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Relating Ground Water and Sediment Chemistry to Microbial Characterization at a BTEX-Contaminated Site**

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ABSTRACT

The National Center for Manufacturing Science is investigating bioremediation of petroleum hydrocarbon at a site near Belleville, MI. As part of this study, we examined the microbial communities to help elucidate biodegradative processes currently active at the site. We observed high densities of aerobic hydrocarbon degraders and denitrifiers in the less-contaminated sediments. Low densities of iron and sulfate reducers were measured in the same sediments. In contrast, the highly contaminated sediments showed low densities of aerobic hydrocarbon degraders and denitrifiers, and high densities of iron and sulfate reducers. Methanogens were also found in these highly contaminated sediments. These contaminated sediments also showed a higher biomass, by the phospholipid fatty acids, and greater ratios of phospholipid fatty acids, which indicate stress within the microbial community. Aquifer chemistry analyses indicated that the highly contaminated area was more reduced and had lower sulfate than the less-contaminated area. These conditions suggest that the subsurface environment at the highly contaminated area had progressed into sulfate reduction and methanogenesis. The less-contaminated area, although less reduced, also appeared to be progressing into primarily iron- and sulfate-reducing microbial communities. The proposed treatment to stimulate bioremediation includes addition of oxygen and nitrate to the subsurface. Ground

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water chemistry and microbial analyses revealed significant differences that resulted from the injection of dissolved oxygen and nitrate. These differences included an increase in Eh, small decrease in pH, and large decreases in BTEX, dissolved iron, and sulfate concentrations at the injection well. Injected nitrate was rapidly utilized by the subsurface microbial communities, and significant nitrite amounts were observed in the injection well and in nearby down-gradient observation wells. Microbial and molecular analyses indicated an increase in denitrifying bacteria after nitrate injection. The activity and population of denitrifying bacteria were significantly increased at the injection well relative to a down-gradient well for as long as 2 mo after the nitrate injection ended.

Index Entries: Microbial characterization; BTEX or petroleum hydrocarbon; bioremediation; subsurface; ground water; oxygen injection; nitrate injection.

INTRODUCTION

Microbial populations capable of degrading petroleum hydrocarbons (BTEX) *(1–6)* can be found at contaminated sites. Microbial biomass, community structure, and biodegradative activities are limited by properties of the subsurface environment, such as moisture, pH, and the availability of carbon, nutrients, and electron donors/acceptors *(6–8)*, and the microbial community can affect these properties. For example, biodegradation at gasoline contaminated sites has been associated with partial depletion of subsurface oxygen, nitrate, and sulfate *(9–11)*, and the addition of oxygen and nitrate has enhanced the biodegradation of BTEX *(1,2,5,6)*. As part of the National Center for Manufacturing Science (NCMS) petroleum hydrocarbon site bioremediation study, we examined microbial communities to help elucidate biodegradative processes currently active at the bioremediation site. Dissolved oxygen and nitrate were injected for about 3 mo to test their effects on subsurface geochemistry, microbiology, and rates of intrinsic bioremediation from aerobic and denitrification processes. The goal of this demonstration was to determine the presence of extant bacteria capable of BTEX biodegradation and to monitor the bioremediation effort. This article examines some of the microbial populations and degradative activities of sediments prior to remedial efforts, and tests ground waters following oxygen and nitrate additions to the subsurface.

MATERIAL AND METHODS

Site Description, Operations, and Sample Collection

The NCMS (Ann Arbor, MI) Advanced *In Situ* Bioremediation study site at the industrial facility near Belleville, MI, was contaminated prior to 1991 by gasoline from a leaking underground storage tank. The site con-

