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Metabolite Formation and Toxicity Measurements in Evaluating Bioremediation of a Jet-Fuel-Contaminated Aquifer

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Abstract

The metabolic capabilities of subsurface, jet-fuel-contaminated, aquifer microbial communities were characterized using an ecological approach to biotreatment assessment. A multifaceted experimental design was used that incorporated quantification of metabolite formation and toxicity screening along with the typical microbial activity measurements and pollutant degradation measurements used for assessing bioremediation potential. For select experiments, dissolved oxygen levels and pH in microcosm systems were also monitored. Results suggest that a sizable, metabolically active microbial community exists in both contaminated and uncontaminated areas of the study site. Time course metabolism analyses indicated that the microbial communities were capable of degrading all three test compounds (amino acids, decane, and toluene) without any apparent adaptation period. Measurements of mineralization, cellular uptake, and metabolite formation indicated that metabolite formation was the predominant fate of the target pollutants in the microcosms. The results of toxicity screening time courses indicated that under oxygen-limiting conditions, the potential for the accumulation of toxic, acidic metabolites that could adversely affect the rates and extent of bioremediation existed. The experimental results indicate that the microbial communities at the site possess the metabolic potential for *in situ* biodegradation of the jet fuel. Care must be taken in the design of an *in situ* biodegradation treatment system (for this site and perhaps other petroleum-contaminated aquifers) to avoid the development of microaerophilic or

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oxygen-depleted zones, which could result in possible self-poisoning owing to acidic metabolite accumulation.

Index Entries: Aquifer; bioremediation; jet fuel; decane; toluene; microcosm.

Introduction

Investigations conducted during the mid and early 1980s have shown that groundwater supplies are susceptible to both biological and chemical pollution. Major groups of chemical pollutants present in groundwater include petroleum products, volatile organic compounds, chlorinated solvents, complex aromatic compounds, and pesticides. In many instances, at sites where groundwater pollution exists, more than a single pollutant is present. Petroleum products are, by nature, complex mixtures of organic compounds including hundreds of individual components and on a total mass basis are by far the most prevalent environmental pollutant *(1,2)*. The major chemical classes present in petroleum products include aliphatic compounds, cycloaliphatic compounds, aromatic compounds, and polyaromatic compounds. Resins and asphaltenes are also present in quantifiable amounts in unrefined petroleum products.

One technology for the cleanup of petroleum contamination in groundwater systems is *in situ* bioremediation. Under optimal environmental conditions, the hydrocarbon constituents normally found in high quantities in petroleum products are readily biodegraded aerobically to carbon dioxide (CO_2) and water. It has been estimated that between 70 and 90% of petroleum product components are biodegradable *(3)*. It is generally recognized that many species of bacteria can metabolize aliphatic fractions of petroleum products via β-oxidation pathways to form products such as CO_2, cellular biomass, and short-chain organic acids *(4,5)*. Bacterial metabolic pathways for biodegradation of the aromatic fraction, including the benzene, toluene, ethylbenzene, and xylene (BTEX) fraction, of petroleum products has been well characterized *(6,7)*. It has also been demonstrated that subsurface microbial communities have the ability to degrade a range of natural and xenobiotic compounds under a wide variety of environmental conditions *(8–13)*.

Select BTEX compounds, particularly benzene, have often been investigated to assess microbial degradation of petroleum contamination in aquifer systems because they are of regulatory concern owing to their toxicity and potential health and ecological effects. Although the biodegradability of petroleum products and their constituents has been well documented, the U. S. Environmental Protection Agency (EPA) and many state regulatory agencies do not consider *in situ* bioremediation a proven technology. One reason for this may be the presence of large quantities of residual saturation that may act as a source of dissolved constituents for long periods of time. Therefore, *in situ* bioremediation must be evaluated on a site-by-site basis for applicability.

The goal of this research was to assess aerobic biodegradation of petroleum hydrocarbon contamination by adopting an ecological approach to biotreatment assessment. A multifaceted experimental design incorporating quantification of metabolite formation and toxicity screening along with standard target compound degradation measurements was utilized. The metabolic fate of a representative aliphatic contaminant, decane, and a representative aromatic contaminant, toluene, were assessed in various aquifer sediment samples using ^{14}C-radiolabeled techniques. In addition, an overall index of metabolic activity in each aquifer sediment sample was assessed by determining the metabolic fate of ^{14}C-mixed amino acids. The metabolic fates assessed for all three test chemicals included mineralization, production of $^{14}CO_2$; cellular uptake, and partial transformation to metabolic intermediates. Changes in microcosm pH, dissolved oxygen (DO), and toxicity were also determined for select experiments to assess the interactions between these parameters and biodegradation.

Materials and Methods

Aquifer sediment samples were collected from a contaminated coastal plain aquifer in Hanahan, near Charleston, South Carolina. An estimated 310,000-L (83,000-gal) spill of JP-4 jet fuel occurred at the site in 1975 from the Tank 1 area (Fig. 1). Approximately 79,500 L (21,000 gal) were recovered by the following year; thus, a great deal of contamination remained sorbed to sediments and dissolved in groundwater. Aquifer sediments at the site consist of predominantly medium-sized sand with interfingering lenses of clay to a depth of approx 6–10 m (20–35 ft). Depth to the water table varies seasonally, but is approx 1–4 m (3–14 ft) below land surface *(14)*.

Aseptic soil core-sampling techniques similar to those described by Dunlap et al. *(15)*, and later modified by Wilson et al. *(16)*, were used. Aquifer sediment samples were collected from the saturated zone and were predominantly neutral to slightly acidic sands *(17)*. The MWGS23 sample was distinct from the other samples, with an alkaline pH, and it contained coarser material and shell fragments. Figure 1 presents approximate sample locations, and Table 1 summarizes the contaminant characteristics and pH of the core sediment samples. Samples with a range of exposure to the jet-fuel contaminants were obtained. The WT7 sample was obtained upgradient of the jet-fuel release, and the MW5A sample was obtained downgradient from the estimated migration of the contaminant plume. Those two samples were judged to be uncontaminated. The MWGS24 sample was exposed to high levels (milligram/kilogram) of contaminants and had visible droplets of free product floating at the top of the sample jar. All remaining samples were collected inside the contaminant plume and were exposed to parts per billion (microgram/kilogram) levels of contaminants.

