J Ind Microbiol Biotechnol (2009) 36:1499–1511
DOI 10.1007/s10295-009-0639-3

ORIGINAL PAPER

Sulfide persistence in oil field waters amended with nitrate and acetate

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Received: 11 July 2009 / Accepted: 9 September 2009 / Published online: 30 September 2009

Abstract Nitrate amendment is normally an effective method for sulfide control in oil field-produced waters. However, this approach has occasionally failed to prevent sulfide accumulation, despite the presence of active nitrate-reducing bacterial populations. Here, we report our study of bulk chemical transformations in microcosms of oil field waters containing nitrate-reducing, sulfide-oxidizing bacteria, but lacking denitrifying heterotrophs. Amendment with combinations of nitrate, acetate, and phosphate altered the microbial sulfur and nitrogen transformations. Elemental sulfur produced by chemotrophic nitrate-reducing bacteria was re-reduced heterotrophically to sulfide. Ammonification, rather than denitrification, was the predominant pathway for nitrate reduction. The application of nitrite led to transient sulfide depletion, possibly due to higher rates of nitrite reduction. The addition of molybdate suppressed both the accumulation of sulfide and the heterotrophic reduction of nitrate. Therefore, sulfidogenesis was likely due to elemental sulfur-reducing heterotrophic bacteria, and the nitrate-reducing microbial community consisted mainly of facultatively chemotrophic microbes. This study describes one set of conditions for continued sulfidogenesis during nitrate reduction, with important implications for nitrate control of sulfide production in oil fields.

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Keywords Ammonification · Elemental sulfur reduction · Petroleum · Souring · Sulfur cycling

Introduction

Sulfide produced by sulfate-reducing bacteria (SRB) is the source of many problems for the oil industry, including corrosion, reservoir plugging, and health risks to workers [44]. The production of biogenic sulfide accompanies waterflooding [31], which is used to increase oil recovery. Amendment of the injected water with nitrate is occasionally used to control sulfidogenesis [9]. This nitrate enables the indigenous, heterotrophic nitrate-reducing bacteria (HNRB) to outcompete SRB for nutrients [18] and drives the consumption of sulfide by nitrate-reducing, sulfide-oxidizing bacteria (NR-SOB) [26]. In addition, the nitrite produced during nitrate reduction is an inhibitor of SRB metabolism [35, 40].

Although nitrate addition has a high rate of success in laboratory and field trials in terms of controlling souring [12], some studies have shown that sulfide can persist in some produced waters amended with nitrate. This can be due to the absence of NRB from some waters, especially from high-temperature oil fields [28]. However, nitrate addition has also failed to control sulfide in mesothermic produced waters containing NRB. Such failures were observed in a flow-through bioreactor inoculated with mixed enrichment cultures (including NRB) from a mesothermic Saudi oil field [32] and in serum-bottle microcosm studies with produced water from an Alberta oil field [8, 10].

Voordouw et al. [45] proposed that the persistence of sulfide in the presence of NRB and nitrate could be due to continued sulfidogenesis as part of a sulfur cycle transferring electrons from oil organics to nitrate. This process was

subsequently demonstrated [42] by growing co-cultures of oil field NR-SOB isolates and either sulfate- or elemental sulfur (S^0)-reducing bacteria in media containing nitrate and either lactate or acetate as an electron donor. Hubert et al. [23] showed that the concentrations of nitrate and lactate, but not sulfate, governed the success of nitrate amendment in bioreactors inoculated with produced water. Because no significant shift in the microbial community was observed [23], the existence of a sulfur cycle between SRB and NR-SOB was proposed. However, sulfur cycling has yet to be unambiguously demonstrated in unenriched, nitrate-amended produced waters, and current accounts of the phenomenon lack detail.

Here we report on the ineffectiveness of nitrate to prevent sulfide persistence in produced water from a mesothermic Alberta oil field containing active NR-SOB. The objectives of this study were, first, to characterize the chemical changes resulting from inorganic and organic microcosm amendments in order to elucidate a detailed mechanistic explanation for sulfide persistence and, second, to infer from this mechanism ways in which the failure of nitrate addition in oil fields could be prevented.

Materials and methods

Site description and sample acquisition

Produced water samples were obtained from an oil field near Brooks, Alberta, Canada. This oil field, with a reservoir temperature of 35°C, is situated over the Glauconitic formation, has been undergoing produced water recirculation since production started in 1993, and has no history of biocide application. The sample used to prepare the microcosms described in this study was obtained in June 2008; the same installation had also been sampled on three previous occasions (September 2006, February 2007, and May 2007). Pertinent chemical and microbiological properties of the May 2007 and June 2008 water sample are provided in Table 1. The sampling vessels used were 4-l screw-cap steel cans lined with a mixture of 70% epoxy and 30% phenolic resin (Central Can Co, Chicago, IL) that had been sterilized by rinsing for 30 min with 30% (v/v) hydrogen peroxide. Hydrogen peroxide concentrations as low as 5 mg/l are known to have antimicrobial effects [3], so the cans were subsequently rinsed with sterile, boiling deionized water until peroxide concentrations in the rinse water were less than 0.1 mg l^{-1}, as determined using a test kit (CHEMets , Calverton, VA), and allowed to air-dry, inverted and uncapped, in a HEPA-filtered laminar-flow hood, prior to being re-capped and shipped to the oil field at Brooks. The prepared cans were completely filled with water from a free water knockout unit associated with a souring well. The filled cans were immediately shipped to our laboratory in refrigerated trucks at 4°C.

Table 1 Chemical properties, MPN estimates for important microbial metabolic groups, and sulfate reduction capability in produced oil field water samples from the Brooks free water knockout unit

	Sample date	
	May 2007	June 2008
Concentration (mM)		
Cl^-	144	140
HS^-	2.5	2.2
SO_4^{2-}	0.15	0.16
NH_4^+	0.99	0.98
NO_3^-	BDL[a]	BDL
MPN estimates (MPN/ml)[b]		
SRB	9,300 (1,500–38,000)	43,000 (7,000–210,000)
NR-SOB	24,000 (3,600–130,000)	210,000[c] (35,000–470,000)
HNRB	7 (1–23)	<0.3[d]
Fermenters	93 (15–380)	1,500[c] (300–4,400)
Methanogens	ND	2,300 (400–12,000)
Sulfate reduction coupled to oxidation of:[e]		
Acetate	–	–
Lactate	+ (to acetate)	+ (to acetate)

MPN, Most probable number; SRB, sulfate-reducing bacteria; NR-SOB, nitrate-reducing, sulfate-reducing bacteria; HNRB, heterotrophic nitrate-reducing bacteria; ND, not determined

[a] Denotes result below the detection limit (BDL) of the quantification method

[b] MPN with 95% confidence intervals in parentheses

[c] Significantly higher than May 2007 sample ($P < 0.05$)

[d] Significantly lower than May 2007 sample ($P < 0.01$)

