Fossil Fuel Biodegradation: Laboratory Studies

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Biodegradation of the polycyclic aromatic hydrocarbons of creosote by undefined bacterial cultures was shown to be accompanied by the accumulation of neutral and acidic oxidation products. Formation of a number of identified neutral products is accounted for by demonstration of anomalous actions of an arene dioxygenase on the benzylic methylene and methylene carbons of napthenoaromatic hydrocarbons. Both neutral and acidic water-soluble fractions are also formed when various mixed bacterial cultures degrade weathered crude oil. While constituents of these fractions are not yet identified, the neutral materials have been shown to be toxic to developing embryos of invertebrates. These observations are discussed in relation to chemical and toxicological assessments of biodegradation of the complex chemical mixtures of fossil fuels. — Environ Health Perspect 103(Suppl 5):79–83 (1995)

Key words: biodegradation, creosote, oil, microorganisms, PAHs, toxicity, metabolites, oxidation

Introduction

Natural processes of biodegradation that return carbon from its various organic forms to the inorganic state are increasingly screened for bioremediation applications. A variety of microbial systems capable of degrading synthetic organic chemicals, from pesticides to polychlorinated biphenyls (PCBs), have been identified for use in processes to clean up environmental contaminants. Before application, any treatment process should be well characterized. Ideally such a characterization will demonstrate, by means of sensitive and exact analytical methods, consistent and effective removal of a target pollutant to levels that are less than those proscribed or regulated by conversion to innocuous or mineralized products. Toxicological evaluations using relevant test species should confirm loss of toxicity due to the pollutant and indicate the absence of toxic products not necessarily evident from mass balance chemistry. The chemical and toxicological consequences of a biotreatment process can be characterized in the laboratory with evaluation of appropriate methods and systems to be used. Few biological treatment systems have been so rigorously characterized before field application.

Chemicals in complex mixtures such as crude and refined petroleum and coal-derived materials such as creosote, coal tar, and gasification wastes offer additional challenges for biodegradation-based technologies. Hundreds, if not thousands, of different chemicals are present in these fossil fuel-related materials, many of which produce adverse human and environmental effects; the chemical composition of these complex mixtures cannot yet be considered adequately established.

Effective biodegradation of the many different chemicals in fossil fuels demands a physiologically diverse and versatile microbial flora. These conditions are prime for the transformation of chemicals, not merely to cellular products or carbon dioxide, but also to organic end products. The term co-metabolism is used in some laboratories to describe these processes. Little is known of the nature or toxicity of products accumulated under these circumstances (*1,2*). Consequently, any characterization of an acceptable biodegradation process for these materials must demonstrate not only substantial depletion of identifiable toxic chemicals, but also must establish the absence of toxicity due to product formation. Until chemical identities and toxicological properties are known for all fossil fuel constituents and organic reaction products, evaluation of treatment effectiveness will rely heavily on toxicological assessment. The work, outlined below, was undertaken to examine some of these aspects of fossil fuel biodegradation.

Pure Culture Studies: Transformation and Degradation of Naphthenoaromatic Hydrocarbons

To define the action of a typical reductive arene dioxygenase on naphthenoaromatic hydrocarbons, a strain of *Pseudomonas aeruginosa* carrying genes encoding naphthalene-1,2-dioxygenase (*3*) was adopted for study. Incubations of induced washed cell suspensions were established with individual test substrates including acenaphthene, acenaphthylene, and fluorene. After incubation with shaking at 30°C, reactions were extracted into ethyl acetate before analysis by gas chromatography–mass spectroscopy (GC-MS) and product purification.

Acenaphthene was transformed to a mixture of products, including acenaphthenol, acenaphthenone (II), acenaphthenequinone (III), and a product recovered as 1,8-naphthalic anhydride (IV) (Figure 1).

This paper was presented at the Conference on Biodegradation: Its Role in Reducing Toxicity and Exposure to Environmental Contaminants held 26–28 April 1993 in Research Triangle Park, North Carolina. Manuscript updated: fall 1994; manuscript received: January 23, 1995; manuscript accepted: February 13, 1995.

