow to pink was devoid of sulfide. Similarly, no sulfide was detected in any of the tubes in which the medium turned pink during the first 30 days of incubation. In freshly prepared modified CSB medium, nitrate is abundant and sulfide (the electron donor) is the limiting nutrient [10]. Thus, the depletion of sulfide is indicative of the growth of NR-SOB. These findings prompted us to calculate MPN values by method B, which considered tubes to be positive for the growth of NR-SOB if the medium was pink or yellow after 30 days of incubation.

Figure 6 compares the NR-SOB MPN results obtained by applying methods A and B to samples from microcosms that contained produced water from oil field N. Data from the unamended and nitrate-amended serum bottles are included. The MPN values determined by method B were always higher than those determined by method A. In general, MPN results from method B gave values that were 100- to 10,000-times higher than MPN results from method A. Indeed, the time zero count, which was simply the NR-SOB counts in the produced water sample collected from oil field N, was 2.3×10^4 ml^{-1} by method B and only 93 ml^{-1} by method A.

Among the samples from the nitrate amendment experiments with produced water from oil field C, only one tube of modified CSB turned yellow, and from oil field P, only two tubes of medium turned yellow. For these two water samples, there was no significant difference ($P<0.05$) between the MPN values for the NR-SOB numbers determined by method A or B.

The MPN results determined by method B (Figure 6, solid symbols) showed that there was a rapid 1000-fold increase in NR-SOB number during the first 7 days of incubation and this elevated count remained high until after day 14, when the numbers dropped to the same levels as those in the unamended microcosm. The most rapid decrease in nitrate concentration also occurred during this 14-day period (Figure 4a).

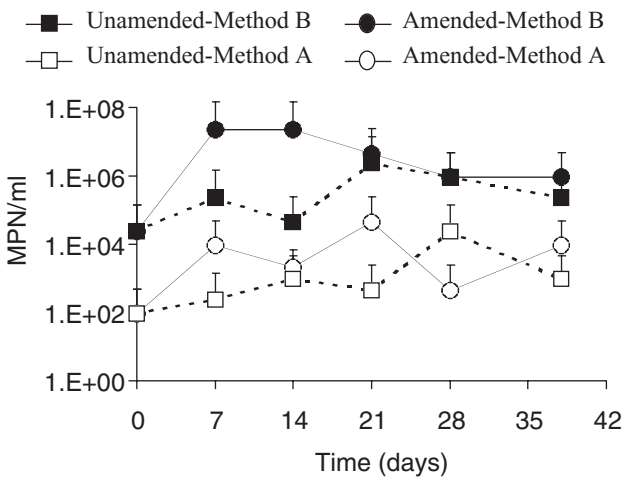

Figure 6 Comparison of NR-SOB MPN values determined by methods A and B, and the error bars show the 95% confidence intervals. Samples were taken from microcosms that contained produced water from oil field N.

Discussion

Any method used to study the composition of a microbial community has its limitations. Madsen [32] recently reviewed many of the major nucleic acid-based methods used for characterizing naturally occurring microorganisms, and he listed limitations for each procedure. No method that relies on cultivation of microorganisms, such as the MPN methods used in this study, can detect all of the microorganisms present in an environmental sample [16]. We used established media formulations, with little or no change to their compositions, for our enumerations. The medium for SRB contained lactate as an electron donor. Thirty-nine of the 54 species (72%) of SRB listed by Stackebrandt *et al* [47] grow on lactate, which is used in the medium recommended by the American Petroleum Institute for the enumeration of SRB [35]. S8 medium is recommended by the American Type Culture Collection for the growth of thiobacilli. The medium and method used to enumerate heterotrophic NRB are standard procedures used for soil analysis [52]. We used only one-half of the concentration of nutrient broth in this medium to make it more suitable for water samples, and comparisons of counts obtained with the half-strength and full-strength media showed that there were no differences in the MPN values (unpublished results). The CSB medium used by others [50] was modified by omitting acetate to make the medium more selective for the chemolithotrophic NR-SOB.

Previous research investigated the presence of NRB and the use of nitrate to control sulfate reduction in produced waters from oil fields C [15,27,28,49] and N [8,10]. Although the number of NRB in oil field P has been reported [10], no previous studies on controlling sulfide production with nitrate amendment to produced waters from this oil field have been done.

