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Assessment of the microbial potential for nitrate-enhanced bioremediation of a JP-4 fuel-contaminated aquifer

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A site that was contaminated with JP-4 jet fuel was characterized microbiologically to assess the feasibility of nitrate-enhanced bioremediation. The results of microcosm studies indicated that the mean pseudo zero-order rate constants for alkylbenzene biodegradation and NO_3^--N removal were 1.2 and 2.4 mg L^{-1} per day, respectively. Several alkylbenzenes were removed to a greater extent in samples contaminated with residual JP-4 than in unexposed samples and samples downgradient of the spill; benzene was recalcitrant in all samples. Numbers of total heterotrophs, JP-4-degraders, oligotrophs, total denitrifiers, denitrifiers growing in the presence of JP-4, estimates of cell number by analysis of phospholipid fatty acids, direct counts and aerobic and anaerobic protozoa were determined; however, numbers of microorganisms were not reliable predictors of alkylbenzene biodegradation activity. The presence of aerobic and anaerobic protozoa suggests that protozoa may be active under a variety of different electron acceptor conditions. The results of the characterization study indicated that the site was amenable to nitrate-enhanced bioremediation.

Keywords: bioremediation; site characterization; nitrate; alkylbenzenes; aromatic hydrocarbons

Introduction

In situ bioremediation of subsurface materials contaminated with petroleum hydrocarbons is a proven technology [5] and has been used commercially [31]. Most contaminants have been bioremediated with oxygen as the terminal electron acceptor because more energy is produced during aerobic than anaerobic respiration; however, there may be contaminant- or site-specific reasons which preclude or limit the success of aerobic treatment. Some compounds, such as tetrachloroethylene and carbon tetrachloride, are biodegraded only under anaerobic conditions [29]. In anaerobic aquifers, the addition of oxygen may cause oxidation and precipitation of reduced iron and excessive microbial growth, both of which may reduce hydraulic conductivity and hinder treatment performance [11]. When hydrogen peroxide is used as the oxygen source, its uncontrolled decomposition to water and oxygen can result in outgassing, which reduces oxygen availability [38] and hydraulic conductivity. In addition, hydrogen peroxide, which is infinitely soluble in water, can be toxic to microorganisms [39]. Another problem associated with aerobic treatment is the poor solubility of oxygen in water: depending on temperature, air and pure oxygen can supply about 8 and 40 mg L^{-1}, respectively.

Nitrate can be used as an electron acceptor in the anaerobic biodegradation of aromatic hydrocarbons present in fuel [16,24,28]. Although saturated hydrocarbons may be biodegraded under sulfate-reducing conditions [2], there have been no reports of their biodegradation with nitrate as the electron acceptor. The use of nitrate as an alternate electron acceptor to oxygen alleviates many of the problems associated with aerobic treatment. Nitrate is more soluble in water and less expensive than oxygen. Also, addition of nitrate to an anaerobic aquifer would not result in reduced hydraulic conductivity from iron precipitation or excessive microbial growth, which may occur with oxygen addition. Several field studies on nitrate-enhanced bioremediation have been conducted [6,15,17]. Although nitrate-enhanced bioremediation does not target saturated hydrocarbons, it does target aromatic compounds, the most toxic fraction of petroleum.

Measurements of microbial activity are essential in determining the feasibility of bioremediation. The feasibility of bioremediation, or biofeasibility, usually is assessed by investigating contaminant biodegradation in laboratory studies and is often coupled with determining microbial numbers. Laboratory tests are conducted to determine the potential for contaminant biodegradation and nutrient amendments that will enhance biodegradation rates. Microbial counts can be used as a preliminary indicator of microbial activity before conducting more expensive treatability tests. However, assessing biofeasibility using determinations of viable counts of microorganisms alone may lead to erroneous conclusions. A review of the literature on the microbiology of the subsurface indicates that viable counts are usually less than direct counts [40] and may not represent the microbial population that is being sampled.

Several papers or documents have been published which describe the microbial characterization of a site to assess the feasibility of bioremediation [10,11,16,32–34]. Most of these studies assessed the use of bioremediation by determining microbial numbers and the biodegradation

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potential of site contaminants in microcosms. Other papers have been published that have demonstrated microbial adaptation to contaminant degradation in the subsurface, but were not conducted to determine the feasibility of bioremediation. In these studies, adaptation was demonstrated by comparing microbial numbers and biodegradation potential in uncontaminated and contaminated samples collected from the same site [3,21,26,27,41,42,47].

In the present study, a site that was contaminated with JP-4 jet fuel was characterized microbiologically to assess the feasibility of nitrate-enhanced bioremediation. Microbial parameters that may be useful in predicting the success of bioremediation were investigated. Numbers of different types of microorganisms and the biodegradation potential of benzene, toluene, ethylbenzene, the xylenes, and the trimethylbenzenes (BTEXTMB) were determined. Microbial numbers were determined by viable and direct counts, cell counts by phospholipid fatty acid (PLFA) determination, the most probable number (MPN) of total denitrifiers, MPN of JP-4-degrading microorganisms with nitrate as the electron acceptor, and aerobic and anaerobic protozoa. Microtox and Mutatox assays were conducted to determine the presence of toxicants and mutagens, respectively.

