Biodegradation **14:** 373–384, 2003.

Effects of heterogeneity and experimental scale on the biodegradation of diesel

Cynthia Davis[1,2], Todd Cort[2,3], Dongping Dai[2], Tissa H. Illangasekare[2] & Junko Munakata-Marr[2,*]

[1]*URS Corporation, 9801 Westheimer, Suite 500, Houston, TX 77042, USA;* [2]*Environmental Science and Engineering Division, Colorado School of Mines, Golden, CO 80401, USA;* [3]*Cameron-Cole, LLC, 5777 Central Avenue, Suite 100, Boulder, CO 80301, USA (*author for corresponding: e-mail: jmmarr@mines.edu)*

Accepted 16 July 2003

Key words: biodegradation, diesel, heterogeneity, NAPL, scale

Abstract

Biodegradation of petroleum hydrocarbon contamination is a common method for remediating soils and groundwater. Due to complexities with field-scale studies, biodegradation rates are typically evaluated at the bench-scale in laboratory studies. However, important field conditions can be difficult to mimic in the laboratory. This study investigates three scaling factors that can impact laboratory biodegradation rates and that are frequently unaccounted for in typical laboratory experimental procedures. These factors are soil heterogeneity, morphology of petroleum hydrocarbon non-aqueous phase liquids (NAPLs) and soil moisture distribution. The effects of these factors on the biodegradation rate of diesel NAPL is tested under a variety of experimental procedures from well-mixed batch studies to four-foot static soil columns. The results indicate that a high degree of variability results from even small-scale heterogeneities. In addition, it appears that as the experimental scale increases, the measured biodegradation rates slow. The results indicate that diesel biodegradation rates derived from small-scale experiments are not necessarily representative of field-scale biodegradation rates.

Introduction

Soil and groundwater contamination resulting from the release of petroleum hydrocarbons has been recognized as one of the principal issues in environmental remediation. The potential health and ecosystem impacts associated with the presence of petroleum hydrocarbons in the subsurface continue to cause concern (Ŝepiĉ et al. 1996). Diesel fuel, which can be released to the environment during fueling, maintenance and storage, is among the most prevalent petroleum hydrocarbons impacting the subsurface. Large volumes of spilled diesel fuel can migrate through unsaturated soil zones and pool on the groundwater table. These non-aqueous phase liquid (NAPL) pools can serve as long-term sources for groundwater contamination as the soluble constituents of the diesel slowly leach into and migrate with the groundwater.

Due to the difficulty and high costs typically associated with extracting residual diesel NAPL from the subsurface and the long timeframes required for traditional pump-and-treat strategies, in-situ bioremediation (degradation of target contaminants by microorganisms present in the subsurface) has become an increasingly common method for treating zones impacted by diesel NAPLs (Goudar & Strevett 1998). The biodegradation of diesel in the subsurface has been well documented under a variety of soil and geochemical conditions (Norris et al. 1994). Successful and cost-effective application of biodegradation is dependent on the accurate measurement of biodegradation rates to predict timeframes and assess the effectiveness of bioremediation approaches.

Typically, diesel and other petroleum hydrocarbons are degraded most rapidly under aerobic conditions (Parker & Burgos 1999; Moller et al. 1996;

Stout & Lundergard 1998; Widrig & Manning 1995). However, accurate measurement of degradation rates under field conditions can be complicated by several factors. Three of the most significant of these factors are the soil and microbial heterogeneity, NAPL entrapment morphology and soil moisture distribution. Soil and microbial heterogeneities can result in differential transport of electron acceptors and nutrients, formation of micro-redox zones and complex distribution of the NAPL (Seagren et al. 1994; Rogers & Logan 2000). Such heterogeneity may result in significant small-scale variation in biodegradation, as concluded in a recent review (Haack & Bekins 2000). This occurs due to the presence of zones of high relative permeability through which these substances will preferentially pass. Outside of these preferential pathways, zones of low permeability are expected to allow little relative passage and exchange of compounds.

Such localized zones of lower permeability are also expected to influence NAPL morphology. NAPL morphology refers to the mode of entrapment of NAPL in the subsurface such as in pools or residual zones. At high saturation, NAPL typically forms pools of free phase NAPL, while at lower saturation, the NAPL forms residual blobs, ganglia and fingers that 'smear' through the soil and remain in small pockets of lower permeability. The NAPL morphology can affect, by three distinct mechanisms, the activity of degrading microbial communities and therefore the overall rate of degradation. The smaller surface area to volume ratio present in pools of NAPL compared to residual zones can decrease the mass flux of soluble constituents from the non-aqueous phase to the water. The result is a decrease in the time-dependent availability of the substrate to nearby organisms (Ramaswami & Luthy 1997; Seagren et al. 1994). Second, the presence of free phase can reduce the potential adhesion sites for microorganisms on the soil particles and pore spaces, resulting in lower numbers of organisms in the potential reactive zone (Rogers & Logan 2000). Finally, the presence of NAPL pools can create large pockets of immobile water (Rogers & Logan 2000; Seagren et al. 1994) that result in decreased substrate delivery to the degrading organisms or decreased bacterial transport rates around the NAPL pool.

The third principal factor influencing biodegradation rate in the subsurface is the moisture distribution of the soil. Moisture is essential to active biodegradation (Bossert & Bartha 1984). However, in fully saturated systems, aerobic biodegradation rates can be reduced due to decreased access to oxygen (Bossert & Bartha 1984; Leeson & Hinchee 1997). Moisture distribution can also influence the accessibility of diesel substrate to the degrading microorganisms. Pools of diesel will tend to sit on top of the saturated zone due to their lower density compared to water. Microorganisms within the saturated zone may therefore have more limited access to the diesel constituents than microorganisms within the capillary fringe just above the groundwater table.