Regardless of the source of the produced water, each microcosm was amended with 10 mM nitrate in our study. This was the concentration used by Davidova *et al* [8] in their studies with samples from oil field N. Various researchers have used different concentrations of nitrate to inhibit sulfate reduction in laboratory studies. For example, Londry and Suflita [30] reported that 16 mM nitrate prevented sulfide accumulation in microcosms that contained oily sludge wastes. Five millimolar nitrate was sufficient to remove sulfide from produced waters from an oil field in Oklahoma [8] and from oil field C [14]. In their studies of four west Texas oil fields, Wright *et al* [55] amended serum bottle cultures with 40 mM nitrate to stimulate sulfide removal. In our study, amendment with 10 mM nitrate was sufficient to control sulfate reduction and remove existing sulfide from each of the three produced waters (Figures 1, 2 and 4). Only microcosms with produced water from oil field P (Figure 1a) consumed all of the nitrate over the duration of the incubations. After 38 days of incubation, there was 4–9 mM nitrate in the serum bottles that contained produced water from oil field C, and 4 mM in the microcosms that contained produced water from oil field N. These results show that there are differences in the amount of nitrate consumed by planktonic microorganisms in waters from different oil fields. Of course, the actual nitrate concentration required in the water handling facilities at an oil field will likely be higher if biofilms have formed in the pipes and storage tanks. Typically, higher concentrations of biocides are required to control microbial activities in biofilms relative to planktonic microorganisms [44], and the same is likely true for nitrate treatment.

Some investigators have supplemented oil field waters with both nitrate and phosphate to stimulate microbial sulfide control

[49,55]. We chose to add only nitrate, which would decrease the costs of amendment in the oil field, and we observed that sulfide removal occurred in all three produced waters without added phosphate.

Laboratory studies with *T. denitrificans* strain F, a sulfide-tolerant strain of NRB, showed that its growth could control biogenic sulfide production [37,48]. Thus, we screened oil field waters for this type of autotrophic NRB, but none was detected in any of the samples taken from the microcosms. The S8 medium used in our studies supported the growth of strain F [10] and *T. denitrificans* ATCC 23642 [9]. These results indicate that the three oil field waters did not contain nitrate-reducing thiobacilli that contributed to sulfide removal.

Several studies estimated the number of NR-SOB in oil field waters using an MPN method, which relies on the oxidation of resazurin by microbially produced nitrous oxide. This turns the medium pink, and the culture tube is then scored positive for growth of NR-SOB. Oil field C has been studied extensively by this method [14,26,27,50], and Telang *et al* [50] enumerated NR-SOB in five additional oil fields using this method. None of the previous publications has reported the MPN medium as having a transient yellow color as we observed with samples of oil field N produced water. After the yellow medium turned pink, we verified the consumption of the sulfide from the modified CSB, indicative of the presence of NR-SOB and justifying the use of method B for enumerating the MPN tubes.

The fact that samples of the produced water from oil field N behaved very differently in the modified CSB medium suggests that a different type of NR-SOB exists in this oil field-produced water. The NR-SOB in oil field C appear to produce nitrous oxide (which oxidizes the resazurin) much more quickly than the NR-SOB from oil field N. The two NR-SOB that have been described in detail, strains CVO and FWKO B [15], were isolated from oil field C, and they do not produce the transient yellow color in the modified CSB medium.

Loka Bharathi *et al* [29] isolated over 100 strains of anaerobic colorless NR-SOB from seawater and a sulfide-rich creek. They presented data showing that different isolates oxidized sulfide at different rates. For example, one isolate oxidized all of the sulfide in the medium within 9 days, whereas another isolate oxidized only 2.9% of the sulfide in the same time. Thus, it is likely that the NR-SOB in the produced water from oil field N oxidize sulfide at a slower rate than the NR-SOB from oil field C. The slower rate of sulfide oxidation would yield a slower rate of nitrate reduction to nitrous oxide. Consequently, the NR-SOB from oil field N would take a longer time to turn the MPN medium pink than those NR-SOB from oil field C. Indeed, this is what we observed.

