

**MICROBIAL ENUMERATION AND LABORATORY-SCALE MICROCOSM
STUDIES IN ASSESSING ENHANCED BIOREMEDIATION POTENTIAL
OF PETROLEUM HYDROCARBONS**

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INTRODUCTION

Biotreatability studies frequently use a phased approach to assess whether enhanced bioremediation may be a suitable strategy for treating petroleum hydrocarbon (PHC) contamination in the subsurface. Enhanced bioremediation involves stimulating intrinsic (passive) bioremediation processes by which indigenous microbes convert contaminants to innocuous end products via electron acceptor and/or inorganic nutrient amendment. Common elements of biotreatability studies include literature searches, microcosm screening studies, and optimization studies. Characterization of site hydrogeology, contaminant distribution, and biogeochemistry are also critical elements in evaluating bioremediation potential, but are beyond the scope of this paper.

Literature reviews are performed first to assess biodegradability potential. Based on literature review results, microcosm respirometry/mineralization screening studies are often performed, under controlled conditions, to evaluate remedial options. Respirometry refers to the measurement of carbon dioxide evolution as an indirect measurement of mass removal based on reaction stoichiometry. Positive results are indicated by significant carbon dioxide evolution over time, relative to controls, indicating PHCs are being utilized in addition to soil organic matter. Aerobic mineralization refers to conversion of chemical reactants (PHCs) to biochemical products (*i.e.*, carbon dioxide, water, and microbial biomass), and is indicated by significantly decreased PHC concentration over time relative to controls. Controls are included to account for different PHC concentrations, subsurface conditions, physical-chemical attenuation processes, and other factors. Microcosm screening studies typically assess contaminant utilization and estimate reaction kinetics

using mass balance techniques. Screening studies may also include *in-situ* testing (Istok, J.D., unpublished observation, 1996).

Laboratory-scale microcosm studies usually require approximately one to three months, and may be performed in conjunction with tests which assess inorganic nutritional requirements and/or inhibitory conditions. Results of microcosm studies are often used to develop remedial pilot test specifications. If microcosm screening study results are positive, additional studies may be performed to optimize PHC biodegradation. These studies evaluate electron acceptor/nutrient loads and other conditions to optimize PHC removal efficiency. Costs for microcosm studies vary with complexity and duration, but are typically on the order of about \$10,000 to \$20,000 each.

Microbial enumeration is a screening-level tool which can be used to evaluate the *in-situ* response of soil bacteria to PHCs. Response of the microbial community, as indicated by significant differences in geometric mean (mean) population density in contaminated locations relative to background (*i.e.*, about one half an order of magnitude), is an indicator of intrinsic bioremediation potential. Because enhanced bioremediation involves stimulating intrinsic bioremediation, the *in-situ* response of the microbial community to PHCs is an important indicator of enhanced bioremediation potential. This paper illustrates the use of microbial enumerations for assessing contaminant biodegradability, particularly by comparison with laboratory-scale microcosm studies, and includes:

- A discussion of the microbial ecology of background and PHC-contaminated soils;

- A review of a technique for enumerating Total Recoverable Heterotrophs (TRHs) from soil; and
- The results of four biotreatability case studies in which both microbial enumeration and microcosm studies were performed to evaluate enhanced bioremediation potential.

Though microbial enumeration studies may be used for assessing microbial activity under oxic as well as anoxic conditions, the focus of this paper is on aerobic biodegradation (with the exception of case study No. 3 described herein) because bio-oxidation is a relatively energetic pathway for PHC mass removal, and most enhanced bioremediation programs involve molecular oxygen amendment. In addition, the technical approach for microbial enumeration discussed in this paper can be use for either soil or groundwater samples; however, the focus is on soils because the bulk of indigenous bacteria are attached to soil grains.

