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Relating Groundwater and Sediment Chemistry to Microbial Characterization at a BTEX-contaminated Site

S. M. PFIFFNER[1], A. V. PALUMBO[1*], T. GIBSON,[2],
D. B. RINGELBERG,[3] and J. F. MCCARTHY[1].

[1]Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831

[2]General Motors Research and Development Center, Warren, MI 48090

[3]Center for Environmental Biotechnology, The University of Tennessee, Knoxville, TN 37932

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

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* Author towhom all correspondence and reprints requests should be addressed.

Telephone: 423-576-8002; Fax: 423-574-0765; Email: avp@ornl.gov

ABSTRACT

The National Center for Manufacturing Science is investigating bioremediation of petroleum hydrocarbon at a site in Belleville, Michigan. 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 phospholipid fatty acids, and greater ratios of phospholipid fatty acids which indicate stress within the microbial community. Aquifer chemistry analyses indicated that the more-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 methanogensis. 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. Groundwater chemistry and microbial analyses revealed significant differences resulted from the injection of dissolved oxygen and nitrate in the subsurface. These differences included increases in pH and Eh and large decreases in BTEX, dissolved iron, and sulfate concentrations at the injection well. Injected nitrate was rapid utilized by the subsurface microbial community and significant nitrite

amounts were observed in the injection well and in nearby downgradient observation wells. Microbial and molecular analyses indicated an increase in denitrifying bacteria after nitrate injection. The activity and population of denitrifying bacteria was significantly increased at the injection well relative to a downgradient well for as long as 2 months after the nitrate injection ended.

INTRODUCTION

Microbial populations capable of degrading petroleum hydrocarbons (BTEX) (1-6) can be focused 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 can affect these properties. For example, biodegradation at gosoline-contaminated sites has been associated with partial depletion of subsurface oxygen, nitrate, and sulfate (10,11), 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 the microbial communities to help elucidate biodegradative processes currently active at the site. Dissolved oxygen and nitrate were injected for about 3 months to test their effects on subsurface geochemistry, microbiology, and rates of intrinsic bioremediation from aerobic and dentrification 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 paper

examined some of the microbial populations and degradative activities of sediments prior to remedial efforts and groundwaters following oxygen and nitrate additions to the subsurface.

MATERIAL AND METHODS

Site Description, Operations, and Sample Collection

The NCMS (Ann Arbor, Michigan) Advanced In Situ Bioremediation study site at the General Motors (GM) Service Parts Operations Facility in Belleville, Michigan was contaminated prior to 1991 by gasoline from a leaking underground storage tank. The site characteristics has a shallow perched aquifer with uniform sandy soil isolated vertically by underlying clay till at 10 to 12 ft and well characterized subsurface contaminant distribution, geology, and hydrology. A total of 100 wells and piezometers were installed at the site and the distribution of contaminants analysed in soil cores and groudwater recovered from the wells. This study focused on four sampling locations. Well (6" diameter) I-2 and R-2 were fully screened across the aquifer. I-2 is approximately 30 ft. down gradient from the source area, and R-2 is 29 ft. down gradient of I-2. Piezometers KV-11 and KV-13 are between the 2 wells, 2.75 and 5.75 ft., respectively, from I-2. Another fully-screened monitoring well, I-2B, is located 1ft. down gradient of I-2.

In Spring 1995, subsurface samples for geochemical and microbiological characterization were recovered from core hole I-2 and R-2 by using the standard split spoon sampling methods. The microbial community structure was analysed in sediments recovered from 2 depths (6-7 ft. and 8-9 ft.) In I-2 and R-2. Oxygen was injected into well I-2 using an innovative passive

diffusion technique (11) and KV-11, KV-13, and R-2 served as down gradient monitoring wells. After the end of oxygen injection experiments (June to August 1995, 70 days), nitrate was injected in I-2 that did not then contain measureable nitrate levels. Sodium nitrate was added at 5.2 g per day for 35 days from August to October 1995. During the nitrate injection, groundwater was monitored for geochemical analyses. In January 1996, two month after nitrate injection ceased, groundwater was collected for complete characterization .

