DOE/BC/15223-1
(OSTI ID: 808526)

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

SEMI-ANNUAL REPORT

(MAY 2001 THRU OCTOBER 2001)

Prepared by

J.R. Paterek
W.W. Bogan
L.M. Lahner
V. Trbovic

GAS TECHNOLOGY INSTITUTE
1700 South Mount Prospect Road
Des Plaines, Illinois 60018
IGT Project 30722

ALL TASKS

For

DEPARTMENT OF ENERGY
and

GAS TECHNOLOGY INSTITUTE

DOE Project Manager
Nancy Comstock

DOE Contract No. DE-AC26-99BC15223

FERC Project Manager
Vipul Srivastava

December 2001

gti

TABLE OF CONTENTS

DISCLAIMER

EXECUTIVE SUMMARY

The results presented within are a summary of research activities from May 2001 until the end of October 2001 between the Gas Technology Institute (GTI), formerly known as Institute of Gas Technology, Department of Energy (DOE), and Federal Energy Regulatory Commission (FERC) funds managed by the Gas Research Institute (now a component of GTI). This report incorporates the data presented not in the previous semiannual reports. Data continues to indicate that Chemically Accelerated Biotreatment (CAB) processes are effective for the restoration of Manufactured Gas Plant (MGP) site wastes and petroleum/natural gas exploration and production (E&P) sites. We developed and applied a number of modifications of bioavailability screens procedure to evaluate the application and efficiency of CAB using risk-based analysis, along with traditional analytical chemistry methods (SW 846, for example), for endpoint determinations. These results will be applied to the development of environmentally acceptable endpoints (EAE) as the indicator of CAB treatment effectiveness. CAB tests focused on issues associated with the *in-situ* application mode.

During this final 6 months of the second project year, research was conducted in five major focus areas:

- Development of mild extraction approaches to estimate bioavailable fraction of crude oil residues in contaminated soils.
- Application of these methods to understand decreases in toxicity and increases in sequestration of hydrocarbons over time, as well as the influence of soil properties on these processes.
- Measurements of the abilities of various bacteria (PAH-degraders and others more representative of typical soil bacteria) to withstand oxidative treatments (*i.e*. Fenton's reaction) which would occur in CBT.
- Experiments into the biochemical/genetic inducibility of PAH degradation by compounds formed by the chemical oxidation of PAH.

RESEARCH SUMMARY

Title	Application of chemically accelerated biotreatment to reduce risk in oil-impacted soils
Contractors	U.S. Department of Energy DOE Contract No. DE-AC26-99BC15223 Gas Technology Institute/Gas Research Institute GRI Contract No. 8054
Principal Investigator	J. Robert Paterek
Report Period	May 2001 through October 2001
Objective	The overall program objective is to develop and evaluate integrated biological/physical/chemical co-treatment strategies for the remediation of wastes associated with the exploration and production of fossil energy. The specific objectives of this project are: chemical accelerated biotreatment (CAB) technology development for enhanced site remediation, application of the risk based analyses to define and support the rationale for environmental acceptable endpoints (EAE) for exploration and production wastes, and evaluate both the technological technologies in conjugation for effective remediation of hydrocarbon contaminated soils from E&P sites in the USA.
Technical Approach	This project is part of the program of IGT/GRI in an effort to develop and enhance the microbiological approach to the degradation of wastes present at natural gas/petroleum production and exploration sites. It is of primary importance to this program to develop the capability to biologically degrade all the mobile or available fraction of the toxic or hazardous hydrocarbons present at these sites, including the "recalcitrant" aromatic moieties, which include those compounds containing elements other than carbon in the rings. To achieve this goal, research efforts focuses on enhancing the growth of microbial cultures with capabilities for biodegrading these compounds, aiding microbial activities by sequential or concurrent chemical oxidation of recalcitrant hydrocarbon moieties, determining the mobility and toxicity of the various hydrocarbon components in E&P wastes, in the conducting parametric studies to maximize detoxification of soils to meet "environmentally acceptable endpoints," and providing scientific and engineering supports for future application of *in-situ*, landfarming, and bioslurry reactor treatment technologies. Research efforts under the GRI project focused on evaluating the performance of selected cultures under a variety of bioremediation conditions in simulated "real world" and field conditions found at representative E&P sites.

Activities Experiments were conducted during this reporting period to extend the application of solvents extractions to model bioavailability of hydrocarbon contaminants in E&P and MGP soils.

EXPERIMENTAL

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 which 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 N_2 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 N_2 stream) had no significant effect on either the TPH content of the soil, or its acute toxicity (Microtox EC_{50}); 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.

Ability of Individual Bacterial Strains to Withstand Fenton's Reaction

Individual bacterial isolates were grown up on R2A agar plates, and suspensions made such that each had an optical density (A_{600}) of 0.1. This was done to ensure that, to the greatest extent possible, equal numbers of all bacteria were initially added to reactions. Aqueous-phase Fenton's reactions, to which bacteria were added, were run as follows: Reactions (40 ml total volume) contained 100 µl of bacterial suspension, 10 mM (final concentration) $FeSO_4$, and 10 mg H_2O_2, and were run in mineral salts medium which had previously been adjusted to pH 3. Samples of reaction mixtures were taken at

time-zero (immediately prior to addition of $FeSO_4$ and H_2O_2) and 30 minutes after the initiation of the reaction; these were diluted in minimal medium and plated on R2A. Bacterial colonies were counted after 3-5 days (longer in the case of *Mycobacterium* species, which tended to require 10-20 days to establish countable colonies).

Inducibility of PAH Degradation

Given that induction of PAH degradation (both low- and high-molecular-weight compounds) has been shown to occur in several species through exposure to Salicylate, we began by screening ten of our previously-isolated strains for this phenotype. Isolates were pre-grown on R2A agar plates, and were then inoculated into culture tubes containing 30 ml of LB medium. These tubes then received 50 μM quantities of either salicylate (as the sodium salt) or glucose (control cultures). After 24 hours of growth, all cultures were pelleted by centrifugation, rinsed once in clean LB medium, and adjusted to the same optical density (A_{600} = 0.3). Phenanthrene mineralization cultures were inoculated using 100 μL of this material per culture; these were set up in 125-ml serum bottles which, as has been previously described, contained NaOH-filled glass vials for the collection of $^{14}CO_2$. Each culture contained *ca*. 20 mg of non-labeled phenanthrene, in addition to ~38,000 dpm of ^{14}C-phenanthrene. Release of $^{14}CO_2$ was assessed after 6 and 20 hours.

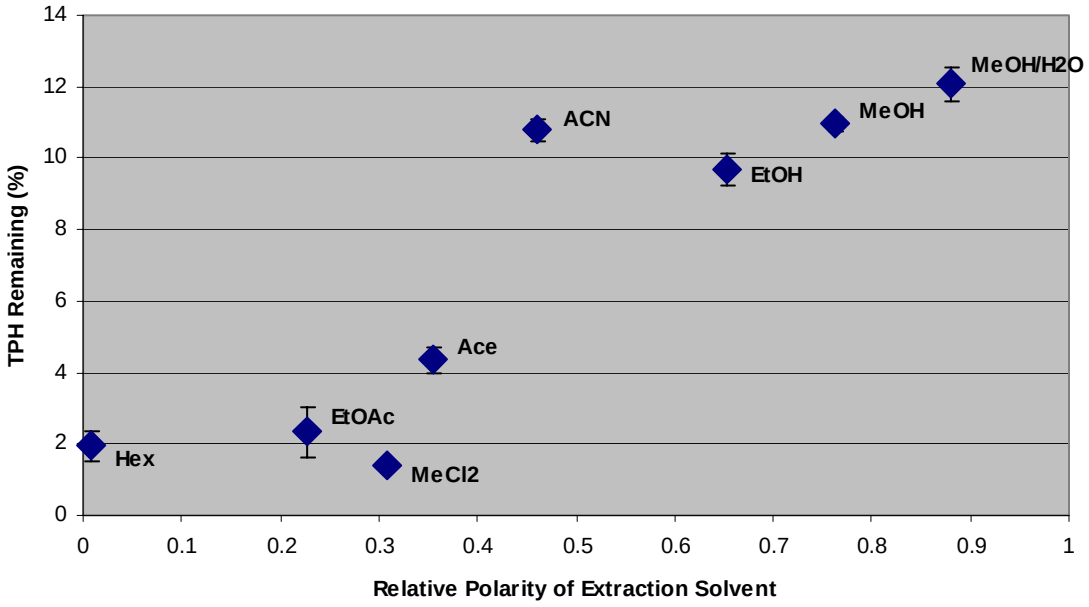
Water-soluble products of Fenton's reagent oxidation of coal tar were generated as follows: Coal tar was coated onto fine-grained sand at a rate of 2.4% (wt/wt basis). 20 grams of this material (500 mg of tar) was added to 250 ml of water, which was adjusted to a pH of 2.8-3.2 with H_2SO_4, followed by addition of $FeSO_4$ (final concentration = 1.6 mM). A dilute stock of H_2O_2 (final concentration = 1.7%) was then added dropwise (30 ml over a total of *ca.* 6 hours). The reaction was then allowed to proceed in the dark, with stirring, for a total of approximately 18 hours. At the conclusion of the reaction, the mixture was neutralized, filtered, and rinsed with 250 ml of water. The combined aqueous phases were then acidified to a pH of approximately 1, and extracted twice with equal volumes (500 ml) of ethyl acetate. This was then evaporated to dryness under N_2, yielding a total of *ca*. 30 mg of oxidized products.

A second experiment was then conducted in which these products were added to LB cultures of various PAH-degrading bacteria (at a concentration of 0.3 mg per 30-ml culture); these bacteria were then collected and washed as before, and examined for possible induction of phenanthrene mineralization (as compared to salicylate-induced cultures and glucose-only controls). Other controls were included in which cultures were supplemented with water-soluble organic products of non-oxidized coal tar, and of clean (*i.e*. non-coal tar-coated) sand treated with Fenton's reagent.

