



Investigation of microbial population biology during the degradation of organic contaminant mixtures
by Eric Anthony Kern

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of
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Abstract:

Spills of contaminants such as oil, diesel, gasoline, and chlorinated solvents result in the introduction complex mixtures of organic contaminants to the environment. I sought to discover relationships between complex mixtures of organic contaminants and niche diversity of contaminant-degrading microorganisms and to determine how the interactions of nonpolar organic contaminants within mixtures influence microbial community structure and function. Denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rRNA gene fragments was combined with traditional cultivation to investigate whether the changing composition of hydrocarbon mixtures could be related to changes in microbial populations that might represent distinct functional groups. Microbial communities associated with the biodegradation of crude oil and a 4-compound hydrocarbon mixture underwent similar patterns of succession based on DGGE analysis, suggesting that different bacterial populations appeared to be associated with the degradation of n- and isoprenoid-alkanes. Since both n- and isoprenoid-alkanes are important nonaqueous phase liquids (NAPLs) that govern the partitioning of other contaminants (e.g. aromatics) in crude oil, enrichments were conducted with phenanthrene partitioned into heptadecane, pristane and a 1:1 mixture (v:v) of heptadecane and pristane. Analysis of these enrichments indicated that the presence of heptadecane as a NAPL results in different kinetics of phenanthrene degradation and community structure in comparison to enrichments with phenanthrene partitioned into pristane. DGGE analyses were also applied to the study of methyl tert-butyl ether (MTBE)-degrading bacteria from a gasoline-contaminated aquifer (Ronan, MT). A consortium was cultivated that could utilize MTBE as a sole carbon and energy source and two MTBE-degrading bacteria were isolated from this consortium. Molecular analyses of the consortium combined with physiological characterization of the isolates suggested that microorganisms in the consortium may be more fit for MTBE degradation than the cultivated isolates. These findings indicate that contaminant mixture complexity may be important in determining microbial niche diversity, which in turn drives selection of contaminant-degrading microbial communities. In addition, describing and explaining the microbial population biology of contaminant degradation is critical to achieving a predictive knowledge of contaminant fate and transport in situ.

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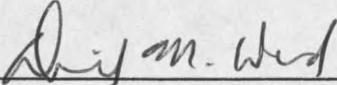
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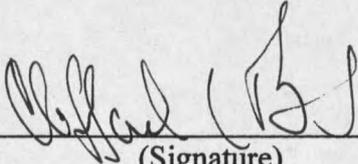
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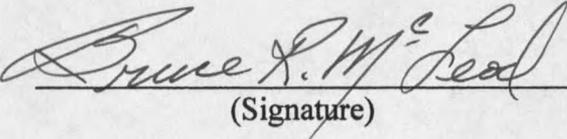
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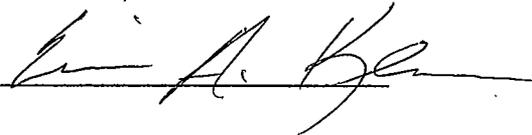
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ABSTRACT

Spills of contaminants such as oil, diesel, gasoline, and chlorinated solvents result in the introduction of complex mixtures of organic contaminants to the environment. I sought to discover relationships between complex mixtures of organic contaminants and niche diversity of contaminant-degrading microorganisms and to determine how the interactions of nonpolar organic contaminants within mixtures influence microbial community structure and function. Denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rRNA gene fragments was combined with traditional cultivation to investigate whether the changing composition of hydrocarbon mixtures could be related to changes in microbial populations that might represent distinct functional groups. Microbial communities associated with the biodegradation of crude oil and a 4-compound hydrocarbon mixture underwent similar patterns of succession based on DGGE analysis, suggesting that different bacterial populations appeared to be associated with the degradation of n- and isoprenoid-alkanes. Since both n- and isoprenoid-alkanes are important nonaqueous phase liquids (NAPLs) that govern the partitioning of other contaminants (e.g. aromatics) in crude oil, enrichments were conducted with phenanthrene partitioned into heptadecane, pristane and a 1:1 mixture (v:v) of heptadecane and pristane. Analysis of these enrichments indicated that the presence of heptadecane as a NAPL results in different kinetics of phenanthrene degradation and community structure in comparison to enrichments with phenanthrene partitioned into pristane. DGGE analyses were also applied to the study of methyl tert-butyl ether (MTBE)-degrading bacteria from a gasoline-contaminated aquifer (Ronan, MT). A consortium was cultivated that could utilize MTBE as a sole carbon and energy source and two MTBE-degrading bacteria were isolated from this consortium. Molecular analyses of the consortium combined with physiological characterization of the isolates suggested that microorganisms in the consortium may be more fit for MTBE degradation than the cultivated isolates. These findings indicate that contaminant mixture complexity may be important in determining microbial niche diversity, which in turn drives selection of contaminant-degrading microbial communities. In addition, describing and explaining the microbial population biology of contaminant degradation is critical to achieving a predictive knowledge of contaminant fate and transport in situ.

CHAPTER 1

INVESTIGATION OF MICROBIAL POPULATION BIOLOGY DURING THE
DEGRADATION OF ORGANIC CONTAMINANT MIXTURES: INTRODUCTIONGoals of Ecology

"Explanation, description, prediction and control". This passage describes the goals of ecology as defined in a leading text (4). The authors define explanation as "a search for knowledge in the pure scientific tradition" and go on to state that "in order to do this... it is first necessary to describe". The authors were clearly referring to the initial description of basic patterns of ecology within complex systems leading to the development of hypothesis-driven explanations. This approach has led to the discovery of basic patterns of ecology in plant and animal communities that have given insight into such processes as niche determination, competition, biogeography and succession, to name only a few. The elucidation of similar patterns in microbial communities has been hindered by the small size and nondescript morphology of microorganisms. The inability to identify and enumerate microbial populations in situ has historically rendered the description of even basic ecological patterns in microbial communities nearly impossible. Therefore, the physiological characterization of bacterial isolates cultivated from natural environments became the predominant approach for study. However, the laboratory conditions under which microorganisms were cultivated often failed to mimic natural

microenvironments and selected for populations that may not be representative of those important in nature. In fact, less than 1% of microorganisms in nature have been cultivated in the laboratory (1) and many cultivated bacteria may not be numerically important in the environment from which they were selected (56). Scientists have long recognized the existence of cultivation bias (5, 57), in part due to the difficulty of designing cultivation conditions that accurately reflect chemical and physical environments at a scale important to microorganisms. The study of pure cultures did yield a wealth of information on bacterial metabolic diversity and furthered the understanding of niche diversity in microbial ecology. Still, studies of natural systems were limited to process level observations and interpretation at the population or community level generally involved making questionable assumptions. Thus, methodological limitations and subsequent reliance on studying pure cultures to infer patterns of distribution and physiology in microbial communities have resulted in a lack of knowledge of basic principles of the ecology of microorganisms in comparison with those of larger organisms. Indeed, while the related fields of bacterial physiology and genetics are currently at stages of prediction and control, the field of microbial ecology is, in many ways, still at the level of description. Fortunately, the knowledge gained from pure culture analysis resulted in a detailed understanding of microbial genetics and physiology, which led in turn to the development of new tools for the microbial ecologist that permit detailed population-level analyses of microbial communities.

Molecular Analysis of Microbial Communities

With the advent of cultivation-independent nucleic acid- and protein-based molecular analyses for the study of microbial ecology, progress in the field is occurring rapidly. The 16S rRNA gene has been widely employed as a phylogenetic marker to describe microbial diversity due to its conservation among all members of the bacterial, archaeal and eukaryal lineages. The current literature contains many examples of 16S rRNA gene surveys describing microbial diversity in many different environments, such as soils (7, 8), sediments (11, 37), freshwater lakes (3, 26), marine waters (19, 20), hot springs (30, 55) and termite hindguts (33, 39). Studies of microbial diversity are starting to reach beyond simply surveying diversity by attempting to elucidate fundamental patterns of microbial ecology. Molecular techniques of community analysis can provide a high level of resolution to differentiate phenotypically similar organisms that may have very specialized physiologies, further aiding the discovery of patterns of ecology within complex communities. For example, these techniques have been used to elucidate patterns of microbial population distribution along environmental gradients in natural environments (15, 16). Ferris et al. (15) and Ramsing et al. (42) reported the distribution of closely related cyanobacteria along temperature and vertical (e.g. possibly light) gradients in hot spring microbial mats. Field et al. (16) demonstrated different vertical positioning of uncultivated proteobacteria in the marine water column. In addition, experiments by Moore et al. (36) revealed the functional specialization of marine cyanobacterial isolates to high and low light conditions, consistent with patterns of

distribution observed with molecular analyses (14). In turn, the description of microbial distribution along these environmental gradients leads to hypothesis-driven explanations that center around basic ecological principles, for instance adaptive radiation in the studies referred to above (55).

A molecular technique particularly well suited for distribution analysis is denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA genes. DGGE has been widely employed to separate 16S rDNA fragments thereby characterizing the composition of microbial communities in natural environments (38) as well as documenting the changing community structure at the population level in a single environment. As with traditional cultivation, these new molecular tools still have inherent biases (24, 43, 49); however, by combining cultivation-independent analyses with more natural cultivation strategies, microbial ecologists are now in an exciting position to be able to describe basic patterns of ecology in microbial communities. In fact, because of the wealth of information on principles of ecology gleaned from other disciplines, we can begin asking ecological questions and let progress in the fields of plant and animal ecology influence our approaches and interpretations. Multi-dimensional strategies are now available for the study of microbial communities; what remains is a need for a change in approach to accompany the change in methodology.

Microbial Ecology of Contaminant Degradation

The field of bioremediation provides a good example of how approaches based solely on cultivation can be problematic. Many bioremediation strategies have been largely unsuccessful because they attempted to use readily cultivated species that may be more fit in the lab than in situ. For example, a single gram of contaminated soil offers an immense amount of niche diversity, which is reflected by an enormous degree of microbial biodiversity, with estimates up to 4000 genetically distinct types of bacteria/g (53). Contaminants may also be presented in various contexts, such as sorbed to organic matter or mineral surfaces, dissolved in water or in nonaqueous phase liquids (NAPLs) and this may lead to a concomitant variety of highly adapted contaminant-degrading species. Yet, the laboratory conditions under which bacteria are cultivated often do not select for populations that are most fit for contaminant degradation in situ, or even in more controlled ex situ systems. For example, Massol-Deya et al. (35) showed that organisms present in tap water were able to displace inoculated populations in a bioreactor treating aromatic hydrocarbons. A steady state biofilm was developed through the inoculation of three readily cultivated toluene-degrading isolates in a fixed-film reactor treating a mixture of benzene, toluene and *p*-xylene. When the reactor biofilm was challenged with tap water, a new, stable community of aromatic-degrading populations indigenous to the groundwater readily displaced the inoculated populations. In addition, the new community showed enhanced removal of aromatics from the effluent in comparison to the inoculated consortium, further reducing the concentration of toluene

in the effluent from $0.140 \text{ mg liter}^{-1}$ to $0.063 \text{ mg liter}^{-1}$ for 98% removal. The rapid displacement of an established contaminant-degrading biofilm (in a controlled reactor environment) by organisms present in tap water reflects our inability to select organisms most fit for contaminant degradation in more complex systems. While the selection of inappropriate contaminant-degrading bacteria (through unnatural cultivation strategies) and competition contribute greatly to the failure of inoculated organisms to enhance contaminant degradation in situ, other factors such as competition for limiting nutrients (48) or other carbon sources (21), predation (41), pH, salinity and temperature may also limit the activity of inoculated populations.

The field of bioremediation is certainly in a descriptive phase with regard to understanding basic ecological factors governing microbial population dynamics in contaminant microenvironments. The use of molecular analyses and more natural cultivation strategies has revealed the importance of microbial population biology in contaminant degradation. For example, the vast majority of polyaromatic hydrocarbon (PAH)-degrading bacteria described in the literature have been cultivated in either mineral salts enrichments with individual PAHs in the aqueous phase (6, 23, 31) or as colonies on solidified medium with PAHs sprayed on the plate surface as crystals (25, 32). These enrichment and isolation strategies may fail to select for organisms that have evolved specialized means to utilize contaminants sorbed to solid phases, a characteristic necessary for contaminant degradation in situ. Recently, several studies have described the differential selection of populations based on the sorption of phenanthrene to solid phases that sequestered phenanthrene to varying degrees (10, 18, 22, 50). Even though

these studies provide an extremely simple representation of a natural contaminant microenvironment (i.e. one sorptive phase and one contaminant), they revealed the existence of functionally specialized populations that traditional cultivation strategies may fail to detect. Additionally, if population selection in these systems is based on differences in phenanthrene bioavailability rather than differences in surface chemistry of the solids, we are guided to other fundamental questions regarding the physiology and ecology of contaminant-degrading populations. Physiological questions may center around explaining cellular mechanisms of enhancing phenanthrene desorption or differences in rates of phenanthrene degradation from various sorptive phases. In contrast, ecological questions may seek to explain the minimum threshold of niche differentiation required for either stable coexistence of contaminant-degrading populations or interspecific competition for the sorbed phase contaminant. The combination of proximal (e.g. physiological) and ultimate (e.g. ecological) explanations is necessary to achieve a predictive knowledge of contaminant degradation in situ. These types of approaches will increase our knowledge of both physiological specialization and ecological distribution of contaminant-degrading populations.

Microbial Degradation of Contaminant Mixtures

Contaminant spills often result in the introduction of complex mixtures of compounds into the environment (e.g. polychlorinated biphenyls, gasoline, diesel and creosote) yet the influence of these mixtures on the structure and function of

contaminant-degrading communities is unknown. Perhaps the increasing complexity of contaminant mixtures is reflected by an increase in niche diversity and subsequently higher diversity of contaminant-degrading microorganisms. Crude oil degradation provides an excellent example of how mixture complexity might relate to population dynamics. Crude oil is a complex hydrocarbon mixture consisting of alkanes (normal and isoprenoid), aromatics (varying in ring number and substitution), asphaltenes (phenols, fatty acids, ketones, esters and porphyrins) and resins (pyridines, quinolines, carbazoles, sulfoxides and amides) (12). Crude oil degradation has been shown to occur in nearly every environment in which it has been investigated. Reports of hydrocarbon degradation (and the presence of hydrocarbon-degrading microorganisms) in soils (44), freshwater sediments (27), marine sediments (51), freshwater (2, 28) and marine waters (58) are widespread in the literature. While the composition of crude oil varies with the physiochemical characteristics of the environment to which it is introduced, a common geochemical pattern of sequential contaminant disappearance has been consistently observed following release into natural systems. Disappearance of crude oil components usually follows a trend of n-alkanes > isoprenoids > low molecular weight aromatics > high molecular weight aromatics > asphaltenes and resins (29, 54). While observations of this geochemical pattern are widespread, the basis for this sequential disappearance remains undetermined. Foght et al. (17) reported that isolates with the capacity for n-alkane degradation could not degrade phenanthrene and isolates with the capacity for phenanthrene degradation could not degrade n-alkanes, suggesting that different microbial populations were specialized to degrade individual classes of hydrocarbons.

More recently, bacteria have been cultivated with the capacity to degrade a broad variety of hydrocarbons, including both n-alkanes and PAHs (46). The interpretation of the structure and function of contaminant-degrading communities based on inferring physiological characteristics of cultivated microorganisms is insufficient to accurately describe patterns of ecology during the degradation of contaminant mixtures. However, the molecular tools described above can provide a means of correlating changing mixture chemistry with population dynamics.

Elucidating factors governing the sequential degradation of components in crude oil may provide insight into understanding the degradation of other hydrocarbon mixtures, such as coal tar, diesel and gasoline. Therefore, in addition to crude oil, other aspects of contaminant mixture degradation investigated in this thesis included the influence of NAPLs on the kinetics of contaminant degradation and population selection. NAPLs are important in partitioning very nonpolar constituents of hydrocarbon mixtures and have been shown to limit their degradation (13, 40). For example, in crude oil, n-alkanes and isoprenoids represent the primary NAPL phases that partition many of the aromatic and resin constituents of the mixture. While degradation rates are reduced when contaminants are partitioned into NAPLs, they often exceed rates of spontaneous contaminant partitioning into the aqueous phase, suggesting that factors other than mass transfer are important in the degradation of contaminants partitioned into NAPLs. Therefore, understanding the influence of microbial population selection in these systems may lead to a stronger predictive knowledge of the fate of contaminant mixtures in natural environments.

The addition of methyl *tert*-butyl ether (MTBE) to gasoline has resulted in a very novel contaminant mixture. MTBE is a synthetically produced chemical added to gasoline at concentrations up to 15% (v/v) to reduce emissions and enhance octane. Largely due to gasoline released from leaking underground storage tanks, MTBE is among the most common contaminants of urban aquifers in the United States (47), yet the environmental fate and transport of the MTBE-gasoline mixture is not well understood. In contrast to gasoline hydrocarbons, MTBE has a relatively low octanol-water partition coefficient ($\log K_{ow} = 0.94$ to 1.16) and a high aqueous solubility ($\sim 50,000$ mg/L at 25°C) resulting in minimal sorption to soil (34, 45) and enhanced mobility in the subsurface environment. Such differences in chemistry and transport will almost certainly influence the selection and activity of MTBE-degrading bacteria. Currently, very few bacteria have been isolated that have the capacity to utilize MTBE as a carbon and energy source and little is known about microorganisms responsible for MTBE degradation in gasoline-contaminated aquifers. Therefore, the study of microbial population dynamics in gasoline-contaminated aquifers must be preceded by the identification and functional characterization of MTBE-degrading populations.

Nearly half a century of research on the microbial degradation of hydrocarbons has revealed the ubiquity of hydrocarbon-degrading microorganisms and environmental factors governing crude oil degradation. Yet, even with a contaminant mixture as well studied as crude oil, basic ecological questions important in predicting and controlling the fate and transport of crude oil in natural environments still remain. For example, is the diversity of contaminant microenvironments reflected by an equally diverse range of

microbial niches in a complex contaminant mixture? If so, what factors govern the breadth of niche availability for contaminant-degrading microorganisms? Answering these questions will not only increase our ability to predict the fate of crude oil in natural environments, but may increase our understanding of the basic ecological principles that govern the degradation of other contaminant mixtures.

Objectives, Rationales and Experiments

The objective of this thesis is to understand the role of microbial population biology (defined here as the study of ecologically distinct populations) in the degradation of hydrocarbons within contaminant mixtures. While the mixtures range from crude oil to reformulated gasoline, the central goal is to describe the response of contaminant-degrading communities to chemically dynamic contaminant environments. The existing body of literature on the particular mixtures studied largely governs the types of questions I have attempted to investigate. For example, the immense amount of literature on crude oil degradation and the physiology of hydrocarbon-degrading bacteria defines a specific question in studying how changing mixtures affect crude oil-degrading communities. In contrast, much less is known about the degradation of contaminants partitioned into NAPL environments, with regard to mechanisms of utilization and selection of contaminant-degrading bacteria. In the last study my work was more descriptive. Since very little is known about the physiology or ubiquity of MTBE-

degrading bacteria, the study of reformulated gasoline mixtures must be preceded by basic studies of MTBE-degrading bacteria.

In all experiments, DGGE analysis of PCR-amplified 16S rDNA fragments was combined with traditional cultivation strategies to elucidate functional contaminant-degrading populations. While the 16S rRNA gene provides valuable phylogenetic information, other approaches must be used in conjunction with this technique to assess functional specialization of populations within a community. The enormous biodiversity of the soil environment necessitated the use of an enrichment strategy to simplify the system and facilitate the observation of basic patterns of microbial population biology during contaminant mixture degradation. The use of an enrichment strategy, rather than soil columns or batch reactors, will undoubtedly affect the selection of specific contaminant-degrading populations. However, it is the *pattern* I seek to elucidate, not the specific species selected, as the pattern may reveal hypotheses about possible mechanisms of population selection based on the changing contaminant microenvironment. While some view enrichments as merely a "*mixed culture [of unknown provenance] on its way to becoming a pure culture*" (9), Beijerinck, one of the inventors of enrichment culture, considered enrichments as systems in which to study variation relative to environmental conditions (52). Hence they are suitable for studying how populations may vary in relation to changing chemical environments. Therefore, with an understanding of the biases inherent in my approach, I sought to observe basic patterns of microbial population biology in my enrichments that are also representative of natural environments. In addition to the characterization of contaminant-degrading

populations, detailed chemical analyses of contaminant-degrading enrichments were performed to determine degradation kinetics and describe the changing chemistry of contaminant mixtures. By comparing patterns of population dynamics with patterns of contaminant chemistry, I hoped to describe trends of microbial population biology that correlated with contaminant degradation activity. Admittedly, this type of approach may be insufficient for identifying all relevant populations and respective physiological mechanisms of contaminant utilization in a mixture environment. However, understanding the basic patterns of microbial population biology will lead to hypothesis-directed approaches to elucidate specific mechanisms in the degradation of nonpolar contaminant mixtures.

Specific chapters of the thesis are introduced in the following sections.

Chapter 2: Crude Oil and Synthetic Mixture Degradation

This study centered around understanding microbial population dynamics during the degradation of a complex contaminant mixture. Using crude oil as a model mixture, I tried to determine whether the sequential disappearance of hydrocarbons during crude oil degradation was the result of a limited number of "generalist" species with broad metabolic capacities or a succession of populations specialized to individual contaminant classes. If a succession of "specialist" populations is indeed the case, I would hypothesize that increases in contaminant mixture complexity should result in increased niche diversity and biodiversity of contaminant-degrading microorganisms. Soil-derived enrichments were constructed with hydrocarbon environments of varying complexity,

including crude oil, a simpler synthetic hydrocarbon mixture and individual hydrocarbons as substrates and patterns of hydrocarbon degradation were compared with patterns of selection of contaminant-degrading populations assayed by DGGE and cultivation. The results indicate a succession of distinct microbial populations, representing separate functional groups of n-alkane and isoprenoid degraders, governing the degradation of these components in complex hydrocarbon mixtures.

Chapter 3: Partitioning Phenanthrene in Different NAPLs

Results presented in Chapter 2 indicated that the degradation of n-alkanes and isoprenoids in crude oil occurs via the selection of functionally unique microbial populations. Recent reports in the literature indicate that NAPLs are important in controlling rates of microbial degradation of nonpolar contaminants, yet the effect of different NAPLs on the ecology of contaminant-degrading microorganisms has not been investigated. Since n-alkanes and isoprenoids partition aromatics and other nonpolar constituents of crude oil, I investigated the influence of partitioning a model aromatic contaminant (phenanthrene) in these NAPL phases on the selection of contaminant-degrading populations. A series of enrichments were constructed with phenanthrene partitioned into heptadecane, pristane, a 1:1 (v/v) mixture of heptadecane and pristane and 2,2,4,4,6,8,8-heptamethylnonane, a reportedly nondegradable NAPL. As described above, enrichments were inoculated with an agricultural soil and selection of functional hydrocarbon-degrading populations was monitored by DGGE and cultivation. The influence of the different NAPLs on phenanthrene degradation was determined by

monitoring the degradation of both the NAPLs and phenanthrene over time. Results indicated that the different NAPLs affect both the kinetics of phenanthrene degradation and microbial community dynamics in the contaminant-degrading enrichments.