Due to the complexities of measuring biodegradation rates resulting from these factors at the field scale, biodegradation rates are more typically measured in laboratories at the bench scale (Møller et al. 1996; Hinchee & Ong 1992; van Eyk & Vreeken 1989; Hogg et al. 1992; Downey et al. 1995; Davis et al. 1998). These bench-scale studies are frequently conducted under more homogeneous soil, NAPL entrapment and moisture conditions established through constant mixing. The rates measured at the bench scale are then commonly used to infer degradation rates that occur in the field. The ability to extrapolate from the bench scale to the field scale is dependent on determining the effect that each of the described factors has on biodegradation rates. The experiments described here were conducted to measure and describe the relative impacts of heterogeneity, NAPL morphology and soil moisture distribution on aerobic diesel biodegradation rates at varying scales. Aerobic biodegradation rates were measured by respirometry in both batch and column studies. All three factors (soil heterogeneity, NAPL morphology and soil moisture distribution) were hypothesized to independently and significantly impact biodegradation rates, with heterogeneity having the largest impact due to its effect on both NAPL morphology and soil moisture distribution. Experimental results are used to discuss the implications of directly using bench scale biodegradation rates to predict effectiveness of degradation at the field scale.

Materials and methods

Rates of aerobic diesel biodegradation were measured using both batch and column studies. Soil was obtained from a railyard impacted by aged diesel. Soil was collected aseptically and shipped on ice to the laboratory. Soil samples were collected from the capillary fringe in areas impacted by diesel in order to obtain microbial cultures pre-adapted to diesel degrad-

ation. Prior to experimentation, soils were stored at 5.5 °C to minimize microbial activity.

A mixture of diesel samples collected from two locations at the site was used in these experiments. Gas chromatograms of the diesel indicated that the samples were enriched in higher molecular weight hydrocarbons compared to fresh diesel. This aged diesel pattern is consistent with the reported time period of diesel impact at the site (approximately 50 years). A nutrient buffer solution (pH 7.2) was used to mimic groundwater conditions at the site and provide nutrients for microbial activity. The nutrient buffer consisted of 16 mg l^{-1} KH_2PO_4, 42 mg l^{-1} K_2HPO_4, 5 mg l^{-1} Na_2HPO_4, 4 mg l^{-1} NH_4Cl, 5 mg l^{-1} $MgSO_4 \cdot 7H_2O$, 0.05 mg l^{-1} $FeCl_3 \cdot 6H_2O$, 5 mg l^{-1} $CaCl_2$, 0.005 mg l^{-1} $MnSO_4 \cdot 4H_2O$, 0.01 mg l^{-1} H_3BO_3, 0.005 mg l^{-1} $ZnSO_4 \cdot 7H_2O$, 0.003 mg l^{-1} $(NH_4)Mo_7O_{24}$ (Gouder & Strevett 1998).

Batch experiments

The experimental conditions for soil batch studies are summarized in Table 1. A total of 19 batch bottles representing six different conditions were tested. Approximately 2 ft^3 (56.6 l) of diesel-impacted soil was homogenized in a methanol-rinsed plastic bucket. All batch studies were conducted using this homogenized soil. The first experimental condition (biotic, well-mixed, containing 20 μl diesel (BM20)) represented the most ideal conditions obtainable in the laboratory. Three replicate soil slurry bottles were created by placing approximately 30 g of sieved (4.75 mm) soil, 60 ml of nutrient solution and 20 μl of diesel mixture (to establish residual NAPL saturations on the soil) in sterilized 250 ml amber glass bottles capped with Mininert valves. The high ratio of liquid to soil promoted mixing during the experiment. During the experiment, the sample bottles were shaken on a rotary shaker at 200 rpm, sufficient to establish a constantly stirred slurry within the bottles. Gas-phase samples (1 ml) were collected from the headspace of the bottles every 6–12 h. The O_2 and CO_2 concentrations were analyzed by gas chromatography (GC) as described below.

A lower ratio of liquid to soil was studied in the second (biotic, partially mixed, 10 μl added diesel (BP10)) and third (biotic, partially mixed, 20 μl added diesel (BP20)) experimental conditions to test less homogeneous conditions. For these batch studies, approximately 60 g of soil and 30 ml of nutrient solution were combined with either 10 μl or 20 μl of diesel mixture in sterilized 250 ml amber glass bottles capped with Mininert valves. Bottles were sampled and shaken as described for the well-mixed bottles (experimental condition 1 – BM20). At the 200 rpm shaking speed, the soil within the bottles was not suspended in the solution.

Three tests were conducted as controls. Two bottles containing biotic soil (60 g), nutrient buffer (30 ml) and no added diesel (biotic, partially mixed, no diesel added (BP0)) were tested to measure the baseline O_2 depletion and CO_2 production rates in the soil. Four additional bottles were tested with sterilized soil (60 g), nutrient buffer (30 ml) and either 10 or 20 μl diesel added (abiotic, partially mixed, 10 μl diesel added (AP10) or abiotic, partially mixed, 20 μl diesel added (AP20)). To sterilize soils for the abiotic tests, a portion of the homogenized soil was autoclaved for one hour at 121 °C and 22 psi on three consecutive days as described by Lotrario et al. (1995).

Column experiments

To test for the potential impacts of experimental scale on diesel biodegradation rates, respirometric tests of soils in three column sizes were conducted. Soil from the same sampling area used in batch studies was used to fill columns of different sizes. The 10, 50 and 120 cm high columns were made of glass, aluminum and Plexiglas, respectively, with a sampling port at the top and a connection at the base to a constant-head reservoir. Each soil column was rinsed with methanol and then packed with soil in increments by alternately filling with soil and packing the soil down with weight. After packing each column with soil to just below the upper end, the columns were slowly filled in an upflow manner over 8 h using an adjustable reservoir of nutrient solution connected to the base of the column. Diesel was then added to each column via syringe loading to the top surface of the packed soil to establish a diesel concentration of approximately 337 mg diesel kg^{-1} soil (to establish residual NAPL saturation in the soil). After addition of the diesel, the water table was dropped to establish a ratio of 30% unsaturated soil to 70% saturated soil and the columns were allowed to equilibrate for at least 18 h prior to initiation of sampling. Samples were collected from the column headspace and analyzed for O_2 and CO_2 similar to those described for batch tests. The experimental conditions for each set of columns are summarized in Table 2.

