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

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EXECUTIVE SUMMARY

The results presented within are a summary of research activities from November 2000 until the end of April 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 first semiannual report or first annual report. Chemically Accelerated Biotreatment (CAB) processes have been proven successful for the treatment of Manufactured Gas Plant (MGP) site wastes; therefore the technology is being investigated for application to wastes associated with petroleum/natural gas exploration and production (E&P) sites. In addition, the application of CAB is being evaluated using risk-based analysis, along with traditional analytical chemistry testing, for endpoint determinations. These results will be applied to the development of environmentally acceptable endpoints (EAE) in comparison to strictly analytical assessments.

During this first 6 months of the second year, work was conducted in five major areas of the project:

- Determining the nutrient limitations which constrain the abilities of indigenous bacteria in two crude oil-contaminated soils to degrade and mineralize aliphatic hydrocarbons (*e.g.* hexadecane) representative of crude oil
- Applying these findings to solid-state (soil flask and soil column) experiments to attempt to increase oil degradation through use of nutrient amendments (especially gaseous N and/or P sources)
- Establishing the Microtox® solid-phase assay as a suitable tool for monitoring toxicity reductions in oil-impacted soils during remediation experiments.
- Determining the ability of individual strains of PAH-degrading bacteria to use triethylphosphate (TEP) as a phosphorus source, and assessing the possible effect of carbon source on this ability.
- Documenting the ability of a strain of *Mycobacterium austroafricanum* to grow on and/or degrade a range of highly-recalcitrant 5- and 6-ring PAHs.

RESEARCH SUMMARY

Title	Application of chemically accelerated biotreatment to reduce risk in oil-impacted soils
Contractors	U.S. Department of Energy DOE Contract No. Institute of Gas Technology GRI Contract No. 8054
Principal Investigator	J. Robert Paterek
Report Period	November 2000 through April 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 <i>in-situ</i> , 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 gaseous nutrient (particularly phosphorus) sources to support bioremediation of crude oil (*i.e.* aliphatic hydrocarbon)-contaminated soils. Toxicological experiments have been used to verify that suitable assays are in place for monitoring the detoxification of these soils during solid-phase bioremediation experiments, which will be run during the next six-month period.

RESEARCH ACTIVITIES

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 which was suitably nutrient-limited. This was accomplished through the use of slurry-phase cultures, as we have used throughout this project 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 cyanocobalamine, 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.

SOLID-STATE REMEDIATION OF E&P SOILS

The preferred configuration for evaluation of gaseous nutrient compounds in solid-phase systems is shown in Figure 1 (in practice, an air inlet line is generally hooked to a bubbler or airstone in the flask, such that air is flushed through the system to facilitate volatilization of the nutrient). Soil is sampled (top and bottom of the column) before and after TEP addition to verify (by analyzing total P) that all supplemented P was captured by the soil column. Periodic sampling can then be done of soil at both ends of the column to assess degradation of TPH and/or PAH, and total bacterial populations (both total heterotrophs and alkane- and/or PAH-degraders) and activity.

Figure 1 – System used for evaluating effectiveness of gasifiable nutrients (such as triethylphosphate) in solid-phase systems (vadose-zone soils).

Because the first experiments of this nature, in which N and P (as ammonium nitrate and either TEP or KH_2PO_4 , respectively) showed very low bacterial populations after one week (4 to 5 orders of magnitude lower than water-only controls), we have switched to an initial evaluation of nutrient-amendment systems prior to initiating column studies. These are being conducted in stoppered flasks, with sidearm reservoirs containing NaOH as a CO_2 trap. Radiolabeled hexadecane (as a model aliphatic hydrocarbon is added to the soil, and evolution of $^{14}\text{CO}_2$ over time is taken as a measure of the performance of the system under various supplementation conditions. Once nutrient-amendment conditions can be defined which appear to support contaminant degradation in this screening system, they will then be applied in the column system shown above to fully study the effects of gaseous nutrients on hydrocarbon degradation and its underlying microbiology.