[e] Results of parallel microcosm studies

Microcosm preparation

Before the sample was received, 158-ml empty serum bottles were flushed with O_2-free N_2, sealed with butyl rubber stoppers and aluminum crimps, autoclaved, and weighed. Immediately upon the arrival of the produced water samples to the laboratory, approximately 150-ml samples were aseptically pumped from the cans into each serum bottle. A hand pump (Nalge Nunc Intl, Rochester, NY; cat. no. 6133-0010) attached to a sterilized length of tubing fitted with an 18-gauge needle was used to draw a vacuum in the bottle, which caused the influx of the sample through a second sterilized needle and tubing connected to a sterile pipette in the sample can with no exposure of the water sample to air. The mass of water in each bottle was adjusted to 148.5 g using a sterile syringe, and bottles were amended with 1.5 ml of one of several sterile 100×

amendment concentrates. These concentrates had previously been prepared in 50-ml serum bottles by dissolving the appropriate sodium salts in boiling, deionized water to which had been added 7 g NaCl/l (to approximately match the chloride content of the sample, 140 mM) and 100 mg resazurin/l (to give a final concentration of 1 mg/l in the microcosms). The bottles were then sparged for 2 min with O_2-free N_2 prior to sealing and autoclaving. Final amendment concentrations in the microcosms were: no amendment; 10 mM nitrate; 10 mM nitrate and 1 mM acetate [hereafter designated NA(1)]; 10 mM nitrate and 10 mM acetate [NA(10)]; 10 mM nitrate and 2 mM phosphate (pH 7; NP); 10 mM nitrate, 5 mM acetate, and 2 mM phosphate (NAP).

During the subsequent incubation, some microcosms received secondary amendment spikes, which were prepared from sodium salts in an analogous manner to the original amendment solutions, excluding the resazurin. These amendments consisted variously of 2 mM molybdate; 2 mM nitrite; approximately 10 mmol S^0/l and 5 mM acetate with (S^0AM) or without (S^0A) 2 mM molybdate; approximately 10 mmol S^0/l, 5 mM acetate, and 10 mM nitrate with (S^0NAM) or without (S^0NA) 2 mM molybdate. S^0, prepared aseptically as a slurry according to the procedure of Frederiksen and Finster [16] using autoclaved glassware and reagents, was added directly to the designated microcosms using a sterile syringe fitted with an 18-gauge needle (0.2 ml of an approx. 7.5 mol S^0/l slurry). Residual sulfate and thiosulfate, each present at <4 mM in the sulfur slurry, became negligible after dilution into the microcosms. The volume of the 100× amendment concentrate required to attain the selected substrate concentration in each microcosm was determined by the mass of liquid remaining in each. Microcosms were sampled immediately after amendment and periodically thereafter using a sterile needle and syringe. Microcosms containing water from the June 2008 sampling were all constructed at the same time; total elapsed time between sample acquisition and the initial microcosm sampling ("time zero") was approximately 48 h.

Rationale behind amendments

Acetate was added to some microcosms because acetate is expected to enhance the growth of NRB, which outcompete SRB for nutrients in the presence of nitrate [18]. However, a countervailing argument asserts that an added organic electron donor could feed the hypothetical sulfur cycle and increase the concentration of nitrate required to successfully control sulfide [23, 42]. We expected our results to reveal the relative merits of acetate application to microcosms of this water sample. Phosphate addition to some microcosms was expected to relieve phosphate limitation for microbial growth [24, 26]. Molybdate is a well-known inhibitor of sulfate reduction [30, 41], and it has also been shown to block S^0 reduction [15]. The added concentration of 2.0 mM molybdate was above the concentration seen by Finster et al. [15] to suppress S^0 reduction and by Nemati et al. [36] to suppress sulfate reduction, thereby increasing the likelihood of complete inhibition of both processes. Nitrite is a known inhibitor of sulfate reduction [49] that has been shown to strongly inhibit sulfidogenesis in oil field-produced water and SRB subcultures [23, 35, 36, 40]. Its effects on S^0 metabolism have not been determined. The nitrite concentration (2.0 mM) added to the NAP microcosms was selected to permit observation of its effects on sulfide concentrations, while still allowing for its complete consumption.

Most probable number analyses

Microbial metabolic types were enumerated using the three-tube most probable number (MPN) method [2, 11]. Briefly, tenfold serial dilutions of a sample were prepared in anoxic 10 mM potassium phosphate buffer (pH approx. 7.2) and introduced into triplicate tubes of selective or differential media. Tubes were scored positive or negative for growth after an 8-week incubation in the dark at room temperature (approx. 21°C). Statistical comparison of MPN values were then performed [5]. The dilution buffer and all media contained 7 g NaCl/l according to the rationale described above. SRB were enumerated using the medium of Collins and Widdel [6], modified to contain a mixture of lactate (2.7 mM), acetate (2.9 mM), benzoate (0.69 mM), ethanol (6.5 mM), decanoate (0.58 mM), and propionate (3.9 mM). Two iron nails, previously washed with dichloromethane to remove a rust-prevention coating, were added to each tube of medium prior to sterilization. Subsequent accumulation of black FeS on the iron nails was taken as a positive score for SRB. Denitrifying HNRB were enumerated [10], and the tubes were scored positive if accumulated N_2O could be detected in the headspace using a gas chromatography (GC) method [14]. The NR-SOB were enumerated [11], and the criterion for a positive score was the appearance of a pink color due to the oxidation of resazurin. Fermentative microorganisms were enumerated in tubes containing tryptic soy broth (30 g/l), cysteine (0.5 g/l), and resazurin (1 mg/l), and scored positive if turbid. Methanogenic microorganisms were enumerated as outlined by Holowenko et al. [21].

Chemical analysis

Microcosm samples were filtered (0.22-μm GS filter; Millipore, Cork, Ireland) into sterile Eppendorf tubes. Sulfide was quantified immediately, and the remaining sample was frozen at −20°C pending the other analyses.

All analyses were completed within 3 weeks of the sampling date.

Soluble sulfide was analyzed using a colorimetric test kit (CHEMetrics, Calverton, VA). Nitrate, nitrite, sulfate, and chloride were determined using an ion-chromatography (IC) method [10]. Thiosulfate was analyzed using a modification of EPA method 300.0 [37] employing a Dionex 2500 ion chromatograph equipped with an NG1 organic guard column, AG14A guard column, AS14A analytical column, ASRS Ultra II suppressor, and CD25 conductivity detector (Dionex Corp, Sunnyvale, CA). The eluent contained 8.0 mM sodium carbonate and 1.0 mM sodium bicarbonate. Ammonium was determined colorimetrically [47] with a modified hypochlorite reagent buffered to a pH of 13 with 70 mM Na_2HPO_4 [29]. Inclusion of the buffer was found to improve color formation and the linearity of the calibration curve relative to unbuffered reagent. Ca^{2+}, Mg^{2+}, NO_2^-, and NO_3^- were not found to interfere in the range of ammonium concentrations encountered. S^0 was quantified using the method of Troelsen and Jørgensen [43], we accurately measured up to 4 mmol S^0/l, with a detection limit of 0.1 mmol S^0/l.

