

**APPLICATION OF CHEMICALLY ACCELERATED BIOTREATMENT TO
REDUCE RISK IN OIL-IMPACTED SOILS**

Final Report

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PUBLIC ABSTRACT

The drilling and operation of gas/petroleum exploratory wells and the operations of natural gas and petroleum production wells generate a number of waste materials that are usually stored and/or processed at the drilling/operations site. Contaminated soils result from drilling operations, production operations, and pipeline breaks or leaks where crude oil and petroleum products are released into the surrounding soil or sediments. In many cases, intrinsic biochemical remediation of these contaminated soils is either not effective or is too slow to be an acceptable approach. This project targeted petroleum-impacted soil and other wastes, such as soil contaminated by: accidental release of petroleum and natural gas-associated organic wastes from pipelines or during transport of crude oil or natural gas; production wastes (such as produced waters, and/or fuels or product gas). Our research evaluated the process designated Chemically-Accelerated Biotreatment (CAB) that can be applied to remediate contaminated matrices, either on-site or in situ. The Gas Technology Institute (GTI) had previously developed a form of CAB for the remediation of hydrocarbons and metals at Manufactured Gas Plant (MGP) sites and this research project expanded its application into Exploration and Production (E&P) sites. The CAB treatment was developed in this project using risk-based endpoints, a.k.a. environmentally acceptable endpoints (EAE) as the treatment goal. This goal was evaluated, compared, and correlated to traditional analytical methods (Gas Chromatography (GC), High Precision Liquid Chromatography (HPLC), or Gas Chromatography-Mass Spectrometry (CG-MS)). This project proved that CAB can be applied to remediate E&P contaminated soils to EAE, i.e. those concentrations of chemical contaminants in soil below which there is no adverse affect to human health or the environment. Conventional approaches to risk assessment to determine “how clean is clean” for soils undergoing remediation have been based on total contaminant concentrations in soil, as determined by laboratory extraction methods that use vigorous physical and chemical procedures. Numerous data collected from bioavailability studies in this study and others carried out by GTI and other organizations conducted on contaminated soils and sediments continue to show that not all contaminants are available to environmental receptors including man or ecologically forms. In short, there exist fractions of contaminants in soil that cannot be released from the soil matrix by normal means. These sequestered contaminant fractions should not be considered a risk to human health or the

environment. This project focused on CAB technology to treat soil contaminants to these acceptable levels. Therefore, the primary objective of this project was to determine what these contaminant levels are and to reach or exceed cleanup standards using CAB. These determinations were demonstrated and verified using toxicity and chemical mobility tests. Based on GTI's experience with a form of CAB for the remediation of soils at Manufactured Gas Plant sites, use of the technology demonstrated in this project could save the oil and gas industry an estimated \$200 million to \$500 million over the next ten years. The merging of CAB with the use of EAE for calibration and evaluation of treatment effectiveness addressed the following research objectives:

- Determination of the kinetics of contaminant desorption and bioavailability
- Further development of CAB technology for the treatment of hydrocarbon-contaminated soils
- Finalization of the methods, procedures and processes needed to apply CAB technology using EAE
- Verification of the applicability of EAE for the remediation of contaminated soils.

Factors influencing treatment effectiveness were determined and quantified in the application of CAB and methods in soil toxicity and bioavailability determination were identified and evaluated. Some of these factors included differences in soil composition, contaminant composition, soil aging and contaminant concentration. Data generated from this project can support site-specific risk assessments by providing evidence of toxicity and bioavailability endpoints among soils contaminated with complex mixtures of contaminants resulting from the production of oil and gas. CAB treatment in our laboratory with EAE proved completely successful at the laboratory-scale and the technology is availability for pilot-scale evaluation.

DISCLAIMER

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STATEMENT OF THE PROBLEM

Natural Gas and Petroleum Industrial Profile

The exploration, production (E&P), and distribution sectors of the energy industry (natural gas and petroleum) are initiated with the wellhead for the exploration and/or production of natural gas/petroleum through processing of the gas/petroleum prior to transmission and distribution. The wastes sites are broken down into waste or reserve pits at the wellhead, dehydrator sites, and gas processing plants (responsible for separating gas liquids from the gas stream and/or remove of nitrogen/sulfur from the gas).¹ A potential site of soil contamination for the petroleum industry follows a similar pattern.

Gas and oil E&P activities are conducted in 33 States under a wide variety of environmental and operational conditions. The pipeline transmission and distribution of natural gas and petroleum involves all 50 states. On the exploration and production sites, there are an excess of 800,000 wells producing gas and oil with 25,985 wells completed or operating annually (Energy Information Administration, U.S. Department of Energy, 2001). Add this number to the 500,000 abandoned wells and a major potential environmental liability is indicated. This research targeted wastes, such as contaminated soils that can be remediated on-site or in situ, as well as soil contaminated by accidental release of petroleum and natural gas-associated organic wastes from pipelines or during transport. Based on GTI's experience with a form of Chemically-Accelerated Biotreatment (CAB) for the remediation of Manufactured Gas Plants (MGP-REM), use of these technologies at E&P sites could save the industry an estimated \$200 Million to \$500 Million over the next ten years (personal estimate, GTI).

The drilling and operation of gas/petroleum exploratory wells and the operation of natural gas and petroleum production wells generate a number of waste materials, which are usually stored and/or processed at the drilling/operations site. The waste materials can include:

- oil-based drilling muds and cuttings;
- water-based drilling muds and cuttings;
- site related soil or sediments contaminated by the drilling and gas recovery activities;
- brines, including drilling and produced water;

- production pit and storage tank sludge;
- produced oily sands and solids;
- residual gas condensates;
- vapors and odors; and
- processing wastes which are or may be commingled with produced formation water.

The most common contaminants associated with energy products E&P wells are:

- hydrocarbons associated with the natural gas condensates, such as **b**enzene, **t**oluene, **e**thyl, and the **x**ylenes which are commonly designated as BTEX;
- **p**olynuclear **a**romatic **h**ydrocarbons (PAH) from the formation's natural gas deposits (liquid or solids under the conditions of different pressures and temperatures in many reservoirs);
- PAH associated with the drilling materials;
- fuel oil and diesel fuel that are often components of the drilling materials;
- heavy metals, such as arsenic, barium, cadmium, chromium, lead, mercury, selenium, silver, and zinc;
- biocides and scale/corrosion inhibitors; and
- organic and inorganic sulfur compounds (mercaptans and hydrogen sulfide, respectively).

The remediation of the contaminated soil and sediments from waste drilling and production pits, soil/sediment contaminated by the operation of the exploratory drilling or production activities, and soil accidentally contaminated by pipeline transmission and distribution hydrocarbons release has been the focus of this project.

This project focused primarily on the organic wastes associated with wellheads used for the exploration and production of natural gas/petroleum. The technologies generated may be applied to the other wastes generated in the processing of hydrocarbons used for energy.

¹ Lawrence, A.W. 1996. "In situ and On Site Bioremediation of Sludges, Soils, and Groundwater at Natural Gas Industry Exploration and Production Facilities," *In Gas, Oil, and Environmental Biotechnology VII*. Ed. By V. Srivastava, R. Markuszewski, J. Smith, and T.D. Hayes. Institute of Gas Technology. Des Plaines, IL. pp. 435-459

Remediation Drivers

Estimates of the number of waste sites generated in the exploration and production of natural gas/petroleum in the continental United States exceed 800,000 potentially contaminated sites that may require some remediation. Exploration, production or reserve wastes may require remediation driven by a number of factors such as:

- condition, or absence of intact pit lining;
- initial closure conditions;
- property value and reuse potential;
- environmental conditions, such as climate, soil type, and threat to ground or surface water; and
- upcoming changes in environmental regulations.

Hydrocarbon contaminants, as well as other organic toxicants associated with the gas/petroleum industry are moved throughout the U.S.A. in pipelines, rail cars, tank trucks, etc. and spills of these materials occur (Lundanes and Greibrokk 1994). A portion of the spilled contaminants can be removed or recovered, but often a residual level of contamination remains in the soil or sediment at the spill site. Chemically-Accelerated Biotreatment that targets Environmentally Acceptable Endpoints (EAE)² for determining site-specific contaminant risk can be applied to these contaminated spill sites.

Regulatory Issues

Currently E&P pit wastes are not classified as hazardous wastes under the Federal Resources Conservation and Recovery Act (RCRA) due to a regulatory exemption from Subtitle C of the Act. The drivers for remedial action are based on state regulations dealing with surface and ground water protection, industrial site cleanup, and natural resources and wildlife protection requirements. At least seventeen states (Alabama, California, Colorado, Kansas, Louisiana, Michigan, Mississippi, Montana, New Mexico, North Dakota, Ohio, Oklahoma, Pennsylvania,

² Environmentally Acceptable Endpoints (EAE): The threshold concentration of chemical substances in soil or other medium, below which there is no adverse affect to human health or the environment.

Texas, Utah, West Virginia, and Wyoming) have regulatory issues involving E&P wastes³. In addition, gas and oil leaseholder obligations on private properties may force remediation.

The Land Disposal Restrictions (LDR) to be imposed by US EPA by April 15, 1998, may restrict landfilling of E&P wastes. In general, the status of the current regulatory environment may be viewed as unacceptable and the E&P wastes may lose their Federal exemption. Additional factors favoring remediation of these sites include property transfer and real estate development or legal actions promulgated by property owners adjacent to the E&P sites. Similar restrictions are or will be applied to soil contaminated by hydrocarbon release from pipelines. These present and potential factors would indicate a need for low-cost and easily implemented remediation technologies. The follow-up project, which will support field experimentation and commercialization/marketing potential development for E&P wastes, focuses on the Chemically-Accelerated Biotreatment and Environmental Acceptable Endpoints technologies to meet these needs.

³ Oil and Gas Exploration and Production Waste Management: A 17-State Study. 1993. Prepared for U.S. Department of Energy and the Interstate Oil and Gas Compact Commission by ICF Resources Incorporated, Washington, DC. pp. II-3.

OBJECTIVES

Permanent, efficient and cost effective solutions for organically (total petroleum hydrocarbons – TPH and polynuclear aromatic hydrocarbons - PAH) contaminated soils typically found at exploration and production (E&P) and other gas industry sites were developed to the point of field demonstration. Two innovative technologies were developed together. The merging of Chemically-Accelerated Biotreatment (CAB) and with the use of Environmental Aceptable Endpoints (EAE) for calibration and evaluation of treatment effectiveness is unique. The CAB process should be effective against other of the organics discussed above, but none were detected in the test samples used in this study.

Specific research objectives were to:

- determine the kinetics of contaminant desorption and availability;
- develop CAB technology for the treatment of hydrocarbon-contaminated soil;
- finalize the methods, procedures, and processes needed to apply the CAB technology using EAE; and
- verify the applicability of EAE in the remediation of contaminated soils.

This project developed techniques used to determine the concentrations of selected residual contaminants in soil, which if left in place, will not adversely affect human health or the environment. In addition, this project resulted in novel soil remediation techniques, site assessment tools and data for supporting this risk assessment approach. Contaminants studied were those of particular interest to the U.S. Department of Energy and the oil and gas industry, such as crude oils, BTEX (although none was detected in the test samples), and exploration and production wastes. Four main technical objectives and deliverables were established as follows:

- Determine the fraction of soil contaminants available for uptake to receptors;
- further develop GTI's CAB Treatment for use in oil-impacted soils;
- demonstrate the effectiveness of GTI's CAB Treatment for soil remediation in removing bioavailable contaminants; and
- develop a rapid, cost-effective method to determine the bioavailable fraction of soil contaminants for establishing EAE.

STATEMENT OF SOLUTION

Over the past ten years, the Institute of Gas Technology, now Gas Technology Institute (GTI) and others have conducted research on the bioremediation of organic contaminants in soil (Institute of Gas Technology, 1997, 1998). Most of this work has been associated with remediation of former **M**anufactured **G**as **P**lant (MGP) sites, but soils from industrial areas and oil production areas have also been studied. The results have shown that: (1) organic contaminants are biodegraded by indigenous soil microorganisms to a concentration that no longer decreases, or that decreases very slowly, with continued treatment; (2) reductions below this concentration are limited by the availability of the contaminants to the microorganisms; and (3) the residual contaminants that remain after biological treatment, regardless of the extent of treatment, are significantly less leachable and significantly less available to other organisms, as measured by simple indicator toxicity tests with bacteria and invertebrates.

Background – Environmental Acceptable Endpoints

Within the past decade, ecological risk assessment methodologies have been developed and used to provide useful descriptions of risk at contaminated sites. More recently, risk-based approaches to contaminated site cleanup, such as ASTM's "**R**isk-**B**ased **C**orrective **A**ction" (RBCA) standards, have been used to determine risk and remediation strategies that are more cost effective than those derived from conventional assessments. While approaches such as RBCA may be a way of distinguishing high-risk sites from low-risk sites, these assessments do not account for the reduced bioavailability of aged or weathered contaminants from soil. Thus, these approaches, while being less conservative than traditional risk models, may sometimes be overly conservative in emphasizing levels of contaminants that have no or very low potential for migrating to ecological receptors.

Risk Assessment

Environmental regulations concerning contaminated-site cleanup have traditionally been based on mere detection or presence of contaminant(s) in soil, air or water. There is a growing body of scientific data showing that the presence of a contaminant in a medium does not necessarily constitute a risk (Linz and Nakles, 1997). Many studies have shown that the total contaminant concentration is frequently not available to receptors. If a contaminant or

contaminant mixture is not available for uptake, then no effect can be shown, and thus, no risk of exposure is likely. Therefore, models used to estimate risk to human or non-human receptors must account for the bioavailable fraction of the contaminant, and not the entire contaminant concentration that may be determined via vigorous chemical and/or physical extraction methods in the laboratory. At this time, no model has been developed to accurately predict the risk associated with individual contaminants or contaminant mixtures on a site-specific basis; therefore, a need exists for developing an assay or group of assays that can indicate exposure risks and effects to biota. Such assays would provide scientifically defensible evidence of the bioavailable fraction of contaminant able to reach environmental receptors, while also establishing a database for calibrating more accurate risk modeling in the future.

Bioavailability

Numerous studies conducted on soils contaminated with organic compounds (i.e., hydrocarbons, oils, pesticides) have shown that as the contaminants persist in soil, they become increasingly resistant to desorption (Kelsey and Alexander 1997, Linz and Nakles 1997). Sorption of organic chemicals to soils often entails an initially rapid and reversible process, followed by a period of slow sorption over weeks, months, or years. This slow sorption leads to a chemical fraction that then resists desorption (Karickhoff 1980, Pignatello 1989, Ball and Roberts 1991). Several mechanisms have been described for the aging of chemicals in soils, including partitioning into humic matter (Chiou *et al.* 1983, Chiou 1989) or diffusion into the soil particles themselves (Steinberg *et al.* 1987). These hypotheses for soil “aging” suggest that chemicals become highly concentrated or crystallized within the organic matrix over time. Diffusion of chemicals may be retarded as these chemicals become more strongly associated with this matrix (Wu and Gschwend 1986, Steinberg *et al.* 1987, Brusseau *et al.* 1991). It is also possible that the formation of strong bonds between organic contaminants and soil constituents may account for resistance to desorption. The result of the slow desorption process is a substantial reduction in the rate of desorption, which correlates with a net reduction in the bioavailability of the contaminant (Kelsey and Alexander 1997). The surrounding environment through sorption and biodegradation processes that operate naturally, often resulting in minimal risk to the surroundings even though analytically detectable quantities of contaminants remain in the soil can often assimilate the fraction that is released slowly.

Current environmental policy assumes that “background” or “pristine” conditions must be achieved to protect human health and the environment for all contaminated sites, for all future uses. The scientific evidence for contaminant aging and lack of bioavailability as a result of this process refutes this assumption. To summarize, there are concentrations of contaminants, and in particular petroleum hydrocarbons, greater than zero that are safe to human health and the environment. However, these “safe” concentrations must be evaluated via risk models and assessments that utilize site-specific analytical and toxicity data. The work proposed in this project sought to evaluate these contaminant concentrations by demonstrating toxicity data derived from a number of assays. The toxicity assays chosen utilized both established protocols currently accepted by the regulatory community, as well as less time-consuming and less expensive novel approaches.

Data from a typical treatability study⁴ performed at GTI on MGP contaminated soil shows a “hockey stick” profile (Figure 1). Reduction in concentration of both carcinogenic and non-carcinogenic PAH generally follows a two-phase rate curve during remediation, with the first phase showing a significant reduction within a few days. However, this rate becomes slower and levels out over time to reach low levels of residual contamination. Many treatability studies conducted at GTI on soils contaminated with mixtures of organic compounds have demonstrated these fast and slow release components. Test protocols used to characterize the relative size of each component and to quantify the kinetics of release are presently being developed and funded by the Gas Research Institute (GRI). One protocol uses batch tests with XAD or C-18 resins. Contaminated soil/water mixtures are combined with resin and shaken over time to release “fast” contaminant components, after which the contaminants bound to the resin are extracted and analyzed (Lane and Loehr 1992). The resin maintains a maximum driving force between the soil-bound contaminants and the water, and it also can enhance contaminant transfer from the soil through direct contact with the contaminated particles. Another protocol uses relatively mild organic solvents (e.g., *n*-butanol, methanol, ethanol) to selectively extract bioavailable PAH from soil (Kelsey *et al.* 1997). This extraction method has shown close correlation with earthworm uptake of PAH. An extraction method that mimics bioavailability is preferred for assessing exposure and risk over one whose sole virtue is the removal of the largest

⁴ Treatability study: A laboratory column or slurry study to determine the potential extent of contaminant degradation in a soil.

percentage of the contaminant from soil. Development of protocols for these partitioning studies has further characterized the slow- and fast-release components from contaminated soils. Determination of these components may be a key factor in understanding bioavailability from a toxicological perspective.

Assuming that the slow-release contaminant fraction from soil particles is not bioavailable, a direct measure of the risk that the fast-release fraction creates, and when this risk occurs, remains to be studied. Fast release of a contaminant from soil may occur as the result of soil disruption via leaching (e.g., flooding events) or when engineered controls are used to eliminate direct contact and other exposure pathways through the use of barriers or other remedial activities at contaminated sites. Leaching protocols have been established for estimating the slow and fast contaminant release fractions from soils (Federal Register 1996 ASTM 1987).

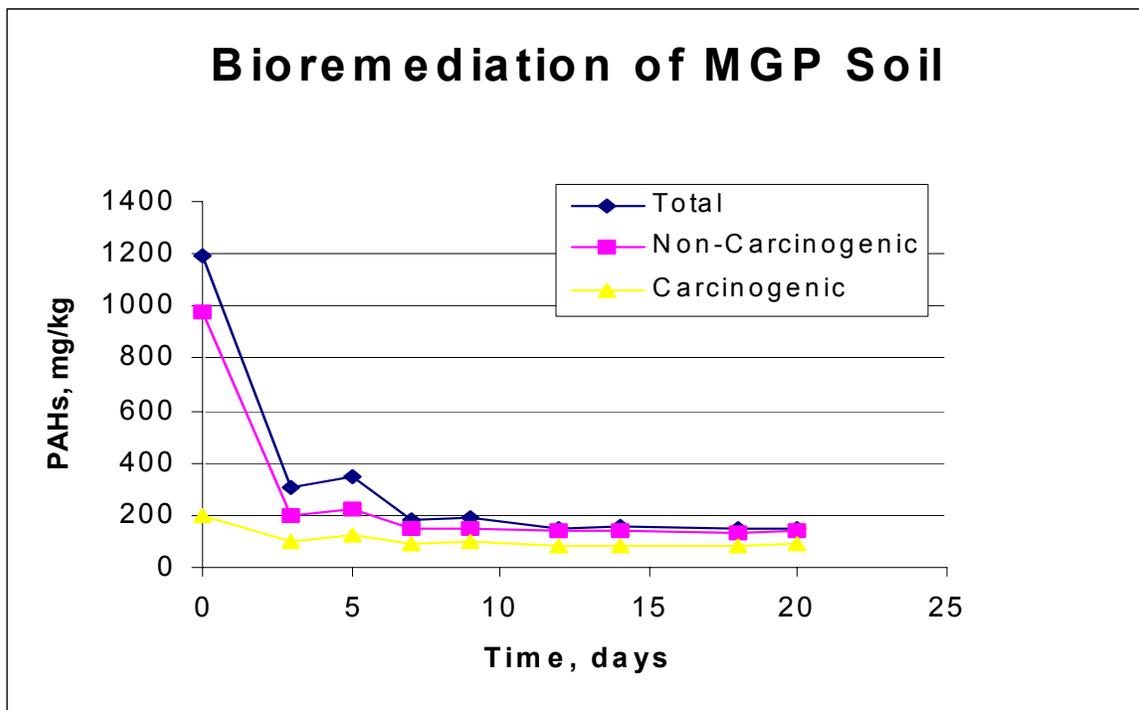


FIGURE 1. TYPICAL “HOCKEY STICK” BIODEGRADATION CURVE IN SOIL TAKEN FROM AN MGP SITE (LIU *ET AL.*, 1996)

Slow reduction in bioavailability as soils age has been documented using plants, microorganisms, and invertebrates such as insects and earthworms (Edwards *et al.* 1957,

Edwards and Neuhauser 1988, Hurle 1977, Scribner *et al.* 1992, Kelsey and Alexander 1997). Protocols have been established to estimate direct bioavailability of soil contaminants (Linz and Nakles 1997). These assays expose test organisms to contaminated soil for various periods of time, after which, contaminants are extracted from tissues, and the total uptake of each contaminant is determined. Data from indicator species, such as earthworms, are generally assumed to be representative of ecological risk. Earthworms directly contact and ingest soil and thus are expected to show the greatest effects of contaminants in soil.

A novel approach to characterize contaminant distributions in soil using passive sampling devices (PSDs) was introduced recently by Johnson *et al.* (1997). These devices use artificial semipermeable membranes to mimic biological membranes, and they present a substantial time and cost savings over conventional bioassays. Artificial membranes have been used successfully in aquatic systems to estimate contaminant bioavailability, and are being marketed by Environmental Sampling Technologies, St. Joseph, Missouri. More recently, artificial membrane devices (PSDs) have been used to determine contaminant distribution in soils and compost (Zabik *et al.* 1992, Johnson *et al.* 1995, Strandberg *et al.* 1997). PSDs are constructed of non-porous polyethylene membranes filled with a C-18 or XAD resin to trap contaminants. The devices are buried in soil for a period of days to weeks. Any contaminant available to pass through the membranes sorbed onto the resin, which was later be eluted and analyzed for the contaminant concentration and/or toxicity. For years, classical non-porous membrane permeation models have described passive diffusion through biological membranes. As such, these membranes have the potential to be used as models of biological membranes to estimate uptake of the bioavailable fraction of a contaminant mixture found in soil, sediment, air, or water.

Both earthworms and PSDs tend to reach equilibrium concentrations after approximately three weeks (Johnson, unpublished data); however, the author is unaware of studies having been conducted that directly compare earthworm uptake with transport of contaminants through these artificial devices. A positive correlation between these two soil contaminant estimation methods would further the use of artificial membrane devices. In addition, easy, inexpensive bacterial toxicity assays such as acute Microtox[®] tests, conducted on eluates from PSD resins after soil exposure could further indicate effects of bioavailable contaminants. Microtox[®] tests that evaluate acute toxicity by measuring a reduction in bioluminescence in a marine bacterium, have

been used successfully as screening tests to determine the presence of contaminants available to elicit a toxic response (Tung *et al.* 1991, Chapman *et al.* 1992, Ross 1993, Ringwood *et al.* 1997). GTI's principal toxicologist has extensive experience in conducting bioassays with invertebrates and microbes for both acute toxicity studies and bioaccumulation assays (Harkey 1993, Harkey *et al.* 1995, Harkey *et al.* 1994 a,b,c, 1995, 1997a, Landrum *et al.* 1995, Kane-Driscoll *et al.* 1997).

At present, GTI is compiling toxicological data from MGP site treatability studies. Screens using bacteria with the Microtox[®] Solid-Phase tests show either an increase or a decrease in toxicity after beginning bioremediation of MGP site soil in laboratory studies (Table 1). These results suggest that mechanisms other than the reduction in total PAH concentration need to be considered to determine bioavailability and contaminant effects on biological systems (Harkey *et al.* 1997b). The results also suggest that a battery of tests (i.e., earthworm, PSDs, chemical extractions) should be used to determine toxicological changes for contaminated soil during remedial activities, because soil composition, nutrient residues, and metabolic by-products may alter toxicity in Microtox[®] assays (Ringwood *et al.* 1997). The battery of tests used in this work included 1) bioavailability assays; 2) Microtox[®] Acute Toxicity assays; and 3) Mutatox[®] Chronic Toxicity assays.

TABLE 1. MICROTOX SOLID-PHASE EC₅₀ AND TOTAL PAH CONCENTRATION CHANGES WITH REMEDIATION DURING TREATABILITY STUDIES AT GTI

Type of Treatment	Contaminant Concentration (mg/kg total PAH)	Period of Treatment	Mean EC₅₀ (percent dry weight)
Phytoremediation	190	0 days	2.86
	83	60 days	6.38
Slurry-phase Bioremediation	1,700	0 days	0.90
	1,650	40 days	2.02
	1,500	51 days	1.02
	1,400	61 days	5.07
Landfarming Bioremediation	21,700	0 days	0.01
	4,000	3 months	0.03

Background – Chemical-Accelerated Biotreatment

Bioremediation is the basis for many of the treatment systems under development at GTI. Microorganisms exhibit the abilities under laboratory conditions to degrade nearly all contaminants examined, but the rates, extent, and dependability of these activities may not meet the requirements of an effective treatment technology. The enhancements under study in GTI’s laboratories are designed to accelerate the rate and/or extent of degradation. The enhancements, such as CAB and its specific modifications including MGP-REM, PCB-REM, and CYAN-REM, for the specific remediation of PAH, polychlorinated biphenyls (PCBs), and cyanides respectively, can add a degree of dependability and predictability necessary for a successful treatment technology with application to full-scale remedial actions.

GTI has developed and is investigating the application of a patented integrated chemical biological chemical treatment (CBT) process for the remediation of soils contaminated with

PAH. This project presents an extension of this work where biotreatment is accelerated by chemical oxidation, but with critical new features. The novelty of the technology proposed within is based on the types of oxidants, sequence of treatments, and the use of a wider variety of microorganisms to execute the biotreatment. The designation of the proposed treatment as **C**hemically-**A**ccelerated **B**iotreatment (CAB) is necessary to differentiate the project technology from the patent CBT. As CAB is based on the chemical-biological treatment, the background information is interchangeable.

The enhanced biotreatment process has been found to be effective in increasing the rate and degree of biological degradation of PAH in soils. This work, as part of GRI's and GTI's Sustaining Membership Program (SMP) is being proven effective, especially in the remediation of sites contaminated by the historical production of town gas by the thermal gasification of coal or petroleum (Srivastava *et al.* 1994). These former Manufactured Gas Plant (MGP) sites are being actively remediated using a form of CBT designated MGP-REM (Liu *et al.* 1993, 1994, Paterek 1993, Paterek *et al.* 1994). The ultimate goal of this development is to provide a cost-effective waste treatment technology that furnishes an efficient alternative to landfilling, thermal treatment (incineration), and others.

Bench-scale and field-scale experiments have indicated that CBT is superior to conventional biotreatment in the common treatment modes - slurry bioreactor, solid-phase treatment, and in situ mode. Soil investigated for CBT was tested in laboratory bioreactors. CBT's removal rate was 3 times greater than conventional biotreatment. CBT-treated soil favors the activity of a diverse microbial population that can degrade PAH to necessary levels. Field-scale evaluation of CBT in soil slurry bioreactors showed a 4-fold improvement over conventional bioremediation, with 95% of total PAH and 90% of carcinogenic PAH removed.

GTI has identified the rate-limiting steps of conventional biological treatment systems, and then developed approaches to overcome these limitations. As a result of extensive bench-scale studies carried out since 1987, GTI has developed and demonstrated a process for PAH-contaminated soils that is a combination of biological and physical/chemical treatment -- the CBT process, in general, and the CBT process specifically for MGP sites known as MGP-REM.

The CAB Process combines two powerful and complimentary remedial techniques: 1) chemical oxidative treatment using Fenton's Reagent (hydrogen peroxide and iron salts); and 2)

biological treatment, primarily using native aerobic microorganisms (Paterek *et al.*, 1994, Srivastava *et al.* 1994; Beltran *et al.* 1998; Bigda 1996). This integrated process generates environmentally benign products including carbon dioxide (CO₂), inorganic salts, biomass, and water. A schematic illustration of the CAB/CBT Process is presented in the Figure 2.

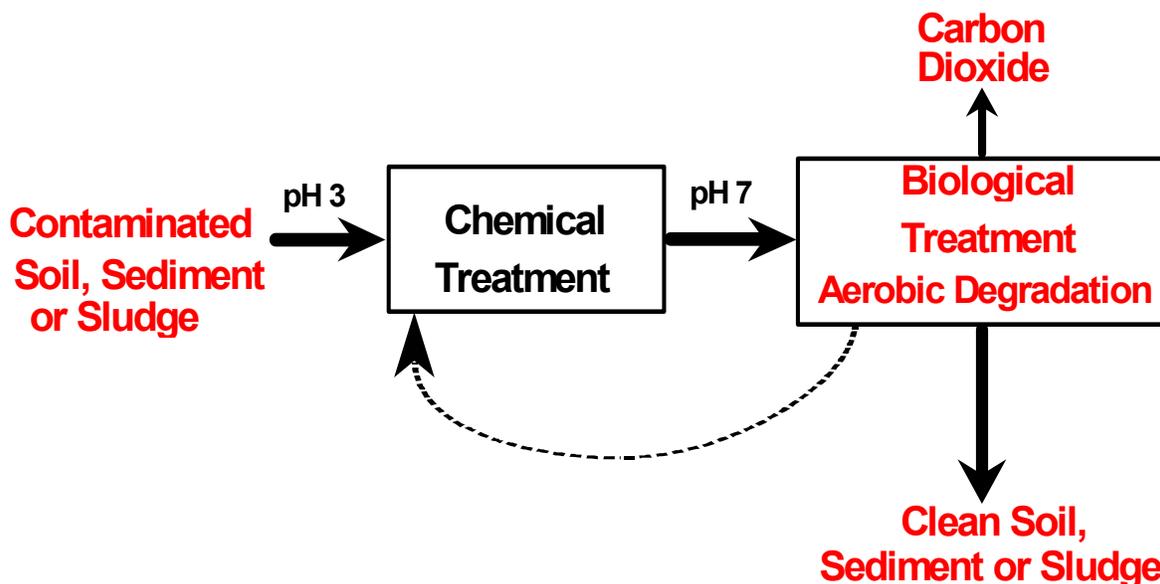


FIGURE 2. SCHEMATIC DIAGRAM OF THE CAB PROCESS

The chemical treatment can be performed as a pre-treatment before biological degradation or can be integrated as a step between biological treatments. The process uses a mild chemical treatment with Fenton's Reagent (H₂O₂ plus Fe²⁺) that produces hydroxyl radicals that start a chain reaction with the organic contaminants. The contaminants are modified or degraded to forms that are more readily degraded by native or supplemented microorganisms. Results with approximately 25 MGP soils show that the CBT Process is capable of enhancing the rate as well as the extent of PAH degradation.

Preliminary application of the standard CBT process to E&P wastes (hydrocarbon contaminated soils, sludge, and sediment) has indicated direct applicability of the basic process to these matrices. Figure 3 shows the removal of more than 94% hydrocarbons from a soil contaminated by E&P activities. These preliminary data, combined with the enhancement of the patented chemical-biological treatment, with treatment goals based on EAE and risk evaluation, indicates that the proposed research described below is effective.

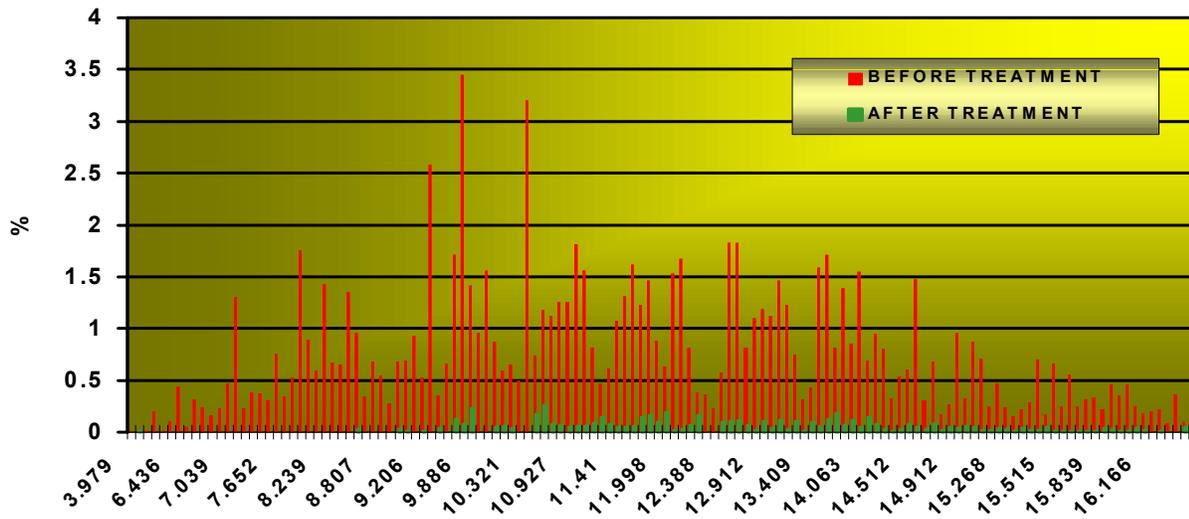


FIGURE 3: CHEMICALLY-ACCELERATED BIOTREATMENT RESULTS WITH AN E&P CONTAMINATED SOIL

TECHNICAL RESULTS

Application of CAB to E&P sites with treatment endpoints based on risk, bioavailability and EAEs is the only task of this research project. This CAB form of bioremediation utilizes engineered systems to supply microbe-stimulating materials and pretreatment chemicals to:

- encourage the growth and activity of targeted microorganisms,
- minimize mass-transfer problems, and
- optimize environmental conditions for degradation and detoxification reactions.

These systems must be effective to decrease the time required to destroy or detoxify the contaminants, thus decreasing the overall liability of the gas/petroleum industry partner that is responsible for the site, as well as his cleanup costs. The technology must also be efficient enough to meet or surpass the regulatory requirements. This technology will also be more reliable than the existing technologies of landfilling, landfarming, and composting. Cost reduction will be realized by shortening the treatment time, due to the option of applying the in situ mode of the CAB process. The use of low-cost and industrially available chemicals will eliminate or minimize the excavation, transport, and land use costs.

The following six major research activities set the parameters and generated the engineering and operating conditions needed to proceed to full-scale remediation.

1. Evaluate Physical, Chemical, and Biological Site Conditions
2. Determination of Baseline Bioavailability
3. Optimization of Biological Treatment Factors
4. Optimization of Chemical Treatment Factors
5. Integration of Treatment Under Simulated Treatment Conditions
6. Evaluate CAB technology using EAE and Risk Reduction Criteria

Soil Collection and Characterization

Soils that represent a variety of soil types and contaminants of interest to the DOE and the gas/petroleum industry were collected and evaluated by the funding is appropriated in this project. Contaminated sites for soil collection were selected based on availability and suitability for the project. A variety of organic chemicals associated with oil and gas production were

represented in samples collected from these areas, including BTEX, PAH, crude oil constituents, and hydrocarbons. Areas of collection were determined according to published contaminant documentation from remedial investigation reports and toxic release inventories.

Sampling

Sample collection was collected following established procedures according to Csuros (1994). All sample containers were be labeled properly and placed in a cooler at 4°C for shipment to the GTI laboratory. In the laboratory, soils were stored in the dark in sealed containers at 4°C throughout the project. Chain-of-custody procedures were in effect from the time of sample collection through completion of analyses at the laboratory. All sampling events were documented, and GTI's chain-of-custody forms were used. Safety precautions were affected during all sampling and sample-handling events, according to protocols established in GTI's Health and Safety Procedures. Hazardous materials collection gear, to include hardhat, goggles, protective suit, gloves and boots were worn at the time of field sampling, as appropriate. Laboratory personnel were required to wear, at a minimum, lab coat, goggles, and gloves when preparing samples for analysis and assays. All samples, which may have contained volatile contaminants, were prepared under a laboratory fume hood.

Evaluate Physical, Chemical, and Biological Site Conditions

This research phase determined the parameters that impact the application of CAB with EAE to E&P site remediation. The physical, chemical, and biological (ecological) site conditions were identified and evaluated under laboratory-scale field simulating conditions. The product of this effort is the expertise to determine, utilize and list the critical parameters, methods to analyze or evaluate the parameters, and effective ranges for operation of CAB/EAE. Additional information on methods to 'fix' parameters that may fall outside the acceptable range was not found to be necessary in any samples that were tested in our laboratory. Due to this broad applicability of the CAB, no additional effort was required. This technology is ready for application at pilot- or full-scale in any treatment modes, (e.g., bioslurry reactor, landfarming, or in situ).

The objective of this research effort was to determine the baseline data that is necessary to apply CAB treatment to reduce the contaminants to an acceptable treatment level. The

detailed chemical, physical, and biological compatibility properties of a contaminated soil were analyzed. Composite samples were prepared for analyses that included the determination of total organic carbon; concentration of total petroleum hydrocarbons; concentration of volatile hydrocarbons including benzene, toluene, ethylbenzene, and xylene (BTEX), diesel fuel, gasoline, and semivolatile hydrocarbons; available nutrients (N, P, K); priority heavy metals; and matrix texture. Analyses were also conducted to determine paraffinic, naphthenic, aromatic and sulfur containing hydrocarbons. Potential factors are listed below.

- Physical - Soil structure/texture, porosity, hydraulic conductivity, bulk density, and particle size distribution
- Chemical - Contaminant concentration and distribution (as a function of particle size), organic matter/organic carbon content, and metals (Mg, Cu, Ni)
- Biological - Microbial count (aerobic and anaerobic), soil moisture, soil pH, and nutrient content (C:N:P)

Physical and Chemical Analyses

All soil samples used in this project were analyzed for contaminant type and concentration, texture, pH, nutrient availability, competing carbon sources, oxygen depleters, metals, porosity, permeability, bulk density, organic matter and carbon content, cation exchange capacity, clay content, dissolved oxygen, redox potential, alkalinity, water field capacity, moisture, particle size distribution, and particle surface area.

Contaminant analysis methods used depended on the type of contaminants present at the sampling site. Soil characterization and analyses were completed according to methods required by regulatory agencies for remedial investigations of hazardous waste sites. Critical variables analyzed used the following methods:

- 1) Sample Preparation, Extraction: EPA Method 3540, Soxhlet Extraction
- 2) Total Petroleum Hydrocarbons (TPH): EPA Method 418.1
- 3) PAH: for Polynuclear Aromatics – Method SW 8310 or 8270, EPA Method 8100
- 4) BTEX: Method SW 5030/8260
- 5) Soil pH: EPA Method 9045
- 6) Total Organic Carbon: Method SW 9060

Particle size distribution and fractionation of soil particles was determined by a modified sedimentation technique described by Harkey *et al.* (1994c).

TABLE 1A. SUMMARY OF DATA TO BE ANALYSED

Contaminant Concentration Analyses (mg/kg)	Soil Characterization	Toxicity Assays (LC₅₀ or ppm)
Standard extraction from soil (Soxlet, before and after treatment)	pH	Microtox Solid Phase tests (before and after CAB treatment)
Extraction of bioavailable contaminant fraction from soil (organic solvent, before and after treatment)	Total organic carbon (%)	Microtox acute tests on PSD resin extracts (before and after CAB treatment)
Contaminant concentration in PSD resin extracts (before and after treatment)	Particle size distribution (% fractions)	
Contaminant concentration in earthworm tissues (before and after treatment)	Soil particle surface area	

It is important to note that the standard contaminant extraction procedures use vigorous physical or chemical methods to determine the total contaminant concentration in the soil. These methods may overestimate the bioavailable fraction of the contaminant, but they will provide concentrations that are normally reported for regulatory purposes. Measures of total soil contaminant concentrations were compared to concentrations in extracts obtained in subsequent research activities. All contaminant concentration analyses were conducted at GTI laboratories.

Based upon the results obtained from this evaluation, soils were selected for bioavailability analyses described below and as indicated. The chemical, physical, and biological properties of potential E&P sites were determined in order to test the CAB process of remediation. Due to these properties, additional chemical tests that may be critical to the effective performance of the microbial community were determined. The tests include, but were not limited to: ammoniac nitrogen, nitrate/nitrite, total nitrogen, phosphorous, potassium, sulfate, and any other existing nutrients that may have limited the microbial population, and potential inhibitors not previously covered in analysis.

Microbiological Analyses

The site microbiological parameters are critical for the application of CAB. The parameters investigated include: total bacterial populations, hydrocarbon-degrading bacterial populations, and fungal populations and activities, in addition to the toxicity of the materials.

The total microbial count was compared to an estimate of the population present that would degrade the contaminants of concern. A preliminary screening for this population of specific contaminant-degrading microorganisms was performed in a growth medium that furnishes inorganic nutrients, nitrogen, phosphate, and possibly vitamins in excess, with no intrinsic carbon sources. The contaminant supplement generated by the extraction of the E&P waste matrix was the sole source of carbon for both metabolic and growth requirements of the contaminant-degrading microorganisms obtained from the wastes unless noted otherwise.

The microbial community that indicated the required degradation activities were isolated and evaluated for the effects of environmental parameters that affect the rate and degree of biodegradation. These factors include those that can be controlled under the various treatment processes, and those that are imposed by the conditions of the contaminated systems. Information is critical for both types of environmental determinants in order to generate simple models required for performance prediction and systems control and monitoring.

The moisture content and parameters of the aqueous phase were evaluated in the test samples. These data was correlated with bacterial consortia levels, microbial activity, chemical treatment efficiency and other factors that controls or influences the CAB process. User-friendly protocols for determining critical factors and controlling them were formulated.

Determination of Baseline Bioavailability

Baseline data on the availability of the toxicants was determined. These data are critical to:

- compare the value of EAE endpoints, as compared to strict analytical targets,
- set the starting point to evaluate the effectiveness of the CAB remediation
- develop ecological and toxicological screening technology to assess contaminated sites and define meaningful treatment goals.

Physicochemical Characterization of Model Soils

Total organic carbon was determined in two ways using the six experimental soils chosen. The first of these, conducted by STAT Environmental, used the low-temperature (*ca.* 400 °C) combustion method. We also used ashing at 900°C in a biological oxidizer. Results were as follows in the table.

TABLE 2. TOTAL ORGANIC CARBON CONTENT OF EXPERIMENTAL SOILS

	1	2	3	4	5	6
Low-temp combustion	2.32	5.78	11.16	24.28	3.58	9.13
High-temp combustion	15.8	11.4	23.8	48.6	13.2	22.6

Total micropore content, total surface area, and pore size distribution were conducted by the Pennsylvania State University Center for Innovative Sintered Products facility. Results for surface area and total pore volume showed a wide range of both of these parameters among the soils tested. Results are as follows.

TABLE 3. PORE SIZE AND DISTRIBUTION OF THE EXPERIMENTAL SOILS

	Soil #					
	1	2	3	4	5	6
"Total" pore volume	0.0508	0.0351	0.0162	0.0114	0.0371	0.0232
"BJH total" pore volume	0.04473	0.02823	0.01754	0.01221	0.03354	0.02125
Total Surface Area	22.18	13.82	6.72	4.58	18.01	10.4

One thing that became clear from this data was that the total surface areas of the soil samples, as well as their total pore volumes (which would be expected to be related quantities) were both a strong function of soil organic matter content for five of the six soils under study. The one exception, soil #4, had a considerably higher total pore volume (and, hence, a higher surface area) than would be expected based on its TOC value, in comparison with the other soils. See figure below.

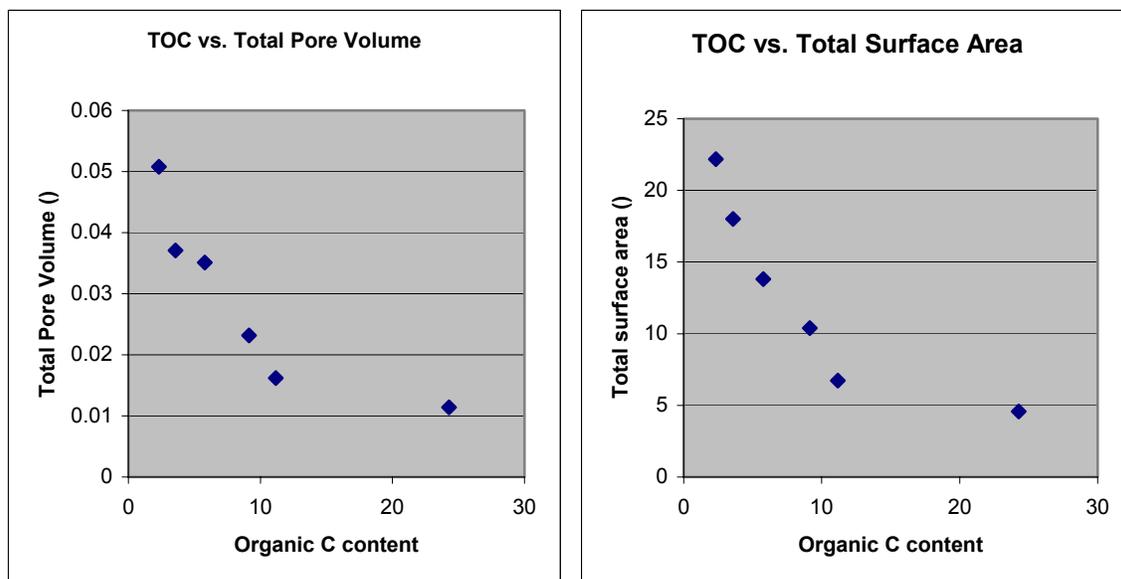


FIGURE 4. TOTAL ORGANIC CARBON OF EXPERIMENTAL SOILS VS TOTAL PORE VOLUME AND TOTAL SURFACE AREA OF SOILS.

With regard to pore size distribution, very little significant differences were seen among the six soils, with the exception of the fact that soils 1, 3, and 4 had a slightly higher content of large (> 80 μm) pores, as shown below in table below.

TABLE 4. PORE SIZE DISTRIBUTION OF EXPERIMENTAL SOILS

Size	1	2	3	4	5	6
< 6 μm (%)	6.8	9.06	13.73	10.36	10.22	15.39
6 - 8 μm (%)	5.27	8.69	6.64	6.86	8.34	7.98
8 - 10 μm (%)	6.81	5.29	3.79	4.08	5.04	4.93
10 - 12 μm (%)	6.96	6.52	4.54	5.16	6.43	6.24
12 - 16 μm (%)	8.83	8.15	6.56	6.41	8.25	7.99
16 - 20 μm (%)	6.92	9.17	7.16	7.28	9.54	8.71
20 - 80 μm (%)	37.23	39.78	33.34	38.75	40.73	38.83
>80 μm (%)	21.19	13.33	24.24	21.1	11.44	9.93

TABLE 5. PARTICLE SIZE DISTRIBUTION AND TEXTURAL CLASSIFICATION

Soil #	% Sand	% Silt	% Clay	Textural classification
1	25.4	51.1	27.5	Clay loam-Loam
2	64.0	25.3	10.7	Sandy loam
3	70.8	18.1	11.1	Sandy loam
4	73.5	10.6	15.9	Sandy loam
5	40.6	33.0	26.4	Loam
6	51.8	26.3	21.9	Sandy clay loam

Humic acid quantitation was done according to methods put forth by the International Humic Substance Society. Fulvic acid was extracted as per the same methods, but was not strictly quantified, as we had no access to a lyophilizer. Instead, relative amounts of fulvic acid extracted from each soil were determined spectrophotometrically (by absorbance at 400 nm), and the relative aromatic contents of the extracted fulvic acid fractions were measured in the same manner (ratio of A_{280} to A_{400}). Relative humin contents of the various soils were determined by ashing the residue remaining after extractions of humic and fulvic acids. Humic acid data showed that soil #2 was very rich in humic acids (HA), and that this fraction in fact made up nearly all of the organic matter in this soil. In contrast, soil #1 showed almost no HA; the other soils were intermediate in value. Five of the six soils showed fulvic acid levels which were strongly a function of their overall organic matter content; however, soil #4, based on these results, had 30-50% more fulvic acid than would be expected. A similar patten was seen for humin content, with the inverse conclusion, namely, that soil #4 had a considerably lower humin/TOC ratio than the other five soils.

TABLE 6. HUMIC ACID CONTENT OF EXPERIMENTAL SOILS

	Soil #					
	1	2	3	4	5	6
Humic acid content (%)	0.1	15.2	6.83	5.9	1.4	10.2

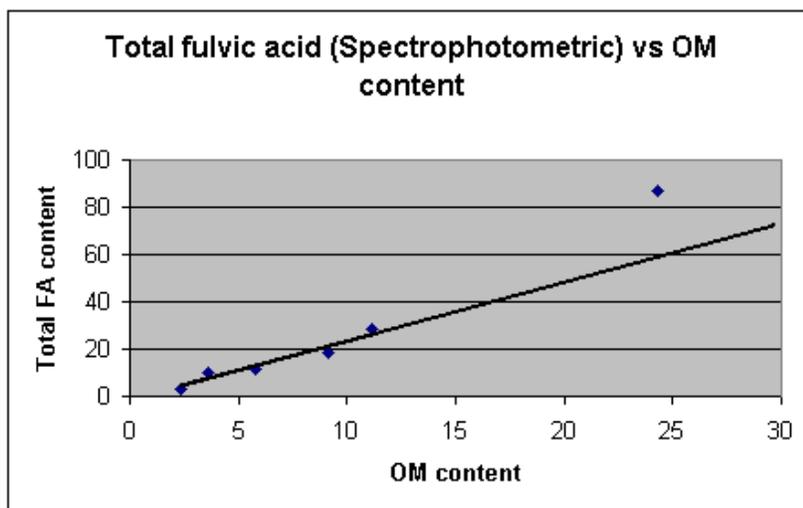


FIGURE 5. FULVIC ACID CONCENTRATIONS IN EXPERIMENTAL SOILS VS ORGANIC MATERIAL CONCENTRATION

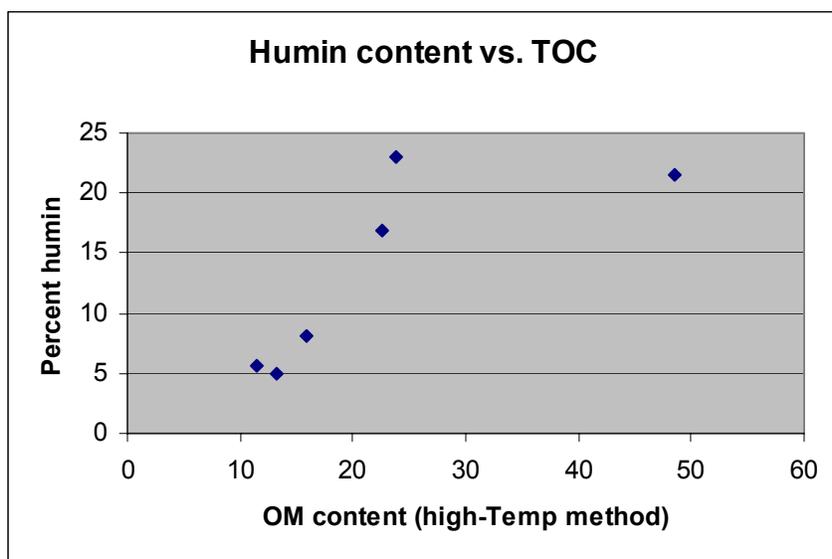


FIGURE 6. HUMIN CONCENTRATION VS. TOTAL ORGANIC CONTENT

Data on the functional groups present in each soil's organic matter (*i.e.* aromatic vs. aliphatic carbon, oxygen content and relative polarity) was collected by solid-state ^{13}C -nuclear magnetic resonance at the Western Research Institute. Because of the low organic C present in

soils 1 and 5, no meaningful data could be collected on these two soils. Results for the other four soils showed, as follows, that there were only slight differences in the chemical makeup of the organic matter in the different soils (values given are percent of the total integrated area from the ^{13}C -NMR signal which fell into each of the functional group ranges given) as shown in the table below.

TABLE 7. FUNCTIONAL GROUPS DETERMINED BY ^{13}C -NMR IN THE SIX EXPERIMENTAL SOILS

ppm	Functional group(s)	2	3	4	6
0-40	Branched & Straight-chain Aliphatics	22	17	20	18
40-90	Ethers	41	38	34	37
90-120	Ethers	13	15	11	11
120-140	Aromatic (Various ring C)	8	9	9	9
140-165	Phenolic	5	9	9	7
165-190	Carboxylic acid/Ester	11	8	10	12
190-250	Carbonyl/Ketone	0	4	7	6

Because of the incompleteness of the NMR data, pyrolysis GC-MS was conducted on all soils in order to better address the question of chemical composition of the various organic matter fractions.

Biodegradability of various hydrocarbon contaminants in soils and impact of increasing sequestration over time was studied. Mineralization of each contaminant measured (using appropriate bacteria) after 0, 40, or 120 days of contact time and the "sequestration ratio" defined as ratio of mineralization after contact to that in systems inoculated immediately after contaminant addition (in reality, there was a delay of ca. 3 days between spiking and inoculation, due to time needed to gamma-sterilize the soil microcosms) was determined.

Degradability of hexadecane by an isolate of *Acinetobacter* was not appreciably affected by 40 days of contact with any of the model soils - as shown below, i.e. sequestration ratio remained near one. This implies that hexadecane is not appreciably sequestered in any soil over this time frame. However, there did appear to be an effect after 120 days of contact: With 5 of the 6 soils, sequestration was strongly dependent on TOC; the exception was soil #4, in which the "sequestration ratio" value was higher than expected, meaning that biodegradation was higher than expected at this timepoint. Thus, there is some physicochemical parameter (or combination

of multiple parameters) that causes soil 4 to exhibit much less sequestration of hexadecane than the other 5 soils.

Mineralization of phenanthrene (model low-molecular weight PAH) by *Mycobacterium austroafricanum* showed a very similar pattern. Again, there was no notable sequestration effect within 40 days, but a very significant sequestration after 120 days, which was, in the case of phenanthrene, strongly TOC-dependent across all five soils examined (Soil #6 could not be included in this experiment).

Pyrene (model HMW PAH) mineralization by *M. austroafricanum* showed some definite trends toward TOC-dependent sequestration, both at 40 and 120 days. However, in both of these cases, soil #4 was, as with hexadecane (above) considerably less prone to sequestration than were the other five model soils.

Both hexadecane and pyrene were less sequestered (more bioavailable) in soil #4 than expected based on the relationships seen in the other soils. Soil #4 is similar to all of the other model soils in many respects (*i.e.* pore-size distribution, particle-size distribution, organic matter chemical composition); however, it does have higher-than expected surface area and pore volume, and also displays considerably lower-than-expected humin content and higher-than-expected fulvic acid content. Other researchers have shown that soluble organic matter (*i.e.* fulvic acids) can increase water "solubility" of PAH in contaminated soils by an order of magnitude or more; thus, we are determining if this is occurring in this system, and planning and conducting experiments on possible fulvic acid uptake by *M. austroafricanum*.

Taken together, these results demonstrate significant reductions in bioavailability of PAH (especially in soils with high organic carbon content) over relatively short periods (*e.g.* 120 days), even when "receptor" organisms are used which are well-adapted to the uptake of PAH. Conversely, results show that biotreatment (alone or as a component of chemical-biological approaches) was progressively less effective in these cases (if strict removal of PAH is the sole criterion).