SUBSURFACE MICROBIAL ECOLOGY

The microbial enumeration method identified in this paper counts those soil microbes capable of colonizing and reproducing utilizing low-nutrient substrate under aerobic conditions. For soil samples, population densities are reported as TRHs which refer to those bacteria recovered from the soil matrix. TRHs include aerobic/facultative anaerobic heterotrophic bacteria, groups which may constitute about one half of total microbial abundance. Under optimal growing conditions, total microbial abundance in background soils can exceed about 10^6 to 10^8 colony forming units per gram (dry weight) of soil (cfu/g) for bacteria, 10^6 cfu/g for actinomycetes, and 10^5 cfu/g for fungi (Turco

et al., 1995; Hazen et al., 1991). However, due to relatively low recovery efficiencies from soils, population densities of TRHs within background soils usually range between about 10^4 and 10^7 cfu/g. The taxonomic richness of the bacterial community in soil can exceed 10^4 species per gram (dry soil) (Turco et al., 1995). Aerobic bacteria within background soils represent about 50 to 70% of the total microbial abundance, while anaerobic bacteria, actinomycetes, and micromycetes represent about 10%, 20% to 30%, and 1 to 2%, respectively (Grunda, 1985).

Factors which control population density and diversity of heterotrophic bacteria in soils include:

- **Physical-chemical factors.** These factors include organic carbon/electron acceptor availability, oxidation-reduction potential, inorganic nutrients, pH, water content, temperature, salinity, and soil texture. Bacterial population density generally decreases with depth as a function of the availability of organic carbon and molecular oxygen, parameters which typically decrease with depth. The spatial distribution of heterotrophic bacteria in soils is also influenced by the distribution of organic carbon with microtopological variations in biogeochemistry; and
- **Biological factors.** These factors include competition for resources, predation by protozoans and microarthropods, and metabolic inhibitors.

At least 30 genera containing over 100 species of microbes are capable of degrading PHCs (Arthur et al., 1991), most of which are bacteria. Gram-negative bacteria genera commonly encountered in PHC-contaminated soils include *Pseudomonas sp.*, *Comamonas sp.*, *Alcaligenes sp.*, and

Acinetobacter sp. (Kämpfer et al., 1991; Tan et al., 1990). Gram-positive genera encountered include *Arthrobacter sp.*, *Nocardia sp.*, and *Bacillus sp.* (Kämpfer et al., 1991).

In background soils, bacteria capable of utilizing contaminants may constitute less than 0.1% of the microbial community, whereas degraders can completely dominate the microbial community in contaminated soils, constituting 100% of total microbial abundance (Arthur et al., 1991). Upon introduction of an environmental stress or change in organic carbon source, microbial community composition shifts to favor those species which can tolerate stress and/or utilize new substrate. Following introduction of PHCs, some microbial species diminish due to cytotoxicity, some are unaffected, and some flourish by obtaining organic carbon and energy from that fraction of contaminant mass which they have the enzymatic capacity to utilize (Foght et al., 1987). For example, in a microbial community exposed to leaded gasoline, taxonomic diversity decreased, and populations exhibited increased metabolic diversity (Atlas et al., 1991). Populations that were dominant in these communities possessed nutritional characteristics related to the contaminant, and were also resistant to many environmental stresses other than the contaminant.

Changes in bacterial community composition, which typically follow introduction of a new organic carbon source such as PHCs, are typically accompanied by substantial increases in total bacterial population density following the release. For instance, Tan et al. (1990) demonstrated a 3,000-fold increase in the indigenous microbial population at a site following a 1,400-gallon diesel fuel spill. Bogardt et al. (1992) demonstrated that soils from sites contaminated with diesel fuel or creosote contained substantially higher population densities of indigenous, phenanthrene-degrading bacteria

than soils from background locations. Similarly, significantly greater population densities of TRHs have been shown to occur in contaminated soils relative to background soils under conditions in which indigenous microbes have been stimulated to utilize the contaminant (Salanitro, 1993; Song et al., 1990; Hickman et al., 1989; Harvey et al., 1984). While organic carbon availability may be the limiting factor to population growth in background soils, lack of molecular oxygen to support aerobic metabolism is probably the most important factor limiting the activity of aerobic/facultative anaerobic heterotrophs in PHC-contaminated soils (Arthur et al., 1991).