Geochemical Analyses

Moisture content, BTEX, total organic carbon (TOC), and total petroleum hydrocarbon (TPH) were measured in sediment and goundwater samples. Groundwater temperature and conductivity were measured at the time of sample collection. Also, Eh was measured by platinum electrode (ORP/Hach), nitrate by using a Hach # 817 or Hach # 353 test kit, and sulfate, ferrous iron, and total iron was measured with a Hach # 380 test kit.

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 salts medium [PBBM] (12) supplemented with 0.1 mL Texaco gasoline. Methanogens were grown on PBBM supplemented with 40 mM acetate and methanol, and with cysteine HCl as a reductant. Denitrifiers in sediments were enumerated with nutrient broth supplemented with KNO_3 (1g/L), but groundwaters were enumerated on a basal salt medium described by Fries et al. (13). Iron reducers were enriched for with a medium described by Lovely, et al. (14). Media pH were adjusted to 7.1 for aerobes and 7.3 for anaerobes.

Total heterotrophic bacterial counts in sediment and groundwater samples were determined by the spread plate enumeration method (11) on plate count agar (Hach, Loveland, CO). Plates were incubated at 25°C and counted at maximum growth (4 to 14 days).

Biological Activity Reaction Tests (BART, Droycon Bioconcepts , Inc., Regina, Saskatchewan, Canada) were performed at GM 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 enuumerated. The test reactor containing nutrient medium was inoculated with 15- 20 mL of groundwater and was incubated for 48 hours at a temperature of 25°C prior to analyses. The tests were used to semiquantitative the densities of various physiological populations.

Microbial Phospholipids

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

The extract was fractionated into specific lipid and transesterified to form phospholipid fatty acid methyl esters (17). A gas chromatograph equipped a mass selective detector was used to indentify and verified individual PLFA (18). Double bond position in the monounsaturated

PLFA was determined as described in Nichols et al. (19). Groundwater samples showed biomass levels near the background detection limit of 2.14 pmol/L (or 5.36 x10^4 cells/L).

Statistical Analyses

Log transformed PLFA mole percent data was used in statistical analyses. Ein*sight pattern recognition software (Infometrix, Inc.; Seattle, WA) was used for hierarchical cluster (HCA) and principal component analyses (PCA). HCA related samples to each other, and PCA determined the factors in the data set that accounted for the most variance (18).

RESULTS AND DISCUSSION

Sediment and Groundwater Analyses Prior to Treatment

The highest total BTEX concentration (2.96 mg/L) was present in I-2 the most western (upgradient) well in the transect, and concentrations decreased toward the east in the direction of groundwater flow. R-2, the most eastern well, contained a very low total BTEX level (0.02 mg/L). Anaerobic conditions were associated with the plume of the hydrocarbon contaminants. Aquifer chemistry analyses (Table 1) indicated that the more-contaminated area was more reduced (Eh -69 mv) and had lower sulfate (37 mg L^{-1}) than the less-contaminated area (Eh -47 mv, sulfate 68 mg L^{-1}). These conditions suggest that the subsurface environment at the highly-contaminated area had progressed into sulfate reduction and methanogensis. The less-contaminated area, although less reduced, also appeared to be progressing into primarily iron- and sulfate-reducing microbial communities.

High densities of aerobic hydrocarbon degraders and denitrifiers, but low densities of iron and sulfate reducers, were observed in the less-contaminated R-2 sediments (Figure 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 (Figure 2). Methanogens were also found in these highly-contaminated sediments (Figure 2). Aquifer material when examined for physiological types of microorganisms (Table 2) revealed 4-5 orders of magnitude difference between the GM and University of Tennessee numbers in heterotrophic counts. The differences seen could be due to the individual sediments utilized, sediment shipment and handling, and the enumeration media used in the two laboratories. Results were comparable between the total aerobic bacteria and the aerobic hydrocarbon degrader, with I-2 and R-2 samples contained 7-60 and 300-4600 cells/g or mL, respectively. Iron-related and sulfate-reducing bacteria were observed at abundances greater that 250 cells/ g or mL.

