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Microbial Activity in Subsurface Samples Before and During Nitrate-Enhanced Bioremediation

J. Michele Thomas*, Virginia R. Gordy, Cristin L. Bruce, Stephen R. Hutchins, Jan M. Sinclair, and C. Herbert Ward

ABSTRACT

A study was conducted to determine the microbial activity of a site contaminated with JP-4 jet fuel, before and during nitrate-enhanced bioremediation. Samples at three depths from six different locations were collected aseptically under anaerobic conditions before and during treatment. Cores were located in or close to the source of contamination, downgradient of the source, or outside the zone of contamination. Parameters for microbial characterization included 1) viable counts of aerobic heterotrophic, JP-4 degrading, and oligotrophic bacteria, 2) the MPN of aerobic and anaerobic protozoa, 3) the MPN of total denitrifiers, and 4) the MPN of denitrifiers in hydrocarbon-amended microcosms. The results indicate that the total number of denitrifiers increased by an order of magnitude during nitrate-enhanced bioremediation in most samples. The number of total heterotrophs and JP-4 degrading microorganisms growing aerobically also increased. In addition, the first anaerobic protozoa associated with hydrocarbon-contaminated subsurface materials were detected.

INTRODUCTION

The effects of *in situ* bioremedial processes on microbial communities are relatively unknown. At best, the only indicator of microbial activity that is generally measured during *in situ* bioremediation operations is viable cell counts. Although viable cell counts may indicate gross changes in population size, changes within the microbial community will not be detected. Of importance will be the selection pressure by the bioremedial process for contaminant-degrading organisms and the fitness traits which allow these microorganisms to survive and effectively compete with other organisms for nutrients.

Knowledge of the effects of bioremediation on the microbial community may provide information that can be used to develop refined methods for process design and enhance the bioremedial process. Of interest in the present study is the effect of nitrate-enhanced bioremediation on microbial populations. We assessed changes in the microbial ecology of the site by determining aerobic viable counts, the MPN of total denitrifiers and JP-4 degrading microorganisms with nitrate as the electron acceptor, and aerobic and anaerobic protozoa.

EXPERIMENTAL PROCEDURES AND MATERIALS

Core Materials

Core material was collected before treatment at three depths from the following six boreholes: 80AA, 80BA, 80DA, 80EB, 80JB, and 80KB (Fig. 1). Boreholes 80AA, 80BA, 80DA, and 80EB were supposed to represent zones where residual JP-4 was located, borehole JB was supposed to represent a zone downgradient but influenced by the contamination, and borehole 80KB was supposed to represent an uncontaminated zone (Table 1). Interim core samples which correspond in depth and location to the intial samples were collected after 5 months of treatment (80Z, 80ZA, 80W, 80X, 80JC and 80KC). Boreholes 80Z, 80ZA, 80W2 and 80X were supposed to represent zones containing residual contamination. The 80JC borehole is downgradient but influenced by the contamination and remedial treatment whereas 80KC represents uncontaminated material that will not be impacted by treatment. The initial and interim samples were collected in March 1993 and August 1994, respectively. The samples were collected using steam-cleaned drilling equipment and were pared aseptically under anaerobic conditions (Hutchins *et al.*, 1991). Core material was subsampled in an anaerobic glovebox. The samples were kept on ice in the field and during shipping, and then stored at 5°C in the laboratory until used. The pilot demonstration of nitrate-enhanced bioremediation was conducted by making two treatment cells 100 ft by 100 ft, one which received recharge amended with 10-20 mg/L NO_3^--N and the second which received the same treatment without nitrate. The recharge was continuously applied through surface sprinklers at a rate of 11 gpm/cell (Hutchins et al., 1995).

Hydrocarbon Biodegradation Under Denitrifying Conditions

Subsurface material collected before treatment was used to assess the denitrification potential of compounds found in JP-4 jet fuel. Microcosms were constructed with the Eglin core samples collected before treatment to evaluate BTEX (benzene, toluene, ethylbenzene, the xylenes) and trimethylbenzenes removal under strictly denitrifying conditions as described previously (Hutchins, 1991). Selected alkylbenzenes were degraded under denitrifying conditions by indigenous aquifer microorganisms. The mean zero-order rate constants were 1.2±0.5 mg/L/d alkylbenzene biodegradation and 2.6 ±1.3 mg/L/d NO_3-N removal.