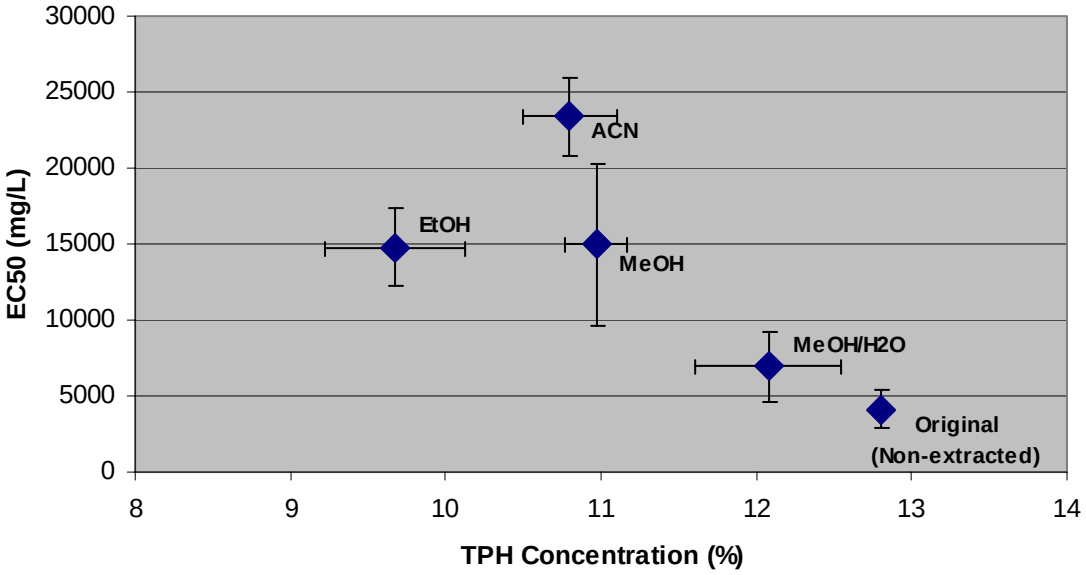
RESULTS

Development of Extraction Methods to Mimic Bioavailability

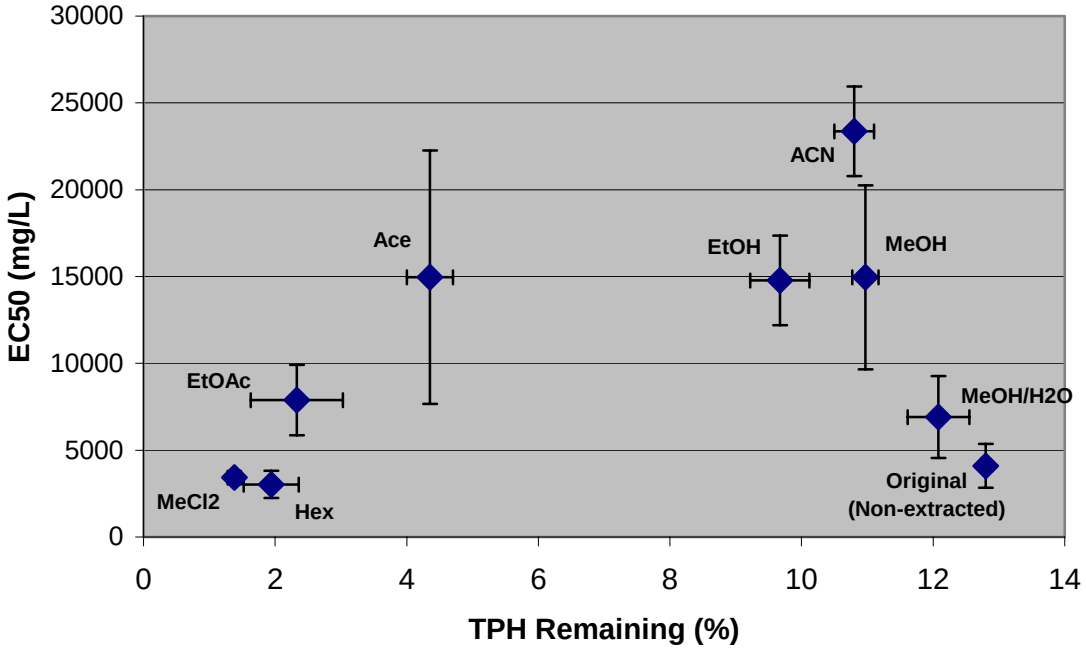
The extent to which TPH was extracted from the soil was, as expected, strongly dependent on the polarity of the solvent, as shown below.



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/H_2O) showed a very good correlation between decreasing amounts of TPH remaining and decreases in acute toxicity:

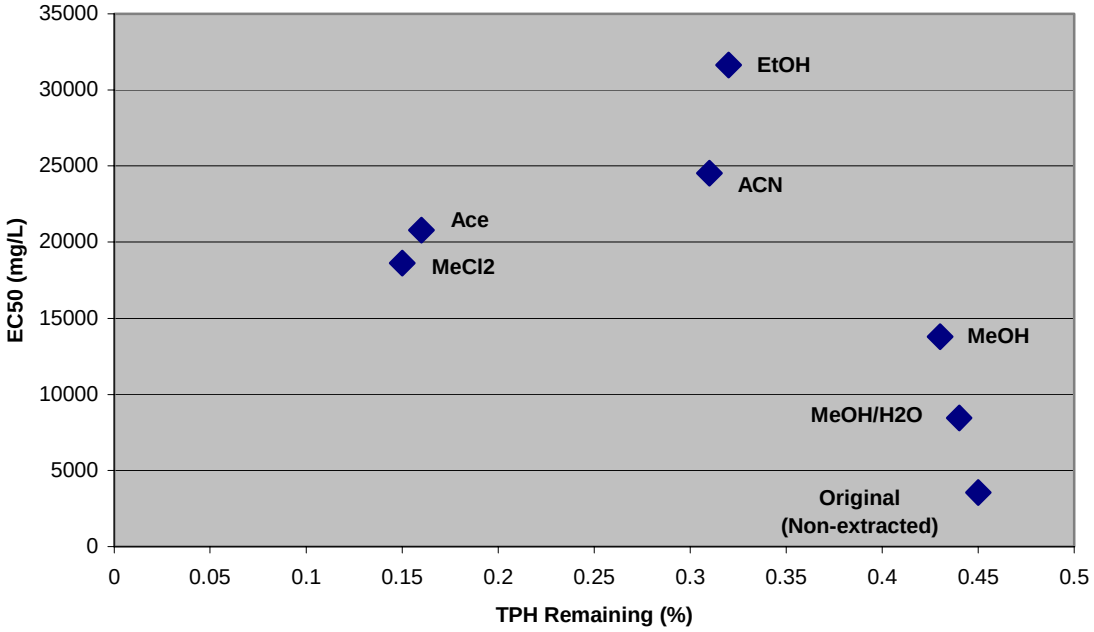


However, when the full range of solvents was included, the observed correlation broke down for those four solvents which were of lowest polarity:



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 EC_{50} values) as the polarity of the solvent decreased. The EC_{50} 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 N_2 after extraction, and removal of this solvent may also have been incomplete. In fact, this EC_{50} 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.

Another observation from the above data was that 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 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 has 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, while 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 ≈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 which was capable of removing the majority of (apparently) bioavailable TPH, with minimal apparent 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):

Crude oil conc. (initial)	**Pre-extraction**		**Post-extraction**	
	TPH conc. (%)	**EC50 (mg/L)**	**TPH conc. (%)**	**EC50 (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:

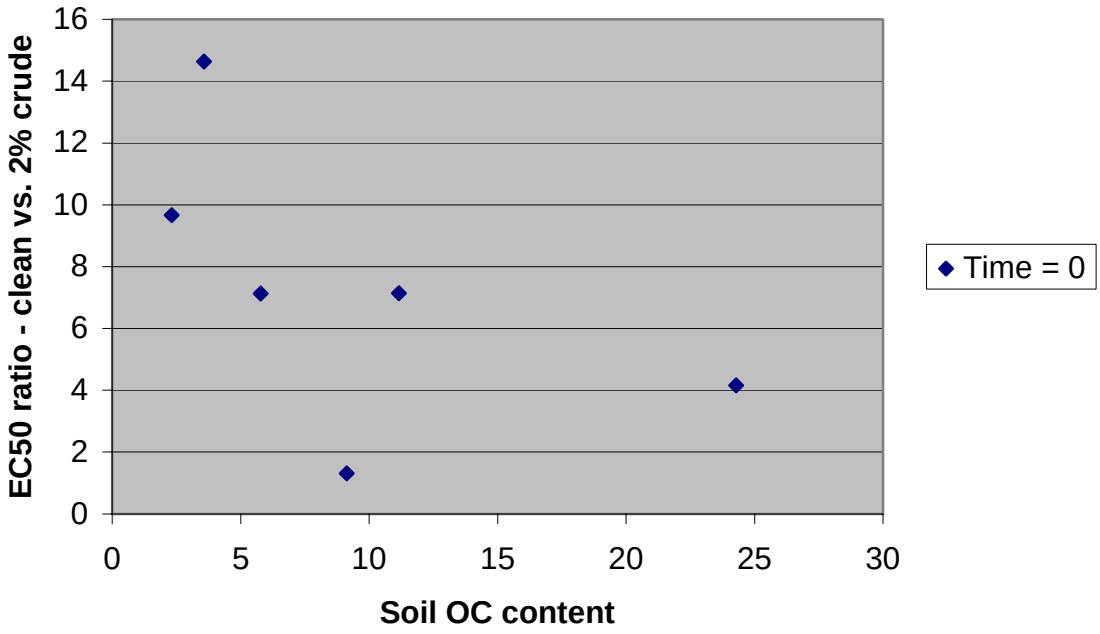
Soil #	% Sand	% Silt	% Clay	Textural classification	OC (%)
1	25.4	51.1	27.5	Clay loam-Loam	2.32
2	64.0	25.3	10.7	Sandy loam	5.78
3	70.8	18.1	11.1	Sandy loam	11.16
4	73.5	10.6	15.9	Sandy loam	24.28
5	40.6	33.0	26.4	Loam	3.58
6	51.8	26.3	21.9	Sandy clay loam	9.13

