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Planktonic nitrate-reducing bacteria and sulfate-reducing bacteria in some western Canadian oil field waters

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Oil fields that use water flooding to enhance oil recovery may become sour because of the production of H_2S from the reduction of sulfate by sulfate-reducing bacteria (SRB). The addition of nitrate to produced waters can stimulate the activities of nitrate-reducing bacteria (NRB) and control sulfide production. Many previous studies have focused on chemolithotrophic bacteria that can use thiosulfate or sulfide as energy sources while reducing nitrate. Little attention has been given to heterotrophic NRB in oil field waters. Three different media were used in this study to enumerate various types of planktonic NRB present in waters from five oil fields in western Canada. The numbers of planktonic SRB and bacteria capable of growth under aerobic conditions were also determined. In general, microbial numbers in the produced waters were very low (<10 ml^{-1}) in samples taken near or at wellheads. However, the numbers increased in the aboveground facilities. No thiosulfate-oxidizing NRB were detected in the oil field waters, but other types of NRB were detected in 16 of 18 produced water samples. The numbers of heterotrophic NRB were equal to or greater than the number of sulfide-oxidizing, chemolithotrophic NRB in 12 of 15 samples. These results showed that each of the oil fields contained NRB, which might be stimulated by nitrate amendment to control H_2S production by SRB.
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Introduction

Relatively few studies of oil field microbiology have considered nitrate-reducing bacteria (NRB). Indeed, no mention of these bacteria was made in a review of the microbiology of petroleum reservoirs [32]. NRB can be classified on the basis of the electron donors that they use. They can be chemolithotrophs that use inorganic compounds such as sulfide, thiosulfate or ferrous sulfide as electron donors [19,30,44], or chemoorganotrophs (heterotrophs) that use organic compounds as electron donors [52].

For decades, the petroleum industry has been plagued by H_2S produced from the reduction of sulfate by sulfate-reducing bacteria (SRB) [13,23]. Hydrogen sulfide causes many problems including souring of gas and oil, corrosion of metals, and plugging of reservoirs by forming precipitates that reduce oil recovery [9,34,36,42]. Of course, H_2S is very toxic.

As the pressure in an oil reservoir decreases, enhanced recovery methods are required to maintain oil production. Water flooding is a commonly used enhanced recovery method in which source water, comprised of surface water or ground water, is injected into the reservoir to help drive the oil to the producing wells. Aboveground, the oil is separated from the produced water, and this water, along with source water, is injected back into the reservoir. Water flooding often stimulates the activities of SRB by introducing these bacteria and/or sulfate into the oil field. As a result, a ''sweet'' crude oil, which has no H_2S, may become a lower-value ''sour'' crude because of the presence of microbially produced H_2S.

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Biocides are often added to the produced waters and injected into oil reservoirs to curtail detrimental microbes [4]. Unfortunately, biocides are not always effective nor do they have long-term inhibitory effects. In some cases, after removal of biocides, regrowth of unwanted bacteria doubled or tripled [41].

The addition of nitrate to anaerobic wastewater [25,40], oil wastes from ships [31], and oil field-produced waters [12,37] has stopped sulfide formation. If heterotrophic NRB and SRB are present in these sulfate-containing waters, the addition of nitrate establishes a competition between these two groups of bacteria. For a given electron donor, the energy gained from nitrate reduction is greater than the energy obtained from sulfate reduction [52]. For example, based on data from Thauer *et al* [47], the free energy change for the oxidation of acetate by NRB and SRB is shown below:

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

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

These reactions show that, per mole of electron acceptor, the NRB have a large thermodynamic advantage over the SRB. Thus, in the presence of nitrate, the heterotrophic NRB will be more active and suppress the activities of SRB, thereby eliminating the production of H_2S.

The presence of chemolithotrophic NRB provides two advantages for oil reservoirs that contain H_2S. First, the nitrate-reducing, sulfide-oxidizing bacteria (NR-SOB) are able to gain energy from oxidizing reduced sulfur. In this way, the H_2S is consumed.

Second, these bacteria produce a variety of products as they use nitrate as a terminal electron acceptor [19], including NO_2^-, NO, N_2O, and N_2 [33]. The production of N_2O by NRB has been shown to raise the redox potential of a given environment to such an extent that strict anaerobic bacteria like SRB are inhibited [26], thereby preventing the production of H_2S. Much of the research to eliminate H_2S in oil fields has centered around using chemolithotrophic NRB, and involves nitrate addition to the reservoir to stimulate existing oil field NRB populations, or the addition of nitrate and cultivated NRB to the reservoir to eliminate H_2S [29]. Heterotrophic NRB also produce N_2O [48,52], having the same effect on the redox potential as the NR-SOB.

Patents have been granted for controlling sulfide production in oil fields by the addition of nitrate [21,22,24]. Two patents [21,22] focused on stimulating NRB by adding acetate, or other organic compounds suitable for denitrifiers, whereas the other patent [24] focused on stimulating NR-SOB. Jenneman *et al* [28] demonstrated that the injection of nitrate into an oil field in Saskatchewan, Canada, substantially reduced sulfide production.

Several studies have enumerated NRB in oil field waters using most probable number (MPN) methods with different media formulations. Most formulations would preferentially, but not exclusively, culture autotrophs. For example, the medium used by Davidova *et al* [12] contained only inorganic compounds except for yeast extract, with thiosulfate serving as the electron donor. The medium used by Telang *et al* [46] contained only inorganic compounds except for acetate, with sulfide serving as the electron donor. Other investigations used sulfide as the electron donor with filter-sterilized produced water from the oil field that was being studied [18,28,45] The filtered produced waters undoubtedly contained some dissolved organic compounds. The only enumerations of heterotrophic NRB in produced water appear to be those of Adkins *et al* [1], who used molasses and sucrose as electron donors in their medium.