Materials and methods

Site history and characterization

In April 1984, a leak was detected in an underground jet fuel pipeline at Eglin Air Force Base, FL [11]. An estimated 75 700 liters of JP-4 jet fuel had leaked into a shallow aquifer, consisting mainly of very-fine-to-coarse quartz sand with scattered gravel and clay lenses. The hydraulic conductivity of the sands was 0.0212 cm s^{-1}. The hydraulic gradient and the saturated thickness of the aquifer were estimated to be 0.015 m m^{-1} and 12.2 m, respectively. Depth to the water table ranged from 1.2 to 1.5 m below ground surface, depending on rainfall.

In 1987, a full-scale field test of *in situ* bioremediation was conducted to assess the use of hydrogen peroxide in the bioremediation of JP-4 jet fuel [11]. The project was only partially successful because of problems with iron precipitation and gas evolution that resulted from oxygenation of an anaerobic aquifer. Because monoaromatic compounds still remained after treatment, the site was assessed for anaerobic treatment with nitrate as the alternate electron acceptor to further remediate these compounds of regulatory interest. The assessment for anaerobic treatment included an extensive characterization of the physical properties of the site that was conducted in March 1993 [18]. Microbiological characterization of the site was the focus of the present study.

Core materials

Subsurface material was collected at three depths from six boreholes: 80AA, 80BA, 80DA, 80EB, 80JB, and 80KB (Figure 1); these are characterized in Table 1. Boreholes 80AA, 80BA, 80DA and 80EB were drilled in a zone known to contain residual contamination from the leak of JP-4 jet fuel that occurred in 1984 [11]. Borehole 80JB was drilled in a location downgradient of the contamination to represent a zone which may be impacted by a plume from the spill and/or metabolites from JP-4 biodegradation upgradient. Borehole 80KB was located outside the influence of the spill zone and was proposed as a control core (Figure 1).

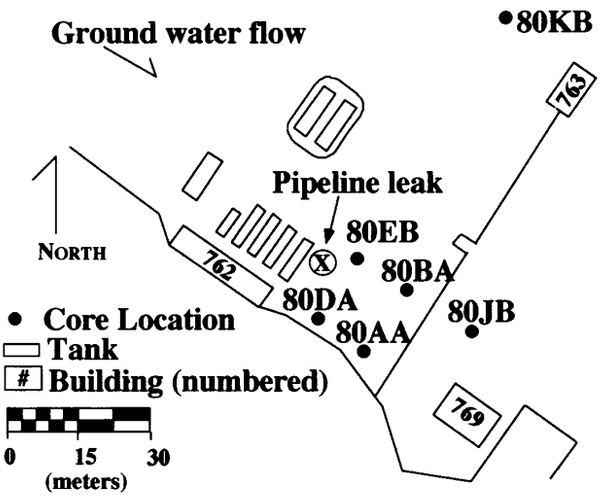

Figure 1 Sampling sites at Eglin Air Force Base.

The top sample of each core was unsaturated while the bottom depths were saturated; however, the water table fluctuates with rainfall. The samples were collected using steam-cleaned drilling equipment and were pared aseptically under anaerobic conditions [25]. The samples were kept on ice in the field and during shipping, stored at 5°C in the laboratory and processed within 6 weeks after collection.

Chemicals and media

All chemicals were reagent grade and of the highest purity available (>99%). 1,3,5-Trimethylbenzene, 1,2,3-trimethylbenzene, 1,2,4-trimethylbenzene and *m*-xylene were purchased from Aldrich Chemical Co (Milwaukee, WI, USA); benzene, *o*-xylene, *p*-xylene, ethylbenzene, and toluene were purchased from Sigma Chemical Co (St Louis, MO, USA).

R2A medium (Difco Industries, Detroit, MI, USA) was used to determine the number of heterotrophs. The number of JP-4 degraders was determined on solid medium prepared with a mineral salts medium and 1.5% Noble Agar (Difco Industries), which was incubated in the presence of JP-4 jet fuel vapors. The mineral salts medium contained per liter of deionized water: 0.8 g KH_2PO_4, 5.58 g Na_2HPO_4, 1.8 g $(NH_4)_2SO_4$, 0.017 g $CaSO_4 \cdot H_2O$, 0.123 g $MgSO_4 \cdot 7H_2O$, 0.5 mg $FeSO_4 \cdot 7H_2O$, 1.54 mg $MnSO_4 \cdot H_2O$, 2.86 mg H_3BO_3, 0.039 mg $CuSO_4 \cdot 5H_2O$, 0.021 mg $ZnCl_2$, 0.041 mg $CoCl_2 \cdot 6H_2O$, and 0.025 mg $Na_2MoO_4 \cdot 2H_2O$. The number of microorganisms that would grow on the mineral salts medium without the presence of fuel vapors was determined. These microorganisms were defined as oligotrophs and were assumed to grow on carbon impurities in the medium or air.