An additional set of experiments involving water table fluctuations was conducted on three of the 120

Table 1. Batch study experimental conditions

Label	Number of replicates	Mixing condition	Soil condition	Approximate soil mass (g)	Nutrient solution volume (ml)	Diesel volume (μl)
BM20	3	Well mixed	Biotic	30	60	20
BP10	5	Partially mixed	Biotic	60	30	10
BP20	5	Partially mixed	Biotic	60	30	20
BP0	2	Partially mixed	Biotic	60	30	0
AP10	2	Partially mixed	Abiotic	60	30	10
AP20	2	Partially mixed	Abiotic	60	30	20

Table 2. Column study experimental conditions

Column height	Soil volume	Diesel volume added	Diesel concentration (mg diesel/kg soil)	Depth unsaturated soil/ depth saturated soil
10 cm	180 ml	0.19 ml	337	2.5 cm/ 5.9 cm
50 cm	875 ml	0.9 ml	337	13 cm/30 cm
120 cm	4.9 L	5 ml	337	30 cm/71 cm

cm columns. During the initial respirometric tests, the water table was located 30 cm from the top of the column. During water table fluctuation studies, respirometric tests were also conducted at water table depths of 0, 15, 45 and 60 cm with no additional diesel added. After visual verification that the desired water table level was established in the columns, the system was aerated from the column base for 30 min to re-establish a high oxygen concentration within the column for aerobic activity. Headspace sampling for each water table depth was conducted for 10 days.

Analytical techniques

Oxygen and carbon dioxide gas-phase samples were measured by Agilent p200 micro GC equipped with dual columns and thermal conductivity detector (TCD). Oxygen was analyzed with a retention time of 25 s and a total run time of 100 s on a 10 m, MS-5Å column at a constant temperature of 50 °C. Carbon dioxide was analyzed with a retention time of 25 s and total run time of 100 s on an 8 m, PPU column at a constant temperature of 70 °C. Oxygen depletion was converted to diesel degradation assuming a stoichiometry of 3.5 mg oxygen to 1.0 mg diesel (Hinchee & Ong 1992; Dupont 1993; Davis et al. 1998; Hickey 1995; Downey et al. 1995; Bregnard et al. 1996; Moller et al. 1996).

Following respirometry experiments, diesel was extracted from batch and column tests using a modified EPA SW-846 method 3550 by varying the methylene chloride to soil ratio based on the extent of contamination in the soil (Environmental Protection Agency 1996a). Extracted diesel was analyzed using EPA SW-846 method 8015 (Environmental Protection Agency 1996b) with a DB-624 rather than a DB-5 column in a Hewlett Packard 6890 Series Gas Chromatograph equipped with a flame ionisation detector. The DB-624 (J&W Scientific) column (30-m × 0.320-mm with a nominal film thickness of 1.8-mm) was used with helium as the carrier gas. The injector and detector temperatures were 255 °C and 260 °C, respectively. Oven temperature conditions were: 60 °C for 3 min, 15 °C min^{-1} to 255 °C and 14 min at 255 °C for an overall run time of 30 min, with integration of data beginning at 5 min and ending at 25 min. Known concentrations of the site diesel (100–50,000 ppm) were used for calibration.

Results

Batch experiments

A high degree of variability was measured in oxygen consumption rates between replicates. This variability was present in all of the batch experiments including the well-mixed, 'homogenous' soil experiments. The oxygen depletion from the three replicates of the well-mixed soil experiments is shown in Figure 1. While the replicates behaved similarly during approximately the first 100 h of the experiment, the oxygen consumption diverged sharply between the three replicates during the last 200 h of the experiment. Average oxygen consumption rates measured over 380 h ranged from 3.1 mg (kg day)$^{-1}$ to 11.2 mg (kg day)$^{-1}$. This large difference is reflected in the calculated standard deviations for both overall oxygen consumption and the calculated diesel degradation rates shown in the first row of Table 3. This marked divergence in oxygen consumption under well-mixed conditions demonstrates that small samples of homogenized soils can exhibit significantly different respiration behavior.

Similar variability was observed in the partially mixed batch experiments. As with the well-mixed systems, biotic experiments initially containing 10 and 20 μl of diesel under partially-mixed conditions showed similar early oxygen depletion followed by significant divergence after approximately 100 h. The results of these studies are summarized in Table 3. Although the standard deviations of the BP10 and BP20 series were similar to the well-mixed soil experiments, the calculated average diesel degradation rates were slower in the partially mixed soil experiments.

Given the variability between replicates, no significant difference (tested at 90% confidence) was observed in degradation rates with initial diesel additions of 10 μl (BP10) and 20 μl (BP20). Therefore, any dependence of the diesel degradation rate on the amount of diesel present cannot be determined. Abiotic controls (AP10 and AP20) showed small oxygen depletion compared to the average oxygen depletion in biotic tests, indicating that the primary mechanism for oxygen depletion was biological oxidation. Relatively low oxygen depletion rates were also observed in controls containing no added diesel (BP0), demonstrating that the biological oxidation of alternate organic substrates (such as background diesel or soil humic and fulvic acids) did not significantly contribute to the oxygen depletion observed in experiments containing added diesel. Based on the results from these two controls, diesel degradation rates, summarized in Table 3, were calculated on the basis of (1) negligible abiotic oxygen uptake; and (2) negligible biological oxidation of alternate organics.

Average diesel degradation rates were also calculated based on recovery of extracted diesel from the batch test materials. A simple batch test to measure extraction efficiency indicated that 83% of diesel added to soil could be recovered (data not shown). Using this efficiency value and the measured remaining diesel, the average diesel degradation rate was calculated, as summarized in Table 3. The degradation rates based on extracted diesel are quite similar to those based on respiration, with no statistically significant difference at 90% confidence with the exception of the AP20 system. The soil in these systems may have been insufficiently sterilized so that bioactivity developed in the time between respiration measurements and diesel extraction, but significant CO_2 production did not occur (data not shown). The most likely cause for this discrepancy in rates is poor extraction efficiency, as seen in the column systems (see below). Though a wide range of diesel degradation rates was observed in the batch studies, the strong correlation between these rates based on the diesel extraction and respirometry measurements suggests that the respirometry method accurately reflected the diesel degradation rates in the batch systems.