More detailed characterization of the soils is in progress, and will include parameters which other authors have linked to the sequestration of hydrophobic organics; included among these will be the soils' relative amounts of humic acids, fulvic acids, and humin, as well as the relative polarities of these fractions, and their aromatic/aliphatic content. In the interest of determining the last of these parameters, GTI has recently contracted the Western Research Institute (Laramie, WY) to conduct solid-state ^{13}C-NMR examination of the six model soils, and expects to have this data within the next few weeks. Soil particle micropore volumes will also be 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 datapoint for this experiment (12 hours after spiking of oil) is currently available; data thus far are as follows:

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):



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 datapoints (over the span of months) will be taken in order to demonstrate sequestration and/or toxicity reductions over time, and to further assess their dependence on soil composition. Also,

this experiment will also be conducted with "aged" oil (extracted from MV AB), which does not contain any appreciable BTEX fraction; this will be done to better understand the behavior of the aliphatic fraction of the oil taken independently.

Ability of Individual Bacterial Strains to Withstand Fenton's Reaction

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

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

Inducibility of PAH Degradation

Ten strains (5 *Sphingomonas*, 3 *Pseudomonas*, one each of *Burkholderia* and *Acidovorax*) were tested for induction of phenanthrene mineralization by salicylate. Results (dpm of $^{14}CO_2$ generated after 6 hours) indicated that there was evidence for higher phenanthrene mineralization following exposure to salicylate in three of these isolates:

	Control (Glucose)	**Salicylate-induced**	**R (S/G)**
Acidovorax	3851 ± 310	3151 ± 400	0.82 ± 0.17
Burkholderia 1	288 ± 131	138 ± 42	0.48 ± 0.36
Pseudomonas 1	273 ± 63	209 ± 0	0.77 ± 0.18
Pseudomonas 2	105 ± 1	188 ± 54	1.79 ± 0.53
Pseudomonas 5	379 ± 197	172 ± 30	0.45 ± 0.32
Sphingomonas 1	149 ± 18	143 ± 15	0.96 ± 0.22
Sphingomonas 2	204 ± 7	391 ± 44	1.92 ± 0.28
Sphingomonas 3	180 ± 12	319 ± 89	1.77 ± 0.61
Sphingomonas 5	197 ± 18	251 ± 72	1.27 ± 0.48
Sphingomonas 12	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 in 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 which 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 which 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 PAHs for which induction of degradation (mineralization) can occur.

REFERENCES CITED

Alley, J.F. and L.R. Brown. 2000. Use of Sublimation to Prepare Solid Microbial Media with Water-insoluble Substrates. Applied and Environmental Microbiology **66:**439-442.

Beltran, F.J., M. Gonzalez, F.J. Rivas, and P. Alvarez. 1998. Fenton Reagent Advanced Oxidation of Polynuclear Aromatic Hydrocarbons in Water. Water, Air, and Soil Pollution **105:**685-700.

Bigda, R.J. 1996. Fenton's Chemistry: An Effective Advanced Oxidation Process. Environmental Technology (May/June):34-39.

Davies, K.G. and R. Whitbread. 1989. *In Vitro* Studies of Siderophore Production by Wild Type and Rifampicin Resistant strains of Fluorescent Pseudomonads. Plant and Soil **116:**123-125.

Gorden, R.W., T.C. Hazen, and C.B. Fliermans. 1993. Rapid Screening for Bacteria Capable of Degrading Toxic Organic Compounds. Journal of Microbiological Methods **18:**339-347.

Lundanes, E. and T. Greibrokk. 1994. "Separation of fuels, heavy fractions, and crude oils into compound classes: A review." J. High Ressolut. Chromatogr. 17: 197-202.

Sheldrick, B.H. and C. Wang. 1993. Particle Size Distribution. Pages 499-512 in M.R. Carter (ed), Soil Sampling and Methods of Analysis. Lewis Publishers, Boca Raton.

APPENDIX – PUBLICATIONS AND PRESENTATIONS

Paper Published in Proceedings of 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

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:

- hydrocarbons associated with the natural gas condensates, such as benzene, toluene, ethyl benzene, and the xylenes which are commonly designated as BTEX;
- polynuclear aromatic hydrocarbons (PAHs) from the formation's natural gas deposits (liquid or solids under the conditions of pressure and temperature in many reservoirs);
- PAHs associated with the drilling materials;

- fuel oil and diesel fuel that is often a component 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 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 (CO_2), inorganic salts, biomass, and water. A schematic illustration of the CAB Process is presented in the Figure 1.

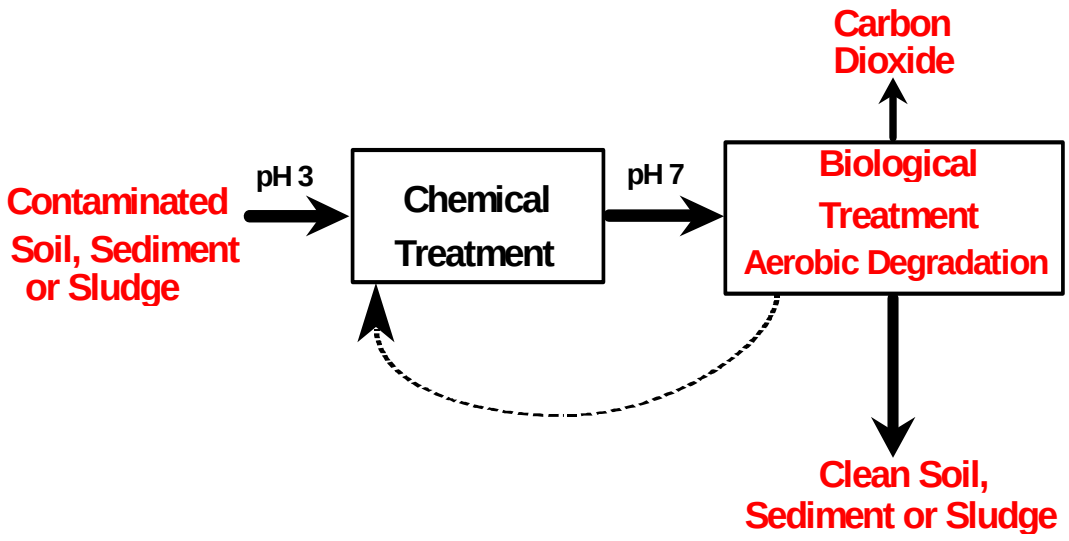


Figure 1: Schematic diagram of the CAB process

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:

- 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 (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 that 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 reseach 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 $mg \cdot l^{-1}$ biotin, 2 $mg \cdot l^{-1}$ folic acid, 10 $mg \cdot l^{-1}$ pyridoxine HCl, 5 $mg \cdot l^{-1}$thiamine HCl, 5 $mg \cdot l^{-1}$ riboflavin, 5 $mg \cdot l^{-1}$ nicotinic acid, 5 $mg \cdot l^{-1}$ pantothenic acid, 0.1 $mg \cdot l^{-1}$ cyanocobalamine, 5 $mg \cdot l^{-1}$ *p*-aminobenzoic acid, 5 $mg \cdot l^{-1}$ thioctic acid), 0.1ml Trace Minerals (100 $mg \cdot l^{-1}$ $ZnSO_4$, 300 $mg \cdot l^{-1}$ H_3BO_3, 300 $mg \cdot l^{-1}$ CoCl, 10 $mg \cdot l^{-1}$ CuCl) and 0.8 ml N- & P-free (pH 7.2) Winogradsky medium (62.5 $g \cdot l^{-1}$ $MgSO_4 \cdot 7H_2O$, 31.25 $g \cdot l^{-1}$ NaCl, 1.25 $g \cdot l^{-1}$ $FeSO_4$, 1.25 $g \cdot l^{-1}$ $MnSO_4$) per 100 ml of sterile deionized water) in 125-ml serum bottles. Cultures were incubated at room temperature (approx. 25 ºC), with constant shaking at 170 rpm.

In order to assess the degree of N- and P-limitation on PAH degradation inherent in each soil, ^{14}C-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 NH_4Cl), P only (as KH_2PO_4), or both N and P. 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- 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: NH_4Cl/KH_2PO_4; N_2O/KH_2PO_4; NH_4Cl/TEP; NH_4Cl/TBP; N_2O/TEP and N_2O/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 N_2O 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 EC_{50} 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 $^{14}CO_2$ 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.