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). 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.

USE OF TEP BY INDIVIDUAL BACTERIAL STRAINS

The abilities of individual PAH-degrading isolates to utilize TEP as a phosphorus source were assessed by generating growth curves in 96-well microplates. Four parallel sets of cultures were run: One set contained glucose as the sole C source and TEP as the sole P source; other sets contained glucose/KH₂PO₄, phenanthrene/TEP, and phenanthrene/KH₂PO₄. All conditions were tested in triplicate. Absorbance measurements were taken periodically, until it could be determined that the stationary growth phase was reached for each culture under each condition. Thus, the data collected in this experiment represents the maximal growth (biomass) attainable under each condition.

PAH GROWTH RANGE OF *MYCOBACTERIUM AUSTROAFRICANUM*

In experiments which we have reported previously, we attempted to determine the range of PAH compounds which could be degraded by *M. austroafricanum* by conducting growth experiments in 96-well microplates. Under a fume hood, individual PAHs are dissolved in solvents and added to the wells, and solvent is allowed to evaporate overnight. The amount of PAH actually deposited into the wells ranged from 0.2 µg to 17.2 µg. A small amount of bacteria (one small colony from a phenanthrene-sublimated gel plate) was added to approximately 20 ml of mineral salts medium and vortexed; 180-µl aliquots of the resultant bacterial suspension were then added to each PAH-containing well. Iodonitrotetrazolium violet (INT) was added to each well at the start of the experiment at a concentration of 0.2 mg·ml⁻¹. In the presence of active respiring bacteria,

INT forms a reddish-purple precipitate when reduced to insoluble formazan. Plates were thus observed visually; wells with red or purple precipitate were scored as positive. Separate wells containing media, INT and bacteria were used as negative controls. During incubation, microplates were sealed in zip-lock bags, to reduce any evaporation loss, and stored at room temperature.

We have also employed several other methods to corroborate the results which we obtained in INT experiments. In the first of these, high-molecular-weight PAHs which emerged from this experiment as probable growth substrates were sublimated onto plates (either noble gel agar or a yeast extract/peptone/starch agar) which had been spread-plated with *M. austroafricanum*. These were then observed over a period of several weeks to detect clearing zones.

An additional experiment was conducted which used the same approach as the INT microplate experiment described above, except that INT was omitted, and growth of the bacterium (assessed as absorbance at $\lambda = 600$ nm) was monitored periodically over a period of approximately 10 days.

RESULTS

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 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 system.

SOLID-STATE REMEDIATION OF E&P SOILS

Thus far, none of the nutrient-amendment regimes tested in solid-phase systems (soil flasks) has had any stimulatory effect on hexadecane mineralization. In fact, all nutrient additions (including either ammonium nitrate or ammonium chloride as N sources) completely abolished the release of ¹⁴CO₂ from cultures grown with radiolabeled hexadecane. Solid-state cultures which received water only did mineralize hexadecane, albeit at a considerably slower rate than in slurry-phase systems. This is true despite the fact that, on a per-soil-mass basis, the amount of N added to the solid-state cultures is very similar to that added to the slurry cultures in earlier experiments.

TOXICITY DETERMINATIONS WITH MICROTOX SOLID-PHASE TEST

The crude oil-contaminated soil which is to be used in the solid-state remediation experiments (Mt. Vernon AB) has been subjected to toxicity testing by the Microtox solid-phase assay system. When the AB soil, diluted 1:1 with clean coarse sand (see above) was subjected to analysis, an EC₅₀ value of 4200 mg/l (± 700 mg/l, n=2) was obtained. For purposes of comparison, the corresponding value derived for the sand itself was 80,000 (± 10,000) mg/l. Thus, the oil-contaminated soil does demonstrate very significant toxicity through the Microtox assay (almost 20-fold more toxic than a comparable clean soil), and this assay is judged suitable for tracking toxicity reductions during treatment of the soil.