The MPN of total denitrifers was determined using Nitrate Broth (Difco Industries). The MPN of organisms that degrade JP-4 using nitrate as the electron acceptor was determined by first sterilizing 40-ml vials containing 20 ml

Table 1 Physical characteristics of samples

Sample	Depth (m)	pH	NH_4-N (mg kg^{-1})	JP-4 (mg kg^{-1})	TOC[a] (%)	Moisture (%)	Texture (%)		
							sand	silt	clay
80AA2	0.7–1.0	5.5	2.4	214	0.12	12.5	96.6	3.4	0
80AA1	1.0–1.4	5.5	3.6	1260	0.06	12.8	97.3	2.7	0
80AA7	1.4–1.7	6.8	1.4	276	0.04	15.9	98.9	1.1	0
80BA3	0.3–0.7	4.9	1.4	ND[b]	0.56	10.8	96.5	3.5	0
80BA2	0.7–1.0	6.2	2.2	355	0.26	8.4	97.1	2.9	0
80BA5	1.4–1.7	7.0	4.0	ND	0.09	13.8	92.6	7.4	0
80DA1	0.8–1.0	5.3	0.9	34.6	0.27	11.2	96.3	3.7	0
80DA5	1.2–1.5	5.8	2.6	377	0.05	13.6	94.6	5.4	0
80DA8	1.8–2.1	6.8	0.5	54.7	0.01	15.2	98.9	1.1	0
80EB2	1.0–1.3	5.3	0.6	1160	0.11	11.4	97.2	2.8	0
80EB1	1.3–1.6	5.5	0.5	1600	0.10	14.4	97.7	2.3	0
80EB5	2.0–2.3	7.2	0.5	ND	0.01	18.4	98.7	1.3	0
80JB2	0.8–1.1	6.7	6.0	ND	0.17	16.4	85.9	14.1	0
80JB1	1.1–1.4	6.9	6.2	ND	0.11	15.1	96.4	3.6	0
80JB5	1.8–2.1	6.6	13.7	ND	0.08	13.1	93.7	6.3	0
80KB2	1.0–1.3	5.1	0.9	ND	0.04	12.5	97.1	2.9	0
80KB1	1.3–1.7	5.8	0.6	ND	0.02	14.3	96.8	3.2	0
80KB6	1.7–2.0	6.0	0.5	ND	0.01	13.5	98.7	1.3	0

[a]TOC, total organic carbon.
[b]ND, not detected; detection limit, 10 mg kg^{-1}.

of mineral salts medium amended with 1 g L^{-1} KNO_3. Then, 200 μl of filter-sterilized JP-4 was added aseptically to the vials, after which the vials were inoculated with serial dilutions of the samples and sealed with Teflon-lined septa and open-top screw caps. Vials prepared identically, except without JP-4, were used as controls to assess denitrification from metabolism of ambient organic carbon. Because the vials containing JP-4 were initially aerobic, any denitrification detected could result from metabolism of oxygenated intermediates of JP-4 biodegradation and/or JP-4.

Microbial enumeration of core material

Serial dilutions of each core sample were prepared in triplicate under aerobic conditions by aseptically adding 10 g of subsurface material to dilution bottles that contained 95 ml of 0.1% sodium pyrophosphate. The bottles were shaken on a wrist action shaker (Burrell Corporation, Pittsburgh, PA, USA) at a setting of 10 (about 324 shakes min^{-1}) for 1 h, after which the rest of the dilution series was prepared using 0.1% sodium pyrophosphate as the diluent. The dilution series was used to determine the number of total heterotrophs, JP-4 degraders, oligotrophs, total denitrifiers, JP-4 degraders that use nitrate as the terminal electron acceptor, and aerobic and anaerobic protozoa in each sample. All plates and incubation vessels used to determine microbial numbers were incubated at room temperature (~25°C) in the dark. The core samples were prepared for acridine orange counts by adding 2.5 g of subsurface material in 22.5 ml of 0.1% sodium pyrophosphate and shaking for 1 h on the wrist action shaker at a setting of 5 (about 282 shakes min^{-1}).

Using the plate count technique, the numbers of total heterotrophs, JP-4 degraders, and oligotrophs that grew aerobically on R2A medium (Difco Industries), a mineral salts medium incubated in the presence of JP-4 vapors, and a mineral salts medium incubated without JP-4 vapors, respectively, were determined. Although the proposed remedial treatment is anaerobic, counts of aerobic microorganisms are important since most denitrifiers are aerobic organisms that switch to anaerobic respiration in the absence of oxygen [4]. The colonies growing on R2A medium were counted after 1.5–2 weeks incubation, whereas colonies growing on the other media were counted after 4 weeks incubation.

The MPN of denitrifiers and the number of JP-4 degraders that use nitrate as the terminal electron acceptor were determined after 3 and 6 weeks, respectively. Denitrification potential was determined calorimetrically by testing for the presence of nitrite with sulfanillic acid and *N*,*N* dimethyl-1-naphthylamine [7].

Direct counts of microorganisms were determined by epifluorescence microscopy [46]. Samples were extracted and analyzed for phospholipid fatty acid concentration by ManTech Environmental Technology, Inc, Ada, OK; an estimate of the number of cells g^{-1} subsurface material was determined from the fatty acid concentration.

The MPN of aerobic and anaerobic protozoa was determined [35] using subsurface sediment or dilutions of the sediment. To enrich for protozoa, the sediment and sediment dilutions were inoculated with a suspension of a 1-day-old culture of *Enterobacter aerogenes* and then 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 for cysts or vegetative protozoa.

Microcosm studies to determine biodegradation activity

Batch microcosm tests were used to determine the potential for biodegradation of BTEXTMB under denitrifying conditions in each of the 18 core samples. Oxygen was excluded by preparing microcosms aseptically in an anaerobic glovebox (oxygen concentration <10 ppm oxygen (vol/vol)); chemical stock solutions were prepared with distilled water that had been sterilized and transferred into the anaerobic glove box [16]. To replicate 12-ml serum bottles were added 10 g wet weight of core material, nutrient solutions to provide final concentrations of 10, 10, or 50 mg L^{-1} NH_4^+-N, PO_4^{2-}-P, and NO_3^--N, respectively, and an aqueous stock of BTEXTMB to provide final concentrations of 1–6 mg L^{-1} for each compound. Abiotic controls were prepared identically, except that 250 and 500 mg L^{-1} mercuric chloride and sodium azide, respectively, were added to inhibit microbial growth. The microcosms were sealed with Teflon-lined butyl rubber septa with no headspace, mixed, inverted, and incubated in an anaerobic glovebox in the dark at 12°C. The initial pH of the microcosms ranged from 7.0 to 7.9.