The degradation experiments were designed to provide information on whether an adaptation period is necessary for degradation of the test

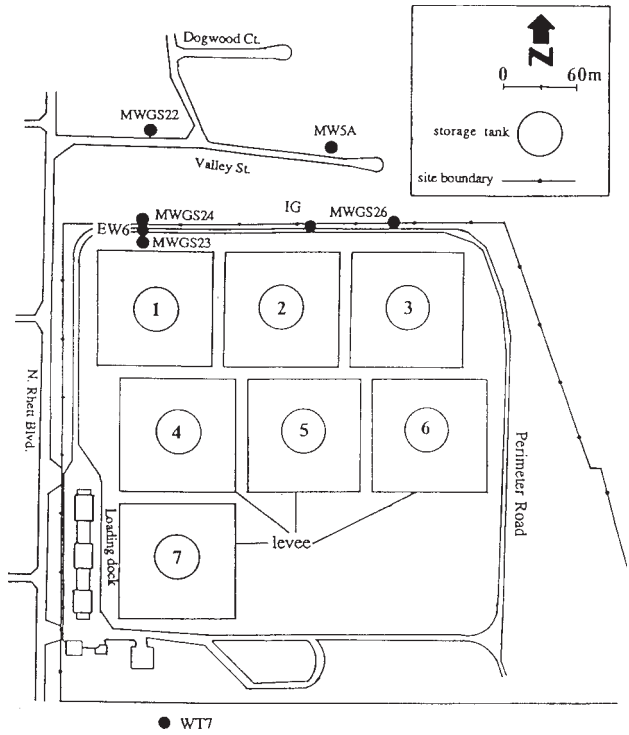

Fig. 1. Schematic of the study site and approximate sample boring locations.

Table 1
Aquifer Soil Core Characteristics

Sample ID	Depth (m)[a]	Contamination level[b]	pH
WT7	2.4 (8)	1	5.1
MW5A	6.1 (20)	1	5.9
MWGS26	5.2 (17)	2	5.9
EW6-1	3.8 (12.5)	2	6.3
EW6-2	3.8 (12.5)	2	6.3
IG	3.5 (11.5)	2	6.3
MWGS23	9.1 (30)	2	9.0
MWSG24	6.2 (20.5)	3	7.0

[a]Numbers in parentheses indicate depth in feet.
[b]1, Uncontaminated; 2, ppb (µg/kg) levels of petroleum exposure; 3, ppm (mg/kg) levels of petroleum exposure.

compounds, and allowed for the amount of mineralization, cellular uptake, and partial transformation of the test substrate(s) to be estimated so that a mass balance of added radiolabeled compound could be calculated.

A microcosm system similar to those used in several other studies of biodegradation potential in aquifer solids was used *(9,11,18,19)*. An initial

slurry of approx 1 g of sediment dry weight/10 mL of water was prepared. The microcosms were prepared by transferring approx 10 mL of slurry into 20-mL Pierce vials (Wheaton, Millville, NJ) with a total capacity of approx 25 mL. The appropriate radiolabeled target compound solution was added, and the microcosms were filled, sealed headspace free, and inverted for incubation. This approach allowed for dilution of background levels of pollutants so that the compounds added to the microcosms predominated for the majority of samples. These experiments were conducted using a radiolabeled target compound concentration of approx 100 ng/g of sediment dry weight (or 100 µg/kg). The radiolabeled solutions of target compounds were L-[^{14}C(U)]-mixed amino acids with a specific activity of 7.137 mg/mCi (New England Nuclear, Boston, MA), *n*-[1-^{14}C]decane with a specific activity of 30 mCi/mmol (Amersham, Arlington Heights, IL), [ring-UL-^{14}C]toluene with a specific activity of 10.9 mCi/mmol (Pathfinder, St. Louis, MO), and $Ba^{14}CO_3$ with a specific activity of 50 mCi/mmol (Amersham). Metabolically inhibited controls were used (0.5% [w/v] sodium azide, Sigma, St. Louis, MO), and bicarbonate controls were conducted to correct for method efficiency.

Time points were sacrificed and analyzed for amino acid time courses after 6, 12, 24, 48, 72, and 96 h and after 7 d of incubation. Decane microcosm time points were analyzed after 2, 4, 7, 14, 21, and 28 d of incubation. Toluene microcosm time points were analyzed after 3, 7, 14, 21, and 30 d of incubation. At these predetermined time points, the microcosm contents were transferred, in triplicate, to 40-mL ultraclean EPA water sample vials (Fisher, Fairlawn, NJ). An aliquot was taken and hexane extracted to determine how much radiolabeled parent compound still remained in the microcosms. The remaining portion was acidified with 20% (v/v) phosphoric acid to drive off the dissolved CO_2, and the vial was fitted with a Goodman-Long base trap containing 2 µL of 1*N* KOH. After 20–24 h, the base was collected and the remaining solution was either subject to soil washing to determine cellular uptake, or fractionated using solid/liquid phase separation techniques. Because of the destructive nature of these procedures, both analyses could not be conducted on the same sample; therefore, each procedure was conducted only on alternating time points. Both metabolically inhibited and bicarbonate control samples were also subject to the previously described analyses. All resulting fractions were subject to liquid scintillation counting (Packard Model 300CD) for quantification.

Cellular incorporation of the radiolabeled substrates was quantified using a soil-washing technique described by Dobbins and Pfaender *(18)*. Cells were released from the soil surface by washing for 30 min with a polyvinylpyrrolidone and polyphosphate solution (final concentrations 0.1 and 1%, respectively). The cells were separated from the soil solids by centrifuging at 120*g* for 15 min. The supernatant was then filtered through 0.2-µm cellulose triacetate filters to separate the microorganisms from the dissolved radiolabeled substrate. Each fraction was subject to liquid scintillation counting.

Metabolite formation from decane and toluene was assessed by fractionating the microcosm supernatant for selected experiments. First, the supernatant was extracted with hexane (high-performance liquid chromatography grade, Fisher) to assess the quantity of parent radiolabeled substrate remaining. The supernatant was then subject to solid/liquid phase separation using solid phase extraction columns (J. T. Baker, Phillipsburg, NJ). The 500-mg columns were used, C18 columns were used with decane experiments, and phenyl columns were used with toluene experiments. A series of washes with deionized water followed by methanol was conducted. Each fraction, supernatant eluate, water wash, and methanol wash was collected separately and quantified using liquid scintillation counting.

