

Effect of Nitrate on Biogenic Sulfide Production

GARY E. JENNEMAN, M. J. MCINERNEY,* AND ROY M. KNAPP

Department of Botany and Microbiology and School of Petroleum and Geological Engineering, University of Oklahoma, Norman, Oklahoma 73019

Received 26 November 1985/Accepted 7 March 1986

The addition of 59 mM nitrate inhibited biogenic sulfide production in dilute sewage sludge (10% [vol/vol]) amended with 20 mM sulfate and either acetate, glucose, or hydrogen as electron donors. Similar results were found when pond sediment or oil field brines served as the inoculum. Sulfide production was inhibited for periods of at least 6 months and was accompanied by the oxidation of resazurin from its colorless reduced state to its pink oxidized state. Lower amounts of nitrate (6 or 20 mM) and increased amounts of sewage sludge resulted in only transient inhibition of sulfide production. The addition of 156 mM sulfate to bottles with 59 mM nitrate and 10% (vol/vol) sewage sludge or pond sediment resulted in sulfide production. Nitrate, nitrite, and nitrous oxide were detected during periods where sulfide production was inhibited, whereas nitrate, nitrite, and nitrous oxide were below detectable levels at the time sulfide production began. The oxidation of resazurin was attributed to an increase in nitrous oxide which persisted in concentration of about 1.0 mM for up to 5 months. The numbers of sulfate-reducing organisms decreased from 10^6 CFU ml⁻¹ sludge to less than detectable levels after prolonged incubation of oxidized bottles. The addition of 10 mM glucose to oxidized bottles after 14.5 weeks of incubation resulted in rereduction of the resazurin and subsequent sulfide production. The prolonged inhibition of sulfide production was attributed to an increase in oxidation-reduction potential due to biogenic production of nitrous oxide, which appeared to have a cytotoxic effect on sulfate-reducing populations.

The use of nitrate to abate odors caused by sulfate-reducing bacteria in waste water treatment was recognized as early as 1929 (1). Since that time, sporadic reports have appeared suggesting that nitrate addition has some success in controlling odors in sewage (1, 5, 8, 16, 31), cannery (33), and pulp mill wastes (25) and in sulfide production in rice paddies (36). However, some workers have felt that nitrate treatment has only a transient effect on sulphide production and that effective control of sulfide production involves repeated treatments that result in high chemical costs. This reasoning was based on the observation that nitrate is reduced preferentially to sulfate under anaerobic conditions. Once nitrate is metabolized, the remaining organic matter is used to reduce sulfate to sulfide unless additional nitrate is added.

Although the transient inhibition of sulfide production by nitrate addition has been observed in many instances, long-term inhibition of sulfide production by nitrate addition has been reported (1, 16, 31). Allen found that the addition of 1 g of nitrate per liter to sewage sludge inhibited sulfide production for at least 29 days (1), which was the longest period tested. This inhibition was attributed to the increase in redox potential caused by the presence of nitrate. Poduska and Anderson (31) found that nitrate addition did control sulfide production in a waste water lagoon so long as enough nitrate was added initially to raise the redox potential of the lagoon above 300 mV. Once the redox potential was above that value, it was easily maintained at that level with little or no additional nitrate. The reason why the redox potential remained high was attributed to a reduction in the amount of organic matter and the production of N₂ gas during denitrification, which allowed for mixing in the lagoon sediment.

Microbially enhanced oil recovery (MEOR) processes rely on the ability of indigenous or injected bacteria to anaerobically metabolize added nutrients and produce useful by-

products such as gases, biosurfactants, polymers, etc., a process that results in additional oil recovery (10, 12). Nitrate is often added to the injected nutrient mixtures because it enhances the energy-yielding capacity of bacteria under O₂-limited conditions. Since many oil reservoirs have high concentrations of sulfate and sulfate-reducing bacteria, the addition of large amounts of organic matter to these environments can potentially stimulate sulfide production. Sulfide production is a major concern to the petroleum industry since it is toxic and corrosive and since it causes plugging due to the formation of insoluble iron sulfides (32). Therefore, it is important to determine whether nitrate addition can effectively control sulfide production.

This study examined the conditions of nitrate and sulfate amendment under which sulfide production occurs. It also provided evidence that the addition of high nitrate concentrations leads to the buildup of N₂O, which raises the redox potential, resulting in the long-term inhibition of sulfide production.

MATERIALS AND METHODS

Sample sites. Sewage samples were obtained from a secondary anaerobic sewage sludge digester in Norman, Okla. Sediment samples were collected at the duck pond on campus and landfill samples were gathered from the aquifer underlying the Norman municipal landfill. Oil reservoir samples were coproduced brine and oil taken from the well-head of producing wells.

Media and conditions of cultivation. Samples were immediately brought to the laboratory and incubated overnight inside the anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.). Samples were dispensed into sterile 160-ml serum bottles containing MOPS (morpholinepropanesulfonic acid) medium: Na₂SO₄, 2.84 g/liter; KH₂PO₄, 0.68 g/liter; MgCl₂ · 6H₂O, 0.41 g/liter; NH₄Cl, 0.32 g/liter; CaCl₂ · 2H₂O, 0.09 g/liter; resazurin, 0.00001 g/liter; 10 mM MOPS (final pH, 7.0). The final liquid volume was 50 ml. Trace metals and vitamin solutions described by Balch et al.

* Corresponding author.

