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Use of nitrate to control sulfide generation by sulfate-reducing bacteria associated with oily waste

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Sulfide is a toxic and corrosive product of sulfate-reducing bacteria that can accumulate in oily waste streams to nuisance levels. Sludge associated with an oily waste stream was collected from a settling tank and used to assess sulfide generation activities. Methanogenesis was a predominant process in sludge in the absence of sulfate, and was suppressed by nitrate. Sulfate reduction and sulfide formation were evident when sulfate was available. Nitrate diminished sulfate reduction and prevented sulfide accumulation under freshwater, brackish, and saltwater conditions. Sodium-, potassium-, and calcium nitrate were equally effective in curtailing sulfide formation. The effects of nitrate on sulfate depletion were concentration-dependent, with 50 mM nitrate diminishing sulfate reduction, yet as little as 16 mM nitrate prevented sulfide accumulation. Sulfide was oxidized in nitrate-reducing incubations, and accumulation of sulfur or sulfate was observed. Nitrate reduction was accompanied by production of nitrite and nitrous oxide, which probably helped prevent sulfate reduction in extended incubations. Our results suggest that nitrate amendments control the formation of sulfide in oily waste streams both by preventing sulfate reduction and by stimulating anaerobic sulfide oxidation.

Keywords: nitrate; sulfide; anaerobic; sulfate-reducing bacteria; petroleum

Introduction

Oily wastes associated with petroleum extraction, refining, utilization, transportation, and disposal are subject to microbiological alteration under anaerobic conditions. During collection and treatment of oily wastes, or subsurface injection of seawater for oil recovery, biological alteration of the physico-chemical properties of oils can occur [16,17,31]. Furthermore, substantial amounts of sulfide can be generated by sulfate-reducing bacteria (SRB) which are virtually ubiquitous in anoxic environments. Whether present in planktonic form or as biofilms, a wide variety of SRB are clearly associated with oil production systems, although the relative prevalence of various species is still under investigation [5,27,34,36]. These nutritionally diverse organisms can use a variety of xenobiotic compounds, including petroleum components, as electron donors, and couple this metabolism to the reduction of sulfate to sulfide [12,30,31]. Sulfide is a serious concern because of its odor, toxicity, corrosiveness, and ability to form insoluble iron sulfide precipitates that plug oil-bearing strata and stabilize undesirable oil-water emulsions [9]. In addition, sulfide contamination increases the sulfur content of fossil fuels and results in the devaluation of energy reserves. Processes for control of sulfide production, as well as other activities of SRB, are needed from both an economic and environmental perspective [16].

Thermodynamically, the microbial reduction of nitrate to nitrite, nitrogen, or ammonia provides more Gibbs free energy than the reduction of sulfate [22], and therefore nitrate can be a preferred electron acceptor when both anions are potentially available. Nitrate may provide a competitive advantage for nitrate-reducing bacteria over SRB during competition for available electron donors. Nitrate can also serve as an alternative electron acceptor for SRB and thus prevent sulfide formation [10]. Nitrate is applied to control odors associated with sewage systems [4,19], resulting in both transient and long-term inhibition of sulfide production [1,3,7,13,15,26]. Nitrate has also been used to control sulfide production in sandstone cores with subsurface formation water from a gas storage facility [23], and in oil fields in which oil is produced by water flooding [34]. The addition of high nitrate concentrations also leads to the buildup of nitrous oxide, which raises the redox potential, contributing to long-term prevention of sulfide production [1,18,26,29]. We investigated whether nitrate could be useful for preventing sulfide formation associated with the collection and treatment of oily wastes produced on board marine vessels.

Materials and methods

Media and experimental conditions

A brackish medium designed for the cultivation of SRB [37] was prepared using strict anaerobic techniques. Resazurin (2 mg L^{-1}) was included as a redox indicator, the sulfate concentration was 20 mM unless otherwise indicated, and the medium was reduced with sodium sulfide (1 mM). The medium was dispensed into 25-ml serum bottles inside an anaerobic chamber and each bottle was inoculated with 0.5 ml of oily sludge. The sludge was collected from a settling tank (Tank No. 63) at the US Navy Craney Island Fuel Depot in Portsmouth, Virginia, and stored in sealed glass bottles at room temperature. Serum bottles were sealed with 1-cm-thick butyl rubber stoppers (Bellco Glass, Vineland, NJ, USA), then removed from the anoxic

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chamber and secured with aluminum crimp seals. The gas phase of the serum bottles was adjusted to N_2:CO_2 (80%:20%). Sterile controls were autoclaved. Sodium lactate was added from a neutralized sterile anoxic stock solution to an initial concentration of 10 mM unless otherwise indicated. Cultures were incubated at room temperature, in the dark, without shaking. Samples were withdrawn periodically using strict anaerobic technique and stored frozen until analyzed. Prior to analysis, the samples were thawed and centrifuged ($10\,000 \times g$) for 5 min to remove particulate debris.