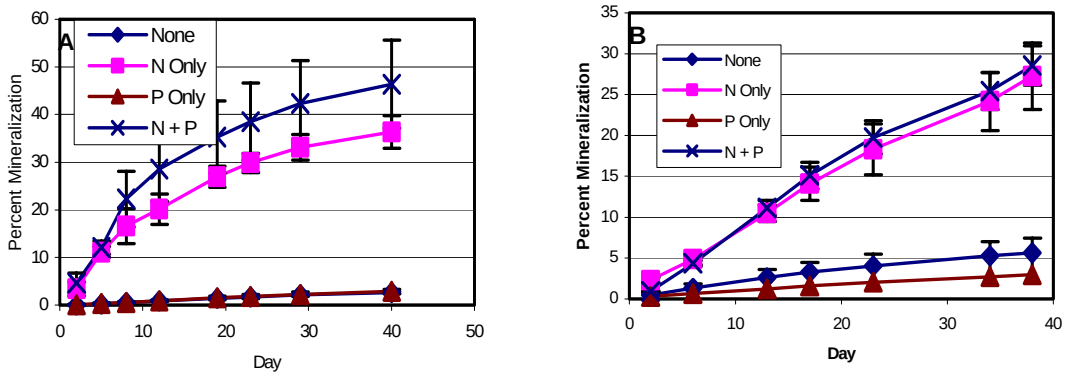


Figure 2 – Effects of N and P addition on mineralization of hexadecane by microbes indigenous to oil-contaminated E&P soils. Graph A = Soil AB; Graph B = Soil C

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.

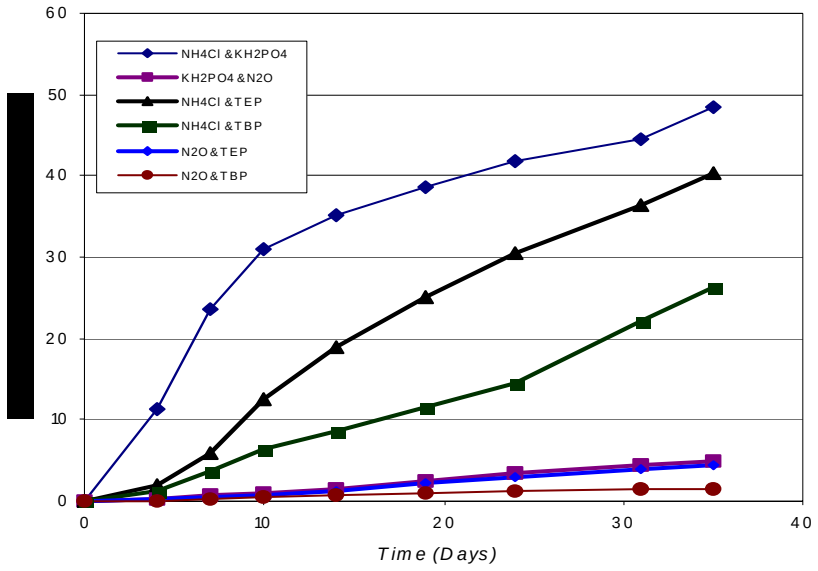


Figure 3 – Ability of gaseous N and P compounds to overcome nutrient limitations and support hexadecane mineralization by indigenous microbes in soil AB.

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 KH_2PO_4. TBP supported considerably less hexadecane mineralization, and use of N_2O 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 there 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®.

Acknowedgements

We would like to acknowledge the support of both the Department of Energy, National Petroleum Technology Office and the Federal Energy Regulatory Without.Commission program managed by the GRI division of Gas Technology Institute. Without their support and participation, this work would have not been possible

References

Harkey, G.A., S. Pradhan, J. Conrad, R. Paterek, and V. Srivastava. (1997). Toxicity changes associated with phytoremediation at Manufactured Gas Plant sites. Abstract, SETAC 18th Annual Meeting, Society of Environmental Toxicology and Chemistry, San Francisco, CA, 16-20 November, 1997.

Paterek, J. R., B. N. Aronstein, L. E. Rice, V. J. Srivastava, J. Chini, and A. Bohrnerud. 1994. "Bioremediation of Soil of a Manufactured Gas Plant Utilizing an *In-situ* Treatment Mode," *in* Seventh International IGT Symposium on Gas, Oil, and Environmental Biotechnology, Colorado Springs, CO. Institute of Gas Technology, Des Plaines, IL. p. 63-75.

Paterek, J. R., B. N. Aronstein, S. Patel, R. L. Kelley, and V. J. Srivastava, T. D. Hayes and D. G. Linz. "Degradation of Polychlorinated Biphenyls Utilizing Sequential Chemical and Microbiological Treatments (IGT's PCB-REM Process); Contaminated Aqueous and Soil Systems," Paper presented at IGT's Sixth International Symposium on Gas, Oil, and Environmental Biotechnology, November 29-December 1, 1993, Colorado Springs, CO.

Srivastava, V. J., R. L. Kelley, J. R. Paterek, T. D. Hayes, G. L. Nelson, and J. Golchin. (1994). "A Field-Scale Demonstration of a Novel Bioremediation Process for MGP Sites." *Appl. Biochem. Biotechnol*. 45/46: 741-755.

Submitted for Publication to *Archives of Microbiology*

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

Bill W. Bogan*, Lisa M. Lahner, and J. Robert Paterek

Gas Technology Institute

1700 South Mount Prospect Rd.

Des Plaines, IL 60018

*Corresponding Author:

FAX: 847-768-0669

e-mail: bill.bogan@gastechnology.org

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 $MgSO_4 \cdot 7H_2O$, 0.05 g $FeCl_3 \cdot 6H_2O$, 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 µl of MSM and 5 µl 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 µl 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 ~ 0.5) with concentrated H_2SO_4, 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 N_2. 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 × 1.5 μm, which is in good agreement with the dimensions (0.5 μm × 2-6 μm) 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 $^{14}CO_2$.

No release of $^{14}CO_2$ 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 $^{14}CO_2$ liberation in the mineralization experiment.

When inoculated into pyrene-spiked soil, GTI-23 was capable of extensively mineralizing ^{14}C-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 $^{14}CO_2$ 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

This work was supported by Research Contract #DE-AC26-99BC15223 from the United States Department of Energy, by Contracts #8054 and #8471 from the Gas Research Institute.

References

Alley JF, Brown LR (2000) Use of sublimation to prepare solid microbial media with water-insoluble substrates. Appl Environ Microbiol 66:439-442.

Arvin E, Jensen B, Godsy EM, Grbić-Galić D (1988) Microbial degradation of oil and creosote related aromatic compounds under aerobic and anaerobic conditions. In: Wu YC (ed) Physicochemical and biological detoxification of hazardous wastes. Technomic Publishing, Lancaster, pp. 828-847.

Bastiaens L, Springael D, Wattiau P, Harms H, deWachter R, Verachtert H, Diels L (2000) Isolation of adherent polycyclic aromatic hydrocarbon (PAH)-degrading bacteria using PAH-sorbing carriers. Appl Environ Microbiol 66:1834-1843.

Berekaa MM, Steinbüchel A (2000) Microbial degradation of the multiply branched alkane 2,6,10,15,19,23-hexamethyltetracosane (squalane) by *Mycobacterium fortuitum* and *Mycobacterium ratisbonense*. Appl Environ Microbiol 66:4462-4467.

Bogan BW, Lahner LM, Trbovic V, Szajkovics AM, Paterek JR (2001) Effects of alkylphosphates and nitrous oxide on microbial degradation of polycyclic aromatic hydrocarbons. Appl Environ Microbiol 67:2139-2144.

Bogan BW, Lamar RT, Hammel KE (1996) Fluorene oxidation in vivo by *Phanerochaete chrysosporium* and in vitro during manganese peroxidase-dependent lipid peroxidation. Appl Environ Microbiol 62:1788-92.

Boldrin B, Tiehm A, Fritzsche C (1993) Degradation of phenanthrene, fluorene, fluoranthene, and pyrene by a *Mycobacterium* sp. Appl Environ Microbiol 59:1927-1930.

Bouchez M, Blanchet D, Vandecasteele JP (1997) An interfacial uptake mechanism for the degradation of pyrene by a *Rhodococcus* strain. Microbiology 143:1087-1093.

Bouchez-Naïtali M, Rakatozafy H, Marchal R, Leveau JY, Vandecasteele JP (1999) Diversity of bacterial strains degrading hexadecane in relation to the mode of substrate uptake. J Appl Microbiol 86:421-428.

Caldini G, Cenci G, Maneti R, Morozzi G (1995) The Ability of an environmental isolate of *Pseudomonas fluorescens* to utilize chrysene and other four-ring polynuclear aromatic hydrocarbons. Appl Microbiol Biotechnol 44:225-229.

Casellas M, Grifoll M, Bayona JM, Solanas AM (1997) New metabolites in the degradation of fluorene by *Arthrobacter* sp. strain F101. Appl Environ Microbiol 63:819-826.

Cerniglia CE (1992) Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3:351-368.

Chen SH, Aitken MD (1999) Salicylate stimulates the degradation of high-molecular weight polycyclic aromatic hydrocarbons by *Pseudomonas saccharophila* P15. Environ Sci Technol 33:435-439.

Churchill SA, Harper JP, Churchill PF (1999) Isolation and characterization of a *Mycobacterium* species capable of degrading three- and four-ring aromatic and aliphatic hydrocarbons. Appl Environ Microbiol 65:549-552.

Grifoll M, Selifonov SA, Chapman PJ (1994) Evidence for a novel pathway in the degradation of fluorene by *Pseudomonas* sp. strain F274. Appl Environ Microbiol 60:2438-2449.

Heitkamp MA, Franklin W, Cerniglia CE (1988a) Microbial metabolism of polycyclic aromatic hydrocarbons: isolation and characterization of a pyrene-degrading bacterium. Appl Environ Microbiol 54:2549-2555.

Heitkamp MA, Freeman JP, Miller DW, Cerniglia CE (1988b) Pyrene degradation by a *Mycobacterium* sp.: identification of ring oxidation and ring fission products. Appl Environ Microbiol 54:2556-2565.

Juhasz AL, Britz ML, Stanley GA (1997) Degradation of fluoranthene, pyrene, benz[*a*]anthracene and dibenz[*a,h*]anthracene by *Burkholderia cepacia*. J Appl Microbiol 83:189-198.