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).

	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		

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.

PAH GROWTH RANGE OF *MYCOBACTERIUM AUSTROAFRICANUM*

A strain of *Mycobacterium austroafricanum* was isolated for its ability to degrade phenanthrene (producing clearing zones on phenanthrene-sublimated gel plates), and was found to also degrade the four-ring PAHs fluoranthene and pyrene as sole carbon and energy sources. As we have previously reported, we then assayed the PAH catabolic range of this strain by using 96-well plates, and assessing growth (respiration) via the deposition of reduced formazan deposits upon incubation with iodonitrotetrazolium violet (INT). This type of experiment led us to conclude that the strain in question was capable of growth on a wide variety of PAHs, including:

3-Ring compounds

Acenaphthene, Acenaphthylene, Anthracene, Fluorene, Phenanthrene

4-Ring compounds

Benz[*a*]anthracene, 2,3-Benzofluorene, Chrysene, Fluoranthene, Perylene, Pyrene, Triphenylene

5-Ring compounds

Benzo[*b*]fluoranthene, 2,3-Benzofluoranthene, Benzo[*a*]pyrene

6- Ring compounds

Benzo[*g,h,i*]perylene

Because of the novelty of this finding (in particular the ability of a bacterium to degrade 6-ring PAHs), we have sought to confirm this through other methods. When *M. austroafricanum* (pre-grown on plates with either phenanthrene or fluoranthene as sole source of carbon) was spread-plated onto gel plates which were then sublimated with 4- to 6-ring PAHs, evidence of clearing zones was seen with the two benzofluoranthenes (benzo[*b*]fluoranthene and 2,3-benzofluoranthene), but not with other compounds. However, when agar plates composed of the yeast extract/peptone/soluble starch (YPS) medium (used for the growth of PAH-degrading *Mycobacterium* species) were employed instead, evidence of at least partial clearing zones was seen with all of the 4-, 5-, and 6-ring compounds listed above; this was frequently very significant, as can be seen in the case of the clearing zones formed by colonies degrading benzo[*g,h,i*]perylene in Figure 4.

The apparent contradiction in these two sets of results may be explained by the fact that some bacteria have been found to be incapable of establishing colonies, when grown on hydrocarbon substrates, from a single cell. Other authors have postulated this to be due to the inability of single cells to attach to hydrocarbon crystals (or liquid/liquid interfaces in the case of oils) strongly enough to initiate their degradation, and have found that, in some cases, as many as ten or more cells may be required to initiate a viable colony under these conditions. Because the bacteria grown on YPS plates are able to use components of this medium as carbon sources, they may be able to overcome this limitation, and give rise to colonies which are capable of degrading the sublimated PAH.

We have also conducted a growth-curve experiment (in 96-well microplates) to determine the ability of *M. austroafricanum* to grow on various high-molecular-weight PAH species. Results of this experiment are given in Figure 5.

Figure 4 – Clearing of benzo[*g,h,i*]perylene by colonies of *Mycobacterium austroafricanum* during growth on yeast extract/peptone/starch agar plates.

Figure 5 – Growth of *Mycobacterium austroafricanum* in 96-well microplates with various three-, four-, or five- and six-ring PAHs serving as a sole source of carbon and energy.

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 Resolution 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 –PUBLICATION

Effects of Alkylphosphates and Nitrous Oxide on Microbial Degradation of Polycyclic Aromatic Hydrocarbons

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We conducted a series of liquid-culture experiments to begin to evaluate the abilities 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 site. 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 three- to five-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 a considerably higher degree of removal of low-molecular-weight PAHs than did PO_4 ; however, loss of high-molecular-weight compounds was impaired under these conditions. The disappearance of most PAHs was significantly less in the oil field 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.