For the initial survey of anaerobic activity, the medium did not receive an electron acceptor (methanogenic conditions), or was amended with either sodium sulfate (20 mM) or sodium nitrate (20 mM) from sterile, anoxic stock solutions. Sodium acetate, when used, was added from a stock solution to an initial concentration of 10 mM. Results from this experiment are averages for cultures established with 0.5, 1, or 2 mM initial sulfide concentration. The effect of nitrate on sulfate-reducing and methanogenic cultures was determined in triplicate cultures containing lactate (10 mM), benzoate (1 mM), or no exogenous substrate, with or without 100 mM nitrate.

To test the effect of sodium nitrate, under different salinity conditions, a freshwater medium was prepared (1 g L^{-1} NaCl), and a salt concentrate was added to give brackish (7 g L^{-1}) or saltwater (20 g L^{-1}) conditions [37]. Incubations without nitrate were compared to those receiving 50 mM $NaNO_3$. Results are averages of triplicates.

To compare the three forms of nitrate, sterile anoxic solutions of $NaNO_3$, KNO_3, and $Ca(NO_3)_2$ were added to cultures to give 50 mM nitrate. Likewise, NaCl, KCl, and $CaCl_2$ were prepared as stock solutions and added to give 50 mM additional chloride, to distinquish effects of the various cations from the nitrate. Results are averages of triplicates except for sterile controls.

For determining the minimum nitrate concentration required to affect sulfate depletion and sulfide accumulation, cultures were amended with 0–80 mM $NaNO_3$. A negative control without lactate, as well as an autoclaved sterile control, were included at each nitrate concentration. Samples were taken initially then after weekly intervals for 7 weeks to follow the rate of transformation of the analytes, and again after 25 weeks to determine long-term effects. Sulfide was analyzed after 6 and 25 weeks incubation; sulfur was measured after >25 weeks incubation.

Sulfate accumulation was measured in sludge incubations amended with nitrate but not sulfate. These cultures were amended with a variety of substrates including lactate (positive controls), *n*-alkanes (which were not degraded), *n*-alkanols (biodegraded), or *n*-alkanoic acids (biodegraded) [21]. Values shown are averages for triplicates after 18–30 weeks incubation, long after lactate and acetate were depleted in positive controls.

Analytical techniques

Sulfate, nitrate, nitrite, lactate, and acetate were analyzed by ion chromatography using a Dionex DX500 system (Dionex, Sunnyvale, CA, USA) with an AS11 4-mm column, a CD20 conductivity detector, and an aqueous mobile phase at 2 ml min^{-1}. The mobile phase was initially a rinse for 5 min with 0.4 mM NaOH. Two min after injection, the mobile phase was changed to 5 mM NaOH over 4 min, and to 18.5 mM NaOH over the next 4 min to elute sulfate. Concentrations were determined by comparison to external standards analyzed the same day. Methane and hydrogen were analyzed by gas chromatography as previously described [25]. The amount of methane or hydrogen in the gas phase of cultures was calculated as percent by volume by comparison to external standards. Sulfide was analyzed by a methylene blue assay as previously described [8]. Elemental sulfur was analyzed spectrophotometrically [35]. Sulfate-reducing bacteria were enumerated according to the method of Tanner [33]. Chemicals were obtained from Aldrich Chemical Co (Milwaukee, WI, USA), were of at least 97% purity, and were used without further purification.

Results

Microbial activity in anaerobic sludge

The oily sludge was a thick, shiny, black, heterogeneous composite with small particulates, and a strong petroleum odor. Analyses of the sludge indicated the presence of sulfide, a suite of alkanes typical of refined petroleum, and methane (data not shown). Microscopic examination of sludge diluted in anaerobic media revealed a diverse assemblage of motile and non-motile rods, cocci, and spirilla. An estimate of SRB in the sludge indicated $>10^6$ cells ml^{-1}.

