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Microbial Ecology of a Shallow Unconfined Ground Water Aquifer Polluted by Municipal Landfill Leachate

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Abstract. The microflora of a shallow anoxic aquifer underlying a municipal landfill in Oklahoma was characterized by direct light microscopy, most probable number determinations of sulfate reducers and methanogens, and measurements of methanogenesis in aquifer samples containing either endogenous or exogenous electron donors and various sulfate concentrations. Acridine orange direct counts of bacteria did not vary significantly with time or between 2 major sampling areas ($1.70 \pm 0.16 \times 10^7$ to $11.2 \pm 2.1 \times 10^7$ cells/gdw). One site (B) was high in organic matter and low in sulfate, and methanogens generally outnumbered sulfate-reducers at most times of the year, whereas the opposite was true for another site (A). Greater than 75% of the theoretical amount of methane was detected within 7 weeks in both site A and B aquifer slurries amended with noncompetitive electron donors like methanol and trimethylamine. However, only site B slurries efficiently converted competitive donors like acetate, H_2, and formate to the expected amount of methane. A mapping of sulfate and methane levels indicated that site A is relatively localized. These results suggest that the predominant flow of carbon and energy is through methanogenesis at aquifer site B whereas sulfate reduction predominated at site A. However, both methanogens and sulfate reducers could be isolated from either site.

Introduction

The pollution of aquifers with organic chemicals is a problem that continues to raise questions regarding the quantity and quality of world water resources. Ground water supplies about one-half of United States drinking water needs, and withdrawal rates are increasing by about 25% per decade [15, 28]. Since about half the U.S. population relies on ground water as a source of potable water, concern over the contamination of this resource has grown considerably in the last 15–20 years.

Accurate risk assessment of this type of contamination requires reliable information on the transport and fate of xenobiotic substances in aquifers. However, the ways in which the subsurface microflora influence these processes are generally unknown or underappreciated, largely because aquifers were historically perceived as lifeless habitats. Current information, using aseptically ob-

tained aquifer material, indicates that the subsurface does harbor a rich assemblage of procaryotic life forms [4, 5, 11, 29, 30, 31]. The small amount of information that is available indicates that subsurface microorganisms can degrade some pollutant chemicals, but the full extent of the biochemical versatility existing in aquifers is simply unknown. In addition, little is known about the ecological conditions governing carbon and electron flow in the subsurface.

This information gap is even more acute when anaerobic conditions develop in aquifers. Anoxia can occur in ground waters whenever the rate of oxygen consumption (i.e., through microbial respiration) exceeds the rate of oxygen diffusion to the site. Thus, shallow aquifers which receive pollution from the disposal of organic matter in or on permeable soil formations are most susceptible to developing anoxic conditions. According to U.S. EPA estimates, there are 18,500 municipal landfills, some of which leach organic chemicals and are major sources of ground water contamination [28]. There is very little characterization of the indigenous anaerobic microorganisms, the prevalent ecology, or the fate of xenobiotic chemicals in anoxic aquifers.

Therefore, this study was designed to biologically characterize a shallow unconfined alluvial aquifer that is polluted by leachate from a municipal landfill. We reasoned that such a characterization would allow us to compare this type of aquifer with surface habitats and help determine if ecological principles could be extrapolated to subsurface environments. Further, we sought to provide baseline information for subsequent studies on the biodegradation of xenobiotic substrates by aquifer microorganisms. Our results show that at least 2 spatially distinct sites occur in close proximity within a single aquifer. One site was actively methanogenic, whereas carbon and electron flow at another site was most likely dominated by sulfate reduction. This conclusion is based on a) our field observations, b) the endogenous rates of methane production in sampled material, c) differences in ground water sulfate concentrations, d) the effect of sulfate additions on the endogenous methane production rates, e) response of the microflora to competitive and noncompetitive electron donors, f) the relative numbers of sulfate-reducing bacteria and methanogens at the sites, g) a mapping of methane and sulfate levels, and h) the isolation of specific types of sulfate reducers and methanogens from each site.

Materials and Methods

Description of Study Area

The aquifer underlying the municipal landfill in Norman, Oklahoma was the site chosen for study (Fig. 1). A comprehensive history of the Norman Landfill and an indication of the organic compounds leaching from it can be found elsewhere [23]. The landfill has been receiving solid waste for approximately 50 years. It was built over a single shallow unconfined alluvial sand aquifer, and native sand has been used as a cover allowing for percolation of rainwater through the refuse mounds. Depth to the water table is 1–1.8 m, and previous analysis showed that the ground water moves in a southerly direction at about 0.6 m/yr and eventually discharges into the South Canadian River (Fig. 1) [22, 23]. The landfill received an estimated 170 t/d of refuse in 1981 [22]. The landfill was closed and a clay capping of the refuse mounds was started in 1985.