Acetate was quantified by GC [14], using 2.0 mM propionate as the internal standard. The specificity of the GC method for acetate was confirmed by performing parallel IC analyses on a sample subset and comparing the results. The IC equipment setup, eluent, and elution program were the same as those in the thiosulfate determination; acetate was quantified against calibration standards (0–1.5 mM) prepared from a stock solution of $CH_3COONa{\cdot}3H_2O$ in deionized water. The IC method had a detection limit of 0.3 mM and was thus considerably less sensitive than the GC method. The peaks obtained using the IC method were also much broader than those obtained using the GC method (peak area:height ratios of 0.35 and 0.06, respectively), leading to a relatively large integration error. Nevertheless, a comparison of 62 samples containing between 1.4 and 15 mM acetate showed that the IC method recovered 97 ± 11% of the acetate determinations by the GC, with a linear R^2 value of 0.97. This was interpreted as evidence that both methods were quantifying the same analyte, thus validating the GC method. Statistical tests, here as elsewhere in the data analysis, were performed using Microsoft Excel 2003.

Results

Characteristics of water samples

Water from the free water knockout unit was brackish, containing approximately 140 mM chloride, and sour, with over 2 mM sulfide present at the time of sampling (Table 1). Sulfate reduction was not a significant process in microcosms containing this water: not only were sulfate concentrations very low (below 0.2 mM; Table 1), but water samples obtained from the free water knockout unit dating back to 2006 (not shown) were characterized by the inability of the native microflora to reduce sulfate using naturally present electron donors, including acetate. This was not due to a lack of SRB, of which MPN estimates were large (Table 1); parallel work showed that SRB activity in the water could be induced by the addition of lactate. Prior to June 2008, water samples from the free water knockout unit also contained detectable populations of NR-SOB, denitrifying HNRB, and fermenters. MPN estimates for the May 2007 sampling are shown in Table 1; previous samplings in September 2006 and February 2007 gave results similar to these.

Microcosm work with the three Brooks samples obtained prior to June 2008 had yielded results typical of similar microcosm studies of nitrate amendment in oil field water samples [8, 10, 26, 36, 50]. Briefly, nitrate amendment led to the rapid consumption of sulfide, which was not oxidized to sulfate but probably to S^0, as evidenced by the appearance of a flaky white precipitate [26, 50]. Following sulfide depletion, nitrate consumption resumed after a lag of approximately 15 days and appeared to be heterotrophic, using acetate. Less than 20% of the consumed nitrate was recovered as nitrite or ammonium, suggesting that denitrification was the dominant metabolic pathway in these microcosms. A representative example of these early results is presented in Fig. 1: triplicate microcosms prepared from water obtained in May 2007 were amended with 10 mM nitrate, 5 mM acetate, and 2 mM phosphate (NAP).

Chemical response of June 2008 microcosms to amended nitrate

Compared to earlier experiments, microcosms prepared from the June 2008 sample displayed a markedly different

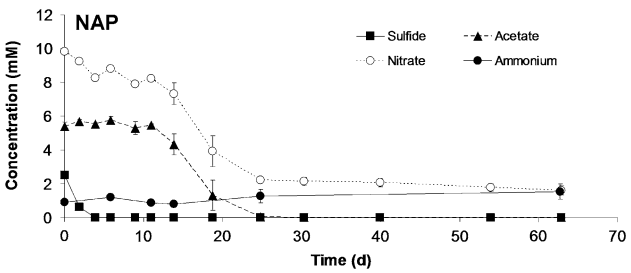

Fig. 1 Bulk chemical changes in May 2007 microcosms containing produced water amended with 10 mM nitrate, 5 mM acetate, and 2 mM phosphate (*NAP*). *Data points* represent the mean ± standard deviation (SD) of triplicate microcosms. Initial sulfate and nitrite concentrations (0.24 ± 0.04 mM and <0.04 mM, respectively) did not change appreciably over the incubation period (not shown)

chemical response to nitrate amendment. Among the novel processes observed was the regeneration of sulfide during nitrate consumption. This was an unprecedented result as well as an unexpected one, especially given the large NR-SOB MPN estimates in June 2008 water relative to previous samplings (Table 1).

Three distinct phases of activity were observed in nitrate-amended June 2008 microcosms (Table 2). Microcosms receiving amendments NAP, NP, and NA(10) are presented in Fig. 2a, b, c, respectively. Phase I was defined as the period between the initial amendment with nitrate and the onset of acetate consumption. It was characterized by a net oxidation of sulfide to S^0, with the concomitant conversion of nitrate to ammonium (Table 2), which is consistent with nitrate-dependent sulfide oxidation by NR-SOB (Eq. 1). The observed nitrate–sulfide stoichiometry was not significantly affected by addition of phosphate ($P > 0.05$; Fig. 2a, b); however, phosphate increased the maximal rates of sulfide and nitrate consumption in Phase I, thereby shortening its duration from approximately 15 to approximately 9 days (Table 2; Fig. 2b, c).

$$NO_3^- + 4HS^- + 6H^+ \rightarrow NH_4^+ + 4S^0 + 3H_2O$$
$$\Delta G^{0'} = -489\,kJ/reaction. \quad (1)$$

Phase II was defined as the period of acetate consumption. S^0 was consumed, and net production of sulfide was observed despite the active reduction of nitrate to ammonium (Table 2). Sulfide regeneration was dependent on the presence of acetate: microcosms that received acetate (e.g., NAP, Fig. 2a) regenerated more sulfide and exhibited a longer Phase II than microcosms receiving no acetate (e.g., NP, Fig. 2b; Table 2). This activity was saturable: 1 mM acetate gave results comparable to 10 mM acetate in terms of the rate and extent of nitrate, sulfide, and acetate consumption over the incubation period (Table 2). We hypothesized that acetate stimulated heterotrophic S^0 reduction to sulfide (Eq. 2), which was subsequently re-oxidized chemotrophically to S^0 by ammonifying NR-SOB (Eq. 1).

$$CH_3COO^- + 4S^0 + 4H_2O \rightarrow 2HCO_3^- + 4HS^- + 5H^+$$
$$\Delta G^{0'} = -6.7\,kJ/reaction \quad (2)$$

The reduction of S^0 to sulfide was significantly faster ($P < 0.001$) in microcosms containing amended phosphate: the maximum rate of sulfide accumulation in NAP microcosms ($+410 \pm 260$ μM HS^-/day) was approximately fourfold higher than the rates in NA(1) and NA(10) microcosms ($+120 \pm 40$ and $+97 \pm 43$ μM HS^-/day, respectively; Table 2).

Summing the equations for S^0 reduction (Eq. 2) and sulfide re-oxidation (Eq. 1) yields Eq. 3, which predicts the consumption of 1 mol acetate for each mole of nitrate ammonified. The actual amount of acetate consumed in Phase II exceeded molar unity with nitrate and ammonium in all microcosm series monitored (Table 2), possibly due to additional sinks, such as biomass formation.

$$NO_3^- + CH_3COO^- + H_2O + H^+ \rightarrow NH_4^+ + 2HCO_3^-$$
$$\Delta G^{0'} = -495\,kJ/reaction \quad (3)$$

Nitrate reduction during Phase II was also significantly accelerated by the addition of phosphate (Table 2): the maximum rate of nitrate consumption in NAP microcosms (-280 ± 190 μM/day) was approximately 15-fold faster than the maximum rate seen in either NA(1) or NA(10) microcosms (both $P < 0.001$). The effects of phosphate on Phase II metabolism are evident from a comparison of Fig. 2a, c.