There appears to be no study that specifically enumerated different nutritional types of NRB in oil field waters, although both chemolithotrophic and heterotrophic NRB have been implicated in controlling sulfide production. Most notably, there is a lack of information on the presence of heterotrophs. Some workers [20,38] have focussed on the abilities of heterotrophic NRB to consume volatile fatty acids, such as acetate, propionate, and butyrate, which are commonly found in produced waters [32]. However, recent studies have demonstrated that many hydrocarbons, such as benzene [6], toluene, ethylbenzene, *m*-xylene, naphthalene, and C_6-C_{12} alkanes can be degraded by heterotrophic NRB (see Ref. [50] for review). Many of these hydrocarbons will dissolve in produced waters that are in contact with petroleum, and provide carbon and energy sources to stimulate heterotrophic NRB in nitrate-amended oil fields.

Our study was designed to selectively enumerate planktonic heterotrophic NRB and chemolithotrophic NRB in waters from five oil fields in western Canada, and to determine the relative abundances of the different types of NRB. We used MPN procedures to enumerate NRB and SRB, and both of these types of bacteria were detected in most of the oil field water samples. The numbers of planktonic bacteria that could grow aerobically on spread plates were also determined. These numbers and the numbers of SRB tended to increase in the aboveground facilities in the oil fields.

Materials and methods

Sampling sites

Five oil fields in western Canada were sampled during this study (Table 1). Four of the sites were in Alberta — one near Edmonton (A), one near Drayton Valley (B), and two near Stettler (P and N) — and one oil field was in Saskatchewan (C). Oil field P was sampled on two occasions, and the samples are designated Pa and Pb (Table 2).

At a typical water flooding oil field site, an emulsion of oil, gas, and water reaches the surface at the producing wells. Some samples were taken directly at the wellhead, and others were taken from a satellite, which is a collecting point for several producing wells. These locations were assigned sample code 1 (Table 2). The gas, oil, and water then flow from several satellites to the oil field battery where the emulsion is broken and the three components are separated using heat and/or gravity at the free water knock out (FWKO) or treater (sample code 2, Table 2). The gas and oil are shipped off site for further processing, and the separated produced water is piped to a storage tank or preinjection site (sample code 3, Table 2) prior to being pumped *via* an injection well into the

Table 1 Some characteristics of the five western Canadian oil fields that were sampled during this study

	A	B	C	N	P
Nearest town or city	Edmonton	Drayton Valley	Coleville	Stettler	Stettler
Production started	1950	1955	1951	1992	1994
Oil-bearing formation	D3A Leduc	Cardium and Belly River	Bakken	Glauconitic[a]	Glauconitic
Field depth (m)	1520	1420	810	1400	1300
Production wells in oil field	98	45	245	40	38
Water injection wells in oil field	2	10	110	7	4
Water flooding started in	1957	1963	1958	1994	1994
Origin of source water	North Sask.[b] River	North Sask. River	Belly River aquifer	Belly River aquifer	Belly River aquifer
Average water cut (%)	95	80–85	95	55	95
Sampling dates	July 2000	September 2000	July 2001	May 2001	December 2000, February 2001

[a]Also referred to as the Upper Mannville formation [12].
[b]Sask. = Saskatchewan.

Table 2 Summary of sample locations within oil fields and some physical, chemical, and microbiological characteristics of the oil field waters

Oil field	Sample location	Temperature (°C)	pH	$S^{=}$ (mM)	$SO_4^{=}$ (mM)	Cl^{-} (mM)	Aerobic plate count (CFU ml^{-1})	SRB (MPN ml^{-1})	Heterotrophic NRB (MPN ml^{-1})	NR-SOB (MPN ml^{-1})	Sample code
A	Storage tank	25	7.0	0.3	8	2400	<10	4.3	4.3	ND[a]	A3
B	Wellhead PW[b]	26	8.0	<0.08	<0.005	70	<10	0.9	2.3	<0.3	B1
	Treater	14	7.5	<0.08	0.005	120	15,000	150	4300	<0.3	B2
	Storage tanks	14	8.0	<0.08	0.005	110	2900	75	930	<0.3	B3
	Source	19	7.0	<0.08	0.4	0.1	15,000	2.1	43	<0.3	B4
C	FWKO[c]	ND	8.5	3	0.6	110	650	930	430	210,000	C2
N	Wellhead PW	20	8.0	0.2	8	700	<10	<0.3	<0.3	<0.3	N1
	FWKO	20	8.0	0.3	4	540	840	230	93	93	N2
	Storage tanks	22	8.5	0.9	4	500	28,000	2300	23,000	93	N3
	Source	13	7.0	<0.08	<0.005	200	4500	43	2300	<0.3	N4
Pa	Satellite PW	24	7.5	2	6	170	<10	2.3	<0.3	<0.3[d]	Pa1
	FWKO	30	7.5	<0.08	4	700	250	750	1.5	<0.3[d]	Pa2
	Preinjection	29	7.5	0.8	6	760	1400	2300	7.5	1500[d]	Pa3
	"Source"[e]	12	9.0	3	13	160	210,000	23,000	1500	4300[d]	Pa4
Pb	Satellite PW	23	8.5	1	0.3	270	<10	9.3	2.3	<0.3	Pb1
	FWKO	32	8.0	0.5	4	620	20	230	43	<0.3	Pb2
	Preinjection	28	8.0	1	4	500	3200	930	2300	93	Pb3
	"Source"[e]	22	9.0	5	12	200	420	93	1.5	930	Pb4

[a]ND, not determined.
[b]PW, produced water.
[c]FWKO, free water knock out.
[d]The formation of nitrite indicated positive MPN tubes.
[e]These "source" waters were actually produced waters from another oil field. See text for details.

reservoir. Source water (sample code 4, Table 2) may be added to the storage tank location and then sent to the oil field, or injected directly into the formation.

Biocides, and scale and corrosion preventors were used in some of the fields that were sampled. Oil fields A and P did not use biocides at the time of sampling. Operators at oil fields B and N turned off the biocide feed 1 week prior to sample collection so the samples would not be influenced by these chemicals. Because of problems associated with SRB, the operators of oil field C were not willing to stop injection of the biocide, and the sample was collected while biocide was being added.