Phospholipid fatty acid methyl ester results show that I-2 sediments (195-259 pmol/g) had 2-4 times the biomass of the 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. Therefore, the abundance of 18:1ω7c and 16:1ω7c indicated the primary pathway utilized in fatty acid synthesis was anaerobic desaturation. This dominant gram negative community expressed higher stress ratios (*trans/cis*, cyclopropyl/mono-unsaturate) in the two samples with the higher biomass (Figure 3). Those samples were the two samples that were from the more contaminated site. High stress was also indicated in the vadoze zone sediments (Figure 3). A substantial level of terminally branched and mid-chain branched saturates were found which may have resulted from sulfate reducers (20-21). However, the ratios

of *iso/anteiso* PLFA are lower than expected for pure cultures of sulfate reducers and these PLFA may be from gram positive influences. Other evidence for sulfate reducers include the presence of 10me16:0 and i17:1w7c. R-2 6-7' showed the largest abundance of 10me16:0 at 20%.

By HCA on the PLFA profiles indicated that, I-2 sediments were closely related, with a distant relationship to R-2 6-7'. R-2 8-9' appeared to be a unique sample with no similarity to the other sediments. These differences between samples demonstrated the spatial beterogeneity seen in the subsurface (22). PCA indicated that 92% of the variance was explained under principle component 1 (PC1). The fatty acid methyl esters under PC1, which were most heavily weighted, were 16:1ώ7c, 16:0, 18:1ώ7c, and 10me16:0 (listed by most weighted). Under PC2, R-2 6-7' was separated from the other sediments by the abundance of 10me16:0,, which may indicate the presence of actinomycetes or sulfate reducers. Likewise, PC2 revealed that R-2 8-9' contained a higher abundance of 16:1ώ7c and 18:1ώ7c, which are indicative of anaerobic desaturation pathways, thus a dominant gram negative microbial community.

Effect of Oxygen Injection

From June to August 1995, high levels of dissolved oxygen (up to 39 mg/L) were injected into the groundwater from a source of pure oxygen in well I-2. Two additional sampling wells KV-11 and KV-13 were installed on the line of groundwater flow between I-2 and R-2. Oxygen injection in the transect caused other significant changes in the geochemistry. The oxidation state of the aquifer materials was strongly affected leading to increases in Eh. In the test area, oxygen injection decreased dissolved ferrous iron in aquifer groundwater, while increasing the ferric iron, presumably in suspended and complexed forms (11). Total BTEX, sulfate and dissolved iron in I-2 decreased probably due to enhanced aerobic biodegradation

processes (Table 3). Carbon dioxide and methane levels were higher in the upgradient end of the transect (I-2) 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. Total heterotrophic count and the level of aerobic bacteria shown by the BART tests increased after tests, 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, its utilization by denitrifying bacteria, and to look for changes in the activity for denitrifying bacteria. Two months after the nitrate injection, groundwater samples were collected over a transect from well I2 to Well R2. Groundwater chemistry results are shown in Table 1. Interestingly, the KV wells, which had higher BTEX concentrations, showed lower abundances of denitrifiers and aerobic heterotrophs than groundwater with lower BTEX concentrations (Table 4). When the concentration of BTEX was compared before and after nutrient addition, it was found that greater that 90% of the BTEX was degraded. Similarly, aerobic and anaerobic degradatiom of diesel fuel was enhanced by the addition of oxygen and nitrate to microcosms (Bregnard et al. 1996).

There were lower Eh values, indicating anaerobic conditions, in groundwater from the KV wells down gradient from the injection well, but higher Eh values were observed furthest from the contaminated area (Table 1 and Figure 3). Athough, ferrous iron decreased due to 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 degraded. This data corresponds with the work of Beller and Reinhard (1995) who showed the

enhancement of anaerobic toluene degradation under sulfate-reducing condition by the addition of ferrous iron. Lower sulfate concentrations were observed in groundwater samples (KV wells) where higher abundances of sulfate-reducing bacteria were seen (Figure 3). These wells also contained the highest levels of contamination, while less-contaminated wells (I-2 and R-2) had higher Eh values, higher sulfate concentrations, and lower numbers of sulfate reducers (Figure 3).