Chapter 4: Identification of Populations Important in MTBE Degradation

The focus of this study was to identify functional MTBE-degrading bacteria in soils and aquifer materials sampled from a gasoline-contaminated aquifer. This study differs in character from chapters 2 and 3, as it originated from a multidisciplinary class project and evolved into an independent study, a part of which fit this thesis as another application of using molecular tools to study the microbial ecology of contaminant degradation. The study site for this research was a gasoline fueling station (Ronan, MT) where an underground storage tank had released an estimated 40,000 L of gasoline over a one-year period. A matrix of enrichments was constructed and amended with ^{14}C -MTBE to assay for MTBE degradation in soil slurries. A consortium was cultivated that could degrade MTBE as the sole carbon and energy source. The consortium and isolates cultivated from it were characterized with respect to kinetics of MTBE-degradation and molecular identification of bacterial members of the consortium. The pattern of MTBE degradation in the consortium prompted an experiment in which the dependence of MTBE degradation on cell density was determined. The resulting observation of a quorum sensing-like pattern may have broad implications for the fate of reformulated gasoline in natural environments.

Literature Cited

1. **Amann, R. I., W. Ludwig, and K. H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev* **59**:143-169.
2. **Atlas, R. M., E. A. Schonfield, F. A. Morelli, and R. E. Cameron.** 1976. Interactions of microorganisms and petroleum in the Arctic. *Environ Pollut* **10**:35-44.
3. **Bahr, M., J. E. Hobbie, and M. L. Sogin.** 1996. Bacterial diversity in an arctic lake: a freshwater SAR11 cluster. *Aquatic Microbial Ecology* **11**:271-277.
4. **Begon, M., J. L. Harper, and C. R. Townsend.** 1996. *Ecology: individuals, populations and communities*, Third. Ed. Blackwell Science Ltd. Cambridge, MA.
5. **Beijerinck, M. W.** 1913. *De infusies en de ontdekking der bacteriën*. Johannes MÅller, Amsterdam.
6. **Boldrin, B., A. Tiehm, and C. Fritzsche.** 1993. Degradation of phenanthrene, flourene, flouranthene, and pyrene by a *Mycobacterium* sp. *Appl Environ Microbiol* **59**:1927-1930.
7. **Borneman, J., P. Skrock, K. M. O'Sullivan, J. A. Palus, N. G. Rumjanek, J. L. Jansen, J. Nienhuis, and E. W. Triplett.** 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. *Appl Environ Microbiol* **62**:1935-1943.
8. **Borneman, J., and E. W. Triplett.** 1997. Molecular microbial diversity in soils from eastern Amazonia: evidence for unusual microorganisms and microbial populations shifts associated with deforestation. *Appl Environ Microbiol* **63**:2647-2653.
9. **Brock, T. D.** 1987. The study of microorganisms in situ: progress and problems. *Symp Soc Gen Microbiol* **41**:1-17.
10. **Calvillo, Y. M., and M. Alexander.** 1996. Mechanism of microbial utilization of biphenyl sorbed to polyacrylic beads. *Appl Microbiol Biotechnol* **45**:383-390.
11. **Coates, J. D., D. J. Ellis, E. L. Blunt-Harris, C. V. Gaw, E. E. Roden, and D. R. Lovley.** 1998. Recovery of humic-reducing bacteria from a diversity of environments. *Appl Environ Microbiol* **64**:1504-1509.

12. **Colwell, R. R., and J. D. Walker.** 1977. Ecological aspects of microbial degradation of petroleum in the marine environment. *Crit Rev Microbiol* **5**:423-445.
13. **Efroymson, R. A., and M. Alexander.** 1995. Reduced mineralization of low concentrations of phenanthrene because of sequestering in nonaqueous-phase liquids. *Environ. Sci. Technol.* **29**:515-521.
14. **Ferris, M. J., and B. P. Palenik.** 1998. Cyanobacterial community structure changes with depth in an oligotrophic ocean environment. *Nature* **396**:226-228
15. **Ferris, M. J., and D. M. Ward.** 1997. Seasonal distributions of dominant 16S rRNA-defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis. *Appl Environ Microbiol* **63**:1375-1381.
16. **Field, K. G., D. Gordon, T. Wright, M. Rappe, E. Urbach, K. Vergin, and S. J. Giovannoni.** 1997. Diversity and depth-specific distribution of SAR11 cluster rRNA genes from marine planktonic bacteria. *Appl Environ Microbiol* **63**:63-70.
17. **Foght, J. M., P. M. Fedorak, and D. W. S. Westlake.** 1990. Mineralization of [¹⁴C] hexadecane and [¹⁴C] phenanthrene in crude oil: specificity among bacterial isolates. *Can. J. Microbiol.* **36**:169-175.
18. **Friedrich, M., R. J. Grosser, E. A. Kern, W. P. Inskeep, and D. M. Ward.** 2000. Effect of model sorptive phases on phenanthrene biodegradation: molecular analysis of enrichments and isolates suggests selection based on bioavailability. *Appl Environ Microbiol* **66**:2703-2710.
19. **Fuhrman, J. A., K. McCallum, and A. A. Davis.** 1993. Phylogenetic diversity of subsurface marine microbial communities from the Atlantic and Pacific Oceans. *Appl Environ Microbiol* **59**:1294-1302.
20. **Giovannoni, S. J., T. B. Britschgi, C. L. Moyer, and K. G. Field.** 1990. Genetic diversity in Sargasso Sea bacterioplankton. *Nature* **344**:60-63.
21. **Goldstein, R. M., L. M. Mallory, and M. Alexander.** 1985. Reasons for possible failure of inoculation to enhance biodegradation. *Appl Environ Microbiol* **50**:977-83.
22. **Grosser, R. J., M. Friedrich, D. M. Ward, and W. P. Inskeep.** 2000. Effect of model sorptive phases on phenanthrene biodegradation: different enrichment conditions influence bioavailability and selection of phenanthrene-degrading isolates. *Appl Environ Microbiol* **66**:2695-2702.

23. **Grund, E., B. Denecke, and R. Eichenlaub.** 1992. Naphthalene degradation via salicylate and gentisate by *Rhodococcus* sp. strain B4. *Appl Environ Microbiol* **58**:1874-7.
24. **Hansen, M. C., T. Tolker-Nielsen, M. Givskov, and S. Molin.** 1998. Biased 16S rDNA PCR amplification caused by interference from DNA flanking the template region. *FEMS Microbiol.Ecol.* **26**:141-149.
25. **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**:2549-55.
26. **Hiorns, W. D., B. A. Methe, S. A. Nierzwicki-Bauer, and J. P. Zehr.** 1997. Bacterial diversity in Aironack mountain lakes as revealed by 16S rRNA gene sequence. *Appl Environ Microbiol* **63**:2957-2960.
27. **Horowitz, A., and R. M. Atlas.** 1978. Crude oil degradation in the arctic: changes in bacterial populations and oil composition during one year exposure in a model system. *Dev Ind Microbiol* **19**:517-522.
28. **Horowitz, A., and R. M. Atlas.** 1977. Response of microorganisms to an accidental gasoline spillage in an arctic freshwater ecosystem. *Appl Environ Microbiol* **33**:1252-8.
29. **Hostettler, F. D., and K. A. Kvenvolden.** 1994. Geochemical changes in crude oil spilled from the Exxon Valdez supertanker into Prince William Sound, Alaska. *Org. Geochem.* **21**:927-936.
30. **Hugenholtz, P., C. Pitulle, K. L. Hershberger, and N. R. Pace.** 1998. Novel division level bacterial diversity in a Yellowstone hot spring. *J Bacteriol* **180**:366-76.
31. **Kiyohara, H., K. Nagao, K. Kouno, and K. Yano.** 1982. Phenanthrene-degrading phenotype of *Alcaligenes faecalis* AFK2. *Appl Environ Microbiol* **43**:458-61.
32. **Kiyohara, H., K. Nagao, and K. Yana.** 1982. Rapid screen for bacteria degrading water-insoluble, solid hydrocarbons on agar plates. *Appl Environ Microbiol* **43**:454-457.
33. **Lilburn, T. G., T. M. Schmidt, and J. A. Breznak.** 1999. Phylogenetic diversity of termite gut spirochaetes. *Environ Microbiol* **1**:331-45.

34. **Mackay, D., W. Y. Shiu, and K. C. Ma.** 1993. Illustrated Handbook of Physical-Chemical Properties and Environmental Fate for Organic Chemicals, Volume III. Lewis Publishers, Ann Arbor, MI.
35. **Massol-Deya, A., R. Weller, L. Rios-Hernandez, J.-Z. Zhou, and J. M. Tiedje.** 1997. Sucession and convergence of biofilm communities in fixed-film reactors treating aromatic hydrocarbons in groundwater. *Appl Environ Microbiol* **63**:270-276.
36. **Moore, L. R., G. Rocap, and S. W. Chisholm.** 1998. Physiology and molecular phylogeny of coexisting *Prochlorococcus* ecotypes. *Nature* **393**:464-7.
37. **Munson, M. A., D. B. Nedwell, and T. M. Embley.** 1997. Phylogenetic diversity of Archaea in sediment samples from a coastal salt marsh. *Appl Environ Microbiol* **63**:4729-4733.
38. **Muyzer, G.** 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. *Curr Opin Microbiol* **2**:317-22.
39. **Ohkuma, M., and T. Kudo.** 1996. Phylogenetic diversity of the intestinal bacterial community in the termite *Reticulitermes speratus*. *Appl Environ Microbiol* **62**:461-468.
40. **Ortega-Calvo, J. J., I. Birman, and M. Alexander.** 1995. Effect of varying the rate of partitioning of phenanthrene in nonaqueous-phase liquids on biodegradation in soil slurries. *Environ. Sci. Technol.* **29**:2222-2225.
41. **Ramadan, M. A., O. M. el-Tayeb, and M. Alexander.** 1990. Inoculum size as a factor limiting success of inoculation for biodegradation. *Appl Environ Microbiol* **56**:1392-6.
42. **Ramsing, N. B., M. J. Ferris, and D. M. Ward.** 2000. Highly ordered vertical structure of *Synechococcus* populations within the one-millimeter-thick photic zone of a hot spring cyanobacterial mat. *Appl Environ Microbiol* **66**:1038-49.
43. **Reysenbach, A.-L., L. J. Giver, G. S. Wickham, and N. R. Pace.** 1992. Differential amplification of rRNA genes by polymerase chain reaction. *Applied and Environmental Microbiology* **58**:3417-3418.
44. **Sexstone, A. J., and R. M. Atlas.** 1977. Response of populations in arctic tundra soils to crude oil. *Can J Microbiol* **23**:1327-1333.
45. **Shaffer, K. L., and C. G. Uehrin.** 1997. Uptake of methyl tertiary butyl ether (MTBE) by groundwater solids. *Bull. Environ. Contam. Toxicol.* **59**:744-749.

46. **Sotsky, J. B., C. W. Greer, and R. M. Atlas.** 1994. Frequency of genes in aromatic and aliphatic hydrocarbon biodegradation pathways within bacterial populations from Alaskan sediments. *Can J Microbiol* **40**:981-5.
47. **Squillace, P. J., J. S. Zogorski, W. G. Wilber, and C. V. Price.** 1996. Preliminary assessment of the occurrence and possible sources of MTBE in groundwater in the United States, 1993-1994. *Environ Sci Technol* **30**:1721-1730.
48. **Steffenson, W. S., and M. Alexander.** 1995. Role of competition for inorganic nutrients in the biodegradation of mixtures of substrates. *Appl Environ Microbiol* **61**:2859-2862.
49. **Suzuki, M. T., and S. J. Giovannoni.** 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl Environ Microbiol* **62**:625-630.
50. **Tang, W.-C., J. C. White, and M. Alexander.** 1998. Utilization of sorbed compounds by microorganisms specifically isolated for that purpose. *Appl Microbiol Biotechnol* **49**:117-121.
51. **Teal, J. M., K. Burns, and J. Farrington.** 1978. Analyses of aromatic hydrocarbons in intertidal sediments resulting from two spills of No. 2 fuel oil in Buzzard's Bay, Massachusetts. *J Fish Res Board Can* **35**:510-520.
52. **Theunissen, B.** 1995. Martinus William Beijerinck and the beginnings of the "Delft" tradition in microbiology, p. 183-192. *In* P. Bos and B. Theunissen (ed.), *Beijerinck and the Delft School of Microbiology*. Delft University Press, Delft, The Netherlands.
53. **Torsvik, V., J. Goksoyr, and F. L. Daae.** 1990. High diversity in DNA of soil bacteria. *Appl Environ Microbiol* **56**:782-787.
54. **Ward, D. M., R. M. Atlas, P. D. Boehm, and J. A. Calder.** 1980. Microbial biodegradation and chemical evolution of oil from the Amoco spill. *AMBIO* **9**:277-283.
55. **Ward, D. M., M. J. Ferris, S. C. Nold, and M. M. Bateson.** 1998. Microbial biodiversity within hot spring cyanobacterial mat communities: an evolutionary ecology view. *Microbiol Mol Biol Rev* **62**:1353-1370
56. **Ward, D. M., C. M. Santegoeds, S. C. Nold, N. B. Ramsing, M. J. Ferris, and M. M. Bateson.** 1997. Biodiversity within hot spring microbial mat communities: molecular monitoring of enrichment cultures. *Antonie van Leeuwenhoek* **71**:143-150.

57. **Winogradsky, S.** 1949. *Microbiologie du sol, problemes et methodes*. Barneoud Freres, Masson, France.
58. **ZoBell, C. E.** 1969. *Microbial modification of crude oil in the sea*, p. 317-326, *Proceedings, Joint Conference on Prevention and Control of Oil Spills*. API Publishing, New York.

CHAPTER 2

MOLECULAR EVIDENCE FOR ECOLOGICAL SUCCESSION DURING THE
MICROBIAL DEGRADATION OF CRUDE OIL AND SYNTHETIC
HYDROCARBON MIXTURES

Introduction

Contamination spills of polychlorinated biphenyls, gasoline, diesel, creosote and crude oil commonly result in the introduction of complex chemical mixtures into the environment. Following contamination, geochemical analyses often reveal a sequential disappearance of compounds or compound classes within hydrocarbon mixtures. For example, Solano-Serena et al. (29) observed the degradation of aromatics before aliphatics in gasoline-amended enrichments. Using activated sludge as an inoculum for batch enrichments, 88% of the aromatic fraction of gasoline was degraded compared to only 14% and 17% degradation of normal (n-) and branched alkanes over the same 2 day period. An opposite trend of alkane degradation before aromatics has been observed during the degradation of both diesel and separated diesel components (26). A sequential disappearance of n-alkanes before isoprenoid alkanes before aromatics before asphaltenes

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has been repeatedly observed in geochemical analyses of crude oil spills in various environments. For instance, geochemical analyses of crude oil following both the Amoco Cadiz and Exxon Valdez spills revealed in situ transformations following such a pattern (20, 31). While such patterns of sequential disappearance of specific chemical classes within contaminant mixtures have been repeatedly observed, no chemical or biological basis has been elucidated.

Such observations raise the question of whether the sequential loss of specific contaminants within a mixture is the result of "generalist" populations simply changing catabolic pathways to degrade different chemical classes of contaminants or the result of a succession of unique populations specialized to the degradation of individual contaminants (or contaminant classes). Evidence exists to support the hypothesis of specialist populations, as McNaughton et al. (21) used molecular methods to observe temporal microbial population changes among treatments including different nutrient regimes or seeded populations in plots of beach sand that were experimentally contaminated with crude oil. However, because crude oil disappearance was monitored only in terms of degradation rate coefficients of total alkanes and total aromatics, it is difficult to infer functional specialization of selected populations with respect to specific chemical compounds or compound classes. Analysis of cultivated hydrocarbon-degrading isolates has also revealed the possibility for niche specialization based on utilization of hydrocarbon substrates. In a study of 138 hydrocarbon-degrading bacteria isolated from a variety of environments, 39% of the isolates could mineralize hexadecane and 4% could mineralize phenanthrene, however none of the isolates had the capability to

degrade both aliphatic and aromatic hydrocarbon substrates (15). We combined cultivation-independent approaches designed to elucidate population changes associated with chemical changes and metabolic analysis of cultivated isolates to provide new insight into the functional specialization of populations selected during crude oil degradation. If succession of specialist populations is indeed the case, one may hypothesize that contaminant mixture complexity reflects ecological niche complexity and is a factor in regulating the functional diversity among contaminant-degrading microorganisms.

Recently, evidence has been shown for the specialization of microbial populations to degrading contaminants sorbed to solid phases (5, 9, 16, 19, 30). Friedrich et al. (16) found variations in community profiles of microbial populations selected on phenanthrene sorbed to a series of solid phases that restricted bioavailability. Microbes isolated on sorbed-phase phenanthrene were more fit to degrade the sorbed-phase contaminant than those isolated on crystalline phenanthrene (18, 30). Such studies provide evidence for degradative populations that exhibit a high level of specificity to unique sorptive microenvironments. Nonpolar contaminants are commonly found in complex mixtures containing nonaqueous phase liquids (NAPLs), which represent additional microenvironments that can partition contaminants and decrease their bioavailability (11, 12, 27). Consequently, the heterogeneity of physical and chemical environments characteristic of complex contaminant mixtures offers a variety of potential unique niches that may be important in selecting for microbes responsible for degrading contaminant mixture components.

Our work focuses on crude oil as a model contaminant mixture and seeks to understand the relationship between temporally changing contaminant mixture complexity and the selection of contaminant-degrading microbial communities, which might either be generalists or specialists with respect to their metabolic function. In attempting to monitor the selection of contaminant-degrading populations, we used an enrichment strategy to evade the complexity of soil and elucidate basic trends of microbial community change during degradation of hydrocarbon mixtures. A series of enrichments were amended with contaminant mixtures ranging from an extremely complex natural contaminant mixture (e.g. crude oil) to a simpler synthetic four-component hydrocarbon mixture to single hydrocarbons. In this way population shifts associated with changes in the chemical composition of the natural mixture could be related to changes in specific compound classes and compounds. These enrichments were monitored over time and sampled regularly for chemical analysis of the contaminant mixtures and molecular analysis of the enriched microbial populations. Denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rRNA gene fragments was used as a culture-independent method to determine enrichment composition (24). However, we also used traditional cultivation strategies to increase our ability to understand the functional attributes of those cultivated populations that were also detected by molecular means. Using these approaches, we observed community shifts corresponding to discrete changes in the chemistry of complex contaminant mixtures during biodegradation. The trends we observed demonstrate the importance of understanding the functional specialization of microbial populations for understanding

the dynamics of degradation rates of specific components present in natural and synthetic contaminant mixtures.

Materials and Methods

Chemicals

The following hydrocarbons were used in this study: n-heptadecane, n-octadecane and pristane, obtained from Aldrich (Milwaukee, WI); methylene chloride, benzene and hexane, obtained from Fisher Scientific (Pittsburgh, PA); phytane, obtained from Protocol Analytical Supplies (Middlesex, NJ); and crude oil, obtained from Conoco Corp., Billings, MT.

Soil/sediment

Crude oil enrichments were inoculated with sediment taken from a freshwater spring on the campus of Montana State University and an agricultural soil (Arthur Post Research Station, Bozeman, MT). Samples (50 g) of the sediment and soil were extracted with methylene chloride using a soxhlet apparatus for 24 hrs (at 5 min/flush). Gas chromatography/mass spectrometry (GC/MS) analyses of the extracts (see below) indicated that neither the soil nor the sediment contained detectable levels of aliphatic or aromatic hydrocarbon contaminants. Synthetic alkane mixture and single substrate enrichments were inoculated only with the agricultural soil. To inoculate enrichments, 1 g soil or sediment was diluted into 9 mL of a modified soil solution equivalent (SSE) medium designed to represent "typical" concentrations of soil pore water constituents (3,

18). The resulting slurry was mixed well and 100 μ L was added to 200 mL enrichments. This resulted in a 10^{-4} dilution of inocula, a concentration that was empirically determined to be below the baseline for PCR/DGGE-based detection of community DNA.

Enrichment Conditions

Aerobic batch enrichments were conducted in 500 mL culture flasks in triplicate containing 200 mL medium and incubated at 25 \pm 2° C on a rotary shaker. A mineral salts medium containing (per liter) 4 g NaCl, 5 g NH₄Cl, 5 g MgSO₄·7H₂O, 5 g KH₂PO₄ and 5 g NaH₂PO₄·H₂O was used in crude oil-amended enrichments inoculated with the freshwater sediment (1). SSE was used in crude oil-, synthetic mixture- and single hydrocarbon-amended enrichments inoculated with the agricultural soil. In all liquid enrichments with SSE, C:N:P ratios were held constant at 100:15:1. Before introduction into the enrichments, the crude oil was physically weathered for 120 hours under a stream of N₂ (g) to remove the more volatile components of the oil. Both sediment- and soil-derived enrichments were amended with 1% (v/v) weathered crude oil. Subsequent enrichments were conducted with pure alkanes and an alkane mixture containing predominant n- and branched alkanes present in weathered crude oil. The pure compounds and the mixture components were at concentrations that were equal to those measured in the weathered crude oil enrichment flasks (n-heptadecane, 70 mg/L; n-octadecane, 44 mg/L; pristane, 40 mg/L; phytane, 17 mg/L). In addition, control enrichments with no hydrocarbon amendment were inoculated with agricultural soil (in

SSE medium) and sediment (in mineral salts medium). Enrichments were subsampled regularly for molecular analysis of microbial populations and chemical analysis of contaminant mixtures.

Hydrocarbon Separation and Quantification

Alkane and aromatic fractions of the crude oil were extracted and separated by low pressure liquid column chromatography using the method of Hostettler and Kvenvolden (20). For the alkane mixture and single substrate experiments, flasks were vigorously shaken to ensure uniform sampling of NAPL-phase hydrocarbons and 2 mL subsamples were taken for extraction (1:1, v/v) with methylene chloride. A variance of less than 5% was observed between subsamples taken from a single flask at a single timepoint (n=4). All hydrocarbons were quantified using GC/MS analyses. Samples (1 μ L, splitless injection) were analyzed on a Hewlett Packard GC equipped with a DB-5ms column (30 m by 0.25 mm, 0.25 μ m film thickness; J & W Scientific) and a flame ionization detector; ($\text{He}_{2(g)}$ was used as the carrier). For synthetic hydrocarbon analyses (e.g. heptadecane, octadecane, pristane and phytane), the initial column head pressure was 70 Kpascals, however the pressure was ramped with the oven temperature to maintain a constant $\text{He}_{2(g)}$ flow rate. The ramping cycle was as follows: the temperature was held initially at 100°C for 2 min, then increased at 25°C/min to 175°C then at 5°C/min to 240°C. For crude oil analyses, the initial column head pressure was 40 Kpascals and the ramping cycles were as follows: the temperature was held initially at 40°C for 4 min, then increased at 5°C/min to a final temperature of 290°C, which was

held constant for 5 min. Ratios of hydrocarbon abundance are based on absolute peak areas of individual hydrocarbons. All samples were subsequently analyzed on a VG 70E-HF double-focusing magnetic sector MS with a mass resolution of 1500 amu. The source temperature was 200°C with an electron energy of 70 eV and an acceleration potential of 5000 V. The mass range was 45 to 350 for synthetic hydrocarbon samples and 45 to 500 for crude oil samples. The scan rate was 0.50 seconds per mass decade.

