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UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

THE ECOLOGY AND BIOGEOCHEMISTRY OF SULFATE REDUCTION IN THE TERRESTRIAL SUBSURFACE

A Dissertation

SUBMITTTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

Doctor of Philosophy

By

Glenn Allen Ulrich
Norman, Oklahoma
1999

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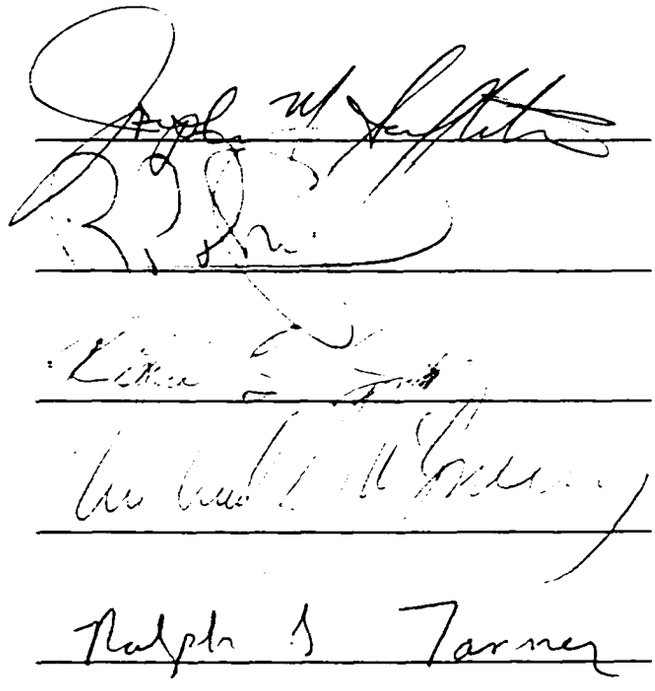
THE ECOLOGY AND BIOGEOCHEMISTRY OF SULFATE REDUCTION IN THE TERRESTRIAL SUBSURFACE

A Dissertation APPROVED FOR THE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY



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Abstract

Interests in the microbiology of subsurface environments continues to grow due to the realization that microbial activities influence a large portion of the Earth's crust by contributing to the biogeochemical cycling of the elements. It is likely that only a fraction of the relations between microbial activities and geochemical phenomena have been identified. It is equally important to obtain an understanding of how microbial activities are regulated in the subsurface, so that these processes can be exploited for bioremedial and biotechnological purposes. The primary focus of this dissertation was to gain an increased understanding of the factors governing anaerobic processes, mainly sulfate reduction, in pristine and contaminated aquifers. A combined microbiological and geochemical approach was used in an effort to evaluate *in-situ* microbial processes rather than potential activities as indicated by more traditional methodologies.

Chapter one describes a relatively simple trapping procedure for measuring sulfate reduction activity and the accumulation of inorganic sulfides in sedimentary environments. The technique yielded results comparable to a traditional and extremely time consuming method. The versatility and reliability of the simplified method has been verified in a wide variety of samples by several independent research groups. Chapter two describes a direct imaging procedure which was developed in order to obtain interpretationaly unambiguous information on the spatial distribution of sulfate-reducing activity in subsurface sediment cores. Experiments in which the method was tested indicated that sulfate-reducing activity and the movement of sulfate-reducing bacteria is influenced by small scale (mm) heterogeneities in sediment structure.

The research presented in chapters 3,4, and 5, indicates that the cycling of sulfur is an important biogeochemical processes that impacts the shallow groundwaters of the Yegua

formation in East Central Texas. The oxidation of pyrite reserves in shallow aquifers produces acidic groundwaters rich in sulfate and reduced iron. This process in conjunction with the transport of sulfate in groundwaters represents a major source of the electron acceptor in sandy formations. Sulfate reduction was highly spatially variable and was detected only in sand intervals. Narrow zones of increased activity were detected in sands adjacent to lignite and clay-rich laminae. The fermentation of organic matter in the latter formations produced organic acids (mainly acetate) which likely supported sulfate reduction in the neighboring sandy regions.

The factors limiting sulfate reduction in a landfill leachate-contaminated aquifer were addressed in chapter 6. Increased sulfate reduction rates were noted in intervals which contained higher sulfate concentrations, and laboratory experiments confirmed this observation. The sources of sulfate that likely impacted intrinsic biodegradation activity in the aquifer included, barite dissolution, iron sulfide oxidation near the water table, and the advective flux of sulfate from upgradient regions.

I believe that the ubiquity and importance of sulfate reduction in subsurface environments is a reflection of the diversity of the hydrological and biogeochemical processes which contribute to the availability of sulfate. Iron reduction and nitrate reduction were of minor importance in the aquifers studied herein due to the decreased availability of nitrate and microbially reducible ferric iron.

Chapter 1

A Rapid and Simple Technique for Estimating Sulfate Reducing Activity and Quantifying Inorganic Sulfides

Abstract

A simplified passive extraction procedure for quantifying reduced inorganic sulfur compounds from sediments and water is presented. This method may be used for the estimation of sulfate reduction rates. Efficient extraction of FeS, FeS_2 and S^{2-} was obtained with this procedure; however, the efficiency for S^0 depended on the form that was tested. Passive extraction can be used with samples containing up to 20 mg reduced sulfur. We demonstrated the utility of this technique in a determination of both sulfate reduction rates and reduced inorganic sulfur pools in marine and freshwater sediments. A side-by-side comparison with the passive extraction method with the established single-step distillation technique yielded comparable results with a fraction of the effort.

Introduction

Sulfate-reducing bacteria are nearly ubiquitously distributed and are major contributors to the degradation of organic matter in both freshwater and marine environments (12). These organisms are known to catalyze desirable processes such as the biodegradation of pollutant chemicals (11). They are also involved in undesirable processes including the souring of oil formations and the corrosion of steel equipment (2). Techniques for the examination of sulfate reduction have led to a better understanding, application, and monitoring of this important process.

As a group, sulfate-reducing bacteria are extremely versatile with the ability to metabolize diverse organic and inorganic electron donors (3, 20). They are also known to utilize a wide variety of electron acceptors (nitrate, iron, oxygen, uranium, thiosulfate, elemental sulfur, and halo- or nitro-aromatic compounds) either in the absence or sometimes in the presence of sulfate (9, 19, 22). Given their metabolic diversity, the mere presence of the requisite bacteria does not necessarily coincide with sulfate-reducing activity. Furthermore, while the consumption of sulfate and the production of sulfide are indicative of sulfate reduction, the absence of changes in these chemical species cannot be taken as evidence excluding this process. The oxidation of sulfide and/or the rapid precipitation of dissolved hydrogen sulfide as metal sulfides can modulate pool sizes such that increases in sulfide concentration may be undetectable. Direct measurements of sulfate reduction rates using $^{35}SO_4^{2-}$ as a tracer can overcome these limitations, but the currently used techniques are time consuming and require

considerable effort. We have adapted these methods and developed a simpler and less time consuming procedure.

Current radiotracer methodology for determination of sulfate reduction rates involves the incubation of $^{35}SO_4^{2-}$ with sediments followed by extraction and quantitation of the produced ^{35}S-sulfides including $H_2{}^{35}S$, $H^{35}S^-$, $Fe^{35}S$, and $Fe^{35}S_2$. It has recently been shown that isotopic exchange between some of these compounds and S^0 occurs, thereby limiting the usefulness of radiotracers in speciation studies (14). Therefore, a single-step method has been proposed in which the total pool of reduced inorganic sulfur (TRIS) is reacted with a solution of Cr(II) and HCl (13). This procedure involves the reduction of TRIS (to H_2S), but not organic S or most sulfuroxy anions (7,13), by heating samples and the HCl/Cr(II) extractants during a reflux distillation. Liberated sulfide is then flushed through the reflux condenser into a sulfide trapping solution (5 to 10% zinc acetate). These distillations must be carefully monitored and are extremely time consuming when the analysis includes multiple samples. Attempts have been made to simplify inorganic sulfide analysis (15) but a simplified radiotracer assay for evaluating sulfate-reducing activity is currently unavailable. Therefore we have adapted the above single-step chromium reduction method (13) for the determination of sulfate reduction rates and inorganic sulfur pools in large numbers of samples.

Materials and Methods

The adapted method involves the extraction of sulfides from samples placed in 120-ml serum bottles. The serum bottles contained an anoxic headspace and a sulfide trap (Fig. 1). All reagents, traps, and samples must also be anoxic to prevent the premature oxidation of the Cr(II) solution. Sample manipulations were conducted in a well working anaerobic glovebox.

Sediment or water samples can be preserved by mixing with an anoxic solution of 20% zinc acetate (boiled and cooled under N_2; ~2ml g^{-1} wet sediment) and stored at -20°C. For analysis, these samples (1-2 g) or iron sulfide minerals were added directly to the serum bottles while inside the anaerobic glove bag. Alternatively, they can be transferred by pipette or injected via syringe into serum bottles. To prevent excessive pressure build up due to CO_2 or H_2S accumulation in the bottles, the sample size should be kept to a minimum; normally < 2g of sediment in the recommended serum bottles is appropriate. Test tubes (12 x 75 mm) containing 2.5 ml of anoxic 10% zinc acetate solution were placed inside the serum bottles as sulfide traps. The serum bottles were fitted with rubber stoppers (Bellco Glass Inc., Vineland, NJ) and removed from the glove bag. If a glove bag is not available, these reagents can be added to the serum bottles under a steady stream of O_2-free N_2 using the Hungate technique (16). Water samples or slurries consisting of fine-grained sediments can also be injected directly into serum bottles without prior fixation of free hydrogen sulfide with zinc acetate. A 1 M Cr(II)-HCl solution (8 ml) (13) followed by anoxic 12N HCl (4 ml) is then injected into the serum bottles through the stoppers. The latter reagent may be prepared by heating 160-ml

serum bottles containing 100 ml of HCl in a water bath (~90°C) while continuously flushing the reagent with N_2 for approximately 40 minutes. The acid fumes from this procedure can be vented through a bicarbonate solution. The bottles are then removed from the hot water bath and stoppered. The HCl and Cr(II) solutions can be stored for several months inside stoppered serum bottles under a N_2 headspace. After addition of the Cr(II) and HCl reagents, serum bottles are placed on a rotary shaker (~150 rpm) for the duration of the extraction. Oxidized samples or samples which contain high concentrations of reduced S may require additional chromium solution. Such cases are evident as the chromium-HCl solution becomes oxidized and changes from a dark blue to a bright green color. The trapping solution precipitates the liberated H_2S as ZnS. After 30 h or as indicated, the sulfide traps were removed from the serum bottles. A homogeneous ZnS suspension was ensured by vigorously vortexing the traps followed by treatment with a sonicating water bath for about 30 s. Sulfide was quantified in aliquots of this suspension using the methylene blue assay (8) or by scintillation counting (10-20% v/v in Ultima-Flo AF scintillation fluid; Packard Instrument Co., Downers Grove, IL).

Results

We have evaluated the time required to extract dissolved sulfide and sulfide from pure (S containing) minerals. At the same time we determined the trapping efficiency for these species (Fig. 2). Recovery of Na_2S was complete within 2.5 h, while comparable recoveries of iron monosulfide (FeS) and pyrite (FeS_2) required 10 and 20 h respectively (Fig. 2A.). Only partial recovery of particulate S^o was

observed with extractions as long as 7 days (Fig. 2B), but S^0 dissolved in acetone was rapidly and completely recovered. Additional experiments indicated that sulfide recovery was linear and complete for concentrations ranging from 1 to 20 mg S (data not shown) above which the recovery decreased. Of course, other forms of S exist in the environment and were not specifically evaluated in our experiments. For instance, polysulfide is commonly found in porewaters of marine sediments (6). Acidification causes polysulfide to decompose to H_2S and S^0 (13), and its recovery will therefore be incomplete and the latter will decrease the recovery of polysulfide recovery.

The applicability of the passive extraction technique for extraction of sulfides from sediments was evaluated by determining TRIS over time in samples from a landfill leachate impacted aquifer (Fig. 3). TRIS recovery from the samples amended with known quantities of pyrite was equivalent to the sum of TRIS recoveries from sediment and pyrite alone. Thus, the sediment had no influence on the extraction of the added pyrite. The additional time required to recover S from the sediment samples relative to the pyrite must be due to the fact that these sulfides are more resistant to chromium and acid extraction than pure S-containing minerals. Thus, an extended extraction time (~70 h) may be necessary for the complete recovery of TRIS in some samples.

In a comparison of the passive extraction technique (30 h) and the distillation method by Fossing and Jorgensen (13), sulfate reducing activity and TRIS pools in two different anaerobic sediment samples were evaluated (Table 1). The two methods yielded virtually identical results for both the highly active brackish sediments from San Diego Bay (21) and the moderately active sediments

from the landfill leachate impacted aquifer (5). While such results are promising, we recognize that they are far from exhaustive and that samples from multiple environments will have to be similarly evaluated.

It is important to point out that we consider the rates of sulfate reduction based on the passive extraction method as estimates only. This is because sulfate reduction rates could be underestimated in some samples where S^0 may represent an important portion of the reduced sulfur pool. S^0 may be formed by the oxidation of reduced S species. It is also known that radioisotopic evaluations of sulfate reduction rates may be biased by exchange reactions between the S^0 pool and $H_2{}^{35}S$ formed by the microbial reduction of $^{35}SO_4^{2-}$ (1,14). The extraction of non-dissolved S^0 is incomplete by chromium reduction techniques, but is more reliably extracted with the heated reflux distillation procedure (13) compared to our proposed passive modifications. Other techniques, such as solvent extractions, have been recommended for circumventing the problems associated with the poor recovery of S^0 in sulfate reduction assays (1,13).

Additional experiments indicated that the modified technique could be used for the speciation of sulfides. Following the addition of 2N HCl, the acid volatile sulfides (H_2S and FeS) were completely recovered from a mixture of pure sulfur containing minerals (data not shown). Pyrite was not extracted under these conditions over a 30 h period but was recovered following the addition of the reduced chromium solution and 12 N HCl.

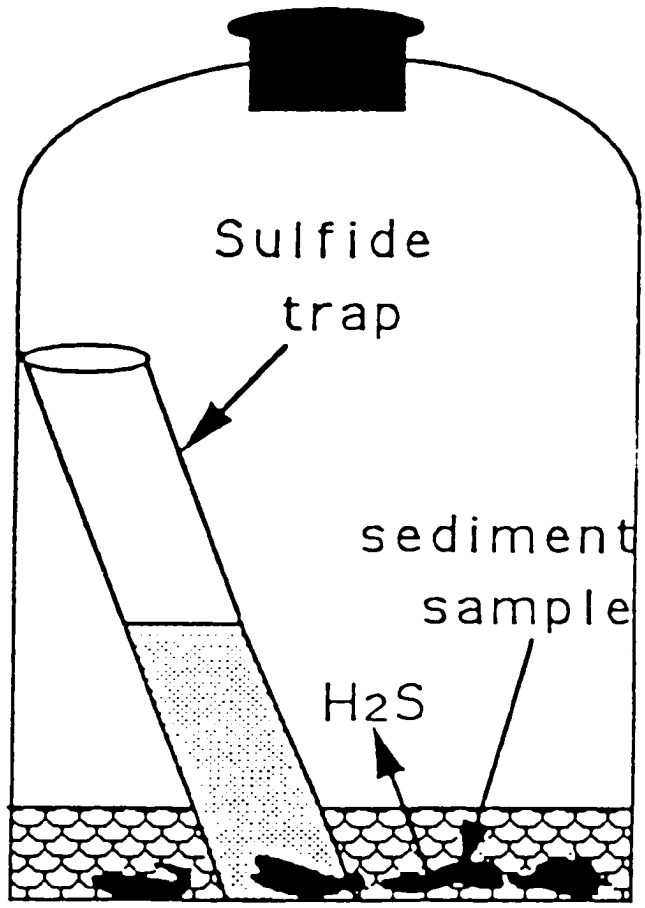

Figure 1. Schematic representation of the passive extraction procedure used for the determination of sulfate reduction activity or TRIS in sediment or water samples. A stoppered 120 ml serum bottle contains Cr(II) (8 ml of 1 M solution), 4 ml of 12 N HCl and the sediment sample. A 12 x 75 mm test tube containing 3 ml of 10% zinc acetate is used to trap the liberated H_2S.

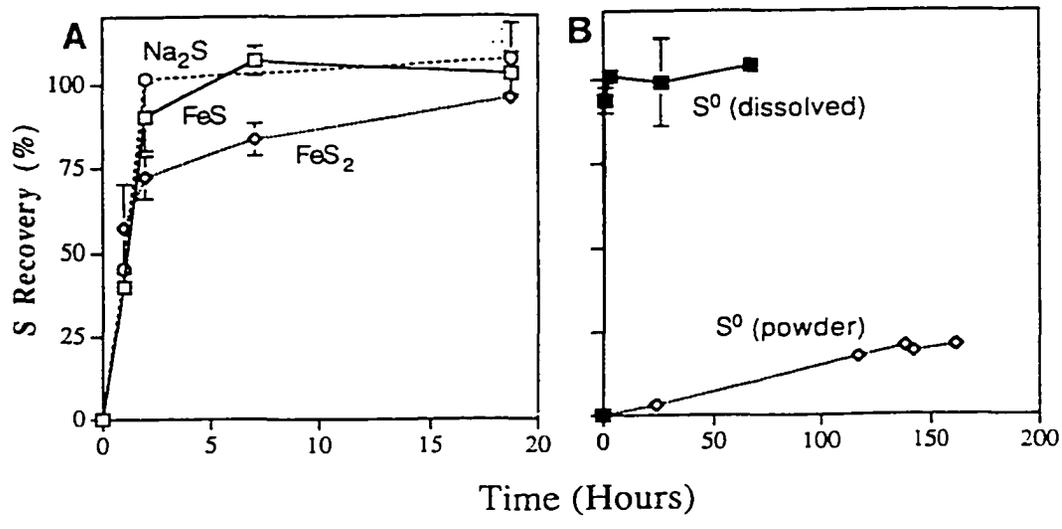

Figure 2. Sulfur recovery as a function of time (< 20h [A] or >20 h [B]) by using the passive extraction procedure with 1 mg sulfur as either Na_2S, powdered elemental sulfur (S^0), or S^0 dissolved in acetone and 5 mg of sulfur as iron monosulfide (FeS) or pyrite (FeS_2). FeS, FeS_2 (99.99 % pure, Alpha AESAR, Ward Hill, Mass.), and elemental sulfur (Sigma Chemical Co., St. Louis Mo.) were added as dry powders. Dissolved S^0 was prepared by dissolving excess particulate S^0 in 100 ml acetone. The solution was filtered to remove particulate S^0. $Na_2S \cdot 9H_2O$ (Aldrich Chemical Co.) stock solutions were prepared with anoxic 0.01 N NaOH. Each point represents the mean of duplicate extractions with the standard error (error bar) given where the value exceeds the dimension of the symbol.

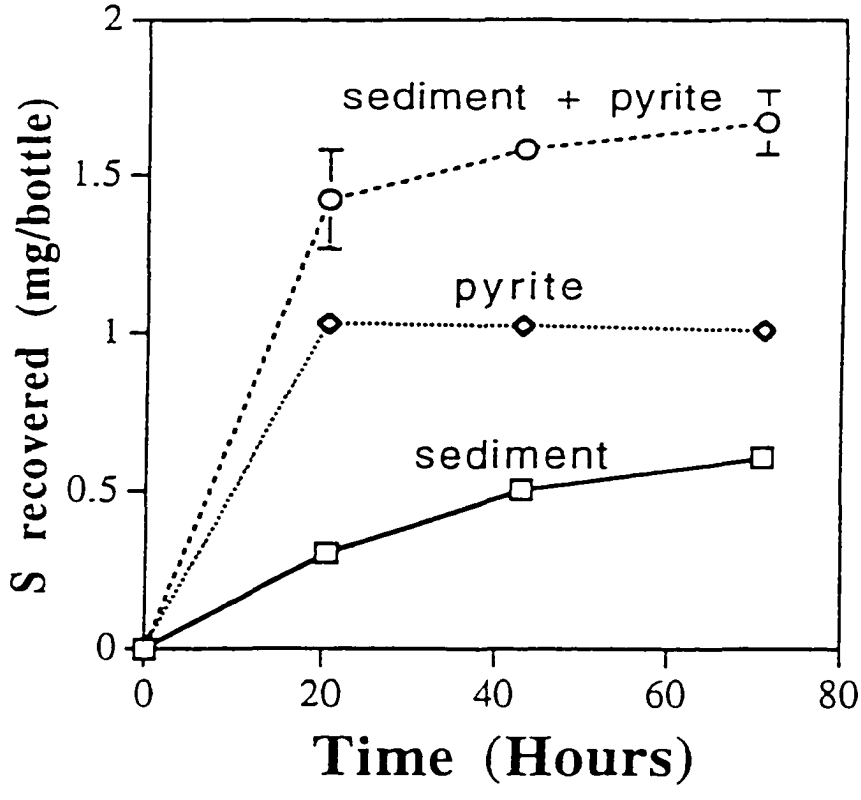

Figure 3. The recovery of TRIS over time for 1g landfill -eachate impacted aquifer sediment , 1mg FeS_2, or a mixture of 1g sediment and 1mg FeS_2. The values represent the mean of duplicate extractions with the standard error (error bar) given where the value exceeds the dimension of the symbol.

Location	Sulfate Reduction Rate[a] nmol · g^{-1} · d^{-1}		TRIS pool umols S · g^{-1}	
	Reflux	Serum Bottle	Reflux	Serum Bottle
Landfill	1.1 (0.001)	1.1 (0.002)	3.8 (0.85)	4.3 (0.72)
San Diego Bay	115.5 (0.32)	116.1 (1.07)	85.2 (19.90)	83.5 (23.9)

[a]Sediments were amended with 2 μCi carrier free $Na^{35}SO_4$ g^{-1} wet sediment, and incubated in serum bottles under 100% N_2 headspace for 48 h in the dark. Bottles were opened at the end of the incubation and weighed aliquots (1 g) of sediment were fixed in 2 ml 10% anoxic zinc acetate. TRIS was extracted from replicate fixed sediment samples using the reflux condenser method or the method described in this paper. Sulfate reduction rates were determined by calculating the fraction of sulfate which was reduced during the incubation (7). Sulfate reduction rates and TRIS determinations represent the mean of three and five determinations respectively; standard deviations are shown in parentheses.

Table 1 Comparison of techniques used for the extraction of total reducible inorganic sulfur (TRIS) and the determination of sulfate reduction rates.

Discussion

The traditional distillation method and the passive extraction chromium reduction techniques gave comparable results. However the passive extraction method has several important advantages over the distillation procedure (13). First, since it is not necessary to monitor the extractions during sulfide distillation, a large number of samples (over 100/day) can be processed over a relatively short time. Furthermore, unlike the reflux extractions, carry over of unreacted $^{35}SO_4^{-2}$ into sulfide traps does not occur when using the passive extraction technique. Thus, it is not necessary to wash unreacted $^{35}SO_4^{-2}$ from samples prior to the sulfide extraction step and time and labor are saved. We have used the modified technique to monitor low levels of sulfate reduction in biodegradation experiments, to determine sulfate reduction rates in natural samples, and to measure reduced S pools in samples from both contaminated and pristine aquifers. The method allows for the relatively rapid processing of thousands of samples which would otherwise require full time personnel and dedicated equipment use.

References

1. **Andersen, S.T., and B. B. Jorgensen.** 1989. Sulfate reduction and the formation of 35S-labeled FeS, FeS_2, and S^0 in coastal marine sediments. Limnology and Oceanography **34:** 793-806.

2. **Azadpour, A., L.R. Brown and A.A. Vadie.** 1996. Examination of thirteen petroliferous formations for hydrocarbon-utilizing sulfate-reducing microorganisms. J. Ind. Microbiol. **16:**263-266.

3. **Bak, F., and H. Cypionka.** 1987. A novel type of energy metabolism involving fermentation of inorganic sulphur compounds. Nature **326:** 891-892.

4. **Bak, F., A. Schuhmann, and K.H. Jansen.** 1993. Determination of tetrathionate and thiosulfate in natural samples and microbial cultures by a new, fast and sensitive ion chromatographic technique. FEMS Microbiology Ecology **12:** 257-264.

5. **Beeman, R.E., and J.M. Suflita**. 1987. Microbial ecology of a shallow unconfined ground water aquifer polluted by municipal landfill leachate. Microb. Ecol. 14:39-54.

6. **Boulegue, J., C.J. Lord III, and T.M. Church.** 1982. Sulfur speciation and assosciated trace metals (Fe, Cu) in the pore waters of Great Marsh Delaware. Geochimica et Cosmochimica Acta **46:**453-464.

7. **Canfield, D.E., R. Raiswell, J.T. Westrich, C.M. Reaves, and R.A. Berner.** 1986. The use of chromium reduction in the analysis of reduced inorganic sulfur in sediments and shales. Chemical Geology **54:**149-155.

8. **Cline, J.D.** 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol. Oceanog. **14:**454-458.

9. **Coleman, M.L., D.B. Hedrick, D.R. Lovely, D.C. White, and K. Pye.** 1993. Reduction of Fe(III) in sediments by sulphate-reducing bacteria. Nature **361:**436-438.

11. **Ensley, B.D., and J.M. Suflita.** 1995. Metabolism of environmental contaminants by mixed and pure cultures of sulfate-reducing bacteria, p. 293-332. *In* L.L Barton (ed.), *Sulfate-reducing Bacteria*.. Plenum Press, New York.

12. **Fauque, G.D.** 1995. Ecology of sulfate-reducing bacteria, p. 217-241. *In* L.L. Barton (ed.), Sulfate-Reducing Bacteria. Plenum Press, New York.).

13. **Fossing, H. and B.B. Jorgensen.** 1989. Measurement of bacterial sulfate reduction in sediments: evaluation of a single-step chromium reduction method. Biogeochem. **8:** 205-222.

14. **Fossing, H., and B.B. Jorgensen.** 1990. Isotope exchange reactions with radiolabeled sulfur compounds in anoxic seawater. Biogeochem. **9:** 223-245.

15. **Hsieh, Y.P., and C.H. Yang.** 1989. Diffusion method for the determination of reduced inorganic sulfur species in sediments. Limnol. Oceanogr. **34:** 1126-1130.

16. **Hungate, R. E.** 1969. A roll tube method for cultivation of strict anaerobes. p. 117-132. *In* J. R. Norris and D. W. Ribbons (Eds.), Methods in Microbiology, Vol. 3B. Academic Press, Inc. New York.

17. **Jorgensen, B.B.** 1990. A thiosulfate shunt in the sulfur cycle of marine sediments. Science **240:**152-154.

18. **Jorgensen, B.B. and F. Bak.** 1991. Pathways and microbiology of thiosulfate transformations and sulfate reduction in a marine sediment (Kattegat, Denmark). Appl. Environ. Microbiol. **57:** 847-856.

19. **Krekeler, D. and H. Cypionka.** 1995. The preferred electron acceptor of *Desulfovibrio desulfuricans* CSN. FEMS Microbiol. Ecol. **17:**271-278.

20. **Lovley, D. R., and E. J. P. Phillips.** 1994. Novel processes for anaerobic sulfate production from elemental sulfur by sulfate-reducing bacteria. Appl. Environ. Microbiol. **60:**2394-2399.

21. **Lovley, D.R., J. D. Coates, J. C. Woodward, and E. J. Phillips.** 1995. Benzene oxidation coupled to sulfate reduction. Appl. Environ. Microbiol. **61:** 953-958.

22. **Widdel, F.** 1988. Microbiology and ecology of sulfate- and sulfur- reducing bacteria, p. 469-585. *In* A.J.B. Zehnder (ed.), Biology of Anaerobic Microorganisms. Wiley, New York, NY.

Sediment Autoradiography for Detecting Sulfate-Reducing Activity in Sediment Cores.

Abstract

Methodologies that preserve the spatial relationship associated with *in-situ* metabolic activities are needed to gain insight on the functioning of microbial communities in sedimentary environments. We used direct sediment autoradiography to evaluate the spatial distribution associated with sulfate reduction in sediment cores that received $^{35}SO_4^{2-}$ as a tracer. The technique relied on the observation that microbially produced sulfide reacted rapidly with iron in the sediments and the resulting precipitate resisted reoxidation under anaerobic conditions. Experiments using $H^{35}S^-$ confirmed that sulfide precipitated where it was introduced to the core and was not removed during an anaerobic washing procedure designed to eliminate $^{35}SO_4^{2-}$. The autoradiographic images of radioactive iron sulfide patterns in the cores correlated with the total amount of reduced radioactive inorganic sulfides determined by other procedures. These analyses revealed that heterogeneities in sediment structure impacted sulfate reduction- activity and also the transport of sulfate-reducing bacteria within cores.

Introduction

Spatial heterogeneity associated with bacterial metabolic activities is a characteristic of microbial communities in many environments (1, 10-12, 14). Understanding the reasons for the distribution of microbial activities can provide insight into the factors which govern these processes. However, studying *in-situ* microbial activities is inherently difficult. In commenting on the constraints associated with current methodologies, Madsen (7) has indicated that most "..... methodologies do not contend well with the complexity of field sites, with the scale differential between microorganisms and humans, and with artifacts that may arise in characterizing microorganisms using laboratory-based physiological, biochemical, genetic, and molecular biological assays".

Given these limitations it would appear to be an advantage to localize microbial processes relative to small scale sedimentary and geochemical features governing microbial activities in sediments. This ability may aid in the development of hypotheses on the factors that control *in-situ* activity. For example, the large variation in denitrification activity in soil can be correlated with the presence of organic matter in isolated pockets (13) as a supply of electron donor or to the development of anaerobic microsites (18, 19). Similarly, 'hot spots' of methanogenesis in an aquifer, were associated with particulate organic matter (i.e. wood fragments) deposited 14,000 to 17,000 years ago (15). Increased sulfate reduction has been detected in sands and sandstones adjacent to fine-grained sediment strata including clay and shales (6, 23). The elevated activity in these regions may explain the occurrence of calcite cements (9) at sediment interfaces.

Extreme small scale (< 1 mm) variability in the isotopic signature and morphology of iron sulfides in thin sections of sediment from the Devonian Nisku Formation in the Western Canada Sedimentary Basin led to the suggestion that sulfate reduction must be a very localized process (17).

The development of techniques for deducing microbial activities at a small scale in minimally disturbed samples can aid in the identification of the factors that govern such processes in sediments. Therefore, we used direct imaging techniques to evaluate sulfate reduction in sediment cores. The procedure relies on the microbial reduction of $^{35}SO_4^{2-}$ as a tracer in cores and the rapid precipitation of $H^{35}S$ as radioactive iron sulfides. The cores are then dissected, unreacted tracer is removed, and the distribution of radioactive iron sulfides is determined by autoradiography. The method allows for an assessment of the spatial distribution of sulfate reduction in unconsolidated sediment cores.

Materials and Methods

Sediment sampling. Sediment cores in plastic core liners (4.4 cm outer diameter) were obtained from a landfill leachate contaminated alluvial aquifer (2) by hollow-stem augering with a split spoon sampler. Cores were also obtained by augering to the shallow water table (~1 m) and driving a hand held sampler approximately 0.5 m into saturated sediments. The cores in the liners were sealed with large rubber stoppers, transported to the laboratory, and stored at 4°C under N_2.

Differential removal of $^{35}SO_4^{2-}$ from sediment cores. The autoradiography of radioactive sulfide precipitates produced via sulfate-reducing activity is dependent on the ability to remove the remaining $^{35}SO_4^{2-}$. Therefore, attempts were made to evaluate whether $^{35}SO_4^{2-}$ could be selectively and efficiently removed from a sediment core receiving both radiotracer and sulfide injections. The $H^{35}S$- sulfide used in this experiment was prepared by dissolving $^{35}S^0$ (specific activity 1 Ci mg^{-1} atom, Amersham Life Science Inc., USA) in a S^0 saturated acetone solution. The $^{35}S^0$ in acetone was then converted to $H^{35}S^-$ and trapped in 1 N NaOH using a previously described method (23). A single injection (0.5 ml) of the ^{35}S-HS^- solution (3.8 μCi $H^{35}S^-$ ml^{-1}) was made near one end of the saturated core by syringe and needle. The needle was withdrawn while making the injection in an effort to leave a "trail" of radioactivity through the diameter of the core. The location of the sulfide injection was marked on the core liner.

Radioactive sulfate was then added to the core by making forty, 25 μl injections of a $^{35}SO_4^{2-}$ stock solution (10 μCi ml^{-1} anoxic water) at multiple locations through small pilot holes previously drilled in the plastic core liner. The injections were designed to distribute the tracer throughout the core. After 3 h of storage at 4° C, the core was dissected, a thin section (~ 1cm) was removed from the glove bag, and imaged using an autoradiographic imager (Instant Imager, Packard Instuments). After imaging, the core section was returned to the glove box. Radioactive sulfate was washed from the section by slowly dripping 500 ml of an anoxic solution of sodium molybdate (10 mM) from a separatory funnel onto the the thin core section which was overlain with 2 layers of filter paper. The filter

paper was used to prevent the dripping solution from distorting the sediment and to distrubute the molybdate wash over the entire section. After the washing procedure, the core section was again removed from the glove bag and imaged. We evaluated whether ^{35}S-iron sulfide was retained during the washing step by determining the amount of radioactivity in the $H^{35}S^-$ region of the autoradiogram before and after removing $^{35}SO_4^{2-}$. The efficiency of $^{35}SO_4^{2-}$ removal was determined in a similar manner.

It was initially not clear if the images produced from cores treated with radioactivity were produced only from the surface or also from the interior of the core slices. To help answer this question, we tested the ability of the sediment to block radiation from ^{35}S. A $^{35}SO_4^{2-}$ solution (10µl) (50 µCi $^{35}SO_4^{2-}$ ml^{-1} water) was spread over absorbant paper and allowed to dry. The paper was then imaged before coverage or with a 0.25 mm and a 0.5 mm thick layer of sand.

Spatial distribution of sulfate reduction activity in sediment cores.

Sulfate reduction assays were done with cores obtained from above and below the water table. The former was used as a method control since since sulfate reduction was previously not detected in this region using more traditional radiotracer assays (not shown). The method control was used to ensure that the signal in the autoradiograms could be attributed to labeled sulfide precipitates and not to residual $^{35}SO_4^{2-}$ remaining in the cores after the washing procedure. The radiotracer was added to cores (~ 10 cm long) by making 30, 0.05 ml injections of an anoxic $^{35}SO_4^{2-}$ stock solution (20 µCi $^{35}SO_4^{2-}$ ml^{-1} water) through pilot holes drilled in the plastic core sleeves. The samples were wrapped with parafilm to seal

the pilot holes and then incubated at 25°C under a N_2/CO_2 (80/20) atmosphere for 3 d.

After incubation, the cores were prepared for image analysis inside the glove bag. Two parallel vertical cuts were made with razor knives approximately 1.5 cm apart through the diameter and length of the cores and liners (Figure 1). The control core section was removed from the glove bag and imaged before the sulfate tracer was removed to determine if the protocol for amending radiotracer was sufficient to distribute $^{35}SO_4^{2-}$ throughout the cores and thus would not contribute to variability in ^{35}S-sulfide precipitation. Unreacted $^{35}SO_4^{2-}$ was then flushed from the core sections inside an anaerobic glove bag using the washing procedure described above prior to being imaged. The image processing time is minimized to prevent substantial iron sulfide oxidation; two to three hours are typical for the cores reported here.

To test if the radioactive signal in autoradiograms represented ^{35}S-sulfide precipitates in sediments, we compared the radioactive signal with the amount of labelled sulfides (total reducible inorganic sulfide - ^{35}TRIS) extracted from 18 sediment samples as previously described (27). The ^{35}TRIS analysis was done on samples of the same cores, but from core faces which were not subject to the washing procedure.