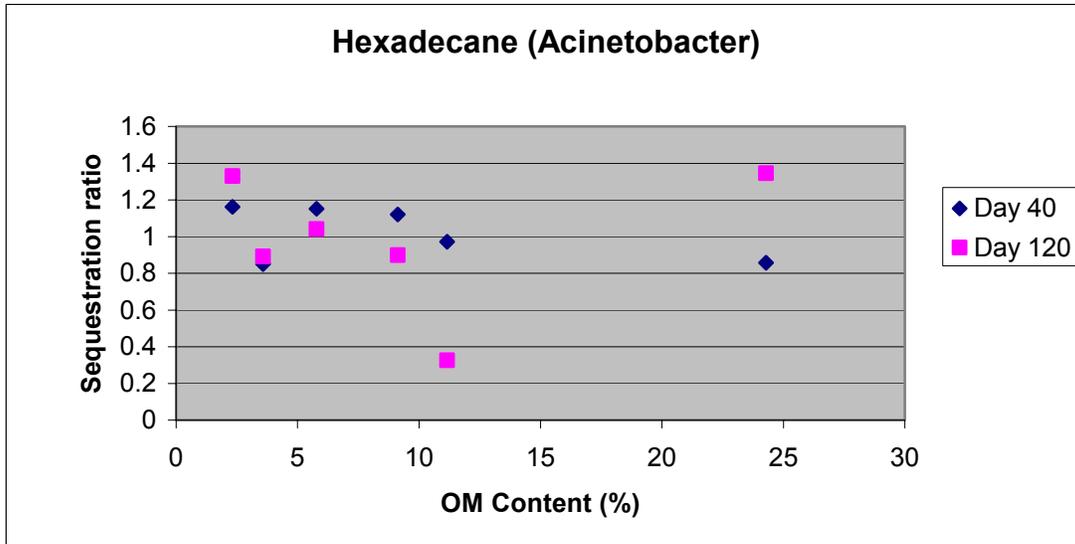


FIGURE 7. HEXADECANES DEGRADATION BY *ACINETOBACTER* SP. RELATED TO ORGANIC MATERIAL CONTENT TO GIVE SEQUESTRATION RATIO

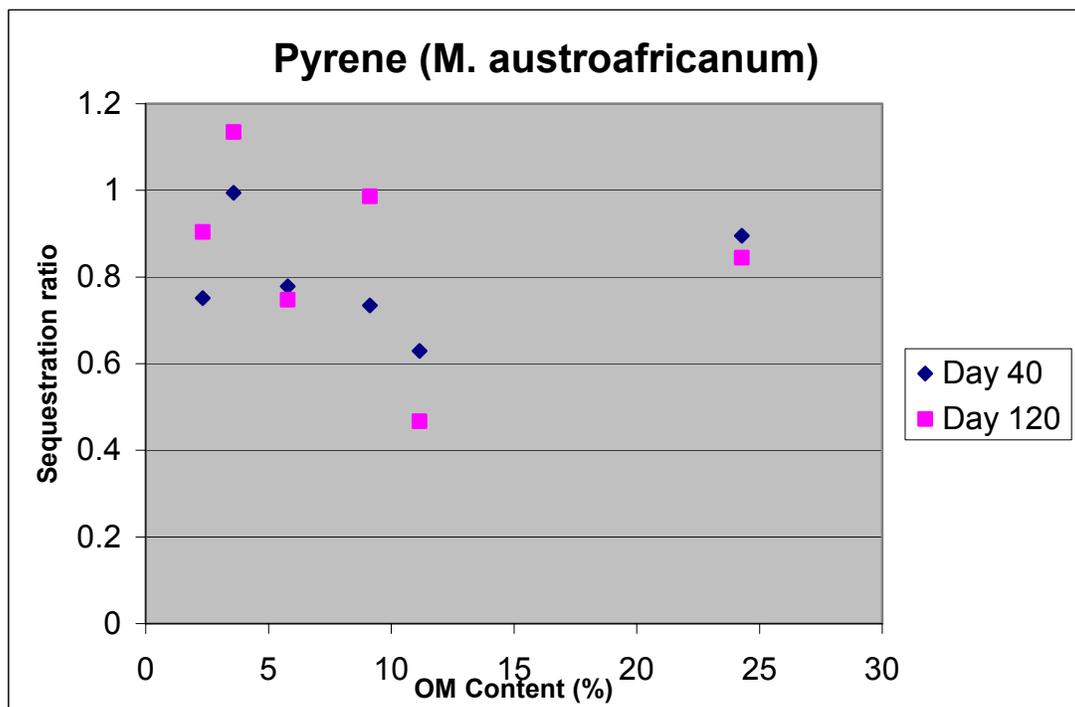


FIGURE 8. PYRENE DEGRADATION BY *MYCOBACTERIUM AUSTROAFRICANUM* RELATED TO ORGANIC MATERIAL CONTENT TO GIVE SEQUESTRATION RATIO

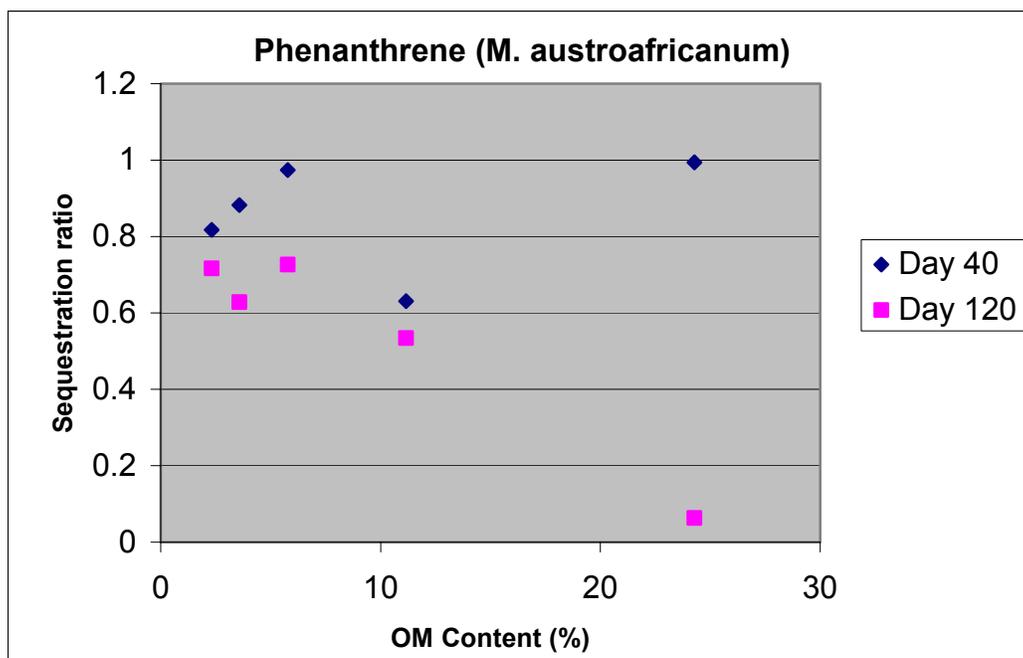


FIGURE 9. PHENANTHRENE DEGRADATION BY *MYCOBACTERIUM AUSTROAFRICANUM* RELATED TO ORGANIC MATERIAL CONTENT TO GIVE SEQUESTRATION RATIO

Extractions Using Mild Organic Solvents

Soil samples were extracted with a mild extractant to determine the bioavailable fraction contained, according to the procedure of Kelsey *et al.* (1997). This procedure, unlike the standard methods of extraction that rely on vigorous extraction by organic solvents, has been positively correlated with bioavailability of PAH to earthworms. Well mixed, 10 g soil samples were transferred to 50-mL Teflon centrifuge tubes. Twenty-five mL of organic extractant (e.g., *n*-butanol, methanol/water, ethanol/water) were added to the tubes. Tubes were agitated for 2 hr at room temperature, then centrifuged at 7600g for 10 min. Supernatant was analyzed for contaminant concentration after solvent reduction under a stream of nitrogen, according to established protocols listed in the prior research activities. The concentrations of contaminants extracted from soils using this procedure were compared to the “total” amount of contaminants extracted from the same soils, using established Soxhlet extraction methods described above.

This contaminant fraction, characterized as the “rapid release” fraction or bioavailable fraction, was compared to data obtained from facets of these studies. Correlation between the concentration of contaminants obtained from organic extractions and other tests were further characterized the bioavailable fraction of soil contaminants for future risk assessments and modeling.

Development of Mild Extraction Approaches to Estimate Bioavailable Fraction of Crude Oil Residues in Contaminated Soils

In order to begin to develop a mild extraction approach that would correlate with the bioavailability of crude oil hydrocarbons, soil (MV AB) from a chronically crude oil-contaminated site (a wellhead area from an actively-producing field in southern Illinois) was extracted with solvents of varying polarity. Extraction was done using a ratio of 20 g of soil to 50 ml of solvent, with shaking for 90 minutes; soil was then allowed to settle (approximately 60 minutes), and bulk solvent was removed by pipetting. Remaining solvent was removed by subjecting soil to a stream of N₂ in a Turbovap apparatus; this was done for a period of approximately two hours, with intermittent mixing. Preliminary experiments using non-extracted soil showed that this process (evaporation by exposure to an N₂ stream) had no significant effect on either the TPH content of the soil, or its acute toxicity (Microtox EC₅₀); this is not surprising, as previous analyses have shown the soil to be very low in those volatile components which are most often associated with high acute toxicity (*e.g.* BTEX). The initial TPH content of the soil in question has been previously determined to be approximately 15% by weight; analyses included in this particular experiment yielded a figure of 12.8%

Solvents employed were as follows: methylene chloride (MeCl₂), hexane (Hex), ethyl acetate (EtOAc), acetone (Ace), ethanol (EtOH), acetonitrile (ACN), methanol (MeOH), and a 1:1 mix of aqueous methanol.

Development of Extraction Methods to Mimic Bioavailability

The extent to which TPH was extracted from the soil was, as expected, strongly dependent on the polarity of the solvent, as shown below.

When Microtox EC_{50} values were determined for each solvent-extracted soil (following removal of solvent by evaporation), results with the four non-polar solvents (ACN, EtOH, MeOH, MeOH/H₂O) showed a very good correlation between decreasing amounts of TPH remaining and decreases in acute toxicity. However, when the full range of solvents was included, the observed correlation broke down for those four solvents, which were of lowest polarity:

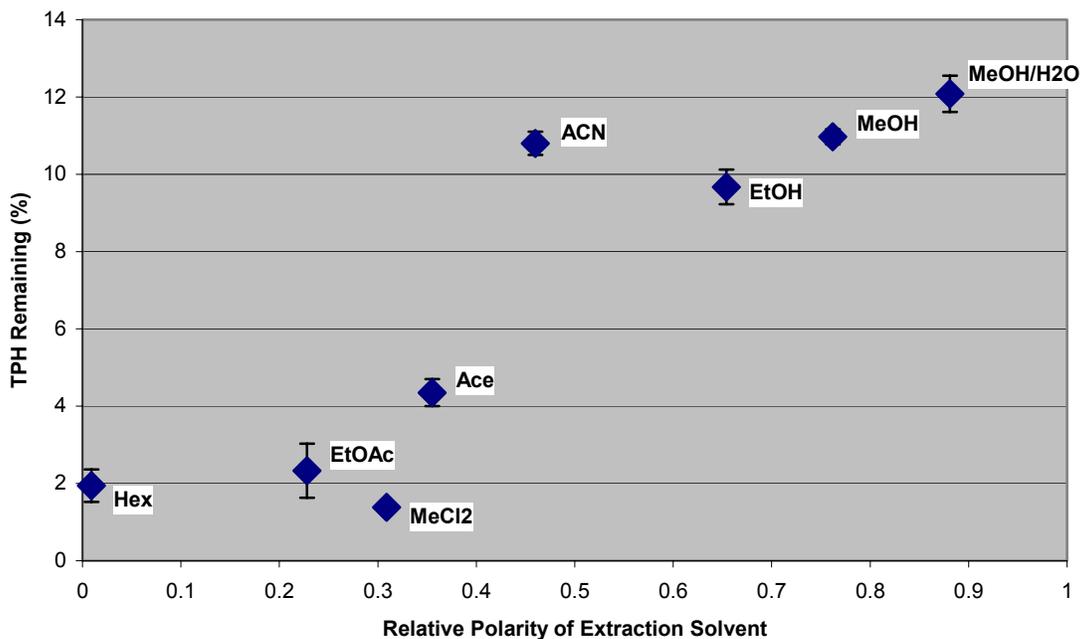


FIGURE 9A. CORRELATION OF SOLVENTS TO EC_{50}

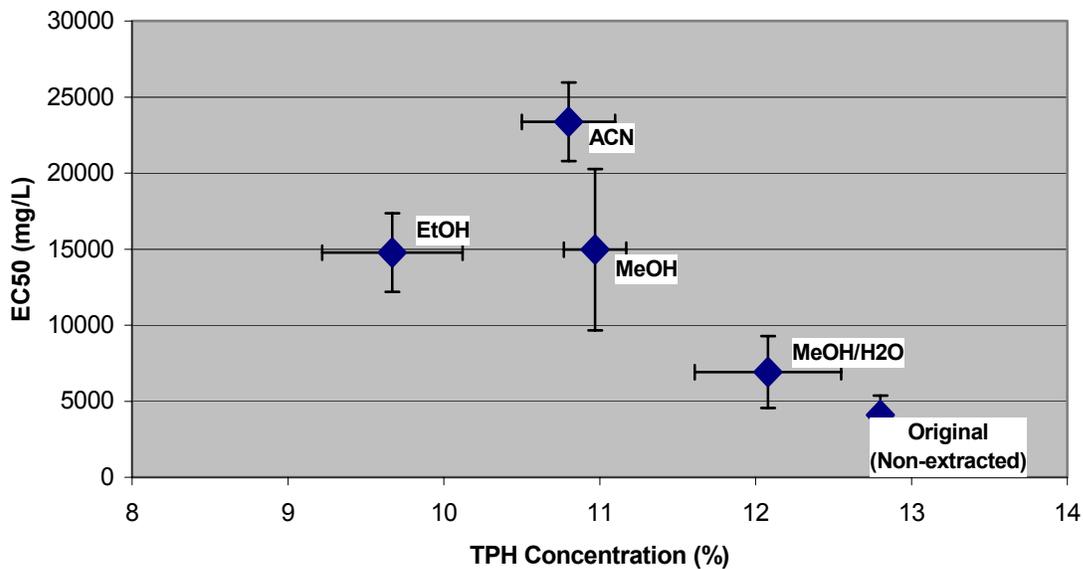


Figure 10. Relation Between Solvent, TPH Concentration and EC₅₀ of Soils

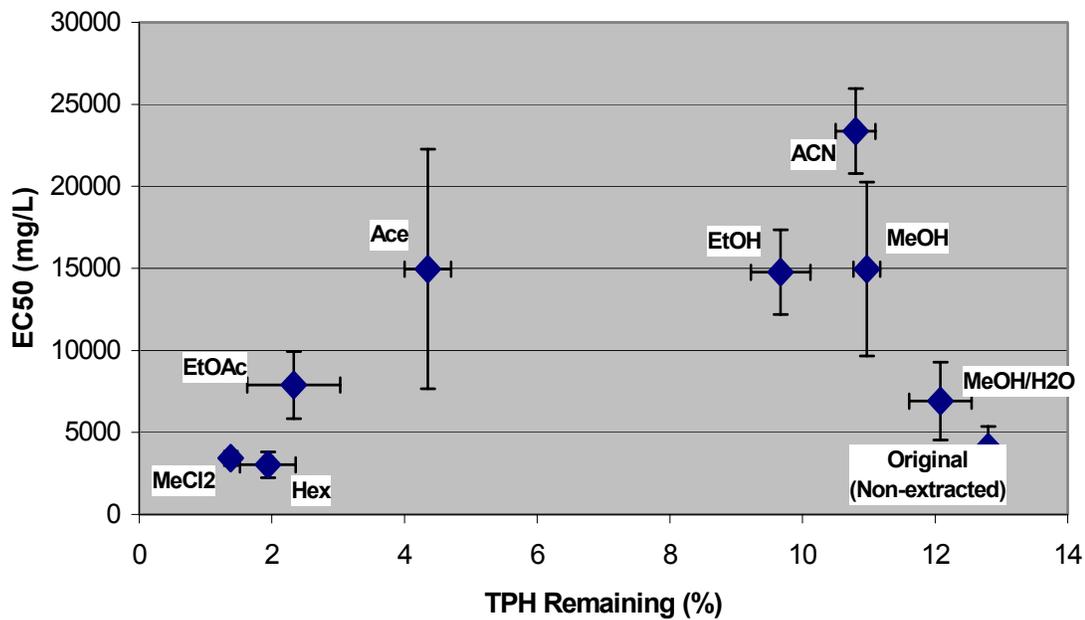


FIGURE 11. RELATIONSHIP OF LOW POLARITY SOLVENTS TO EC₅₀ AND TPH REMAINING

The likely explanation for this observation is that, in the case of the highly hydrophobic solvents (acetone, methylene chloride, hexane, and ethyl acetate), the interactions between the solvent and the soil organic matter were sufficiently strong as to prohibit full removal of the solvent by evaporation. Under this scenario, residual solvent in the soil would account for the higher-than-expected toxicities of the soils extracted with these solvents. This seems particularly likely, given that the toxicities of soils extracted with these solvents increased (lower EC₅₀ values) as the polarity of the solvent decreased. The EC₅₀ of MV AB soil from which TPH was exhaustively extracted (by sonication in tetrachloroethylene, as per EPA methods) was found to be 10,700 mg/L. The same caveat applies in this case, as tetrachloroethylene was evaporated under N₂ after extraction, and removal of this solvent may also have been incomplete. In fact, this EC₅₀ value would mean that acetone-, ethanol- and methanol-extracted soils were less toxic than soil which was completely “clean” (at least with regard to TPH); this would seem to give further weight to the likelihood of interferences due to residual solvent.

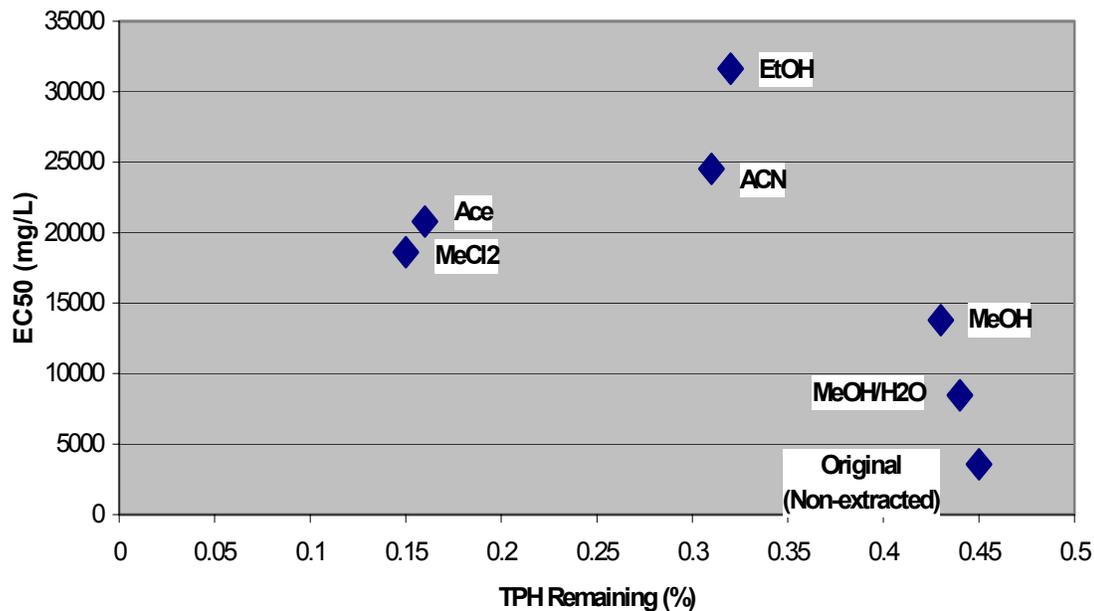


FIGURE 12. RELATIONSHIP BETWEEN TOTAL PETROLEUM HYDROCARBON REMOVAL AND TOXICITY REDUCTION

Another observation from the above data was that there were very significant reductions in toxicity (up to almost six-fold in the case of ACN-extracted soils), although relatively little of

the TPH in the soil was actually removed (approximately 15% in this case). This implied that significant amounts of oil-derived TPH may be able to remain in soil, with relatively low toxicity; again, it must be emphasized that at least some portion of the TPH in this soil was “aged”, as leakage of oil into the soil had taken place over the period of at least several years. The same pattern was observed in soil (commercial topsoil) which was spiked with 0.5% fresh crude oil (“sweet” Texas crude). Again, solvents of relatively higher polarity gave a good correlation between TPH removal and toxicity reduction; this pattern did not hold for more-hydrophobic solvents.

Again, significant decreases in toxicity were realized with only small removals of total hydrocarbons: Methanol-extracted soil was three-fold less toxic than the original spiked soil, although its TPH content was only $\approx 5\%$ lower; similarly, ethanol extraction yielded a nearly 7-fold toxicity decrease, corresponding to a TPH reduction of 29%. However, this may be due in part to the presence in fresh crude oil of fractions, such as BTEX, which, although relatively minor by weight, may account for a significant portion of the oil’s overall toxicity. Further experiments are planned to better elaborate the differences in toxicity and in sequestration behavior, between fresh and aged crude oil, and to elucidate the dependence of these behaviors on the hydrocarbon composition of the oil.

Based on the above data, ethanol was chosen as a mild extractant that was capable of removing the majority of (apparently) bioavailable TPH, with apparent minimal interference due to its retention in extracted soil. Using commercial topsoil as a model soil, fresh crude oil (“sweet” Texas crude) was spiked into soil at various rates, and ethanol extractability was assessed after a short period (12 hours). Results, along with corresponding Microtox EC_{50} values, were as follows (for comparison, EC_{50} for clean topsoil was determined to be 9870 mg/L).

TABLE 8. CORRELATION OF PERCENT CRUDE OIL IN SOIL TO EC₅₀

Crude oil conc. (initial)	Pre-extraction		Post-extraction	
	TPH conc. (%)	EC ₅₀ (mg/L)	TPH conc. (%)	EC ₅₀ (mg/L)
1%	1.35 ± 0.03	6159 ± 789	1.22 ± 0.04	12674 ± 3236
2%	2.53 ± 0.02	2109 ± 182	2.15 ± 0.02	6391 ± 211
4%	4.13 ± 0.16	1248 ± 128	3.52 ± 0.02	6271
8%	6.10 ± 0.11	704 ± 24	6.50 ± 0.28	3419 ± 384

Again, very slight reductions in TPH content in all cases accounted for significant reductions in toxicity, implying that a significant fraction of the hydrocarbon was non-bioavailable (or much less-toxic). Very significant amounts of input TPH were also, after only 12 hours contact time with the soil, non-extractable using ethanol.

The final experiment, which is currently ongoing, seeks to correlate the extent and rapidity of sequestration, as well as toxicity effects, with soil properties. Six model soils have been obtained from grassy and wooded areas in northeastern Illinois. Textural classification and organic carbon content have been determined for all soils, and are as follows:

TABLE 9. TEXTURAL CLASSIFICATION AND ORGANIC CONTENT OF SOILS

Soil #	% Sand	% Silt	% Clay	Textural classification	OC (%)
1	25.4	51.1	27.5	Clay loam-Loam	2.32
2	64.0	25.3	10.7	Sandy loam	5.78
3	70.8	18.1	11.1	Sandy loam	11.16
4	73.5	10.6	15.9	Sandy loam	24.28
5	40.6	33.0	26.4	Loam	3.58
6	51.8	26.3	21.9	Sandy clay loam	9.13

In the interest of determining the last of these parameters, GTI has contracted the Western Research Institute (Laramie, WY) to conduct solid-state ¹³C-NMR examination of the six model soils, and expects to have this data shortly. Soil particle micropore volumes were also measured for each soil.

In a first experiment with the six soils, crude oil was spiked into each at the 2% rate, and ethanol extractability and acute toxicity are being tracked over time. The “initial” toxicity data-

point for this experiment (12 hours after spiking of oil) is currently available; data thus far are as follows:

TABLE 10. EC₅₀ OF SOILS WITH AND WITHOUT OIL ADDITION

Soil #	Baseline EC ₅₀	EC ₅₀ w/oil (2%)	EC ₅₀ Ratio (Baseline/Oil)
1	4890 ± 430	515	9.67
2	7906 ± 854	1109	7.13
3	14035 ± 375	1966	7.14
4	24290 ± 370	5843	4.16
5	11484 ± 1556	785	14.63
6	7863 ± 1509	6037	1.30

The ratio of a soil’s baseline (“clean”) EC₅₀ to its EC₅₀ after spiking with oil provides an index of the toxicity of the oil in that particular soil; the higher the ratio, the greater the increase in toxicity when oil is added. When these ratios are plotted for each soil, in comparison with the organic matter contents of the soil, the following correlation becomes apparent (at least for this initial time point):

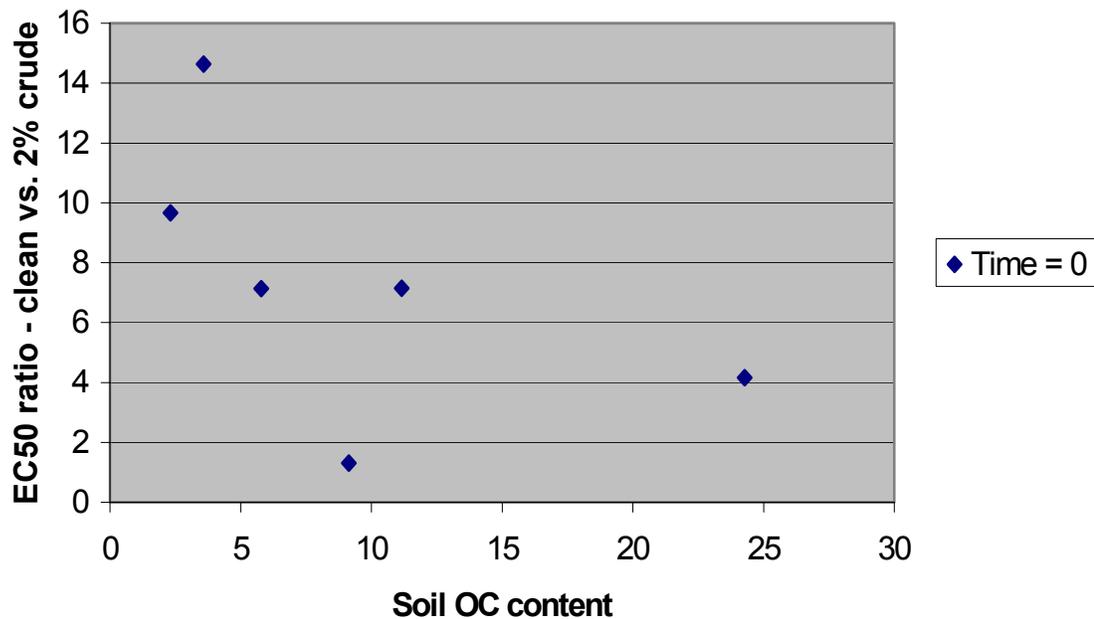


FIGURE 13. CORRELATION OF 2 PERCENT OIL CONTAMINATED SOIL VS ORGANIC CONTENT

It appears that soils with higher organic matter contents are less impacted by crude oil (in terms of toxicity increases) than are soils with lower OC content. This phenomenon has been observed many times in the case of herbicide toxicity to plants; in fact, it was studies of this nature which first led to the notion of sequestration of hydrophobic pollutants taking place through physicochemical interactions with soil organic matter. It is not immediately clear as of yet the extent to which this phenomenon has been previously described for crude oil hydrocarbons. Further data-points (over the span of months) were taken in order to demonstrate sequestration and/or toxicity reductions over time, and to further assess their dependence on soil composition. Also, this experiment was also conducted with “aged” oil (extracted from MV AB), which does not contain any appreciable BTEX fraction; this was done to better understand the behavior of the aliphatic fraction of the oil taken independently.

Susceptibility to chemical oxidation of coal tar hydrocarbons in soils and impact of increasing sequestration over time

The model soils were spiked with coal tar (1000 ppm total tar concentration, resulting in total PAH content of ~250 ppm). Degradability of priority pollutant tar PAHs was determined in $H_2O_2/Fe(II)$ and $CaO_2/Fe(II)$ reactions (abiotic) after 0, 40, and 80 days contact time. Time-zero data showed that, for all six of the six soils examined, TOC was the major determinant governing PAH degradability. This is most likely due to two factors, namely sorption of PAH onto SOM retarding its oxidation and the fact that the soils organic matter consumes some portion of the oxidizing equivalents (OH) generated by the reaction.

As hydrophobicity of individual PAH increases, the linearity of this function increases TOC (or some other factor which it represents) becomes the only governing factor over PAH degradability. Experiments conducted at day 40 showed this relationship still held for 4 of the 6 soils, but that notable deviations were occurring, particularly in soils 1 and 5. Both of these soils showed considerably poorer removal of PAH than expected based on TOC content alone. Deviations at Day 40 appeared to be larger for low-molecular-weight PAH. Note that these two soils also have the highest pore content (see above) of the six. Day 80 degradability data is similar in pattern to that at day 40, but the slope of the line formed by soils 1 and 5 (i.e. the deviation from the line based on TOC) appears to have increased for most PAH - this is

apparently indicative of increasing migration of PAH into the pore spaces in these soils, which further retards the accessibility of the PAH to the chemical oxidant.

Following completion of the day-120 experiment, all data from this experiment were subjected to further mathematical modeling to generate a manuscript on the effects of PAH sequestration on their chemical oxidizability. This publication is included in the Appendix.

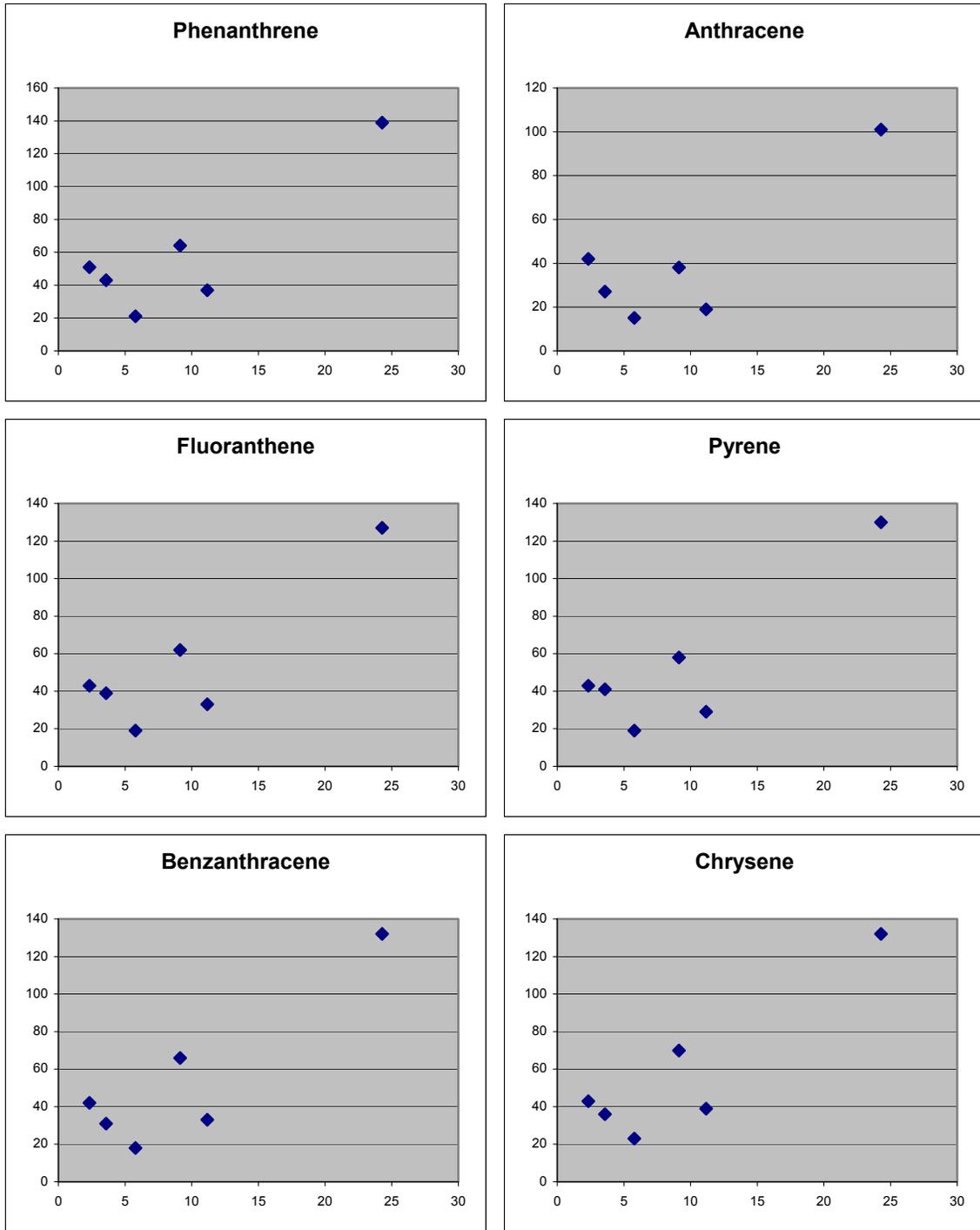


FIGURE 14A. PAH RECOVERED (% OF NO-OXIDANT CONTROL – “Y” AXIS) VS. TOC. TIMEPOINT #1 – “0” DAYS SEQUESTRATION

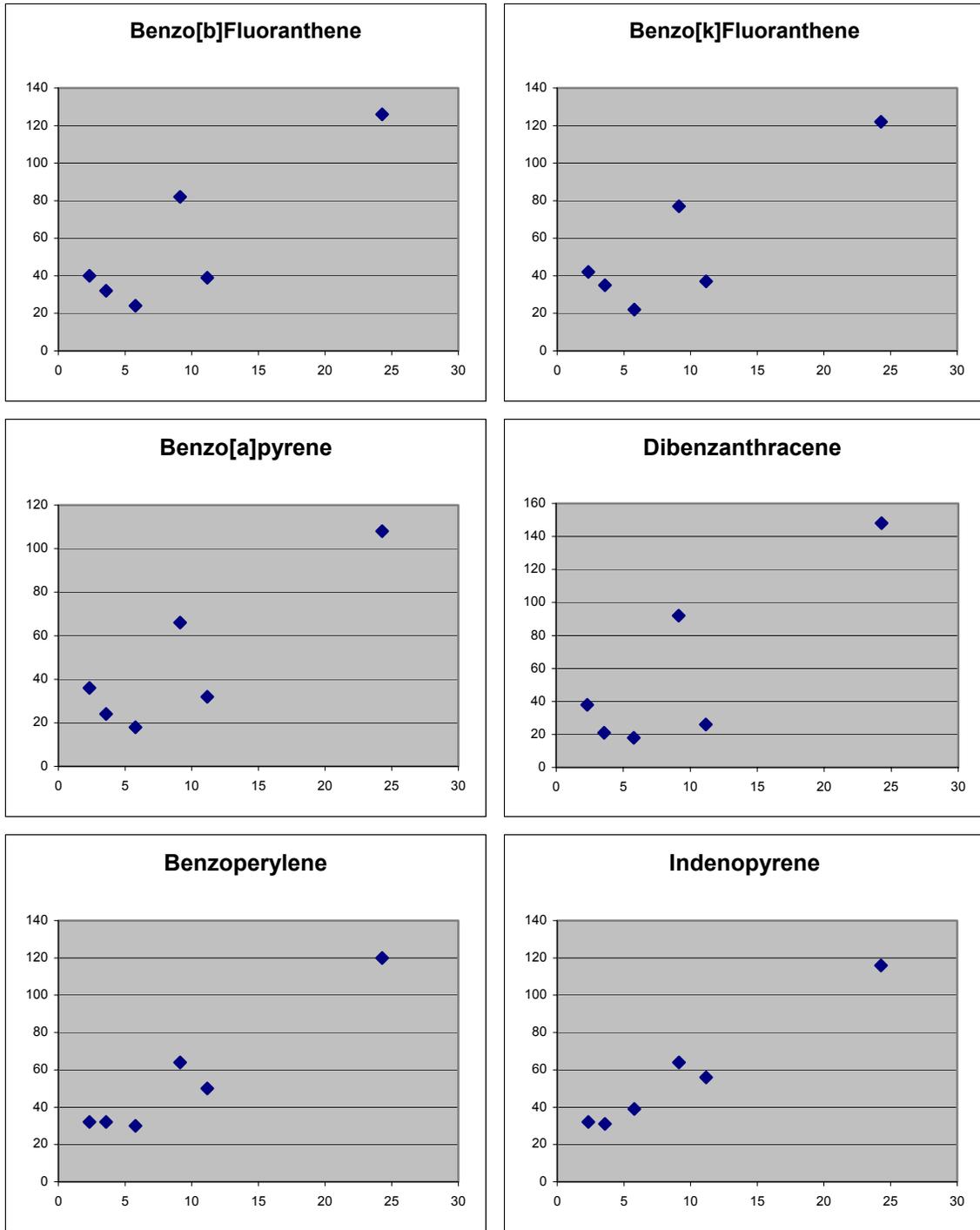


FIGURE 14B. PAH RECOVERED (% OF NO-OXIDANT CONTROL – “Y” AXIS) VS. TOC. TIMEPOINT #1 – “0” DAYS SEQUESTRATION

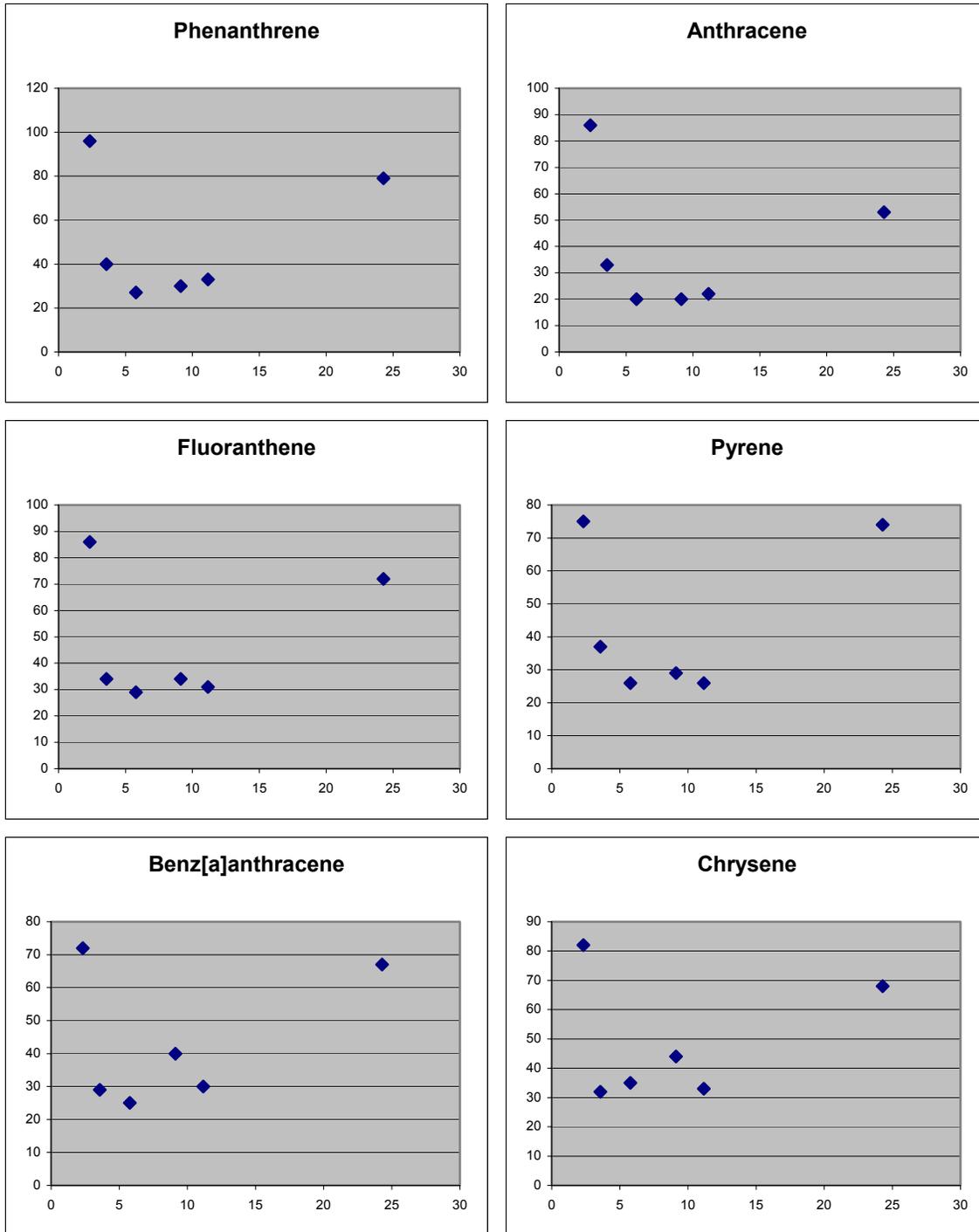


FIGURE 15A. PAH RECOVERED (% OF NO-OXIDANT CONTROL – “Y” AXIS) VS. TOC. TIMEPOINT #2 – 40 DAYS SEQUESTRATION

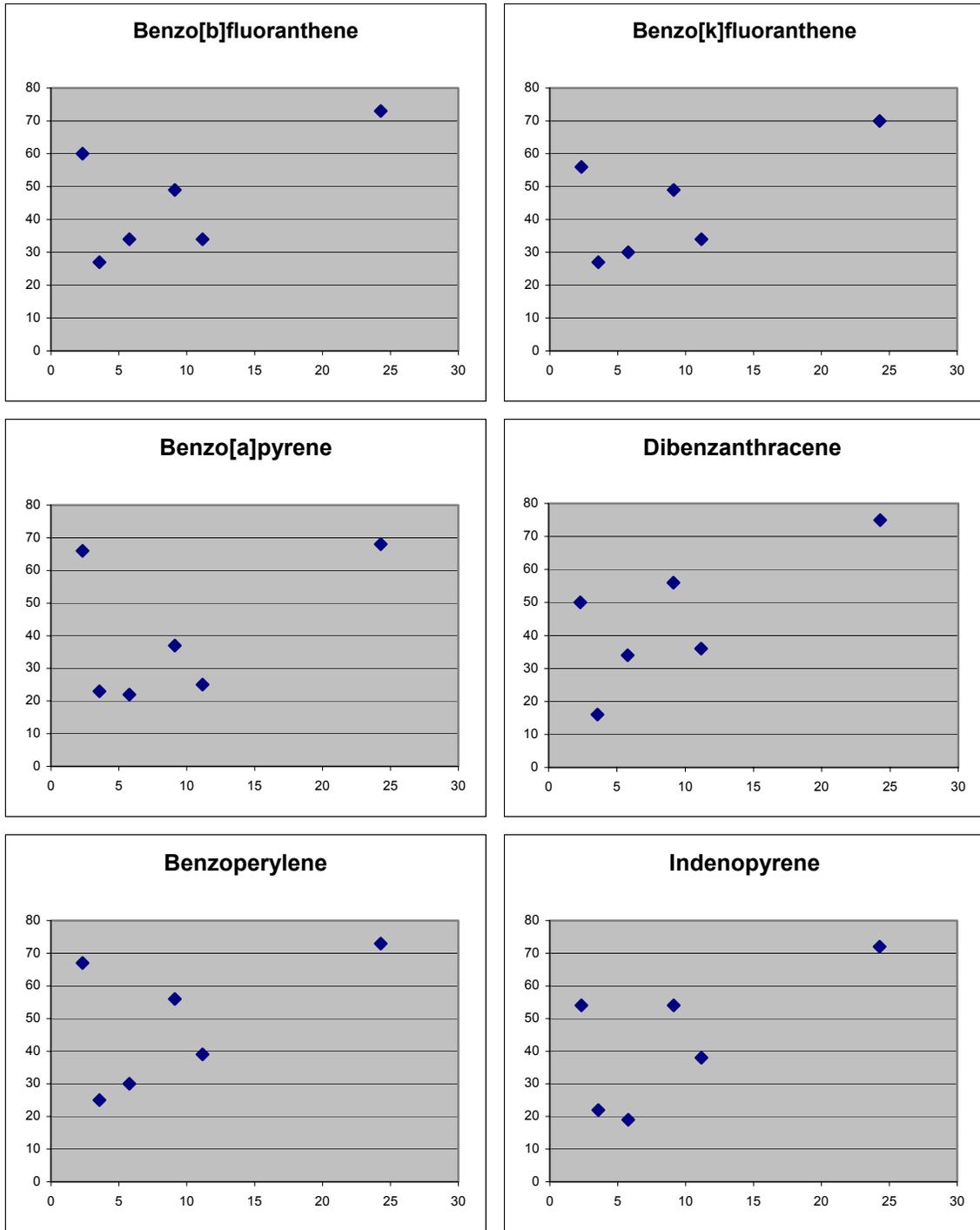


FIGURE 15B. PAH RECOVERED (% OF NO-OXIDANT CONTROL – “Y” AXIS) VS. TOC. TIMEPOINT #2 – 40 DAYS SEQUESTRATION

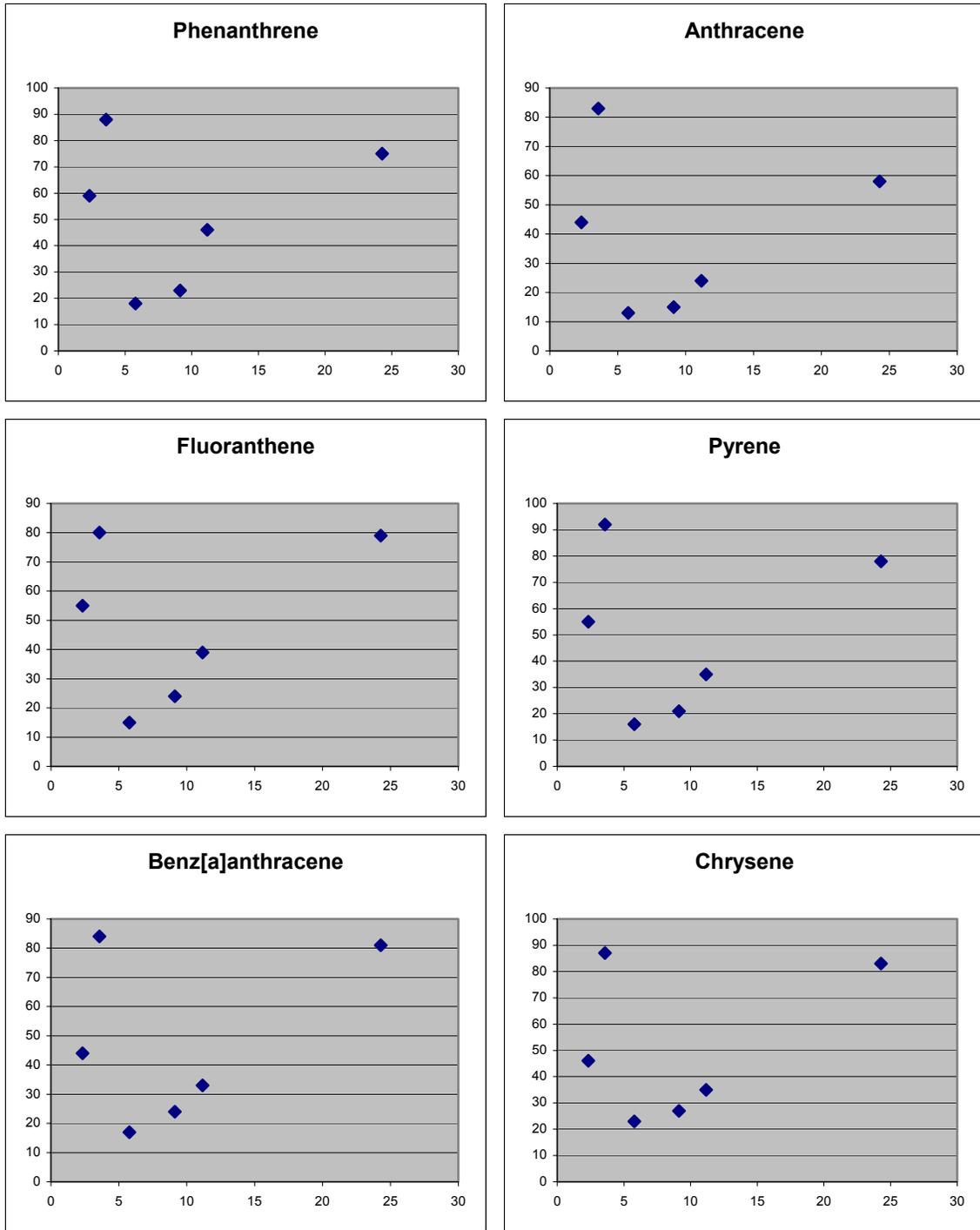


FIGURE 16A. PAH RECOVERED (% OF NO-OXIDANT CONTROL – “Y” AXIS) VS. TOC. TIMEPOINT #3 – 80 DAYS SEQUESTRATION

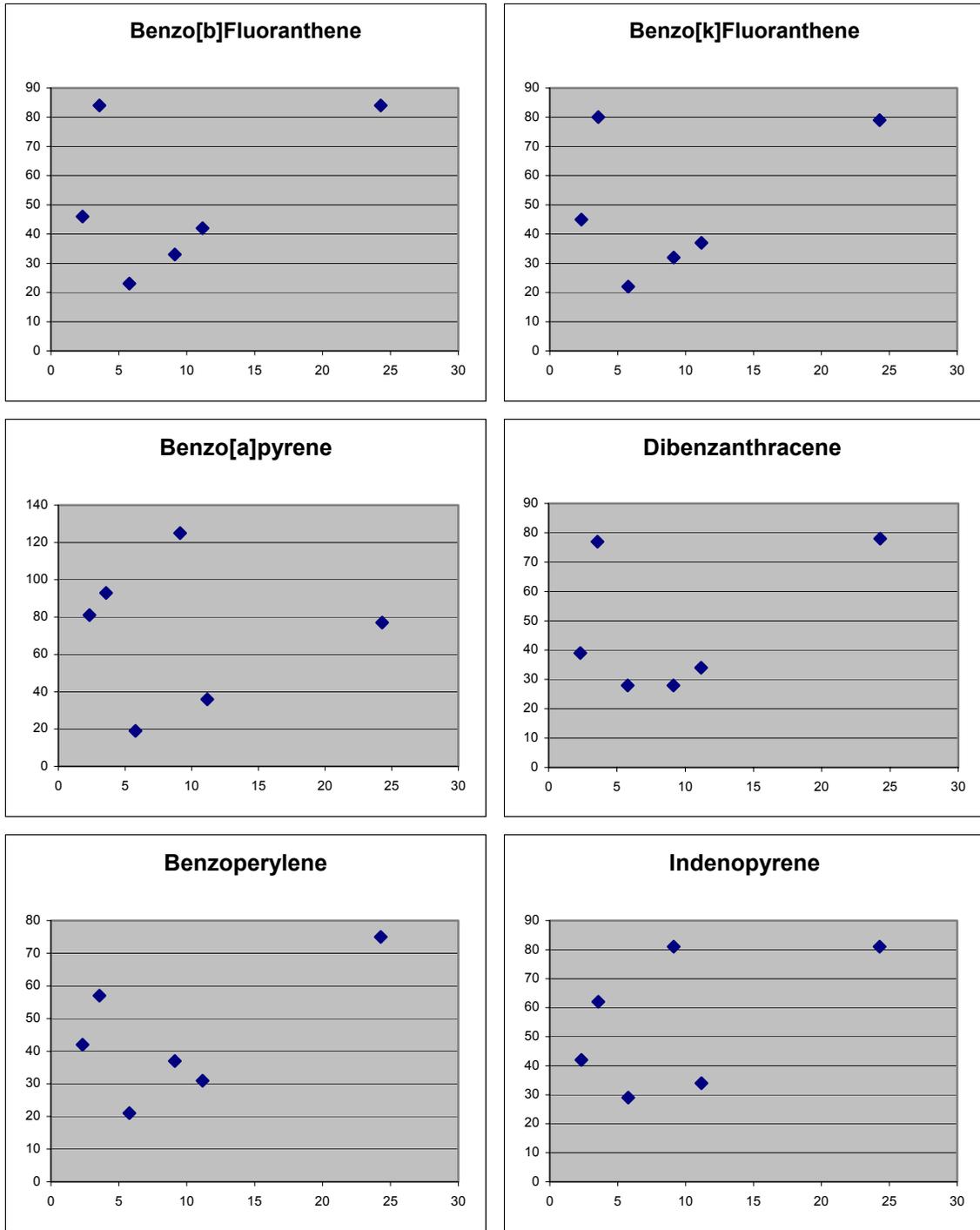


FIGURE 16B. PAH RECOVERED (% OF NO-OXIDANT CONTROL – “Y” AXIS) VS. TOC. TIMEPOINT #3 – 80 DAYS SEQUESTRATION

Roles of Soil Physicochemical Parameters on Contaminant Sequestration and Effect of Sequestration on Biodegradability and Chemical Treatability

Humic Coverage Index (HCI) has been defined as a new parameter for each of our model soils that we have used in biodegradability and chemical degradability studies. HCI is defined as the content of humic and fulvic acids (summed together, on a weight basis), divided by the specific surface area of the soil. Derivation of this quantity is rationalized by current conceptual models of soil particles which postulate that such particles consist of cores of humin and minerals, overlain by a combined layer of humic and fulvic acids. According to these models, the process of sequestration of organic chemicals in soil consists of (A) adsorption on and dissolution in the HA/FA outer layer, and (B) entrapment in micropores in the humin/mineral cores, which most likely requires diffusion through the HA/FA overlayer.

HCI values were calculated based on two different surface area measurements, namely, the N₂ (BET) adsorption method, and the CO₂ adsorption method. Results from the CO₂ method are still pending. The latter of these has recently been argued (de Jonge and Mittelmeijer-Hazeleger, 1996) to give a better estimate of the real surface area and pore volume of soil particles and similar materials, due to its much higher diffusibility through the HA/FA layer. According to our results, the six model soils represent a wide range of different values for humic coverage when defined in this way.

TABLE 11. HUMIC COVERAGE INDEX (HCI) OF TEST SOIL DETERMINED BY TWO METHODS

SOIL	By N₂ Adsorption		By CO₂ Adsorption	
	Surface Area (m ² /g soil)	HCI (mg humic material/m ²)	Surface Area (m ² /g soil)	HCI (mg humic material/m ²)
1	22.18	6.8		
2	13.82	55		
3	6.72	347		
4	4.58	895		
5	18.01	14		
6	10.40	172		

These values have been applied to several different biodegradation (mineralization) experiments that have been conducted with various bacteria (representatives of the genera *Mycobacterium*, *Burkholderia*, *Acidovorax*, and *Acinetobacter*), catabolizing PAH (pyrene or phenanthrene) or aliphatic hydrocarbons (hexadecane) spiked into model soils. In almost all cases, there appears to be an optimal HCl value (which varies somewhat from one bacterium to another) for contaminant biodegradation to occur. Results for these experiments are as shown below.