The authors acknowledge the following sources of support: U.S. EPA Cooperative Agreement #CR-818998 with the University of West Florida (M.S.); Postdoctoral Fellowship from the Ministry of Education and Science, Spain (M.G.); U.S. EPA Cooperative Agreement CR-817770 with the University of West Florida (S.A.S.). Contribution no. 871 from the Environmental Research Laboratory, U.S. Environmental Protection Agency, Gulf Breeze, FL. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

The authors also thank the following individuals for their assistance and contributions: W. Fisher, S. Foss, and W. Gilliam, U.S. Environmental Protection Agency, Environmental Research Laboratory, Gulf Breeze, FL; Semen Akkerman, University of West Florida, Pensacola, FL; B. Blattmann, M. Downey, C. Gatlin, and S. Resnick, Technical Resources Inc., Environmental Research Laboratory, Gulf Breeze, FL.

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Apparently, naphthalene 1,2-dioxygenase can initiate attack on a benzylic carbon of acenaphthene-forming acenaphthenol, which can then undergo a second hydroxylation at the adjacent benzylic carbon or alternatively undergo oxidation to acenaphthenone through action of nonspecific alcohol dehydrogenases. The same range of products, with the exception of acenaphthenol, was formed from acenaphthylene in which initial methyne monooxygenation and dioxygenation appear to account for the observed conversions (*4*). These products are also observed in creosote-polycyclic aromatic hydrocarbons (PAH) biodegradation studies (*5*) in which product formation can be explained as being due to the action of dioxygenases elaborated by PAH-utilizing bacteria. With fluorene (V) a somewhat more complicated result was obtained; again, benzylic oxidation was a favored reaction, as evidenced by the presence of 9-fluorenol (VI) and 9-fluorenone (VII). Other products presumably formed by dehydration of different labile dihydrodiols are hydroxylated fluorenes, fluorenols, and fluorenones (VIII) (Figure 1). Benzylic oxidation of fluorene is a reaction also found as an obligatory step in the pathway of degradation in certain fluorene-utilizing bacteria. A recent study (*6*) has rigorously identified a 1,1*a*-diol product of angular dioxygenation of fluorenone, confirming the existence of an alternate biochemical strategy for fluorene catabolism that provides for its complete degradation (*7*). Whether acenaphthenone is as readily accommodated by pathways of acenaphthene catabolism is under investigation.