A modified method was used for adding and removing radiotracer when longer cores (~ 20 cm) were used. Groundwater collected adjacent to the sediment coring location was transported to the laboratory and supplemented with $^{35}SO_4^{2-}$ (to 1.4 $\mu Ci\ ml^{-1}$). The cores and groundwater were placed inside an anaerobic chamber containing 100% N_2. Approximately three pore volumes (assuming a 15 %

sediment moisture content) of the $^{35}SO_4^{2-}$ -supplemented groundwater was passed through the cores which were then sealed with stoppers and stored at 25°C for 30 d. Unreacted $^{35}SO_4^{2-}$ was then washed from the cores by passing 3 liters of an anaerobic solution of sodium molybdate (10 mM) through each core. The cores were then dissected and imaged as described above. To test if sulfate-reducing activity in cores was correlated with sediment grain size distribution, samples were obtained and assayed for grain size by sieve analysis.

Microbial transport experiment. To evaluate the utility of the technique for monitoring microbial transport experiments, sediment cores were constructed in an anaerobic glove bag by adding autoclaved (for 1h) sand to plastic core sleeves (4.4 cm outer diameter, ~12 cm long) that were sealed at one end. An anaerobic basal medium (8) containing ferrous sulfate (15 mM), $^{35}SO_4^{2-}$, and sodium lactate (5 mM) was periodically added to slurry the sediment. Approximately two pore volumes of the $^{35}SO_4^{2-}$-supplemented growth medium was then allowed to pass through the cores. Based on the estimated pore volume (assuming a 15 % moisture content), each core contained approximately 100 μCi of $^{35}SO_4^{2-}$. A control core was supplemented with sodium molybdate (5 mM) to inhibit sulfate reduction. *Desulfovibrio vulgaris*, strain hildenborough, was grown in the medium described above but with 30 mM sodium lactate and sodium sulfate. The cells were harvested by centrifugation during exponential growth and suspended in 10 ml of anaerobic lactate-free medium. An aliquot (0.5 ml) of the cell suspension was added drop-wise to the top of the cores. The upper layer of the cores was gently mixed to ensure that the inoculum was evenly distributed. The cores were sealed with rubber

stoppers, and incubated under N_2 upright without flow for 98 h at 37°C. The cores were dissected, washed, and imaged as described above. The distribution of *D. vulgaris* cells in the cores were determined by removing sediment samples from several locations of the core face opposing the imaged core face. The samples were then subjected to a 3 tube MPN enumeration using the described cultivation medium.

Results

Differential removal of $^{35}SO_4^{2-}$ from sediment cores. Radioactivity was relatively evenly distributed in the core section that contained both $^{35}SO_4^{2-}$ and $H^{35}S^-$ (Figure 2 A). However, after the section was washed to remove $^{35}SO_4^{2-}$, the location of the injected radioactive sulfide could easily be visualized (Figure 2 B). The majority of the radioactive sulfate (90-100%) was removed from the cores with the washing protocol. When $^{35}SO_4^{2-}$ remained in sediment cores, it produced a faint and relatively uniform radioactive signal slightly above background. The amount of radioactivity in the rectangular insert was determined using computer software before and after the washing procedure and corrected for background levels of radioactivity. The location of the insert corresponded to the $H^{35}S^-$ injection point marked on the core liner. The region within the insert harbored 4.2 CPM and 4.9 CPM detected by the imager before and after the washing step respectively, indicating that the sulfide precipitated rapidly in the sediment and was not removed during the washing procedure.

Autoradiography of ^{35}S in sediments. We found that a 0.25 mm thick layer of sand blocked 98% of the radioactivity from $^{35}SO_4^{2-}$ (data not shown). Therefore, we considered it unlikely that radioactive signals from beneath the immediate surface of the core sections interfered with the autoradiographic procedure.

Distribution of sulfate reduction in sediment cores. The autoradiography procedure was further evaluated by comparing the distribution of radioactivity in imaged core sections with sulfide (^{35}S-TRIS) extracted from the opposing core surface. This procedure was done on a core taken from above the water table where sulfate reduction was known to be minimal and a saturated core taken below the water table. Autoradiography indicated that radioactivity was relatively evenly distributed in the former core prior to the washing procedure (Figure 3 A). After the section was washed to remove $^{35}SO_4^{2-}$, only trace amounts of residual radioactivity at a level slightly above background remained (Figure 3B). The amount of signal in autoradiograms could be correlated with the total amount of radioactive sulfides extraced from the corresponding core sections (plot in Figure 3). Thus, insight on the spatial distribution associated with sulfide deposition could be obtained by autoradiography of the core surface.

In contrast to the control core, radioactive sulfide was indicated by the sediment extractions and by sediment autoradiography of sulfate reduction in the core obtained from beneath the water table (Figure 3). ^{35}S-TRIS was extracted from all of the sediment samples except those from the upper right portion of the imaged core face where very little sulfate reduction was detected in the

autoradiogram. The distribution of sulfate-reducing activity in various regions of the autoradiograms (indicated by the grids in Figure 3) of the control and active cores correlated in a linear fashion with ^{35}S-TRIS extracted from sediment samples.

The pattern of sulfate-reducing activity in a longer core correlated with differences in sediment structure (Figure 4). Very little activity was detected at the top of the core that consisted of finer grained sediments as seen by the insert showing the grain size distribution. A sharp increase in activity was detected in the sediments immediately beneath this interval. The grain size distribution of 7 sediment samples taken from other regions of the core, were similar as represented by the other insert in Figure 4. Four distinct bands of decreased sulfate reduction were oriented parallel to the depositional layering of the sediments in the core. The grain size of sediments in these regions was similar to the grain size of sediments in active areas. The reasons for the decreased sulfate reduction activity oriented as bands within the core is not clear.

Migration of sulfate-reducing bacteria through sediment cores. Autoradiograms of the sediment sections from the microbial transport study are shown in Figure 5. The images represent sulfate reduction which occurring during the 98 h incubation as a consequence of the transport of sulfate-reducing bacteria. Substantial sulfate reducing activity was only detected in the core that was inoculated and not treated with molybdate (Figure 5A). The upper section of the core exhibited the greatest amount of sulfate reduction. Despite the extensive sulfate reduction, dissolved sulfide was not detected in liquid samples removed from the cores as it most likly precipitated as iron sulfide. Radioactivity above

background was not detected in the the lower portion of the core indicating that sulfate reduction did not occur in that region and that unreacted $^{35}SO_4^{2-}$ was completely removed during the washing procedure. Radioactive sulfide was not detected in a small (~ 0.5 cm) clay inclusion near the left edge of the core. There was also less activity in the sands immediately beneath the clay.

The number of sulfate-reducing bacteria *Desulfovibrio vulgaris* cells detected in various regions of the active core ranged from 2.4×10^6 to 4.6×10^{10} cells g^{-1}. The large region of the core which showed intense sulfate reduction contained a very high and essentially constant number of cells. The number of cells just below this region dropped between 3 and 4 orders of magnitude over approximately 3 cm distance where a signal was not detected in the autoradiogram. The sulfate-reducing bacteria were not detected by cultivation assays at the bottom of the core where radioactivity was not detected. Sulfate reduction was not detected and sulfate-reducing bacteria were not cultivated from the molybdate control core.

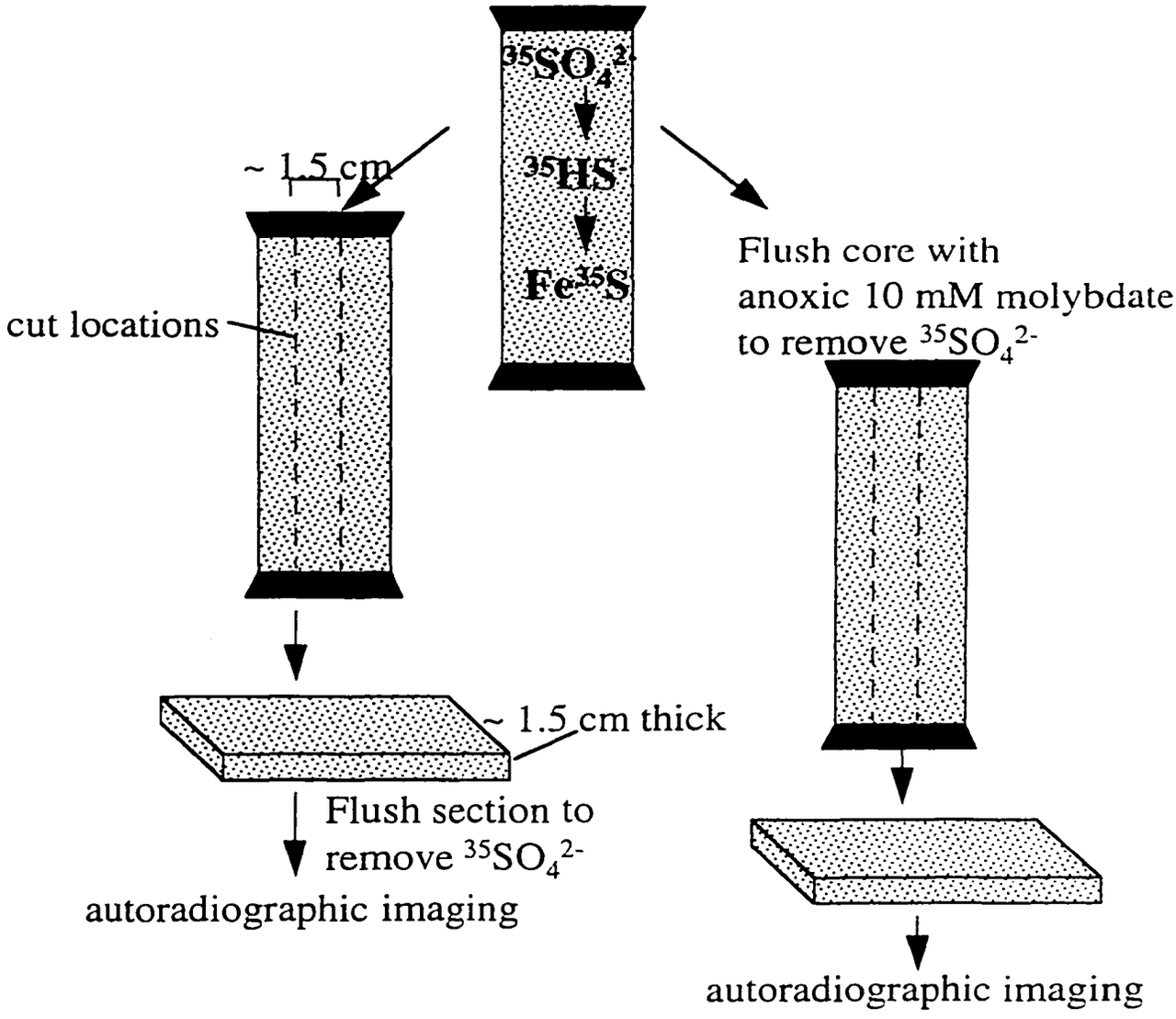

Figure 1. Schematic showing an abbreviated protocol of the sediment autoradiography technique for detecting sulfate-reducing activity in sediment cores.

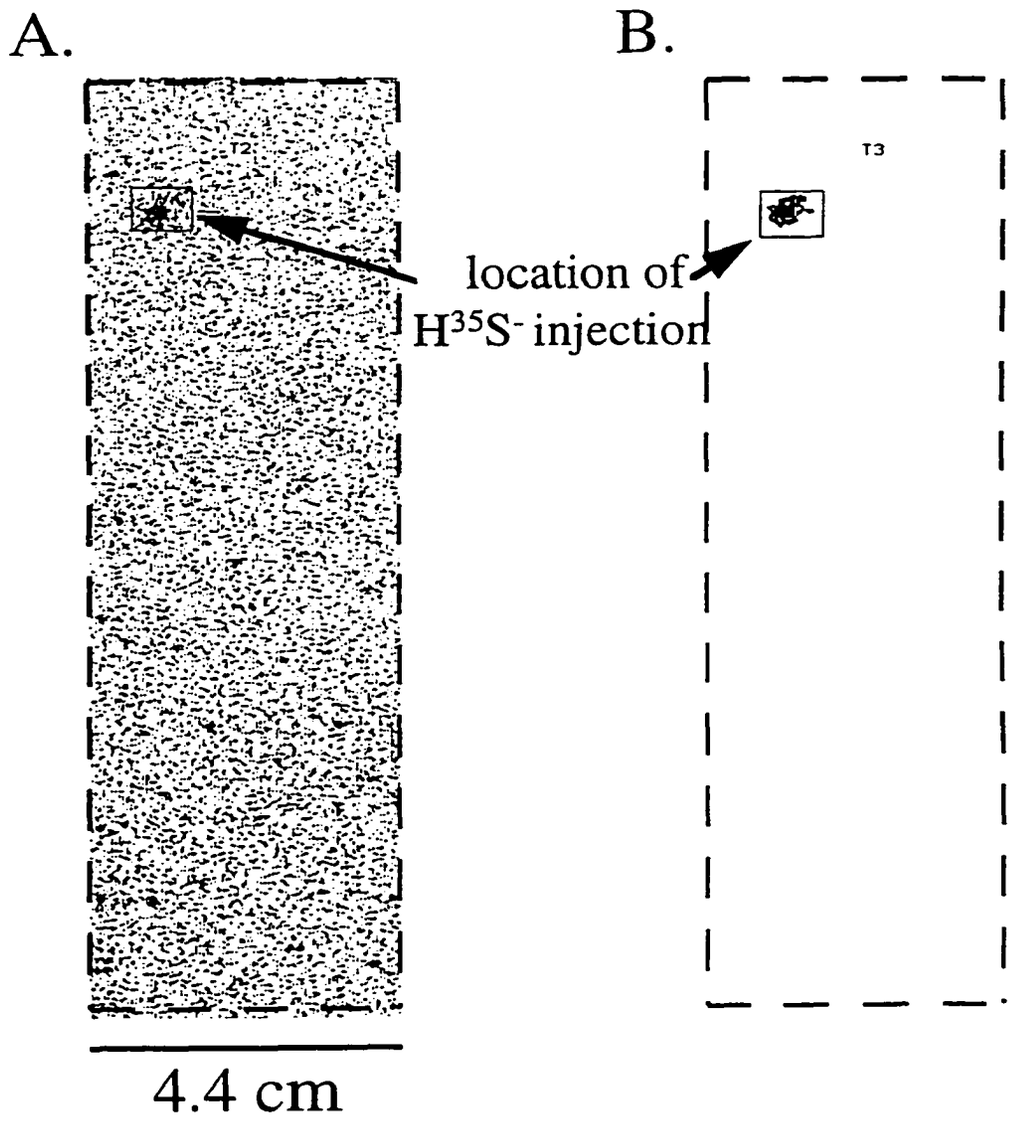

Figure 2. Autoradiograms showing the localization of radioactive sulfide deposition and the selective removal of $^{35}SO_4^{2-}$ from a sediment core that received a single injection of $H^{35}S$- and multiple $^{35}SO_4^{2-}$ injections. Autoradiograms A and B were obtained before and after a washing procedure for removing $^{35}SO_4^{2-}$, respectively.

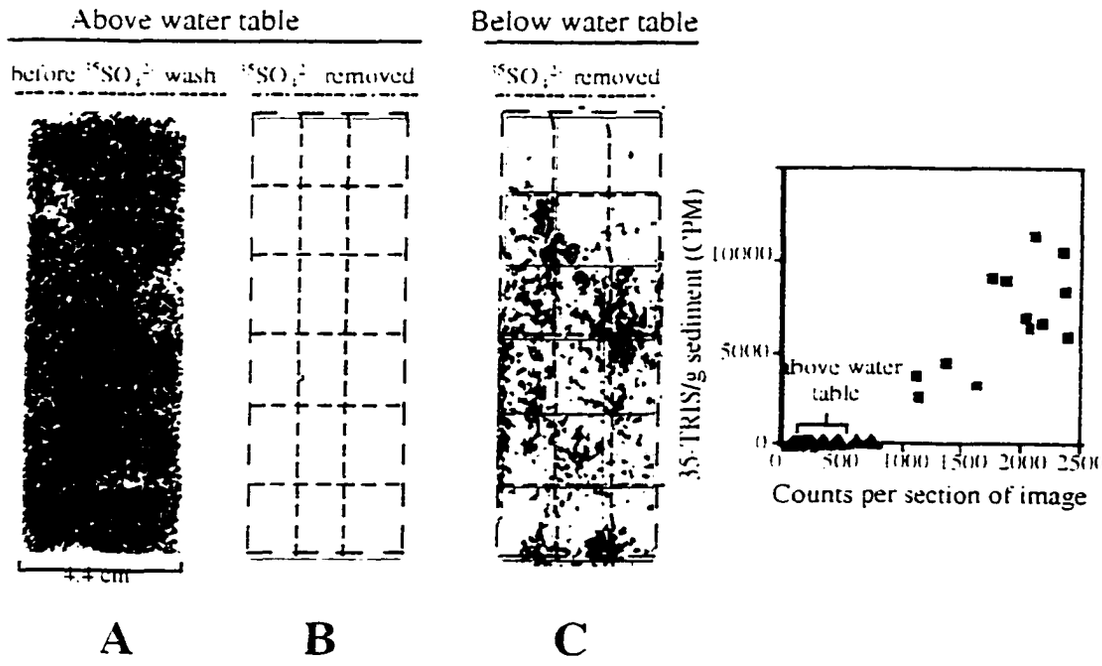

Figure 3. Autoradioagrams A and B show the distribution of the injected $^{35}SO_4^{2-}$ before and after the washing procedure for removing unreacted $^{35}SO_4^{2-}$ in a core where sulfate reduction was not detected. Yellow and red coloration indicate areas of increased levels of radioactivity in the autoradiograms. Autoradiogram C was obtained after removing $^{35}SO_4^{2-}$ from a core where sulfate reduction was detected. The plot shows the relationship between 35S-sulfide extracted from sediments obtained from various regions in the cores before the sediment was washed and the radioactivity in the corresponding regions (indicated by the grids) of images B and C.

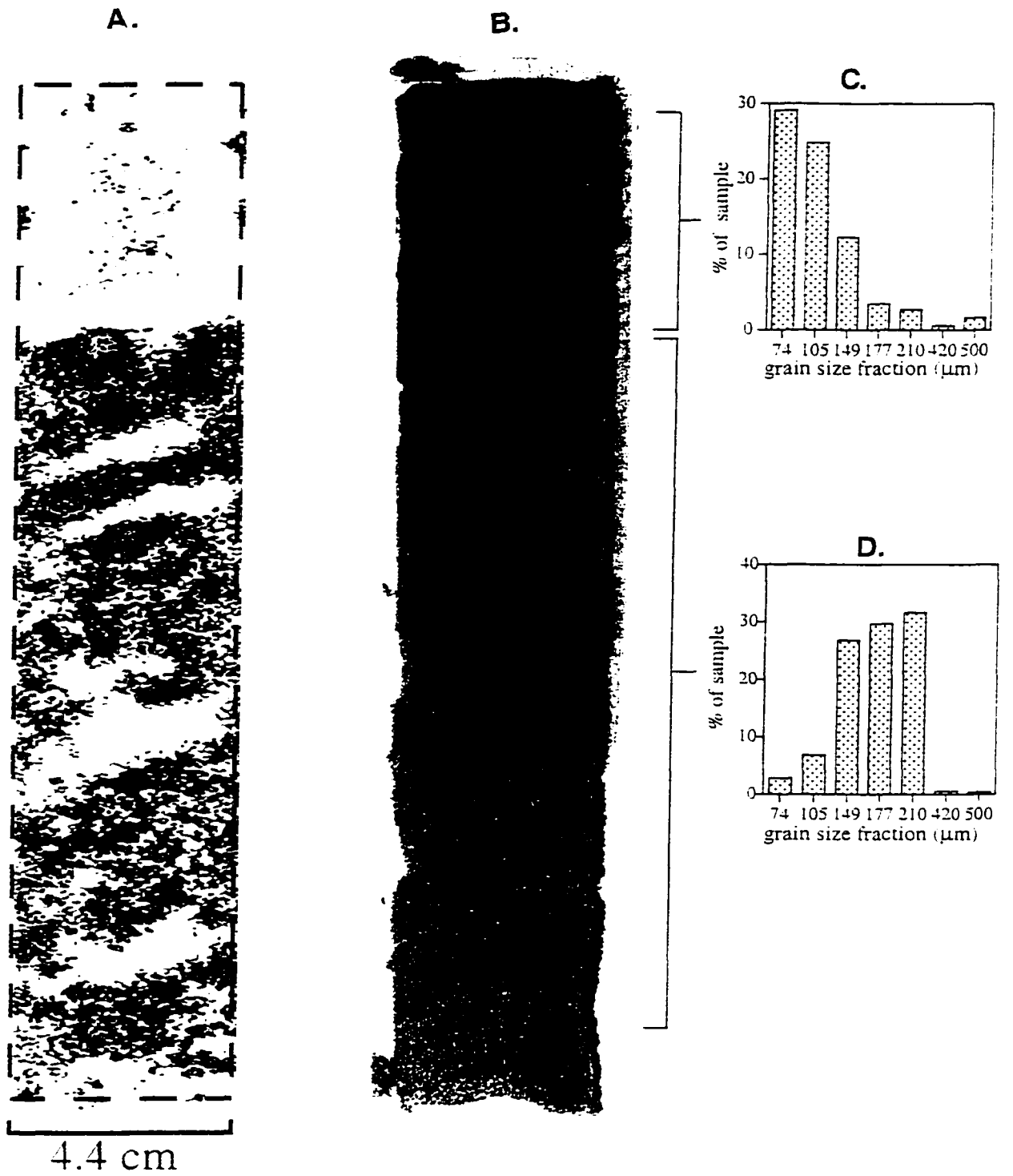

Figure 4. Autoradiogram (A) showing the distribution of sulfate reduction in a sediment core from a contaminated aquifer. Yellow and red coloration indicate areas of increased levels of radioactivity in the autoradiogram. A photograph of the imaged core face (B) is seen to the right. The inserted graphs indicate the sediment grain size distribution at the top of the core (C) and a sample representative of the remaining portions.

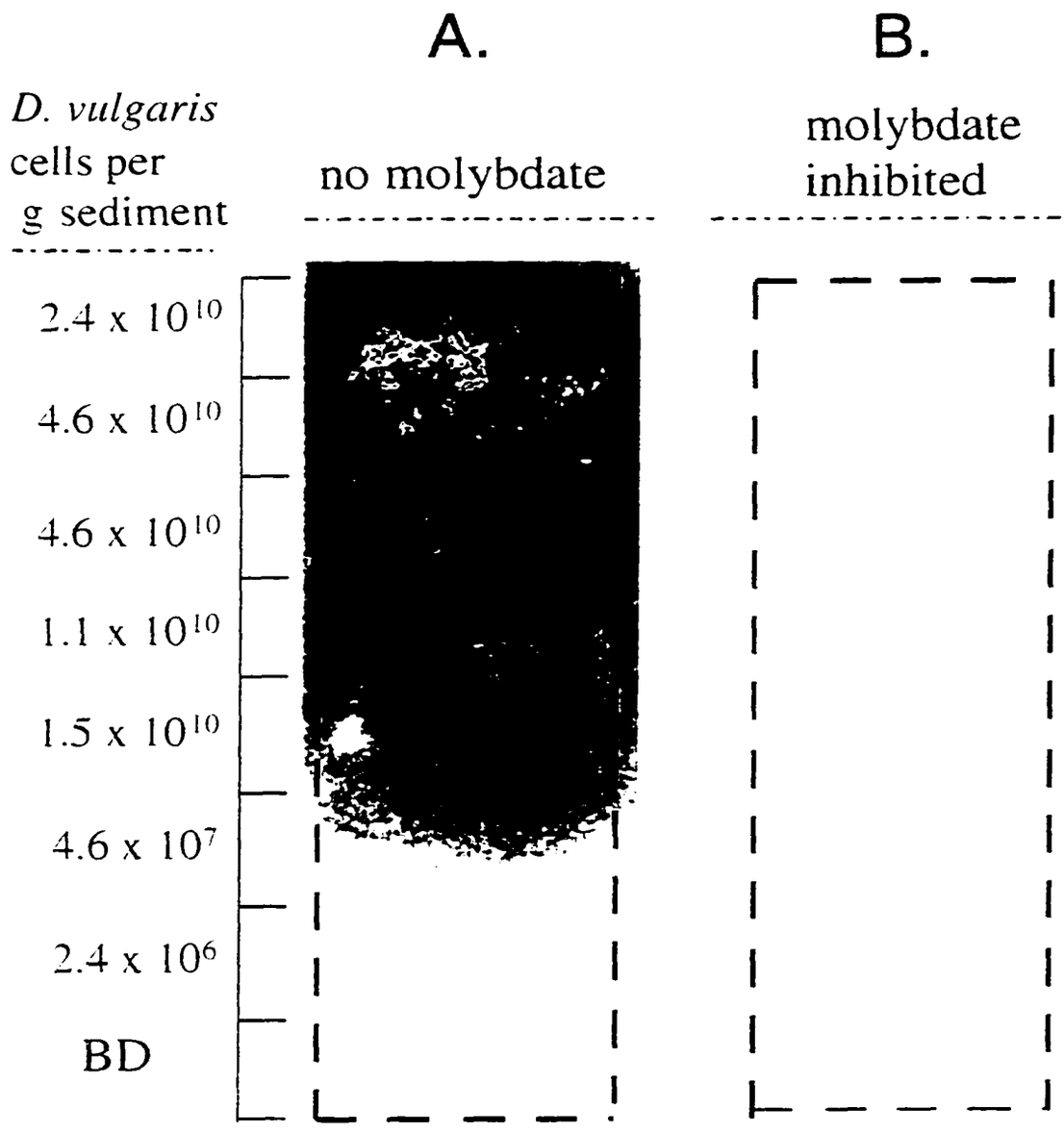

Figure 5. Autoradiograms showing the distribution of sulfate reduction and sulfate- reducing bacteria as a consequence of the migration of cells from the top of an uninhibited (A) and a molybdate treated core (B).

Discussion

Insight on the factors governing metabolic activity in subsurface environments has been acquired by evaluating the spatial distribution of sulfate reduction relative to physical and chemical heterogeneities (6, 23). Therefore, the continued development of methodologies capable of detecting the spatial distribution of this process while preserving sample integrity, may provide information on the ecology of this important process. To that end, we developed a sediment autoradiography technique to determine the spatial variability of sulfate reduction in minimally disturbed cores.

The method was founded on the observation that dissolved sulfide was below detection in groundwater samples from a leachate contaminated aquifer including intervals where sulfate reduction was detected (Chapter 6). Similarly, dissolved sulfide is often not detectable in the pore water of marine sediments provided that the rates of sulfate reduction do not exceed the capacity of reactive iron species to precipitate H_2S (3, 21). Our current results confirm that hydrogen sulfide precipitates rapidly in the aquifer sediment where it was produced (Figure 3.4.5) or injected (Figure 2) in cores. Thus, it is possible that the distribution of sulfide precipitates, unlike most soluble endproducts of anaerobic metabolism, can be used to track the distribution of metabolic activity in sediment. Indeed, the distribution of sulfate reduction activity could be evaluated by determining the pattern of ^{35}S-iron sulfide deposition during incubation with $^{35}SO_4^{2-}$ as a tracer since the removal of unreacted $^{35}SO_4^{2-}$ was sufficient to allow for the autoradiography of the ^{35}S precipitates. These precipitates were not removed

during the washing procedure (Figure 2). Furthermore, only radioactive sulfides near the surface of the samples are imaged since beta radiation from ^{35}S was efficiently blocked by overlying sediment. This aspect contributes to the resolution of the technique. The distribution of sulfate-reducing activity in various regions of the autoradiograms correlated in a linear fashion with ^{35}S-TRIS extracted from corresponding sediment samples (Figure 3) confirming that the method was useful for identifying the spatial pattern of sulfate reduction in cores.

These laboratory observations are consistent with our previous finding that the distribution of iron sulfides in contaminated aquifer sediments tracked the sulfate reduction rate measurements (Chapter 6). This was especially true in deeper portions of the aquifer where the periodic influx of oxygen is less likely. The sulfides were found to be stable in sediment slurries under anoxic conditions even when a variety of potential electron acceptors (other than oxygen) were made available. Thus, the distribution of sulfide deposition in aquifer sediments and in sediment cores was useful for identifying the spatial variability of sulfate reduction at the meter and mm scales respectively. The capacity to determine the distribution of microbial activities at multiple scales is important since the controlling variables and resulting heterogeneity may occur over an equally large spatial range. For example, sulfate-reducing activity as indicated by gradients in iron sulfides with depth in an aquifer was influenced by differences in sulfate concentration over meter distances (Chapter 6). As described below, mm-scale heterogeneities in activity judged by the pattern of ^{35}S-sulfide deposition in sediment cores have also been identified.

There are important advantages of sediment autoradiography relative to more traditional radiotracer assays of sulfate reduction. First, the spatial pattern of this process can easily be related to sedimentary and geochemical features. For instance, by comparing the image of sulfate reduction in leachate contaminated sediments with a photograph of the corresponding core (Figure 4), it is apparent that activity was reduced in the fine grained interval near the top of the core. This observation is consistent with several other studies indicating that sulfate reduction (6, 23), and several other microbial activities and cell numbers (4, 5, 16, 20, 24), are depressed in fine grained sediments. The bands of decreased sulfate reduction in the remainder of the core appear to be related to the depositional layering of the sediments but could not be correlated with decreased grain size. The specific cause for these heterogeneties is unknown but it is unlikely that differences in soluble chemistry were responsible since the pore water was exchanged with $^{35}SO_4^{2-}$ amended groundwater for this particular experiment. The soluble phase chemistry in the cores was likely homogenous after exchanging the pore water.

The sediment autoradiography procedure in conjunction with SRB enumerations was useful for detecting the migration and growth of a motile culture of *D. vulgaris* through sediment cores. For instance, the images of sulfate reduction (Figure 5) suggest that the cells moved as a band and that the migration of SRB was influenced by a small (~ 1 cm) clay inclusion in a sediment core. The upper portion of the inoculated core exhibited the greatest amount of sulfate reduction probably because SRB were present near the inoculated end for the greatest length of time. Increased sulfate-reducing activity was also associated

with regions of the core where SRB apparently grew to very high (~ 10^{10} cells g^{-1} sediment) densities.

We previously used silver foils as a ^{35}S-sulfide trap to evaluate the spatial distribution associated with sulfate reduction in consolidated rock and unconsolidated cores (6, 23). While useful, the technique is indirect and the resolution and interpretation of the images are occasionally reduced by the inability to ensure even contact between the sediment and foil. As illustrated in the current study, an important advantage of the direct autoradiography technique is that it allows for the determination of sulfate reduction in whole sediment cores rather than activity at a disturbed sample face. Furthermore, the silver foil could serve as a surface for bacterial attachment thereby increasing the potential for disturbing the rates and distribution of sulfate-reducing activity. It is our contention that the sediment autoradiography technique may provide more direct information on the spatial distribution of sulfate reduction. However, we recognize that additional work will be required to evaluate the utility of the method to other sediments and applications. For instance, the technique would require modifications for evaluating sulfate reduction in sediments that do not contain sufficient iron to precipitate radioactive sulfide in place.

References

1.**Adrian, N. R., J.A. Robinson, and J.M. Suflita.** 1994. Spatial variability in biodegradation rates as evidenced by methane production from an aquifer. Appl. Environ. Microbiol. **60:**3632-3639.

2.**Beeman, R. E., and J.M. Suflita.** 1987. Microbial ecology of a shallow unconfined ground water aquifer polluted by municiple landfill leachate. Microb. Ecol. **14::**39-54.

3.**Chanton, J. P., C.S. Martens, and M.B. Goldhaber.** 1987. Biogeochemical cycling in an organic-rich coastal marine basin, 7. Sulfur mass balance, oxygen uptake and sulfide retention. Geochim. Cosmochim. Acta. **51:**1187-1199.

4.**Fredrickson, J. K., T.R. Garland, R.J. Hicks, J.M. Thomas, S.W. Li, and K.M. McFadden.** 1989. Lithotrophic and heterotrophic bacteria in deep subsurface sediments and their relation to sediment properties. Geomicrobiol. J. **7:**53-66.

5.**Fredrickson, J. K., J. P. McKinley, B. N. Bjornstad, P. E. Long, D. B. Ringelberg, D. C. White, L. R. Krumholz, J. M. Suflita, F. S. Colwell, R.M.Lehman, and T. J. Phelps.** 1997. Pore-size constraints on the activity and survival of subsurface bacteria in a late Cretaceous shale-sandstone sequence, northwestern New Mexico. Geomicrobiol. J. **14:**183-202.

6.**Krumholz, L. R., J.P. McKinley, G.A. Ulrich, and J.M. Suflita.** 1997. Confined subsurface microbial communities in Cretaceous rock. Nature. **386:**64-66.

7.**Madsen, E. L.** 1998. Epistemology of environmental microbiology. Environ. Sci. Technol. **32:**429-439.

8.**McInerney, M. J., M.P. Bryant, and N. Pfennig.** 1979. An anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. Arch. Microbiol. **122:**129-135.

9.**McMahon, P. B., F. H. Chapelle, W. F. Falls, and P. M. Bradley.** 1991. Role of microbial processes in linking sandstone diagenesis with organic-rich clays. J. Sediment. Petrol. **62:**1-10.

10.**Parkin, T. B., S.T. Chester, and J.A. Robinson.** 1990. Calculating confidence intervals for the mean of a lognormally distributed variable. Soil Sci. Soc. Am. J. **54:**321-326.

11.**Parkin, T. B., J.J. Meisinger, S.T. Chester, J.L. Starr, and J.A. Robinson.** 1988. Evaluation of statistical estimation methods for lognormally distributed variables. Soil Sci. Soc. Am. J. **52:**323-329.

12.**Parkin, T. B., J.L. Starr, and J.J. Meisinger.** 1987. Influence of sample size on measurement of soil denitrification. Soil Sci. Soc. Am. J. **51:**1492-1501.

13.**Parkin, T. B.** 1987. Soil microsites as a source of denitrification variability. Soil Sci. Soc. Am. **51:**1194-1199.

14.**Parkin, T. B.** 1993. Spatial variability of microbial processes in soil-a review. J. Environ. Qual. **22:**409-417.

15.**Parkin, T. B. a. W. W. S.** 1995. Contemporary groundwater methane production from Pleistocene carbon. J. Environ. Qual. **24:**367-372.

16.**Phelps, T. J., E.G. Raione, D.C. White, and C.B. Fliermans.** 1989. Microbial activities in in deep suvsurface environments. Geomicrobiol. J. **7:**79-92.

17.**Riciputi, L. R., D.R. Cole and H.G. Machel.** 1996. Sulfide formation in reservoir carbonates of the Devonian Nisku formation, Alberta, Canada: An ion microprobe study. Geochim. Comochim. Acta. **60:**325-336.

18.**Seech, A. G. a. E. G. B.** 1988. Denitrification in soil aggregates of different sizes. Soil Sci. Soc. Am. J. **52:**1616-1621.

19.**Sexstone, A. J., N.P. Revsbech, T.B. Parkin, and J.M. Tiedje.** 1985. Direct measurement of oxygen profiles and denitrification rates in soil aggregates. Soi. Sci. Soc. Am. J. **49:**645-651.

20.**Sharma, P. K., and M.J. McInerney.** 1994. Effect of grain size on bacterial penetration, reproduction, and metabolic activity, in porous glass bead chambers. Appl. Environ. Microbiol. **60:**1481-1486.