Approximately 1,500 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 United States are contaminated with creosote as a result of improper handling and disposal of materials during wood-preserving activities (18). Both of these classes of sites are considered to pose a significant potential health risk for humans and wildlife, since 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) is 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 U.S. Environmental Protection Agency (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 two-, three-, and four-ring PAHs as sole sources of carbon and energy (for a review, see reference 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 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 trimethylphosphate). 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 C_4 - C_{10} alkanes and monoaromatic hydrocarbons (e.g., benzene, toluene, ethylbenzene, and 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 of the abilities of organic phosphates (TEP and TBP) and N_2O to support degradation of PAH 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

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TABLE 1. Initial levels of PAH in the soils incorporated in this study, as well as the *n,n*-dimethylformamide-solubilized extract of MGP soil which was added as a supplemental source of available PAH

PAH	Concn (ppm) of PAH in:		
	Soils		Supplemental extract
	MGP	Oil field	
Fluorene	88	1.0	1,172
Phenanthrene	239	3.5	2,140
Anthracene	359	0.7	4,494
Fluoranthene	643	0.8	1,102
Pyrene	940	1.1	666
Benz[a]anthracene	168	<0.4	377
Chrysene	182	4.3	387
Benzo[b]fluoranthene	82	<0.4	316
Benzo[k]fluoranthene	51	1.2	247
Benzo[a]pyrene	106	<0.5	277
Dibenz[ah]anthracene	26	<0.4	147
Benzo[ghi]perylene	14	<0.4	64
Indeno(123-cd)pyrene	20	<0.4	140

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 oil field soil is a crude oil-contaminated sandy loam (63% sand, 3% clay, 34% silt) obtained from the vicinity of a wellhead in a drilling field in southern Illinois. PAH concentrations for the two soils are given in Table 1. The oil field 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 of sterile media (0.1 ml of Wolfe's vitamins [2-mg · liter⁻¹ biotin, 2-mg · liter⁻¹ folic acid, 10-mg · liter⁻¹ pyridoxine HCl, 5-mg · liter⁻¹ thiamine HCl, 5-mg · liter⁻¹ riboflavin, 5-mg · liter⁻¹ nicotinic acid, 5-mg · liter⁻¹ pantothenic acid, 0.1-mg · liter⁻¹ cyanocobalamin, 5-mg · liter⁻¹ *p*-aminobenzoic acid, 5-mg · liter⁻¹ thioctic acid], 0.1 ml of trace minerals [100-mg · liter⁻¹ ZnSO₄, 300-mg · liter⁻¹ H₃BO₃, 300-mg · liter⁻¹ CoCl₂, 10-mg · liter⁻¹ CuCl₂], and 0.8 ml of N- and P-free Winogradsky medium [pH 7.2] [62.5-g · liter⁻¹ MgSO₄ · 7H₂O, 31.25-g · liter⁻¹ NaCl, 1.25-g · liter⁻¹ FeSO₄, 1.25-g · liter⁻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 N and P supplementation: NH₄Cl plus KH₂PO₄; N₂O plus KH₂PO₄; NH₄Cl plus TEP; NH₄Cl plus TBP; N₂O plus TEP; and N₂O plus 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 of total PAH; Table 1) dissolved in *n,n*-dimethylformamide. One set of cultures (duplicates of each condition) was further supplemented with [¹⁴C]-phenanthrene for mineral-

ization determinations (see below), while one received no radiolabel and was used to simultaneously measure the extent of disappearance of multiple PAHs. Both sets of cultures were incubated at room temperature (approximately 25°C) with shaking at 170 rpm. Poisoned controls (10 mg of HgCl₂ per culture) were also conducted in duplicate.

Mineralization of [¹⁴C]-PAH. CO₂ traps were made by wrapping stainless steel wire around the necks of 12-by-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 0.5 M 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 liquid scintillation cocktail (Packard, Meriden, Conn.) and counted in a liquid scintillation counter (Packard model 2200CA Tri-Carb). Fresh NaOH was then added to the CO₂ traps. Cultures containing [¹⁴C]-phenanthrene typically received ca. 80,000 to 100,000 dpm of PAH in 20 µl of methanol.