Only one sample was collected for chemical and bacterial analyses from the site near Edmonton (A3, Table 2). This came from the preinjection line. Similarly, only one sample was collected from the Saskatchewan field. This was from the FWKO (C2). For the sites near Drayton Valley and Stettler, four oil field water samples were collected on each sampling trip (Table 2).

Sample collection and chemical analysis

Oil field water samples were collected by completely filling sterile, 4-l plastic bottles. The samples were taken immediately to a work area in the field and, as quickly as possible, the water samples were tested for temperature, pH (using color pHast indicator strips; EM Science, Gibbstown, NJ), and sulfide (using the methylene blue method; CHEMetrics, Calverton, VA). To minimize exposure of the collected water samples to O_2 during transport, portions of the samples were transferred into sealed, sterile 158-ml serum bottles that had previously been made anoxic with O_2-free nitrogen. The transfer was done using a hand pump to create a slight negative pressure in the serum bottle. A piece of tubing was attached to the vacuum pump, and there was a sterile needle on the other end of this tubing. A second piece of sterile tubing with a sterile needle on one end and a sterile pipette attached to the other end was used to transfer the sample to the serum bottle. The pipette was lowered into the 4-l bottle that contained the water sample. Then, the two needles were simultaneously inserted through the stopper in the serum bottle, and the vacuum pump was used to pull the water sample into the serum bottle. A reduced pressure was maintained using the hand pump until the serum bottle was filled.

In addition, about 100 ml of oil field water was filtered using 0.2-μm pore size Millex-GS Millipore filters (Bedford, MA) in preparation for ion chromatography analysis. All samples were packed on ice before being transported to the University of Alberta in Edmonton. Sulfate, nitrate, and chloride were determined by ion chromatography [14].

Bacterial enumeration and MPN culture analyses

Media for enumerations were inoculated within 24 h of sample collection. Dilutions for the MPN and plate count procedures were made to 10^{-11} using 10-fold serial dilutions of the oil field waters in serum bottles with 10 mM phosphate buffer (pH=7.2). O_2-free nitrogen was used to prepare the phosphate buffer anaerobically and to flush serum bottles and syringes. The phosphate buffer and all media contained the chloride concentration of the oil field water being tested (Table 2). Nitrogen-flushed syringes (1 ml) were used to dispense the appropriate oil field water dilutions to the media. Sterile medium controls were prepared by adding 1 ml of sterile phosphate buffer to each type of medium. These controls were used as references to assess growth and chemical changes that occurred in the medium containing viable cultures.

NRB and SRB were enumerated by a three-tube MPN procedure. The inoculated media were incubated for 30 days at room temperature (approximately 21°C) in the dark before being scored for growth. The resulting MPN values were compared by the statistical method of Cochran [8].

Heterotrophic NRB were enumerated using a nutrient broth–nitrate medium (heterotrophic NRB medium) [14] in sealed 16×125-mm Hungate-type anaerobic culture tubes (Bellco Glass, Vineland, NJ) with air in the headspace [48]. Acetylene (25% vol vol^{-1} of headspace gas) was added to each tube to block nitrate reduction at N_2O [48]. After incubation, the growth of NRB and other bacteria produced turbidity in the culture tubes. To verify that growth in the cultures was due to the presence of heterotrophic NRB, the medium was tested for nitrate loss using a second derivative UV absorbance method [14]. When a decrease of >20% of the nitrate concentration in the culture medium was observed, the MPN tube was considered positive for growth of NRB. This threshold was easily detected by the second derivative UV method, and the decrease of 20% was large enough to ensure that variability in the nitrate concentrations among culture tubes with no growth would not lead to some tubes being falsely scored positive [14]. Cultures were also analyzed for nitrite using sulfanilamide and *N*-(1-naphthyl)-ethylenediamine dihydrochloride [7] and for N_2O production [16]. *Pseudomonas stutzeri*, a known denitrifying bacterium [52], was used as a positive control in the heterotrophic NRB medium.

Chemolithotrophic NRB were enumerated using two types of media to cultivate sulfide-oxidizing or thiosulfate-oxidizing bacteria. Both media were prepared anaerobically in sealed, Hungate-type anaerobic culture tubes. Thiosulfate medium was modified ATCC 295 S8 medium [2] (S8 medium) used to cultivate thiobacilli [14]. Inoculated medium with *Thiobacillus denitrificans* (ATCC 23642) was used as the positive control for growth. After 30 days of incubation, MPN tubes that had >20% of the nitrate consumed from the S8 medium (as determined by the second derivative UV absorbance method [14]) were scored positive for thiosulfate-oxidizing nitrate reducers. Cultures were also analyzed for nitrite.

To enumerate NR-SOB, the CSB medium [46] was modified by omitting the acetate in order to make it selective for only chemolithotrophic NRB that oxidize sulfide. The medium (pH 7.5) contained, per liter: 0.027 g of KH_2PO_4, 0.68 g of $MgSO_4{\cdot}7H_2O$, 0.24 g of $CaCl_2{\cdot}2H_2O$, 0.02 g of NH_4Cl, 0.13 g of $(NH_4)_2SO_4$, 1.9 g of $NaHCO_3$, 1.0 g of KNO_3, 1 mg of resazurin, and 50 ml of trace elements-1 solution [46]. The medium was dispensed into Hungate-type anaerobic culture tubes, sealed, and autoclaved. After cooling, 0.25 ml of 0.1 M $Na_2S{\cdot}9H_2O$ was injected into each tube to give a final sulfide concentration of 2.5 mM, as used by Telang *et al* [46]. Then acetylene was injected into each tube to give 25% (vol vol^{-1}) in the headspace to block nitrate reduction at N_2O. The sealed, inoculated MPN tubes from the first two samplings were incubated on the laboratory bench. Because of a concern that some of the septa on the Hungate-type anaerobic culture tubes might leak and allow O_2 into these tubes (thereby oxidizing the medium), the sealed, inoculated tubes from the final three samplings were incubated in a Coy anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) filled with 5% CO_2, 10% H_2, and balance N_2. This ensured that the change in the redox indicator, resazurin, and loss of sulfide was not due to O_2 contamination. The

cell yields of the NR-SOB in the modified CSB medium were so low that turbidity could not be observed. The reduced (colorless) medium turned pink due to oxidation of resazurin by the microbially produced N_2O [46]. All MPN tubes with modified CSB medium that turned pink were scored positive. Strains CVO and FWKO B, which are known NR-SOB [19], were obtained from Dr. G Voordouw's laboratory (University of Calgary) and used as positive controls for the modified CSB medium.