21.**Thandrup, B., H. Fossing, and B. B. Jørgensen.** 1994. Manganese, iron, and sulfur cycling in a coastal marine sediment, Aarhus Bay, Denmark. Geochim. Cosmochim. Acta. **58:**5115-5129.

22.**Ulrich, G. A., L. R. Krumholz, and J. M. Suflita.** 1997. A rapid and simple method for estimating sulfate reduction activity and quantifying inorganic sulfides. Appl. Environ. Microbiol. **63:**1627-1630.

23.**Ulrich, G. A., D. Martino, K. Burger, J. Routh, E. L. Grossman, J. W. Ammerman, and J. M. Suflita.** 1998. Sulfur cycling in the terrestrial subsurface: commensal interactions, spatial scales, and microbial heterogeneity. Microb. Ecol. **36:**141-151.

24.**Zhang, C., A.V. Palumbo, T.J. Phelps, J.J. Beauchamp, F.J. Brockman, C.J. Murray, B.S. Parsons, D.J.P. Swift.** 1998. Grain size and depth constraints on microbial variability in coastal plain subsurface sediments. Geomicrobiol. J. **15:**171-185.

Chapter 3

Microbial Abundance and Activity in a Low Hydraulic Conductivity Aquifer System in East-Central Texas

Abstract

The influence of sediment properties and groundwater geochemistry on microbial abundance and activity was examined in a Gulf Coast aquifer system. Three boreholes were drilled into the sands, silts, clays, and lignite of the Eocene Yegua formation and wells were installed in all water-bearing sands. Total numbers of microorganisms ranged from 10^6 to 10^8 cells per gram dry weight (gdw^{-1}), and viable counts ranged from 0 to 10^6 cells gdw^{-1}. The highest densities of anaerobic heterotrophs and sulfate reducing bacteria (SRB) (10^5 and 10^6 cells gdw^{-1}, respectively) were measured in the deepest aquifer sands (28-31 m) even though the total organic carbon content was very low. Rates of anaerobic H_2, lactate, and formate consumption were also high in aquifer sands relative to the other strata. The higher microbial numbers and activities in the aquifer sediments likely reflect the importance of increased electron donor and acceptor transport in higher hydraulic conductivity sands relative to other strata. The presence of sulfate and near absence of other electron acceptors (O_2, NO_3^-, and Fe^{3+}), absence of methanogens, high numbers of SRB, and relatively high sulfate reduction activity suggest that sulfate reduction is the dominant terminal electron accepting process in

Introduction

Many aquifers are contaminated with xenobiotic compounds from anthropogenic sources (17). Evaluation of the potential for bioremediation in these aquifers requires a better understanding of the microbial ecology of pristine as well as contaminated formations (15). The prospects for bioremediation appear good, as numerous studies have shown that nutritionally diverse microorganisms are present in pristine subsurface environments (2, 7, 20, 21) and that their activities are influenced by physical and chemical conditions within the subsurface environment (20, 21, 23). The vast majority of aquifer remediation efforts in the past ten years have involved aerobic biodegradation processes. However, it is becoming increasingly apparent that, due to the limitations of oxygen transport in the subsurface, studies on the potential for anaerobic biodegradation of contaminants are required (5).

Microbiological investigations of reducing aquifer sediments are uncommon (8, 13, 18, among others) and few if any studies have focused on the Gulf Coast Aquifer system. The aquifer under study, the Yegua aquifer, becomes anaerobic at shallow depth (< 30 m) and remains so over the remainder of its flow path. The anoxic conditions are attributable to low hydraulic conductivity sediments in the recharge zone which limit the amount of recharge to the system (35), thus increasing the residence time of the groundwater and consequently restricting the amount of dissolved oxygen.

Our objective was to determine if low conductivity aquifer systems of the Gulf Coast exhibit the same microbiological and geochemical patterns as aquifers

recharged through higher conductivity sediments. For example, will aquifers exhibit greater microbial activity and abundance than aquitards as suggested by others (13)? Further, do aquitards serve as sources of sulfate for SRB as proposed for the Atlantic Coastal Plain aquifers (8)? Thus we pose three specific hypotheses: (1) Sediment properties (i.e., grain size, organic carbon content) will influence the distribution of microorganisms throughout the formation; (2) sulfide oxidation products will be the primary source of sulfate for anaerobic respiration within the aquifer; and, (3) sulfate reduction will have a significant impact on the aquifer geochemistry. To investigate these hypotheses core material was collected from three boreholes in the Yegua formation and analyzed for physical, chemical, and microbiological properties.

Materials and Methods

Sampling site The sampling site (Figure 1) lies within the Texas coastal uplands aquifer system which consists of four aquifers and two confining units of the Eocene Claiborne and Wilcox Groups (31). This site is located within the recharge zone of the Yegua aquifer. The Yegua is the uppermost aquifer within the coastal upland system and is comprised of silty to clayey, fine- to medium-grained sand thinly interbedded with clay, silt, and lignite. These sediments were deposited in a fluvial to deltaic system that was periodically influenced by marine flooding (3). The aquifer is characterized by abrupt vertical and lateral lithologic changes resulting in uncorrelatable sands, even over short distances. Groundwater flow primarily occurs through sands that are hydraulically connected despite separation

by finer-grained sediments (11, 35). The aquifers studied here mostly involve shallow groundwater flow controlled by local topography; intermediate flow, perhaps influencing the deepest aquifer (NP-3), is controlled by the Brazos and Navasota rivers, 17 km to the west and 48 km to the east, respectively. Regional groundwater flow is toward the southeast (35).

Sediment sampling Three boreholes (NP-1, NP-2, and NP-3) were drilled by hollow-stem auger and cores were obtained using a split spoon sampler with core liners. Borehole NP-1 (18.6 m below land surface, mbls) was drilled on a knoll and boreholes NP-2 (16.1 mbls) and NP-3 (30.8 mbls) were drilled about 280 m to the southeast in the dip direction (Figure 1). NP-2 is located 15 m south of NP-3 and was drilled to provide stratigraphic control for borehole NP-3. Samples obtained from these boreholes, especially NP-3, were used for sedimentological, microbiological, and geochemical analyses.

Upon collection, cores were immediately wrapped in plastic and chilled with ice. Within 4 h, cores were transported to the laboratory for processing in a glove bag inflated and constantly purged with Ar. The potentially contaminated exterior of each core was pared away using sterile knives. The core interior was aseptically sampled for geochemical and microbiological analyses. The samples were placed in Whirl-Pak™ bags and in Ar-filled jars then stored at 4°C.

Multiple tracers were used to evaluate the prospects for chemical and microbiological contamination of samples that may have occurred during drilling and sample processing. Carboxylated fluorescent microspheres (Polysciences, Inc., Warington, Pa.) were used as microbiological tracers (29). Samples showing

levels of microsphere contamination above the detection limit (>10^3 microspheres gdw^{-1}) or samples that did not remain intact during handling, such as brittle lignites or extremely wet sands, were designated non-aseptically obtained.

Physical and chemical analyses of sediments Grain size was determined by a combination of wet sieving and pipetting. Percent moisture was determined from the weight loss of 2 g samples after heating at 100°C for 24 hours. Carbon isotopic composition of total organic carbon (TOC) was determined using the sealed tube combustion method (4). The CO_2 yields from combustion were used to determine percent TOC in the sediments.

Acid volatile sulfides (AVS) and chromium reducible sulfides (TRS) were analyzed using a passive extraction technique (34). Anions in groundwater samples or extracted from sediments with distilled water (1:1; wt:vol) were analyzed using a Dionex ion chromatographic system equipped with an AS4A-SC (4 mm) column, a CD 20 conductivity detector, and a carbonate:bicarbonate mobile phase (1.8:1.7 mM) at 2 ml min^{-1}.

For sulfur isotope values, sulfides were converted to H_2S, trapped as ZnS, acidified and reprecipitated as Ag_2S, and shipped to Coastal Science Laboratories, Inc. (Austin, TX) for isotope ratio mass spectrometer (IRMS) analysis.

Microbial enumeration assays Total organisms were determined by epifluorescence microscopy using the method of Sinclair and Ghiorse (31), with the exception that fifty fields of view were counted per slide. Two slides were

prepared for each core interval. For each set of samples, sterile controls consisting of sterilized water and sediment baked at 550°C for 5 h were prepared. Slides were counted at 1250X total magnification, with a minimum detection limit of 1.0×10^{4} cells g^{-1} wet wt sediment. Microorganisms in groundwater samples were determined the same way, but without the initial slurry procedure and heat inactivated control.

Viable aerobic heterotrophs were determined by the spread plate method of Balkwill (2), on a 1% peptone, trypticase, yeast extract, and glucose (PTYG) medium. Samples were incubated aerobically at 25°C for four weeks and colonies were counted weekly. Sulfur oxidizing microorganisms were enumerated in sediment and water samples by the most probable number (MPN) method of Fredrickson et al. (13). A decline in pH and visible growth was scored as positive for this group of organisms. Iron oxidizing microorganisms were enumerated using the medium of Kuenen et al. (22). Tubes were scored by either visual growth or by Fe^{2+} disappearance as measured photometrically at 510 nm (10).

Sulfate-reducing and methanogenic bacteria were enumerated in sediment and water samples by a three-tube MPN procedure. The medium used for the cultivation of methanogens was that of Mormile et al. (26) with the exception that both acetate and H_2 were included as electron donors. The medium of Tanner (33) was used for the assay of SRB. Anaerobic heterotrophs were assayed the same way as the aerobic heterotrophs, but the plates were spread and incubated for at least a month in an anaerobic glovebag containing a 1:9 H_2:N_2 atmosphere and a palladium catalyst. *Methanobacterium thermoautotrophicum, Desulfovibrio*

desulfuricans, and *Clostridium kluyveri* were used as method control organisms for the methanogenic, sulfate reducing, and heterotrophic media, respectively.

Microbial activity assays Microbial activity was assessed by monitoring the utilization of (1) H_2, (2) a variety of low molecular weight organic acids including lactate, formate and acetate, and (3) several methoxylated aromatic compounds such as the mono-, di- and tri-methoxylated derivatives of benzoate and cinnamate. H_2 consumption assays were initiated by adding 0.026% (~ 5 µmol) H_2 to 25 ml sealed serum bottles containing 10 g of freshly obtained sediment under a N_2 atmosphere. H_2 was periodically monitored with a reduction gas analyzer (26). Anaerobic biodegradation of the organic acids and the methoxylated aromatic compounds was evaluated with sediment slurries (20%; wt:vol) as previously described (18). For the latter substrates the inocula were slurried with a mineral medium (25) and the incubation was placed under H_2:CO_2 (80:20) (24). The depletion of the parent substrates was assessed by high pressure liquid chromatography (HPLC; 18, 25). Methane was monitored by flame ionization gas chromatography.

Sulfate reduction rates were determined using $^{35}SO_4^{2-}$ as a tracer in intact sediment samples taken from the center of cores. Subsamples were taken while the cores were inside an anaerobic glovebox. Approximately 1 g of sediment was dispensed into pre-weighed anaerobic culture tubes, closed with a septum, and removed from the glovebox. The headspace composition in the tubes was adjusted to N_2:CO_2 (80:20). Five µCi of carrier-free $Na^{35}SO_4$ (ICN; Irvine, Ca.) dissolved in 0.05 ml of anoxic distilled water was added to each incubation. Duplicate incubations were periodically sacrificed over the course of a month. Sulfate

reduction was stopped with 2 ml of an anoxic solution of zinc acetate (10%) and frozen prior to analysis. Sulfides were extracted by the procedures outlined in Chapter 1 and the radioactivity associated with the extracts was determined by scintillation counting.

Well installation Monitoring wells were installed in the three water-bearing sands encountered in these boreholes (Figure 2). Wells NP-1, NP-2, and NP-3 are screened from 11.3 to 12.8 m, 11.2 to 14.2 m, and 27.6 to 30.7 m, respectively. The wells were constructed using polyvinyl chloride casing and screen, Colorado silica sand filter pack, and granular bentonite annular seal.

Groundwater sampling and analyses Groundwater samples were collected from wells NP-2 and NP-3 using a submersible stainless steel pump. Well NP-1 was sampled with a bailer because of its limited water production. Water temperature, dissolved oxygen content, pH, and specific conductivity were monitored while pumping. Dissolved oxygen was measured by probe for concentrations in excess of 6 μM, and by a Hach spectrophotometer with AccuVac™ reagent ampoules (Indigo Carmine method; 16) for concentrations <6 μM. Field analyses and samples were performed and collected after at least two well volumes had been removed and after all above measurements were stable. H_2S was fixed in the field using Cline's reagent and analyzed in the laboratory with a DMS 100S UV visible spectrophotometer. Fe^{2+} was analyzed in the field using a Hach spectrophotometer and prepackaged reagents (16). Water samples were

collected for analysis of major ions and analyzed at the Soil, Water and Forage Testing Laboratory, Texas A&M University. For cation analysis, samples were acidified to pH 2 with 10% HCl and measured by inductively-coupled-plasma spectrometry. Sulfate was measured by the turbidimetric method and alkalinity was measured by titration (1). For isotopic analyses of dissolved inorganic carbon (DIC), five ml of groundwater was reacted with one ml phosphoric acid in an evacuated vessel; the CO_2 produced was measured manometrically and analyzed for $\delta^{13}C$ on an IRMS. Groundwater sulfate was precipitated as $BaSO_4$ (1) and analyzed for $\delta^{34}S$ at Coastal Science Laboratories, Inc. Samples collected for tritium and ^{14}C were sent to the University of Waterloo and Lawrence Livermore National Laboratory, respectively, for analysis.

Microbiological samples were collected in presterilized containers and stored on ice until transferred to the laboratory for processing. All analyses except for pH, conductivity, probe-determined dissolved oxygen, DIC, and microbiological analyses were performed on water filtered using an in-line capsule filter (0.45 μm).

Results

Sediment NP-1 sediments consisted of interbedded silts and clays with minor lignite, a 2 m sand layer at the surface, and a 2 m aquifer sand centered at 12 m depth (Figure 2). NP-2 and NP-3 cores contained mostly interlayered sand and silt, with little lignite or clay. Aquifer sands were found at 9 to 24 m (probably interlayered with silts) and at 27 m to >31 m. Borehole sediments exhibited

stratigraphic continuity on a scale of 10's of meters (NP-2 and NP-3), but not on a scale of 100s of meters (NP-1 and NP-2/NP-3). Abrupt color changes from orange to gray occurred at a depth of ~10 m in NP-1 and at 4 to 5 m depth in NP-2 and NP-3, which apparently mark the transition from oxidizing to reducing environment. The aquifer sands were fine- to medium-grained and quartzose. Percent TOC in NP-1 and NP-3 sediments ranged from 33 - 63% in lignites, 0.4 - 2.7% in silts and clays, and 0.01-0.3% in sandy sediments (Figure 3).

In NP-3, sulfate concentrations were ~0.6 μmol·gdw^{-1} in the soil zone, 1.9 to 7.9 μmol·gdw^{-1} from 3 to 10 m, and 0.7 to 1.1 μmol·gdw^{-1} from 17 to 31 m (Figure 3). The AVS and TRS concentrations ranged from 0.3 to 5.2 μmol·gdw^{-1} and from 0.6 to 1974 μmol·gdw^{-1}, respectively, with TRS being the dominant form of inorganic sulfur in the system (Figure 3). This TRS is dominantly pyrite based on petrographic observations and low to undetectable concentrations AVS and elemental sulfur (data not shown). The TRS maximum at 6.4 m coincides with the sulfate maximum and occurs just below the interval where the sediments become reducing. The $\delta^{34}S_{TRS}$ value for this interval was -7.3‰. The deepest intervals, 30.2 m and 30.6 m, have unusually high $\delta^{34}S_{TRS}$ values of +58‰ and +43‰ respectively.

NP-3 soil zone sediments yielded the highest direct microscope counts (9.6 x 10^6 cells gdw^{-1}; Figure 3), the greatest numbers of culturable organisms, and the highest activity measurements. Aerobic and anaerobic heterotrophs in the soil zone numbered 9.0 x 10^4 and 1.0 x 10^4 cells gdw^{-1}, respectively. Sulfate reducing bacteria numbered 1.0 x 10^5 cells gdw^{-1}. Methanogens and anaerobes capable of catalyzing O-demethylation of a variety of methoxylated aromatic compounds were

easily detected in the surface soil (Figure 3). Microbial activities were also high in these samples as evidenced by the fast rates of H_2 consumption and the rapid metabolism of lactate, formate, and to a lesser degree acetate. Similar to the NP-3 soil, the soil zone in the NP-1 core (0.6-1.2 m) also contained 10^5 SRB gdw^{-1}, but no methanogens were detected.

Yegua formation sediments generally exhibited lower microbial abundances and activities than the soil samples. In the NP-3 borehole, total direct counts were 0.6×10^6 to 6.9×10^6 cells gdw^{-1} while aerobic heterotrophs were 0.04×10^4 to 9.0×10^4 cells gdw^{-1} (Figure 3). These two measures of microbial biomass followed the same general trend with depth, but neither correlated with lithology nor with any other measured physical or geochemical parameter. Anaerobes were detected in many NP-3 intervals (Figure 3). Anaerobic heterotrophs ranged from undetectable to 1.0×10^6 cells gdw^{-1} and were more prevalent in the deeper intervals (23.7, 27.3, and 30.8 m). SRB were found in the same intervals as the anaerobic heterotrophs as well as in two additional intervals (8.5 and 17.1 m, Figure 3). In the NP-1 borehole, SRB were found at three depths (6.7, 11.5, and 14.6 m), but were not cultured in other strata. Within the Yegua formation, SRB and anaerobic heterotrophs both occurred in greatest abundance in the deep aquifer (27 - 31 m). Furthermore, sulfate reduction activity was only detected within these sediments (up to 2 nmol SO_4^{2-} reduced $gdw^{-1} \cdot d^{-1}$). In contrast to the surface soil, neither methanogens nor O-demethylating anaerobes could be cultivated from any of the formation sediment samples taken from NP-1 and NP-3 (Figure 3).

The metabolic activity profiles and the anaerobe enumeration data agree reasonably well for the NP-3 borehole (Figure 3). That is, strata that harbored

anaerobic heterotrophs or SRB are those where the metabolism of H_2, lactate, formate, or acetate was noted (Figure 3). Of the nine subsurface samples tested, only six, three, three, and one harbored microorganisms capable of metabolizing lactate, formate, H_2 and acetate, respectively. In general, sediments with higher numbers of the aforementioned anaerobic groups were associated with more rapid microbial activity or a more diverse array of substrates utilized (Figure 3).

While only a few strata contained organisms capable of consuming H_2, slurries of 30.5 m sediment produced H_2 in excess of that added to the incubation system (Figure 4). Autoclaved control slurries showed no ability to produce or consume H_2. Barring an abiotic explanation for the observation, such findings suggest that endogenous organic matter reserves in the samples were amenable to anaerobic decay and that H_2 was produced as a consequence of this metabolism. This anaerobic decay occurred despite the low organic carbon content of the sediment (0.32 and 0.04 wt %, respectively). After its production in the 30.5 m sample, H_2 was consumed relatively rapidly.

Groundwater Wells NP-2 and NP-3 are effectively a shallow (11 m) and deep (30 m) well cluster. NP-2 water levels average 85.4 meters mean sea level (mmsl). and NP-3 levels averaged 82.9 mmsl (Figure 3). NP-2 and NP-3 are confined and under artesian conditions. NP-1 has a water level of 79 mmsl and is unconfined (Figure 2).

Tritium contents for NP-1 and NP-2 waters are below the detection limit (<0.8 tritium units; TU), whereas that for NP-3 waters is at the detection limit, 0.8 TU (Table 1). These data indicate that the waters are at least 40 to 50 years old. Carbon-14 content for the three waters are 57, 53 (N = 2), and 35 percent modern carbon (N = 3), respectively (Table 1), yielding uncorrected ages of 4,720, 5,320 and 8,590 years. These ages are overestimates because carbonate mineral dissolution adds "dead" carbon to the groundwater. Using the ^{13}C mass balance model of Fontes (12) we obtain "corrected" ages for NP-1, NP-2 and NP-3 waters of 2,700, 4,300, and 3,700 years, respectively. Though closer to the true ages, these ages are also likely to be overestimates because the models can not correct for dead inorganic carbon added by respiration of sedimentary organic matter from the formation.

Using Darcy's law and measurements of water level and hydraulic conductivity (by slug test), lateral advective flow is estimated to be on the order of 10^{-5} cm/s. The lack of connectivity of the sands probably results in a composite advective flow rate that is lower than that calculated from measured hydraulic conductivities; thus groundwater flow rates are most probably less than the calculated 10^{-5} cm/s. Using advective flow rates of 10^{-5} to 10^{-8} cm/s and corrected ^{14}C groundwater ages, calculated travel distances from recharge area range from 10 m to 10 km.

Considering that the water table height and consequently shallow groundwater flow are controlled by local topography, the recharge zone for these waters is likely on the order of 2 km from the site, suggesting flow rates of 10^{-5} to 10^{-6} cm/s.

The three waters studied are geochemically distinct. There is a sharp contrast between NP-2 water with high total dissolved solids (TDS; 2960 mg/L) and underlying NP-3 water with the low TDS (455 mg/l; Table 1). NP-1 waters to the north are intermediate in TDS with 2000 mg/l. With regard to redox indicators, dissolved oxygen concentrations are relatively high for the unconfined NP-1 water (38 μM) and much lower for the other waters (Table 1). All of the waters have low nitrate concentrations (<4 μM). Sulfide content is significantly above the detection limit (5 μM) only in NP-1 (13 μM), whereas Fe (II) content is high in NP-2 water, intermediate in NP-1 water, and low in NP-3 water. The pH values are 6.2, 5.7, and 7.1 for NP-1, NP-2, and NP-3 waters, respectively.

Of note, NP-2 sulfate concentrations are exceptionally high (14 mM) compared with those of other groundwaters (Table 1). NP-2 waters also have high sulfate/chloride ratios (1.0) relative to NP-1 and NP-3 waters (0.10 and 0.12, respectively), suggesting addition of sulfate in NP-2. Sulfate $\delta^{34}S$ values show that sulfate in NP-2 waters (-4.5‰) is derived from a different source than that of NP-1 and NP-3 waters (7.3 and 8.0‰, respectively).

Not only are TDS and sulfate contents low for NP-3 water, but DIC content is also low (3.0 mM). More importantly, the $\delta^{13}C$ for this water (-11.3‰) is much higher than those for NP-1 and NP-2 waters (-18.8 and -19.5‰, respectively).

This reflects either differences in carbon sources or differences in the degree of atmospheric exchange in the soil zone (9).

Direct counts of microorganisms in NP-1 and NP-2 groundwaters were an order of magnitude higher than in NP-3 water (Table 1). Viable aerobic and anaerobic heterotrophs showed a similar trend between the wells, with similar values for NP-1 and NP-2 waters, and lower values for NP-3 waters. O-demethylating anaerobes were detected only in NP-3 waters and methanogens were not detected in any waters. Sulfur oxidizing bacteria were cultured from the shallower NP-1 and NP-2 groundwaters but not in deeper NP-3 groundwater. Iron oxidizing bacteria occurred only in NP-2 waters with an abundance of 1.0×10^{2} ml^{-1}. Sulfate reducing bacteria were 100 X more abundant in NP-3 waters than in NP-2 waters, not surprising considering the high SRB numbers in NP-3 aquifer sediments (Table 1; Figure 3).

	NP-1	NP-2	NP-3
Depth of screened interval (m)	13.1–16.4	11.2–14.3	27.4–30.8
Temperature (°C)	22.0	22.0	21.8
Specific conductance ($\mu S\ cm^{-1}$)[a]	2920	4110	630
Total dissolved solids ($mg\ l^{-1}$)	2000	2960	455
DO_2 (μM)	38	9	7
pH	6.21	5.71	7.10
DIC (mM)	7.78	4.42	3.02
H_2S (μM)	13	<0.24	<0.24
Cl^- (mM)	21.0	14.0	3.2
SO_4^{2-} (mM)	2.06	14.24	0.39
Mg^{2+} (mM)	1.1	3.1	0.1
Fe^{2+} (μM)	13.7	122	5.1
ΣMn (μM)	12.7	56.4	1.8
$\delta^{13}C_{DIC}$ (‰)	−18.8	−19.5	−11.3
$\delta^{34}S$ (‰)	7.3	−4.5	8.0
^3H (TU)	<0.8	<0.8	0.8
^{14}C % modern carbon	57.9	50.5	33.7
Direct counts (cells ml^{-1})	10^6	10^6	10^5
Aerobic heterotrophs (cells ml^{-1})	10^5	10^5	10^2
Anaerobic heterotrophs (cells ml^{-1})	10^2	10^2	10^1
Sulfur-oxidizing bacteria (cells ml^{-1})	10^3	10^4	ND
Iron-oxidizing bacteria (cells ml^{-1})	ND	10^2	ND
SRB (cells ml^{-1})	ND	10^2	10^4

[a] Abbreviations: $\mu S\ cm^{-1}$, microsiemens cm^{-1}; DO_2, dissolved oxygen; TU, tritium units; BDL, below detection level; ND, not determined.

Table 1 Hydrochemical properties and microbiology of NP-4 and NP-5 ground waters

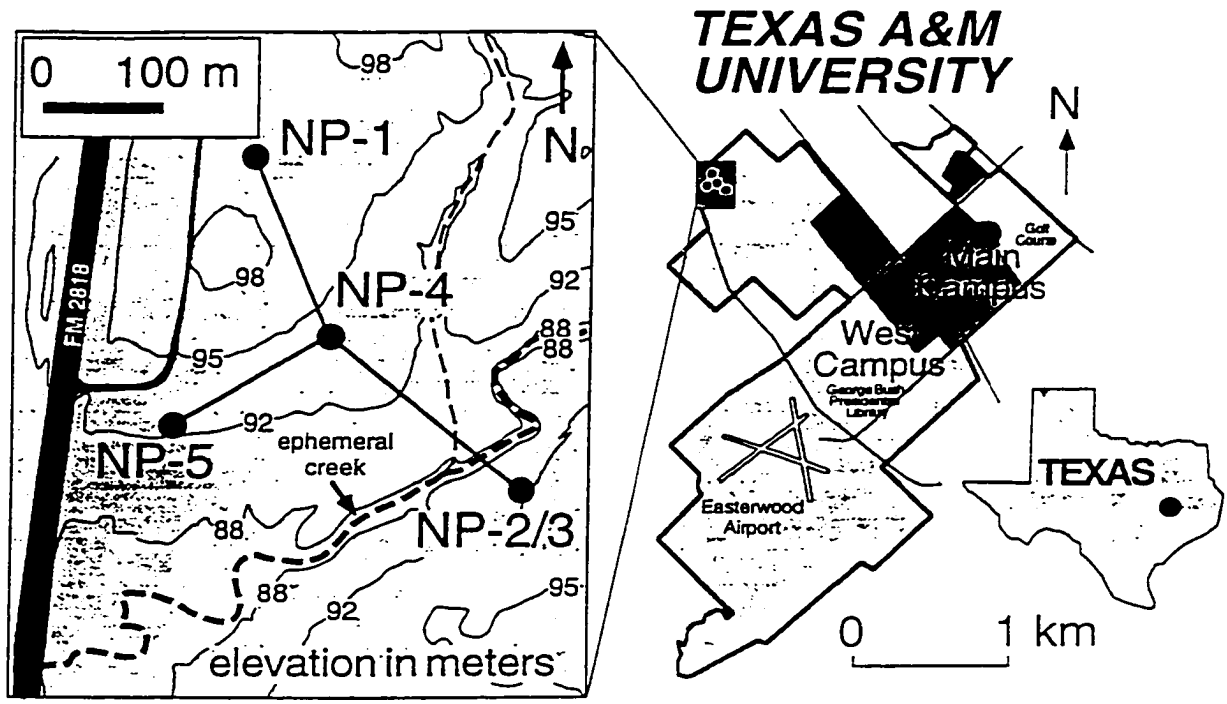

Figure 1. Map of the study site, its relation to the TAMU campus, and position of various wells (NP-1 through NP-5).

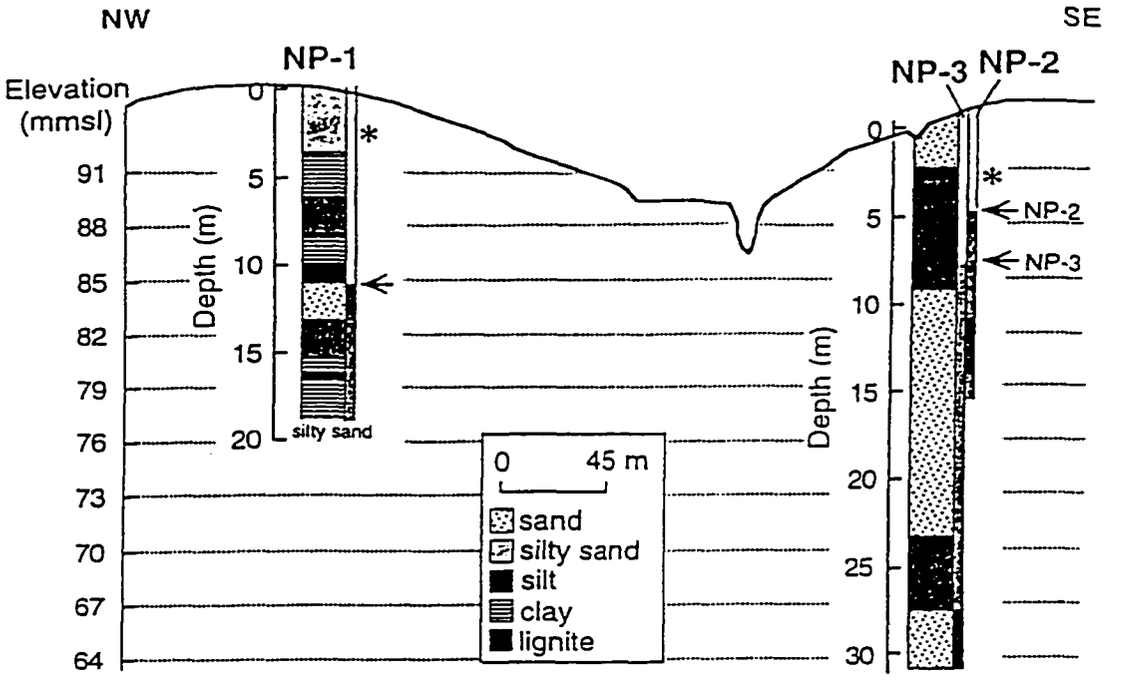

Figure 2. Lithologic cross-section of study area showing NP-1 and NP-3 boreholes, and water levels (←) of all three wells. Vertical exaggeration = 10X; * = transition from oxidizing to reducing sediments.

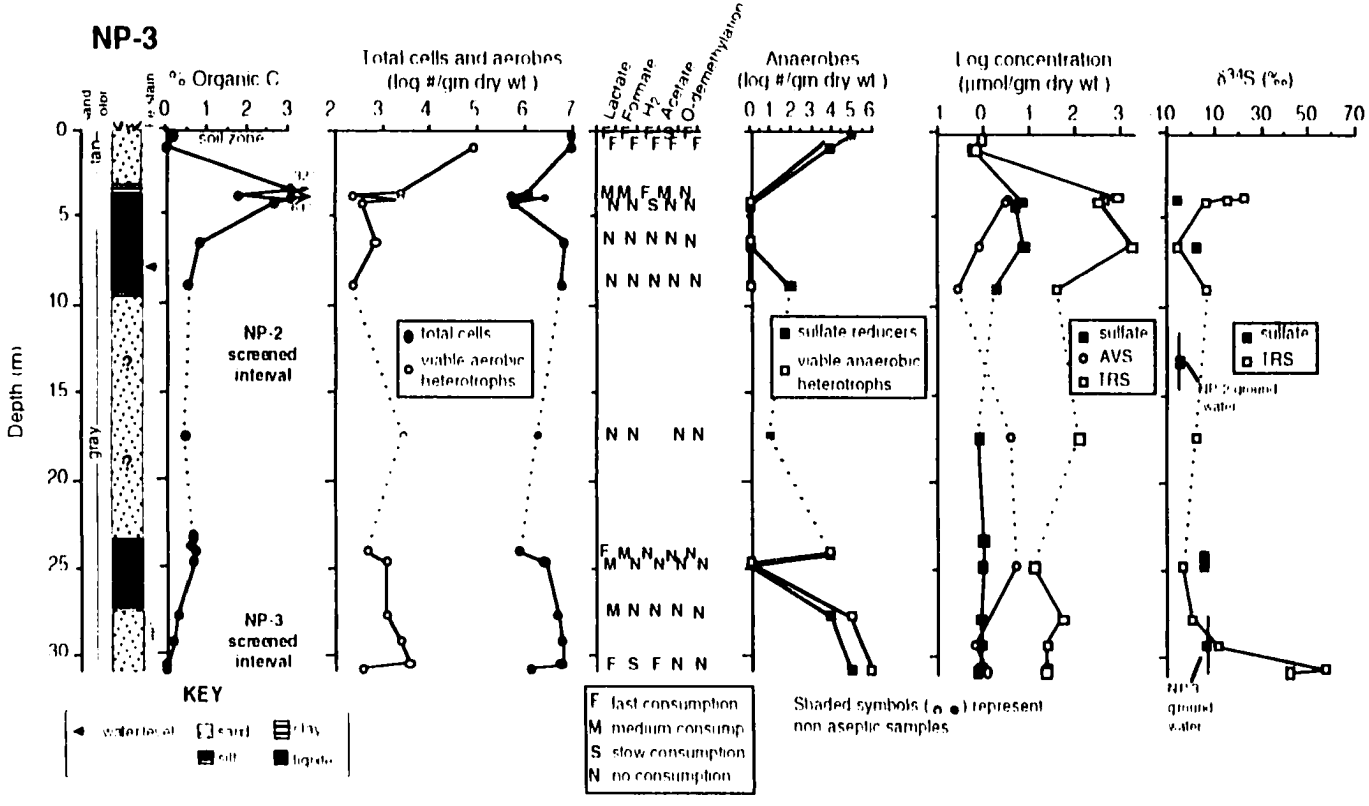

Figure 3. Geochemical and microbiological data from NP-3 core (Yegua formation and soil zone) in east-central Texas. AVS = acid volatile sulfur, TRS = total reduced sulfur (pyrite), gdw = gram dry weight sediment. Light stippled area at top is soil zone; other stippled areas are water-bearing sands. Note that the greatest activity and highest numbers of anaerobes occur where % organic C is lowest.