Microcosm pHs were measured using a pH meter with a combined calomel electrode (Fisher), and were measured at each time point of selected experiments to assess the changes in pH associated with contaminant metabolism. Microcosm DOs were measured using a YSI dissolved oxygen meter fitted with a self-mixing probe (YSI, Yellow Springs, CO). Changes in DO were also measured at each time point of selected experiments to assess the level of oxygen consumption and the development of oxygen-limited conditions in the microcosms as contaminant metabolism progressed. Care was taken not to introduce atmospheric air into microcosm contents during DO measurements. Microcosms were opened, and the DO probe was fitted with a Teflon-lined septa, and an open ring top was placed into the microcosm at an angle to avoid entrapping air bubbles. Excess microcosm contents were displaced and the ring top was tightened.

A separate set of microcosms was prepared and amended with target compound solutions, amino acids and decane (Sigma), and toluene (Fisher) that were not radiolabled for selected experiments. At identical time points, as for degradation experiments, the microcosm contents were each transferred to a 35-mL glass centrifuge tube (Kimble Glass, Vineland, NJ) and centrifuged at 250*g* at 4°C to separate the supernatant from the sediment. The supernatants were tested using the Microtox® 100% assay procedure, and the pellets were tested using the Microtox solid phase test (Microbics, Carlsbad, CA). Results provide an EC_{50} value. Sediments exhibiting higher toxicities will provide lower EC_{50} values, reflecting a lower effective dose. Since this is counterintuitive, EC_{50} Index values were calculated and presented here. The EC_{50} Index is equal to the reciprocal of the EC_{50} value $\times 100$. Including a toxicity screening assay in the experimental approach allowed us to determine whether biodegradation may be limited by toxic effects of the target pollutants or by their metabolites.

Statistical analyses and appropriate confidence intervals for all data presented were based on pooled error values (s^2_{pooled}) for grouped samples in contaminant Levels 1 and 2 to calculate 95% confidence limits so that they could be directly compared to the group 3 single sample results. *t*-Statistics for independent samples using pooled values (s^2_{pooled}) and nonparametric tests were used to determine statistical significance of the different measurements of metabolic potential among soil sample groups (Levels 1, 2, and 3).

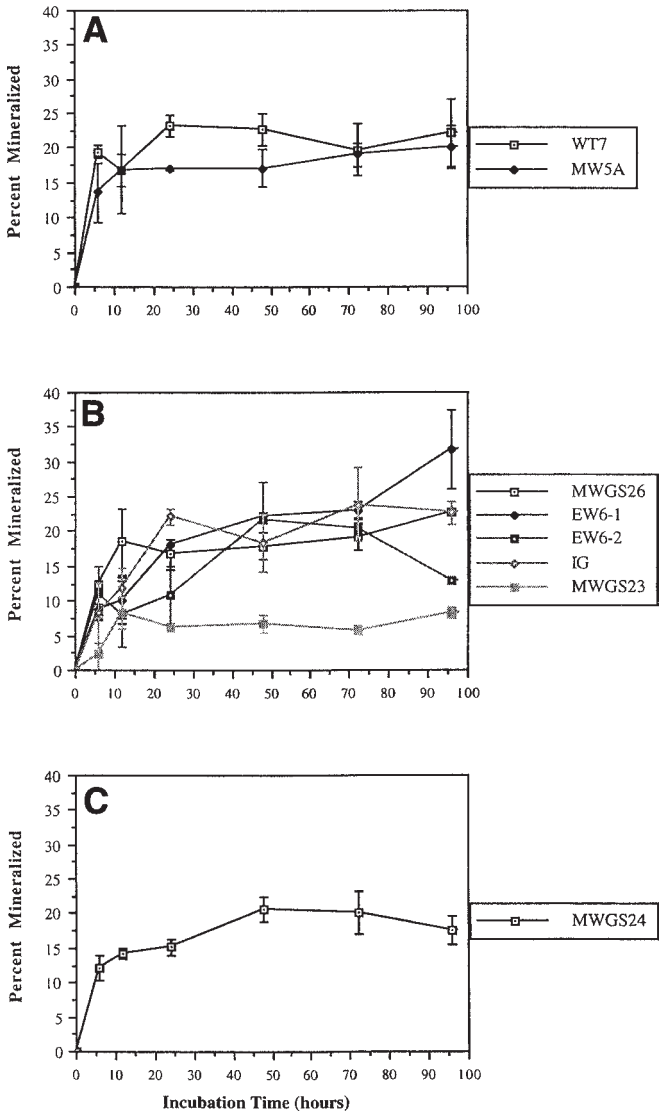

Fig. 2. Amino acid mineralization time course results for each sample group.

Results and Discussion

Mineralization

The results of all time course experiments are presented by target compound and soil pollutant exposure group. Figure 2 presents the amino acid mineralization time course data for the Level 1, 2, and 3 soil pollutant exposure groups, parts a, b, and c, respectively. The results indicated that no apparent adaptation period, or an adaptation period less than the first time point, was required before metabolism began. For amino acid metabolism, a maximum of 24% conversion to CO_2 was measured for both

Level 1 samples; a maximum of 22–34% conversion to CO_2 was measured for the Level 2 samples except for the alkaline MWGS23 sample, in which only 11% conversion to CO_2 was measured; and 20% conversion to CO_2 was measured in the Level 3 sample. Thus, it can be noted that for all samples, amino acid mineralization proceeded rapidly and appeared to be mostly completed by 2–4 d of incubation. The amount of amino acids converted to CO_2 for the MWGS23 sample was statistically different from the other samples in the Level 2 group, whereas the levels of hydrocarbons converted to CO_2 were statistically similar ($\alpha < 0.05$). Only 11% of added amino acids were metabolized in that sample when compared to an average of 25% mineralization for the other four samples in the Level 2 group.

Figure 3 presents the decane mineralization time course data, with parts a, b, and c of the figure presenting the results for the Level 1, 2, and 3 soil pollutant exposure groups, respectively. The peak level of decane conversion to CO_2 was 12 and 15% for the Level 1 samples, between 11 and 16% for the Level 2 samples, and 4% for the Level 3 sample. For decane conversion to CO_2, the alkaline MWGS23 sample demonstrated one of the highest levels of activity, 15%. Similar to amino acid mineralization, decane mineralization proceeded rapidly in the Level 1 and 2 samples and was mostly completed by 14–21 d of incubation. Decane mineralization proceeded slowly in the Level 3 sample, and gradually increased for the first 8 d of incubation. Overall, the mineralization of decane was similar between the Level 1 and 2 samples. The Level 3 sample demonstrated a different pattern and lesser extent of decane degradation when compared with the other two groups.