Extraction and high-pressure liquid chromatography (HPLC) analysis. Soil samples were centrifuged (10 min, 5,000 × *g*) in stainless-steel containers to separate solid and aqueous phases. Soil solids were mixed with anhydrous sodium sulfate (1:1) and ground with 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 N₂ in a Turboprep evaporator (Zymark, Hopkinton, Mass.) and exchanged into acetonitrile (ACN) (1 ml). Ten microliters of this solution was analyzed by reverse-phase HPLC (EPA method 8310 [27]) using a Supelcosil LC-PAH column (15 cm by 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 five-point standard curves (all *r*² values were >0.988).

Chemicals. [¹⁴C]phenanthrene (reported purity, 98%) was purchased from Sigma (St. Louis, Mo.). TEP (99% pure) and TBP (98%) were purchased from Aldrich (Milwaukee, Wis.); N₂O (ultra-high purity) was from Matheson Gas Products (Joliet, Ill.). NH₄Cl and KH₂PO₄ were purchased from Mallinckrodt Chemicals (Paris, Ky.), and NaOH and HPLC-grade solvents were purchased from Fisher Chemicals (Fairway, N.J.). Authentic PAH standards for use in HPLC analysis were obtained from Ultra Scientific (Kingstown, R.I.).

RESULTS AND DISCUSSION

Nutrient limitations were assessed for each site 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 Fig. 1. Both soils were strongly nutrient limited. The MGP soil showed an especially strong N limitation and a P limitation which was also significant, while the oil field soil was greatly limited by both N and P; in this case, neither nutrient alone was capable of enhancing [¹⁴C]CO₂ release at all relative to results with 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 three- to six-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 Fig. 2. All nutrient regimes examined sustained between 50 and 70% conversion to CO₂, with the only significant difference between the different treatments

FIG. 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 and KH_2PO_4 were added either singly or in combination.

being a slightly longer lag time prior to mineralization in the three conditions in which N_2O served as the nitrogen source. Cultures receiving HgCl_2 (10 mg) were still capable of mineralizing 7.3% of the input phenanthrene.

Under conventional nutrient additions (NH_4 and PO_4), the microbial community present in the MGP soil displayed sig-

nificant removal of all three-ring compounds examined, as well as some elimination of several four- and five-ring PAHs (Table 2). A separate experiment, in which mineralization of ^{14}C -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 cometabolic

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

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

PAH ^a	% Recovery of PAH after treatment with ^b :						Poison (killed controls)
	NH ₄ + PO ₄	N ₂ O + PO ₄	NH ₄ + TEP	NH ₄ + TBP	N ₂ O + TEP	N ₂ O + TBP	
FLU	16 ± 0	36 ± 11	46 ± 6	12 ± 4	51 ± 13	51 ± 5	76 ± 6
PHE	24 ± 2	35 ± 2	52 ± 4	7 ± 2	45 ± 8	36 ± 1	107 ± 1
ANT	25 ± 6	45 ± 7	44 ± 3	6 ± 0	51 ± 7	102 ± 8	124 ± 4
FLA	63 ± 5	109 ± 12	118 ± 5	67 ± 13	111 ± 14	131 ± 9	112 ± 4
PYR	86 ± 15	116 ± 5	137 ± 8	104 ± 21	128 ± 11	132 ± 15	118 ± 4
BAA	90 ± 11	108 ± 7	140 ± 7	120 ± 24	128 ± 7	123 ± 6	105 ± 4
CHR	90 ± 10	143 ± 14	140 ± 11	122 ± 21	128 ± 3	115 ± 12	97 ± 2
BBF	99 ± 15	95 ± 12	162 ± 15	144 ± 26	143 ± 9	145 ± 8	135 ± 20
BKF	68 ± 12	125 ± 5	105 ± 10	94 ± 18	101 ± 4	97 ± 10	85 ± 10
BAP	86 ± 13	132 ± 13	149 ± 14	124 ± 28	128 ± 4	121 ± 5	105 ± 8

^a FLU, fluorene; PHE, phenanthrene; ANT, anthracene; FLA, fluoranthene; PYR, pyrene; BAA, benz[a]anthracene; CHR, chrysene; BBF, benzo[b]fluoranthene; BKF, benzo[k]fluoranthene; BAP, benzo[a]pyrene.