Anaerobic cultures were established using the oily sludge as inoculum with various substrates and electron acceptors. A variety of metabolic types including lactate-degrading, hydrogen-consuming, and to a lesser extent acetate-degrading microorganisms, as well as nitrate-reducing, sulfate-reducing, and methanogenic bacteria were found in the oily sludge. Lactate and acetate were degraded under nitrate-reducing conditions within 2 weeks although there was a 4–5 day delay prior to substrate utilization (Figure 1). The active nitrate-reducing cultures turned pink after 1 week, indicating resazurin oxidation as a result of nitrous oxide production, whereas sterile controls and cultures without nitrate remained colorless. Under sulfate-reducing con-

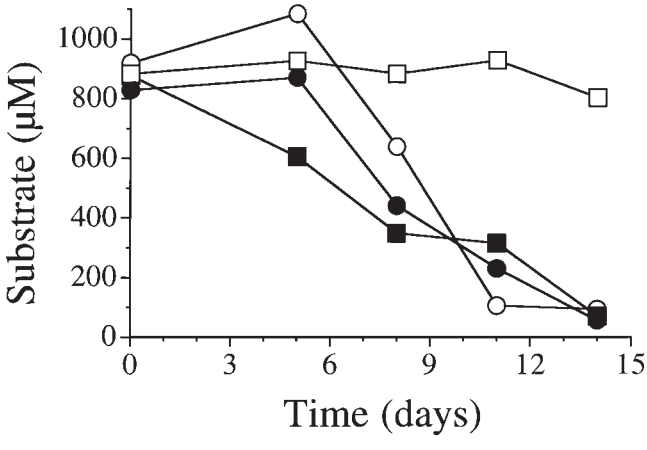

Figure 1 Degradation of fatty acids by oily sludge incubations. Lactate (○, ●) and acetate (□, ■) were degraded under nitrate-reducing conditions (closed symbols), and lactate was transformed under sulfate-reducing conditions (open symbols), whereas acetate was not immediately degraded under sulfate-reducing conditions.

ditions, acetate was degraded more slowly than lactate, with no appreciable decrease until after 2 weeks (Figure 1). In cultures with either sulfate or nitrate as electron acceptor, hydrogen in the headspace of cultures was consumed, although a transient increase in hydrogen concentration was associated with the degradation of lactate or acetate under sulfate-reducing conditions (data not shown). In the absence of sulfate or nitrate, endogenous components in the sludge as well as added lactate were transformed eventually to methane. Reducing the medium with 0.5–2 mM sodium sulfide did not affect microbial activity, and no degradation of lactate or acetate was observed in sterile controls.

Prevention of sulfidogenesis and methanogenesis by nitrate

Nitrate addition diminished sulfate reduction in anaerobic cultures established with oily sludge, sulfate, and various substrates. In sulfate-amended cultures with only endogenous substrates, 11.4 mM sulfate was consumed in 18 weeks, following an 8-week delay. In substrate-amended cultures, sulfate reduction paralleled the degradation of lactate (10.3 mM sulfate in 6 weeks) or benzoate (16.3 mM sulfate in 18 weeks). In nitrate-amended cultures, lactate or benzoate addition resulted in nitrate reduction, whereas nitrate loss was minimal with only endogenous substrate. Less than 1 mM sulfate was reduced in the presence of 100 mM nitrate. To confirm the apparent inhibition of sulfate reduction by nitrate addition, the sulfide concentration in cultures was measured after 10 weeks incubation. Cultures given lactate or benzoate had almost 4 mM sulfide, and even cultures with just endogenous substrate had more sulfide than sterile controls (Figure 2). In contrast, cultures that received nitrate as well had less sulfide than even the corresponding sterile controls (Figure 2). In sulfate-free cultures, methane was produced from endogenous substrates (0.5% of gas phase), or with benzoate (1%), or lactate (5%) as substrates. If sulfate or nitrate were added, little or no methane was produced (<0.1%). Thus, nitrate also limited methanogenesis in these cultures. No substrate degradation, sulfate or nitrate reduction, or methanogenesis occurred in sterile controls.