Media and Culture Conditions

Serial dilutions of each sample were prepared in triplicate under aerobic conditions by aseptically adding 10 grams of subsurface material to dilution bottles that contained 95 mL of 0.1% Na4P2O7 • 10H2O. The bottles were shaken on a wrist action shaker (Burrell Corporation, Pittsburgh, PA) at a setting of 10 for 1 hour, after which the rest of the dilution series was prepared using 0.1% $Na_4P_2O_7$ • $10H_2O$ as the diluent. The dilution series were used to determine the number of total heteroptrophs, JP-4 degraders, oligotrophs, total denitrifiers, microorganisms that denitrify with JP-4 as the sole carbon source, and aerobic and anaerobic protozoa in each sample. The number of total heterotrophs, JP-4 degraders, and oligotrophs in each sample was determined under aerobic conditions on R2A medium (Difco Industries, Detroit, MI), a mineral salts medium incubated in the presence of JP-4 vapors, and a mineral salts medium incubated without JP-4 vapors, respectively. The colonies growing on R2A medium were counted after 1- 1.5 weeks of incubation, whereas colonies growing on the other media were counted after 2 weeks of incubation. Counts of aerobic microorganisms are important because most denitrifiers are aerobic and only switch to anaerobic respiration in the absence of oxygen. The mineral salts medium (pH 7)contained per liter of deionized water: 0.8 g KH_2PO_4, 5.58 g Na_2HPO_4 or 6.99 Na_2HPO_4 • $2H_2O$, 1.8 g $(NH_4)_2SO_4$, 0.017 g $CaSO_4$ • $2H_2O$, 0.123 g $MgSO_4$ • $7H_2O$, 0.5 mg $FeSO_4$ • $7H_2O$, 1.54 mg $MnSO_4$ • H_2O, 2.86 mg H_3BO_3, 0.039 mg $CuSO_4$ •

$5H_2O$, 0.021 mg $ZnCl_2$, 0.041 mg $CoCl_2 \cdot 6H_2O$, and 0.025 mg $Na_2MoO_4 \cdot 2H_2O$. The total number of denitrifiers was determined using Nitrate Broth (Difco Industries). The number of organisms that denitrify with JP-4 as the sole carbon source was determined in the mineral salts medium amended with 1 g/L KNO_3^- and 200 µL of JP-4 jet fuel. Vials (40-mL volume) containing 20 mL of sterile aerobic mineral salts medium were amended aseptically with 200 µL of filter-sterilized JP-4, inoculated with serial dilutions of the samples, and sealed. Equal numbers of samples were incubated in the same medium without JP-4 to determine the effect of ambient carbon on denitrification potential. Because some oxygen was present, the denitrification detected in these samples could be the result of biodegradation of JP-4 or JP-4 degradation intermediates produced during the initial aerobic phase of incubation. Denitrifying activity was measured colorimetrically by testing for NO_2^- using sulfanic acid and N,N dimethyl-1-naphthylamine. The total number of denitrifiers and the number of JP-4 degraders that use NO_3^- as the terminal electron acceptor were determined after 3 and 6 weeks, respectively.

The number of aerobic and anaerobic protozoa was determined (Sinclair and Ghiorse, 1987) using subsurface sediment or dilutions of the sediment. Plates containing the protozoan enrichments were incubated aerobically or anaerobically in an anaerobic glovebox. The aerobic enrichments were observed at 2 weeks, 1 month, and 2 months. The anaerobic enrichments were observed every 3 weeks for 3 months.

Physical Analysis of Samples

The pH was determined with U. S Environmental Protection Agency method 9045 (U. S. Environmental Protection Agency, 1986). Texture analysis was conducted by Law Engineering, Houston, TX. The initial samples were found to consist of at least 92% sand and the rest silt. The interim samples have not been analyzed yet.