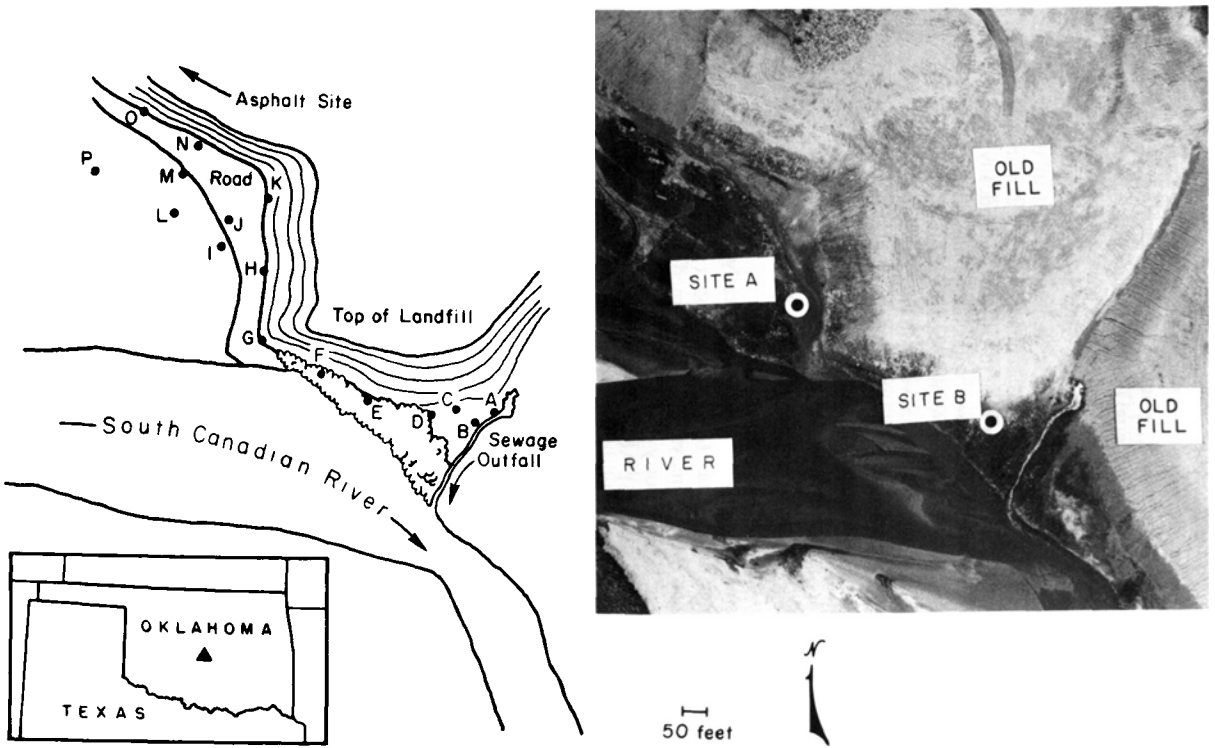

Fig. 1. Map of the Norman Municipal Landfill. The insert in the lower left corner shows the location of Norman, Oklahoma. The upper left drawing depicts sites A–P subsampled on 4/29/84. The upper right of the figure is an aerial photograph of the southern base of the landfill showing locations of the major sampling sites A and B.

Sample Collection

Samples of aquifer solids and ground water were obtained from sites bordering the landfill (Fig. 1). Unless otherwise noted, samples for microbiological analysis were taken by digging to the top of the water table with shovels and inserting stainless steel core barrels (7.5 cm i.d. × 43 cm) into the aquifer. The core barrels and contents were removed by excavation, and the techniques of Wilson et al. [34] were used to obtain aquifer material uncontaminated by surface microorganisms. Briefly, aquifer material was extruded from the core barrel and passed over a sterile paring device to remove the potentially contaminated outer few cm of the core. The pared core was then received in a sterile glass vessel. The extrusion and paring steps were conducted in the field inside an ATMOS glovebag (Aldrich Chem. Co., Milwaukee, Wisconsin) which was partially inflated and constantly purged with N_2 to minimize sample exposure to O_2. When aseptically obtained material was not required, ground water and aquifer solids were collected by handfilling sterile glass jars to capacity and tightly capping the jars with sterile lids. All field samples were placed on ice, transported to the laboratory within 3 hours, and placed inside a well-working anaerobic glovebox containing an atmosphere of N_2-H_2 (90:10). The temperature in the glovebox ranged from 24°–27°C.