Column experiments

The oxygen concentration observed over time in the headspace of each column is shown in Figure 2. The highest degree of variability was observed in the 120 cm columns, with overall diesel degradation rates based on respirometry ranging from 0.04 mg (kg day)$^{-1}$ to 0.34 mg (kg day)$^{-1}$ and an average of 0.20 mg (kg day)$^{-1}$ (see Table 4). Like the batch experiments, oxygen concentrations decreased initially and then appeared to stabilize somewhat. Oxygen and diesel concentrations upon stabilization were sufficiently high so that respiration rates were not limited, but nitrogen may have been a limiting nutrient. Estimates based on CO_2 data (not shown) and typical cell growth stoichiometry suggest that some, though not all, of the experimental systems may have been nitrogen-limited, though no experiments were conducted to determine a limiting growth factor.

The rates of diesel biodegradation based on extraction of diesel from the column materials are substantially higher than those calculated from respirometry

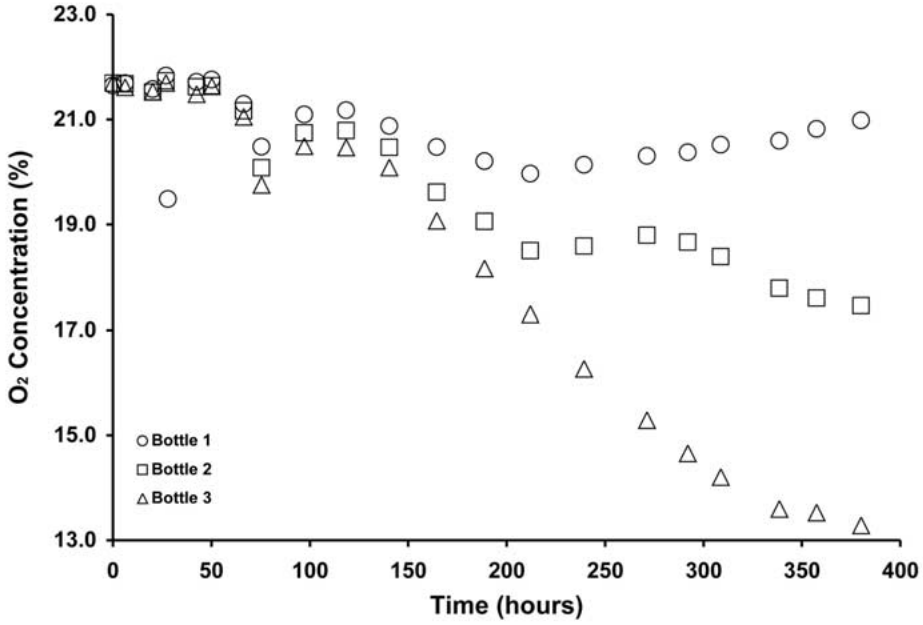

Figure 1. Oxygen depletion in three replicates of biotic, well-mixed bottles with 20 μL diesel added.

Table 3. Batch experiment oxygen consumption and calculated diesel degradation rates

Condition	Label	Initial [O_2] (%)	Final [O_2] (%)	Diesel degradation rate based on respiration (mg/kg/day)	Diesel degradation rate based on diesel extraction (mg/kg/day)
1	BM20	21.7 (0.001)	18.3 (0.11)	6.9 (0.6)	6.5 (0.5)
2	BP10	21.3 (0.001)	19.3 (0.11)	2.3 (1.0)	2.4 (0.1)
3	BP20	21.4 (0.001)	18.3 (0.13)	3.6 (0.8)	5.3 (0.0)
4	BP0	21.4 (0.001)	21.1 (0.02)	0.3 (1.4)	−0.8 (0.0)
5	AP10	21.4 (0.001)	20.1 (0.02)	0.5 (1.0)	0.8 (0.1)
6	AP20	21.3 (0.001)	21.2 (0.00)	0.2 (0.4)	3.8 (0.2)

Numbers in parentheses represent the coefficient of variation between experimental replicates.

Table 4. Column experiment calculated diesel degradation rates

Column height	Number of replicates	Overall (10 day) diesel degradation rate based on respiration (mg/kg/day)	Overall diesel degradation rate based on diesel extraction (mg/kg/day)
10 cm	2	0.26 (0.01)	33 (0.06)
50 cm	2	0.13 (0.00)	15 (0.20)
120 cm	4	0.20 (0.62)	33 (0.03)

Numbers in parentheses represent the coefficient of variation between experimental replicates.

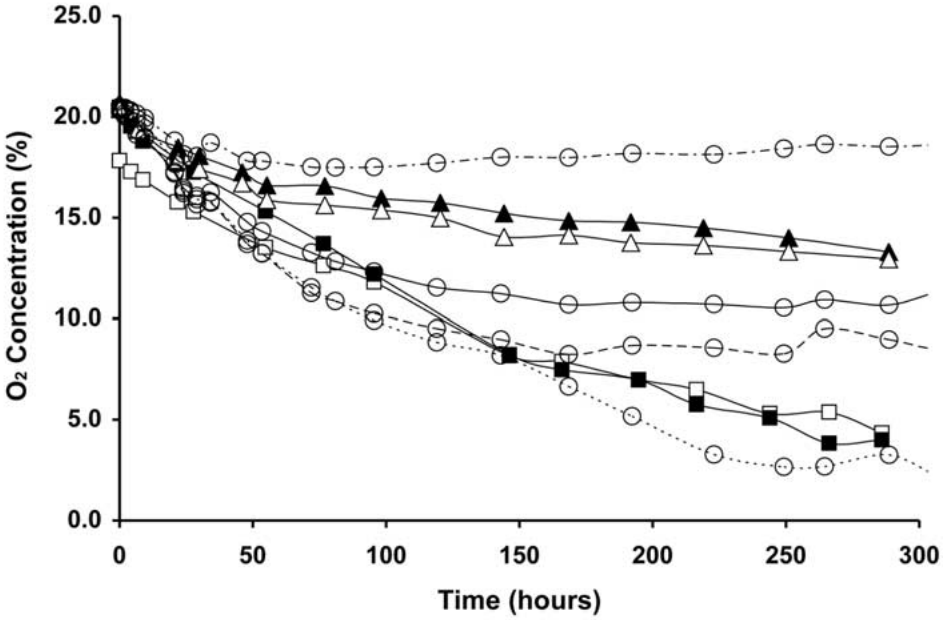

Figure 2. Oxygen depletion in column headspaces for 10 cm column replicates (squares), 50 cm columns (triangles) and 120 cm columns (circles).