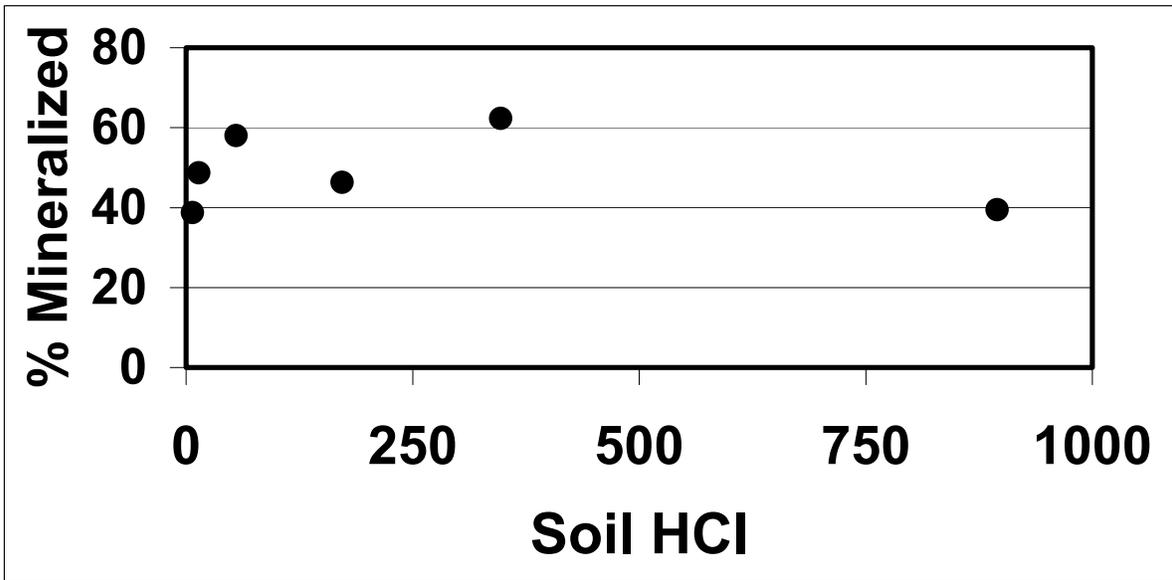


FIGURE 17. PYRENE MINERALIZATION (*MYCOBACTERIUM*) RELATED TO SOIL HCl

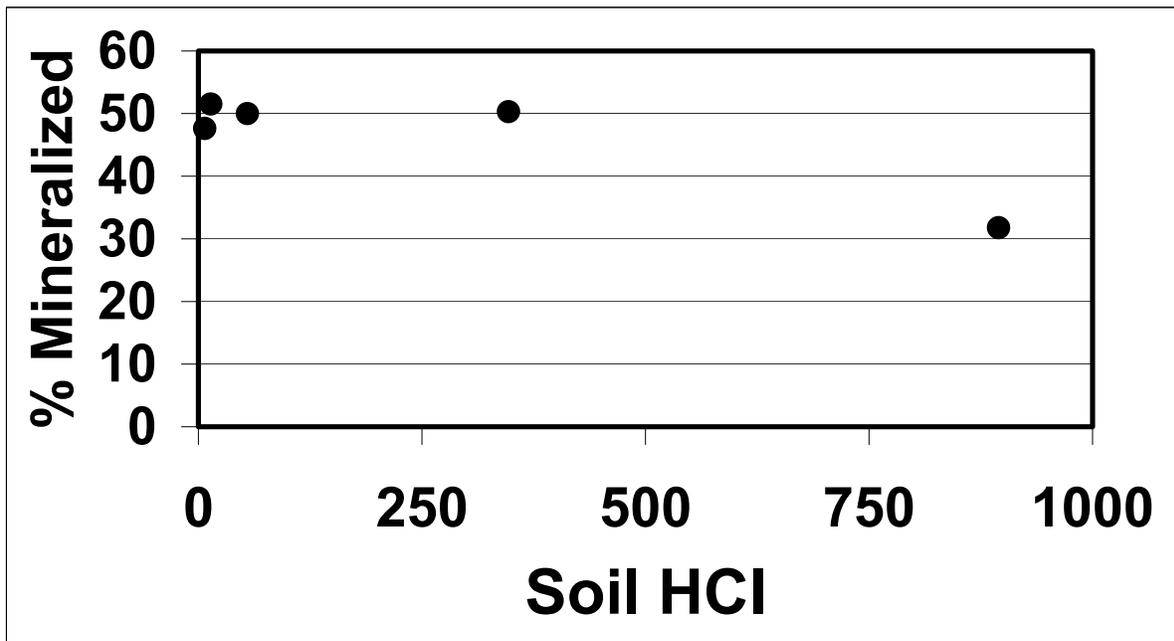


FIGURE 18. PHENANTHRENE MINERALIZATION (*MYCOBACTERIUM*)

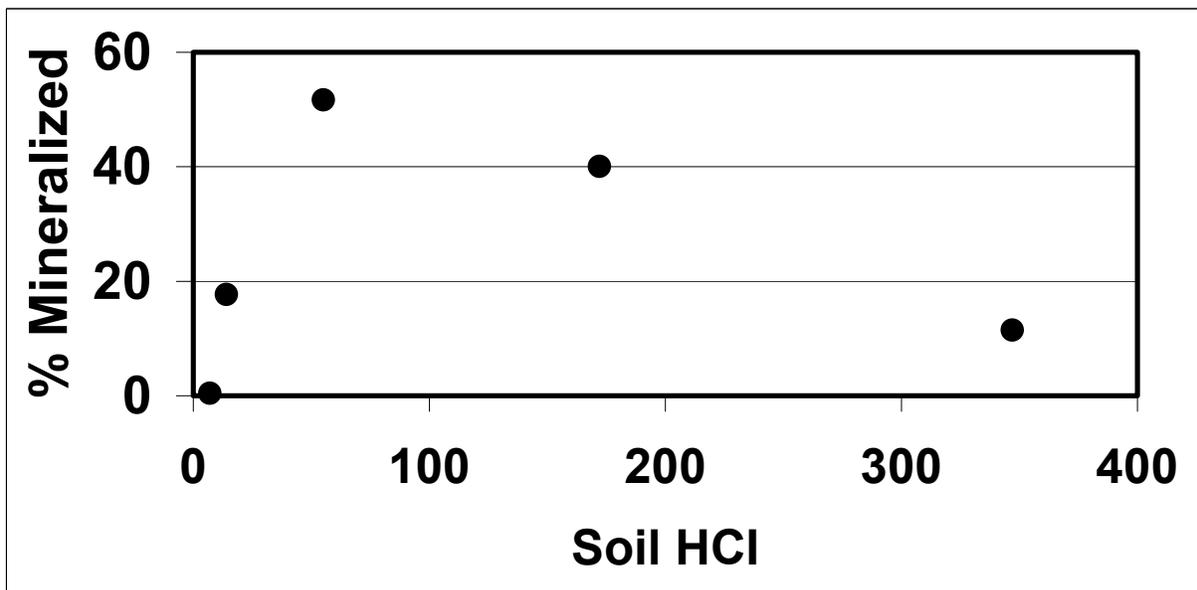


FIGURE 19. PHENANTHRENE MINERALIZATION (*BURKHOLDERIA*)

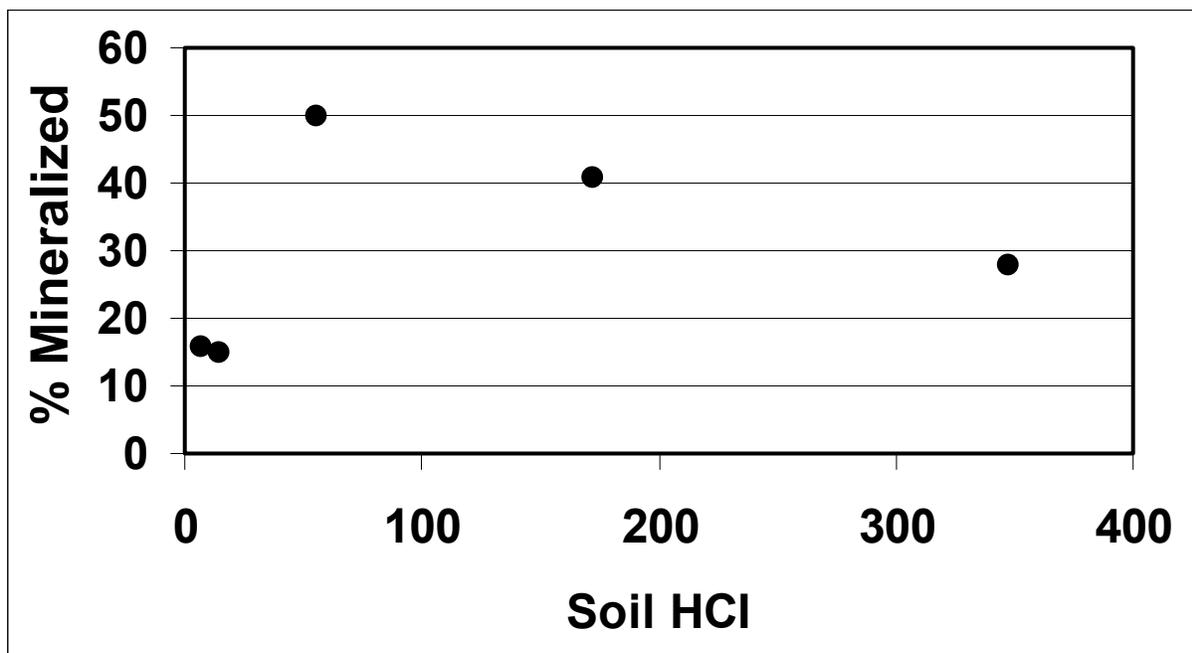


FIGURE 20. PHENANTHRENE MINERALIZATION (*ACIDOVORAX*)

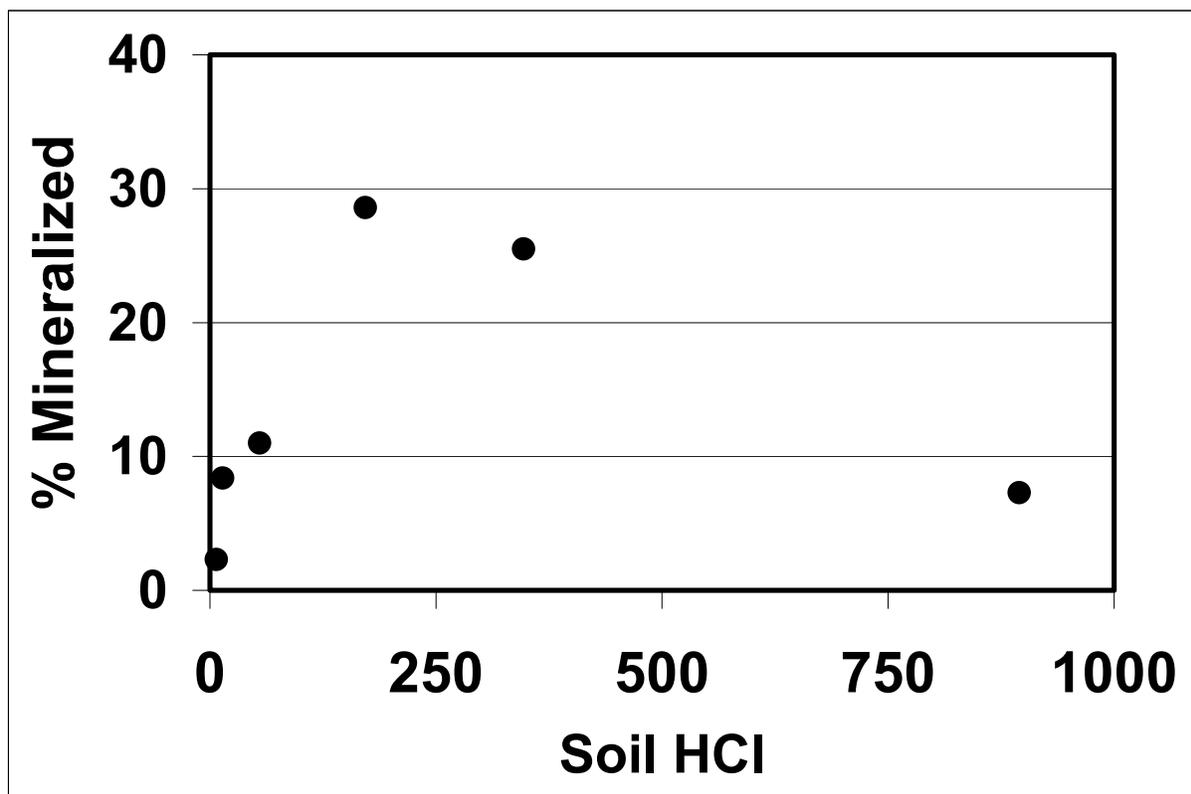


FIGURE 21. HEXADECANE MINERALIZATION (*ACINETOBACTER*)

The fact that each bacterial strain apparently has an optimal value for the extent of soil-particle coverage by HA and FA with respect to contaminant biodegradation can be explained as follows. At HCl values below the optimal for a particular bacterium/contaminant combination, diffusion into humin micropores is more rapid and extensive, because the HA/FA layer is insufficient to retard this process. As entrapment within these pores becomes more pronounced, biodegradation is retarded. Conversely, above the optimal HCl, the HA/FA complex itself becomes the main sequestering site, through a combination of hydrophobic interactions (adsorption) and steric constraints (dissolution and “burying”).

It is interesting to note that there are several differences in the various bacteria with respect to their biodegradation profiles. For example, *Mycobacterium*, both on pyrene and phenanthrene, has a very broad optimal HCl; only soil #1, which has the lowest HA/FA content (is essentially completely dominated by humin) shows reduced PAH degradation. This is consistent with the fact that *Mycobacterium* species are known to be much more hydrophobic than other genera, and

therefore are much more adept at degrading strongly-sorbed contaminants (such as those which would be present in a high-HCI soil). Several other bacteria also display a reduced tendency in this direction, as the slopes of the downward lines (above optimal HCI) are much more shallow than others. An example of this is the fact that *Acidovorax* mineralization of phenanthrene drops off much less steeply at high HCI values than does that by *Burkholderia*; one possible explanation for this is that *Acidovorax* is, as *Mycobacterium* has already been established to be, better adapted to degradation of sorbed contaminants.

In order to test if these differences could be mechanistically attributed to the sorption of contaminant to humic acid, we conducted mineralization studies, using 8 different bacteria, to compare degradation of free phenanthrene with that of phenanthrene that had been first sorbed onto humic acid. All of these experiments were conducted under solid-state conditions (on agar surfaces in culture bottles), so as to avoid effects of PAH dissolution in bulk liquid phase, and require the bacteria themselves effect that contaminant solubilization. Results were as shown below, and indicated that bacteria could be divided into three categories: those which showed decreases in PAH mineralization when the PAH was sorbed to HA (the two *Burkholderia* strains), those which were unaffected by HA (two *Sphingomonas* species), and those which showed higher PAH degradation when HA was present (*Pseudomonas*, *Acidovorax*, two *Mycobacterium*). With respect to the last of these, we note that other authors (Holman *et al.* 2002) have observed the same behavior among *Mycobacterium* species, and have begun to model the process with the aid of synchrotron IR-spectroscopy experiments. With respect to our biodegradation results, it is interesting to note that strains which have either (A) broad optimal HCI values (*Mycobacterium*) or (B) reduced effects of high HCI (*Acidovorax*) tend to be those in which PAH degradation is enhanced *in vitro* by humic acid; apparently, this is true in soil systems as well. Conversely, *Burkholderia*, which (above) shows a steeper decrease in phenanthrene mineralization with increasing soil HCI, is the sole genus among those which we studied in which phenanthrene mineralization seems to be slightly impeded by humic acid; thus, these results agree as well.

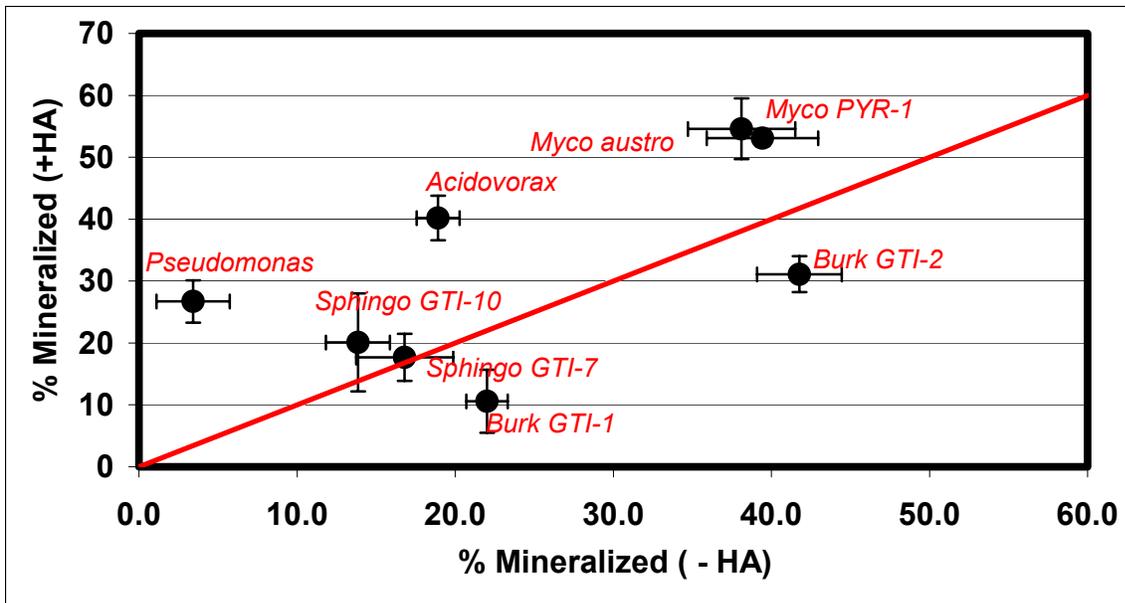


FIGURE 22. OXIDATION SUSCEPTIBILITY OF COAL TAR HYDROCARBONS IN SOILS

HCI concept is being used to explain PAH susceptibility to Fenton’s reaction over time in various soils. All data that has been collected to date (*see results previously reported for Fenton’s/sequestration experiments*) implies that there is an optimal HCI for chemical treatment of contaminants, with treatment efficiency decreasing above and below this value, similar to biodegradation. Mechanisms (pore entrapment at sub-optimal HCI, HA adsorption and dissolution at supra-optimal HCI) may be the main controlling means and all are probably similar as well.

Susceptibility to chemical oxidation of coal tar hydrocarbons in soils exhibits a response based on the effect of soil nanoporosity on sequestration. As reported previously, one model soil (#2) of intermediate initial porosity was subjected to 3 or 6 cycles of autoclaving; this was done in an attempt to alter soil pore structure and content with minimal disruption of other parameters (TOC content, chemical composition of OM, etc). Soils were sent to outside lab (Pennsylvania State University) for porosity measurements. These indicated that while soil micropore content in the soil was slightly decreased by autoclaving; average pore diameter was significantly increased (implying that the total number of pores, especially small ones, must have been decreased). The surface area was also dramatically decreased.

As in previous experiments, model soils spiked with coal tar (1000 ppm total tar concentration, resulting in total PAH content of ~250 ppm) were used. Degradability of priority

pollutant tar PAH was determined in H₂O₂/Fe(II) reactions (abiotic) after 40 days contact time – results were very inconsistent, and did not show any clear correlation with the porosity changes which were measured in the soil. One possible explanation is that the different autoclaving treatments caused substantial changes in other physicochemical characteristics of the soil that complicated this interpretation.

Effects of Fulvic Acid Supplementation on Contaminant Biodegradability

Various authors have observed conflicting results as to whether or not addition of soluble or semi-soluble humic material (FA or HA) enhances contaminant degradation; some studies with PAH and PCB have concluded that such supplementation is beneficial, while others have found it to be non-effective or even detrimental. An HCI model can be applied to explain these conflicting results. As previously reported, addition of either 10 or 20 milligrams of Minnesota peat fulvic acid to soil #1 prior to pyrene spiking greatly enhanced the ability of *M. austroafricanum* GTI-23 to mineralize pyrene in this soil. On the other hand, such enhancements were much lower (or nonexistent) when the experiment was repeated with pyrene in two other soils (#2 and #5), or with phenanthrene in soil #1. The HCI concept appears to explain this, as only soil #1 was found (see above) to be below the optimal HCI value for pyrene mineralization by our strain of *Mycobacterium*. Thus, addition of more FA to the overlayer would not be expected to enhance contaminant degradation. Similarly, all soils (except #4), including soil #1, were found to be at the optimal HCI for phenanthrene mineralization by this strain; thus, inclusion of more FA in this case would also not be expected to enhance PAH biodegradation.

In contrast, addition of FA to the same three soils prior to spiking with hexadecane had much different effects on the mineralization of this hydrocarbon by *Acinetobacter*.

Table 5. Percentage Hexadecane mineralized with with Various Amounts of Fulvic Acid by *Acinetobacter*

Soil	0% FA	0.5% FA	1.0 % FA
1	1.14 ± 0.15	1.41 ± 0.10	1.52 ± 0.01
5	2.16 ± 0.17	2.87 ± 0.41	3.68 ± 0.38
2	2.50 ± 0.15	3.14 ± 0.71	5.68 ± 0.52

Again, this is explainable in light of the HCI model. All three of these soils are below the optimal HCI for hexadecane biodegradation by *Acinetobacter*, and would therefore all be expected

to show some mineralization enhancement through addition of FA. Furthermore, from the results shown above, the steepness of the increase in mineralization is much greater at values which are closer to the HCI; thus, small increases in FA (or HA) would result in larger increases in mineralization in this region of the “curve” than at lower HCI values. Since soil #2 is the closest of these three to the optimal HCI (followed by #5 and then #1), it is not surprising that FA addition to this soil would produce the most dramatic increase in hexadecane mineralization.

Microtox® Acute Assays

Microtox® Acute assays (aqueous phase) have been applied for the last 10 years as an easy, cost-effective, and rapid toxicity screen for aqueous eluates from various waste samples. Microtox assays evaluate the toxicity of samples by measuring a reduction in bioluminescence in a marine bacterium, *Vibrio fischerii*. To perform the acute Microtox® assay, an aqueous sample is serially diluted and placed into test vials. After osmotic adjustments, the bacteria are introduced in each vial and exposed for 5 to 30 minutes. A toxic response is quantified by measuring the reduction in bioluminescence in the dilution series using a specially designed spectrophotometer. The endpoint measured by the assay is the amount of bioluminescence inhibition after incubation with the aqueous sample. This is expressed as an EC₅₀, or the effective concentration of the aqueous sample that results in a 50% reduction in light production compared with a negative, water control. Microtox® Acute assays were performed in duplicate on eluates from the studies, as indicated above.

Microtox® Solid-Phase Assays.

Microtox® Solid-Phase assays, marketed as test kits by Azur Environmental, Carlsbad, California, were used to obtain additional soil toxicity data. In these tests, toxicity is sensed by a decrease in metabolism, and thus a decrease in light output, which can be sensed by a modified spectrophotometer. Microtox® software then calculates the EC₅₀ and 95% confidence intervals from duplicates of each sample analyzed. The difference between the Microtox® Acute assay and the Microtox® Solid Phase test is in the way that the bacteria are combined with the sample. Bacteria are combined with an aqueous extract of the soil sample in the acute phase test, while actual particles of contaminated soil are combined with bacteria in the solid phase test. Microtox®

Solid-Phase tests were used to determine baseline toxicity in the soil samples selected for study. The manufacturer’s protocols were used for all Microtox[®] tests.

Toxicity Analyses

As an examination into the toxicity of aqueous extracts (simulated leachates) of crude oil, three different extractions were run. Crude oil (1 ml, 10 ml, or 25 ml) was extracted (via shaking for 24 hours) with 100 ml of distilled water. Each of these extracts was then subjected to Microtox toxicity analysis. The EC₅₀ values thus determined were as follows:

TABLE 12. TOXICITY OF AQUEOUS EXTRACTS OF CRUDE OIL BY MICROTOX

Oil – Water Ratios	EC₅₀ (5 min. exposure)	EC₅₀ (15 min. exposure)
1 ml oil extracted w/100 ml H ₂ O	34.85% v/v	40.31% v/v
10 ml oil extracted w/100 ml H ₂ O	10.12% v/v	11.74% v/v
25 ml oil extracted w/100 ml H ₂ O	9.18 % v/v	8.25% v/v

Thus, it is evident that, even at an oil:water ratio (on a volume basis) of 1:100, a 24-hour aqueous extraction of sweet crude oil resulted in the solubilization of toxic products. However, although extraction of the same oil at the 1:10 or 1:4 ratio yielded an extract that was more toxic (as evidenced by lower EC₅₀ values), this relationship was not linear when compared to the volume of oil extracted. One possible explanation for this observation is that the solubility limit of some subset of the toxic factors is reached at a ratio of oil to water somewhere between 1:100 and 1:10; increasing the amount of oil extracted therefore does not result in further increases in the concentrations of these factors. Alternatively, a greater amount of “clumping” may take place with higher oil:water ratios, which would be expected to impose mass-transfer limitations on the solubilization of the toxic components of the oil.

In the interest of method development and validation, we have run Microtox solid-phase tests on soil taken from a New Jersey MGP (manufactured gas plant) site. In order to estimate the accuracy and precision of this method, we ran this analysis on the same soil four times in-house. Our average EC₅₀ value was 1073 mg of soil/L; in other words, slightly over 1 gram of this soil per liter of medium was sufficiently toxic as to reduce the light output from *Vibrio fischeri* by 50%. The pertinent data for EC₅₀ values and statistical analyses of our four trials were as follows:

TABLE 12A. FOUR TRIALS TO VERIFY THE MICROTOX ANALYSES

Trial	EC₅₀ (mg/L)	95% Conf. range	95 % Conf factor	R² of data points
#1	1812	1136-2893	1.596	0.838
#2	806	573-1133	1.406	0.903
#3	544	386-769	1.412	0.947
#4	1130	908-1405	1.244	0.958

We also shipped a subsample of the soil to a contract lab (CH2M Hill, Corvallis, OR) with significant experience in Microtox solid-phase analysis for validation of our results. Their analysis yielded an EC₅₀ value of 1092 mg/L (95% confidence interval 821-1454 mg/L), which is clearly in very good agreement with our results. Comparisons of CH2M Hill's 95% confidence factor (1.332 vs. our average 1.414) and R² of data points (0.938 vs. our 0.912) with our figures also increases our confidence that we were able to run this assay over the course of this project and obtain meaningful results.

Optimization of Biological Treatment Factors

The ultimate removal of the total petroleum hydrocarbons and any other organic contaminants by CAB is due to enhanced biological treatment. This research activity identified, defined, and maximized co-treatments, additions, and supplements needed to reach complete remediation of the site using CAB.

The deliverables of this task were:

- a collection of operational conditions for optimum biological activity;
- analytical processes and supplies for field evaluation;
- analytical processes and supplies for laboratory evaluation; and
- critical features for application of CAB with EAE evaluation.

This research activity used the data collected in the earlier research tasks to optimize the application of the biological phase of the CAB process. Based on experience gained in this project and others at GTI, ease of use at field scale was also determined. Operational protocols that maximize the biological effectiveness at field and full-scale operation were generated at this point of the research.

Soil Collection and Characterization

Numerous representative crude oil-contaminated soils were obtained from the vicinity of wellheads in an oil field in south central Illinois, in addition to experimental soils that were artificially contaminated with TPH and PAH. The first of these, designated Soil AB, was reportedly contaminated with oil pumped from the Salem limestone formation (Mississippian Age), at a depth of approximately 3400 feet. The second soil, designated C, contained oil which originated in the Aux Vases sandstone formation (also Mississippian), at a depth of 2700 feet. Based on conversations with various individuals in the oil industry, these oils should be very representative of crude oils produced throughout the central U.S., including Oklahoma and Texas.

The following baseline physicochemical analyses have been conducted on the samples: textural classification, field moisture holding capacity, pH, oil and grease content, and diesel-range organics (DRO). Soil textural classification was done using the hydrometer method (Sheldrick and Wang 1993). Soil pH was measured according to EPA Method 9045. Oil and grease content of the soils was determined by EPA Method 9071A (four-hour Soxhlet extraction with hexane, followed by evaporation of solvent and gravimetric determination of extracted material). Diesel-range organics measurement was based on the “modified DRO” method published by the Wisconsin DNR, in which samples are dehydrated with sodium sulfate, ground, and Soxhlet extracted (18 hours) in methylene chloride; analysis of the extracts is then conducted by GC.

TABLE 13. A PRELIMINARY TEXTURAL CLASSIFICATION OF THE TWO HYDROCARBONS-CONTAMINATED SOILS

Sample	% Sand	% Clay	%Silt
Soil AB	72 (+/- 2)	7.73 (+/- 0.01)	20 (+/- 2)
Soil C	75 (+/- 2)	4 (+/- 1)	20.81 (+/- 0.01)

Thus, each of the two soils would be classified as loamy sands according to the USDA’s Guide to Textural Classification. Unfortunately, these tests were conducted before the oil and grease analyses were carried out; the hydrometer procedure described by Sheldrick and Wang is not recommended for soils with a total organic carbon content of greater than 2%. The TOC of both of these soils was clearly much higher than this, as the oil and grease content alone (see below) exceeds these values.

The pH of the soils was found to be as follows: Soil AB was found to have a pH of 5.9 (+/- 0.2), whereas that of Soil C was found to be 7.6 (+/- 0.1). Thus, Soil AB is somewhat acidic, and Soil C is very slightly alkaline.

Both soils contained very significant levels of oil and grease, indicative of being significantly impacted by past hydrocarbon spills. Soil AB contained 12.48% (+/- 0.08%, n=3) oil and grease by weight. Soil C was actually somewhat higher still, with a total oil & grease content of 13.7% (+/- 0.3%, n=2). Diesel-range organics (DRO) have also been quantitated in the two soils, totaling 960 ppm (+/- 100 ppm, n=2) in Soil AB, and 1540 ppm (+/- 30 ppm, n=2) in Soil C.

Microbial analysis

Isolation of PAH-degrading bacteria from the two soils has been done by standard techniques, based on the formation of “clearing zones” in a layer of pure PAH which has been overlaid onto a plate of agar media previously inoculated with bacteria isolated from the soil in question. This method eliminates the possible introduction of other potential carbon sources (solvents or aerosol carriers) for the bacteria. Briefly, a Pyrex petri dish is filled with sand and heated to a temperature which is sufficiently high for the PAH of interest to sublime. An aluminum dish containing the pure PAH is placed on the sand. A previously inoculated plate of minimal media is then placed upside down above the dish containing the hydrocarbon, until an even layer of precipitated PAH (that is to be the only carbon source) forms on the agar. The sublimated plates are incubated at 30° C and observed for zones of clearing around the bacterial colonies. A zone of clearing, such as those shown in Figure 1, indicates that the bacterial colony is utilizing the hydrocarbon for its growth; this colony can then be sub-cultured for further study. Colonies with zones of clearing were carefully sub-cultured onto new plates until a pure culture (*i.e.* one in which microscopic observation shows only one type of bacteria) was obtained.

Bacterial cultures, once isolated, were tested for degradation of other hydrocarbons. This was done in 96-well polystyrene plates, in a variation of a previously published method (Gordon, *et al* 1993). The wells of the plate contain individual hydrocarbons (anthracene, benzo[*a*] pyrene, benzo[*b*]fluoranthene, chrysene, 1,2,5,6-dibenzanthracene, fluoranthene, fluorene, phenanthrene, and pyrene). These hydrocarbons are dissolved in solvent and when transferred to the microplate, the solvent is allowed to evaporate. Growth utilizing these hydrocarbons was then quantified.

Microbial Analysis

We have been able to isolate several PAH-degrading bacteria from the E & P soil samples that are being used in this work. Using plates sublimated with phenanthrene, we have thus far isolated two species, which have been sent out for identification. A minimum of two (perhaps three) further species are in the process of being isolated. Identifications of these species are expected shortly. In addition, we have to date isolated and tentatively identified 5 species from a series of PAH- and hydrocarbon-contaminated manufactured gas plant (MGP) sites. Included among these are the following:

TABLE 14. ISOLATED MICROORGANISMS FROM HYDROCARBON-CONTAMINATED SOILS

Isolate	Level of match	Source
<i>Pseudomonas veronii</i>	Species (MicroSeq & GenBank)	New Jersey MGP soil
<i>Burkholderia</i> sp.	Genus (MicroSeq & RDP)	New Jersey MGP soil
<i>Burkholderia</i> sp.	Genus (MicroSeq & GenBank)	French MGP soil
<i>Sphingomonas</i> sp.	Genus (MicroSeq, GenBank, RDP)	New Jersey MGP soil
Soil bacterium is110	Genus (GenBank)	Iowa MGP soil

All isolates were evaluated using the microplate method, as described above, to determine their PAH substrate ranges. To date, we have observed growth of the two *Burkholderia* species, *Sphingomonas* sp., and Soil Bacterium is110 on all PAH tested. In none of these cases was significant growth (as scored by production of purple formazan) detected in wells that received only medium (no carbon source), INT and bacteria. This analysis has been complicated somewhat by the fact that our automated microplate reader has given higher numbers for wells that do not show any purple color than wells that are clearly purple. One possible explanation for this is that the hydrocarbon leaves an opaque film on the bottom of the well, increasing the A_{405} of that well. Thus, the plates must be visually scored, in order to avoid false positives for growth. New microplates have been purchased that clearly do not react with the solvent used, but these still give higher readings in the cases of some PAH, due to the absorbance of the hydrocarbon itself. At this point, it is therefore very difficult (if not impossible) in many cases to make *quantitative* statements comparing growth under different conditions (*e.g.* on phenanthrene *vs.* benzo[*a*]pyrene) using this method.

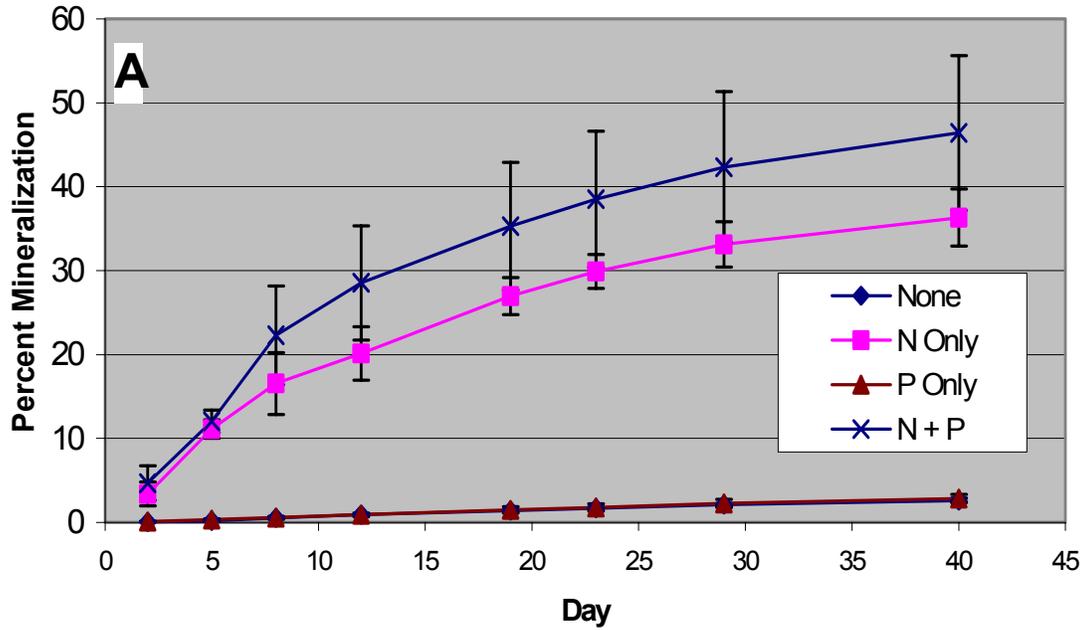
Nutrient Requirements and Delivery

The effect of macro- and micronutrients were tested. Phosphorus content and other nutrients influenced bioremediation of soils contaminated with crude oil. The rate of biodegradation was enhanced with the addition of nitrogen compounds and phosphorus compounds. All soil tested, however, was capable of biodegradation of the hydrocarbons tested without addition of either nutrient, but at often markedly lower rates and extents. Due to the availability and low-cost of soil fertilizers, we recommend their use in applications of CAB. No micronutrients were found to be needed.

Determination of Nutrient Limitations in E&P Soils

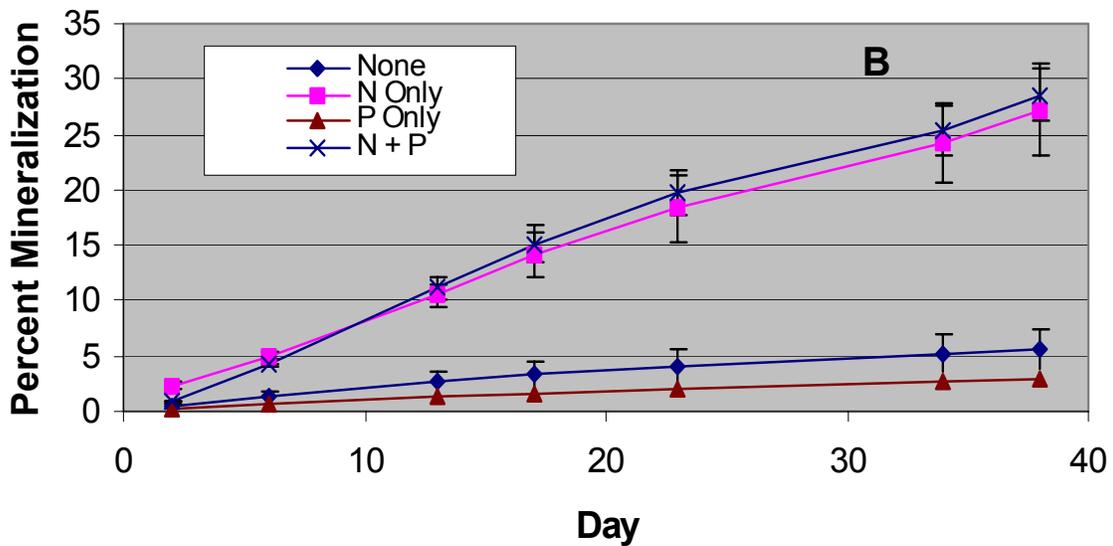
The extents of hexadecane mineralization in slurry microcosms containing indigenous microbes from the two oil-contaminated E&P soils are shown in the figure below. Both of the two soils tested evidenced very significant limitations for nitrogen; this was most notable in soil AB, in which the amount of $^{14}\text{CO}_2$ released through hexadecane mineralization was approximately 17-fold higher in N-supplemented conditions versus those with no nutrient addition. Similar patterns were seen in soil C, although the degree of stimulation by supplemental N was not as high as in soil AB. The other notable difference between the two soils was in the significance of the phosphorus limitation: While soil AB supported significantly higher mineralization when both N and P were added (vs. N only), the two conditions behaved virtually identically in soil C.

FIGURE 23A. EFFECTS OF N AND P ADDITION ON MINERALIZATION OF HEXADECANE BY MICROBES INDIGENOUS TO OIL-CONTAMINATED E&P SOILS.



Graph A = Soil AB; Graph B = Soil C

FIGURE 23B. EFFECTS OF N AND P ADDITION ON MINERALIZATION OF HEXADECANE BY MICROBES INDIGENOUS TO OIL-CONTAMINATED E&P SOILS.



Graph A = Soil AB; Graph B = Soil C

Thus, it can be concluded that, in terms of degradation of oil-related hydrocarbons, the microbes in soil AB are strongly limited by their available N supply, and, when N is added in sufficient levels, a significant P limitation also becomes apparent. In contrast, soil C contained sufficient P supplies that N alone was the limiting factor for hydrocarbon degradation. Given that the goal of the experiments undertaken here was to evaluate potential gaseous N and P sources for their effects on hydrocarbon remediation, soil AB was selected for further work, due to the fact that it was found to be limited for both nutrients. The ability of the indigenous microbes in soil AB to mineralize hexadecane was then investigated under different conditions of nitrogen and phosphorus supplementation. The results of this experiment are shown in figure below.

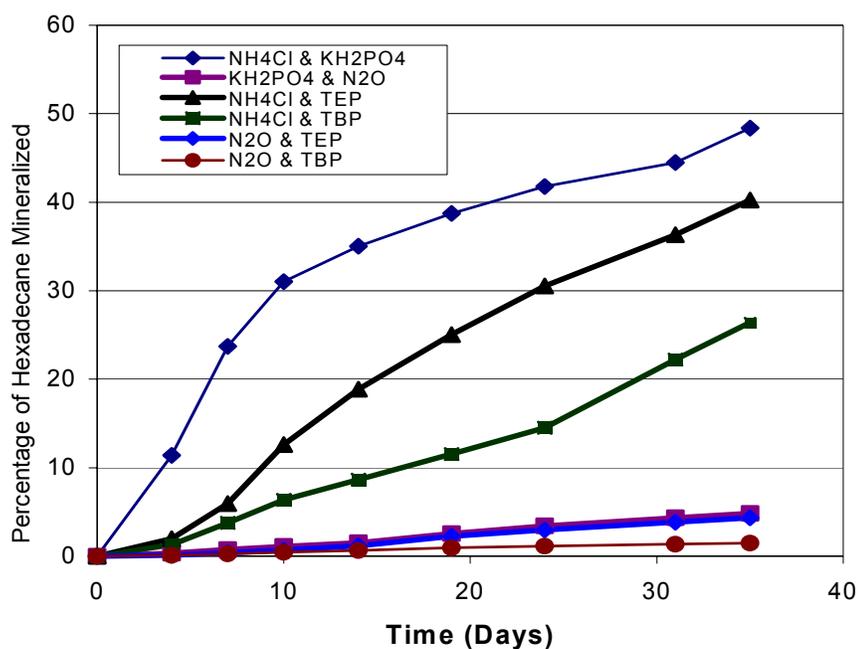


FIGURE 24. ABILITY OF GASEOUS N AND P COMPOUNDS TO OVERCOME NUTRIENT LIMITATIONS AND SUPPORT HEXADECANE MINERALIZATION BY INDIGENOUS MICROBES IN SOIL AB.

Ability of Gaseous Nutrients to Support PAH Degradation

The amounts of phenanthrene and pyrene mineralized by cultures of the microbial community from Soil AB during the first 8 days of incubation are shown below. In the case of

phenanthrene, the data thus far indicate that the “traditional” nutrient amendment regime (*i.e.* solutions of ammonia nitrogen and inorganic phosphate) have given the best results, although several other conditions (most notably NH_4Cl and triethylphosphate) have also supported significant conversion of phenanthrene to CO_2 . It seems from this data that either NH_4Cl or inorganic phosphate can be replaced by a gaseous nutrient; however, it should be noted that replacement of both (*i.e.* those cultures with N_2O and either TEP or TBP) results in mineralization totals that are very much reduced from the optimal conditions. In contrast, in the case of pyrene mineralization, it can be seen from the data below that both $\text{NH}_4\text{Cl}/\text{TBP}$ and $\text{N}_2\text{O}/\text{TBP}$ have (thus far) outperformed the combination of NH_4Cl and inorganic phosphate. One possible explanation for the significant differences in the patterns of phenanthrene and pyrene mineralization across the spectrum of nutrient conditions studied in this experiment is that different bacterial species are involved in the two processes. Whether or not this is in fact the case, it is noteworthy that, among three soils we have examined in experiments such as these (two MGP site soils and the present E&P soil), this is the first in which an actual strong “preference” has been shown for an organic phosphate (TBP) by a PAH-degrading soil bacterium.

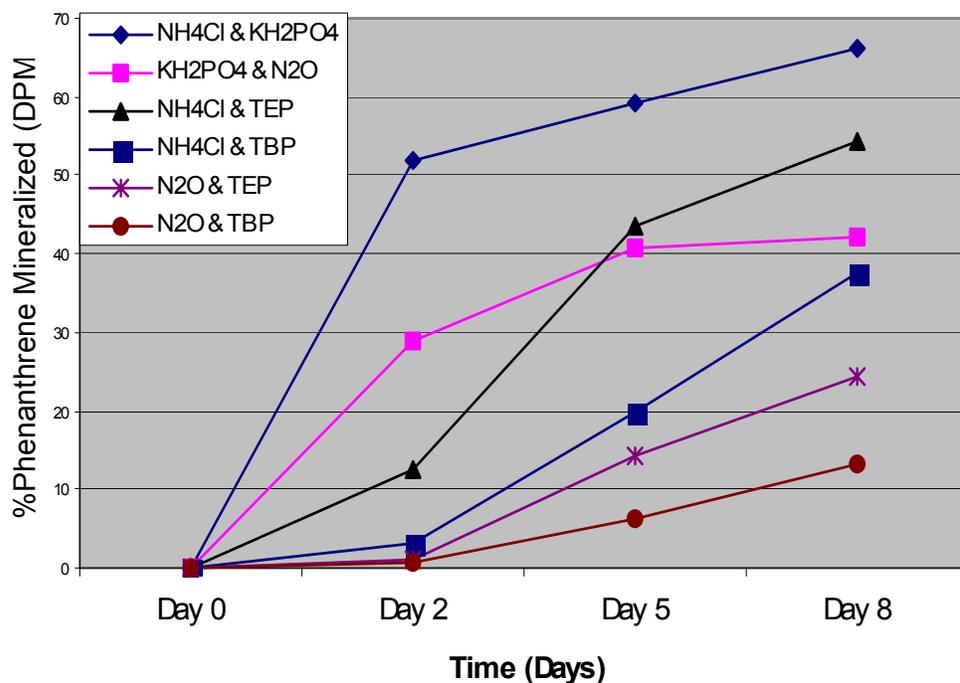


FIGURE 25. GROWTH OF ISOLATE ON PHENANTHRENE IN THE PRESENCE OF GASEOUS NUTRIENTS

Use of TEP by Individual Bacterial Strains

The abilities of various bacterial strains to grow, on either glucose or phenanthrene as a sole source of carbon and energy, were compared with potassium phosphate (KH₂PO₄) or TEP serving as the source of phosphorus. Results for this experiment were as follows (all data are absorbance readings at $\lambda = 600$ nm during stationary phase of culture, and represent averages and standard deviations of triplicate determinations).

Thus, several bacterial strains show marked preferences for KH₂PO₄ over TEP. The most notable examples of these include *Acidovorax* (when growing on glucose) and *Pseudomonas* strain #6 and *Burkholderia* strain #2 on phenanthrene; the latter is particularly significant, with nearly 5-fold better growth occurring on inorganic phosphate than on TEP.

TABLE 15. USE OF TEP BY INDIVIDUAL BACTERIAL STRAINS

	Glucose		Phenanthrene	
	KH ₂ PO ₄	TEP	KH ₂ PO ₄	TEP
<i>Acidovorax</i>	1.041 ± 0.11	0.63 ± 0.12	0.20 ± 0.01	0.23 ± 0.02
<i>Burkholderia 1</i>	0.80 ± 0.05	0.86 ± 0.21	0.74 ± 0.01	0.49 ± 0.03
<i>Burkholderia 2</i>	0.57 ± 0.16	0.63 ± 0.18	0.54 ± 0.09	0.11 ± 0.01
<i>Burkholderia 3</i>	0.54 ± 0.09	0.49 ± 0.01		
<i>Pseudomonas 1</i>	0.19 ± 0.02	0.13 ± 0.01	0.18 ± 0.02	0.13 ± 0.01
<i>Pseudomonas 2</i>	0.20 ± 0.02	0.25 ± 0.02	0.15 ± 0.07	0.19 ± 0.16
<i>Pseudomonas 4</i>	0.73 ± 0.02	0.93 ± 0.07	0.72 ± 0.23	0.52 ± 0.19
<i>Pseudomonas 6</i>	0.18 ± 0.02	0.11 ± 0.02	0.11 ± 0.01	0.06 ± 0.02
<i>Sphingomonas 1</i>	0.71 ± 0.08	0.49 ± 0.02	0.36 ± 0.12	0.24 ± 0.02
<i>Sphingomonas 3</i>	0.74 ± 0.01	0.58 ± 0.04		

Bacterial Survival during Chemical Pretreatment

We are currently in the process of preparing antibiotic-resistant mutants of PAH-degrading strains from two soils for use in this experiment. The first of these is an MGP site, located in Iowa; the second is the southern Illinois oil field site that we are studying as a representative E&P site. As an illustration of how this is done, preliminary studies on the former of these indicated that concentrations of streptomycin in the range of 2.5 mg/ml of agar were required to restrict

growth of all microorganisms (including wild-type PAH-degraders) in site soil when plated on R2A agar. Thus, we prepared two-layer agar plates by the method of Davies and Whitbread (1989), in which this concentration of streptomycin was present in the lower layer, with a non-streptomycin-containing overlayer as shown in the following cross-section.

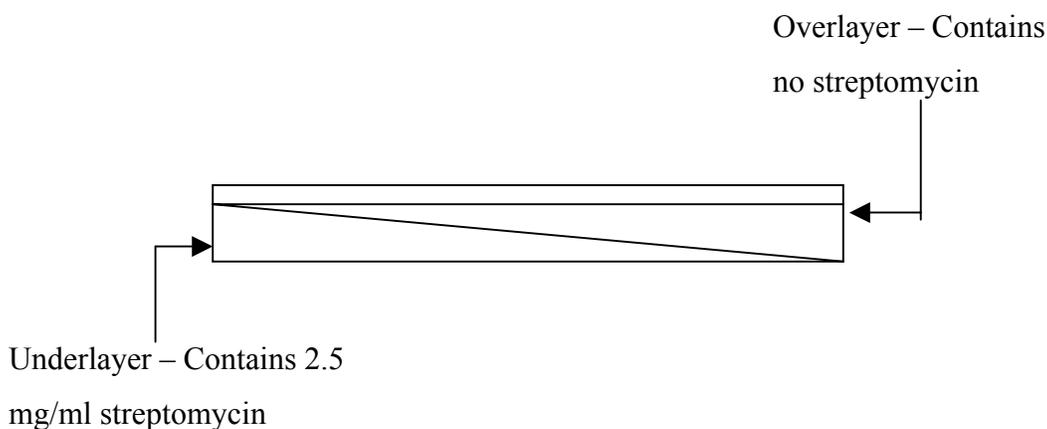


FIGURE 26. METHOD TO ISOLATE MICROORGANISMS RESISTANT TO CHEMICAL TREATMENT

After an appropriate equilibration period (*ca.* 24 hours), the surface of the plate contains a gradient of very low (approx. 0) to high (approx. 2.5 mg/ml) of streptomycin. PAH-degraders from the site soil are then spread-plated, and (spontaneously arising) mutants are sought which grow nearest the high-antibiotic side of the plate. These can then be tested to ensure that they are capable of growth on the concentration of streptomycin that is required to kill all other microbes from the soil; if necessary, the selection process can be re-run on plates with a higher concentration of antibiotic in the underlayer. We have isolated mutants of one PAH degrader from the Iowa MGP soil, and three from the Illinois E&P soil. Once isolated, these were used in experiments such as those described above.

The extent to which various bacterial species and strains are capable of surviving aqueous-phase Fenton's reactions is shown below.

TABLE 16. SURVIVAL OF VARIOUS BACTERIAL SPECIES TO AQUEOUS FENTON'S REACTION

Bacteria	# of Trials	% Survival
<i>Acidovorax sp.</i>	1	8.5
<i>Bacillus simplex</i> **	1	98.1
<i>Burkholderia 1</i>	1	0.2
<i>Burkholderia 2</i>	2	25 ± 13
<i>Burkholderia 3</i>	1	0
<i>Mycobacterium austroafricanum</i>	2	85 ± 13
<i>Mycobacterium phlei</i>	1	61
<i>Pseudomonas 2</i>	2	5 ± 5
<i>Pseudomonas aeruginosa R75</i> **	1	0.5
<i>Pseudomonas fluorescens R111</i> **	1	0
<i>Rhodococcus sp.</i>	3	11 ± 10
<i>Sphingomonas 1</i>	1	0.3
<i>Sphingomonas 4</i>	1	0
<i>Sphingomonas 8</i>	1	0

** Indicates non-PAH-degrading strain included as a representative of general soil bacteria.

There is no clear pattern for survivability of oxidative conditions (presence of hydroxyl radical generated through Fenton's reaction) across PAH degraders vs. non-degraders. The ability of *Bacillus simplex* to withstand •OH is probably related to endospore formation, as opposed to any other biochemical characteristic; we are currently in the process of obtaining non-sporulating *Bacillus* strains (*B. subtilis*) in order to verify this. Apart from *B. simplex*, the only isolates that have shown the ability to consistently withstand Fenton's reaction conditions are Mycobacteria (*Mycobacterium austroafricanum* and *Mycobacterium phlei*). We believe this to be due to the presence in this genus of an extensive, waxy hydrophobic outer coat; one other Mycobacterium (*Mycobacterium sp.* PYR-1) is also being evaluated to better determine the distribution across the genus of the ability to withstand •OH.

Co-Metabolites

The more recalcitrant hydrocarbons may be degraded at a higher rate and to a lower endpoint when the phenomenon of co-metabolism is utilized. Co-metabolism is when a microorganism or community of microorganisms transforms a contaminant that does not serve as

an energy/carbon source. Other compounds act as primary substrates for the growth and activity of the microbial communities. The activities against the 4-6 ringed PAH moieties in CAB for MGP sites seen in previous experimentation may have been due to the effects of co-metabolism.

Primary substrates to be investigated include short chain hydrocarbons and fatty acids, Fenton's oxidation products of hydrocarbons, and microbial metabolite pathway intermediates in the degradation of hydrocarbons, such as acetate and succinate. Preliminary CAB experiments have shown dramatic improvement in biological activity against benzo(a)pyrene with the addition of two possible primary substrates, succinate and phenanthrene. Figure 5 indicates the increase in rate and extent of benzo(a)pyrene degradation when phenanthrene is added to the system. Data indicate that both the rate and extent of degradation is more than triple that of benzo(a)pyrene alone. Additional experimentation is required to improve both the rate and extent of degradation. Concentration effects, multiple co-metabolites, environmental conditions, the presence of soil, and multiple hydrocarbon moieties have been investigated.

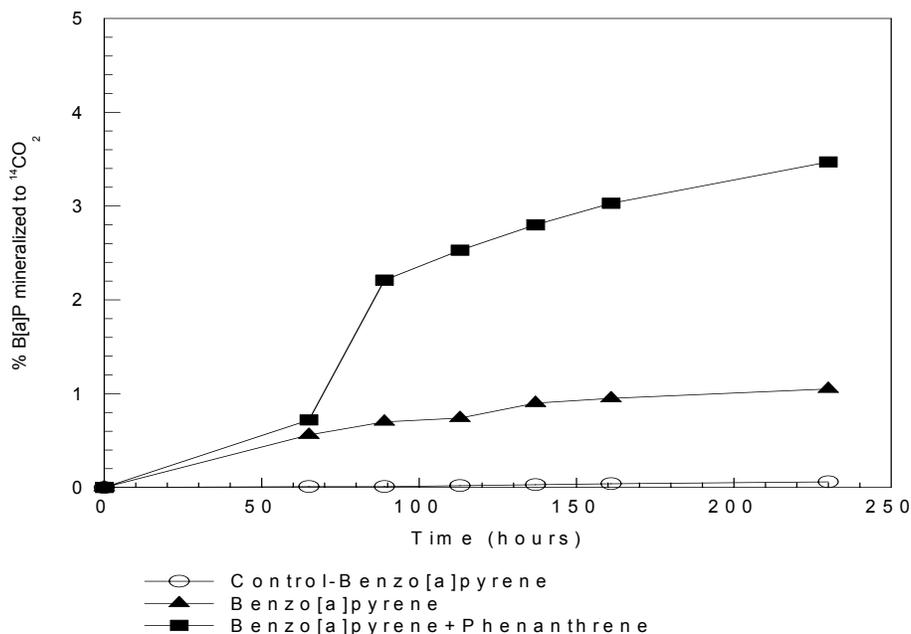


FIGURE 27. CO-METABOLIC DEGRADATION OF BENZO(A)PYRENE

Ten strains (5 *Sphingomonas*, 3 *Pseudomonas*, one each of *Burkholderia* and *Acidovorax*) were tested for induction of phenanthrene mineralization by salicylate. Results (dpm of ¹⁴CO₂

generated after 6 hours) indicated that there was evidence for higher phenanthrene mineralization following exposure to salicylate in three of these isolates.

TABLE 17. SALICYLATE INDUCTION OF POLYNUCLEAR HYDROCARBON DEGRADATION OF VARIOUS STRAINS OF MICROORGANISMS.