(3) were added separately at 0.1% (vol/vol) and 0.5% (vol/vol), respectively. Sodium nitrate was added at final concentrations of 6, 20, and 59 mM. Sodium acetate and glucose were added at concentrations of 15 and 5 mM, respectively. A 2.5% (wt/vol) cysteine hydrochloride–2.5% (wt/vol) $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ reducing agent (17) was added at a final concentration of 0.05% (wt/vol) of each component. The serum bottles were capped with sterile black rubber stoppers and aluminum crimp seals and were removed from the glove box. The atmosphere inside the bottles was replaced with 100% O_2 -free N_2 (123.6 kPa) by evacuating and purging the bottles three consecutive times. Bottles with H_2 as the electron donor were prepared as above, with 100% H_2 added instead of N_2 .

The bottles were incubated in the dark at 25°C without agitation. Samples were withdrawn at weekly intervals with a 5-ml sterile glass syringe that had been previously degassed with O_2 -free N_2 . A portion of the sample was used immediately for sulfide analysis while the remainder of the sample was centrifuged ($12,100 \times g$, 10 min, 4°C) and then frozen (–20°C) in sealed tubes until other analyses were performed.

Sulfate-reducing bacteria were enumerated with plate-counting techniques by using the MOPS medium described above with 15 g of Bacto-Agar per liter (Difco Laboratories, Detroit, Mich.), 0.5 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ per liter and 15 mM acetate. The medium was boiled under a stream of 100% N_2 , 1 mM dithiothreitol was added, and the medium was dispensed into Balch tubes (3) under the same gas phase. The tubes were stoppered with black rubber stoppers and autoclaved for 20 min in a press. After cooling to 60°C, the tubes were placed inside the glove box, and the contents were poured into petri dishes containing 0.1 ml of 10-fold serial dilutions of the appropriate environmental sample. Nitrous oxide-producing bacteria were enumerated by using the MOPS agar medium without $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ or dithiothreitol and with a 2.0% (vol/vol) cysteine-sulfide solution (17) and 0 to 59 mM NaNO_3 . Nitrous oxide-producing bacteria were identified by the presence of a pink, diffusible halo that surrounded the colony, owing to the localized oxidation of resazurin (20). The diluent was 10 mM MOPS buffer (pH 7.0), which had been preincubated overnight in the anaerobic chamber.

Enumeration of hydrogen-using sulfate reducers was done by the three-tube most-probable-number method. The MOPS medium without agar or $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was anaerobically dispensed into Balch tubes containing a small amount of reduced iron powder (J. T. Baker Chemical Co., Phillipsburg, N.J.). Each tube was inoculated with 1 ml of the appropriate dilution, stoppered, and the gas phase replaced with 100% H_2 (123.6 kPa) by evacuating and purging the tube three times. Positive tubes were identified by a blackening of the medium. Hydrogen-using N_2O -producing bacteria were enumerated by using the above medium with 59 mM NaNO_3 added and the iron powder deleted. Positive tubes were identified by the pink coloration of the medium, which is indicative of N_2O formation (20). Most probable numbers were calculated with the computer program described by Hurley and Roscoe (18). Sulfate-reducing bacteria from oil reservoir brines were assayed according to the methods of the American Petroleum Institute (2).

Analytical techniques. A modified version of the colorimetric method of Truper and Schlegel (40), using 0.02% zinc acetate in 0.2% acetic acid, was chosen to measure sulfide concentrations. The standard curve was prepared with $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ crystals that were washed with distilled water and blotted dry before being weighed. The turbidimetric

procedure was used to measure sulfate (14). Sulfate standards were obtained from Hach Chemical Co. (Ames, Iowa).

Nitrate was measured with a nitrate ion-specific electrode (Corning Glass Works, Corning, N.Y.) and an expanded-scale pH/ion meter (Fisher Scientific Co., Pittsburgh, Pa.). The addition of known concentrations of NO_3^- to replicate samples indicated that no interfering substances were present. Nitrite was determined by using the colorimetric method described by Hanson and Phillips (15).

Glucose was measured colorimetrically by the glucose-oxidase method (Sigma Chemical Co., St. Louis, Mo.). Acetate was quantitated by gas chromatography. Samples were acidified by adding 0.1 ml of 6 N hydrochloride to 0.9 ml of sample. A 1.0- μl sample was then injected into a gas chromatograph (model 3400; Varian Instruments Group, Walnut Creek, Calif.) equipped with a flame ionization detector and a fused silica column (diameter, 530 μm ; length, 10 m; Hewlett-Packard, Palo Alto, Calif.) packed with Carbowax 20 M (Union Carbide). The carrier gas was helium (11.5 ml/min). The oven temperature was set at 60°C for 3 min, increased by 4°C/min until 100°C was reached, increased again by 7.5°C/min until 200°C was reached, and then held at 200°C for 5 min. Acetate was quantitated by comparing peak area to that of known standards.

Oxygen, CO_2 , CH_4 , NO_2 , and H_2 were measured with a gas chromatograph (model 427; Packard Instrument Co., Downers Grove, Ill.) equipped with a thermal conductivity detector and a Porapak Q column (Supleco, Inc., Bellefonte, Penn.). The oven, detector, and injector temperatures were 70, 90, and 90°C, respectively. Peak identification was made by comparing peak retention times with those obtained by external standards prepared from high purity gases (Supelco). Calibration curves were prepared by adding high-purity gases with a gas-tight syringe to 160-ml serum bottles flushed with O_2 -free nitrogen and sealed with a black rubber stopper. Peak areas were determined either by an integrator (model 3390A; Hewlett-Packard) or by use of a computer (Model IIe; Apple Computer, Inc., Cupertino, Calif.) equipped with a graphics tablet and an electronic planimeter.