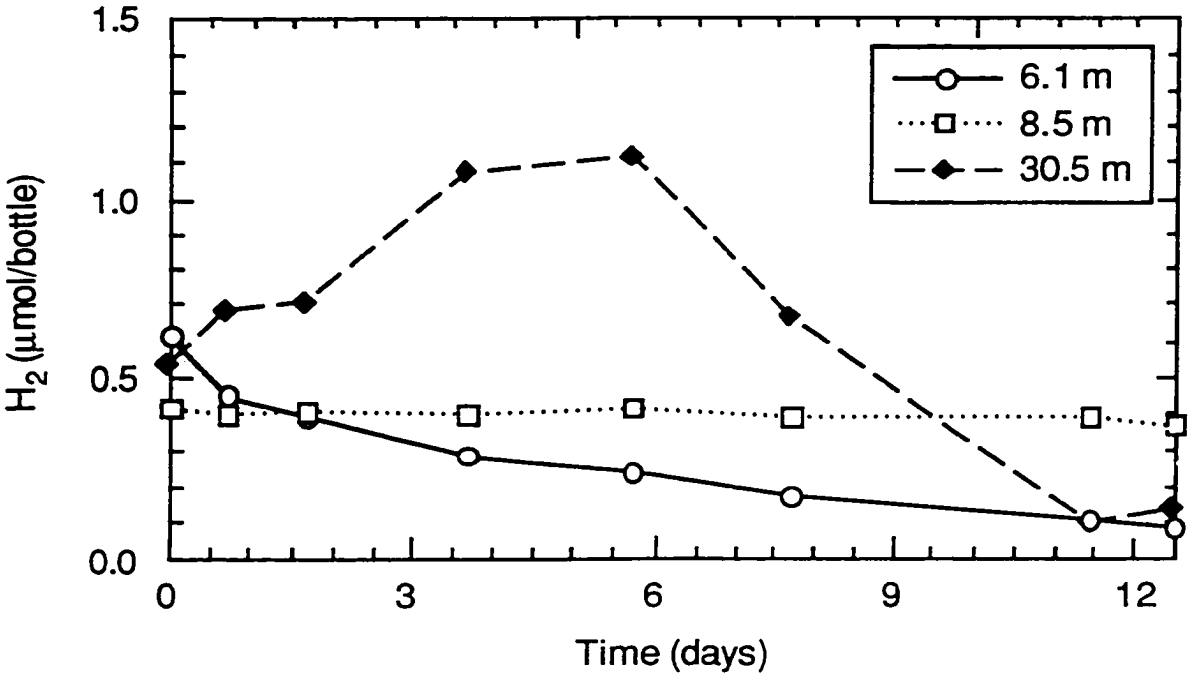

Figure 4. Anaerobic hydrogen metabolism in NP-3 sediments. Note hydrogen production in the 30.5 m sample.

Discussion

Influence of Sediment Properties on the Distribution of Microorganisms The low hydraulic conductivity of the recharge zone sediments in the study area creates an aquifer system that is anoxic over much of its flow path, resulting in sharp geochemical gradients. We observed the presence of large and metabolically diverse microbial communities in aquifer and confining bed sediments similar to those found in studies of the Atlantic Coastal Plain aquifers (2, 13, 30). Unlike previous studies that reported fewer viable organisms in confining clays, we observed that the numbers of total cells and culturable aerobic heterotrophs were evenly distributed with depth in the formation (i.e., below the soil zone), regardless of sediment grain size. Not surprisingly, the soil zone showed the highest activity and largest number of aerobic and anaerobic microorganisms.

In contrast to the aerobic microorganisms, the numbers and activity of the anaerobic microorganisms exhibited much higher heterogeneity. The interval associated with the NP-3 aquifer had the highest numbers of SRB. The presence of SRB in the Atlantic Coastal Plain sediments is well documented (18), but throughout the formations aerobes were more abundant than anaerobes by two to five orders of magnitude (18). In the Yegua sediments associated with the NP-3 aquifer (28 - 31 m), anaerobic heterotrophs and SRB were two to four orders of magnitude more abundant than aerobes. Sulfate reduction activities in the Yegua

sediments are highest in the NP-3 aquifer. H_2 as well as numerous organic acids stimulated sulfate reduction in sediment slurries and enrichment cultures obtained from this aquifer. Increased rates of H_2 consumption were found in the NP-3 aquifer sediments and water, consistent with MPN counts and other activity measurements. Clearly, the largest population of active anaerobes exists within the NP-3 aquifer. The diversity of this interval is further demonstrated by the production of H_2 in aquifer sediments (Figure 4). This hydrogen likely resulted from the fermentation of organic matter contained in the aquifer. The lack of H_2 consumption under anoxic conditions in some incubations is consistent with other assays indicating that some shallow intervals did not harbor a significant population of active anaerobic microorganisms (Figure 3).

TOC was determined for each sediment interval to assess whether this property influenced microbial activity within the geologic strata. Aerobic and anaerobic heterotroph counts did not correlate with TOC (Figure 3). Furthermore, there was a statistically significant *negative* correlation between log SRB and TOC ($r = 0.64$, $p = 0.05$), suggesting that organic carbon does not limit SRB abundance. The high TOC lignite layers showed little or no microbial activity (sulfate reduction, hydrogen oxidation, organic acid oxidation) (Figure 3). Similar observations were made by Murphy et al. (27) and attributed to the recalcitrant nature of lignite.

Impact of Sulfide Oxidation on Sulfate Reduction The NP-2 aquifer possesses high numbers of sulfur oxidizing bacteria *and* SRB. The co-occurrence of aerobic sulfur oxidizers and anaerobic SRB in the same groundwaters seems contradictory. However, Fredrickson et al. (13) noted that SRB and sulfur

oxidizing bacteria were present in the same cores from the Middendorf Aquifer of the Atlantic coastal plain though no correlation between the populations was found (18). The presence of both types of bacteria may indicate the existence of anaerobic microsites in an otherwise aerobic aquifer. Murphy et al. (27) interpreted this as a syntrophic relationship where sulfur oxidizing bacteria aerobically produce sulfate which is used by SRB within anaerobic microsites. The idea of a syntrophic relationship occurring in the NP-2 aquifer leads us to consider whether sulfur cycling in shallow portions of the formation provides electron acceptors for sulfate reduction in the deeper portions.

The unsaturated sediments above the NP-2 aquifer are rich in sulfate and ferric iron and could supply sulfate to NP-2 and NP-3 waters through diffusive and advective transport. Hydrochemical data indicate that the sulfate in NP-2 waters is derived from sulfide oxidation in the unsaturated zone. NP-2 waters have low pH, high sulfate and iron contents, and high sulfate/chloride ratios. Furthermore, $\delta^{34}S_{SO4}$ values for NP-2 waters (-4.5‰) are similar to those of TRS (pyrite) in the TRS-rich layer at 6.4 m (-7.3‰) (Figure 3), and different from the $\delta^{34}S_{SO4}$ of NP-1 and NP-3 waters or even typical gypsum and anhydrite (10 to 35‰; 28). Mass balance calculations show that NP-2's $\delta^{34}S_{SO4}$ value can be reproduced (within 0.6‰) by adding to recharge water, represented by NP-1 water, 12.1 mmol/L of sulfate from sulfide oxidation in the 6.4-m layer. The presence of sulfur oxidizing microorganisms in NP-2 waters and unsaturated zone sediments is additional evidence for sulfide oxidation and possible microbial participation.

Having gained sulfate from sulfide oxidation in the unsaturated zone, NP-2 water likely acquired additional sulfate along its flow path from overlying confining beds that also contain sulfide oxidation products. Water levels at well cluster NP-2/NP-3 indicate vertical water movement in the downward direction. Darcy's law and hydraulic conductivity estimates based on aquitard lithology (silts and clays) suggest velocities on the order of 10^{-8} cm/s. The high vertical gradients in sulfate concentration would enhance the effect of vertical transport (Figure 3). The time (t) required to transport sulfate into the aquifers from the overlying aquitard by vertical advection can be calculated by starting with the advective mass transport equation:

$$J = V \cdot C_{aqt} \cdot n \qquad (1)$$

where J = flux, C_{aqt} = sulfate concentration in aquitard, V = advective velocity, and n = effective porosity. Assuming no initial sulfate present, the sulfate concentration in the aquifer (C_{aqf}) can be calculated from flux, aquifer thickness (z), and time:

$$C_{aqf} = \frac{J}{z \cdot n} \cdot t \qquad (2)$$

Substituting equation 1 into equation 2 and solving for t, we get:

$$t = \frac{C_{aqf} \cdot z}{V \cdot C_{aqt}} \qquad (3)$$

The calculation assumes that all sulfate is derived from vertical transport, and that the effective porosity in the aquifer and aquitard are the same. Using sulfate concentrations from sediment slurries, the respective equations for NP-2 and NP-3 are:

$t = (2.88\ \mu mol \cdot gdw^{-1} \times 400\ cm)/(1 \times 10^{-8}\ cm/s \times 7.9\ \mu mol \cdot gdw^{-1}) = 462\ yr$

and $t = (0.73\ \mu mol \cdot gdw^{-1} \times 300\ cm)/(1 \times 10^{-8}\ cm/s \times 0.9\ \mu mol \cdot gdw^{-1}) = 772\ yr$

The 772-yr time for NP-3 represents a minimum because only the upper 300 cm of that aquifer were drilled; thus the full aquifer thickness is unknown. These calculated times are reasonable considering the estimated groundwater age (100s to a few thousand years) and suggest that vertical transport of shallow sulfide oxidation products can be an important source of sulfate to deeper aquifers. In an analogous study, Chapelle and McMahon (8) presented evidence of vertical *diffusive* transport of sulfate from confining beds to explain observed CO_2 production rates by sulfate reduction in the Black Creek aquifer.

Impact of Sulfate Reduction on Aquifer Geochemistry As previously mentioned, SRB numbers are highest in the deep NP-3 aquifer. Does sulfate reduction activity in this aquifer significantly impact the chemistry of aquifer water and sediment? The low sulfate content and relatively high $\delta^{34}S_{SO4}$ values of NP-3 waters could indicate (1) consumption of sulfate by sulfate reduction, and/or (2) comparatively minor influence by sulfide oxidation during recharge and along the flow path. NP-3 waters show none of the characteristics of sulfide oxidation, such as low pH, high sulfate and Fe contents, and high TDS (Table 1). The 8‰ $\delta^{34}S_{SO4}$ value provides additional evidence that the low sulfate content of NP-3 waters is not due to sulfate reduction. Sulfate reduction will cause ^{34}S enrichment of unreduced sulfate pool. Because the fractionation associated with sulfate reduction in nature is high (typically -25‰ to -50‰; 19), even minor sulfate reduction can have major impacts on groundwater sulfate $\delta^{34}S$. For example, if NP-3 water initially had the sulfate content and $\delta^{34}S_{SO4}$ of NP-1 water (2.1 mM and 7.3‰, respectively), and

sulfate reduction lowered the sulfate content to its current value of 0.4 mM, the accompanying isotope fractionation (e.g., -25‰) would increase $\delta^{34}S_{SO4}$ from 7.3‰ to 50‰! The $\delta^{34}S_{SO4}$ of NP-3 waters can be explained by minor oxidation of ^{34}S-rich sedimentary sulfides (e.g., at 4 and 9 m; Figure 3). Thus, the $\delta^{34}S$ of NP-3 sulfate supports the conclusion that sulfate reduction does not significantly alter the sulfate pool in the aquifer.

Although the isotopic effects of progressive sulfate reduction are not obvious in NP-3 groundwater sulfate, they are seen in the high $\delta^{34}S_{TRS}$ values of NP-3 aquifer sediments. The TRS at 30 - 31 m has values of 42‰ and 58‰, compared with 8‰ for the dissolved sulfate. Though these aquifer sediments have high SRB numbers, the TRS undoubtedly did not form in the present aquifer system. Instead, the sulfide likely formed by sulfate reduction within a closed, early diagenetic, presumably marginal marine system. Even though sulfate reducing bacteria are active in the NP-3 aquifer, they appear to have had little impact on the sediment or groundwater chemistry. Conversely, compared with microbiological assays, aquifer sediment and groundwater chemistry are not sensitive indicators of sulfate reducing activity and SRB distribution.

Summary In contrast to many studies of higher conductivity sediments, we found (1) no lithologic influence on the distribution of total cells and aerobic heterotrophs, and (2) anaerobe abundances that are two to four orders of magnitude greater than aerobe abundances. Furthermore, we found that lithology influences

anaerobic heterotroph and SRB abundances, with higher numbers in the aquifer sands. Sulfate reduction activity is also higher in the aquifer sands. Microbial activity is apparently not limited by the quantity of sedimentary organic matter, but more likely by its quality.

Shallow (NP-2) waters show geochemical evidence for sulfide oxidation including high sulfate and Fe^{2+} contents, low pH, and low sulfate $\delta^{34}S$. Sulfur oxidizers are present in NP-2 groundwater and appear to play a role in sulfide oxidation. This sulfide oxidation supplies sulfate to deeper sediments through lateral and vertical transport.

Although the deeper aquifer (NP-3) provides a favorable environment for sulfate reduction, with high SRB numbers and activity, the impact of this modern sulfate reduction on either the aquifer's groundwater and sediment geochemistry is negligible.

References

1. **American Public Health Association** (1985) Standard Methods for the Examination of Water and Wastewater. American Public Health Assoc., Washington DC.
2. **Balkwill DL** (1989) Numbers, diversity, and morphological characteristics of aerobic, chemoheterotrophic bacteria in deep subsurface sediments from a site in South Carolina. Geomicrobiol. J. **7:**33-52.
3. **Berg RR** (1970) Outcrops of the Claiborne group in the Brazos Valley southeast Texas. In Geological Society of America: Fourth annual meeting of the South-Central Section, pp. 79.
4. **Boutton T** (1991) Stable carbon isotope ratios of natural materials: 1. Sample preparation and mass spectrometric analysis. In D. C. Coleman, B. Fry (eds.). Carbon isotope techniques, Academic Press, Inc., San Diego. pp. 155-171.
5. **Brubaker GA** (1995) The boom in *in situ* bioremediation. Civil Engineering **10:**38-41.
6 **Chambers LA, Trudinger PA** (1978) Microbial fractionation of stable sulfur isotopes: A review and critique. Geomicrobiol. J. **1:**249-293.
7. **Chapelle FH, Lovley DR** (1990) Rates of microbial metabolism in deep coastal plain aquifers. Appl. Environ. Micro. **56:**1865-1874.
8. **Chapelle FH, McMahon PB** (1991) Geochemistry of dissolved inorganic carbon in a Coastal Plain aquifer. J. Hydrol. **127:**85-108.
9. **Dörr H., Sonntag C., and Regenberg W.** (1987) Field study of the initial C-14 content as a limiting factor in C-14 groundwater dating: In Isotope

Techniques in Water Resource Development, Proceedings of an International Symposium on the use of Isotope Techniques in Water Resources Development. International Atomic Energy Agency, Vienna, 30 March-3 April.

10. **Ehrenreich A, Widdel F** (1994) Anaerobic oxidation of ferrous iron by purple bacteria, a new type of phototrophic metabolism. Appl. Environ. Micro. **60:**4517-4526.
11. **Follett CR** (1974) Ground-water resources of Brazos and Burleson counties: Texas Water Development Board Report 185, Texas Water Development Board. Austin, TX. 193 p.
12. **Fontes JC** (1992) Chapter 17. Chemical and isotopic constraints on ^{14}C dating of groundwater. *In* Radiocarbon after four decades, an interdisciplinary perspective. Taylor RE, Long A, Kraceds RS (eds), Springer-Verlag, New York. p. 242-261.
13. **Fredrickson JK, Garland TR, Hicks RJ, Thomas, JM, Li, SW, McFadden KM,** (1989) Lithotrophic and heterotrophic bacteria in deep subsurface sediments and their relation to sediment properties. Geomicrobiol. J. **7:**53-66.
14. **Ghiorse WC, Balkwill DL** (1983) Enumeration and morphological characterization of bacteria indigenous to subsurface environments. Devel. Indust. Micro. **24:**213-224.
15. **Ghiorse WC, Wobber FJ** (1989) Introductory Comments. Geomicrobiol. J. **7:**1-3.
16. **Hach Company** (1993) Instruction manual for DR/2000 spectrophotometer. Hach Corporation.

17. Hicks RJ, Fredrickson JK (1989) Aerobic metabolic potential of microbial populations indigenous to deep subsurface environments. Geomicrobiol. J. **7**:67-78.

18. **Jones RE, Beeman RE, Suflita JM** (1989) Anaerobic metabolic processes in the deep terrestrial subsurface. Geomicrobiol. J. **7**:117-130.

19. **Kaplan, IR** (1983) Stable isotopes of sulfur, nitrogen and deuterium in recent marine environments. In Arthur et al., Stable Isotopes in Sedimentary Geology, SEPM Short Course No. 10, p. 2-1 to 2-108.

20. **Kieft TL, Amy PS, Brockman FJ, Fredrickson JK, Bjornstad BN, Rosacker LL** (1993) Microbial abundance and activities in relation to water potential in the vadose zones of arid and semiarid sites. Microb. Ecol. **26**:59-78.

21. **Kieft TL, Fredrickson JK, McKinley JP, Bjornstad BN, Rawson SA, Phelps TJ, Brockman FJ, Pfiffner SM** (1995) Microbiological comparisons within and across contiguous lacustrine, paleosol, and fluvial subsurface sediments. Appl. Environ. Micro. **61**:749-757.

22. **Kuenen JG, Robertson LA, Tuovinen OH** (1992) The Genera *Thiobacillus, Thiomicrospira, and Thiosphaera.* In Balows A, Truper HG, Dworkin M, Harder W, Schleifer S (eds). The Prokaryotes, A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications . pp. 2638-2657.

23. **Lovley DR** (1991) Dissimilatory Fe(III) and Mn (IV) reduction. Microbiol. Rev. **55**:259-287.

24. **Liu S, Suflita JM** (1993) H_2-CO_2-dependent anaerobic O-demethylation activity in subsurface sediments and by an isolated bacterium. Appl. Environ. Microbiol. **59**:1325-1331.

25. **Liu S, Suflita JM** (1994) Anaerobic biodegradation of methyl esters by *Acetobacterium woodii* and *Eubacterium limosum*. J. Indust. Microbiol. **13:**321-327.

26. **Mormile MR, Gurijala KR, Robinson JA, McInerney MJ, Suflita JM** (1996) The importance of hydrogen in landfill fermentations. Appl. Environ. Micro. **62:**1583-1588.

27. **Murphy EM, Schramke JA, Fredrickson JK, Bledsoe HW, Francis AJ, Sklarew DS, Linehan JC** (1992) The influence of microbial activity and sedimentary organic carbon on the isotope geochemistry of the Middendorf Aquifer. Water Resources Research **28:**723-740.

28. **Nielsen H** (1979) Sulfur isotopes. in Jager E, Hunziker J (eds). Lectures in isotope geology, pp. 283-312. Springer, 329.

29. **Phelps TJ, Fliermans CB, Garland TR, Phiffner SM, White DC** (1989) Methods for recovery of deep terrestrial subsurface sediments for microbiological studies. J. Microbiol. Meth. **9:**267-280.

30. **Phelps TJ, Raione EG, White DC, Fliermans, CB** (1989) Microbial activities in deep subsurface sediments. Geomicrobiol. J. **7:**79-91.

31. **Ryder PD, Ardis AF** (1991) Hydrology of the Texas Gulf Coast Aquifer Systems: US Geological Survey Open-File Report 91-64.

32. **Sinclair JL, Ghiorse WC** (1989) Distribution of aerobic bacteria, protozoa, algae, and fungi in deep subsurface sediments. Geomicrobiol. J. **7:**15-31.

33. **Tanner RS** (1989) Monitoring sulfate reducing bacteria: comparison of enumeration media. J. Microbiol. Meth. **10:**83-90.

34. **Ulrich GA, Krumholz LR, Suflita JM** (1997) A rapid and simple method for estimating sulfate reduction activity and quantifying inorganic sulfides. Appl. Environ. Microbial. **63:**1627-1630.

35. **Williamson AK, Grubb HF, Weiss JS** (1990) Ground-Water flow in the Gulf Coast aquifer systems, south central United States -- A preliminary analysis: US Geological Survey Water-Resources Investigations Report 89-4071.

Chapter 4

S-CYCLING IN THE TERRESTRIAL SUBSURFACE: COMMENSAL INTERACTIONS, SPATIAL SCALES, AND MICROBIAL HETEROGENEITY

Abstract

Microbiological, geochemical, and isotopic analyses of sediment and water samples from the unconsolidated Yegua formation in east-central Texas were used to assess microbial processes in the terrestrial subsurface. Previous geochemical studies suggested that sulfide oxidation at shallow depths may provide sulfate for sulfate-reducing bacteria (SRB) in deeper aquifer formations. The present study further examines this possibility and provides a more detailed evaluation of the relationship between microbial activity, lithology, and the geochemical environment on meter to millimeter scales. Sediment of varied lithology (sands, silts, clays, lignite) was collected from two boreholes to depths of 30 m. Our findings suggest that pyrite oxidation strongly influences the geochemical environment in the shallow sediments (~5 m) and produces acidic waters (pH 3.8) that are rich in sulfate (28 mM) and ferrous iron (0.3 mM). S- and Fe- oxidizing bacteria are readily detected in shallow sediments and likely play an indirect role in pyrite oxidation. In consistent fashion, there is a relative paucity of pyrite in shallow sediments and a low $^{34}S/^{32}S$-sulfate ratio (0.2‰), reflecting contributions from ^{34}S-depleted sulfides, in shallow regions. Pyrite oxidation likely provides a sulfate source for

both oxic and anoxic aquifers in the region. A variety of assays and direct imaging techniques of ^{35}S-sulfide production in sediment cores indicates that sulfate reduction occurs in both the oxidizing and reducing portions of the sediment profile with a high degree of spatial variability. Narrow zones of activity were detected in sands that are juxtaposed to clay or lignite-rich sediments. The fermentation of organic matter in the lignite-rich laminae provides small molecular weight organic acids to support sulfate reduction in neighboring sands. Consequently, sulfur cycling in shallow sediments and the transport of sulfate represents an important mechanism for commensal interaction among subsurface microorganisms by providing electron donors for chemoautotrophic bacteria and electron acceptors for SRB. The activity of SRB is linked to the provision of suitable electron donors from spatially distinct zones.

Introduction

Geochemical conditions prevalent in the terrestrial subsurface are often a function of the metabolic activity of the resident microflora. As microorganisms metabolize various electron donors at the expense of the available electron acceptors, redox zones form, usually along groundwater flowpaths (28,40). As microbial metabolism ensues, the changed geochemical conditions allow for the succession of different dominant microbial assemblages. Thus, the regulated interlinkage of oxidation-reduction reactions influences groundwater quality and the latter governs the distribution and activity of indigenous microorganisms. Microbial activity and geochemical conditions, in turn, are controlled by the physical and chemical properties of the sediments such as hydraulic conductivity and the abundance of soluble and reactive materials (6,30). Understanding these interactions is critical for predicting the transport and fate of materials, the cycling of nutrients and the harnessing of microbial activities for *in situ* biotechnological applications.

We combined microbiological and geochemical techniques to assess the interactions and distribution of microorganisms in unconsolidated sediments of the Yegua formation in east-central Texas. The Yegua consists of sands, silts, clays, and lignite of Eocene age (~45 Ma). The low hydraulic conductivity and moderate organic carbon content of the sediments result in anoxic conditions prevailing at relatively shallow depths. A previous study of the area showed a relationship between lithology and microbial abundance (29). The greatest number of anaerobic heterotrophs and sulfate reducing bacteria (SRB) were associated with aquifer

sands while total cells did not vary with lithology. Additional factors which control microbial activity and heterogeneity in the subsurface were evaluated. Our findings show enhanced microbial activities at sand-silt/clay lithologic boundaries, presumably due to the diffusion of electron donors from organic-rich fine grained sediments. Furthermore, sulfate generated by sulfide oxidation in shallow regions is not only transported to deeper regions where it can be reduced, but also is reduced in shallow sediments. This cycling of sulfur contributes to the diversity and overall activity of the subsurface microbial communities in the Yegua formation.

Materials and Methods

Sediment and Ground-Water Sampling. Four boreholes (NP-1,3,4,5) were drilled in the Yegua formation by hollow stem auger in an orthogonal pattern parallel and perpendicular to the dip direction of the beds (Figures 1 and 2). Well clusters were installed at three of the four sites to sample water-bearing sands at different depths. Our previous study focused on boreholes NP-1 and NP-3 (29). This study represents a detailed analysis of boreholes NP-4 (27.8 m) and NP-5 (30.3 m). Drilling was done as previously described (29) and we used carboxylated fluorescent microspheres to assess microbial contamination of the cores (24). Within minutes of collection, the outer portions of cores were pared away using sterile knives to remove potential microbial contaminants. For microbiological assays, cores were sectioned and stored in sterile plastic bags. Core sections for anaerobic assays were also placed in metal ammunition boxes which

were purged and pressurized with N_2 through a rubber septum in the lid. All core sections were stored at 4^0C and pared again before analysis.

Geochemistry. Amorphous iron(III) oxyhydroxides were determined using a hydroxylamine-HCl extraction procedure (27) and analyzed by atomic absorption spectroscopy and by spectrophotometry. Sulfides were sequentially extracted from sediment samples using 1N HCl for acid volatile sulfides followed by 1M Cr(II) in 6N HCl for chromium reducible sulfides (CRS) using a passive extraction technique (45) which is a modification of a previous published diffusion-based method (20). The CRS fraction was presumed to be pyrite. For sulfur isotope analysis the sulfur from pyrite (CRS fraction trapped as ZnS) was reprecipitated as AgS and sulfate from sediments and groundwater was precipitated as $BaSO_4$. The solids were converted to SO_2 for $^{34}S/^{32}S$ determinations using standard techniques (5) at Coastal Science Laboratories, Inc. (Austin, TX). ^{14}C and ^{13}C contents of dissolved inorganic carbon were measured by accelerator mass spectrometer and isotope ratio mass spectrometer respectively. Anions in groundwater samples or duplicate water extracts of sediments (1 g/ml) were quantified by ion chromatography as previously described (29). The pH of the sediment extracts was determined using a standard electrode. Groundwaters were analyzed for reduced iron, dissolved oxygen, soluble sulfide, pH, and specific conductance on site (29).

Enumeration of Microorganisms. A three-tube most probable number assay was used to enumerate sulfur-oxidizing (12), iron-oxidizing (12, with medium in 41), and SRB (43). Inocula were prepared by blending 10 g of

sediment with 95 mL of 0.01M $Na_4P_2O_7$ for one minute. Iron oxidation was indicated by an increase in turbidity and the disappearance of Fe^{2+} as measured spectrophotometrically at 510 nm (7). Sulfur oxidation was evident by an increase in turbidity and a drop in pH. Sulfate reduction was indicated by the blackening of the medium. Enumeration assays were scored after a six-week incubation period.

Activity of Sulfur and Iron Oxidizing Microorganisms. The oxidation of S^0, thiosulfate, and pyrite was evaluated in sediment slurries (6.7 m, NP-4; 1:1 weight/volume groundwater), and in defined medium (42) inoculated with S- and Fe-oxidizing microorganisms obtained from the same sediments. The oxidation of the sulfur forms was evaluated by measuring a decrease in pH relative to autoclaved controls. Iron oxidation by the enrichments was evaluated by measuring the accumulation of hydroxylamine-reducible Fe(III) while cell growth was monitored using an acridine orange direct counting procedure (18).

Fermentation studies and organic acid extractions. To examine the fermentation of endogenous carbon in NP-4 sediments, lignite from 60 m and clay-rich sediments from 60.5 m were slurried (40% wt:vol.) with anoxic filter-sterilized groundwater (from well NP-4B) in sterile 25-mL serum bottles. The slurries were amended with 10 μCi $^{35}SO_4^=$ (carrier free) and bicarbonate (2 g/L) from anoxic filter-sterilized stock solutions. The headspace consisted of a N_2/CO_2 (80/20) atmosphere. Autoclaved incubations served as sterile controls. Organic acids were determined as previously described (46) at the beginning of the incubation period

and after 270 days. Radioactive sulfides were extracted at the end of the incubation to evaluate sulfate reduction activity as previously described (45).

Organic acids were extracted from the pared exterior portions of the sediment (as described above) and treated with 7.4 mM $HgCl_2$ (~ 20% wt/v) to inhibit microbial activity. The samples were sealed in Nalgene centrifuge tubes and stored at -20° C. Prior to analysis, sediment samples were thawed, dried in a N_2-filled-glove bag, and homogenized by crushing with mortar and pestle. Ten grams of homogenized sediment were slurried with 25 mL of deionized distilled water. The slurries were shaken at 200 rpm for 24 h prior to centrifugation at 12,000 x g for 10 min. The aqueous extracts were analyzed on a gas-chromatograph equipped with a flame ionization detector (Hewlett-Packard 6890) and a free-fatty acid-phase column [19] within 48 h of extraction.

Sulfate Reduction. Sulfate reduction was determined using $^{35}SO_4^=$ as a tracer in duplicate sediment samples taken from the center of the cores as previously described (29). Rates were calculated from the amount of sulfide produced after 30 days of incubation. The localization of sulfate reduction activity in sediment cores was determined by trapping radioactive sulfide on silver foils (26) juxtaposed to a flat surface carved into the cores using sterile razor knives. Depending on core size, 100-400 µL of sterile anoxic stock solution of $Na^{35}SO_4$ (10 µCi/0.1 mL anoxic H_2O) was added drop-wise to the flat side of the core with a micropipettor and the solution was quickly absorbed. To maximize contact, the silver foil was forced against the core surface with foam padding. The cores with associated foils were placed in sterile plastic bags and incubated at room temperature in the dark

under a N_2/CO_2 (80/20) atmosphere. By maintaining the relative orientation of the foils, core features associated with SRB activity could be visualized. After 13 d, the foils were recovered and washed with running water for at least 5 h to remove any unreacted or adherent $^{35}SO_4^=$ and the radioactive sulfide precipitated on the foils was visualized by autoradiography (Packard Instant Imager, Meriden, CT).

Results

Sediment description and site hydrogeology. Sediment characteristics greatly influence subsurface microbial environments. Sediments at the site consisted of unconsolidated, thinly-interbedded sand, silt, clay, and lignite (Fig. 2). Water-bearing units (aquifers) were 0.9 to 8.2 m thick and consisted of silty to clayey, very fine- to medium-grained sand. Fine-grained sediments were sandy silts and clays (usually <3 m thick) containing thin laminae and occasional leaf fossils and root casts. Both sands and fine-grained sediments contained lignitic lenses and laminae. Sediment texture and composition suggest a nearshore, low energy environment of deposition such as a delta plain, as evidenced by preserved laminae, abundant terrestrial organic matter (including lignite), and rapid lateral and vertical facies changes. Widespread iron sulfide minerals associated with these sediments suggest early sulfate reduction under brackish or saline conditions, probably during periods of marine flooding (38). A color transition from tan to gray in boreholes NP-4 and NP-5 indicates transitions from oxidizing to reduced conditions at 6.1 and 4.5 m, respectively. Furthermore, mottled iron staining is evident in the upper oxidized sediment zone.

Sand bodies formed in a fluvial or delta plain system occur as discontinuous, sinuous to linear bodies abruptly terminated against fine-grained deposits (3). This results in an indeterminate, tortuous, and consequently complex flow system like that in our study area (Fig. 2). Groundwater from the well clusters at NP-4 and NP-5 are confined and under artesian pressure. We have used various approaches to try to constrain the age of groundwater in the aquifers studied to support estimates of sulfate flux. Hydraulic conductivities measured in aquifer sands by slug tests are relatively low, 10^{-4} to 10^{-3} cm·s^{-1}. Using Darcy's law, these hydraulic conductivities, along with water level and porosity data, yield horizontal advection rates of 1 to 10 m·yr^{-1} to the southwest. Considering local topography and probable distances to recharge sites, these estimated velocities suggest water ages on the order of hundreds of years. Vertical flow velocities at the study area are estimated at 0.001 to 0.02 m·yr^{-1} (downward) based on measured water levels and aquitard hydraulic conductivities derived from lithology. This results in travel times between aquifers on the order of 600 to 10,000 yr. Consistent with the age calculations, tritium contents are all below the detection limit (<0.8 TU), indicating water ages of at least 40 to 50 years. Groundwater ^{14}C contents decrease downward from 52 and 42% modern carbon for shallow wells NP-4A and NP-5A, to 17 and 15% modern carbon for deeper wells NP-4C and NP-5C (Table 1). Modeled ^{14}C ages (9) increase with depth, ranging from 4000 yr for NP-5A to about 10,500 yr for NP-4C and NP-5C. This trend is consistent with the downward hydraulic gradient in the study area. However, the ^{14}C ages are an order of magnitude greater than those inferred from velocity calculations. The true age likely lies between the estimates based on horizontal flow rates (100s of years).

which cannot account for low hydraulic conductivies between sand units, and the ^{14}C ages (1000s of years), which cannot be adequately corrected for the "dead" inorganic carbon added by the microbial respiration of Eocene sedimentary organic matter.

Geochemical Indications of Microbial Activity Geochemical parameters in both sediments and groundwater indicate that sulfur and likely iron cycling are dominant processes occurring in Yegua sediments. Pyrite is the major form of reduced inorganic sulfur in these sediments and averages about 30 μmol $S \cdot g^{-1}$ (Fig. 3B). In contrast, acid volatile sulfides range from 0.05 to 0.4 μmol $S \cdot g^{-1}$ (data not shown). Pyrite concentrations are nearly three orders of magnitude lower in shallow sediments relative to deeper sediments and sharply increase in the saturated sand of the NP-4A aquifer. This pyrite, together with the more oxidizing appearance of the sediments, suggest that pyrite oxidation is responsible for the diminution of pyritic S in shallow regions. If true, the products of sulfide oxidation (including sulfate, ferrous iron, and acidity) should differentially impact shallow regions and adjacent formations. Consistent with this suggestion, sulfate is found in higher concentrations in shallow unsaturated sediments and underlying aquifer sands (6-7 m depth) relative to deeper sediments (Fig. 3B). Further, an inverse relationship between pH and sulfate concentration is evident throughout the borehole, with the lowest pH (3.8) associated with the highest $SO_4^=$ content (40 $\mu mol \cdot g^{-1}$)(Fig. 3A).

Geochemical profiles of groundwaters from the area are also consistent with pyrite oxidation as exemplified by the NP-4 wells (Table 1). The shallowest

groundwater (NP-4A) exhibits a relatively high dissolved oxygen content (112 μM), low pH (pH 3.7), and high sulfate and Fe^{2+} concentrations (28.1 and 0.6 mM, respectively). The acidic conditions likely prevent the rapid abiotic oxidation and precipitation of the reduced iron in the water. Deeper groundwaters (NP-4C, NP-5C) contain progressively less dissolved oxygen, higher pH values, and lower sulfate and Fe^{2+} concentrations (Table 1, Fig. 2). Further, the similar chloride concentrations in the three NP-4 waters indicate that the elevated sulfate concentration in the shallow groundwater is not due to increased influences of connate marine fluids. Dissolved sulfide, a product of sulfate reduction, was below the detection limit (<5 μM) in all study area wells except NP-1 (13 μM), which produces from an unconfined aquifer at ~15 m (Fig. 1; 29).

We determined the ^{34}S composition of the major forms of S in the region to further examine the source of sulfate and the prospects for the migration of $SO_4^=$ from the presumed pyrite oxidation zone. Typically, sulfide minerals from clastic sediments are depleted in ^{34}S with $\delta^{34}S$ values less than 15‰ (23,33). The $\delta^{34}S$ values of pyrite from NP-4 and NP-5 sediments range -4.1 to 35‰ and -4.3 to 32‰, respectively (Fig. 3D). The uncommonly high $\delta^{34}S$ values (>30‰) occur only at depth (>20 m) in relatively pyrite-poor sediments. The weighted average for $\delta^{34}S$ values of pyrite from Yegua sediments is more typical at 6‰ (also see 29). The $\delta^{34}S$ values of sulfate in groundwaters and sulfate-rich porewaters range from -1.1 to 10.2‰ (Fig. 3D). In general, sulfate $\delta^{34}S$ values increase with depth and correlate positively with pH and negatively with sulfate content. These isotopic

trends are consistent with oxidation of ^{34}S-depleted pyrite in shallow sediments. The increased $\delta^{34}S$ values for sulfate at depth, combined with the decrease in sulfate content, can be modeled as sulfate reduction, which results in preferential consumption of $^{32}SO_4^{=}$, along with dilution. However, because of the variability of the pyrite $\delta^{34}S$ values (Fig. 3D; 29), we cannot rule out the possibility that ^{34}S-enriched sulfate at depth is derived from ^{34}S-enriched pyrite which is oxidized either upgradient within the aquifer, or above the aquifer with downward transport of sulfate.