A modulation in nitrate metabolism between Phase I and Phase II was also apparent in NAP microcosms (Table 2): the conversion of nitrate to ammonium in NAP microcosms was significantly more efficient during Phase II (3.6 ± 1.0 mM nitrate reduced to 3.3 ± 0.7 mM ammonium; conversion efficiency of all five replicates averaged $94 \pm 9\%$) than during Phase I (1.2 ± 0.4 mM nitrate reduced to 0.72 ± 0.19 mM ammonium; average efficiency of $60 \pm 14\%$ among 13 replicates) ($P < 0.0001$). The extent of nitrate metabolism in the other microcosm series (Fig. 2b, c; Table 2) was not large enough to permit an accurate comparison of nitrate conversion efficiencies. At no time during any of our incubations did the resazurin in the microcosm turn pink, indicating that the redox potential in each microcosm remained below −100 mV.

Phase III comprised all activity following acetate depletion. Due to the extended duration of Phase II in acetate-amended microcosms, only microcosms unamended with acetate entered Phase III during the period of observation, i.e., series N (two of three replicates; Table 2) and NP (Fig. 2b). Acetate-amended microcosms would also be expected eventually to enter Phase III, contingent on a stoichiometric excess of nitrate relative to organic electron donors [42]. The predominant process in Phase III was the nitrate-dependent re-oxidation of regenerated sulfide to S^0 (Table 2; Fig. 2b). Sulfide oxidation occurred in concert with ammonification, which accounted for all Phase III nitrate consumption in N and NP microcosms (Table 2), although the source of the electron donor appeared to differ between the two microcosm series. The calculation of the molar ratio of nitrate consumed to sulfide consumed during Phase III in each of the NP replicates yielded an average of 0.25 ± 0.04 mol nitrate/mol sulfide, matching the molar ratio of 0.25 predicted from Eq. 1 and indicating stoichiometric chemotrophic nitrate reduction. One of the N microcosms gave a similar result, with the consumption of 0.22 mol NO_3^-/mol HS^-; however, the

Table 2 Substrate consumption or accumulation in microcosms containing Brooks June 2008 produced water amended with one of five amendments during each phase of sulfur metabolism (see Fig. 2)

Series	*n*	Phase duration (days)	Change in substrate concentration (mM)[a]					Maximum rate of change (μM/day)	
			ΔHS^-	ΔS^0	ΔNO_3^-	ΔNH_4^+	ΔCH_3COO^-	ΔHS^-	ΔNO_3^-
Phase I[b]									
N	3	20 ± 8	−1.9 ± 0.3	+1.7 ± 0.3	−0.99 ± 0.19	+0.95 ± 0.21	−0.06 ± 0.09	−98 ± 29	−55 ± 23
NP	4	9 ± 1	−1.5 ± 0.5	+2.0 ± 0.9	−1.0 ± 0.2	+0.89 ± 0.13	−0.15 ± 0.03	−360 ± 150	−150 ± 10
NA(1)	3	15 ± 0	−1.4 ± 0.5	+1.4 ± 0.5	−0.85 ± 0.19	+0.71 ± 0.25	+0.20 ± 0.16	−90 ± 30	−56 ± 12
NA(10)	3	15 ± 0	−1.4 ± 0.6	+1.6 ± 0.5	−0.71 ± 0.23	+0.49 ± 0.23	+0.34 ± 0.12	−88 ± 40	−46 ± 15
NAP	13	8 ± 1	−1.9 ± 0.5	+2.2 ± 0.3	−1.2 ± 0.4	+0.72 ± 0.19	−0.11 ± 0.18	−320 ± 100	−200 ± 70
Phase II									
N	3	15 ± 17	+0.46 ± 0.19	−0.62 ± 0.39	−0.11 ± 0.29	+0.29 ± 0.16	−0.32 ± 0.09	+93 ± 50	−3 ± 19
NP	4	7 ± 1	+0.40 ± 0.17	−0.26 ± 0.13	−0.22 ± 0.06	+0.28 ± 0.06	−0.35 ± 0.02	+140 ± 130	−17 ± 47
NA(1)	3	>50[e]	+1.5 ± 0.5	−1.7 ± 0.5	−0.29 ± 0.14	+0.46 ± 0.28	−1.2 ± 0.3	+120 ± 40	−15 ± 16
NA(10)	3	>50[e]	+1.5 ± 0.6	−1.9 ± 0.5	−0.17 ± 0.06	+0.60 ± 0.05	−1.3 ± 0.3	+97 ± 43	−20 ± 11
NAP	5[c]	>72[e]	+1.8 ± 0.5	−2.1 ± 0.4	−3.6 ± 1.0	+3.3 ± 0.7	−4.8 ± 1.1	+410 ± 260	−280 ± 190
Phase III									
N	2[d]	37 ± 12	−0.78 ± 0.31	+0.86 ± 0.09	−0.31 ± 0.13	+0.29 ± 0.02	−0.08 ± 0.06	−35 ± 21	−8 ± 3
NP	4	32 ± 20	−0.75 ± 0.26	+0.40 ± 0.10	−0.20 ± 0.09	+0.20 ± 0.08	−0.02 ± 0.03	−33 ± 9	−4 ± 5
NA(1)	3	–[f]	–	–	–	–	–	–	–
NA(10)	3	–	–	–	–	–	–	–	–
NAP	5[c]	–	–	–	–	–	–	–	–

All values are given as the mean ± standard deviation (SD)

Ammendments: 10 mM nitrate (N); 10 mM nitrate and 2 mM phosphate (NP); 10 mM nitrate and either 1 or 10 mM acetate [NA(1) and NA(10), respectively]; 10 mM nitrate, 2 mM phosphate, and 5 mM acetate (NAP)

[a] S^0 concentrations given in mmol/l

[b] See text for definition of each phase. Phase I transition to Phase II was considered to occur at the last sampling point at which acetate concentrations were not significantly different from the concentration at 0 days ($P > 0.05$). Phase II was considered to end either when acetate was no longer detectable, or at the end of the incubation (90 days for NAP; 65 days for all others)

[c] NAP microcosms that did not receive spikes at 20 days

[d] Only two of three N microcosms entered Phase III during the incubation period

[e] All microcosms in these series were in Phase II at the end of the incubation period. The concentration changes in this row are calculated from this truncated Phase II, ending at 65 days [NA(1) and NA(10)] or 90 days (NAP)

[f] No microcosms in these series entered Phase III during the incubation period

other replicate microcosm to enter Phase III consumed 0.72 mol NO_3^-/mol HS^-. This observation suggests an additional source of electrons for nitrate reduction, other than sulfide, but this source was not identified. Phase III sulfide oxidation therefore appeared to be coupled to the ammonification of nitrate—according to Eq. 1—with the possibility of further ammonification with other, unidentified electron donors.