Small amounts of nitrous oxide were measured with a gas chromatograph (model 3400; Varian) equipped with a Porapak Q column and a ^{63}Ni -electron capture detector. Oven and injection temperatures were set at 55°C while the detector temperature was set at 300°C. Carrier gas was 5% CH_4 –95% Ar at a flow rate of 15 ml/min. Samples (5 μl) were taken with a gas-tight syringe. The pressure of each tube was measured with a pressure transducer (Validyne Engineering Corp., Northridge, Calif.). The minimum detectable pressure of N_2O was 12.1 Pa when a 5- μl sample was used. Concentration of N_2O in the liquid phase was estimated by measuring the amount of N_2O in the gas phase and calculating with the published values for the Ostwald coefficient (41).

Total residue in environmental samples was determined by drying 3 ml of the sample at 50°C in a tared aluminum weighing dish to measure constant weight. The reported values were means from three replicate samples.

RESULTS

The effect of nitrate addition on sulfide production in different environmental samples was studied. No sulfide was produced during the 14-day incubation period when 59 mM nitrate was added to diluted samples of anaerobic sewage sludge or pond sediment that were amended with 20 mM sulfate and 15 mM acetate (Table 1). No sulfide was detected when two oil reservoir brines were treated as above (data not

TABLE 1. Effect of nitrate and sulfate addition on biogenic sulfide production^a

Addition	Sulfide concn (mg/liter)	
	Sewage sludge	Pond sediment
20 mM SO ₄ ²⁻	41.2 ± 18.4	130.8 ± 34.1
20 mM SO ₄ ²⁻ + 59 mM NO ₃ ⁻	<1.5	<1.5
156 mM SO ₄ ²⁻	56.7 ± 4.3	73.9 ± 35.0
156 mM SO ₄ ²⁻ + 59 mM NO ₃ ⁻	86.6 ± 19.9	231.0 ± 47.9

^a Serum bottles containing MOPS medium supplemented with 15 mM sodium acetate and 3.5 g of NaHCO₃ per liter were inoculated with 10% (vol/vol) of the environmental sample. An 80% N₂-20% CO₂ gas phase was used. Sulfide concentration was determined after 2 weeks of incubation and values are means of 4 or 5 replicates ± standard error. Initial sulfide concentrations were 2.0 mg/liter and less than the detection limit (1.5 mg/liter) for bottles inoculated with sewage sludge and pond sediment, respectively.

shown). In these experiments, nitrate, sulfate, and acetate were added to the undiluted brines. The presence of viable sulfate-reducing bacteria in the inoculum was confirmed by plating onto solid medium or by liquid enrichment. When the sulfate concentration was increased to 156 mM, large amounts of sulfide were produced even in the presence of 59 mM nitrate (Table 1). In fact, much higher sulfide concentrations were found in bottles that received 156 mM sulfate and 59 mM nitrate compared with bottles that received 156 mM sulfate. No attempt was made to follow the fate of nitrate in these samples to determine if and when nitrate was used in relation to sulfide production. Because of its availability, anaerobic sewage sludge was used as the inoculum source for the remainder of the study.

Two different patterns of sulfide inhibition were observed when the amount of sewage sludge added to each bottle was varied (Fig. 1). In the bottles containing undiluted or 50% (vol/vol) sewage sludge, the addition of 59 mM nitrate

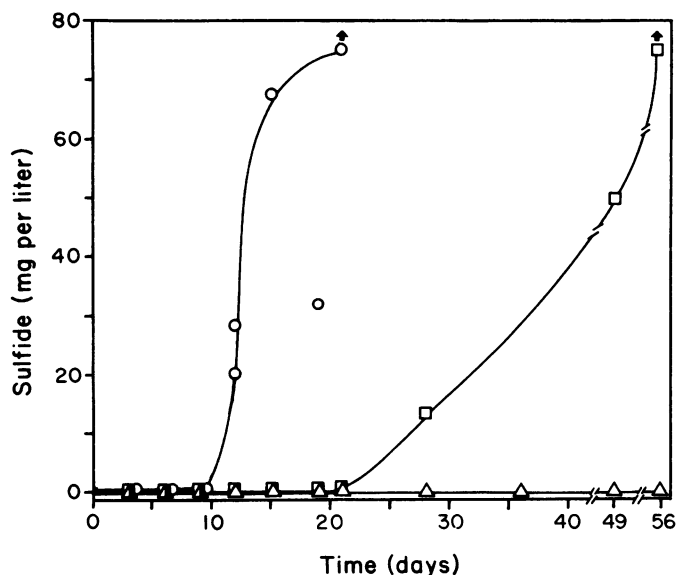


FIG. 1. Effect of sewage sludge concentration on sulfide production. Serum bottles containing MOPS medium amended with 20 mM Na₂SO₄, 15 mM sodium acetate, and 59 mM NaNO₃ were inoculated with the indicated volume of sewage sludge. The average total residue of the sewage sludge was 3.2% (dry weight/vol) and values are corrected for the initial sulfide levels in the sludge (15 mg/liter). Symbols: Δ, and ▲, 10% (vol/vol) sludge; □, 50% (vol/vol) sludge; ○, 100% (vol/vol) sludge.