	Control (Glucose)	Salicylate-induced	R (S/G)
<i>Acidovorax</i>	3851 ± 310	3151 ± 400	0.82 ± 0.17
<i>Burkholderia 1</i>	288 ± 131	138 ± 42	0.48 ± 0.36
<i>Pseudomonas 1</i>	273 ± 63	209 ± 0	0.77 ± 0.18
<i>Pseudomonas 2</i>	105 ± 1	188 ± 54	1.79 ± 0.53
<i>Pseudomonas 5</i>	379 ± 197	172 ± 30	0.45 ± 0.32
<i>Sphingomonas 1</i>	149 ± 18	143 ± 15	0.96 ± 0.22
<i>Sphingomonas 2</i>	204 ± 7	391 ± 44	1.92 ± 0.28
<i>Sphingomonas 3</i>	180 ± 12	319 ± 89	1.77 ± 0.61
<i>Sphingomonas 5</i>	197 ± 18	251 ± 72	1.27 ± 0.48
<i>Sphingomonas 12</i>	354 ± 5	412 ± 36	1.16 ± 0.12

Thus, these three strains (*Pseudomonas 2*, *Sphingomonas 2* and 3) are being examined, in ongoing experiments, to determine if their capability to mineralize phenanthrene is increased through prior exposure to intermediate products generated by Fenton's reagent treatment of coal tar. Thus far, an initial experiment in this direction has failed to show any induction of PAH (phenanthrene) mineralization due to pre-exposure to oxidized coal tar products. However, this may be due to the fact that possible individual inducer compounds may be present at insufficient levels in the overall product mixture to stimulate degradation at the addition rates that have so far been tested. Further experiments are seen as necessary to clarify this possibility, as is the chemical characterization of the oxidized coal tar products (which is underway using GC/MS). If induction of PAH degradation in one or more bacterial strains by intermediate products of chemical oxidation of coal tar can be shown, we expect to conduct further experiments as follows:

- Parallel experiments examining strains that showed no evidence of salicylate induction – this would be of interest because several of our strains show little to no ability to grow on salicylate, which may explain the lack of inducibility. A pool of oxidized PAH intermediates may contain one or more compounds which would be inducers, even for this group of bacteria.

- Experiments to determine the range of PAH for which induction of degradation (mineralization) can occur.

Optimization of Chemical Treatment Factors

The CAB process involves advanced oxidation to enhance, expand, and accelerate the biological facet of the treatment. Fenton's Reagent and ozone were evaluated to maximize the process. Fenton's Reagent has proven successful to enhance TPH degradation, but methods to make it 'user friendly' in the field were investigated. Ozone was not evaluated due to the efficiency of the Fenton's reaction.

The CAB process is composed of both an advanced oxidation step and a biological or biodegradation step. Individual steps or stages must operate at optimum rates and extents for the entire treatment process to be effective. Advanced oxidation has previously been carried out using the Fenton's reaction. The application protocols have been determined for the field application of CAB and are available for the next stage of this process.

This chemical treatment has proven, in previous studies with MGP and E&P soils, to be effective in: complete oxidation of some of the organic components, including the organic contaminants; partial oxidation of organic compounds in the soil, including the organic contaminants which increase the solubility of the contaminants; the increased solubility and partial oxidation that allow an increase in the biodegradative activity associated with the components; and structural changes in the soil or sediment that allow access of the microbial community to the contaminants.

Fenton's Treatment

The concentrations of both components of the Fenton's Reagent were varied to determine the maximum rate and extent of oxidation of the contaminants in the test matrices. Also, the advantages of order of addition and time between addition of the iron component and the hydrogen peroxide component of the treatment were determined.

Other metals and reduced organic and inorganic compounds may be present in the waste materials, but they appeared to have no effects on the treatment of the organic phase of the contaminants. Variations in the chemical treatment were examined and generated the most effective characteristics for the first phase of treatment.

Integration of Treatment under Simulated Treatment Conditions

The integrated treatment of biological-chemical-biological and chemical-biological-chemical were be evaluated. The objective was to maximize the degradation of hydrocarbons in the contaminated soils under the optimal combination of biological and chemical treatment. The biological treatment used with the indigenous population, and, in some cases, the addition of isolated and enriched biological agents. Chemical treatment was done with H₂O₂ (2-5%); FeSO₄ and other iron compounds; and sodium citrate or other chelating compounds. The eventual field application of a sequence of treatments will be determined after a potential field site have been identified and evaluated.

Fenton's Reagent Generation of Intermediates and Their Identification

Instrumentation

The GC/MS system that we have available in the Environmental Science and Technology Center is a ThermoFinnigan Trace GC PolarisQ ion trap system equipped with an AS2000 liquid sample autosampler. This GC/MS system can be operated in the electron ionization (EI) mode as well as positive and negative chemical ionization (+/- CI) modes. In addition, this GC/MS is capable of an MSⁿ analysis up five times. All of these features are highly valuable for the identification and confirmation of unknown compounds. The samples are introduced into the GC through a programmed temperature vaporizing (PTV) injector. This type of injector can be used as a regular split/splitless injector or as a PTV injector, which is ideal for large volume injection as well as for the analysis of polycyclic aromatic hydrocarbon (PAH).

Quantitative Analysis

The protocol that we use for the quantification of PAH compounds is the modified EPA method for the analysis of semivolatile organic compounds (Method 8270C) by GC/MS selected ion mass (SIM) mode. This method is used to determine the concentrations of PAH as well as other semivolatile organic compounds in extracts from soil, sediment and water samples. The column that is used for this analysis is a 30 m x 0.25 mm ID 0.25 μm film thickness silicone-coated fused silica capillary column (Restek DB-5 MS or equivalent). Five internal standards are

used to quantify the compounds of interest (naphthalene-d₈, acenaphthene-d₁₀, phenanthrene-d₁₀, chrysene-d₁₂, and perylene-d₁₂).

Qualitative Analysis

The same GC/MS conditions are used for the qualitative analysis of semivolatile organic compounds. The peaks in the total ion chromatography (TIC) for each sample are identified using interactive chemical information system (ICIS™) algorithm. The spectrum of each identified peak is enhanced using the combined algorithm and searched with the spectra in the Nation Institute of Standards and Technology (NIST) library. The spectra indices and probability for the matches are used as a guideline for the identification of the unknown compound. The GC elution time as well as the spectra generated by +/- CI and MSⁿ modes of a known standard can all be used to confirm the identity of the unknown compound.

100 mg of five individual PAHs (phenanthrene, anthracene, fluoranthene, pyrene, and benzo(a)pyrene) were added to Fenton's reactions (25 mL water, 0.85 mL 30% H₂O₂, 250 μL 1 M FeSO₄·7H₂O) as granules in duplicating serum bottles. The first set was taken down from the shake table after 2 weeks, while the second set was taken down after 4 weeks. Two different extractions were made with the separatory funnel liquid-liquid extraction (EPA Method 3510C), once when at the pH of 7 (adjusted with 0.5 M NaOH) and once at the pH of 2 (adjusted with 1M H₂SO₄). Water was removed from these extracts with hexane rinsed anhydrous NaSO₄. Methylene chloride in each extract was evaporated down to a final volume of approximately 2 mL.

Each extract was analyzed with modified EPA Method 8270. Using Xcalibur 1.2 using ICIS peak identification algorithm (parameters: peak smoothing = 7, area noise factor = 2, and peak noise factor = 30) the chromatogram generated by each extract was qualitatively analyzed by. The identified peaks were limited to peaks that were at least 1% of the highest peak height. The spectra of the identified peaks were enhanced using the combined algorithm (parameters: peak top region = 4 points, background subtraction = 5 points on the left and right of the peak start and peak end regions, respectively). The enhanced spectra were searched with spectra in the NIST library; five top hits are displayed with the spectra indices and probability for the matches.

Neutral Extracts

Of the 5 PAH (phenanthrene, anthracene, fluoranthene, pyrene, and benzo(a)pyrene), fluoranthene appears to be most easily degraded by Fenton's reaction (~60% left), possibly because not all made up of benzene rings; however, pyrene was the second most easily degraded by Fenton's reaction (~70% left). A hypothesis is under development. For some reason, there appears to be more degradation after 2 weeks than after 4 weeks. The cause of this unusual observation is unknown.

For phenanthrene, all intermediates are composed of 2 benzene rings and some "break-down" structure (e.g., 5-C ring to 6-C ring that is not aromatic). For anthracene, there was some rearrangement → get phenanthrene, some intermediates found in phenanthrene reaction, and some methylation (C1-of 3-ring benzene). For fluoranthene, the most abundant intermediate was more complex (4-ring benzene); other intermediate were products of hydration, rearrangement, & methylation. For pyrene, all intermediates were composed of 2-3 benzene ring, some oxygenated. For benzo(a)pyrene, there was an elimination of 1 benzene ring, and most intermediates were seen in pyrene reaction.

Phenanthrene Intermediates

In the 2-week extract, 5 chemical fragments were identified, while only 3 fragments were identified in the 4-week extract. Phenanthrene was 94% of the total peak area in the 2-week extract and was 97% of the total peak area in the 4-week extract. The most abundant intermediate of phenanthrene was dibenzothiophene (2-3% of total peak area). The second most abundant intermediate of phenanthrene was anthracene-maleic anhydride Diels-Alder adduct or pentacyclo[6.6.5.0(2,7).0(9,14).0(15,19)]nonadeca-2,4,6,9,11,13,16-heptaen-16-ol-18-one (~1% of total peak area). The other 2 fragments that were identified in the 2-week extract but not in the 4-week extract were relatively small; they are fluorene or 1H-phenalene and anthracene, 9,10-dihydro-.

Anthracene Intermediates

In the 2-week extract, 7 chemical fragments were identified, while 6 fragments were identified in the 4-week extract. Anthracene was 87% of the total peak area in the 2-week extract and was 94% of the total peak area in the 4-week extract.

The most abundant intermediate of anthracene was N-hydroxymethylcarbazole or carbazole (3-6% of total peak area). The second most abundant intermediate of anthracene was phenanthrene (1-3% of total peak area). The third most abundant intermediate of anthracene was C1-anthracene or C1-phenanthrene (~ 1% of total peak area). The fourth most abundant intermediate of anthracene was dibenzothiophene (~ 1% of total peak area). The fifth most abundant intermediate of anthracene was fluorene or 1H-phenalene (~ 1 % of total peak area). The peak that was only identified in the 2-week extract but not in the 4-week extract was quite small; it was identified as dibenzofuran-2-sulphonic acid.

Fluoranthene Intermediates

In the 2-week extract, 9 chemical fragments were identified, while 7 fragments were identified in the 4-week extract. Fluoranthene was ~ 60% of the total peak area.

The most abundant intermediate of fluoranthene was pyrene (~ 26% of total peak area). The second most abundant intermediate of fluoranthene was acephenanthrylene, 4,5-dihydro- or 1H-Indene, 1-(phenylmethylene)- or anthracene, 9-ethenyl- or naphthalene, 1-phenyl- (6-8% of the total peak area). The rest of the intermediates were less than 0.5% of the total peak area; they were identified as: anthracene, phenanthrene, methyl-phenanthrene/methyl-anthracene, and methyl-pyrene/methyl-fluoranthene.

Pyrene

In the 2-week treatment and extract, 12 chemical fragments were identified, while 9 chemical fragments were identified in the 4-week extract. Pyrene was ~ 70% of the total peak area.

The most abundant intermediate of pyrene was most likely Benzene, 1,1'-(1,3-butadiyne-1,4-diyl)bis- (~3% of the total peak area). The second most abundant intermediate of pyrene was not identifiable with great certainty; but may possibly be phenanthrene, 9-methoxy (~1% of total peak area). The third most abundant intermediate of pyrene was benzo[b]naphtho[2,1-d]furan or benzo[k]xanthen (~1% of total peak area). The rest of the intermediates were less than 0.5% of the total peak area; they were identified as: phenanthrene, anthracene, 4H-cyclopenta[def]phenanthrene, 1H-Indene, 1-(phenylmethylene)- or acephenanthrylene, 4,5-

dihydro- or anthracene, 9-ethenyl- or 1,9-dihdropyrene or 3,10B-dihydrofluoranthene (C₁₆H₁₂; MW = 204 compound).

Benzo(a)pyrene

In the 2-week treatment and extract, 9 chemical fragments were identified, while 7 chemical fragments were identified in the 4-week extract. Benzo(a)pyrene was 90-97% of the total peak area.

The most abundant intermediate of benzo(a)pyrene was pyrene (~7% in the 2-week extract; but <0.5% in the 4-week extract). The second most abundant intermediate of benzo(a)pyrene was identified as perylene, 3-methyl- or 11H-indeno[2,1-a]phenanthrene or 13H-dibenzo[a,h]fluorene (C₂₁H₁₄; MW=266 compound) (~1% of the total peak area in both extracts). The rest of the intermediates were less than 0.5% of the total peak area; they were identified as: fluoranthene, triphenylene or benz[a]anthracene or chrysene (C₁₈H₁₂; MW=228 compounds), and possibly dibenz[a,h]anthracene, 5,6-dihydro- or phenanthrene, 9-ethyl-3,6-dimethoxy-10-methyl-

Fenton's Reagent Generated Intermediate Identification for Anthracene

Five milligrams of anthracene was completely dissolved in 50 mL ethanol in duplicating serum bottles. Five milliliters of 1M FeSO₄.7H₂O was then added to each bottle. The pH of each solution was subsequently adjusted to 3.5 - 4.5 using 1M H₂SO₄. Finally, 5-mL of 30% H₂O₂ was added to initiate Fenton's reaction. Fenton oxidation of the first bottle was terminated after 30 minutes, while the reaction in the second bottle was allowed to react for 75 minutes.

Each of these two samples was then extracted with 50-mL methylene chloride two times using the separatory funnel liquid-liquid extraction (EPA Method 3510C). Water was removed from these extracts with hexane rinsed anhydrous NaSO₄. Methylene chloride/ethanol in each extract was completely evaporated down with high purity nitrogen TurboVap system. The extracts were re-dissolved in 2-mL acetone.

Each extract was analyzed with modified EPA Method 8270. The chromatogram generated by each extract was qualitatively analyzed by Xcalibur 1.2 using ICIS peak identification algorithm (parameters: peak smoothing = 7, area noise factor = 2, and peak noise factor = 30). The identified peaks were limited to peaks that were at least 1% of the highest peak height. The spectra of the identified peaks were enhanced using the combined algorithm (parameters: peak top region

= 4 points, background subtraction = 5 points on the left and right of the peak start and peak end regions, respectively). The enhanced spectra were searched with spectra in the NIST library; five top hits are displayed with the spectra indices and probability for the matches.

The first following figure shows the results of total ion chromatography (TIC) of GC/MS after 30-minute Fenton oxidation of anthracene. Anthracene was not completely oxidized at this time. The main oxidation product of anthracene was 9,10-anthracenedione (28.46 minute). Traces of anthrone (28.00 minute) and 2-hydroxy-9,10-anthracenedione (35.45 minute) were also observed. The chemical properties of anthracene and its Fenton oxidation products are listed in the following table.

The second figure shows the results of total ion chromatography (TIC) of GC/MS after 75-minute Fenton oxidation of anthracene. After 75 minutes of reaction, anthracene was completely oxidized. The main oxidation product at the 75-minute mark was still 9,10-anthracenedione (28.42 minute). The only other oxidation product that was measurable was 2-hydroxy-9,10-anthracenedione (35.45 minute).

After the addition of 30% H₂O₂, the pH of the solution was drastically reduced to as low as 1.7. This low pH is much lower than the optimal pH for the Fenton's reaction. The optimal pH may be maintained if 30% H₂O₂ is slowly introduced to the reaction, for instance, with the use of a syringe pump. We will also investigate and identify the Fenton oxidation products of 9,10-anthracenedione (i.e., anthracene main Fenton oxidation product) by: (1) using 9,10-anthracenedione as the starting material and (2) add more Fe²⁺ and/or H₂O₂ to the 75-minute reaction. These similar conditions and strategies will also be used to explore the Fenton oxidation products of other PAH such as phenanthrene, fluoranthene, pyrene, and benzo(a)pyrene in future studies and projects.

FIGURE 28. TOTAL ION CHROMATOGRAPHY OF GC/MS AFTER 50-MINUTE FENTON OXIDATION OF ANTHRACENE

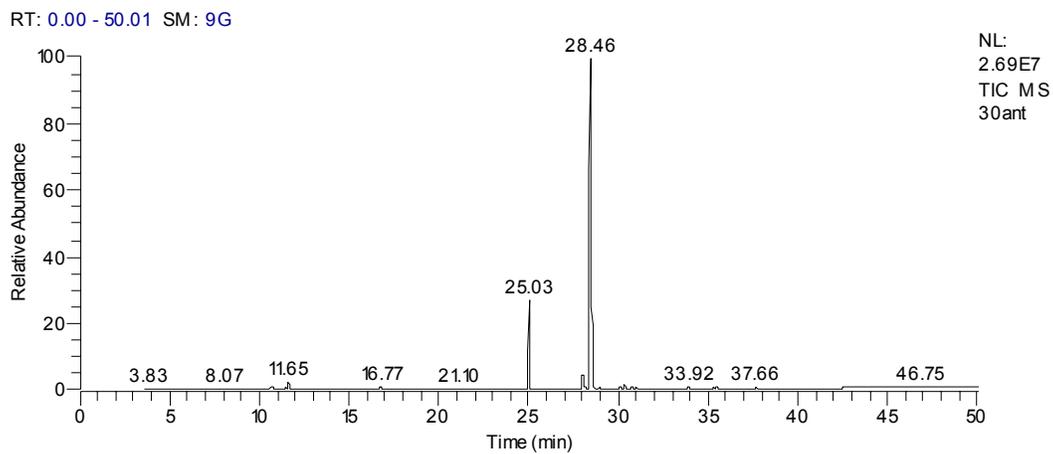


FIGURE 29. TOTAL ION CHROMATOGRAPHY OF GC/MS AFTER 75-MINUTE FENTON OXIDATION OF ANTHRACENE

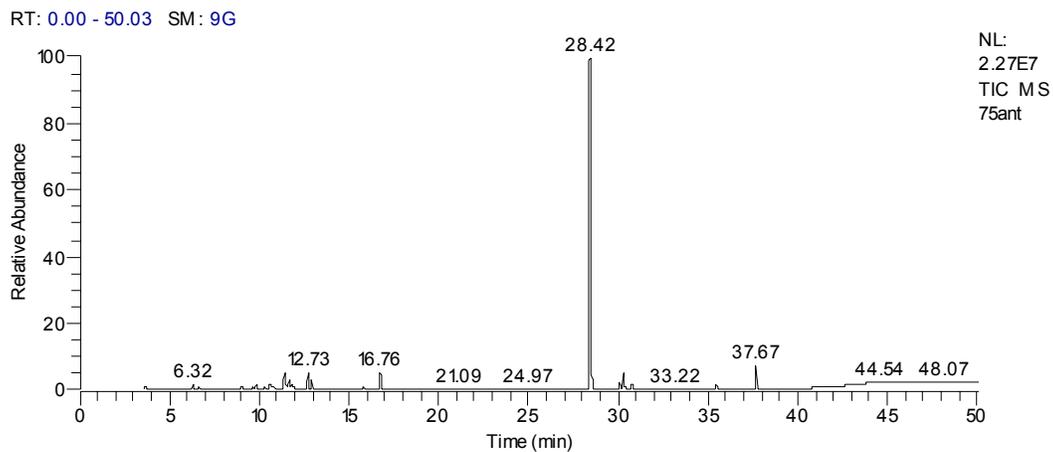
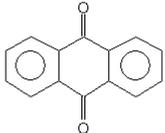
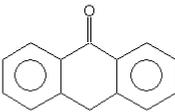
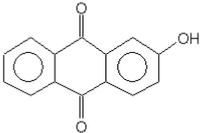


TABLE 18. CHEMICAL PROPERTIES OF ANTHRACENE AND OXIDATION PRODUCTS

Name	Structure	Molecular Formula	Formula Weight
anthracene		C ₁₄ H ₁₀	178.23
9,10-anthracenedione		C ₁₄ H ₈ O ₂	208.21
anthrone		C ₁₄ H ₁₀ O	194.23
2-hydroxy-9,10-anthracenedione		C ₁₄ H ₈ O ₃	224.21

Sequential Treatment and Delivery

Application of CAB at MGP sites has been carried out using soluble peroxide solutions and soluble salts of iron as the catalyst. This application has been applied to E&P wastes.

Engineering procedures for future field trials will be formulated for CAB application. The methods will cover both applications with traditional delivery systems, such as flooding with vertical and horizontal wells, and with GTI's Foam Assisted Remediation technology. Actual field-testing will not be accomplished under this project.

Concurrent Chemical and Biological Treatment and Delivery

The application of the Fenton's Reagent as a pre- or post-treatment has proven effective in all the treatment modes tested. In an effort to improve the process and to decrease costs, a novel approach is under investigation, the concurrent Chemical-Biological Treatment.

This study is based on the use of the CAB Process for in situ and land treatment modes where control of the soil pH is difficult, and potentially expensive. Preliminary experiments indicate that the Fenton's Reagent can be prepared and exhibit activity in E&P soil at ambient pH. This would allow the chemical transformation of the PAH to occur while the microbial community biodegrades both the parent PAH and the intermediate forms. The advantages of this approach are:

- shorter treatment periods,
- less soil handling,
- fewer chemicals applied, and
- decreased movement of hydrocarbon intermediates.

The first three advantages decrease the overall cost of applying this technology. The last point decreases the potential risk of the treatment.

Slow Release Peroxide

The peroxide component of the Fenton's Reagent is traditionally added as an aqueous solution of hydrogen peroxide. This has proven effective in all modes of the CAB Process, but a slow release form may be a distinct advantage in the in situ and land treatment applications. A variety of peroxide compounds are available, including sodium peroxide, magnesium peroxide, calcium peroxide, and barium peroxide. The magnesium form is commercially available and has been used in the field as source of oxygen in groundwater remediation.

Slow release of the peroxide may allow the production of more free radicals (OH•) because of the availability of catalytic iron, decreased temperature, and fewer local pH extremes. This may allow for more PAH to be converted to biodegradable forms and which may be less injurious to the microbial community.

Chelated Iron

The Fenton's reaction is reported to operate best at a pH range of 3 to 4. This is needed to maintain the iron in solution, but it is not optimal for the biological component of CAB. A variety

of chelating agents will be reviewed for future field applications of this technology and studied to keep the iron in solution at a pH approaching neutrality. These agents include: EDTA, sodium citrate and trinitrolotriacetic acid.

Estimating the efficiency of the Fenton's reaction in PAH transformation was the primary goal of this task. In addition, effects on the microbial community and this community's ability to degrade PAH was determined and optimized.

Biological Recycle of Iron

The combination of iron in the form of Fe^{3+} and iron-respiring bacteria (microorganisms that use iron, not oxygen, as their primary terminal electron acceptor) can add a new facet to concurrent chemical-biological treatment. The iron-respiring bacteria convert the iron into the form that acts as the catalyst for the Fenton's reaction, and the Fenton's reaction produces the form needed by the iron-respiring bacteria. Experiments will be executed to determine if this potentially patentable process is effective in degrading the PAH in MGP soils in future GTI work.

Laboratory Systems to Simulate in situ Conditions

Various columns constructed to simulate in situ conditions were tested for degradation of E&P hydrocarbon compounds. Hydrocarbon removal and dissolved oxygen was determined by the effect of column size. A correlation was developed between residual dissolved oxygen concentration and contaminant degradation. This correlation was positive, so oxygen was found to be a factor in using this technology in the field.

The objective was to determine the effect of column size on degradation of the contaminants. The reactors were monitored for any observed clogging due to bacterial growth and feed conditions, and will be modified to minimize the problem of clogging. No problems with clogging were observed. The results were supportive of our hypotheses and will be verified in field trials of these technologies.

Determination of Bioavailability and Toxicity of CAB-Treated Soils

Baseline data on the availability of the toxicants after CAB application were determined. These data are critical to:

- Evaluation of EAE endpoints, as compared to strict analytical targets in remedial action.
- Set the terminal EAE point to evaluate the effectiveness of the CAB remediation.
- Develop ecological and toxicological screening technology to define meaningful treatment goals.

Data from toxicity and accumulation studies in this task demonstrated the loss of the bioavailable fraction of contaminants from soils after CAB treatment. Tests to determine bioavailability (e.g., extractions using mild organic solvents, membrane studies, earthworm studies, Microtox[®] acute assays, and Microtox[®] Solid Phase assays) were conducted on treated soils. The data are presented within and with the attached publications. Procedures for these tests will be followed as described above.

This phase of the project also led to the availability of technically defensible data that can support risk-based regulatory decisions associated with contaminated soils from oil and gas production. Analytical and toxicological data was generated that will demonstrate the fraction of soil contaminants available to ecological and human receptors. These contaminant fractions were, in many cases, significantly less than the “total” amount of contamination present, as detected by vigorous chemical and physical extraction techniques. Development of such techniques and subsequent data generated from the use of protocols generated in this project will support cost-effective remediation strategies and endpoints for contaminants of interest to the U.S. Department of Energy and the gas/petroleum industry. Field trials of the CAB technology with EAE as the target should verify this assertion.

Toxicity Effect of Soil Contaminant Extraction Method

Background

Numerous studies conducted on soils contaminated with organic compounds have shown that as the contaminants persist in soil, they become increasingly resistant to desorption (Scribner et al. 1992, Hatzinger and Alexander 1995, Pignatello and Xing 1995, Linz and Nakles 1997). Sorption of organic contaminants to soils often entails an initially rapid and reversible process,

followed by a period of slow sorption over weeks, months, or years. This slow sorption leads to a chemical fraction that then resists desorption (Karickhoff 1980, Pignatello 1989, Ball and Roberts 1991). The result of this slow desorption process in aged soils is a substantial reduction in the rate of desorption, which correlates with a net reduction in bioavailability of the contaminant (Kelsey *et al.* 1997, Chung and Alexander 1998).

Manufactured gas plants (MGP) typically operated in the United States from the mid 1800s to the late 1940s or early 1950s (Hayes *et al.* 1996). As such, contaminants associated with MGP sites are typically organic compounds, including polynuclear aromatic hydrocarbons (PAH) that have been in contact with soil particles for a considerable amount of time. Risk assessments and determination of cleanup levels of contaminants at MGP sites are typically based on chemical analyses of samples collected at the site. However, chemical data alone provides no direct indication of the potential risks and effects of site contaminants. When soils are analyzed for contaminant concentrations, they are extracted using vigorous chemical and physical means (e.g., supercritical carbon dioxide, sonication, Soxhlet extraction), procedures that do not occur in the natural environment. These methods tend to extract and isolate both bioavailable and non-bioavailable contaminants from soil particles, resulting in the removal of otherwise sequestered contaminants from the particles. Consequently, concentrations of “total” contaminants determined by these extractions can overestimate the amount of contaminant available to biota in the environment.

Evidence of decreased contaminant bioavailability with soil aging has created the need for estimating bioavailability as a function of extractability. In order to determine site specific “Environmentally Acceptable Endpoints” (EAEs), or contaminant concentrations in soils below which no adverse effects are seen, information concerning bioavailability must be generated. Tests that determine the toxicity of historically contaminated soils are based on the availability for the contaminants to be taken up by indicator species such as bacteria, invertebrates and vertebrates. If the contaminant is available, uptake occurred to a point where it will produce an effect. However, if the contaminant is sequestered, no contaminant will be available for uptake by the organisms. Application of CAB process lead to soil that contaminants were below the toxicity levels.

Toxicity tests designed to rapidly screen environmental media are usually extracted into an aqueous solution prior to organism exposure. Therefore, extraction protocol and type of solvent used were a major factor in the extent of contaminant extraction and subsequent bioavailability to

the indicator species. These results will be verified in the proposed field-scale trial of these technologies.

In this study, changes in the toxicity of hydrocarbons-contaminated site soils resulting from various extraction techniques was examined. Aged soils contaminated with PAH from a number of HYDROCARBONS-CONTAMINATED sites were extracted and subjected to two forms of Microtox[®] tests: acute aqueous tests and solid phase tests (SPTs). The hypothesis that toxicity significantly decreases with less vigorous extraction methods was tested. Possible correlation between toxicity and soil PAH concentration was also examined. Such a correlation between the toxicity of a soil extract using a particular extraction method and soil contaminant concentration would be valuable in determining EAEs for historically contaminated sites.

Soil Collection

Five archived soils collected from various hydrocarbons-contaminated (MGP) sites and stored in the dark at 4 to 10°C were used. The soils were originally collected for contaminant treatability studies in 1996 and 1997 and were characterized for pH, particle size distribution and total organic carbon shortly after that time. The properties of the soils are presented in Table 1. For the analysis of soils, pH was measured in a 1:1 (w/w) soil-water suspension, the particle-size distribution was by hydrometer method (Sheldrick and Wang 1993), and organic carbon was determined by combustion at 1000°C. Soil GH-1 was collected from the bottom of a gasholder in early 1997. Soil SBO28 was taken from a soil boring in 1997 where HYDROCARBONS-CONTAMINATED residuals had been landfilled into a former creek channel. SoCal 2 and SoCal 3 were lampblack-based soils collected from approximately the same area in 1996 and 1997, respectively. SoCal Ed soil was collected in 1997. Additional information regarding these soils and associated treatability studies are included in project reports (IGT 1997, 1998).

TABLE 19. CHARACTERISTICS OF HYDROCARBON-CONTAMINATED SOILS USED IN THE STUDY

Soil ID	% Sand	% Silt	% Clay	Soil Type	% Total Organic Carbon	pH
SBO 28	83	12	5	Loamy sand	2.80	8.5
GH-1	55	28	17	Sandy loam	3.50	8.0
SoCal Ed	86	7	7	Sand	1.10	7.9
SoCal 2	78	10	12	Sandy loam	1.50	6.0
SoCal 3	81	12	7	Loamy sand	1.10	6.7

Supercritical Fluid Extraction

Soils were shipped to the Civil Engineering Department at the University of California, Davis, for supercritical fluid extraction (SFE). Extraction followed the procedures of U.S. EPA Method 3561 (USEPA 1995). Two to four g dry weight soil samples were extracted using a Suprex[®] Flexprep supercritical fluid extractor with an Accutrap[®] sample collector and MP-1 modifier pump (Isco, Lincoln, NE). Method 3561 is designed to recover three fractions of extracts. The more volatile PAH are extracted and recovered in the first fraction, while the lesser volatile PAH are removed in the second fraction. The third fraction is designed to purge the system of modifier and recover any residual PAH that have not been extracted in the first two fractions. Methanol was used as a modifier, with dimethyl sulfoxide (DMSO) as the rinse solvent. The first fraction (fraction A) was recovered by extracting at 80°C at 1750 psi. Static equilibration time was set at 10 min, with a dynamic extraction time of 10 min at 2.0 mL/min. The second fraction (fraction B) was then extracted by setting the pressure to 4900 psi at 120°C, with static equilibration time set at 10 min followed by a dynamic extraction time of 30 min at 4.0 mL/min. The third fraction (fraction C) was extracted at 4900 psi at 120°C. Static equilibration time was 5 min, followed by a dynamic extraction time of 10 min at 4.0 mL/min. A final volume of approximately 0.8 mL sample in DMSO was recovered for each fraction. Actual sample volumes for calculating PAH concentrations and EC₅₀ equivalents were determined from solvent weights in the individual sample vials.

Extract Analysis

All SFE extracts were analyzed without any additional sample preparation, except for the addition of an internal standard (2-fluorobiphenyl and *d*₁₄ *p*-terphenyl) prior to gas chromatography

(GC)/mass spectrometry-selected ion monitoring (MS-SIM). Extracts were analyzed for 15 individual PAH compounds and 1 compound group: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[*a*]anthracene, chrysene/triphenylene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, indeno[*1,2,3-cd*]pyrene, dibenz[*a,h*]anthracene, and benzo[*g,h,i*]perylene. Quantification of individual PAH was performed using a Hewlett Packard 5890 II gas chromatograph/5971 mass selective detector. The GC analysis employed a 30 m × 0.25 i.d. Restek XTI-5 column, with an oven temperature profile of: 40°C for 4 min, 40°C to 300°C at 10°C min⁻¹, and isothermal at 300°C for 20 min. Recovery of surrogate standards averaged 97.78% ± 7.40 SD. Extracts from fractions A, B and C were analyzed individually. The PAH concentrations reported were calculated as the sum of these fractions for each soil sample tested.

Microtox Testing

Both Microtox solid phase tests (SPTs) and Microtox acute toxicity basic tests (acute) were used in this study and followed the manufacturer’s protocols (Azur Environmental 1995 *a,b*). Microtox is a commercial toxicity bioassay based on the reduction in bioluminescence of the marine bacterium *Vibrio fischerii* following exposure to toxicants (Bulich 1986). Soil contaminants are extracted into an aqueous medium prior to acute testing. Alternatively, the SPT was developed as a standard procedure to test complex solid samples that allows soil particles to come into direct contact with bacteria for a fixed period of time. Table 2 describes the particular soil samples/extracts that were tested.

TABLE 20. MICROTOX TESTS PERFORMED ON SOILS AND SOIL EXTRACTS.

Sample	Description	Test Used
Dry soil	Soil samples tested prior to SFE	Microtox SPT
Dry soil	Soil residual material after SFE	Microtox SPT
Aqueous extract (2% NaCl)	Soil sample tested prior to SFE	Microtox Acute
Aqueous extract (DMSO)	Fraction A recovered after SFE	Microtox Acute
Aqueous extract (DMSO)	Fraction B recovered after SFE	Microtox Acute
Aqueous extract (DMSO)	Fraction C recovered after SFE	Microtox Acute

Acute tests were performed on soil samples following saline-based extraction procedures outlined in the American Society for Testing and Materials’ Designation D 5660-95 (ASTM 1995). Briefly, 2 g dry soil was combined with 20mL Microtox diluent (2% NaCl solution) in 50 mL Oak

Ridge Teflon[®] tubes. The mixture was mixed on an orbital rotator at 200 rpm for 16 h. The samples were then centrifuged at 2000 g for 20 min at 4°C and the supernatant decanted and subjected to acute testing. Acute tests were also performed on fractions A, B and C after SFE. Microtox SPTs were performed on soils before SFE and on the residual soil material recovered from the extraction vessels after SFE. Sample preparation for fractions A, B and C was as follows: 150 ul of the DMSO extract was added to 10 mL of Microtox diluent to make up a 1.5% DMSO dilution. Serial 1:1 dilutions were then made from this initial dilution. Reconstituted cells of *V. fischerii* were exposed in duplicate to eight different dilutions of DMSO extract. A Microtox control blank of 1.5% pure DMSO to diluent was prepared to offset any effect from the DMSO and to set the light measurement of the Microtox analyzer. The resulting decrease in bioluminescence was measured after 15 min at a constant temperature of 15°C. A zinc sulfate reference toxicant was run as a daily internal quality control procedure, whenever acute tests were performed. All results from the zinc sulfate toxicant were within ± 2 standard deviations of established means.

SPTs were performed by combining soil samples with Microtox solid phase diluent in a 1:5 (w:v) ratio and mixing for 10 min. Eleven serial 1:1 dilutions, in duplicate, were prepared and the bacteria exposed to these dilutions for 20 min. Microorganisms were then separated from the soil suspensions with a filter column for analysis using the Microtox analyzer.

Data analysis

All Microtox test data were expressed in EC_{50s} , defined as the effective concentration of a test sample that causes a 50 percent decrease in light output (Qureshi *et al.* 1984, Kwan and Dutka 1992); the lower the EC_{50} , the greater the toxicity. To determine toxicity among test methods, all sample concentrations were reported in units of mg dry weight soil per mL of extraction diluent. This represents the mass of dry soil that, if extracted into an aqueous solvent, would result in a 50% reduction in bioluminescence. Comparisons among EC_{50} values were conducted using Pearson product-moment correlation coefficients, r , (Zar 1984). Comparisons between EC_{50} values generated with the same soils and extraction method were determined via student's t-tests, using Microsoft Excel Analytical ToolPak[®] software package.

RESULTS

Supercritical fluid extraction and subsequent GC/MS-SIM analyses of the five soils used in this study revealed total PAH concentrations ranging from 60 to 2592 mg/kg (Table 3), with 3- and 4-ring compounds making up the largest fraction of compounds in each sample (Table 4). These compound classes approximated the proportion of PAH that were expected to be extracted into each of fractions A, B and C from SFE. The relative proportion of contaminants from each of fractions A, B and C paralleled toxicity of the SFE extracts (Table 5). For all of the soils, most of the PAH were found in fraction B, which was the most toxic.

Results from Microtox acute tests conducted before SFE (tests following the ASTM method of extraction) showed a substantial reduction in toxicity, compared to tests run after SFE on fractions A and B (t test, $p < 0.05$; Table 3). For three of the soils (SoCal 2, SoCal 3 and SoCal Ed), acute EC_{50} s obtained via the ASTM extraction method were greater than negative saline controls, indicating no toxicity of the sample extract. Acute EC_{50} values from the ASTM method were also significantly greater than those obtained from fraction C for soil samples GH-1, SoCal 2 and SoCal 3. However, no statistical differences were seen between EC_{50} s for the two extraction methods from SBO-28 and SoCal Ed. This is most likely due to the relative proportion of PAH contained in fraction C. SBO-28 and SoCal Ed contained the least amount of PAH in fraction C of the five soils studied (Table 5).

EC_{50} values compared between pre- and post-SFE for Microtox SPTs were not statistically different for three of the samples tested: SoCal 2, SoCal 3 and SoCal Ed (t test, $p < 0.05$). This indicates that soil toxicity did not change after a vigorous extraction with SFE, even with total PAH concentrations up to over 700 mg/kg (Table 3). The remaining two soils showed a significant decrease in toxicity after SFE with the Microtox SPT. Solid phase test EC_{50} values showed forty-fold and nineteen-fold reductions in toxicity for SBO-28 and GH-1, respectively. These samples also demonstrated the highest acute EC_{50} values with the ASTM extraction method, indicating that some PAH were available for desorption with mild extraction procedures.

Correlation coefficients (r values) for comparison of EC_{50} values generated for the different extraction procedures and total PAH concentrations are listed in Table 6. No significant positive correlations were found between total PAH concentration of the soils and any of the toxicology tests.

TABLE 21. COMPARISON OF MICROTOX EC₅₀ VALUES OBTAINED WITH VARIOUS EXTRACTION METHODS ON HYDROCARBONS-CONTAMINATED SITE SOILS. VALUES ARE MEAN ± 1 STANDARD DEVIATION OF DUPLICATE OR TRIPPLICATE SAMPLES

Soil ID	EC ₅₀ (mg/mL extractant)						
	Total PAH mg/kg ¹	SPT ² Pre-SFE	Acute ASTM method ³	Acute fraction A post-SFE	Acute fraction B post-SFE	Acute fraction C post-SFE	SPT Post-SFE
SBO-28	2592 (235)	0.06 (0.04)	4.47 (0.01)	0.59 (0.79)	0.04 (0.02)	5.89 (1.98)	2.45 (0.72)
GH-1	188 (11)	0.47 (0.10)	18.54 (4.61)	2.39 (1.41)	1.33 (1.94)	2.33 (0.05)	9.05 (1.90)
SoCal Ed	60 (2)	14.39 (4.24)	>100 (0.00)	20.52 (18.68)	10.23 (2.11)	138.15 (88.46)	41.96 (19.12)
SoCal 2	387 (21)	22.24 (0.57)	>100 (0.00)	6.81 (0.02)	1.79 (0.27)	37.32 (9.64)	32.97 (5.87)
SoCal 3	712 (126)	12.89 (5.49)	>100 (0.00)	5.42 (2.91)	0.64 (0.35)	2.15 (0.41)	35.37 (8.20)

¹Total PAH are reported as the sum of 16 compounds extracted via SFE and analyzed via GC/MS.

²Microtox Solid Phase Tests

³Microtox protocol followed ASTM designation D 5660-95.

TABLE 22. CONTAMINANT CONCENTRATIONS (MG/KG) IN HYDROCARBONS-CONTAMINATED SITE SOILS. VALUES REPRESENT CONCENTRATIONS FOR 2 REPLICATE SAMPLES.

PAH compound	SOIL ID				
	SBO-28	GH-1	SoCal Ed	SoCal 2	SoCal 3
Naphthalene	7.7, 11.6	11.6, 9.3	<0.2, ¹ <0.2	4.4, 2.0	11.8, 10.0
Acenaphthylene	296.4, 363.1	7.1, 9.0	0.4, <0.2	2.6, 2.0	4.5, 3.7
Acenaphthene	59.9, 77.9	7.7, 7.6	<0.2, <0.2	<0.2, <0.2	0.2, <0.2
Fluorene	73.8, 297.9	14.3, 16.2	<0.2, <0.2	0.6, 0.3	1.6, 0.9
Phenanthrene	725.0, 768.5	49.2, 52.1	3.3, 3.6	53.8, 43.4	169.3, 117.2
Anthracene	223.5, 255.4	9.7, 10.7	0.4, 0.5	4.4, 4.1	10.0, 7.4
Fluoranthene	295.6, 287.3	24.1, 28.6	12.0, 12.6	88.5, 78.6	211.4, 155.9
Pyrene	387.0, 369.8	27.9, 30.2	16.5, 17.7	98.6, 90.5	215.9, 170.3
Benzo(a)anthracene	121.8, 110.8	8.6, 9.2	3.0, 2.8	15.5, 14.8	30.3, 24.8
Chrysene + Triphenylene	115.4, 103.8	8.6, 9.3	4.6, 4.4	26.4, 24.3	48.8, 41.8
Benzo(b)fluoranthene	25.3, 24.1	2.6, 2.8	4.0, 3.3	19.7, 19.0	27.3, 24.3
Benzo(k)fluoranthene	32.4, 28.4	2.9, 3.2	3.5, 2.7	15.8, 16.3	22.4, 19.9
Benzo(a)pyrene	42.0, 39.3	3.6, 4.0	4.5, 3.5	18.0, 18.5	18.0, 17.6
Indeno(1,2,3-cd)pyrene	8.3, 8.3	0.9, 1.0	3.3, 2.4	19.5, 21.1	12.2, 12.3
Dibenz(a,h)anthracene	2.0, 3.8	0.4, 0.6	0.7, 0.4	2.9, 2.4	1.5, 1.6
Benzo(g,h,i)perylene	9.3, 8.7	1.2, 1.2	5.2, 3.9	31.1, 34.4	16.4, 16.0

¹Method detection limit was 0.2 mg/kg

TABLE 23. COMPARISON OF TOTAL PAH CONCENTRATIONS WITH MICROTOX ACUTE EC₅₀

Soil ID	Fraction A		Fraction B		Fraction C	
	PAH %	EC ₅₀	PAH %	EC ₅₀	PAH%	EC ₅₀
SBO-28	7.6	.59	91.6	0.04	0.9	2.45
GH-1	16.7	2.39	77.8	1.33	6.0	9.05
SoCal Ed	8.3	20.52	88.4	10.23	3.1	41.96
SoCal 2	12.8	6.81	77.8	1.79	9.5	32.97
SoCal 3	6.7	5.42	82.2	0.64	11.0	35.37

^avalues for SFE fractions A, B and C.

^bPAH concentrations reported for the fractions are percent of the total sample concentrations.

^cEC₅₀ values are in mg dry weight soil per mL extractant.

^dValues represent the mean of 2 replicate samples.

Microtox EC₅₀ values. Instead, relatively non-linear negative relationships were seen for all combinations of Microtox tests and total PAH concentration (for correlations, *r* values indicate the degree of the linear relationship between two variables; an *r* value of ± 1 represents a perfect linear relationship, while a 0 value indicates no relationship). However, some positive correlations between Microtox tests and extraction methods were seen. For example, as EC₅₀ values increased from Microtox SPTs after SFE, EC_{50s} also increased using the acute test according to the ASTM procedure prior to SFE (*r* = .92, Table 6).

Further correlation analyses was completed for individual PAH compounds to determine if any relationships existed between individual compound concentration and EC₅₀ values obtained with a particular extraction procedure. Table 7 shows little correlation between the majority of PAH compounds and Microtox EC_{50s}. Five exceptions are apparent from Table 7, where *r*-values are greater than ± 0.7 . Naphthalene concentration correlated well with EC₅₀ values obtained from acute tests on post-SFE fractions B and C, with *r* = -0.78 for both tests. Acenaphthene concentration correlated with EC_{50s} obtained from the ASTM extraction method

for the acute test ($r = -0.74$, Table 7). EC_{50s} obtained from the SPT tests prior to SFE showed r values of 0.74 and 0.81 for indeno(1,2,3-cd)pyrene and benzo(g,h,i)perylene, respectively.

TABLE 24. CORRELATIONS (*R*) BETWEEN MEASURED CHEMICAL AND TOXICITY PARAMETERS OF HYDROCARBON-CONTAMINATED SITE SOILS STUDIED. VALUES ARE CALCULATED FROM THREE REPLICATE SAMPLES IN EACH CATEGORY

	Total PAH	Microtox SPT EC₅₀, Pre SFE¹	Microtox SPT EC₅₀, Post SFE	Microtox Acute EC₅₀, ASTM Method²	Microtox Acute EC₅₀, SFE Fraction A³	Microtox Acute EC₅₀, SFE Fraction B
Microtox SPT EC ₅₀ , Pre SFE	-0.456					
Microtox SPT EC ₅₀ , Post SFE	-0.581	.684				
Microtox Acute EC ₅₀ , ASTM Method	-0.562	.870	.924			
Microtox Acute EC ₅₀ , SFE, Fraction A	-0.418	.340	.684	.516		
Microtox Acute EC ₅₀ , SFE, Fraction B	-0.480	.264	.688	.440	.811	
Microtox Acute EC ₅₀ , SFE, Fraction C	-.357	.325	.635	.431	.940	.912

¹EC₅₀ values prior to supercritical fluid extraction.

²Microtox protocol followed ASTM designation D 5660-95.

³EC₅₀ values after supercritical fluid extraction.

TABLE 25. CORRELATIONS (*R*) BETWEEN MEASURED INDIVIDUAL PAH COMPOUNDS AND TOXICITY PARAMETERS OF HYDROCARBONS-CONTAMINATED SITE SOILS STUDIED. VALUES ARE CALCULATED FROM THREE REPLICATE SAMPLES IN EACH CATEGORY

PAH compound	Microtox SPT EC₅₀, Pre SFE¹	Microtox SPT EC₅₀, Post SFE	Microtox Acute EC₅₀, ASTM Method²	Microtox Acute EC₅₀, SFE Fraction A³	Microtox Acute EC₅₀, SFE Fraction B	Microtox Acute EC₅₀, SFE Fraction C
Naphthalene	-0.547	-0.577	-0.466	-0.603	-0.780	-.778
Acenaphthylene	-0.540	-0.637	-0.675	-0.372	-0.358	-.252
Acenaphthene	-0.601	-0.694	-0.740	-0.400	-0.375	-.278
Fluorene	-0.456	-0.537	-0.564	-0.319	-0.288	-.208
Phenanthrene	-0.524	-0.618	-0.626	-0.412	-0.450	-.338
Anthracene	-0.547	-0.646	-0.680	-0.382	-0.375	-.268
Fluoranthene	-0.279	-0.392	-0.302	-0.410	-0.576	-.449
Pyrene	-0.334	-0.461	-0.393	-0.413	-0.545	-.434
Benzo(a)anthracene	-0.482	-0.602	-0.596	-0.412	-0.460	-.341
Chrysene + Triphenylene	-0.379	-0.524	-0.473	-0.422	-0.520	-.392
Benzo(b)fluoranthene	0.175	-0.089	0.121	-0.324	-0.564	-.420
Benzo(k)fluoranthene	-0.089	-0.331	-0.187	-0.399	-0.576	-.426
Benzo(a)pyrene	-0.213	-0.476	-0.382	-0.410	-0.515	-.362
Indeno(1,2,3-cd)pyrene	0.741	0.217	0.509	-0.110	-0.323	-.165
Dibenz(a,h)anthracene	0.118	-0.258	-0.121	-0.353	-0.436	-.286
Benzo(g,h,i)perylene	0.813	0.286	0.569	-0.037	-0.232	-.084

¹EC₅₀ values prior to supercritical fluid extraction.

²Microtox protocol followed ASTM designation D 5660-95.

³EC₅₀ values after supercritical fluid extraction.

DISCUSSION

In this study, we showed that the toxicity of soils extracted according to ASTM procedures was significantly less than that seen in the same soils extracted via SFE. This confirms our hypothesis that toxicity significantly decreases with less vigorous extraction methods. A detailed examination of the toxicity produced by various fractions of the extractant after SFE showed that toxicity was relative to the amount of PAH extracted. Further testing on the combined fractions A, B and C from SFE would have likely shown toxicity to be much greater than that extracted following ASTM protocols. These hydrocarbons-contaminated soils data indicate much variation in the bioavailability of contaminants from (MGP) site soils, which is not dependent on total contaminant concentration. For example, total PAH concentration in SoCal Ed soil was the lowest of any soils tested. However, SoCal 2 soil contained over six times this concentration of PAH, but showed only about half of the toxicity of the SoCal Ed soil in pre-SFE SPTs.

The data show that contaminant concentrations, either as total PAH or as individual PAH compounds, determined by a vigorous extraction procedure were not correlated with the toxicity of MGP site soils. Bioavailability of the contaminants, evidenced by Microtox tests run on soils prior to SFE, showed toxicity to be greatest in the sample that contained the greatest concentration of PAH (SBO-28). However, this trend did not continue; the soil that contained the second greatest concentration of PAH (SoCal 3) was one of the least toxic samples. Previous studies completed on contaminated soils and sediments have also shown lack of consistent correlation between the results of chemical analyses and Microtox toxicity (True and Heyward 1990, Donnelly *et al.* 1991, Jacobs *et al.* 1993), as well as toxicity determined with fish and invertebrates (Athey *et al.* 1989, Hoke *et al.* 1990). Vigorous physical and chemical methods of contaminant extraction from soils tend to pull contaminants from particles that would not ordinarily occur in the natural environment. Extraction procedures and chemical analyses that rely on more “natural” methods (i.e., physiologically-based saline, fluids mimicking gastrointestinal fluids) have been suggested as more accurate means of estimating risk to human health and the environment (Linz and Nakles, 1997).

Comparisons between chemical analyses and soil toxicity bioassays can be complex. Soils from typical MGP sites can be contaminated with hundreds of individual compounds in addition to PAH. Further, contaminants may exist in concentrations below instrument detection limits but may still elicit toxicity. Interactions among chemical compounds may affect toxicity tests in synergetic or antagonistic ways. Of the detectable contaminants routinely analyzed, PAH were the class of compounds found to be most significant in the MGP site soils used in this study. Toxicity seen in these soils can only be assumed to be due to the PAH contained in them. Despite the uncertainty of contaminant species or concentrations contained in the soils used in this study, the lack of correlation of relatively highly contaminated soils with toxicity indicates differential bioavailability not due to chemical concentration alone.

Soil aging, particle size distribution, pH and organic carbon content are factors known to be responsible for differential bioavailability among soils and sediments (Linz and Nakles 1997). No trends between toxicity and any of these factors were readily apparent for the soils examined in this study. However, a larger database that incorporates more samples and greater percentages of silts and clays may show correlation between Microtox EC_{50s} and one or more physical parameters.

Two questions arose during this study: 1) What is a “toxic” soil? and 2) What extraction method is best when using Microtox with contaminated soils? A previous study conducted with sediments reported EC_{50} from Microtox SPTs by percent dry weight of the sample (Day et al. 1995). Sample concentrations greater than 2% were regarded as a negative response and sample concentrations between 1 and 2% were doubtful or suggestive of toxicity. More recently, SPT EC_{50} values have been reported in mg equivalent soil or sediment weight per mL of extract (Johnson 1997, Johnson and Long 1998). Johnson and Long used an EC_{50} of 5.2 mg equivalent sediment extract/mL as a reference point for determining sediment toxicity. Sample EC_{50} values significantly less than this value were considered toxic. To normalize EC_{50} values for both acute and SPTs in this study, we reported toxicity in units of mg equivalent soil weight per mL of extract. If calculated by percent dry weight of the sample, SPT EC_{50} results are all >1% for both pre- and post SFE samples in SoCal Ed, SoCal 2 and SoCal 3 soils. Using the criteria for toxicity suggested by Day et al. (1995), toxicity can be considered significant in soils SBO-28 and GH-1, where EC_{50s} for SPTs ranged from 0.03 to 1.0% dry sample weight. Using the criteria of Johnson and Long (1998), toxicity can also be considered significant in SBO-28 and

GH-1 soils, except for the post-SFE extract from GH-1 where the mean EC₅₀ was reported as 9.05 (Table 3).

The two types of Microtox tests used in this study employ different methods of organism exposure. Acute tests were performed on aqueous extracts of soils, while SPTs allowed for the bacteria to come into direct contact with soil particles during the test, increasing the probability for the measurement of responses to particle bound and marginally soluble toxicants. Comparison of SPTs and acute tests performed prior to SFE showed substantially greater toxicity produced in the SPT (Table 3). This indicates that a large portion of the PAH present in the MGP site soils were not soluble and extracted into the saline-based media used in the acute test. However, if combined with actual soil particles, PAH availability to bacteria increased, resulting in increased toxicity. This information leads to the second question of which type of Microtox test should be used for determining the toxicity of contaminated soils. To answer this question, one must know the potential routes of exposure from which estimates of risk will be significant. This will differ for each contaminated site under consideration and will thus depend on site-specific factors. For example, if leaching to groundwater is of particular concern, and then the Microtox acute test could be used to estimate the potential toxicity of leachable contaminants to the aqueous phase. However, if soil ingestion or dermal contact to contaminated soil is an issue, then the Microtox SPT would be a logical choice for estimating toxicity via the direct contact pathway.

Summary of Results

Data from this project provide evidence of sequestration and reduced availability of polycyclic aromatic hydrocarbons (PAH) from soils extracted via physiologically based procedures, compared to vigorous physical extraction protocols. Toxicity tests using two types of Microtox procedures on PAH-contaminated soils showed toxicity to be minimal after extraction with a saline-based solvent, even though mean total PAH concentrations in these soils ranged from 60 to 712 mg/kg. However, these soils were considered “toxic”, when Microtox was used to test extracts after supercritical fluid extraction, considered a vigorous extraction procedure.

Data from this project also show that contaminant concentrations, either as total PAH or as individual PAH compounds, determined by a vigorous extraction procedure, were not correlated with the toxicity of MGP site soils. This information provides further evidence that the removal of otherwise sequestered contaminants from soil particles as determined by conventional extraction procedures can overestimate the amount of contaminant available to biota in the environment.

Future plans for study include using Microtox acute and SPTs on additional contaminated site soils to further develop the site-specific toxicity database initiated in the project. This data will contribute to the bank of data supporting the EAE approach for defining acceptable concentrations of soil contaminants at contaminated sites. Proposals for additional funding from GTI's Internal Research and Development will be solicited to compare the Microtox test with other newly developed toxicity tests that can be used as cost-effective surrogates of environmental risk.

Data Analysis and Quality Control

Table 26 describes the soil samples to be analyzed in this project. Negative and positive control soils were analyzed, characterized and run as test samples in each phase of the project. Since it is difficult to obtain uncontaminated soil samples with the same physical and chemical characteristics as each of the test soils, six test "uncontaminated" soils were obtained. Contaminants (TPH and PAH) were added a "aged" to various degrees. (USEPA 1988).

The following table summarizes the complete data set to be analyzed. Since a goal of this project was to develop a rapid, cost-effective method to determine the bioavailable fraction of soil contaminants for establishing EAE, statistical tests will determine significant correlation among the assays performed, as well as between soils before and after CAB treatment. This correlation will include the fraction of bioavailable contaminant detected via organic solvent extraction, earthworm bioaccumulation, and membrane eluates. This bioavailable fraction will be compared to the "total" fraction of contaminants in soils as detected via traditional Soxhlet extraction and analyses. Toxicity, reported as EC₅₀ or LC50 concentrations, will be compared between Microtox[®] tests and earthworm survival tests, both before and after treatment of contaminated soil samples. These comparisons will be made using the appropriate parametric or nonparametric tests at the 95% level of significance.