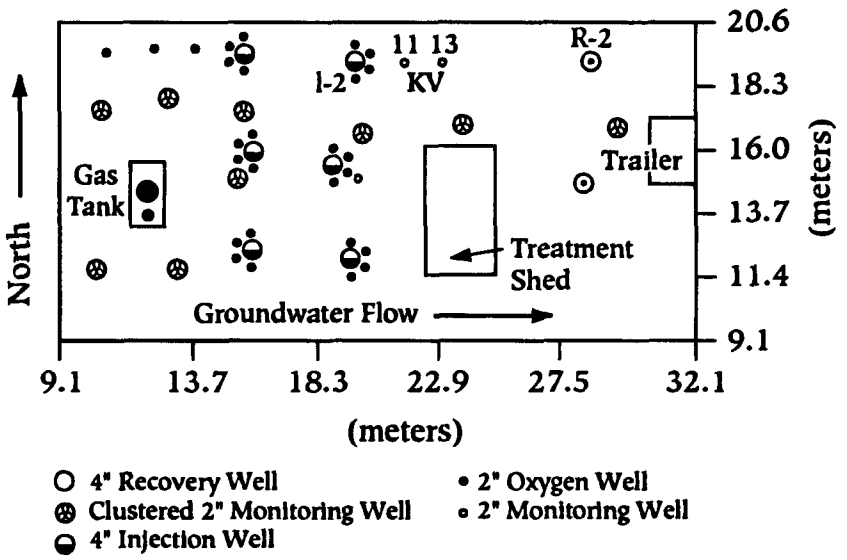

Fig. 1. The site map shows the location of the injection and recovery wells (I-2 and R-2) and the monitoring wells (KV-11 and KV-13). Ground water flows eastwardly from the highly contaminated up-gradient well (I-2) to the less-contaminated down-gradient well (R-2).

tains a shallow perched aquifer with uniform sandy soil isolated vertically by underlying clay till at 3.0–4.3 m below land surface, and has been well characterized for subsurface contaminant distribution, geology, and hydrology. A total of 100 wells and piezometers were installed at the site, and the distribution of contaminants was analyzed in soil cores and ground water recovered from the wells. This study focused on four sampling locations shown in Fig. 1. The injection and recovery wells (4-in. diameter), I-2 and R-2, were fully screened across the aquifer. Well I-2 is approx 9.1 m down-gradient from the source area, and well R-2 is 8.8 m down-gradient of I-2. Monitoring wells (2-in diameter) KV-11 and KV-13 were between the injection and recovery wells, and were 0.8 and 1.7 m, down-gradient from well I-2, respectively. Another fully screened monitoring well, I-2B, is located 0.3 m down-gradient of well I-2.

In spring 1995, subsurface samples for geochemical and microbiological characterization were recovered from core hole I-2 and R-2 using the standard split-spoon sampling technique. The microbial community structure was analyzed in sediments recovered from two depths (1.8–2.1 and 2.4–2.7 m) in both bore holes. For the first bioremediation treatment, oxygen was injected into well I-2 using an innovative passive diffusion technique *(11)*, and wells KV-11, KV-13, and R-2 served as down-gradient monitoring wells. After the end of oxygen injection experiments (June–August 1995, 70 d), nitrate was injected into well I-2. Prior to nitrate injection, nitrate levels in the ground water

were below detection limits (0.44 mg/L). Sodium nitrate was added at 5.2 g/d for 35 d (August–October 1995). During the nitrate injection, ground water was monitored for geochemical analyses. In January 1996, 2 mo after nitrate injection ceased, ground water was collected for complete characterization.

Geochemical Analyses

Moisture content, BTEX, total organic carbon, and total petroleum hydrocarbon were measured in sediment and ground water samples at the on-site field laboratory. Ground water temperature and conductivity were measured at the time of sample collection. Also, Eh was measured by platinum electrode (ORP/Hach); nitrate was measured by using a Hach #817 or Hach #353 test methods; and sulfate, ferrous iron, and total iron were measured with other Hach methods.

Microbial Analyses

Denitrifiers, methanogens, iron reducers, and aerobic hydrocarbon degraders were assessed by a turbidimetric most probable number (MPN) three-tube technique *(12)*. Aerobic hydrocarbon degraders were grown in phosphate-buffered mineral salt medium (PBBM) *(12)* supplemented with 0.1 mL Texaco gasoline. Methanogens were grown on PBBM supplemented with 40 m*M* acetate and methanol, and with cysteine HCl as a reductant. Denitrifiers in sediments were enumerated with nutrient broth supplemented with KNO_3 (1 g/L), but ground water samples were enumerated on a basal salt medium described by Fries et al. *(13)*. Iron reducers were enriched for with a medium described by Lovley et al. *(14)*. Media pHs-were adjusted to 7.1 for aerobes and 7.3 for anaerobes. These microbial enumerations were performed at the University of Tennessee.