Nucleic Acid Extraction, PCR, DGGE and Sequence Analysis

Cell lysis and DNA extraction protocols were based on those of Moré et al. (23). Briefly, cells were lysed by mechanical disruption at 6.5 m/s for 45 s using a beadbeater (Savant Instruments, Bio 101). The crude cell lysates were precipitated with ammonium acetate and the DNA in the resulting supernatant was precipitated with isopropanol. PCR conditions and primers were identical to those previously described (14). The primers used (nucleotide positions 1070 to 1392 based on *E. coli* numbering) have been shown to be highly conserved for numerous bacterial lineages, including the proteobacterial, Gram-positive, cyanobacterial, green nonsulfur and *Cytophagales-Flavobacterium-Bacteriodes* lineages. DGGE conditions were similar to those used by Ferris et al. (14), with the addition of a concentration gradient of acrylamide (6-11%) to enhance band sharpness by reducing diffusion of DNA (8). DGGE bands were purified for sequence analysis by gently pressing a disposable 10 µl plastic pipette tip (Molecular Bio-Products, San Diego, CA) into the center of a band and immersing the tip into a series of previously prepared PCR mixtures containing all reagents except template DNA. This method differs from

other previously described purification strategies in that the band DNA is transferred directly to a PCR reaction and no visible core or gel fragment is sampled. Band purity was confirmed by analyzing the resulting PCR products on a DGGE gel in comparison to the sample from which the band was obtained. Bidirectional sequencing reactions of isolate DNA and DGGE bands were performed using primers 1114f and 1392r and an ABI Prism 310 Genetic Analyzer with a 47 cm capillary (PE Applied Biosystems, Foster City, CA) and Prism BigDye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA). The sequences were aligned using the Sequencher 3.1.1 software program (Gene Codes Corporation, Ann Arbor, Mich.) and compared to sequences deposited in GenBank by performing a BLAST search (2).

Cryostage Scanning Electron Microscopy

Individual octadecane crystals of approximately 3 mm x 5 mm (2-4 mm in thickness) were removed at day 12 from soil-derived enrichments on pure octadecane. Immediately following sampling, the crystals were attached to a pre-frozen, beveled aluminum coupon using O.C.T. compound (Miles Inc., Elkhart, IN). Colloidal graphite was placed on 2 to 3 points along the edges of the sample to minimize sample charging under exposure to the electron beam. The coupons with mounted samples were quickly frozen in liquid nitrogen and introduced into an Oxford CT1500 Cryostation system for coating under high vacuum (< 10 to 2 mbar) to affect sublimation of any surficial water. The sample-bearing coupon was then introduced into the cryostage within the SEM

chamber of a JEOL JSM-6100 scanning microscope for imaging. All images were collected at an accelerator voltage of 15.0 kV and a filament current of 3.2 A.

Isolation of Hydrocarbon-degrading Bacteria

Hydrocarbon-degrading bacteria were isolated from the agricultural soil after serial dilution in SSE by direct plating onto SSE agar plates. The plates were then sprayed with 2% alkane hydrocarbon dissolved in acetone. Each dilution from the series was plated on heptadecane, octadecane, pristane and phytane plates. Individual colonies exhibiting zones of clearing on the respective hydrocarbons were picked from the highest dilution plates and transferred to 25% YEPG (0.05 g of yeast extract liter⁻¹, 0.25 g of peptone liter⁻¹, 0.5 g of glucose liter⁻¹, and 0.05 g of NH₄NO₃ liter⁻¹) plates. After three transfers on YEPG plates to ensure isolate purity, isolates were again plated on SSE hydrocarbon spray plates and assayed for zones of clearing around individual colonies to confirm degradative ability.

Results

Crude Oil Enrichments

Results of the chemical analysis of crude oil degradation by the freshwater sediment enrichment are shown in Figure 1. After an initial lag time of approximately 14 days, biodegradation of the alkane fraction proceeded most notably with the loss of n-alkanes in preference to isoprenoid alkanes at approximately day 25. At day 42, only

isoprenoid and cyclic alkanes persisted in the alkane fraction of the crude oil. Analysis of individual n-alkanes revealed that these compounds were degraded at approximately the same rate regardless of chain length. The enrichments inoculated with agricultural soil showed the same trend of n-alkane disappearance before isoprenoid alkanes, as indicated by nC_{17} /pristane and nC_{18} /phytane ratios (Figure 2). However, a much shorter lag phase was observed (~ 6 days) in addition to a more rapid degradation of n-alkanes in this enrichment relative to the sediment enrichment. As in the sediment enrichments, no differences were observed with respect to chain length for n-alkane degradation. DGGE analysis of 16S rDNA fragments amplified from the sediment-derived enrichment revealed a temporal pattern of population change that accompanies the changing chemistry of the crude oil (Figure 3). Some bands (e.g., A, C and D) appeared early and persisted throughout the incubation period. Other bands (e.g., B, E and F) appeared early but disappeared at approximately day 25, which corresponded to the disappearance of n-alkanes in the crude oil. A third group of bands appeared only when n-alkanes were largely degraded (e.g., band G appeared at day 25 and bands H and I appeared at day 33). Samples taken from both sediment- and soil-inoculated control enrichments (with no hydrocarbons added) did not yield PCR products at any time point, indicating that cell growth in these enrichments was below the detection limit of our methods. Therefore, the populations detected were responding to the addition of hydrocarbons, which were the sole carbon and energy sources in all other enrichments.

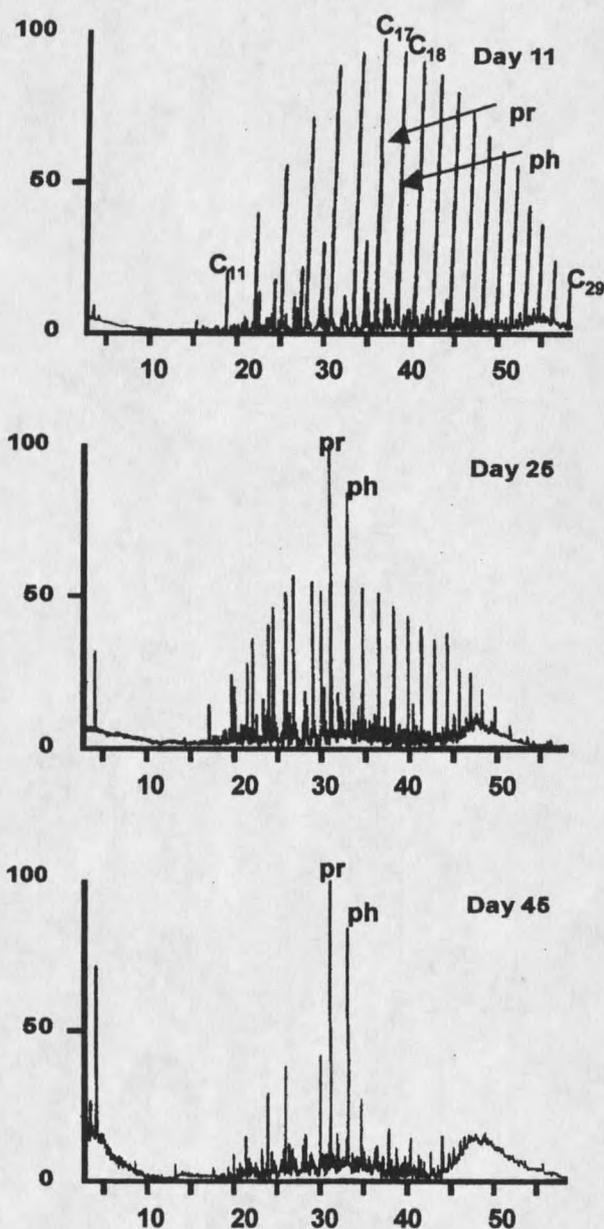


Figure 1. GC/MS analysis of the alkane fraction of a physically weathered crude oil during biodegradation by a freshwater sediment-derived enrichment culture. Major peaks in the day 11 profile are n-alkanes increasing in C₁ increments from n-C₁₁ to n-C₂₉; pristane (pr) and phytane (ph) peaks are shown. Retention times of hydrocarbons in day 11 profile are ~7 min longer than day 25 and day 42 resulting from differences in GC conditions; however, peak identities were confirmed by MS.

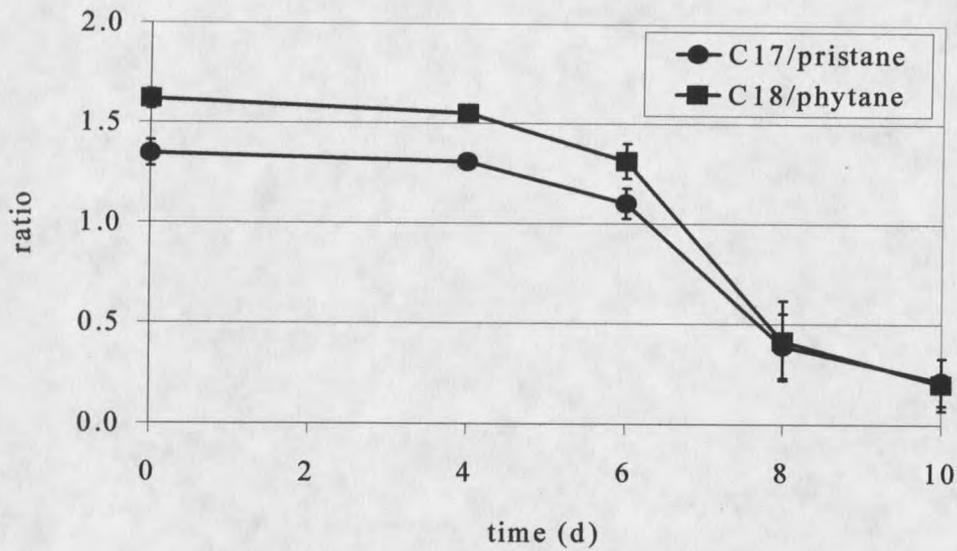


Figure 2. Changing ratios of n-alkanes to branched alkanes during the degradation of crude oil in a soil-derived enrichment culture.

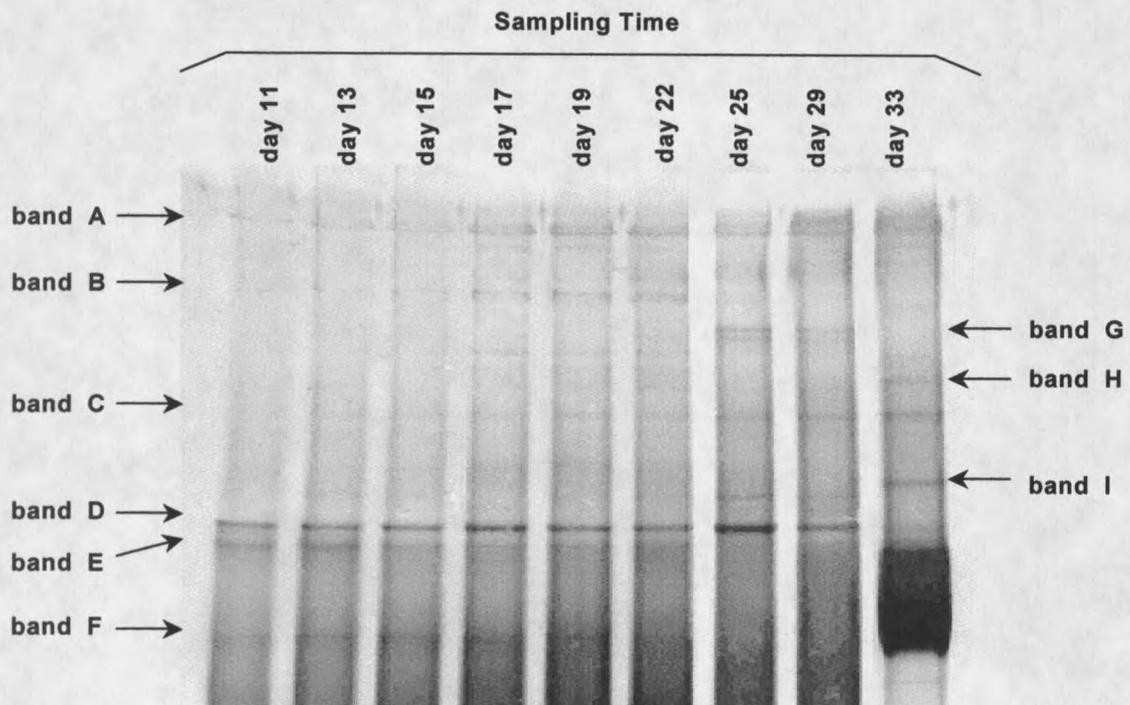


Figure 3. DGGE profiles of a freshwater sediment-derived crude oil-degrading enrichment culture through time. The negative image of the gel photo is shown.

The soil-derived enrichment showed a similar trend of population changes during crude oil degradation (Figure 4), although, based on band migration, different populations were observed in the soil- and sediment-derived enrichments. Band 1 was initially the most intense band in the soil-derived enrichment profile and remained present through day 20. The primary shift in banding pattern in this enrichment occurred at day 8 with the appearance of two new intense bands 5 and 6, corresponding to the point at which isoprenoid alkanes dominated the mixture. Sequence analysis of DGGE bands from soil enrichments revealed that band 1 is contributed by an *Acinetobacter calcoaceticus*-like population, whereas bands 5 and 6 are contributed by *Rhodococcus koreensis*-like populations (Table 1). Bands 2 and 3, which appeared early in the timecourse and remained present through the 20 day enrichment, both show a high degree of sequence similarity (98% and 100%, respectively) to the same nearest relative, *Variovorax* sp. WFF52. Band 4, contributed by a distant relative of *Cytophagales* str. MBIC4147, was present early and disappeared by day 8.

Synthetic Mixture and Pure Hydrocarbon Enrichments

A simpler four-component hydrocarbon enrichment and a series of single hydrocarbon enrichments were also conducted to discern which populations were responsible for the degradation of individual contaminants or contaminant classes during crude oil degradation. The simpler hydrocarbon mixture consisted of heptadecane,

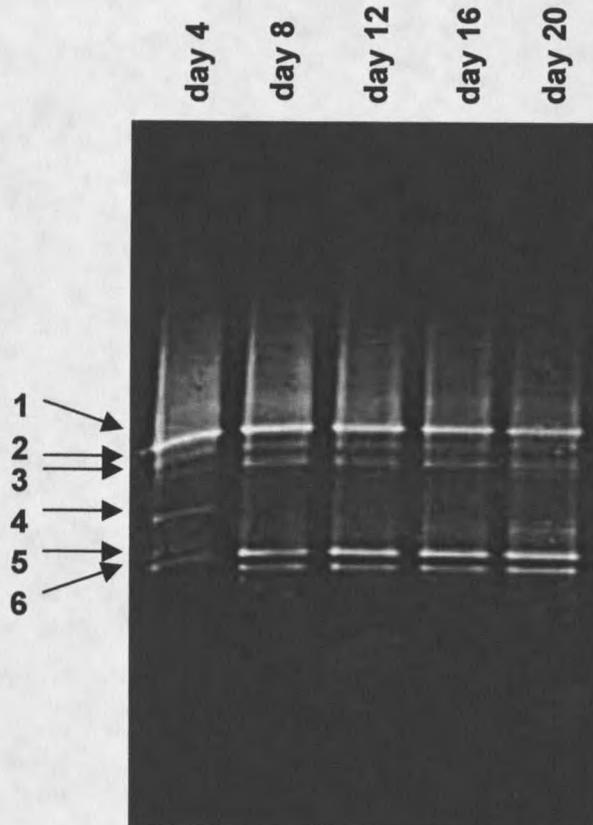


Figure 4. DGGE profiles of a soil-derived crude oil-degrading enrichment culture through time. Numbers correspond to bands whose sequences are described in Table 1.

Table 1. Comparison of DGGE band sequences from soil-derived enrichments and hydrocarbon-degrading isolates cultivated directly from the agricultural soil.

DGGE Band ^a	Isolate ^b	Closest GenBank relative	Percent similarity ^c	Genbank accession number	Hydrocarbon degradation ^d	
					n-alk	iso
Proteobacteria						
1		<i>Acinetobacter calcoaceticus</i>	99	AF159045		
2		<i>Variovorax</i> sp. WFF52	98	AB003627		
3		<i>Variovorax</i> sp. WFF52	100	AB003627		
7		<i>Leptothrix discophora</i>	100	Z18533		
8	m	<i>Bartonella vinsonii</i>	99	U26258		X
	f	<i>Bartonella quintana</i>	96	AJ250247	X	
Cytophagales-Flavobacterium-Bacteroides (CFB) Group						
4		<i>Cytophagales</i> str. MBIC4147	90	ABO22889		
	g	<i>Taxeobacter gelupupurascens</i>	96	Y18836		X
	I	<i>Dyadobacter fermentans</i>	99	AF137029	X	
	h	<i>Cytophaga</i> sp. P1	100	AF260716		X
High G+C Gram-positive bacteria						
5	a	<i>Rhodococcus koreensis</i>	100	AF124342	X	X
6		<i>Rhodococcus koreensis</i>	99	AF124342		
	j	<i>Rhodococcus zopfii</i>	99	AF191343		X
	l	<i>Rhodococcus koreensis</i>	96	AF124342	X	
	b	<i>Streptomyces mirabilis</i>	99	AF112180	X	X
	c	<i>Streptomyces bikiniensis</i>	100	X79851	X	X
	o	<i>Streptomyces bikiniensis</i>	94	X79851	X	
	e	<i>Streptomyces pseudovenezuelae</i>	100	AJ399481	X	X
	p	<i>Streptomyces bicolor</i> str. ISP	100	AJ276569		X
	k	<i>Streptomyces subrutilus</i>	99	X80825	X	
	d	<i>Arthrobacter keyseri</i>	99	AF256196		X
	n	<i>Nocardioides fulvus</i>	99	AF005017	X	

^a Corresponds to marked bands in Figures 4, 6 and 7.

^b Isolates listed on the same line as a corresponding band are identical to that band in the 322 bp region sequenced.

^c Percent similarity between band and/or isolate and the closest GenBank relative (accession numbers shown are those of Genbank relatives).

^d Degradation based on ability of isolates to grow and form zones of clearing on individual hydrocarbon spray plates.

octadecane, pristane and phytane, the most predominant n-alkanes and branched alkanes in our weathered crude oil. These compounds were added to the simple mixture and single hydrocarbon enrichments at concentrations and ratios equivalent to those measured in the weathered crude oil. The degradation of n-alkanes and isoprenoids in the four-component mixture followed a pattern similar to that for the same components of the crude oil enrichments, with n-alkanes degraded preferentially to isoprenoid alkanes (Figure 5a). In single component enrichments, the degradation of heptadecane was rapid following a brief lag phase of 4 days; pristane and phytane were again degraded at slower rates (Figure 5b). Degradation kinetics were not quantified in octadecane enrichments, where the presence of solid phase octadecane prevented representative sampling.

Figure 6 shows DGGE patterns of replicate soil-derived alkane mixture-degrading enrichments. The pattern of temporal population changes with the changing mixture chemistry, including selection of intense bands contributed by *Acinetobacter*- and *Rhodococcus*-like populations (bands 1 and 5), resembled that of the crude oil enrichments. Bands 1,2,3,5 and 6 were 100% identical (in the region analyzed) to those detected in the crude oil-degrading enrichments. Two bands not observed in the crude oil enrichment (e.g., bands 7 and 8, contributed by populations closely related to *Leptothrix discophora* and *Bartonella vinsonii*, respectively; see Table 1) appeared in later stages in one replicate. Interestingly, enrichments with heptadecane or octadecane alone (Figure 7) selected primarily for the *A. calcoaceticus*-like population (band 1) with

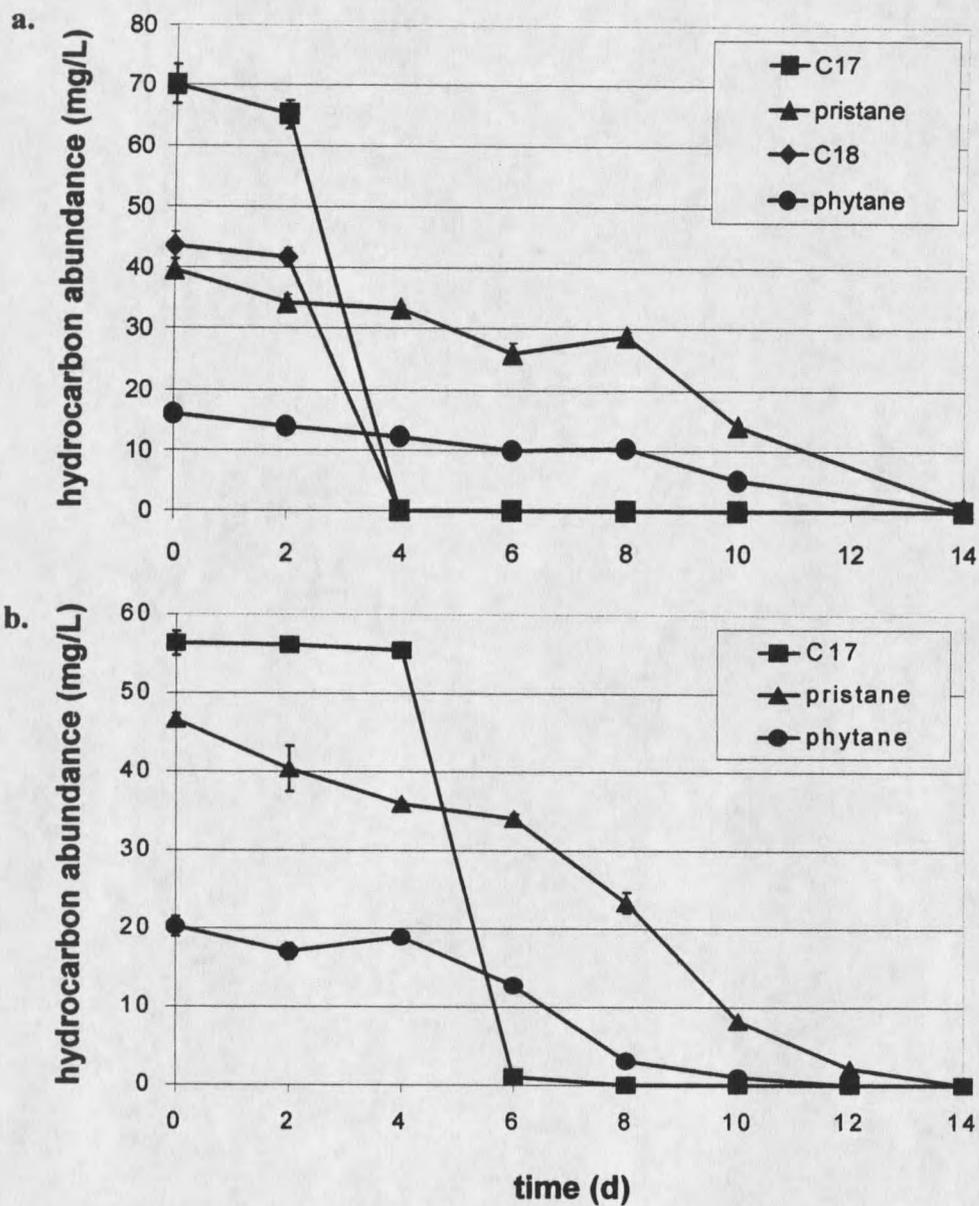


Figure 5. Concentrations of key n-alkanes and branched alkanes in (a) a 4-compound synthetic hydrocarbon mixture or (b) added as single substrates by a soil-derived enrichment culture.