TABLE 26. SOIL SAMPLES TO BE ANALYZED

Test Samples	Negative Control	Positive Control
6 soils – Before Accelerated Biological Treatment	Uncontaminated mixture of sand, sphagnum peat and kaolinite clay	6 soils spiked and aged
6 soils – After Accelerated Biological Treatment		

The Project Manager is responsible for review of the final study report to assure that the report accurately describes the methods and SOPs and that the reported results accurately reflect the raw data of the study. When appropriate, parametric statistical tests will be used to describe differences between results generated from the same soil samples using different assays (i.e., t-tests, ANOVA, regression analysis) at the 95% level of significance. Sample variability will be determined by measuring the standard deviation of the mean. Mean differences will be measured by ANOVA. If the overall F ratio is significant, appropriate post-hoc testing will be undertaken to determine where the differences lie.

All protocols provided by the manufacturer of the Microtox[®] assays will be followed for QA/QC requirements. Sample handling will follow guidelines under GTI’s standard Health and Safety Procedures. In addition to careful documentation of equipment calibration and assay controls, general good laboratory practices will include the following:

- 1) Disposable gloves, protective clothing including sleeve protectors and safety glasses will be used when handling potentially toxic substances;
- 2) All toxicological transfers will be conducted under a properly vented ventilation hood;
- 3) Proper provisions for the collection and disposal of contaminated soil, aqueous waste, and other toxic waste will be arranged with the safety officer at GTI; and
- 4) Supervision of all technicians will be the responsibility of the Project Manager and, in the case of technician training, careful supervision will continue until the technicians are well trained and capable of performing procedures independently.

DATA INTERPRETATION

The aims of this research were to address the following specific hypotheses:

- A bioavailable fraction can be extracted from soils contaminated with a mixture of chemicals that is lower in concentration than that extracted with conventional methods.
- The bioavailable fraction can be positively correlated with earthworm toxicity and bioaccumulation. Toxicity of the bioavailable fraction is decreased after crossing artificial membranes. Significant correlation exists between earthworm bioaccumulation and contaminant permeation through artificial (PSD) membranes and bacterial (Microtox[®]) assays.
- Chemically-Accelerated Biotreatment is more effective and dependable than traditional biotreatment.
- Chemically-Accelerated Biotreatment is effective when evaluated by standard chemical analyses of contaminants concentration, as well as by toxicity reduction based on environmental acceptable endpoints.
- Data interpretation was performed with the purpose of either supporting or negating these hypotheses after appropriate statistical data analyses. The data generated from this project are intended to support site-specific risk assessments by providing evidence of bioavailability changes among soils contaminated with complex mixtures of contaminants generated from the production of oil and gas. Factors influencing such bioavailability changes may include differences in soil composition, contaminant composition, soil aging and contaminant concentration. Data from soil characterization will be used to assess these factors.

FINAL STUDY CONCLUSIONS

The final conclusions of this study are:

- Chemically Accelerated Biotreatment (CAB) is an effective alternative to other commercially available and experimental technology,
- Toxicity endpoints (EAE) are an effective endpoint for the treatment, and
- This technology is ready for field-scale testing or evaluation.

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APPENDIX – PUBLICATIONS AND PRESENTATIONS

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**ENVIRONMENTAL RISK REDUCTION AFTER APPLICATION OF
CHEMICALLY ACCELERATED BIOTREATMENT TO HYDROCARBON-
CONTAMINATED SOILS UNDER WATER-SATURATED AND
UNSATURATED CONDITIONS**

J. Robert Paterek, William Bogan, Lisa Lahner, and Vesna Trbovic, GTI and Nancy Comstock, National Energy Technology Laboratory, US EPA

Abstract

Our laboratory is studying various forms and factors of chemically accelerated biotreatment (CAB) for hydrocarbons contaminated soils, i.e. polynuclear aromatic hydrocarbons; benzene, ethylbenzene, toluene, and xylenes (BTEX); and aliphatic moieties. As the biodegradation capacity of the contaminated soils is the decisive parameter in the CAB technology, we are investigating the effects of delivering nutrients (nitrogen and phosphorus moieties) to soils under simulated in situ conditions to maximize biodegradation. Nitrogen and phosphorus containing compounds that are gases under expected field conditions are a major research area. In order to determine effectiveness of these additions it was necessary to first identify candidate soil that is suitably nutrient-limited. Slurry-phase bioreactors were the method to assess nutrient effects on contaminant degradation under “ideal” conditions, *i.e.* in systems where issues such as nutrient and contaminant bioavailability are minimized. In order to determine whether the Microtox[®] solid-phase test would be suitable for assessing the potential toxicity of the E&P soils to be used in remediation experiments and in determinations of environmentally acceptable endpoints, this assay was investigated. All procedures were followed according to manufacturer’s instructions, and clean coarse sand (the same sand used to dilute the soil for column experiments) was used as a control. Solid-phase Microtox[®] analyses proved to be applicable to the experimental systems under study. In addition, soil moisture content (a_w) was evaluated in both the nutrient study and the Microtox[®] evaluation.

Keywords

Bioremediation, environmentally acceptable endpoints, risk-based technology, chemically accelerated biotreatment.

Introduction

Gas and oil exploration and production (E&P) activities are conducted in 33 U.S. states under a wide variety of environmental and operational conditions. The pipeline transmission and distribution of natural gas and petroleum involves all 50 states. On the exploration and production sites, there are an excess of 800,000 wells producing gas and oil with 30,000 new wells completed annually (based on 1991 surveys). Add this number to the 500,000 abandoned wells and a major potential environmental liability is indicated. The research reported is part of a DOE/FERC project that targets waste pits that can be remediated on-site or in situ, as well as soil contaminated by accidental release of petroleum and natural gas-associated organic wastes from pipelines or during transport. Based on GTI's experience with a form of Chemically-Accelerated Biotreatment (CAB) for the remediation of Manufactured Gas Plants (MGP-REM), use of these technologies at E&P sites could save the industry an estimated \$150 Million to \$200 Million over the next ten years.

The most common contaminants associated with E&P sites are:

- 0 hydrocarbons associated with the natural gas condensates, such as benzene, toluene, ethyl benzene, and the xylenes which are commonly designated as BTEX;
- 1 polynuclear aromatic hydrocarbons (PAHs) from the formation's natural gas deposits (liquid or solids under the conditions of pressure and temperature in many reservoirs);
- 2 PAHs associated with the drilling materials;
- 3 fuel oil and diesel fuel that is often a component of the drilling materials;

- 4 heavy metals, such as arsenic, barium, cadmium, chromium, lead, mercury, selenium, silver, and zinc;
- 5 biocides and scale/corrosion inhibitors; and
- 6 organic and inorganic sulfur compounds (mercaptans and hydrogen sulfide, respectively).

The technology under development, i.e. CAB to mitigate possible contaminated sites, combines two powerful and complimentary remedial techniques: 1) chemical oxidative treatment using Fenton's Reagent (hydrogen peroxide and iron salts); and 2) biological treatment, primarily using native aerobic microorganisms (Paterek *et al.*, 1994, Srivastava *et al.* 1994). This integrated process generates environmentally benign products including carbon dioxide (CO₂), inorganic salts, biomass, and water. A schematic illustration of the CAB Process is presented in the Figure 1.

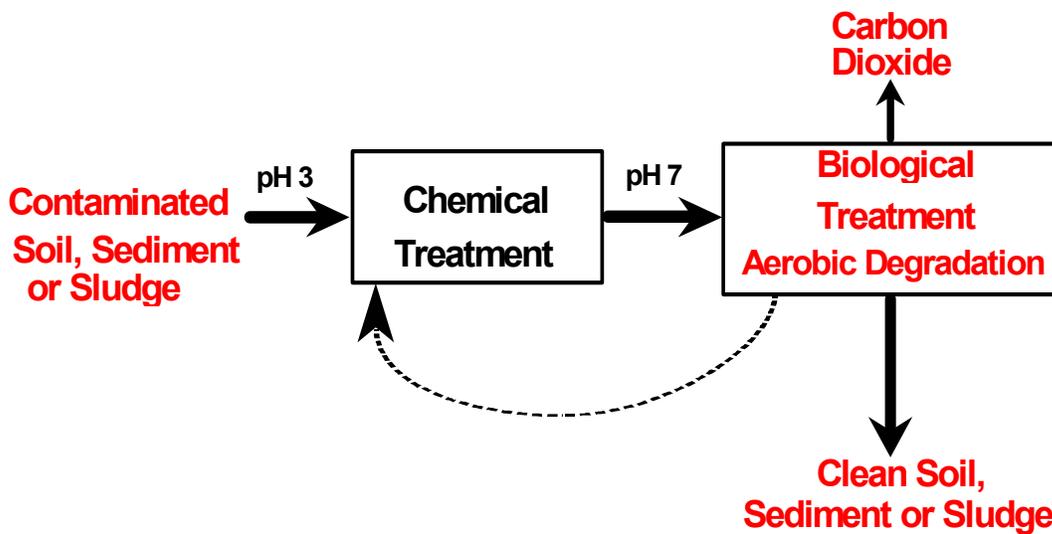


Figure 1: Schematic diagram of the CAB process

Application of CAB to E&P sites and other gas industry sites (Paterek *et al.* 1993) with treatment endpoints based on risk, bioavailability and EAEs (Harkey *et al.* 1997)

utilizes engineered systems to supply microbe-stimulating materials and pretreatment chemicals to:

- 0 encourage the growth and activity of targeted microorganisms,
- 1 minimize mass-transfer problems, and
- 2 optimize environmental conditions for degradation and detoxification reactions.

These systems must be effective to decrease the time required to destroy or detoxify the contaminants (Grey *et al.* 2000), thus decreasing the overall liability of the gas/petroleum industry partner that is responsible for the site, as well as his cleanup costs. The technology must also be efficient enough to meet or surpass the regulatory requirements. This technology will also be more reliable than the existing technologies of landfilling, landfarming, and composting. Cost reduction will be realized by shortening the treatment time, due to the option of applying the in situ mode of the CAB process. The use of low-cost and industrially available chemicals will eliminate excavation, transport, and land use costs.

Materials and Methods

Determination of Nutrient Limitations in E&P Soils

As a first step towards determining the ability of gaseous nutrient sources to support degradation of oil-derived aliphatic hydrocarbons in petroleum-contaminated E&P soils, it was necessary to first identify a candidate soil that is suitably nutrient-limited. This was accomplished through the use of slurry-phase cultures, as we have used throughout this research to assess nutrient effects on contaminant degradation under “ideal” conditions, *i.e.* in systems where issues such as nutrient and contaminant bioavailability were minimized.

Homogenized soil samples (500 mg) were mixed with 50 ml sterile media (0.1ml Wolfe’s Vitamins (2 mg·l⁻¹ biotin, 2 mg·l⁻¹ folic acid, 10 mg·l⁻¹ pyridoxine HCl, 5 mg·l⁻¹ thiamine HCl, 5 mg·l⁻¹ riboflavin, 5 mg·l⁻¹ nicotinic acid, 5 mg·l⁻¹ pantothenic acid, 0.1 mg·l⁻¹ cyanocobalamin, 5 mg·l⁻¹ *p*-aminobenzoic acid, 5 mg·l⁻¹ thioctic acid), 0.1ml

Trace Minerals (100 mg·l⁻¹ ZnSO₄, 300 mg·l⁻¹ H₃BO₃, 300 mg·l⁻¹ CoCl, 10 mg·l⁻¹ CuCl) and 0.8 ml N- & P-free (pH 7.2) Winogradsky medium (62.5 g·l⁻¹ MgSO₄·7H₂O, 31.25 g·l⁻¹ NaCl, 1.25 g·l⁻¹ FeSO₄, 1.25 g·l⁻¹ MnSO₄) per 100 ml of sterile deionized water) in 125-ml serum bottles. Cultures were incubated at room temperature (approx. 25 °C), with constant shaking at 170 rpm.

In order to assess the degree of N- and P-limitation on PAH degradation inherent in each soil, ¹⁴C-hexadecane mineralization was measured in cultures of each of two crude oil-contaminated wellhead soils which received no supplemental N or P, N only (as NH₄Cl), P only (as KH₂PO₄), or both N and P. CO₂ traps were made by wrapping stainless steel wire around the necks of 12 x 32 mm borosilicate glass autosampler vials and pushing the wire through 20-mm Teflon silicone-lined septa. These assemblies were placed in the serum bottles, which were then crimped with aluminum seals. Syringes were used to inject 1 ml of 0.5M NaOH into each CO₂ trap. Periodically, the CO₂-containing NaOH solution was withdrawn from the traps, mixed with 5 ml of Ultima Gold® high-flashpoint LSC cocktail solution (Packard, Meriden, CT), and counted in a liquid scintillation counter (Packard Model 2200CA Tri-Carb). Fresh NaOH was then added to the CO₂ traps. Cultures containing ¹⁴C-hexadecane typically received *ca.* 60,000-80,000 dpm of labeled hexadecane in 20 µl of methanol.

Six combinations were then investigated for nitrogen (N) and phosphorus (P) supplementation: NH₄Cl/KH₂PO₄; N₂O/KH₂PO₄; NH₄Cl/TEP; NH₄Cl/TBP; N₂O/TEP and N₂O/TBP. Within each condition, duplicate cultures were employed. In all cases, addition of N and P sources was normalized on a molar basis to provide 9.2 mM N and 3.7 mM P. When N₂O was used, it was added by injection to sealed bottles.

Toxicity Determinations with Microtox® Solid-Phase Test

In order to determine whether the Microtox® solid-phase test would be suitable for assessing the potential toxicity of the E&P soils to be used in solid-state remediation experiments and in determinations of environmentally acceptable endpoints, this assay was run on one of the oil-contaminated soils (AB). Microtox® Solid-Phase assays, marketed as test kits by Azur Environmental, Carlsbad, California, will be used to obtain soil toxicity data. In these tests, toxicity is determined by a decrease in metabolism, and

thus a decrease in light output, which can be sensed by a modified spectrophotometer. Microtox[®] software then calculates the EC₅₀ and 95% confidence intervals from replicates of each sample analyzed. Microtox[®] Solid-Phase tests was used to determine baseline toxicity in the soil samples selected for study. The manufacturer's protocols were used for all Microtox[®] tests. Coarse sand was used as a control.

Results And Discussion

Determination of Nutrient Limitations in E&P Soils

The extent of hexadecane mineralization in soil-slurry bioreactors containing indigenous microbes from the two oil-contaminated E&P soils are shown in Figure 2 below. Both of the two soils tested evidenced very significant limitations for nitrogen; this was most notable in soil AB, in which the amount of $^{14}\text{CO}_2$ released through hexadecane mineralization was approximately 17-fold higher in N-supplemented conditions versus those with no nutrient addition. Similar patterns were seen in soil C, although the degree of stimulation by supplemental N was not as high as in soil AB. The other notable difference between the two soils was in the significance of the phosphorus limitation: While soil AB supported significantly higher mineralization when both N and P were added (vs. N only), the two conditions behaved virtually identically in soil C.

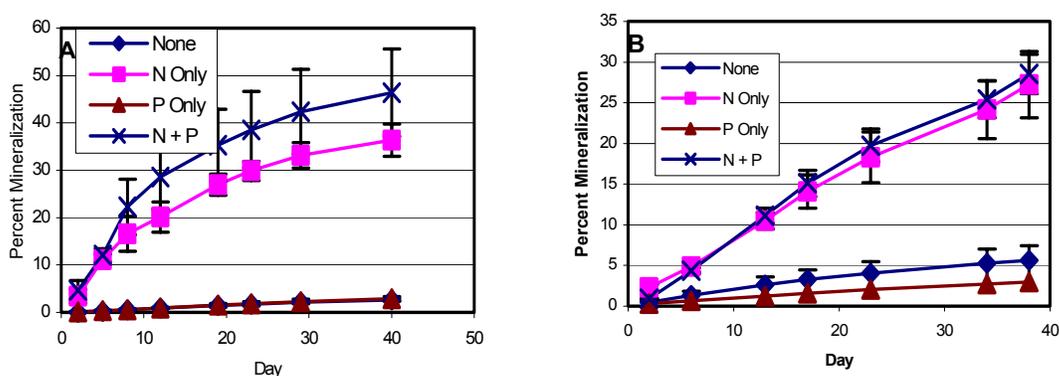


Figure 2 – Effects of N and P addition on mineralization of hexadecane by microbes indigenous to oil-contaminated E&P soils. Graph A = Soil AB; Graph B = Soil C

Thus, it can be concluded that, in terms of degradation of oil-related hydrocarbons, the microbes in soil AB are strongly limited by their available N supply, and, when N is added in sufficient levels, a significant P limitation also becomes apparent. In contrast, soil C contained sufficient P supplies that N alone was the limiting factor for hydrocarbon degradation. Given that the goal of the experiments undertaken here was to evaluate potential gaseous N and P sources for their effects on hydrocarbon remediation, soil AB was selected for further work, due to the fact that it was found to be limited for both nutrients.

The ability of the indigenous microbes in soil AB to mineralize hexadecane was then investigated under different conditions of nitrogen and phosphorus supplementation. The results of this experiment are shown in Figure 3 below.

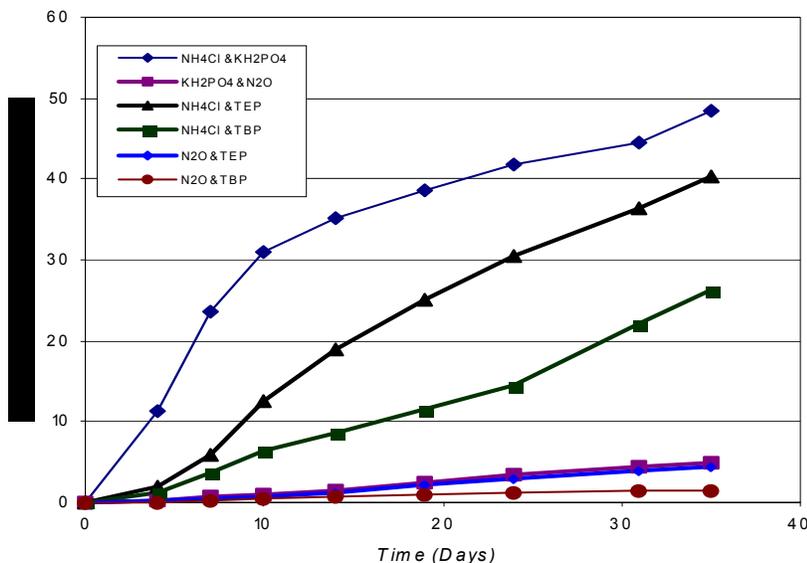


Figure 3 – Ability of gaseous N and P compounds to overcome nutrient limitations and support hexadecane mineralization by indigenous microbes in soil AB.

These results clearly show that use of TEP as an alternative to inorganic phosphate to support remediation of aliphatic hydrocarbons is quite feasible in this soil. Mineralization of hexadecane with TEP as a phosphorus source was nearly equal to that with KH₂PO₄. TBP supported considerably less hexadecane mineralization, and use of N₂O as a source of N was found to be extremely deleterious to the performance of the microorganisms in this soil. Other tests with soils with PAHs as the target for biodegradation indicated the opposite effect. Environmental conditions of the treatment regime, such as aw, pH, application of chemical oxidants, i.e. Fenton's reagent impact the effectiveness. These factors can be determined and incorporated into the treatment technology.

Conclusions

These results support our previous observation that each soil and its associated microorganisms is unique. Due to the unique features of these soils and their response to

nutrient stimulation, each soil with its comittent contaminants must be evaluated to determine the most effective treatment parameters to met or exceed the treatment endpoints. These endpoints can be determined using traditional analytical methods or risk or toxicity associated techniques, such as solid-phase Microtox[®].

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**DEGRADATION OF POLYCYCLIC AROMATIC AND STRAIGHT-
CHAIN ALIPHATIC HYDROCARBONS BY A NEW STRAIN OF
*MYCOBACTERIUM AUSTRROAFRICANUM***

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Abstract

We have isolated, identified, and characterized of a new strain of *Mycobacterium austroafricanum*, obtained from manufactured gas plant (MGP) site soil, which we have designated GTI-23. This strain is capable of mineralizing the polycyclic aromatic hydrocarbons (PAHs) fluoranthene and pyrene in liquid culture. It is also capable of degrading, but not mineralizing, fluorene and benzo[*a*]pyrene (although the extent of degradation of the latter of these is relatively minor). Extensive and rapid mineralization of pyrene by GTI-23 was also observed in pyrene-amended soil. When grown in liquid culture, GTI-23 was also found to be capable of growing on and mineralizing two aliphatic hydrocarbons (dodecane and hexadecane). Taken together, these findings indicate that this isolate of *M. austroafricanum* may be useful for bioremediation of soils and sediments contaminated with complex mixtures of aromatic and aliphatic hydrocarbons.

Introduction

Bioremediation has long been proposed and applied as a treatment technology for the decontamination of hydrocarbon-contaminated soils. Many different bacteria are known which are capable of degrading, and in many cases, completely mineralizing, various individual xenobiotic compounds. For example, in the case of polycyclic aromatic hydrocarbons (PAH), numerous bacteria are known which are capable of catabolizing various PAH as sole sources of carbon and energy, making them good candidate species for site-remediation applications. The ability to degrade low molecular weight PAH compounds, such as naphthalene and phenanthrene, is widespread, and numerous researchers have identified bacteria capable of utilizing these compounds for growth (Cerniglia 1992; Sutherland et al 1995). Growth on PAH containing four fused aromatic rings (*e.g.* chrysene, fluoranthene, pyrene, benz[*a*]anthracene) is somewhat more rare, although organisms are known which can utilize each of these as growth substrates (Heitkamp et al 1988a & b; Churchill et al 1999; Schneider et al 1996; Mueller et al 1990; Boldrin et al 1993; Bastiaens et al 2000, Walter et al 1991, Bouchez et al 1997, Juhasz et al 1997; Caldini et al 1995; Weissenfels et al 1991; Vila et al 2001). Currently, very few bacteria have been reported to be capable of growth using solely PAH with five or more benzene rings (Juhasz et al 1997), although some cases have been

reported in which bacteria co-metabolize five-ring PAHs during growth on simpler substrates (Chen and Aitken 1999; Schneider et al 1996).

Many contaminated sites are characterized by the presence of complex mixtures of pollutants. For example, creosotes and the coal tars from which they are derived typically comprise a wide range of aromatic hydrocarbons, aliphatics, heterocyclic (N-, S- and O-containing) compounds, phenols and amines (Rhodes 1951; Nestler 1974; Nishioka et al 1986). Crude and refined oils present a similar situation; for example, a typical fuel oil (#2) consists of 45% cycloalkanes, 30% linear (straight-chain and branched) aliphatics, and 25% aromatics (Arvin et al 1988). It is, therefore, clear that the success of bioremediation approaches to soil treatment will hinge in part on the ability of bacteria (either versatile single strains or consortia in which multiple members can be simultaneously maintained) to degrade all of the components of complex hydrocarbon mixtures.

Members of the genus *Mycobacterium* may be particularly well-suited to this role. Mycobacteria, for example, are well known to possess extremely lipophilic cell surfaces (Rehmann et al 1988), which may make them better suited to the direct uptake (Bouchez-Naïtali et al 1999) of highly hydrophobic hydrocarbons, including high-molecular-weight PAHs (Kelley and Cerniglia 1995; Schneider et al., 1996) and highly branched aliphatic hydrocarbons (Berekaa and Steinbüchel 2000; Solano-Serena et al., 2000). This paper presents an initial characterization of a newly-isolated strain (GTI-23) of *Mycobacterium austroafricanum*, which is capable of growth on, and/or degradation of, various PAHs (with up to 5 fused rings), as well as straight-chain aliphatic hydrocarbons (decane, dodecane, and hexadecane).

Methods and Materials

Chemicals

Fluorene (98%), phenanthrene (98%), and pyrene were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). Fluoranthene (Practical grade) was obtained from Eastman Organic Chemicals (Rochester, N.Y.). Benzo[*a*]pyrene (98%), *n*-decane (99%+), *n*-dodecane (99%), *n*-hexadecane (99%+), and gelrite gellan gum were from

Sigma Chemical Co. (St. Louis, MO), as were the following ^{14}C -radiochemicals: 7- ^{14}C -benzo[*a*]pyrene (26.6 mCi/mmol), 9- ^{14}C -fluorene (14.2 mCi/mmol), 4,5,9,10- ^{14}C -pyrene (58.7 mCi/mmol), 1- ^{14}C -dodecane (4.1 mCi/mmol), and 1- ^{14}C -hexadecane (2.2 mCi/mmol). 3- ^{14}C -fluoranthene (45 mCi/mmol) was from Moravek Biochemicals (Brea, CA).

Isolation, Identification, and Characterization

A small amount of homogenized soil (~1 g), obtained from a former manufactured gas plant site in Iowa, was reconstituted with 25 ml of sterile deionized water and serial 10-fold dilutions were made; appropriate dilutions (from 10^{-5} through 10^{-8}) were spread onto gel plates (1.0 g KNO_3 , 0.38 g K_2HPO_4 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 17.0 g gelrite gellan gum (Sigma Chemical Co., St. Louis, MO) per liter of distilled water, pH 7.0). Pyrene was then sublimated onto the plates (Alley and Brown 1999), which were then incubated at 30 °C. A clearing zone was observed around a smooth, yellowish colony within approximately 8-10 days.

This colony was further subcultured until cellular and colonial morphologies were uniform. Both pyrene- and phenanthrene-sublimated plates were employed during this procedure; the latter was occasionally used due to the fact that growth of the isolate was more rapid on these plates (generally requiring 2-3 days or less). Culture purity was assessed microscopically, and by streaking onto R2A agar (Difco Products, Detroit, MI). Once an axenic culture was obtained, it was identified by sequencing of 16s rRNA gene (MIDI Labs, Newark, DE). The resultant sequence was matched using the GenBank database. Fatty acid determinations were done (MIDI Labs) using bacteria grown on R2A agar plates.

Hydrophobicity of the isolate was determined according to the “BATH” (bacterial adhesion to hydrocarbons) method, as described by Bouchez-Naïtali et al (1999). Briefly, cultures were grown in medium (YPS for *Mycobacterium* strains, MSM for other isolates), supplemented with crystalline phenanthrene. When cultures became visibly turbid, cells were collected by centrifugation, washed once, and resuspended in phosphate buffer. Cell suspensions were adjusted to a uniform A_{600} value (0.1 – 0.2), and 100 μl of hexadecane was added to 2-ml aliquots of cell suspension. Cells and

hexadecane were extensively mixed (vortexed for 2 minutes); the hexadecane layer was then given 60 minutes to partition away from the aqueous phase. A_{600} measurements of the remaining aqueous layer were taken; hydrophobicity is expressed as the ratio of this value to the initial A_{600} .

Liquid-Culture Studies

Two primary growth media were employed in this work. The media used in studies assessing the ability of GTI-23 to grow on hydrocarbons as sole sources of carbon and energy was the previously-described (Bogan et al 2001) mineral salts medium (MSM). The second medium, used for studies of co-metabolism and mineralization studies, was the *Mycobacterium* mineral salts medium, supplemented with peptone, yeast extract and soluble starch (each at 250 mg·l⁻¹), as described by Kelley and Cerniglia (1995).

Studies examining growth of GTI-23 on alkanes were conducted in 96-well microplates, which contained 150 μ l of MSM and 5 μ l of liquid hydrocarbon. Growth was monitored (A_{600}) in an automated microplate reader (Dynex Technologies, Chantilly, VA). Liquid-culture experiments on degradation and mineralization of individual PAHs and alkanes were conducted in YPS medium (50 ml) in sealed serum bottles with NaOH traps as previously described (Bogan et al 2001). In the cases of PAHs, 20 mg were added to each bottle as previously described (Mueller et al 1990); for alkanes, 10 μ l of liquid hydrocarbon was added to each bottle. Experiments were typically run for 3-4 weeks; photodegradation of PAH (Miller et al 1988) was avoided by wrapping bottles in aluminum foil.

Analyses of fluorene- and benzo[*a*]pyrene-supplemented cultures were conducted as follows. Cultures were acidified (pH \sim 0.5) with concentrated H₂SO₄, and disrupted in a sonicator bath for *ca.* 3 minutes. Cultures were extracted twice with ethyl acetate (50 ml), which was then pooled. Aliquots of the remaining aqueous phase and the organic-extractable fractions were subjected to scintillation counting to determine the distribution of radioactivity between the two phases. To further characterize the organic-soluble fraction, ethyl acetate was evaporated (to dryness) under N₂. Following dissolution in acetonitrile (1 ml), the organic-soluble material was subjected to reverse-phase HPLC

analysis as previously described (Bogan et al 2001), with fractions (1-minute intervals) of the HPLC eluent collected for quantitation of ^{14}C .

Pyrene Mineralization in Soil

A commercial potting soil was screened, and allowed to air-dry. This material was then weighed into flasks (60 g/flask), and spiked with pyrene and ^{14}C -pyrene (dissolved in 20 ml methylene chloride), such that the final soil pyrene concentration was 100 ppm, with a total of 150,000 dpm of ^{14}C -pyrene per flask. After evaporation of solvent (approximately 48 hours), these were then adjusted to a moisture content of *ca.* 25%, and inoculated with an MSM suspension of *M. austroafricanum* GTI-23 (1 ml per flask of a suspension with $A_{600} \sim 2.7$). Flasks were attached to a constant air flow (*ca.* 25 ml/min); air exiting the microcosms was flushed through CO_2 traps containing 20 ml of 0.5 M NaOH. Every 2-4 days, NaOH in the traps was replaced, and 5 ml was added to 15 ml of scintillation cocktail and subjected to liquid scintillation counting. Water was periodically (every 1-2 days) added to microcosms to replace evaporative losses.

Results

Identification and Morphology

Searches of the GenBank database using the sequence data determined for the 16S rRNA gene of the GTI-23 isolate resulted in a 100% match to *Mycobacterium austroafricanum*. GTI-23 was found to be a gram-positive rod, typically in the range of $0.6 \times 1.5 \mu\text{m}$, which is in good agreement with the dimensions ($0.5 \mu\text{m} \times 2-6 \mu\text{m}$) recognized for *M. austroafricanum* (Sneath et al 1989). The fatty acid profile of GTI-23 is given in Table 1.

Hydrophobicity

Strain GTI-23 was considerably more hydrophobic than various strains of PAH-degrading soil bacteria (*Acidovorax*, *Burkholderia*, *Pseudomonas*, and *Sphingomonas*) which we have previously isolated. Of the tested strains, only one *Sphingomonas* (GTI-

8) was at all close to GTI-23 in net hydrophobicity; *Mycobacterium* sp. strain PYR-1 (obtained from Dr. C. Cerniglia) had approximately the same hydrophobicity value as GTI-23 (52% vs. 48%) when both were cultured on phenanthrene-supplemented YPS medium.

Degradation of PAH and Alkanes

Mycobacterium austroafricanum GTI-23 was originally recognized by, and chosen for, its ability to degrade phenanthrene, sublimated onto minimal agar (gelrite) medium as sole sources of carbon and energy. It was also found, in liquid culture, to be capable of mineralizing pyrene and fluoranthene, as shown in Figure 1. Release of ^{14}C from labeled pyrene was rapid, and totaled essentially 100%, implying (because of the distribution of the label across four positions within the molecule) near-complete mineralization. Mineralization of fluoranthene displayed a slight lag (5-7 days), and leveled off after approximately 60% of the input radioactivity had been released as $^{14}\text{CO}_2$.

No release of $^{14}\text{CO}_2$ was observed from either fluorene or benzo[*a*]pyrene at these same initial concentrations. Essentially all of the input radioactivity was recovered at the conclusion of the experiment; for both compounds, the vast majority (>95%) remained in the organic-soluble fraction. However, analysis of these fractions by reverse-phase HPLC indicated that extensive transformation of fluorene had, in fact, occurred (Figure 2a); the products thus formed were somewhat more polar than fluorene, and may have been 9-fluorenone and/or 9-hydroxyfluorene. These compounds have previously been reported as dead-end products of fungal oxidation of fluorene (Bogan et al 1996), and as isolable, accumulated intermediates in bacterial fluorene metabolism (Grifoll et al 1994; Casellas et al 1997). Figure 2b shows reverse-phase HPLC analysis of the ethyl acetate-extractable products of *M. austroafricanum* cultures which contained ^{14}C -benzo[*a*]pyrene. The starting material, when analyzed the same way, was found to be 97% radiochemically pure, with the only significant impurity (2-3% of total label) eluting at approximately 18 minutes. In the *M. austroafricanum*-inoculated cultures, approximately 16-20% of the starting material was converted to more-polar products. One major product (*ca.* 8% of input label) had a slightly lower retention time than

benzo[*a*]pyrene (24 minutes vs. 26-27), and was most likely a mono-hydroxylated metabolite; whereas the second (7-8 % of input label, 18 min. RT) may have been a dihydrodiol. Approximately 5% of the input label was recovered in more-polar fractions.

Strain GTI-23 was found to be capable of growth on both decane and hexadecane (Figure 3), and of mineralization of hexadecane and dodecane (Figure 4). Growth on both of these began to level off after approximately 20 days, a figure which coincided well with the leveling-off of ¹⁴CO₂ liberation in the mineralization experiment.

When inoculated into pyrene-spiked soil, GTI-23 was capable of extensively mineralizing ¹⁴C-pyrene, as is shown in Figure 5. As in liquid culture, the onset of mineralization is rapid, with no significant lag time, and persists for several weeks of incubation time. Mock-inoculated (MSM, no GTI-23) microcosms released insignificant levels (<1,000 dpm) of ¹⁴CO₂ over this time frame, indicating (as expected) that no pyrene-degrading microorganisms were present in the absence of inoculation.

Discussion

The ability to catabolize PAHs with more than three fused benzene rings is relatively rare among bacteria, particularly when compared to the ability to grow on lower-molecular-weight polycyclic aromatics. To date, growth on pyrene has been observed in some species of *Rhodococcus* (Walter et al 1991; Bouchez et al 1997) and strains of *Burkholderia cepacia* (Juhasz et al 1997); additionally, cometabolism of pyrene has been observed in *Pseudomonas saccharophila* (Chen and Aitken 1999). However, the ability to grow on high-molecular-weight PAH substrates may be most widespread in the genus *Mycobacterium*, where this result has been reported for several different species and strains (Heitkamp et al 1988b; Churchill et al 1999; Schneider et al 1996; Boldrin et al 1993; Bastiaens et al 2000; Vila et al 2001). At least one of these, *Mycobacterium* PYR-1 (Kelley and Cerniglia 1995; Rafii et al 1992) is, based on recently-published evolutionary distance trees (Solano-Serena et al 2000), very closely related to *M. austroafricanum*.

Among the PAH-degrading Mycobacteria, some are also known to degrade aliphatic hydrocarbons. Solano-Serena et al (2000) reported that *Mycobacterium austroafricanum* strain IFP2173, originally isolated from gasoline-contaminated

groundwater, was able to degrade some monoaromatics (e.g. toluene, *m*- and *p*-xylene), as well as a wide range of straight-chain and branched alkanes. They did not, however, examine degradation of polycyclic aromatics. Several other strains which were identified as *Mycobacteria* were able to degrade and grow on alkanes (dodecane and hexadecane) as well as 3- and 4-ring PAHs (phenanthrene, fluoranthene, pyrene), although the authors (Lloyd-Jones and Hunter 1997) suggested that some of these strains might actually belong to a new genus, rather than *Mycobacterium*. Growth substrates of *Mycobacterium* strain AP-1 included phenanthrene, fluoranthene, pyrene and hexadecane (Vila et al 2001). In addition to our findings with GTI-23, we have also observed mineralization (in YPS medium) of dodecane and hexadecane by *Mycobacterium* strain PYR-1 (data not shown), further extending within the genus *Mycobacterium* the ability to degrade both of these contaminant classes.

To our knowledge, GTI-23 is the first strain which has been reported to degrade both aliphatics and PAHs with five benzene rings (benzo[*a*]pyrene). Inasmuch as this latter compound is among the most hazardous individual PAHs (Smucker 2000), this finding has some significance. Also, in contrast to the studies described above, the results reported herein provide clear evidence of GTI-23's ability to survive and degrade PAH in a soil system. Failure of inoculated bacteria to persist in soil is a frequent impediment to successful bioremediation (Van Dyke and Prosser 2000). Further examination of strain GTI-23 is underway, with a goal of better delineating its ability to survive, compete, and function in soil environments under various conditions.

The relative rarity of bacterial species capable of growth on PAHs containing four or more fused rings is most likely due to the difficulties inherent in uptake of highly hydrophobic compounds of this nature. This is generally accomplished through specialized adaptations, such as lipid-rich outer cell walls and/or production of biosurfactants (Bouchez-Naïtali et al 1999; Bastiaens et al 2000). Based on our results, *M. austroafricanum* GTI-23, as well as the closely-related *Mycobacterium* sp. PYR-1, are considerably more hydrophobic than any of the other PAH-degrading soil bacteria which we tested. The precise nature of the adaptations which allow uptake of high molecular weight PAH compounds by *M. austroafricanum* GTI-23 is the subject of ongoing

investigations in this laboratory, as is the possible use of this strain as an inoculum for soil remediation.

Acknowledgments

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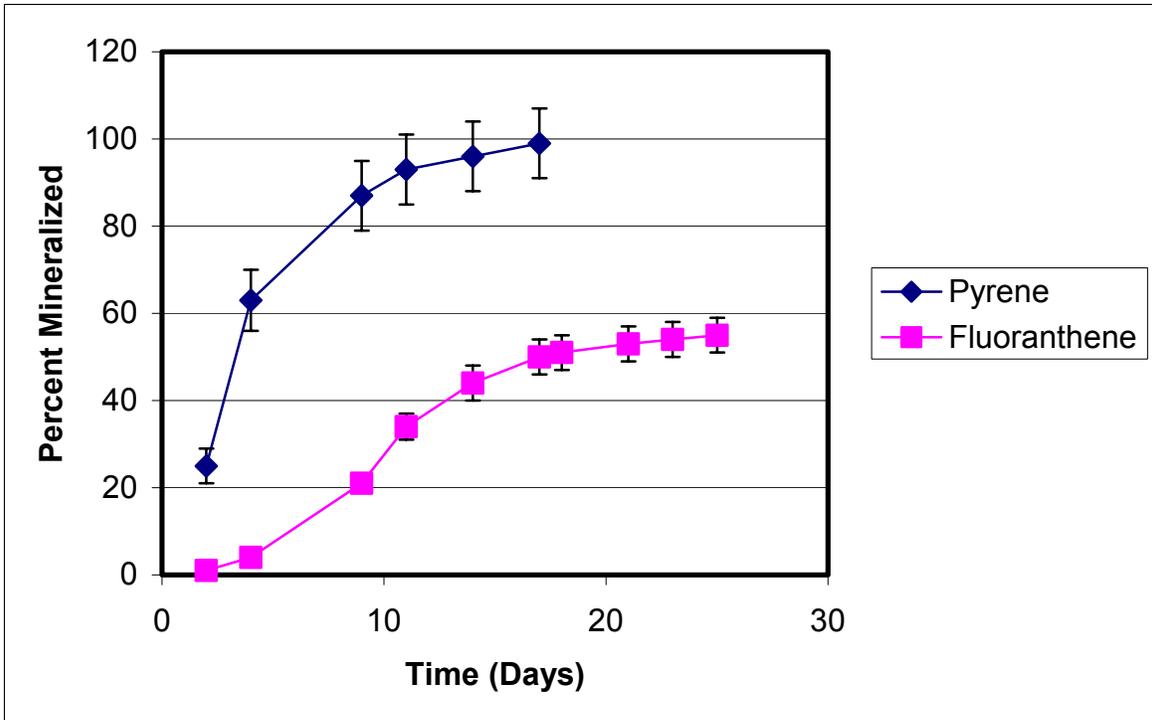
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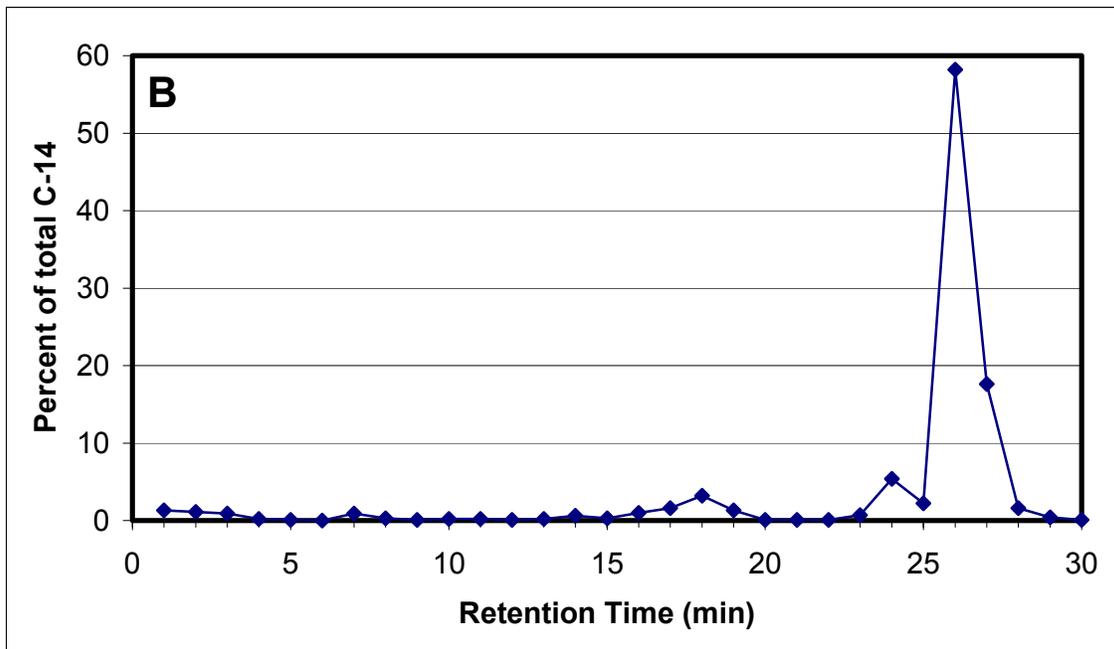
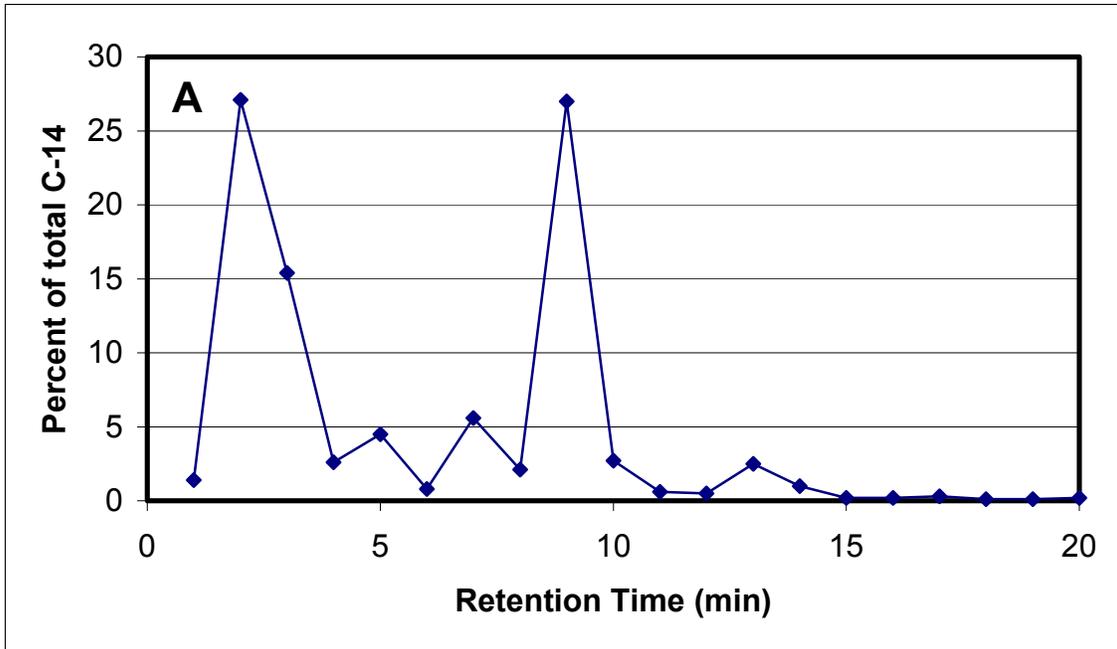
Component	% of Total
14:0	3.20
15:0	0.97
16:1 w9c	1.70
16:1 w6c	13.78
16:0	34.63
8-Me-16:0 + 10-Me-16:0	1.09
17:1 w8c	0.87
17:0	0.60
18:2 w6,9c	0.51
18:1 w9c	26.94
18:0	2.66
10-Me18:0	12.39
20:0	0.67

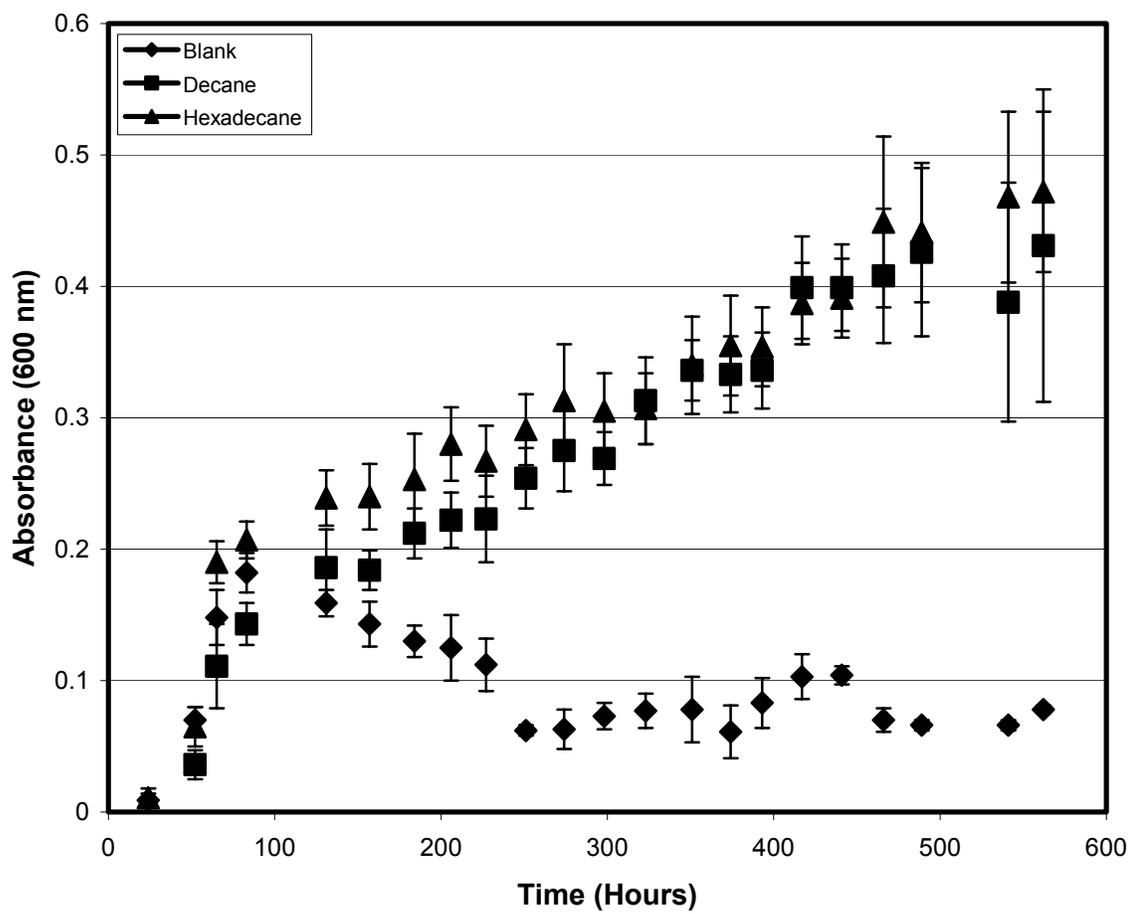
Table 1 – Fatty acid profile of *Mycobacterium austroafricanum* strain GTI-23.

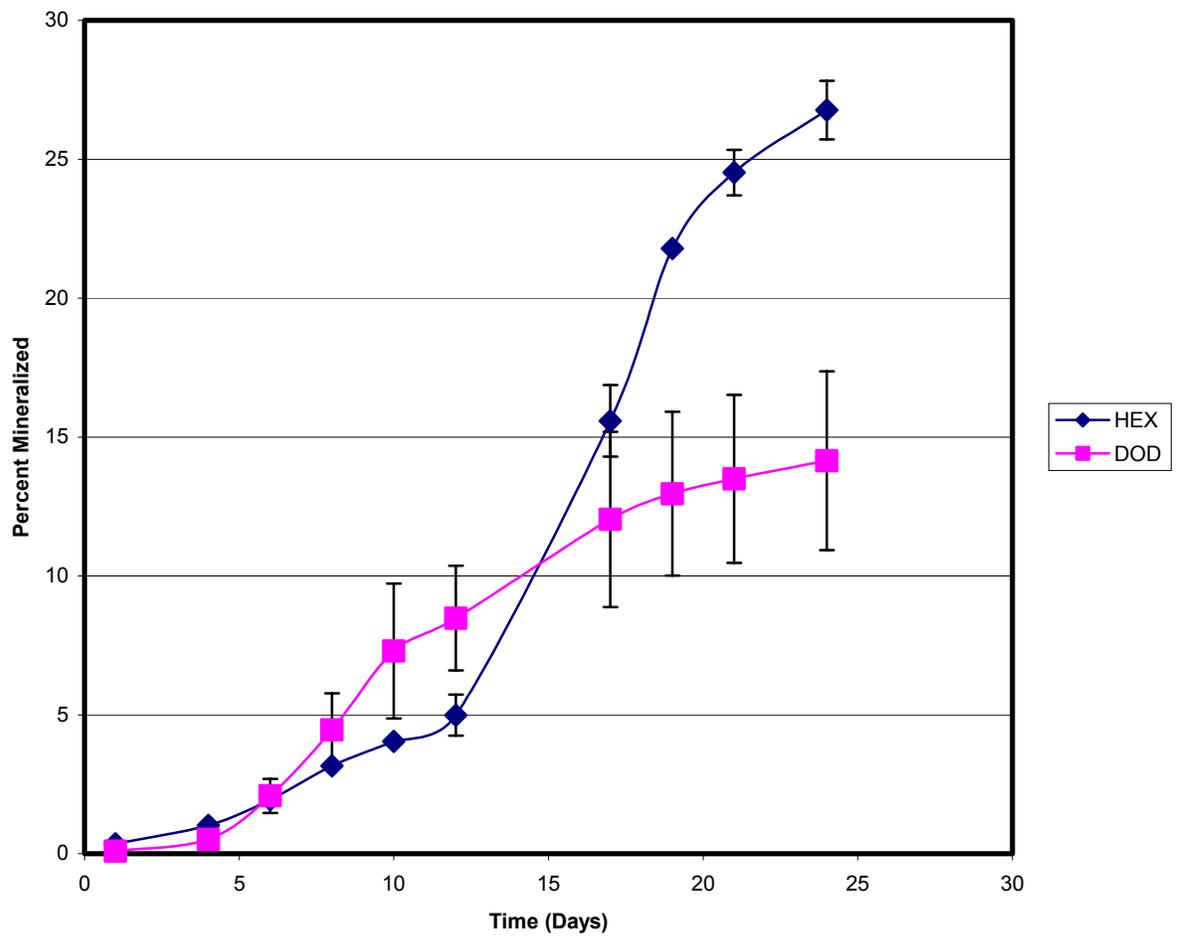
Isolate	Medium	Culture Age	Hydrophobicity
Acidovorax temperans GTI-19	MSM/phenanthrene	1 day	3%
Burkholderia sp. GTI-3	MSM/phenanthrene	6 days	15%
Pseudomonas viridiflava GTI-5	MSM/phenanthrene	6 days	4%
Sphingomonas sp. GTI-7	MSM/phenanthrene	2 days	15%
Sphingomonas sp. GTI-8	MSM/phenanthrene	6 days	26%
Sphingomonas sp. GTI-10	MSM/phenanthrene	6 days	0%
Sphingomonas sp. GTI-11	MSM/phenanthrene	6 days	18%
Sphingomonas subarctica GTI-12	MSM/phenanthrene	2 days	0%
Mycobacterium austroafricanum GTI-23	YPS/phenanthrene	6 days	48%
Mycobacterium sp. PYR-1	YPS/phenanthrene	6 days	52%

Table 2 – Hydrophobicity of *M. austroafricanum* GTI-23, in comparison with various other PAH-degrading isolates









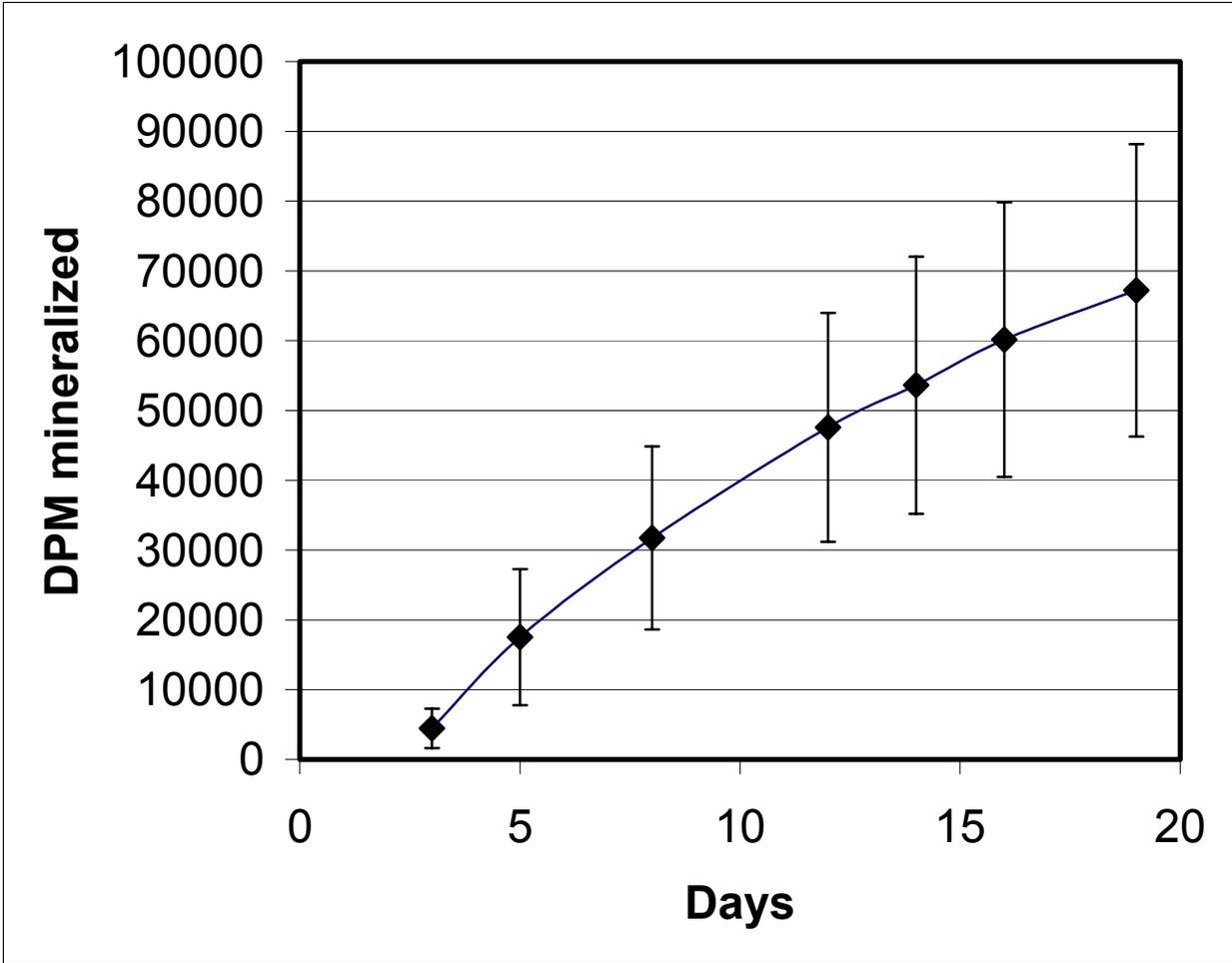


Figure 1 – Mineralization of fluoranthene and pyrene (Initial = 20 mg/50 ml) by *Mycobacterium austroafricanum* GTI-23 grown in YPS medium. Data are means of duplicate cultures, with 95% confidence limits.