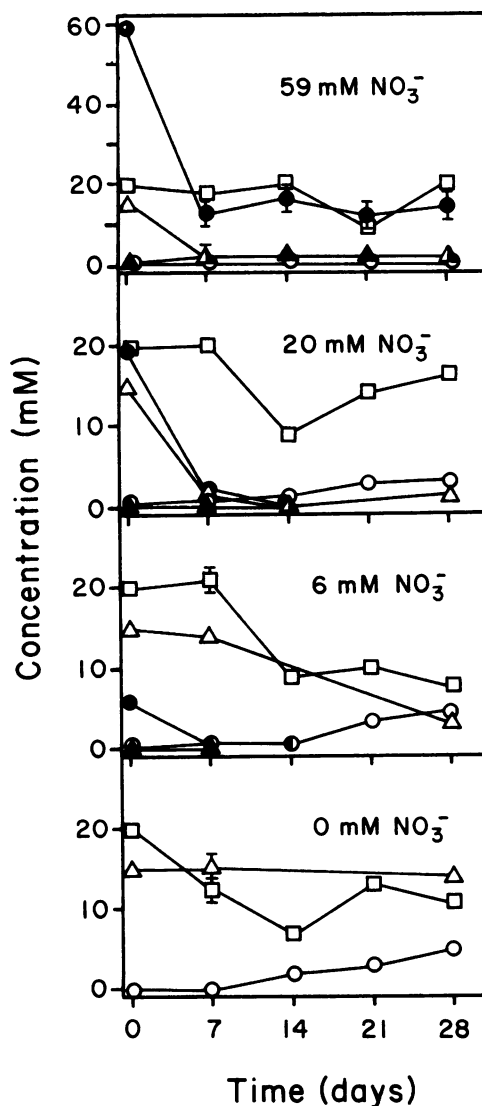


FIG. 2. Effect of acetate and different nitrate concentrations on sulfide production in sewage sludge. Serum bottles containing 20 mM Na₂SO₄ and 10% (vol/vol) sewage sludge were amended with the indicated nitrate concentration and 15 mM sodium acetate. Values are averages of concentrations in three bottles. Standard error bars are shown for cases in which the standard error was larger than width of the symbol. Symbols: ●, NO₃⁻; ▲, NO₂⁻; □, sulfate; ○, sulfide; Δ, acetate.

temporarily delayed the onset of sulfide production by 9 and 25 days, respectively. Sulfide was detected within 3 days of incubation in bottles without added nitrate (data not shown). However, when a 10% (vol/vol) sewage sludge inoculum was used, sulfide was not detected in bottles containing 59 mM during the 56-day period. In other experiments, sulfide production was not observed for periods of 4 to 6 months in bottles containing 10% (vol/vol) sewage sludge and amended with nitrate, acetate, and sulfate as described above.

A series of experiments was conducted to determine whether the length of sulfide inhibition depended on nitrate concentration or the type of electron donor. Bottles with 10% (vol/vol) sewage sludge were amended with 20 mM sulfate, 0 to 59 mM nitrate, and 15 mM acetate (Fig. 2). Sulfide production began by week 1 or 2 of incubation in

bottles with 20 mM nitrate or less. No sulfide was detected (< 0.1 mM) during the 4-week incubation period in bottles that were amended with 59 mM nitrate. When sulfide was produced in bottles amended with nitrate, no nitrate, nitrite, or nitrous oxide was detected at the time that sulfide production occurred. In bottles with 59 mM nitrate added, residual levels of nitrate (about 11 to 16 mM) and nitrite (about 2 mM) remained throughout the sampling period. These bottles also contained about 0.1 to 2 mM soluble concentrations of nitrous oxide (the importance of this compound will be discussed below). About 85 to 95% of the added acetate was used within 1 week in bottles with 20 or 59 mM nitrate. In bottles with lower levels of added nitrate, less than 10% of the added acetate was used after 1 week, and acetate concentrations of 0.2 to 14 mM were detected after 4 weeks of incubation. Sulfate levels remained high (15 to 20 mM) until sulfide production began. Similar results were obtained when 5 mM glucose or 100% H₂ (123.5 kPa) was added as the electron donor instead of acetate (data not shown).

These results suggest that the temporary inhibition of sulfide production is due to the preferential use of nitrate as an electron acceptor, a finding that has been reported by others (30, 32). When nitrate is completely used, sulfate is reduced to sulfide. However, the redox state of resazurin during these incubations suggests that another mechanism for sulfide inhibition also occurs. In all cases in which sulfide production was inhibited for long periods of time (greater than 30 days), the redox indicator, resazurin, changed from its colorless reduced state to its pink oxidized state. This oxidation started at the bottom of the bottle (i.e., at the liquid/sediment interface) and then spread throughout the entire sample, unlike oxygen contamination, in which oxidation starts at the air/liquid interface and diffuses downward.

The following sequence of color changes was consistently observed in all bottles amended with nitrate. Initially, the medium turned green or yellow-green and then pink. These changes were followed by the development of a white cloudy precipitate. In bottles with acetate added, a yellow-green color followed the pink color, and the pink color and white cloudy precipitate occurred simultaneously. The white cloudy precipitate was not observed when dithiothreitol replaced cysteine-sulfide as the reducing agent, suggesting that the precipitate was most likely the result of oxidation products of cysteine, such as the cysteine sulfur dioxides, which are insoluble in water. The onset and duration of each color change depended on the source of inoculum, the electron donor present, and the amount of nitrate added to the bottles. The sequence of color changes was usually completed within 2 weeks of incubation in bottles with 20 mM nitrate or less. These bottles turned gray or black, indicating the production of sulfide. Bottles without nitrate added and bottles containing 10% (vol/vol) sewage sludge that were autoclaved for 30 min at 121°C before the addition of the electron donors and acceptors did not change color throughout the 4-week incubation period.