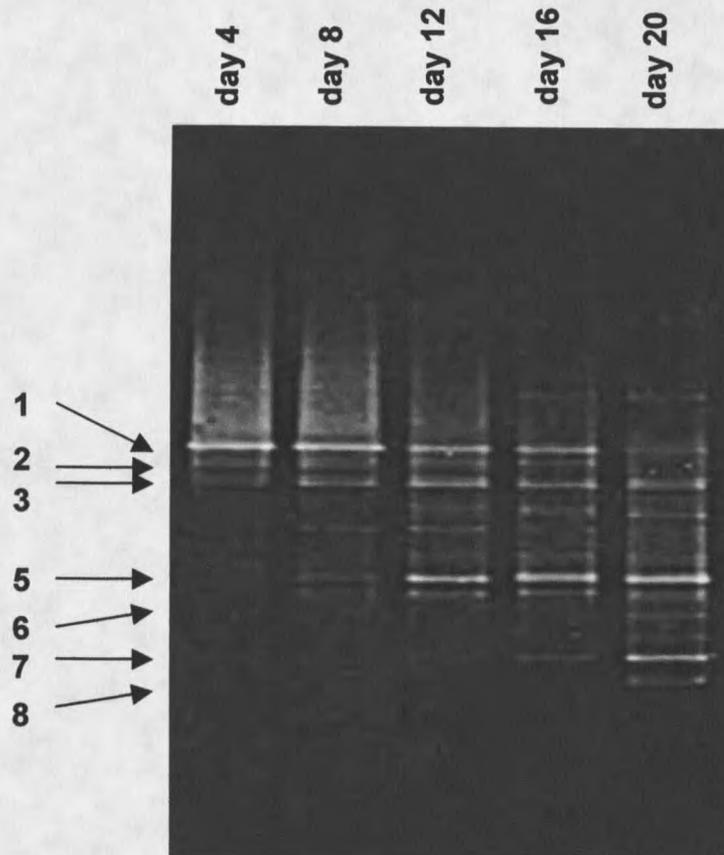


Figure 6. DGGE profiles depicting successional population changes in soil-derived synthetic alkane mixture enrichments. Numbers correspond to bands whose sequences are described in Table 1.

a less intense band corresponding to a *Cytophaga*-like organism (band 4). In one of the two octadecane replicates, one of the *R. koreensis*-like 16S rRNA gene fragments (band 5) was selected in addition to the *A. calcoaceticus*-like population (band 1) and the *Cytophaga*-like organism (band 4). Scanning electron micrographs of octadecane crystals from this enrichment are shown in Figure 8. Cells observed colonizing the octadecane crystal were filamentous with club-shaped ends resembling the pleimorphic characteristics indicative of various cultivated actinomycetes. Both pristane- and phytane-amended enrichments selected for the *R. koreensis*-like populations (bands 5 and 6) as well as the *Cytophaga*-like organism contributing band 4 and many other less intense bands. Sequences corresponding to bands from these enrichments are described in Table 1.

Characterization of Hydrocarbon-degrading Isolates

72 hydrocarbon-degrading isolates were cultivated from a serial dilution of the agricultural soil (Table 1). Of the total, 18 were cultivated on heptadecane, 15 on octadecane, 17 on pristane and 22 on phytane. Sequence analysis of the 16S rRNA gene revealed 16 genetically unique isolates with nearest relatives from proteobacterial, Gram-positive and *Cytophaga-Flavobacterium-Bacteroides* lineages (Table 1). The majority of isolates were Gram-positive bacteria that grouped within the *Rhodococcus* lineage of the high G+C division of Gram-positive bacteria.

A comparison of DGGE band and hydrocarbon-degrading isolate sequences along with hydrocarbon degradation capacity of isolates is shown in Table 1. Only two

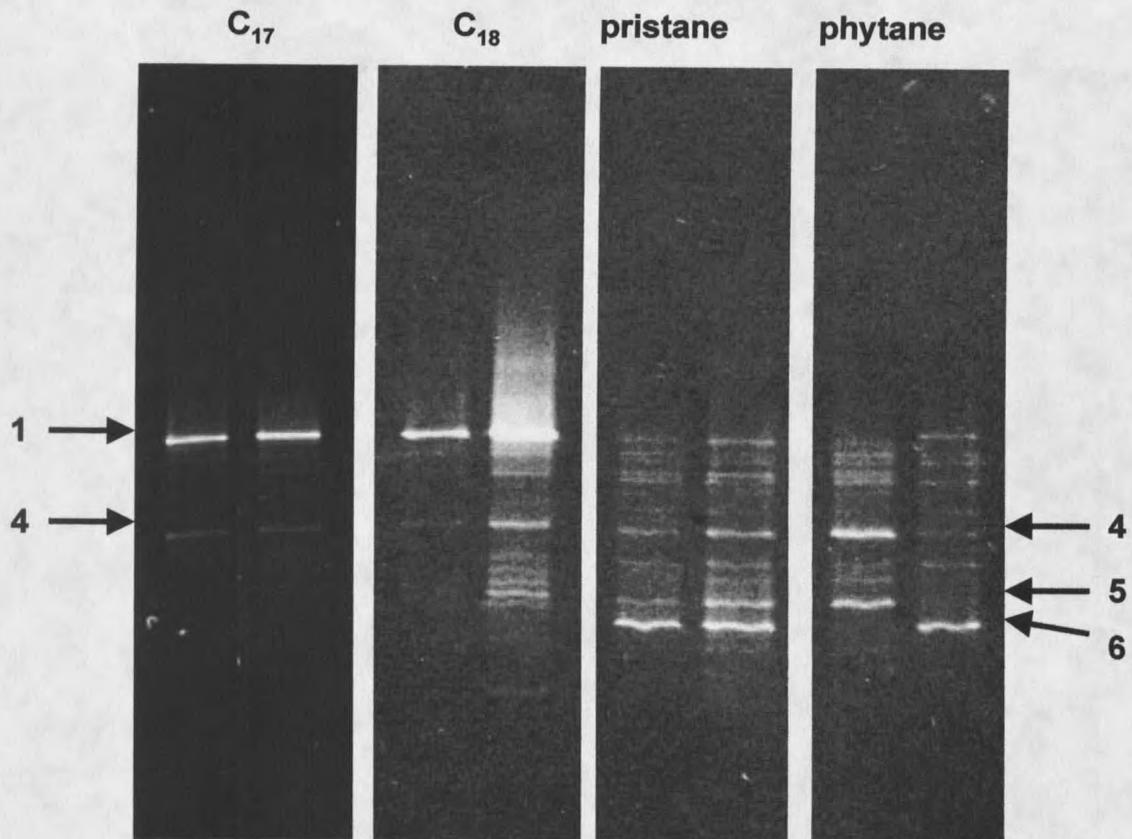
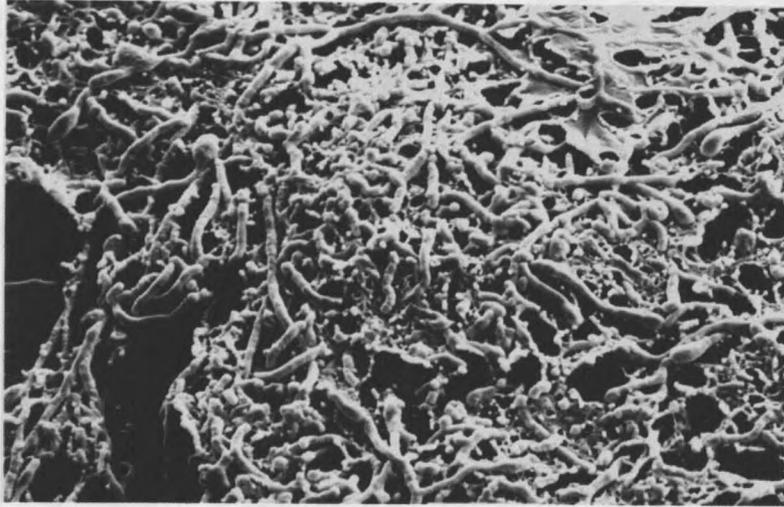
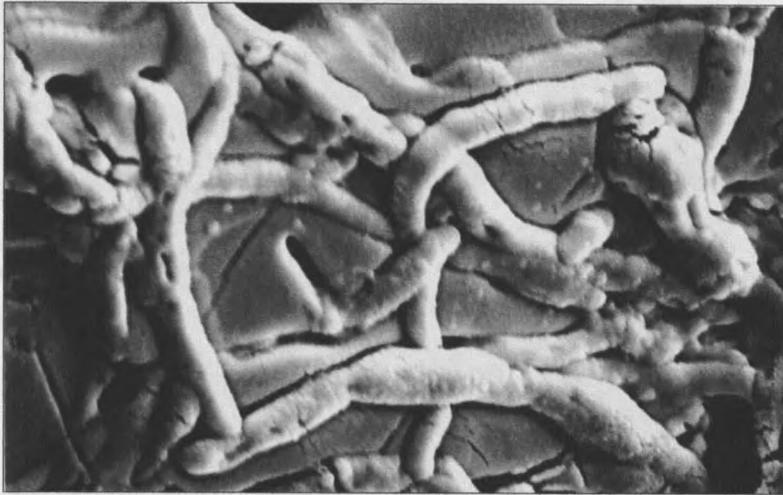


Figure 7. DGGE profiles of soil-derived liquid enrichments on individual hydrocarbon substrates. Numbers correspond to bands whose sequences are described in Table 1.



— 10



— 1 μm

Figure 8. Cryostage scanning electron micrographs of microbial colonization of solid-phase n-octadecane during degradation in a single-substrate liquid enrichments.

hydrocarbon-degrading isolates (*Rhodococcus koreensis*, a; *Bartonella vinsonii*, m) corresponded to bands observed in the enrichments. The *B. vinsonii*-like isolate corresponding to band 8 degraded only branched alkanes, consistent with its late appearance in enrichments with the synthetic hydrocarbon mixture. Interestingly, the *R. koreensis*-like isolate corresponding to band 5 was able to degrade both normal and branched alkanes, even though it was observed only in later stages of crude oil and synthetic hydrocarbon mixture and when branched alkanes were provided as sole substrates. Most isolates did not represent populations observed in DGGE profiles of crude oil and hydrocarbon enrichments and no isolates were obtained for many of the prominent bands observed in the enrichments (bands 1, 2, 3, 4, 6, 7; Table 1).

Discussion

The chemical pattern of sequential hydrocarbon disappearance in our enrichments is similar to that observed in previous studies of crude oil disappearance in both natural and laboratory systems. Changes in ratios of n- to isoprenoid alkanes show similar trends in both the sediment and soil-derived crude oil-amended enrichments, although specific population shifts occurred over different time intervals (Figures 1 and 2). Based on band migration in DGGE profiles, it appears that different populations were selected in the sediment- and soil-derived enrichments (Figures 3 and 4). The timecourses of hydrocarbon degradation in the enrichments were also notably different. This could have been due to the difference in mineral salts media used in each enrichment series. The

basic salts medium used in the sediment-derived enrichments has an ionic strength of 0.6 M, while the soil solution equivalent medium used in the soil-derived enrichments has an ionic strength of 0.03 M. Such differences in salinity have been shown to affect the selection of hydrocarbon-degrading populations and may have resulted in a much longer lag phase before the onset of n-alkane degradation in the sediment-derived enrichment (13). However, other factors, such as inoculum type, may also explain the difference in patterns of n-alkane degradation.

Despite differences between freshwater sediment and soil enrichments, similar successional patterns of community change during the timecourse of crude oil degradation were observed. The primary changes in community profiles coincided with the disappearance of n- and isoprenoid alkanes in each enrichment series. For instance, in the sediment-derived enrichment series, several DGGE bands (Figure 3) appear to fade in intensity or disappear completely, while other bands appear around the time of n-alkane disappearance. In the soil enrichment a band shift occurred between days 4 and 6 correlating with the disappearance of n-alkanes in the crude oil enrichment and the subsequent dominance of isoprenoid alkanes as the primary contaminant type in the remaining crude oil mixture.

In an attempt to elucidate which populations were active in degrading individual compounds (or compound classes) in the soil-derived crude oil enrichments, these enrichments were examined in greater detail. A simpler synthetic hydrocarbon enrichment was constructed in addition to a series of single hydrocarbon enrichments. The chemical transition of n- before isoprenoid alkanes in the synthetic mixture was very

similar to that observed in the crude oil-amended enrichments. Furthermore, analysis of population changes during the time course of hydrocarbon degradation in the synthetic mixture revealed a pattern nearly identical to that of the soil-derived crude oil-amended enrichments. Sequence analysis of DGGE bands in both of these enrichment series confirmed the selection of several populations that were common to both types of treatments. An *A. calcoaceticus*-like population was present in each treatment during the period of n-alkane degradation, while *R. koreensis*-like populations were selected during the period of isoprenoid alkane degradation.

Molecular analysis of the single hydrocarbon enrichments supported the importance of the *A. calcoaceticus*- and *R. koreensis*-like populations in degrading n- and isoprenoid-hydrocarbons, respectively. The *A. calcoaceticus*-like population was selected in both the heptadecane and octadecane single hydrocarbon enrichments and corresponded to the most intense band present in the heptadecane treatment. The *R. koreensis*-like populations were selected in each of the pristane and phytane single hydrocarbons enrichments. Interestingly, a hydrocarbon-degrading isolate with 100% partial 16S rRNA sequence identity to *R. koreensis*-like band 5 was able to degrade both normal and isoprenoid alkanes in pure culture.

A common trend observed in all the hydrocarbon-amended enrichments was the increase in complexity of the banding patterns over time. Many sequences from bands appearing later in the time course of degradation did not correspond with cultivated alkane-degrading isolates (e.g. bands 2, 3, 7 and 6), possibly indicating that some of these bands were associated with organisms that degrade intermediates produced by

hydrocarbon degraders and not the hydrocarbons themselves. The emergence of organisms with multiple unique ribosomal operons may also explain the increase in band complexity. It is possible that the very similar *Variovorax*-like (bands 2 and 3) and *R. koreensis*-like (bands 5 and 6) band sequences observed in crude oil- and synthetic mixture-amended enrichments result from sequence heterogeneities of 16S rRNA operons within single organisms (25). However, this is speculative as bands 5 and 6 do not co-occur in enrichments on phytane alone. The closest genbank relative of bands 5 and 6 is a 2,4-dinitrophenol-degrading bacterium with two unique 16S rRNA genes that are 99.3% similar (32).

Our DGGE observations by themselves appear to support the hypothesis that specialist organisms are important in the degradation of contaminant mixtures, however, the situation may be more complex. A *B. vinsonii*-like isolate corresponding to a DGGE band that appeared when branched alkanes dominated some hydrocarbon mixtures seems to specialize on branched alkanes. However, the *R. koreensis*-like isolate, which also corresponded with an intense band that was consistently associated with branched alkane metabolism, can metabolize both normal and branched alkanes. It can therefore be considered a generalist with respect to metabolism of both classes of hydrocarbons. Other isolates were found to metabolize either strictly n-alkanes (e.g. isolate f, i, l, k and n; Table 1), branched alkanes (e.g. isolate m, g, h, j, p and d; Table 1) or both types of hydrocarbons (e.g. isolate a, b, c and e; Table 1). Hence, the enrichments on hydrocarbon mixtures may be comprised of both generalists and specialists, with interspecific competition governing the selection of n-alkane-degrading bacteria. The basis of

selection and succession of different organisms on the two predominant hydrocarbon classes in crude oil is still unresolved. The influence of NAPL phase sequestration of less abundant hydrocarbons on population selection and contaminant mixture degradation was investigated by varying ratios of n- and branched alkanes. However, n-alkanes degradation always preceded that of branched alkanes even with contaminant mixture ratios (v/v) of branched alkanes:n-alkanes up to 100:1 (data not shown). This suggests that bioavailability limitations resulting from partitioning of less abundant NAPL hydrocarbons into more abundant NAPL hydrocarbons were not responsible for the sequential degradation of n- and branched alkanes observed in our system.

Focusing on the populations represented by the most intense bands in our enrichments, it is interesting to note reports in the literature that suggest that *Rhodococcus* and *Acinetobacter* species use different modes of uptake for alkanes. Many *Rhodococcus* species rely on interfacial uptake of alkanes and are often characterized by cell surfaces that are very hydrophobic (4). Scanning electron micrographs correlate with the molecular analyses of our octadecane enrichments in that they confirm the presence of organisms with *Rhodococcus*-like morphology on the surface of octadecane crystals, possibly indicative of interfacial incorporation of the hydrocarbons. In contrast, *Acinetobacter* species have been shown to commonly produce biosurfactants that emulsify and solubilize the hydrocarbon substrates, a mechanism well-suited for organisms that have characteristically low cell surface hydrophobicity (22, 28).

One possible basis of succession during the degradation of these hydrocarbon mixtures is facilitation (7). Perhaps the *Acinetobacter*-like population produces

biosurfactants or bioemulsifiers during the degradation of n-alkanes that decrease droplet size of the NAPL-phase hydrocarbons, a phenomenon observed qualitatively in the crude oil enrichments. If the *Rhodococcus*-like species selected in our enrichments are in fact dependent upon interfacial incorporation of the substrate, higher surface area-to-volume ratios of the NAPL-phase contaminants would theoretically favor the proliferation of these organisms. An ionic biosurfactant might, however, render the surface of the micelle-bound NAPL droplets more hydrophilic which may inhibit attachment of potential branched alkane degraders. Colores et al. (6) observed distinct microbial population changes in response to surfactant amendment in phenanthrene- and hexadecane-degrading soil microcosms; surfactant addition caused displacement of Gram-positive hydrocarbon degraders by Gram-negative hydrocarbon (and surfactant) degraders. Such competitive selection may explain the emergence of a relatively small fraction of alkane-degraders in our enrichments in contrast to a much higher diversity of isolates cultivated by direct plating from our inoculum (Table 1). The higher diversity of isolates may be a result of isolation conditions that avoid the direct competition between organisms present in an enrichment environment (10).

Our findings suggest that the sequential disappearance of n- and branched alkanes during crude oil degradation is the result of a distinct succession of microbial populations. The population shifts observed were reproducible in replicates of both crude oil and four component synthetic mixtures. Molecular analysis of the single hydrocarbon enrichments correlate with the presence of several populations (most notably *Acinetobacter*- and *Rhodococcus*-like populations) selected in the more complex

mixtures with the degradation of individual n- and branched alkanes. Other recent studies have shown similar patterns of population shifts occurring during contaminant degradation. Fries et al. (17) used cultivation and molecular analyses to document microbial succession following cosubstrate addition in a trichloroethylene-contaminated aquifer. Other studies have shown evidence for population shifts in response to hydrocarbon contamination and surfactant addition in soils (6, 21). All of these studies emphasize the need for coupling the study of environmental chemistry with microbial population biology for understanding contaminant mixture degradation. Such approaches enhance our understanding of the basic ecology of hydrocarbon-degrading communities and may ultimately allow for better prediction and control of in situ remediation efforts for contaminant mixtures.

Literature Cited

1. **Aaronson, S.** 1970. *Experimental Microbial Ecology*. Academic Press, New York.
2. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**:3389-3402.
3. **Angle, J. S., S. P. McGrath, and R. L. Chaney.** 1991. New culture medium containing ionic concentrations of nutrients similar to concentrations found in the soil solution. *Appl. Environ. Microbiol.* **57**:3674-3676.
4. **Bouchez-Naitali, M., H. Rakatozafy, R. Marchal, J.-Y. Leveau, and J.-P. Vandecasteele.** 1999. Diversity of bacterial strains degrading hexadecane in relation to the mode of substrate uptake. *J Appl Microbiol* **86**:421-428.
5. **Calvillo, Y. M., and M. Alexander.** 1996. Mechanism of microbial utilization of biphenyl sorbed to polyacrylic beads. **45**:383-390.
6. **Colores, G. M., R. E. Macur, D. M. Ward, and W. P. Inskeep.** 2000. Molecular analysis of surfactant-driven microbial population shifts in hydrocarbon-contaminated soil. *Appl Environ Microbiol* **66**:2959-64.
7. **Connell, J. H., and R. O. Slatyer.** 1977. Mechanisms of succession in natural communities and their role in community stability and organization. *Amer. Natur.* **111**:1119-1144.
8. **Cremonesi, L., S. Firpo, M. Ferrari, P. G. Righetti, and C. Gelfi.** 1997. Double-gradient DGGE for optimized detection of DNA point mutations. *Biotechniques* **22**:326-30.
9. **Crocker, F. H., W. F. Guerin, and S. A. Boyd.** 1995. Bioavailability of naphthalene sorbed to cationic surfactant-modified smectite clay. *Environ. Sci. Technol.* **29**:2953-2958.
10. **Dunbar, J., S. White, and L. Forney.** 1997. Genetic diversity through the looking glass: effect of enrichment bias. *Appl Environ Microbiol* **63**:1326-1331.
11. **Efroymsen, R. A., and M. Alexander.** 1995. Reduced mineralization of low concentrations of phenanthrene because of sequestering in nonaqueous-phase liquids. *Environ. Sci. Technol.* **29**:515-521.

12. **Efroymsen, R. A., and M. Alexander.** 1994. Role of partitioning in biodegradation of phenanthrene dissolved in nonaqueous-phase liquids. *Environ. Sci. Technol.* **28**:1172-1179.
13. **Emerson, D., and J. A. Breznak.** 1997. The response of microbial populations from oil-brine contaminated soil to gradients of NaCl and sodium \square p-toluate in a diffusion gradient chamber. *FEMS Microbiol.Ecol.* **23**:285-300.
14. **Ferris, M. J., G. Muyzer, and D. M. Ward.** 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. *Appl Environ Microbiol* **62**:340-346.
15. **Foght, J. M., P. M. Fedorak, and D. W. S. Westlake.** 1990. Mineralization of [14 C] hexadecane and [14 C] phenanthrene in crude oil: specificity among bacterial isolates. *Can. J. Microbiol.* **36**:169-175.
16. **Friedrich, M., R. J. Grosser, E. A. Kern, W. P. Inskeep, and D. M. Ward.** 2000. Effect of model sorptive phases on phenanthrene biodegradation: molecular analysis of enrichments and isolates suggests selection based on bioavailability. *Appl Environ Microbiol* **66**:2703-2710.
17. **Fries, M. R., G. D. Hopkins, P. L. McCarty, L. J. Forney, and J. M. Tiedje.** 1997. Microbial succession during a field evaluation of phenol and toluene as the primary substrates for trichloroethene cometabolism. *Appl Environ Microbiol* **63**:1515-1522.
18. **Grosser, R. J., M. Friedrich, D. M. Ward, and W. P. Inskeep.** 2000. Effect of model sorptive phases on phenanthrene biodegradation: different enrichment conditions influence bioavailability and selection of phenanthrene-degrading isolates. *Appl Environ Microbiol* **66**:2695-2702.
19. **Guerin, W. F., and S. A. Boyd.** 1997. Bioavailability of naphthalene associated with natural and synthetic sorbents. *Wat. Res.* **31**:1504-1512.
20. **Hostettler, F. D., and K. A. Kvenvolden.** 1994. Geochemical changes in crude oil spilled from the Exxon Valdez supertanker into Prince William Sound, Alaska. *Org. Geochem.* **21**:927-936.
21. **MacNaughton, S. J., J. R. Stephen, A. D. Venosa, G. A. Davis, Y. J. Chang, and D. C. White.** 1999. Microbial population changes during bioremediation of an experimental oil spill. *Appl. Environ. Microbiol.* **65**:3566-3574.