Kelley I, Cerniglia CE (1995) Degradation of a mixture of high-molecular-weight polycyclic aromatic hydrocarbons by a *Mycobacterium* Strain PYR-1. J Soil Contam 4:77-91.

Lloyd-Jones G, Hunter DWF (1997) Characterization of fluoranthene- and pyrene-degrading *Mycobacterium*-like strains by RAPD and SSU sequencing. FEMS Microbiol Lett 153:51-56.

Miller RM, Singer GM, Rosen JD, Bartha R. 1988. Photolysis primes biodegradation of benzo[*a*]pyrene. Appl Environ Microbiol 54:1724-1730.

Mueller JG, Chapman PJ, Blattmann BO, Pritchard PH (1990) Isolation and characterization of a fluoranthene-utilizing strain of *Pseudomonas paucimobilis*. Appl Environ Microbiol 56:1079-1086.

Mueller JG, Chapman PJ, Pritchard PH (1989) Creosote-contaminated sites: their potential for bioremediation. Environ Sci Technol 23:1197-1201.

Nestler FHM (1974) The characterization of wood-preserving creosote by physical and chemical methods of analysis. USDA Forest Service Research Paper FPL 195. USDA Forest Service Forest Products Laboratory, Madison, WI.

Nishioka M, Chang HC, Lee ML (1986) Structural characteristics of polycyclic aromatic hydrocarbon isomers in coal tars and combustion products. Environ Sci Technol 20:1023-1027.

Rafii F, Butler WR, Cerniglia CE (1992) Differentiation of a rapidly growing, scotochromogenic, polycyclic-aromatic-hydrocarbon-metabolizing strain of *Mycobacterium* sp. from other known *Mycobacterium* species. Arch Microbiol 157:512-520.

Rehmann K, Noll HP, Steinberg CE, Kettrup AA (1998) Pyrene degradation by *Mycobacterium* sp. strain KR2. Chemosphere 36:2977-2992.

Rhodes EO (1951) History of changes in chemical composition of creosote. Proc Amer Wood-Pres Assoc 47:40-61.

Schneider J, Grosser R, Jayasimhulu K, Xue W, Warshawsky D (1996) Degradation of pyrene, benz[*a*]anthracene, and benzo[*a*]pyrene by *Mycobacterium* sp. strain RJGII-135, isolated from a former coal gasification site. Appl Environ Microbiol 62:13-19.

Smucker SJ 2000. Region 9 Preliminary Remediation Goals – R9 PRG Tables. www.epa.gov/docs/region09/waste/sfund/prg/files/PRG2000.pdf USEPA Region 9 Office, San Francisco.

Sneath PHA, Mair NS, Sharpe ME, Holt JG (eds) (1986) Bergey's Manual of Systematic Bacteriology – Volume 2. Williams & Wilkins, Baltimore.

Solano-Serena F, Marchal R, Casarégola S, Vasnier C, Lebeault JM, Vandecasteele JP (2000) A *Mycobacterium* strain with extended capacities for degradation of gasoline hydrocarbons. Appl Environ Microbiol 66:2392-2399.

Sutherland JB, Rafii F, Khan AA, Cerniglia CE (1995) Mechanisms of polycyclic aromatic hydrocarbon degradation. In: Young LY, Cerniglia CE (eds) Microbial transformation and degradation of toxic organic chemicals. Wiley-Liss, New York, pp. 269-306.

Van Dyke MI, Prosser JI (2000) Enhanced survival of *Pseudomonas fluorescens* in soil following establishment of inoculum in a sterile soil carrier. Soil Biol Biochem 32:1377-1382.

Vila J, Lopez Z, Sabate J, Minguillon C, Solanas AM, Grifoll M (2001) Identification of a novel metabolite in the degradation of pyrene by *Mycobacterium* sp. strain AP1: actions of the isolate on two-and three-ring polycyclic aromatic hydrocarbons. Appl Environ Microbiol 67:5497-5505.

Walter U, Beyer M, Klein J, Rehm HJ (1991) Degradation of pyrene by *Rhodococcus* sp. UW1. Appl Microbiol Biotechnol 34:671-676.

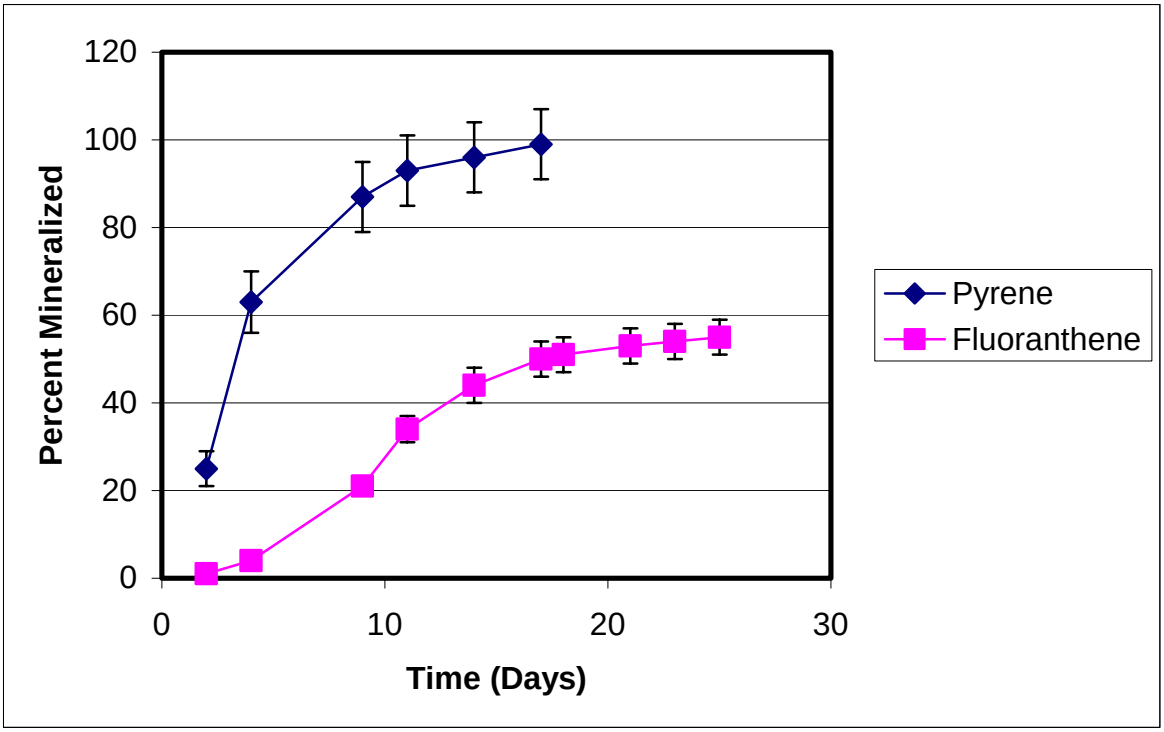
Weissenfels WD, Beyer M, Klein J, Rehm HJ (1991) Microbial metabolism of fluoranthene: isolation and identification of ring-fission products. Appl Microbiol Biotechnol 34:528-535.

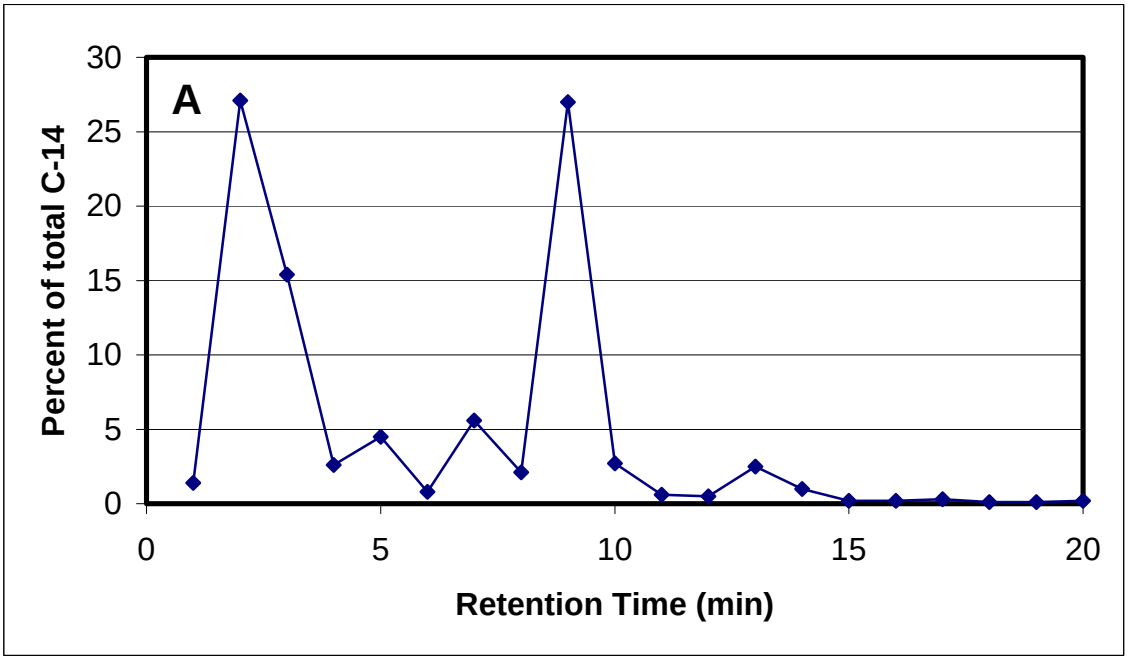
Component	% of Total
14:0	3.20
15:0	0.97
16:1 w9c	1.70
16:1 w6c	13.78
16:0	34.63
8-Me-16:0 + 10-Me-16:0	1.09
17:1 w8c	0.87
17:0	0.60
18:2 w6,9c	0.51
18:1 w9c	26.94
18:0	2.66
10-Me18:0	12.39
20:0	0.67