Sulfur metabolism in NAP microcosms after the addition of molybdate

We tested whether Phase II nitrate reduction in NAP microcosms was dependent on a reductive sulfur metabolism by spiking triplicate NAP microcosms in early Phase II with 2.0 mM molybdate. Its addition to NAP microcosms was expected to stop sulfidogenesis and lead to the accumulation of the product of sulfide oxidation produced by NR-SOB. Within 5 days after molybdate addition, the decrease in sulfide concentration (1.8 ± 0.3 mM) corresponded with the appearance of S^0 (2.5 ± 0.5 mmol/l), while nitrate (2.4 ± 1.0 mM) was reduced to ammonium (2.5 ± 0.6 mM), and acetate concentrations declined by 2.2 ± 1.4 mM (Fig. 3). The rate of ammonification was significantly accelerated, from 85 ± 71 μM NH_4^+/day in the 5 days prior to the spike to 510 ± 130 μM NH_4^+/day in the 5 days following ($P < 0.05$). The continued consumption of acetate, despite the inhibition of heterotrophic S^0 reduction, suggested that nitrate-dependent acetate oxidation (i.e., heterotrophic nitrate reduction) became significant after the addition of molybdate. Over the 5 days following the addition of molybdate, the nitrate consumed in the

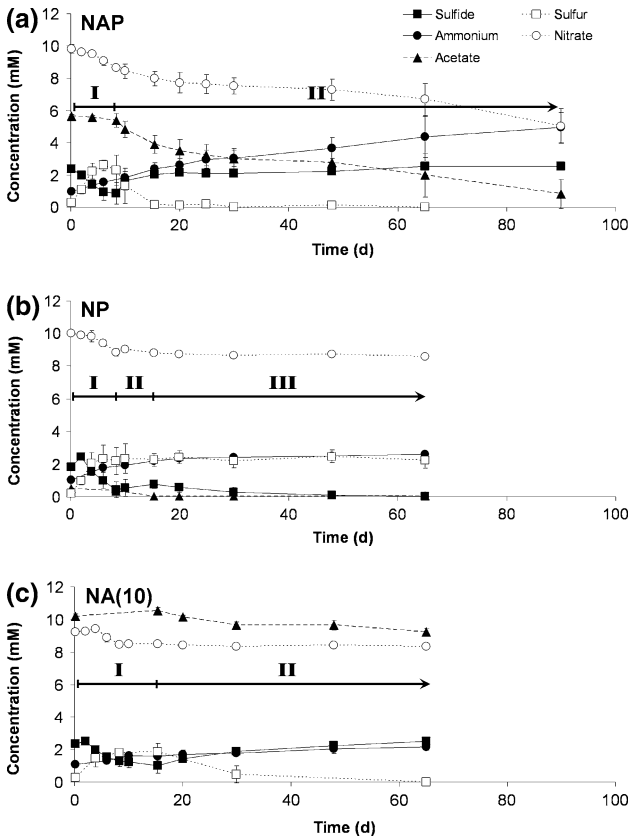

Fig. 2 Chemical changes in June 2008 microcosms containing produced water amended with 10 mM nitrate, 5 mM acetate and 2 mM phosphate (*NAP*, **a**); 10 mM nitrate and 2 mM phosphate (*NP*, **b**); 10 mM nitrate and 10 mM acetate [*NA(10)*, **c**]. *Data points* represent the mean ± SD of quintuplicate (**a**) or triplicate (**b**, **c**) microcosms. Divisions marked with *Roman numerals* show the phases of sulfur transformation, as defined in the text. Data are summarized in Table 2. Initial sulfate and nitrite concentrations (<0.2 mM and <0.04 mM, respectively) did not change appreciably over the incubation period (not shown)

microcosms averaged 99% of the stoichiometric predictions of Eqs. 1 and 3. Between 0.4 and 3.3 mM nitrite accumulated in the microcosms after acetate concentrations decreased below 1 mM (Fig. 3).

Sulfur metabolism in NAP microcosms after the addition of nitrite

Nitrite was added at 90 days to two NAP microcosms in Phase II, shown in Fig. 4a, b. Within 1 day of amendment, sulfide began to be converted to S^0. No evidence of a chemical reaction between sulfide and nitrite was seen in the abiotic controls (not shown). For the duration of its persistence in the microcosms (days 90–95), nitrite appeared to be preferred to nitrate as an electron acceptor: nitrate consumption virtually ceased following the nitrite spike (Fig. 4). In both microcosms, all amended nitrite was completely converted to ammonium within 5 days of the

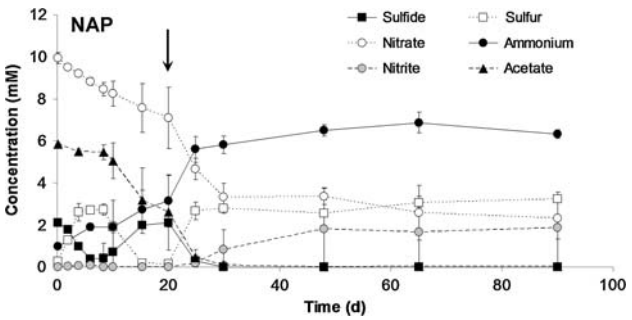

Fig. 3 Chemical changes in June 2008 microcosms containing produced water amended with 10 mM nitrate, 5 mM acetate, and 2 mM phosphate (*NAP*), spiked with 2 mM molybdate after a 20-day incubation (*arrow*). *Data points* represent the mean ± SD of triplicate microcosms. The initial sulfate concentration (0.15 ± 0.01 mM) did not change appreciably over the incubation period (not shown)

spike. However, net sulfide regeneration only resumed in the microcosm that still contained acetate (days 95–100; Fig. 4a), and even then, only until depletion of the acetate by day 95, demonstrating again the acetate-dependence of sulfidogenesis in these microcosms.