Samples were analyzed for JP-4 at the R.S. Kerr Environmental Research Laboratory, U. S. Environmental Protection Agency, Ada, OK using the standard operating procedure, RSKSOP-72 (U.S. Environmental Protection Agency, 1991).

Statistics

The data were compared using the t-test for equal or unequal variances, depending on the samples (95% confidence). The samples were compared in different ways. The entire depth intervals of initial and interim samples within the treatment cell (all cores except for JB, JC, KB, KC) were compared to determine the overall effect of remediation. Proximate cores that could be paired by depth and compared statistically were 80EB and 80X, 80BA and 80Z, 80JB and 80JC, and 80KB and 80KC.

RESULTS

Denitrification Potential

The average total denitrifier population in the treatment zone (all samples except the J and K cores) increased from $\log_{10}$ 5.8 to 6.9 during the 5 month period of nitrate-enhanced bioremediation (Table 2). The number of total denitrifiers in the entire interval of the K (control) region did not increase, suggesting that the treatment stimulated denitrification potential within the nitrate cell.

A comparison of the average number of denitrifiers in the entire depth interval of initial BA core with the average number in interim Z core indicated that numbers were higher after treatment. When individual samples within the initial BA core were paired by depth to those in interim core Z, counts were also higher after treatment. The average number of total denitrifiers in the entire interval of initial EB and interim X cores was not different. When individual samples within BA and Z, or EB and X cores were paired by depth and compared, the data indicated that there was a significant increase in total denitrifiers after treatment in all samples except for the lowest depth, X4, in which there was a significant decline.

Comparison of the entire depth interval of initial JB and interim JC samples indicated numbers of total denitrifiers were not different; however, when individual samples within cores were paired by

depth and compared, stimulation was observed in the upper and lower depths but the middle depth was not different.

Since the individual samples in cores KB and KC did not correspond directly with depth (Table 1), the number of total denitrifiers in initial sample KB6 were compared with the average of those in interim samples KC2 and KC1 combined. Numbers of total denitrifiers did not increase at this depth interval.

In contrast to an increase in the number of total denitrifiers, numbers of denitrifiers growing on JP-4 or JP-4 degradation products were lower after 5 months of treatment (Table 2). The MPN of many samples incubated in the presence of JP-4 could not be calculated because they were below the detection limit of the assay ($\log_{10}$ 2 cell/g dry wt). The results of control samples (no added JP-4) indicated that ambient carbon could be responsible for some of the denitrification that was detected in some samples.

Aerobic Viable Counts

When the entire depth interval of every core in the treatment zone was considered, there was an overall increase in hetrotrophic, JP-4-degrading, and oligotrophic microorganisms after treatment (Table 3). Numbers of heterotrophs, JP-4 degraders and oligotrophs in the treatment zone increased from $\log_{10}$ 5.8 to 6.9, 4.7 to 5.4, and 4.3 to 5.5, respectively; however, numbers of these organisms in the K cores (control) increased from 5.6 to 5.8, 4.0 to 4.8, and 4.1 to 5.0, respectively, suggesting that some of the growth resulted from other factors, such as seasonal influences. The K location is downgradient of the water from the control cell which may have stimulated growth. There were no consistent trends when pairs of samples were compared by depth.

Protozoa

Numbers of aerobic protozoa in the initial samples ranged from $\log_{10}$ 6.2 in shallow samples from the contaminated zone to less than detection limit (<10 cells/ g) for some KB (control) samples (data not shown). Of interest is that anaerobic protozoa were detected in low to moderate numbers (not

greater than 10000 cells/g). Data sets for the interim sampling were not available when this paper was written.

CONCLUSIONS

Denitrification potential was stimulated in the treatment zone after 5 months of nitrate-enhanced bioremediation. The number of total denitrifiers was higher in the interim than initial samples and did not increase in the control core. The increase in the number of viable counts may have been the result of treatment; however, other factors may have affected growth.