data, as shown in Table 4. However, the total mass of oxygen present in the columns in both gas and aqueous phases was only sufficient to allow for oxidation of 3–11% of the diesel to CO_2. In addition, CO_2 data (not shown) also does not support oxidation of that much diesel but instead accounts for about 62% of the oxygen depletion observed in both batch and column tests. Significant anaerobic diesel degradation is unlikely due to continuous presence of oxygen in the column headspace (Figure 2), though mass transfer limitations may have led to the formation of anoxic zones. Thus, extraction of diesel from column materials appears to be much less efficient than from batch materials. This could be due in large part to the mechanical difficulty in removing soils from the columns, which could have resulted in significant volatilization of diesel components and loss of solid materials to the columns, weighing apparatus and extraction tools. Based on the difficulties associated with the diesel extraction, respirometry data appears to more accurately reflect actual degradation rates and was used exclusively to assess degradation rates in the soil columns.

The effect of soil moisture distribution was also investigated by fluctuating the depth to water table in three of the 120 cm columns. The sequence of depths tested in the water table fluctuation experiments was: 30 cm, 0 cm, 15 cm, 45 cm and 60 cm.

Table 5. Calculated degradation rates at varied water table depths

Depth to water table (cm)	Overall (10 day) O_2 depletion (%)	Overall (10 day) degradation rate (mg/kg/day)
0	3.4 (0.90)	0.02 (0.91)
15	37 (0.71)	0.15 (0.70)
30	65 (0.31)	0.26 (0.31)
45	56 (0.50)	0.24 (0.49)
60	58 (0.44)	0.25 (0.42)

Numbers in parentheses represent the coefficient of variation between experimental replicates.

The average oxygen consumption data for the three columns is shown in Figure 3. Calculated diesel degradation rates, assuming equilibrium between gas- and aqueous-phase oxygen, are summarized in Table 5. While dissolved oxygen (DO) levels were not measured, the mass of oxygen present in the aqueous phase constituted only 4.3% of the total oxygen in the system with the most water. Therefore, assuming complete DO consumption would not significantly increase the estimates of total diesel degraded. Essentially no oxygen depletion occurred when the water table was set at the soil surface, indicating that degradation occurred very slowly in the fully saturated system. For depths

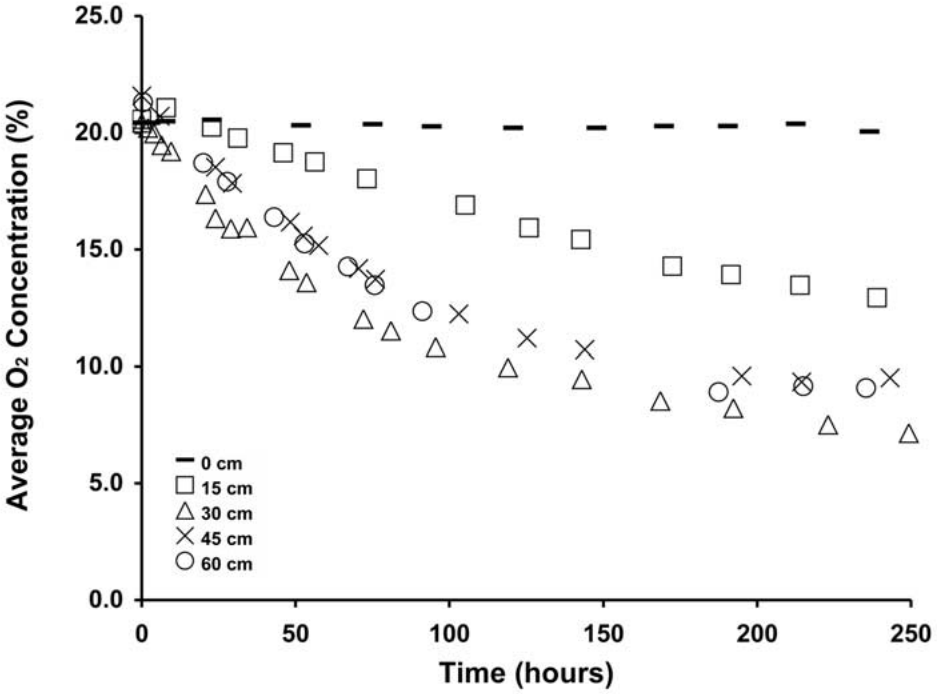

Figure 3. Average oxygen depletion at varied water table depths.

between 0 and 30 cm, the rate of oxygen depletion increased with increasing depth to water table (correlation coefficient of 0.84). Though absolute rates varied between columns (again, likely due to soil and microbial heterogeneities), similar relative trends were observed within each of the columns. However, this rate increase was not observed at the 45 and 60 cm depths (correlation coefficient of −0.03). Instead, the oxygen depletion rates were similar at 30, 45 and 60 cm.

Discussion

Effect of heterogeneities and NAPL morphology

The observed rates of oxygen consumption decreased as the scale of the system increased in partially mixed and un-mixed systems. The implication is that the increased presence of soil and microbial heterogeneities at larger scales reduces the efficiency of biodegradation by one or more mechanisms. One potential mechanism is that the presence of less permeable soil zones could decrease the access of degrading populations to electron acceptors and substrate (Kao & Prosser 1999). Similarly, different soil zones could have different levels of degrading microbe populations. Also, the presence of differential zones of NAPL morphology can reduce degradation rates by concentrating substrate in localized areas or by creating zones with contaminant concentrations that are bioinhibitory (Seagren et al. 1994; Rogers & Logan 2000).