The different response of the NR-SOB from oil field N in the CSB medium suggests that these bacteria grow more slowly than those from oil field C. This slower growth may have contributed to the low initial nitrate reduction rate in the microcosms that contained water from oil field N (Table 2) and the slower depletion of sulfide from these microcosms (Table 2). The NR-SOB from oil field N may be more sensitive to soluble organics that are known to sometimes inhibit chemolithotrophs, or they might be inhibited by sulfide concentrations in the produced water and modified CSB medium.

Very few other oil fields have been studied for the presence of NR-SOB. Using molecular biology techniques, Voordouw *et al* [53] detected sulfide oxidizers in several oil field waters from western Canada, but the growth characteristics of these bacteria were not determined. Using a MPN method with acetate-containing CSB medium, Telang *et al* [50] detected NRB in five of the six oil fields that they sampled.

Our work is the first investigation to specifically monitor the changes in number of heterotrophic NRB and NR-SOB in nitrate-amended oil field waters. Some studies have monitored changes in the NR-SOB population size by RSGP [38,49,50], whereas other studies have used culture methods with a medium that was not selective for a particular nutritional type of NRB because it contained reduced sulfur species along with organic compounds from filter-sterilized oil field brine [14,27], yeast extract [8], or acetate [50]. Many simple hydrocarbons, such as benzene, toluene, ethylbenzene, *m*-xylene, naphthalene, and C_6–C_{12} alkanes that will dissolve in produced waters can be degraded by heterotrophic NRB [4,54]. Acetate and other short-chain fatty acids are common in oil field waters [1,33] and these are known to serve as a substrate for heterotrophic nitrate reduction [2]. Our modified formulation of CSB medium lacked acetate and contained only inorganic compounds to select for chemolithotrophic NR-SOB.

Bacterial counts on samples from the microcosms that contained produced water from oil field P were done only twice (Table 3). These showed large increases in numbers of heterotrophic NRB (57,000-fold) and NR-SOB (2,900-fold) after 38 days of incubation (Table 2). To gain a better understanding of the community dynamics, bacterial counts were done on six occasions over the 38-day incubation of microcosms with waters from the other two oil fields (Figures 3 and 5). The initial MPN of NR-SOB in produced water from oil field C was 2.1×10^5 ml^{-1} (Table 2), determined using modified CSB medium. Previously, the number of "NR-SOB" has been determined with medium containing filter-sterilized brine from this oil field or acetate. Using filtered brine, the values were about 10^4–10^5 ml^{-1} at injection wells and <10 ml^{-1} at oil-producing wells [27]. Using acetate-containing medium, the number of "NR-SOB" was reported to be 10^6 ml^{-1} [50]. These counts were typically higher than what we observed with our organic-free modified CSB medium.

Figure 3a and b shows that the increases in NRB number occurred during the first 7 days of incubation, with the numbers of heterotrophic NRB increasing 22,000-fold while the increase in NR-SOB was only 440-fold (Table 2). A portion of the reservoir in oil field C was experimentally amended with nitrate in 1996 [27,28], and during nitrate amendment, the number of NRB enumerated in filter-sterilized brine often exceeded 10^8 ml^{-1} [27]. The highest count of NR-SOB observed in our nitrate-amended microcosm from this oil field was 9.3×10^7 ml^{-1} (Table 2), in reasonable agreement with the observed field data after nitrate amendment.

Telang *et al* [49] collected produced water samples from oil field C before and after nitrate amendment to follow the changes in the microbial community by RSGP. Their master filter had DNA from 47 bacterial isolates including the NR-SOB strain CVO, 26 different SRB, and three heterotrophic bacteria that reduced nitrate to nitrite. By subjecting the total DNA extracted from the produced water to RSGP, they concluded that isolate CVO became the dominant community member immediately after nitrate injection, and that no significant enhancement of other community members, including SRB, was observed [49]. Our data from the nitrate-amended microcosm that contained produced water from oil field C also showed an increase in the numbers of NR-SOB (from 2.1×10^5 to 9.3×10^7 ml^{-1}) during the first 7 days of incubation

(Figure 3b) and no increase in the numbers of SRB (Figure 3c), in agreement with the previous study [49].