Based on these observed trends in subsurface microbial ecology, enumerating soil bacteria from samples collected from both contaminated and background locations provides a means to assess whether indigenous soil bacteria are metabolizing PHCs in accordance with the following:

- Mean population densities of TRHs in samples from contaminated soils, significantly greater than in samples from background locations, suggest PHCs are being utilized as substrate;
- Similar population densities of TRHs between samples from contaminated and background locations suggest that PHCs are not being utilized as substrate; and
- Smaller population densities of TRHs within PHC-contaminated samples relative to background suggest inhibitory conditions exist.

Microbial enumeration studies provide data on bacterial viability and activity, but cannot identify the

nature of inhibitory conditions, if present. In addition, while TRH enumeration data are evidence of utilization of a single compound (*e.g.*, toluene), TRH results for soils contaminated by mixtures (*e.g.*, fuel oil No. 2) should be interpreted as indicators of utilization of the mixture, but not of specific constituents. For example, increased population densities of TRHs within gasoline-contaminated soil may be due to stimulation by the aliphatic fraction of the fuel, and not the aromatic fraction.

MICROBIAL ENUMERATION METHOD

Soil samples for microbial enumeration are collected aseptically from contaminated and background locations. Samples from background locations, collected from the same depth and unit as contaminant locations, serve as controls for microbial processes occurring within contaminated locations. Aseptic sampling procedures are described by Wilson et al. (1983) and Leach et al. (1988).

Enumerations are performed for soil bacteria anticipated to be viable degraders given existing subsurface conditions. For example, soil samples from areas where molecular oxygen amendment is considered feasible for stimulating PHC degradation are enumerated for obligate aerobic/facultative anaerobic heterotrophs or TRHs, whereas soil samples from areas where amendment with an alternative electron acceptor is considered feasible are enumerated for facultative anaerobic/obligate anaerobic heterotrophs (*e.g.*, denitrifiers, sulfate reducers, methanogens). Because most enhanced bioremediation strategies focus on the aerobic pathway, subsequent discussion will concern

enumeration of obligate aerobic/facultative anaerobic TRHs via the serial dilution plate count method. TRH enumerations cost about \$50 per sample and have turn around times of about a week.

Enumeration procedures are as follows:

- The soil sample is homogenized under a fume hood to prevent contamination from exogenous bacteria;
- A one-gram aliquot of the sample is added to 9 milliliters (ml) of buffered peptone water from which a series of aqueous solutions representing ten-fold dilutions of the sample are prepared. Each sample dilution is then swirled for 30 seconds to extract adsorbed bacteria from soil grains;
- Eight 0.01-ml aliquots from each dilution are inoculated onto sterile, disposable 15-ml Petri dishes containing R2A agar. R2A agar is a low nutrient content growth medium marketed by DIFCO for performing TRH plate counts; and
- The culture plates are incubated at ambient temperature for up to five days, and appearance of colonies on the plates is interpreted as a positive test (*i.e.*, heterotrophic microbes are present). Population densities are evaluated based on the observed distribution of positive and negative tests, and the number of colonies observed on the respective culture plates.

Though only a small fraction of total biomass is recovered using this method, TRH data are

meaningful when used comparatively to evaluate microbial activity under different conditions (Zuberer, 1994).

Microbial enumeration studies have also begun to include enumerations for contaminant-degraders, which count that fraction of recoverable biomass capable of utilizing the contaminant of concern. Methods for enumerating contaminant degraders utilizing Most Probable Number (MPN) techniques are discussed by Song and Bartha (1990) and Kämpfer et al. (1991). These methods involve culturing bacteria in a liquid growth medium containing the contaminant. We believe that enumeration of potential degraders would be extremely helpful in evaluating biodegradation potential, in particular for mixtures where constituents of concern may be targeted.