^b Values are given as means ± the standard deviations.

effects (7), which may also account for the loss of other four- and five-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 results with NH₄ and PO₄); however, losses of fluorene and fluoranthene are no greater under these conditions, and four- and five-ring compounds are unaffected. Most other nutrient regimes supported degradation of only the most labile compounds (fluorene, phenanthrene, and anthracene).

In the crude oil-contaminated wellhead soil, as in the MGP soil, mineralization of phenanthrene was greatest in the NH₄Cl-plus-KH₂PO₄ cultures (Fig. 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, since all three of the NH₄-containing cultures outperformed their N₂O-containing counterparts. Furthermore, the trend in which PO₄ outperformed TEP which, in turn, outperformed 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 oil field soil was considerably higher than that 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-plus-PO₄ supplementation; this includes five-ring PAHs. Again, inasmuch as no significant mineralization of pyrene was

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

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

PAH ^a	% Recovery of PAH after treatment with ^b :						Poison (killed controls)
	NH ₄ + PO ₄	N ₂ O + PO ₄	NH ₄ + TEP	NH ₄ + TBP	N ₂ O + TEP	N ₂ O + TBP	
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

^a DAA, dibenz[ah] anthracene. For other abbreviations, see Table 2, footnote a.

^b Values are given as means ± the standard deviations.

seen in this case (data not shown), it seems possible that some of the losses of high-molecular-weight (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, since the N₂O-PO₄ combination supports a broader and more extensive removal of HMW species than do any conditions including either of the alkylphosphates.

Among the soils examined in this work, it is clear that the bacteria present in the oil field soil were more adept at removing PAHs, including the HMW species. This is interesting because although the soil in question has very high levels of total petroleum hydrocarbons (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. Coinjection of TEP and methane into a solvent-contaminated aquifer thus caused up to 1,000-fold increases in the activities of these bacteria, while the total bacterial biomass remained essentially unchanged (4, 21). Literature on the taxonomic distribution and extent of TEP and 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 or 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 results with NH₄-plus-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 the solubility of TEP and TBP on their availability to PAH-degrading bacteria. As a consequence of its longer aliphatic groups and resultant greater hydrophobicity, TBP is more resistant to solubilization than TBP; 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 the effects of solubility alone, since 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 (Table 2), as well as the high mineralization of phenanthrene supported by both alkylphosphates in the MGP soil (Fig. 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 a 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 (3a); 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 5 orders of magnitude higher in the gaseous phase than when these compounds are dissolved in water (4). Thus distribution would be expected to improve somewhat in vadose soil (4, 21), and the issue of the availability of P to soil surface adherents 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 of 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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REFERENCES