In order to evaluate the impact of experimental scale on observed diesel biodegradation rates, batch and column results were compared to experiments conducted in an intermediate-scale tank system (245 × 122 × 8 cm) previously reported by Cort et al. (2001). The same soil and diesel mixture were used in both the column/batch experiments reported here and in the intermediate-scale tank experiments described by Cort et al. (2001). Diesel mixture was added to the soil-packed tanks and aqueous-phase oxygen concentrations were measured over a period of several days. The average oxygen depletion rate observed in the intermediate-scale tanks is listed along with the average column and batch oxygen depletion rates in Table 6. Aqueous oxygen depletion data for the tank system was converted to total mass depletion using the soil porosity and total soil volume. Gaseous oxygen depletion in the batch and column systems was converted to total oxygen depletion based on the headspace volume. This method does not report the actual

depletion rate, as the headspace concentration is limited by oxygen diffusion, so the value represents a conservative rate estimate.

When the oxygen depletion rates are normalized to the total mass of soil in the system, a clear trend of decreasing rate with increasing experimental scale is observed. Assuming first-order diesel degradation and using average rates at each scale, the time required to reduce diesel concentrations from 1000 mg kg^{-1} to 100 mg kg^{-1} was calculated. The time ranges from 64 days based on batch rates, to 1047 days based on the 50-cm columns, to 79 years based on the intermediate-scale tank. Similar results have been noted when comparing predicted rates and field-scale biodegradation rates of petroleum products. Sturman et al. (1995) noted that half lives of petroleum products (gasoline, fuel oil, BTEX) under aerobic or denitrifying conditions were 4 to 10 times longer in the field than in the lab.

The effects of heterogeneity on the oxygen uptake and corresponding diesel degradation rate were evident in both batch and column experiments. The large observed variability between replicates suggests that small-scale heterogeneities can significantly impact degradation rates, even in well-mixed batch systems. Assuming that diesel degradation rate is first order with respect to the concentration of diesel, the time required to reduce diesel concentrations from 1000 mg kg^{-1} to 100 mg kg^{-1} was calculated using the highest and lowest rates (mg $(kg\ day)^{-1}$) determined for each experimental scale. The time required ranges from 24 to 113 days based on batch-scale rates, to 317 to 1389 days based on the 120-cm columns. These calculations indicate that to overcome the effects of these small-scale heterogeneities and measure representative bioactivity, large sample sizes are required. The exact size of soil sample required to capture representative activity depends primarily on the degree of heterogeneity present in the soil sample and in the field. Such variability in microbial activity within a single site has been attributed to the presence of microbiological 'hotspots' (areas of high microbial density or degradation activity) (Brockman & Murray 1997; Vroblesky & Chapelle 1994). The presence of these 'hotspots' is hypothesized to result from differential substrate and electron acceptor availability due to small- and larger scale heterogeneities. In this study, the 13 kg soil used in the 120-cm columns appears to be insufficient to capture representative diesel biodegradation, though differences in column packing may also have contributed to the observed variability. Measured soil mass in the different columns was quite different from that expected based on typical soil bulk density. The measured bulk density varied substantially between column scales (CV = 0.50), suggesting that column packing variability may have been important, though replicate column masses were comparable (CVs 0.0007–0.18).

The impacts of heterogeneity on microbial growth and activity have been observed in numerous studies at different scales. In studies examining bacterial communities associated with microbial activity, in general, activity and bacterial density were only loosely correlated (Kao & Borden 1997; Hickman & Novak 1989) and highly variable (Beloin et al. 1988; Zhang et al. 1998; Ulrich et al. 1998). Ulrich et al. (1998) suggest that such microscale variability may account for the majority of total heterogeneity associated with subsurface sulfate reduction and, possibly, other microbial processes.

Within a single site, high variability has been observed in bioactivities ranging from BTEX degradation to denitrification. A study on the degradation of BTEX in a contaminated aquifer showed that more permeable sediment materials responded to bioremediation with higher biomass and significant toluene degradation while less permeable sediments contained lower microbial populations and had no significant degradation (Kao & Prosser 1999). Parkin et al. (1987) observed a coefficient of variation (CV) in measured denitrification rates ranging from 1.28–3.83, with the standard deviation of small cores 3–10 times lower than that of large cores. Most variability occurred at distances <10 cm, and a computerized random resampling algorithm suggested that 10–15 kg of soil would be required to obtain "representative" rates from a single sample. Highly variable methanogenesis rates within single sites have also been observed, with CV 0.40–1.80 (Wachinger et al. 2000) and 3.40–4.90 (Adrian et al. 1994) for replicates, and significant differences at 95% level in a MANOVA (Pedersen & Sayler 1981).

Finally, a recent review of pesticide biodegradation concluded that *a priori* prediction of pesticide biotransformation is not possible, even within a single site, due to subsurface heterogeneities (Hoyle & Arthur 2000). Another review of microbial communities associated with organic biodegradation concluded that studies indicate that many organic-degrading bacteria with different degradative capabilities may exist within a single site, and that significant small-scale heterogeneity in biodegradation may be expected due to heterogeneity in aquifer materials (Haack & Bekins

Table 6. Calculated oxygen depletion rates at various experimental scales

System type	Total oxygen depletion	Soil mass (kg)	Normalized oxygen depletion (mg/kg/day)
Partially-mixed batch	2.19	0.06	12
Ten-cm column	1.85	0.31	2.5
Fifty-cm column	5.11	0.81	3.1
120-cm column	35.1	13	0.7
Tank (2.4 m × 1.2 m × 7.6 cm)	49.4	600	0.08

2000). Based on the results reported in this study, it appears that the impacts of heterogeneity and inherent variability observed in other systems are also present in the biodegradation of diesel in bench-scale soil systems.

Effect of soil moisture distribution

Oxygen uptake rates in fully saturated columns (0 cm water table depth) indicated little aerobic microbial activity. The lack of activity indicates that the lack of oxygen in the saturated zone and/or lack of accessible substrate (due to the majority of the diesel remaining above the water table) severely inhibited the diesel degradation. Regardless of the exact mechanism, it appears that the soil moisture content, particularly under static conditions, can have significant impacts on the observed degradation rates.