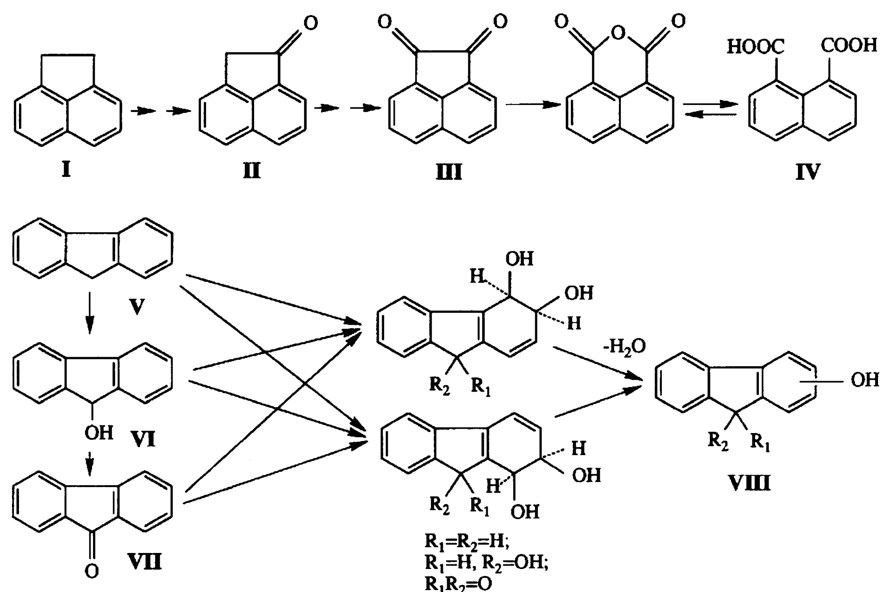

Figure 1. Oxidation of the naphthenoaromatic compounds, acenaphthene (I) and fluorene (V), by *Pseudomonas aeruginosa* PAO 1(pRE681) carrying naphthalene 1,2-dioxygenase genes from the naphthalene catabolic plasmid, NAH7 (*3*). See text for identities of II, III, IV, VI, VII, and VIII.

Creosote–PAH Biodegradation

PAH-degrading enrichment cultures were established with microorganisms washed from creosote-contaminated soils of local lumber treatment facilities. PAHs, purified from creosote by chromatography on neutral alumina (*8*), were used as a carbon source at 0.1% in shaken cultures containing 25 ml of mineral salts media (*9*) and 0.02% DMSO. Cultures were maintained at 20 to 22°C and transferred every 14 days. Growth, evident as visible increases in turbidity, was confirmed by significant increases in viable cell counts; results of biological activity were seen as color changes from yellow, through orange and brown, to a gray-black suspension of finely divided particles. Consistently reproducible degradation of PAHs was observed in cultures routinely transferred for over a year. Uninoculated controls showed no such visible changes. GC-analyses indicated some losses by volatilization.

PAHs with lower molecular weights than fluoranthene/pyrene are readily degraded by these enrichments, as shown by capillary gas chromatography–flame ionization detection (GC–FID) analysis of methylene chloride extracts of entire cultures. Approximately 72% of the measured PAHs are removed, accounting for 52.5% of the weight of initial PAHs. Losses of unmeasured PAHs may contribute another 5 to 10% to this assessment. GC traces of fluoranthene, pyrene, and later emerging PAHs are indistinguishable from the corresponding regions of GC traces of starting PAHs showing the greater persistence of these components (Figure 2).

To better characterize this process, large scale (400 ml) cultures were established in replicates and, after 14 days incubation, extracted by one of two alternative procedures. The first involved sequential extraction of entire incubations with hexane (for PAHs), methylene chloride (for polar neutrals), and, after filtration and acidification, ethyl acetate (for acids). The second procedure involved extraction of the centrifuged and washed cell material with methylene chloride after treatment with anhydrous sodium sulfate. The supernatant and washings were extracted as in the first procedure. When data are compared, it can be seen that, overall, each procedure gave similar results (Table 1); about 50% of the weight of initial PAHs is recovered in neutral extracts. Acidic products are significant. Procedure #1 recovers a significant proportion of the low molecular weight neutrals in methylene chloride, whereas hexane extraction of uninoculated controls is essentially quantitative. It should also be noted that the bulk of starting material and polar neutral products is essentially water-insoluble and can be readily overlooked if entire cultures or separated insoluble debris are not efficiently extracted (Procedure 2). A continuing line of work examines the neutral and acidic products. The neutral material is comprised of both low molecular weight oxidation products and high molecular weight polymeric material, possibly formed by oxidative coupling of phenolic compounds (*10*). Among the low molecular weight compounds, acenaphthenone (II), acenaphthenequinone (III), and 1,8-naphthalic anhydride (IV) have been rigorously characterized and probably are derived from acenaphthene and acenaphthylene by reactions described above. Capillary GC of the acid fraction as methyl esters shows that it is complex; GC–MS shows evidence of the presence of *ortho*-hydroxylated aromatic carboxylic acids, products presumably formed by aromatic ring cleavage and subsequent reactions.