Enumeration of Microorganisms

Total numbers of microorganisms were enumerated on aseptically obtained samples of aquifer material using the acridine orange direct count (AODC) technique of Ghiorse and Balkwill [11]. Five-tube most probable number (MPN) analysis was performed with aquifer solids for both methanogens and sulfate reducers. A previously described sulfate-free mineral salts medium was used for the enumeration of methanogens [36]. Additions to the medium included 10 mM of either acetate, trimethylamine, methanol, formate, or a H_2-CO_2 (80:20; 1.8 ATM) headspace. Sulfate reducers were enumerated in modified Postgate's medium B [21] which did not contain yeast extract or lactate but did contain Pfennig's vitamins (1 ml/liter) and minerals (1 ml/liter) [17]. Additions to the enumeration medium for sulfate reducers were the same as those noted for the methanogens except that methanol and trimethylamine were not tested.

Within 48 hours of collection, aquifer samples were diluted inside an anaerobic glovebox containing a 100% N_2 atmosphere. The MPN tubes were incubated at room temperature (24–26°C) for a minimum of 1 month. Positive results were scored if methane concentrations were at least twice background or if a black ferrous sulfide precipitate formed in the tubes during the enumeration of methanogens and sulfate-reducers, respectively.

Experiments with Aquifer Slurries

For methane production or sulfate depletion expeirments, 50 ± 1 g samples of nonaseptically obtained aquifer material were placed in sterile 160 ml serum bottles. This manipulation was performed in an anaerobic glovebox under a nitrogen atmosphere. The sulfate-free mineral salts medium (above) (72 ml) was added to the bottles using the modified Hungate technique [7] to bring the total volume occupied by the slurries to 100 ml. The initial headspace was adjusted to N_2-CO_2 (80:20). The bottles were closed with 1 cm thick rubber septa which were held in place with aluminum crimp seals. Methanogenesis from either 1 mM or 10 mM acetate, formate, methanol, trimethylamine, or H_2-CO_2 (80:20; 1.8 ATM) was measured when these substrates were added as exogenous electron donors to the incubation mixtures. To demonstrate sulfate-reduction, sulfate depletion was monitored in aquifer slurries amended with 4 mM acetate and compared with unamended controls. At the end of the incubation period, the endogenous methane production or sulfate depletion which occurred in unamended controls was subtracted from the concentrations observed in substrate amended samples. Each experiment was performed in duplicate and repeated at least twice. When required, the serum bottles were also amended with 20 mM Na_2SO_4. The

bottles were incubated at room temperature for up to 3 months and periodically assayed for methane production in the headspace by gas chromatography (GC) or for sulfate depletion from the liquid phase by high pressure liquid chromatography (HPLC).

Mapping of Methane and Sulfate Levels

To better define the major sampling areas, methane evolution from the aquifer and sulfate levels in ground water were surveyed at the points detailed in Fig. 1. To accomplish this, the soil overburden was removed by shovel at each location point to approximately 0.5 m from the water table. A hand soil corer (2.5 cm i.d.) was then used to bore the remaining distance to the water table. The tip of a syringe was placed in the hole and repeatedly flushed before a 25 cc sample of the bore hole atmosphere was taken and placed in a prevacuated test tube for subsequent methane analysis. Samples for background methane determinations were similarly taken at the surface and used for comparison. As ground water seeped into the small bore holes, 3–5 ml samples were taken by syringe, transported to the laboratory, and stored frozen until analyzed for sulfate by HPLC. Once the sampling areas were crudely defined, sulfate levels were periodically monitored in ground water from the different portions of the aquifer for approximately 1 year. With time, we avoided resampling the same areas by triangulating sampling locations relative to defined reference points.

Isolation of Microorganisms

Aseptically obtained core material was used for all isolations of bacteria from the aquifer. To isolate methanogens, core material was serially diluted in the sulfate-free mineral salts medium (above). Roll tubes (3% agar) were made from these dilutions after amendment with either 10 mM methanol, formate, acetate, trimethylamine or H_2-CO_2 (80:20). After 30 days incubation at room temperature, colonies were picked from those tubes exhibiting methane levels greater than background levels. The colonies were subcultured in liquid media on the same enrichment substrate, incubated for 1 month, and analyzed for methane production. Methane-producing enrichments were then used to inoculate roll tubes, and the resulting isolated colonies were again transferred to liquid media. This procedure was repeated at least 3 times. Isolates obtained in this manner were examined using a Leitz D phase contrast/epifluorescent microscope equipped with a DQ filter as previously described [10, 18].

Sulfate-reducing bacteria were similarly isolated, except the modified Postgate/Pfennig medium (above) was used as the diluent and 10 mM acetate served as the electron donor in all roll tubes and liquid transfers. Inoculated roll tubes not receiving acetate served as controls. Black colonies that developed in roll tubes were picked and subcultured on liquid media. This process was repeated at least 4 more times before the isolates were examined microscopically.