Elemental sulfur metabolism in produced water microcosms

The capability of the June 2008 Brooks oil field microflora to couple S^0 reduction to acetate oxidation was tested by spiking an unamended microcosm with approximately 10 mmol S^0/l and 5 mM acetate (S^0A) at 48 days (Fig. 5a). (The concentrations of S^0 in all S^0-amended microcosms overwhelmed the assay for S^0 and could not be accurately determined.) Thereafter, sulfide was produced at an average rate of 160 μM HS^-/day in a 3.3:1 molar ratio to consumed acetate, which is close to the 4:1 ratio predicted from Eq. 2. Neither sulfide production nor acetate consumption was observed in either of duplicate microcosms that received 2 mM molybdate in addition to S^0 and acetate (S^0AM, Fig. 5b). Instead, molybdate resulted in a rapid decrease in sulfide concentration of 2.2 ± 0.2 mM and the development of a deep orange-red color. These observations are consistent with chemical scavenging of the sulfide by molybdate (Eq. 4) [13], forming thiomolybdates, which are red or orange in color [34]. The reaction is catalyzed by NH_4^+, which was present in the native Brooks water (Table 1). The production of a reddish-brown color in sulfidic water amended with ammonium molybdate was also reported by Nemati et al. [36]; no effect on sulfide concentration was reported by those authors due to interference of the colored compound with their sulfide assay.

$$MoO_4^{2-} + 4H_2S \rightarrow [MoO_{4-x}S_x]^{2-} + x\,H_2O + (4-x)H_2S. \quad (4)$$

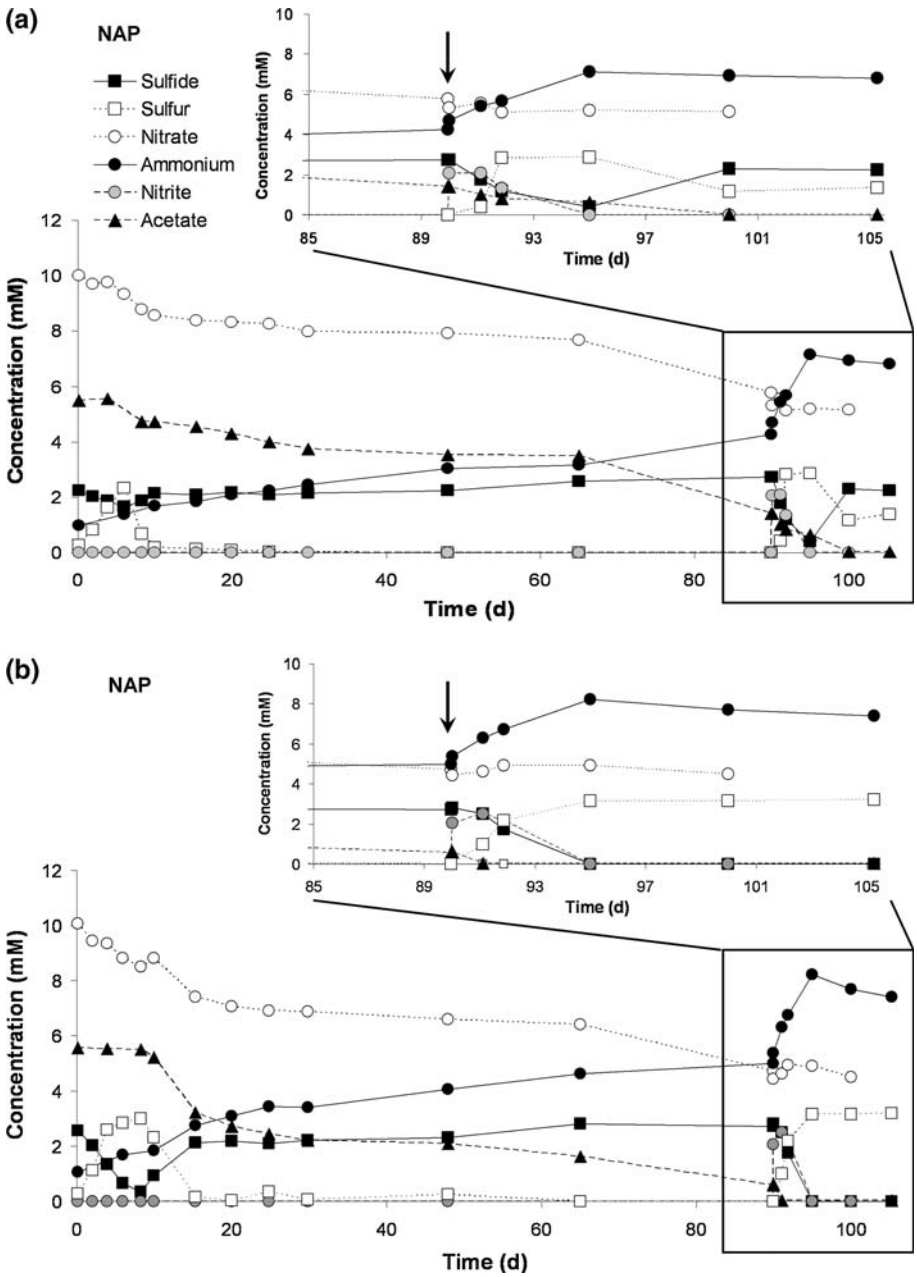

Fig. 4 Chemical changes in two replicate June 2008 microcosms (**a**, **b**) containing produced water amended with 10 mM nitrate, 5 mM acetate, and 2 mM phosphate (*NAP*), spiked with 2 mM nitrite after a 90-day incubation (*arrow*). The final 30 days of the incubation for each microcosm are shown expanded in an *inset*. The initial sulfate concentration in each replicate (0.17 mM) did not change appreciably over the incubation period (not shown)

A microcosm receiving 10 mM nitrate in addition to approximately 10 mmol S^0/l and 5 mM acetate (S^0NA) exhibited simultaneous ammonification, acetate consumption, and sulfidogenesis (Fig. 5c). Nitrate (4.9 mM) was converted to ammonium (4.7 mM) concurrently with the depletion of acetate (5.1 mM) and the production of sulfide (2.9 mM) at an average rate of 140 μM HS^-/day, or 84% of the rate seen in the S^0A microcosm (Fig. 5a). When molybdate was included in the amendment applied to duplicate microcosms (S^0NAM; data not shown), neither nitrate reduction nor acetate consumption was seen, and the observed decline in sulfide concentration was not significantly different from that seen in the S^0AM microcosms ($P = 0.97$). The S^0NAM microcosms also developed the same orange-red color observed in S^0AM microcosms.

Discussion

Sulfide persistence mechanisms

Although the persistence of sulfide in nitrate-amended produced waters has been noted by some researchers [8, 10, 32], mechanistic explanations of the phenomenon have been speculative. Evidence supporting a hypothesis involving a sulfur cycle linking organotrophic sulfidogenesis to