22. **Marin, M. M., A. M. Pedregosa, M. L. Ortiz, and F. Laborda.** 1995. Study of a hydrocarbon-utilizing and emulsifier-producing *Acinetobacter calcoaceticus* strain isolated from heating oil. *Microbiologia* 11:447-454.
23. **More, M. I., J. B. Herrick, M. C. Silva, W. C. Ghiorse, and E. L. Madsen.** 1994. Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment. *Appl Environ Microbiol* 60:1572-1580.
24. **Muyzer, G., E. C. De Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59:695-700.
25. **Nubel, U., B. Engelen, A. Felske, J. Snaidr, A. Wieshuber, R. I. Amann, W. Ludwig, and H. Backhaus.** 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *J Bacteriol* 178:5636-43.
26. **Olson, J. J., G. L. Mills, B. E. Hebert, and P. J. Morris.** 1999. Biodegradation rates of separated diesel components. *Environ. Toxicol. Chem.* 18:2448-2453.
27. **Ortega-Calvo, J. J., I. Birman, and M. Alexander.** 1995. Effect of varying the rate of partitioning of phenanthrene in nonaqueous-phase liquids on biodegradation in soil slurries. *Environ. Sci. Technol.* 29:2222-2225.
28. **Rosenberg, M., and E. Rosenberg.** 1981. Role of adherence in growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. *J. Bacteriol.* 148:51-57.
29. **Solano-Serena, F., R. Marchal, M. Ropars, J.-M. Lebeault, and J.-P. Vandecasteele.** 1999. Biodegradation of gasoline: kinetics, mass balance and fate of individual hydrocarbons. *J. Appl. Microbiol.* 86:1008-1016.
30. **Tang, W.-C., J. C. White, and M. Alexander.** 1998. Utilization of sorbed compounds by microorganisms specifically isolated for that purpose. *Appl. Microbiol. Biotechnol.* 49:117-121.
31. **Ward, D. M., R. M. Atlas, P. D. Boehm, and J. A. Calder.** 1980. Microbial biodegradation and chemical evolution of oil from the Amoco spill. *AMBIO* 9:277-283.
32. **Yoon, J. H., Y. G. Cho, S. S. Kang, S. B. Kim, S. T. Lee, and Y. H. Park.** 2000. *Rhodococcus koreensis* sp. nov., a 2,4-dinitrophenol-degrading bacterium. *Int J Syst Evol Microbiol* 50:1193-201.

CHAPTER 3

INFLUENCE OF NONAQUEOUS PHASE LIQUIDS ON KINETICS OF
PHENANTHRENE DEGRADATION AND MICROBIAL POPULATION
SELECTIONIntroduction

Partitioning of nonpolar organic contaminants into natural organic matter has been shown to affect the kinetics of microbial degradation and the selection of contaminant-degrading microbial populations (4, 6, 11-14). Several studies have provided evidence supporting the hypothesis that mass transfer of a contaminant from sorbed domains to the aqueous phase can control rates of contaminant biodegradation (3, 5, 21). In complex mixtures, the partitioning of contaminants in nonaqueous phase liquids (NAPLs) can also limit microbial degradation. Although degradation rates are reduced when contaminants are partitioned into NAPLs, the degradation rates often exceed rates of contaminant dissolution into the aqueous phase. For example, Efraymson and Alexander (8) observed rates of phenanthrene mineralization by a *Pseudomonas* sp.

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that exceeded the rates of phenanthrene dissolution from three NAPLs tested (hexadecane, 2,2,4,4,6,8,8-heptamethylnonane and di-2-ethylhexyl phthalate) into the aqueous phase. However, in the same study, mineralization of phenanthrene by a soil slurry was slower than phenanthrene dissolution from the NAPLs into the aqueous phase. In addition, Ortega-Calvo et al. (19) reported that while mineralization rates of phenanthrene in a series of soil slurries exceeded the rates of phenanthrene dissolution from NAPL to water, phenanthrene degradation in these slurries was enhanced by treatments that increased mass transfer rates of phenanthrene from NAPL to water. The inconsistency in these observations suggests that mass transfer limitation alone may not control the degradation of contaminants partitioned in NAPLs and other biological factors may be important in these systems.

Microbial attachment to the NAPL surface may be an important mechanism for utilization of contaminants partitioned into NAPLs. Efromyson and Alexander (7) studied the effect of bacterial attachment on the degradation of naphthalene and n-hexadecane partitioned into 2,2,4,4,6,8,8-heptamethylnone by an *Arthrobacter* sp. that could not degrade the NAPL. Interestingly, the addition of 0.1% Triton X-100 inhibited cell attachment and prevented hexadecane degradation, yet it increased the rate and extent of naphthalene mineralization. Additionally, in a later study with the same organism, cells attached to the NAPL-water interface degraded naphthalene at a rate four times greater than the rate of naphthalene dissolution into the aqueous phase (20). These studies indicate the importance of attachment as a mechanism for utilization of

contaminants partitioned in NAPLs, but also suggest that there may be variation in utilization of different types of contaminants in different NAPL contexts.

Several recent studies have shown that contaminant sorption to solid phases is an important factor governing microbial selection, and subsequent kinetics of contaminant degradation (11, 12, 22). Friedrich et al. (11) observed the selection of different bacterial populations in a series of enrichment environments that differentially limited the bioavailability of phenanthrene sorbed to solid-phase organic resins. In addition, microbes isolated on sorbed-phase phenanthrene have shown more rapid rates of degradation of sorbed-phase contaminants than isolates cultivated on crystalline phenanthrene (12, 22). We hypothesized that the partitioning of phenanthrene in different NAPLs may also affect the selection of contaminant-degrading bacteria and ultimately influence kinetics of contaminant degradation.

The chemical and physical characteristics of complex contaminant mixtures are dynamic during microbial degradation, as certain components of a mixture are degraded before others. For example, in crude oil the most abundant contaminant classes are usually normal (n-) alkanes, followed by isoprenoids, aromatics and asphaltenes. Therefore, aromatic contaminants in crude oil are initially partitioned in a NAPL comprised mainly of n-alkanes; however, as n-alkanes are degraded the primary NAPL will then consist of isoprenoid hydrocarbons. Since the degradation of n-alkanes in crude oil often precedes that of isoprenoids and aromatics, a shift in NAPLs during crude oil degradation may affect mass transfer of aromatic contaminants to the aqueous phase and may alter the microenvironment for the selection of aromatic-degrading bacteria.

In this study we used phenanthrene as a model aromatic contaminant and sought to understand the influence of NAPLs on the kinetics of phenanthrene degradation and the selection of contaminant-degrading bacteria. Enrichment cultures were suitable for describing how population selection correlates with changing mixture environments, especially since such changes would be difficult to discern against the complexity of native species in situ. In an attempt to simulate the predominant NAPLs in which phenanthrene is partitioned during crude oil degradation, heptadecane and pristane (an n-alkane and an isoprenoid among the most abundant in crude oil) and a mixture of the two were chosen as NAPLs. In addition, the more recalcitrant 2,2,4,4,6,8,8-heptamethylnonane was used as a "control" NAPL to elucidate populations selected on phenanthrene alone. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments was used in conjunction with traditional cultivation strategies to not only track microbial populations in general, but also identify functional contaminant-degrading populations selected in soil-derived enrichments. Using this approach, we observed differences in patterns of phenanthrene degradation and population selection in enrichments with phenanthrene partitioned in different NAPL environments.

Materials and Methods

Chemicals

The following hydrocarbons were used in this study: n-heptadecane and pristane, purchased from Aldrich (Milwaukee, WI); methylene chloride, purchased from Fisher Scientific (Pittsburgh, PA); and phenanthrene and 2,2,4,4,6,8,8-heptamethylnonane, purchased from Sigma (St. Louis, MO).

Soil/Sediment

Enrichments were inoculated with an agricultural soil (Arthur Post Research Station, Bozeman, MT). Previous chemical analysis indicated the soil did not contain detectable levels of aliphatic or aromatic hydrocarbon contaminants (15). To inoculate enrichments, 1 g soil or sediment was diluted into 9 mL of a modified soil solution equivalent (SSE) medium designed to represent concentrations of inorganic nutrients present in soil pore water (2, 12). The resulting slurry was mixed well and 100 μ L was added to 200 mL enrichments. This resulted in a 10^{-4} dilution of inocula, a concentration that was empirically determined to be below the baseline for PCR/DGGE-based detection of community DNA.

Enrichment conditions

Aerobic batch enrichments were conducted in 500 mL culture flasks containing 200 mL SSE medium and incubated at 25 \pm 2° C on a rotary shaker. In all liquid

enrichments, C:N:P molar ratios were held constant at 100:15:1. NAPLs were added to enrichments with equal concentrations (250 mg L^{-1}) of heptadecane or pristane, 2,2,4,4,6,8,8-heptamethylnonane, or as a 1:1 (v/v) mixture of heptadecane and pristane. Phenanthrene was dissolved into each NAPL (~8% w/v, a concentration below the empirically determined solubility limit in each of the organic solvents) prior to addition to enrichments. Enrichments were also conducted with individual substrates of pure heptadecane (250 mg L^{-1}), pristane (250 mg L^{-1}) and phenanthrene ($15 \text{ mg crystals flask}^{-1}$) present as (i) solid phase crystals and (ii) a liquid phase partitioned into a recalcitrant NAPL carrier (2,2,4,4,6,8,8-heptamethylnonane). Degradation kinetics were not quantified in crystalline phenanthrene enrichments, where the presence of solid phase phenanthrene prevented representative sampling. In addition, control enrichments with no hydrocarbon amendments were inoculated with agricultural soil in SSE medium. Enrichments were subsampled regularly for molecular analysis of microbial populations and chemical analysis of contaminant mixtures.

Hydrocarbon separation and quantification

Enrichment flasks were vigorously shaken to ensure uniform sampling of NAPL hydrocarbons and 2 mL subsamples were taken for extraction (1:1, v/v) with methylene chloride. A variance of less than 5% was observed between subsamples taken from a single flask at a single timepoint (n=4). All hydrocarbons were quantified using GC/MS analyses as previously described (15).

Nucleic acid extraction, PCR, DGGE and sequence analysis

Cell lysis and DNA extraction protocols were based on those of Moré et al. (16). PCR and sequencing conditions and primers were identical to those previously described (9, 15). DGGE conditions and band purification were identical to that described by Kern et al. (15). The sequences were aligned using the Sequencher 3.1.1 software program (Gene Codes Corporation, Ann Arbor, Mich.) and compared to sequences deposited in GenBank by performing a BLAST search (1).

Isolation of hydrocarbon-degrading bacteria

Bacteria were isolated from hydrocarbon-degrading enrichment flasks after serial dilution in SSE by direct plating onto SSE or R2A (Difco Corp., Detroit, MI) agar plates. The SSE plates were then sprayed with 2% hydrocarbon dissolved in acetone. Individual colonies exhibiting zones of clearing of the respective hydrocarbons were picked from the highest dilution plates and transferred to R2A plates for purification. After three transfers on R2A plates to ensure isolate purity, isolates were again plated on SSE hydrocarbon spray plates and assayed for zones of hydrocarbon clearing around individual colonies to confirm degradative ability.

Results and Discussion

Phenanthrene partitioned into heptadecane

Chemical analyses of enrichments with phenanthrene partitioned into heptadecane are shown in Figure 9. Heptadecane degradation began after day 4 and complete degradation was observed by day 12. Significant phenanthrene degradation was first observed at day 4 and approximately 60% of the phenanthrene was degraded by day 12. However, phenanthrene degradation stopped at day 12 and an extended lag was observed between day 12 and day 20. Degradation resumed after day 20 and complete disappearance of phenanthrene was noted at day 32.

Results from DGGE analysis of 16S rDNA fragments amplified from enrichments with phenanthrene partitioned into heptadecane are shown in Figure 10. Sequences corresponding to bands from these enrichments are described in Table 2. Interestingly, most of the bands sequenced were closely related (>97% similarity) to SSU rRNA gene sequences of organisms in the database. Band 1 appeared earliest at day 4, which correlates with the disappearance of heptadecane, and while its relative intensity decreased significantly beginning at day 20, the band remained present through day 32. Sequence analysis indicated this band is contributed by an *Acinetobacter calcoaceticus*-like population that is 100% similar to a heptadecane-degrading isolate obtained in this study (Table 2). In an attempt to elucidate functional populations responsible for degradation of NAPL hydrocarbons and phenanthrene, enrichments were conducted with

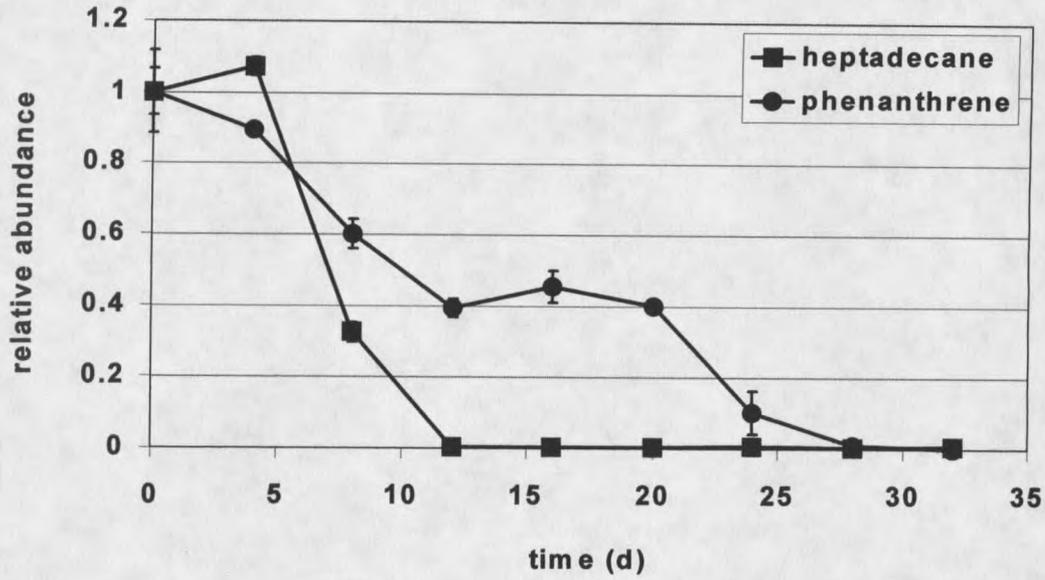


Figure 9. Relative abundance of hydrocarbons in enrichments with phenanthrene partitioned into heptadecane.

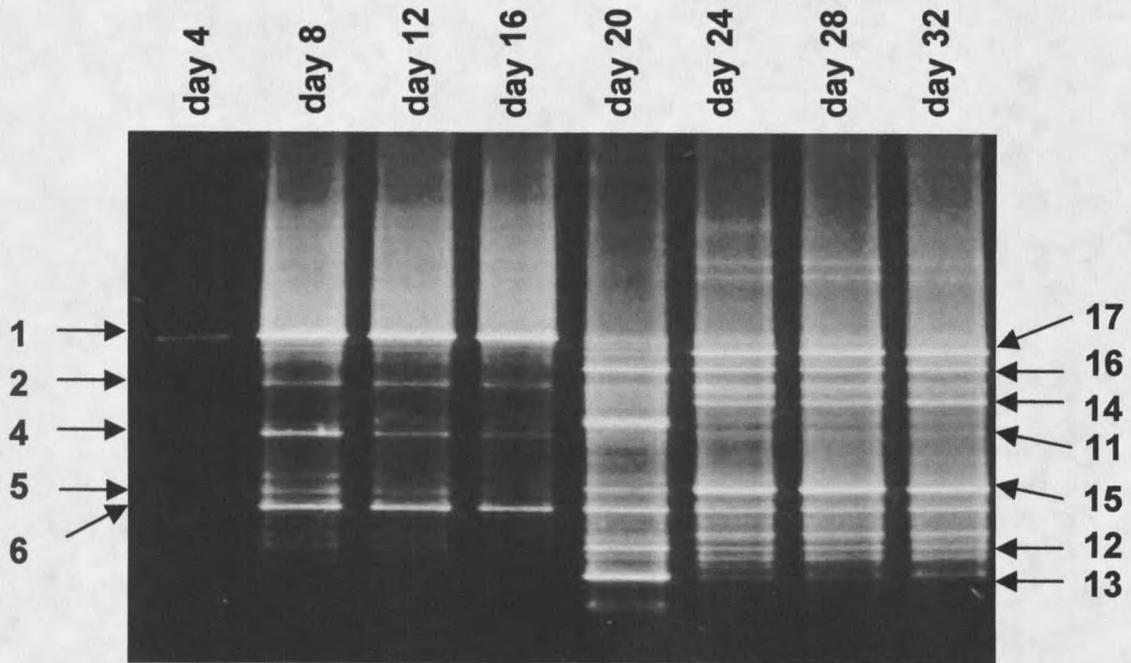


Figure 10. DGGE profiles of a soil-derived enrichment culture with phenanthrene partitioned into heptadecane through time. Numbers correspond to bands whose sequences are described in Table 2.

Table 2. Comparison of DGGE band sequences from soil-derived enrichments and hydrocarbon-degrading isolates cultivated from enrichments.

DGGE Band ^a	Isolate ^b	Closest Genbank relative	Percent similarity ^c	Genbank accession number	Hydrocarbon degradation ^d		
					n-C ₁₇	pr	ph
Proteobacteria							
1	a	<i>Acinetobacter calcoaceticus</i>	99	AF159045	y	n	n
2		<i>Variovorax paradoxus</i>	99	D88006			
3		<i>Alcaligenes sp.</i> strain 05-51	100	X86584			
4	b	<i>Alcaligenes sp.</i> isolate R6	99	AJ002809	y	n	y
5		<i>Sphingomonas aromaticivorans</i>	100	U20774			
7		<i>Ralstonia sp.</i>	91				
10		<i>Alcaligenes sp.</i> isolate R6	98	AJ002809			
14		<i>Bdellovibrio sp.</i> SRP1	97	AF148940			
15		<i>Sphingomonas aromaticivorans</i>	99	U20774			
Cytophagales-Flavobacterium-Bacteroides (CFB) Group							
11		<i>Flexibacter elegans</i>	91	M58783			
High G+C Gram-positive bacteria							
6	c	<i>Rhodococcus koreensis</i>	99	AF124342	y	y	n
8		<i>Rhodococcus koreensis</i>	97	AF124342			
9		<i>Nocardioides sp.</i> OS4	100	U61298			
12	d	<i>Paenibacillus cf. polymyxa</i>	98	AF355463	n	n	n
13		<i>Paenibacillus sp.</i> TK	97				
16		<i>Paenibacillus sp.</i> TK	98				
17		<i>Paenibacillus cf. polymyxa</i>	98	AF355463			

^a Corresponds to marked bands in Figures 10, 12, 13, 15 and 17.

^b Isolates listed with a corresponding band are identical to that band in the 322 bp region sequenced.

^c Percent similarity between band and/or isolate and the closest GenBank relative.

^d Degradation based on ability of isolates to grow and form zones of clearing on individual hydrocarbon spray plates.

heptadecane, pristane and phenanthrene as individual substrates. Changes in contaminant concentrations over time are shown in Figure 11 and corresponding DGGE analyses are shown in Figure 12, which also demonstrates the high reproducibility of our DGGE results. Enrichments on heptadecane as an individual substrate revealed degradation kinetics that were similar to those observed in enrichments with phenanthrene partitioned into heptadecane and DGGE analysis revealed these enrichments selected only for the *Acinetobacter calcoaceticus*-like population contributing band 1 (Figure 12). This population was previously observed during periods of n-alkane degradation in enrichments on crude oil, n- and isoprenoid alkane mixtures and heptadecane and octadecane (15), indicating its involvement in heptadecane degradation in the enrichments shown in Figures 9 and 10.

Returning to the enrichment with phenanthrene in heptadecane (Figure 10), bands 2, 4, 5 and 6 appeared on day 8 and all except the band 5 population remained present through day 32. An *Alcaligenes* sp. R6-like isolate (corresponding to band 4) could utilize phenanthrene on spray plates and was present during both the early and the late phases of phenanthrene degradation, suggesting its likely role in phenanthrene degradation in these enrichments. This population was also observed in enrichments with phenanthrene as the sole substrate (Figure 12). As an aside, the enrichments on phenanthrene as crystals and in 2,2,4,4,6,8,8-heptamethylnonane were conducted to

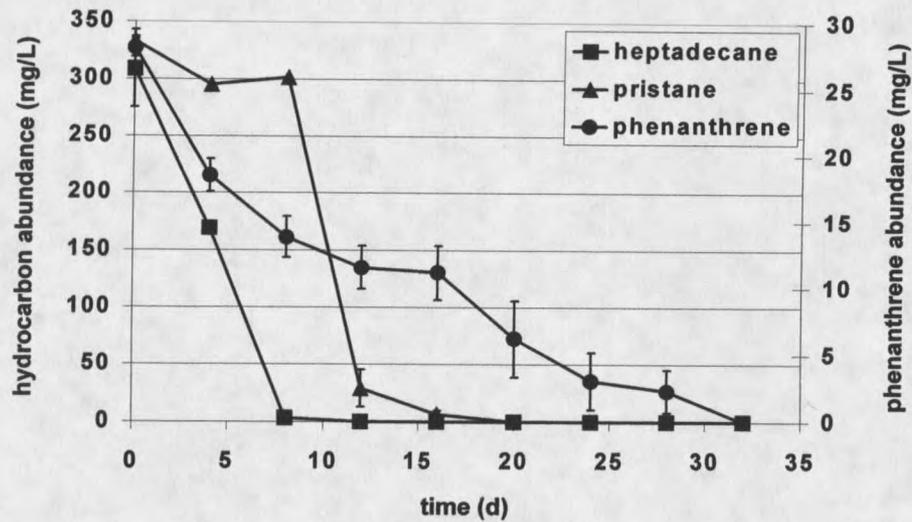


Figure 11. Relative abundance of heptadecane, pristane and phenanthrene (in the presence of heptamethylnonane) in enrichments on individual hydrocarbon substrates.