To determine the effect of nutrient amendments on BTEXTMB biodegradation, microcosms were prepared as described above except that 10 mg L^{-1} NH_4^+-N, 10 mg L^{-1} PO_4^{2-}-P, 10 mg L^{-1} each of NH_4^+-N and PO_4^{2-}-P, or no nutrients were added. A 1 : 1 mixture of samples AA1 and AA2 was used.

Triplicate microcosms from each sample set were sacrificed at intervals to determine the concentration of volatile aromatic hydrocarbons. Each microcosm was mixed by inverting it three times and centrifuged at $510 \times g$ for 30 min to clarify the supernatant fluid. Teflon septa were removed and glass syringes were used to withdraw 5- to 7-ml portions of the aqueous phase for BTEXTMB and other analyses. The volatile aromatic hydrocarbons contained in the aqueous phase were analyzed by purge-and-trap gas chromatography using a liquid sample concentrator (model LSC-2000; Tekmar Company, Cincinnati, OH, USA) and a gas chromatograph (model HP5890; Hewlett Packard, Avondale, PA, USA) equipped with a flame ionization detector. Hydrocarbons were purged onto a Tenax (Tekmar Company) trap for 6 min at 34°C followed by a 2-min dry purge and desorbed for 4 min at 180°C. Samples were transferred onto a 30-m megabore DB-wax capillary column (J&W Scientific, Folsom, CA, USA) with a 0.53-mm i.d. and 1.0-μm film thickness. The gas chromatograph was programmed to hold initially at 50°C for 4 min, increase at a rate of 8°C min^{-1} to 120°C, and then increase at a rate of 30°C min^{-1} to 180°C. The sensitivity for each of the compounds was 0.2 μg L^{-1}. The injector and detector temperatures were 200°C and 250°C, respectively. Samples were also analyzed for pH, nitrate, nitrite, ammonia, and phosphate using standard Environmental Protection Agency methods [23]. The residual solids were not analyzed.

Toxicity and mutagenicity assays

Each sample was extracted using methylene chloride at ABC Laboratories, Columbus, MO, USA (Johnson BT, personal communication, 1995). The extracts were used in Microtox and Mutatox assays to determine the toxicity [30] and mutagenicity [19,20], respectively, of the samples. Both assays rely on changes in bacterial luminescence when *Photobacterium phosphoreum* is exposed to toxins or mutagens. Dr B Thomas Johnson, National Fisheries Contaminant Research Center, Columbia, MO, conducted the assays. Toxicity was determined by comparison of a subsurface sample to an uncontaminated control soil provided by Dr Johnson. A sample was classified as genotoxic, suspect mutagen, or negative, in decreasing order of potency, by the Mutatox assay [20].

Physical analysis of samples

The pH was determined using method 9045, US Environmental Protection Agency [43]. The texture analysis of the subsurface materials was conducted by Law Engineering, Houston, TX, USA. The concentration of JP-4 in the subsurface materials was determined using gas chromatography as described previously [44].

Statistics

Student's *t*-test for equal or unequal variances was used to compare microbial numbers in contaminated and uncontaminated zones. The relationship between biodegradation rate and microbial numbers was determined by one-tailed linear correlation using Student's *t*-test. Differences in biodegradation activity between samples were determined by comparing individual points on the biodegradation curves. Points were different if the means were separated by more than one standard deviation. Differences in biodegradation rates could not be determined because the initial rates were derived from a section of the curve which contained only two points.

Results and discussion

Physical properties of subsurface material

Core samples were composed of at least 92% sand while the remainder was silt (Table 1). The samples were slightly acidic and contained nitrate concentrations below the detection limit (0.5 mg kg^{-1}). The total organic carbon levels were low. A more comprehensive characterization of the physical properties of the site has been published [18].

Biodegradation studies

In general, toluene and ethylbenzene were the most biodegradable while benzene and 1,2,3-trimethylbenzene were recalcitrant in core samples in which alkylbenzene biodegradation occurred. For example, toluene, ethylbenzene and *m*-xylene were degraded rapidly, while *p*-xylene, *o*-xylene, 1,3,5-trimethylbenzene and 1,2,4-trimethylbenzene were degraded less rapidly, and benzene and 1,2,3-trimethylbenzene were more recalcitrant in sample 80EB5 (Figure 2). Similar patterns of BTEXTMB biodegradation under denitrifying conditions were observed in samples of aquifer material from Park City, Kansas [15]. Alkylbenzene biodegradation coincided with nitrate removal and nitrite production in sample 80EB5; however, denitrification preceded alkylbenzene removal in some samples (data not shown), presumably because of the presence of additional electron donors.