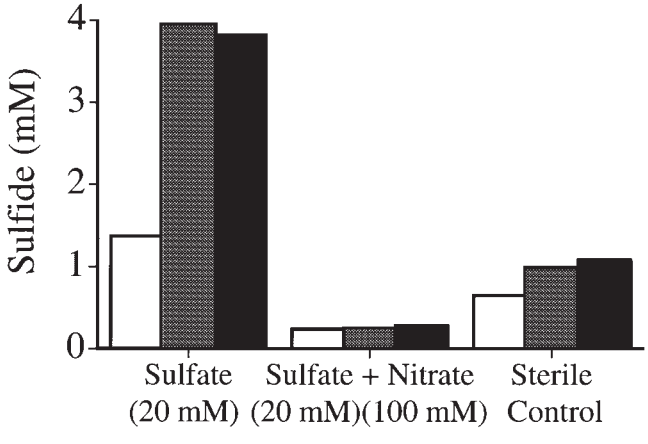

Figure 2 Sulfide concentrations after 10 weeks incubation in cultures amended with sulfate, or sulfate and nitrate, compared to sterile controls. Cultures were inoculated with oily sludge that contained endogenous substrate (□), or were also amended with the exogenous electron donors lactate (▨) or benzoate (■).

Comparison of the effectiveness of three different nitrate salts for preventing sulfate reduction and sulfide production

The effects of sodium-, potassium- and calcium nitrate on lactate degradation, sulfate reduction, sulfide production, nitrate reduction, and nitrite accumulation in cultures established with oily sludge were compared with the effects of the corresponding chloride salts. Lactate was depleted in all incubations except sterile controls. However, in the chloride-amended cultures, lactate degradation was incomplete since acetate accumulated up to a stoichiometric conversion (data not shown). Thus, there was variation between cultures in the amount of electrons released from the oxidation of lactate, and the amount of electron acceptor reduced. Using Equations 1–2, the theoretical amount of electrons released by the oxidation of lactate to acetate and by the degradation of acetate were calculated for each culture. The recovery of electrons accepted was calculated based on the actual amount of sulfate and nitrate reduced, correcting for nitrite accumulation (Equations 3–5). All values were corrected for slight changes in sterile controls due to analytical variations. The average recovery of electron equivalents was 122%, indicating that the assumed complete reduction of sulfate and nitrate did not occur. This leads to an overestimation of actual electron equivalents accepted, although contribution of electrons from components in the sludge were not accounted for.

Lactate oxidation: (1)

$$CH_3CHOHCOOH + H_2O \rightarrow CH_3COOH + CO_2 + 4\,H^+ + 4\,e^-$$

Acetate oxidation: (2)

$$CH_3COOH + 2\,H_2O \rightarrow 2\,CO_2 + 8\,H^+ + 8\,e^-$$

Sulfate reduction: (3)

$$SO_4^{2-} + 8\,e^- + 8\,H^+ \rightarrow S^{2-} + 4\,H_2O$$

Nitrate reduction: (4)

$$NO_3^- + 2\,e^- + 2\,H^+ \rightarrow NO_2^- + H_2O$$

Nitrite reduction: (5)

$$2\,NO_2^- + 6\,e^- + 8\,H^+ \rightarrow N_2 + 4\,H_2O$$

SRB were active in cultures that received the chloride salts, since sulfate was reduced and sulfide was produced (Table 1). The decrease in sulfate consumption was greater than the increase in sulfide production by 3 mM in all cultures except sterile controls, suggesting that an intermediate oxidation state of sulfur might accumulate independent of nitrate addition. The relative amount of activity of SRB in these cultures was determined by calculating the recovery of electrons based on the average of the measured sulfate depletion and sulfide production. The recovery of electron equivalents was 112%, 100%, and 89% for cultures that received sodium, potassium, and calcium chloride, respectively, for an overall average of 100% in the absence of nitrate. Therefore, SRB were responsible for most of the activity observed in these cultures. The results were independent of whether sodium, potassium, or calcium chloride were added, confirming that the cations were not respon-

Table 1 Comparison of the effects of sodium, potassium, or calcium salts of chloride and nitrate on lactate degradation, sulfate reduction, and nitrate reduction in cultures established with oily sludge

Conditions	Change in concentration (mM)[a]				
	lactate	acetate	nitrate	sulfate	sulfide
Chloride salts (50 mM Cl^-)					
sodium	−11.73	+4.17	–	−15.87	+11.96
potassium	−11.65	+9.78	–	−7.47	+3.67
calcium	−11.54	+5.49	–	−10.41	+6.48
Nitrate salts (50 mM NO_3^-)					
sodium	−11.01	+0.03	−19.88	−4.34	−0.57
potassium	−11.01	+0.04	−17.02	−5.53	−0.58
calcium	−11.24	+0.09	−29.77	−2.80	−0.43

[a]Averages of triplicates; (−) decrease, (+) increase; corrected for sterile controls.