The geochemical profiles foreshadow the types of microorganisms to be expected in various regions of the subsurface. For instance, the evidence outlined above suggests that sulfur oxidizing microorganisms should inhabit shallow Yegua sediments. In contrast, the reducing nature of the lower sediments, the relatively depressed sulfate concentrations, and our previous findings (29) suggest that sulfate reduction might be more prevalent in deeper formations. Similarly, we found amorphous Fe(III) iron oxyhydroxides in shallow unsaturated sediments at concentrations ranging from 1 to 13 $\mu mol \cdot gdw^{-1}$ (Fig. 3C), but these iron oxyhydroxides were not detected below the wet sands (>7 m). This suggests that aerobic iron oxidizing bacteria proliferate in the upper regions of the sediment profile, consuming ferrous iron produced during pyrite oxidation.

Enumeration and Activity Profiles of Selected Subsurface Microorganisms In light of the geochemical evidence, we assayed for Fe- and S-oxidizing bacteria in the NP-4 sediment formations and associated groundwaters.

These organisms were easily cultured at relatively low numbers ($\sim 10^2$ cells g^{-1}) in the shallowest groundwater (Table 1), and in 6 of 7 sediment samples taken from the 0 - 13.5 m interval (Fig. 3E). In contrast, only 2 of 21 sediment samples taken from deeper strata, and none of the deeper groundwaters, harbored these organisms. Thus, the organisms were more evident in the presumed pyrite oxidation zone. Despite their relatively low numbers, the activity of Fe- and S-oxidizing bacteria in shallow aerobic sediment slurries was relatively easy to demonstrate. The oxidation of S^o and thiosulfate resulted in a pH drop within 10 days that continued for up to 60 d (Fig. 4A). In contrast, the addition of pyrite to the incubations did not result in a pH drop even after 120 d. Similarly, no change in pH occurred in sterile control incubations which received thiosulfate or S^o. S-oxidizing microorganisms enriched on thiosulfate were also unable to oxidize pyrite. However, these cells were able to grow with reduced iron serving as an electron donor as indicated by the accumulation of both cells and ferric iron (Fig. 4B).

SRB activity was suggested by the bulk geochemical parameters. Our assay showed that SRB activity was found in sandy sediment samples throughout the NP-4 depth profile (Fig. 3F), but not in the hard silty sediments at 10.3 m, 10.6 m, and 18.3 m or in the lignite sampled at 18.4 m. A more detailed analysis of sulfate reduction in a single sediment core indicated increased sulfate reduction immediately below a layer of lignite and clay-rich sediment which extended from 13.55 to 13.70 m (Fig. 3F, inset). Sulfate reduction was not detected in the nine sediment samples taken immediately above this region (from 12.95 to 13.47 m). While sulfate reduction was expected in the more reducing portions of the NP-4

borehole, activity was also detected in the shallow oxidized and acidic sediments. Despite the slightly elevated sulfate reduction in the shallow sediments, about an order of magnitude fewer SRB could be cultured from shallow relative to deeper groundwaters (Table 1), suggesting that cell abundance does not equate with activity.

We observed wide variations in SRB activity at several spatial scales. For instance, differences of several orders of magnitude in rates were found throughout the 28 m depth profile (Fig. 3F). The detailed profile from 12.95 to 13.70 m revealed the heterogeneity associated with this microbiological process on a decimeter spatial scale. Sulfate reduction rates reported in Figure 3 are the means of duplicate determinations, but a greater than usual variation between replicates was noted in many subsamples. These observations prompted us to examine SRB activity on an even finer scale using a silver foil autoradiography assay. This technique maps SRB activity in sectioned cores (26) (Fig. 5).

A photograph of a portion of the NP-5 core (25.8 m) is compared with the corresponding image of SRB activity (dark area) in Figure 5A,B. Note that a band of SRB activity is confined to a sandy region of the core immediately beneath a clay layer. Similarly, another NP-5 core section (18.6 m) shows a band of activity in a sandy area between a lignite lens at the bottom of the core and clayey sediments near the top (Fig. 5C,D). SRB activity was very heterogeneously distributed in all eight core sections tested by this procedure. In one NP-4 core section (13.3 m), no SRB activity could be detected with either the silver foil assay or sediment incubations containing $Na^{35}SO_4$ (Fig. 3F).

Based on the sulfate reduction assays, we hypothesized that one of the factors governing sulfate reduction in the subsurface is the delivery of electron donors from localized clay or lignitic lenses. We therefore chose core samples that were rich in either clay or lignite and extracted organic acids with water immediately after a mercuric chloride treatment to inhibit microbial activity. Acetate and propionate were detected in extracts of both a clay-rich sample (NP-4, 18.4 m; 0.23 and 0.02 $\mu mol \cdot g^{-1}$, respectively) and a lignite-rich sample (NP-4, 18.3 m; 0.69 and 0.04 $\mu mol \cdot g^{-1}$, respectively). Other fatty acids were below detection limits.

We also observed acetate and propionate accumulation in non-$HgCl_2$-treated sediment slurries made from the two core samples mentioned above. Organic acids were not produced in the corresponding autoclaved controls. Acetate and propionate accumulated over a 270 d incubation to 200 and 66 μM, respectively, in the clay slurry and to 198 and 111 μM in the lignite slurry. In the latter incubation, formate also accumulated to 171 μM. The detection and production of these organic acids in the aforementioned samples likely indicates that the fermentation of endogenous carbon reserves occurs in these sediments. However, the production of acetate, formate, and propionate was not coupled to either sulfate reduction or methanogenesis (data not shown). Consequently, we believe the organic acids diffuse to adjacent subsurface regions where they may serve as electron donors for sulfate reducing bacteria.

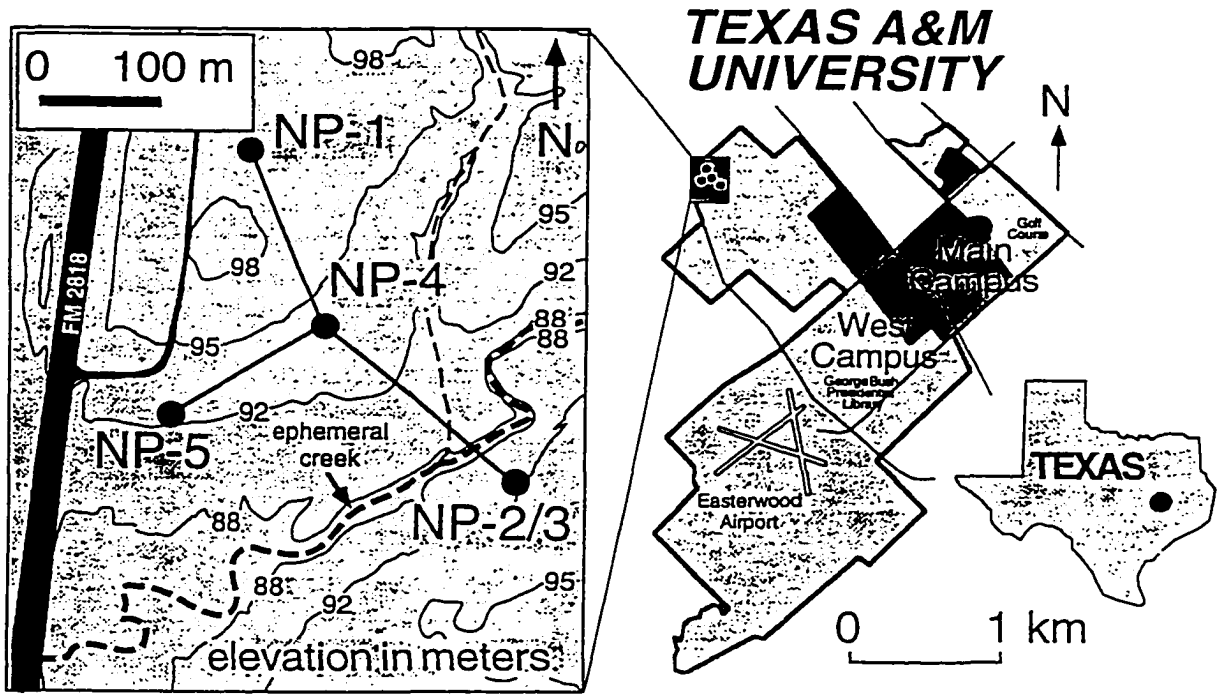

Figure 1. Map of the study site, its relation to the TAMU campus, and position of various wells (NP-1 through NP-5).

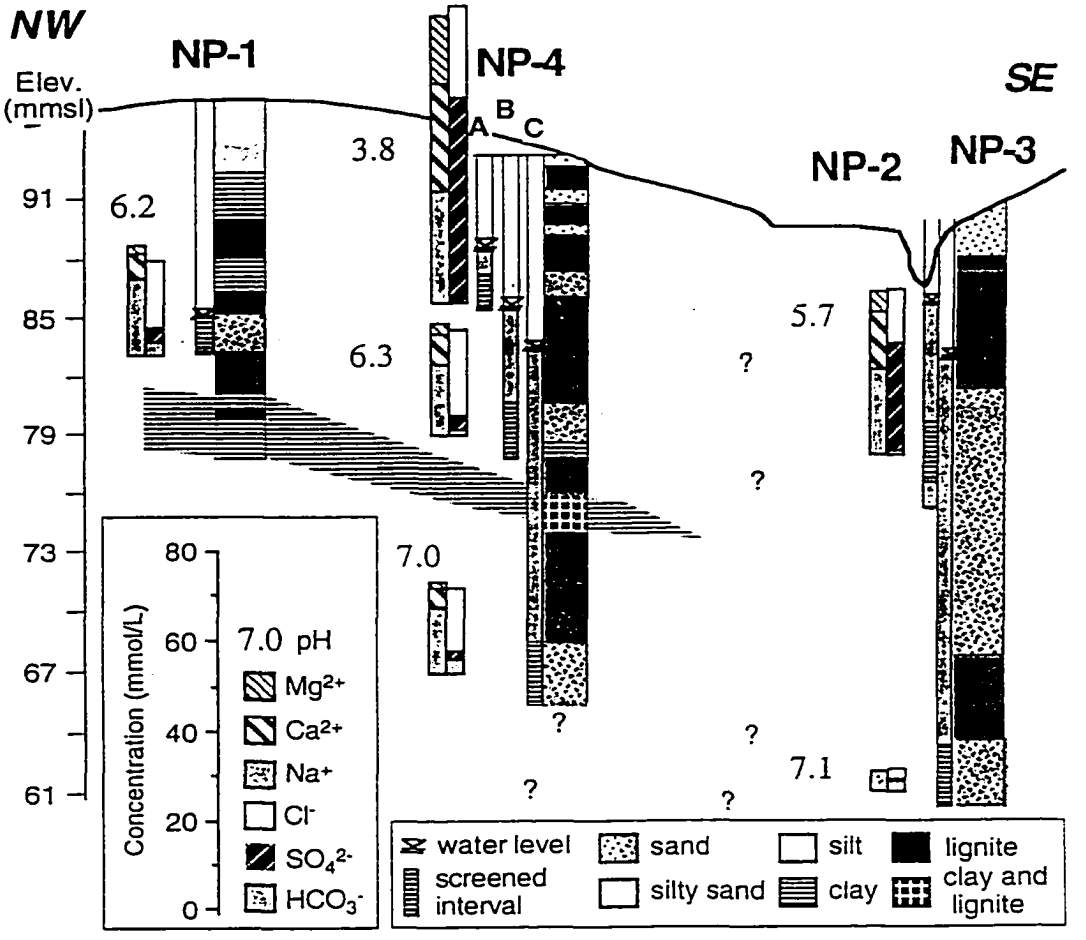

Figure 2. Cross-section of study area showing lithologies of boreholes NP-1, NP-4, and NP-3, water levels, and water chemistry. Note the poor connectivity of the sands as indicated by the lateral variation in water chemistry.

Table 1. Hydrochemical properties and microbiology of NP-4 and NP-5 waters.

	well designations[a]				
	NP-4A	NP-4B	NP-4C	NP5A	NP-5C
Well Depth (m)	7	13	26.5	10	26.5
Diss. O_2 (μM)	112	35	11	54	14
pH	3.7	6.4	7.0	6.4	7.2
$SO_4^=$ (mM)	28.1	2.3	1.3	4.7	1.1
Fe^{+2} (mM)	0.6	0.1	0.01	0.03	0.00
NO_3^- (μM)	4.4	< 1.3	< 1.3	< 1.3	< 1.3
Cl^- (mM)	24.2	23.6	16.7	23.8	10.3
$\delta^{34}S$ (‰)	0.2	3.8	10.2	8.4	8.3
$\delta^{13}C$	−23.4	−17.5	−16.5	−16.7	−15.5
^{14}C (%modern C)	52.3	33.6	16.5	42.3	14.7
^{14}C age, corr. (yr)	5,400	6,300	10,300	4,000	10,700
Sulfate reducing bacteria · ml^{-1}	460	4300	2400	150000	240
S-oxidizing bacteria · ml^{-1}	460	ND[b]	ND	ND	ND

[a] See Fig. 1 for well locations
[b] ND—not detected

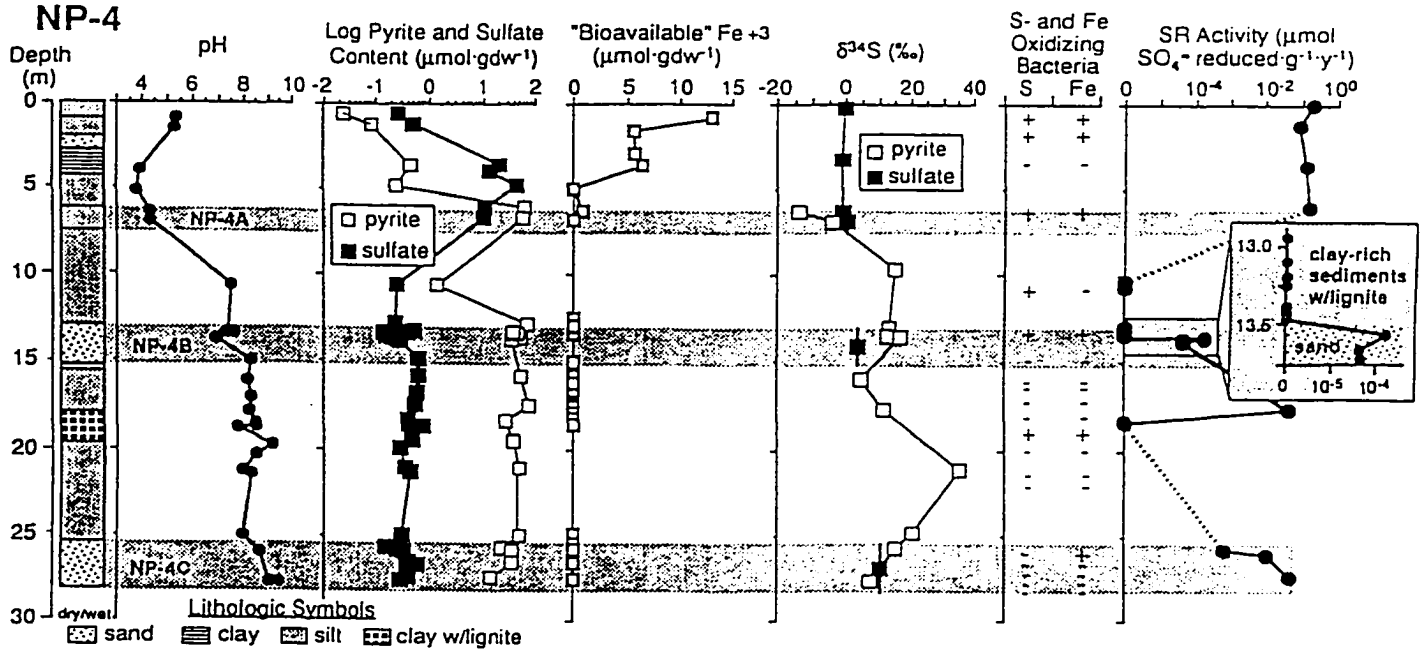

Figure 3. Sedimentology, sediment geochemistry (A,B,C,D) and microbiology (E,F) depth profiles of the NP-4 borehole. Stippled bands show screened intervals of wells. SR = sulfate reduction.

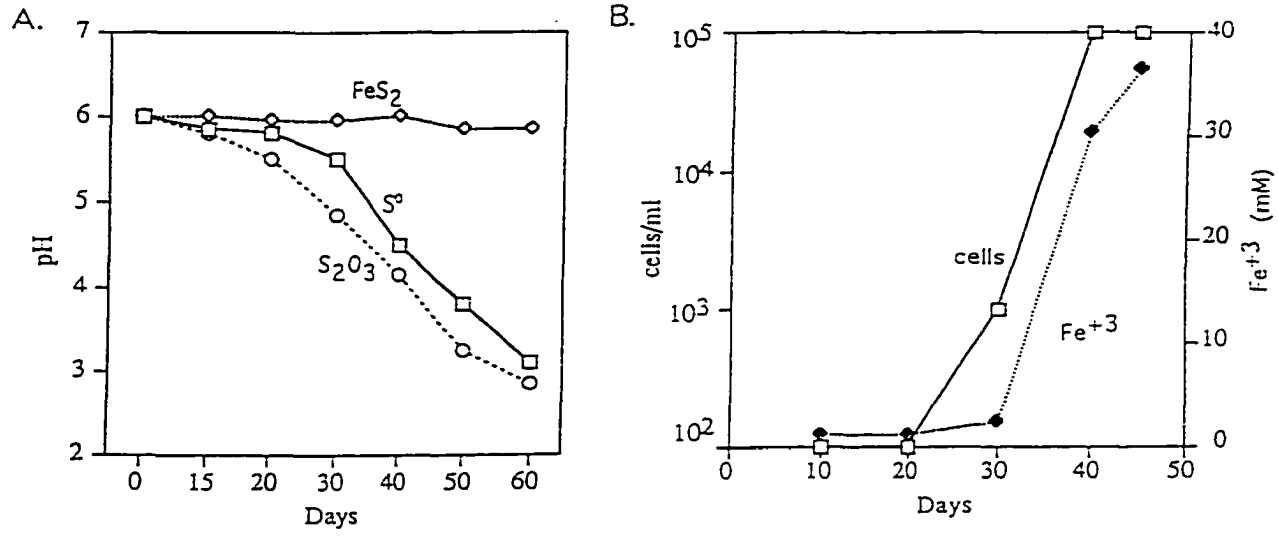

Figure 4. (A) S oxidizing activities in sediment slurries using S^0, $S_2O_3^{2-}$, and FeS_2 as reduced S sources. (B) Iron oxidation and cell growth of an isolate from the 6.4 m interval.

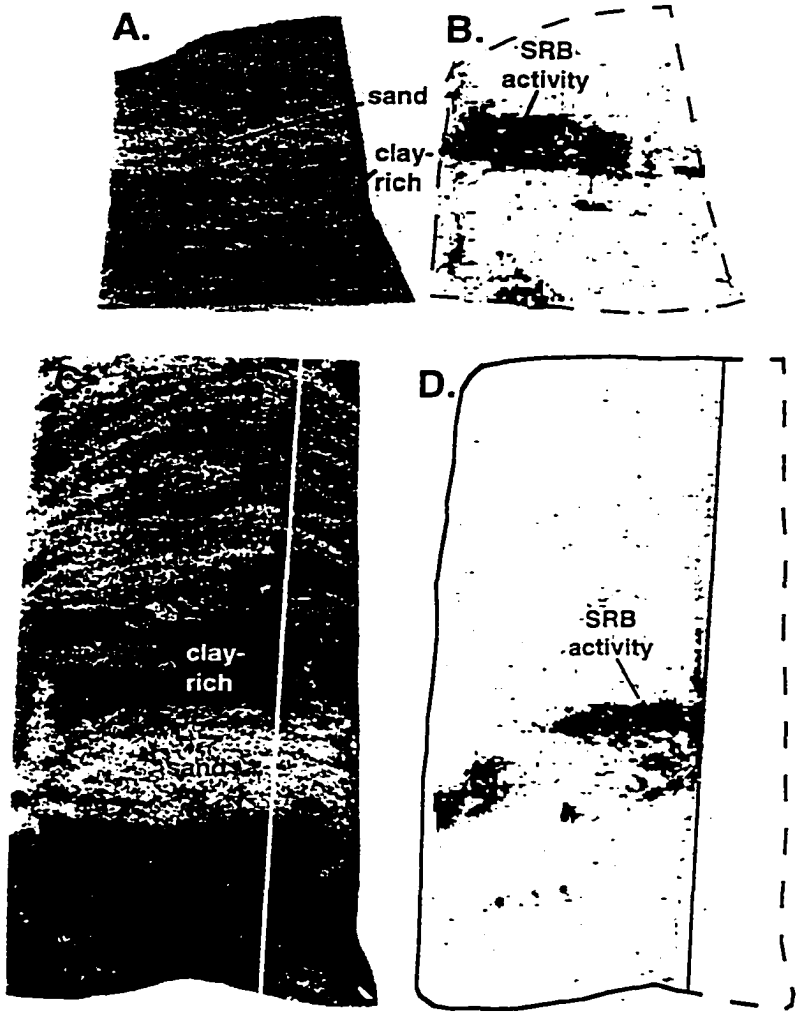

Figure 5. Core segments (left) and complementary autoradiographic images (right) of sulfate reduction activity in samples NP-5 (25.8 m; A, B) and NP-5 (18.6m; C, D).

Discussion

A combination of microbiological, geochemical, and hydrological techniques suggest that sulfur, iron, and carbon cycling are intimately linked in the low conductivity Yegua aquifer system. Differences in geochemistry between the shallow and deeper subsurface regions are clearly evident. Pyrite oxidation was indicated in the shallow, more oxidizing zone that possessed higher amounts of oxygen and sulfate, lower pH, more amorphous Fe(III) iron oxyhydroxides, and less reduced sedimentary S relative to underlying reduced zone.

The oxidizing zone also harbored more Fe- and S-oxidizing bacteria than the reducing zone. The activity profiles of these organisms revealed that they were capable of oxidizing S°, thiosulfate, and soluble Fe^{+2}, but not pyrite (Fig. 4A). These profiles and the general acidic conditions in the shallow regions argue that such organisms probably play an indirect role in the oxidation of pyrite. That is, they may oxidize ferrous to ferric iron which can then abiotically oxidize pyrite. The role of iron oxidizing microorganisms in this process is believed to be especially important under acidic conditions (pH < 5), where the rate of abiotic Fe^{+2} oxidation is relatively low and where pyrite oxidation is more rapid with Fe^{+3} than with O_2 (8,32).

Pyrite oxidation also represents an oxygen sink and probably helps maintain anaerobic conditions in deeper formations. In consistent fashion, sulfate and oxygen values are depressed in the more reducing deeper sediments where pyrite is

more plentiful. The lack of a depletion of pyrite in the shallowest water-bearing sand suggests that pyrite oxidation does not occur to an appreciable degree in and below this formation, despite the presence of low levels of oxygen and the occasional detection of the requisite microorganisms (Fig. 3E). Presumably, pyrite oxidation is restricted below the shallow aquifer (NP-4A) due to decreased oxygen diffusion in water saturated regions (8,37).

We believe that sulfate produced by pyrite oxidation in shallow subsurface areas is advectively transported to deeper aquifers by downward vertical transport and by down dip horizontal transport. Hydraulic gradients indicate local downward flow. Furthermore, regional flow is in the down dip (southeast) direction, with beds dipping about 20 m per km. The time required to vertically advect enough NP-4A dissolved sulfate to provide the sulfate concentration of NP-4B water (time = NP-4B concentration x thickness/flux, where flux = NP-4A concentration x advective velocity) is only 78 years. Likewise, vertical transport of NP-4B water could account for the sulfate content of NP-4C water in 538 years. Based on the measured sulfate reduction rates which should be viewed only as upper limits of the actual in-situ rates, it would take greater than 2000 years and just over 10 years to deplete sulfate in the NP-4B and NP-4C aquifers, respectively. These calculations suggest that vertical advection of sulfate is sufficient to supply electron acceptor for sulfate reduction where the aquifer is overlain by sulfate-rich sediments (such as in the NP-4B aquifer), but not in cases where overlying sediments are relatively sulfate-poor (as in NP-4C). Of course, we considered only one point along the flow path and likely there are many opportunities for shallow lenses of sulfate-rich waters to interact with NP-4C waters along their flow path. Furthermore, sulfate

acquired from a shallow pyrite oxidation zone will likely be transported laterally along the flow path. Thus, it is reasonable to assume that sulfate produced in the shallow subsurface by pyrite oxidation is a significant sulfate source for SRB in shallow reducing microenvironments and deeper, more distant anaerobic sediments.

The sulfate reducing activity measured in the shallow acidic sediments (pH 3.5 - 5) was somewhat surprising. However, the dissimilatory reduction of sulfate has been observed in other highly acidic environments (pH ~ 2.3 - 4.5), particularly those impacted by acid mine drainage (15,16,44). Relatively high numbers of SRB have been recovered from acidic mine tailings (10,11), but isolates were unable to grow under acidic conditions. In these instances, sulfate reduction may occur in microenvironments with favorable environmental conditions (44) or the requisite organisms may be able to metabolize but not grow at low pH.

Similarly, the requisite bacteria and sulfate reduction have been reported in oxidized environments (4,14), but a pure culture capable of reducing sulfate in the presence of oxygen has yet to be obtained (25). Our measurement of sulfate reduction in even the shallow regions were conducted under anaerobic conditions. Thus, the prospects for sulfate reduction under aerobic conditions in this zone were not specifically evaluated.

The lack of culturable methanogens or methane formation (29) and the absence of potentially competing electron acceptors are evidence that sulfate reduction is a predominant process in the deeper, more reducing Yegua sediments. Sulfate reduction has been found to be an important process in many other pristine subsurface environments (6,21,22,26,34). This study indicates that the process was highly spatially skewed regardless of the scale examined or assay employed.

In this respect, the underlying frequency distribution associated with sulfate reduction may be far from normal and perhaps similar to other highly skewed biological processes including, denitrification (35) and methanogenesis (1). Future studies will be designed to evaluate this prospect. A variety of assays indicated the importance of both sediment interfaces and composition in governing the distribution of this activity. Consistent with previous findings (29), sulfate reduction activity was most readily detected in sands, but not in silt- and clay-rich sediments (Fig. 3F). This observation is consistent with many previous studies, mostly of aerobic microorganisms, which have shown higher microbial numbers and metabolic potential in sandy sediments (2,12,17,22,36,39) relative to other formations. Detailed sampling of a single sediment core indicated that sulfate reducing activity was confined to the interface between a sand underlying a lignite and clay-rich sediment (see inset in Fig. 3F). Comparable results were obtained by investigators probing the exploratory drilling site in Cerro Negro, New Mexico. In these studies, most microbial activity including sulfate reduction was associated with consolidated porous sandstones immediately underlying low permeability organic rich shales (26,13).

The silver foil procedure also attested to the importance of interfaces and sediment composition. Bands of sulfate reducing activity were noted in sands that were juxtaposed to sediments rich in clay or lignite (Fig. 5). Much of the variability in SRB activity occurred at the subcentimeter scale. If this proves to be generalizing, it may be that microscale variability accounts for the majority of the total heterogeneity associated with subsurface sulfate reduction, and by extrapolation, possibly other microbial processes.

Beyond the question of where 'hot spots' of sulfate reduction occur, is the question of why they exist. Increased sulfate reduction in sands near lignite or clay laminae suggest that nutrients, perhaps electron donors, emanate from the latter sediments and stimulate microbial respiration in sands. In consistent fashion, the production of low molecular weight fatty acids was detected in clay and lignite laminae, but sulfate reduction was not. This in turn suggests that organic matter was fermented in these deposits. In fact, the highest concentrations of acetate and propionate were detected in strata with low sand contents (J. Routh, unpublished data) and common fermentation products were consumed in sandy formations (29). Similarly, it was shown that fermentation in aquitards exceeded respiration and resulted in the accumulation of low molecular weight fatty acids in the pore waters of confining (ie. clay-rich) sediments (30). In contrast, respiration outpaced fermentation in high-permeability sandy aquifers. Diffusion of electron donors from confining layers or sediments which contain high concentrations of organic matter would account for the spatially discrete zones of sulfate reduction detected at sedimentary interfaces.

While the interlinkage between the oxidative and reductive portions of the S-cycle in this shallow aquifer system is recognizable, such interactions represent a model for other subterranean regions where anaerobic microbial communities depend on the generation and transport of electron acceptors from shallow or other oxidized regions. Our results also have practical implications for geochemical models for determining terminal electron accepting processes in aquifers. For instance, the lack of sulfate depletion along groundwater flow paths cannot necessarily be taken as evidence for the absence of sulfate reduction. The vertical

transport of the anion into aquifers could prevent its net loss even if sulfate reduction occurs. Similarly our data indicate that Fe^{+2} produced during pyrite oxidation can be a substantial source of reduced iron in groundwaters which are hydraulically linked to a pyrite oxidation zone. This could confound interpretations of biological iron reduction based solely on the presence of Fe^{+2} in groundwaters. Clearly the mere spatial separation of microbial assemblages cannot be construed as evidence for the absence of metabolic interactions.

References

1. **Adrian NR, Robinson JA, Suflita JM** (1994) Spatial variability in biodegradation rates as evidenced by methane production from an aquifer. Appl Environ Microbiol **60:**3632-3639
2. **Balkwill DL** (1989) Numbers, diversity, and morphological characteristics of aerobic, chemoheterotrophic bacteria in deep subsurface sediments from a site in South Carolina. Geomicrobiol J **7:**33-52
3. **Berg RR** (1970) Outcrops of the Claiborne group in the Brazos Valley, southeast Texas. In: Geological Society of America. Fourth annual meeting of the South-Central Section, p. 79
4. **Canfield DE, Des Marais DJ** (1991) Aerobic sulfate reduction in microbial mats. Science **251:**1471-1473
5. **Chambers LA, Trudinger PA** (1978) Microbiological fractionation of stable sulfur isotopes: A review and critique. Geomicrobiol J **1:**249-293

6. **Chapelle FH, McMahon PB** (1991) Geochemistry of dissolved inorganic carbon in a Coastal Plain aquifer. 1. Sulfate from confining beds as an oxidant in microbial CO_2 production. J Hydrol **127:** 85-108
7. **Ehrenreich A. and F. Widdel** (1994) Anaerobic oxidation of ferrous iron by purple bacteria, a new type of phototrophic metabolism. Appl Environ Microbiol **60:**4517-4526
8. **Evangelou VP, Zhang YL** (1995) A review: pyrite oxidation mechanisms and acid mine drainage prevention. Critical Reviews in Environmental Science and Technology. **25:**141-199
9. **Fontes JC** (1992) Chapter 17. Chemical and isotopic constraints on ^{14}C dating of groundwater. In: Radiocarbon after four decades, an interdisciplinary perspective. Taylor RE, Long A, Kraceds RS (eds), Springer-Verlag, New York. pp 242-261
10. **Fortin D, Davis G, Southam G, Beveridge TJ** (1995) Biogeochemical phenomena induced by bacteria within sulfidic mine tailings. J Ind Microbiol **14:**178-185
11. **Fortin D, Beveridge TJ** (1997) Microbial sulfate reduction within sulfidic mine tailings: formation of diagenetic Fe sulfides. Geomicrobiol J **14:**1-21
12. **Fredrickson JK, T.R. Garland TR, Hicks RJ, Thomas JM, Li SW, McFadden KM** (1989) Lithotrophic and heterotrophic bacteria in deep subsurface sediments and their relation to sediment properties. Geomicrobiol J **7:**53-66
13. **Fredrickson JK, McKinley JP, Bjornstad BN, Long PE, Ringelberg DB, White DC, Krumholz LR, Suflita JM, Colwell FS, Lehman RM, Phelps TJ** (1997) Pore-size constraints on the activity and survival of subsurface

bacteria in a late Cretaceous shale-sandstone sequence, northwestern New Mexico. Geomicrobiol J **14:**183-202

14. **Frund C, Cohen Y** (1992) Diurnal cycles of sulfate reduction under oxic conditions in cyanobacterial mats. Appl Environ Microbiol **58:**70-77
15. **Gyure RA, Konopka A, Brooks A, Doemel W** (1990) Microbial sulfate reduction in acidic (pH 3) strip-mine lakes. FEMS Microbiol Ecol 73:193-202
16. **Herlihy AT, Mills AL** (1985) Sulfate reduction in freshwater sediments receiving acid mine drainage. Appl Environ Microbiol **49:**2878-2920
17. **Hicks RJ, Fredrickson JK** (1989) Aerobic metabolic potential of microbial populations indigenous to deep subsurface environments. Geomicrobiol J **7:** 67-77
18. **Hobbie JE, Daley RJ, Jasper S** (1977) Use of Nucleopore filters for counting bacteria by fluorecence microscopy. Appl. Environ. Microbiol. **33:**1225-1228
19. **Hordjik CA, Burgers I., Phylipsen GJM, Cappenberg TE** (1989) Trace determination of lower volatile fatty acids in sediments by gas chromatography with chemically bonded FFAP columns. J. Chromatography **511:**317-323
20. **Hsieh YP, Shieh YN** (1997) Analysis of reduced inorganic sulfur by diffusion methods: improved apparatus and evaluation for sulfur isotopic studies. Chem Geol **137:** 255-261
21. **Jakobsen R, Postma D** (1994) In situ rates of sulfate reduction in an aquifer (Romo, Denmark) and implications for the reactivity of organic matter. Geology **22:**1103-1106
22. **Jones RE, Beeman RE, Suflita JM** (1989) Anaerobic metabolic processes in the deep terrestrial subsurface. Geomicrobiol J **7:**117-130

23. **Kaplan IR** (1983) Stable isotopes of sulfur, nitrogen and deuterium in recent marine environments. In: Arthur MA, et al., Stable Isotopes in Sedimentary Geology, SEPM Short Course No. 10, pp 2-1 to 2-108

24. **Kieft TL, Fredrickson JK, McKinley JP, Bjornstad BN, Rawson SA, Phelps TJ, Brockman FJ, Pfiffner SM** (1995) Microbiological comparisons within and across contiguous lacustrine, paleosol, and fluvial subsurface sediments. Appl Environ Microbiol **61:**749-757

25. **Krekeler D, Sigalevich P, Teske A, Cypionka H, Cohen Y** (1997) A sulfate-reducing bacterium from the oxic layer of a microbial mat from Solar Lake (Sinai), *Desulfovibrio oxyclinae* sp. nov. Arch Microbiol **167:**369-375

26. **Krumholz LR, McKinley JP, Ulrich GA, Suflita JM** (1997) Confined subsurface microbial communities in Cretaceous Rock. Nature **386:**64-66

27. **Lovley DR, Phillips EJP** (1987) Rapid assay for microbially reducible ferric iron in aquatic sediments. Appl Environ Microbiol **53:**1536-1540

28. **Lovley DR, Chapelle FH, Woodward JC** (1994) Use of dissolved H_2 concentrations to determine distribution of microbially catalyzed reactions in anoxic groundwater. Environ Sci Technol **28:**1205-1210

29. **Martino DP, Grossman EL, Ulrich GA, Burger KC, Schlichenmeyer JL, Suflita JM, Ammerman JW** (1997) Microbial abundance and activity in a low conductivity aquifer system in East-Central Texas. Microb Ecol **35:**224-234.

30. **McMahon PB, Chapelle FH** (1991) Microbial production of organic acids in aquitard sediments and its role in aquifer geochemistry. Nature **349:**233-235

31. **McMahon PB, Chapelle FH, Falls WF, Bradley PM** (1991) Role of microbial processes in linking sandstone diagenesis with organic-rich clays. J Sediment Petrol **62:**1-10

32. **Moses CO, Nordstrom DK, Herman JS, Mills AL** (1987) Aqueous pyrite oxidation by dissolved oxygen and by ferric iron. Geochim Cosmochim Acta **51:**1561-1571

33. **Nielsen H** (1979) Sulfur isotopes. In: Jäger E and Hunziker J (eds) Lectures in Isotope Geology, Springer, Berlin, pp 283-312.

