FINAL REPORT

BIOREMEDIATION OF PETROLEUM HYDROCARBONS IN HETEROGENEOUS SOILS

**Topical Final Report Task 35
Jointly Sponsored Research Proposal
Under DE-FC26-98FT40323**

March 2006

**By
Song Jin
Paul Fallgren
Terry Brown**

**For
University of Wyoming
Laramie, Wyoming**

**And
U.S. Department of Energy
National Energy Technology Laboratory
Morgantown, West Virginia**

**By
Western Research Institute
Laramie, Wyoming**

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

ABSTRACT

Western Research Institute (WRI) in conjunction with the University of Wyoming, Department of Renewable Resources and the U.S. Department of Energy, under Task 35, conducted a laboratory-scale study of hydrocarbon biodegradation rates versus a variety of physical and chemical parameters to develop a base model. By using this model, biodegradation of Petroleum hydrocarbons in heterogeneous soils can be predicted.

The base model, as developed in this study, have been tested by both field and laboratory data. Temperature, pH, and nutrients appear to be the key parameters that can be incorporate into the model to predict biodegradation rates. Results to date show the effect of soil texture and source on the role of each parameter in the rates of hydrocarbon biodegradation. Derived from the existing study, an alternative approach of using CO₂ accumulation data has been attempted by our collaborators at the University of Wyoming. The model has been modified and fine tuned by incorporating these data to provide more information on biodegradation.

TABLE OF CONTENTS

	<u>Page</u>
DISCLAIMER	ii
ABSTRACT	iii
EXECUTIVE SUMMARY	vi
BACKGROUND	1
RESULTS	2
SUMMARY	23
REFERENCES	24

LIST OF TABLES

<u>Table</u>	<u>Page</u>
Table 1 Soil Physical Parameters	2
Table 2 Soil Physical Properties.....	3
Table 3 Temperature vs. Respiration Rate; X=Lob (T), Y= Log (Respiration Rate) ..	16
Table 4 Moisture vs. Respiration Rate; X= Moisture Content (W), Y= Log (Respiration Rate)	17
Table 5 Salinity vs. Respiration Rate; X= (Salt Concentration), Y= Log (Respiration Rate)	18
Table 6 N/C Ratio (NH ₄ NO ₃) vs. Respiration Rate; X=(N/C), Y=Log (Respiration Rate)	18
Table 7 P/C (K ₂ PO ₄) vs. Respiration Rate; X= (P/C), Y= Log (Respiration Rate)	18
Table 8 pH vs. Respiration Rate for the Three Soils	19
Table 9 Base Model Coefficients for Various Water Contents (Includes Correlation).....	20
Table 10 Base Model Coefficients for Sodium Chloride Concentration	22

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
Figure 1 M-1 Soil Moisture Release Curve	3
Figure 2 R-1 Soil Moisture Release Curve	4
Figure 3a Microbial Respiration vs. Temperature (S-1 Soil)	5
Figure 3b Microbial Respiration vs. Temperature (M-1 Soil).....	6
Figure 3c Microbial Respiration vs. Temperature (R-1)	7

LIST OF FIGURES (cont'd)

<u>Figure</u>	<u>Page</u>
Figure 4a Microbial Respiration vs. Moisture (S-1 Soil)	8
Figure 4b Microbial Respiration vs. Moisture (M-1 Soil).....	9
Figure 4c Microbial Respiration vs. Moisture (R-1 Soil).....	9
Figure 5a Microbial Respiration vs. Salinity (S-1 Soil)	10
Figure 5b Microbial Respiration vs. Salinity (M-1 Soil).....	11
Figure 5c Microbial Respiration vs. Salinity (R-1 Soil).....	12
Figure 6a Microbial Respiration vs. Nitrogen (S-1 Soil)	12
Figure 6b Microbial Respiration vs. Nitrogen (M-1 Soil)	13
Figure 6c Microbial Respiration vs. Nitrogen (R-1 Soil).....	14
Figure 7a Microbial Respiration vs. P (S-1 Soil)	14
Figure 7b Microbial Respiration vs. P (M-1 Soil)	15
Figure 7c Microbial Respiration vs. P (R-1 Soil).....	16
Figure 8 CO ₂ Accumulation vs. Hours for Various Water Contents.....	20
Figure 9 Regression Curves Relating Coefficients with Water Content	21
Figure 10 CO ₂ Accumulation vs. Hours for Sodium Chloride Concentration	22
Figure 11 Regression Curves Relating Coefficients with Sodium Chloride Conc.....	22

EXECUTIVE SUMMARY

Bioremediation of petroleum, contaminated soils is a viable and cost-effective technology. However, its success is dependent on accurate predictions of timeframes and effectiveness of bioremediation approaches. Important parameters that control the biodegradation rates of hydrocarbons in soil include temperature, pH, moisture content, hydrocarbon concentrations, soil texture, sorption, bioavailability, salinity, and the presence of microbial toxins. It is difficult to apply laboratory biodegradation rate measurements to the field due to complications caused by soil heterogeneity. Accurate laboratory scale measurements of biodegradation rates versus physical, chemical, and biological parameters are pertinent to estimate field-scale biodegradation.