Sulfide was the limiting substrate in the modified CSB medium, so complete consumption of sulfide provided strong evidence of NR-SOB in the MPN culture. An alkaline sodium nitroprusside spot test [17] was used to detect sulfide in the cultures. Cultures in the modified CSB medium were also analyzed for nitrite and N_2O production.

SRB were enumerated using the method of Fedorak *et al* [15]. The medium, in tubes with Kaput® closures (Bellco Glass), contained lactate as the growth substrate, 1 mg l^{-1} resazurin, and two iron finishing nails in each tube. Tubes in which the nails turned black because of the formation of FeS precipitate were scored positive for growth of SRB.

To determine the numbers of bacteria in the oil field waters that would grow under aerobic conditions, 0.1 ml of the dilutions prepared for the MPN procedure was inoculated to triplicate R2A (Becton Dickinson, Sparks, MD) agar plates. The plates were incubated at approximately 21°C in the dark. After a 7-day incubation period, the plates were examined for growth and triplicate plates at the dilution giving between 30 and 300 colonies were used for estimations of bacterial numbers.

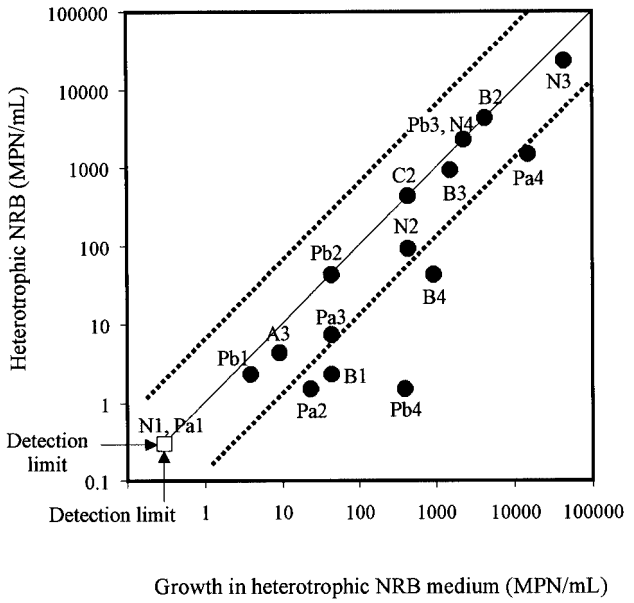

Figure 1 Comparison of numbers of heterotrophic NRB, as determined by nitrate consumption, and growth, as determined by turbidity, in heterotrophic NRB medium. Each point is designated by the sample code given in Table 2. Data from the samples in which the counts were below our detection limit are plotted as open squares. Counts that are equal fall on the solid equivalence line. Those data that fall between the parallel dashed lines have MPN values that are indistinguishable from each other by the statistical method of Cochran [8] ($P<0.05$).

Results

Evaluation of counting methods, and the absence of thiosulfate-reducing bacteria

The criteria used to score MPN tubes for growth of NRB were established for the heterotrophic NRB and the NR-SOB. For the former group, either the loss of nitrate from the medium or the production of N_2O was a candidate for the criterion used to score for growth of NRB. When the denitrifier *P. stutzeri* grew in this medium, it consumed nitrate and produced N_2O as expected. After 30 days of incubation of the medium inoculated with oil field water samples, both of these parameters were measured and they were individually used to determine the MPN values. For each of the 16 samples that yielded growth in the heterotrophic NRB medium, the MPN results based on the consumption of nitrate and the MPN results based on the N_2O analysis were the same ($P<0.05$) [14].

The medium used for enumerating heterotrophic NRB was the medium recommended by Tiedje [48] for enumerating denitrifying bacteria (modified to contain only one-half the amount of nutrient broth). Because denitrification is a facultative trait [52], the medium was not prepared using anaerobic methods. Thus, over a 30-day incubation time, growth in the medium could have been a succession of aerobes, facultative anaerobes, and even microaerophilic or aerotolerant anaerobes. In addition to measuring nitrate loss and N_2O production, the MPN tubes were also scored for growth based on turbidity. Figure 1 compares the heterotrophic NRB medium for the numbers of heterotrophic NRB, based on nitrate consumption, with the numbers of bacteria that grew in the medium based on the production of turbidity. If the counts generated by the two methods were the same, the data points fell on the solid, equivalence line shown in Figure 1. Those counts that were indistinguishable based on the Cochran statistical method ($P<0.05$) fell within the parallel dashed lines. In total, of the 16 samples that showed growth, 11 counts fell within the dashed lines, indicating that in most cases, the number of heterotrophic NRB in a given sample was essentially the same as the number of heterotrophs that grew in this medium. In five samples (Pa2, B1, B4, Pa4, and Pb4), the number of heterotrophic NRB was less than the number of heterotrophs that grew in this medium.

Of course, there is no reason why the MPN values should fall on the equivalence line (Figure 1) because the figure is simply comparing the numbers of heterotrophic NRB with the numbers of heterotrophs that grew in the medium. However, given that so many different types of heterotrophic bacteria could grow in this undefined medium, it is interesting that nitrate loss was observed in so many of the MPN series. In addition, the consumption of nitrate in so many tubes indicates that the MPN values do not include only the strictly aerobic and fermentative bacteria that could grow in this medium.