Table 2 Effects of sodium nitrate addition on lactate degradation, sulfate reduction, and sulfide accumulation in cultures established with oily sludge under different salinity conditions

Conditions	Change in concentration (mM)[a]				
	lactate	acetate	nitrate	sulfate	sulfide
Without nitrate					
freshwater	−11.21	+6.05	–	−11.97	+6.09
brackish	−10.17	+6.96	–	−8.67	+7.20
saltwater	−10.48	+6.29	–	−9.31	+6.00
With nitrate (50 mM)					
freshwater	−11.38	+0.07	−25.93	−9.32	−0.58
brackish	−10.54	+0.03	−20.11	−5.75	−0.63
saltwater	−10.22	0.00	−18.44	−1.48	−0.49

[a]Averages of triplicates; (−) decrease, (+) increase; corrected for sterile controls.

sible for the inhibitory effects observed with the various nitrate salts.

Nitrate addition consistently diminished both sulfate reduction and sulfide production. With nitrate, lactate was degraded but acetate did not accumulate (Table 1). Substantial nitrate reduction occurred in all cultures regardless of which nitrate salt was added. However, slight differences between the effects of the three forms of nitrate were observed. Nitrite accumulated in cultures to which $NaNO_3$ (2.43 mM NO_2^-) and KNO_3 (0.78 mM NO_2^-) were added, but not in cultures with $Ca(NO_3)_2$. Nitrous oxide accumulated in all cultures containing nitrate as evidenced by the conversion of resazurin to its pink oxidized state. Sulfate reduction occurred even in the presence of nitrate, but sulfide did not accumulate, and in fact the sulfide levels decreased below initial amounts and values in sterile controls (Table 1). In contrast to the cultures without nitrate, the average recovery of electrons based on sulfate depletion and sulfide production was only 12%, 16% and 7% for sodium-, potassium-, and calcium nitrate, respectively. Therefore, all three forms of nitrate limited microbial sulfate reduction in these incubations.

Effects of nitrate under different salinity conditions

The effects of nitrate on sulfate reduction under different salinity conditions was tested with $NaNO_3$. As with the previous experiment, cultures without nitrate sometimes accumulated acetate, so that not all the reduction potential was realized during the incubation period. Nevertheless, transformation of lactate was associated with both sulfate reduction and sulfide production under all three salinity regimes (Table 2). Accounting for the expected electron transfers from the conversion of lactate to acetate as well as acetate degradation, recovery of electrons by reduction of sulfate to sulfide was 92%, 112%, and 100% under freshwater, brackish, and saltwater conditions, respectively. Therefore, the degradation of substrates was accounted for by SRB activity.

At the salinities tested, the addition of nitrate consistently diminished sulfate reduction and sulfide accumulation relative to controls without nitrate. Lactate was degraded and no acetate accumulated in cultures containing nitrate (Table 2). Nitrate was reduced (Table 2) and 0.47 mM, 1.92 mM, and 1.19 mM nitrite accumulated under freshwater, brackish, and saltwater conditions, respectively. Nitrous oxide production in all nitrate-reducing cultures was evidenced by the oxidation of resazurin. Sulfate reduction also occurred even though sulfide concentrations decreased below initial levels and values for sterile controls (Table 2). The decrease in sulfate concentration was greater than any increase in sulfide production in cultures with nitrate, particularly under freshwater conditions (8.76 mM), compared to brackish (5.19 mM) and saltwater conditions (0.92 mM). The average recovery of electrons associated with sulfate reduction to sulfide was 28%, 17%, and 2% under freshwater, brackish, and saltwater conditions, respectively. As in the previous experiment, the net recovery of electron equivalents assuming complete reduction of electron acceptors was 124%, suggesting that endogenous substrate contributed electrons during the incubation or that incomplete reduction of electron acceptors occurred. No degradation of lactate or reduction of sulfate or nitrate occurred in sterile controls under the three salinity conditions.