The above experiments suggested that the oxidation of the medium was likely the result of biological nitrate reduction. The NO₃⁻/NO₂⁻ redox couple has an E₀' of +433 mV, whereas the E₀' of the NO/N₂O and N₂O/N₂ redox couples is +1,175 and +1,355 mV, respectively (37). The addition of 380 nM of N₂O or about 10 nM of NO to reduced medium with 0.0025% (wt/vol) each of cysteine hydrochloride and Na₂S · 9H₂O completely oxidized the medium (20). However, the addition of up to 72 mM sodium nitrate or 59 mM

sodium nitrite (highest concentration tested) did not oxidize the medium (20). When N₂O levels were followed by gas chromatography, it was observed that N₂O accumulation (0.1 to 2.0 mM) immediately preceded the oxidation of the medium and N₂O levels declined before the re-reduction of the medium (data not shown). N₂O concentrations remained high in bottles containing 10% sewage sludge and amended with 59 mM nitrate, even after extended incubation (21 weeks). Oxygen was not detected in the bottles when they became oxidized. Although nitrite accumulated at the time the bottles became oxidized, the addition of large amounts of nitrite to reduced medium did not affect the redox state of resazurin. Also, nitrite was not detected in oxidized bottles with H₂ as the electron donor (data not shown). These observations strongly argue against nitrite accumulation as the cause of the oxidation. They also strongly indicate that the biological production of N₂O caused the oxidation of the medium. However, it is possible that NO production was also important in this regard, since our gas chromatographic procedures may not have been sensitive enough to detect this compound. Sulfide production was not observed in bottles containing N₂O even after prolonged incubation. Thus, the prolonged inhibition of sulfide production could, at least in part, be attributed to the increased redox potential of the medium caused by N₂O production.

Bottles with 10% (vol/vol) sewage sludge amended with 15 mM acetate, 20 mM sulfate, and 59 mM nitrate with either 1 mM dithiothreitol or 2.5% (wt/vol) each of cysteine hydrochloride and Na₂S · 9H₂O as the reducing agent were vented to the atmosphere of the glove box by using sterile filters. These bottles also demonstrated the above sequence of color changes, and no sulfide production was observed in the 4-week incubation period. Sterile, reduced medium exposed to the glove box atmosphere remained reduced, indicating that little or no oxygen was present. These experiments showed that the oxidation of the medium occurs even in an open system.

Although the above data strongly indicate that N₂O production results in the oxidation of the medium, oxygen was detected in the bottles after prolonged incubation and may have contributed to the prolonged oxidation of the medium. To test this possibility, three bottles originally amended with 59 mM nitrate, 15 mM acetate, and 20 mM sulfate that had remained pink (oxidized) after 122 days were subjected to the following treatments. The headspace of one bottle (bottle B in Fig. 3) was replaced with O₂-free nitrogen by evacuating and purging the bottle three times with this gas. Another bottle (A) was treated in this manner and also received 2 ml of cysteine-sulfide solution which turned resazurin back to its colorless reduced state. The third bottle (C) served as the untreated control. Figure 3 shows the changes in sulfate, sulfide, nitrate, nitrite, and acetate concentrations in each bottle. No sulfide was detected in any of these bottles after 147 days of incubation, 25 days after the nitrogen and cysteine-sulfide treatments, even though sufficient levels of sulfate and acetate were present to support sulfide production. In fact, bottles A and B were reoxidized only a few days after the above treatment was applied. Oxygen was not detected in bottles A and B, but N₂O was detected in concentrations of 1.0 and 0.9 mM in bottles A and B, respectively. After 21 weeks of incubation, bottles A and B were again treated as described above, but these bottles became oxidized and produced N₂O within a few days. No sulfide was detected in them. These experiments show that the prolonged oxidation of the medium was the result of N₂O production rather than O₂ contamination.