The average degradation rates tend to increase as depth to water table increases up to a depth of 30 cm (with a significant correlation coefficient of 0.84). A similar trend of increasing aerobic diesel degradation rates with increasing depth to water table was observed for two field depths by Davis et al. (1998), while the opposite trend was observed for anaerobic methane production by Wachinger et al. (2000). It is possible that a greater volume of diesel became available to degrading microorganisms as the water table was lowered, due to smearing of the LNAPL over the exposed soil (Saba 1999; Bulman et al. 1993). This effect would have been coupled to a higher oxygen concentration in the exposed soil due to higher expected diffusion rates in the soil:gas zones (Hupe et al. 1998). Subsequent extractions of the column soil confirmed that the diesel was concentrated at the water table with lower concentrations in the capillary fringe and vadose zones and very low concentrations below the water table (data not shown).

From 30 to 60 cm depth to water table, the observed average oxygen depletion rate no longer increased as the depth increased (with a correlation coefficient close to zero (−0.03)). This behavior may be attributed to preferential degradation of diesel components or decreased microbial activity as a result of the ten-week period of soil saturation during the previous soil column scale studies when the water table depth was held at 30 cm below the soil surface. However, the added diesel was well aged (at least 20 years) and is thus unlikely to have exhibited preferential degradation behavior. In addition, chromatograms of the diesel generated before and after biodegradation show no significant preferential degradation of specific diesel components (data not shown). With regard to a potential decline in microbial activity, the microorganisms below the 30-cm water table may have either decreased in population or acclimated to a different substrate source (Dineen et al. 1990) during the previous ten-week column experiment. The experimental protocol using ten days of observation may not have been sufficient to observe a potential lag period during which such microorganisms re-acclimated to diesel degradation. However, no experiments were completed to test this hypothesis.

Conclusions

Respirometry data was used to evaluate the impacts of soil and microbial heterogeneity, NAPL morphology and soil moisture content on diesel biodegradation rates. Although rates calculated based on diesel extractions corroborated the respirometry data observed in batch-scale experiments, difficulties with removal of soil from columns precluded meaningful comparison of respirometry and extraction data from column-scale experiments. The differences in diesel degrad-

ation rates observed within replicates at each scale have significant implications for the extrapolation of biodegradation rates determined in the laboratory to field-scale applications. The high degree of variability observed at the various experimental scales indicates that small-scale soil and microbial heterogeneities significantly impact biodegradation rates. In addition, the biodegradation rates determined in small-scale batch or column systems are enhanced by more efficient diffusion rates of substrate and electron acceptors and will be susceptible to variability introduced by small-scale heterogeneities. The results of this study indicate that bench-scale studies may not sufficiently capture representative microbial and soil heterogeneity and diffusion limitations, such that larger-scale studies, which also incorporate large-scale heterogeneities, are required to effectively predict field-scale bioremediation outcomes.

Acknowledgements

The authors wish to acknowledge the funding and support provided by Union Pacific Railroad and Cameron-Cole, LLC, in particular to Mr Shawn Leppert for his assistance in acquiring the funds. Dr. Frank Barranco assisted in the design of the experiments. His contributions are gratefully acknowledged.

References

Adrian N, Robinson JA & Suflita JM (1994) Spatial variability in biodegradation rates as evidenced by methane production from an aquifer. Appl. Environ. Microbiol. 60: 3632–3639

Beloin RM, Sinclair JL & Ghiorse WC (1988) Distribution and activity of microorganisms in subsurface sediments of a pristine study site in Oklahoma. Microbial Ecol. 16: 85–97

Bossert I & Bartha R (1984) The fate of petroleum in soil ecosystems. In: Atlas RM (Ed) Petroleum Microbiology (pp 435–473). MacMillan Publishing Company, New York

Bregnard TP-A, Hohener P, Haner A & Zeyer J (1996) Degradation of weathered diesel fuel by microorganisms from a contaminated aquifer in aerobic and anaerobic microcosms. Environ. Toxicol. Chem. 15: 299–307

Brockman FJ & Murray CJ (1997) Subsurface microbiological heterogeneity: Current knowledge, descriptive approaches and applications. FEMS Microbiol. Rev. 20: 231–247

Bulman TL, Newland M & Wester A (1993) *In-situ* bioventing of a diesel fuel spill. Hydrol. Sci. 38: 297–308

Cort T, Davis CA, Dai D, Illangasekare TH & Munakata-Marr J (2001) Intermediate-scale evaluation of bioremediation technologies in heterogeneous LNAPL-contaminated soil. Proc. Conf. Environ. Research, Hazardous Substance Research Center, Manhattan, KS, May 21–24

Davis GB, Johnston CD, Patterson BM, Barber C & Bennett M (1998) Estimation of biodegradation rates using respiration tests during in-situ bioremediation of weathered diesel NAPL. Ground Water Monit. Remediat. 18: 123–132

Dineen D, Slater JP, Hicks P, Holland J & Clendening LD (1990) *In-situ* biological remediation of petroleum hydrocarbons in unsaturated soils. In: Kostecki PT & Calabrese EJ (Eds) Petroleum Contaminated Soils (pp 177–187). Lewis Publishers, Inc. Boca Raton FL

Downey DC, Guest PR & Ratz JW (1995) Results of a two-year in-situ bioventing demonstration. Environ. Progress. 14: 121–125

Dupont RR (1993) Fundamentals of bioventing applied to fuel contaminated sites. Environ. Progress. 12: 45–53

Environmental Protection Agency (1996a) SW-846, Test Methods for Evaluating Solid Wastes: Physical/Chemical Methods: Method 3550b Ultrasonic Extraction, Revision 2, http://www.epa.gov/epaoswer/hazwaste/test/pdfs/3550b.pdf (July 1, 2003)

Environmental Protection Agency (1996b) SW-846, Test Methods for Evaluating Solid Wastes: Physical/Chemical Methods: Method 8015b Nonhalogenated Organics using GC/FID, Revision 2, http://www.epa.gov/epaoswer/hazwaste/test/pdfs/8015b.pdf (July 1, 2003)