Numbers of viable microorganisms also increased in the control core, which could have been a seasonal effect. In general, operation of the pilot system resulted in increased pH, nitrate, ammonia, and orrthophosphate levels throughout the nitrate cell. Total Kjeldahl nitrogen and total phosphate levels have generally decreased. This probably results from the combined effects of nitrate assimilation and decomposition, denitrification, and leaching of minerals. This has also resulted in slightly higher cell counts, as determined by phospholipid fatty acids (PFLA). Other parameters (TOC, BTEXTMB, JP-4) are too variable in concentrations to generalize. In summary, these data indicate that the microbial activity at the site has been increased as a result of the pilot operation.

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LIST OF FIGURE AND TABLE CAPTIONS

KEYWORD LIST

Bioremediation

Nitrate

Hydrocarbon

Denitrifiers

FIGURE 1. Soil core locations at Eglin Air Force Base

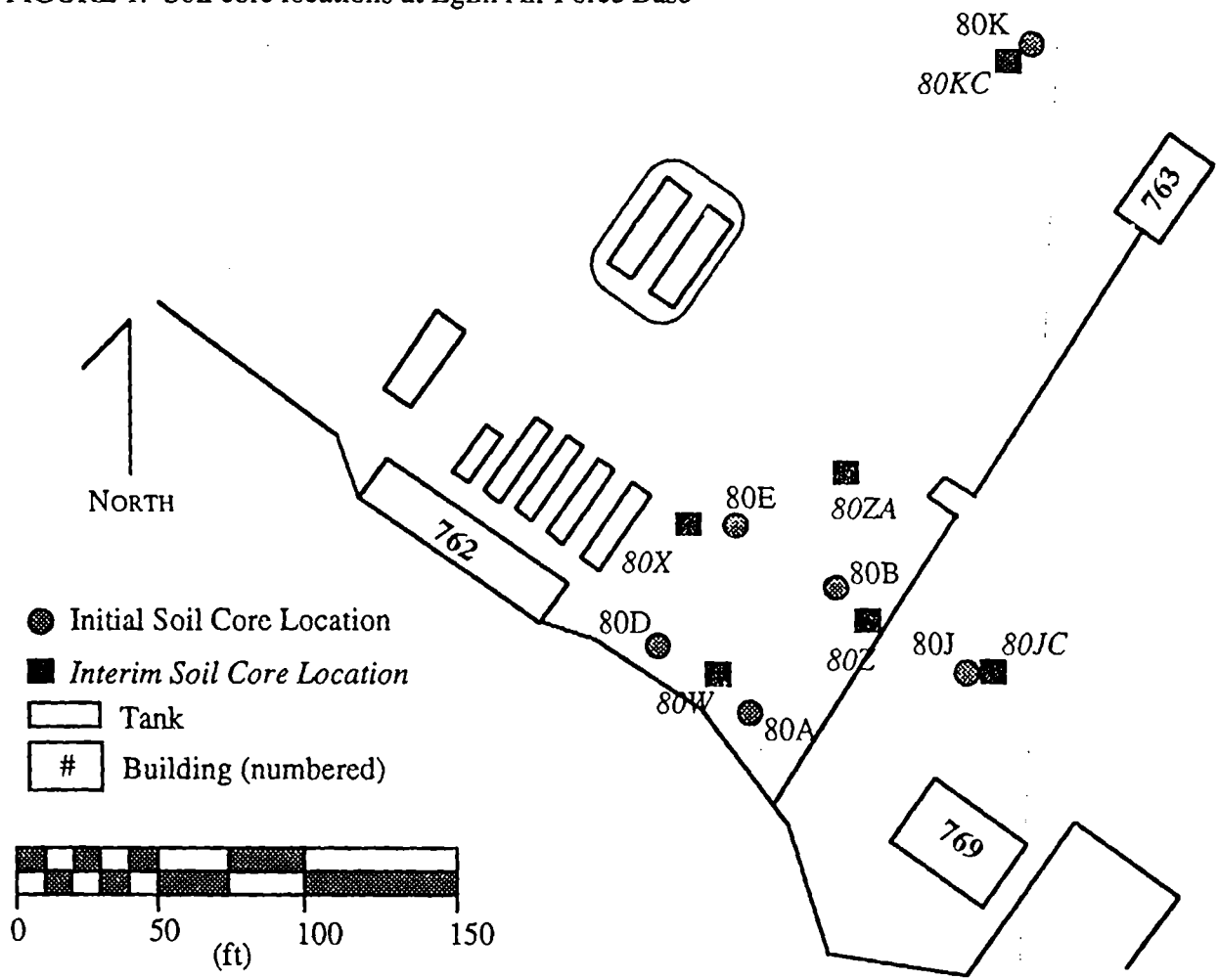