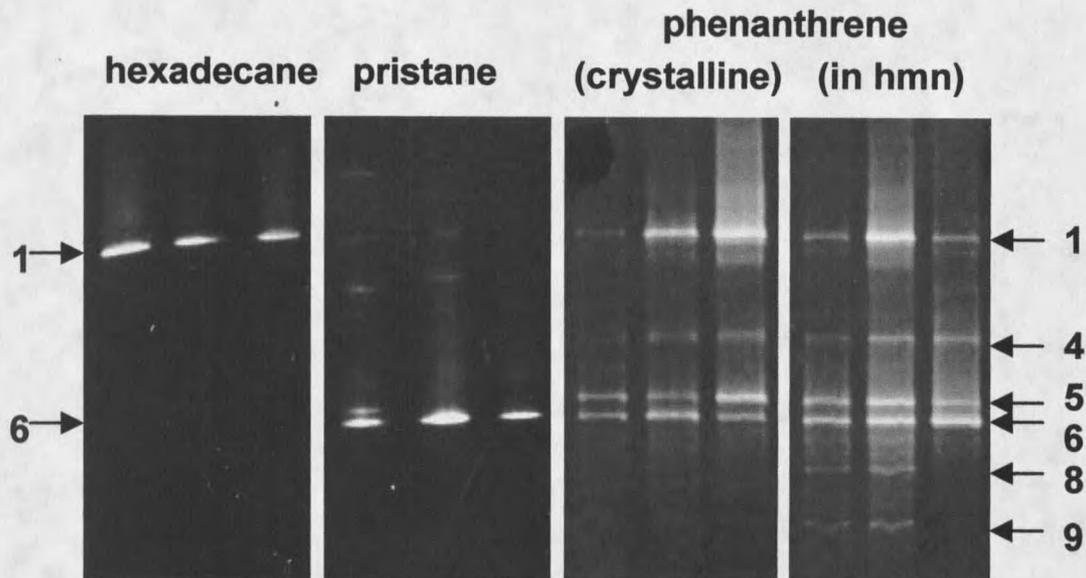


Figure 12. DGGE profiles of soil-derived liquid enrichments on individual hydrocarbon substrates. Numbers correspond to bands whose sequences are described in Table 2. (hmn is 2,2,4,4,6,8,8-heptamethylnonane)

determine differences in microbial population selection based on different phases of a single contaminant. Previous studies have reported that 2,2,4,4,6,8,8-heptamethylnonane is refractory to degradation by some aromatic-degrading bacteria (7) and these enrichments were conducted to provide a "liquid phase" phenanthrene control; however, a significant degree of 2,2,4,4,6,8,8-heptamethylnonane degradation (~40%) was observed during the time period of phenanthrene degradation (data not shown). Therefore, we cannot disregard the possible influence of 2,2,4,4,6,8,8-heptamethylnonane degradation on the kinetics of phenanthrene degradation or the selection of microbial populations in these enrichments. A *Sphingomonas aromaticivorans*-like population contributing band 5 (Table 2) appeared on day 8, decreased in intensity at day 12 and disappeared completely by day 16, exhibiting a pattern similar to the initial phase of phenanthrene degradation. We feel less confident about inferring phenotypes from bands such as this for which we did not observe corresponding isolates. However, since very close relatives of the *Sphingomonas aromaticivorans*-like populations have been shown to degrade aromatic contaminants (10), it is plausible that these populations may be candidates for phenanthrene degradation in our enrichments as well. Further evidence comes from the observation of this band in enrichments with phenanthrene as the sole substrate (Figure 12).

Bands 2 and 6 are contributed by *Variovorax paradoxus*- and *Rhodococcus koreensis*-like populations, respectively (Table 2). Isolate c showed 100% sequence similarity to the *Rhodococcus koreensis*-like population and was capable of degrading heptadecane, but not phenanthrene. The *Variovorax paradoxus*-like population

contributing band 2 was consistently observed during early and late periods of phenanthrene degradation. The role of this population in phenanthrene degradation is unclear, as this band was not observed in enrichments with phenanthrene alone and no isolate was cultivated to confirm its ability to degrade phenanthrene. Interestingly, a previous study reported the transfer (by conjugation) of a plasmid containing genes for 2,4-dichlorophenoxyacetic acid degradation from an *Alcaligenes* sp. to *Variovorax paradoxus* (17). Since our enrichments harbored a phenanthrene-degrading *Alcaligenes* sp. and since genes for phenanthrene degradation are commonly plasmid borne, horizontal gene transfer between these populations may be a possibility.

A major shift in banding pattern in this enrichment occurred at day 20, approximately when phenanthrene degradation resumed, with the appearance of numerous new bands (bands 11-17) (Figure 10). This shift included the appearance of bands 12, 13, 16 and 17, which all showed a high degree of similarity to the 16S rRNA sequence of *Paenibacillus* species; which were noted previously to possess multiple 16S rRNA operons with small sequence heterogeneities (18). DGGE analysis of isolate d indeed revealed the presence of multiple 16S rRNA genes with sequence similarities between 98 and 99% in the region analyzed (Figure 13). Thus, this sudden increase in band diversity probably does not reflect an equivalent increase in community diversity. The failure of the *Paenibacillus* isolate to metabolize heptadecane, pristane and phenanthrene suggests that this organism may degrade metabolites produced during the degradation of these contaminants. While the cultivation of this isolate provides valuable insight into accurately interpreting changes in community structure, the inability to

cultivate isolates corresponding to many other bands makes attributing functions to these populations difficult. The emergence of another *Sphingomonas aromaticivorans*-like population (band 15) corresponded with the late period of phenanthrene degradation. As in the case of the *S. aromaticivorans*-like population contributing band 5 (mentioned above), we are forced to rely on this correlation and the ability of close relatives to degrade aromatics to infer that this population may participate in phenanthrene degradation. Band 14 (which appeared at day 24 and remained throughout) was 97% similar to an ectoparasitic *Bdellovibrio* isolate, possibly indicating the selection of a bacterial parasite in the enrichment. The function of band 11, which was the most intense band in the community profile at day 20, is also difficult to resolve, especially since the low degree of similarity with sequences in the database prohibits any inference of phenotype (Table 2).

Phenanthrene partitioned into pristane

Enrichments were conducted with phenanthrene partitioned into pristane to determine whether an isoprenoid NAPL influenced phenanthrene degradation kinetics or the selection of hydrocarbon-degrading populations. Degradation of pristane was initially slow with only approximately 20% degradation occurring by day 8, however, a rapid period of degradation began at day 8 and by day 12 less than 10% of the pristane remained (Figure 14). Following a similar lag phase of about 8 days phenanthrene was rapidly and continuously degraded, and degradation was nearly complete by day 16.

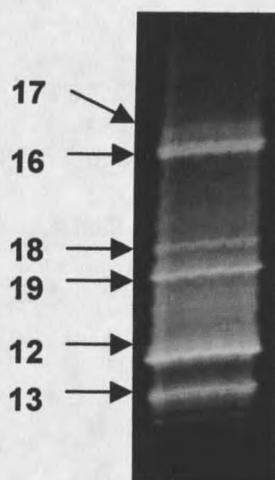


Figure 13. DGGE profile of a *Paenibacillus* species isolated from a hydrocarbon-degrading enrichment. Numbers correspond to bands whose sequences are described in Table 1.

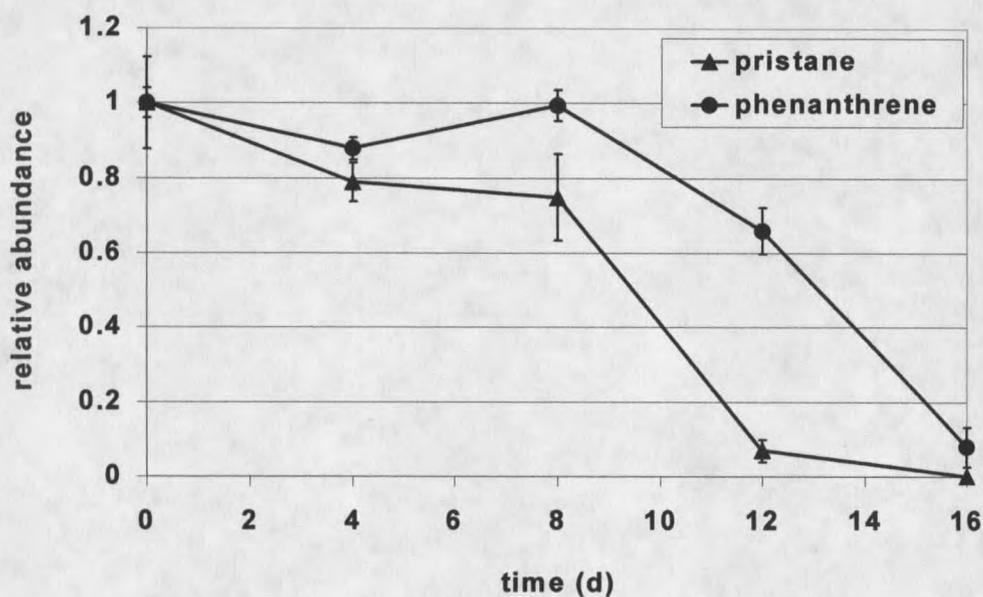


Figure 14. Relative abundance of hydrocarbons in enrichments with phenanthrene partitioned into pristane.

DGGE analysis of these enrichments indicated a much simpler banding pattern (Figure 15) in comparison to enrichments with phenanthrene partitioned into heptadecane. Bands 1, 2 and 6 again appeared early in the profile (day 4), during the period of slow pristane degradation. Band 6 was the most intense band in the profile at day 4 and remained predominant through day 16, suggesting its involvement in pristane degradation. Enrichments on pristane as an individual substrate also selected for the *Rhodococcus koreensis*-like population contributing band 6, in addition to several other more faint bands that could not be purified sufficiently for sequence analysis (Figure 12). In addition, the *Rhodococcus koreensis*-like isolate corresponding to band 6 is capable of pristane degradation (Table 2) and is 100% similar to a population previously shown to be important in isoprenoid degradation (15). The *Acinetobacter calcoaceticus*-like population corresponding to band 1 decreased in intensity at day 8 and disappeared completely by day 16. The isolate corresponding to this population could not degrade either pristane or phenanthrene as substrates on spray plates, suggesting this population may be metabolizing intermediates derived from the catabolism of pristane and/or phenanthrene. This hypothesis is strengthened by the presence of the *Acinetobacter calcoaceticus*-like population in enrichments on crystalline phenanthrene. The *Variovorax paradoxus*-like population corresponding to band 2 was again observed during a period of phenanthrene degradation, indicating that this uncultivated population may be a candidate for phenanthrene degradation.

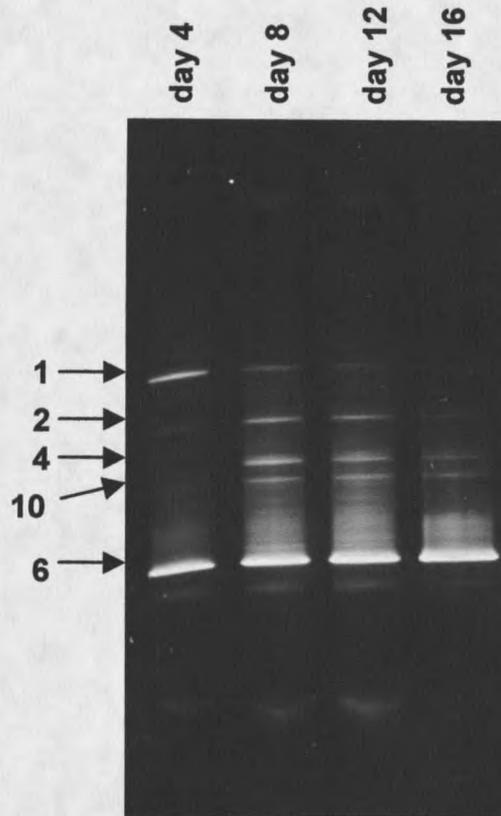


Figure 15. DGGE profiles of a soil-derived enrichment culture with phenanthrene partitioned into pristane through time. Numbers correspond to bands whose sequences are described in Table 1.

Bands 4 and 10 first appear at day 8 and remain present through day 16, which correlates with the period of rapid pristane and phenanthrene degradation. Both these bands are contributed by *Alcaligenes*-like populations, one of which (band 10) was not observed in any other enrichments. Since the isolate corresponding to band 4 has the capacity to degrade phenanthrene, the high degree of similarity (99%) between the two *Alcaligenes*-like populations suggests the possibility that the organism that contributes band 10 may also be important in phenanthrene degradation. Interestingly, neither of the *Sphingomonas aromaticivorans*-like populations that were observed in enrichments with phenanthrene partitioned into heptadecane were detected when phenanthrene was partitioned into pristane.

Phenanthrene partitioned into a NAPL mixture

In an attempt to simulate the NAPL environment present in crude oil, enrichments were conducted with phenanthrene partitioned into a 1:1 (v/v) mixture of heptadecane and pristane. Heptadecane degradation kinetics were very similar to those observed in enrichments with phenanthrene partitioned into heptadecane, with degradation beginning at day 4 and complete by day 8 (Figure 16). In a pattern similar to that observed in enrichments with phenanthrene partitioned into heptadecane, phenanthrene degradation began at day 4, stopped at day 12 and did not resume until after day 28. A similar and more pronounced interruption was observed in pristane degradation. Approximately 50% of the pristane in the contaminant mixture was degraded between days 4 and 12;

however, degradation stopped at day 12 and significant degradation was not observed again until after day 28.

DGGE analysis again revealed the selection of the *Acinetobacter calcoaceticus*-like population corresponding to band 1 at day 4, followed by the emergence of *Alcaligenes*-, *Sphingomonas aromaticivorans*- and *Rhodococcus koreensis*-like populations at day 8 (bands 4, 5 and 6, respectively; Figure 9). Since isolates corresponding to the *Acinetobacter calcoaceticus*- or *Rhodococcus koreensis*-like populations can degrade heptadecane, and heptadecane and pristane, respectively, these populations are presumably responsible for the degradation of these NAPL components, as previously observed in other alkane enrichments (15). The best candidates for the early phase of phenanthrene degradation are the *Alcaligenes* R6- (band 4) and *Sphingomonas aromaticivorans*-like (band 5) populations. The emergence and disappearance of *Sphingomonas aromaticivorans*-like population between days 8 and 12 corresponded with the pattern of early phenanthrene degradation (as in enrichments with phenanthrene partitioned into heptadecane), but its reemergence on day 32 (together with band 7) occurred after phenanthrene was completely degraded. The *Variovorax paradoxus*-like population corresponding to band 2 also appeared later in the time course, during the later phase of pristane and phenanthrene degradation.

Implications

The results of this study indicate the importance of NAPL phases in governing the degradation of hydrophobic organic compounds. The presence of heptadecane as a

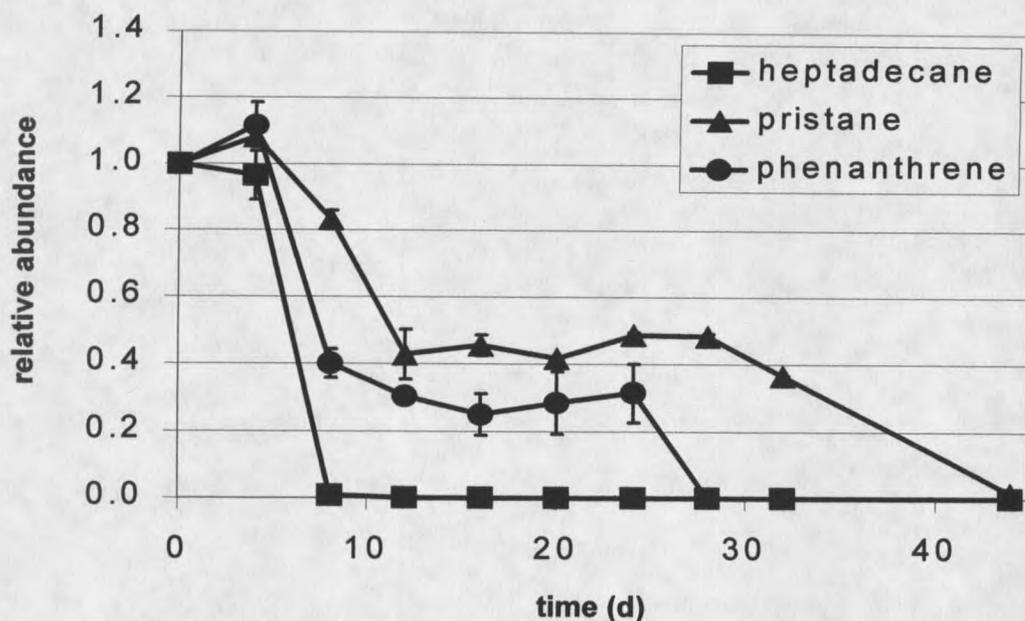


Figure 16. Relative abundance of hydrocarbons in enrichments with phenanthrene partitioned into a mixture of heptadecane and pristane.

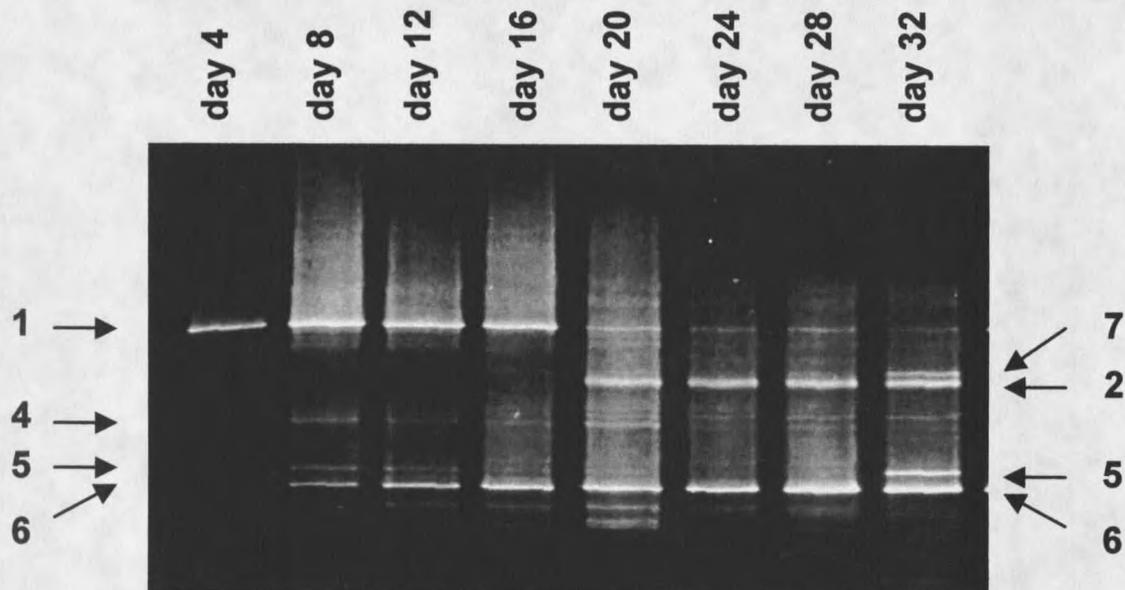


Figure 17. DGGE profiles of a soil-derived enrichment culture with phenanthrene partitioned into a 1:1 mixture of heptadecane and pristane. Numbers correspond to bands whose sequences are described in Table 1.

NAPL phase resulted in a biphasic pattern of phenanthrene degradation and decreased rates of pristane degradation in the NAPL mixture enrichments. A biphasic pattern of a long, slow phase of naphthalene degradation followed by a very rapid period of mineralization has been previously reported with naphthalene partitioned into di(2-ethylhexyl)phthalate and 2,2,4,4,6,8,8-heptamethylnonane (20). Based on this observation, Ortega-Calvo and Alexander (20) hypothesized that two physiologically different populations may be responsible for naphthalene mineralization. In this hypothesis, a population is initially selected whose activity is limited by mass transfer of the aromatic to the water phase, then a second population is selected that can grow on the NAPL-water interface and degrade the substrate at a faster rate than that of contaminant dissolution into the aqueous phase. Our enrichments with phenanthrene partitioned into heptadecane exhibit a rapid period of degradation, followed by a long lag and a secondary phase of rapid degradation. The lag in phenanthrene degradation consistently observed in enrichments containing heptadecane may have been due to phenanthrene partitioning into other sorptive phases. Specifically, the enrichment flasks became noticeably turbid during the period of heptadecane degradation, indicating substantial cell growth and therefore the possibility for phenanthrene to partition into hydrophobic cell lipids. Phenanthrene may have also precipitated as a crystalline solid phase as the solubility limit in heptadecane was exceeded. These types of shifts in phenanthrene distribution would influence mass transfer of phenanthrene to the aqueous phase and may affect microbial population selection.

The importance of microbial population biology in the degradation of contaminants partitioned into different NAPLs is suggested by the presence of some populations only in enrichments with phenanthrene partitioned into pristane (e.g. band 10), the presence of others in enrichments on phenanthrene partitioned into heptadecane (or the heptadecane/pristine mixture) (bands 5 and 15) and abrupt shifts in banding patterns correlating with changing chemical dynamics and degradation rates. The mechanisms controlling the selection of hydrocarbon-degrading populations in these enrichments are difficult to elucidate, due to the problem of inferring which populations were responsible for the degradation of specific constituents present in the mixture environments. The cultivation of isolates corresponding to bands selected in the enrichments provides valuable insight into specialized physiologies, however, numerous populations were selected during periods of hydrocarbon degradation whose capacity for contaminant degradation is uncertain (e.g. bands 2, 5, 10, 11 and 15). In order to gain a more detailed understanding of the functional specialization of selected populations, methods must be developed that definitively link activity with phylogeny of hydrocarbon-degrading populations.

Our results suggest that chemically different NAPL phases may be important in controlling the degradation kinetics of contaminants partitioned in NAPLs. In addition, molecular analyses indicate that the presence of different NAPL phases may influence the structure and function of contaminant-degrading microbial communities. The mechanisms controlling the degradation of phenanthrene partitioned into the structurally different NAPLs were not elucidated. We speculate that mass transfer rates of

contaminants from NAPL to the aqueous phase and/or the varying surface chemistries of the NAPLs are important factors governing the kinetics of contaminant degradation and the selection of contaminant-degrading microorganisms adapted to specific contaminant microenvironments. The observation that different NAPL phases are important in controlling the selection of contaminant-degrading populations will guide our understanding of mechanisms governing the degradation of contaminants partitioned into NAPLs.