Even though more BTEXTMB was removed in the non-

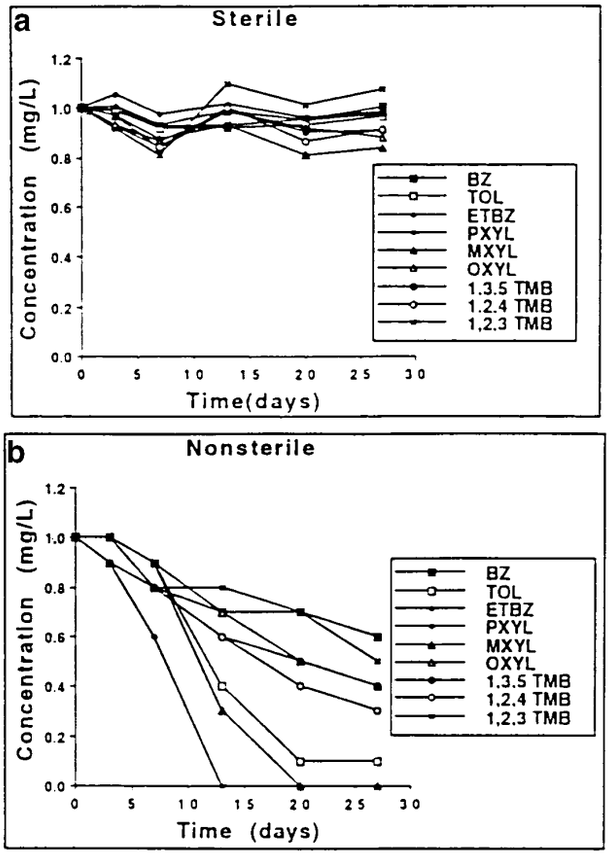

Figure 2 Removal of alkylbenzenes in sterile (a) and nonsterile (b) sample 80EB5 under denitrifying conditions (BZ, benzene; TOL, toluene; ETBZ, ethylbenzene; PXYL, *p*-xylene, MXYL, *m*-xylene; OXYL, *o*-xylene; TMB, trimethylbenzene).

sterile than the sterile controls, the data do not conclusively prove that these compounds are biodegraded under denitrifying conditions. Previous work showed that abiotic removal of benzene is enhanced when a biodegradable substrate, such as toluene, is metabolized under denitrifying conditions, possibly in response to enhanced sorption to increased biomass or metabolites [13]. Since benzene is the least hydrophobic of the BTEXTMB compounds, sorption of the alkylbenzenes would also be expected. However, removal of the compounds in sample 80EB5 (Figure 2b) and at the other borehole locations (data not shown) was not related to hydrophobicity (ie benzene, the least hydrophobic, and 1,2,3-trimethylbenzene, the most hydrophobic of the compounds, were both recalcitrant), which strongly suggests that biodegradation is primarily responsible for removal of the alkylbenzenes. In addition, alkylbenzene removal generally coincided with nitrate removal and nitrite production. Results from studies using microcosms in which radiolabeled benzene, toluene, *m*-xylene and *o*-xylene were used indicated 1.9, 53.0, 84.6 and 0.39 percent mineralization, respectively, in samples collected in the zone of contamination after nitrate treatment had started (unpublished data). These data provide additional evidence that some of the removal could be attributed to biodegradation.

To examine the overall biodegradation activity at the site, the individual alkylbenzene concentrations were totaled (excluding benzene) (Figure 3). Of the 18 core samples, eight (80AA7, 80EB2, 80EB1, 80JB2, 80JB1, 80JB5, 80KB2 and 80KB1) did not exhibit any biodegradation potential in comparison to sterile controls. Except for samples 80AA7, 80EB2 and 80EB1, the inactive samples did not contain detectable JP-4 contamination (Table 1, Figure 1). Although sample 80AA7 contained 276 mg JP-4 kg^{-1}, the low potential for alkylbenzene biodegradation may have resulted from the low numbers of microorganisms detected in this sample in comparison to the other samples with similar or higher JP-4 concentrations (Figure 3a, Table 2). The control curve for core 80EB was highly variable which made it difficult to determine significant biodegradation activity in those samples (Figure 3d). These data suggest that the microorganisms in the contaminated zone have adapted to degrade alkylbenzenes. Other researchers have reported an adaptation response of subsurface microorganisms after exposure to contamination [3,21,26,27,42,47].

The most active samples at the site were 80BA3, 80DA8, 80EB5 and 80KB6, in which alkylbenzene removal was 80% or greater. Although JP-4 was not detected at any depth in the 80KB core, which was located outside the zone of influence of the pipeline spill, the activity in sample 80KB6 may have been the result of prior exposure to a soluble BTEX plume (Table 1, Figure 3f). Analysis of subsurface material just below sample 80KB6 indicated the presence of 0.031 and 0.321 mg kg^{-1} BTEXTMB at depth intervals of 2.0–2.2 and 2.2–2.4 m, respectively [18].

Biodegradation activity was not related to depth or saturation when means of individual points between biodegradation curves were compared (Figure 3). The deepest sample (saturated) yielded the most activity in cores 80DA, 80EB, and 80KB and the least activity in sample 80AA; there was a lag phase before biodegradation commenced in the bottom sample of core 80BA, after which biodegradation potential was about the same as the other core samples. The top core (usually unsaturated) was most active in core 80BA and exhibited an intermediate activity in cores 80AA and 80DA. There was too much variation in the data to discern differences in biodegradation in the upper samples of core 80EB. There was no biodegradation activity in core 80JB or the top samples of core 80KB in comparison to the sterile controls.