Analytical

Methane was monitored by flame ionization GC as previously described [27] or with a Varian model 3300 gas chromatograph using a 1.8 × 0.32 cm stainless steel column packed with 80/100 mesh poropack Q. Nitrogen (N_2) was used as the carrier gas at 30 ml/min and the operating temperatures for the injector, column, and detector were 100°C, 105°C, and 120°C, respectively.

Aqueous samples for sulfate analysis were stored frozen, thawed when necessary, centrifuged at 20,000 × *g* for 25 min, and analyzed for sulfate or nitrate by anion exchange HPLC. The HPLC system consisted of a model 396 LDC/Milton Roy minipump, a Wescan anion exchange column (269-001), and a Wescan (Santa Clara, California) conductivity detector (model 213-505). The mobile phase was 4.0 mM potassium hydrogen phthalate (pH 4.5) at 2.43 ml/min.

Table 1. Physicochemical characteristics of samples taken from 2 sites within a shallow aquifer

Characteristics	Sample type	Site A	Site B
Dissolved organic matter (ppm)	W[a]	80–160	325–1,100
Chloride (ppm)	W	250	2,000
pH	W/S	6.2–7.5	6.9–7.6
Temperature (°C)	W/S	1–21	4–22
Dissolved oxygen (ppm)	W/S	UN[b]–2	UN
Nitrate (ppm)	W	UN	UN
Sulfate (ppm)	W	52–537	UN–230
Rate of methane formation[c] ($ppm \cdot d^{-1} \cdot g^{-1}$)	W/S	0.06–1.0	3–17
Rate of methane formation with[c] SO_4^{-2} amendment ($ppm \cdot d^{-1} \cdot g^{-1}$)	W/S	0.06–1.0	1–3

[a] W = ground water sample; W/S = ground water-aquifer solids slurry
[b] UN = undetectable
[c] Range observed in experiments performed with samples obtained on 7/83 and 2/84, respectively

Results

Aquifer material sampled over a 2 year period at 2 sites bordering a municipal landfill showed distinct differences in physicochemical characteristics. Both sites were polluted by landfill leachate as evidenced by the high levels of dissolved organic matter and chloride in the ground water (Table 1). However, site B had twice the amount of organic matter and almost 10 times the amount of chloride as site A.

The availability of potential electron acceptors was also measured at these sites. Dissolved oxygen was generally low to undetectable at both sites. This determination was made with a portable O_2 probe placed below the water table without regard to possible atmospheric oxygen contamination (Table 1). Similarly, nitrate was below detectable concentrations (<0.25 mM) in the ground water from either site when assayed by HPLC (Table 1). However, sulfate ranged from 0.6–5.6 mM at site A and was often an order of magnitude more than the levels detected at site B. Sulfate was generally higher in samples from site A than site B most times of the year (Fig. 2). During the summer months, sulfate was often undetectable at site B.

The high levels of sulfate presumably account for the large differences observed in the endogenous rate of methanogenesis at the 2 sites. Despite the presence of adequate organic matter at both sites, site B incubations generally produced methane at a rate that was 3–1,000 times greater than the rates measured in site A samples (Table 1).

These results, together with our previously reported field observations [27], suggested that sulfate might be inhibiting methanogenesis at site A and that the slow rate observed in these samples was likely due to the metabolism of noncompetitive electron donors. To test this possibility, slurries of aquifer solids and ground water from the various sites were amended with 20 mM

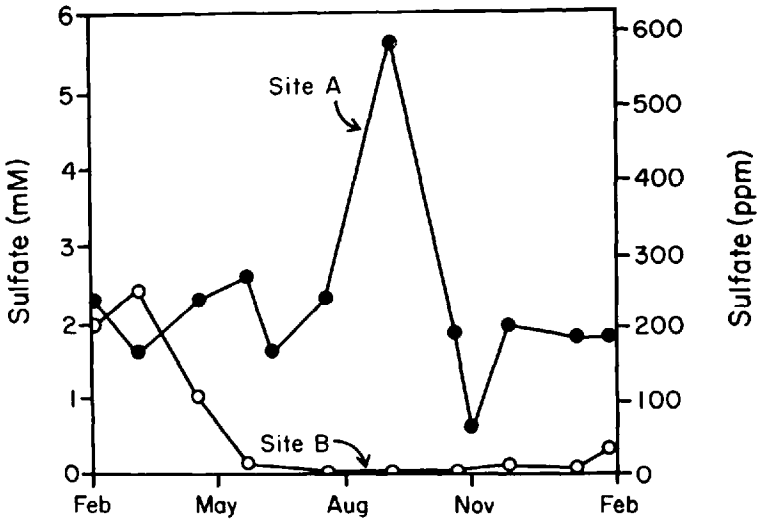

Fig. 2. Sulfate concentration in ground water sampled over a year (1984–85) from sites A and B.

sulfate. The endogenous rate of methanogenesis was inhibited by at least 60% in site B incubations, but no significant influence of sulfate was measured in site A incubations (Table 1).