Western Research Institute (WRI), in conjunction with the University of Wyoming Department of Renewable Resources and the U.S. Department of Energy, under Task 35, conducted a laboratory-scale study of hydrocarbon biodegradation rates versus a variety of physical and chemical parameters to develop a model from which would serve as a foundation to estimate field-scale biodegradation of petroleum hydrocarbons in heterogeneous soils. Biodegradation rates were indirectly measured by carbon dioxide production. Carbon dioxide was measured by respirometry, and from the data, relationships between rates and parameters were developed.

A base model has been established based on the data obtained from this study. The model has been tested by both field and laboratory data. Temperature, pH, and nutrients appear to be the key parameters that can be incorporated into the model to predict biodegradation rates. The model can be used under various site conditions to provide more information on biodegradation.

BACKGROUND

Bioremediation has been studied and applied as a cost-effective technology in environmental cleanup. The process relies on microorganisms to break down or transform contaminants to harmless end products such as carbon dioxide and water. Microorganisms in nature metabolize the petroleum hydrocarbons by extracting carbon and energy from them.

It has been determined that biodegradation is a complicated mechanism that dominates the destruction of organic contaminants in the environment and typically occurs most rapidly under both aerobic conditions (Parker and Burgos, 1999; Moller et al., 1996; Stout and Lundergard, 1998; Widrig and Manning, 1995). Therefore, information on biodegradative pathways is critical to assess the fate of environmental contaminants. Numerous mathematical and statistical models have been used to predict the migration or fate of environmental contaminants; however, most of these models fail to include a complete set of parameters that are closely associated with microbial activities or presume biodegradation as a simple first order process (Geerdink et al., 1996; Jin and Bethke, 2003; Nakhla, 2003; Wang et al., 2003).

The biodegradation of hydrocarbons in a variety of soil and geochemical conditions, have been well documented (Norris et al., 1994). For successful, cost-effective application of bioremediation, the accurate measurement of biodegradation rates under a variety conditions is an important factor. The structural complexity of the hydrocarbons usually determines the degradation rate. Other important parameters that control the biodegradation rates of hydrocarbons in soil include temperature, pH, moisture content, hydrocarbon concentrations, soil infiltration rate, sorption, bioavailability, salinity, and the presence of microbial toxins. However, heterogeneities make it difficult to extrapolate biodegradation kinetics from laboratory studies to field applications. Soil heterogeneities result in significant variation in biodegradation (Haack and Bekins, 2000), and due to these complexities at the field-scale, biodegradation rates are often first measured in bench-scale studies (Davis et al., 2003; Davis et al., 1998; Downey et al., 1995; Hinchey and Ong, 1992; Moller et al., 1996). We expect this work to lay a foundation to accurately predict *in situ* biodegradation from laboratory research data.

WRI, in conjunction with the University Of Wyoming Department Of Renewable Resources and the U.S. Department of Energy, under Task 35, conducted a laboratory-scale study of hydrocarbon biodegradation rates versus a variety of physical and chemical parameters. The goal of this project was to develop a model from bench-scale biodegradation vs. parameter results, which, would serve as a foundation to estimate field-scale biodegradation of petroleum hydrocarbons in heterogeneous soils.

RESULTS

Sample Collection and Physical Analysis

Three types of soil samples were collected from different sources. Petroleum contaminated soil samples (S-1) were collected from a petroleum-contaminated site in Egypt. Soils M-1 and R-1 originated in southeast Wyoming were obtained from Dr. Larry Munn and Dr. Renduo Zhang, respectively (Department of Renewable Resources, University of Wyoming).

Textural analysis showed S-1 to be mostly sand, when petroleum constituents were removed through hydrogen peroxide wash. M-1 soil is distinctively different from the others in its black color (both dry and wet) and its high clay content. R-1 soil is a sandy-loam and separates from the other soils in that it is yellowish brown when dry and pale brown when wet. The percentages of total clay, silt, and sand are presented in Table 1. Total clay was found by dividing 5-cm deep pipette sample dry weight over dry weight of soil. Likewise, total silt content of the soil was calculated by dividing 10-cm deep pipette sample over the total dry sample weight.

Table 1. Soil Physical Parameters

10 cm depth samples(silt+clay)								
sample	tare, gr	dry-wt+t,gr	dry-wt, gr	tot silt,S	% clay	%silt	%sand	%solids
S-1	0.989	1.044	0.055	0.65	8.58	2.71	94.51	105.80
M-1	0.997	1.388	0.391	6.25	53.16	25.02	19.21	97.39
R-1	1.015	1.234	0.219	6.9	16.11	27.59	57.28	100.98
5-cm depth samples (clay)								
sample	tare, gr	dry-wt+t,gr	dry- wt, g	tot clay, C	texture			
S-1	0.997	1.039	0.042	2.06	Sand			
M-1	1	1.266	0.266	13.28	Clay			
R-1	1.029	1.11	0.081	4.03	SandyL			

Table 2 summarizes soil physical properties including soil water characteristics and the how these characteristics change with increasing suction forces (Figures 1 and 2) in soils M-1 and R-1. Similar, studies were performed earlier by our group for S-1 soil in a different project.

In Figures 1 and 2, the suction values on the x-axis are logarithms of the suctions, which give a smooth curve. To determine field capacity, wilting point, and near saturation values of soil suction (absolute values of soil water potential), the following conversion values are used.