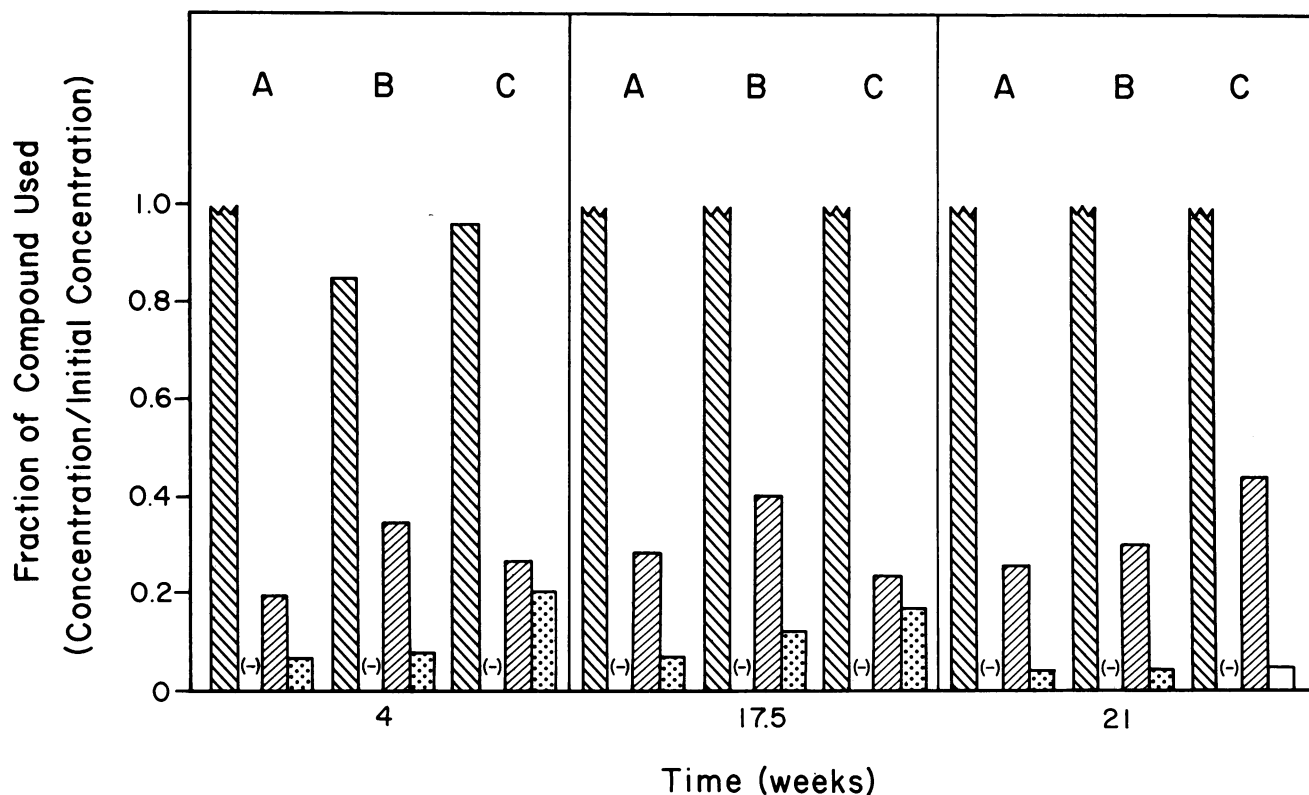


FIG. 3. Effect of gas purging and addition of reductant on sulfide production in oxidized bottles. Three oxidized bottles amended with 59 mM NaNO_3 (shown in the uppermost panel of Fig. 2) were treated as described in the text. The posttreatment concentrations of sulfate, acetate, and nitrate are represented as fractions of the concentrations shown in Fig. 2 for bottles A, B, and C. Nitrite concentration is represented as a fraction of the initial nitrate concentration. Jagged lines on the top of the bars indicate that the concentration exceeded the initial value. Symbols: \square , NaSO_4 ; \boxtimes , $\text{NO}_3^- + \text{NO}_2^-$; \boxdot , sodium acetate. Sulfide is represented in each case by a minus sign.

When large amounts of glucose (10 mM) were added to bottles that had been oxidized for 14 weeks, the bottles became reduced after 2 days, and sulfide production was observed. This suggests that electron donor concentration was limiting and that this factor contributed to the prolonged oxidation of the medium.

Initial populations of nitrous oxide-producing bacteria in sewage sludge were $1.5 \pm 0.28 \times 10^6$, $3.5 \pm 0.7 \times 10^6$, and 7.4×10^4 bacteria per ml when glucose, acetate, and hydrogen, respectively, were used as electron donors. Sulfate-reducing bacteria were found in concentrations of $9.3 \pm 0.5 \times 10^5$ and 10^6 cells per ml when acetate and H_2 , respectively, were used as electron donors. Population levels of nitrous oxide-producing bacteria remained high in oxidized bottles after 17.5 weeks of incubation, ranging from 10^5 to 10^6 cells per ml. However, less than 10 sulfate-reducing bacteria per ml were found in these bottles. In another experiment, the number of sulfate-reducing bacteria in oxidized bottles decreased from 10^6 to 10^3 cells per ml after 9 weeks of incubation.

DISCUSSION

Our results support the contention of Poduska and Anderson (31) that prolonged inhibition of sulfide production is due to the increase in the redox potential of the environment as a result of the action of nitrate-using bacteria. Biological sulfide production does not occur when the redox potential is above -100 mV (32). However, we attribute the cause of the increased redox potential to the accumulation of N_2O , or possibly of NO , rather than to the decrease in

soluble organic matter (31). The oxidation of the medium was associated with the accumulation and persistence of N_2O . Also, the addition of low levels of either NO or N_2O oxidized reduced medium (20) or diluted sewage sludge. The rereduction of the medium was associated with a decrease in N_2O concentrations (20). This should not be unexpected, since the E_0' of $\text{NO}/\text{N}_2\text{O}$ (+1,175 mV) and $\text{N}_2\text{O}/\text{N}_2$ (+1,355 mV) (37) redox couples is very high, much higher than that of resazurin (-42 mV) (17). These data and observations are consistent with the hypothesis that N_2O production results in the oxidation of the medium. Our results may explain the unexpected high redox potential (+222 mV) observed by Sorensen (35) in marine sediments amended with nitrate and the lack of sulfide production observed by Balderston and Sieburth (4) in their aqua-culture system. They may also explain why strict anaerobes such as sulfate-reducing bacteria that respire nitrate reduce it to ammonium and not to N_2O or N_2 (9, 21, 27), as well as why addition of nitrate to anaerobic environments can cause inhibition of methanogenesis in some cases.

The transient inhibition of sulfide production which we observed with low nitrate levels (6 or 20 mM) (Fig. 2) and the lack of sulfide inhibition when high sulfate concentrations (156 mM) were added (Table 1) are probably caused by competition among sulfate-reducing bacteria and denitrifiers, as discussed by other workers (30, 32, 42). Many sulfate-reducing bacteria can use nitrate as well as sulfate as an electron acceptor (21, 27). Thus, it is possible that transient sulfide inhibition was the result of the preferential use of nitrate rather than sulfate by sulfate-reducing bacte-

ria. This would explain why nitrate did not inhibit sulfide production when sulfate levels were high (156 mM).

Inhibition of sulfide production by nitrate addition was more pronounced with low sludge concentrations (10% vol/vol) than with high sludge concentrations. With increasing sludge concentrations, the electron donor to electron acceptor ratio increased. Dissimilatory nitrate reduction rather than denitrification is the predominant pathway for nitrate use when electron donor concentrations are high (22, 28, 34, 39), which may be the reason why prolonged oxidation of the medium and inhibition of sulfide production were not observed at higher sludge concentrations. It is also possible that the turnover rates of NO or N₂O were more rapid at higher sludge concentrations, thus preventing the buildup of NO and N₂O.