Determination of minimum nitrate concentrations to prevent sulfate reduction and sulfide accumulation

To determine how much nitrate must be added to control sulfate reduction, cultures were established with lactate as a substrate and varying amounts of nitrate. Again, the fate of substrates depended on nitrate addition. Without nitrate, lactate was depleted within 4 weeks, coupled with accumulation of acetate. After 25 weeks incubation, acetate values decreased to <2 mM. With nitrate, lactate degradation occurred primarily between 2 and 4 weeks incubation, but lactate was not completely removed in 7 weeks, although fatty acids could not be detected after 25 weeks. Acetate accumulated transiently during the first 4 weeks, up to 6 mM, with greater concentrations accumulating with

Table 3 Effects of sodium nitrate on lactate degradation and nitrate and sulfate reduction in cultures with oily sludge inoculum, 20 mM sulfate, and varying concentrations of nitrate

Nitrate addition	Change in concentration (mM)[a]						
	lactate	nitrate		nitrite		sulfate	
	6 wks	6 wks	25 wks	6 wks	25 wks	6 wks	25 wks
0 mM	–7.4	–	–	–	–	–4.0	–4.8
17 mM	–6.3	–10.7	–18.1	+1.6	+4.3	–3.9	–0.0
35 mM	–4.7	–10.5	–30.1	+3.0	+8.8	–1.5	–0.0
50 mM	–3.7	–13.0	–34.2	+2.7	+4.8	–1.0	–0.8
70 mM	–5.3	–11.4	–40.0	+0.4	+2.6	–0.8	–0.3

[a]Averages of triplicates; (–) decrease, (+) increase; corrected for sterile controls.

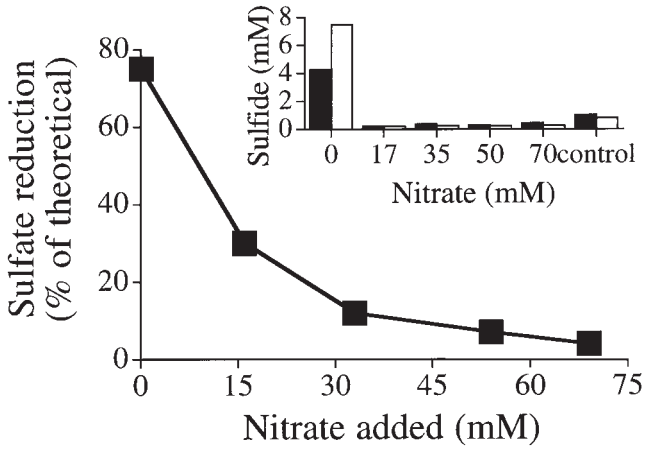

Figure 3 Effect of nitrate concentration on sulfate depletion and sulfide accumulation. Increased nitrate concentration resulted in decreased sulfate reduction as indicated by the percent of the theoretical amount expected. The concentration of sulfide in cultures (insert) after 6 weeks (■) and 25 weeks (□) was also affected by the addition of nitrate.

decreased nitrate concentration (data not shown). There was no evidence for lactate degradation, sulfate or nitrate reduction, or sulfide production in sterile controls.

Nitrate was reduced in these cultures, and accounted for most of the electron-accepting processes in these cultures. The total reduction of electron acceptors was 75–108% of expected amounts based on substrate decay. The amount of nitrate reduced relative to the potential amount predicted, if all the reducing potential from the degradation of lactate were transferred to nitrate (based on Equations 1,2,4, and 5), was 75%.

The degradation of lactate was associated with both sulfate reduction and sulfide production in cultures lacking nitrate. Over a 6-week incubation period, 4.0 mM sulfate was reduced, and 3.5 mM sulfide accumulated above sterile controls (Table 3). Based on Equations 1–3, this represents 75% of potential sulfate reduction based on the substrate decay observed. However, sulfate depletion was affected by the concentration of nitrate added, with greater nitrate concentrations decreasing the amount of sulfate reduced (Figure 3). Only cultures lacking nitrate accumulated sulfide to amounts greater than in sterile controls; cultures with nitrate had sulfide concentrations of less than 0.5 mM even after 25 weeks incubation (Figure 3).

Sulfide oxidation associated with nitrate amendments

In all of the previous experiments, cultures to which nitrate was added also contained sulfate, so it was difficult to determine whether sulfide oxidation resulted in sulfate accumulation. In separate oily sludge incubations without added sulfate, sulfate accumulation was indeed associated with nitrate reduction and nitrite formation. In sterile controls and cultures degrading exogenous substrates, the sulfate concentration was <0.1 mM between 18 and 30 weeks incubation, whereas in substrate-unamended controls sulfate was 2.23 mM during this time. Sulfate accumulation was found only in cultures that were not actively degrading substrates like lactate or fatty acids, and was always associated with nitrite accumulation. Cultures established with *n*-alkanes, which were not degraded, had accumulated 2.04 mM sulfate. In these same cultures there was <0.1 mM, 1.3 mM, and 1.8 mM nitrite, respectively. Sulfide added to the medium is presumably the source for sulfate production, and the production of nitrous oxide as indicated by the oxidation of resazurin also occurred under these conditions.