Telang *et al* [49] stated that, based on the RSGP analysis, none of the three heterotrophic isolates that produced nitrite from nitrate showed a strong increase as a result of nitrate amendment. They wrote, "It thus appears that of the community members represented on the filter, CVO is the primary benefactor from nitrate addition." Similarly, no increase in heterotrophic NRB was reported in nitrate-amended serum bottle experiments using produced water from oil field C and the same RSGP analysis [38]. In contrast, our results showed that the number of heterotrophic NRB increased sharply during the first 7 days of incubation (from 4.3×10^2 to 9.3×10^6 ml^{-1}) as shown in Figure 3a. Although the number of heterotrophic NRB on day 7 was only one-tenth that of the number of NR-SOB, nitrate amendment caused a larger increase in the number of heterotrophic NRB (a 22,000-fold increase) than the NR-SOB (a 440-fold increase) (Table 2). Interestingly, the number of heterotrophic NRB remained high over the duration of the incubation (Figure 3a), whereas the number of NR-SOB decreased after 21 days (Figure 3b).

The RSGP method is limited by the types of DNA standards that are spotted onto the master filter. For example, if the DNA from the heterotrophic NRB that proliferated in our microcosms did not hybridize with the DNA of the three "standard" heterotrophic nitrate reducers, then the increase in population of heterotrophic NRB could not be detected by the RSGP method. The MPN method that we used is a more general approach, which detects any heterotrophic NRB that are culturable in the heterotrophic NRB medium under the incubation conditions that were used in this study. Our results (Figure 3a) clearly demonstrated that the heterotrophic NRB also benefited from nitrate addition to the water from oil field C.

The ability of nitrate amendment to stimulate and increase the number of NRB in microcosms that contained produced water from oil field N was demonstrated by Davidova *et al* [8]. However, the MPN medium that they used was different from the media used in our study, and it is not clear which group of NRB proliferated in that study, resulting in a 570-fold increase in number after 42 days of incubation [8]. In that study, there was no increase in the numbers of SRB in the nitrate-amended microcosms, consistent with the results presented in Figure 5c.

Sulfide was present until between days 20 and 27 in the nitrate-amended microcosms that contained produced water from oil field N (Figure 4a), in sharp contrast to the 3–4 days in the microcosms that contained samples from oil fields P and C (Table 2). Davidova *et al* [8] observed active nitrate reduction in nitrate-amended microcosms that contained produced water from oil field N, and sulfide persisted at near 0.3 mM in their microcosms for 9 weeks. Similarly, an incubation time of nearly 14 weeks was required before the sulfide concentration in nitrate-amended microcosms that contained produced water from an oil field in Oklahoma decreased from approximately 4 mM to below detection limit [8]. Thus, although the activities of NRB control the net production of sulfide in produced waters, nitrate reduction does not always result in rapid sulfide consumption (for example, see Figure 4).

Voordouw *et al* [53] presented a model for an anaerobic sulfur cycle that might exist in an oil field. The cycle may be driven by the diffusion or convection of nitrate from surface layers into a reservoir. Nitrate provides an electron acceptor for the NR-SOB that oxidize sulfide to sulfate. This sulfate serves as an electron acceptor for SRB that use H_2, organic acids, or hydrocarbons as electron donors to reduce the sulfate back to sulfide, thereby completing the sulfur cycle [53].

Assuming that the media formulations that we used would grow and clearly distinguish different types of NRB, the results presented in Figure 4a appear to support the notion of anaerobic sulfur cycling in these microcosms that contained an abundant supply of nitrate. For the first 20 days, the concentrations of sulfate and sulfide in the water from oil field N remained nearly constant, while there was a decrease in nitrate concentration. These observations suggest that NR-SOB consumed nitrate and produced sulfate, which was reduced back to sulfide by the SRB. Figure 6 shows that the number of NR-SOB increased substantially during the first week of incubation, presumably because of the added source of nitrate. In addition, there was no increase in the number of heterotrophic NRB (Figure 5a), suggesting that they were not responsible for the decrease in nitrate concentration. Furthermore, the produced water in these microcosms turned yellow on day 2, lost this color on day 3, and became yellow again on day 13 and remained yellow for 7 days thereafter. Others have attributed the yellow color in nitrate-amended microcosms to polysulfides [23]. The transient presence of polysulfides would indicate that transformations of inorganic forms of sulfur occurred, while the sulfate and sulfide concentrations remained essentially constant, consistent with sulfur cycling. Although we propose that the SRB were active in these nitrate-amended microcosms, no increase in their numbers was observed (Figure 5c). However, it is possible that there were increases in the numbers of SRB and heterotrophic NRB in the nitrate-amended microcosms, but the MPN media used for their enumeration may not have had the correct formulations to detect these types of NRB and SRB.