Figure 4 presents the toluene mineralization time course data. Again, parts a, b, and c of the figure present the results for the Level 1, 2, and 3 soil pollutant exposure groups, respectively. The levels of toluene conversion to CO_2 were 6 and 8% for the Level 1 samples, between 2 and 20% for the Level 2 samples, and 3% for the Level 3 sample. Mineralization activity was essentially completed in the microcosms by 21 and 30 d of incubation in Level 1 samples. Two of the Level 2 samples, EW6-1 and IG, exhibited a maximum of about 20% toluene mineralization by 14 d of incubation. Two of the Level 2 samples, MWGS26 and EW6-2, exhibited low levels of toluene mineralization, similar to that observed with the Level 3 sample with a gradual increase in mineralization through 30 d of incubation. For the alkaline MWGS23 sample, a peak of 6.5% mineralization was measured after 30 d of incubation.

The mineralization results from each of the soil sample groups were pooled and averaged. The 72-h, 14-d, and 21-d time points were chosen for amino acid, decane, and toluene mineralization, respectively, because mineralization in the microcosms appeared to have plateaued for each test compound and significant artifacts of microcosm effects did not appear to have affected CO_2 recoveries (data not shown). Figure 5 summarizes the average level of each target compound mineralized for each sample group, as discussed previously. The error bars represent 95% confidence levels for

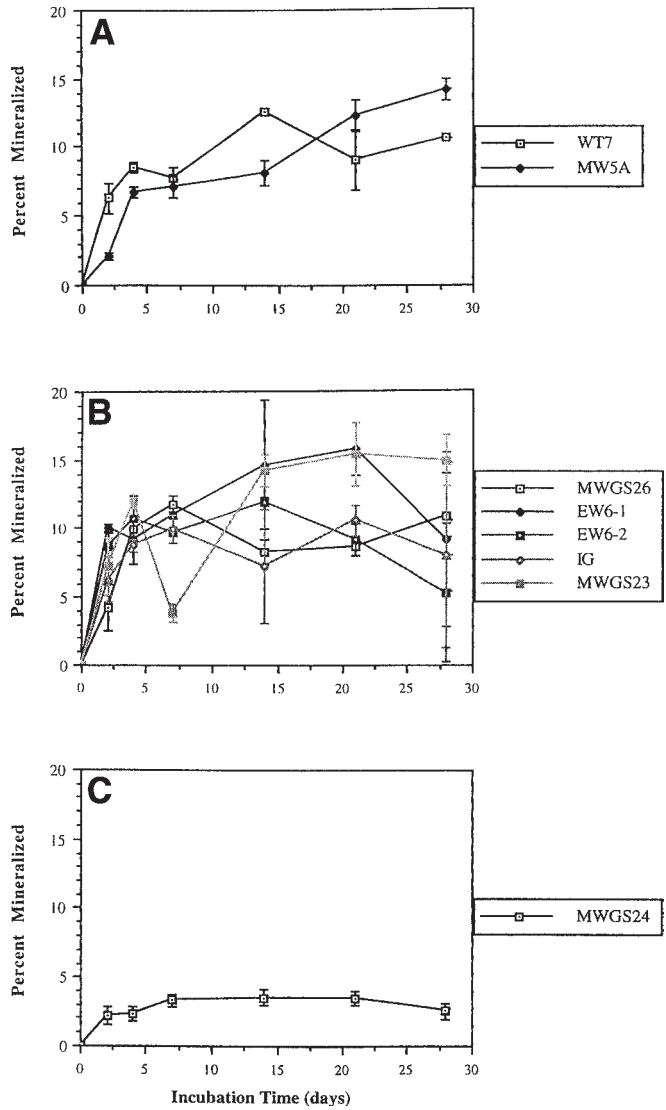

Fig. 3. Decane mineralization time course results for each sample group.

each sample group. Amino acids were mineralized to a greater extent than decane, which in turn was metabolized to a greater extent than toluene. No significant difference in amino acid mineralization after 72 h of incubation was observed among the sample groups—26, 22, and 20.5% for the Level 1, 2, and 3 samples, respectively. For decane, mineralization levels after 14 d of incubation were similar for the Level 1 and 2 samples, 10.4 and 11.1%, and both were significantly different ($\alpha < 0.05$) from the Level 3 sample, 3.5%. Toluene mineralization after 21 d of incubation was calculated to be 3% for Level 1 samples, 8% for Level 2 samples, and 1.8% for the Level 3 sample. However, the difference in the extent of toluene mineral-

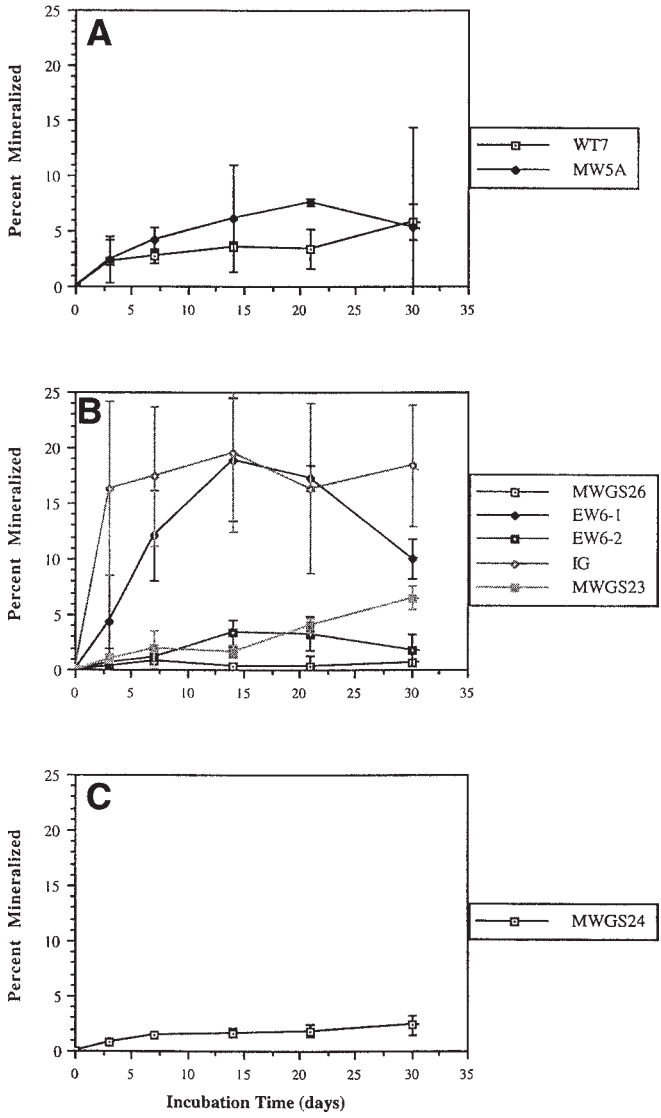

Fig. 4. Toluene mineralization time course results for each sample group.

ization was only statistically significant ($\alpha < 0.05$) when Level 1 and 2 samples were compared. Level 2 and 3 samples were not statistically different.