The mean pseudo zero-order rate (linear) constants for alkylbenzene biodegradation and NO_3^--N removal in the 10 active samples were 1.2 ± 0.5 mg L^{-1} day^{-1} and 2.4 ± 1.1 mg L^{-1} day^{-1}, respectively (Table 3). These rates are lower than those determined at other field sites undergoing nitrate-enhanced bioremediation [14,16]. Nutrient addition had no effect on alkylbenzene biodegradation in the mixture of samples 80AA1 and 80AA2 (data not shown).

Characterization of microorganisms

Aquifer sediments at the site contain variable, but generally high, numbers of denitrifying bacteria, many of which can grow using constituents or breakdown products of JP-4 as carbon sources (Table 2). The MPN of total denitrifiers ranged from 10^4 to 10^7 per g of soil, about the same as that observed in JP-4-contaminated aquifer material from

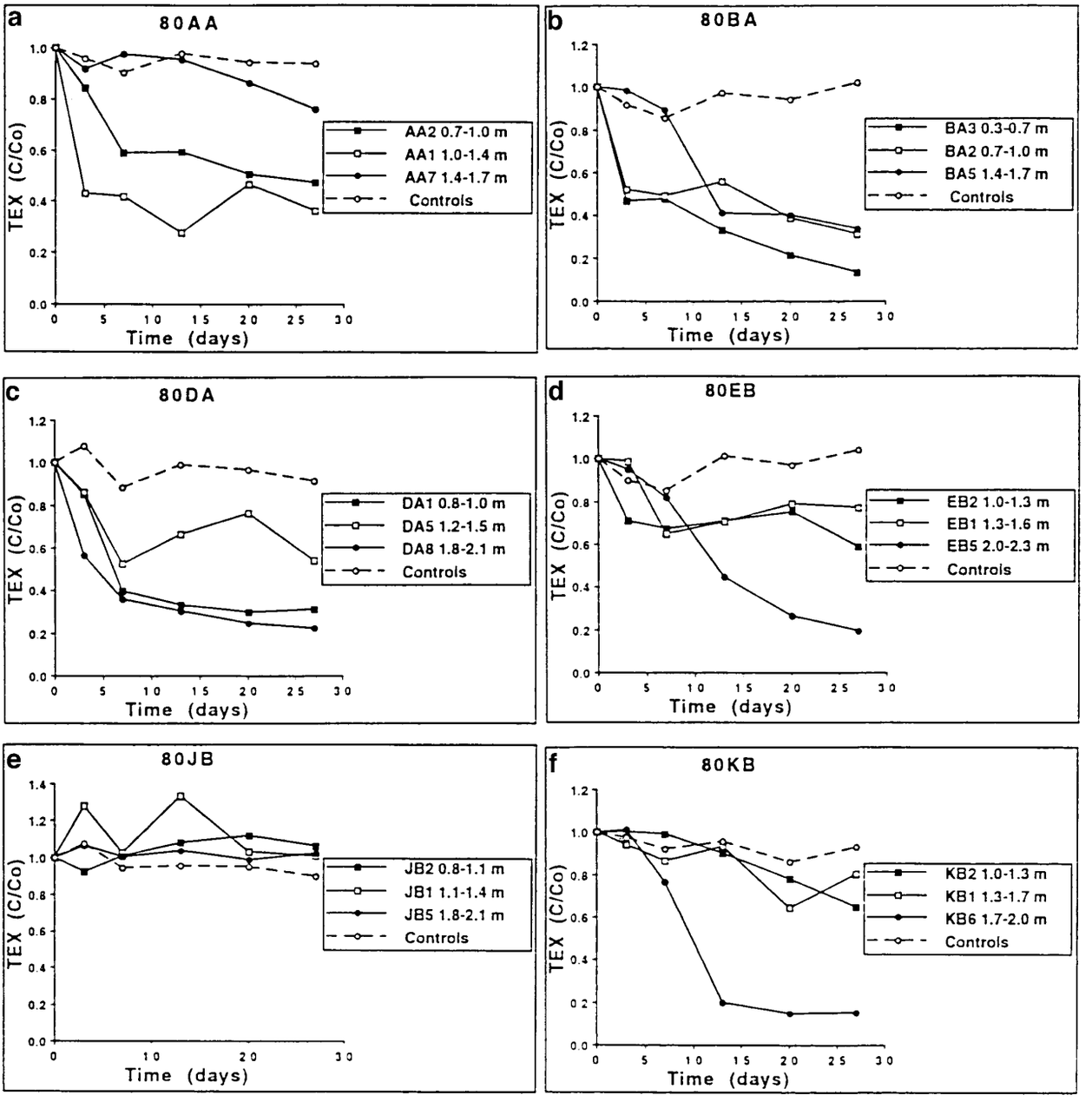

Figure 3 Biodegradation of alkylbenzenes (TEX) under denitrifying conditions at selected depths below ground surface in cores (a) 80AA, (b) 80BA, (c) 80DA1, (d) 80EB, (e) 80JB, and (f) 80KB.

Traverse City, MI [17]. Viable counts ranged from below detection to 10^6 per g and direct counts ranged from $10^{7.2}$ to $10^{9.0}$ per g, which is within the range observed for other subsurface materials [8,21]. Although the number of microorganisms growing on a mineral salts medium in the presence of JP-4 vapor (JP-4 degraders) or without JP-4 vapor (oligotrophs) were about the same in many samples, the size of many colonies in the former were larger. Those microorganisms producing large colonies most likely were growing on the JP-4 whereas those producing small colonies may have grown on carbon impurities in the medium. Cell numbers estimated by phospholipid fatty acid analysis were usually less than, but positively correlated ($\alpha = 0.05$) to direct count (Table 2).