The toxic effects of these and other products of PAH catabolism have yet to be examined. Such information is relevant in

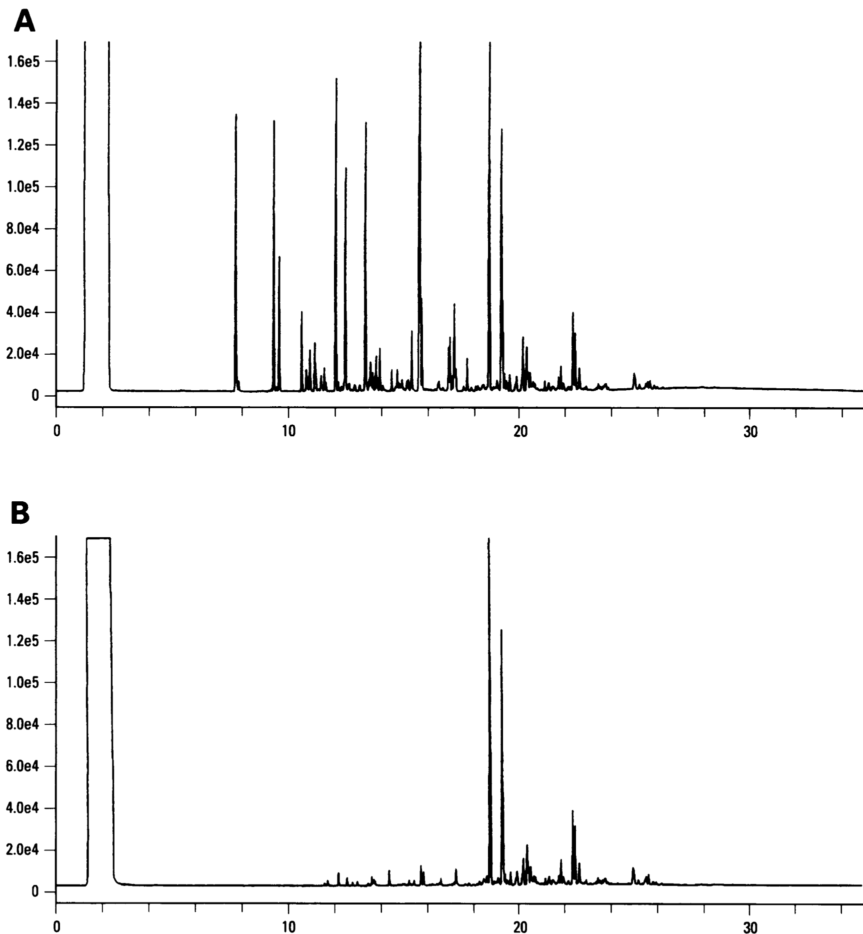

Figure 2. Gas chromatographic profiles of creosote–PAHs extracted by procedure #1 after 14 days incubation (*A*) without inoculation or (*B*) after inoculation with enrichment culture.

determining, for example, why little change in toxicity is observed when creosote undergoes biodegradation in groundwater (*11*).

Crude Oil Biodegradation

The action of a variety of bacterial cultures on an artificially weathered Prudhoe Bay crude oil, Alaskan North Slope (ANS) 521*, was studied under optimized biodegradation conditions. Cultures included: *a*) defined cocultures of bacteria isolated for their ability to utilize specific oil components, *b*) enrichment cultures on 521 established from Pacific Ocean and Arabian Gulf source materials, and *c*) indigenous bacterial populations from Gulf of Mexico seawater used without enrichment. Microorganisms were grown at 20°C (or 30°C for the Arabian Gulf source system) in 16.5 liters seawater (sterilized except for condition *c*) supplemented with NH_4NO_3 (1.0 g/l) and Na_2HPO_4/Na_2HPO_4 (1.0 g/l, pH 7.5), and vigorously stirred in 20-liter carboys for 7 and 14 days with large bar magnets (1.0" × 5.0") to create top-to-bottom vortices.

Degraded oil was separated by passage of entire cultures, first through diatomaceous earth (Hyflo Supercel, Celite Corp, Lompoc, CA) and then through 0.7 μ glass fiber filters. The resulting filtrate (pH 7.0–7.2) was extracted with methylene chloride to recover water-soluble neutrals and acids. Uninoculated controls provided water-soluble neutral (WSN) and acidic fractions and demonstrated the effectiveness with which oil can be recovered from Hyflo Supercel after elution with methylene chloride (> 99%).