TABLE 1. Physical characteristics of initial and interim samples

Initial Sample	Initial Sample Depth (ft)	pH	JP4 Conc. mg/kg	Interim Sample	Interim Sample Depth (ft)	pH	JP4 Conc. mg/kg
80AA2	2.3-3.4	5.46	214	80ZA2	2.3-3.4	7.81	138.0
80AA1	3.4-4.5	5.49	1260	80ZA1	3.4-4.5	8.08	2630.0
80AA7	4.5-5.6	6.77	276	80ZA4	4.8-5.9	8.11	55.4
80BA3	1.0-2.2	4.88	2.8	80Z2	1.3-2.4	7.81	1.1
80BA2	2.2-3.4	6.23	355	80Z1	2.4-3.5	7.13	3750.0
80BA5	4.5-5.6	6.95	8.3	80Z4	4.9-6.0	8.32	34.1
80DA1	2.5-3.2	5.26	34.6	80W2	2.3-3.4	7.77	10.0
80DA5	4.0-5.0	5.77	377	80W1	3.4-4.5	7.76	1310.0
80DA8	6.0-6.8	6.82	54.7	80W4	4.8-5.9	7.75	4.1
80EB2	3.2-4.2	5.29	1160	80X2	2.5-3.8	8.75	4560.0
80EB1	4.2-5.2	5.46	1600	80X1	3.8-5.0	8.46	2620.0
80EB5	6.5-7.5	7.18	6.8	80X4	5.3-6.4	8.38	5780.0
80JB2	2.5-3.5	6.69	8.5	80JC2	2.3-3.4	6.87	11.1
80JB1	3.5-4.5	6.87	4.0	80JC1	3.4-4.5	6.90	1.7
80JB5	6.0-7.0	6.59	ND	80JC3	5.9-7.0	7.87	0.0
80KB2	3.2-4.4	5.07	6.4	80KC2	5.0-6.0	5.63	0.2
80KB1	4.4-5.5	5.80	3.3	80KC1	6.0-7.5	7.01	0.0
80KB6	5.5-6.7	5.98	3.6	80KC4	7.8-8.9	7.55	0.3