The levels of amino acid mineralization after 72 h of incubation, ranging from 11 to 34%, were similar in magnitude to those reported in aquifer samples studied by Armstrong et al. *(20)*. No literature values for decane mineralization were located for aquifer sediment microcosms. However, in a study of vadose zone soils, maxima of 20–30% mineralization were measured for C_6-C_{16} *n*-alkanes after an incubation time of 4 wk *(21)*. The results in this study, which ranged from 3 to 20% mineralization after 14 d of incubation, were lower than literature values but along the same magnitude.

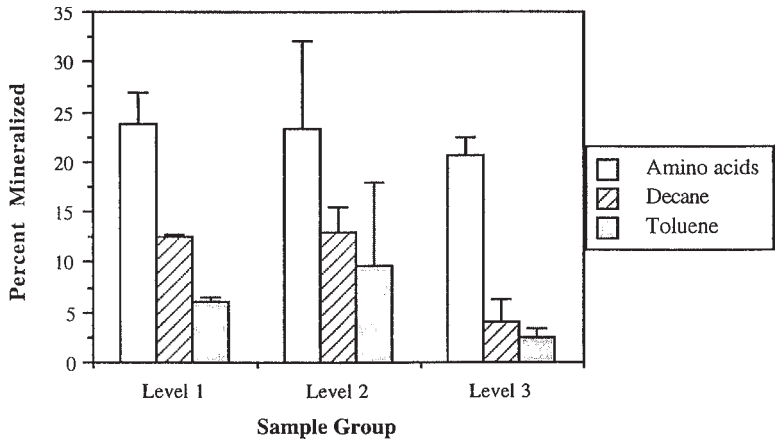

Fig. 5. Comparison of mineralization of target compounds among sample groups.

Several reports of toluene mineralization in aquifer microcosms were reviewed *(11,14,20,22–24)*. Levels of toluene mineralization measured after 21 d of incubation in this study were generally low, but were within the range reported in the literature for both the uncontaminated and contaminated sample groups. Wide variations in metabolic response to toluene were noted for all samples, with observed variation within sample groups being larger than variation across sample groups, as discussed previously.

Metabolic Fate

Proportions of radiolabeled contaminants metabolized and incorporated into cellular material and extracellular material were also measured. The metabolic fates delineated included mineralization (conversion to CO_2) and incorporation into cellular materials for amino acid microcosms. In addition, the proportion of target compound converted to water-soluble metabolites was also measured for the decane and toluene microcosms. These analyses were conducted for the 24-h, 14-d, and 21-d incubation times for amino acids, decane, and toluene, respectively. For this discussion, the total amount of amino acids metabolized includes conversion to CO_2, incorporation into cellular materials, and incorporation into extracellular materials. The total among decane and toluene metabolites includes conversion to CO_2, incorporation into cellular materials, incorporation into extracellular materials, and transformation to water soluble metabolites.

Figure 6 presents the results of the metabolic ^{14}C fate analyses for amino acids, decane, and toluene metabolism in parts a, b, and c, respectively. Overall, mass balance recoveries of radiolabel was approx 88% for added amino acids, with recoveries ranging from 67 to 107%. The mass balance recoveries for decane experiments averaged 61%, with recoveries ranging from 51 to 102%. The mass balance recoveries for toluene averaged 54% of added radiolabel and ranged from 37 to 72%. These lower recoveries

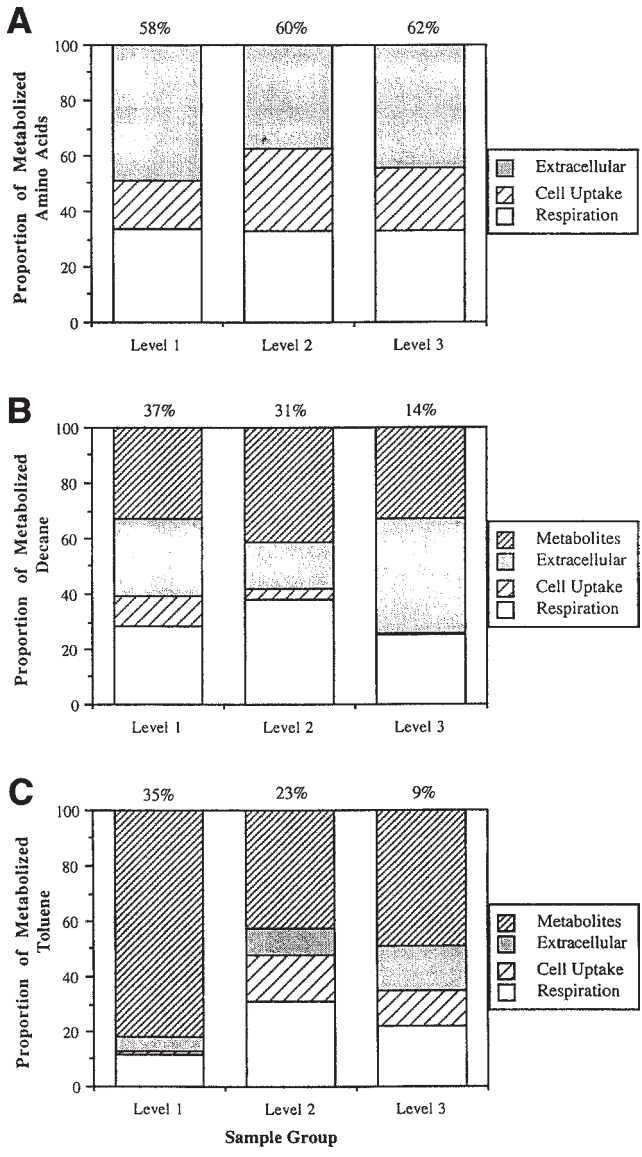

Fig. 6. Comparison of target compound metabolic fates among sample groups. Fractions of each classification normalized to 100% for comparison purposes. Numbers at the top represent percentage of compound initially added that was metabolized.

may be owing to losses from volatilization of target compound, sorption to microcosm surfaces, subsample errors (since small aliquots of each fraction were subject to scintillation quantification), and loss of volatile metabolites (CO_2 and short-chain acids) as a result of microcosm artifacts. Mineralization and metabolism measurements were corrected using abiotic controls and carbonate recovery controls. However, the metabolic inhibitor sodium azide is alkaline in nature, and the bicarbonate stock

was dissolved in 2*N* sodium hydroxide making the control microcosms less susceptible to decreases in pH to significantly below the pK_a of carbonate than the live microcosms. Nevertheless, nearly all samples exhibited recoveries >50%.