- Allen-Hoffmann, B. L., and J. G. Rheinwald. 1984. Polycyclic aromatic hydrocarbon mutagenesis of human epidermal keratinocytes in culture. *Proc. Natl. Acad. Sci. USA* **81**:7802-7806.
- Amin, S., J. Krzeminski, A. Rivenson, C. Kurtzke, S. S. Hecht, and K. El-Bayoumi. 1995. Mammary carcinogenicity in female CD rats of fjord region diol epoxides of benzo[c]phenanthrene, benzo[g]chrysene and dibenzo[a,l]pyrene. *Carcinogenesis* **16**:1971-1974.
- Bastiaens, L., D. Springael, P. Wattiau, H. Harms, R. deWachter, H. Verachtert, and L. Diels. 2000. Isolation of adherent polycyclic aromatic hydrocarbon (PAH)-degrading bacteria using PAH-sorbing carriers. *Appl. Environ. Microbiol.* **66**:1834-1843.
- Bogan, B. W., L. M. Lahner, and J. R. Paterek. Limited roles for salicylate and phthalate in bacterial PAH bioremediation. *Bioremediat. J.*, in press.
- Brockman, F. J., W. Payne, D. J. Workman, A. Soong, S. Manley, and T. C. Hazen. 1995. Effect of gaseous nitrogen and phosphorus injection on in situ bioremediation of a trichloroethylene-contaminated site. *J. Hazard. Mater.* **41**:287-298.
- Cerniglia, C. E. 1992. Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation* **3**:351-368.
- Chadwick, R. W., S. E. George, M. J. Kohan, R. W. Williams, J. C. Allison, D. L. Talley, Y. O. Hayes, and J. Chang. 1995. Potentiation of 2,6-dinitrotoluene genotoxicity in Fischer 344 rats by pretreatment with coal tar creosote. *J. Toxicol. Environ. Health* **44**:319-336.
- Chen, S.-H., and M. D. Aitken. 1999. Salicylate stimulates the degradation of high-molecular weight polycyclic aromatic hydrocarbons by *Pseudomonas saccharophila* P15. *Environ. Sci. Technol.* **33**:435-439.
- Djomo, J. E., V. Ferrier, L. Gauthier, C. Zoll-Moreux, and J. Marty. 1995. Amphibian micronucleus test *in vivo*: evaluation of the genotoxicity of some major polycyclic aromatic hydrocarbons found in a crude oil. *Mutagenesis* **10**:223-226.
- Eng, R. 1985. Survey of town gas and by-product production and locations in the US (1880-1950). U.S. Environmental Protection Agency Air and Energy Engineering Research Laboratory, Research Triangle Park, N.C.
- Ghisalpa, O., M. Kuenzi, G. M. Ramos Tombo, and H. P. Schar. 1987. Microbial degradation and utilisation of selected organophosphorus compounds: strategies and applications. *Chimia* **41**:206-215.
- Glatt, H., A. Piee, K. Pauly, T. Steinbrecher, R. Schrode, F. Oesch, and A. Seidel. 1991. Fjord- and bay-region diol-epoxides investigated for stability, SOS induction in *Escherichia coli*, and mutagenicity in *Salmonella typhimurium* and mammalian cells. *Cancer Res.* **51**:1659-1667.
- Heitkamp, M. A., W. Franklin, and C. E. Cerniglia. 1988. Microbial metabolism of polycyclic aromatic hydrocarbons: isolation and characterization of a pyrene-degrading bacterium. *Appl. Environ. Microbiol.* **54**:2549-2555.
- Keith, L. H., and W. A. Telliard. 1979. Priority pollutants. I. A perspective view. *Environ. Sci. Technol.* **13**:416-423.
- Levin, W., D. R. Thakker, A. W. Wood, R. L. Chang, R. E. Lehr, D. M. Jerina, and A. H. Conney. 1978. Evidence that benzo[a]anthracene 3,4-diol-1,2-epoxide is an ultimate carcinogen on mouse skin. *Cancer Res.* **38**:1705-1710.
- Looney, B. B., S. M. Pfiffner, T. J. Phelps, K. H. Lombard, T. C. Hazen, and J. W. Borthen. May 1998. Apparatus and method for phosphate-accelerated bioremediation. U.S. patent 5,753,109.