ND means not detected

TABLE 2. Denitrification potential in initial and interim samples

INITIAL Sample	Log Denitrifiers MPN/g dry wt (SD) Total	JP-4	No JP-4	INTERIM Sample	Log Denitrifiers MPN/g dry wt (SD) Total	JP-4	No JP-4
80AA2	7.1 (0.4)	6.8 (0.2)	3.4 (0)	80ZA2	6.8 (0.6)	2.0, 1.7, <2	2.7 (0.3)
80AA1	7.2 (0.6)	6.4 (0.1)	<1	80ZA1	6.5 (0.9)	2.0, <2, <2	<1
80AA7	4.2 (0.2)	3.2 (0.5)	<1	80ZA4	6.5 (0.2)	<2, 1.7, 2.0	0.7, <1, <1
80BA3	5.2 (0.7)	1.6 (0.3)	1.9 (0.5)	80Z2	7.2 (0.2)	6.3 (0.2)	2.1 (0.1)
80BA2	6.0 (0.4	4.5 (0.2)	3.2 (0.1)	80Z1	7.9 (0.2)	3.6 (0.4)	2.2 (0.4)
80BA5	4.3 (0.4)	2.9 (0.3)	<1	80Z4	7.5 (0.2)	1.7, <2, <2	<1
80DA1	6.5 (0.2)	3.9 (0.2)	2.5, 2.9, <1	80W2	6.0 (0.5)	3.8 (0.5)	1.2 (0.3)
80DA5	6.1 (0.1)	4.1 (1.2)	1.6 (0.2)	80W1	7.1 (0.4)	<2	<1
80DA8	6.3 (0.4)	5.6 (0.3)	<2	80W4	6.6 (0.9)	4.0 (1.0)	0.7 (0)
80EB2	6.6 (0.5)	5.5 (0.9)	<1	80X2	8.4 (0.3)	3.6 (1.1)	0.6, <1, <1
80EB1	4.4 (0.9)	2.3 (0.5)	<1	80X1	7.0 (0.8)	2.3 (0.5)	<1
80EB5	6.4 (0.2)	5.1 (0.3)	<1	80X4	4.8 (0.2)	<2	0.7, <1, <1
80JB2	4.7 (0)	3.7 (0.3)	<1	80JC2	7.5 (0)	3.1 (0.4)	0.7, 0.7, <1
80JB1	7.1 (0.4)	6.0 (0.4)	<1	80JC1	6.9 (0.2)	2.9 (0.5)	<1
80JB5	4.4 (0.2)	3.0 (0.2)	1.5, 1.5, <1	80JC3	5.2 (0.1)	2.6 (0.2)	<1
80KB2	4.4 (0.2)	0.9 (0.2)	<1	80KC2	4.7 (0.6)	<2, <2, 2.0	<1
80KB1	5.7 (0.4)	1.4 (0.7)	<1	80KC1	5.6 (0.2)	0.7, <2, 1.0	<1
80KB6	5.3 (0.2)	2.0 (0.2)	<1	80KC4	5.6 (0.4)	2.9 (0.3)	<1

TABLE 3. Aerobic viable counts in initial and interim samples

INITIAL Sample	Viable Counts (LOG 10)			INTERIM Sample	Viable Counts (LOG 10)		
	R2A	JP4	no JP-4		R2A	JP4	no JP-4
80AA2	6.73 (0.05)	6.57 (0.08)	6.59 (0.07)	80ZA2	6.97 (0.09)	4.88 (0.12)	4.99 (0.14)
80AA1	6.84 (0.16)	5.48 (0.1)	5.45 (0.15)	80ZA1	6.97 (0.07)	5.00 (0.17)	5.04 (0.1)
80AA7	4.68 (0.22)	2.43 (0.13)		80ZA4	6.84 (0.08)	4.95 (0.14)	5.72 (0.17)
80BA3	5.69 (0.05)	4.53 (0.14)	3.64 (0.09)	80Z2	6.12 (0.04)	5.12 (0.1)	5.47 (0.14)
80BA2	6.01 (0.08)	4.38 (0.18)	3.94 (0.08)	80Z1	7.59 (0.05)	6.61 (0.2)	6.99 (0.05)
80BA5	4.42 (0.08)	3.57 (0.08)	3.61 (0.13)	80Z4	7.11 (0.08)	6.05 (0.07)	6.09 (0.07)
80DA1	5.86 (0.04)	4.87 (0.13)	5.07 (0.1)	80W2	6.55 (0.07)	5.16 (0.26)	5.04 (0.05)
80DA5	5.91 (0.03)	3.8 (0.12)	3.39 (0.1)	80W1	6.42 (0.04)	5.84 (0.17)	5.93 (0.1)
80DA8	5.76 (0.07)	5.23 (0.08)	5.15 (0.1)	80W4	7.21 (0.13)	5.70 (0.04)	5.76 (0.13)
80EB2	6.80 (0.06)	5.65 (0.33)	6.18 (0.03)	80X2	7.57 (0.14)	6.93 (0.04)	6.79 (0.1)
80EB1	4.55 (0.06)	4.16 (0.10)	3.78 (0.09)	80X1	7.61 (0.09)	5.19 (0.56)	4.67 (0.37)
80EB5	5.61 (0.12)	5.65 (0.08)	5.8 (0.09)	80X4	6.17 (0.04)	3.46 (0.13)	3.46 (0.17)
80JB2	5.58 (0.07)	3.33 (0.09)	3.32 (0.11)	80JC2	6.92 (0.03)	6.00 (0.22)	5.99 (0.17)
80JB1	6.64 (0.06)	6.26 (0.08)	6.29 (0.05)	80JC1	6.90 (0.04)	5.28 (0.14)	5.71 (0.16)
80JB5	4.75 (0.08)	4.15 (0.04)	4.15 (0.04)	80JC3	5.54 (0.06)	4.17 (0.41)	4.55 (0.17)
80KB2	5.8 (0.1)	4.2 (0.1)	4.2 (0.1)	80KC2	5.6 (0.1)	5.3 (0.1)	5.1 (0.1)
80KB1	5.2 (0.02)	4.4 (0.03)	4.9 (0.1)	80KC1	6.0 (0.1)	4.8 (0.2)	5.5 (0.03)
80KB6	5.9 (0.2)	3.5 (0.1)	3.2 (0.1)	80KC4	5.8 (0.3)	4.1 (0.1)	4.0 (0.1)