Several criteria could be used to score the modified CSB medium positive for growth of NR-SOB, which carry out the following reaction [46]:

$$5HS^- + 2NO_3^- + 7H^+ \rightarrow 5S^0 + N_2 + 6H_2O \qquad \Delta G^{o'} = -491 \text{ kJ (mol } NO_3^-)^{-1} \quad (3)$$

The criteria include the depletion of sulfide from the medium (which contained a fourfold molar excess of nitrate); the change in the medium from colorless to pink due to the oxidation of the redox indicator, resazurin, by the production of the intermediate N_2O [26,46]; the formation of nitrite as an intermediate of nitrate reduction; and the accumulation of N_2O in the headspace gas.

The use of the MPN method for NR-SOB evolved as this project progressed. Work with the first samples taken (from oil field B) showed no indication of color change in the modified CSB medium after incubation. Work with the second samples (designated Pa) showed that 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 for nitrite, and those tubes that contained nitrite were scored positive. The nitrite analysis was used as the basis for MPN results given for the Pa samples in Table 2.

MPN cultures in the modified CSB medium for the last three samplings (Pb, C, and N) were incubated in an anaerobic chamber to ensure that O_2 contamination could not cause the redox indicator to oxidize, as was suspected for sample Pa. After incubation, the color of the MPN cultures for these three samplings was noted and the medium was assayed for nitrite and sulfide. The MPN values were then determined with each individual parameter. No evidence of NR-SOB activity was observed in four of these samples (N1, N4, Pb1, and Pb2; Table 2). That is, there was no nitrite detected in any of these MPN tubes, and sulfide was detected in each tube. The use of the spot test for measuring sulfide precluded the detection of small decreases in sulfide concentration that might have occurred by biotic or abiotic reactions in the medium.

For the remaining five samples (C2, N2, N3, Pb3, and Pb4), the NR-SOB MPN values obtained by measuring the loss of sulfide were the same as those determined by the color change of the medium ($P<0.05$). Four (samples N2, N3, Pb3, and Pb4) of the five MPN values based on N_2O accumulation in the headspace gas yielded lower MPN values than those based on color change or sulfide loss. These results were consistent with other studies, which found that sulfide inhibited acetylene blockage [5,11]. In the fifth case (sample C2), the MPN values based on these two parameters were equal. In addition, for the same four samples, the MPN values based on the detection of nitrite in the medium were the same as those based on sulfide consumption and pink color formation. Using the criterion of nitrite accumulation in the medium to score for MPN, sample C2 gave a lower MPN ($P<0.05$) than when sulfide depletion or oxidation of the redox indicator was used to score the tubes. Thus, scoring positive tubes based on the color change of the resazurin [26,46] was the easiest procedure, and it agreed completely with the depletion of sulfide from the medium. The NR-SOB strains CVO and FWKO B both grew in the modified CSB medium, consuming sulfide and turning the medium pink.

Although elevated sulfide concentrations are inhibitory to some chemolithotrophic NRB [43], the well-characterized NR-SOB strains, CVO and FWKO B, grow at 10 and 15 mM sulfide, respectively [46]. The sulfide concentration in the modified CSB medium was 2.5 mM, which was higher than most sulfide concentrations in the water samples collected (Table 2), except "source" waters Pa4 and Pb4 and sample C2. Telang *et al* [46] used 2.5 mM sulfide in their CSB medium formulation, and we did not attempt to optimize the sulfide concentration in the modified medium.

There were no thiosulfate-oxidizing NRB cultivated from any of the oil field water samples. However, when each set of oil field samples was inoculated, the S8 medium amended with the same chloride concentration observed in the oil field water samples was inoculated with *T. denitrificans* (ATCC 23642) as the positive control for growth. The reference culture grew well in the medium with chloride concentrations below 760 mM. In addition, Dr. KL Sublette (University of Tulsa, OK) verified that *T. denitrificans* strain F grew in the S8 medium.

Temperature and pH of oil field waters

None of the oil reservoirs was very hot, and the temperatures of the produced waters from the wellheads and the satellites were between 20 and 26°C (Table 2). Source waters were generally colder than waters from the wellheads or satellites. The highest temperature recorded was 32°C in the FWKO at oil field P (sample Pb2, Table 2). The sample from oil field C was taken by the oil field operators and transported to us; thus, no temperature reading was available.

The pH measurements were done on the samples prior to transferring them to the serum bottles because exposure of the produced waters to a reduced pressure may have caused a loss of dissolved CO_2, which would have affected the pH. The pH values of the water samples were generally between 7.0 and 8.5. The only exceptions were the "source" waters from oil field P (samples Pa4 and Pb4, Table 2), which were pH 9. These data indicate that neither the temperature nor pH of these waters would adversely affect microbial growth.

Oil field A

This oil field was only a short distance from our laboratory, and it was the first field sampled. Only one sample, from the water storage tank, was collected, and this was used to test our methods. Although the sulfate concentration was high (8 mM, Table 2), the sulfide concentration was low (0.3 mM), and the operators did not consider the field to be "souring." This field had the highest chloride concentration (2400 mM) of any that was studied. The number of aerobic bacteria was below the detection limit of the plate count method, and some SRB were detected in the produced water (Table 2). We had not implemented the method for enumeration of NR-SOB at that time, but a small number of heterotrophic NRB (4.3 ml^{-1}) were detected.

Oil field B

The operators at this oil field said that H_2S production occurred "seasonally." Sulfide was not detected in any of the four sample locations, and the sulfate concentrations were very low in the produced waters. The source water used in this field is from the North Saskatchewan River (Table 1), which has a higher sulfate concentration (0.4 mM) than the produced waters. SRB were detected in all four samples, with MPN values ranging from 0.9 to 150 ml^{-1} (Table 2). No colonies were observed in the aerobic plate count of the wellhead produced water, but bacteria able to grow aerobically were abundant in the other three samples, with counts ranging from 2.9×10^3 to 1.5×10^4 ml^{-1}. No NR-SOB were detected in any of the samples from field B, but heterotrophic NRB were found in each of the samples. The highest heterotrophic NRB counts were in samples from the treater and storage tanks.