For the above scenario for anaerobic sulfur cycling (Figure 4a) to be feasible, the predominant electron donor used by the SRB in the produced water from oil field N could not serve as an electron donor for the heterotrophic NRB; otherwise, the heterotrophic NRB would have a thermodynamic advantage (e.g., Eqs. (2) and (3)) and they would outcomplete the SRB for that electron donor. When the electron donor used by the SRB became depleted in these batch cultures, sulfate reduction would stop, but sulfide oxidation would continue because nitrate and sulfide were still available. This would lead to the depletion of the sulfide. The results in Figure 4a suggest that the electron donor for the SRB was consumed after about 20 days of incubation because sulfide was not detected on day 27. The nature of the electron donor in these microcosms is unknown.

The number of NR-SOB was stimulated by 440- to 2900-fold in the nitrate-amended microcosms that contained the three different oil field samples used in this study (Table 2). Nitrate addition to waters from oil fields P and C caused a much larger increase in the numbers of heterotrophic NRB, which were stimulated 57,000- and 22,000-fold, respectively (Table 2). These gave initial nitrate reduction rates of 0.68 and 1.4 mM day^{-1}, respectively. The only microcosm in which there was no increase in heterotrophic NRB was that which contained produced water from oil field N (Table 2, Figure 5a). In addition, this sample gave the lowest initial nitrate reduction rate of 0.4 mM day^{-1} (Table 2) and the longest time before sulfide depletion (approximately 27 days). These observations suggest that the activities of heterotrophic NRB play a key role in the overall removal of sulfide from produced waters, and that when only the NR-SOB are stimulated, sulfide persists for a longer time, as was observed with produced water from oil field N. These occurrences may be related to the presence of electron donors that can be used by both the heterotrophic NRB

and the SRB. Under this condition, the heterotrophic NRB would outcompete the SRB for the electron donor (based on thermodynamic considerations shown in Eqs. (2) and (3)), thereby stopping sulfate reduction. This would terminate the anaerobic sulfur cycle because the sulfide that was oxidized to sulfate by the NR-SOB would not be reduced back to sulfide by the SRB. Thus, the sulfide would be quickly depleted from the produced waters as observed in Figures 1a and 2a. In their survey of produced waters from oil field C, Gevertz *et al* [14] observed sulfide removal within 2 days in five of the six sample locations studied. We did not take a sample from our microcosms on day 2, but by day 3, the sulfide was depleted from the produced water from oil field C (Figure 2a). Thus, our data agree with those of Gevertz *et al* [14].

Without specifically enumerating heterotrophic NRB, Wright *et al* [55] showed the importance of heterotrophic activity in sulfide removal. They studied waters from four west Texas oil fields to determine which amendments were required to stimulate sulfide removal. In two of the samples, addition of nitrate and phosphate was not sufficient to promote biological removal of sulfide over a 28-day incubation. However, sulfide removal was observed when acetate or formate plus vitamins or yeast extract were added to two of these waters that had been supplemented with nitrate and phosphate. Hitzman and Sperl [18] observed that acetate and propionate were used by the heterotrophic denitrifying populations in several oil field waters and this activity prevented the growth of SRB. They reported that heterotrophic denitrifiers became the dominant group in the microbial community after nitrate amendment. Unfortunately, the description of the enumeration method was not adequate to evaluate the validity of their results.