34. **Parkes RJ, Cragg BA, Bale SJ, Getliff JM, Goodman K, Rochelle PA, Fry JC, Weightman AJ, and Harvey SM** (1994) Deep bacterial biosphere in Pacific Ocean sediments. Nature **371:**410-413

35. **Parkin TB, Starr JL, and Meisinger JJ** (1987) Influence of sample size on measurements of soil denitrification. Soil Sci Soc Am J **51:**1492-1501

36. **Phelps TJ, Raione EG, White DC, Fliermans CB** (1989) Microbial activities in deep subsurface environments. Geomicrobiol J **7:**79-92

37. **Pionke HB, Rogowski AS, De Angelis RJ** (1980) Controlling the rate of acid loss from strip mine spoil. J Environ Qual **9:**694-699

38. **Postma D** (1982) Pyrite and siderite formation in brackish and freshwater swamp sediments. American Journal of Science **282:**1151-1185

39. **Sinclair JL, Ghiorse WC** (1989) Distribution of aerobic bacteria, protozoa, algae, and fungi in deep subsurface sediments. Geomicrobiol J **28:**369-377

40. **Smith RL** (1997) Determining the terminal electron accepting reaction in the saturated subsurface. In: Hurst CJ, Knudsen GR, McInerney MJ, Stetzenbach LD,

and Walter MV (eds) Manual of Environmental Microbiology. American Society for Microbiology, Washington, D.C., pp 577-585

41. **Sugio T, Wada K, Mori M, Inagaki K, Tano T** (1988) Synthesis of an iron-oxidizing system during growth of *Thiobacillus ferroxidans* on sulfur-basal salt medium. Appl Environ Microbiol **54:**150-152

42. **Suzuki I, Takeuchi TL, Yuthasastrakosol TD, Oh JK** (1990) Ferrous iron and sulfur oxidation and ferric iron reduction activities of *Thiobacillus ferroxidans* are affected by growth on ferrous iron, sulfur, or a sulfur ore. Appl Environ Microbiol **56:**1620-1626

43. **Tanner RS** (1989) Monitoring sulfate reducing bacteria: comparison of enumeration media. J Microbiol Meth **10:**83-90

44. **Tuttle JH, Dugan PR, Macmillan CB, Randles CI** (1969) Microbial dissimilatory sulfur cycle in acid mine water. J Bacteriol **97:**594-602

45. **Ulrich GA, Krumholz LK, Suflita JM** (1997) A rapid and simple method for estimating sulfate reduction activity and quantifying inorganic sulfides. Appl Environ Microbiol **63:**1627-1630

46. **Warikoo V, McInerney MJ, Robinson JA, and Suflita JM** (1996) Interspecies acetate transfer influences the extent of anaerobic benzoate degradation by syntrophic consortia. Appl Environ Microbiol **62:**26-32.

Chapter 5

Volatile Organic Acids and Microbial Processes in the Yegua Formation, East-Central Texas

Abstract

Geochemical and microbiological evidence indicate the presence of viable microorganisms which produce or consume volatile organic acids (VOA) in the Yegua formation. Acetic and propionic acid concentrations in mudstones range from 200 - 1270 and 20 - 38 nmol·gdw^{-1} respectively, whereas concentrations in sands are 50 - 200 and less than 20 nmol·gdw^{-1}. VOA concentrations in sediments and in laboratory incubations suggest net production of VOAs by microorganisms in mudstones, and net consumption of VOAs by sulfate reducing bacteria (SRB) in sands. Notably, SRB activity is mostly confined to aquifer sands. Although mudstones have high total organic carbon, VOA, and sulfate concentrations, absence of SRB activity implies that some parameter(s) other than electron donor and acceptor availability limits sulfate reduction.

We modeled vertical diffusion and advection to estimate acetic acid transport from aquitard to aquifer. Assuming that SRB completely respire the acetic acid transported into the aquifer (5.4 μmol·l^{-1}· yr^{-1}), the CO_2 production rate in the Yegua aquifer sands is 9.2 μmol·l^{-1}·yr^{-1}. This value is within the range obtained for deep

aquifers, but is still 10^3 to 10^5 times slower than near-surface microbial processes. This slow mineralization of *in situ* organic matter likely accounts for the long-term survival of subsurface microorganisms. Lignite, like mudstone, may also serve as a source of electron donors for microorganisms because laboratory incubations yield similar VOA production in lignite and mudstone. The net production of VOAs in mudstones and lignite supports previous studies contending that subsurface microbial communities exhibit a loose commensalism, with microorganisms in aquitards providing VOAs for respiratory processes (i.e., sulfate reduction) in aquifers.

Introduction

The discovery of diverse microbial communities in the terrestrial subsurface has important ramifications for bioremediation and sediment diagenesis (Ehrlich, 1996). Microbes in the soil-zone rapidly consume soluble nutrients, rendering much of the recharge waters oligotrophic (Madsen and Ghiorse, 1993). Therefore, *in situ* sedimentary organic matter (OM) is most likely the primary source of nutrients (electron donors) for microbial activity in the deep subsurface. Under anoxic conditions typical of subsurface environments, microbes degrade large polymeric compounds in a sequence of oxidation and reduction reactions. The fermentation product acetic acid is a key intermediate in these processes, serving as a substrate for sulfate reducing bacteria (SRB) and methanogens (Sansone and Martens, 1982).

Volatile organic acids such as acetic, propionic, *iso*-butyric, *n*-butyric, *iso*-valeric, and *n*-valeric acids are hydrophilic, short-chain carboxylic acids and range in concentrations from less than 1 $mg \cdot l^{-1}$ in groundwater to thousands of $mg \cdot l^{-1}$ in oil-field brines (Leenheer *et al.*, 1974; Carothers and Kharaka, 1978). VOAs are important in nature because of their role as: 1) substrates for a variety of microorganisms (Balba and Nedwell, 1982; Monetti and Scranton, 1992), 2) complexing agents of metals and radio-nuclides (Giordano, 1994), 3) diagenetic agents affecting secondary porosity (Surdam *et al.*, 1984), and 4) precursors for hydrocarbons (Carothers and Kharaka, 1978). VOAs in the soil-zone and in shallow marine and continental sediments are mostly generated by biotic processes. In contrast, VOA sources in deeply buried sediments and formation fluids are considered to be mostly abiotic (Lundegard and Kharaka, 1994). The nature of VOA production in sedimentary basins, however, is still in question. For example, Kawamura and Ishiwatari (1985) note that abiotic production of VOAs in sedimentary basins at low temperature is problematic and difficult to verify in laboratory experiments. In addition, many researchers question whether high temperature, short-duration experiments provide a reasonable simulation for VOA generation at low temperatures (Lewan and Fisher, 1994; Chapelle and Bradley, 1996).

The role of organic acids in microbial processes and OM mineralization in soils and marine sediments has received much attention (Sansone *et al.*, 1987; Shaw and McIntosh, 1990; Fox and Comerford, 1990; Lundström, 1993; among others), but few researchers have studied their role in aquifer systems. Jones *et al.* (1989) noted acetic acid production and consumption in deep Atlantic Coastal Plain

sediments, and Liu and Suflita (1993) further demonstrated the presence of acetogenic microorganisms in these sediments. The first comprehensive study of the relationship between VOA distribution and subsurface microbial activity in aquifer systems was reported by McMahon and Chapelle (1991). Also studying Atlantic Coastal Plain sediments, these authors presented evidence that microbial production of organic acids in aquitards and consumption in aquifers results in diffusive transport of VOAs from aquitards to aquifers. Calculations of diffusive flux indicated that rates of organic acid transport are sufficient to account for observed rates of microbial respiration in the aquifers. In recent studies of deep Atlantic Coastal Plain sediments, Chapelle and Bradley (1996) confirmed the higher concentrations of VOAs in fine-grained sediments compared with sandy sediments. More importantly, these authors found and characterized viable acetogenic microorganisms within fine-grained sediments in the deep subsurface. Except for these few studies, little is known about the relation between organic acids and microbial activity in the terrestrial subsurface.

The objectives of this study were: 1) to understand the microbial and geochemical processes controlling the distribution of VOAs in Gulf Coast sediments, and 2) to test whether fine-grained sediments (aquitards) serve as VOA sources for microorganisms living in sands (aquifers), and 3) to evaluate the components of this groundwater system that govern the *in situ* microbial activities.

Materials and Methods

Sediment Sampling Sediments were collected from four 19 - 31 m deep boreholes drilled with hollow-stem augers, and eight water wells were installed in clusters at the four borehole sites (Figure 1, Chapter 4). Cores were removed from the split-spoon sampler, wrapped in plastic, stored on ice, and brought back to the laboratory within 3 h. The oxidized and contaminated core exteriors were pared with sterile knives in an Ar-filled glove bag. The inner portions of the cores were used for the microbiological assays. The sediment pairings were stored in Nalgene® centrifuge tubes which contained 5 ml of 7.4 mmol·l^{-1} $HgCl_2$ (~20% wt/v) to inhibit microbial activity. Samples of the pairings were at -20°C, and were later used for organic acid analysis. To check for possible contamination during drilling, carboxylated fluorescent microspheres (Polysciences Inc., Warington, Pennsylvania) were used as microbiological tracers. Samples showing levels of microsphere contamination of less than 10^1 microspheres·g^{-1} (detection limit) were designated as aseptically attained and used in this study.

Geochemical analyses An aqueous extraction technique (Routh, 1999) was used for analyzing organic acids. Grain size, percent moisture, and total organic carbon in sediments were determined as described in chapter 4.

Microbiology Microbial activities were evaluated in a mudstone and lignite-rich sediment layer in the NP-4 core at 18.38 m. A mudstone and lignite sample and a 1:1 mixture of both lithologies were slurried with NP-4B groundwater (40% wt : vol) and incubated at 25 °C. VOAs, methane, and sulfate reduction were measured in the incubations. SRB activity was determined for several NP-4

sediments using radiotracer methodology (Ulrich *et al.*, 1998). Additional experiments were performed to evaluate the possibility that VOAs were produced by SRB. The slurries to test this hypothesis were prepared with NP-3 groundwater and sand 27 - 30 m because high SRB activity was previously reported in this interval (Martino *et al.*, 1998). Autoclaved incubations served as sterile controls. Sodium molybdate (1 mM) was added as an inhibitor of sulfate reduction. Sulfate and VOAs were determined during the incubation period.

Experiments were performed to determine the major fermentation products produced by enrichments and pure cultures of microorganisms obtained from NP-3 and NP-4 sediments. Enrichments were obtained by preparing anoxic sediment slurries (5 g / 10 ml) in a 20% peptone, trypticase, yeast extract, glucose (PTYG) medium (Balkwill *et al*, 1985). Hydrogen and organic acids were determined after a 30 d incubation at 25 °C.

Pure cultures were obtained from the enrichments using the agar roll tube technique. Colonies were transferred to anoxic spread plates (with 20% PTYG medium) two times before assuming the cultures were pure. The cultures were then transferred to an anoxic phosphate buffered basal medium containing 1 g/L glucose as the sole carbon source and 0.2 mM cysteine and sulfide as the reducing agent. After 4 d, hydrogen and organic acids were determined.

Results

Geochemistry TOC and VOA concentrations in Yegua sediments were high in mudstones, but low in sands. TOC content in aquifer sands was < 0.25%, whereas

in mudstones TOC content is > 0.50%. Acetic acid was the dominant organic acid measured followed by propionic, *iso*-butyric, *n*-butyric, *iso*-valeric, and *n*-valeric acids. VOAs greater than C_8-carboxylic acids were below the detection limit. Acetic and propionic acid concentrations in mudstones ranged from 200 - 1270 and 20 - 38 nmol·gdw^{-1} respectively, whereas in sands they were 50 - 200 and less than 20 nmol·gdw^{-1}. Typically, unpoisoned Yegua sediments yielded VOA concentrations below the detection limit or at the low end of the concentration range. Occasionally, lignite stringers associated with sands (*e.g.*, NP-4 at 17.95 and NP-5 at 23.27, 27.49 and 30.27 m) significantly increased the TOC and VOA concentrations in these samples. Excluding these samples, TOC and percent sand in the NP-4 and NP-5 cores showed a negative correlation ($r^2 = 0.53$, data not shown). Consistent with this, acetic acid and percent sand also correlated negatively ($r^2 = 0.50$, Fig. 1).

Microbiology Incubation experiments indicated that acetic and propionic acids were produced by the biological degradation of endogeneous carbon in mudstone and lignite-rich sediments. These acids accumulated in lignite and mudstone slurries during incubation (Fig. 2), but were not produced in autoclaved controls. When mudstone and lignite-rich sediments were mixed, the acetic acid production rate increased 30-fold. Molybdic acid (a sulfate reduction inhibitor) did not prevent the production of acetic and propionic acids (Fig. 3). Sulfate reduction did not occur in the incubations of fine-grained sediments and was only measured in sandy sediment slurries.

Acetic and propionic acid were also the major fermentation products produced in sediment slurries ammended with PTYG (Fig. 4). Similarly, acetate and propionate were the dominant glucose fermentation products produced by pure cultures (Fig. 5). Organic acids other than acetate and propionate were either not detected or produced in trace quantities in these experiments (data not shown).

Sulfate reduction activity, as measured using $^{35}SO_4$ as a tracer, was detected in the sands throughout the NP-4 depth profile. In fact, a strong positive correlation between sulfate reduction and sand content ($r^2 = 0.61$) was observed (Fig. 6). Sulfate reduction occurred only in sediments with greater than 60% sand. Sandy sediments which exhibited increased sulfate reducing activity contained less acetic acid (Fig. 7).

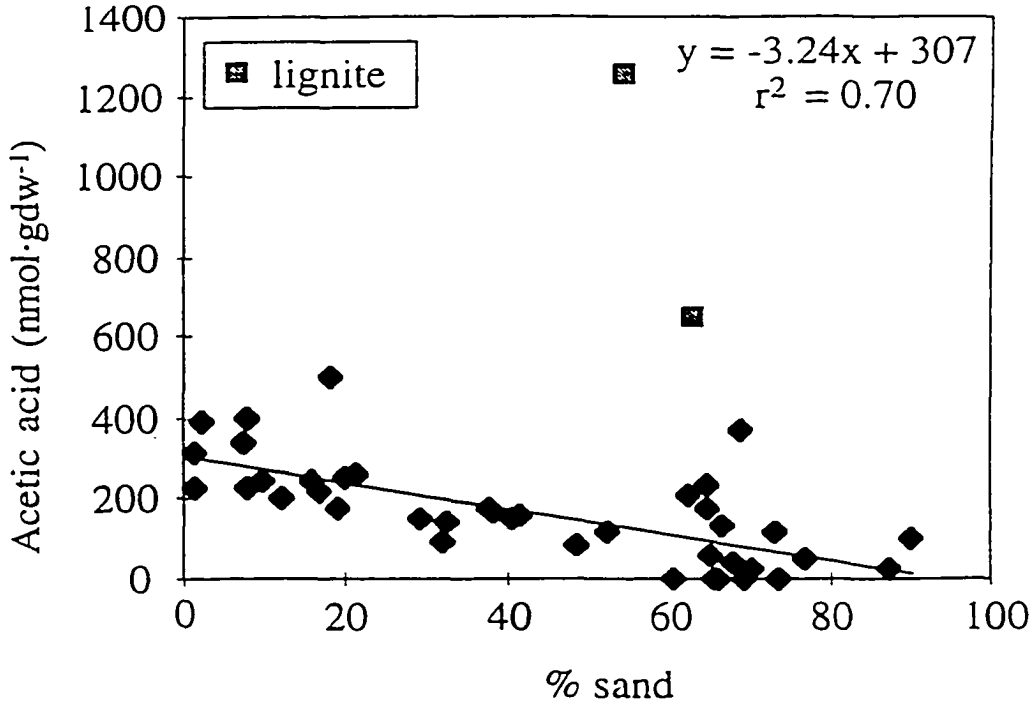

Figure 1. Acetic acid concentrations versus percent sand in NP-4 and NP-5 sediments. (Regression does not include samples with visible lignite laminae).

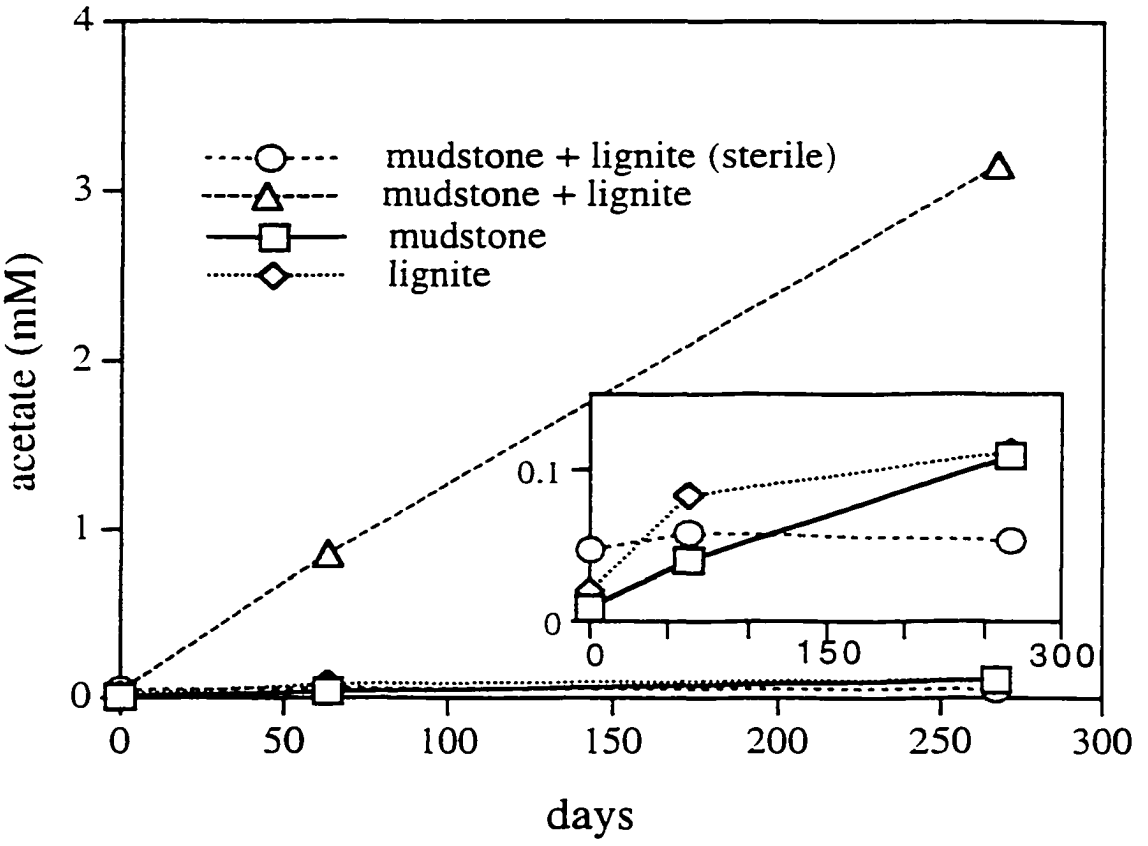

Figure 2. Production of acetic acid in NP-4 Yegua sediments (18.38 m).

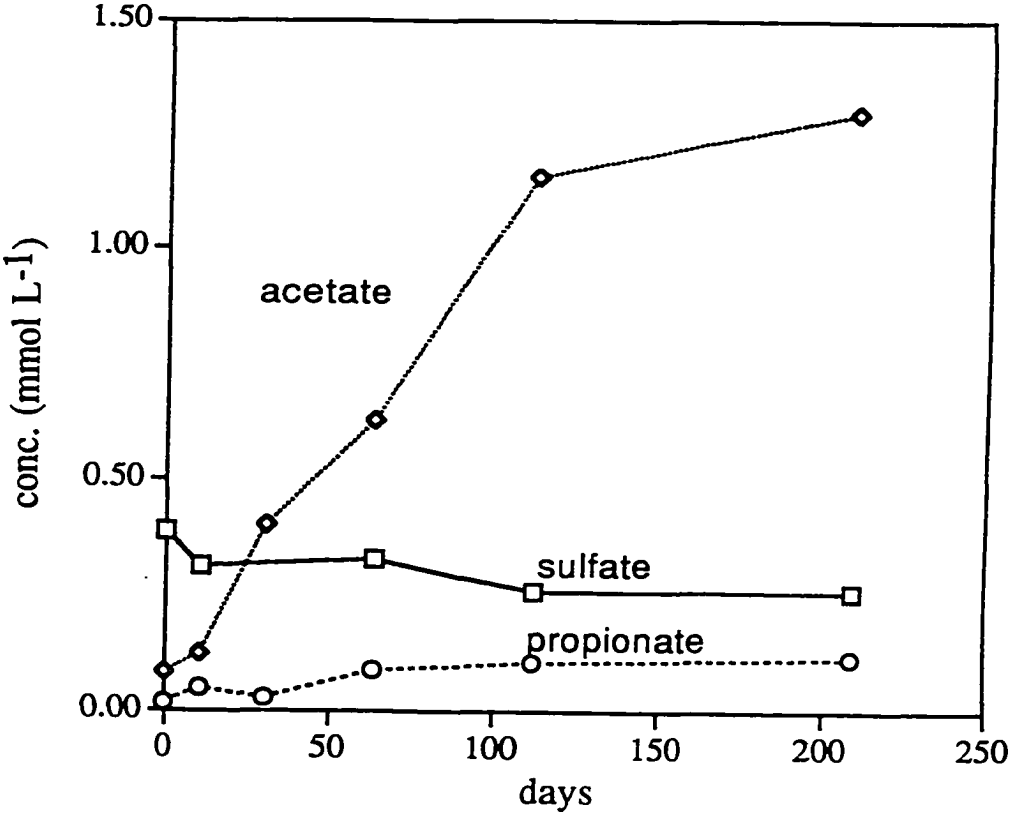

Figure 3. Production of volatile organic acids in microbial cultures from Yegua sediments treated with molybdate (sample NP-3 at 30.48 m).

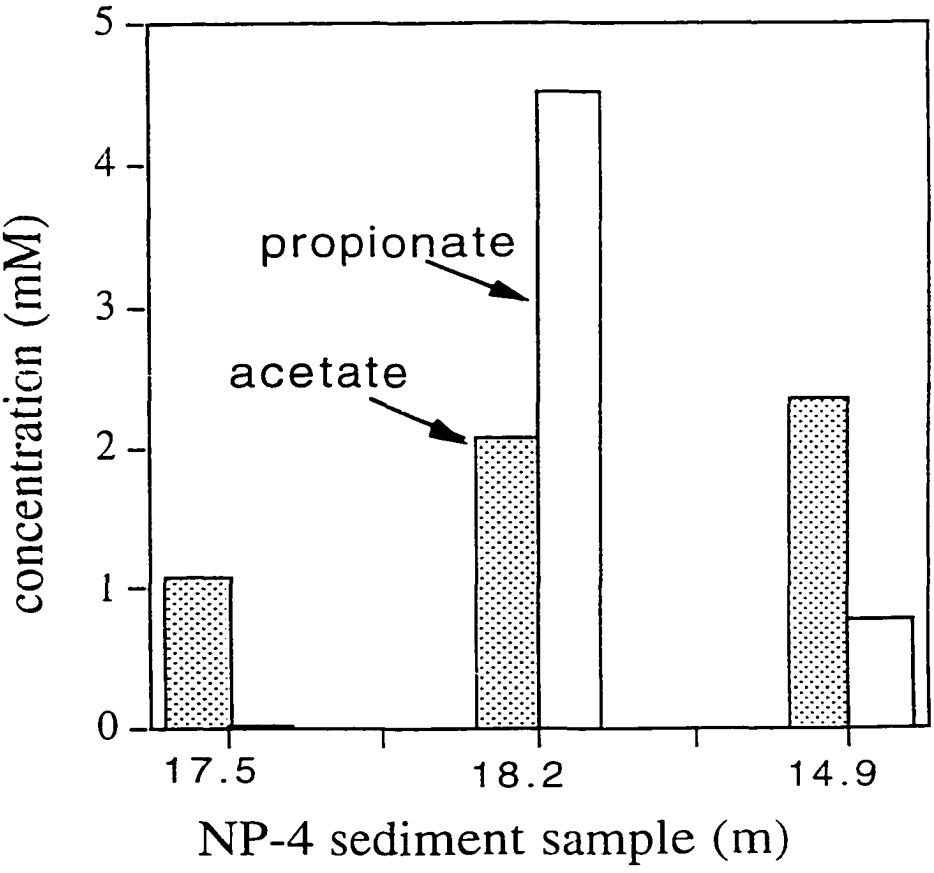

Figure 4. Organic acids produced in sediment incubations supplemented with peptone, trypticase, yeast extract, and glucose.

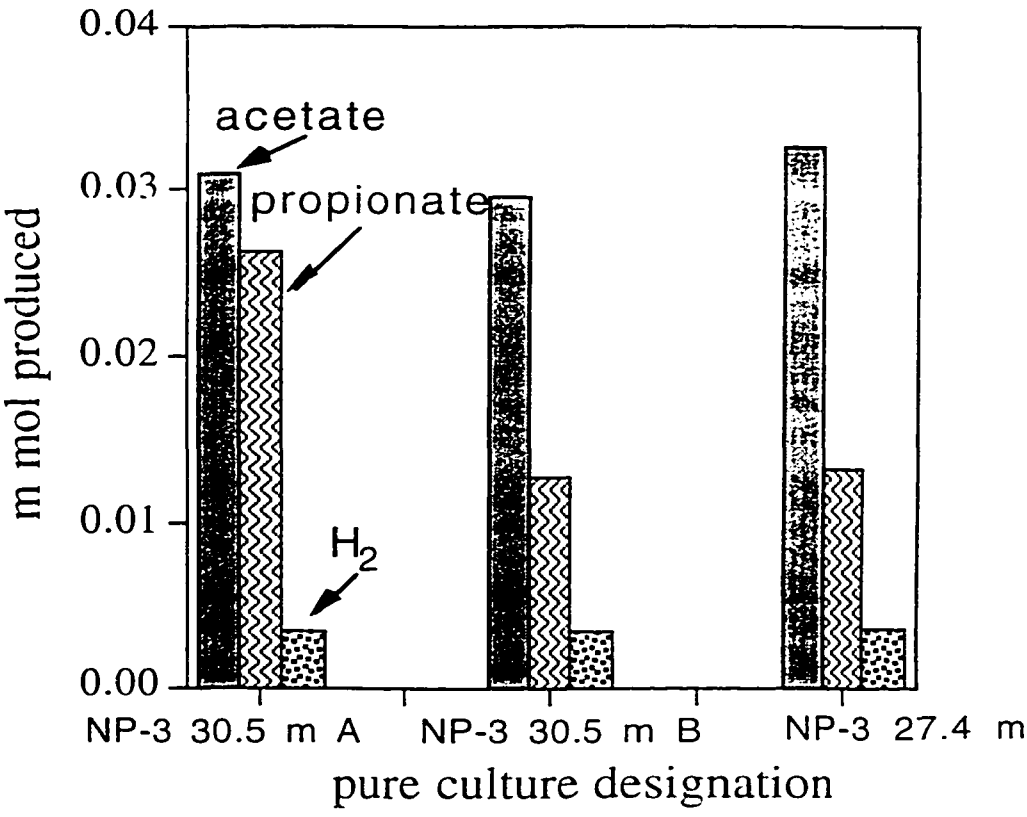

Figure 5. Products of glucose fermentation by pure cultures of microorganisms isolated from Yegua sediments.

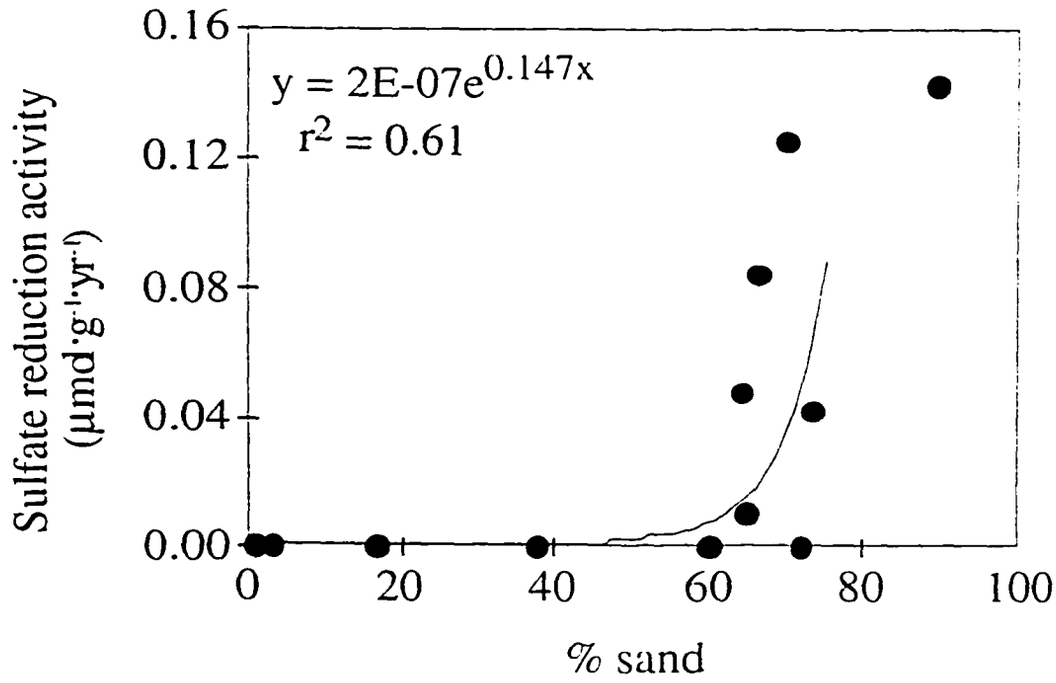

Figure 6. Percent sand versus sulfate reducing activity in NP-4 sediments. Sulfate reduction activities from Chapter 4.

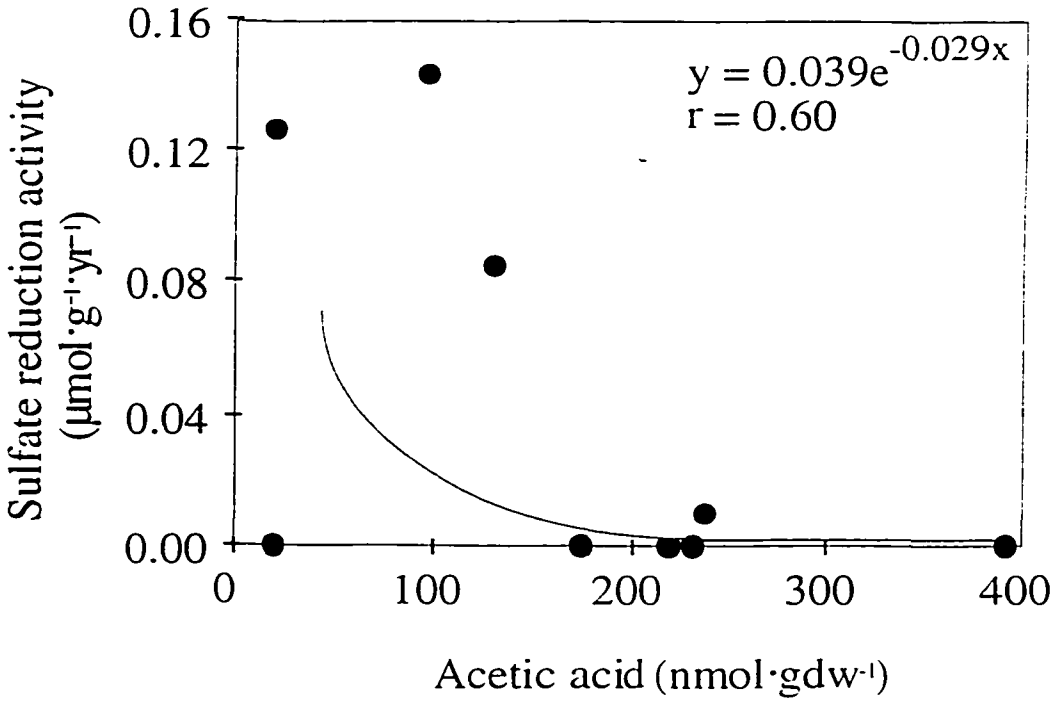

Figure 7. Acetic acid versus sulfate reducing activity in NP-4 sediments. Sulfate reduction activities from Chapter 4.

Discussion

VOA concentrations VOA concentrations in natural waters vary greatly depending on environmental factors like OM quality, nutrient and electron acceptor availability, and temperature. With one exception, acetic acid concentrations in Yegua sediments ranged from 50 - 694 nmol·gdw^{-1}, equivalent to 200 to 2780 μmol·l^{-1} (assuming a 25 % moisture content). These values are slightly higher than those obtained by Chapelle and Bradley (1996) for Atlantic Coastal Plain sediments (2 - 1800 μmol·l^{-1}). In the Yegua formation, acetic acid concentrations are higher than concentrations in anoxic marine pore waters (1 - 44 μmol·l^{-1}; Shaw and McIntosh, 1990; Novelli *et al.*, 1988), but are at least an order of magnitude lower than those commonly found in deep (> 1800 m) formation fluids (> 50,000 μmol·l^{-1}; Carothers and Kharaka, 1978). Carothers and Kharaka (1978) suggested that these hydrocarbon-associated VOAs form through catagenetic processes during sediment diagenesis.

Origin of VOAs It is difficult to prove that subsurface VOAs form solely by biotic or abiotic processes. Evidence for abiotic formation of VOAs at low temperature is based on extrapolation of high temperature experiments to low temperature, using the Arrhenius relationship applied to experimentally determined reaction rates (Lewan and Fisher, 1994). However, others have suggested that microbial processes may produce VOAs in sedimentary basins at low temperature

(Jones *et al.*, 1989; Chapelle and Bradley, 1996). We have both geochemical and microbiological evidence that the organic acids (mainly acetic and propionic acids) in Yegua sediments are produced by fermentation of endogeneous OM. Evidence from Routh *et al.* (1998) argues against an abiotic origin of VOAs in Yegua sediments because sedimentary OM in the formation is immature (Type III OM with low thermal alteration index), and has not undergone sufficient burial for catagenetic degradation. Furthermore, non-sterilized and anaerobic incubations of mudstone and lignite-rich samples produced acetic and propionic acids, but sterilized slurries did not.

The microbial process responsible for VOA production in these sediments is likely fermentation. Sulfate reduction is not responsible since VOAs are produced in clay and lignite-rich strata where sulfate reduction apparently does not occur. Furthermore, the inhibition of sulfate reduction did not prevent the production of acetic and propionic acids (Fig. 7). The presence of acetogenic bacteria in subsurface sediments combined with potential activity measurements (Liu and Suflita, 1993; Chapelle and Bradley, 1996; Krumholz *et al.*, 1997) has been used to suggest that acetogenesis is an important process in the formation of acetic acid. This does not appear to be the case in our studies of the Yegua formation. Radiolabeled acetate was not produced in mudstone and lignite-rich sediment incubations which were amended with ^{14}C-bicarbonate (data not shown) even when acetate was produced at relatively rapid rates (as shown in Fig. 2). This indicates that acetogenesis was not the dominant acetate producing pathway in these incubations. Further, the observation that the dominant organic acids *in-situ*, acetate and propionate, were also the major products of glucose fermentation in the

laboratory studies with sediments and pure cultures obtained from several different locations, suggests that fermentation rather than acetogenesis is the dominant organic acid producing mechanism in the Yegua formation. The relative role of acetogenesis and fermentation in subsurface acetate production needs to be further evaluated since acetate is a key intermediate of the carbon cycle in the subsurface.