Biodegradation of oil was somewhat limited (ranging from 6–18% of initial oil), probably due to the use of weathered 521 oil free of a significant proportion of more degradable components. Nonetheless, amounts of neutral and acid extractives were significantly greater than those recovered from uninoculated controls and showed increases with increasing time of incubation. The amounts of products formed as a percentage of the observed losses in oil weight are significant, varying from 4.3 to 6.9% for neutrals, from 6.4 to 9.2% for acids, and from 10.7 to 16.1% for totals. The larger proportions of neutral products go hand in hand with the larger proportions of acids and are highest where the microbial diversity of the cultures employed is restricted, as with a defined coculture of four microbial isolates.

Examination of the neutral extracts from all incubations by FID–GC showed their composition to be quite distinct from that of oil and degraded oil recovered at the same sampling times (Figure 3*A*,*B*). Preliminary fractionation, by alumina chromatography, of the neutral fractions from 14-day cultures has shown that they possess a fractional composition that is markedly different from that of oil and its WSN fraction. Acidic materials, after conversion to methyl esters and GC analysis, are observed as complex mixtures. Work continues to identify classes, and even individual constituents, of neutral and acidic products. Toxicological assessments of neutral and acidic products were carried out using two different test organisms and systems. The first used embryos of the grass shrimp, *Palaemonetes pugio*, because of its local availability and its conservative use of products (*12*). The second test was the 7-day chronic estimator test with larvae of the mysid shrimp, *Mysidopsis bahia* (*13*).

*521 Oil is artificially weathered by distillation to resemble crude oil after several days of environmental exposure. It is useful for study because it is essentially free of volatile components and its weight is, therefore, a simple measure of its quantity.

Table 1. Recovery of PAHs and metabolic products by alternative extraction procedures.

	Percent of initial PAHs recovered as			
	Neutrals			Acids
Extraction procedure	Hexane extracted	CH_2Cl_2 extracted	Combined	EtOAc extracted
# 1 Entire reaction	29.8	18.2	48.0	12.0
# 2 Cells/debris	—	41.8	—	—
Supernatant	4.2	3.8	49.8	22.4