Oil field C

Produced waters from this oil field have been studied extensively [27,28,45] because of its severe souring problem, and it was the source of novel NR-SOB [19]. The only sample available was from the FWKO, which contained 3 mM sulfide (Table 2). Because of the problems caused by microbial activities in this field, the operators were not willing to interrupt the addition of biocides, so these inhibitors were being added at the time of sampling. Nonetheless, all four groups of bacteria were found in the produced water (Table 2), with the NR-SOB being the most abundant (2.1×10^5 ml^{-1}).

Oil field N

Over the past few years, souring has become a problem at this field. The sulfide concentrations in the produced waters ranged from 0.2 to 0.9 mM (Table 2). The source water for this field is the Belly River aquifer (Table 1), which has a very low sulfate concentration. As shown in Table 2, sulfate originates from the oil reservoir. The source water contained SRB, heterotrophic NRB, and bacteria that grew aerobically on plates. However, no NR-SOB were detected in this water. None of the four groups of bacteria was detected in the produced water from the wellhead, but all four groups were detected in the samples from the FWKO and the storage tanks (Table 2).

Oil field P

This "souring" oil field was sampled on two occasions. Normally, the source water for oil field P is also the Belly River aquifer, but for a period of time, this ground water source was not available. Operators of another oil field in the vicinity trucked produced water to oil field P for disposal by injecting it into this field. At both sampling times, the only "source" water being injected into the field was actually produced water from a neighboring oil field. This "source" water contained more sulfide and sulfate than any of the produced waters from oil field P (Table 2). On the first sampling trip (Pa), the "source" water had a very high number of colonies on the aerobic plate count medium (2.1×10^5 ml^{-1}) and high numbers of SRB (2.3×10^4 ml^{-1}). It also contained relatively high numbers of heterotrophic NRB and NR-SOB (Table 2).

No colonies grew on the aerobic plate count medium, and no NR-SOB were detected in either produced water from the satellite at field P (Table 2). Similarly, no heterotrophic NRB were found in the satellite sample taken on the first trip (Pa), but 2.3 ml^{-1} was found in the second sampling trip (Pb). Various numbers of SRB and heterotrophic NRB were found in the FWKO, the preinjection, and "source" waters. NR-SOB were present in the preinjection and "source" waters from both sampling times (Table 2).

Comparison of SRB numbers

Figure 2 summarizes the numbers of SRB (enumerated with lactate as the carbon and energy source) in samples taken from oil fields B, P, and N. These MPN values all show a common trend. The lowest numbers of SRB were found at the wellhead (fields B and N) or the satellite (field P), and these ranged from <0.3 ml^{-1} (sample N1) to 9.3 ml^{-1} (sample Pb1). These samples provide the best estimate of the numbers of SRB just as the oil–water emulsions leave the reservoirs. Adkins *et al* [1] also found low numbers of SRB in samples taken as near as possible to wellheads. Their MPN values of SRB were $\leq$5 ml^{-1}.

Figure 2 shows that as the waters move through the above-ground handling facilities, such as treaters or FWKO units, the numbers of planktonic SRB increase markedly. For example, in oil field B, the number increased from 0.9 ml^{-1} in the wellhead sample (B1) to 150 ml^{-1} in treater sample (B2), and in oil field P, the number increased from 2.3 ml^{-1} in the satellite produced water sample (Pa1) to 750 ml^{-1} in FWKO sample (Pa2). The numbers of SRB in the storage tanks or preinjection waters were essentially the same as those in the treater or FWKO. For instance, the MPN values for samples B2 and B3 (Figure 2) were the same ($P<0.05$), and there was no difference between the MPN values for samples Pa2 and Pa3 ($P<0.05$). In oil field N, the number of SRB in the storage tank (N3) was slightly greater than ($P<0.05$) the number in the FWKO (N2).

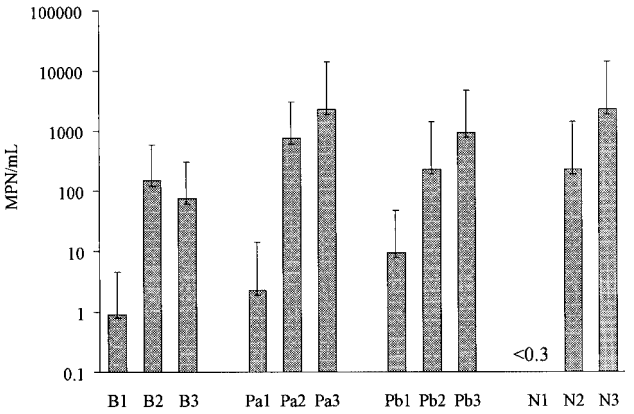

Figure 2 SRB counts in various waters from oil fields B, P (sampled on two occasions), and N. The small bars represent the 95% confidence interval of the MPN values. See Table 2 for sample codes.

Discussion

Three of the oil fields studied during this project began operation in the early 1950s, and the other two began operating in the early 1990s (Table 1). These represent four different oil formations at depths between 810 and 1520 m. All have been operated with water flooding for many years. The number of production wells varies from 38 to 245, and the number of injection wells varies from 2 to 110 (Table 1). The average water cut (the proportion of water recovered from the wellheads) varies from 55% to 95%. Thus, more water than oil is being handled at these facilities.

The major focus of this study was to enumerate planktonic NRB from oil field waters. In particular, we aimed to differentiate among the types of NRB present in these fields to assess which type was most abundant, and might be stimulated by nitrate amendment to control sulfide production. The enumeration methods used differentiated between chemolithotrophic and heterotrophic NRB in oil field waters.

Laboratory studies with *T. denitrificans* strain F, a sulfide-tolerant strain of NRB, showed that its growth could control biogenic sulfide production [35,37,44]. Thus, the western Canadian oil field waters were screened for this type of chemolithotrophic NRB. However, none was detected in any of the samples, which is consistent with the work of McInerney *et al* [35], who detected no denitrifying thiobacilli in formation water from a gas storage field.