CASE STUDIES

CASE STUDY NO. 1 - NEWINGTON, NEW HAMPSHIRE Releases of aviation turbine fuel (grade JP-4) and lesser amounts of aviation gasoline resulted in contamination of overburden with the aromatic PHCs benzene, toluene, ethylbenzene, and total xylenes (BTEX) as well as aliphatic volatile PHCs (VPHCs) in the carbon number range of C₄ to C₁₆. Contaminant concentrations in fine-grained site soils range from about 5 to 15 parts per million (ppm) total BTEX, and about 300 to 9,000 ppm total VPHCs.

TRH population densities in eleven samples obtained from the contaminated soils ranged from 1.2×10^3 to 6.0×10^7 cfu/g and had a geometric mean population density of 1.9×10^6 cfu/g, whereas

population densities in four samples obtained from background soils ranged from 1.9×10^5 to 1.1×10^6 cfu/g and had a mean of 6.5×10^5 cfu/g, suggesting that soil bacteria were stimulated by the PHCs.

A 41-day microcosm study was performed on aliquots of these soil samples with the following results:

- Biotransformation of PHCs to carbon dioxide was stimulated by molecular oxygen amendment in test columns (varying contaminant concentrations) relative to control columns (biocide amendment to control for biodegradation, restricted air flow to control for natural attenuation, and background site soil to control for carbon dioxide evolution owing to natural soil organic matter degradation); and
- Microbial degradation was significantly more effective than volatilization for attenuating volatile organic compounds (VOCs). In terms of VOC removal efficiency, 27% of attenuation was attributed to volatilization in contrast to 69% attributed to biodegradation. In terms of VPHC removal efficiency, 71% of attenuation was attributed to enhanced volatilization in contrast to 13% attributed to enhanced biodegradation.

The positive results of the microbial enumeration study were consistent with the results of the microcosm study, suggesting that indigenous bacteria are metabolizing PHCs and that molecular oxygen amendment stimulates that process. Presently, *in-situ* bioventing is being performed at the site.

CASE STUDY NO. 2 - NASHUA, NEW HAMPSHIRE A fuel oil No. 2 release site contains PHC-contaminated soils at concentrations of up to 10,000 ppm total petroleum hydrocarbons (TPH) based on analytical laboratory testing by gas chromatography using flame ionization detection. Unsaturated zone soils at the site consist of about 7 to 13 feet of granular fill ranging from fine sands and silts, to fine to medium sands and gravels. Two soil samples, which were collected from within contaminated soils, contained a mean TRH population density of 9.9×10^5 cfu/g, whereas two samples collected from background locations contained a mean density 3.7×10^4 cfu/g, suggesting that soil bacteria were stimulated by the PHCs.

A 82-day microcosm study was performed on aliquots of these soils with the following results:

- Degradation of PHCs to carbon dioxide was significant, indicating there were no apparent limiting environmental factors; and
- The observed reduction in contaminant concentration (approximately 26% in 82 days) suggested that bioventing could be effective for treating PHC-contaminated site soils.

The positive results of microbial enumeration were in agreement with the microcosm study, suggesting that indigenous bacteria are metabolizing PHCs and that molecular oxygen amendment stimulates that process. Continuation of the project is contingent on the results of future groundwater monitoring at the site.

CASE STUDY NO. 3 - BRATTLEBORO, VERMONT A release of aromatic PHCs, esters, and ketones resulted in contamination including as much as several feet of light, non-aqueous phase liquid. Subsurface soils consisted of lacustrine clays and silty clays inter-bedded with thin lenses of fine sand and silt of relatively low hydraulic conductivity (10^{-5} to 10^{-6} centimeters per second). Based on the relatively low permeability of the groundwater system in combination with very high biochemical oxygen demand exerted by the elevated contaminant concentrations, an enhanced denitrification biotreatability program was developed for the site. Both microbial enumeration and microcosm studies were performed to evaluate whether *in-situ* bioremediation may be a suitable treatment strategy for the site. In total, 18 soil samples were enumerated for denitrifying bacteria using MPN technique (Schaffner, 1996). A series of ten-fold dilutions of the samples were inoculated into stoppered test tubes containing nitrate salt and pH color indicator. The test tubes were incubated for seven days and a color change indicated a positive test (*i.e.*, denitrifying bacteria were present). Population densities were evaluated based on the observed distribution of positive and negative tests, and the use of published statistical MPN tables.