The concentration of nitrate and nitrite was monitored in the injection well (I-2) and adjacent wells (I-1B, KV-11). Nitrate concentration increased rapidly in the injection well (I-2) and rose more slowly (I-2B) or not at all (KV-11) in down gradient wells (Figure 4). Significant nitrate concentration could only be tracked about 1 ft from the injection well after 35 days of injection. In contrast, a conservative tracer (bromide) migrated to all the monitoring points by 11 days after injection in concentrations of at least 260 mg/L, 65% of the concentration injected. Using the bromide tracer, the groundwater flow velocity was estimated at 0.8 ft/day. Nitrite was not detected in any of the groundwater at day 11. By day 16, nitrite was detected in I-2 at 1 mg/L, and by day 35, both I-2 and I-2B showed 2-3 mg/L nitrite. Nitrate was not detected in KV-11 groundwater. The low recovery (1-5%) of nitrite 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 upon injection and the low recovery of nitrite observed at the GM site was similar to that experienced in laboratory microcosms by Ball and Reinhard (24).

Denitrifying populations were increased by nitrate addition and the subsurface community was still ability to utilize nitrate 2 months after nitrate injection ceased.

The denitrifing bacteria, as estimated by the BART test, were enumerated at < 100 cells/mL for day 0 at I-2, and for days 0, 12, and 35 at KV-11. For sampling days 12 and 35 at I-

2, denitrifiers were estimate at 100-100,000 cells/mL and 10,000-1,000,000 cells/mL, respectively. Toluene-degrading denitrifiers were isolated from I-2 groundwater and confirmed by PCR amplification of primers specific to *Azoarcus toluluticus*, 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 a electron donor and nitrate as an electron acceptor as observed in well I-2. Thus, the denitrifying populations were stimulated by nitrate injection, and BTEX degradation was enhanced.

SUMMARY/CONCLUSION

Microbial characterization of both sediment and groundwater revealed an anaerobic microbial community which 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 groundwater chemistry which showed more reduced conditions (lower redox potentials) and less sulfate in the highly-contaminated area as compared to the less contaminated areas. Microbial analyses of groundwater indicated changes in the microbial community composition as a result of oxygen amendment to I-2. Decreases in strict anaerobic bacteria along with increases in aerobic bacteria was demonstrated in I-2 groundwater. The geochemical data of a higher redox potential and sulfate concentration supports these observations. Microbial characterization indicates 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 acceptor were utilized by the subsurface microbial populations.

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Figures

Figure 1. 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) coreholes.

Figure 2. 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 6-7' depth sediment are from the vadose zone and the 8-9' depth sediments are from the aquifer.

Figure 3. Sulfate reducers, sulfate concentrations and Eh values observed in groundwater from monitoring wells two months after the oxygen and nitrate treatments ceased. The top portion of the graph represents observed values indicative of anaerobic conditions, while the. bottom protion of the graph indicates aerobic conditions.

Figure 4. Changes in groundwater nitrate concentrations during the injection of nitrate. Well I-2 was the injection well followed downgradient by wells I-2B and KV-11 at distances from I-2 of 1 ft. and 2.75 ft., respectively.