Total heterotrophic bacterial counts in sediment and ground water samples were determined at the field site laboratory by the spread plate enumeration method *(11)* using plate count agar (Hach, Loveland, CO). Plates were incubated at 25°C and counted at maximum growth (4–14 d).

Biological Activity Reaction Tests (BART, Droycon Bioconcepts, Inc., Regina, Saskatchewan, Canada) were performed at the industrial plant laboratory and were used to determine the specific reactions that are catalyzed by the bacterial enzymes *(15)*. Thus, the activity tests can be used to estimate the capacity of the native microbial population to perform oxidation and reduction reactions that can biodegrade contaminants under the natural subsurface conditions. Total aerobes, sulfate reducers, denitrifiers, iron-related bacteria (aerobic and anaerobic), and fluorescent pseudomonads were enumerated. The test reactor containing nutrient medium was inoculated with 15–20 mL of ground water and was incubated for 48 h at a temperature of 25°C prior to analyses. The tests were used to determine the semiquantitative densities of various physiological populations.

Microbial Phospholipids

Microbial biomass and community structure were estimated using ester-linked phospholipid fatty acids (PLFA) *(16)*. Total PLFA was recovered from 75 g of sediment or 1 L of ground water filtered through 0.2-μm pore size inorganic filters (Anodisc 47, Whatman, Maidstone, England). PLFA was quantitatively extracted from the frozen samples (–50°C) as previously described *(16)*.

The extract was fractionated into specific lipid classes and transesterified to form phospholipid fatty acid methyl esters *(17)*. A gas chromatograph equipped with a mass-selective detector was used to identify and verify individual PLFA *(18)*. Double-bond position in the monounsaturated PLFA was determined as described in Nichols et al. *(19)*. Ground water samples showed biomass levels near the background detection limit of 2.14 pmol/L (or 5.36×10^4 cells/L).

Statistical Analyses

Log-transformed PLFA mole percentages were used in statistical analyses. Ein*sight pattern recognition software (Infometrix, Inc., Seattle, WA) was used for hierarchical cluster (HCA) and principal component analyses (PCA). These analyses were used to determine sample relatedness and factors that may account for variance in the data set *(18)*.

RESULTS AND DISCUSSION

Sediment and Ground Water Analyses Prior to Treatment

The highest total BTEX concentration (2.96 mg/L) was present in I-2 the most western (up-gradient) well in the transect. Concentrations decreased toward the east in the direction of ground water flow. R-2, the most eastern well, contained a very low total BTEX level (0.02 mg/L). Anaerobic conditions were associated with the hydrocarbon contaminant plume. Aquifer chemistry analyses (Table 1) indicated that the highly contaminated area was more reduced (Eh –69 mV) and had lower sulfate (37 mg/L) than the less-contaminated area (Eh –47 mV, sulfate 68 mg/L).

High densities of aerobic hydrocarbon degraders and denitrifiers, but low densities of methanogens, iron reducers, and sulfate reducers were observed in the less-contaminated R-2 sediments (Fig. 2, Table 2). In contrast, the highly contaminated I-2 sediments showed low densities of aerobic hydrocarbon degraders and denitrifiers and high densities of iron and sulfate reducers (Fig. 2, Table 2). Methanogens were also found in these highly contaminated sediments. The chemical and microbial analyses indicate that the subsurface environment at the highly contaminated area had progressed into sulfate reduction and methanogenesis. The less-contaminated area, although less reduced and containing more sulfate,

Table 1
Ground Water Geochemical Characterization

	Prior to treatment				Two months after both oxygen and nitrate injection ended			
Monitoring well	I-2	KV-11	KV-13	R-2	I-2	KV-11	KV-13	R-2
Screen zone or distance from injection well	2–3 m depth	2.7 m depth	2.7 m depth	2–3 m depth	0 m from injection	0.8 m from injection	1.8 m from injection	8.8 m from injection
Eh (mV)	–69.5	–65.2	–55.4	–47.0	–32.0	–101.3	–65.4	+72.7
Fe(II) (mg/L)	21.2	49.9	26.0	2.15	7.33	21.7	18.3	4.6
total Fe (mg/L)	25.8	56.6	32.5	3.25	9.17	31.3	22.1	5.1
Sulfate (mg/L)	37.0	8.0	12.0	68.0	1.0	0.00	0.00	67
Nitrate (mg/L)	<0.88	0	0	<0.44	0.00	0.00	0.00	0.00
Total BTEX (mg/L)	2.96	2.91	2.58	0.022	0.19	1.01	0.909	0.0058