About 60% of the added amino acids was metabolized across all groups of soil samples. Approximately one-third of the total metabolized amino acids was mineralized, and two-thirds was incorporated into cellular or extracellular carbon. Similar to measurements of mineralization, total metabolism of amino acids observed for the MWGS23 (coarse and alkaline) sample was different from the other Level 2 samples. When comparing the total amount of amino acids metabolized, approx 39% total amino acid metabolism was measured in the MWGS23 sample, whereas an average of 57% total amino acid metabolism was measured for the other four samples in the Level 2 group. It can be hypothesized that owing to the physical (heterogeneous composition) and chemical (alkaline pH) differences in the MWGS23 sample, a different microbial community with different characteristics might be present. The differences in the microbial community and environmental conditions could result in the differences in metabolic activity observed.

The proportion of metabolized decane measured as water-soluble metabolites was high, accounting for one-third to one-half of the total metabolized decane. The amount of decane metabolized and proportions incorporated into each measured carbon pool varied with sample group. The proportion of decane mineralized was highest among the parts per billion level contaminated samples. Decane metabolism was lowest in the highly contaminated sample, MWGS24, at about 14% of the added decane. These results indicated that a sizable portion of decane, and potentially other aliphatic contaminants, can be metabolized to water-soluble metabolites by the microbial communities present at the site. However, the proportion of mineralization was greatest among samples with moderate levels of contaminant exposure, thus indicating that aerobic microbial biodegradation could significantly reduce the levels of aliphatic contaminants present. In a previous study of the site, Aelion and Bradley *(25)* also found preferential degradation of *n*-aliphatic compounds when compared to branched aliphatic compounds of comparable molecular weight.

A large portion of metabolized toluene was measured as water-soluble metabolites, 30–80% of the metabolized amount. The total amount of toluene metabolism varied with sample group, from 9 to 35%. Similar to decane metabolism, the lowest level of toluene metabolism was measured in the highly contaminated sample, MWGS24. The highest level of toluene metabolism was noted among the moderately contaminated group of samples. With the exception of the uncontaminated sample group, partitioning of metabolized toluene between mineralization and cellular carbon was approximately equal. These results indicated that aerobic microbial biodegradation could be effective for reducing the concentration of toluene, and potentially aromatic contaminants, at the study site.

Whereas amino acid degradation was similar (58 to 62%) for all levels, pollutant degradation was significantly lower ($\alpha < 0.05$) in the MWGS24 sample, which was highly contaminated, when compared with Level 2 samples. The total levels of metabolism for decane were 14% for MWGS24 and 31% for the Level 2 sediments. The total levels of metabolism for toluene were 9% for MWGS24 and 23% for the Level 2 sediments. It can be hypothesized that the high level of contaminants or associated metabolic products in the MWGS24 sample may be inhibitory to the microbial metabolic activity of the community in the sample.

The absolute mass of contaminants degraded was not calculated, since the naturally present levels of contaminants were not quantified for each microcosm. However, statistically similar percentages of radiolabeled decane metabolism (converted to CO_2, incorporated into cellular and extracellular material, and converted to water-soluble metabolites) were measured when a three-log range (1–1000 ng/g) of added radiolabeled decane was tested with the same Level 2 soil sample (data not shown).

Microcosm Environmental Parameters

Figure 7 summarizes the levels of DO consumption for selected experiments during incubation with added decane and toluene. Data for the MWGS23 sample were not included in Level 2 statistical analyses owing to its unique physical and chemical characteristics. Oxygen consumption was higher in the parts per billion contaminated sample group (Level 2) than in the Level 1 or 3 samples. In the toluene incubations, the DO in Level 2 was completely depleted by the end of the time courses. This is consistent with findings in other microcosm and field studies that concluded that oxygen availability controlled aerobic BTEX degradation in the subsurface *(22,26,27)*. The levels and rates of DO consumption were higher for toluene metabolism than for decane metabolism. The theoretical stoichiometric oxygen demands for the added decane and toluene in the microcosms were 0.35 and 0.31 µg of O_2, respectively. The total available O_2 in microcosms, assuming a starting DO of 6 ppm, was 150 µg of O_2. It may be hypothesized that the metabolism of petroleum constituents already present in the samples and endogenous respiration along with the decane or toluene metabolic activity accounted for the observed oxygen utilization. These results have important implications for the design of *in situ* bioremediation systems. Simple stoichiometric calculations of oxygen delivery in the field based on contaminant mass balances may result in underestimates of oxygen demand.

Figure 8 summarizes changes in pH during microcosm incubations with added decane and toluene. Data for the MWGS23 sample were again separated from the other Level 2 samples owing to its unique physical and chemical characteristics. In all samples tested, a greater decrease in pH was noted in toluene incubations than in the corresponding decane incubations. Decreases in pH corresponded temporally to decreases in DO in Level 2 toluene samples. Acidic metabolite accumulation occurred in

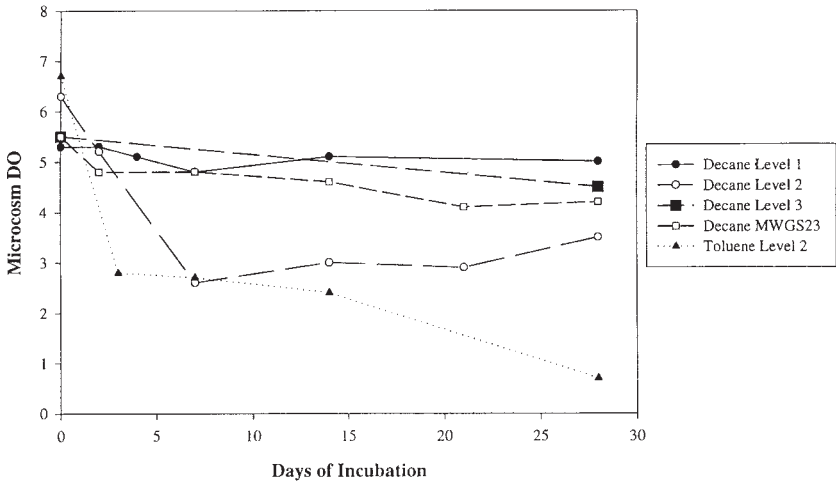

Fig. 7. Changes in microcosm DO during metabolism of decane and toluene for selected experiments.