Elemental sulfur was also detected in the nitrate-reducing cultures. For example, cultures that received 17 mM, 35 mM, 50 mM, or 70 mM $NaNO_3$ (as described in Table 3) contained 0.23 g L^{-1}, 0.20 g L^{-1}, 0.22 g L^{-1}, and 0.30 g L^{-1} elemental sulfur, respectively. In contrast, controls without nitrate, in which sulfate was reduced to sulfide, had only 0.02 g L^{-1} elemental sulfur, and sterile controls had 0.10 mg L^{-1}. The average increase in elemental sulfur in the nitrate-reducing cultures would be equivalent to 7.4 mM sulfate reduced, compared to 13.6 mM predicted for the reduction of sulfate to sulfur instead of sulfide, based on the average amount of lactate degraded as corrected for sterile controls.

Discussion

Generation of sulfide in the petroleum industry is associated with a myriad of detrimental consequences, so industries have invested in strategies to manage this nuisance gas and the organisms that produce it, including the use of biocides, special coatings, and mechanical cleaning [16]. However, rarely is an ecological approach to sulfide control employed. The addition of nitrate as a preferred electron acceptor is one approach that has been successful in preventing sulfide formation in contaminated sediments [6,23]. Unlike other electron acceptors, nitrate is readily soluble in water and does not form precipitates. We attempted to determine the feasibility of using nitrate for the treatment of oily waste streams that produce copious amounts of sulfide.

Our studies confirmed that SRB were indigenous to the oily wastes, which are typical of those encountered on board Navy ships and at treatment facilities. When oily sludge was incubated, these organisms oxidized exogenous substrates, reduced sulfate, and produced sulfide. Lactate,

a preferred substrate for incomplete oxidizing SRB, was readily degraded in these cultures; acetate, which is degraded by complete oxidizers, was removed after a long delay, if at all. This may indicate that complete oxidizing SRB were less numerous or active in our sludge incubations. Hydrogen, a source of electrons for many anaerobes, was readily depleted in our cultures regardless of an available electron acceptor. Benzoate was also metabolized in these cultures, although a substantial lag period was typically encountered. These results are consistent with the nutritional diversity of SRB [12], and indicate a metabolic capacity to couple the degradation of a wide variety of compounds in oily sludge to sulfate reduction. In addition, physiological diversity was indicated by observing sulfate-reducing activity under freshwater as well as saltwater conditions.

In the absence of sulfate or nitrate, the sludge inoculum converted lactate initially to acetate, and the latter was eventually converted to methane. Methanogenesis was also detected in the containers used to store the oily waste, suggesting that methanogens are abundant in the highly reduced oily sludge. Methanogens would not be expected to compete well with other organisms for common electron donors in oily waste streams, in the presence of sulfate or other electron acceptors, but could predominate if other electron acceptors were depleted [28]. Our results indicate that sulfate and especially nitrate curtail methanogenic activity, so that the nitrate additions proposed here for prevention of sulfide formation would effectively preclude methane production as well. Intermediate products of nitrate reduction (nitrite, N_2O, NO) may also be responsible for preventing methanogenesis in these incubations [20].

Nitrate limited sulfate reduction and sulfide formation in cultures, regardless of substrate, salinity, or nitrate form. Differences were noted for the amount of nitrate reduced and the amount of nitrite accumulating depending on the nitrate salt added. Nevertheless, all three forms of nitrate tested would be effective for preventing sulfidogenesis. Our research indicates that nitrate-reducing organisms in oily sludge were sufficiently active that additional inoculation would not be required.

Due to the heterogeneous nature of oily wastes, any remediation strategy would have to be applicable to a variety of environmental conditions. We tested the effects of nitrate under different salinity regimes, as freshwater, marine, and mixed wastes are all encountered on board ships and at treatment facilities. The addition of 50 mM nitrate prevented sulfide accumulation in lactate-degrading cultures under all the salinity conditions. Less sulfate reduction was noted under saltwater conditions in the presence of 50 mM nitrate relative to freshwater conditions. The SRB may have been more active under freshwater conditions, or conversely the nitrate-reducing or sulfide-oxidizing bacteria may have been relatively more active under saltwater conditions. Nevertheless, salinity was not a limiting environmental factor for using nitrate for sulfide prevention.