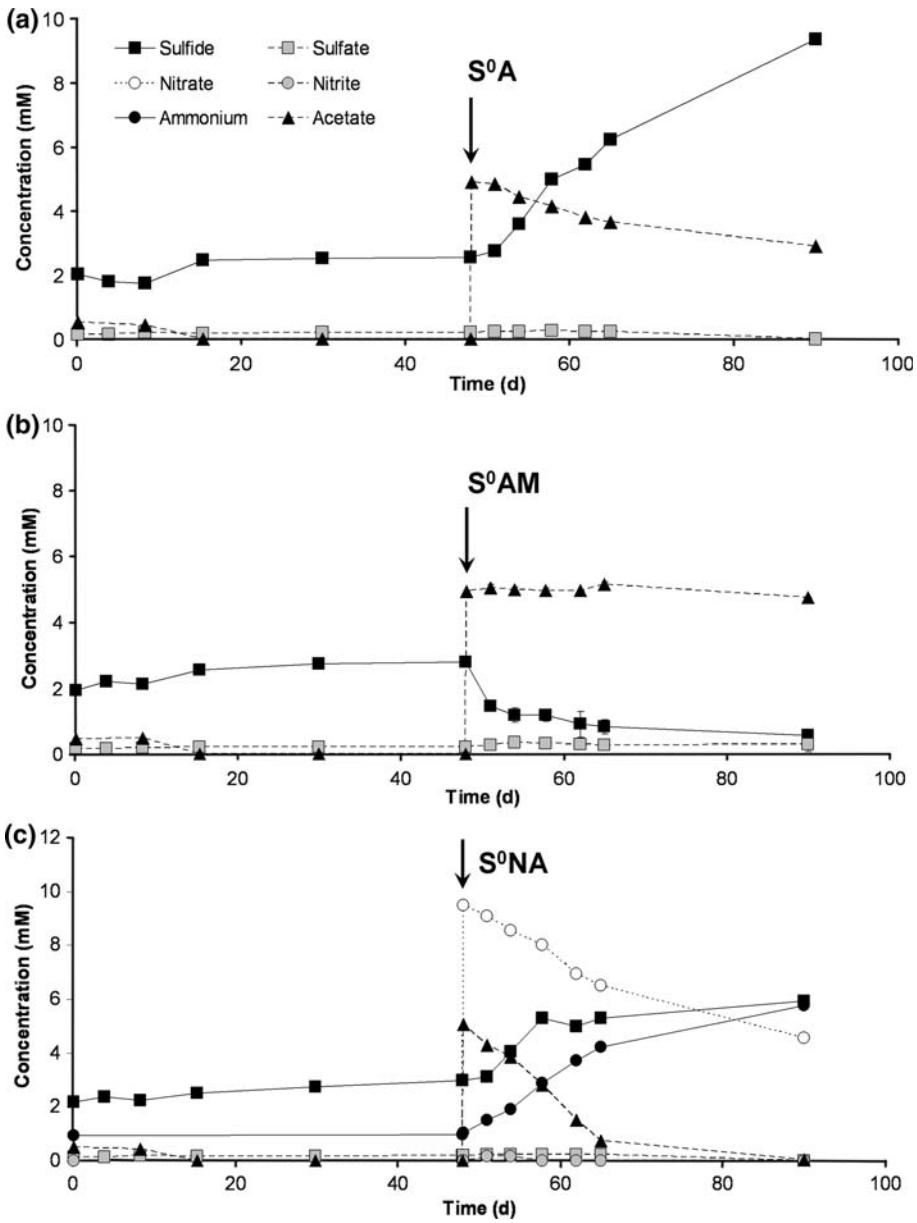

Fig. 5 Chemical changes in unamended June 2008 microcosms containing produced water spiked after 48 days (*arrow*) with one of the following: approximately 10 mmol S^0/l and 5 mM acetate (S^0A) (**a**); approximately 10 mmol S^0/l, 5 mM acetate, and 2 mM molybdate (S^0AM) (**b**); approximately 10 mmol S^0/l, 5 mM acetate, and 10 mM nitrate (S^0NA) (**c**). *Data points* in **a** and **c** are measurements of single microcosms; those in **b** represent the mean ± SD of duplicate microcosms

chemotrophic nitrate reduction [48] has been obtained in co-cultures of oil field isolates and produced water enrichments [42], but our study is the first to detect this mechanism in unenriched, nitrate-amended produced water.

A diagram of the proposed metabolic relationships is presented in Fig. 6. The reduction of the electron acceptor nitrate is coupled with the incomplete oxidation of sulfide to S^0, which is then re-reduced with acetate. Sulfide will accumulate if the rate of acetate-dependent S^0 reduction exceeds the rate of nitrate-dependent sulfide oxidation.

The oxidation of sulfide to S^0 was characteristic of Phases I and III of microcosm metabolism (Fig. 2a, b, c). This reaction was linked to the conversion of nitrate to ammonium, presumably by the NR-SOB present in the sample (Table 1), and this type of metabolism has been observed in a NR-SOB isolated from an oil field [22]. The coupling of acetate conversion to S^0 reduction in nitrate-amended microcosms during Phase II was inferred from the regeneration of higher concentrations of sulfide (1.5–1.8 mM) and its longer persistence (>50 days) in the presence of amended acetate (Table 2). This process was seen in isolation in S^0A microcosms (Fig. 5a), which produced sulfide and consumed acetate in the approximate proportions predicted by Eq. 2. Microbial catalysis of acetate-dependent S^0 reduction was evidenced by the higher maximum rates of Phase II sulfide accumulation in the presence of phosphate (NAP microcosms; Table 2) than in its absence (NA microcosms) and by the complete suppression of sulfidogenesis and acetate consumption in the presence of molybdate [15] (Fig. 5b).

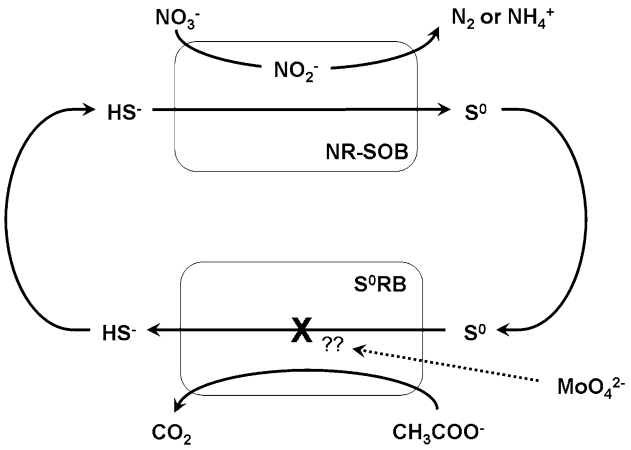

Fig. 6 Schematic of S^0 cycling inferred from June 2008 microcosms containing produced water amended with nitrate and phosphate. *Solid lines* denote flux, *broken line* and *X* indicate inhibition. *S^0RB*, S^0-reducing bacteria; *NR-SOB*, nitrate-reducing, sulfide-oxidizing bacteria. *Question marks* denote a process with an unknown mechanism

Nitrate did not suppress the reduction of exogenous S^0 in S^0NA microcosms (Fig. 5c): the persistence of sulfide during nitrate reduction in these microcosms was consistent with the role of sulfide as an electron conduit in the proposed sulfur cycle (Fig. 6). The chemical changes in S^0NA microcosms were also similar to the Phase II chemical changes in NAP microcosms between days 8 and 90 (Fig. 2a), when the sulfur cycle was thought to be active.

The absence of substantial Phase II nitrate metabolism in microcosms which did not receive amended phosphate [series N, NA(1), NA(10); Table 2] may have been due to slower nitrate reduction under phosphate-limiting conditions. With respect to the mechanism of nitrate metabolism itself, the predominance of ammonification in NAP microcosms during Phase II versus Phase I may have been due to the effects of accumulated sulfide, which has been shown to inhibit denitrification [27] and to encourage ammonification in NRB capable of both metabolisms [4].