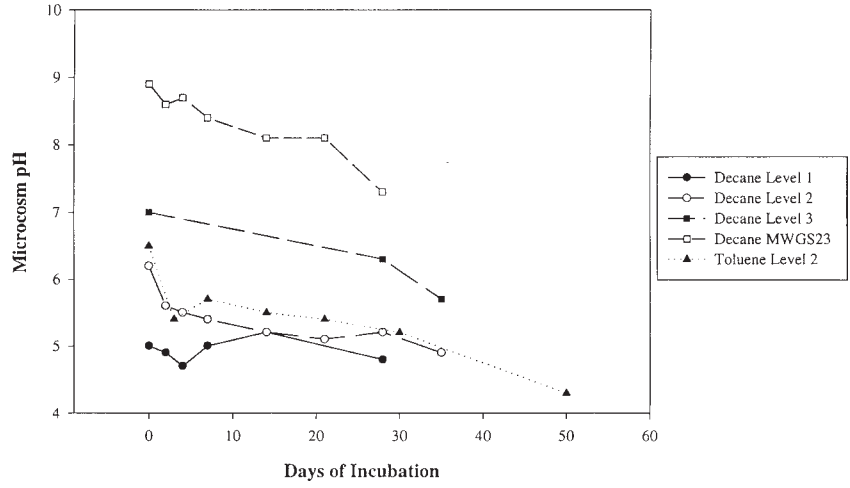

Fig. 8. Changes in microcosm pH during metabolism of decane and toluene for selected experiments.

decane microcosms under aerobic conditions (residual DO > 3 mg/L). Since microorganisms are known to degrade *n*-aliphatic compounds via β-oxidation, the formation of organic acids would be expected. Acidic metabolites also accumulated under aerobic conditions in toluene microcosms. Higher magnitudes of change in pH appeared to be discernible when DO levels also were low. It would seem that acidic metabolite formation increased in the toluene microcosms as DO approached microaerophilic conditions. The acidic metabolites formed during metabolism in microaerophilic toluene microcosms may have been fermentation products or intermediates of

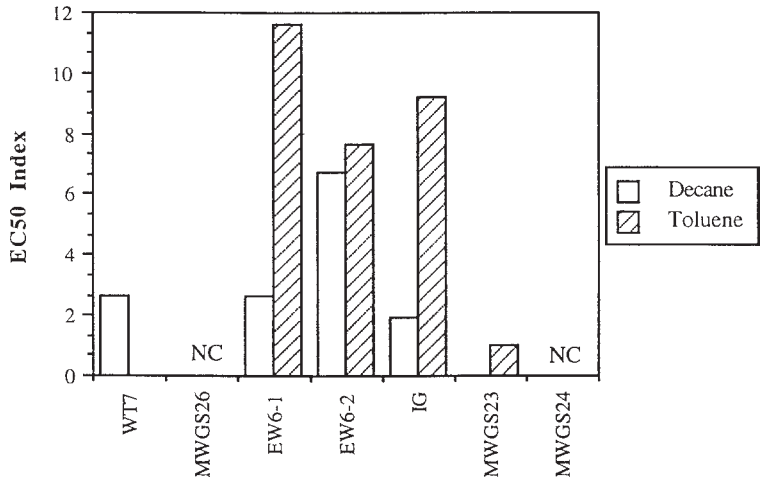

Fig. 9. Comparison of microcosm supernatant toxicity changes during metabolism with decane or toluene. NC, no change.

microbial decay products in addition to toluene metabolites. These experimental results indicated that a greater production of acidic metabolites occurred during low-oxygen or fermentative toluene metabolism. The phenomenon of organic acidic metabolite accumulation during monoaromatic hydrocarbon metabolism in groundwater was reported to occur in the field when anoxic conditions developed *(28)*. Noted also that formation of acidic metabolic products continued even though mineralization had plateaued.

Figure 9 presents comparisons of changes in microcosm toxicity among sample incubations. EC_{50} Index values for supernatant tests from the last time point of the time courses are presented. For all samples except the highly contaminated sample, MWGS24, time zero supernatants were not toxic in the Microtox assay. The level of toxicity in MWGS24 microcosms was statistically similar throughout the incubation. Increases in toxicity were noted for all Level 2 samples except the MWGS26 sample. A greater amount of toxicity was observed for the metabolism of decane than for toluene for the uncontaminated sample (WT7) that was tested.

The Microtox assay can be sensitive to pH changes. Similar levels of pH changes were noted in the MWGS26 microcosms as in other microcosms, but no toxicity was noted in MWGS26 microcosms. To the contrary, toxicity was detected in the MWSG23 sample microcosms in which the pH was within the range (6.0–8.0) specified by Microbics. In previous studies using Savannah River Site sediment, side-by-side Microtox tests with and without pH adjustment produced statistically similar toxicity values (data not shown). Thus, it can be concluded that the decane and toluene metabolites were the probable cause of the toxicity detected and that toxicity differences were not owing only to differences in pH.

Blum and Speece *(29)* reported that Microtox toxicity correlated with aerobic heterotroph toxicity with an R^2 value of 0.70 for a comparison of

34 chemicals and that it correlated with standard acute toxicity bioassay and fathead minnow bioassay—results with an R^2 value of 0.85 for 31 chemicals. The majority of *in situ* pollutant degradation is carried out by heterotrophic bacteria indigenous to aquifer systems; therefore, if toxic metabolites are allowed to accumulate in the subsurface, self-poisoning of an *in situ* system may occur and toxicity results may have significant implications for the design and operation of an *in situ* bioremediation scheme at the study site.