Figure 2 – Reverse-phase HPLC profiles of ethyl acetate-soluble radioactivity derived from *M. austroafricanum* cultures supplemented with fluorene or benzo[*a*]pyrene.

Figure 3 – Growth of *M. austroafricanum* GTI-23 in MSM supplemented with either decane or hexadecane. Data are means of triplicate cultures, with 95% confidence limits.

Figure 4 – Mineralization (in YPS liquid cultures, n=2) of ¹⁴C-dodecane and hexadecane by GTI-23.

Figure 5 – Mineralization of ¹⁴C-pyrene (Initial = 100 ppm; 150,000 dpm) in non-sterile soil microcosm (n=3) by *M. austroafricanum* GTI-23.

**PRESENTATION AT GTI'S SITE REMEDIATION TECHNOLOGIES &
ENVIRONMENTAL MANAGEMENT IN THE UTILITY INDUSTRY.**

DECEMBER 2-6, 2001

**Environmental Risk Reduction After
Application of Chemically Accelerated
Biotreatment to Hydrocarbon-
Contaminated Soils Under Water-
Saturated and Unsaturated Conditions**

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Contaminants

- Benzene, Toluene, Ethyl Benzene & Xylenes (BTEX)
- Fuel Oil and Diesel Fuel
- Biocides and Corrosion/Scale Inhibitors
- Polynuclear Aromatic Hydrocarbons (PAH)
- Heavy Metals - arsenic, barium, cadmium, chromium, lead, mercury, selenium, silver, and zinc
- Mercaptans and Hydrogen Sulfide

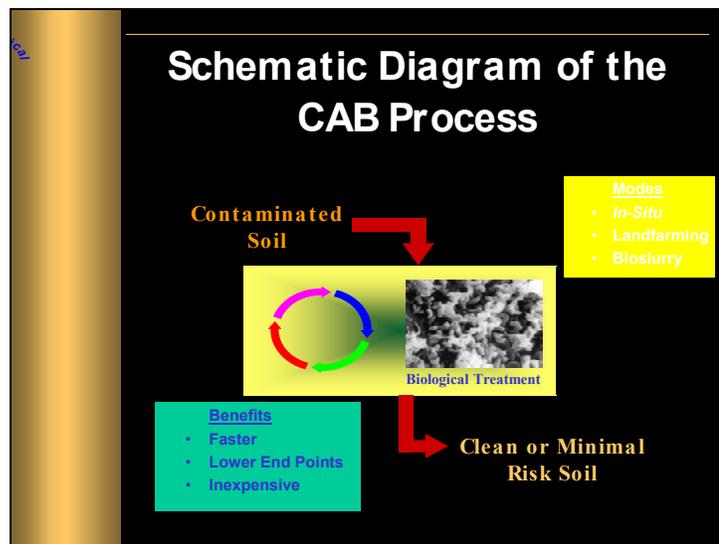
Slide 3

Contaminated Site Remediation Approaches

- Chemically Accelerated Biological Treatment (*In-Situ* & *Ex-Situ*)
- Accelerated Intrinsic Remediation (*In-Situ*)
- Biosparging/Bioventing (*In-Situ*)
- Enhanced Bioremediation (*In-Situ* & *Ex-Situ*)
- Chemical Treatment/Oxidation (*In-Situ* & *Ex-Situ*)
- *In-Situ* Thermal Desorption
- Thermal Oxidation (*Ex-Situ*)
- Phytoremediation (*In-Situ*)
- Electrokinetic Remediation (*In-Situ*)
- Various Combinations



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Environmental Acceptable Endpoints (EAE)



There are concentrations of chemicals in soil greater than "zero" that are safe to humans and the environment



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Risk-Based Site Management Strategies

- Site Assessment for Bioavailability & Toxicity
- Risk Assessment Using Innovative Approaches such as "bioavailable" fractions
- Active and Intrinsic Treatment Based on "rapid release" and "slow release" fractions
- Remedial Alternative(s) Development & Cost/Benefit Analysis

Slide 7

Consortium Engineering 

"Manipulating microbial consortia, which have so far been treated as black boxes, for constructing more efficient biotechnological processes..."
- K. Watanabe

Basics of Consortium Engineering

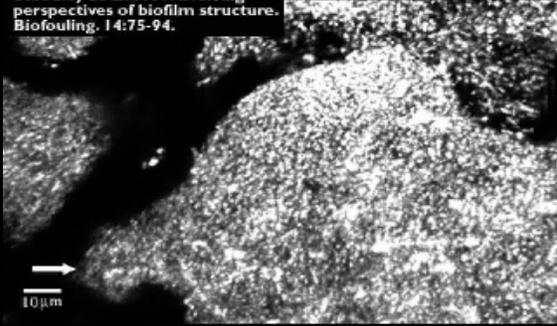
- Microbial Population Structure
- Function and Activities of these Populations
- Controlling or Forcing Function Engineering

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Microbial Consortia Are Complex in Space and Time

Stoodley et al. 1999. Evolving perspectives of biofilm structure. *Biofouling*, 14:75-94.



10 µm

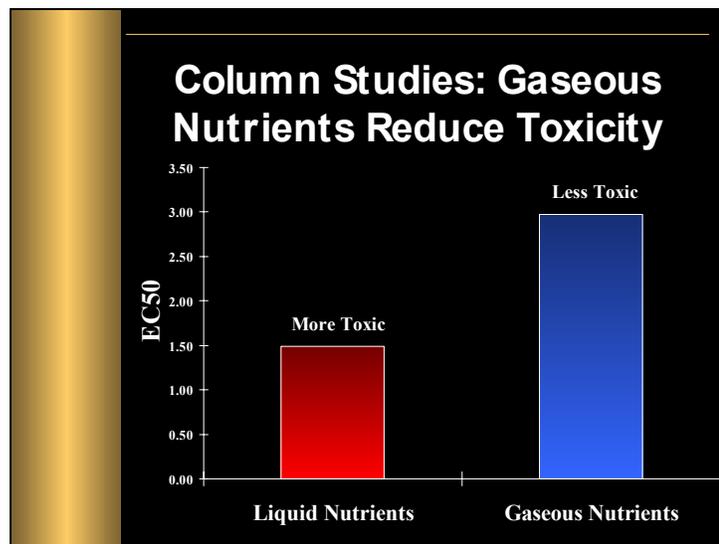
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Consortium Engineering

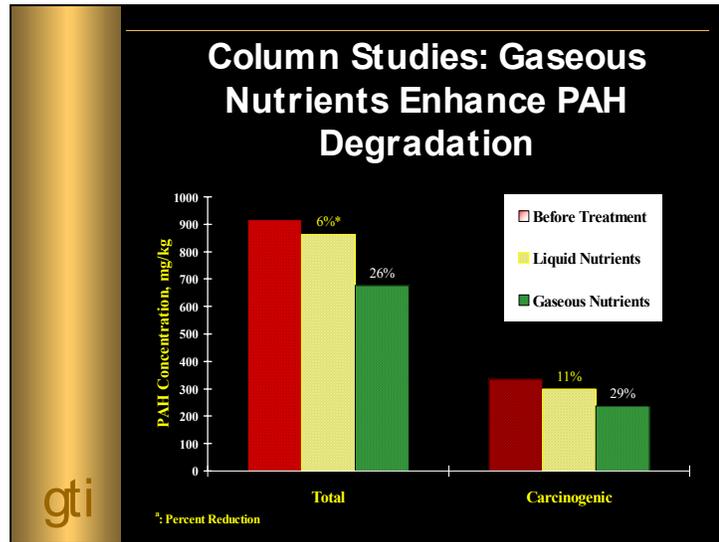
- Encourage the growth and activity of targeted microorganisms
- Minimize mass-transfer problems
- Optimize environmental conditions for degradation and detoxification reactions

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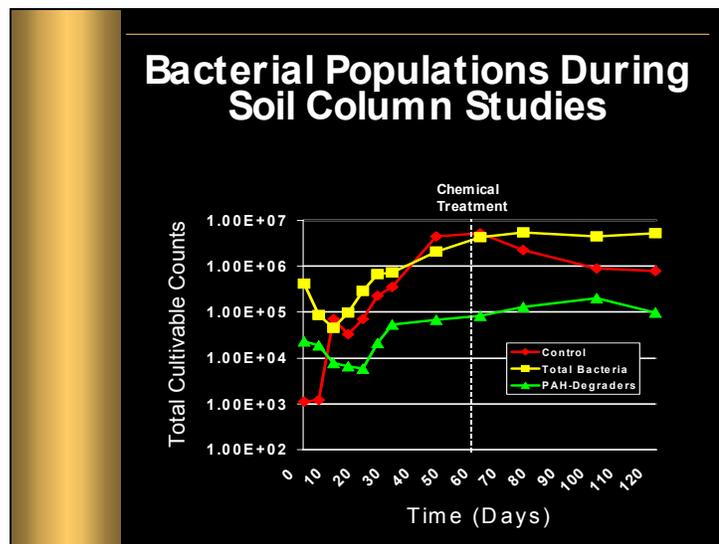
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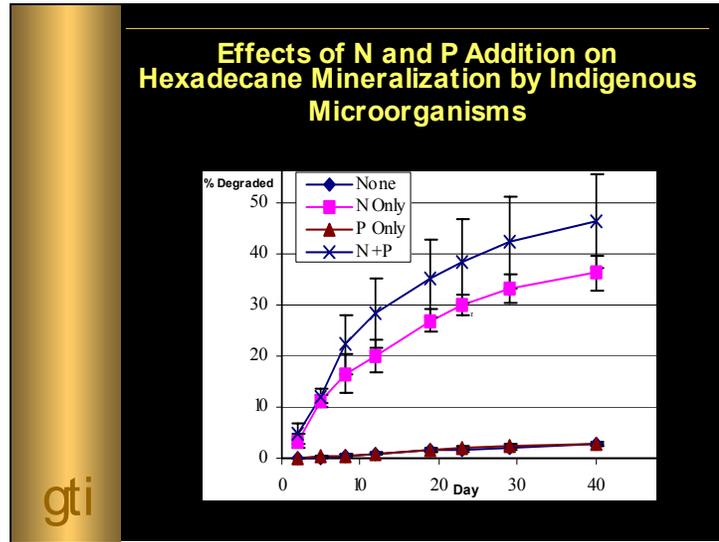
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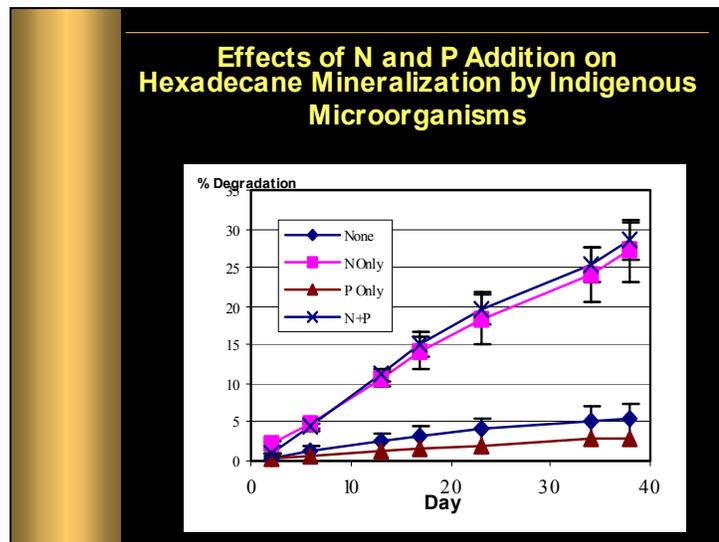
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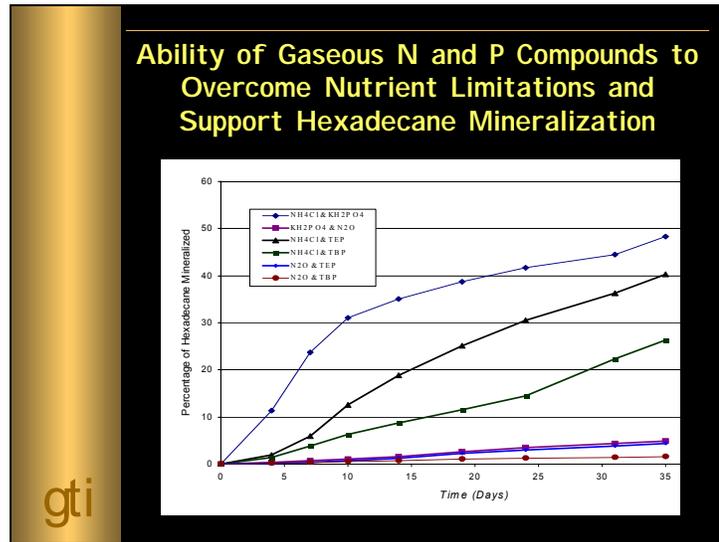
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- ### *In-situ* Chemically-accelerated Biodegradation Gaseous Nutrients Summary
- Gaseous nutrients can replace waterflood to deliver microbial nutrients
 - Decrease cost in material and operations
 - Minimizes moving contaminants off-site or into groundwater
 - Gaseous nutrients can stimulate selectively members of the microbial community
 - Large-ring PAHs (usually toxic or carcinogenic)-degrading bacteria enhanced in number and activity
 - Bacteria that degrade low-ring number PAHs (usually considered non-toxic) are less enriched
 - Gaseous nutrients can allow more air into soil or nutrients can be added to air sparging stream (oxygen of biodegradation)
 - Increase rate and extent of removal to toxic PAHs
 - Reduces risk from contaminants

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Various Bacterial Species Ability to Resist the Effect of Fenton's Treatment

Bacteria	# of trials	% Survival
<i>Acidovorax</i> sp.	1	8.5
<i>Bacillus simplex</i> **	1	98.1
<i>Burkholderia</i> 1	1	0.2
<i>Burkholderia</i> 2	2	25 ± 13
<i>Burkholderia</i> 3	1	0
<i>Mycobacterium austroafricanum</i>	2	85 ± 13
<i>Mycobacterium phlei</i>	1	61
<i>Pseudomonas</i> 2	2	5 ± 5
<i>Pseudomonas aeruginosa</i> R75**	1	0.5
<i>Pseudomonas fluorescens</i> R111**	1	0
<i>Rhodococcus</i> sp.	3	11 ± 10
<i>Sphingomonas</i> 1	1	0.3
<i>Sphingomonas</i> 4	1	0
<i>Sphingomonas</i> 8	1	0

** Indicates non-PAH-degrading strain included as a representative of general soil bacteria

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Inducibility of PAH Degradation

	Control (Glucose)	Salicylate-induced	R (S/G)
<i>Acidovorax</i>	3851 ± 310	3151 ± 400	0.82 ± 0.17
<i>Burkholderia</i> 1	288 ± 131	138 ± 42	0.48 ± 0.36
<i>Pseudomonas</i> 1	273 ± 63	209 ± 0	0.77 ± 0.18
<i>Pseudomonas</i> 2	105 ± 1	188 ± 54	1.79 ± 0.53
<i>Pseudomonas</i> 5	379 ± 197	172 ± 30	0.45 ± 0.32
<i>Sphingomonas</i> 1	149 ± 18	143 ± 15	0.96 ± 0.22
<i>Sphingomonas</i> 2	204 ± 7	391 ± 44	1.92 ± 0.28
<i>Sphingomonas</i> 3	180 ± 12	319 ± 89	1.77 ± 0.61
<i>Sphingomonas</i> 5	197 ± 18	251 ± 72	1.27 ± 0.48
<i>Sphingomonas</i> 12	354 ± 5	412 ± 36	1.16 ± 0.12

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 - Southern California Gas
 - Pacific Gas and Electric
 - others
 - MidAmerican Energy
 - Southern California Edison



***ALKANINDIGES ILLINOISENSIS* GEN. NOV., SP. NOV., AN
OBLIGATELY HYDROCARBONOCLASTIC, SQUALANE-DEGRADING
BACTERIUM ISOLATED FROM OILFIELD SOILS**

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Summary

An alkane-degrading bacterium, designated MVAB Hex1, was isolated from chronically crude oil-contaminated soil from a Southern Illinois oilfield. The isolate grew very weakly or not at all in minimal and rich media without hydrocarbons. Straight-chain aliphatics such as hexadecane and heptadecane greatly stimulated growth; shorter-chain ($\leq C_{15}$) hydrocarbons did not. Growth was also greatly enhanced by the branched aliphatic hydrocarbons pristane and squalane. The latter of these was most intriguing, as catabolism of squalane has hitherto been reported only for *Mycobacterium* species. Although unable to utilize mono- or polycyclic aromatics as sole carbon sources, the isolate did show slight fluorene-mineralizing capability in LB medium, which was partially repressed by hexadecane. In contrast, hexadecane supplementation greatly increased mineralization of ^{14}C -dodecane, which was not a growth substrate. BIOLOG testing further emphasized the isolate's extremely narrow substrate range, as only Tween 40 and Tween 80 supported significant growth. Microscopic examination (SEM and TEM) revealed a slightly polymorphic coccoidal to bacillar morphology, with hydrocarbon-grown cells tending to be more elongated. When grown with hexadecane, MVAB Hex1 accumulated large numbers of electron-transparent intracytoplasmic inclusion bodies. These were also prevalent during growth in the presence of squalane. Smaller inclusion bodies were occasionally observed with pristane supplementation; they were, however, absent during growth on crude oil. On the basis of 16s rRNA sequence data and range of growth substrates, we propose classification of this isolate as the type strain of a new genus, *Alkanindiges illinoisensis*, which is most closely related (*ca.* 94% sequence similarity) to *Acinetobacter*.

Bacterial biodegradation of crude oil first gained widespread attention as a potential remediation approach in the 1970's (Atlas and Bartha 1972; Soli and Bens 1972). Many bacteria degrade the aliphatic constituents of crude oils, including straight-chain aliphatics up to C₄₄ (Sakai *et al.*, 1994; Radwan *et al.*, 1996). Branched-chain alkanes, particularly those with anteiso-terminal branching (substitution on carbons immediately adjacent to the terminal carbon(s)), are much less susceptible to β -oxidation (Schaeffer *et al.*, 1979), and thus more recalcitrant than their unbranched counterparts. Thus, pristane (2,6,10,14-tetramethylpentadecane) was often used as an internal standard in determining biodegradation and weathering of other oil fractions (Alvarez *et al.*, 2001). More recently, however, species of several genera have been shown to biodegrade pristane (McKenna and Kallio 1971; Pirnik *et al.*, 1974; Nakajima and Sato 1983; Lal and Khanna 1996; Alvarez *et al.*, 2001). The longer and more heavily substituted squalane (2,6,10,15,19, 23-hexamethyltetracosane) has to date only been shown to be catabolized by two *Mycobacterium* species (Berekaa and Steinbüchel 2000).

The work presented herein describes the isolation and initial characterization of a bacterium with a very narrow range of growth substrates, restricted essentially to long-chain linear and branched aliphatic hydrocarbons, from a chronically heavily-contaminated oilfield site in southern Illinois. The soil from this site was very heavily contaminated with crude oil hydrocarbons, with TPH (total petroleum hydrocarbon) levels averaging 150,000 ppm. Samples (4 grams) of soil were added to 50 ml of mineral salts medium (MSM) (Bogan *et al.*, 2001a), containing 800 μ l of hexadecane. After 3 days of shaking, enriched cultures were subcultured into fresh hexadecane-supplemented MSM (300 μ l of primary culture to 50 ml medium). After one additional day, cultures were plated onto gelrite plates (Bogan *et al.*, 2001b), overlaid with a thin layer of liquid hexadecane (100 μ l). Colonies were subcultured onto R2A agar, overlain with hexadecane, until pure; the isolate, which was given the initial designation MVAB Hex1, was identified by sequencing 1533 base pairs of its 16S rRNA gene, using primers spanning positions 5 to 1540 in the corresponding *E. coli* sequence (MIDI Labs, Newark, DE). The resultant sequence was matched against GenBank using BLASTN 2.2.2 (Altschul *et al.*, 1997).

Based on the 16S rRNA gene sequence of the MVAB1 isolate, the highest degree of similarity (94% homology) observed was to *Acinetobacter junii*. This is at the lower limit of sequence homology among the 21 recognized *Acinetobacter* genospecies (Ibrahim *et al.*, 1997),

leaving some ambiguity as to whether a confident genus-level match can be made. However, when the 21 sequences used in the above phylogenetic analysis were aligned with the MVAB Hex1 sequence using Clustal W (Thompson *et al.*, 1994), we observed 36 individual nucleotides, distributed throughout the sequences, which, although strictly conserved among all *Acinetobacter* strains, diverged in MVAB Hex1.

Growth data on decane, dodecane, tetradecane, pentadecane, hexadecane, heptadecane, pristane and squalane (all > 98 % purity) were obtained using the following method. Wells in a 96-well plate received 150 μ L of LB or MSM media, 50 μ L of a bacterial suspended (in MSM) with an $A_{600} \sim 0.2$, and 5 μ L of hydrocarbon. Non-inoculated controls (with hydrocarbon) and hydrocarbon-only (no inoculum) were included, as were media-only blanks. Plates were incubated at room temperature with shaking; readings were done at A_{600} (MRX-II Plate Reader, Dynex Technologies). The ability of the isolate to utilize individual PAHs as sole carbon sources was checked on PAH-sublimated gel plates (Alley and Brown 2000; Bogan *et al.*, 2001b). Gel plates were supplied with benzene, toluene, or naphthalene vapors to assess possible growth on these substrates. Growth on non-petrochemical compounds was assessed using the BIOLOG procedure; this test was conducted, using standard methods, by Geneva Laboratories, Inc. (Elkhorn, WI).

The MVAB Hex1 isolate grew poorly on plates in the absence of hydrocarbon, producing small (~ 0.5 mm), translucent off-white colonies on R2A agar. No growth was observed on MacConkey agar. Growth was vigorous with an overlayer of liquid hexadecane. When the isolate was subcultured from hexadecane-overlain plates to hydrocarbon-less R2A plates, growth remained vigorous for 1-2 successive transfers, then decreased, eventually reaching the same poor level seen when cultured directly to these plates. Results were the same in liquid culture: MVAB Hex1 exhibited essentially no growth in LB broth without liquid hydrocarbon. Growth was greatly enhanced by hexadecane; the isolate was also able to grow in MSM with hexadecane as a sole carbon source. Similar results were observed (Table 1) for heptadecane and octadecane, as well as the branched-chain aliphatics pristane and squalane. No growth or PAH-clearing ability was observed on PAH (fluorene, anthracene, phenanthrene, or pyrene) - sublimated plates, or on gel plates exposed to benzene, toluene, or naphthalene vapors.

Of the 95 BIOLOG growth substrates, strong growth was evident only on Tween 40 and Tween 80. Interestingly, these are the only substrates included in the BIOLOG system which contain long-chain alkyl moieties within their molecular structures. Weak growth was seen on acetic acid, and equivocal results were obtained in the cases of α -hydroxybutyric acid, muconic acid, α -keto butyric acid, and sebacic acid. Growth was not observed substrates which are diagnostic (Bernards *et al.*, 1995) for any of the 14 recognized DNA-DNA hybridization groups of *Acinetobacter*. For example, of 14 *Acinetobacter* genomospecies, 11 have at least one substrate for which every known strain gives a positive reaction; for some cluster groups (*e.g.* genomospecies 2), the number of such substrates is as high as 24. The only two groups for which this is not true, genomospecies 8 and 12, still have several substrates which give positive reactions for at least 60% of known strains. The closest “match” to our results is *Acinetobacter johnsonii*. In fact, however, the substrate range of the MVAB Hex1 isolate is considerably narrower than that enumerated in the BIOLOG database for *A. johnsonii*, which generally ($\geq 70\%$ frequency) gives positive reactions with Tween 40, Tween 80 and acetic acid, but also methyl pyruvate (100% of isolates), α - and β -hydroxybutyric acid, lactic acid, propionic acid, bromosuccinic acid, D- and L-alanine, L-proline, L-glutamic acid, and L-pyroglutamic acid (BIOLOG database). Bernards *et al.* (1995) state that 100% of *Acinetobacter* genomospecies 7 strains grew on lactic acid, bromosuccinic acid, alanine, asparagine, and pyroglutamic acid; none of these yielded a positive reaction for our strain.

One mineralization experiment examined conversion of ^{14}C -hexadecane to $^{14}\text{CO}_2$ in different media; a second assessed mineralization of an alkane which did not support growth (dodecane) with or without hexadecane. All mineralization experiments were conducted as previously described (Bogan *et al.*, 2001a). Cultures containing ^{14}C -hydrocarbons received *ca.* 50,000-100,000 dpm of ^{14}C ; added in 10 μl of methanol. Non-labeled hydrocarbons (*ca.* 10 mg per culture bottle) were added aseptically just prior to inoculation.

MVAB Hex1 mineralized ^{14}C -hexadecane in both MSM and LB; mineralization was more rapid and extensive in the latter (Figure 1). Mineralization of dodecane was very low in LB cultures which were supplemented only with the C_{12} hydrocarbon, approximately 2% mineralization in 53 days (Figure 2). This agrees well with the growth-substrate data, as dodecane, when added alone, is a poor substrate for growth. In contrast, when 10 μl of

hexadecane was added to ^{14}C -dodecane-spiked cultures, the amount of $^{14}\text{CO}_2$ generated increased by approximately six fold.

Sample preparation for Scanning Electron Microscopy (SEM) was based on previously-published methods (Glauert 1991; Bozzola and Russell 1999). Samples were prepared on 0.2-micron black polycarbonate filters, sputter-coated with gold before the bacteria were introduced. Bacteria were harvested from agar plates in 50 mM phosphate buffer (pH 7). Media and extracellular debris were removed with five exchanges of phosphate buffer through vacuum filtration. Samples were fixed with 3% glutaraldehyde in phosphate buffer for one hour, then washed with phosphate buffer. The secondary fixative was 1% osmium tetroxide in phosphate buffer for 20 minutes, again followed by a buffer rinse. After the secondary fix, dehydration was accomplished through a series of ethanol solutions (30%, 70%, 90%, 100%, and 100%); each change of dehydrant was held for 20 minutes. The samples were then dried in a Model K-850 Critical Point Drier (Emitech Ltd., Ashford, Kent, UK), with liquid CO_2 as a transitional fluid. The samples were then gold sputter-coated and viewed using a Hitachi S3500-N scanning electron microscope. For transmission electron microscopy, cells on agar plates were fixed (room temperature, 45 minutes) by flooding with 2.5 % glutaraldehyde buffered with 0.1M sodium cacodylate. After buffer washing, cells were fixed with 1% osmium tetroxide in the same buffer on ice for 45 minutes, washed with distilled water, and stained with 1% aqueous uranyl acetate (30 minutes). Samples were dehydrated in an ethanol series and embedded in Spurr resin. Sections were cut on an LKB Nova ultramicrotome, post-stained with uranyl acetate and lead citrate, and observed and photographed on a Zeiss EM-10CA transmission electron microscope.

SEM examination of MVAB Hex1 revealed a somewhat polymorphic morphology (Figure 3), with both cocci (diameter \approx 0.6-0.8 μm), and short bacilli (0.6-0.8 x 1.0-1.2 μm) present. When hexadecane-grown cells were visualized by transmission electron microscopy, they were found to contain large numbers of electron-transparent inclusion bodies. These were of two types, as seen with *Acinetobacter* (Scott and Finnerty 1976a & b; Ishige *et al.*, 2002) and *Rhodococcus* (Alvarez *et al.*, 1996). Using the nomenclature from the latter work, we observed both spherical “ET1” inclusions, which were the predominant inclusion type (Figure 4a-c, e-f), and non-spherical “ET2” bodies; the latter were sometimes disc-shaped (Figure 4b) or irregular

in structure (Figure 4c-d). The largest and most prevalent inclusion bodies were seen in hexadecane-grown cells; frequency and size decreased in squalane-grown cells (Figure 4e), and further diminished in cells grown on pristane (Fig. 4f). Cells grown in the presence of crude oil contained no inclusions, as did those cultured without hydrocarbon (Figure 4g & h).

Growth of this isolate on pristine and squalane is of particular interest; to date, the only identified species capable of growth on squalane have been *Mycobacterium fortuitum* and *Mycobacterium ratisbonense* (Berekaa and Steinbüchel 2000). The weakness or absence of growth observed with shorter-chain (C₁₀-C₁₅) hydrocarbons is analogous to *Arthrobacter nicotianae* KCC B35, which grows better on long-chain (C₂₀-C₄₀) alkanes than on shorter (C₁₀-C₁₈) aliphatics (Radwan *et al.*, 1996). This culture was isolated from a post-Gulf War environment in which many of the short-chain aliphatics from oil spills had been biodegraded and/or removed via weathering, leaving a disproportionate amount of heavy alkanes. The MVAB Hex1 isolate can clearly take up and catabolize short-chain alkanes (at least dodecane - Figure 2b); failure to use these hydrocarbons as sole growth substrates may indicate that one or more of the strain's alkane-degrading enzymes are only induced by aliphatics with chain length of C₁₆ or above.

Such a limited growth-substrate range for a bacterium is fairly rare. Thermophilic bacteria are known to occasionally possess this trait (Merkel *et al.*, 1978; Zarzilla and Perry 1984). Among non-thermophiles, however, we are only aware of two instances in which a bacterial strain has been documented to be as obligately hydrocarbonoclastic as MVAB Hex1. One such strain, related to *Alcaligenes*, grew on C₉-C₁₈ alkanes, but was unable to utilize any of the sugars or amino acids against which it was tested (Bertrand *et al.*, 1976b); this strain did, however, grow on propionic acid (Bertrand *et al.*, 1976a), which MVAB Hex1 is unable to utilize. Furthermore, the range of potential substrates against which this strain was screened was far less broad than the 95 substrates employed in the BIOLOG procedure. Cabezali and Cubitto isolated "many" strains of hydrocarbon-degraders from the Bahía Blanca estuary; one of these was only capable of growth on linear alkanes, fatty acids, and ethanol (Cabezali and Cubitto 1990; Cubitto and Cabezali 1994). Like MVAB Hex1, both of the above-described strains were isolated from chronically oil-polluted sites. Taken together, these findings suggest that

chronically petrochemical-contaminated sites may have a high propensity for giving rise to bacteria which can only grow on petrogenic hydrocarbons.

The 16S rRNA gene sequence of MVAB Hex1 is outside existing genera, differing from the closest *Acinetobacter* species (*A. junii*) by approximately 6%, which roughly equals the maximum amount of divergence seen among the 21 genospecies of *Acinetobacter* (Ibrahim *et al.*, 1997). However, our isolate diverged at 36 positions which are strictly conserved among all *Acinetobacter* sequences; we are thus confident that it belongs outside the genus. Only one 16S rRNA sequence in the GenBank database exhibits sufficient identity to MVAB Hex1 so as to be considered possibly congeneric; the partial (339 bp) sequence (Accession #Z69268) of a groundwater bacterium (JN3c) from an alkaline spring at Maqarin, Jordan, displayed 99.1% homology to MVAB Hex1. Furthermore, a BLAST search reveals that the JN3c sequence, like that of MVAB Hex1, exhibits no more than 94% homology to any other sequences in the GenBank database; included among those which matched JN3c at this level were numerous *Acinetobacter* strains. The JN3c bacterium, however, has so far not been cultured, and the sequence information was obtained exclusively by isolating environmental DNA (Dr. Karsten Pedersen, Göteborg University, personal communication); therefore, the strain is unavailable for direct comparison.

DESCRIPTION OF *ALKANINDIGES* GEN. NOV.

Aerobic cocci to short rods. Gram-variable, probably due to extensive extracellular coat (see species description below). Sporulation has not been observed. In both liquid and solid media, growth is very poor in the absence of linear alkane hydrocarbons with chain length below C₁₆. The type and only species described to date is *Alkanindiges illinoisensis*, although an as-yet-uncultured bacterium, JN3c, may be congeneric.

Description of *Alkanindiges illinoisensis* sp. nov.

In addition to the properties described above for the genus, we report the following. Cells are, non-motile, and catalase-positive. Colonies formed on R2A agar in the absence of hydrocarbon are small and colorless to translucent cream-colored. Sugars are not hydrolyzed, and the isolate is non-hemolytic. Demonstrated substrates supporting good growth include hexadecane, heptadecane, pristane, squalane, and Tween surfactants. Weak growth occurs on acetic acid; no other substrate in the BIOLOG test gave unequivocal positive growth. Morphologically, cells occur as both spheres and short rods, with dimensions of 0.9-1.6 μm x 1.5-2.5 μm . Based on TEM observations of hydrocarbon-grown cells, plasma membrane, peptidoglycan, and outer membrane structures are similar to Gram-negative strains, with an extensive layer of extracellular matrix often present. During rapid growth on hydrocarbons (especially hexadecane), extensive accumulation of large intracellular inclusion bodies is observed. The type strain is GTI MVAB Hex1.

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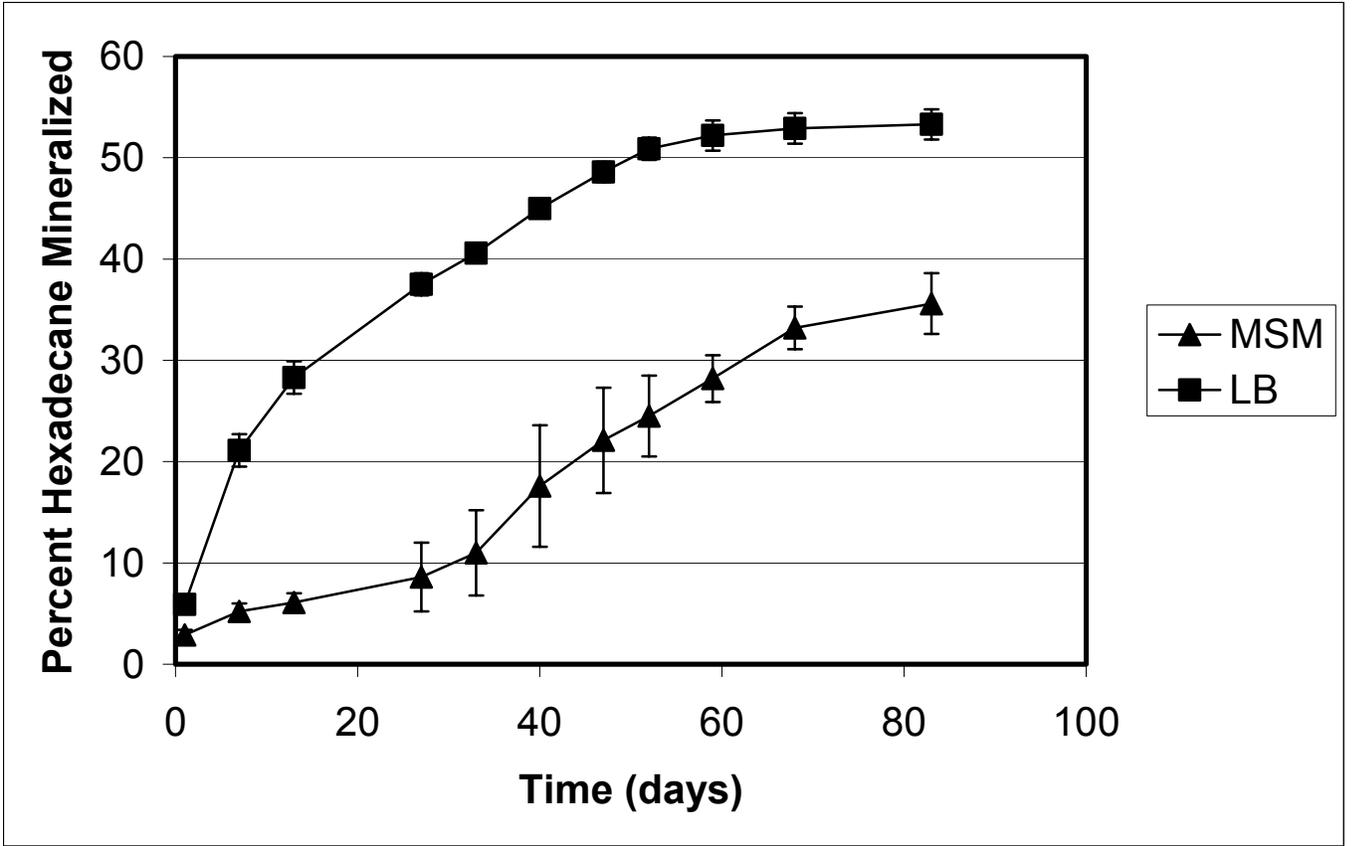
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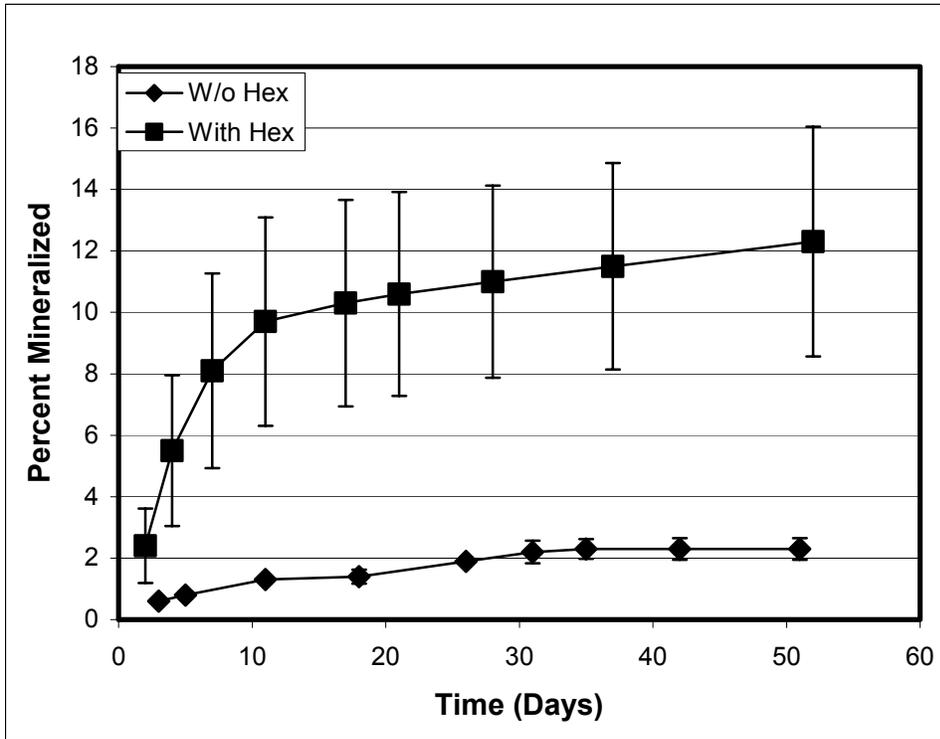
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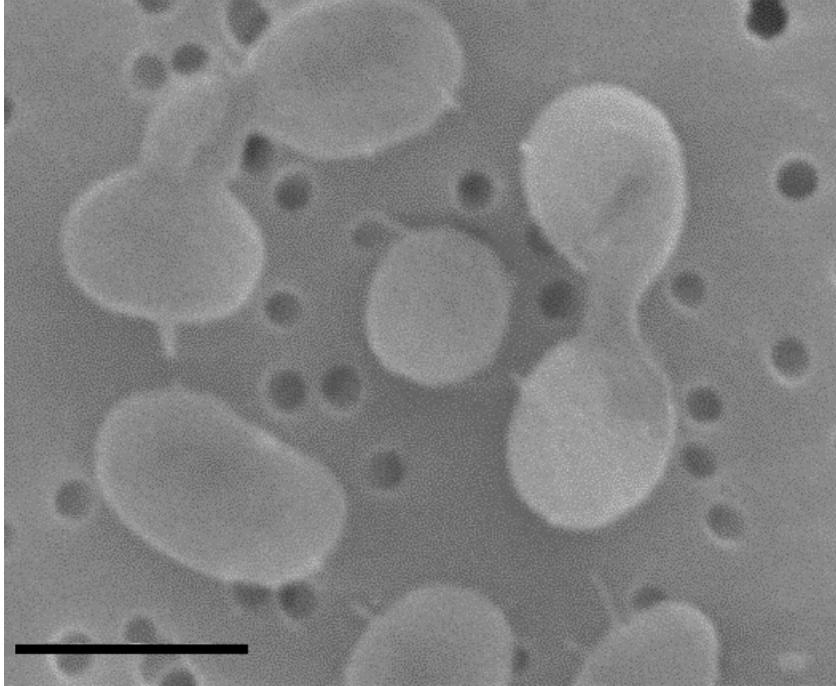
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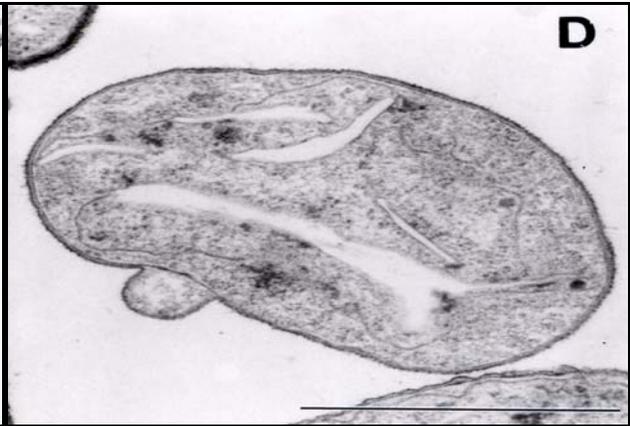
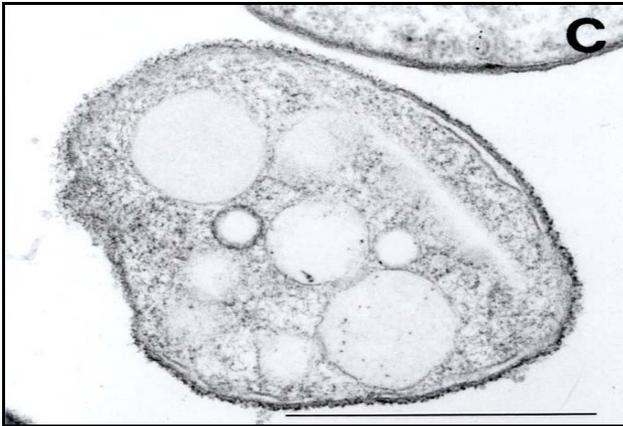
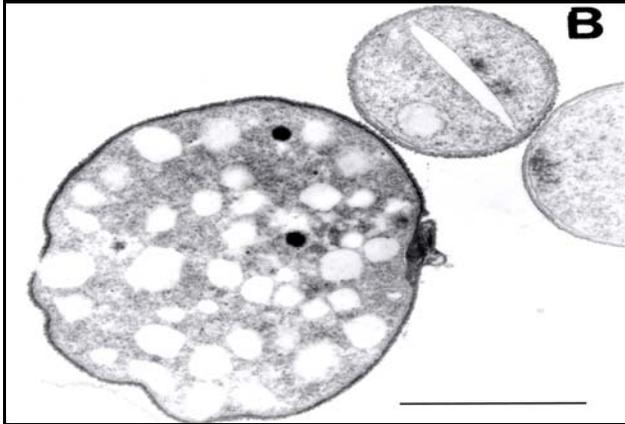
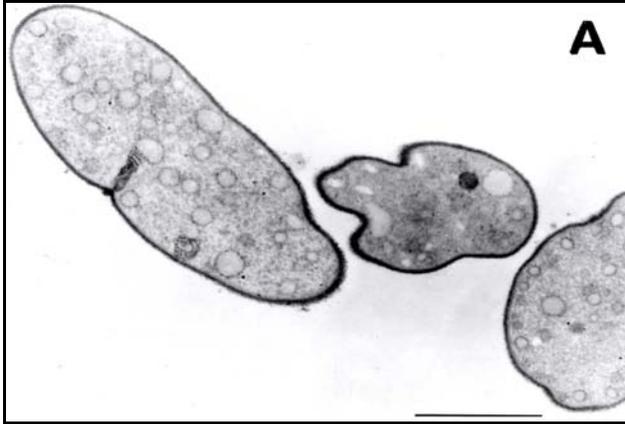
TABLE 1 – Effect of hydrocarbon supplementation on liquid-culture growth (measured as increase in A_{600}) of the MVAB HEX1 isolate. Experiments were conducted in LB broth and in MSM; hydrocarbon addition rate was ~ 5mg/well (total culture volume per well \approx 150 μ l); “media-only control” indicates wells to which no hydrocarbon was added. Non-inoculated (hydrocarbon + media) control wells were included for each individual condition; no growth occurred in any of these (data not shown). “-“ indicates that no measurable growth occurred.

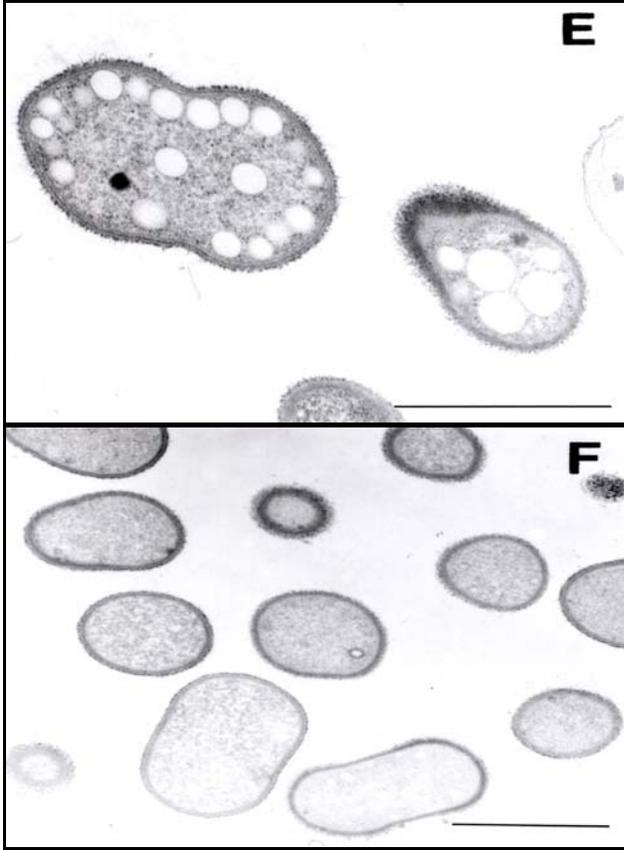
	LB Medium		MSM
	Day 2	Day 4	Day 4
<i>n</i> -Decane	-	-	-
<i>n</i> -Dodecane	-	-	-
<i>n</i> -Tetradecane	-	-	-
<i>n</i> -Pentadecane	-	-	-
<i>n</i> -Hexadecane	0.267	0.815	0.112
<i>n</i> -Heptadecane	0.221	0.983	0.140
Pristane	0.055	0.736	0.344
Squalane	0.284	0.529	0.232
(Inoculated)Media-only control	- (-0.004)	- (-0.004)	- (-0.002)











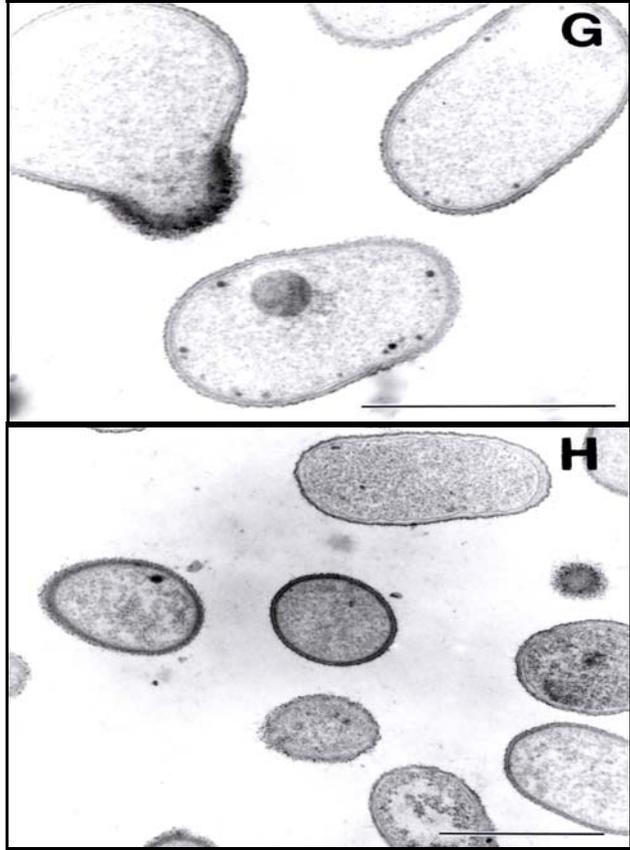


Figure 1 – Mineralization of ^{14}C -hexadecane (initial hexadecane concentration = 1.2 mM) by MVAB Hex1 grown in either mineral salts medium or LB broth.

Figure 2 – Mineralization of dodecane, added at the rate of 10mg/40 ml, by MVAB Hex1 grown in LB broth in the presence or absence of 1.2 mM hexadecane.

Figure 3 – SEM image of MVAB Hex1, taken from R2A agar plates. Bar denotes 1 micron.

Figure 4 – Transmission electron microscopy of MVAB Hex1. All cells were taken from R2A agar plates after 3-5 days growth in the presence or absence of liquid hydrocarbon overlay. (A) Hexadecane-grown cells showing large number of small spherical “ET1”-type inclusion bodies. Hexadecane-grown cells were observed to also accumulate disc-shaped to amorphous “ET2” wax ester inclusions, either alone (B) or together (C) with ET1 bodies. (D) ET2-containing cell in which inclusion bodies show evidence of intracellular membranes. (E) Squalane-supplemented cells showing ET1 inclusions, which also occurred, albeit smaller and more rarely, in cells cultured on pristane (F). Crude oil-grown cells (G) and those grown in the absence of hydrocarbon (H) lacked detectable inclusions. In all frames, bar denotes one micron.

**INCLUSION OF VEGETABLE OILS IN FENTON'S CHEMISTRY FOR
REMEDICATION OF PAH-CONTAMINATED SOILS**

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ABSTRACT

Pre-treatment with vegetable oils prior to treatment with Fenton's reagent led to increased oxidation by the latter of polycyclic aromatic hydrocarbons (PAHs) in a pair of model manufactured gas plant soils. This effect was frequently most pronounced in the cases of high-molecular-weight (HMW) PAH species, indicating a preferential "targeting" of oxidative equivalents toward these compounds. In both cases, addition of oils - either corn oil containing unsaturated lipids or palm kernal oil comprised primarily of saturated fats - at the 5% dosage was required; supplementation with 1% oil apparently did not sufficiently facilitate PAH desorption and mass-transfer to have a notable effect on degradation efficiency. In palm kernal oil-supplemented reactions, replacement of H₂O₂ with calcium peroxide (CaO₂) further increased the extent of PAH removal. Again, this was most pronounced in the cases of several HMW PAHs; among a suite of four 5- and 6-ring PAH (benzo[*a*]pyrene, dibenz[*a,h*]anthracene, benzo[*g,h,i*]perylene and indeno[*c,d*]pyrene), average removal efficiency increased from 5% in palm kernal oil-supplemented reactions in which H₂O₂ served as the oxidant, compared to 44% in CaO₂-containing reactions. This last finding is consistent with other reports which have indicated that slower-release oxidants are better suited to degradation of contaminants which, despite vegetable oil treatment, remain soil-sorbed.

Keywords

HYDROGEN PEROXIDE, CORN OIL, PALM KERNAL OIL, LIPID PEROXIDATION, BIOREMEDIATION, CALCIUM PEROXIDE

INTRODUCTION

It has been estimated that at least 1500 former manufactured gas plant (MGP) sites in the U.S. have sustained soil and groundwater contamination through the improper disposal of coal tar and related waste products. A similar number have been impacted by creosote-related wastes generated by the wood-preserving industry. The most notable class of hazardous compound present in coal tar and creosote (which is generally derived from coal tar) is the polycyclic aromatic hydrocarbons (PAHs). Sixteen PAHs have been included on the priority pollutant list established by the U.S. EPA (Keith and Telliard, 1979). Thus, PAH-contaminated MGP sites are considered to pose a significant public health risk, and effective, economical technologies to remediate these sites are needed.

Fenton's chemistry (the generation of hydroxyl radical via the homolytic cleavage of hydrogen peroxide by Fe^{2+}) has been studied by numerous groups for remediation of organic-contaminated soils and other solid matrices (Watts et al., 1993 & 1994; Srivastava et al., 1994; Watts & Dilly, 1996; Li et al., 1997; Pradhan et al., 1997; Nam et al., 2001). These researchers have shown that hydroxyl radicals generated through Fenton's reaction are capable of oxidative degradation of numerous xenobiotics, including fuel hydrocarbons (Watts, 1992; Chen et al., 1995; Kong et al., 1998; Watts et al., 2000), chlorinated aliphatic (Howsawkeng et al., 2001) and aromatic (Sedlak and Andren, 1991; Watts et al., 1994) hydrocarbons, pentachlorophenol (Ravikumar, 1992), pesticides (Pignatello and Baehr, 1994), nitrobenzenes and nitrophenols (Lipczynska-Kochany, 1992), trinitrotoluene (Li et al., 1997), benzene, toluene and other monoaromatics (Lou and Lee, 1995; Chen et al., 1995), and PAHs (Srivastava et al., 1994; Pradhan et al., 1997; Lee and Hosomi, 2001; Nam et al., 2001).

Data obtained in field trials of Fenton's-based chemical-biological soil treatment clearly show that very significant removals of all PAHs examined could be achieved through this process (Pradhan et al., 1997). It is, however, also clear that 5- and 6-ring PAH species were more slowly removed. This is most likely due to the fact that these compounds are notably more hydrophobic than their lower-molecular-weight counterparts, and therefore are more prone to remain tightly bound to soil organic matter. It is well known that soil-bound contaminants are more resistant to oxidative attack during chemical treatments than contaminants in solution

(Watts et al., 1993; Watts and Dilly, 1996). The significance of this lies in the fact that the majority of the total genotoxicity and mutagenicity of PAH-contaminated soils and sediments is due to these compounds (Marvin et al., 1995); this is reflected in the fact that the EPA-promulgated cleanup levels for several 5- and 6-ring PAHs (including especially benzo[*a*]pyrene and dibenz[*a,h*]anthracene) are the most stringent of all individual PAHs (Smucker, 2000). Tolerable levels of these two compounds may be 10,000-fold lower than those of relatively non-hazardous low-molecular-weight PAH compounds such as phenanthrene and anthracene. Thus, remediation processes are needed which can directly address these compounds; this is particularly true when cleanup goals are based entirely on the presence and levels of contaminant compounds, as opposed to risk-based, site-specific determinations.