<u>Near Saturation</u>	<u>Field capacity</u>	<u>Wilting point</u>
< 100 cm	300-500 cm	>15000 cm
Log-scale: 2.477	2.477-2.698	4.176

M1 soil has a water content (volumetric) range 0.028 – 0.564. Similarly, the R1 soil has a range from 0.013 to 0.583. The range offered minimum and maximum water holding capacities of the soil samples. The water content in Table 2 is the gravimetric water content and is defined as:

$$w = \frac{W_w}{W_s} \quad (1)$$

in which W_w and W_s are weight of water and weight of soil solid in a sample, respectively.

Table 2. Soil Physical Properties.

Soil	S	M1	R1
Color		Black	Yellow
Clay (%)	8.11	54.58	15.96
Silt (%)	2.56	25.69	27.32
Sand (%)	89.33	19.73	56.72
Texture	Sand	Clay	Sandy loam
Bulk Density (g/cm ³)	1.60	1.119	1.454
Residual Water Content (g/g)	Not detected	0.025	0.009
Field Water Holding Capacity (g/g)	0.10	0.36	0.34
Saturated Water Content (g/g)	0.260	0.504	0.401

Figure 1. M-1 Soil Moisture Release Curve

Figure 2. R-1 Soil Moisture Release Curve

Soil Treatments and Monitoring

Based on the definition and information in Table 2, the following equations were set up to prepare soil samples with different water contents. If we change X g of soil sample with initial water content a to a final water content b ($b > a$), the following equation can be used to calculate the amount of water (g) to be added:

$$W_a = \frac{(b - a)X}{(1 + a)} \quad (2)$$

Biodegradation of hydrocarbons can be indirectly measured by the carbon dioxide formed from microbial respiration. Carbon dioxide, measurements, by respirometry, is a common method in bench-scale biodegradation studies (Davis et al., 2003; Davis et al., 1998; Goudar and Strevett, 1998; Hinchee and Ong, 1992; Moller et al., 1996). A respirometer with oxygen and carbon dioxide (CO₂) sensors and biometer flasks were used to measure microbial respiration rates of soil samples. Biometer flasks were also used in the monitoring of microbial respiration. CO₂ evolution was trapped in the alkali solution and titrated with acid. Results from the respirometer and biometer flasks were calibrated to ensure the sensitivity and comparability. Microbial respiration measured as CO₂ ul/min is the key indicator of biodegradation. A water bath was used to control soil temperature.

In addition to the heterogeneities of the three soil samples, as summarized in soil physical analysis, other parameters investigated included temperature, moisture content, salinity and nutrient content. Each variable was studied in the soil treatments that were established by fixing all other parameters.

Parameter vs. Biodegradation

Each sample bottle contained 20 grams of soil with a, fixed moisture content equaling their respective field water holding capacity. No nutrients were supplemented

and the soil samples were incubated in the dark in a water bath with temperature control. The microbial respiration rates were monitored by respirometry and the samples were incubated in the temperature range of 4.0°C – 92°C.

In S-1 soil samples, the active temperature range for normal microbial activities was observed at 20°C – 8°C, with the optimal temperature at 80 °C. When the temperature was below 20°C or above 82°C, microbial respiration declined significantly (see Fig 3a). The data indicates that the dominant microbial population in S-1 soil is hyperthermophilic, with an optimal temperature at 80°C. This may be attributed to the microbial adaptation to the warm climate at the sampling site.

In M-1 soil samples, the active temperature range for biodegradation was observed at 15°C – 66°C. At two temperatures, 46°C and 66°C, the microbial respiration rates reached peaks. When the temperature was below 15°C or above 6°C, microbial respiration rates declined significantly (see Fig 3b).

M-1 soil samples originated from surface soil in the state of Wyoming, where surface soil temperature varies from subzero to over 50°C. The data indicate that a variety of microbial populations may exist in the soil samples due to the exposure to a wide temperature range in the region. It appeared that the dominant population adapts to a mild temperature range of 15°C – 46°C. The activities of the thermophilic microbes became prevailing when the temperature rose above 4°C. The presence of thermophilic microbes in M-1 soils may be partially attributed to the high clay content in M-1 soil. Clay enhances the water and nutrient containing capacity of the soil, offering favorable conditions for the co-existence of diversified microbial populations.

In R-1 soil samples, the active temperature range for microbial respiration was observed at 12°C – 66°C. At two temperatures, 30°C and 60°C, the microbial respiration rates reached peaks. When the temperature was below 12°C or above 60°C, microbial respiration rates declined significantly (see Fig 3c).

Similar to M-1 soil, R-1 soil samples originated from surface soil in the state of Wyoming, where surface soil temperature ranges varies from subzero to over 60 °C. The data indicates that a variety of microbial populations may exist in the soil samples due to the exposure to a wide temperature range in the region. It appeared that the dominant population adapts to a lower to mild temperature range of 12 °C – 30 °C. The activities of some thermophilic microbes became prevailing when the temperature rose above 30 °C. The presence of thermophilic microbes in R-1 soils may be partially attributed to the high clay content in R-1 soil. Clay enhances the water and nutrient containing capacity of the soil, offering favorable conditions for the co-existence of diversified microbial populations.

When compared to S-1 and M-1 soils, a significant drop of microbial respiration at 50°C was observed in R-1 soil samples. The loss of microbial activities at 50°C suggested that the microbial respiration stay most active when the temperature is in the lower range. Taking microbial respiration rates at 30°C for example, S-1, M-1 and R-1 soils were measured at 0.19 μl/min, 0.27μl/min and 0.53μl/min, respectively. The difference in microbial respiration rates at lower (R-1 soil), mild (M-1 soil) to high (S-1 soil) temperatures indicated the difference of prevailing microbial populations in the three soils under study. The difference may also be attributed to the heterogeneities in soil physical structures.