Thiosulfate was the major electron donor in the medium used by Davidova *et al* [12], but their medium also contained yeast extract. They enumerated NRB in water samples from field N, and found these bacteria in each of the six samples they examined. The MPN values were about 100 NRB ml^{-1}. In contrast, using S8 medium, we detected no thiosulfate-oxidizing NRB in this oil field. Our medium was devoid of any utilizable carbon source, which suggests that yeast extract may have been supporting growth of heterotrophic NRB in the medium used by Davidova *et al* [12].

Adkins *et al* [1] used nitrate-containing media with molasses or sucrose to enumerate heterotrophic NRB in produced waters from some petroleum reservoirs. Each sample was taken as near the

wellhead as possible. They incubated their cultures at 37°C and detected heterotrophic NRB in each of the five samples they collected. The numbers were very low, with the highest count being 4 ml^{-1}. Our results showed that the highest heterotrophic NRB count at the wellhead or satellite samples was only 2.3 ml^{-1} (Table 2), in good agreement with the findings of Adkins *et al* [1].

Figure 3 shows a comparison of numbers of planktonic heterotrophic NRB and NR-SOB in the oil field waters. Any point that appears in the region enclosed by the dashed line had heterotrophic NRB and NR-SOB counts that were indistinguishable from each other ($P<0.05$). In the cases in which no MPN value could be determined because there was no growth in any of the MPN tubes that contained heterotrophic NRB medium or CSB medium, the value was plotted at 0.3 ml^{-1}, the detection limit of the method. Data from 17 water samples are plotted in Figure 3. Three samples (C2, Pa3, and Pb4) contained higher numbers of NR-SOB than heterotrophic NRB. The MPN values for the NR-SOB and heterotrophic NRB in three of the samples (Pa4, N2, and Pa2) were equal ($P<0.05$). Neither group of NRB was detected in two produced water samples (N1 and Pa1). The remaining nine samples contained higher numbers of heterotrophic NRB than NR-SOB. In six of these samples (B1, Pb1, B4, Pb2, B3, N4, and B2), no NR-SOB were detected. The results in Figure 3 show that heterotrophic NRB were more abundant than the NR-SOB in 9 of 15 oil field waters that yielded NRB counts.

The sample that contained the highest number of NR-SOB (C2) was from oil field C. This field has been the focus of several studies by other workers [28,29,39,45], and in 1996, nitrate was injected into a portion of this field for 50 days to demonstrate that this amendment could control sulfide production [28,29]. Jenneman

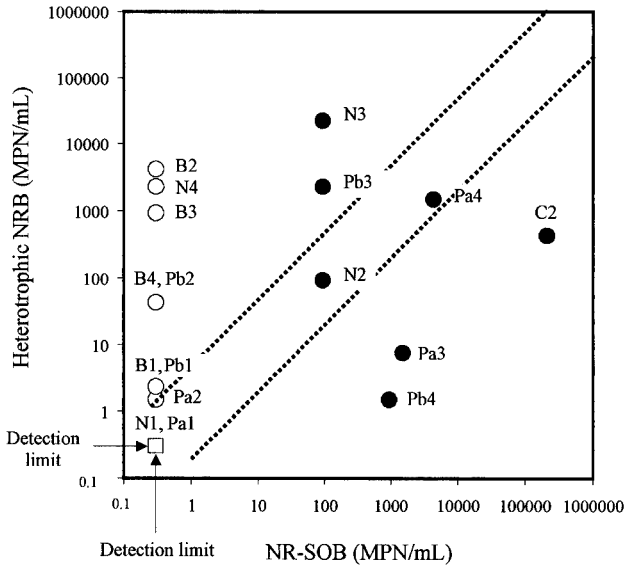

Figure 3 Comparison of the MPN values of heterotrophic NRB and NR-SOB in 17 oil field waters examined in this study. Each point is designated by the sample code given in Table 2. Data from the samples in which both heterotrophic NRB and NR-SOB were below our detection limit are plotted as open squares. Data from the samples in which NR-SOB were below our detection limit, but heterotrophic NRB were detected, are plotted as open circles. Data from the samples in which both heterotrophic NRB and NR-SOB were detected are plotted as solid circles. Those data that fall between the parallel dashed lines have heterotrophic NRB and NR-SOB counts that are indistinguishable from each other by the statistical method of Cochran [8] ($P<0.05$).

et al [28] supplemented filter-sterilized produced water from this field with nitrate and used this preparation as the growth medium for enumerating NRB at various locations in the oil field. Before nitrate injection into the oil field, they found 10^4–10^5 NRB ml^{-1} at the injector wells, and <10 NRB ml^{-1} at the producing wells. During the period of nitrate injection, the numbers of NRB increased to as high as 10^8 ml^{-1} [28]. Using CSB with acetate, Telang *et al* [46] found 10^6 NRB ml^{-1} in a produced water sample from oil field C.

Due to limited resources, we could only study one sample from oil field C, and this sample was taken while biocides were being injected into the field. The sample came from the FWKO, which was the origin of the novel NR-SOB described by Gevertz *et al* [19]. Undoubtedly, the filter-sterilized produced water used by Jenneman *et al* [28] contained dissolved organic compounds, and the CSB medium used by Telang *et al* [46] contained acetate, so it is very likely that both heterotrophic and chemolithotrophic NRB were enumerated in these media. Acetate is known to serve as a substrate for heterotrophic nitrate reduction [3]. The modified CSB medium used in this study contained no organic carbon and was designed to select for chemolithotrophic NR-SOB. This medium gave a count of 2.1×10^5 NR-SOB in the FWKO-produced water (Table 2). Using the medium for heterotrophic NRB, we found 430 heterotrophic NRB per milliliter in the FWKO water (Table 2). Telang *et al* [45] used the reverse sample genome probe method to monitor the effects of nitrate addition to oil field C. Among their reference DNA preparations were "standards" of three heterotrophs that reduced nitrate to nitrite, and they detected these heterotrophic NRB in the produced waters from oil field C [45]. Our detection of viable heterotrophic NRB in this oil field illustrates that our MPN method can detect the same physiological group of NRB that Telang *et al* [45] detected using genome probing methods.