One potential approach to this problem is to tailor the chemical portion of the remediation process, through inclusion of other amendments, such that higher-molecular-weight, more hydrophobic contaminants are preferably attacked. We conducted experiments to examine the potential utility of vegetable oils in this regard. Vegetable oils have long been used as carriers for hydrophobic herbicides (Sonntag, 1988). Solutions of free fatty acids similar to those found in vegetable oils (*e.g.* potassium laurate) have molar solubility ratios (MSRs) for PAHs (phenanthrene and pyrene) which are very similar to those of synthetic chemical surfactants (Klevens, 1950; Tiehm, 1994), as do esterified fatty acids derived directly from coconut oil (Grimberg et al., 1995). Paraffin oil, which is chemically similar to some vegetable oils, has been shown to increase the aqueous solubility of pyrene by 5 orders of magnitude (Jimenez and Bartha, 1996). Recently, protocols have been developed in which soil is “washed” *ex situ* with a microemulsion of vegetable oils, causing hydrophobic pollutants to desorb from the soil and allowing their subsequent treatment and disposal (Isosaari et al., 2001). This process, which further illustrates the high affinity of PAH for vegetable oils, even in a soil system, is the subject of a recent U.S. Patent (Haegel et al., 1998). Finally, inclusion of vegetable oil-based formulations have been shown (Bogan and Lamar, 1999) to greatly enhance the degradation of PAH in solid-phase (soil) cultures of several white-rot fungi, a process which, in at least some cases, is mediated by metal ions (Mn^{3+}) (Moen and Hammel, 1994; Böhmer et al., 1998).

Our objective in the research described herein was to determine if the incorporation of vegetable oils could improve chemical treatment processes for PAH-contaminated soils by improving the efficiency of Fenton’s reaction. Our results indicate that vegetable oils do, in fact,

allow the oxidative reactions of such a system to more efficiently “target” high-molecular-weight PAHs, and do improve their overall performance.

Materials and Methods

Soils

The two soils used in the work described herein were obtained from former MGP sites. The first of these originated in Central New Jersey, while the second was from East-central Pennsylvania. Total PAH levels in the New Jersey soil were slightly more than double those in the Pennsylvania site soil (*ca.* 7700 ppm vs. 3100 ppm, respectively).

Reaction Conditions

We examined the effect of lipid addition to slurries of PAH-contaminated soils, which were then treated using Fenton’s reagent. Soils were slurried in water (10 grams of soil to 50 ml of water), pH was adjusted to 3.0 (\pm 0.2) with H₂SO₄, reactions were poisoned with 100 mg HgCl₂ (in order to ensure that reactions remained abiotic), and vegetable oils were added (where appropriate) at the 1% or 5% dosage (per dry wt. of soil). Following a 2- to 3-hour contact time (in slurry mode) between the lipids and soils, Fenton’s reagent (1% wt/vol H₂O₂, 10 mM (final concentration) FeSO₄) was added in a single dose. Soils were incubated at room temperature, with constant shaking (~200 rpm); two separate experiments were run; total duration of the treatment was 5 days (New Jersey soil) or 14 days (Pennsylvania soil). At the conclusion of the incubation, soils were extracted (see below) and analyzed for PAH level. In parallel with this experiment, corn oil-supplemented reactions were run, with New Jersey soil, which were not poisoned with HgCl₂. This parallel experiment was conducted to determine if there could be any further biological contribution to degradation under these conditions (despite unfavorable pH). Reactions which examined substitution of CaO₂ for H₂O₂ (Arienzo, 2000) were conducted with Pennsylvania soil. These were not pH adjusted; the pH of slurry reactions which received 1% (by weight) of CaO₂ was approximately 11.8.

Extraction and Analysis

Soil samples were centrifuged (10 min, 5000 x g) in stainless-steel containers to separate solid and aqueous phases. Soil solids (~2 g) were mixed with anhydrous sodium sulfate (1:1)

and ground to a fine powder. Sonication was performed according to EPA Method 3550A, using 1:1 methylene chloride/acetone (20 ml) as the solvent, and was repeated twice. The extracts were combined and vacuum filtered, and were evaporated to dryness under a stream of N₂ in a Turbovap Evaporator (Zymark, Hopkinton, MA) and exchanged into 1 ml of acetonitrile (ACN). 10 µl of this solution was analyzed by reverse-phase HPLC (EPA Method 8310) using a Supelcosil LC-PAH column (15 cm x 4.6 mm) and a Waters HPLC system coupled to a diode-array detector (Waters Model 996). The following gradient was used, with a flow rate of 1.5 ml min⁻¹ throughout: 0 min - 60% H₂O/40% ACN; 25 min - 100% ACN (hold for 2 min); 33 min - 60% H₂O/40% ACN. Identities of individual PAHs were verified by comparing the retention times and the absorbance spectra, and quantified by comparison with 5-point standard curves (all $r^2 > 0.988$).

RESULTS

Effects of Vegetable Oil Addition

Figure 1 shows the results of abiotic Fenton's reactions, with and without vegetable oil supplementation (5% by wt), for the New Jersey (Fig. 1a) and Pennsylvania (1b) soils, respectively. In both cases, Fenton's reagent treatment alone was unable to bring about any significant removal of the carcinogenic, high-molecular-weight PAHs. In the absence of lipids, there was significant removal (compared to no-treatment controls) of only a few low-molecular-weight PAHs (fluorene, phenanthrene, anthracene, and pyrene); furthermore, this effect was essentially restricted to the New Jersey soil. No-treatment controls with the New Jersey soil retained 106% ($\pm 20\%$) of the 13 PAHs examined (Fig. 1a). Under the longer duration of the Pennsylvania soil experiment, untreated controls had 76% ($\pm 11\%$) of initial PAH levels (Fig. 1b).

However, in both soils, the effects of vegetable oil addition on the effectiveness of Fenton's reactions were considerable. Essentially all PAHs were significantly removed, even during the short five-day treatment phase used with the New Jersey soil, from reactions which contained vegetable oil. The magnitude of this effect (when compared to the no-oil Fenton's treatment) was most significant in the cases of the high-molecular-weight PAHs. Addition of

1% corn oil (CO) to the Pennsylvania soil, under abiotic conditions, resulted in much lower removal rates, as is also shown in Fig. 1b.

Abiotic vs. Biotic Treatments

Figure 2 shows the difference in PAH recovery from HgCl₂-poisoned abiotic reactions, as compared to those which were not poisoned, in the presence of corn oil. Recoveries of low-molecular-weight PAHs (fluorene through pyrene) were essentially the same under both sets of conditions. However, for a suite of higher-molecular-weight (4- and 5-ring) compounds, recovery was slightly (but consistently) lower in biotic reactions. Substitution of a mineral salts medium or 2% Lennox Luria-Bertani (LB) broth for water in slurry reactions yielded a slight, but not statistically significant, increase in PAH removal in biotic reactions (data not shown).

Incorporation of Calcium Peroxide

Average recovery of the 13 target PAHs from the corn oil-only (no FeSO₄ or H₂O₂) reactions with Pennsylvania soil were 78% (\pm 8%), identical to the no-treatment recoveries in earlier experiments with this soil. Patterns of PAH removal in Fenton's/palm kernal oil (PKO) reactions in this experiment were also similar to those seen in the similarly-supplemented reactions in previous experiments. In all cases, however, reactions supplemented with 1% CaO₂ showed greater extents of PAH removal than those containing the same amount of H₂O₂ (Fig. 3). Overall, the average percentage of PAH remaining (relative to controls which contained PKO and no oxidant) at the conclusion of the treatment period was 80% (\pm 13%) for reactions containing PKO and H₂O₂, compared with 53% (\pm 5%) in the case of reactions supplemented with CaO₂ and PKO. Enhancement of removal was most evident in the cases of several higher-molecular-weight PAHs, such as benzo[a]pyrene (49% removal with CaO₂ vs. 12% in H₂O₂), dibenz[a,h]anthracene (45% vs. 5%), benzo[g,h,i]perylene (40% vs. 0%) and indeno[c,d]pyrene (43% vs. 5%).

DISCUSSION

Interaction of hydrophobic organic chemicals, such as PAHs, with soils entails an initially rapid, reversible sorption process, followed by a longer period which is characterized by

increasingly strong interactions. This results in an increasing recalcitrance of contaminants, which become less bioavailable, and therefore less amenable to biodegradation. Several mechanisms have been described for this “aging” of chemicals in soils, including partitioning into or onto humic matter (Chiou et al., 1983) – a process whose speed and extent is apparently governed by the polarity and aromatic content of the soil organic matter (Kopinke et al., 2001) – or diffusion into three-dimensional micropores of soil particles themselves (Steinberg et al., 1987; Ball and Roberts, 1991). Diffusibility and release of contaminant molecules becomes retarded as they become more strongly sorbed to, or entrained within, this matrix (Wu and Gschwend, 1986; Steinberg et al., 1987; Brusseau et al., 1991).

PAHs which are solubilized in vegetable oil lipids (either in solution as micelles, or adhered to soil surfaces in admicelles) are expected to be very subject to the types of free-radical reactions inherent in a Fenton or Fenton-like chemical system. Free-radical based reactions can be preferentially localized to such structures, as has been demonstrated in the case of polymerization of styrene monomers into ultrathin films (Wu et al., 1987). Both H_2O_2 and Fe(II) are somewhat soluble and diffusible within a lipid bilayer, perhaps at levels which would support Fenton’s reaction (Schaich and Borg, 1988), generating at least some amount of $\bullet\text{OH}$ “in situ”. Alternatively, $\bullet\text{OH}$ generated in the aqueous phase can initiate peroxidative reactions within lipid aggregates. In either of these scenarios (*e.g.* regardless of the site of $\bullet\text{OH}$ generation), contaminants within micelles may be preferentially attacked, relative to those outside the lipid phase, as the peroxidative “cascade” sweeps through the micelle. In this regard, it should be noted that one or more species of lipid-derived radicals, although not as yet conclusively identified, have been implicated as causative agents in the initial extracellular oxidations of high-ionization potential PAHs (*e.g.* phenanthrene, chrysene, and benzo[*b*]fluoranthene) by white-rot fungi, a process which is apparently based on electron-abstraction reactions (Bogan and Lamar, 1995).

In cases where PAH is initially sorbed onto soil particles, desorption is expected to significantly enhance Fenton-like soil treatment, as desorption rate has been found (Watts and Dilly, 1996) to be the limiting factor in oxidation of contaminants in low- H_2O_2 systems. These authors have further found that Fenton-like systems in which endogenous Fe-containing minerals function as the sole catalyst for $\bullet\text{OH}$ generation (presumably resulting in lower but more sustained $\bullet\text{OH}$ levels) are incapable of degrading soil-sorbed pentachlorophenol (Watts et al.,

1993) and hexachlorobenzene (Watts et al., 1994). Inclusion of vegetable oils facilitates desorption of hydrophobic contaminants from the soil matrix into lipid micelles. Sorption of organic chemicals onto soil organic matter is correlated with organic carbon partition coefficients (K_{oc}) and octanol-water partition coefficients (K_{ow}), both of which, among PAHs, generally increase with molecular weight (Walters and Luthy, 1984; Chiou et al., 1998). Thus, degradation (either biotic or abiotic) of high-molecular-weight (HMW) compounds should be most hampered by sorption. This provides one possible explanation for the finding that vegetable oil addition seems to preferentially stimulate the removal of HMW PAHs in soil-slurry Fenton's reactions. Alternatively, dissolution of PAH aggregates (either on or between soil particles) may be stimulated by vegetable oil addition. In either of these cases (desorption or dissolution), partitioning of PAHs into lipid micelles should mimic partitioning into organic solvent, or onto solid organic matter; proportionately more HMW PAH should partition into micelles, which should contribute to the preferential removal of these species in oil-supplemented reactions. Addition of lower amounts of oil (< 1%) apparently does not, at least in the case of the Pennsylvania MGP soil, facilitate sufficient mass-transfer of contaminants into lipid aggregates, thus resulting in less-than-optimal contaminant degradation. It is also possible, however, that use of excess lipid (e.g. above the 5% range) may, however, also negatively impact performance, as a higher percentage of lipid aggregates, which would still consume the initial oxidant, would be expected to contain little to no contaminant – thus, this portion of the initial oxidant would be consumed unproductively.

It is of interest that all vegetable oils tested gave very similar results in the abiotic Fenton's systems: PAH removal with a polyunsaturated oil (corn oil) was not greatly different from that with an oil composed primarily of saturated fats (palm kernel oil), or from a monounsaturated oil (olive oil – data not shown). In fact, in the Pennsylvania soil, PKO performed considerably better than CO, when the two were added at equal dosages (Fig. 1b).

Our results indicate that inclusion of lipids, in the form of readily-available, inexpensive, non-toxic vegetable oils, can significantly enhance the effectiveness of Fenton's reagent for degradation of PAH contaminants in MGP soils. There is some evidence that this enhancement is preferentially manifested against high-molecular-weight compounds, including PAHs such as benzo[*a*]pyrene and dibenz[*a,h*]anthracene, which are often the most significant compounds

from the standpoint of risk assessment. As such, this technique may help in the cost-effective remediation of MGPs and other PAH-contaminated sites.

Acknowledgments

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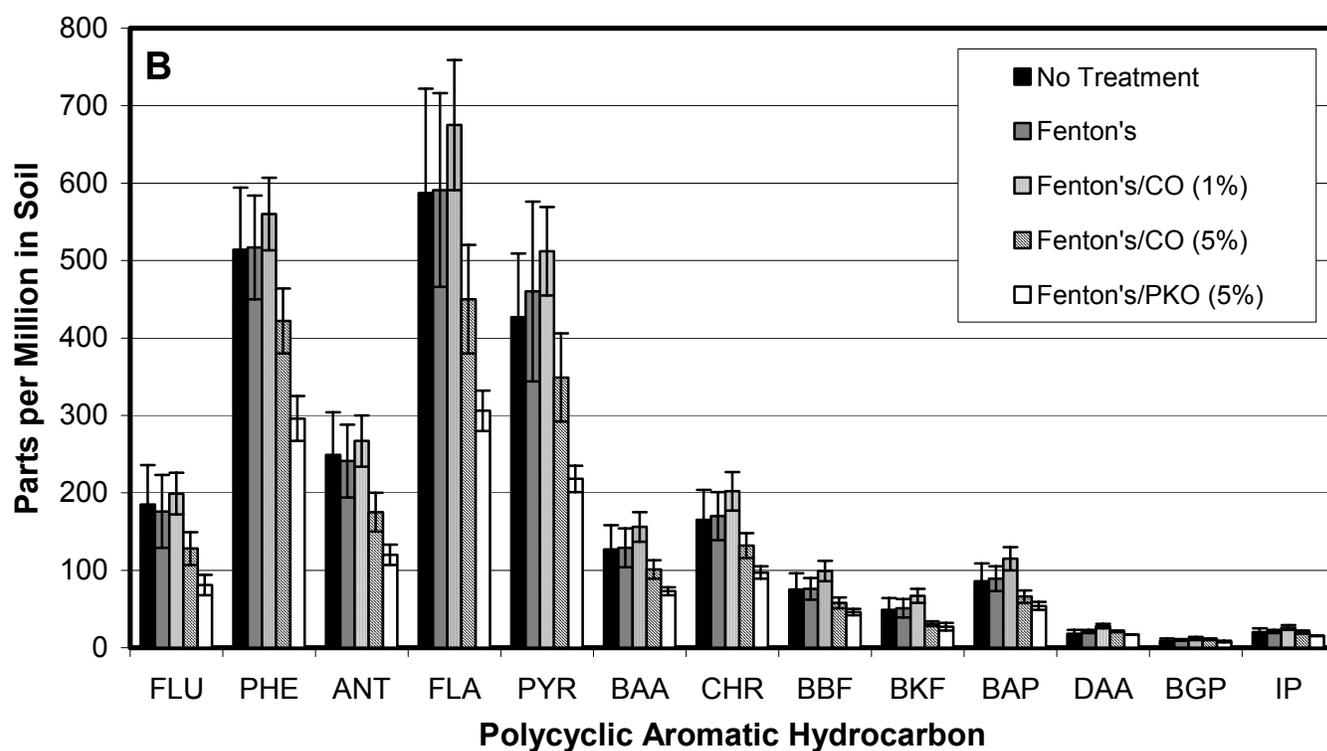
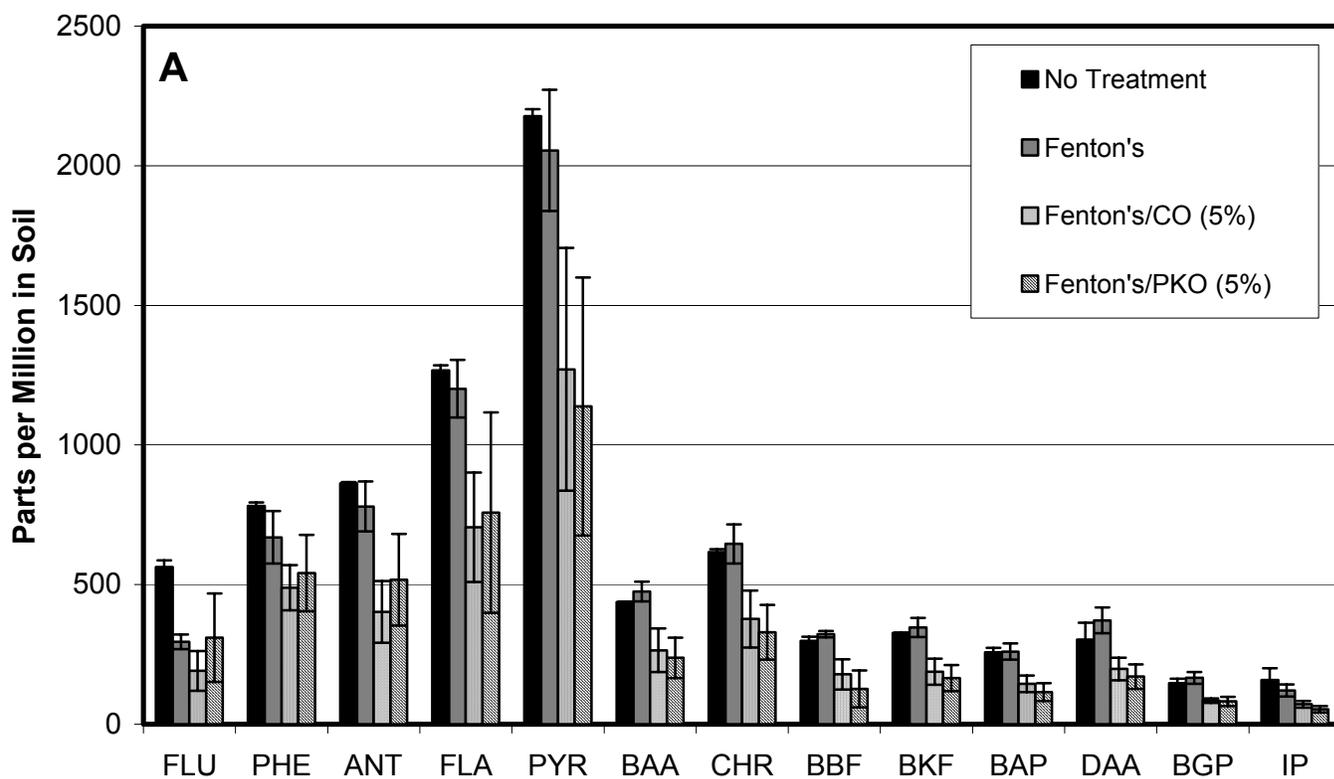
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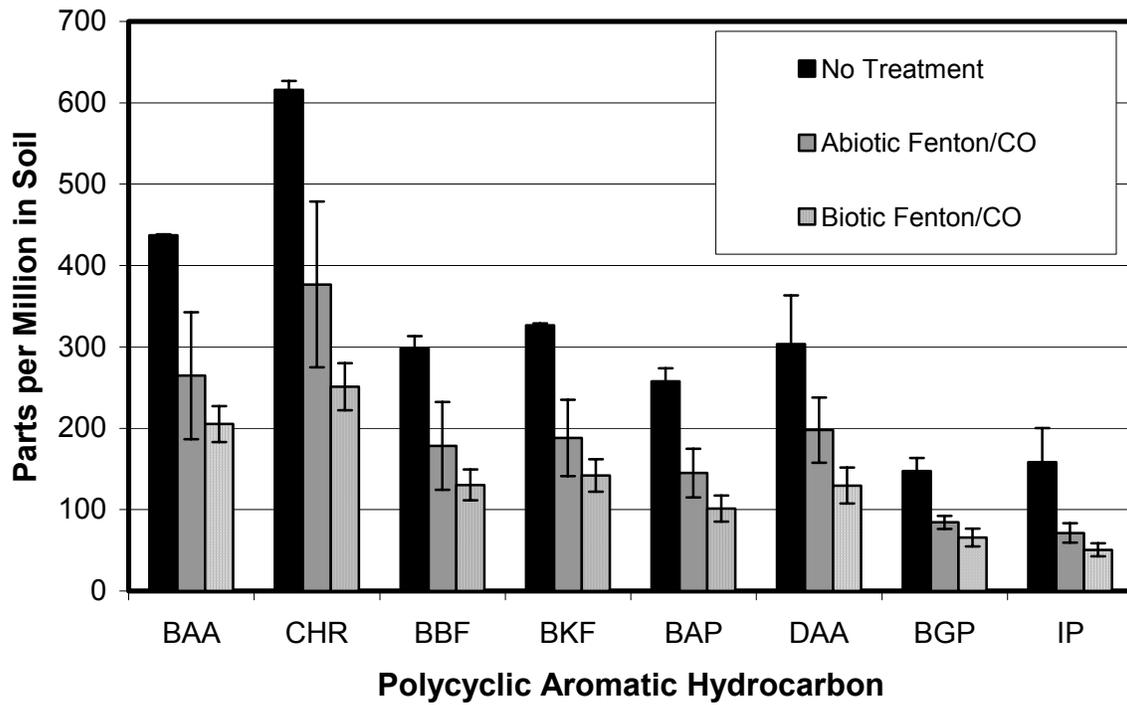
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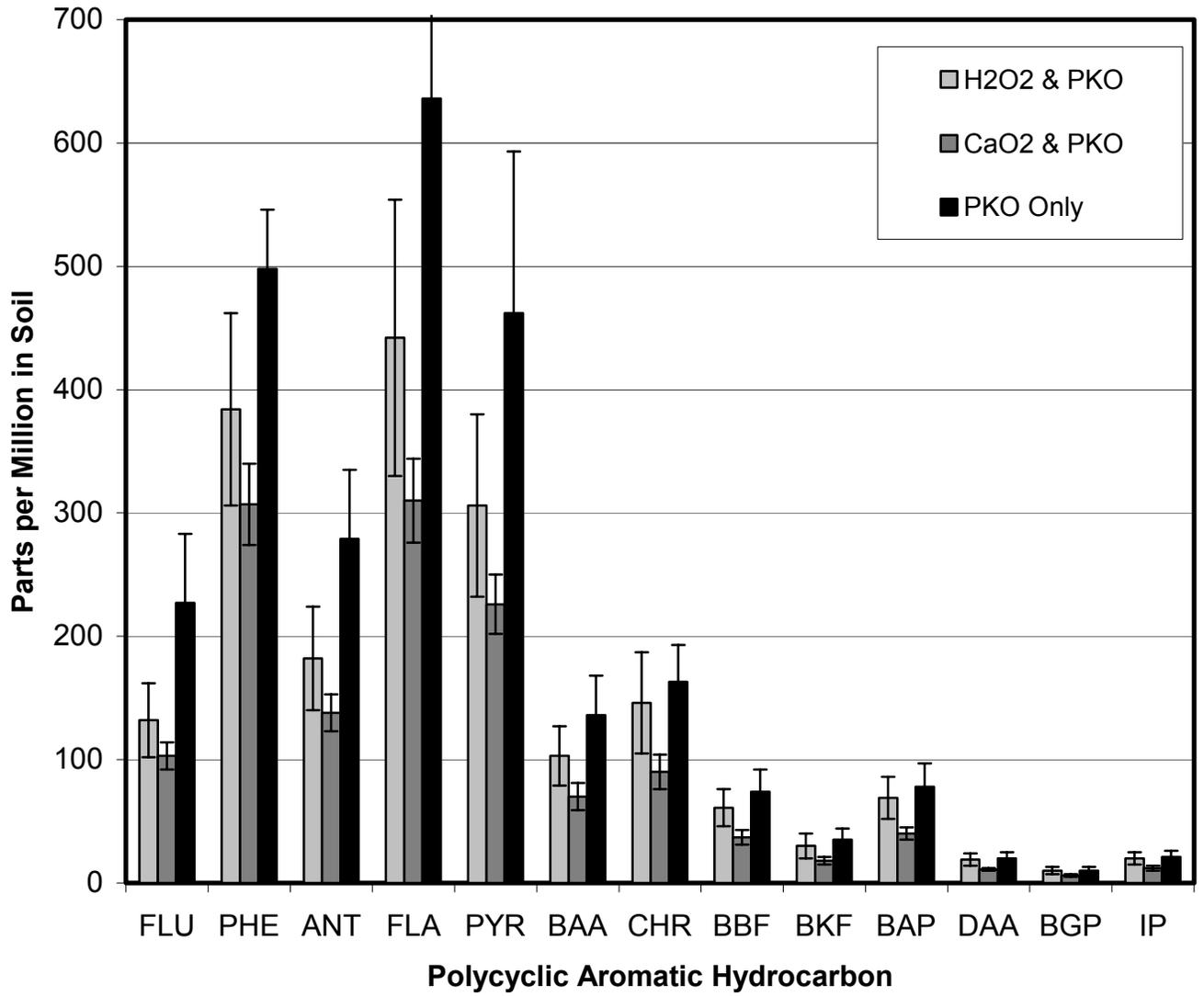


Figure 1 – Levels of target 3- to 6-ring PAH compounds remaining after treatment in abiotic (*i.e.* HgCl₂-poisoned) Fenton’s reaction treatment, or in reactions supplemented with corn oil (CO) or palm kernal oil (PKO) at the indicated dosages. **(A)** New Jersey soil – treatment time 5 days; **(B)** Pennsylvania soil – treatment time 14 days.

Figure 2 – Recovery of PAH from abiotic versus biotic (non-poisoned) corn oil-supplemented Fenton’s treatment of New Jersey soil.

Figure 3 – Comparison of PAH levels remaining after abiotic treatment of Pennsylvania soil with palm kernal oil (5%) and either hydrogen peroxide or calcium peroxide (both 1%).

**PHYSICOCHEMICAL SOIL PARAMETERS AFFECTING SEQUESTRATION
AND MYCOBACTERIAL BIODEGRADATION OF POLYCYCLIC AROMATIC
HYDROCARBONS IN SOIL**

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Abstract

Six soils, obtained from grasslands and wooded areas in Northeastern Illinois, were extensively physicochemically characterized. Measured parameters included total organic carbon (TOC) content, contents of humic acid, fulvic acid and humin, pore volume and pore size distribution, and chemical makeup of soil organic matter (determined using solid-state ^{13}C -NMR). Moistened, gamma-sterilized soils were then spiked with ^{14}C -phenanthrene or pyrene; following 0, 40, or 120 days of aging, the contaminant-spiked soils were then inoculated with *Mycobacterium austroafricanum* strain GTI-23, and evolution of $^{14}\text{CO}_2$ was assessed over a 28-day period. Results for both phenanthrene and pyrene indicated that increased contact time led to increased sequestration and reduced biodegradation, and that TOC content was the most important parameter governing these processes. One soil in particular consistently showed significantly lower-than-expected sequestration (higher-than-expected mineralization), despite a very high TOC value (> 24%); the primary distinguishing feature of this soil was its considerably elevated fulvic acid content. Further experiments showed that addition of exogenous fulvic acid to a soil with very low HA/FA content greatly enhanced pyrene mineralization by *M. austroafricanum*. Extractability of all priority pollutant coal tar PAHs in *n*-butanol from the six soils after 120 days of sequestration was strongly TOC-dependent; although there was no discernible correlation between *n*-butanol extractability and mineralization across the entire suite of soils, these correlations (especially phenanthrene) were greatly improved when “hard” or “soft” carbon-dominated soils – operationally defined primarily by their humin contents relative to HA and FA – were considered separately.

INTRODUCTION

It is well known that organic compounds which have been in contact with soil for an extended period of time display reduced bioavailability, whether the latter is measured as microbial biodegradability (Kelsey and Alexander, 1997; White et al., 1999; Reid et al., 2001), extractability under mild conditions (Kelsey et al., 1997; Tang and Alexander, 1999), uptake by earthworms or other soil-inhabiting organisms (Kelsey and Alexander, 1997), toxicity to insects

(Edwards et al., 1957), or phytotoxicity (Hurle, 1977; Scribner et al., 1992). It was in the last of these contexts that the phenomenon of sequestration of organic chemicals (*e.g.* herbicides) was first described, and a mechanism proposed. It was observed, for example, in the late 1950's and early 1960's that application in soils with high organic matter contents resulted in substantial reductions in the herbicidal efficacy of simazine (Grover, 1966 & refs. therein) and numerous other herbicides (Upchurch and Mason, 1962), and that the magnitude of this effect was strongly correlated with the TOC content of the soil.

Several mechanisms have since been proposed and described for the "aging" of chemicals in soils, including partitioning into or onto humic matter (Chiou et al., 1983), or diffusion into three-dimensional micropores of soil particles themselves (Steinberg et al., 1987). More recent models have recognized that the overall sequestration process most likely encompasses both of these individual mechanisms. Weber and co-workers (Weber et al., 1992; Weber and Huang, 1996) and Pignatello and others (Xing and Pignatello, 1997) have each described two-site models to describe the processes inherent in interactions between hydrophobic contaminants and soil particles, in which sequestration begins with partitioning of contaminant molecules into (and onto) humic and fulvic acid polymer layers at the surface of soil particles, followed by diffusion into micropores. The latter structures most likely occur in the humin core of the particles, and are rendered partially inaccessible to the bulk solution phase by the overlayer of polymeric HA and FA.

There has recently been considerable interest in the phenomenon of contaminant sequestration due to the anticipated impact of this process, because of its effect on contaminant bioavailability, on the risk-assessment paradigms used to guide management and cleanup of contaminated sites and soils. The reasoning which has been put forth (Kelsey and Alexander, 1997) is that sequestered contaminants, because of their reduced bioavailability, pose no inherent risk to human health, or to other eco-receptors; thus, their remediation carries an unnecessarily high economic burden, with minimal ecological, health, or safety benefits.

Members of the genus *Mycobacterium* have recently been widely studied as possible bioremediation agents. Mycobacteria are known to have extremely lipophilic surfaces (Rehmann et al., 1988; Bastiaens et al., 2000), which may make them better suited to direct uptake of hydrophobic pollutants (Bouchez-Naitali et al., 1999). Furthermore, *Mycobacterium* strains are known to have a wide range of exceptional catabolic capabilities, including

degradation of polycyclic aromatics with up to five fused benzene rings (Khan et al., 2002; McClellan et al., 2002; Bogan et al., submitted) and highly-branched alkanes (Berekaa and Steinbüchel, 2000). To our knowledge, however, none of the previous studies which have examined the effects of sequestration on microbial contaminant degradation have made use of mycobacterial strains. This, therefore, was one of the primary aims of the present study. Additionally, we sought to gather more information on the physicochemical parameters of soils which are responsible for governing the rate and extent of the sequestration process, and which therefore impact contaminant bioavailability and biodegradability.

METHODS AND MATERIALS

Soils

Six model, non-contaminated soils were collected from vegetated areas in the Northwestern Greater Chicago area. Particle size analyses were performed by the hydrometer method (Sheldrick and Wang, 1993). Total organic carbon (TOC) was conducted by ashing soil samples at 440 °C, according to ASTM Method D2974-87. Soil organic matter was fractionated into humic acids (HA), fulvic acids (FA) and humin as follows. 15 grams of each soil material (except #4, the high TOC of which allowed a 2-gram sample to be used) were stirred (using a Teflon-coated stir-bar) for 24 hours with 100 mL of 0.1M NaOH. Samples were centrifuged (30 minutes at 2000 x g). The supernatant (containing the humic acid and fulvic acid) was decanted. The residue (which represents the humin fraction) was shaken with 100 mL of 0.1M NaOH and centrifuged again to remove residual humic acid and fulvic acid; this supernatant was decanted and added to that previously obtained for that sample. Reagent-grade water (100 mL) was added to each humin sample and shaken. The suspension was acidified ($\text{pH} \leq 2$) with concentrated HCl, centrifuged, and the supernatant discarded. The humin was then subjected to similar rinses with water until the rinsate gave a negative test for chloride with AgNO_3 . The humin was dried in a 60°C oven. The HA/FA-containing supernatants were acidified ($\text{pH} \leq 2$) and centrifuged. The resulting supernatants – the fulvic acid fractions – were individually loaded onto an XAD-8 column, which was washed with reagent water to remove salt. Fulvic acid was eluted with 0.1M NaOH, converted to the acid form using a hydrogen-saturated cation exchange resin, and

recovered by lyophilization. The humic acid residue was treated as described above for the humin fraction (acid- and water-rinsed) until the water tested negative for chloride. The humic acid was dried in a 60°C oven. All solid samples (whole soil materials, humic acid and humin) were measured using a Shimadzu TOC-V/SSM-5000A analyzer; aqueous fulvic acid samples were determined using a Shimadzu TOC-500 analyzer.

Relative contents of aromatic and aliphatic functional moieties in soil organic matter were determined by ^{13}C -solid-state nuclear magnetic resonance (NMR). NMR measurements were made on a Chemagnetics CMX solids NMR spectrometer using the technique of cross polarization (CP) with magic-angle spinning (MAS). NMR spectra were obtained using a ceramic NMR probe that uses a 7.5-mm zirconia pencil rotor. The ceramic probe eliminates background signals that could arise from the NMR probe when running samples that have low organic carbon contents for which signal averaging over long periods of time is required. The spectra were recorded at a ^{13}C frequency of 25 MHz, using a 90° pulse width of 5 μs , a contact time of 1 ms, a pulse delay of 1 s, and a spinning rate of 4.5 kHz. Signal averaging times ranged from 8–15 hrs per sample (28,800 to 54,000 transients). Samples with TOC values of *ca.* 5 % or less (soils 1, 2, and 5) gave noisy spectra, even after signal averaging for 15 hours. Consequently, these samples were washed with 6N HCl to remove paramagnetic materials (mainly Fe^{+3} ions) and carbonates in an attempt to improve the S/N ratio of the spectra of these samples. This procedure yielded a much less-noisy spectrum for sample 2 (5.78% TOC), but was not successful for samples 1 (2.32% TOC) and 5 (3.58% TOC). Integrations of the spectra were performed as follows: Signals in the 0-40 ppm chemical shift range were attributed to aliphatic carbons (methyl, methylene, methine, and quaternary), 40-90 ppm to oxygenated aliphatic carbons, 90-120 ppm to di-*o*-alkyl carbons, 120-140 to aromatic carbons (protonated, bridgehead and branched), 140-165 to phenolic and aromatic ether carbons, 165-190 ppm to carboxyl carbons, and 190-220 ppm to carbonyl carbons in aldehyde and ketone moieties.

Chemicals

Phenanthrene (98%), and pyrene were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). *n*-hexadecane (99%+) was from Sigma Chemical Co. (St. Louis, MO), as were the following ^{14}C -radiochemicals: 9- ^{14}C -phenanthrene (46.9 mCi/mmol), 4,5,9,10- ^{14}C -

pyrene (58.7 mCi/mmol), and 1-¹⁴C-hexadecane (2.2 mCi/mmol). Minnesota peat fulvic acid was purchased from the International Humic Substances Society (St. Paul, MN).

Bacteria

Isolation and characterization of *M. austroafricanum* GTI-23 (Bogan et al, submitted) and *Burkholderia* sp. GTI-2 (Bogan et al., 2001a) have been previously reported elsewhere.

Biodegradation Experiments

Biodegradation (mineralization) experiments were conducted in 30-ml crimp-top serum bottles, fitted with suspended 1-ml NaOH traps for CO₂ as previously described (Bogan et al., 2001b). Soil (9 g dry wt.) was weighed into bottles, spiked with 1.8 mg/bottle (∴200 ppm) of hydrocarbon (phenanthrene or pyrene), containing 50,000-80,000 dpm ¹⁴C, in 4 ml methylene chloride, and allowed to evaporate overnight in a fume hood. Soils were then mixed with 2 ml water, and bottles were sealed and gamma-sterilized. Sterilization by gamma rays was done using a ⁶⁰Co source, with an exposure time of approximately 4 hours, and a total applied dose of 30-40 kGy. Sterile soil-filled bottles were then stored at room temperature until inoculation. “Time-zero” inoculations were performed immediately upon return of the soils from sterilization; hence, approximately 72 hours elapsed between spiking/watering of soils and the actual initial inoculation. Subsequent groups of samples were inoculated 40 and 120 days post-spiking. All inoculations were done as follows: Bacteria were collected from R2A agar plates (which had been streaked 3-10 days earlier) in mineral media (Bogan et al., 2001a & b), and adjusted to an A₆₀₀ of 1.2 (± 0.1); one ml of this suspension was then used to inoculate each bottle of soil.

Effect of Exogenous Fulvic Acid on Contaminant Degradation

A second series of biodegradation experiments were conducted with soil #1 which was amended with fulvic acid prior to spiking with pyrene. This soil was chosen because it had the lowest TOC; it was reasoned that this soil would be most likely to show effects of exogenously-added FA. Two-gram quantities of sterile soil were weighed into 30-ml serum bottles; fulvic

acid was dissolved in de-ionized water (1 ml/bottle), filter-sterilized, and added to give a final dosage of either 10 or 20 mg FA per 2 grams of soil. The bottles containing FA-amended soils were allowed to sit for *ca.* 72 hours in a sterile transfer hood, at which time pyrene (1000 ppm total concentration, $\approx 90,000$ dpm ^{14}C per bottle) was added in one ml of methylene chloride, which was then allowed to evaporate overnight in a fume hood. Pyrene was then allowed to age (in the dark) in the soil for 14 days; inoculation and tracking of $^{14}\text{CO}_2$ release were then done as described above. Soil bottles were kept in the dark during this experiment to minimize the potential for photochemical pyrene degradation.

Extractability of Aged Coal Tar PAHs in *n*-Butanol

Gamma-sterilized soils (air-dried) were spiked with coal tar (1000 ppm), which resulted in a total concentration of the 16 EPA priority pollutant PAHs of ~ 145 ppm; these were then allowed to age for 120 days. At the conclusion of this time, a mild *n*-butanol extraction was used to extract readily-available hydrocarbons. Approximately 2.0 grams of sample was weighed into a 20-ml glass sample vial. Ten ml of *n*-butanol were added to each vial, and each vial was shaken by hand for 30 seconds. Immediately following the shaking, 1 ml of the *n*-butanol supernatant was removed and filtered through a 0.45- μm Acrodisc HT “Tuffryn” filter prior to analysis by gas chromatography/ mass spectroscopy (GC/MS), which was conducted as described below.

GC/MS

PAH quantification was done using a DB-5MS capillary column (0.25mm x 30m with 0.25 μm film thickness). Samples were injected using a ThermoFinnigan AS2000 Autosampler (Injection port temperature 35°C for 0.05 min, ramped @ 10°C/sec to 250 °C, held for 1 min), into a TRACE gas chromatograph (gas flow = 1 cfs; split ratio = 20) with the following temperature profile: 40°C for 5 min, ramped @ 10 °C/min to 150 °C, ramped @ 5°C/min to 270°C, ramped @ 10°C/min to 300 °C, held for 7 min. A PolarisQ mass spectrometer was operated according to the following parameters: EI+ Full scan 50 – 450 m/z, 0.3 microscan, ion source temperature of 250°C. The 16 PAH-mixture were quantified using fully-deuterated internal standards for each PAH. Five concentrations (1, 5, 20, 50, and 100 ng/ μl) were used to

generate each calibration curve. All calibration curves had r^2 values of at least 0.999, with the exception of pyrene ($r^2 = 0.994$).

RESULTS

Soil Characteristics

Table 1 summarizes the results of all characterizations done on the six model soils in this work. The six soils were found to encompass a wide range of TOC contents, with the lowest (2.32 %) found in soil #1 (a clay loam-loam), and the highest (24.28%) in a sandy loam (soil #4). When the organic matter in the soils was fractionated into HA, FA, and humin, other significant differences were observed. Organic matter fractionation data showed that soils #1 and #5 were markedly lower in summed HA+FA (6.5% and 7.1%, respectively) than the other four soils, which contained between 13.2 % (soil #2) and 20.9% (soil #3) of these acids. The humic material in soil #4 was highly enriched in fulvic acid (4.0% of the TOC) when compared to the other five soils. Soil pore volumes and surface areas, measured by the BET method, also covered a wide range of values; upon closer inspection, these quantities were found to both be highly inversely correlated (increasing pore volume and surface area with decreasing TOC) with TOC content for five of the soils. The exception to the linear correlation was soil #4, which displayed higher-than-expected surface area and total pore volume. Only minor differences were found (among the four soils which gave interpretable spectra) in the contributions of individual functional moieties to the total integrated area of overall ^{13}C -NMR signals; it should be noted, however, that the two soils which could not be integrated were the very humin-rich, HA- and FA-poor soils (#1 and #5). When these values – with 120-165 ppm considered as aromatic and 0-120 ppm taken as aliphatic (approximately the same values used by Chefetz et al., 2000) – were used to determine aromatic/aliphatic ratios for the different soils, the differences in chemical makeup did appear to be somewhat more significant; the ratio of aromatic/aliphatic carbons ranged from .146 (soil #2) to .217 (soil #4).

Hydrocarbon Mineralization and Sequestration

Table 2 displays the extents to which phenanthrene and pyrene were mineralized (both 7- and 28-day $^{14}\text{CO}_2$ evolution) by *Mycobacterium austroafricanum* in the six model soils after various residence times in the soils (0, 40, or 120 days). For phenanthrene, there is very little effect of 40-day contact time on mineralization, with the exception of soil #3. In contrast, phenanthrene which had been aged for 120 days showed considerable evidence for sequestration in all soils, especially when the 28-day cumulative mineralization data is examined. The magnitude of the sequestration effect is maximal in soil #4 (2.0% mineralization after 120 days of sequestration vs. 31.8% at time-zero), and minimal in soils #1 and #2. In both of these latter soils, mineralization of freshly-spiked phenanthrene is approximately 50%, which drops off to 34.1 % (soil 1) or 36.3% (soil 2) after 120 days of sequestration. In the case of pyrene, significant sequestration was noted for only two of the soils, namely #2 and #3; of these, the magnitude of the decrease in mineralization was the greater for soil #3, in which 61.9% of newly-added pyrene was mineralized, compared to 28.9% of aged pyrene. In the other four soils, a 120-day aging period had essentially no effect on pyrene mineralization, either in terms of the 28-day “total” mineralization or the 7-day “rapid-release” mineralization.

Recent similar studies to ours have employed both subtractive analyses (e.g. mineralization after sequestration minus mineralization at time-zero) and raw (post-sequestration) mineralization values in attempts to correlate the magnitude of sequestration with soil physicochemical properties (Chung and Alexander, 2002). These authors note that sequestration measurements which necessitate a “time-zero” measurement may not be applicable to field sites, given that such measurements are often not possible in these situations. We suggest, however, that an approach which incorporates time-zero baseline data is necessary to a mechanistic study of the factors governing sequestration, as it negates possible effects of the soil itself on the health, proliferation, or performance of the microorganisms.

“Sequestration ratio” or “SR” was defined as the ratio of mineralization after aging to that when bacterial inoculation took place prior to sequestration. An SR of 1.0 therefore indicates that mineralization after aging is equal to that prior to aging, and that no sequestration has occurred; SR values less than 1 are indicative of reduced bioavailability, as assessed by bacterial mineralization. The dependence of SR_{total} , defined as the SR based on cumulative contaminant mineralization in 28 days, on organic carbon content, both after 40 and 120 days of aging, for the

six soils is shown in Figure 1. Figure 2 repeats this analysis, using the 7-day mineralization data to determine SR_{fast} , or the effect of sequestration on the rapidly-biodegradable contaminant fraction. These analyses show that, for five of the six soils (excluding #4), correlations can be drawn between SR and organic matter content. This is particularly true after 120 days of aging; correlations between SR and TOC were significantly lower after 40 days of aging.

Effect of Fulvic Acid on PAH Mineralization

As shown in Figure 3, addition of either 10 or 20 milligrams of Minnesota peat fulvic acid to soil #1 prior to spiking greatly enhanced the ability of *M. austroafricanum* GTI-23 to mineralize pyrene in this soil. In the absence of exogenously-added fulvic acid, mineralization of pyrene totaled approximately 12.4% of the input ^{14}C over a period of 32 days. The apparent discrepancy between this data and that in Table 2 – the fact that this total is approximately three-fold lower than the time-zero mineralization totals reported in Table 2 for pyrene in soil #1 (38.7%) – is most likely due to the fact that the mass of pyrene added to soil for the sequestration time-course experiment was one-fifth that added to this fulvic acid experiment. When this is taken into account, the actual masses of pyrene mineralized in the two experiments is very similar. In contrast, pyrene mineralization was increased by approximately 2.5-fold, to just below 30% of the input total, in cultures containing fulvic acids at the levels of 10 and 20 mg; these two sets of cultures were indistinguishable from each other.

PAH Extractability in *n*-Butanol

Table 3 shows the amounts of each of 13 three- to six-ring PAH species which could be extracted from the six coal tar-spiked model soils after 120 days of sequestration. For each of the PAH compounds, the percentage of the total burden (determined by comparison with the time-zero recoveries from a rigorous methylene chloride/acetone sonication (EPA method 3550)) which is recovered in a rapid *n*-butanol extraction is also given. As expected, recoveries of each individual PAH are, in general, inversely correlated to the organic matter content of the soil; recoveries from soil #4 (24% TOC) are approximately 1/3 to 1/5 those from soil #1 (2.3% TOC). Interestingly, percent recoveries of the higher-molecular-weight compounds were almost always higher than those of the lower-MW PAHs; this was most evident in soils 1, 6, 3, and 4, and less so in soils 2 and 5. This would most likely indicate differences in the kinetics of

sequestration between freshly-spiked low- and high-molecular-weight PAHs in the coal-tar mixture.

DISCUSSION

Availability to, and degradability by, *Mycobacterium* is unlikely to directly approximate bioavailability of contaminants to higher organisms. Other research has shown, for example, that uptake of phenanthrene by earthworms decreases faster than does the degradability of this PAH by *Pseudomonas* strains (Kelsey and Alexander, 1997). If, in fact, *Mycobacterium* species are better-suited to uptake of hydrophobic pollutants – for example, by virtue of their greater lipophilicity (Rehmann et al., 1988; Bastiaens et al., 2000) – then this discrepancy is likely to be even more accentuated. However, if *Mycobacterium* species are important in the natural remediation of contaminated sites, which is logical to infer given the frequency of their isolation from such environments, an understanding of the physicochemical soil parameters which impact their performance in this regard is clearly of interest.

Recent studies on PAH sequestration have found significant correlations between microbial biodegradability of sequestered hydrophobic contaminants and the organic carbon content of the soil in which the contaminant is present (Chung and Alexander 1998 & 2002), confirming the early herbicide bioavailability studies which implicated OM content (or some parameter which is covariant with OM content) as the primary determinant of sequestration. The latter of these studies (Chung and Alexander 2002), recognizing that OM content is not the sole contributor to the sequestration process, sought to identify other parameters, and investigated the possible roles of cation exchange capacity (CEC), micropore volume, soil texture, and surface area, among others. The work which we describe herein supports the centrality of TOC, in that we find that changes in the degradability (by *Mycobacterium austroafricanum* strain GTI-23) of phenanthrene and pyrene which have been aged in different soils are strongly dependent on the organic matter content of the individual soil. We further find that the impact of soil TOC is greater on the rapidly-biodegradable “fast-release” fraction than on the total biodegradable hydrocarbon pool (as exemplified by a comparison of the slopes of the regression lines in Figure 1 with those in Figure 2), and that reductions in the size of the rapidly-degradable fraction with

increasing soil TOC content are greater with a higher-molecular-weight PAH (pyrene) than with a lower-molecular-weight compound (phenanthrene).

Clearly, our data (as those of Chung and Alexander) support the conclusion that soil OM content *per se* is not the sole factor governing sequestration. The most compelling instance of this in our data set was the case of soil #4, which consistently evidenced much higher-than-expected rates of mineralization than would be expected based on its TOC content. In examining other possible factors, we found micropore volume and surface area (as determined by N₂ adsorption) to be strongly covariant with OM content within our group of soils, with the one exception being soil #4, which displayed slightly higher-than-expected values for both of these parameters based on the linear regression determined for the other five soils. This soil also displayed considerably higher fulvic acid content than any of the other soils.

Other workers have shown that dissolved organic matter (DOM) can increase water "solubility" of PAH in contaminated soils by an order of magnitude or more (Johnson and Amy, 1995; Villholth, 1999; MacKay and Gschwend, 2001). From the standpoint of bioavailability, interaction between dissolved organic matter (*e.g.* fulvic acids) and PAH has been reported to decrease PAH uptake and bioconcentration by, and/or toxicity to, higher organisms, for example *Daphnia magna* (Akkanen and Kukkonen, 2001; Perminova et al., 2001) and other aquatic organisms (Haitzer et al., 1999 & references therein). This, however, is in direct contrast to a spate of studies which have recently reported that addition of humic substances significantly stimulates microbial degradation of both polychlorinated biphenyls (Fava and Piccolo, 2002) and PAH (Haderlein et al., 2001; Holman et al., 2002). In the present study, addition of exogenous fulvic acids to a very low-TOC soil greatly enhanced pyrene mineralization by *M. austroafricanum*. Thus, our results clearly demonstrate that, if all other physicochemical properties of a soil are held constant, higher fulvic acid content (or supplementation with endogenous FA-rich material) can definitely result in a higher rate (and/or extent) of contaminant biodegradation.

Conversely, it has been shown that extraction of soluble organic matter (humic and fulvic acids), leaving humin, increased the rate and extent of PAH sequestration in a model soil (White, et al., 1999). Other workers have modeled sequestration of organic compounds in soil as a two-step process, with adsorption onto hydrophobic material at the surfaces of soil particles (primarily HA and FA) occurring first, and partitioning into pores, access to which is partially

blocked by the HA/FA overlayer, occurring later (Weber et al 1992; Weber and Huang 1996; Xing and Pignatello 1997, Amellal et al 2001 & refs. therein); the majority of these “internal pores” are believed to be in the humin fraction of soil. Although we did not find any clear case where the humin content of a soil could be shown to directly affect PAH degradability in that soil, humin content does clearly play a role in the sequestration process, as became evident in attempts to correlate the *n*-butanol extractabilities of PAH with their biodegradabilities.

When the full suite of six soils was considered together, the ability of *n*-butanol extractability of phenanthrene to serve as a predictor of biodegradability by *Mycobacterium austroafricanum* GTI-23, appears poor (Figure 4a). However, upon closer examination, it becomes clear that, if soils 3, 4, and 6 are considered as one subgroup, and soils 1, 2, and 5 as a second subgroup, the correlations between extractability and mineralization in fact become excellent, both for the 7-day “fast release” mineralization data and the 28-day “total release” data. We suggest that the significance of this observation lies in the fact that the three soils in the first category (3, 4, and 6) have the lowest N₂-accessible pore volumes and surface areas, highest TOC values, and lowest humin contents; thus, they can be considered as being dominated by highly-flexible “soft” carbon (humic and fulvic acids). In contrast, the second group of soils (1, 2, and 5) can be considered as more “hard” in nature, as they display higher pore volumes and surface areas by the N₂ method, less TOC, and more humin than the soils in the first group. Correlations are considerably poorer in the case of pyrene (Figure 4b). A similar pattern to that in Figure 4a was seen in the results of a second experiment, in which mineralization of phenanthrene by *Burkholderia* sp. strain GTI-2 (Bogan et al., 2001a) was measured; this data is presented in Figure 5. These results indicate that, although *n*-butanol extractability does show excellent promise as an indicator of biodegradative potential for at least some PAHs (*e.g.* phenanthrene), it may not work well for all PAH (*e.g.* pyrene in our data); furthermore, the ability to determine absolute correlations between the two properties may require some *a priori* knowledge of soil physicochemical parameters.

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TABLE 1 – PHYSICOCHEMICAL CHARACTERISTICS OF THE SIX MODEL SOILS USED IN THIS WORK.

SOIL	Particle Size Distribution			Classification	Organic Carbon Analyses ¹			
	% Sand	% Silt	% Clay		% TOC	% FA	% HA	% Humin
1	25.4	51.1	27.5	Clay loam-Loam	2.32	0.5	6.0	93.5
2	64.0	25.3	10.7	Sandy loam	5.78	0.9	12.3	86.7
3	70.8	18.1	11.1	Sandy loam	11.16	1.4	19.5	79.0
4	73.5	10.6	15.9	Sandy loam	24.28	4.0	12.9	83.1
5	40.6	33.0	26.4	Loam	3.58	0.7	6.4	92.9
6	51.8	26.3	21.9	Sandy clay loam	9.13	1.1	18.5	80.4

¹ – Values for FA (fulvic acid), HA (humic acid), and humin are expressed as % of the TOC (total organic carbon) in each soil.

SOIL	Pore Volume (ml/g soil)	Surface Area (m ² /g soil)	¹³ C-NMR chemical shift (% of total integrated signal) ²							Aromatic/Aliphatic Ratio ³
			0-40	40-90	90-120	120-140	140-165	165-190	190-250	
1	0.0508	22.18	nd	nd	nd	nd	nd	nd	nd	-
2	0.0351	13.82	22	41	13	8	5	11	0	0.146
3	0.0162	6.72	17	38	15	9	9	8	4	0.204
4	0.0114	4.58	20	34	11	9	9	10	7	0.217

5	0.0371	18.01	nd	-						
6	0.0232	10.40	18	37	11	9	7	12	6	0.195

² – Chemical shifts from ¹³C-NMR were assigned to the following functional group(s): Branched & straight-chain aliphatics (0-40 ppm), ethers (40-90 and 90-120 ppm), aromatic rings (120-140 ppm), phenolic (140-165 ppm), carboxylic acid/ester (165-190 ppm), and carbonyl/ketone (190-250 ppm). nd = not determined.

³ – Ratio of total aromatic C (120-165 ppm) to total aliphatic C (0-120 ppm).

TABLE 2 – Mineralization (expressed as percent of input ¹⁴c) of 200 ppm phenanthrene and pyrene by *mycobacterium austroafricanum* gti-23 during incubations of six model soils, following contaminant/soil contact times of 0, 40, or 120 days. Data given are ¹⁴C released in 7 days (corresponding to the “fast release” fraction) and 28 days (total biodegradable fraction); data are means and standard deviation (in parentheses) of duplicate cultures, except in the case of pyrene soil #4, for which one of the cultures was lost.