Thirteen soil samples collected from within the contaminant plume source area contained a mean recoverable denitrifier population density of 1.7×10^2 MPN per gram of dry soil (MPN/g). Five soil samples from background soils contained a mean population density of 1.3×10^2 MPN/g. These results were interpreted as not demonstrating that a viable population of denitrifiers existed at the site, nor that the indigenous population was stimulated by the contaminants. Moreover, the negative results of the microbial enumeration study were in agreement with the results of the microcosm study, in which no significant decrease in either nitrate concentration or chemical oxygen demand

was observed during the 120-day study attributed to nitrate salt and/or inorganic nutrient amendment. For additional information on this study, refer to Schaffner and Autery (1995).

CASE STUDY NO. 4 - CONCORD, NEW HAMPSHIRE A release of approximately 250 gallons of fuel oil No. 2 resulted in the contamination of soils with PHCs at concentrations on the order of 500 to 5,000 ppm. Subsurface soils at the site consists of about 10 to 15 feet of glacial till (very dense, fine to medium sands with some silt).

Less than three months after the spill, eight soil samples collected from fuel oil-contaminated soils contained TRH population densities ranging from 9.1×10^5 to 4.7×10^6 cfu/g with a mean of 1.7×10^6 cfu/g. Whereas, three samples collected from background locations contained 2.2×10^5 to 2.1×10^6 cfu/g with a mean of 5.1×10^5 cfu/g. Significantly, the population only had three months to acclimate to the fuel oil.

A microcosm study was performed on aliquots of these soil samples. Initial results of the respirometry study were not encouraging--background soils evolved significantly greater quantities of carbon dioxide than contaminated soils; however, on Day 20 of the study, carbon dioxide evolution from contaminated soils surpassed that from background soils. Contaminated soils continued to evolve significantly greater carbon dioxide relative to background soils to the conclusion of the 61-day study. The conclusions of the respirometry/mineralization study were as follows:

- Microbial degradation of TPH to carbon dioxide was greatly accelerated by molecular oxygen amendment in test columns (varying TPH concentrations) relative to control columns (biocide-amended soils to control for biodegradation, restricted air flow to control for natural attenuation, and background soil to control for carbon dioxide evolution owing to natural soil organic matter degradation); and
- Microbial degradation was significantly more effective than volatilization for attenuating PHCs. In terms of TPH removal efficiency, 26% of removal was attributed to enhanced volatilization in contrast to 33% attributed to enhanced biodegradation. During the 61-day study, a 9% reduction in TPH was attributed to natural attenuation.

Presently, *in-situ* bioventing is being performed at the site.

CONCLUSIONS

Microbial enumeration is a screening-level tool for evaluating enhanced bioremediation potential of PHCs. As illustrated by the case studies, results of laboratory-scale microcosm studies are consistent with the results of the microbial enumeration studies. In cases where microbial population densities are greater in contaminated soils relative to background (*e.g.*, Case Studies 1, 2, and 4), microcosm studies demonstrated that indigenous microbes could be stimulated by electron acceptor amendment to increase the rate of mass removal. Conversely, in the case where population densities in contaminated materials were similar to background materials (Case Study 3), the microcosm study

indicated that indigenous microbes could not be stimulated by electron acceptor and/or inorganic nutrient amendment to utilize the contaminants. Based on these results, we believe microbial enumerations should be a fundamental element of biotreatability studies due to their low cost (~\$50 per sample), relatively rapid turn around time (about a week), and good agreement with microcosm studies which cost about an order of magnitude more and take up to three months to complete.

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