(a) Samples were plated on low nutrient agar (R2A), and on a mineral nutrient agar with or without JP-4 vapors
(b) The detection limit for the assay is $\log_{10} 2$

TABLE 4. Aerobic protozoa in initial and interim samples

Initial Sample	Log_{10} no.cells/g dry wt	Interim Sample	Log_{10} no.cells/g dry wt
80AA2	4.4 (0.2)	80ZA2	5.7 (0.5)
80AA1	3.0, <1, <1	80ZA1	2.7 (0.1)
80AA7	2.9 (0.1)	80ZA4	3 (0.1)
80BA3	6.2 (0.1)	80Z2	5.9 (0.2)
80BA2	5.8 (0.4)	80Z1	3.8 (0.2)
80BA5	2.9 (0.5)	80Z4	2.4 (0.1)
80DA1	5.7 (0.4)	80W2	2 (0)
80DA5	6.0 (0.3)	80W1	<1, 3.7, 3.0
80DA8	>6	80W4	2.9 (0.1)
80EB2	2.3 (0.3)	80X2	3.9 (0.3)
80EB1	2.7 (0.2)	80X1	2.7 (0)
80EB5	3.5 (0.1)	80X4	2.7 (0)
80JB2	3.6 (0.7)	80JC2	>6.2
80JB1	2.5 (0.2)	80JC1	3.5 (0.2)
80JB5	2.8 (0.4)	80JC3	<1, 2.3, 2.3
80KB2	3.2 (0.2)	80KC2	3.3 (0.2)
80KB1	<1, <1, <1	80KC1	3.3 (0.1)
80KB6	2.5 (0.2)	80KC4	>6.2

(a) Detection limit for the assay, log_{10} 1

(b) Replicate Subsamples were averaged unless a replicate was greater or less than the detection limit

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16. ABSTRACT

A study was conducted to determine the microbial activity of a site contaminated with JP-4 jet fuel, before and during nitrate-enhanced bioremediation. Samples at three depths from six different locations were collected aseptically under anaerobic conditions before and during treatment. Cores were located in or close to the source of contamination, downgradient of the source, or outside the zone of contamination. Parameters for microbial characterization included 1) viable counts of aerobic heterotrophic, JP-4 degrading, and oligotrophic bacteria, 2) the MPN of aerobic and anaerobic protozoa, 3) the MPN of total denitrifiers, and 4) the MPN of denitrifiers in hydrocarbon-amended microcosms. The results indicate that the total number of denitrifiers increased by an order of magnitude during nitrate-enhanced bioremediation in most samples. The number of total heterotrophs and JP-4 degrading microorganisms growing aerobically also increased. In addition, the first anaerobic protozoa associated with hydrocarbon-contaminated subsurface materials were detected.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field, Group
AQUIFER GROUND WATER ANAEROBIC BIOREMEDIATION MICROORGANISM ECOLOGY PETROLEUM HYDROCARBON	DENITRFICATION PROTOZOA JET FUEL AEROBIC MPN	

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