The prolonged oxidation of the medium could indirectly be caused by nitrate or the accumulation of nitrite. It has been observed that under certain nutrient conditions both NO₃⁻ and NO₂⁻ inhibit the reduction of NO and N₂O (7, 13, 29). However, Betlach and Tiedje did not observe this inhibition in their experiments (6). In our experiments, NO₃⁻ and in some instances NO₂⁻ were present after 21 weeks of incubation in the oxidized bottles. Kucera et al. found that the buildup of NO₂⁻ and N₂O can inhibit nitrate reductase in *Paracoccus denitrificans* by channeling electrons through nitrite and nitrous oxide reductases (24). This may explain why NO₃⁻ was present after 5 months of incubation in our bottles, since large amounts of N₂O were also present, which could have inhibited the further reduction of nitrate.

The persistence of NO₃⁻, NO₂⁻, and N₂O could also be a result of the limitation of an electron donor necessary to reduce these compounds. The addition of 10 mM glucose to oxidized bottles after 14.5 weeks of incubation resulted in the rereduction of the redox indicator resazurin. Although glucose itself is a reducing sugar, the medium was not reduced until 2 to 4 days after glucose addition. This implies that glucose metabolism was required for the reduction to occur. However, bottles that were vented to the atmosphere of the anaerobic chamber containing H₂ remained oxidized for prolonged periods of time. The presence of an oxidized environment or nitric oxide (23) may have prevented the use of H₂ in these bottles.

Another reason for the lack of sulfide production and the prolonged oxidation of the medium may be the reduced level of sulfate-reducing bacteria. Numbers of these bacteria decreased by several orders of magnitude during the prolonged incubation of oxidized bottles. The accumulation of some product of denitrification or the long-term exposure to high redox conditions may be toxic to the sulfate-reducing bacteria. Nitric oxide is known to be bacteriostatic to certain bacteria (26), and N₂O can be cytotoxic (38). However, the effects of these chemicals on sulfate-reducing bacteria are not known.

Our results suggest that there are two different reasons why sulfide production was inhibited for prolonged periods by nitrate addition. The first reason is that the redox potential increased. This increase was due to the buildup of N₂O or NO or both, which resulted in an oxidized environment. This buildup will occur in environments with high denitrification capacities and in instances in which the ratio of electron donor to electron acceptor (NO₃⁻) is low. The relative importance of NO₃⁻, NO₂⁻, and N₂O in maintaining these oxidized conditions is not understood at this time, although the addition of high concentrations of the electron donor, glucose, does result in rereduction of the oxidized medium. However, simply reducing the medium without

adding an external electron donor does not result in sulfide production. Therefore, continued input of organic matter can shorten the period in which the environment remains oxidized. The second reason is that the levels of sulfate-reducing bacteria decreased during the prolonged exposure to an oxidizing environment and high N₂O concentration.

Our work suggests that the use of high nitrate concentrations may prevent sulfide production during microbially enhanced oil recovery processes and groundwater reclamation projects and may be useful in controlling biogenic sulfide production in other anaerobic environments. Jack et al. found that the addition of 2.4 mM NO₃⁻ inhibited sulfide production in samples obtained from oil field filters for 4 months (19). However, further work is needed on the control of electron flow during denitrification as well as on the effect of N₂O and NO on sulfate-reducing bacteria.

ACKNOWLEDGMENTS

This work was supported by contracts DE-AC19-80BC10300 and DE-AS05-83ER-13053 from the U.S. Department of Energy.

LITERATURE CITED

- Allen, L. A. 1949. The effect of nitro-compounds and some other substances on production of hydrogen-sulphide by sulphate-reducing bacteria in sewage. *Proc. Soc. Appl. Bacteriol.* 2:26-38.
- American Petroleum Institute. 1975. American Petroleum Institute recommended practice for biological analysis of subsurface injection waters, 3rd ed. American Petroleum Institute, Washington, D.C.
- Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe. 1979. Methanogens: reevaluation of a unique biological group. *Microbiol. Rev.* 43:260-296.
- Balderston, W. L., and J. M. Sieburth. 1976. Nitrate removal in closed-system aquaculture by columnar denitrification. *Appl. Environ. Microbiol.* 32:808-818.
- Beardsley, C. W., N. J. Krottinger, and J. H. Rigdon. 1956. Removal of sewer odors by scrubbing with alkaline solutions. *Sewage Ind. Wastes* 28:220-225.
- Betlach, M. R., and J. M. Tiedje. 1981. Kinetic explanation for accumulation of nitrite, nitric oxide, and nitrous oxide during bacterial denitrification. *Appl. Environ. Microbiol.* 42:1074-1084.
- Blackmer, A. M., and J. M. Bremner. 1978. Inhibitory effect of nitrate on reduction of N₂O to N₂ by soil microorganisms. *Soil Biol. Biochem.* 10:187-191.
- Carpenter, W. T. 1932. Sodium nitrate used to control nuisances. *Water Works Sewerage* 79:175-176.
- Caskey, W. H., and J. M. Tiedje. 1979. Evidence for clostridia as agents of dissimilatory reduction of nitrate to ammonium in soils. *Soil Sci. Soc. Am. J.* 43:931-936.
- Clark, J. B., D. M. Munnecke, and G. E. Jenneman. 1981. *In situ* microbial enhancement of oil production. *Dev. Ind. Microbiol.* 22:695-701.
- Fales, A. L. 1929. Treatment of industrial wastes from paper mills and tannery on Neponsit River. *J. Indust. Eng. Chem.* 21:216.
- Finnerty, W. R., and M. E. Singer. 1983. Microbial enhancement of oil recovery. *Bio/technology* 1:47-54.
- Firestone, M. K., M. S. Smith, R. B. Firestone, and J. M. Tiedje. 1979. The influence of nitrate, nitrite, and oxygen on the composition of the gaseous products of denitrification in soil. *Soil Sci. Soc. Am. J.* 43:1140-1144.
- Hach Chemical Co. 1975. Hach water and wastewater analysis procedures manual, 3rd edition. Hach Chemical Co., Ames, Iowa.
- Hanson, R. S., and J. A. Phillips. 1981. Chemical composition, p. 328-364. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), *Manual of methods for general bacteriology*. American