Although application of a nitrite spike to sulfur-cycling NAP microcosms resulted in temporary cessation of sulfide regeneration (Fig. 4), this was not necessarily due to the inhibitory effects of nitrite on S^0 reduction. Nitrite inhibits sulfate reduction by competitively binding the enzyme dissimilatory sulfite reductase [49]. S^0 reduction is not thought to require this enzyme [39]. Instead, nitrite may simply be a more favorable electron acceptor for sulfide oxidation, possibly because of a higher abundance or activity of nitrite reductase relative to nitrate reductase [1]. Consequently, sulfide depletion following the nitrite spike may be due to increased rates of sulfide oxidation rather than to the inhibition of sulfidogenesis. Continued acetate consumption (possibly due to S^0 reduction) in the presence of amended nitrite and the rapid resumption of sulfide accumulation after nitrite depletion (Fig. 4a) provide tentative support for this hypothesis. Increased rates of sulfide oxidation with nitrite reduction relative to nitrate reduction may also explain why the commencement of Phase II sulfide accumulation was always dependent on the total depletion of nitrite produced during Phase I.

The addition of molybdate to sulfide-regenerating NAP microcosms in Phase II led to the rapid depletion of sulfide and the accumulation of S^0, coincident with marked increases in the rates of ammonification and of acetate consumption (Fig. 3). Molybdate is a effective inhibitor of sulfidogenesis [9, 36]. However, inhibition of sulfidogenesis alone does not explain the disappearance of sulfide from molybdate-amended NAP microcosms. Nor is chemical scavenging plausible: no color change indicative of thiomolybdate production (Eq. 4) was observed in molybdate-amended NAP microcosms, and sulfide was quantitatively converted to S^0 (Fig. 3). A more likely hypothesis is that the interruption of the reductive arm of the sulfur cycle by molybdate revealed concomitant sulfide consumption by NR-SOB as the oxidative arm continued unabated (Fig. 6).

This explanation accounts for the observed sulfur transformations after the molybdate spike, but it does not explain the increased rates of ammonification and acetate consumption (Fig. 3), both of which should have been impeded by a break in the sulfur cycle (Fig. 6). The stoichiometric changes in acetate, nitrate, and ammonium concentrations after the addition of molybdate were still consistent with acetate-dependent ammonification (Eq. 3), even though this had not been observed in the absence of an intervening sulfur cycle. The emergence of a previously inactive and unnoticed HNRB population was contraindicated by the rapid onset of the increased ammonification rates following the molybdate spike and by the absence of HNRB activity from all other microcosms. The data therefore led us to propose that the NR-SOB community active prior to the molybdate spike was facultatively chemotrophic and thus able to ammonify nitrate heterotrophically once the sulfur cycle was broken. This contention, although unsupported in this work by any determinative microbiology, has some precedent: Hubert and Voordouw [22] demonstrated the predominance of just such facultatively chemotrophic ammonifying NRB in a nitrate-amended produced water system.

Implications of sulfide regeneration for souring control of oil fields

Several mechanisms exist whereby the suppression of sulfidogenesis is thought to be induced in produced waters by nitrate amendment [12]. These include (1) the production of nitrite [17]; (2) "biocompetitive exclusion" [18], in which HNRB outcompete sulfidogenic microbes for electron donors and nutrients; (3) increased redox potential

[25]. The persistence of sulfide in our nitrate-amended microcosms shows that these mechanisms were either inactive or otherwise insufficient for the prevention of microbial sulfidogenesis. With respect to the first mechanism, we did not detect nitrite in our microcosms at any point (Fig. 2)—except following molybdate or nitrite treatment of NAP microcosms. The absence of nitrite may be explained by its rapid metabolism relative to nitrate, as seen in the nitrite-spiked NAP microcosms (Fig. 4). The second mechanism of "biocompetitive exclusion" failed due to a lack of HNRB activity in nitrate-amended microcosms. This was consistent with the absence of denitrifying HNRB from the water sample (Table 1) and supports the speculation of Eckford and Fedorak [10] that HNRB activity is an important competitive check on sulfidogenic processes that could otherwise sustain a sulfur cycle. Third, the lack of development of a pink color in any nitrate-amended microcosm showed that in no instance did the redox potential rise above −100 mV, the approximate threshold for sulfate reduction [38] (S^0-reducing bacteria are less stringent in their redox requirements [48]). The low redox potential was consistent with the lack of denitrifying HNRB in the water sample (Table 1) and the predominance of ammonification, rather than denitrification, in the microcosms (Fig. 2). Although ammonifying NRB have been neglected in studies of oil field waters, here they appear to be important by virtue of their abundance relative to denitrifying microbes.

The results of this study highlight several important practical considerations for the design of nitrate amendment solutions. Some patented methods for nitrate-mediated sulfide control [19, 20] advocate the inclusion of acetate or other organic carbon sources to serve as an additional electron donor and/or carbon source for NRB, which are then assumed to outcompete SRB. However, the addition of acetate to our nitrate-amended microcosms obviously counteracted the short-term goal of sulfide control: acetate was used for the production of sulfide (Fig. 5c, 6), which accumulated (Fig. 2a, c); the addition of acetate also prolonged sulfur cycling and increased the amount of nitrate required to successfully remove sulfide (Table 2). Although we did not determine the long-term effects of acetate on NRB populations, the fact that the acetate amendment stimulated sulfidogenesis and not heterotrophic nitrate reduction is not salutary.

In our nitrate-amended microcosms, sulfide was generated exclusively through S^0 reduction using acetate as electron donor. Studies of produced water microbiology tend to ignore this pathway in favor of sulfate reduction [11, 33, 46] because sulfate is quantitatively a far more important substrate for de novo sulfidogenesis [7, 31]. However, S^0-reducing bacteria are far more phylogenetically diverse than SRB, with minimal overlap between the two groups [39], and their abundance in oil field produced waters has not been systematically examined.

The generation of S^0 within the oil field environment as a result of nitrate amendment could conceivably lead to serious practical problems. In addition to its corrosivity towards iron and its solubility in petroleum, S^0 is a particulate that could accumulate and reduce reservoir porosity. Even assuming long-term suppression of in situ sulfidogenesis, deposits of S^0 would constitute relatively static reservoirs of sulfidogenic substrate, awaiting the return of favorable conditions.

In summary, we propose a mechanism by which sulfide can persist in produced water after nitrate amendment, despite the presence of a significant NR-SOB population (Fig. 6). The results of this study contribute to a growing body of evidence suggesting that nitrate-mediated sulfide control is most effective when sulfide oxidation by NR-SOB is complemented by processes that inhibit sulfidogenesis. They also demonstrate that the production of sulfide from S^0 can be practically significant, even in the absence of actively sulfidogenic SRB. This indicates, first, the need for more comprehensive microbial enumeration and classification strategies in the characterization of produced water, and second, a more holistic approach to the inhibition of sulfidogenesis, one that encompasses the possibility of S^0 reduction.

Acknowledgments This work was funded by Schlumberger Cambridge Research Ltd. and the Natural Sciences and Engineering Research Council of Canada. We thank EnCana Corporation for the water samples, and we thank Sara Ebert and Debbi Coy for technical assistance.

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