Water was added to each type of soil sample to the respective saturation point, as determined in the soil physical analysis. Samples were established in biometer flasks and incubated in the dark at 35°C. Each sample contained 20 g of soil. No nutrients were supplemented and oxygen in the samples was maintained at adequate concentrations through the large headspace of the biometer flask. Microbial respiration was measured by titrating CO₂ evolution that was trapped in the NaOH solution.

In S-1 soil samples, microbial respiration showed a parallel trend with the increased moisture contents, until the field water holding capacity of approximately 10% was reached (Fig 4a). At a water content above 7%, microbial respiration rates stabilized with no further increase.

Due to the sandy texture, field water holding capacity of S-1 soil is relatively low. Indigenous microorganisms have adapted to the arid environment during the long-term exposure to the petroleum contamination in the desert. Consequently optimal microbial activities may be achieved rapidly and maintained at a relatively low water content. As shown on Fig 4a, microbial respiration rate rose from, 0.025 μl/min to 0.25 μl/min at a water concentration of 7% (g/g).

Similar treatments were established for M-1 soil samples. Results were shown on Fig 4b. In M-1 soil samples, microbial respiration rates increased with the moisture contents; however, this trend is much less observable than that in S-1 soils. The optimal microbial respiration rate was reached when moisture content was at 35.7%. The gap between the lowest (0.03 µl/min) and the highest (0.06 µl/min) rates of microbial respiration is much less significant than that in S-1 soils (0.025 µl/min to 0.25 µl/min). Also different from S-1 soils, microbial respiration rates at water concentrations above 35.7% decreased.

The high clay content of M-1 soils results in the high water saturation point as well as elevated residual water content. The abundance of water held in M-1 soil may have compromised the significance of the role of water content in microbial respiration. Consequently optimal microbial activities were achieved slowly when water content is the isolated variable, as shown on Fig 4b. The higher water holding capacity of M-1 soil and the clay texture also attribute to the recharge and circulation of oxygen in the soil samples. When water content was added at the saturation point of M-1 soil, the water residence time was longer than that in soils with less clay, therefore microbial respiration was adversely affected due to oxygen limitations.

Results from R-1 soil samples were shown in Fig 4c. Microbial respiration rates increased with the rise of moisture contents. The correlation of water content and microbial respiration was as evident as that in S-1 soils (Fig 4a). Similar to S-1 samples, microbial respiration rates rose from 0.10 $\mu\text{l}/\text{min}$ to 0.41 $\mu\text{l}/\text{min}$ when water content was increased from 0.09% to 0.11%. The optimal microbial respiration rate was reached when moisture content was at 22.9%. At water content above 11.4%, microbial respiration rates stabilized with no further increase.

Due to the sandy loam texture, the field water holding capacity of R-1 soil is between S-1 and M-1 soils. Indigenous microorganisms may have adapted to the environment with limited moisture content but water content remains as a rate limit for microbial respiration. Consequently optimal microbial activities may be achieved rapidly and maintained when water content increases until the need for water is saturated. Water content appears to be a key variable in microbial respiration in R-1 soil.

Artificial seawater (“AS”, 0.425 M sodium chloride, 0.009 M potassium chloride, 0.0093 M calcium chloride, 0.0255 M magnesium sulfate, 0.023 M magnesium chloride, and 0.002 M sodium bicarbonate, pH 8.0) was used to evaluate the relationship between salinity and microbial respiration. Different concentrations of sodium chloride (NaCl) were used to obtain different salinities. AS, was added to samples at their respective field water holding capacities. All samples were incubated in the dark at 30 °C. Each treatment contained 20 g of soil. No nutrients were supplemented and oxygen in the samples was maintained at adequate concentrations through the large headspace of the biometer flask. Microbial respiration was measured by titrating CO₂ evolution that was trapped in the NaOH solution in the biometer flasks.

Effects of salinity on biodegradation rates are presented in Figure 5. In S-1 samples (Fig 5a), microbial respiration increased sharply when the water source was switched from di-H₂O to AS (0.025 $\mu\text{l}/\text{min}$ to 0.29 $\mu\text{l}/\text{min}$). When NaCl concentration increased beyond that in AS (0.43 M), microbial respiration rates decreased, indicating

that microbes are not highly halophilic even though they seem to be able to maintain normal respiration at the sea water equivalent salinity (0.43 M of NaCl).

The results suggest that the indigenous microbial population has adapted to seawater equivalent saline environments. However, NaCl concentrations higher than seawater may have adverse effects on microbial respiration.

Effects of salinity on microbial respiration rates in M-1 soil samples are presented on Fig 5b. No trend was observed between the microbial respiration rates vs. increased concentrations of NaCl. Microbial respiration rate was 0.12 µl/min when AS was added to the field water holding capacity (36%). This rate did not change significantly when NaCl concentrations increased (Fig 5b). When freshwater was added to the field water holding capacity (36%), as shown on Fig 4b and 5b, microbial respiration rate was 0.06 µl/min. The presence of salts in the AS seemed to slightly enhance the microbial activities in M-1 soil.