Nitrate did not affect sulfate removal and sulfide accumulation equally. Addition of even the lowest concentration of nitrate tested (16 mM) prevented sulfidogenesis, and reduced the sulfide concentration to below initial values and the values in sterile controls. The lower limit of nitrate required to prevent sulfide formation is therefore unknown. Previous reports from studies with cultures established with dilute sewage sludge, pond sediment, or oil field brines, and amended with acetate, glucose, or hydrogen, indicate that 59 mM nitrate inhibits biogenic sulfide production [17]. Inhibition was incomplete with lower amounts of nitrate (6 or 20 mM), or if the sulfate concentration was increased from 20 mM to 159 mM [17]. Recent studies have indicated that nitrate and nitrite can prevent sulfide production at concentrations as low as 0.71 mM in sandstone columns containing a biofilm of SRB [29], so the potential to use low concentrations of nitrate is promising from an economic point of view. Further studies would be required to determine the lower limit required for prevention of sulfidogenesis in actual oil-water separators and storage tanks, but our results suggest that the amount could be lower than equimolar to the amount of sulfate present.

In contrast to sulfide production, prevention of sulfate reduction was concentration dependent over the range of nitrate concentrations tested. Nitrate at 50 mM reduced sulfate reduction by 90% whereas greater than 80 mM would be required for total prevention of sulfate reduction. At $<$50 mM nitrate, the flow of electrons was divided between sulfate- and nitrate-reduction which indicates that both acceptors are available. Our results demonstrate that it is important to distinguish whether sulfate depletion or sulfide accumulation is used as the indicator of the activity of SRB. Further investigation into the competition for electron donors by sulfate- and nitrate-reducing bacteria, as well as the relative contribution of sulfide-oxidizing bacteria to sulfide removal in cultures is needed.

The dichotomy between the effects of nitrate on sulfate reduction and sulfide accumulation suggested that intermediate sulfur oxidation state products were probably formed, the most likely of which are elemental sulfur and thiosulfate. We have not detected thiosulfate in our incubations, although this does not exclude a role for thiosulfate transformations as observed elsewhere [2]. Elemental sulfur was detected in the nitrate-reducing reducing cultures. Sulfate-reducing bacteria produce elemental sulfur as an intermediate product of sulfide oxidation [14]. The addition of nitrate could either prevent complete reduction of sulfate to the fully reduced sulfide, or sulfide may be produced but then re-oxidized to elemental sulfur biologically or abiotically. Further research will be required to establish the mechanisms and rates of elemental sulfur production in these systems.

In all cultures established with nitrate, sulfide levels decreased during the incubation relative to initial values and sterile controls. In addition, sulfate was detected in cultures containing nitrate in which biological activity was substrate-limited. Therefore, nitrate can also be used for the oxidation of pre-existing sulfide in these systems. Some species of bacteria including *Thiobacillus denitrificans* can use nitrate as an electron acceptor to oxidize sulfide to sulfur or sulfate [24]. Several SRB are also able to oxidize sulfide with nitrate as the electron acceptor [11,14], although studies at an oil field indicate that nitrate injections cause a dramatic increase in a sulfide-oxidizing, nitrate-reducing *Campylobacter* sp, without increasing SRB populations [34]. High concentrations of sulfide inhibit

growth of *T. denitrificans* [32] and cause incomplete dinitrification to gaseous nitrogen oxides, as well as dissimilatory nitrate reduction to ammonia, in nitrate-amended sediment slurries that oxidize reduced sulfur compounds [6]. However, concentrations of sulfide up to 2 mM (65 ppm) did not affect nitrate utilization in our cultures. Bacteria able to utilize nitrate for the oxidation of sulfide are apparently naturally present in the oily wastes we have used, so that inoculation with sulfide-tolerant sulfide-oxidizing bacteria would not be required as part of a treatment strategy.

Several intermediates of nitrate reduction were detected in these cultures. Nitrite was detected in concentrations up to 3 mM in cultures with sodium nitrate, although it did not uniformly accumulate in all cultures. Nitrite has the effect of raising the redox potential of the medium and preventing the activity of SRB [18]. Nitrous oxide was also detected as indicated by the conversion of the redox indicator resazurin to its pink oxidized state [18]. Nitrite and nitrate do not oxidize resazurin, and there was no evidence for contamination of these cultures with oxygen because sterile controls and cultures established without nitrate remained colorless (reduced). Resazurin might be useful in oil-water separator and storage containers as an indicator of nitrate reduction and the existence of an elevated redox potential in the system that would preclude sulfide production.

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