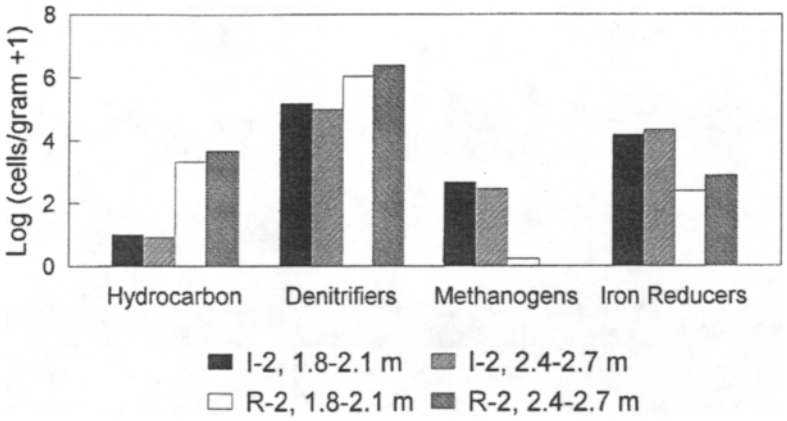

Fig. 2. Physiological types of bacteria present in the four sediments sampled at two depths from the highly contaminated (I-2, 10–16 mg/kg BTEX) and the less-contaminated (R-2, 0.02 mg/kg BTEX) boreholes.

Table 2
Aquifer Microbial Characterization Performed by the Field Site Laboratory (CFU/mL for Spread Plate Counts and BART)

Analysis	Location I-2	Location R-2
Total heterotrophs	1900	500
Fluorescent pseudomonads	Present	Present
Total aerobic bacteria	~60	~320
Iron-related bacteria	~500	~250
Sulfate-reducing bacteria	~1300	~250
Denitrifying activity	Weak positive	Negative

also appeared to be progressing into primarily iron- and sulfate-reducing microbial communities.

Aquifer material, when examined for physiological types of microorganisms (Table 2), revealed a four to five orders of magnitude difference between the heterotrophic densities observed by the field site laboratory and by the University of Tennessee laboratory. The differences could be attributed to sample heterogeneity, but may also be influenced by sediment shipment and handling, and the types of enumeration media used in the two laboratories. Results between the total aerobic bacteria and the aerobic hydrocarbon degraders were comparable for the two laboratories. I-2 exhibited 7 hydrocarbon degraders and 60 total aerobes/g of sediment, whereas R-2 sediment contained 300 total aerobes and 4600 hydrocarbon degraders/g of sediment (Fig. 2, Table 1). Iron-related and sulfate-reducing bacteria were observed at abundances >250 cells/g of sediment or mL of aquifer material by both laboratories.

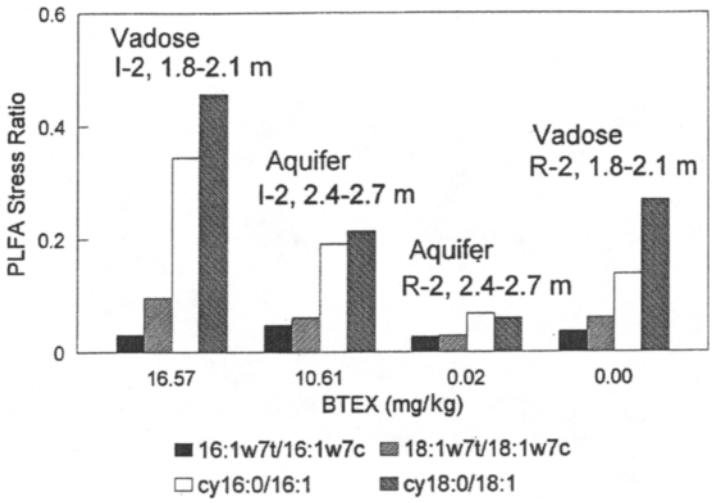

Fig. 3. PLFA stress indicators, based on four types of PLFA ratios, found in the four sediment samples and related to BTEX concentration. Larger stress ratios indicated more stress was experienced in the microbial community. The 1.8–2.1 m depth sediments were from the vadose zone and the 2.4–2.7 m depth sediments were from the aquifer.