Conclusion

The multifaceted research approach was aimed at evaluating the indigenous subsurface microbial community and determining its inherent degradation potential. Amino acid metabolism was measured to determine variability and the level of activity of the microbial communities present in different sediment cores at the study site. Measurement of representative hydrocarbon degradation was used to assess whether the indigenous microbial communities have the ability to degrade the groundwater pollutants present at the study site. Degradation experiments conducted from samples contaminated over a several order-of-magnitude range indicated that high levels of pollutants may either select for a different degrader community or were inhibitory to degrader community activity. This indicated that mechanisms to reduce concentrations of contaminants in highly contaminated areas may need to be incorporated into an *in situ* treatment scheme in order to effect more efficient biodegradation/bioremediation.

Metabolite formation measurements coupled with DO and pH measurements indicated that complete degradation of aromatic hydrocarbons may be limited when oxygen availability is less than optimum. When oxygen became depleted in microcosms, measurable proportions of water-soluble metabolites were measured and pH became acidic. Necessary field oxygen delivery rates could be calculated from laboratory results in addition to mass balance estimates of contaminant levels to minimize acidic metabolite accumulation.

Toxicity analyses were coupled with both decane and toluene microcosm time courses. An increase in acute toxicity was measured at the longer incubation times tested. This has implications for the potential use of *in situ* remediation strategies. If toxic metabolites accumulate in the field, self-poisoning of the system may result. Groundwater recovery is a common aspect of *in situ* bioremediation schemes, and it is recommended that groundwater from the recovery wells be monitored for toxicity.

Overall, the results of this study indicate that a multifaceted experimental approach is useful when evaluating the potential for successful *in situ* bioremediation of a contaminated site. Without the integration of metabolite quantification and toxicity assessment into a site investigation, valuable information regarding operational constraints for an *in situ* system can be overlooked.

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References

1. American Petroleum Institute. (1992), *Basic Petroleum Data Book: Petroleum Industrial Statistics*, vol. 8, no. 3., API, Washington, DC.
2. Welch, J. and Yanado, F. (1993), *Proceedings of the 1993 International Oil Spill Conference*, American Petroleum Institute, Washington, DC, p. 811.
3. Prince, R. C. (1993), *Crit. Rev. Microbiol.* **19,** 217–242.
4. Atlas, R. M. and Bartha, R. (1972), *Can. J. Microbiol.* **18,** 1851–1855.
5. Dobbins, D. C., Aelion, C. M., and Pfaender, F. K. (1992), *CRC Crit. Rev. Environ. Control* **22,** 67–136.
6. Sachenen, A. N. (1954), in *Chemistry of Hydrocarbons*, vol. 1, Reinhold, New York, pp. 5–36.
7. King, R. W. (1988), *Occupational Medicine: The Petroleum Industry* **3(3),** 409–430.
8. Long, S. C. (1986), Masters Thesis, University of North Carolina, Chapel Hill, NC.
9. Aelion, C. M., Swindoll, C. M., and Pfaender, F. K. (1987), *Appl. Environ. Microbiol.* **53,** 2212–2217.
10. Lee, M. D., Thomas, J. M., Borden, R. C., Bedient, P. B., Ward, C. H., and Wilson, J. T. (1988), *CRC Crit. Rev. Environ. Control* **18,** 29–89.
11. Swindoll, C. M., Aelion, C. M., Dobbins, D. C., Jiang, O., Long, S. C., and Pfaender, F. K. (1988), *Environ. Toxicol. Chem.* **7,** 291–299.
12. Lovley, D. R., Baedecker, M. J., Lonergan, D. J., Cozzarelli, I. M, Phillips, E. J. P, and Siegel, D. I. (1989), *Nature* **339,** 297–300.
13. Hutchins, S. R, Sewell, G. W., Kovacs, D. A., and Smith, G. A. (1991), *Environ. Sci. Technol.* **25,** 68–76.
14. Aelion, C. M. (1996), *J. Contaminant Hydrol.* **22,** 109–121.
15. Dunlap, W. J., McNabb, J. F., Sccalf, M. R., and Cosby, R. L. (1977), EPA-600/2-77-176, U. S. Environmental Protection Agency, Washington, DC.
16. Wilson, J. T., McNabb, J. F., Balkwill, D. L., and Ghiorse, W. C. (1983), *Ground Water* **21,** 134–142.
17. Aelion, C. M. and Long, S. C. (1994), in *Hydrocarbon Bioremediation*, Hinchee, R. E., Alleman, B. C., Hoeppel, R. E., and Miller, R. N., eds., Lewis Publishers, Boca Raton, FL, pp. 192–200.
18. Dobbins, D. C. and Pfaender, F. K. (1988), *Microb. Ecol*. **15,** 257–273.
19. Richards, R. J., Ostendorf, D. W., and Switzenbaum, M. S. (1992), *HWHM* **9,** 397–410.
20. Armstrong, A. Q., Hodson, R. E., Hwang, H. M., and Lewis, D. L. (1991), *Environ. Toxicol. Chem.* **10,** 147–158.
21. Miles, R. A., Doucette, W. J., Bugbee, B., Dupont, R. R., McLean, J. E., and Sorensen, D. L. (1993), Paper presented at the 14th Annual Meeting of the Society of Environmental Toxicology and Chemistry, Nov. 14–18, Houston, TX.
22. Wilson, J. T., McNabb, J. F., Wilson, B. H., and Noonan, M. J. (1983), *Dev. Ind. Microbiol.* **24,** 225–233.
23. Thomas, J. M., Gordy, V. R., Fiorenza, S., and Ward, C. H. (1990), *Water Sci. Technology* **22,** 53–62.

24. Lee, M. D. and Hoepple, R. E. (1991), in *In Situ Bioreclamation, Applications and Investigations for Hydrocarbon and Contaminated Site Remediation*, Hinchee, R. E. and Olfenbuttel, R. F, eds., Butterworth-Heinemann, Stoneham, MA, pp. 570–580.
25. Aelion, C. M. and Bradley, P. M. (1991), *Appl. Environ. Microbiol.* **57,** 57–63.
26. Barker J. F., Patrick, G. C., and Major, D. (1987), *GWMR* **7,** 64–71.
27. Leahy, J. G. and Colwell, R. R. (1990), *Microbiol. Rev.* **54,** 305–315.
28. Cozzarelli, I. M., Eganhouse, R. P., and Baedecker, M. J. (1990), *Environ. Geol. Water Sci.* **16,** 135–141.
29. Blum, D. J. and Speece, R. E. (1991), *Res. J. Water Pollut. Control Fed.* **63,** 198–207.