- Society for Microbiology, Washington, D.C.
16. Heukelelekian, H. 1943. Effect of the addition of sodium nitrate to sewage on hydrogen sulfide production and B.O.D. reduction. *Sewage Works J.* **15**:255-261.
 17. Hungate, R. E. 1969. A roll tube method for cultivation of strict anaerobes, p. 117-132. *In* J. R. Novic and D. W. Ribbons (ed.), *Methods in microbiology*, vol. 5B. Academic Press, Inc., New York.
 18. Hurley, M. A., and M. E. Roscoe. 1983. Automated statistical analysis of microbial enumeration by dilution series. *J. Appl. Bacteriol.* **5**:159-164.
 19. Jack, T. R., E. Lee, and J. Mueller. 1985. Anaerobic gas production from crude oil, p. 167-180. *In* J. E. Zajic and E. C. Donaldson (ed.), *Microbes and oil recovery*, vol. 1. Bioresource Publications, El Paso, Tex.
 20. Jenneman, G. E., A. D. Montgomery, and M. J. McInerney. 1986. Method for the detection of microorganisms that produce gaseous nitrogen oxides. *Appl. Environ. Microbiol.* **51**:776-780.
 21. Keith, S. M., and R. A. Herbert. 1983. Dissimilatory nitrate reduction by a strain of *Desulfovibro desulfuricans*. *FEMS Microbiol. Lett.* **18**:55-59.
 22. King, D., and D. B. Nedwell. 1985. The influence of nitrate concentration upon the end-products of nitrate dissimilation by bacteria in anaerobic salt marsh sediment. *FEMS Microbiology Ecol.* **31**:23-28.
 23. Krasna, A. I., and D. Rittenberg. 1954. The inhibition of hydrogenase by nitric oxide. *Proc. Natl. Acad. Sci. USA* **40**:225-227.
 24. Kucera, I., V. Dadak, and R. Dobry. 1983. The distribution of redox equivalents in the anaerobic respiratory chain of *Paracoccus denitrificans*. *Eur. J. Biochem.* **130**:359-364.
 25. Lawrance, W. A. 1950. The addition of sodium nitrate to the Androscoggin River. *Sewage Ind. Wastes* **22**:820-832.
 26. Mancinelli, R. L., and C. P. McKay. 1983. Effects of nitric oxide and nitrogen dioxide on bacterial growth. *Appl. Environ. Microbiol.* **46**:198-202.
 27. McCready, R. G. L., W. D. Gould, and F. D. Cook. 1983. Respiratory nitrate reduction by *Desulfovibro* sp. *Arch. Microbiol.* **135**:182-185.
 28. Nedwell, D. B. 1982. Exchange of nitrate, and the products of bacterial nitrate reduction, between seawater and sediment from a U.K. saltmarsh. *Estuarine Coastal Shelf Sci.* **14**:557-566.
 29. Payne, W. J. 1973. Reduction of nitrogenous oxides by microorganisms. *Bacteriol. Rev.* **37**:409-452.
 30. Payne, W. J. 1981. Denitrification, p. 214. John Wiley & Sons, Inc., New York.
 31. Poduska, R. A., and B. D. Anderson. 1981. Successful storage lagoon odor control. *J. Water Pollut. Control Fed.* **53**:299-310.
 32. Postgate, J. R. 1979. The sulphate-reducing bacteria. Cambridge University Press, Cambridge.
 33. Ryan, W. A. 1945. Experiences with sodium nitrate treatment of cannery wastes. *Sewage Works J.* **17**:1227-1230.
 34. Sorensen, J. 1978. Denitrification rates in a marine sediment as measured by the acetylene inhibition technique. *Appl. Environ. Microbiol.* **36**:139-143.
 35. Sorensen, J. 1978. Occurrence of nitric and nitrous oxides in a coastal marine sediment. *Appl. Environ. Microbiol.* **36**:809-813.
 36. Takai, Y., and T. Kamura. 1966. Mechanism of reduction in waterlogged paddy soil. *Folia Microbiol.* **11**:304-313.
 37. Thauer, R. K., K. Jungermann, and K. Decker. 1977. Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol. Rev.* **41**:100-180.
 38. Thom, S. R., and R. E. Marquis. 1984. Microbial growth modification by compressed gases and hydrostatic pressure. *Appl. Environ. Microbiol.* **47**:780-787.
 39. Tiedje, J. M., A. L. Sexstone, D. D. Myrold, and J. M. Robinson. 1982. Denitrification: ecological niches, competition and survival. *Antonie van Leeuwenhoek J. Microbiol.* **48**:569-583.
 40. Truper, H. G., and H. G. Schlegel. 1964. Sulphur metabolism in Thiorhodaceae. 1. Quantitative measurement on growing cells of *Chromatium okenii*. *Antonie van Leeuwenhoek J. Microbiol.* **30**:225-238.
 41. Wilhelm, E., R. Tattino, and R. J. Wilcock. 1977. Low-pressure solubility of gases in liquid water. *Chem. Rev.* **77**:225-231.
 42. Winfrey, M. R. 1984. Microbial production of methane, p. 153-219. *In* R. M. Atlas (ed.), *Petroleum microbiology*. Macmillan Publishing Co., New York.