Goudar CT & Strevett KA (1998) Comparison of relative rates of BTEX biodegradation using respirometry. J. Ind. Microbiol. Biotechnol. 21: 11–18

Haack SK & Bekins BA (2000) Microbial populations in contaminant plumes. Hydrogeol. J. 8: 63–76

Hickey WJ (1995) In situ respirometry: Defining the limits. Environ. Sci. Technol. 30: 398A–401A

Hickman GT & Novak JT (1989) Relationship between subsurface biodegradation rates and microbial density. Environ. Sci. Technol. 23: 525–532

Hinchee RE & Ong SK (1992) A rapid *in-situ* respiration test for measuring aerobic biodegradation rates of hydrocarbons in soil. J. Air Waste Manag. Assoc. 42: 1305–1312

Hogg DS, Burden RJ & Riddell PJ (1992) *In-situ* vadose zone bioremediation of soil contaminated with non-volatile hydrocarbons. Presented at HMCRI Conference, February 4, 1992, San Francisco, CA

Hoyle BL & Arthur EL (2000) Biotransformation of pesticides in saturated-zone materials. Hydrogeol. J. 8: 89–103

Hupe K, Heerenklage J, Woyczechowske H, Bollow S & Stegmann R (1998) Influence of oxygen on the degradation of diesel fuel in soil bioreactors. Acta Biotechnologica 18: 109–122

Kao C-M & Borden RC (1997) Site specific variability in BTEX biodegradation under denitrifying conditions. Groundwater 35: 305-311

Kao C-M & Prosser J (1999) Influence of aquifer biogeochemical distribution on BTEX biodegradation under aerobic/anaerobic conditions. J. Chinese Institute Environ. Engin. 9:187–197

Leeson A. & Hinchee RE (1997) Soil Bioventing: Principles and Practice. Lewis Publishers, Boca Raton, FL

Lotrario JB, Stuart BJ, Lam T, Arands RR, O'Connor OA & Kosson DS (1995) Effects of sterilization methods on the physical characteristics of soil: Implications for sorption isotherm analyses. Bull. Environ. Contam. Toxicol. 54: 668–675

Moller J, Winther B, Lund K, Kirkebjerg K & Westermann P (1996) Bioventing of diesel oil-contaminated soil: Comparison of degradation rates in soil based on actual oil concentration and on respirometric data. J. Indus. Microbiol. 16: 110–116

Norris RD, Hinchee RE, Brown R, McCarty PL, Semprini L, Wilson JT, Kampbell DH, Reinhard M, Bouwer EJ, Borden

RC, Vogel TM, Thomas JM & Ward CH (1994) Handbook of Bioremediation. Lewis Publishers, Boca Raton, FL

Parker EF & Burgos WD (1999) Degradation patterns of fresh and aged petroleum-contaminated soils. Environ. Eng. Science 16: 21–29

Parkin TB, Starr JL & Meisinger JJ (1987) Influence of sample size on measurement of soil denitrification. Soil Sci. Soc. Am. J. 51: 1492–1501

Pedersen D & Sayler GS (1981) Methanogenesis in freshwater sediments: inherent variability and effects on environmental contaminants. Can. J. Microbiol. 27: 198–205

Ramaswami A & Luthy RG (1997) Mass transfer and bioavailability of PAH compounds in coal tar NAPL-slurry systems 1. Model Development. Environ. Sci. Technol. 31: 2260–2267

Rogers B & Logan BE (2000) Bacterial transport in NAPL-contaminated porous media. J. Environ. Engin. 126: 657–666

Saba TA (1999) Upscaling of mass transfer from entrapped NAPLs under natural and enhanced conditions. Dissertation, University of Colorado, Boulder

Seagren EA, Rittmann BE & Valocchi AJ (1994) Quantitative evaluation of the enhancement of NAPL-pool dissolution by flushing and biodegradation. Environ. Sci. Technol. 28: 833–839

Sepic E, Trier C & Leskovsek H (1996) Biodegradation studies of selected hydrocarbons from diesel oil. Analyst 121: 1451–1456

Stout SA & Lundergard PD (1998) Intrinsic biodegradation of diesel fuel in an interval of separate phase hydrocarbons. Appl. Geochem. 13: 851–859

Sturman PJ, Stewart PS, Cunningham AB, Bouwer EJ & Wolfram JH (1995) Engineering scale-up of in situ bioremediation processes: A review. J. Contam. Hydrol. 19: 171–203

Ulrich GA, Martino D, Burger K, Routh J, Grossman EL, Ammerman JW & Suflita JM (1998) Sulfur cycling in the terrestrial subsurface: Commensal interactions, spatial scales, and microbial heterogeneity. Microbial Ecol. 36: 141–151

Van Eyk J & Vreeken C (1989) Venting mediated removal of diesel oil from subsurface soil strata as a result of stimulated evaporation and enhanced biodegradation. In: Hazardous Waste and Contaminated Sites, Envirotech Vienna, Vol. 2, Session 3 (pp 475–485). Westarp Wiss., Essen

Vroblesky D A & Chapelle FH (1994) Temporal and spatial changes of terminal electron-accepting processes in a petroleum hydrocarbon-contaminated aquifer and the significance for contaminant biodegradation. Wat. Resour. Res. 30: 1561–1570

Wachinger G, Fiedler S, Zepp K, Gattinger A, Sommer M & Roth K (2000) Variability of soil methane production on the microscale: Spatial association with hot spots of organic material and archaeal populations. Soil Biol. Biochem. 32: 1121–1130

Widrig DL & Manning JF (1995) Testing bioremediation in the field. In: *In-Situ* Bioremediation: When Does it Work? (pp 160–184). National Research Council; National Academy Press, Washington DC

Zhang C, Palumbo AV, Phelps TJ, Beauchamp JJ, Brockman FJ, Murray CJ, Parsons BS & Swift DJP (1998) Grain size and depth constraints on microbial variability in coastal plain subsurface sediments. Geomicrobiol. 15: 171–185