The results suggest that the indigenous microbial population either has adapted to saline environments or the salt influence is shielded by the clay texture in the M-1 soil. Clay material restricts transportations of water, nutrients and microorganisms. It also absorbs certain ions from the water. High NaCl concentration may disperse the soil particulates and further minimize the transportation channels in soils. The relatively low microbial respiration rates (0.06 µl/min – 0.15 µl/min) and the seemingly non-influence from salinity supported the presumptions. More assessments are necessary to decipher the relationship between salinity and microbial respiration in M-1 soil.

The relationship between salinity and microbial respiration in R-1 soil samples was shown in Fig 5c. The data were difficult to interpret. No trend was observed between the microbial respiration change rates vs. concentrations of NaCl. When freshwater was added to the field water holding capacity (34%), as shown in Fig 4c and 5c, microbial respiration rate was 0.10 µl/min. The presence of salts in the AS increased the microbial respiration rate to 0.25 µl/min. However when NaCl concentrations increased beyond that in AS (0.43 M), no enhancement or inhibition of microbial activities were observed. The microbial respiration rates presented a vibration of increases and decreases as shown on Fig 5c.

The results suggest that the indigenous microbial population either has adapted to saline environments or the salt influence is shielded by clay texture in the R-1 soil. Clay material restricts transportations of water, nutrients and microorganisms. High NaCl concentration may disperse the soil particulates and further minimize the transportation channels in soils. The cyclic changes of microbial respiration rates along with increased salinity as shown on Fig 5c indicated that the influence of high salt concentration on microbial activities in R-1 soil is built on a complicated mechanism. Further studies are warranted to elucidate the observed results.

Theoretical optimal C: N: P molar ratio of 100:10:1 was used as the benchmark in nutrient amendments. Total carbon and phosphorus were analyzed in S-1, M-1 and R-1 soils. Due to the nature of sandy soil, contamination types, and the infeasibility of nitrogen speciation in one of the soil types, background nitrogen concentration was not included in the calculations of nitrogen amendments. Nitrogen was added as ammonium nitrate in the samples to different nitrogen/carbon ratios. All samples were incubated in the dark at 30°C. Microbial respirations were, measured by bio-meter flasks as described in the previous sections.

In S-1 samples, nitrogen was added as ammonium nitrate in the treatments to nitrogen/carbon ratios at 0-0.5. No other nutrients were added to the soil samples. Water content was maintained at the field water holding capacity of S-1 soil (10%, g/g). As shown in Fig 6a, the addition of nitrogen nutrient observably elevated the microbial respiration rates. The increases of microbial activities were linear proportional to the increased amount of nitrogen. The results suggest that nitrogen in S-1 soil is a limiting factor to microbial respiration. No saturation effects were observed for nitrogen in the enhancement of microbial respiration rates in S-1 soil. This might be attributed to the high reproduction rates of microorganisms in S-1 soil.

Similar samples were established for M-1 soil. Nitrogen was added as ammonium nitrate in the samples to nitrogen/carbon ratios at 0-0.4. No other nutrients were added to the soil samples. Water content was maintained at the field water holding capacity of M-1 soil (36%, g/g). As shown in Fig 6b, the increase of nitrogen/carbon from the background to 0.05:1 raised the microbial respiration rates from 0.06 $\mu\text{l}/\text{min}$ to 0.16 $\mu\text{l}/\text{min}$. However, when nitrogen/carbon ratio was increased to above 0.05, microbial the respiration rates stabilized and decreased gradually. At nitrogen/carbon=0.4, microbial respiration rate declined to 0.10 $\mu\text{l}/\text{min}$. The results suggest that nitrogen in M-1 soil is not a limiting factor to microbial respiration. This may be attributed to the clay texture of M-1 soil, in which high clay content adsorbed ammonium and restricted its bioavailability. The increased amendments of nitrogen may not be accessible to microorganisms in the soils, resulting in a stabilized pattern of microbial respiration.

Nitrogen was added as ammonium nitrate in the R-1 soil samples to reach nitrogen/carbon ratios at 0-0.4. No other nutrients were added to the soil samples. Water content was maintained at the field water holding capacity of R-1 soil (34%, g/g). As shown in Fig 6c, the increase of nitrogen/carbon ratio did not have observable effects on microbial respiration rates. At nitrogen/carbon = 0.4, microbial respiration rate was 0.09 $\mu\text{l}/\text{min}$, compared to the background of 0.10 $\mu\text{l}/\text{min}$. The results suggest that ammonium nitrogen may not be the preferred nitrogen source to be metabolized by the microbial populations in R-1 soils. In addition, the clay in the R-1 soil might have adsorbed ammonium and restricted its bioavailability.

Studies have shown the influences of phosphorus on biodegradation tend to be site specific. In our study, different amounts of phosphate were added to the three soils to evaluate the effect of phosphorus on microbial respiration. K_2HPO_4 was used as the phosphorus source. The molar ratio of P/C was added to the range of 0.003 to 0.05. Nitrogen was maintained at N/C = 0.1:1. All samples were incubated in the dark at 30°C.

In S-1 samples, the initial addition of phosphorus showed significant enhancement in microbial respiration rates. As shown in Fig 7a, samples with C:P = 100:2 achieved an 117% increase in CO₂ evolution rate, compared to the samples with C:P = 100:0.3. However, when C:P was increased to above 100:2, no additional enhancement of microbial respiration was detected (see Fig 7a).