Table 1. Groundwater Geochemical Characterization								
	Prior to Treatment				Two Months after both Oxygen and Nitrate Injection Ended			
Corehole or 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	6.5-10 ft depth	9 ft depth	9 ft depth	6.5-10 ft depth	0 ft. from injection	2.75 ft. from injection	5.75 ft. from injection	29.0 ft. 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

Table 2. Aquifer Microbial Characterization (cfu/mL) (Spread plate counts and BART tests)		
Analysis:	Location I-2	Location R-2
Total heterotrophs	1,900	500
Fluorescent pseudomonads	present	present
Total aerobic bacteria	~60	~320
Iron-related bacteria	~500	~250
Sulfate-reducing bacteria	~1,300	~250
Denitrifying activity	weak positive	negative

Table 3. Groundwater Microbial Characterization (cells/mL)				
Location	I-2	KV-11	KV-13	R-2
Denitrifiers (NO_3)	28	4	4	28
Nitrogen-utilizers (NH_4Cl)	1	10	100	1,000
Heterotrophs (aerobic)	100,000	100	100	100,000
Heterotrophs (anaerobic)	10	1	100	1,000
Sulfate reducers	10	100	100	1
Iron reducers	ng	ng	ng	ng
Methanogens	1,ng	1	10,1	1,ng
PLFA biomass	68	277	84	377

ng = no growth

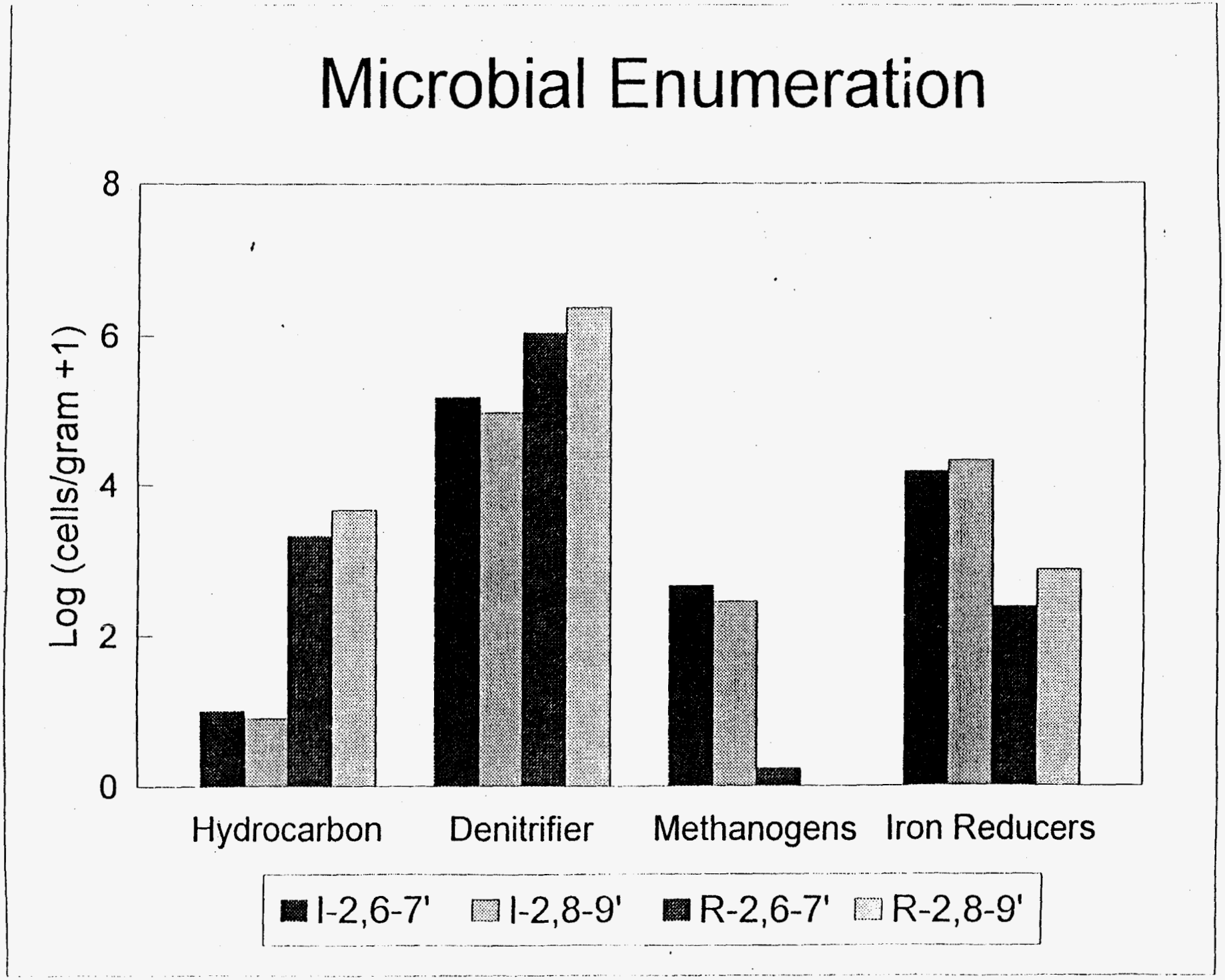
Microbial Enumeration
Log (cells/gram +1)
8
6
4
2
0
Hydrocarbon
Denitrifier
Methanogens
Iron Reducers
I-2,6-7'
I-2,8-9'
R-2,6-7'
R-2,8-9'

