

## **Continuation of an Investigation into the Anaerobic Intrinsic Bioremediation of Whole Gasoline – Final Report Executive Summary**

**Period Covered by the Report:** March 1, 2001 to August 31, 2002

**Date of Report:** October 28, 2002

**EPA Grant Number:** R827015-01-0

**Title:** Continuation of an Investigation into the Anaerobic Intrinsic Bioremediation of Whole Gasoline

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**Research Category:** Intrinsic bioremediation/natural attenuation

**Project Period:** March 1, 2001 to February 28, 2002 (N/C Ext. to August 31, 2002)

**Objectives of the Research Project:** The primary objective of this project was to investigate the ability of microorganisms from contaminated aquifers to degrade a variety of hydrocarbon molecules under different electron accepting conditions. Specifically, the degradation of several alicyclic compounds with either sulfate or carbon dioxide as electron acceptor was examined. In addition, the ability of hydrocarbons in several crude oils to stimulate sulfate reduction and methanogenesis was examined. Compounds contained in the oils were noted for their relative susceptibility to anaerobic microbial decay and the preferred electron acceptor was similarly examined.

**Summary of Findings:** Petroleum mixtures are the primary feedstock meeting the energy demands of the world. Crude oil production in the United States is approaching 1 billion liters per day with worldwide production more than an order of magnitude greater. The extraction, transport, and storage of petroleum-based fuels have resulted in numerous contamination episodes around the globe. Environmental insults from contamination episodes occur as a result of marine accidents, pipeline breaches, and unknown numbers of small-scale releases associated with day-to-day exploration and distribution activities. Indeed, it is estimated that over 400,000 underground storage tanks in the United States are leaking hydrocarbons into the environment. While various fates exist for hydrocarbons in the environment, one of the most promising is microbial degradation to innocuous products. Indeed, microbial activity is the only route for complete mineralization of pollutant chemicals in situ. Microbial degradation of hydrocarbons with molecular oxygen as electron acceptor has been studied previously, and various aspects of aerobic biodegradative metabolism have been described. However, many aquifers and sedimentary environments contaminated with hydrocarbons are limited for oxygen. Thus, the resident microorganisms must use alternate electron acceptors when oxidizing the reduced molecules they encounter. Sulfate or carbon dioxide is typically available as electron acceptor in many environments and several genera of microorganisms are able to utilize these molecules as electron acceptors. Thus, microbial degradation of various hydrocarbon molecules was examined in the presence of either sulfate or carbon dioxide. Since alkanes, cycloalkanes, and various aromatic compounds can constitute up to 98% of the mass of crude oil, laboratory incubations containing

compounds representative of these classes were examined for their susceptibility to microbial attack.

Sediment from a gas condensate contaminated aquifer was used as the inoculum in laboratory incubations in which gasoline was used as the substrate. The gasoline mixture contained several alicyclic hydrocarbons allowing us to assess the ability of the aquifer microorganisms to degrade individual compounds. Because of solubility differences, various methods were necessary to quantitate hydrocarbon depletion. The fate of the following compounds was followed by analysis of the headspace composition: cyclopentene, methylcyclopentene, methylcyclopentane, cyclohexane, and methylcyclohexane. In the presence of sulfate, all compounds listed above were degraded without a lag and to levels below the detection limit within 100 days. Biodegradation under methanogenic conditions also occurred for the compounds as well although to a lesser extent. The gasoline mixture also contained five C<sub>2</sub>-substituted cyclopentanes that were examined by purge and trap. The results were similar in that incubations containing sulfate were able to degrade the C<sub>2</sub>-substituted compounds to a greater extent and rate than samples incubated under methanogenic conditions.

In a separate experiment, the alicyclic composition of the natural gas condensate was also examined after 200 days of incubation. Similar biodegradation profiles were observed. Generally, the naturally occurring alicyclic compounds were more amenable to decay when sulfate was present as electron acceptor. Four isomers of dimethylcyclopentane were present in the gas condensate. Interestingly, the four isomers showed a different degree of susceptibility to microbial attack. None of the isomers were degraded in the absence of sulfate. Similarly, five dimethylcyclohexane isomers were identified in our incubations and demonstrated a dramatic preference for biodegradation with only the *cis*-1,3-dimethylcyclohexane isomer showing substantial degradation over the incubation period. We believe this is the first laboratory report demonstrating the anaerobic biodegradation of alicyclic hydrocarbons. These findings suggest that similar to aerobic systems, complex anaerobic microbial communities exist and are active in hydrocarbon contaminated environments. These anaerobic communities appear to be only slightly less metabolically diverse in terms of the range of alicyclic hydrocarbons that can be degraded.