The data demonstrated that phosphorus is a very important factor in microbial activity. But this enhancing effect saturates at relatively low phosphorus content, presumably due to the limited needs for phosphorus in microbial metabolisms. The potential interactions between phosphorus and nitrogen nutrients in S-1 soil are under investigation.

In M-1 samples, similar results were obtained as in S-1 soil. The initial addition of phosphorus showed significant enhancement in microbial respiration rates. But the enhancement occurred at much lower phosphorus concentrations. As shown in Fig 7b, samples with C: P=100:0.3 achieved an 117% increase in CO₂ evolution, compared to C:P=100:2 for same increase of microbial respiration rate in S-1 samples. When C:P was increased to above 100:0.3, no additional enhancement of microbial respiration was detected (see Fig 7b).

Results indicated that phosphorus effect may be, more sensitive in M-1 soils. This may be attributed to different microbial populations than in S-1 soils.

Although data in R-1 samples showed similar patterns as in M-1 soil, the enhancement of phosphorus in microbial respiration was less significant. The initial addition of phosphorus resulted in an observable increase in microbial respiration rates. As shown on Fig 7c, samples with C: P=100:0.5 achieved a 30% increase in CO₂ evolution. When C: P was increased to above 100:0.5, no additional enhancement of microbial respiration was detected (see Fig 7c).

It appeared that the role of phosphorus on microbial respiration is less significant in R-1 soils. The mechanism is unknown but presumably related to the heterogeneity of R-1 soil when compared to M-1 and S-1 soils. Further studies are needed to decipher the mechanisms and to investigate the potential interactions between phosphorus and nitrogen nutrients.

Linearity Assessments of Raw Data

As an initial step, the data was analyzed mathematically to determine if there is any linear relationship between each parameter and the microbial respiration rates. Under some circumstance, data was transformed logarithmically. The results from this assessment, was partially used to establish a model equation that summarize key factors and possesses predicting power for microbial respiration.

Temperature

Table 3. Temperature vs. Respiration Rate; X= Log (T), Y= Log (Respiration Rate).

Soil	Log-Log	R ²
S-1	Y=1.1684X-2.4375	0.903
M-1	Y=1.1956X-2.3581	0.8208
R-1	Y=1.3309X-2.3565	0.9096

As shown in Table 3, S-1 soil demonstrates significant linear relationship between the log-transformed respiration rates and temperature ($p < 0.005$). The relationship fits all temperature values tested except for temperature at or above 83°C. The decline of microbial respiration is probably due to the elevated temperature that is detrimental to enzyme activities even for thermophilic microbes as identified in S-1 soil.

In M-1 soil, a significant linear relationship between the log-transformed respiration rate and temperature was also shown ($p < 0.005$). The relationship fits all temperature values tested except for readings at or above 70°C, at which the decline of microbial respiration is probably due to the elevated temperature that is detrimental to enzyme activities for mesothermophilic microbes as identified in M-1 soil. The R-1 soil showed a significant linear relationship between the log-transformed respiration rate and

temperature, as is the case for the other two soils ($p=0.005$). However, the linear relationship in R-1 soil is less significant when temperature reached 50°C or above. As shown on Fig 3c, a different trend of microbial respiration was observed when temperature was above 50°C. It is possible that the activities of the dominant mesothermophilic species were shut down at the temperature and a thermal species was activated, resulting in a subsequent elevation in microbial respiration. The relationship is not significant between the temperature and microbial respiration rates when the temperature increased above 50°C.

Moisture

The relations between moisture content and respiration rate for the three soils are shown in Table 4. The natural log transformation of respiration rate resulted in a basically linear relationship with moisture content. The most significant linear relation is shown in R-1 soil while the other two soils show slightly lower linearity between the parameters. The effect of moisture content on respiration is steady after moisture content 0.2 (g/g) for all the soils in this experiment.

Table 4. Moisture vs. Respiration Rate; X= Moisture Content (W), Y=Log (Respiration Rate).

Soil	Regression	R ²
S-1	$Y=0.1681\ln(X) - 0.3236$	0.654
M-1	$Y= 0.0767\ln(X)- 1.2365$	0.638
R-1	$Y=0.1732\ln(X)-0.1268$	0.91

Data from S-1 soil suggest that moisture content has a significant effect on respiration ($p<0.004$). The respiration rates were less significantly affected by moisture contents in M-1 soil samples ($p>0.05$). R-1 soil demonstrated a significant effect of moisture content on respiration rate ($p<0.012$), illustrated by increasing respiration rates with increasing moisture contents. However, when moisture content reached 20% (g/g) and above, respiration rates also reached a steady peak and did not change with further increased moisture contents.

Data shown in Table 4 indicate that soil physical features such as porosity and texture might have defined the different levels of linearity in the three soils. Moisture content appears to be significantly affecting microbial respirations in sandy soils such as S-1 and R-1, when compared to the more clay type M-1 soil. Physical parameters will be incorporated into the mathematic analysis for their roles in the relationship to microbial degradation.

Salinity

Table 5. Salinity vs. Respiration Rate; X= (Salt Concentration), Y=Log (Respiration Rate).

Soil	Regression	R ²
S-1	$Y = -0.1907 \ln(X) - 0.6855$	0.8625
M-1	$Y = -0.0336X - 1.0409$	0.295
R-1	$Y = -0.0282X - 0.555$	0.2518

In S-1 soil samples, there is a significant linear relationship between the transformed salinity and respiration rates. The respiration rate of S-1 soil decreased log-linearly with increased salt concentrations and this relationship is significant ($p < 0.001$). This is probably due to the presence of halophilic populations in S-1 soils with longterm exposure to salty conditions. The logarithmic transformation for R-1 and M-1 soil parameters did not improve the linear relationship.