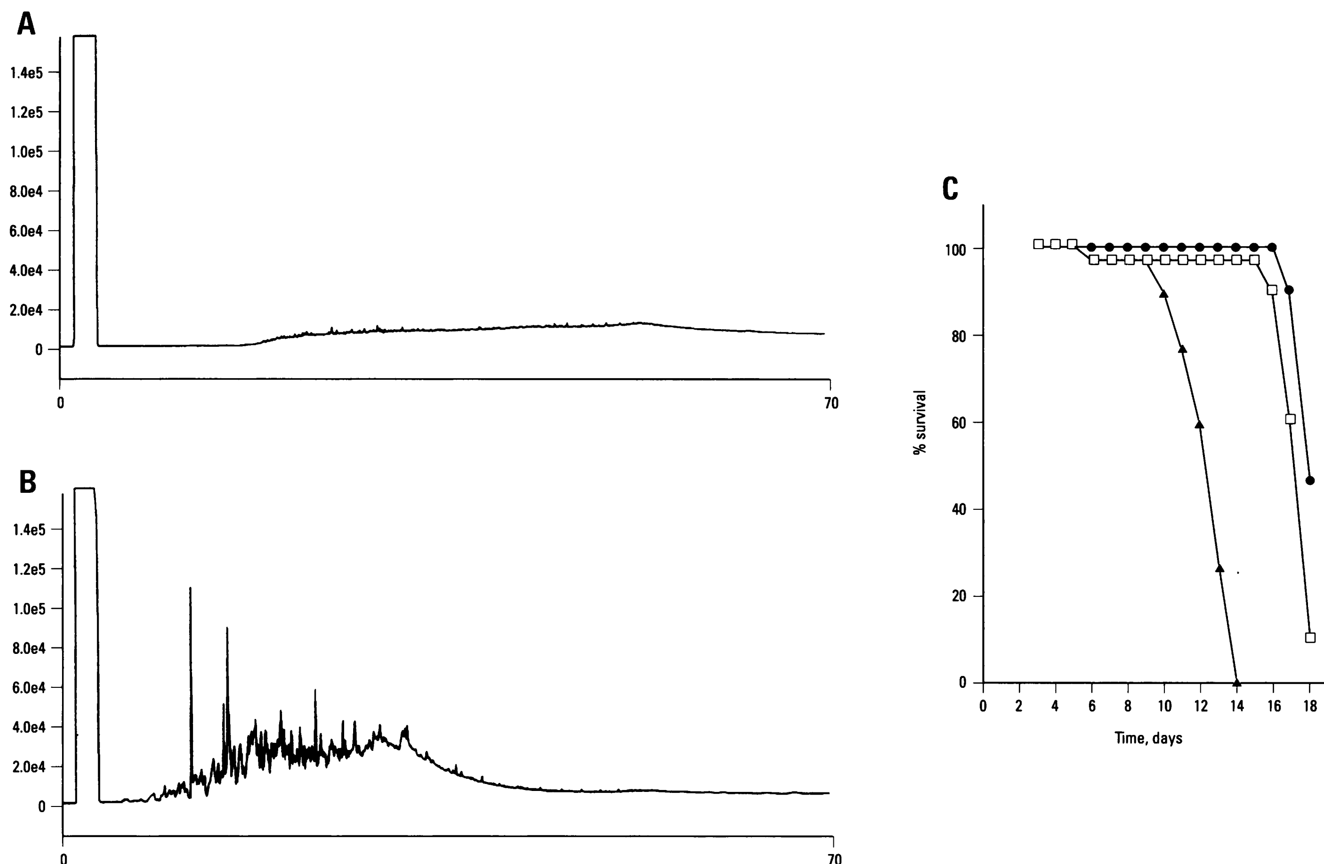

Figure 3. Comparison of gas chromatography profiles of oil (*A*), and neutral products (*B*), recovered after 14 days incubation with defined four-member coculture, G04. Also shown (*C*) is the result of a grass shrimp toxicity test of the same neutral fraction (–▲–), of test water alone (–●–), and of water-soluble neutral (WSN) from uninoculated (–□–) incubations.

Neutral and acid products were reconstituted in test media (at pH 7.2) at concentrations matching those found in cultures. Controls used materials from uninoculated incubations. In all cases, neutral products showed toxicity to grass shrimp embryos (Figure 3*C*) generally resulting in 100% mortality at or around the hatch time (*14*). The onset of this response was observed earlier with neutral material from 14-day incubations than with material from 7-day incubations. Effects of neutrals from uninoculated incubations were no different from controls. No observable effect has been found with the one acidic product examined to date.

In the few cases where the mysid test has been used, results have reinforced those obtained with grass shrimp. At 3-fold dilutions, toxic effects on both test species were abolished. Currently, it is not clear whether the observed responses are due to the cumulative effects of many different weakly toxic chemicals or associations with specific chemical groups or classes of metabolic products. Work is continuing to resolve this question and to examine the precursors, agents, and biochemical routes involved.

Summary

Laboratory studies of microbial degradation of complex chemical mixtures, such as in crude oil and in coal-derived products, showed that significant accumulations of neutral and acidic products accompany losses of parent compounds. Preliminary characterization of a neutral fraction formed from creosote showed that the structures of many metabolic products can be anticipated based on studies of compound biotransformations by organisms with relevant catabolic activities. Many other metabolic products however await characterization. Toxicological assessment, also at a preliminary stage, indicated that concentrations of neutral products formed during oil biodegradation in the laboratory were mildly toxic to mysids and grass shrimp. These findings point to the need for more complete characterization of biodegradation and bioremediation processes, particularly those of complex chemical mixtures, by addressing not only the effectiveness of pollutant removal but also product accumulation and any attendant toxicological consequences.

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