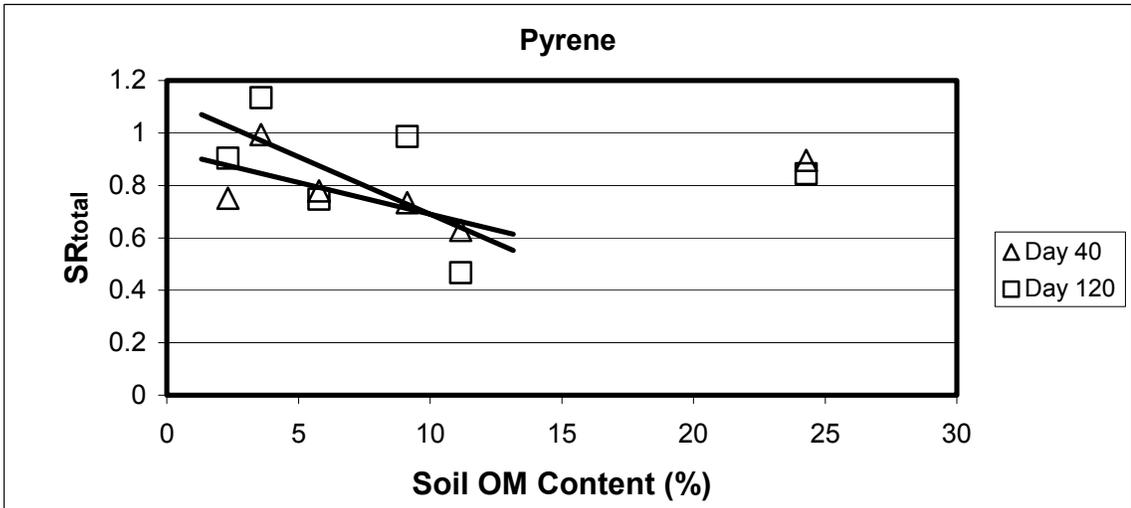
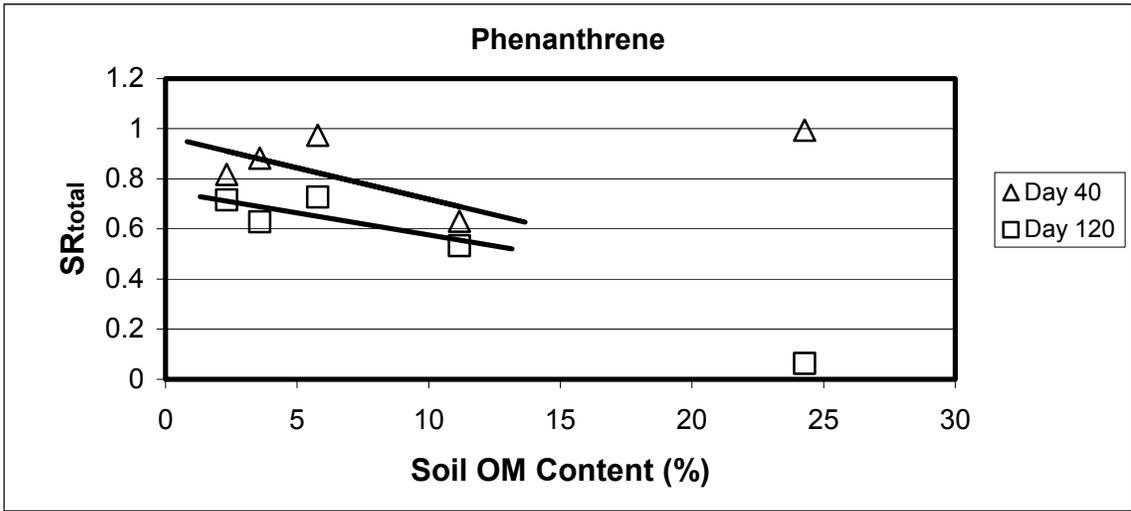
	Contact Time = 0 d		Contact Time = 40 d		Contact Time = 120 d	
	7-Day	28-Day	7-Day	28-Day	7-Day	28-Day
Phenanthrene						
Soil 1	16.3 (0.2)	47.6 (2.4)	16.5 (2.9)	39 (5.7)	19.6 (0.7)	34.1 (0.4)
Soil 2	33.2 (0.9)	50.0 (1.7)	31.4 (1.0)	48.7 (0.5)	25.5 (4.1)	36.3 (9.9)
Soil 3	32.2 (0.1)	50.3 (0.6)	14.4 (11.6)	31.7 (16.9)	11.5 (2.6)	26.8 (11.0)
Soil 4	25.6 (0.2)	31.8 (0.9)	24.1 (0.3)	31.6 (0.4)	0.2 (0.1)	2.0 (2.7)
Soil 5	25.0 (0)	51.5 (0.3)	22.0 (0.3)	45.4 (1.7)	12.7 (0.6)	32.3 (1.5)
Soil 6	nd	nd	32.3 (1.5)	49.5 (2.5)	29.9 (2.2)	44.2 (1.6)
Pyrene						

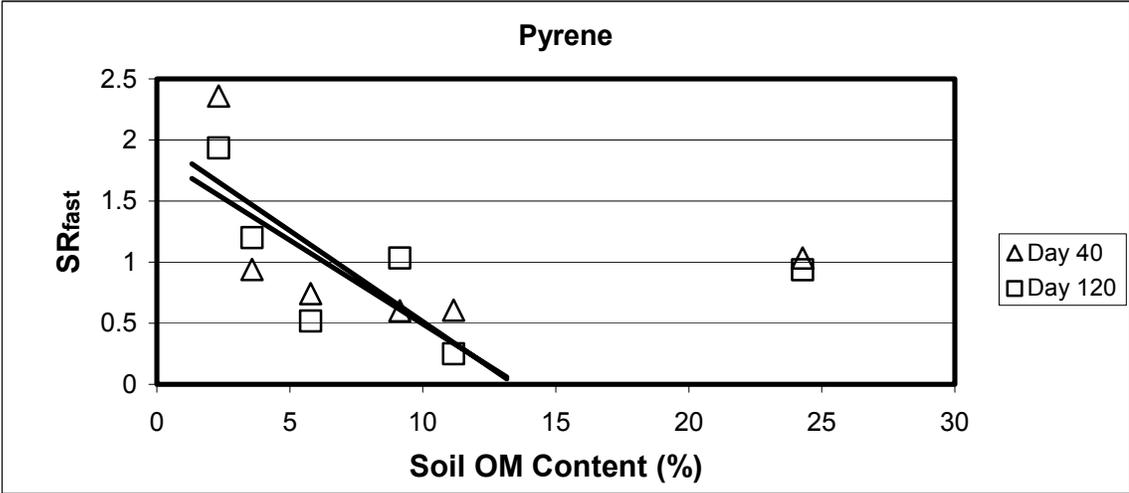
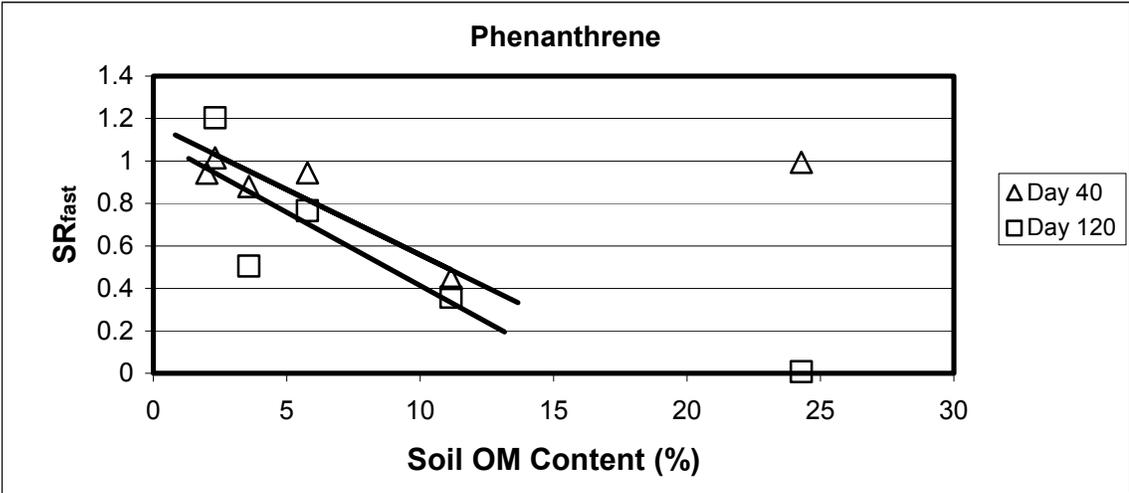
Soil 1	3.8 (1.8)	38.7 (2.1)	9.0 (1.4)	29.1 (5.6)	7.3 (0.2)	35.0 (2.9)
Soil 2	29.1 (3.6)	57.3 (3.8)	21.6 (5.0)	44.6 (4.5)	15.1 (2.2)	42.8 (5.3)
Soil 3	27.1 (5.3)	61.9 (3.5)	16.4 (1.5)	38.9 (2.3)	6.8 (2.7)	28.9 (8.7)
Soil 4	17.4 (-)	39.4 (-)	18.0 (-)	35.2 (-)	16.3 (-)	33.3 (-)
Soil 5	14.1 (4.2)	44.0 (0.5)	13.3 (1.7)	43.8 (1.7)	16.9 (1.2)	50.0 (0.5)
Soil 6	22.0 (0.2)	45.9 (1.3)	13.2 (5.5)	33.7 (9.7)	22.7 (6.0)	45.3 (8.8)

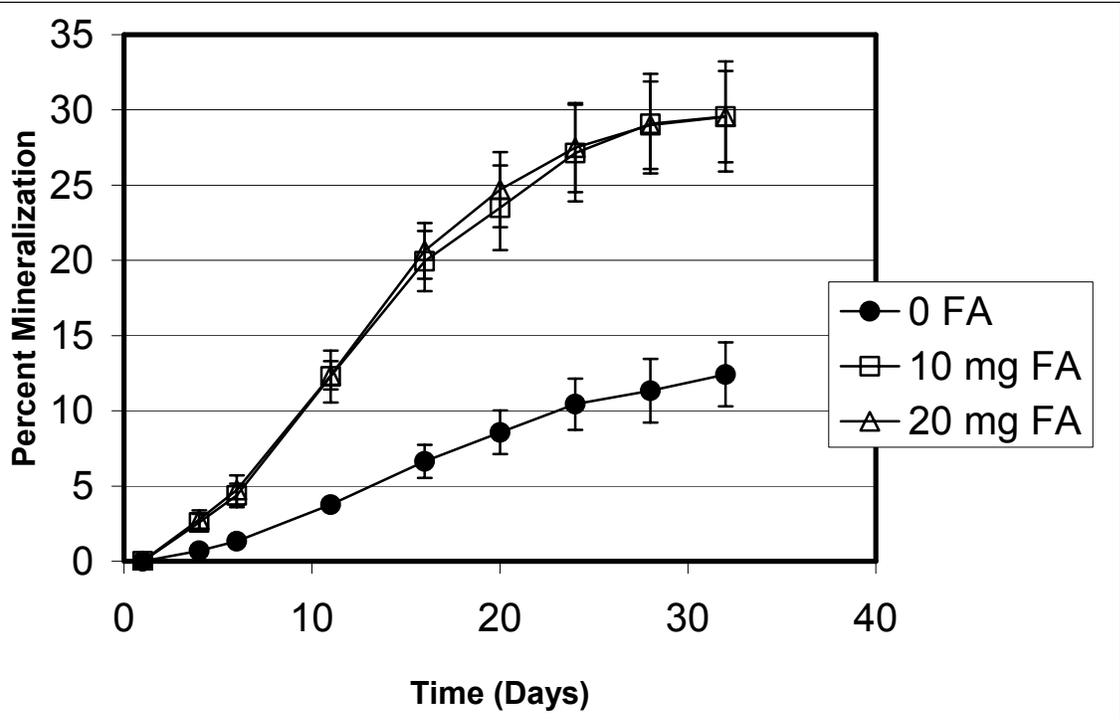
Table 3 – Extractability (after 120 days) in *n*-butanol of various pahs from six model soils. All data are given as parts per million of each pah which were recovered by rapid *n*-butanol extraction (see text). Numbers in bold represent the fraction (in percent) of each pah (determined by comparison with time-zero amounts measured using rigorous methylene chloride/acetone sonication extraction) which is *n*-butanol-extractable.

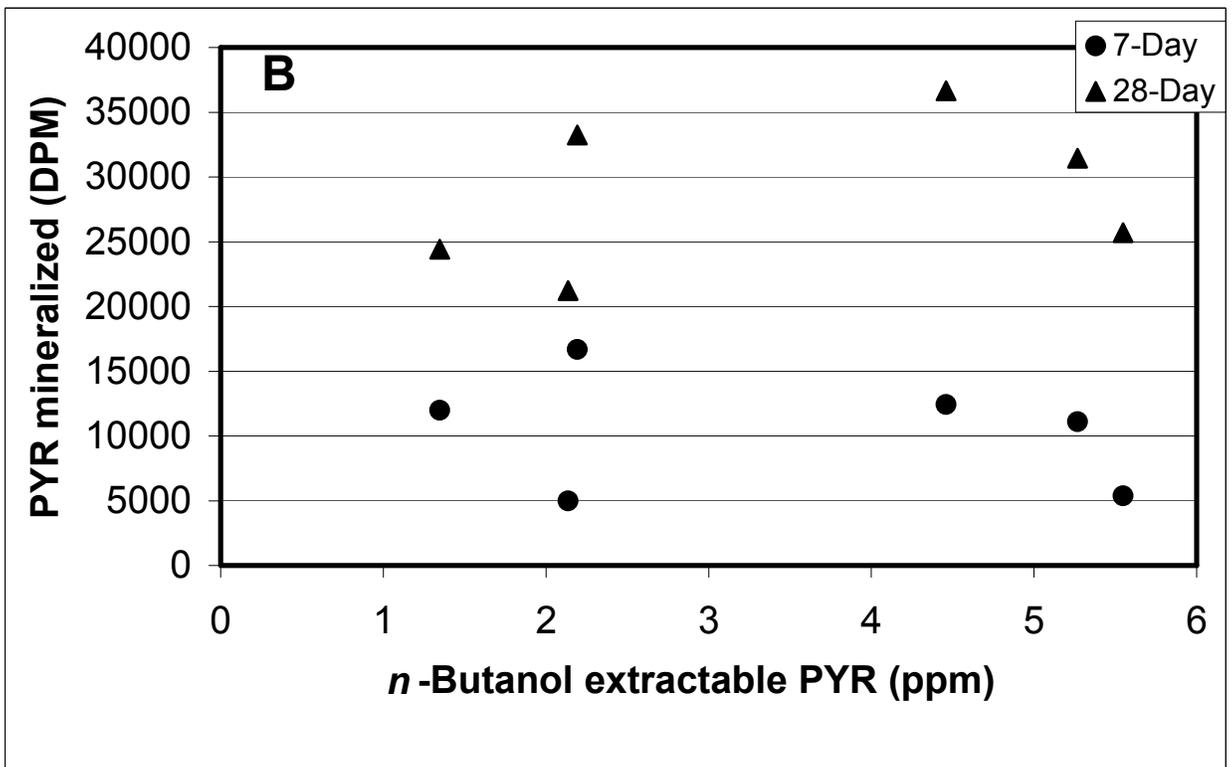
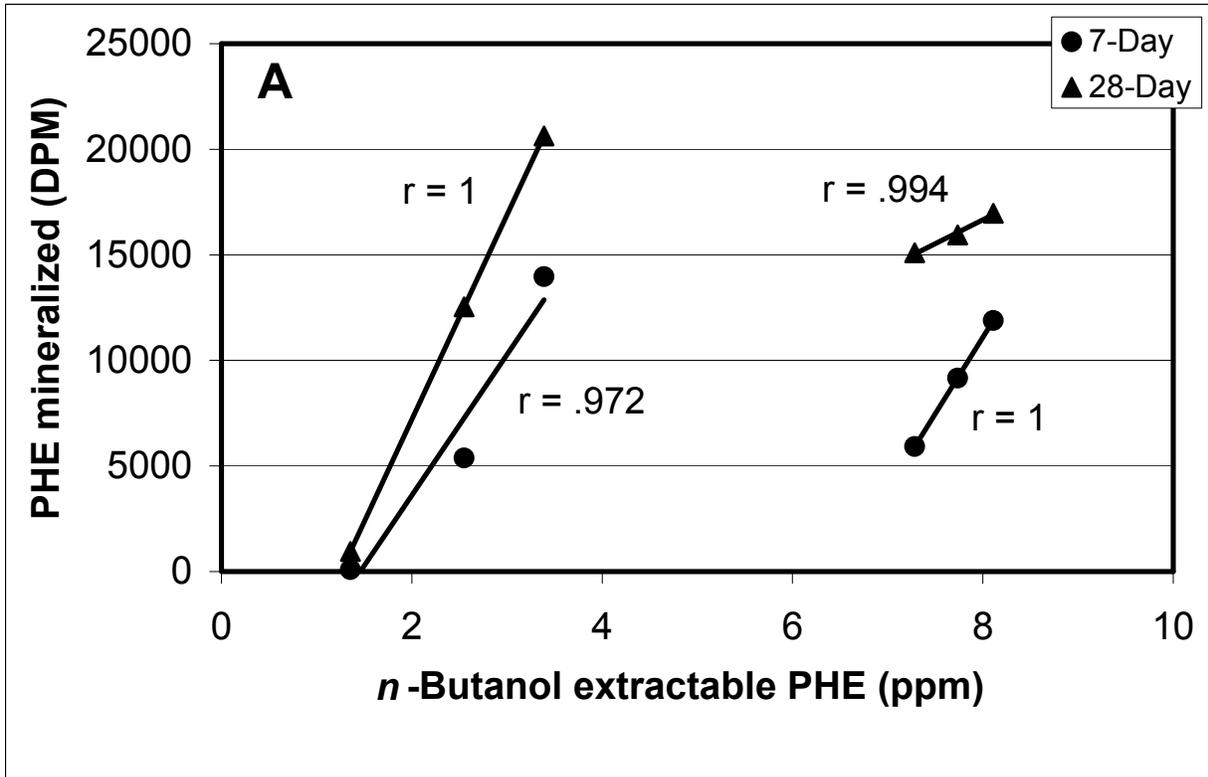
	Soil (TOC Content)					
	1 (2.32%)	5 (3.58%)	2 (5.78%)	6 (9.13%)	3 (11.16%)	4 (24.28%)
Fluorene	1.56 51.91	1.16 38.63	1.41 46.89	0.69 22.80	0.53 17.79	0.29 9.79
Phenanthrene	7.73 20.06	7.28 18.90	8.11 21.04	3.39 8.79	2.55 6.61	1.35 3.51
Anthracene	2.10 15.26	0.69 5.00	1.18 8.61	0.71 5.15	0.74 5.41	0.46 3.36
Fluoranthene	4.22 13.81	3.51 11.50	4.17 13.67	1.65 5.39	1.43 4.70	0.85 2.79
Pyrene	5.55 24.88	4.46 20.00	5.27 23.62	2.19 9.83	2.14 9.58	1.35 6.04
Benz(a)anthracene	1.88 33.52	1.24 22.02	1.31 23.23	0.67 11.96	0.60 10.62	0.41 7.32
Chrysene	1.91 22.05	1.46 16.85	1.47 16.98	0.75 8.67	0.64 7.44	0.45 5.22
Benzo(b)fluoranthene	2.31	1.45	1.13	0.90	0.69	0.55

	Soil (TOC Content)					
	1	5	2	6	3	4
	46.29	29.05	22.53	17.99	13.79	10.97
Benzo(<i>k</i>)fluoranthene	1.61	0.78	0.77	0.60	0.54	0.49
	74.22	35.87	35.36	27.83	24.95	22.74
Benzo(<i>a</i>)pyrene	1.68	1.00	0.98	0.72	0.70	0.57
	38.37	22.86	22.35	16.51	15.92	13.10
Indeno(1,2,3- <i>c,d</i>)pyrene	1.85	1.02	1.04	0.71	0.67	0.58
	43.87	24.09	24.78	16.78	15.99	13.67
Dibenz(<i>a,h</i>)anthracene	1.31	0.46	0.42	0.44	0.40	0.39
	47.19	16.54	15.23	15.76	14.36	13.95
Benzo(<i>g,h,i</i>)perylene	1.80	0.86	0.88	0.63	0.65	0.56
	48.88	23.37	24.00	17.10	17.75	15.19









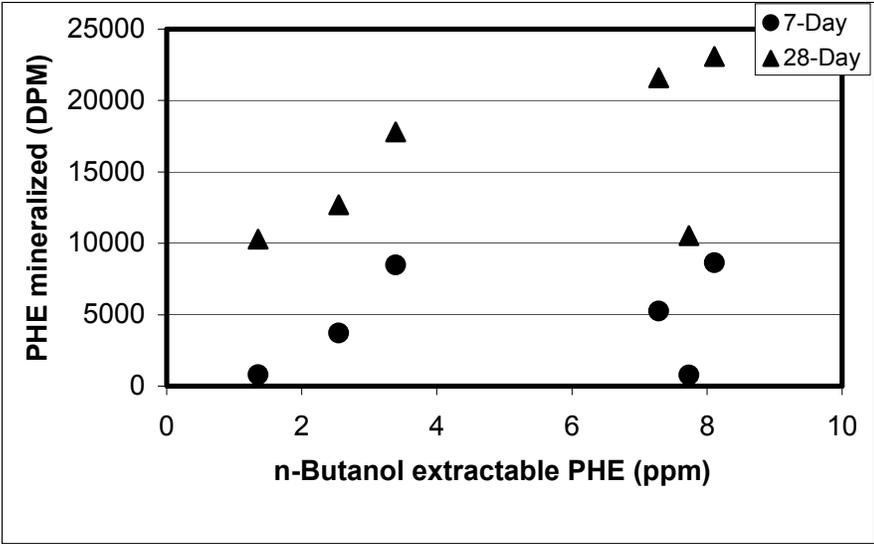


Figure 1 – Sequestration ratios as determined using 28-day cumulative mineralization data (SR_{total}), as a function of soil organic matter content for phenanthrene and pyrene in the six model soils. Data is given for soil cultures which were aged for 40 or 120 days prior to inoculation with *Mycobacterium austroafricanum* GTI-23.

Figure 2 – Sequestration ratios determined using the 7-day “fast release” mineralization data from *M. austroafricanum* soil cultures.

Figure 3 – Effect of exogenous Minnesota peat fulvic acid supplementation (10 or 20 mg per 2-gram soil culture) on pyrene mineralization in soil #1 (2.32% TOC) by *M. austroafricanum*.

Figure 4 – Relationships between *n*-butanol extractable levels of phenanthrene (A) and pyrene (B) (after 120 days of sequestration) and mineralization of the same compounds by *M. austroafricanum* (after 80 days of sequestration) in the six model soils.

Figure 5 – Relationship between *n*-butanol extractable levels of phenanthrene and mineralization of phenanthrene by *Burkholderia* sp. strain GTI-2 (after 80 days of sequestration) in the six model soils.

**EFFECTS OF ALKYLPHOSPHATES AND NITROUS OXIDE ON MICROBIAL
DEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS**

Running Title: Alternative N and P Sources for PAH Bioremediation

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ABSTRACT

We conducted a series of liquid-culture experiments to begin to evaluate the ability of gaseous sources of nitrogen and phosphorus to support biodegradation of polycyclic aromatic hydrocarbons (PAHs). Nutrients examined included nitrous oxide, as well as triethylphosphate (TEP) and tributylphosphate (TBP). Cultures were established using the indigenous microbial populations from one manufactured gas plant (MGP) site, and one crude oil-contaminated drilling-field soil. Mineralization of phenanthrene was measured under alternative nutrient regimes, and was compared to that seen with ammoniacal nitrogen and PO_4 . Parallel cultures were used to assess removal of a suite of 3- to 5-ring PAHs. In summary, the abilities of the different communities to degrade PAH when supplemented with N_2O , TEP and TBP were highly variable. For example, in the MGP soil, organic P sources, especially TBP, supported considerably higher removal of low-molecular-weight (LMW) PAHs than did PO_4 ; however, loss of high-molecular-weight (HMW) compounds was impaired under these conditions. Disappearance of most PAHs was significantly lower in the oilfield soil when organophosphates were used. These results indicate that the utility of gaseous nutrients for PAH bioremediation in situ may be limited, and will very likely have to be assessed on a case-by-case basis.

INTRODUCTION

Approximately 1500 former manufactured gas plant (MGP) sites in the United States are estimated to exhibit contaminated soil and groundwater due to coal and oil gasification and liquefaction operations (9). Similarly, at least 700 identified sites in the U.S. are contaminated with creosote as a result of improper materials handling and disposal during wood-preserving activities (18). Both of these classes of sites are considered to pose a significant potential health risk for humans and wildlife, as the wastes generated in these processes (primarily coal tars and related substances) contain numerous toxic, carcinogenic, and/or mutagenic compounds.

The most notable class of hazardous compounds found in both coal tar and its derivatives (*e.g.* creosote) are the polycyclic aromatic hydrocarbons, or PAHs (19, 20), which consist of two or more benzene rings fused into a single aromatic structure. Mammalian liver enzymes (cytochromes P-450 and epoxide hydrolase) oxidize certain PAHs to fjord- and bay-region diol-epoxides (2, 11, 17, 29, 30); these moieties form covalent adducts with DNA (17, 28). Therefore, many PAHs are genotoxic and/or carcinogenic (1, 2, 8, 14, 23), and promote similar effects of other compounds (6). Thus, a total of 16 PAHs have been included on the United States EPA's priority pollutant list (13).

Bioremediation has long been proposed as a treatment technology for the decontamination of PAH-contaminated soils. Numerous bacteria are known to catabolize various 2-, 3-, and 4-ring PAHs as sole sources of carbon and energy (For review, see (5)), thus making them good candidate species for site-remediation applications. The efficacy of bioremediation approaches, particularly when applied *in situ*, depends on overcoming any potential nutrient limitations within the soil system to be remediated. In the case of hydrocarbon-contaminated soils, the limiting nutrient is most frequently either phosphorus or nitrogen or, in some cases, both of these. This can be ameliorated via the subsurface injection of soluble nutrients; however, the resultant very high concentrations of nutrients in the immediate vicinity of such injection wells has been observed to lead to excessive localized microbial growth, with concomitant "biofouling" of the wells (4).

The use of gaseous nutrients (N and P compounds with sufficiently high vapor pressures so as to allow their conversion to a gas under environmental conditions) has been demonstrated in situ as a means of better distributing nutrients throughout the system in support of soil bioremediation. Triethylphosphate (TEP) and tributylphosphate (TBP) although mildly toxic and corrosive irritants, are nonetheless the safest phosphorus compounds which can readily be gasified (in comparison with, for example, phosgene or the carcinogenic trimethyl phosphate). They have thus been utilized as phosphorus sources (4, 21) in a patented process (15, 16). Similarly, gaseous nitrous oxide has been used to supply nitrogen (4, 21). The delivery of gaseous nutrients has been shown to enhance the in situ remediation of chlorinated solvents and volatile organic compounds (4, 21), as well as C₄-C₁₀ alkanes and monoaromatic hydrocarbons (*e.g.* benzene, toluene, ethylbenzene, xylene) (24). It has not, however, been documented as a means of enhancing the remediation of PAH-contaminated soils.

This paper presents the results of liquid-culture studies into the ability of organic phosphates (TEP and TBP) and N₂O to support PAH degradation by bacteria present in MGP and other petroleum-contaminated site soils. Liquid-culture conditions, although clearly not representative of field conditions, were chosen in order to evaluate microbial performance under conditions of optimal bioavailability. In general, we have found that, while removal of some PAHs in some soils does appear to be significantly stimulated through the use of alternative sources of N and P, this effect is not universal. There appears to be considerable site-specific variability based on differences in microbiology, soil chemistry, and/or soil structure, implying that soil treatability evaluations will have to be conducted on a case-by-case basis. Our results will serve as a starting point for studies on the use of gaseous N and P sources to support PAH bioremediation in soil-column microcosms which are more representative of site conditions.

MATERIALS AND METHODS

Soils - Soil samples were obtained from sites with a history of industrial activities leading to PAH contamination. The MGP soil is a loamy sand (86% sand, 5% clay, 9% silt) from a New Jersey site, whereas the oilfield soil is a crude oil-contaminated sandy loam (63% sand, 3% clay, 34% silt) obtained from the vicinity of wellhead in a drilling field in Southern Illinois. PAH concentrations for the two soils are given in Table 1. The oilfield soil, although very high (*ca.* 16% - data not shown) in total petroleum hydrocarbons, actually contained relatively modest levels of PAH, as can be seen in Table 1. Each of these soils was air-dried (to *ca.* 3% moisture) and homogenized immediately prior to use.

Culture Conditions - Homogenized soil samples (500 mg) were mixed with 50 ml sterile media (0.1ml Wolfe's Vitamins (2 mg·l⁻¹ biotin, 2 mg·l⁻¹ folic acid, 10 mg·l⁻¹ pyridoxine HCl, 5 mg·l⁻¹ thiamine HCl, 5 mg·l⁻¹ riboflavin, 5 mg·l⁻¹ nicotinic acid, 5 mg·l⁻¹ pantothenic acid, 0.1 mg·l⁻¹ cyanocobalamine, 5 mg·l⁻¹ *p*-aminobenzoic acid, 5 mg·l⁻¹ thiocctic acid), 0.1ml Trace Minerals (100 mg·l⁻¹ ZnSO₄, 300 mg·l⁻¹ H₃BO₃, 300 mg·l⁻¹ CoCl, 10 mg·l⁻¹ CuCl) and 0.8 ml N- & P-free (pH 7.2) Winogradsky medium (62.5 g·l⁻¹ MgSO₄·7H₂O, 31.25 g·l⁻¹ NaCl, 1.25 g·l⁻¹ FeSO₄, 1.25 g·l⁻¹ MnSO₄) per 100 ml of sterile deionized water) in 125-ml serum bottles. In order to assess the degree of N- and P-limitation on PAH degradation inherent in each soil, ¹⁴C-phenanthrene mineralization was measured in cultures of each of the three soils which received no supplemental N or P, N only (as NH₄Cl), P only (as KH₂PO₄), or both N and P. Six combinations were then investigated for nitrogen (N) and phosphorus (P) supplementation: NH₄Cl/KH₂PO₄; N₂O/KH₂PO₄; NH₄Cl/TEP; NH₄Cl/TBP; N₂O/TEP and N₂O/TBP. Within each condition, duplicate cultures were employed. In all cases, addition of N and P sources was normalized on a molar basis to provide 9.2 mM N and 3.7 mM P. When N₂O was used, it was added by injection to sealed bottles. In order to ensure the presence of at least some bioavailable PAH, all cultures were also supplemented with 50 µl of a PAH-containing extract from a second MGP soil (approx. 12,000 ppm total PAH – see Table 1), dissolved in *n,n*-dimethylformamide (DMF). One set of cultures (duplicates of each

condition) was further supplemented with ^{14}C -phenanthrene for mineralization determinations (see below), while one received no radiolabel, and was used to simultaneously measure extent of disappearance of multiple PAHs. Both sets of cultures were incubated at room temperature (approx. 25 °C), with shaking at 170 rpm. Poisoned controls (10 mg HgCl_2 per culture) were also conducted in duplicate.

Mineralization of ^{14}C -PAH - CO_2 traps were made by wrapping stainless steel wire around the necks of 12 x 32 mm borosilicate glass autosampler vials and pushing the wire through 20-mm Teflon silicone-lined septa. These assemblies were placed in the serum bottles, which were then crimped with aluminum seals. Syringes were used to inject 1 ml of 0.5M NaOH into each CO_2 trap. Periodically, the CO_2 -containing NaOH solution was withdrawn from the traps, mixed with 5 ml of Ultima Gold® high-flashpoint LSC cocktail solution (Packard, Meriden, CT), and counted in a liquid scintillation counter (Packard Model 2200CA Tri-Carb). Fresh NaOH was then added to the CO_2 traps. Cultures containing ^{14}C -phenanthrene typically received *ca.* 80,000-100,000 dpm of PAH in 20 μl of methanol.

Extraction and HPLC Analysis - Soil samples were centrifuged (10 min, 5000 x g) in stainless-steel containers to separate solid and aqueous phases. Soil solids were mixed with anhydrous sodium sulfate (1:1), and ground in a mortar and pestle to form a fine powder. Sonication was performed according to EPA Method 3550A (27), using 1:1 hexane/acetone (30 ml) as the solvent, and was repeated three times. The extracts were combined and vacuum filtered before evaporation. The aqueous phases of various cultures were extracted threefold with methylene chloride as per EPA Method 3510B (27). These extracts were then dried by passage through anhydrous sodium sulfate. Both solid and aqueous extracts were evaporated to dryness under a stream of N_2 in a Turbovap Evaporator (Zymark, Hopkinton, MA) and exchanged into acetonitrile (1 ml). 10 μl of this solution was analyzed by reverse-phase HPLC (EPA Method 8310 (27)) using a Supelcosil LC-PAH column (15 cm x 4.6 mm) and a Waters HPLC system coupled to a diode-

array detector (Waters Model 996). The following gradient was used, with a flow rate of 1.5 ml·min⁻¹ throughout: 0 min - 60% H₂O/40% ACN; 25 min - 100% ACN (hold for 2 min); 33 min - 60% H₂O/40% ACN. Identities of individual PAHs were verified by comparing the retention times and the absorbance spectra, and quantified by comparison with 5-point standard curves (all r² >0.988).

Chemicals - Phenanthrene (9-¹⁴C, reported purity = 98%) was purchased from Sigma (St. Louis, MO). TEP (99% pure) and TBP (98%) were purchased from Aldrich (Milwaukee, WI); N₂O (ultra high purity) was from Matheson Gas Products (Joliet, IL). NH₄Cl and KH₂PO₄ were purchased from Mallinckrodt Chemicals (Paris, KY), and NaOH and HPLC-grade solvents were purchased from Fisher Chemicals (Fairway, NJ). Authentic PAH standards for use in HPLC analysis were obtained from Ultra Scientific (Kingstown, RI).

Results and Discussion

Nutrient limitations were assessed for each soil by determining the degree of phenanthrene mineralization which occurred in the absence of any supplemental N or P; this was then compared to that which was supported by either nutrient singly, or when the two were combined. Data for these trials are shown in Figure 1. Both soils were strongly nutrient-limited. The MGP soil showed an especially strong N limitation and a P limitation which was also very significant, while the oilfield soil was greatly limited by both N and P; in this case, neither nutrient alone was capable of enhancing ¹⁴CO₂ release at all relative to unsupplemented conditions.

Cultures of the microbial communities from the two soils were examined for their ability to mineralize phenanthrene under various conditions of N and P supplementation. We also determined, using reverse-phase HPLC, disappearance of a range of 3- to 6-ring PAHs from cultures under the same set of nutrient-supplementation conditions.

In the case of the MGP soil, there was little effect of varying nutrient compositions on mineralization of spiked ¹⁴C-phenanthrene, as can be seen in Figure 2. All nutrient regimes examined sustained between 50 and 70 percent conversion to CO₂, with the only significant difference between the different treatments being a slightly

longer lag time prior to mineralization in the three conditions in which N₂O served as the nitrogen source. Cultures receiving HgCl₂ (10 mg) were still capable of mineralizing 7.3% of the input phenanthrene.

Under “conventional” nutrient additions (NH₄ and PO₄), the microbial community present in the MGP soil displayed significant removal of all 3-ring compounds examined, as well as some elimination of several 4- and 5-ring PAHs (Table 2). A separate experiment, in which mineralization of ¹⁴C-pyrene was measured (data not shown) indicated that no mineralization of this PAH occurred; thus, it seems possible that the loss of pyrene seen in this soil may be due to co-metabolic effects (7), which may also account for the loss of other 4- and 5-ring compounds. No loss of six-ring PAHs was observed in this soil (data not shown). The data in Table 2 clearly show that substitution of alternative N and P sources does not, in most cases, enhance microbial PAH degradation in this soil. The combination of NH₄ and TBP results in enhanced removal of phenanthrene and anthracene (relative to NH₄/PO₄); however, losses of fluorene and fluoranthene are no greater under these conditions, and 4- and 5-ring compounds are unaffected. Most other nutrient regimes supported degradation of only the most labile compounds (fluorene, phenanthrene, anthracene).

In the crude oil-contaminated wellhead soil, as in the MGP soil, mineralization of phenanthrene was greatest in the NH₄Cl/KH₂PO₄ cultures (Figure 3), again exceeding 80%. In this soil, use of any of the alternative nutrients substantially reduced the mineralization of phenanthrene. Pairwise comparisons of the different conditions showed clear trends, as all three of the NH₄-containing cultures outperformed their N₂O-containing counterparts. Furthermore, the trend of PO₄>TEP>TBP was true for both the NH₄ and N₂O sets of cultures. Killed controls evolved 1.7% of the input ¹⁴C as CO₂.

The extent of PAH removal from the oilfield soil was considerably higher than from the MGP site soil. Table 3 shows that nearly all compounds examined were significantly removed from this soil (which, as described above, is strongly N- and P-limited for mineralization of phenanthrene) under the NH₄/PO₄ supplementation; this includes 5-ring PAHs. Again, inasmuch as no significant mineralization of pyrene was seen in this case (data not shown), it seems possible that some of the losses of HMW PAHs are co-metabolic in nature. As with the MGP soil, inclusion of an alternative

source of either N or P significantly impeded PAH removal for many compounds, although essentially complete removal of some (phenanthrene, anthracene and fluorene) did still occur under all nutrient regimes. In the case of this soil, it appears that N₂O is more capable of serving as a nitrogen source than either TBP or TEP is as a phosphorus source, as the N₂O/PO₄ combination supports broader and more extensive removal of HMW species than do any conditions including either of the alkyphosphates.

Among the soils examined in this work, it is clear that the bacteria present in the oilfield soil were most adept at removing PAHs, including the higher-molecular-weight species. This is interesting because, although the soil in question has very high levels of total petroleum hydrocarbons (TPH of approximately 16.2%), the levels of PAHs are actually quite low. As can be seen in Table 1, the initial levels of total PAH in this soil were no higher than approximately 20 ppm. The superior ability of the microbial community in this soil to degrade PAH may therefore be the result of a long period of constant or recurring low-level exposure to these compounds.

It has been stated by other authors that the P contained in organophosphorus molecules such as TEP is not available to all microbes (4). In fact, the selective pressure exerted through the addition of TEP was looked upon as an advantage in the case of cometabolic remediation of TCE- and PCE-contaminated groundwaters by Type II methanotrophs, which are capable of utilizing TEP as a phosphorus source. Co-injection of TEP and methane into a VOC-contaminated aquifer thus caused up to 1000-fold increases in activities of these bacteria, while total bacterial biomass remained essentially unchanged (4, 21). Literature on the taxonomic distribution and extent of TEP/TBP utilization is limited. A mixed culture of *Pseudomonas* strains was capable of releasing PO₄ from TEP; this was examined for the removal (via precipitation of H₂UO₂PO₄) of UO₂²⁺ from uranium-contaminated mine wastewaters (26). Similarly, other *Pseudomonas* strains (22), as well as strains of *Hyphomicrobium* (10) and *Acinetobacter* (25) are known to be able to utilize TBP, TEP, and/or trimethylphosphate as sole sources of P.

Our results, however, imply that the ability to utilize TEP/TBP and N₂O, at least under slurry conditions, is not a universal attribute among PAH-degrading bacteria. The ability of alternative nutrient combinations to actually enhance PAH removal relative to

NH₄/PO₄-supplemented cultures seems to have been restricted to the MGP soil, and was, even in this case, seen only with lower-molecular-weight compounds.

It is possible that some of our results were due to the effects of solubility of TEP and TBP on their availability to PAH-degrading bacteria. As a consequence of its longer aliphatic groups, and resultant greater hydrophobicity, tributylphosphate is more resistant to solubilization than triethylphosphate; for example, we observed that the former tended to form persistent globules in the culture media, whereas the latter did not. It is, however, difficult to attribute these observations to solubility effects alone, as the same adaptations (e.g. lipid-rich outer cell layers, production of biosurfactants) which confer the capability to take up HMW PAHs upon bacteria such as *Mycobacterium* (3, 12) and *Sphingomonas* (3) would be expected to have the same effect on TBP. It is therefore possible that the increased removal of some PAHs which was occasionally seen with TBP (Tables 1 & 2), as well as the high mineralization of phenanthrene supported by both alkylphosphates in the MGP soil (Figure 2) may reflect the participation of some of these bacteria. These species may actually be favored by the use of more hydrophobic nutrient sources, both because of increased compatibility with their uptake systems and more favorable distribution within the microcosm. Hydrophobic nutrient sources such as TBP might be expected to partition onto soil organic matter, which might be beneficial to organisms such as those listed above, many of which tend to be adherent in nature (3). We have isolated several *Sphingomonas* strains from these two soils (Bogan et al, in press); further examination of these isolates' relative abilities to utilize these alternative nutrient sources may help to address these questions.

The diffusivity of TEP and TBP is approximately five orders of magnitude higher in the gaseous phase than when dissolved in water (4). Thus, distribution would be expected to improve somewhat in vadose soil (4, 21), and the issue of availability of P to soil-surface adherent should be less of a factor than it may have been in these preliminary experiments. The results of the experiments described here indicate that it may not always be feasible to support remediation of PAH-contaminated soils with gaseous sources of N and P, and that a great deal of site-specific variability can be expected. It is clear, however, that a true conclusion regarding the applicability of gaseous N and P supplements to in situ soil remediation will require soil-column experiments which will

better approximate the environmental conditions and behavior of bacteria in the vadose zone. It will also require a better understanding as to which members of microbial communities involved in PAH degradation thrive and function under the different regimes, and whether or not conditions can be devised to better optimize the performance of the entire community. Experiments in these areas are ongoing in this laboratory.

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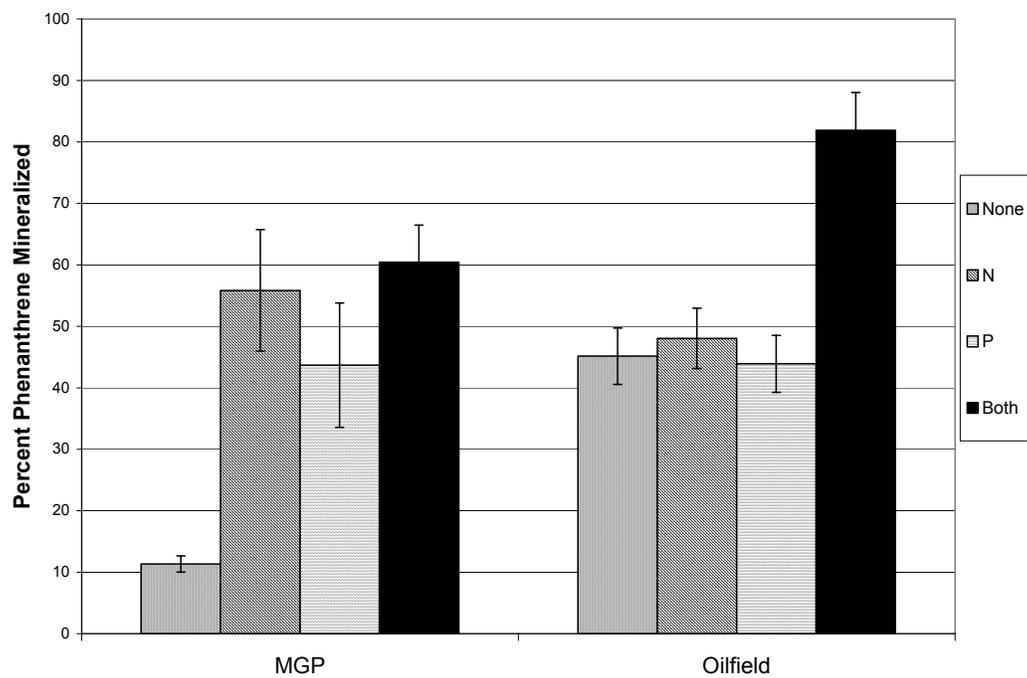
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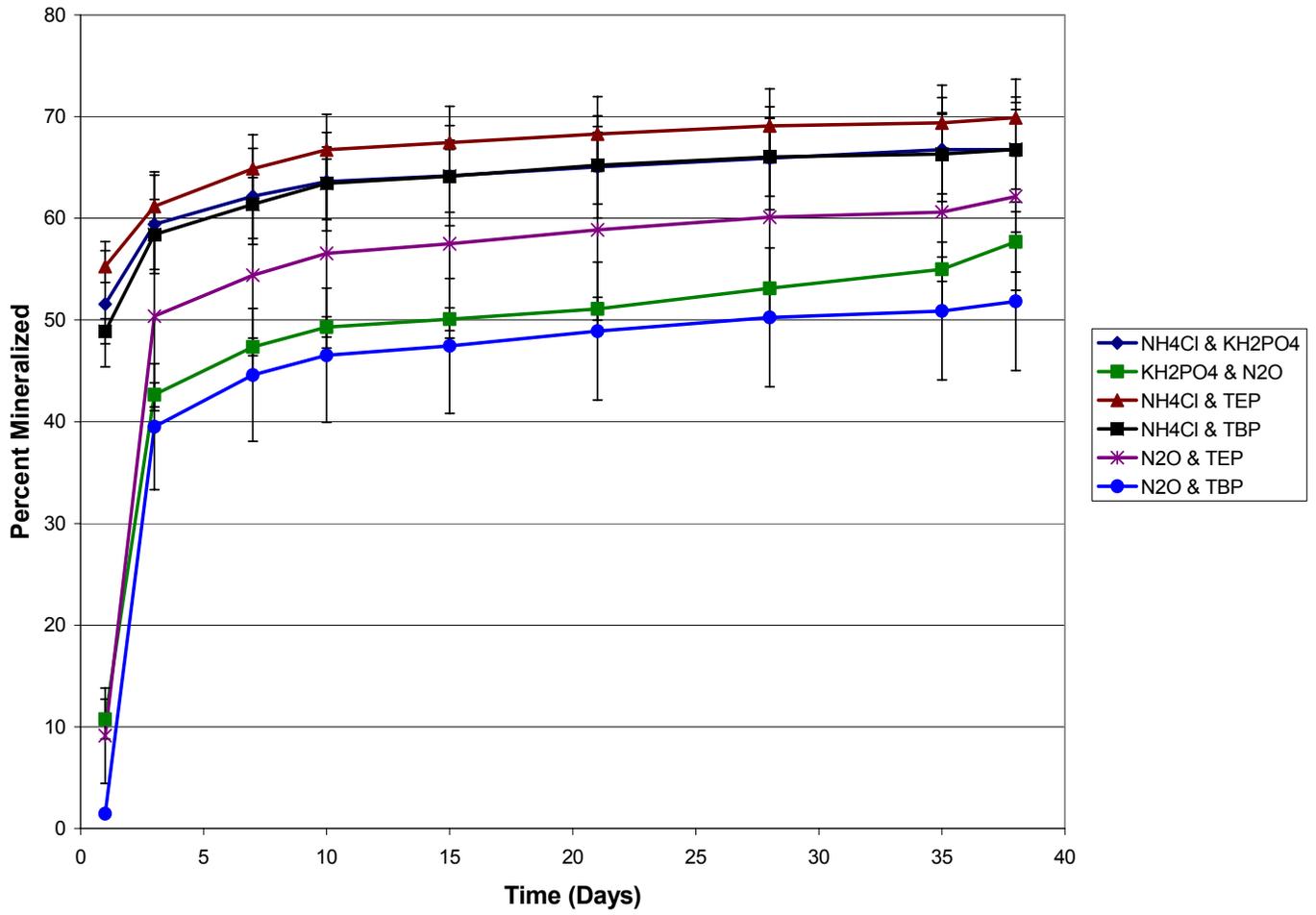
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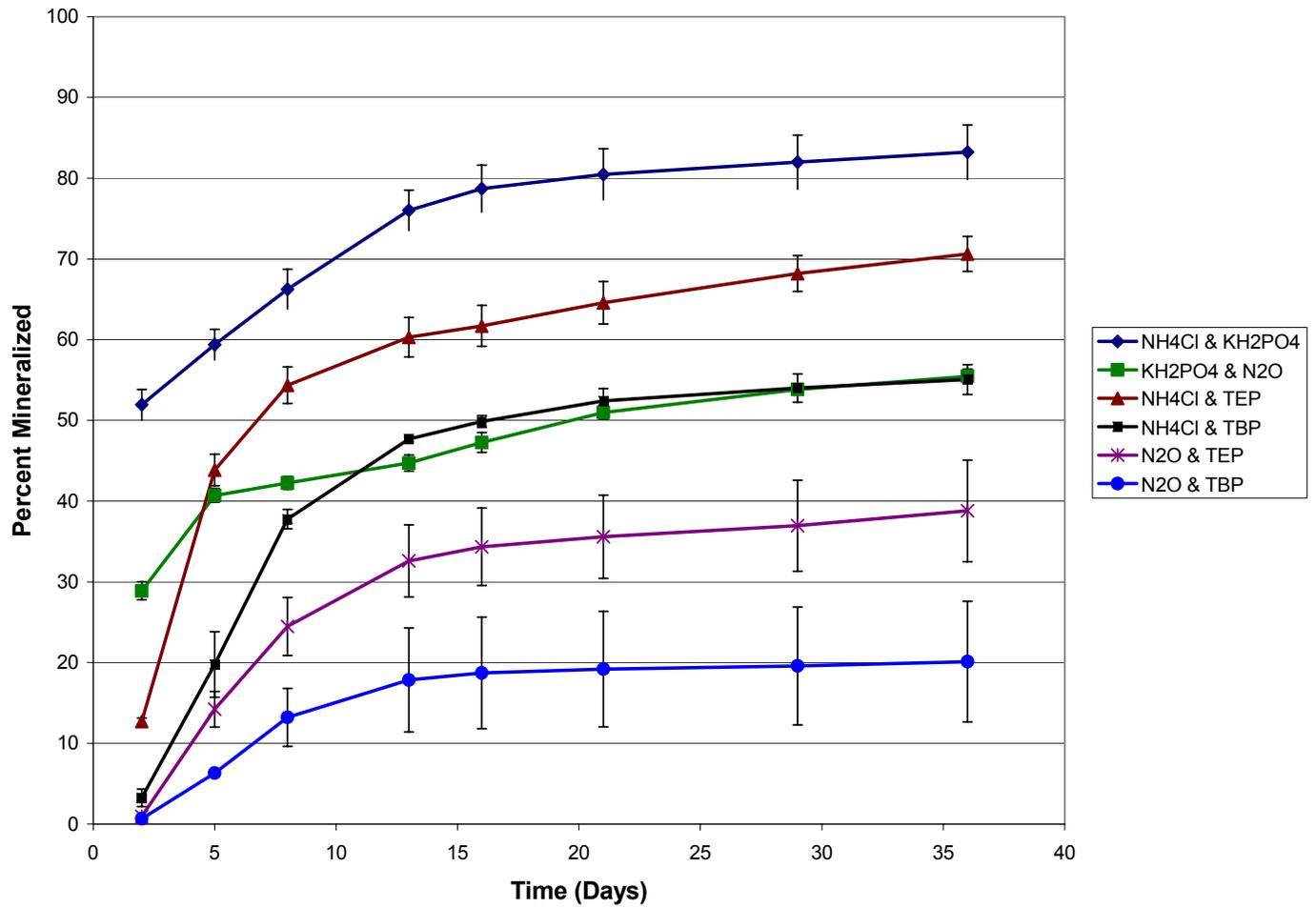


TABLE 1. Initial levels (parts per million) of PAH in the three soils incorporated in this study, as well as the DMF-solubilized extract of MGP soil #1 which was added as a supplemental source of available PAH.

	Soils		Supplemental Extract
	MGP #2	Oilfield	
Fluorene (FLU)	88	1.0	1172
Phenanthrene (PHE)	239	3.5	2140
Anthracene (ANT)	359	0.7	4494
Fluoranthene (FLA)	643	0.8	1102
Pyrene (PYR)	940	1.1	666
Benz[<i>a</i>]anthracene (BAA)	168	<0.4	377
Chrysene (CHR)	182	4.3	387
Benzo[<i>b</i>]fluoranthene (BBF)	82	<0.4	316
Benzo[<i>k</i>]fluoranthene (BKF)	51	1.2	247
Benzo[<i>a</i>]pyrene (BAP)	106	<0.5	277
Dibenz[<i>ah</i>]anthracene (DAA)	26	<0.4	147
Benzo[<i>ghi</i>]perylene (BGP)	14	<0.4	64
Indeno(123- <i>cd</i>)pyrene (IP)	20	<0.4	140

TABLE 2. Percent recovery (versus time-zero levels) of selected 3- to 5-ring PAHs from the MGP soil after 38 days of treatment under various conditions of N and P supplementation.

	<i>NH₄/PO₄</i>	<i>N₂O/PO₄</i>	<i>NH₄/TEP</i>	<i>NH₄/TBP</i>	<i>N₂O/TEP</i>	<i>N₂O/TBP</i>	<i>Killed</i>
FLU	<i>16 ± 0</i>	<i>36 ± 11</i>	<i>46 ± 6</i>	<i>12 ± 4</i>	<i>51 ± 13</i>	<i>51 ± 5</i>	<i>76 ± 6</i>
PHE	<i>24 ± 2</i>	<i>35 ± 2</i>	<i>52 ± 4</i>	<i>7 ± 2</i>	<i>45 ± 8</i>	<i>36 ± 1</i>	<i>107 ± 1</i>
ANT	<i>25 ± 6</i>	<i>45 ± 7</i>	<i>44 ± 3</i>	<i>6 ± 0</i>	<i>51 ± 7</i>	<i>102 ± 8</i>	<i>124 ± 4</i>
FLA	<i>63 ± 5</i>	<i>109 ± 12</i>	<i>118 ± 5</i>	<i>67 ± 13</i>	<i>111 ± 14</i>	<i>131 ± 9</i>	<i>112 ± 4</i>
PYR	<i>86 ± 15</i>	<i>116 ± 5</i>	<i>137 ± 8</i>	<i>104 ± 21</i>	<i>128 ± 11</i>	<i>132 ± 15</i>	<i>118 ± 4</i>
BAA	<i>90 ± 11</i>	<i>108 ± 7</i>	<i>140 ± 7</i>	<i>120 ± 24</i>	<i>128 ± 7</i>	<i>123 ± 6</i>	<i>105 ± 4</i>
CHR	<i>90 ± 10</i>	<i>143 ± 14</i>	<i>140 ± 11</i>	<i>122 ± 21</i>	<i>128 ± 3</i>	<i>115 ± 12</i>	<i>97 ± 2</i>
BBF	<i>99 ± 15</i>	<i>95 ± 12</i>	<i>162 ± 15</i>	<i>144 ± 26</i>	<i>143 ± 9</i>	<i>145 ± 8</i>	<i>135 ± 20</i>
BKF	<i>68 ± 12</i>	<i>125 ± 5</i>	<i>105 ± 10</i>	<i>94 ± 18</i>	<i>101 ± 4</i>	<i>97 ± 10</i>	<i>85 ± 10</i>
BAP	<i>86 ± 13</i>	<i>132 ± 13</i>	<i>149 ± 14</i>	<i>124 ± 28</i>	<i>128 ± 4</i>	<i>121 ± 5</i>	<i>105 ± 8</i>

TABLE 3. Percent recovery (versus time-zero levels) of selected 3- to 5-ring PAHs from the oilfield soil after 38 days of treatment under various conditions of N and P supplementation.

	NH_4/PO_4	N_2O/PO_4	NH_4/TEP	NH_4/TBP	N_2O/TEP	N_2O/TBP	Killed
FLU	0 ± 0	3 ± 2	0 ± 0	5 ± 1	2 ± 1	5 ± 4	95 ± 8
PHE	0 ± 0	1 ± 0	1 ± 0	1 ± 1	1 ± 1	2 ± 2	97 ± 12
ANT	0 ± 0	1 ± 1	0 ± 0	1 ± 0	3 ± 1	1 ± 1	72 ± 7
FLA	2 ± 1	4 ± 1	12 ± 1	43 ± 4	58 ± 4	43 ± 13	101 ± 10
PYR	6 ± 5	35 ± 9	104 ± 5	71 ± 7	132 ± 14	99 ± 29	100 ± 13
BAA	7 ± 4	37 ± 13	36 ± 1	74 ± 12	85 ± 5	82 ± 6	74 ± 7
CHR	14 ± 9	12 ± 2	34 ± 2	82 ± 11	110 ± 20	102 ± 14	108 ± 11
BBF	17 ± 7	72 ± 9	90 ± 4	87 ± 18	104 ± 6	92 ± 15	68 ± 7
BKF	7 ± 4	48 ± 13	99 ± 10	104 ± 14	107 ± 15	107 ± 14	93 ± 10
BAP	27 ± 19	76 ± 15	90 ± 8	72 ± 11	86 ± 10	87 ± 16	73 ± 14
DAA	58 ± 43	108 ± 38	154 ± 22	65 ± 20	79 ± 20	98 ± 17	72 ± 14

Figure 1 – Extent of ^{14}C -phenanthrene mineralization (during a total incubation time of 5 weeks) in each soil in the absence of N or P supplementation, or when NH_4Cl or KH_2PO_4 were added either singly or in combination.

Figure 2 – Mineralization of phenanthrene and pyrene by the microbial community associated with MGP soil #2 under various conditions of N and P supplementation.

Figure 3 – Mineralization of phenanthrene and pyrene by the microbial community associated with the crude oil-contaminated oilfield soil under various conditions of N and P supplementation.