Phospholipid fatty acid methyl ester results showed that the highly contaminated I-2 sediments had two to four times the biomass (195–259 pmol/g) exhibited by the less-contaminated R-2 sediments (55–97 pmol/g). A large variety of PLFA were detected in the sediments, but monounsaturated PLFA were the most abundant fatty acid methyl ester representing ~40% of the total PLFA. The abundance of 18:1ω7c and 16:1ω7c indicated the utilization of a pathway in fatty acid synthesis known as anaerobic desaturation, which is typically employed by Gram-negative bacteria. This dominant (~40%) Gram-negative community expressed higher stress ratios (trans/cis, cyclopropyl/monounsaturate) in the two samples with the higher biomass (Fig. 3). Those two samples were from the highly contaminated area. Physiological stress was also indicated in the vadose zone sediments compared to the saturated aquifer sediments (Fig. 3). A substantial level of terminally branched and mid-chain-branched saturates was found, which indicated the presence of sulfate reducers *(20,21)*. However, the ratios of iso/anteiso PLFA are lower than expected for pure cultures of sulfate reducers; these PLFA may be from Gram-positive influences as well. Other evidence for sulfate reducers included the presence of 10me16:0 and i17:1ω7c. R-2 sediment (1.8–2.1 m) showed the largest percentage or relative abundance (20%) of 10me16:0, thus indicating the presence of sulfate reducers.

The HCA of the PLFA profiles indicated that the I-2 sediments were closely related at a similarity index of 0.65, where 0 represents no similarity

and 1 represents an identical match. A distant relationship (0.13 similarity index) was exhibited between the R-2 sediment (1.8–2.1 m) and both I-2 sediments. The R-2 sediment (2.4–2.7 m) appeared to be a unique sample with no similarity to the other sediments. These differences between samples demonstrated the spatial heterogeneity seen in the subsurface *(22)*. PCA indicated that 92% of the variance could be explained with a single principal component (PC1). The fatty acid methyl esters under PC1, which were most heavily weighted or assigned the greatest positive correlation coefficients, were 16:1ω7c, 16:0, 18:1ω7c, and 10me16:0 (listed in descending order). These PLFAs indicated the dominance of Gram-negative bacteria and sulfate-reducing bacteria as previously described. Under PC2, sediment R-2 (1.8–2.1 m) was separated from the other sediments by the abundance of 10me16:0, which as already indicated suggests the presence of sulfate reducers. Likewise, PC2 revealed that sediment R-2 (2.4–2.7 m) contained a higher abundance of 16:1ω7c and 18:1ω7c, indicative of the anaerobic desaturation pathways, thus containing a dominant Gram-negative microbial community.

Effect of Oxygen Injection

From June–August 1995, high levels of dissolved oxygen (up to 39 mg/L) were injected from a source of pure oxygen into the ground water at well I-2. Two additional monitoring wells (KV-11 and KV-13) were installed along the ground water flow path between wells I-2 and R-2. Oxygen injection caused other significant changes in the geochemistry for wells in this transect. The oxidation state of the aquifer materials was strongly affected, leading to increases in Eh. Oxygen injection in the test area decreased dissolved ferrous iron in the aquifer ground water while increasing the ferric iron, presumably in suspended and complexed forms *(11)*. Total BTEX, sulfate, and dissolved iron in I-2 decreased, probably because of enhanced aerobic biodegradation processes (Table 1). Carbon dioxide and methane levels were higher in the up-gradient end (I-2) of the transect (data not shown), probably as a result of the aerobic and methanogenic processes coexisting in the subsurface. Ammonium and phosphate both decreased in the transect, probably as a result of enhanced microbial growth during the oxygen injection experiment (data not shown). Total heterotrophic counts and the level of aerobic bacteria shown by BART increased with oxygen addition to the subsurface, but these increases continued only while increased dissolved oxygen was maintained *(11)*.