Literature Cited

1. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**:3389-3402.
2. **Angle, J. S., S. P. McGrath, and R. L. Chaney.** 1991. New culture medium containing ionic concentrations of nutrients similar to concentrations found in the soil solution. *Appl. Environ. Microbiol.* **57**:3674-3676.
3. **Brusseau, M. L., R. E. Jessup, and P. S. C. Rao.** 1991. Nonequilibrium sorption of organic chemicals: elucidation of rate-limiting processes. *Environ. Sci. Technol.* **25**:134-142.
4. **Calvillo, Y. M., and M. Alexander.** 1996. Mechanism of microbial utilization of biphenyl sorbed to polyacrylic beads. *Appl. Microbial Biotechnol.* **45**:383-390.
5. **Chung, G.-Y., B. J. McCoy, and K. M. Scow.** 1993. Criteria to assess when biodegradation is kinetically limited by intraparticle diffusion and sorption. *Biotechnol. Bioeng.* **41**:625-632.
6. **Crocker, F. H., W. F. Guerin, and S. A. Boyd.** 1995. Bioavailability of naphthalene sorbed to cationic surfactant-modified smectite clay. *Environ. Sci. Technol.* **29**:2953-2958.
7. **Efroymsen, R. A., and M. Alexander.** 1991. Biodegradation by an *Arthrobacter* species of hydrocarbons partitioned into an organic solvent. *Appl. Environ. Microbiol.* **57**:1441-1447.
8. **Efroymsen, R. A., and M. Alexander.** 1994. Role of partitioning in biodegradation of phenanthrene dissolved in nonaqueous-phase liquids. *Environ. Sci. Technol.* **28**:1172-1179.
9. **Ferris, M. J., G. Muyzer, and D. M. Ward.** 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. *Appl Environ Microbiol* **62**:340-346.
10. **Fredrickson, J. K., D. L. Balkwill, G. R. Drake, M. F. Romine, D. B. Ringelberg, and D. C. White.** 1995. Aromatic-degrading *Sphingomonas* isolates from the deep subsurface. *Appl Environ Microbiol* **61**:1917-1922.
11. **Friedrich, M., R. J. Grosser, E. A. Kern, W. P. Inskeep, and D. M. Ward.** 2000. Effect of Model Sorptive Phases on Phenanthrene Biodegradation:

- Molecular Analysis of Enrichments and Isolates Suggests Selection Based on Bioavailability. *Appl Environ Microbiol* **66**:2703-2710.
12. **Grosser, R. J., M. Friedrich, D. M. Ward, and W. P. Inskeep.** 2000. Effect of Model Sorptive Phases on Phenanthrene Biodegradation: Different Enrichment Conditions Influence Bioavailability and Selection of Phenanthrene-Degrading Isolates. *Appl Environ Microbiol* **66**:2695-2702.
 13. **Guerin, W. F., and S. A. Boyd.** 1997. Bioavailability of naphthalene associated with natural and synthetic sorbents. *Wat Res* **31**:1504-1512.
 14. **Guerin, W. F., and S. A. Boyd.** 1992. Differential bioavailability of soil-sorbed naphthalene to two bacterial species. *Appl Environ Microbiol* **58**:1142-1152.
 15. **Kern, E. A., W. P. Inskeep, and D. M. Ward.** 2001 Chapter 2 Ph.D.Dissertation, Montana State Univ. Bozeman, MT
 16. **More, M. I., J. B. Herrick, M. C. Silva, W. C. Ghiorse, and E. L. Madsen.** 1994. Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment. *Appl Environ Microbiol* **60**:1572-1580.
 17. **Neilson, J. W., K. L. Josephson, I. L. Pepper, R. B. Arnold, G. D. Di Giovanni, and N. A. Sinclair.** 1994. Frequency of horizontal gene transfer of a large catabolic plasmid (pJP4) in soil. *Appl Environ Microbiol* **60**:4053-4058.
 18. **Nubel, U., B. Engelen, A. Felske, J. Snaidr, A. Wieshuber, R. I. Amann, W. Ludwig, and H. Backhaus.** 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *Journal of Bacteriology* **178**:5636-5643.
 19. **Ortega-Calvo, J. J., I. Birman, and M. Alexander.** 1995. Effect of varying the rate of partitioning of phenanthrene in nonaqueous-phase liquids on biodegradation in soil slurries. *Environ. Sci. Technol.* **29**:2222-2225.
 20. **Ortega-Calvo, J.-J., and M. Alexander.** 1994. Roles of bacterial attachment and spontaneous partitioning in the biodegradation of naphthalene initially present in nonaqueous-phase liquids. *Appl Environ Microbiol* **60**:2634-2646.
 21. **Pignatello, J. J., and B. Xing.** 1995. Mechanisms of slow sorption of organic chemicals to natural particles. *Environ. Sci. Technol.* **30**:1-11.
 22. **Tang, W.-C., J. C. White, and M. Alexander.** 1998. Utilization of sorbed compounds by microorganisms specifically isolated for that purpose. *Appl. Microbiol Biotechnol.* **49**:117-121.

CHAPTER 4

CHARACTERIZATION OF METHYL *tert*-BUTYL ETHER (MTBE)-DEGRADING
BACTERIA FROM A GASOLINE CONTAMINATED AQUIFERIntroduction

Alkyl ethers such as methyl *tert*-butyl ether (MTBE), ethyl *tert*-butyl ether (ETBE), and *tert*-amyl methyl ether (TAME) have been used as gasoline additives in the United States since the late 1970s to reduce vehicle emissions. MTBE, the most common fuel oxygenate, has been added at concentrations up to 15% (v/v) to more than 30% of all gasoline sold in the United States. Due to the widespread use of MTBE in reformulated gasoline, there have been numerous reports of releases into the subsurface from point sources such as leaking underground storage tanks. In addition, non-point source contamination by motorized watercraft (22) and urban air and rainwater

[This chapter was initiated by a class project to investigate the fate and transport of MTBE in a contaminated aquifer. My involvement included the design and monitoring of the MTBE-degrading enrichment, molecular characterization of the enrichment and MTBE-degrading isolates, and design of the spent culture medium experiment. It has been submitted for publication as "Characterization of methyl *tert*-butyl ether (MTBE)-degrading bacteria from a gasoline-contaminated aquifer" E. A. Kern, R.H. Veeh, H.W. Langner, R.E. Macur, and A.B. Cunningham, Montana State Univ., Bozeman, MT]

(11, 19, 31) may also contribute significantly to detectable levels of MTBE in groundwater. A 1993-1994 survey conducted by the USGS identified MTBE as the second most common contaminant of urban aquifers in the U.S. (28). Despite the work completed by the USGS in the early 1990s, only a few states were testing for MTBE prior to 1995. Most MTBE plumes were not characterized, because states had no action level for remediating the compound in groundwater or drinking water. Most states have only recently initiated routine sampling for MTBE and are now identifying MTBE plumes from petroleum releases at service stations.

The physical and chemical properties of MTBE contribute to its mobility in groundwater. In contrast to gasoline hydrocarbons, MTBE has a relatively low octanol-water partition coefficient ($\log K_{ow} = 0.94$ to 1.16) and a high aqueous solubility ($\sim 50,000$ mg/L at 25°C), which limit partitioning into soil organic matter (16, 26). While MTBE has a higher vapor pressure than benzene, its Henry's Law constant (0.022 at 25°C) is an order of magnitude lower than that of benzene, resulting in minimal losses of aqueous phase MTBE to the atmosphere (23). Due to significant partitioning of MTBE into the aqueous phase and limited sorption to soil, MTBE plumes in contaminated groundwater are generally longer and more stable than coexisting gasoline plumes (14, 25).

Traditional pump-and-treat technologies, such as air stripping, used to eliminate gasoline constituents from groundwater, have not been as successful for MTBE removal. However, bioremediation remains a viable strategy for removal of MTBE from gasoline-contaminated aquifers. Recent studies suggest that biodegradation of MTBE and its

degradation products of concern, *tert*-butyl alcohol (TBA) and *tert*-butyl formate (TBF), occur primarily under aerobic conditions (2, 4, 5, 12, 20, 24, 29). Hanson et al. (12) recently reported a bacterial isolate (PM1) capable of utilizing MTBE as its sole carbon and energy source. In addition, cometabolism of MTBE may be a potentially important mechanism of degradation. Steffan et al. (29) isolated several propane-oxidizing bacteria that could degrade MTBE, ETBE and TAME after growth on propane or 2-propanol. MTBE degradation by *Pseudomonas aeruginosa* has been shown to occur in the presence of pentane (10); and cometabolism of MTBE (in the presence of gaseous n-alkanes) by a filamentous fungus has also been reported (13).

MTBE research is in its infancy and expanded knowledge of the fate and transport of MTBE in natural systems is essential as more risk-based approaches to contaminant remediation such as natural attenuation are evaluated. Therefore, the goals of this study were (i) to assay for the presence of MTBE-degrading bacteria at a contaminated aquifer as an initial step in assessing the potential for natural attenuation of MTBE and (ii) to identify specific bacteria involved in MTBE degradation and their associated rates of biodegradation. In addition to traditional cultivation strategies, denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments was used to determine the composition of MTBE-degrading consortia.

Materials and Methods

Chemicals

HPLC-grade MTBE and 2-propanol were purchased from Fisher Scientific (Bridgewater, NJ). Uniformly labeled [^{14}C]-MTBE (> 99% purity; 10.1 mCi mmol $^{-1}$) was synthesized by DuPont New England Nuclear Products (Boston, MA). TBA (99.6%) and TBF (99%) standards were obtained from J.T. Baker (Phillipsburg, NJ, USA) and Aldrich Chemical Co. (Milwaukee, WI), respectively.

Site Characterization

The contaminated site is located at a fuel station east of U.S. Highway 93, one mile south of Ronan, MT where an underground storage tank (removed in April, 1994) released an estimated 40,000 L of gasoline over a 1-year period. The Montana Department of Environmental Quality established 20 monitoring wells at the site to identify plume boundaries, and 8 additional wells were installed in 1997 for free product removal. Figure 18 shows the free product and dissolved contaminant plumes at the site and Table 3 (MSE/HKM, unpublished data) presents selected well monitoring results of contaminant concentrations over a 3-year period. The maximum depth to groundwater is approximately 5 m at the point of release. The dominant lithology identified from bore holes was silt and fine sand with scattered clay lenses, typical of glacial lacustrine deposits common to the area. Remediation at the site has included a combination of passive recovery skimmers, air sparging systems coupled with soil vapor extraction and an interceptor trench.

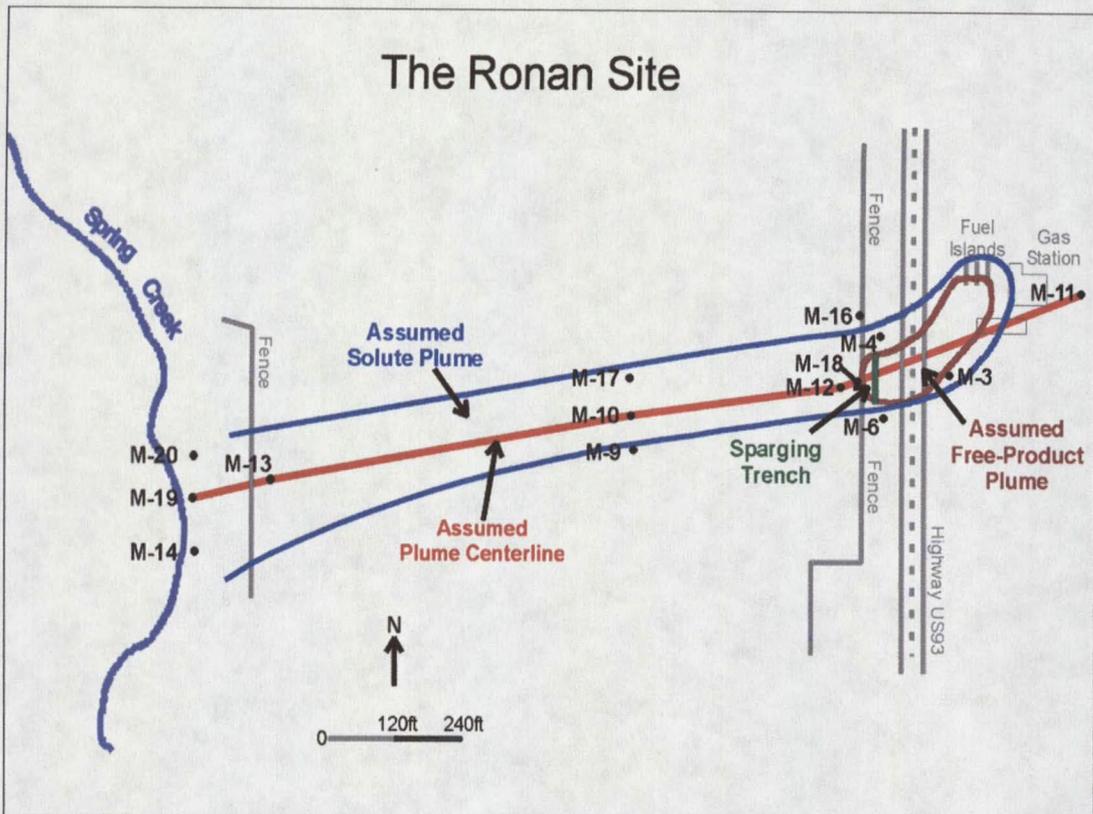


Figure 18. Schematic map of the MTBE-contaminated site in Ronan, MT.

sampling date	well M-12		well M-10		well M-13	
	TPH*	MTBE*	TPH*	MTBE*	TPH*	MTBE*
5/96	101000	19800	1570	1460	nd	nd
12/96	94100	15800	3880	2900	100	221
12/97	98500	9540	8270	4570	201	204
11/98	68900	4950	9760	4650	50	57
6/99	21900	3500	5770	3580	58	72

Table 3. Site concentrations of total petroleum hydrocarbons (TPH) and MTBE

* TPH and MTBE concentrations are given in $\mu\text{g/L}$

nd not determined

Soil/Aquifer Materials

Soil and aquifer materials used as inocula for MTBE-degrading bacterial enrichments were collected from two bore holes drilled to a depth of 1 m below the groundwater surface. One bore hole was near the down-gradient edge of the nonaqueous phase liquid (NAPL) gasoline plume at well M-12 and the second was adjacent to well M-19 near Spring Creek. Samples were retrieved from above, at the interface and below the groundwater surface, and were stored at 4°C for one week prior to inoculation of enrichments. A pristine agricultural soil (Arthur Post Research Station, Bozeman, MT) was used as inoculum in several control enrichments.

Enrichment Conditions

Degradation experiments were conducted in closed 125-mL Erlenmeyer flasks under aerobic conditions. The flasks contained 25 mL of a mineral medium (SSE) designed to simulate a typical soil solution (1) and contained NH_4NO_3 (1.25 mM), CaSO_4 (2 mM), MgCl_2 (2 mM), KH_2PO_4 (10 μM), KOH (1.25 mM), FeCl_2 (5 μM), supplemented with 100 $\mu\text{L L}^{-1}$ of micronutrient solution (27). Subsamples of the composite soil/aquifer material were used as inocula (1% w/v) for the MTBE-degrading microcosms. MTBE was added to enrichment flasks to a final concentration of 10 mg L^{-1} MTBE (~100,000 dpm flask⁻¹ [¹⁴C]-MTBE), which was the approximate concentration of MTBE measured at well M-12 at the time of inoculum sampling. Radiolabeled ¹⁴CO₂ evolved from the enrichments was captured and used to track MTBE degradation. Solutions of 0.5 M NaOH (0.3 mL) were utilized as ¹⁴CO₂-traps and were placed in cups

suspended from the stoppers. The NaOH was removed weekly and $^{14}\text{CO}_2$ was quantified using liquid scintillation analysis. Disappearance of MTBE from the aqueous phase was monitored by gas chromatography (GC). Treatments were tested in triplicate and compared to autoclaved controls that contained $250 \text{ mg L}^{-1} \text{ HgCl}_2$.

Due to potential loss of vapor phase MTBE during sampling of the base traps, subsequent kinetic degradation experiments with one consortium were performed in 120-mL serum bottles with sealed teflon-coated septa. MTBE was introduced into 40 mL of SSE medium at concentrations ranging from $10\text{-}70 \text{ mg L}^{-1}$ and 2 mL samples were aseptically removed for GC analysis. At each sampling time, 3 mL of sterile air was injected into the serum bottle headspace to maintain aerobic conditions.

In studies with spent culture medium amendment, duplicate MTBE-degrading cultures of RS24 were centrifuged at 8,000 rpm for 10 min at 4°C . The supernatant was decanted and saved, and the cell pellets were re-suspended in 3 mL of 0.85% phosphate buffered saline. Aliquots (1 mL) from each cell suspension were used as inocula for duplicate cultures with 40 mL spent growth medium, and 2 mL aliquots were used as inocula for duplicate cultures with 40 mL of fresh SSE medium. The four new cultures were amended with MTBE to a final concentration of $32 \pm 6 \text{ mg L}^{-1}$.

Gas Chromatography

GC analysis of 2-propanol, MTBE, TBA, and TBF was conducted using a Hewlett-Packard 5890 Series II gas chromatograph with a FID detector. A Porapak PS 80/100 mesh packed column (2 mm I.D. x 1.22 m glass; Supelco, Bellefonte, PA) was

used with a He carrier gas flow rate of 18 mL min^{-1} at 100°C and a head pressure of 28 psi. The injector temperature was set at 190°C and the detector temperature was 250°C . The initial oven temperature was set at 100°C for 4 min then ramped at $10^\circ\text{C min}^{-1}$ to a final temperature of 200°C . The 2-propanol, TBA, MTBE, and TBF peaks showed retention times of approximately 2.7, 4.5, 6.3, 8.3 min, respectively.

Bacterial Cell Counts

0.5-mL samples of MTBE-degrading enrichments were placed above a $0.2\text{-}\mu\text{m}$, 25 mm-dia black polycarbonate membrane (Poretics Products; Livermore, CA) clamped in a filter chimney assembly and vacuum manifold apparatus. A $100\text{-}\mu\text{L}$ aliquot of 10 mg L^{-1} DAPI was added, cells were stained for 2 min and then rinsed 2-3 times with dH_2O to remove any unbound stain. Direct counts of DAPI-stained cells were conducted using a Nikon Eclipse E800 epifluorescence microscope with a mercury lamp UV source. The filter block used had an excitation bandwidth of 340-380 nm and an emission bandwidth of 435-495 nm. Total cell counts were based on a calibrated ocular grid, calculated total membrane area, and an average of 20 enumerated grid fields.

Isolation of MTBE-Degrading Bacteria

Samples from an MTBE-degrading consortium were spread on R2A agar plates (Difco Corp., Detroit, MI) and Noble agar plates amended with SSE. Noble agar plates were incubated in sealed metal cans containing MTBE vapors produced by volatilization from MTBE-saturated filter paper. Half of the Noble agar plates also received $50 \mu\text{L}$ of 20 mg L^{-1} 2-propanol applied to the plate surface prior to inoculation with bacteria.

Single colonies from both types of plates were purified through repeated transfer under identical cultivation conditions. Isolated colonies were selected by differences in colony color and morphology and used to inoculate 120 mL serum bottles containing 40 mL of SSE amended with 10 mg L⁻¹ MTBE. 2-Propanol (20 mg L⁻¹) was added as a cosubstrate in selected treatments. Gas chromatography was used to monitor MTBE disappearance and accumulation of degradation products.

Nucleic Acid Extraction, PCR, DGGE and Sequence Analysis

Cell lysis and DNA extraction protocols were based on those of Moré et al. (18). Briefly, cells were lysed by mechanical disruption at 6.5 m s⁻¹ for 45 s using a beadbeater (Savant Instruments, Qbiogene; Carlsbad, CA). The crude cell lysates were precipitated with 12 M ammonium acetate and the DNA in the resulting supernatant was precipitated with isopropanol. PCR conditions and primers were identical to those previously described (9). These primers amplify a region of the 16S rRNA gene from nucleotide positions 1070 to 1392 (based on *E. coli*) and have been shown to be highly conserved for numerous bacterial lineages, including the proteobacterial, Gram-positive, cyanobacterial, green nonsulfur and *Cytophagales-Flavobacterium-Bacteriodes* lineages. DGGE conditions were similar to those used by Ferris et al. (9), with a 6-11% concentration gradient of acrylamide used in addition to a 45-70% urea/formamide gradient (8). DGGE bands were purified for sequence analysis by touching individual bands with a 10 µL pipet tip and immersing the tip into previously prepared PCR reaction mixtures. Band purity was confirmed by analyzing the re-amplified bands on a DGGE

gel in comparison to the parent community. Sequencing of isolate DNA and DGGE bands was performed on an ABI Prism 310 Genetic Analyzer using a 47-cm capillary (PE Applied Biosystems, Foster City, CA) and Prism BigDye terminator cycle sequencing ready reaction kits (PE Applied Biosystems, Foster City, CA).

Results

Soil-Derived Enrichments

A series of enrichment cultures amended with 10 mg L^{-1} MTBE was established to assay the presence of MTBE-degrading bacteria at the gasoline-contaminated site. The treatment matrix consisted of three inoculum types (M-12, M-19 and pristine) and two concentrations of inorganic nutrients (1x and 0.1 x SSE). In addition, a set of 0.1x SSE-treated enrichments was amended with 2-propanol. After an initial lag time of approximately 15 days, one flask (RS24) inoculated with soil from well M-12 and amended with 0.1x SSE and 2-propanol displayed significant MTBE degradation compared to sterile controls and other treatments (Figure 19). $^{14}\text{CO}_2$ analyses from this enrichment indicated a maximum of 33% MTBE degradation after 35 days. All other treatments displayed only minimal $^{14}\text{CO}_2$ evolution over the 35-day trial relative to the sterile controls.

MTBE Degradation by Consortium RS24

The MTBE-degrading enrichment (RS24) was transferred (10% v/v) and enriched further with 14 mg L^{-1} MTBE and 20 mg L^{-1} 2-propanol. GC analysis revealed

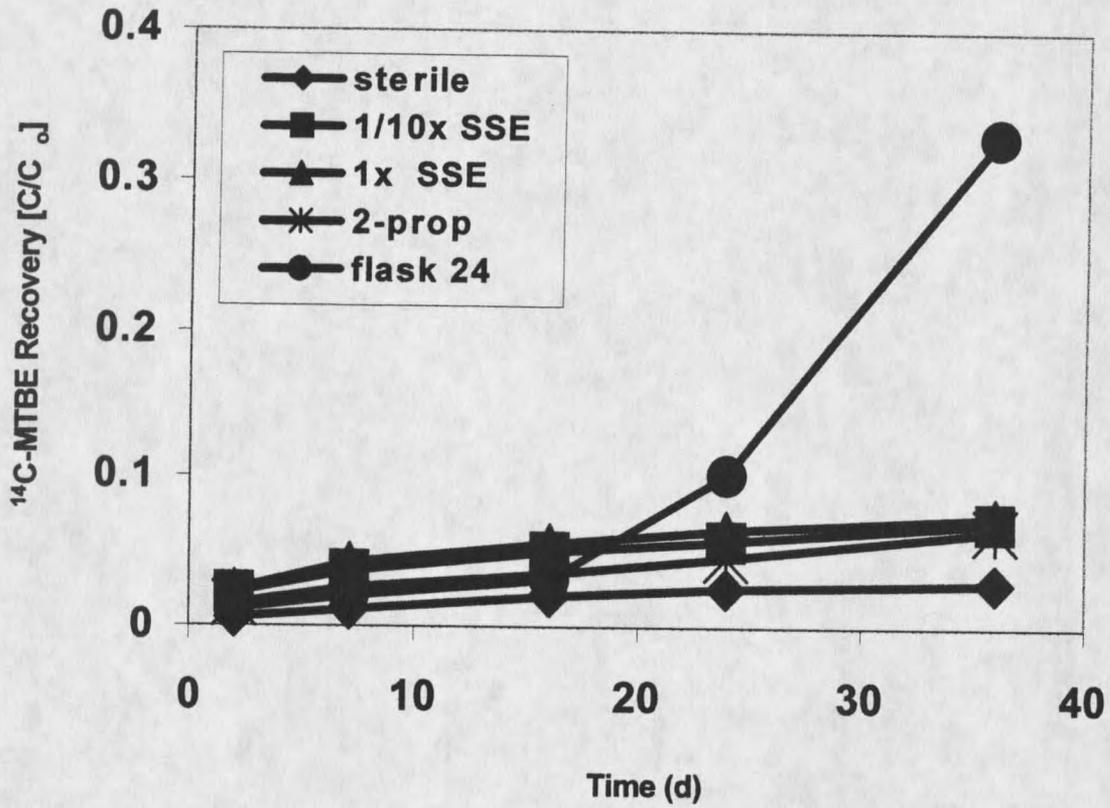


Figure 19. Recovery of $^{14}\text{CO}_2$ from ^{14}C -MTBE amended soil enrichments.

the disappearance of 2-propanol and accumulation of two metabolites identified by mass spectrometry as TBA and TBF after 7 days. TBA and TBF were still present at day 14 and disappeared by day 21. Over the 21-day period, the MTBE concentration decreased to 12 mg L^{-1} and continued to decrease steadily at a rate of about $0.1 \text{ mg L}^{-1} \text{ d}^{-1}$. Following the complete disappearance of MTBE after 146 days, another transfer (10% v/v) was made and the enrichment was amended with 6 mg L^{-1} MTBE without the addition of 2-propanol. An extended lag time (~ 18 days) was again observed before significant degradation occurred (Figure 20). However, with subsequent addition of MTBE on days 40, 47 and 53 the onset of degradation became increasingly rapid. As amendments of MTBE increased from 10.3 mg L^{-1} to 14.7 mg L^{-1} to 66.4 mg L^{-1} , no significant decrease in degradation rates were observed. Beginning on day 47, MTBE disappearance was monitored daily and MTBE degradation rates of 0.15 mg d^{-1} and 0.66 mg d^{-1} were calculated from days 47-53 and days 53-57, respectively. In contrast to the previous enrichments in the presence of 2-propanol, no metabolites were observed at any point in the degradation. Figure 21 shows increasing bacterial cell density during MTBE degradation in duplicate transfer cultures (10% v/v) of RS24, indicating that microbial growth was coupled to MTBE degradation. A gross estimate of biomass yield based on maximum MTBE utilization rates represented in the graphs was calculated. Assuming an approximate dry mass of $2 \times 10^{-10} \text{ mg cell}^{-1}$, a yield of $0.25 \pm 0.02 \text{ mg dry biomass (mg MTBE)}^{-1}$ was observed.