Many recent studies on controlling sulfide production in oil fields have focussed on NR-SOB [15,27,49,50], with the emphasis on the strictly anaerobic *Arcobacter* sp. strain FWKO B and the microaerophilic *Thiomicrospira* sp. strain CVO. Both of these strains are obligate chemolithotrophs [15], so they would not grow in the medium that we use for enumerating heterotrophic NRB, which was devoid of sulfide. Although we are not aware of any reports of oil field waters containing NR-SOB that can grow heterotrophically, Robertson and Kuenen [42] described a bacterium with this capability. The bacterium, now known as *Paracoccus pantotrophus* [40] (formerly *P. denitrificans* [31] and *Thiosphaera pantotropha* strain GB17 [42]), was isolated from a denitrifying effluent treatment system. It is a facultative anaerobe and a facultative autotroph that uses nitrate as an electron acceptor. It grows autotrophically with sulfide as an electron donor, or heterotrophically with a variety of organic compounds (including acetate, lactate, glucose, and casamino acids) as electron donors [42].

The presence of NR-SOB that are facultative autotrophs (like *P. pantotrophus*) in our microcosms would confound our assessment of the roles of the strictly autotrophic NR-SOB and the heterotrophic NRB in controlling sulfide in produced waters. This is because a facultative autotroph would likely grow in both the modified CSB medium used to enumerate the autotrophic NRB and in the medium used to enumerate heterotrophic NRB. Although our methods would not clearly indicate the presence of facultative autotrophic NRB, our results suggest that these microorganisms were not abundant in the microcosms. That is, if facultatively autotrophic NRB constituted the dominant portion of the community, then nitrate amendment would markedly increase their numbers, and this increase should be reflected to essentially the same extent in both modified CSB medium and the medium for heterotrophic NRB. However, the data in Table 2 do not show this trend. For example, in the samples from oil field P, the maximum increase in MPN values for the heterotrophic NRB was 57,000-fold and that for the NR-SOB was only 2900-fold. The data from oil field P show an even greater difference between the increases in the MPN values, with that of the heterotrophic NRB increasing 22,000-fold and that of the NR-SOB increasing only 440-fold. The data from oil field N (Table 2) present a different situation in that there was no increase in the heterotrophic NRB number, while there was a 1000-fold increase in the NR-SOB number. Clearly, none of these sets of data shows a similar increase in the numbers of heterotrophic NRB and NR-SOB, suggesting that facultatively autotrophic NRB are not the major type of NRB in these produced waters. However, further specific studies are required to better assess whether facultatively autotrophic NRB play a role in controlling sulfide concentrations in oil field waters.

The objective of this research was to use different media to determine which groups of planktonic NRB were stimulated in laboratory microcosms containing produced waters amended with nitrate. This was the first study to specifically monitor the number of heterotrophic NRB in this type of experimental system. We anticipated that their number would increase with time, but the results presented in Table 2 were somewhat surprising. That is, in oil fields P and C, stimulation of heterotrophic NRB far exceeds that of autotrophic NR-SOB, whereas in oil field N the converse was true. In addition, our findings strengthen the notion that the activities of the heterotrophic NRB help control sulfide removal by interrupting the anaerobic sulfur cycle. In retrospect, determining the types and quantities of the organic compounds dissolved in the produced waters would have provided valuable information to help assess which electron donors were key to the anaerobic processes that took place after nitrate amendment. However, this was not done in this study. Now that the importance of the heterotrophic NRB has been clearly demonstrated, characterization of the dissolved organic electron donors should be addressed in subsequent studies.

In summary, our study showed that the MPN technique used for enumerating NR-SOB in oil field waters P and C (method A) gave much lower counts when applied to produced water from oil field N. The NR-SOB in the latter field appeared to be much slower-growing, so longer incubation times or the application of method B was required, resulting in much higher counts than were obtained by method A after 30 days of incubation. In addition, this investigation demonstrated that sulfide removal was much faster in produced waters from oil fields P and C compared to produced water from oil field N. In the former two waters, heterotrophic NRB were stimulated by nitrate amendment, whereas in the latter water, heterotrophic NRB were not stimulated by nitrate amendment. These results suggest that an active heterotrophic NRB population is required to outcompete heterotrophic SRB and disrupt anaerobic sulfur cycling, thereby hastening sulfide removal. Further studies are required to specifically prove this hypothesis.

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