Figure 1

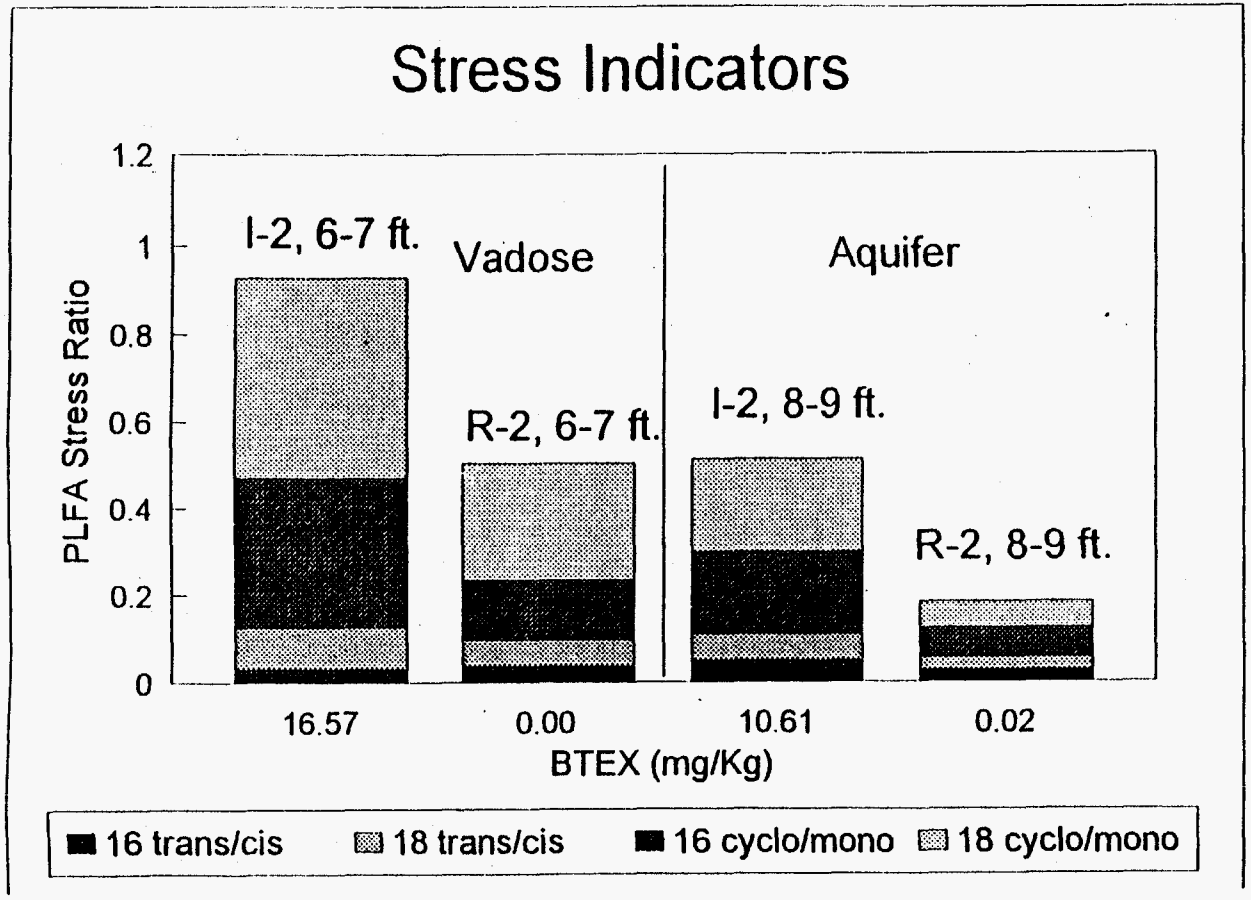
Stress Indicators
PLFA Stress Ratio
1.2
1
0.8
0.6
0.4
0.2
0
I-2, 6-7 ft.
Vadose
Aquifer
R-2, 6-7 ft.
I-2, 8-9 ft.
R-2, 8-9 ft.
16.57
0.00
10.61
0.02
BTEX (mg/Kg)
16 trans/cis
18 trans/cis
16 cyclo/mono
18 cyclo/mono