Effect of Nitrate Injection

The objective of the nitrate addition was to monitor the migration of nitrate and its utilization by denitrifying bacteria, and to look for changes in activity for denitrifying bacteria. Two months after the nitrate injection, ground water samples were collected over a transect from well I-2 to well

Table 3
Ground Water Microbial Characterization Performed by the University of Tennessee (Cells/mL)

Location	I-2	KV-11	KV-13	R-2
Denitrifiers (NO_3)	28	4	4	28
Ammonium utilizers (NH_4Cl)	1	10	100	1000
Heterotrophs (aerobic)	100,000	100	100	100,000
Heterotrophs (anaerobic)	10	1	100	1000
Sulfate reducers	10	100	100	1
Iron reducers	0	0	0	0
Methanogens	0–1	1	1–10	0–1
PLFA biomass	68	277	84	377

R-2. Ground water chemistry results are shown in Table 1. Interestingly, the KV wells, which had higher BTEX concentrations (Table 1), showed lower abundances of denitrifiers and aerobic heterotrophs than ground water with lower BTEX concentrations (Table 3). When the concentration of BTEX was compared before and after nutrient addition, it was found that >90% of the BTEX was degraded. Similarly, aerobic and anaerobic degradation of diesel fuel has been enhanced by the addition of oxygen and nitrate to microcosms *(2)*.

There were lower Eh values in ground water from the KV wells, indicating anaerobic conditions down-gradient from the injection well, but higher Eh values were observed furthest down-gradient from the contaminated area (Table 1, Fig. 4). Although, ferrous iron decreased because of abiotic interaction with the oxygen addition, the nitrate injection stimulated the biological conversion to ferrous iron as was seen in the KV wells as sulfate was utilized and BTEX was degraded. This data correspond with the work of Beller and Reinhard *(23)*, who showed the enhancement of anaerobic toluene degradation under sulfate-reducing condition by the addition of ferrous iron. Lower sulfate concentrations were observed in ground water samples (KV wells) where higher abundances of sulfate-reducing bacteria were seen (Fig. 4). These wells also contained the highest levels of contamination. Ground water recovered from wells (I-2 and R-2) had lower levels of contamination (Table 1) and exhibited higher Eh values, higher sulfate concentrations, and lower numbers of sulfate reducers (Fig. 3).

The concentrations of nitrate and nitrate were monitored in the injection well (I-2) and in down-gradient wells (I-2B, KV-11). Nitrate concentrations increased rapidly in the injection well (I-2) and rose more slowly (I-2B) or not at all (KV-11) in down-gradient wells (Fig. 5). Significant nitrate could only be tracked about 0.3 m from the injection well after 35 d of injection. In contrast, a conservative tracer (bromide injection at concentrations of at least 260 mg/L) had migrated to all the monitoring points by

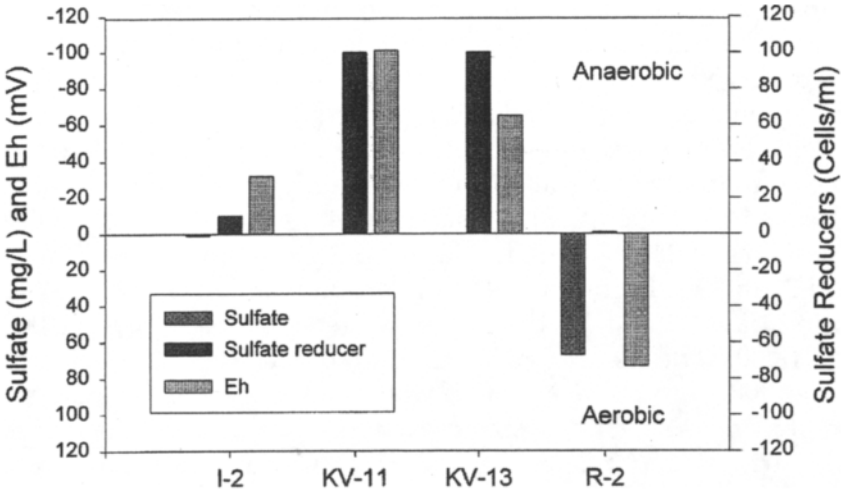

Fig. 4. Sulfate reducers (top portion of right axis), sulfate concentrations (bottom portion of left axis), and Eh values (full scale on left axis) observed in ground water from monitoring wells 2 mo after the oxygen and nitrate treatments ceased. The top portion of the graph represents observed values indicative of anaerobic conditions, whereas the bottom portion of the graph indicates aerobic conditions.