To further characterize the sites, a variety of both competitive and noncompetitive electron donors were added to aquifer slurries under different sulfate regimes. Rapid methanogenesis evolved from site B aquifer slurries when amended with competitive electron donors like acetate, H_2, and formate (Table 2). Based on the stoichiometries listed in Table 2, the methane recoveries for these substrates ranged from 51–100% of that theoretically expected. However, when exogenous sulfate (20 mM) was added to these incubations, the methane recoveries for these compounds dropped to less than 10% of that theoretically possible. The incubations containing the competitive electron donors and exogenous sulfate blackened with time, presumably due to the production of H_2S and reaction with ferrous iron to form insoluble precipitates.

Only 3% and 1% of the expected amount of methane was detected when site A aquifer slurries were amended with 1 mM acetate or formate respectively (Table 2). However, the conversion of hydrogen to methane in site A slurries was only slightly less efficient than that detected in site B slurries (Table 2). The addition of exogenous sulfate to site A incubations decreased the degree of methanogenesis from the 3 competitive electron donors to about 1% of that theoretically possible (Table 2).

When a high concentration (10 mM) of a competitive electron donor like acetate was added to site A aquifer slurries, 39% of the expected amount of methane could be recovered. However, a lag of 18–30 days and a blackening of aquifer solids (relative to unamended and autoclaved controls) were observed before rapid methane production ensued. Presumably, during this time, the endogenous levels of sulfate were essentially depleted and no longer available

Table 2. Methane recoveries obtained from aquifer slurries amended with 1 mM substrate additions unless otherwise noted

Additions	CH_4 recovery (%)[a]	
	Site A	Site B
Acetate		
10 mM	39	87
1 mM	3	92
Acetate + SO_4^{-2}		
10 mM	7	21
1 mM	1	4
Formate	1	100
Formate + SO_4^{-2}	1	9
$H_2 + CO_2$	41	51
$H_2 + CO_2 + SO_4^{-2}$	1	4
Methanol		
10 mM	98	100
1 mM	70	100
Methanol + SO_4^{-2}		
10 mM	91	92
1 mM	75	74
Trimethylamine	89	96
Trimethylamine + SO_4^{-2}	89	87

[a] Following a 2 month incubation and assuming the following stoichiometries: $CH_3COOH \rightarrow CH_4 + CO_2$; $4CHOOH \rightarrow CH_4 + 3CO_2 + 2H_2O$; $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$; $4CH_3OH \rightarrow 3CH_4 + 2H_2O + CO_2$; $4(CH_3)_3N + 6H_2O \rightarrow 9CH_4 + 3CO_2 + 4NH_4^+$

to support dissimilatory sulfate reduction. Carbon and energy flow then shifted toward methanogenesis. This view would be supported by the fact that site A incubations which received exogenous acetate (10 mM) and excess sulfate showed only a 7% methane recovery.

Experiments using exogenous noncompetitive electron donors like methanol and trimethylamine showed greater than 70% conversion to the theoretical amount of methane regardless of the substrate concentration used, type of aquifer slurry, or sulfate concentration (Table 2). Generally, slightly longer lag times and lower methane recoveries were observed in site A incubations compared with site B samples. The same general influence was also noted in slurries amended with noncompetitive electron donors and exogenous sulfate.

Experiments designed to demonstrate sulfate reduction in site A aquifer slurries in response to added electron donors were less extensive. Endogenous rates of sulfate depletion in these samples were variable and difficult to measure accurately. However, the addition of 4 mM acetate to site A slurries resulted in a 3.9 mM decrease in endogenous sulfate levels in 7 weeks. This would