N/C Ratio

Table 6. N/C Ratio (NH₄NO₃) vs. Respiration Rate; X= (N/C), Y=Log (Respiration Rate).

Soils	Log-Log	R ²
S-1	$Y = 0.2347 \ln(X) + 0.1983$	0.916
M-1	$Y = -0.755X - 0.9743$	0.643
R-1	$Y = -0.3237X - 0.8659$	0.5151

The relationship between N/C ratio and respiration varies in different soils. Data from S-1 soil show a linear relationship between the log-transformed respiration rates and N/C ratio, and this relationship is significant ($p < 0.001$). For R-1 soil, the linear relationship between transformed N/C ratio and respiration rates is insignificant. Other factors such as soil physical features a presumably overweighed N/C ratio on respiration rates in R-1 soil. Data from M-1 soil show reasonably linear relations between the transformed parameters, but the relationship remains insignificant. The respiration rates fluctuated with increasing N/C ratios. The insignificant relationship between N/C and microbial respiration rates may be attributed to other factors such as soil physical features that presumably overweighed N/C ratio in M-1 soil.

P/C Ratio

Table 7. P/C (K₂PO₄) vs. Respiration Rate; X= (P/C), Y=Log (Respiration Rate).

Soil	Log-Log	R ²
S-1	$Y = 0.0647 \ln(X) + 0.1469$	0.395
M-1	$Y = -0.0121 \ln(X) - 0.9467$	0.13
R-1	$Y = -0.0089 \ln(X) + 0.9447$	0.048

The relationship between the transformed respiration rates and P/C ratios is unclear in this analysis, as shown in Table 7. Data from S-1 soil, which is mostly sandy, show a relative linear relationship among the three soils under test, while data from the clay M-1 and sandy loam R-1 soils show low linearity between the parameters. The generally low linearity between the transformed P/C and microbial respiration suggest that other factors may outweigh P/C on microbial respiration. Presumably pH and soil textures are the responsible factors.

pH

Table 8. pH vs. Respiration Rate for the Three Soils .

Soil	Log-log	R ²
S-1	Res=0.5717pH-0.5594	0.995
M-1	Res=0.0094pH-1.269	0.0016
R-1	Res=0.5726pH-0.5607	0.996

Compared to other parameters, pH is a unique one that is expected with no observable linear relationship with microbial respirations. All three soils demonstrated the highest microbial respiration when pH was in neutral to slightly alkali range. This pH requirement is applicable to most soil microorganisms except for ones from an environment with extreme pH.

Development of Base Model Based on CO₂ Accumulation

Due to the complexities of the rate equations developed above, a different approach was established using CO₂ accumulation data over a 200-hour period. A base model was developed based on CO₂ accumulation data collected from previous studies. The base model that was established is defined as,

$$V_c(t) = at^b$$

where V_c is the cumulative volume (μ l) of CO₂ produced through microbial respiration, t is the incubation time (h), and a and b are coefficients that are functions of various physical and chemical parameters. Determination of coefficients based on specific varied parameters is the focus of this study. Coefficient, values may be determined by varying one parameter and keeping others constant. Since the coefficients are functions of parameters, it may be possible to relate one parameter to another.

Biodegradation rates at any point in time can be determined by taking the derivative of the base model,

$$R(t) = V_c'(t) = abt^{b-1}$$

The M1 soil was used in the following studies, and CO₂ accumulation was measured by respirometer. The soil temperature was kept constant at 30°C in both cases, but water content was kept constant at 0.504 for the sodium chloride concentration study.

Soil Water Content

CO₂ accumulation from the biodegradation of diesel and motor oil constituents over time is illustrated in Figure 8. It appears that the microbial degradation rates increase significantly at gravimetric water contents (w) between 0.20 and 0.30. Microbial activity is maximized at water contents greater than or equal to 0.40. Coefficients for the base model are tabulated with corresponding gravimetric water contents in Table 9. These coefficient values were plotted versus water content (Figure 9) and relationships between a , and w , and b and w were determined by regression. Substituting the relationships into the base model yields,

$$V_c(w, t) = (2395w^{0.8686})t^{0.0684w + 0.0618}$$

Figure 8. CO₂ Accumulation vs. Hours for Various Water Contents

Table 9. Base Model Coefficients for Various Water Contents (Includes Correlation)

0.025	89.065	0.074	0.918
0.100	438.560	0.053	0.829
0.200	416.980	0.075	0.881
0.300	943.620	0.090	0.885
0.400	1272.700	0.088	0.949
0.504	1144.800	0.095	0.931

(a)

(b)

Figure 9. Regression Curves Relating Coefficients with Water Content

Sodium Chloride Concentration

CO₂ accumulation from the biodegradation of diesel and motor oil constituents over time is illustrated in Figure 10. It appears with increasing concentrations (*C*) of sodium chloride the biodegradation activity decreases. The plots suggest halophilic bacteria are present in the soil; however, the threshold of sodium chloride concentration is between 19,480 and 38,960 mg / kg. Coefficients for the base model are tabulated with corresponding sodium chloride concentrations in Table 10. These coefficient values were plotted versus water content (Figure 11) and relationships between *a*, and *w*, and *b* and *w* were determined by regression. Substituting the relationships into the base model yields,

$$V_c(C, t) = [(1.73 \times 10^{-7}) C^2 - 0.0136C + 474]t^{[(2.19 \times 10^{-12})C^2 - (3.59 \times 10^{-7})C + 0.0198]}$$