Sulfate reduction Lack of culturable methanogens and dissolved methane in groundwater, as well as the absence of other electron acceptors, suggest that sulfate reduction is the dominant terminal electron accepting process in deeper reducing sediments. SRB activity in the Yegua formation correlates positively with % sand and negatively with VOAs (Fig. 8), implying net VOA consumption in aquifer sands. In addition, radiotracer measurements of sulfate reduction in sediments from the NP-4 core indicate that SRB activity is restricted to aquifer sands (Fig. 3D). This is consistent with previous work on deep subsurface sediments which reported high SRB activity and a great diversity of microorganisms in aquifer sands (Jones *et al.*, 1989; Sinclair and Ghiorse, 1989; McMahon and Chapelle, 1991; among others).

The underlying reasons for the stratification of microbial processes (mainly sulfate reduction in sands and fermentation in aquitards) and the absence of sulfate reduction in fine-grained sediments is unclear. In mudstones, TOC, VOA, and sulfate concentrations are high yet SRB activity is absent, suggesting that some parameter(s) other than availability of electron donors and acceptors limits sulfate reduction in fine-grained sediments. These parameter(s) may be water potential, pH, pore throat diameter, or availability of nutrients (*e.g.*, Ghiorse and Wilson,

1988; Lovley and Chapelle, 1995). In contrast, enhanced SRB activity in sands or sandstones at aquifer-aquitard interfaces in Yegua and Atlantic Coastal Plain sediments likely reflects the diffusion of electron donors and perhaps electron acceptors from aquitards (McMahon and Chapelle, 1991; Lovley and Chapelle, 1995; Krumholz *et al.*, 1997; Ulrich *et al.*, 1998).

Our results support the contention that OM in lignite and mudstones may be a potential source of electron donors (Fredrickson *et al.*, 1991; Murphy *et al.*, 1992), and may be less refractory than previously suggested. This has important implications for survival of microbial communities in oligotrophic environments where the microorganisms depend upon endogenous carbon sources.

VOA fluxes and microbial activity Direct evidence for microbial sulfate reduction in the terrestial subsurface has recently been obtained (Chapelle *et al.*, 1987; McMahon and Chapelle 1991; Ulrich *et al.*, 1998, and the present study) by either providing geochemical and/or microbiological evidence for SRB activity, or by recovering SRB from laboratory incubations of aquifer sediments. As mentioned earlier, McMahon and Chapelle (1991) modeled the acetic acid flux from aquitards to aquifers and its impact on groundwater chemistry in Atlantic Coastal Plains. Assuming that all of this acetic acid is transported by diffusion and mineralized to CO_2 by SRB activity, the authors obtained a HCO_3^- production rate of 3.6×10^{-5} mmol·l^{-1}·yr^{-1}. HCO_3^- increased along the flowpath, yielding a production rate of 3.7×10^{-5} mmol·l^{-1}·yr^{-1}, very close to the rate calculated for the diffusive flux of acetic acid.

As indicated in the Atlantic Coastal Plain aquifers, we observed net production of VOAs in the Yegua mudstones. To estimate the impact of this process, we modeled vertical diffusive and advective transport of acetic acid from aquitards to aquifers in the Yegua formation. Diffusive transport was calculated from Fick's first law:

$$J_d = -D \cdot (dC/dz) \cdot n \qquad (1)$$

where J_d = diffusive flux into aquifer in $\mu mol \cdot l^{-1} \cdot m \cdot yr^{-1}$, D = diffusivity of acetic acid (8.7×10^{-3} $m^2 \cdot yr^{-1}$; McMahon and Chapelle, 1991), C = concentration in $\mu mol \cdot l^{-1}$, z = depth in meters, and n = effective porosity of aquitard (0.4). The concentrations used were for the transition from aquitard to aquifer in NP-4 well, 960 $\mu mol \cdot l^{-1}$ at 26.09 m and 680 $\mu mol \cdot l^{-1}$ at 26.63 m. Acetic acid concentrations ($nmol \cdot gdw^{-1}$) were converted to $\mu mol \cdot l^{-1}$ by assuming that 1 gdw sediment contains 0.25 gm (25% water by weight) of water. The estimated diffusive flux is 1.8 $\mu mol \cdot l^{-1} \cdot m \cdot yr^{-1}$.

The vertical advective flux of acetic acid (J_a) from the overlying aquitard into an aquifer can be estimated from:

$$J_a = V \cdot C_{aqt} \cdot n \qquad (2)$$

where V = vertical advective velocity and C_{aqt} = average VOA concentration in the aquitard (1130 $\mu mol \cdot l^{-1}$). Vertical advective velocity was estimated as 0.003 $m \cdot yr^{-1}$ using Darcy's law, hydraulic conductivity estimates from lithology and water level measurements (Martino *et al.*, 1998). Equation (2) yields an acetic acid flux of 3.6 $\mu mol \cdot l^{-1} \cdot m \cdot yr^{-1}$. Assuming that the diffusively and advectively transported acetic acid is completely consumed by SRB activity, the rate of microbial CO_2 production

in aquifers (R_{CO2}) can be estimated by:

$$R_{CO2} = \frac{2(J_d + J_a)}{h \cdot n} \qquad (3)$$

where h = aquifer thickness (3 m minimum). The rate of CO_2 production calculated from equation (3) is 9.2 $\mu mol \cdot l^{-1} \cdot yr^{-1}$.

Lateral advection may be a source of fermentable dissolved organic carbon and therefore acetic acid in aquifers. It is unclear, however, how much of this carbon survives in groundwater hundreds to thousands of years old. VOA concentrations decrease with depth in lake sediments and the soil-zone because of microbial consumption (Fox and Comerford, 1990; Hordijk *et al.*, 1994), suggesting that only a small fraction of VOAs survive this degradation and is potentially available for recharge waters. Moreover, radiocarbon dating of dissolved organic carbon in the Milk River aquifer suggests that the low molecular weight fraction (including acetic acid) is derived predominantly from sedimentary OM in the aquifer (Murphy *et al.*, 1989; Wassenaar *et al.*, 1990a).

McMahon and Chapelle (1991) obtained a close match between the CO_2 production rate calculated from flowpath modeling of HCO_3^- contents, and those calculated assuming diffusive transport of acetic acid and acetic acid oxidation coupled to sulfate reduction. In the absence of carbonate mineral precipitation, sulfate reduction in aquifers coupled to fermentation in aquitards should increase HCO_3^- concentrations along the flowpath. Thus, for the case of vertical advection, HCO_3^- should increase downward. HCO_3^- concentrations in the study area do not uniformly increase with depth, and uncertainty in groundwater flowpaths makes

independent mass balance calculation of CO_2 production difficult. Moreover, CO_2 volatilization in shallow acidic intervals may cause loss of HCO_3^-.

The CO_2 production rate for the Yegua (9.2 $\mu mol \cdot l^{-1} \cdot yr^{-1}$) is at the high end of the values for deep aquifers (10^{-3} to 10 $\mu mol \cdot l^{-1} \cdot yr^{-1}$), and much lower than rates for shallow marine sediments (10^2 to 10^5 $\mu mol \cdot l^{-1} \cdot yr^{-1}$; Plummer *et al.*, 1990; Lovley and Chapelle, 1995). The Yegua CO_2 production rate is similar to that for the Miocene Hawthorn aquifer in South Carolina (Chapelle *et al.*, 1988), but higher than rates estimated for Cretaceous aquifers in Maryland (0.01 to 1.0 $\mu mol \cdot l^{-1} \cdot yr^{-1}$; Chapelle *et al.*, 1987). As with other deep aquifers, the metabolic rates in the Yegua formation are comparable to the rate estimates for highly oligotrophic deep ocean waters (Williams and Carlucci, 1976). The low (relative to surface environments) rates of OM oxidation in the Yegua formation could be a consequence of the low OM content in sands or the low reactivity of OM compared with that in recent organic-rich sediments (Westrich and Berner, 1984). However, our research indicates that there are factors in addition to organic carbon availability which limit microbial activity. For instance, when sediments from two vertically juxtaposed strata were mixed, the biological production of acetic acid occurred at a rate roughly 1000 times faster than the *in situ* rates based on acetic acid flux calculations. This clearly indicates the presence of bioavailable carbon in the sediments, and that some factor(s) prevents its utilization *in situ*. Similarly, sulfate reduction does not occur in clay-rich strata such as mudstones where ample electron donor and sulfate were present. As discuused previously, these observations indicate that lithological characteristics of fine-grained sediments may limit microbial activity.

The Yegua sediments support microorganisms (Martino *et al.*, 1998) that are clearly metabolically active. Microorganisms in the Yegua formation have developed a spatially separated commensalism; SRB in aquifers benefit from microorganisms in aquitards that provide VOAs produced by sedimentary OM degradation. Microorganisms most likely develop these complex associations as an adaptation for survival in subsurface oligotrophic environments.

Microbiological and geochemical evidence indicate that fermentation processes are active in mudstone and lignitic sediments, and result in production of VOAs. In contrast, SRB activity is mostly confined to aquifer sands and likely results in consumption of VOAs. Geochemical modeling suggests that diffusion and vertical advection transports VOAs into the aquifer where SRB consume the VOAs during respiration. CO_2 production rates in Yegua sediments are within the range obtained for deep aquifers, but are still 10^3 to 10^5 times slower than near-surface microbial processes. Finally, we conclude that these microorganisms survive in oligotrophic aquifer systems through breakdown of sedimentary OM, VOA cycling, and commensal relationships between microbial communities.

References

Balba M.T. and Nedwell D.B. (1982) Microbial metabolism of acetate, propionate and butyrate in anoxic sediment from the Colne Point Saltmarsh, Essex, U.K. *J. Gen. Microbiol.* **128:**1415-1422.

Balkwill, D.L. and W.C. Ghiorse. 1985. Characterization of subsurface bacteria associated with two shallow aquifers in Oklahoma. Appl. Environ. Microbiol. **50:**580-588.

Berg R.R. (1970) Outcrops of the Claiborne group in the Brazos Valley southeast Texas. *Geological Society of America, 4th Annual Meeting of the South-Central Section*, p. 79.

Boutton T. (1991) Stable carbon isotope ratios of natural materials: 1. Sample preparation and mass spectrometric analysis. In *Carbon Isotope Techniques* (eds D.C. Coleman and B. Fry), pp. 155-171, Academic Press Inc., San Diego.

Carothers W.W. and Kharaka Y.K. (1978) Aliphatic acid anions in oil-field waters-implications for origin of natural gas. *Bull. Am. Assoc. Petrol. Geol.* **62**, 2441-2453.

Chapelle F.H. and Bradley P.M. (1996) Microbial acetogenesis as a source of organic acids in ancient Atlantic Coastal Plain sediments. *Geology* **24**, 925-928.

Chapelle F.H., Morris J.T., McMahon P.B. and Zelibor J.L. Jr. (1988) Bacterial metabolism and the $\delta^{13}C$ composition of groundwater, Floridan aquifer South Carolina. *Geology* **16**, 117-121.

Chapelle F.H., Zelibor J.L. Jr., Grimes J.D. and Knobel L.L. (1987) Bacteria in deep coastal plain sediments of Maryland: A possible source of CO_2 to groundwater. *Water Resour. Res.* **23**, 1625-1632.

David M.B., Vance G.F., Rissing J.M. and Stevenson F.J. (1989) Organic carbon fractions in extracts of O and B horizons from a New England spodosol: Effects of acid treatment. *J. Environ. Qual.* **18**, 212-217.

Ehrlich H.L. (1996) *Geomicrobiology*. Marcel Dekker, New York.

Fisher W.L. (1990) Delta systems and oil-gas occurrence. *South Texas Geol. Soc. Bull.* **31**, 13-26.

Fontes J.C. (1992) Chemical and isotopic constraints on ^{14}C dating of groundwater. In *Radiocarbon After Four Decades, An Interdisciplinary Perspective* (eds R.E. Taylor, A. Long and R.S. Kraceds), pp. 242-261. Springer-Verlag, New York.

Fox T.R. and Comerford N.B. (1990) Low-molecular-weight organic acids in selected forest soils of the southeastern USA. *Soil Sci. Soc. Am. J.* **54**, 1139-1144.

Fredrickson J.K., Balkwill D.L., Zachara J.M., Li S.W., Stewart D.L. and Workman D.J. (1991) Physiological diversity of heterotrophic bacteria associated with deep cretaceous sediments of the Atlantic Coastal Plain. *Appl. Environ. Microbiol.* **57**, 402-411.

Ghiorse W.C. and Wilson, J.T. (1988) Microbial ecology of the terrestrial subsurface. *Adv. Appl. Microbiol.* **33**, 107-172.

Giordano T.H. (1994) Metal transport in ore fluids by organic ligand complexation. In *Organic Acids in Geological Processes* (eds E.D. Pittman and M.D. Lewan), pp. 319-354. Springer-Verlag, New York.

Herbert B.E. and Bertsch P.M. (1995) Characterization of dissolved and colloidal organic amtter: A review. In *Carbon Forms and Functions in Forest Soils*. pp. 63-87. Soil Science Society of America, Madison, Wisconsin.

Hordijk C.A., Kamminga H. and Cappenberg T.E. (1994) Kinetic study of acetate in freshwater sediments: Use of stable isotopic tracers. *Geochim. Cosmochim. Acta* **58**, 683-694.

Jones R.E., Beeman R.E. and Suflita J.M. (1989) Anaerobic metabolic processes in the deep terrestrial subsurface. *Geomicrobiol. J.* **7**, 117-130.

Kawamura K. and Ishiwatari R. (1985) Conversion of sedimentary fatty acids from extractable (unbound + bound) to tightly bound form during mild heating. *Org. Geochem.* **8**, 197-201.

Krumholz L.R., McKinley J.P., Ulrich G.A. and Suflita J.M. (1997) Confined subsurface microbial communities in Cretaceous rock. *Nature* **386**, 64-66.

Liu S. and Suflita J.M. (1993) H_2-CO_2- dependent anaerobic O-demethylation activity in subsurface sediments and by an isolated bacterium. *Appl. Environ. Microbiol.* **59**, 1325-1331.

Lundegard P.D. and Kharaka Y.K. (1994) Distribution and occurrence of organic acids in subsurface waters. In *Organic Acids in Geological Processes* (eds E.D. Pittman and M.D. Lewan), pp. 40-69. Springer-Verlag, New York.

Madsen E.L. and Ghiorse W.C. (1993) Groundwater microbiology: Subsurface ecosystem processes. In *Aquatic Microbiology an Ecological Approach* (ed T.E. Ford), pp. 167-213. Blackwell Scientific Publications, London.

Martino D.P., Grossman E.L., Ulrich G.A., Burger K.C., Schlichenmeyer J.L., Suflita J.M. and Ammerman J.W. (1998) Microbial abundance and activity in a low conductivity aquifer system in east-central Texas. *Microbial Ecol.* **35, 224-234.**

McMahon P.B. and Chapelle F.H. (1991) Microbial production of organic acids in aquitard sediments and its role in aquifer geochemistry. *Nature* **349**, 233-235.

Mendoza Y.A., Gülaçar F.O. and Buchs A. (1987) Comparison of extraction techniques for bound carboxylic acids in recent sediments. *Chem. Geol.* **62**, 307-319.

Monetti M.A. and Scranton M.I. (1992) Fatty acid oxidation in anoxic marine sediments: The importance of hydrogen sensitive reactions. *Biogeochem.* **17**, 23-47.

Murphy E.M., Schramke J.A., Fredrickson J.K., Bledsoe H.W., Francis A.J., Sklarew D.S. and Linehan J.C. (1992) The influence of microbial activity and sedimentary organic carbon on the isotope geochemistry of Middendorf Aquifer. *Water Resour. Res.* **28**, 723-740.

Novelli P.C., Michelson A.R., Scranton M.I., Banta G.T., Hobbie J.E. and Howarth R.W. (1988) Hydrogen and acetate cycling in two sulfate-reducing sediments: Buzzards Bay and Town Cove, Mass. *Geochim. Cosmochim. Acta* **52**, 2477-2486.

Parkes R.J. and Taylor J. (1983) Analysis of volatile fatty acids by ion-exclusion chromatography, with special reference to marine pore water. *Mar. Biol.* **77**, 113-118.

Plummer L.N., Busby J.F., Lee R.W. and Hanshaw B.B. (1990) Geochemical modeling of the Madison Aquifer in parts of Montana, Wyoming, and South Dakota. *Water Resour. Res.* **26**, 1981-2014.

Routh J., McDonald T.J. and Grossman E.L. (1998) Sedimentary organic matter sources and depositional environment in Yegua formation (Brazos County, Texas). *Org. Geochem. (submitted).*

Sansone F.J., Andrews C.C. and Okamoto M.Y. (1987) Adsorption of short-chain organic acids onto nearshore marine sediments. *Geochim. Cosmochim. Acta* **51**, 1889-1896.

Sansone F.J. and Martens C.S. (1982) Volatile fatty acid cycling in organic-rich marine shales. ***Geochim. Cosmochim. Acta* 46, 1575-1589.**

Shaw D.G. and McIntosh D.J. (1990) Acetate in recent anoxic sediments: Direct and indirect measurements of concentration and turnover rates. *Estuar., Coast. and Shelf Sci.* **31**, 775-788.

Sinclair J.L. and Ghiorse W.C. (1989) Distribution of aerobic bacteria, protozoa, algae, and fungi in deep subsurface sediments. *Geomicrobiol. J.* **7**, 369-377.

Surdam R.C., Boese S. and Crossey L.J. (1984) The chemistry of secondary porosity. In *Clastic Diagenesis* (eds D.A. McDonald and R.C. Surdam) *Mem. Am. Assoc. Petrol. Geol.* **37**, 127-149

Takijima Y. (1964) Studies on organic acids in paddy field soils with reference to their inhibitory effects on the growth of rice plants Part 1. Growth inhibitory action of organic acids and adsorption and decomposition to them by soils. *Soil Sci. Plant Nutr.* **10**, 14-21.

Ulrich G.A., Martino D., Burger K.C., Routh J., Grossman E.L., Ammerman J.W. and Suflita J.M. (1998) Microbial interactions in the terrestrial subsurface: Sulfur cycling, heterogeneity, and survival. *Microbial Ecol.* **36**, 141-151.

Wassenaar L.I., Hendry M.J., Aravena R. and Fritz P. (1990a) Organic carbon isotope geochemistry of clayey deposits and their associated porewaters, southern Alberta. *J. Hydrol.* **120**, 251-270.

Westrich J.T. and Berner R.A. (1984) The role of sedimentary organic matter in bacterial sulfate reduction: The G model tested. *Limnol. Oceanog.* **29**, 236-249.

Williams P.M. and Carlucci A.F. (1976) Bacterial utility of organic matter in the deep sea. *Nature* **262**, 810-811.

Winfrey M.R. (1984) Microbial production of methane. In *Petroleum Microbiology* (ed R.M. Atlas), pp. 57-77. MacMillan Publishing, London.

Yamane I. and Sato K. (1967) Method for extracting organic acid from flooded soil. *Soil Sci. Plant Nutrition* **13**, 113-120.

Chapter 6

Barite Dissolution and Electron Acceptor Availability on the Rate of Sulfate Reduction in a Polluted Aquifer

Abstract

Field and laboratory studies were used to evaluate the biogeochemical factors governing biodegradation activity linked to sulfate reduction in a shallow aquifer contaminated with landfill leachate. Depth profiles revealed that sulfate reduction in the aquifer correlated with the concentration of sulfate. Experimental manipulation of the sulfate status in aquifer samples revealed a Michaelis Menten relationship with a K_m of 80 μM SO_4^{2-}. The ambient concentration of sulfate in most regions of the aquifer were generally below this value. This led to the hypothesis that the concentration and sources of sulfate helped govern *in-situ* sulfate-reducing activity. Since the mineral barite ($BaSO_4$) was found to be a major constituent of the aquifer solids, we investigated the possibility that barite dissolution was a source of electron acceptor. Laboratory incubations of unamended and barite-amended landfill slurries accumulated barium as the result of sulfate consumption to low levels (~ 1 μM). Evidence that barite dissolution occurred *in-situ* included the finding that barium concentrations were higher (up to 100 μM) in contaminated groundwater where sulfate was depleted (< 10 μM). This suggests that active sulfate reduction decreased sulfate to levels that allowed barite dissolution and a resupply of a low level of sulfate to the aquifer. The second

identified source of sulfate was iron sulfide oxidation in shallow regions where despite relatively high rates of sulfate reduction, sulfate was increased and the concentration of iron sulfides were comparatively low. Another important source of electron acceptor in the aquifer is the advective flux of sulfate in a highly permeable region just above the confining layer at the bottom of the aquifer where increased sulfate reduction was measured. These observations indicate that the concentration of sulfate and the processes which supply this electron acceptor, are major determinants governing the biodegradation of contaminants via sulfate reduction in the leachate-contaminated aquifer.

Introduction

Anaerobic microbial processes are prevalent in many subsurface environments due to limited oxygen availability. For instance, increased organic loading in contaminated aquifers typically leads to the relatively rapid depletion of oxygen and the onset of anaerobic respiration coupled to the reduction of NO_3^-, Mn-oxides, Fe-oxides, SO_4^{2-}, and HCO_3^-. While the importance of these processes in the degradation of a variety of contaminants in subsurface regions has been recognized (17, 21), the factors which limit anaerobic biodegradation in aquifers are largely unknown.

Sulfate reduction is a major contributor to the degradation of naturally occurring organic matter and contaminant molecules in subsurface environments. Therefore, it is important to consider the factors which govern this ecologically important process. The availability of organic matter as electron donor is often an

important determinant of sulfate-reducing activity. For instance, increased sulfate reduction has been detected in sands and sandstone formations adjacent to organic-rich lignite, clay, and shale (18, 36). The elevated activity near these sedimentary interfaces was attributed to increased organic carbon and fermentation products (i.e. organic acids)(18, 32), which diffuse from more consolidating formations (27, 28). Similarly, Jakobsen and Postma attributed the variability of sulfate reduction activity in a noncontaminated sandy aquifer to differences in the reactivity of the endogenous organic matter (15).

Several studies have found higher microbial populations and metabolic potential in sandy sediments (14, 16, 30) relative to less permeable subsurface regions. In the clay-rich formations of the Yegua formation in Texas, fermentable carbon, organic acids, and sulfate concentrations are high yet SRB activity is absent relative to adjacent sandier formations (32). This suggests that the lithological characteristics such as water potential, pH, pore throat diameter, or availability of nutrients (*e.g.*, Ghiorse and Wilson, 1988; Lovley and Chapelle, 1995) rather than the availability of electron donors and acceptor can limit sulfate reduction in fine-grained sediment strata. The decreased pore throat diameter of shales has been implicated to be an important limitation of sulfate-reduction in consolidated rock formations (10).

Subsurface microorganisms must integrate the turnover of organic matter and pollutants into the existing biogeochemical cycles and the hydrological characteristics of aquifers. Sulfate reduction is generally of decreased importance when thermodynamically more favorable electron acceptors such as nitrate and ferric iron are available in sufficient quantities (20, 24). Therefore, the

biogeochemical processes which contribute to the source of electron acceptor ultimately select for and sustain respiratory process in the subsurface. For example, sulfate derived from the oxidation of pyrite in shallow oxidized subsurface areas in conjunction with advective transport of the anion, serves as an important source of electron acceptor to anaerobic regions of an unconsolidated aquifer (25, 36). The aim of the current study was to evaluate the ecological factors that regulate anaerobic metabolism, mainly sulfate reduction, in a landfill-leachate contaminated aquifer. A combined microbiological and geochemical approach was used in an effort to evaluate *in-situ* microbial activity.

Materials and Methods

Field Site. The aquifer study site is located on the alluvium of the South Canadian River in Norman, Oklahoma and underlies a now closed municipal landfill (Figure 1). The aquifer is contaminated with leachate from the landfill. The alluvium consists primarily of medium grained sand with interbedded layers of clay which are typically only a few cm thick with the exception of a 0.6 m-thick clay layer at approximately 4 m below land surface. The alluvium is 10 to 15 m deep and is confined by the underlying shale and mudstone sediments of the Hennessey Group. Sediments immediately above this consolidating layer consist of larger grained sands, gravel, and some clay. Hydraulic conductivity estimates based on slug tests range from 8.4×10^{-7} to 2.5×10^{-4} m s^{-1}, with a median value of 6.6×10^{-5} m s^{-1} (33). The highest and lowest values were measured in the deep gravel layer and clay-rich interval respectively. A more detailed description of the hydrology is available (33).

Ground Water Sampling. Groundwater samples were collected in April 1996. using a network of small diameter temporary wells. The wells were driven to the desired sampling depth and water was pumped through 8.7 cm long screens using teflon tubing and a peristaltic pump. Samples were collected from three locations immediately downgradient from the landfill (sites 35, 38, and 40; Figure 1), from a leachate impacted slough, and from a 'background' location (site NPD; Figure 1). Samples were stored in glass bottles at 4 ^{0}C and transported to the laboratory. Dissolved sulfide was determined on site as previously described (7).

Sediment Sampling. Sediment cores were obtained in March of 1996 using a Geoprobe Macro-Core Soil Sampler. Immediately after retrieving the samples in the field, each end of the cores was flushed with N_2 and capped with large butyl rubber stoppers. The cores were stored at 4 °C under N_2. Sediments used to evaluate the kinetics of sulfate reduction and the dissolution of barite were obtained from beneath the water table at site 35 (Figure 1) as previously described (3).

Sulfate reduction activity, iron sulfide, and anion analyses. Sulfate reduction rate measurements and geochemical analyses were performed within 2 days after obtaining the cores. Sediments were handled inside an anaerobic glove bag filled with 100% N_2 . For sulfate reduction assays, small sediment samples were obtained by pushing 5 ml syringes (with distal end cut and removed) into the cores. An anoxic solution of $^{35}SO_4$ (100 µL)(20 µCi ml^{-1}; carrier free; ICN) was injected into the samples with a syringe and needle. The incubations were sealed with butyl rubber stoppers and stored under a N_2/CO_2 (80:20) headspace. The

samples incubated for 17 h at 18°C which was the approximate *in-situ* temperature of the aquifer at the time of sampling. Sulfate reduction rates, iron monosulfides extractable with 6N HCl, and total reducible inorganic sulfides (TRIS) were determined as previously described (35). Pore waters were centrifuged from 20 g aliquots of sediment and analyzed for SO_4^{2-}, NO_3^-, and Cl^- in the pore waters were determined by ion chromatography (36).

Sulfate reduction kinetics. We evaluated the factors which limit sulfate reduction including electron acceptor and donor availability using contaminated sediments and groundwater obtained from the aquifer. Sediment for these experiments was first flushed with anoxic and sulfate free groundwater until the concentration of sulfate in the effluent was below 5 μM. Aliquots (5 g) of this sediment and 2 ml of sulfate-free groundwater were placed inside 25 ml serum bottles. The incubations were sealed with butyl rubber septa and flushed with N_2:CO_2 (80:20). Triplicate bottles were amended with various levels of sulfate ranging from 5 μM to 1 mM. Other incubations received amendments of both 1 mM SO_4^{2-} and an electron donor as either acetate (1 mM), lactate (1 mM), or H_2 (as a H_2:CO_2 / 80/20 headspace). Radiolabeled sulfate was added (2.5 μCi per incubation) to measure sulfate reduction activity as described. The bottles were incubated at 25°C for 26 h in the dark.

Barite Dissolution experiments. Naturally occurring or synthetic barite dissolution in aquifer sediments and in enrichments of sulfate-reducing bacteria was evaluated. The former experiments consisted of sediment slurries (20 g / 20 ml anoxic groundwater) prepared in 60 ml serum bottles amended with 20 mg of

$BaSO_4$. The slurries were amended with sodium lactate (to 20 mM) in an effort to stimulate sulfate reduction. The synthetic $BaSO_4$ was prepared by reacting H_2SO_4 with $BaCl_2$ (19). The $BaSO_4$ suspension was heated and stirred for 4 days to increase the crystallinity in an effort to imitate naturally occurring barite. The solid was rinsed thoroughly with deionized water and dried. Sodium molybdate was amended (to 5 mM) to some of the incubations to inhibit sulfate reduction. Autoclaved incubations served as abiotic controls. The bottles were incubated at 25 °C in the dark. Liquid samples were withdrawn from the slurries over the course of the experiment using N_2-flushed syringes, filtered (0.25 μm) and stored at -20°C. Barium concentrations in the liquid samples were determined using atomic adsorption spectroscopy. Sulfate concentration was analyzed by ion chromatography as previously described (36).

Barite dissolution experiments were also performed in a more defined system consisting of sulfate-reducing enrichment cultures containing synthetic $BaSO_4$ as the sole source of barium. Inocula for the enrichment experiments were obtained by adding approximately 1 g of aquifer sediment to each of several 60 ml serum bottles containing sulfate amended groundwater (2 mM SO_4^{2-}) and a H_2:CO_2 (80:20) headspace. The enrichments turned black within 2 days due to precipitation of iron sulfides. After a 2-week incubation, the liquid phases of the replicate enrichments were combined (yielding ~ 200 ml) and diluted to 500 ml in sulfate-free groundwater. The dissolution of synthetic $BaSO_4$ was evaluated in anoxic incubations consisting of 30 ml of this culture suspension supplemented with 20 mM sodium lactate, 5 mg of synthetic $BaSO_4$, and a N_2:CO_2 (80:20) headspace.

Controls were amended with sodium molybdate (5 mM final concentration) to inhibit sulfate reduction.

Iron sulfide oxidation experiments. The conditions which supported the oxidation of reduced sulfur species to sulfate were evaluated using sediments obtained from site CS-1 (6.4 m depth, Figure 1). Since elemental sulfur and dissolved sulfide were not detected in the sediments and groundwaters respectively (data not shown), the source of potentially oxidizable sulfur in these experiments was assumed to be iron sulfide minerals which were easily detected. Anoxic sediment slurries (10 g / 10 ml) were prepared with groundwater. The potential sulfide oxidants tested included 1 ml of a poorly crystalline manganese oxide suspension (22), NO_3^- (5 mM final concentration), 1 ml of an iron oxyhydroxide suspension (23), and O_2. The aerobic incubations were closed with sterile cotton to allow for air exchange, while the other incubations were sealed in typical fashion. Anoxic incubations which were not supplemented with potential oxidants served as background controls. Sodium chloride (20% w/v) was added as a microbial inhibitor to aerobic and anaerobic control incubations.

Results

Sulfate reduction and associated sulfur geochemistry. The sulfate reduction rates with depth ranged from 0.06 to 5.6 μM SO_4^{2-} day^{-1} (Figure 2). The highest activity occurred immediately beneath the water table. Sulfate reduction was not detected in the highly oxidized sediments above the water table or in the

clay layer at ~ 4 m. Sulfate concentrations ranged from 10 μM to 9 mM. Dissolved sulfide was below detection in all of the groundwater samples including intervals where sulfate reduction was detected. However, the concentration of TRIS correlated with the sulfate reduction rates, suggesting that sulfide precipitated rapidly as iron sulfide minerals. This correlation was most pronounced beneath the clay interval at 4 m. The amount of TRIS in the sediments ranged from 0.01 to 16.8 μmol S g^{-1} (Figure 2). TRIS increased from the water table to just above the clay layer, and from below the clay to the interval above the consolidating bedrock. Nitrate was below detection (~5 μM) in all of the samples except those taken at the water table (data not shown). Excluding results obtained from and immediately beneath the clay layer, the sulfate reduction rates correlated positively with sulfate concentration throughout the depth profile (Figure 2) suggesting that sulfate was an important determinant of sulfate-reducing activity. In consistent fashion, sulfate reduction activity in aquifer sediment slurries amended with various concentrations of SO_4^{2-} exhibited Michaelis-Menten kinetics (Fig. 3A) with an apparant K_m and V_{max} of 84 μM and 0.13 nmol SO_4^{2-} g^{-1} d^{-1} (0.83 μM SO_4^{2-} d^{-1}), respectively. Sulfate concentrations in most regions of the aquifer (Figure 2) are below the apparent Km for SO_4^{2-}. Sediment slurries which were amended with saturating levels of SO_4^{2-} (1000 μM) and hydrogen as an electron donor, reduced sulfate at an increased rate (~ 3x) relative to incubations which received only SO_4^{2-} (1000 μM) (Figure 3B). This suggests that electron donor availability limited sulfate-reducing activity when saturating levels of SO_4^{2-} were provided. The addition of acetate and lactate did not stimulate sulfate reduction activity in incubations containing saturating levels of SO_4^{2-} during the 26 h incubation period.

Barium Geochemistry. It was important that the sources of sulfate be identified given the observation that the concentration of this electron acceptor helped govern *in-situ* sulfate-reducing activity. We examined whether the dissolution of barite ($BaSO_4$ ------> $Ba^{2+} + SO_4^{2-}$) served as a source of electron acceptor since the mineral is a common constituent of sediments in the Canadian River drainage area. Scanning electron microscopy of the sediment grains confirmed the presence of barite and small (< 2 μm) pyrite grains on the surfaces of quartz (Figure 4). Barium can also be found in potassium containing minerals including feldspar, micas, and clay. However, the sediments of the alluvium contained a much higher content of barium (0.03 to 0.06 wt. %) relative to potassium levels (1-2 wt.%) (data not shown) which would be expected if potassium containing minerals were a substantial source of barium .

Dissolved barium was higher in contaminated groundwater samples that were depleted in sulfate. Barium concentrations were typically between 50 μM and 100 μM in contaminated areas, and from 0.5 μM to 3 μM in background ground waters (data not shown). Vertical profiles indicated that Ba^{2+} and SO_4^{2-} were inversely related in groundwater samples obtained from two highly contaminated regions adjacent to the east (site 35) and west (site 40) landfill mounds (Figure 5). The highest concentration of dissolved Ba^{+2} at both sampling locations was detected near the center of the aquifer where the SO_4^{2-} concentrations were lowest. A sharp decrease in Ba^{2+} occurred just beneath the water table at site 35 where the SO_4^{2-} concentration was highest.

Barite Dissolution. Dissolved barium accumulated in lactate-amended aquifer slurries, while sulfate was consumed relatively rapidly to a concentration of approximately 1 μM (Figure 6A). Barium accumulated at a faster rate and to a greater extent when $BaSO_4$ was added to the slurries (Figure 6B). Sulfate decreased at essentially the same rate as in the $BaSO_4$ unamended incubations, and again was not depleted below 1 μM. This suggests that a near steady state sulfate concentration exists where sulfate depletion is essentially balanced by sulfate production via barite dissolution. Consistent with this suggestion, barium levels continued to increase in nonsterile incubations during the course of the experiments. This was not evident in molybdate treated or sterile controls. Dissolved barium was not produced in slurries treated with 5 mM molybdate to inhibit sulfate reduction (e.g. Figure 6B) or in the sterile controls (e.g. Figure 6 A).

Dissolved barium also accumulated in the sulfate-reducing enrichment cultures containing synthetic $BaSO_4$ as the sole source of sulfate and barium (Figure 7). Dissolved barium did not accumulate in the molybdate-amended or sterile controls.

The oxidation of iron sulfide containing minerals in sediment. We evaluated whether the oxidation of iron sulfides contributed to the supply of sulfate. *In situ* evidence for this process was found at the water table, where despite relatively high sulfate reduction activity, the concentration of iron sulfides and sulfate were comparatively low and high respectively. Laboratory studies indicated that sulfate was produced in aerobic sediment slurries at relatively high rates (750 μM over 20 d) compared to the anaerobic incubations (Figure 8). Anaerobic

slurries containing manganese oxides produced sulfate but at comparatively much lower levels (250 μM over 140 d). Less than 75 μM sulfate was produced above the unamended controls in anaerobic incubations amended with nitrate and ferric iron (Figure 8). Sulfate was not produced in the NaCl inhibited controls (data not shown).