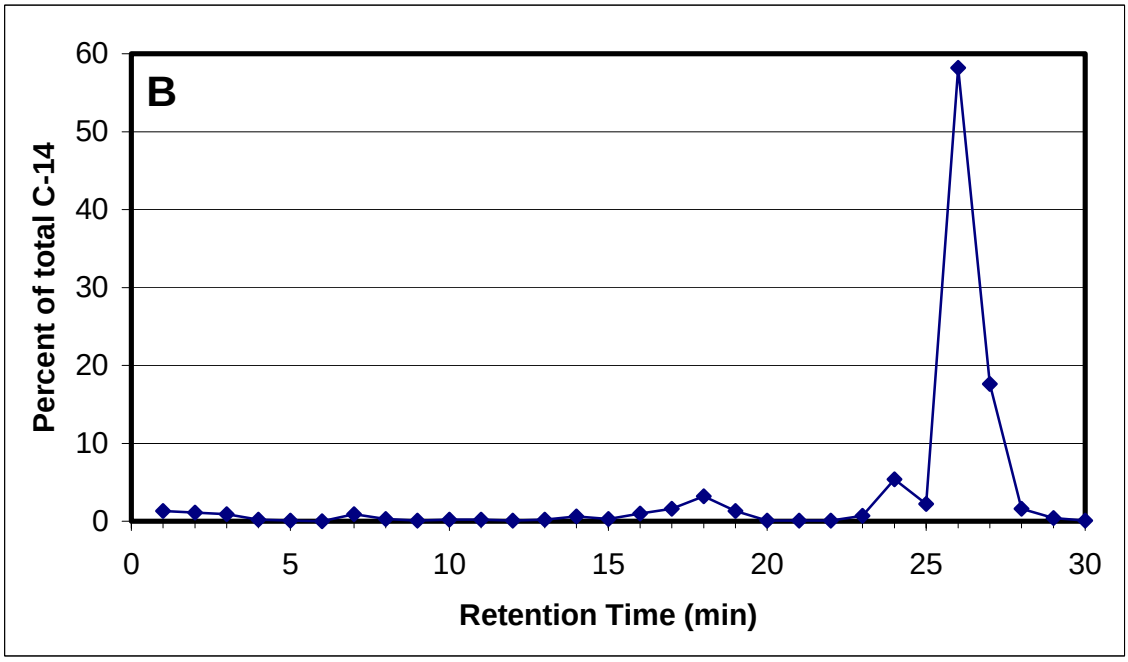
Table 1 – Fatty acid profile of *Mycobacterium austroafricanum* strain GTI-23.

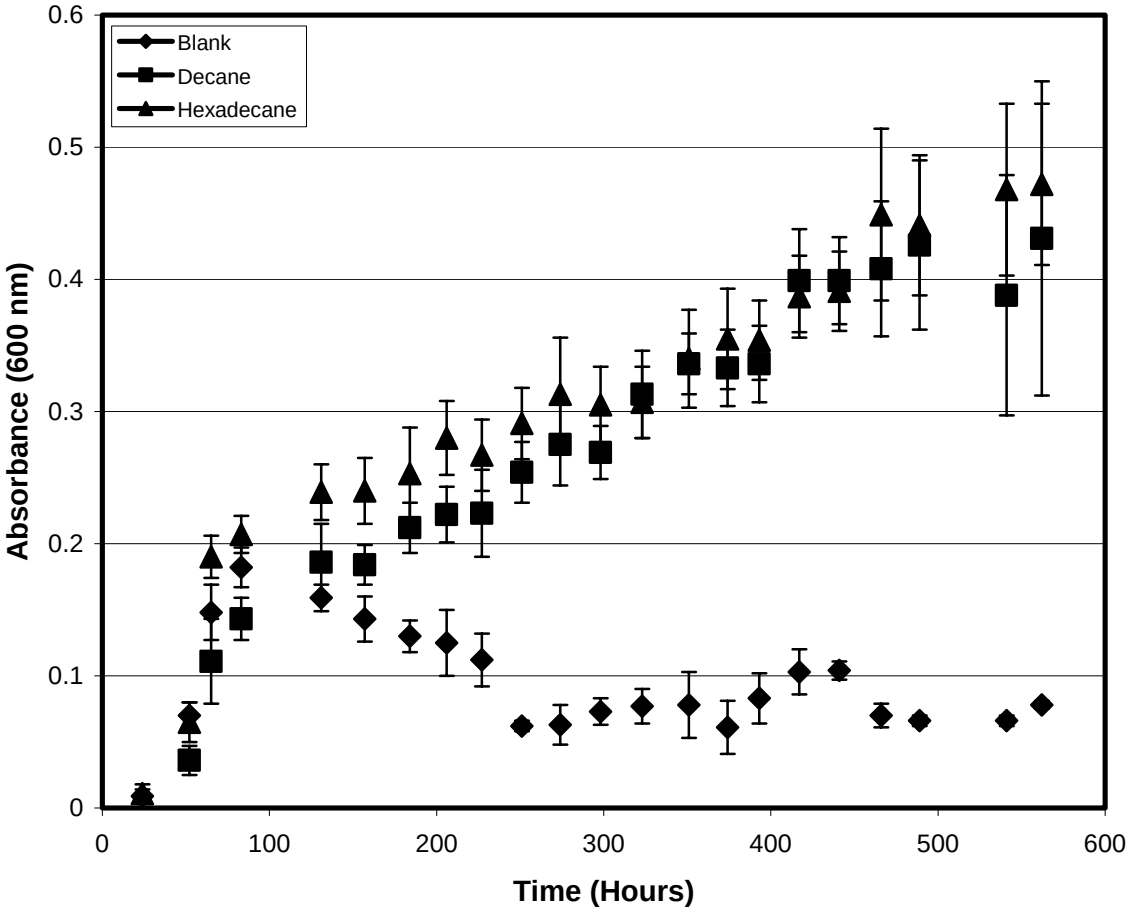
Isolate	Medium	Culture Age	Hydrophobicity
Acidovorax temperans GTI-19	MSM/phenanthrene	1 day	3%
Burkholderia sp. GTI-3	MSM/phenanthrene	6 days	15%
Pseudomonas viridiflava GTI-5	MSM/phenanthrene	6 days	4%
Sphingomonas sp. GTI-7	MSM/phenanthrene	2 days	15%
Sphingomonas sp. GTI-8	MSM/phenanthrene	6 days	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%

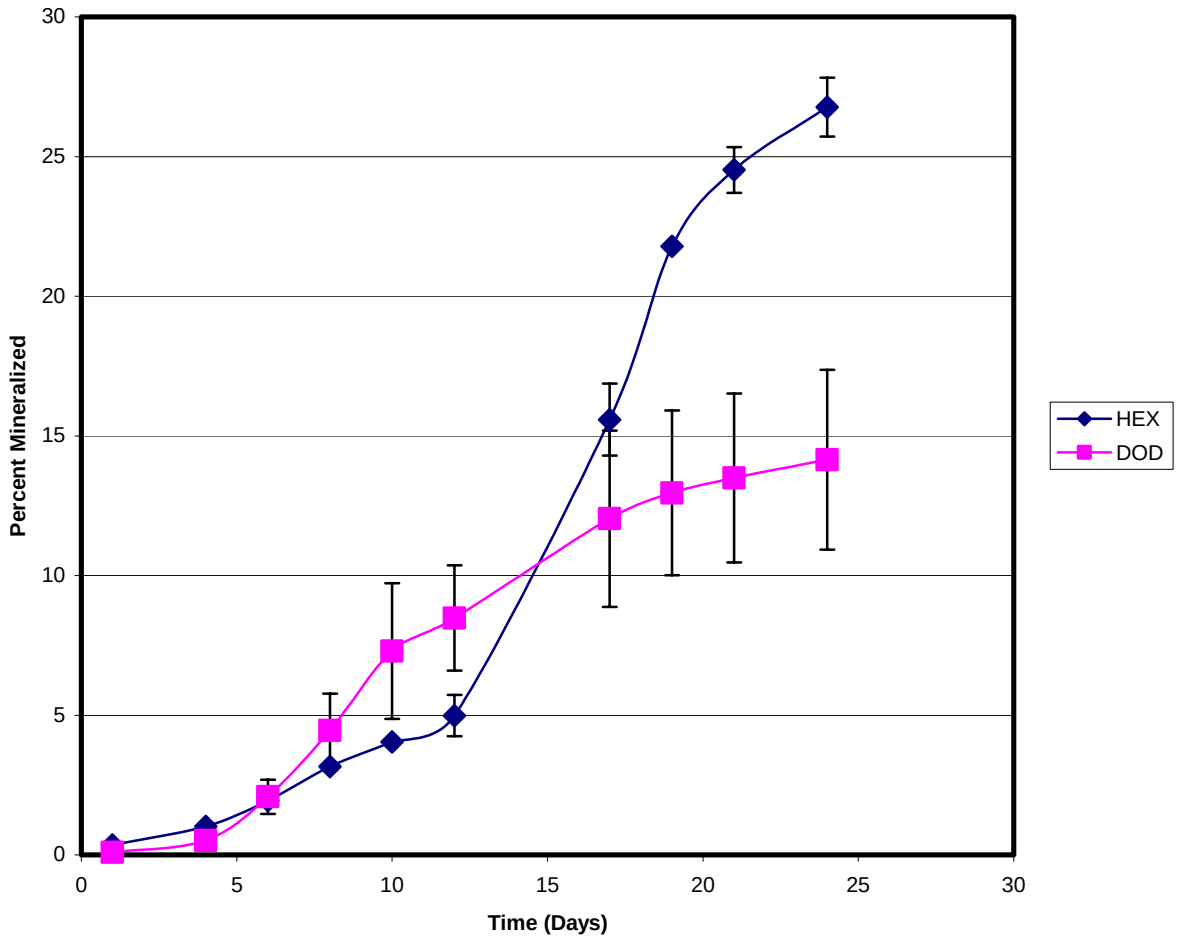
Table 2 – Hydrophobicity of *M. austroafricanum* GTI-23, in comparison with various other PAH-degrading isolates.