Figure 10 CO₂ Accumulation vs. Hours for Sodium Chloride Concentration

Table 10 Base Model Coefficients for Sodium Chloride Concentration

C	a	b	r^2
0	447.520	0.019	0.989
8825	455.300	0.017	0.985
19480	183.000	0.016	0.967
38960	250.310	0.006	0.909
58440	261.350	0.007	0.909

(a)

(b)

Figure 11 Regression Curves Relating Coefficients with Sodium Chloride Concentration

The low correlations between the, *a* coefficient with NaCl concentration and the *b* coefficient with water content illustrate the complications other factors can have on predicting biodegradation. Studying one parameter, while holding the others constant must be done with as many parameters as possible to accurately model biodegradation in soils. It is difficult to compare the model from the water content study with the model from the NaCl concentration study, but it may be easier to compare water content model with another parameter model. Further studies with other parameters are undergoing by the University of Wyoming Department of Renewable Resources to develop an accurate model predicting biodegradation of petroleum contaminants in soils.

SUMMARY

WRI in conjunction with the University of Wyoming, Department of Renewable Resources and the U.S. Department of Energy, Under Task 35, conducted a laboratory-scale study of hydrocarbon biodegradation rates versus a variety of physical and chemical parameters to develop a base model. By using this model, field-scale biodegradation of petroleum hydrocarbons in heterogeneous soils can be predicted.

The base model, as developed in this study, have been tested by both field and laboratory data. Temperature, pH, and nutrients appear to be the key parameters that can be incorporated into the model to predict biodegradation rates. Results, to date, show that there is an effect of soil texture and source on the role of each parameter in the rates of hydrocarbon biodegradation. Additional parameters such as microbial species and populations may be included in the model in the future. Derived from the existing study, an alternative approach of using CO₂ accumulation data has been attempted by our collaborators at the University of Wyoming. The goal is to modify and fine-tune the model to provide more information on biodegradation.

REFERENCES

- Davis, C. T. Cort, D. Dai, T.H. Ilangasekare, and J. Munakata-Marr. 2003. Effects of heterogeneity and experimental scale on the biodegradation of diesel. *Biodegradation*. 14:373-384.
- Davis, G.B., C.D. Johnson, B.M. Patterson, C. Barber, and M. Bennett. 1998. Estimation of biodegradation rates using respiration tests during in-situ bioremediation of weathered diesel NAPL. *Ground Water Monit. Remediat.* 18:123-132.
- Downey, D.C., P.R. Guest, and J.W. Ratz. 1995. Results of a two-year in-situ bioventing demonstration. *Environ. Progress*. 14:121-125.
- Geerdink, M.J., M.C.M. van Loosdrecht, and K.C.C.A.M. Luyben. 1996. Model for microbial degradation of nonpolar organic contaminants in a soil slurry reactor. *Environ. Sci. Technol.* 30:779-786.
- Goudar, C.T., and K.A. Strevett. 1998. Comparison of relative rates of BTEX biodegradation using respirometry. *J. Ind. Microbiol. Biotechnol.* 21:11-18.
- Haack, S.K., and B.A. Bekins. 2000. Microbial populations in contaminant plumes. *Hydrogeol. J.* 8:63-76.
- Hinchee, R.E., and S.K. Ong. 1992. A rapid in-situ respiration test for measuring aerobic biodegradation rates of hydrocarbons in soil. *J. Air Waste Manag. Assoc.* 42:1305-1312.
- Jin, Q., and C.M. Bethke. 2003. A new rate law describing microbial respiration. *Appl. Environ. Microbiol.* 69:2340-2348.
- Moller, J., B. Winther, K. Lund, K. Kirkebjerg, and P. Westernman. 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.
- Nakhla, G. 2003. Biokinetic modeling of in situ bioremediation of BTX compounds—impact of process variables and scaleup implications. *Water Res.* 37:1296-1307.
- Norris, R.D., R.E. Hinchee, R. Brown, P.L. McCarty, L. Semprini, J.T. Wilson, D.H. Kampbell, M. Reinhard, E.J. Bouwer, R.C. Borden, T.M. Vogel, J.M. Thomas, and C.H. Ward. 1994. *Handbook of bioremediation*. Lewis Publishers, Boca Raton, FL.
- Parker, E.F., and W.D. Burgos. 1999. Degradation patterns of fresh and aged petroleum-contaminated soils. *Environ. Eng. Science.* 16:21-29.

- Stout, S.A. and P.D. Lundergard. 1998. Intrinsic biodegradation of diesel fuel in an interval of separate phase hydrocarbons. *Appl. Geochem.* 13:851-859.
- Wang, W.J., R.C. Dalal, P.W. Moody, and C.J. Smith. 2003. Relationships of soil respiration to microbial biomass, substrate availability and clay content. *Soil Biol. Biochem.* 35:273-284.
- Widrig, D.L. and J.F. Manning. 1995. Testing bioremediation in the field. In: *In-situ Bioremediation: When Does it Work?* pp. 160-184. National Research Council; National Academy Press, Washington, D.C.