Figure 2

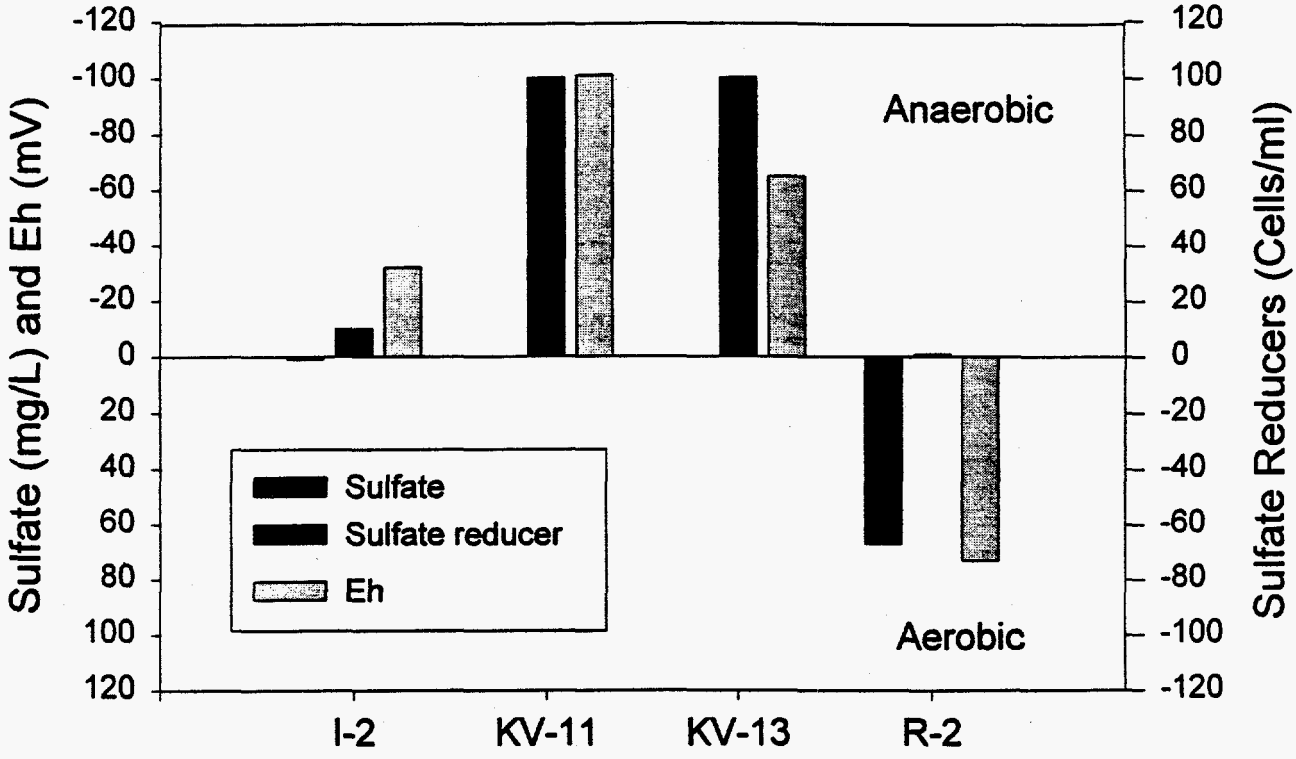
Sulfate (mg/L) and Eh (mV)
Sulfate Reducers (Cells/ml)
-120
-100
-80
-60
-40
-20
0
20
40
60
80
100
120
Anaerobic
Aerobic
Sulfate
Sulfate reducer
Eh
I-2
KV-11
KV-13
R-2