Both aerobic and anaerobic protozoa were detected, suggesting that protozoa may be active under a variety of different electron acceptor conditions. The anaerobic protozoa were detected at numbers of $10^{2.7}$ or less per g dry weight. Previously, Kinner *et al* [22] reported anaerobic protozoa in an aquifer contaminated with municipal wastewater at Cape Cod, MA. A flagellate isolated from sample 80DA1 grew at 0 ppm oxygen, but also was able to grow in the presence of atmospheric oxygen.

The aerobic protozoa ranged from below detection to 10^6

Table 2 Numbers of different types of microorganisms in subsurface samples[a]

Sample	Depth (m)	Log No. g^{-1} dry wt (s.d.)			Phospholipid fatty acid count (Log cells g^{-1})	Direct counts log No. g^{-1} dry wt (s.d.)	Total denitrifiers[b] Log MPN g^{-1} dry wt (s.d.)	Denitrifiers[b] Log MPN g^{-1} dry wt (s.d.)		Protozoa[b] Log No. g^{-1} dry wt	
		Total heterotrophs	JP-4 degraders	Oligotrophs				JP-4	no JP-4	Aerobic	Anaerobic
80AA2	0.7–1.0	6.7 (0.1)	6.6 (0.1)	6.6 (0.1)	8.5	9.0 (0.1)	7.1 (0.4)	6.8 (0.2)	3.4 (0)	4.4 (0.2)	0.8 (0.2)
80AA1	1.0–1.4	6.8 (0.2)	5.5 (0.1)	5.5 (0.2)	8.1	8.9 (0.1)	7.2 (0.6)	6.4 (0.1)	<1	3.0, <1, <1	2.0 (0.1)
80AA7	1.4–1.7	4.7 (0.2)	2.4 (0.1)	<2	7.3	7.2 (2.3)	4.2 (0.2)	3.2 (0.5)	<1	2.9 (0.1)	0.4 (0)
80BA3	0.3–0.7	5.7 (0.1)	4.5 (0.1)	3.7 (0.1)	8.5	8.9 (0.2)	5.2 (0.7)	1.6 (0.3)	1.9 (0.5)	6.2 (0.1)	2.7 (0.4)
80BA2	0.7–1.0	6.0 (0.1)	4.4 (0.2)	3.9 (0.1)	8.6	9.1 (0.1)	6.0 (0.4)	4.5 (0.2)	3.2 (0.1)	5.8 (0.4)	2.2 (0.4)
80BA5	1.4–1.7	4.4 (0.1)	3.6 (0.1)	3.6 (0.1)	7.8	7.5 (2.4)	4.3 (0.4)	2.9 (0.3)	<1	2.9 (0.5)	0.7 (0.6)
80DA1	0.8–1.0	5.9 (0.04)	4.9 (0.1)	5.1 (0.1)	8.5	8.8 (0.1)	6.5 (0.2)	3.9 (0.2)	2.5, 2.9, <1	5.7 (0.4)	>2, >2, 1.6
80DA5	1.2–1.5	5.9 (0.03)	3.8 (0.1)	3.4 (0.1)	8.0	8.5 (0.1)	6.1 (0.1)	4.1 (1.2)	1.6 (0.2)	6.0 (0.3)	<0, <0, 0.7
80DA8	1.8–2.1	5.8 (0.1)	5.2 (0.1)	5.2 (0.1)	7.7	8.5 (0.2)	6.3 (0.4)	5.6 (0.3)	<2	>4	1.6 (0.2)
80EB2	1.0–1.3	6.8 (0.1)	5.7 (0.3)	6.2 (0.03)	8.1	8.4 (0.3)	6.6 (0.6)	5.5 (0.9)	<1	2.3 (0.3)	0.9, <0, 1.0
80EB1	1.3–1.6	4.6 (0.1)	4.2 (0.1)	2.6 (1.9)	8.0	8.3 (0.4)	4.4 (0.9)	2.3 (0.5)	<1	2.7 (0.2)	<0
80EB5	2.0–2.3	5.6 (0.1)	5.7 (0.1)	5.8 (0.1)	7.3	8.4 (0.3)	6.4 (0.2)	5.1 (0.3)	<1	3.5 (0.1)	<0, 0.4, 0.4
80JB2	0.8–1.1	5.6 (0.1)	3.3 (0.1)	3.3 (0.1)	7.5	8.3 (0.3)	4.7 (0)	3.7 (0.3)	<1	3.6 (0.7)	1.7 (0.2)
80JB1	1.1–1.4	6.6 (0.1)	6.3 (0.1)	6.3 (0.1)	7.6	8.3 (0.2)	7.1 (0.4)	6.0 (0.4)	<1	2.5 (0.2)	<0, 0.7, 1.0
80JB5	1.8–2.1	4.8 (0.1)	4.2 (0.1)	4.2 (0.04)	7.0	8.1 (0.2)	4.4 (0.2)	3.0 (0.2)	1.5, 1.5, <1	2.8 (0.4)	<0, <0, 1.4
80KB2	1.0–1.3	5.8 (0.1)	4.2 (0.1)	4.2 (0.05)	7.6	8.5 (0.2)	4.8 (0.2)	0.9 (0.2)	<1	3.2 (0.2)	1.4 (0.2)
80KB1	1.3–1.7	5.2 (0.02)	4.4 (0.03)	4.9 (0.1)	7.3	7.8 (1.5)	5.7 (0.4)	1.4 (0.7)	<1	<1	0.8 (0.2)
80KB6	1.7–2.0	5.9 (0.2)	3.5 (0.1)	3.2 (0.1)	6.8	8.1 (0.4)	5.3 (0.2)	2.0 (0.2)	<1	2.5 (0.2)	1.3 (0.7)

[a]<log 0, log 1, or log 2; >log 2, or 4 were the detection limits of the assays.
[b]Triplicate subsamples were averaged unless a replicate was greater or less than the detection limit.