120
100
80
60
40
20
0
Percent Mineralized
0
10
20
30
Time (Days)
Pyrene
Fluoranthene


A
Percent of total C-14
30
25
20
15
10
5
0
0
5
10
15
20
Retention Time (min)


B
Percent of total C-14
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Retention Time (min)


Blank
Decane
Hexadecane
Absorbance (600 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
100
200
300
400
500
600
Time (Hours)


30
25
20
15
10
5
0
Percent Mineralized
0
5
10
15
20
25
30
Time (Days)
HEX
DOD

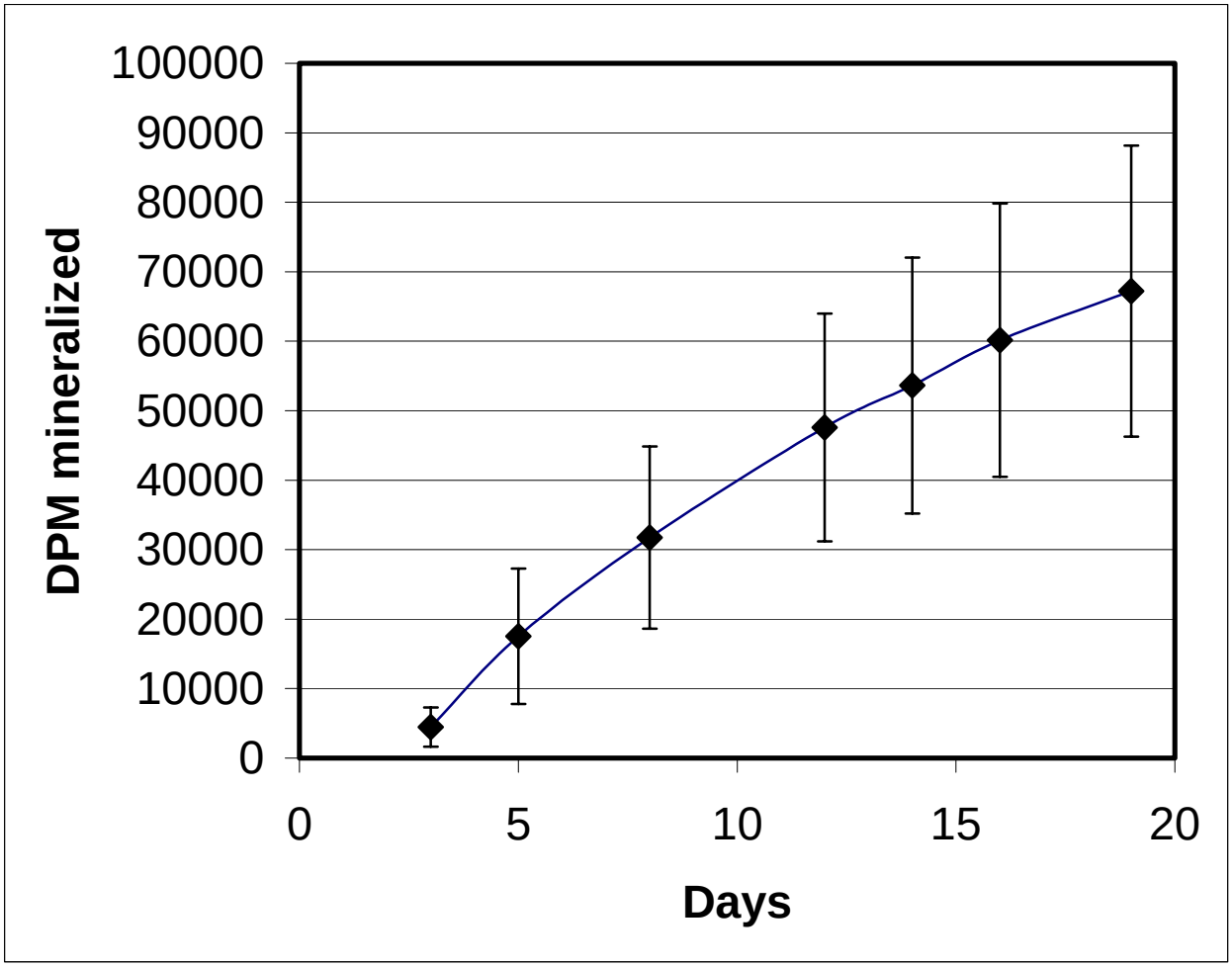

DPM mineralized
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
0
5
10
15
20
Days

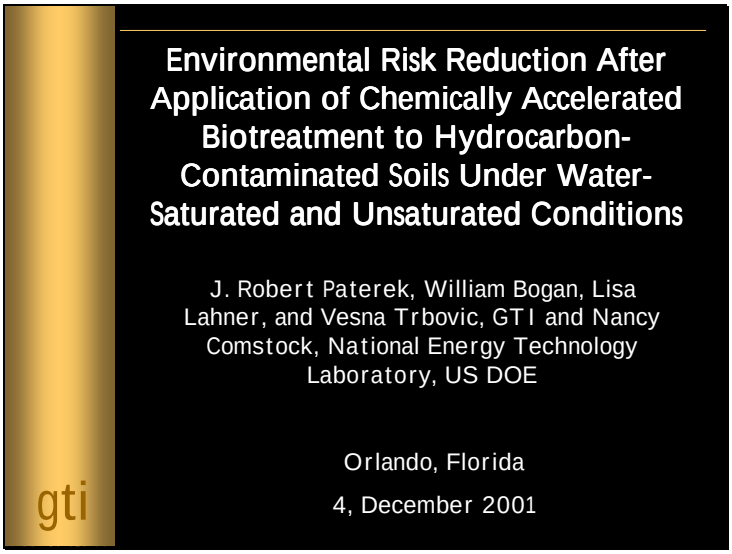
Figure 1 – Mineralization of fluoranthene and pyrene (Initial = 20 mg/50 ml) by *Mycobacterium austroafricanum* GTI-23 grown in YPS medium. Data are means of duplicate cultures, with 95% confidence limits.

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

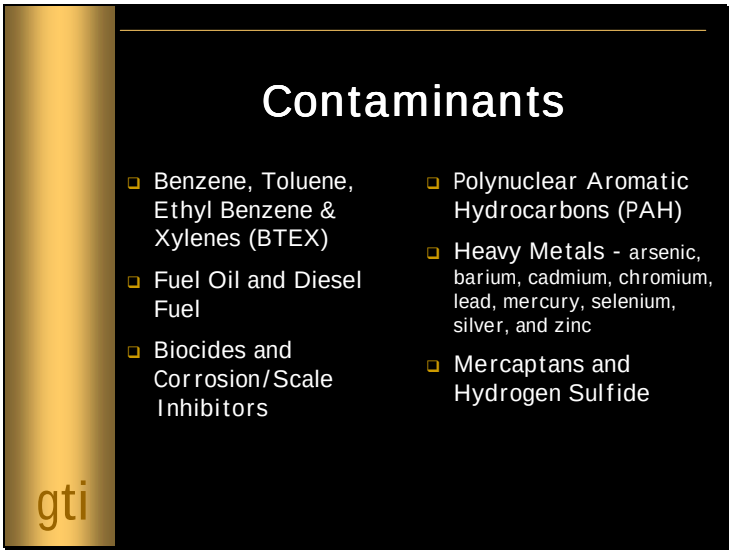
Figure 3 – Growth of *M. austroafricanum* GTI-23 in MSM supplemented with either decane or hexadecane. Data are means of triplicate cultures, with 95% confidence limits.

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

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

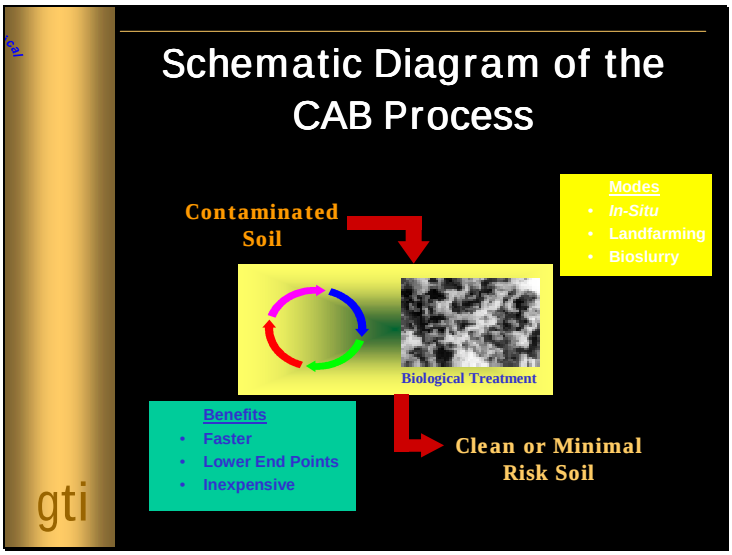

Environmental Risk Reduction After Application of Chemically Accelerated Biotreatment to Hydrocarbon-Contaminated Soils Under Water-Saturated and Unsaturated Conditions
J. Robert Paterek, William Bogan, Lisa Lahner, and Vesna Trbovic, GTI and Nancy Comstock, National Energy Technology Laboratory, US DOE
Orlando, Florida
4, December 2001
gti

Slide 2


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
gti

Slide 3

Slide 4



Slide 5

Environmental Acceptable Endpoints (EAE)