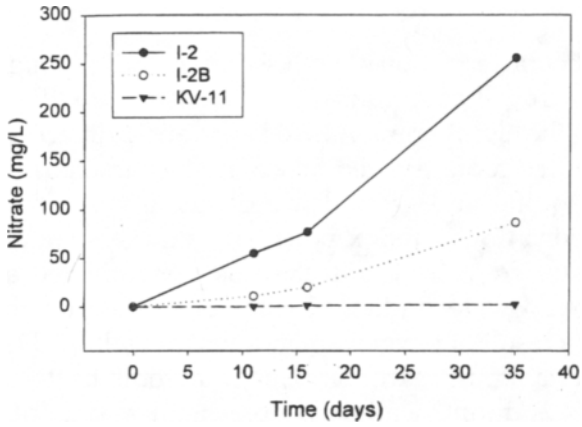

Fig. 5. Changes in ground water nitrate concentrations during the injection of nitrate. Well I-2 was the injection well with down-gradient monitoring wells I-2B and KV-11 at distances from I-2 of 0.3 and 0.8 m, respectively.

11 d after the injection and was detected at 65% of the concentration injected. Using the bromide tracer, the ground water flow velocity was estimated at 0.24 m/d. Nitrite was not detected in any of the ground water at day 11. By day 16, nitrite was detected in I-2 ground water at 1 mg/L, and by day 35, both I-2 and I-2B ground water samples showed 2–3 mg/L nitrite. Nitrate was not detected in KV-11 ground water. The low recovery of nitrite (1–5%) may be attributed to the continued reduction of nitrite by

the subsurface microbial communities to nitric oxide, nitrous oxide, or nitrogen. The utilization of nitrate on injection and the low recovery of nitrite observed at the industrial site was similar to that experienced in laboratory microcosms by Ball and Reinhard *(24)*.

Denitrifying populations increased after nitrate addition, and the subsurface community was still able to utilize nitrate 2 mo after nitrate injection ceased. The denitrifing bacteria, as estimated by BART, were enumerated at <100 cells/mL for day 0 in I-2 ground water, and for days 0, 12, and 35 in KV-11 ground water. For ground water from well I-2 for the sampling days 12 and 35, denitrifiers were estimate at 100–100,000 and 10,000–1,000,000 cells/mL, respectively. Toluene-degrading denitrifiers were isolated from well I-2 ground water and confirmed by PCR amplification of primers specific to *Azoarcus tolulyticus*, a known toluene-degrading denitrifier, and related strains *(13)*. These denitrifiers were not observed in well KV-11 or further down-gradient in KV-13 or R-2. The detection of toluene degradation activity correlated with the presence of BTEX as an electron donor and nitrate as an electron acceptor as observed in well I-2 ground water. Thus, the denitrifying populations were stimulated by nitrate injection, and BTEX degradation was enhanced.

CONCLUSIONS

Microbial characterization of both sediment and ground water revealed an anaerobic microbial community that consisted of sulfate reducers and methanogens in the highly contaminated area, and primarily iron and sulfate reducers in the less-contaminated area. These characterizations were supported by the aquifer and ground water chemistry, which showed more reduced conditions (lower redox potentials) and less sulfate in the highly contaminated area compared with the less-contaminated areas. Microbial analyses of ground water indicated changes in the microbial community composition as a result of oxygen amendment at well I-2. Decreases in strict anaerobic bacteria along with increases in aerobic bacteria were demonstrated in well I-2 ground water. The geochemical data of a higher redox potential and sulfate concentration support these observations. Microbial characterization indicated that several electron acceptors were important in implementation and treatment to achieve effective and efficient bioremediation. Monitoring the microbial community resulted in direct evidence for the changes seen in the geochemical parameters as different electron acceptors were utilized by the subsurface microbial populations.

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