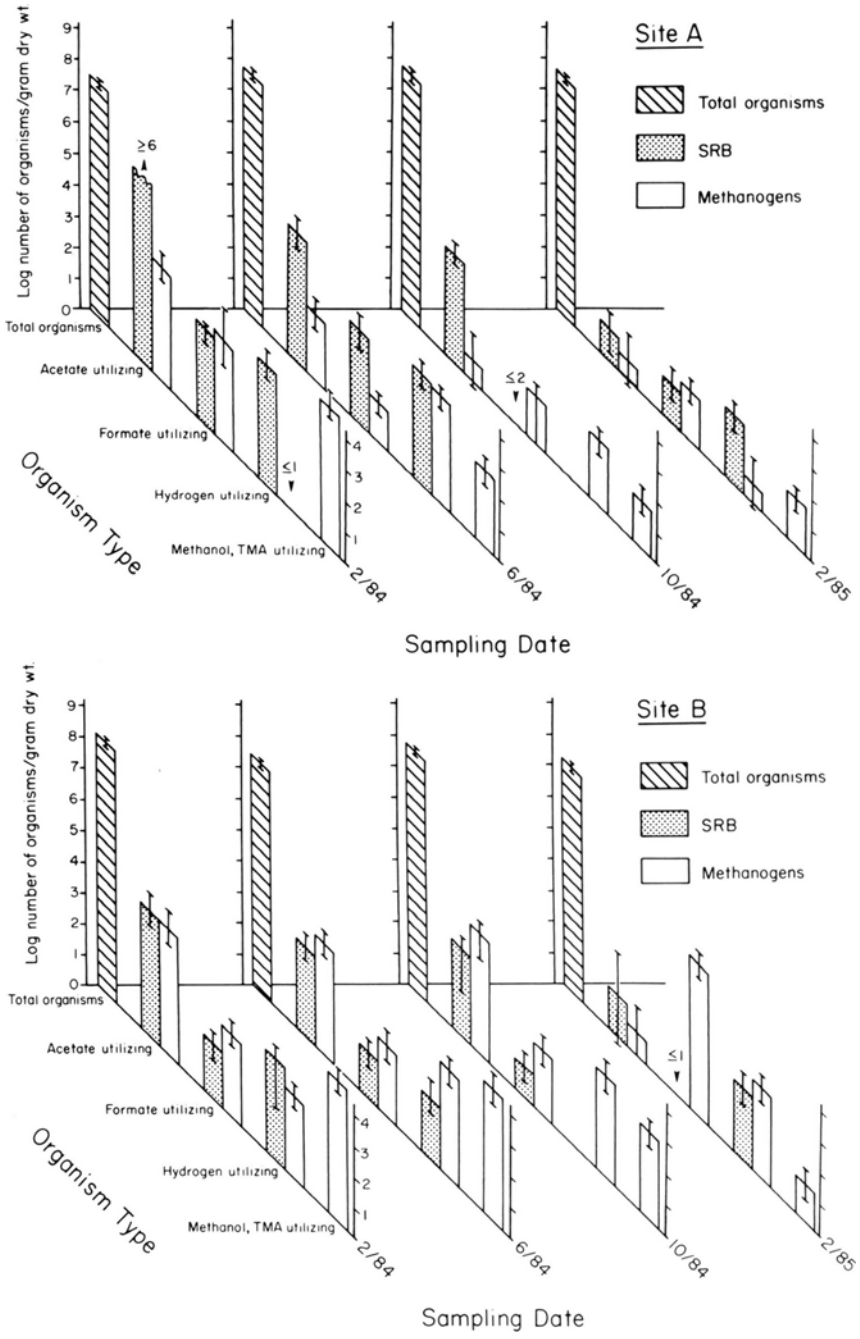

Fig. 3. **A.** The log number of total microorganisms and specific physiological groups of sulfate-reducing bacteria (SRB) and methanogenic bacteria at different sampling times in site A samples. **B.** The log number of total microorganisms and specific physiological groups of sulfate-reducing bacteria (SRB) and methanogenic bacteria at different sampling times in site B samples.

Table 3. Methane and sulfate concentrations at the various sampling locations shown in Figure 1

Sampling location	SO_4^{-2}(mM)	CH_4[a]
River	5	–
A	2	N.D.[b]
B	1	N.D.
C	3	1+
D	0	4+
E	2	1+
F	1	3+
G	3	1+
H	0	4+
I	1	3+
J	2	1+
K	0	1+
L	2	N.D.
M	2	N.D.
N	0	4+
O	0	4+
P	1	N.D.

Data was collected on a single sampling date, 4/29/84
[a] Methane content: 1+ = 1–10 ppm, 2+ = 10–50 ppm, 3+ = 50–100 ppm, 4+ = ≥100 ppm
[b] None detected

account for more than 90% of the theoretically predicted sulfate removal assuming the following stoichiometry [21]:

$$CH_3COO^- + SO_4^{-2} \rightarrow H_2O + CO_2 + HCO_3^- + S^{-2}$$

Mapping of Methane and Sulfate Levels

To help define the general extent of the sampling areas, methane content evolving from the top of the water table was measured on a single sampling date (4/29/84) at various sites (Fig. 1) and compared with ground water sulfate concentrations. As can be seen in Table 3, the sulfate levels varied from 0–3 mM. However, in areas where the level of sulfate was high, very little methane could be measured. Similarly, when sulfate was below 1 mM, methane evolution from the top of the water table could easily be detected. These results suggest that site A is not sulfate limited and that it is localized to a relatively narrow portion of the aquifer (locations J, L, and M, Fig. 1). The exact boundaries of site A are still not precisely defined, nor is it known whether they change with time. However, we have returned to the general area on more than 25 occasions over the past 2 years and have never failed to collect samples of ground water rich in sulfate.