Figure 3

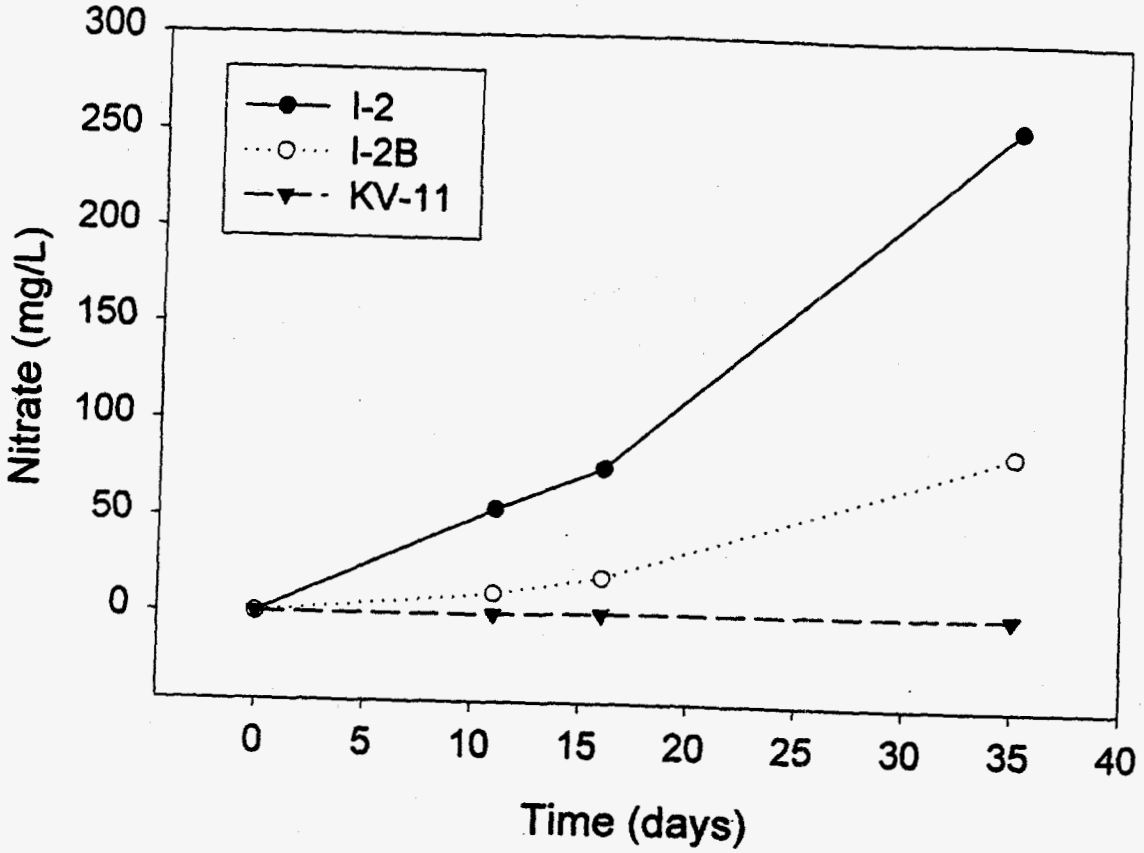

Figure 4