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

gti

Slide 6

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

gti

Slide 7

Consortium Engineering

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

- K. Watanabe

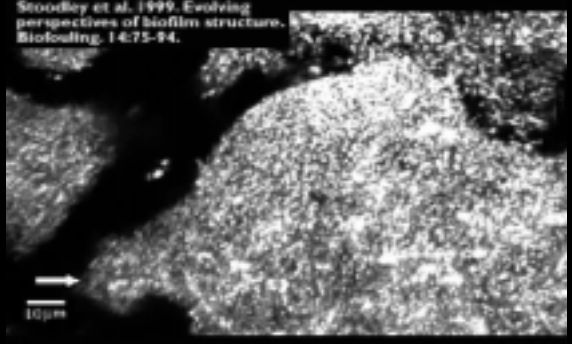
Basics of Consortium Engineering

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

gti

Slide 8

Microbial Consortia Are Complex in Space and Time



gti

Slide 9

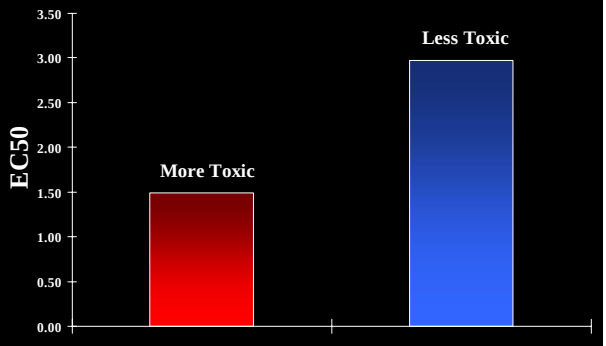
Consortium Engineering

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

gti

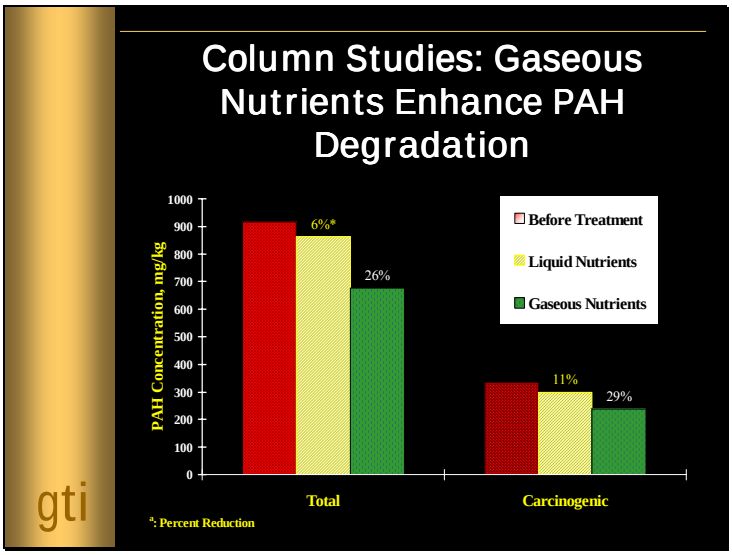
Slide 10

Column Studies: Gaseous Nutrients Reduce Toxicity

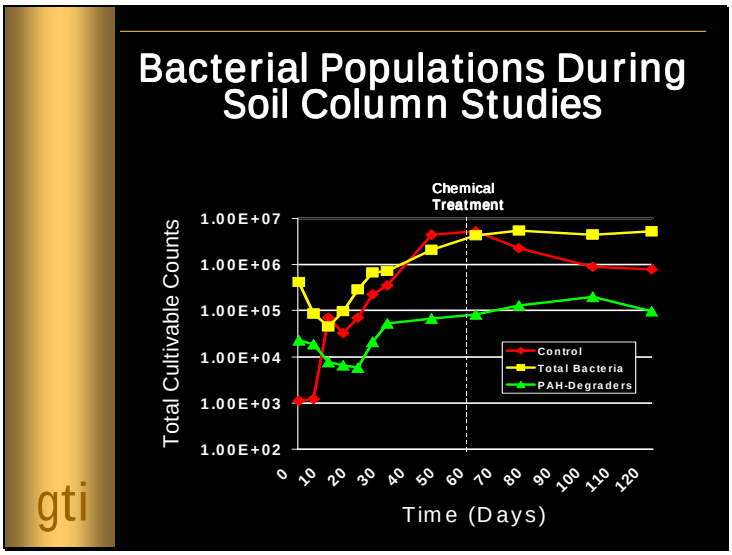


gti

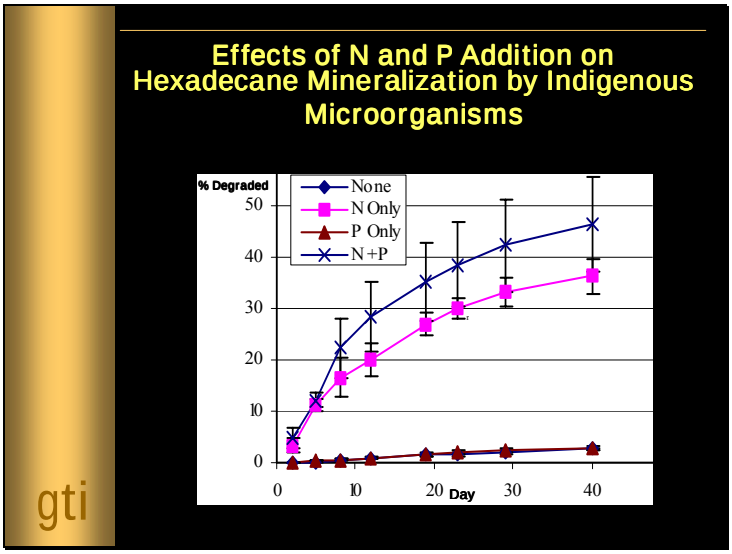
Slide 11



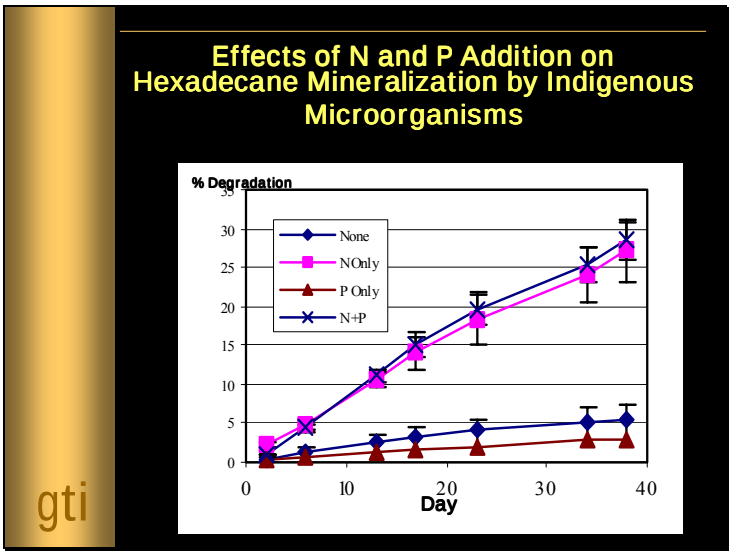
Slide 12



Slide 13

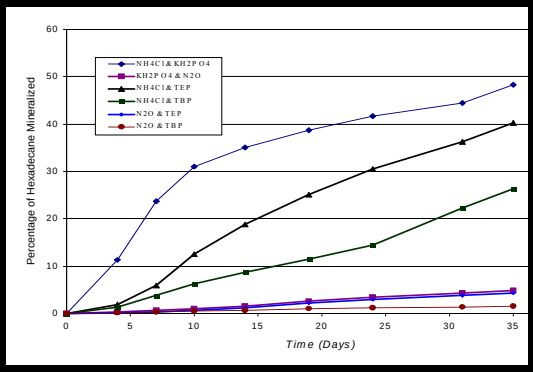


Slide 14



Slide 15

Ability of Gaseous N and P Compounds to Overcome Nutrient Limitations and Support Hexadecane Mineralization



gti

Slide 16

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

gti

Slide 17

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

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

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

gti

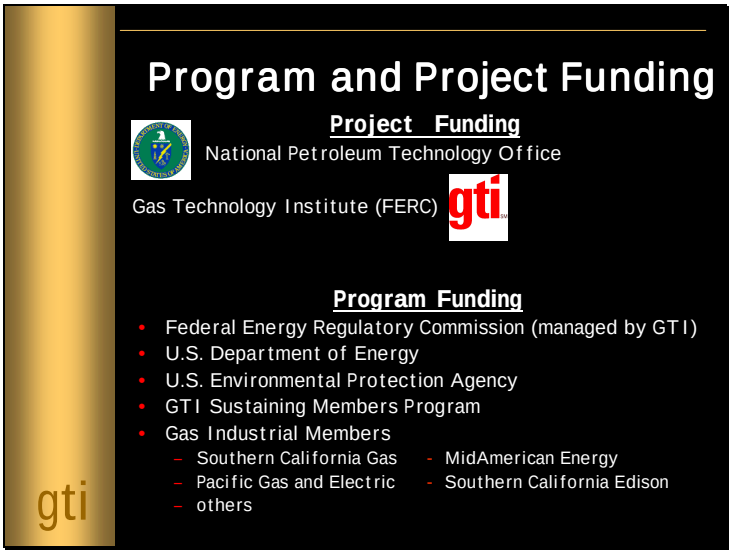
Slide 18

Inducibility of PAH Degradation

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

gti

Slide 19


Program and Project Funding
Project Funding
National Petroleum Technology Office
Gas Technology Institute (FERC)
gti
Program Funding
Federal Energy Regulatory Commission (managed by GTI)
U.S. Department of Energy
U.S. Environmental Protection Agency
GTI Sustaining Members Program
Gas Industrial Members
Southern California Gas
Pacific Gas and Electric
others
MidAmerican Energy
Southern California Edison
gti