Enumeration of Microorganisms

Total numbers of microorganisms were determined by acridine orange direct counting procedures [11] on aseptically obtained aquifer solids. As can be seen in Fig. 3A and 3B, total numbers of microorganisms did not vary appreciably between sites or throughout the year. Total numbers of organisms ranged from $3.02 \pm 0.75 \times 10^7$ to $4.79 \pm 1.82 \times 10^7$ cells/gdw during various times of the year at site A and from $1.70 \pm 0.16 \times 10^7$ to $11.2 \pm 2.1 \times 10^7$ cells/gdw at site B. Bacterial endospores were typically noted at both sites and represented less than 10% of the total counts. Most cells were rods and had a clear orange fluorescence possibly suggesting a high RNA content and therefore active protein synthesis. Filamentous microorganisms were only rarely observed.

The numbers of methanogens and sulfate-reducing bacteria were determined using an MPN technique. Specific populations were enumerated on competitive and noncompetitive electron donors known to support the growth of these organisms. Figure 3A and 3B show that various populations of sulfate reducers and methanogens fluctuated with time but were generally present at 10^2–10^3 cells per gram of dry weight of aquifer material. The acetate and hydrogen utilizing sulfate-reducing bacteria were consistently more numerically dominant than methanogens through the year in site A samples (Fig. 3A). However, in site B samples (Fig. 3B), the sulfate reducers were outnumbered by the methanogens utilizing these electron donors at all times of the year. The same general trend was also noted when formate served as the assay substrate, but the sulfate reducers were only occasionally more predominant in site A samples (Fig. 3A).

The number of methanogens detected with methanol or trimethylamine as growth substrates was equal in all MPN assays, so they are reported together in Fig. 3A and 3B. In general, site B harbored more methanogens than site A regardless of the assay substrate or time of the year. Similarly, more sulfate-reducing bacteria were generally detected in site A samples compared with site B samples.

Isolation of Sulfate Reducers and Methanogens

To isolate methanogens, roll tubes containing either H_2-CO_2, acetate, formate, trimethylamine, or methanol were inoculated with aseptically obtained aquifer material from the 2 sites. After 30 days of incubation, colonies were observed in all roll tubes inoculated with site B material and methane was easily detected above uninoculated and unamended controls. In contrast, roll tubes inoculated with site A material only produced significant amounts of methane from methanol and trimethylamine, even though colony growth was evident with all substrates.

Isolated colonies from selected roll tubes were picked, subcultured to liquid media containing the same enrichment substrate, incubated, and assayed for methane production. The positive enrichments were inoculated into roll tubes and the fastest growing colonies isolated for microscopic characterization.

White grape-like colonies were picked from methanol containing roll tubes inoculated with organisms originally derived from either site A or site B. These cells were large, gram-positive coccoidal packets that were autofluorescent and produced methane from methanol. Accordingly, a *Methanosarcina* sp. could be easily detected at either site. Roll tubes containing formate and inoculated with organisms originally obtained from site B material yielded colonies containing gram-negative slender rods which were autofluorescent and also produced methane from formate. The most probable genus for this organism is *Methanobacterium.* However, despite our attempts, this organism could not be demonstrated in site A material.

To isolate sulfate-reducing bacteria, roll tubes containing either H_2-CO_2, acetate, or formate were inoculated with either site A or site B material. Large black colonies quickly grew and were easily isolated from roll tubes inoculated with material from either site. Examination of some of the isolates by phase-contrast microscopy revealed most colonies contained vibrio or rods usually growing singly and occasionally in pairs. Since the acetate-utilizing colonies were generally quickest to develop, several from site A were picked, purified, and examined microscopically. The predominant organism isolated in this manner was a pleomorphic, gram-negative spore-forming bacillus which reduced sulfate using acetate as an electron donor and therefore most likely belonged to the genus *Desulfotomaculum* [32, 33].

Discussion

The landfill in Norman, Oklahoma is located on an alluvial sand formation which was deposited by the South Canadian River (Fig. 1). Leachate from this landfill pollutes the unconfined aquifer of the area. Our chemical and microbiological characterization of the aquifer revealed the existence of 2 spatially distinct sites that differed with respect to the dominant metabolic process governing carbon and energy flow.

Site B (Fig. 1) is 3–10 m from the southern base of a refuse mound, and ground water is generally low in oxygen and sulfate but high in dissolved organic matter. Methane could be detected evolving from the top of the water tables in this area and aquifer samples efficiently converted both competitive and noncompetitive electron donors to the expected amount of methane. Methanogens capable of utilizing both types of electron donors could be isolated from this site and as a group were more numerous than the sulfate-reducing bacteria. These results suggest that the dominant flow of carbon in this area of the aquifer was through methanogenesis.