- Looney, B. B., K. H. Lombard, T. C. Hazen, S. M. Pfiffner, T. J. Phelps, and J. W. Borthen. January 1996. Method for phosphate-accelerated bioremediation. U.S. patent 5,480,549.
- Melikian, A. A., K. A. Prahalad, S. Amin, and S. S. Hecht. 1991. Comparative DNA binding of polynuclear aromatic hydrocarbons and their dihydrodiol and bay region diolepoxide metabolites in newborn mouse lung and liver. *Carcinogenesis* **12**:1665-1670.
- Mueller, J. G., P. J. Chapman, and P. H. Pritchard. 1989. Creosote-contaminated sites: their potential for bioremediation. *Environ. Sci. Technol.* **23**:1197-1201.
- Nestler, F. H. M. 1974. The characterization of wood-preserving creosote by physical and chemical methods of analysis. U.S. Department of Agriculture Forest Service research paper FPL 195. U.S. Department of Agriculture Forest Service Forest Products Laboratory, Madison, Wis.
- Nishioka, M., H.-C. Chang, and M. L. Lee. 1986. Structural characteristics of polycyclic aromatic hydrocarbon isomers in coal tars and combustion products. *Environ. Sci. Technol.* **20**:1023-1027.
- Palumbo, A. V., S. P. Scarborough, S. M. Pfiffner, and T. J. Phelps. 1995. Influence of nitrogen and phosphorus on the in situ bioremediation of trichloroethylene. *Appl. Biochem. Biotechnol.* **51/52**:635-647.
- Rosenberg, A., and M. Alexander. 1979. Microbial cleavage of various organophosphorus insecticides. *Appl. Environ. Microbiol.* **37**:886-891.
- Salagovič, J., I. Kalina, and K. Dubayová. 1995. Induction of single strand DNA breaks in workers professionally exposed to polycyclic aromatic hydrocarbons. *Neoplasma* **42**:115-118.
- Shamory, B. D., A. W. Lawrence, D. L. Miller, J. A. Miller, R. L. Weightman, R. M. Raetz, and T. D. Hayes. 1995. In situ bioremediation of a former natural gas dehydrator site using bioventing/biosparging, p. 587-599. *In Proceedings of the SPE/EPA Exploration & Production Environmental Conference*. Society of Petroleum Engineers, Richardson, Tex.
- Stoner, D. L., and A. J. Tien. September 1995. Method and compositions for the degradation of tributyl phosphate in chemical waste mixtures. U.S. patent 5,453,375.
- Thomas, R. A. P., and L. E. Macaskie. 1998. The effect of growth conditions on the biodegradation of tributyl phosphate and potential for the remediation of acid mine drainage waters by a naturally-occurring mixed microbial culture. *Appl. Microbiol. Biotechnol.* **49**:202-209.
- U.S. Environmental Protection Agency. 1994. Test methods for evaluating solid waste. Vol. IB: Laboratory manual physical/chemical methods. Office of Solid Waste and Emergency Response, U.S. Environmental Protection Agency, Washington, D.C.
- Walsh, P., C. el Aldouni, M. J. Mukhopadhyay, G. Viel, D. Nadeau, and G. G. Poirier. 1995. ³²P-postlabeling determination of DNA adducts in the earthworm *Lumbricus terrestris* exposed to PAH-contaminated soils. *Bull. Environ. Contam. Toxicol.* **54**:654-661.
- Wood, A. W., R. L. Chang, W. Levin, R. E. Lehr, M. Schaefer-Ridder, J. M. Karle, D. M. Jerina, and A. H. Conney. 1977. Mutagenicity and cytotoxicity of benz[a]anthracene diol epoxides and tetrahydro-epoxides: exceptional activity of the bay region 1,2-epoxides. *Proc. Natl. Acad. Sci. USA* **74**:2746-2750.
- Wood, A. W., R. L. Chang, W. Levin, D. E. Ryan, P. E. Thomas, H. D. Mah, J. M. Karle, H. Yagi, D. M. Jerina, and A. H. Conney. 1979. Mutagenicity and tumorigenicity of phenanthrene and chrysene epoxides and diol epoxides. *Cancer Res.* **39**:4069-4077.