Davidova *et al* [12] studied two oil fields (one of which was oil field N), and reported that the majority of sulfide production appeared to occur after the oil was pumped aboveground, rather than in the reservoir. The distribution of SRB in the oil fields examined in this study (Figure 2) is consistent with that observation [12]. Disregarding the "source" waters in oil field P (which were actually produced waters from another oil field), elevated sulfide concentrations in oil fields N and P (Table 2) were found in the storage tank water (sample N3) and the preinjection waters (samples Pa3 and Pb3). These observations also suggest that the activities of SRB aboveground are increasing the sulfide being reinjected into the reservoir, which will contribute to souring the petroleum recovered from these fields. Thus, the aboveground facilities would be potential targets for nitrate amendment to control sulfide production. As shown in Table 2, each of the produced water samples collected from the aboveground facilities contained heterotrophic NRB and/or the chemolithotrophic NR-SOB, which would likely be stimulated by nitrate amendment. However, we did not determine the concentrations of dissolved organic carbon in the produced waters, so there is no measure of the amounts of potential electron donors for the heterotrophic NRB that might be stimulated by nitrate amendment.

A plate count method was used to enumerate heterotrophic bacteria that could be cultivated under aerobic conditions. None was detected (i.e., <10 ml^{-1}) in any of the wellhead or satellite samples (Table 2). Adkins *et al* [1] also enumerated heterotrophic aerobes in oil field waters taken as near the wellhead as possible. Using an MPN method, their counts were between 0.1 and 20 ml^{-1}

in four of five samples. No aerobes (<0.1 ml^{-1}) were detected in the fifth sample. Thus, none of the wellhead oil field waters examined in our survey and in the study of Adkins *et al* [1] had very high numbers of bacteria capable of growing under aerobic conditions.

The data in Table 2 show that the numbers of bacteria detected by the aerobic plate count increased markedly as the produced waters move through the aboveground facilities. For example, in the oil field N samples, the aerobic counts increased from <10 ml^{-1} at the wellhead (N1) to 840 ml^{-1} in the FWKO (N2) to 2.8×10^4 ml^{-1} in the storage tanks (N3). This was the same trend that was observed for the SRB (Figure 2), again illustrating the increase in microbial numbers through the aboveground facilities.

There is another group of NRB that has not been discussed in this work. This group carries out dissimilatory nitrate reduction to ammonium [49], and *Citrobacter freundii* is an example of a bacterium that reduces nitrate in this manner. We are currently developing a selective medium for use in a MPN method to determine the relative abundance of this group of NRB to help determine whether nitrate amended to an oil field might lead to ammonium production.

In laboratory studies, Wright *et al* [51] investigated the effects of nitrate amendment to bacteria in four produced brines from west Texas oil fields. These fields had not been subject to extensive water flooding and have reservoir temperatures of 40–60°C. The addition of nitrate stimulated bacterial oxidation of sulfide in three of the brines. However, the rate of oxidation was increased significantly by addition of glucose, organic acids (acetate and formate), and vitamins. These results implied that heterotrophic bacteria played a key role in the oxidization of sulfide, although no mechanism for this process was suggested [51].

Our studies have demonstrated that NRB were detected in each of the five western Canadian oil fields that were studied (Table 2). In a few cases, these were not detected in the produced waters from the wellheads or the satellites, but NRB were always found in produced waters from the aboveground operations. Heterotrophic NRB were often more abundant than NR-SOB (Figure 3).

Nitrate amendment to oil field waters provides the potential to stimulate both groups of NRB, and control sulfide production by SRB. From Eq. (1), the oxidation of acetate by heterotrophic NRB has a $\Delta G^{o\prime}=-495$ kJ (mol $NO_3^-)^{-1}$. Based on the work of Burland and Edwards [6], oxidation of benzene by heterotrophic NRB has a $\Delta G^{o\prime}=-498$ kJ (mol $NO_3^-)^{-1}$. From Eq. (3), the oxidation of sulfide by NR-SOB has a $\Delta G^{o\prime}=-491$ kJ (mol $NO_3^-)^{-1}$. The energy yields from these reactions are quite similar, so amending nitrate to produced waters that contain both heterotrophic NRB and NR-SOB should stimulate both groups, with no competition for electron donors between the two. To date, there has been little direct evidence that heterotrophic NRB play a role in controlling sulfide production. However, the results of investigations by Jenneman *et al* [28], Wright *et al* [51], Telang *et al* [46], and Davidova *et al* [12] provide indirect evidence that heterotrophic NRB may be stimulated when nitrate is added to oil field waters.

Although heterotrophic NRB were detected in the produced waters (Table 2), understanding the roles of these bacteria and the SRB in produced waters is crucial to assessing the utility of nitrate amendment to control sulfide. Indeed, different scenarios are possible during nitrate amendment to an oil field. For example, the heterotrophic NRB may outcompete the NR-SOB for nitrate needed to oxidize sulfide, thereby hindering sulfide removal. It is also known that some SRB use nitrate as an electron acceptor [10]. Pure culture studies with different species of SRB have shown that in the presence of both sulfate and nitrate, either sulfate or nitrate may be the preferred electron acceptor, or both electron acceptors can be reduced concomitantly [10]. Thus, the addition of nitrate might increase the numbers of SRB in produced waters, which may become problematic after nitrate amendment ceases. However, there was no indication that either of these scenarios occurred in the Saskatchewan oil field studied by Jenneman *et al* [28,29] and Telang *et al* [45].

Clearly, there is much to be learned about the roles of the heterotrophic NRB in controlling sulfide production in oil fields. In laboratory studies, we have amended oil field waters with nitrate and used the MPN methods described in this paper to monitor the responses of the heterotrophic NRB and the NR-SOB, and to assess the roles of these two groups of NRB. These results will be reported in a later paper.

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