Table 3 Removal rates of alkylbenzenes (TEX) and nitrate under denitrifying conditions

Sample	Time period for TEX and nitrate removal (days)	Pseudo zero-order rate constant for TEX removal (mg L^{-1} per day)	Pseudo zero-order rate constant for nitrate removal (mg L^{-1} per day)
80AA2	0–7	0.5	1.5
80AA1	0–3	1.2	3.7
80AA7	NA[a]	ND[b]	0.4
80BA3	0–3	1.7	2.7
80BA2	0–3	1.6	3.3
80BA5	7–13	1.1	1.6
80DA1	3–7	1.3	3.4
80DA5	3–7	0.6	1.0
80DA8	0–3	2.1	3.6
80EB2	NA	ND	5.4
80EB1	NA	ND	2.3
80EB5	7–13	1.0	1.4
80JB2	NA	ND	<0.3
80JB1	NA	ND	<0.3
80JB5	NA	ND	<0.3
80KB2	NA	ND	<0.3
80KB1	NA	ND	0.7
80KB6	3–13	1.1	1.4

[a]NA, not active.
[b]ND, not determined.

per g and varied in numbers between boreholes and with depth in each borehole. The highest aerobic protozoan numbers were similar to those reported for a site contaminated with aviation gasoline [36] but higher than that encountered at a site contaminated with coal tar [26]. Variable numbers of aerobic protozoa between boreholes and with depth were also observed in aviation gasoline and coal tar ground water plumes [26,36] and may reflect the relation between contaminants, oxygen and contaminant-degrading bacterial populations.

Protozoa may affect the bioremediation of organic contaminants in ground water plumes by limiting the population size of the bacteria and thereby may prevent biomass from clogging the aquifer [12]. They may stimulate contaminant-degrading bacteria by mineralizing organic-bound limiting minerals [9] or by other grazing effects. Finally, protozoa may reduce the number of specific contaminant-degrading bacteria and thereby slow biodegradation [45].

Numbers of the different types of microorganisms in the zone of residual contamination (cores 80AA, 80BA, 80DA, and 80EB) were statistically compared to the uncontaminated samples, 80KB2 and 80KB1, of the control core (Figure 1); sample 80KB6 was not used as an uncontaminated control since it probably had been exposed to contamination. Microbial numbers were significantly higher in the zone of residual contamination than in the uncontaminated control samples, except for the number of oligotrophs, total denitrifiers, and an estimate of anaerobic protozoa (α = 0.05). These data suggest that the biomass increased as a result of the contamination event. The high

numbers in the contaminated zone of JP-4 degraders and microorganisms that can use JP-4 under denitrifying conditions suggest that these microorganisms have adapted to degrade the JP-4 fuel. Others reported that contamination often increases biomass and biodegradation activity [1,37,42].

Predicting biodegradation activity with microbial counts

Rates of alkylbenzene biodegradation and numbers of different types of microorganisms were compared by core location and depth (Tables 2 and 3). There was no significant correlation ($\alpha = 0.05$) between biodegradation rate and microbial numbers except for numbers of JP-4 degraders, aerobic protozoa, and anaerobic protozoa in core 80BA only. These data suggest that measurements of microbial numbers alone do not reliably predict biodegradation activity.

Toxicity and mutagenicity assessment

Using the Microtox assay, the only samples which exhibited toxicity were 80AA1 and 80EB1 (data not shown), which were the samples that contained the highest JP-4 concentrations (Table 1). However, sample AA1 exhibited the highest biodegradation activity in the 80AA core, suggesting that the JP-4 fuel was not toxic to the contaminant-degrading microorganisms. The biodegradation activity in sample 80EB1 was not different from the sterile control; however, this inactivity may have been the result of the variability of the 80EB control rather than toxicity. Sample 80EB2, which was not toxic, also had high JP-4 concentrations and was inactive compared to the controls. In addition to being toxic, 80EB1 was a suspect mutagen. Although two samples contained toxic and one contained suspect mutagenic material, the data were too few and variable to show a correlation between JP-4 fuel concentration and contaminant biodegradation.

Conclusions

The results of the microbial characterization study indicated that the site is amenable to nitrate-enhanced bioremediation. Biomass increased as a result of the JP-4 contamination and the subsurface microflora was capable of degrading several alkylbenzenes in JP-4 jet fuel under denitrifying conditions. Although nine estimates of microbial biomass were determined, no single measure was predictive of biodegradation activity at the site. We believe the variability and inconsistencies observed were probably influenced by subsurface heterogeneity. However, the cost of sampling to statistically describe the heterogeneity would have been prohibitive. These findings suggest that more time and effort should be invested in developing new, better and more cost-effective methods to predict biodegradation activity in heterogeneous environments.

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