In contrast, another site (site A, Fig. 1), is about 30 m from the western edge of the same refuse mound and the ground water is low in oxygen but high in sulfate and organic matter. Little or no methane could be detected evolving from the water table, and samples from this area could not efficiently convert competitive electron donors to methane. However, significant sulfate depletion was noted in site A samples that were amended with acetate. Only methanogens capable of using noncompetitive electron donors and sulfate-reducing bacteria

could be easily isolated from this site. The sulfate-reducing bacteria were generally more numerically dominant than methanogens in this area. These results suggest that the predominant process for carbon dissimilation in this region of the aquifer is through sulfate reduction rather than methanogenesis. This suggestion would also account for the observed dark grey to black coloration of aquifer solids previously noted at site A [27]. Presumably, sulfide produced during sulfate reduction reacts with ferrous iron to produce insoluble precipitates that color the aquifer sands.

Sulfate is known to inhibit methanogenesis in a variety of other environments where sulfate reducers and methanogens coexist [2, 8, 14, 19, 26, 35]. Presumably, subsurface sulfate-reducing organisms outcompete the indigenous methanogens for the available electron donors in site A. A variety of studies have shown that sulfate-reducing bacteria possess more efficient kinetic systems compared with methanogens for the consumption of substrates like acetate and hydrogen [2, 3, 13, 16, 20, 24, 25]. In addition, methanogenesis at site A could be inhibited because the endogenous sulfate effectively shifts the metabolism of sulfate reducers from the production to consumption of hydrogen [3, 9, 24]. Therefore, the pool sizes of electron donors at site A may possibly be too low to permit methanogens to effectively compete with the sulfate-reducing bacteria.

Nevertheless, a low rate of methanogenesis could be detected in site A samples. Our studies suggest that methanogens coexist with sulfate reducers in this sulfate-rich site by utilizing electron donors not immediately available to sulfate-reducing microorganisms. Alternately, methanogens could possibly exist in sulfate-poor microhabitats.

It is interesting to note that aquifer slurries amended with sulfate and a noncompetitive electron donor like methanol or trimethylamine produced less methane than comparable slurries without added sulfate. It is generally believed that sulfate-reducing bacteria do not efficiently compete for such electron donors except at possibly low substrate concentrations [6, 12]. The decrease in methane production that we observed could possibly be due to the consumption of reducing equivalents generated during the metabolism of those substrates by methanogens and transferred to sulfate reducers via interspecies hydrogen transfer [9]. Our periodic sampling and analysis of ground water indicates that sulfate does impact site B, particularly during the winter months (Fig. 2). Perhaps low temperatures (Table 1) adversely affect carbon dissimilation during winter which results in sulfate accumulation [1]. As the aquifer warms in summer, sulfate may get depleted and carbon and energy flow may shift toward methanogenesis. The organic matter-to-sulfate ratio may be too low at site A to result in a similar shift toward methanogenesis in summer.

Questions remain about the origin of the ground water sulfate at the sites, in particular site A. Several possibilities or combinations exist. Usually, the aquifer flows toward the South Canadian River which in turn drains several gypsum soils in parts of western Oklahoma and therefore contains high levels of sulfate. Depending on the time of the year and hydrogeologic conditions, river water can conceivably infiltrate the aquifer sites and thus contribute to the ground water sulfate burden. Alternately, an asphalt manufacturing plant

is located close to site A, and leached sulfate from this material could differentially impact this region of the aquifer. Further work is required in order to make such a determination.

However, our summer mapping effort indicates that the site A is relatively narrow, and our experience has been that this region contains high levels of sulfate at all times of the year. A third possibility is that the sulfate could be emanating from buried refuse in the landfill (i.e., gypsum building materials) and slowly leaching into the aquifer creating a localized plume rich in sulfate.

In conclusion, this work demonstrates that two spatially distinct sites exist in close physical proximity in a shallow anoxic aquifer. Total numbers of bacteria are comparable at each site, and the organisms generally do not show signs of being nutritionally stressed as in other pristine alluvial aquifers of Oklahoma. The dominant ecological factor governing the flow of carbon and energy at these sites is the nature of the terminal electron acceptor. The influence of sulfate on methanogenesis in this shallow aquifer appears to be quite similar to findings in surface habitats, suggesting that ecological principles can be cautiously extrapolated to subsurface environments. The biological and chemical characterization of these sites should prove useful for subsequent studies on the decomposition of xenobiotic compounds under both methanogenic and sulfate-reducing conditions.

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