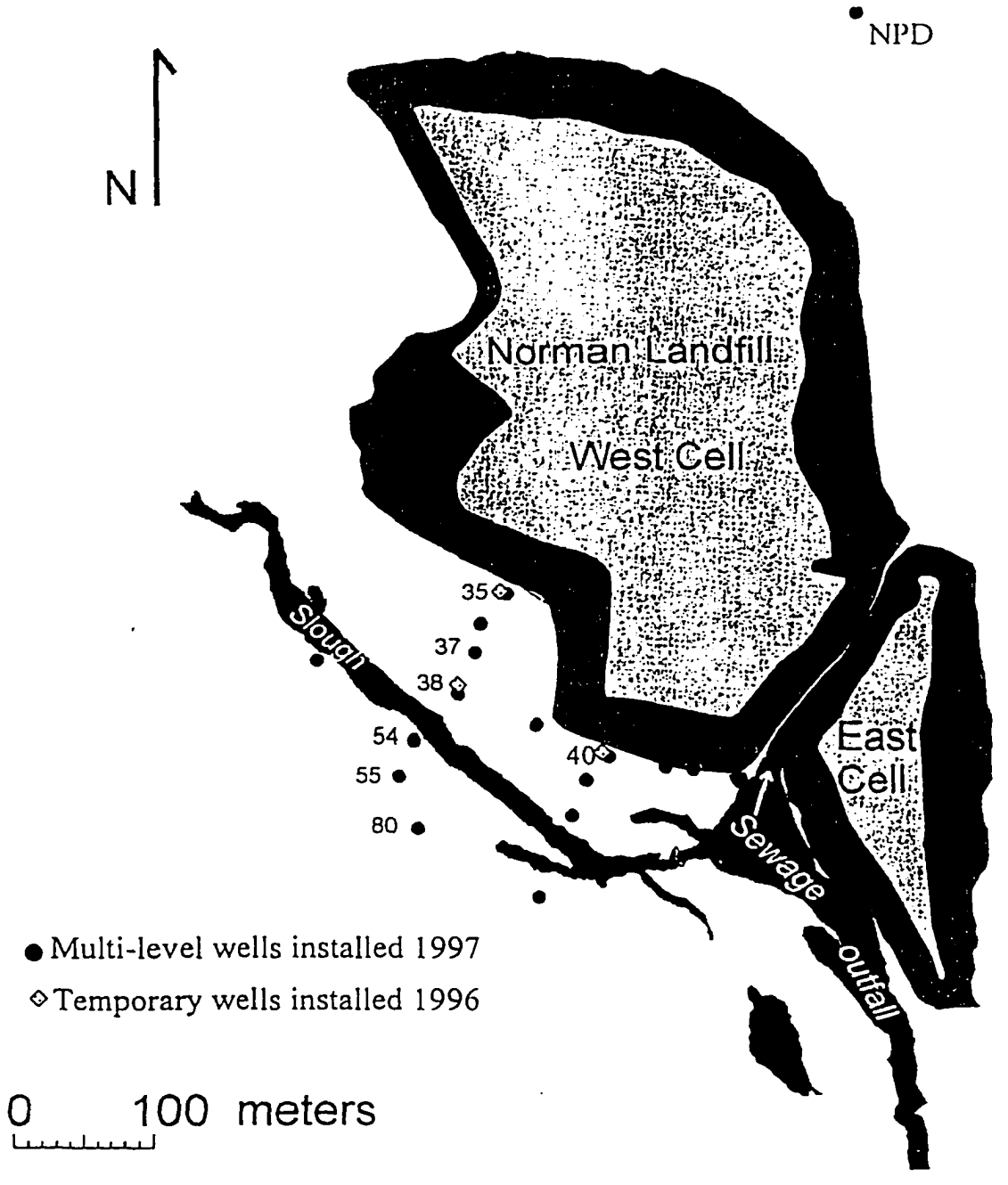

Figure 1. Map of the Norman, OK Landfill and the sampling locations.

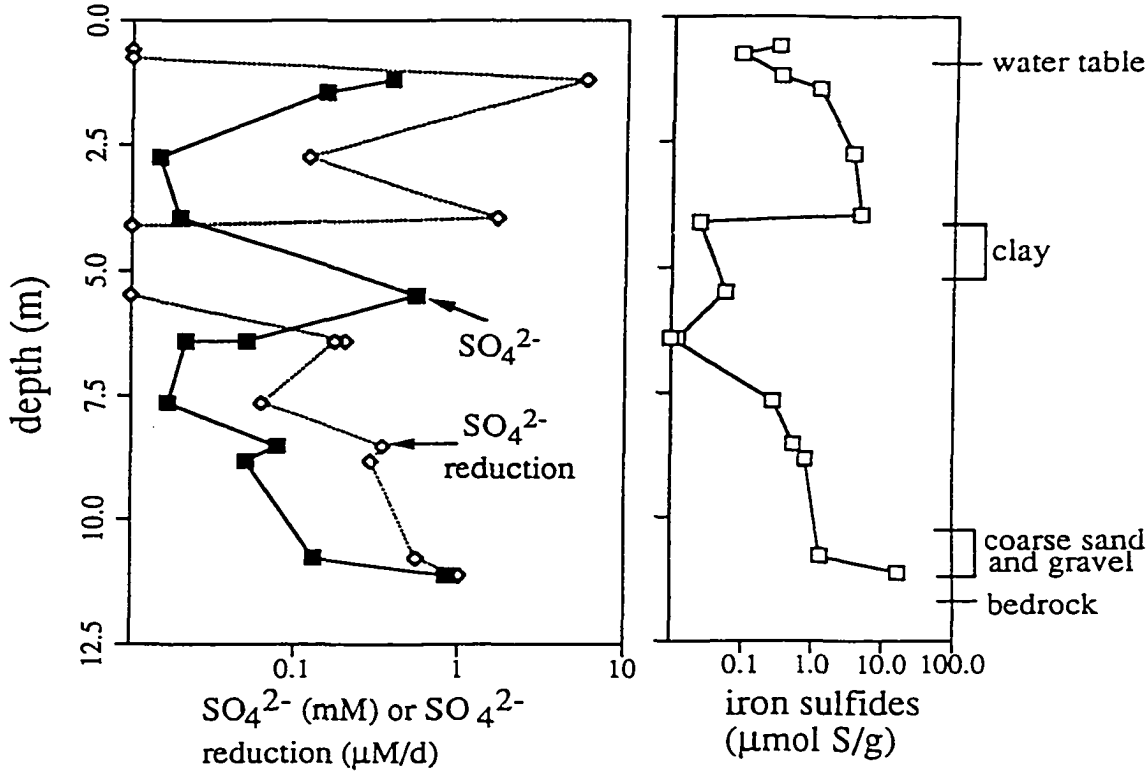

Figure 2. Depth profile of sulfate reduction rates, sulfate concentration, and iron sulfide content of sediments at site 40. The sulfate reduction and iron sulfide data represent the average of duplicate incubations. Depth is in units of meters below land surface.

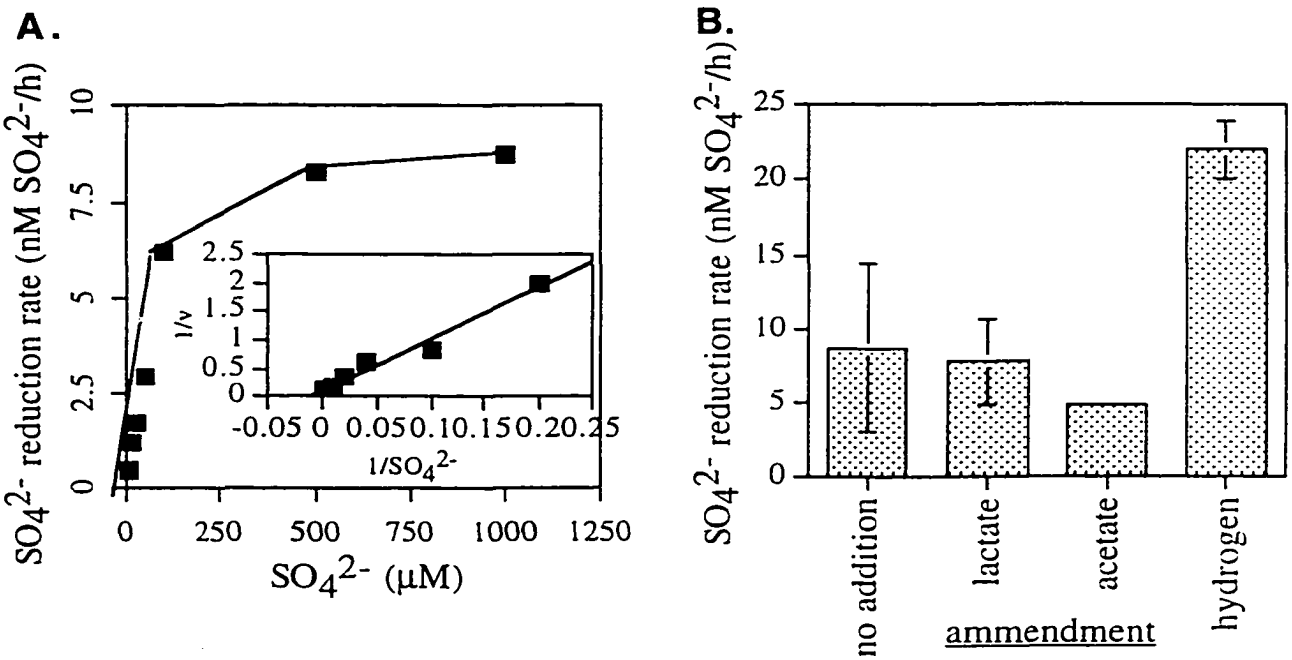

Figure 3. Initial rates (A) of sulfate reduction in sediment slurries at various sulfate concentrations. The insert shows the corresponding Lineweaver-Burk plot. Sulfate reduction rates (B) at a saturating level of sulfate (1 mM) with the addition of potential electron donors. The values represents the mean of triplicate determinations, with the standard errors (error bars) illustrated.

Figure 4. SEM micrograph of sediment showing the surface of quartz with small grains of framboidal FeS_2 and a fragment of barite in the upper right. The sample was obtained from site 37 at a depth of 11.3 m.

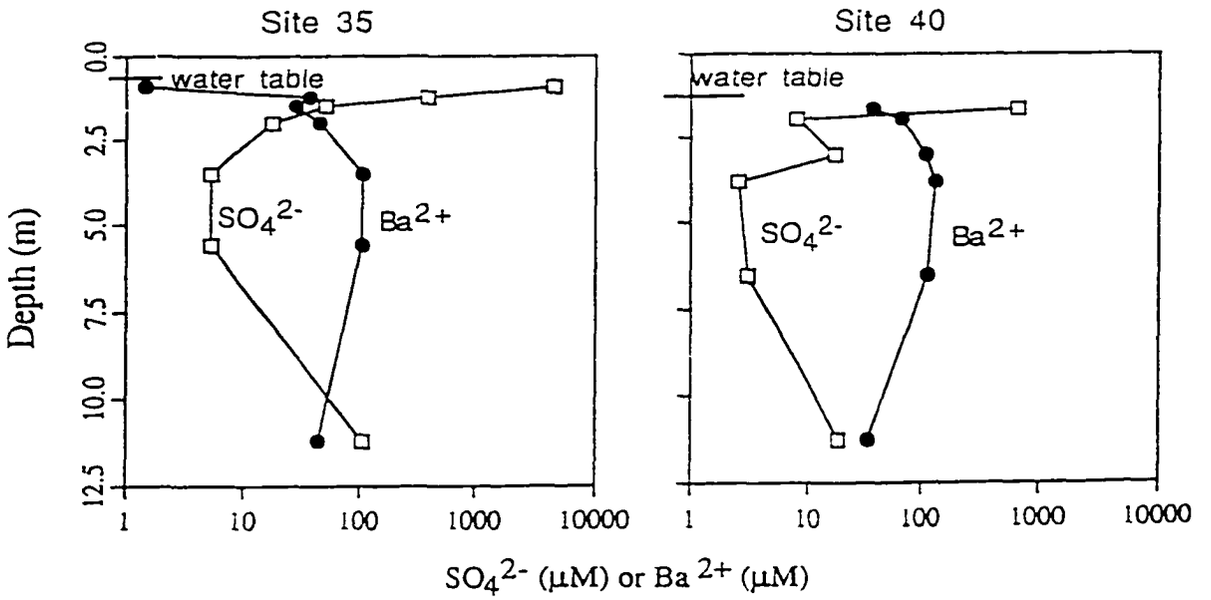

Figure 5. Depth profiles of sulfate and barium in groundwaters at sites 35 and 40. Depth is in units of meters below land surface.

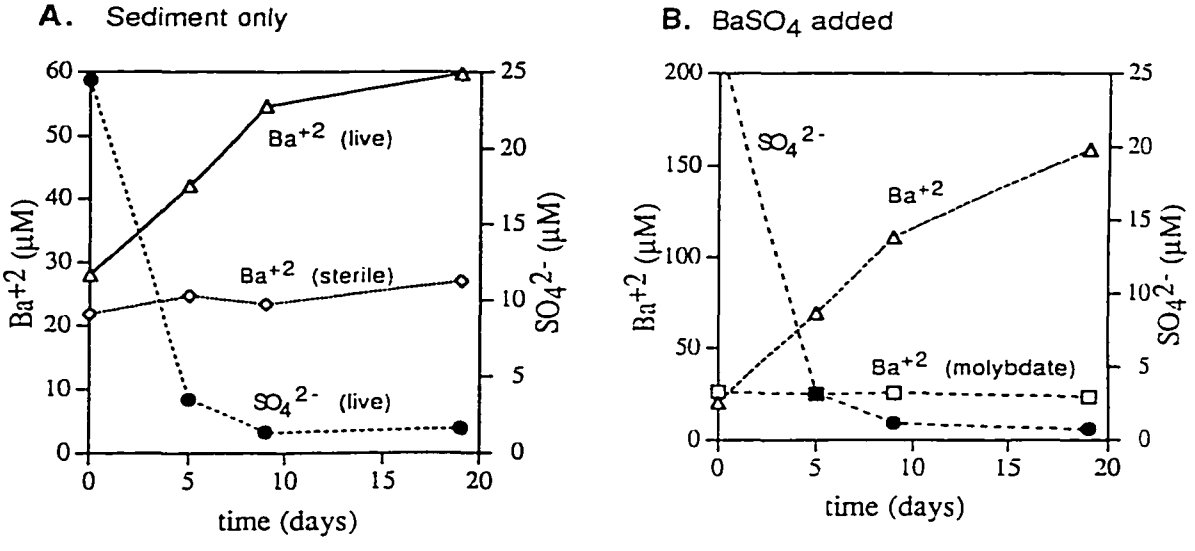

Figure 6. Barium accumulation and sulfate depletion in (A) sediment slurries and (B) sediment slurries amended with $BaSO_4$.

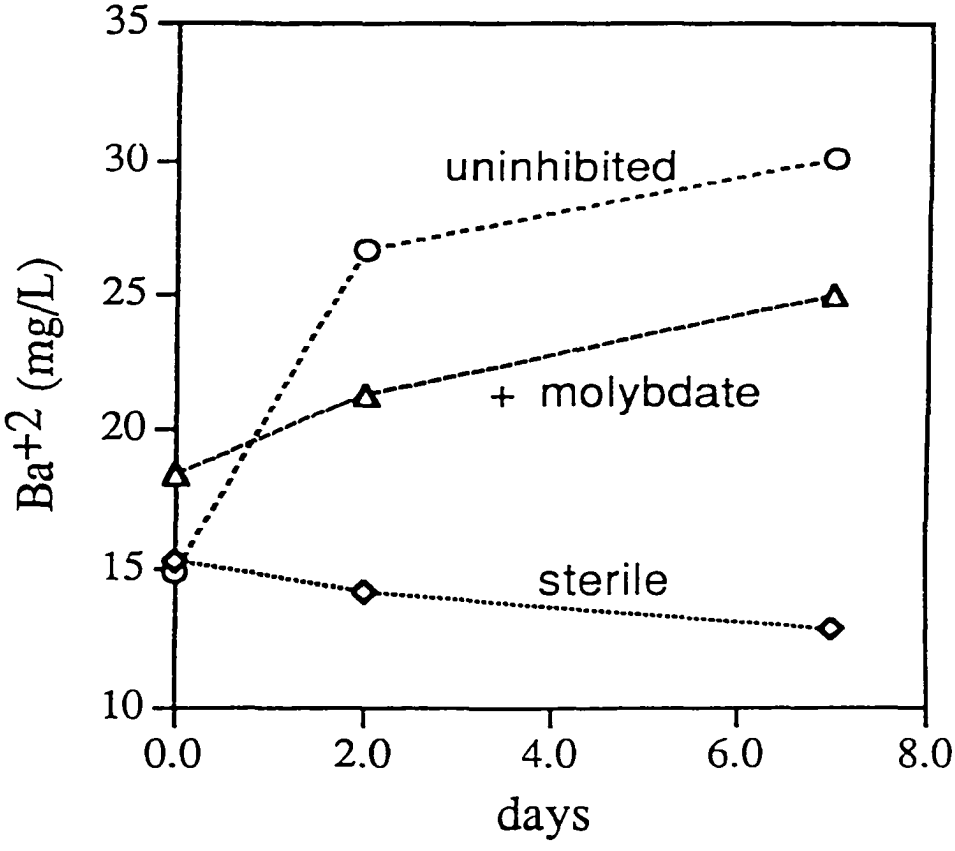

Figure 7. Barium accumulation in sulfate-reducing enrichments amended with $BaSO_4$.

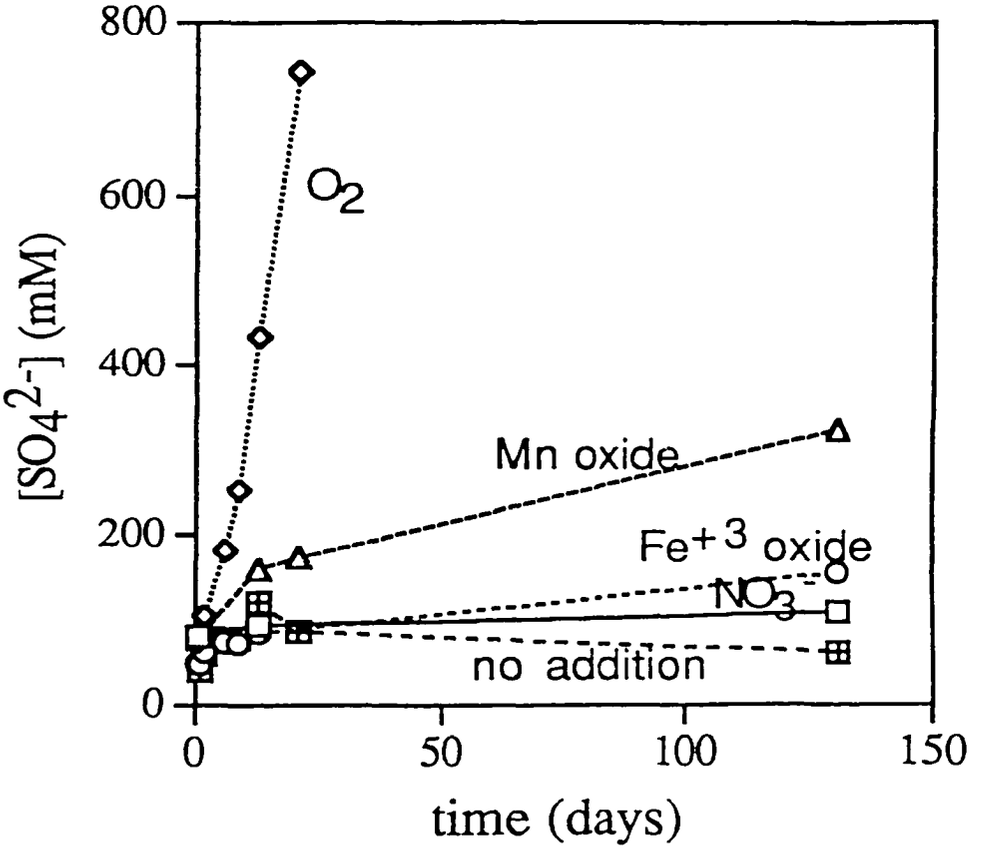

Figure 8. Production of sulfate in sediment slurries in response to the addition of potential iron sulfide oxidants.

Discussion

We used soluble and solid phase geochemical profiles coupled with laboratory rate experiments to evaluate the ecological factors governing the *in-situ* biodegradation activities in a landfill leachate contaminated aquifer. In a previous study (12), oxygen and nitrate were reported to be below detection and iron reduction was observed in only a few samples in the study region near the base of the landfill. Methanogenesis was mainly important in a zone of low sulfate near the center of the aquifer depth profile. In the current study, sulfate reduction was found to be widely distributed. Overall, the sulfate reduction activity measurements paralleled the concentration of sulfate in the sediment profile (Figure 2). Indeed during periodic sampling events over the past three years, higher sulfate was always present in shallow regions and in deeper areas above the consolidating layer where the highest rates of sulfate reduction were detected (Figures 2 and 5). In consistent fashion, we observed Michalis-Menten saturation kinetics with an apparent Km of approximately 80 μM. This value and lies within the wide range of the sulfate affinity constants obtained with pure cultures of SRB (4.8 - 244 μM SO_4^{2-}) (11). It is interesting to note that the ambient concentrations of sulfate in most regions of the aquifer were generally below this apparent Km value. Thus, the concentration of sulfate was likely a major variable controlling sulfate-reducing activity. It was important that the sources of sulfate be identified since they likely impact the intrinsic biodegradation capacity in the aquifer.

We have been able to identify three sources of sulfate to the aquifer. In shallow regions, the oxidation of iron sulfides to sulfate is apparently an important source of electron acceptor. Experiments have shown that hydrogen sulfide, produced as an endproduct of sulfate reduction, precipitates rapidly as iron sulfide minerals in the aquifer (Chapter 7). Such minerals were found to be rather stable and not easily oxidized under anaerobic conditions (Figure 8). In the presence of a variety of potential electron acceptors, little sulfate formation was evident in these experiments except when manganese oxide was present. Anaerobic sulfide oxidation catalyzed by microorganisms using manganese has been shown in marine environments mainly at the aerobic-anaerobic interface (1). The *in-situ* oxidation of iron sulfides with manganese was not specifically evaluated in our experiments. However, when aerobic conditions prevail, reduced sulfides can be readily reoxidized and sulfate is observed to accumulate.

In situ evidence for this process was found at the water table, where despite relatively high rates of sulfate reduction, the concentration of iron sulfides were comparatively low (Figure 2). We typically observed larger quantities of sulfides with increased depth and decreased levels of sulfate and sulfate reduction. Further, sulfate concentrations were highest just above and below the water table, and decreased rapidly with depth (Figure 5). These observations suggest that the oxidation of iron sulfides occurs at the water table where periodic inputs of oxygen allow sulfate regeneration and restoration to the aquifer. The observation that iron sulfide oxidation near the water table contributes to the supply of SO_4^{2-} is analogous to findings from an uncontaminated aquifer in the Yegua formation of East Central Texas (36).

Another important source of sulfate in the aquifer is through advective flux occurring just above the confining layer at the bottom of the aquifer. Hydraulic conductivity is relatively high (33) in this region where course grained sands and gravel are the predominate sediment types, allowing sulfate-laden uncontaminated groundwater to mix with the sinking leachate plume. The decreased Cl^- and lower specific conductance (33) of groundwater in deeper portions of the aquifer is consistent with the increased influence of uncontaminated waters. If sulfate was an important determinant of sulfate reduction one might expect to find increased rates of sulfate reduction in this region. In fact, increased rates of sulfate reduction could be measured at locations near the bottom of the aquifer.

Finally, we investigated the possibility of barite ($BaSO_4$) dissolution as an additional sulfate source based on the observations of the presence of this mineral (Figure 4). Under conditions of low sulfate (<10 μM), as in many aquifer locations, barite will dissolve resulting in a sulfate resupply mechanism and the production of soluble barium. Laboratory incubations of unamended and barite-amended sediment slurries accumulated barium as the result of sulfate consumption to low levels (~1 μM) (Figure 5). Barium did not accumulate in sterile controls or in live bottles that contained 5 mM molybdate as an inhibitor of sulfate reduction. These data suggest that the activity of sulfate-reducing bacteria diminishes the *in situ* sulfate concentration to levels that favor barite dissolution, and a resupply of a low level of sulfate to the aquifer. The observation that barium was produced at a faster rate and to a greater extent in sediment slurries amended with synthetic $BaSO_4$ (Figure 5), suggests that either the abundance or the reactivity of the natural barite may potentially limit dissolution *in-situ* .

The dissolution of barite has previously been linked to sulfate reduction in microbial studies with pure and mixed cultures of sulfate-reducing bacteria (2, 4, 26) and in geochemical investigations mainly of marine systems (34). Our study, with respect to barium biogeochemistry, focused on the biotic and abiotic factors that impact the mobilization and immobilization of barium in shallow aquifers. This is of significance because barite and dissolved Ba^{2+} are commonly found in sedimentary and igneous rock formations and associated groundwaters respectively (5, 9, 29). The toxicity of barium is well documented (31) and Ba^{2+} can approach toxic levels in groundwaters. An additional reason for the interest in the biogeochemistry of barium is that the chemical behavior of this element is very similar to radium which is a naturally occurring radioactive element commonly found in subsurface formations (8, 9). Thus, the factors that govern radium and barium mobilization and immobilization in the subsurface are likely similar. For example, radium and barium can both be mobilized via sulfate reduction from $(Ba,Ra)SO_4$ sludge produced during uranium mining operations (8).

Our results indicate that the sources and supply of sulfate were important variables controlling the levels of dissolved barium. A strong inverse correlation between dissolved barium and sulfate occurred within the aquifer (Figure 5). Dissolved barium was found at much lower levels where the supply of sulfate was increased in shallow regions where iron sulfide oxidation occurred, and near the bottom of the aquifer where the advective flux of sulfate was increased. Barite dissolution is extremely unfavorable above μM levels of sulfate. In fact barium precipitation as barite, is more favorable under high sulfate conditions (9). Our

observations indicate that the barium cycle is dynamic and highly interrelated with the cycling of sulfur in the aquifer.

The utility of combining solid phase geochemical and microbiological investigations to clarify the important microbial processes was demonstrated in this study. Dissolved sulfide and very little sulfate were ever measured in groundwater despite the biogeochemical importance of sulfate reduction. Furthermore, when resupply mechanisms for sulfate exist, methods for evaluating the oxidation (electron accepting) capacity of aquifers that are based on the ability of aquifer sediments to reduce certain chemical indicators (13) would discount the importance of sulfate as an electron acceptor.

The distribution of sulfides in contaminated sediments vs. background zones was useful in determining the localization of *in-situ* sulfate reduction. For example, in deeper portions of the aquifer under study, the distribution of solid phase inorganic sulfides tracked the sulfate reduction rate measurements and sulfate concentration. Iron sulfide content would not however be a useful indication of sulfate reduction in regions where substantial sulfide oxidation occurs, in aquifers that do not contain sufficient reactive iron (6) to precipitate hydrogen sulfide, or in sediments which contain high background levels of iron sulfides. Clearly, the analysis of groundwater geochemistry is an essential part of evaluating the dominant electron accepting conditions in an environment, but such information is most useful when it is bolstered by multiple lines of evidence on both the solid and liquid phases of an aquifer.

Our findings suggest that the level of sulfate in the aquifer is a dominant factor modulating the destruction of contaminants linked to sulfate reduction. In

addition, several resupply mechanisms serve to buffer the sulfate status in this environment. The importance of sulfate reduction in subsurface environments may be a reflection of the diversity of the hydrological and biogeochemical processes which contribute to the availability of sulfate.

References

1.**Aller, R. C., and P.D. Rude.** 1987. Complete oxidation of solid phase sulfides by manganese and bacteria in anoxic marine sediments. Geochim. Cosmochim. Acta. **52:**751-765.

2.**Baldi, F., M. Pepi, D. Burrini, G. Kniewald, D. Scali, and E. Lanciotti.** 1996. Dissolution of barium from barite in sewage sludges and cultures of *Desulfovibrio desulfuricans*. Appl. Environ. Microbiol. **62:**2398-2404.

3.**Beeman, R. E., and J. M. Suflita.** 1987. Microbial ecology of a shallow unconfined groundwater aquifer polluted by municipal landfill leachate. Microb. Ecol. **14:**39-54.

4.**Bolze, C. E., P. G. Malone, and M. J. Smith.** 1974. Microbial mobilization of barite. Chem. Geol. **13:**141-143.

5.**Breit, G. N., M.B. Goldhaber, D.R. Shawe, and E.C.Simmons.** 1990. Authigenic barite as an indicator of fluid movement through sandstones within the Colorado plateau. J. Sed. Petrology. **60:**884-896.

6.**Canfield, C. E.** 1989. Reactive iron in marine sediments. Geochim. Cosmochim. Acta. **53:**619-632.

7.**Cline, J. D.** 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol. Oceanogr. **14:**454-458.

8.**Fedorak, P. M., D.W.S. Westlake, C. Anders, B. Kratochvil, N. Motkosky, W.B. Anderson, and P. M. Huck.** 1986. Microbial release of $^{226}Ra^{+2}$ from $(Ba,Ra)SO_4$ sludges from uranium mine wastes. Appl. Environ. Microbiol. **52:**262-268.

9.**Fisher, R. S.** 1995. Naturally occurring radioactive materials (NORM) in produced water and scale from Texas oil, gas, and geothermal wells: geographic, geologic, and geochemical controls, Geological Circular, vol. 95. Bureau of economic geology, The University of Texas at Austin, Austin, TX.

10.**Fredrickson, J. K., J.P. McKinley, B.N. Bjornstad, P.E. Long, D.B. Ringelberg, D.C. White, L.R. Krumholz, J.M. Suflita, F.S. Colwell, R.M. Lehman, T.J. Phelps, and T.C. Onstott.** 1997. Pore-size constraints on the activity and survival of subsurface bacteria in a late Cretaceous shale-sandstone sequence, northwestern New Mexico. Geomicrobiol. J. **14:**183-202.

11.**Fukui, M., and S. Takii.** 1994. Kinetics of sulfate respiration by free-living and particle-associated sulfate-reducing bacteria. FEMS Microbiol. Ecol. **13:**241-248.

12.**Harris, S. H., G.A. Ulrich, and J.M. Suflita.** 1999. Predominant terminal electron accepting processes occuring at a landfill leachate-impacted site as

indicated by field and laboratory and field measures.. U.S. Geological Survey Water Resources report, 99-4018C ed, vol. 3, Charleston, South Carolina.

13.**Heron, G., T. H. Christensen, and J. C. Tjell.** 1994. Oxidation capacity of aquifer sediments. Environ. Sci. Technol. **28:**153-158.

14.**Hicks RJ, F. J.** 1989. Aerobic metabolic potential of microbial populations indigenous to deep subsurface environments. 7:67-77.

15.**Jakobsen, R., and D. Postma.** 1994. In situ rates of suflate reduction in an aquifer (Romo, Denmark) and implications for the reactivity of organic matter. Geology. **22:**1103-1106.

16.**Jones RE, B. R., Suflita JM.** 1989. Anaerobic metabolic processes in the deep terrestrial subsurface. Geomicrobiol J. **7:**117-130.

17.**Krumholz, L. R., M. E. Caldweil, and J. M. Suflita.** 1996. Biodegradation of 'BTEX' hydrocarbons under anaerobic conditions. Cambridge University Press, New York.

18.**Krumholz, L. R., J.P. McKinley, G.A. Ulrich, and J.M. Suflita.** 1997. Confined subsurface microbial communities in Cretaceous rock. Nature. **386:**64-66.

19.**L. S. Clesceri, A. E. G., and R. H. Trussel (ed.).** 1989. Standard methods for the examination of water and wastewater, 17 ed. American Public Health Association, Washington DC.

20.**Lovley, D. R., and E.J. Phillips.** 1987. Competetive mechanisms for inhibition of sulfate reduction and methane production in the zone of ferric iron reduction in sediments. Appl. Environ. Microbiol. **53:**2636-2641.

21.**Lovley, D. R.** 1997. Potential for anaerobic bioremediation of BTEX in petroleum-contaminated aquifers. J. Ind. Microbiol. Biotechnol. **18:**75-81.

22.**Lovley, D. R., and E. J. P. Phillips.** 1988. Novel mode of energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. Appl. Environ. Microbiol. **54:**1472-1480.

23.**Lovley, D. R., and E. J. P. Phillips.** 1986. Organic matter mineralization with reduction of ferric iron in anaerobic sediments. Appl. Environ. Microbiol. **51:**683-689.

24.**Ludvigsen, L., H.-J. Albrechtsen, G. Heron, P. L. Bjerg, and T. H. Christensen.** 1998. Anaerobic microbial redox processes in a landfill leachate contaminated aquifer (Grindsted, Denmark). J. Contam. Hydrol. **33:**273-291.

25.**Martino, D. P., E.L. Grossman, G.A. Ulrich, K.C. Burger, J.L. Schlichenmeyer, J.M. Suflita, and J.W. Ammerman.** 1998. Microbial abundance and activity in a low conductivity aquifer system in East-Central Texas. Microb. Ecol. **35:**224-234.

26.**McCready, R. G. L., and H. R. Krouse.** 1980. Sulfur isotope fractionation by *Desulfovibrio vulgaris* during metabolism of $BaSO_4$. J. Geomicrobiol. **2:**55-63.

27.**McMahon, P. B., and F.H. Chapelle.** 1991. Microbial production of organic acids in aquitard sediments and its role in aquifer geochemistry. Nature. **349:**233-235.

28.**McMahon, P. B., F. H. Chapelle, W. F. Falls, and P. M. Bradley.** 1991. Role of microbial processes in linking sandstone diagenesis with organic-rich clays. J. Sediment. Petrol. **62:**1-10.

29.**Pettijohn, F. J., P. E. Potter, and R. Siever.** 1973. Sand and sandstone. Springer-Verlag, New York.

30.**Phelps TJ, R. E., White DC, Fliermans CB.** 1989. Microbial activities in deep subsurface environments. Geomicrobiol J. **7:**79-92.

31.**Reeves, A. L.** 1986. Handbook on the toxicology of metals, 2 ed. Elsevier Science Publishers Biomedical Press, Amsterdam.

32.**Routh, J., E.L. Grossman, G.A. Ulrich, and J.M. Suflita.** 1999. Volatile organic acids and microbial processes in the Yegua formation, east-central Texas. Biogeochemistry. **submitted**.

33.**Scholl, M. A., and S. Christenson.** 1998. Spatial variation in hydraulic conductivity determined by slug tests in the Canadian River alluvium near the Norman Landfill, Norman, Oklahoma. 97-4292. U.S. Geological Survey Water-Resources Investigations Report.

34.**Torres, M. E., H. J. Brumsack, G. Bohrmann, and K. C. Emeis.** 1996. Barite fronts in continental margin sediments: A new look at barium remobilization in the zone of sulfate reduction and formation of heavy barites in diagenetic fronts. Chem. Geol. **127:**125-139.

35.**Ulrich, G. A., L. R. Krumholz, and J. M. Suflita.** 1997. A rapid and simple method for estimating sulfate reduction activity and quantifying inorganic sulfides. Appl. Environ. Microbiol. **63:**1627-1630.

36.**Ulrich, G. A., D. Martino, K. Burger, J. Routh, E. L. Grossman, J. W. Ammerman, and J. M. Suflita.** 1998. Sulfur cycling in the terrestrial subsurface: commensal interactions, spatial scales, and microbial heterogeneity. Microb. Ecol. **36:**141-151.