Sediment from the gas condensate contaminate aquifer was used as an inoculum to assess the ability of microorganisms to degrade two crude oils under methanogenic and sulfate reducing conditions in the laboratory. Alaska North Slope (ANS) crude oil stimulated methane production and sulfate consumption in sediment incubations. GC analysis revealed that the entire n-alkane fraction of the ANS oil was consumed under both electron accepting conditions. In addition, naphthalene, 2-methylnaphthalene, and 2-ethylnaphthalene were also naturally present and degraded under sulfate reducing but not methanogenic conditions. A second crude oil, Alba is naturally depleted in n-alkanes was used in similar incubations of sediment from the contaminate site. Methanogenesis and sulfate reduction were also stimulated in the presence of the Alba oil but to a lesser extent. Similarly, polyaromatic hydrocarbons in Alba oil, including several naphthalenes, were degraded but only if sulfate was included as electron acceptor. These findings

indicate that n-alkanes are relatively labile under either sulfate reducing or methanogenic conditions. Thus, in most cases the in situ biodegradation of the n-alkane fraction of crude oil should not be limited by electron acceptor availability. However, polycyclic aromatic hydrocarbons are relatively more recalcitrant and their degradation appears to be dependent on the presence of sulfate. This information should be useful for assessing the limits of crude oil biodegradation in terrestrial environments and in making predictions regarding the capacity of the resident microflora to mitigate the contamination levels of particular pollutants.

Sediment and groundwater from the gas condensate contaminated aquifer was used to develop an enrichment capable of degrading ethylcyclopentane (ECP) with sulfate as electron acceptor. ECP was degraded with the consumption of a stoichiometric amount of sulfate. Several putative intermediates of ECP metabolism were identified by gas chromatography/mass spectrometry (GCMS). These intermediates were identified by GCMS and used to propose a pathway for the anaerobic biodegradation of ECP in the sulfate-reducing enrichment culture. The pathway appears to be initiated by an anaerobic fumarate addition mechanism. Fumarate addition has been identified as the mechanism by which several other hydrocarbons are degraded, including alkylbenzenes, alkanes, methyl-naphthalene and toluene. Ethylcyclopentylsuccinic acid was one of the metabolites identified in enrichment cultures degrading ECP. Interestingly, a similar putative metabolite was found in all contaminated groundwater samples obtained from the gas condensate contaminated aquifer from which the enrichment was developed. This metabolite could be the result of biodegradation of other another alicyclic compound present in the aquifer. However, ethylcyclopentylsuccinic acid was not detected in groundwater samples taken from several background locations. Thus, these results suggest that alicyclic hydrocarbons like ECP could be subject to biodegradation by anaerobic activation mechanisms only in the contaminated portion of the aquifer. This suggests that these metabolites could be used as indicators of in situ biodegradation extending our knowledge of the biodegradative capacities of native populations at hydrocarbon-contaminated sites.

Analysis of the microbial community in the ECP degrading enrichment was analyzed by denaturing gradient gel electrophoresis (DGGE). At least three different organisms were identified base on sequence analysis of 550 base pairs of the 16S rRNA gene. The organisms are related to the genera *Syntrophobacter*, *Desulfotomaculum*, and the *Cytophaga-Flexibacter-Bacteroides* group. The sequence belonging to the *Bacteroides* group is closely related to an uncultured clone obtained from groundwater contaminated with oil from an underground storage cavity. The genus *Syntrophobacter* is comprised of organisms that are best known for their ability to degrade fatty acids and other small organic molecules by syntrophic association with a hydrogen consuming organism or using sulfate as electron acceptor. This organism was isolated in pure culture and did not demonstrate the ability to degrade ECP. However, the isolate could degrade several short chain organic acids with sulfate as electron acceptor, suggesting its role in the enrichment is to oxidize low-molecular-weight fatty acids. Finally, the organisms in the genus *Desulfotomaculum* are not known for their ability to degrade hydrocarbons but they do oxidize aliphatic monocarboxylic and dicarboxylic acids coupled to sulfate reduction.

These results support the suggestion that the *Desulfotomaculum* sp. is responsible for the degradation of ECP.

**Publications/Presentations:**

Rios-Hernandez, L. A., Gieg, L. M., and Suflita, J. M. Biodegradation of an Alicyclic Hydrocarbon by a Sulfate-Reducing Enrichment from a Gas Condensate-Contaminated Aquifer. (2003) *Appl. Environ. Microbiol.* 69 (1), 434-443

Townsend, G. T., Prince, R. C., and Suflita, J. M. The Anaerobic Oxidation of Crude Oil Hydrocarbons by the Resident Microorganisms of a Contaminated Anoxic Aquifer. In preparation.

Townsend, G. T., Prince, R. C., and Suflita, J. M. Anaerobic Biodegradation of Alicyclic Constituents of Gasoline and Natural Gas Condensate by Bacteria from an Anoxic Aquifer. In preparation.

**Supplemental Keywords:**

groundwater, hydrocarbons, biodegradation, alicyclic, alkanes, sulfate reduction, methanogenesis, ethylcyclopentane, anaerobic, community analysis, denaturing gradient gel electrophoresis, gas chromatography/mass spectrometry (GCMS), PAH's, bacteria, bioremediation, pathway, intermediates, microbial ecology, aquifer, oil, gas condensate, contamination, gasoline, fumarate addition