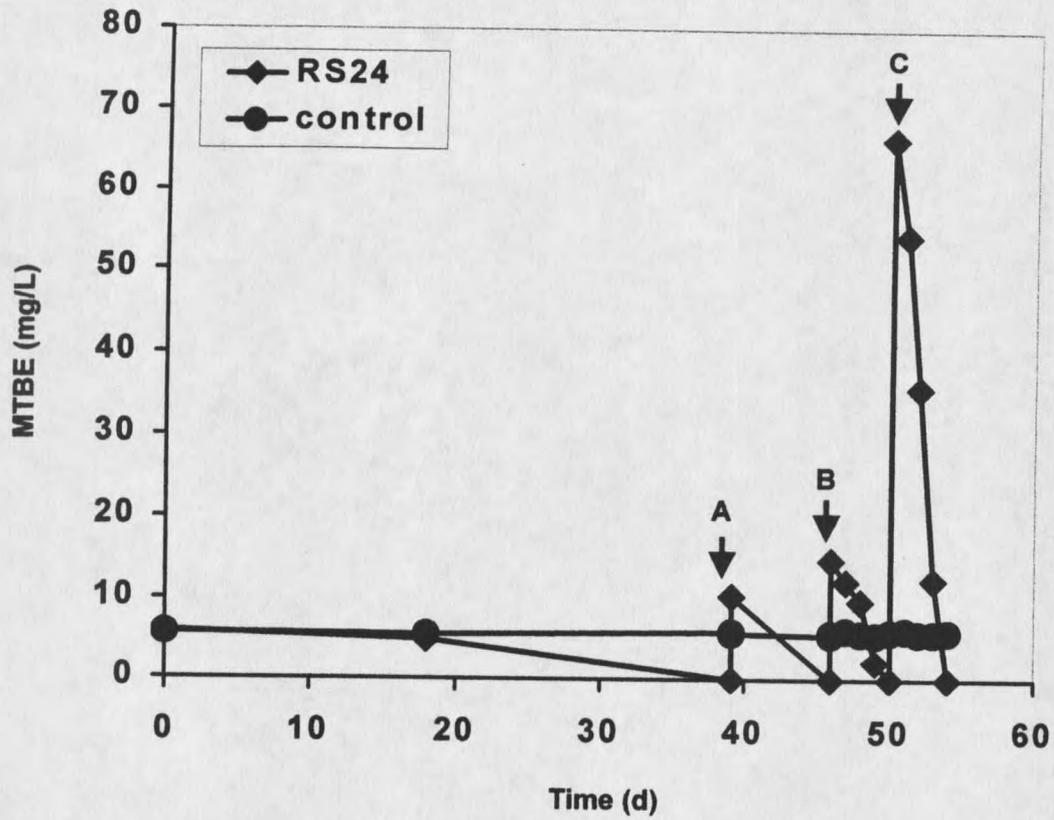


Figure 20. Degradation of increasing concentrations of MTBE by enrichment culture RS24. Letters correspond to MTBE additions of A, 10.3 mg L^{-1} ; B, 14.7 mg L^{-1} ; and C, 66.4 mg L^{-1} .

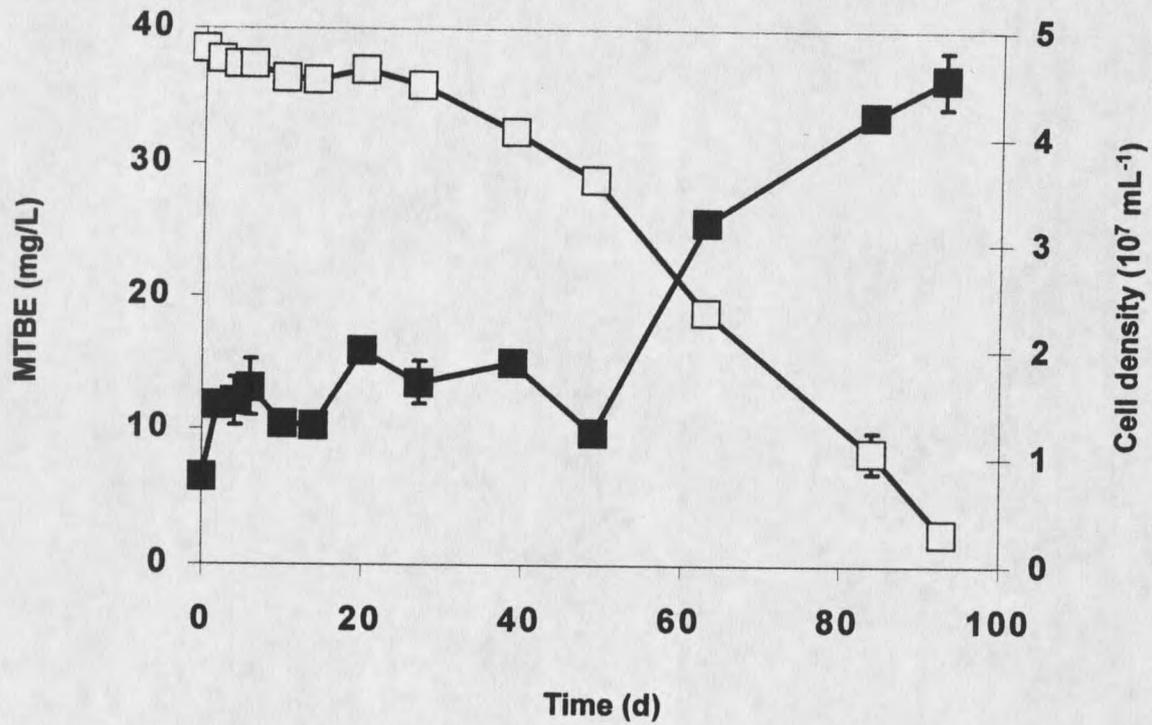


Figure 21. Degradation of MTBE with corresponding increases in cell density with enrichment culture RS24. MTBE concentration (\square) and bacterial cell density (\blacksquare) were monitored over time. Error bars represent standard deviation from the mean of duplicate cultures.

Growth Factor Experiments

A significant lag phase was repeatedly observed upon transfer of active MTBE-degrading cultures of RS24 to fresh medium. However, when active cultures were spiked with MTBE no lag in degradation was observed (Figure 20). To determine if a growth-related factor in the medium may be involved in the metabolism of MTBE by enrichment RS24, degradation experiments were conducted with fresh SSE and spent culture medium (i.e. supernatant from active MTBE-degrading cultures). Figure 22A shows MTBE degradation with corresponding increases in cell density for the cultures with spent growth medium. Despite a two-fold higher initial cell density, the cultures with fresh SSE showed reduced MTBE degradation and cell growth over the 60-day trial period (Figure 22B).

Isolation of MTBE-Degrading Bacteria

Thirteen MTBE-degrading isolates were cultivated from enrichment RS24 and MTBE degradation kinetics were determined for each isolate in the presence and absence of 2-propanol. Degradation of MTBE was more rapid and complete in the presence of 2-propanol for all isolates tested. Since many isolates had similar colony morphologies and MTBE degradation kinetics, nucleotide sequence analysis of a 340-bp region of the 16S rRNA gene was performed to identify unique isolates. Of the nine isolates characterized, four isolates showed 100% sequence similarity to *Pseudomonas* sp. Ant9 and five isolates were 100% similar to *Rhodococcus koreensis*. Figure 23 shows degradation kinetics of MTBE in the presence and absence of 2-propanol for representative isolates

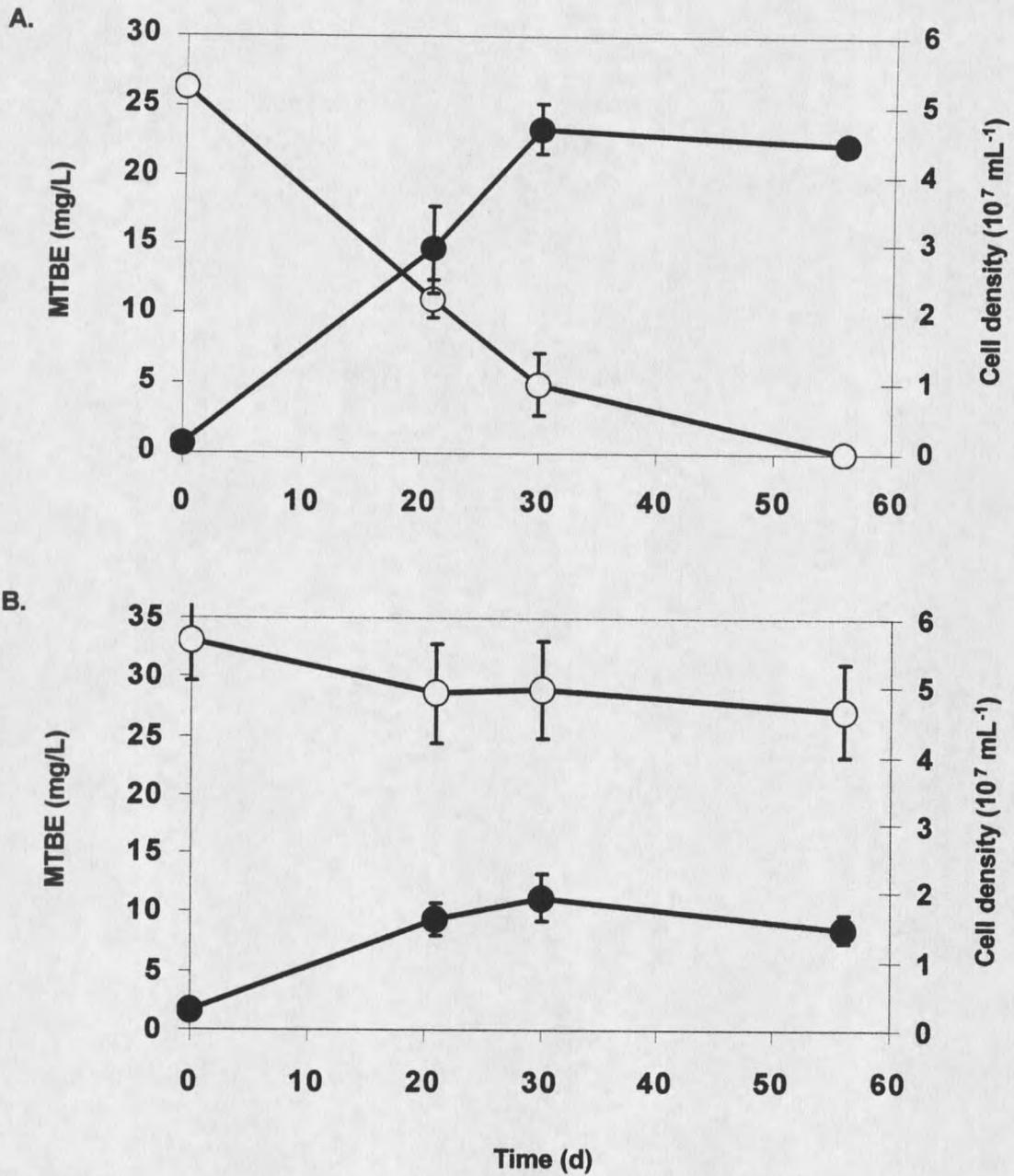


Figure 22. Degradation of MTBE by enrichment culture RS24 in the presence of spent (A) and fresh culture medium (B). MTBE disappearance (open symbols) and bacterial cell density (closed symbols) were monitored over time. Error bars represent standard deviation from the mean of duplicate cultures.

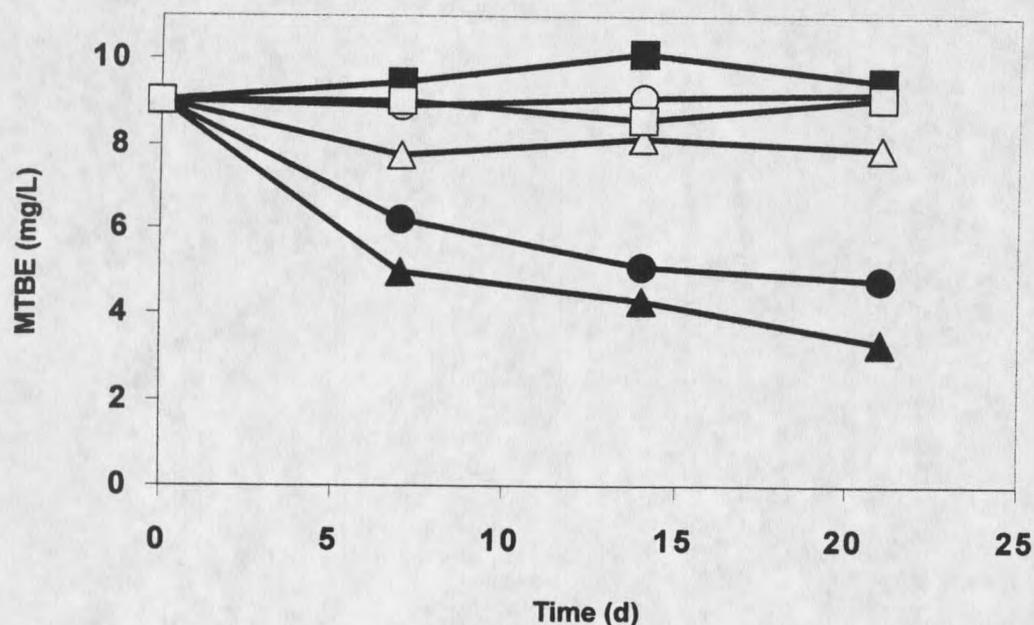


Figure 23. MTBE degradation by bacterial isolates in the presence and absence of 2-propanol as a cosubstrate. Symbols: *Pseudomonas* sp. Ant9 with (●) and without (○) 2-propanol; *Rhodococcus koreensis* with (▲) and without (△) 2-propanol; sterile controls with (■) and without (□) 2-propanol.

identified as *Pseudomonas* sp. Ant9 and *Rhodococcus koreensis*. Both isolates had similar MTBE degradation rates in the presence of 2-propanol as a cosubstrate and displayed minimal MTBE degradation in the absence of 2-propanol. GC analysis of 20 mg L⁻¹ 2-propanol treatments revealed an initial decrease in 2-propanol concentration prior to the onset of MTBE disappearance, indicating a probable cometabolic induction of MTBE degradation. Accumulation of TBA and TBF was observed during MTBE degradation with both isolates in the presence of 2-propanol.

Molecular Characterization of MTBE-Degrading Consortia

Figure 24 illustrates DGGE profiles of enrichment RS24 and two successive subcultures taken from this consortium. Sequence analysis of purified DGGE bands revealed a phylogenetically diverse group of bacteria present in the MTBE-degrading consortium, which was comprised of members of Gram-positive, proteobacterial and *Cytophaga-Flavobacterium-Bacteroides* lineages. A decrease in the total number of bands in the community profiles was observed with successive subculturing of enrichment RS24. DGGE bands identified as *Pseudomonas* sp. Ant9 and *Rhodococcus koreensis* showed 100% sequence similarity to the two cultivated MTBE-degrading isolates identified above. Interestingly, the *Rhodococcus koreensis* band disappeared completely by the second subculture, while the band corresponding to *Pseudomonas* sp. Ant9 decreased in intensity with subculturing. However, due to bias inherent in the PCR, differences in DGGE band intensity may not reflect population abundance in the consortium. The band corresponding to *Rhodoferax fermentans* was present in the original consortium and appeared stable in both subcultures. After numerous attempts, band A could not be sufficiently purified for sequence analysis. Bands H1 and H2 are believed to be heteroduplex molecules formed during the PCR reaction as single strands of DNA from different parent molecules combine to form a double-stranded product (Ferris et al., 1996). Purification and re-amplification of bands H1 and H2 always yielded band A and the band corresponding to *Rhodoferax fermentans*, suggesting these are the parent molecules that form the heteroduplexes. Since heteroduplexes are often formed from

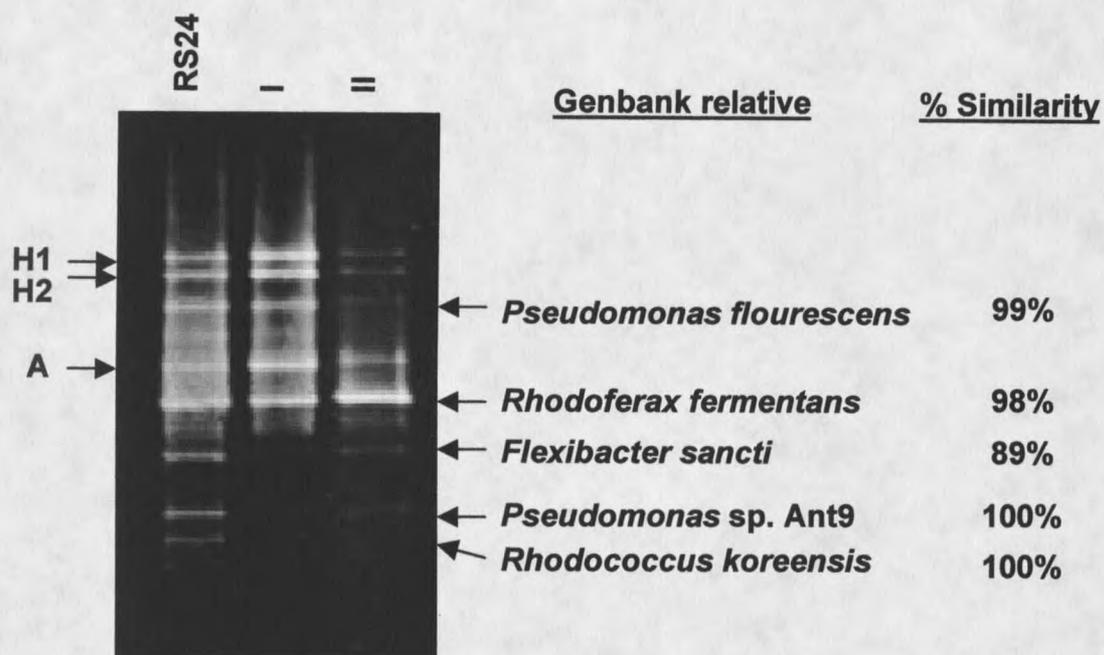


Figure 24. DGGE profiles of RS24 and two subcultures taken from RS24 at different times. Genbank accession numbers for the sequences above are *Pseudomonas fluorescens* bv. G (AF228366), *Rhodoferrax fermentans* (D16212), *Flexibacter sancti* (M62795), *Pseudomonas sp. Ant9* (AF184219) and *Rhodococcus koreensis* (AF134343).

very similar parent molecules, this indicates that the organism contributing band A may be a close relative of *Rhodofex fermentans*.

Discussion

Initial screening of the gasoline/MTBE-contaminated soils yielded a single enrichment capable of degrading MTBE in the presence of 2-propanol. Maintenance and transfer of this enrichment resulted in a bacterial consortium (RS24) that could degrade MTBE at rates of 0.66 mg d^{-1} in the absence of 2-propanol as a cosubstrate. In this consortium, cell growth coupled to MTBE degradation as the sole carbon source and the lack of metabolite accumulation suggested that the bacterial consortium derived energy from the mineralization of MTBE. Calculated gross biomass yields for enrichment RS24 ($\sim 0.25 \text{ mg dry biomass (mg MTBE)}^{-1}$) were comparable to the estimated biomass yield of $0.18 \text{ mg cells (mg MTBE)}^{-1}$ reported by Hanson et al. (12) and to the estimated biomass yields of $0.21\text{-}0.28 \text{ mg dry biomass (mg MTBE)}^{-1}$ reported by Salanitro et al. (1994). Bacterial strain PM1 isolated by Hanson et al. (12) was identified by 16S rRNA gene sequence analysis as a member of the Beta subgroup of Proteobacteria. In our study, sequence analysis of a purified DGGE band identified *Rhodofex fermentans*, also a member of the Beta subgroup of proteobacteria, as an important member of the consortium RS24 due to its stability over subculturing.

While no metabolites were detected during degradation of MTBE by enrichment RS24 in the absence of 2-propanol, accumulation of both TBA and TBF was consistently observed when RS24 was grown with 2-propanol, or in the presence of 10 mg L^{-1}

benzene and 20 mg L⁻¹ 2-propanol (data not shown). In addition, both TBA and TBF were repeatedly observed during MTBE degradation by both isolates, *Pseudomonas* sp. Ant9 and *Rhodococcus koreensis*, in the presence of 2-propanol. The presence of these organisms in the community profile of RS24 and their subsequent disappearance with subculturing suggests that these two isolates may be limited to cometabolic MTBE-degradation. Mo et al. (17) reported a reduction in MTBE degradation by a *Rhodococcus* sp. in the presence of butyl formate, *t*-butanol, and several other simple organic compounds. These results coupled with results from our study, showing an accumulation of TBA and TBF by *Rhodococcus koreensis* and *Pseudomonas* sp. Ant9, suggest an inhibitory effect caused by accumulation of these metabolites.

The production of TBA and TBF support an MTBE degradation pathway such as that proposed by Hardison et al. (13) in which the enzyme, cytochrome P-450, oxidizes MTBE to *tert*-butoxymethanol, which then may be converted to TBF via an alcohol dehydrogenase. The TBF may then undergo hydrolysis to TBA. This latter hydrolysis step was also proposed by Church et al. (7); however, they suggested that the initial formation of TBF may be due to abiotic chemical oxidation by atmospheric oxygen. Our results do not support chemical oxidation as a major MTBE degradation step as no disappearance of MTBE or accumulation of TBA or TBF was observed in our sterile controls in the presence or absence of 2-propanol.

The reduction of a lag phase in MTBE degradation by RS24 transfer cultures grown in spent supernatant versus fresh SSE medium suggests the production of a factor in the medium that stimulates MTBE degradation. The mechanism governing this

regulation may involve either a repressor-regulated transcriptional system or activator-dependent regulation. In the first case, a constitutively expressed repressor protein would prevent transcription of genes involved in MTBE degradation. Presumably, production or activation of an inducer protein could be initiated by the presence of MTBE or an effector protein. Production of the inducer over time would increase its concentration and affect more binding to and release of the repressor protein, thus allowing transcription of genes responsible for MTBE degradation. This type of mechanism has already been proposed for other genes involved in contaminant biodegradation. Leisinger et al. (15) constructed a model for dichloromethane (DCM) degradation in *Methylobacterium* sp. in which production of the protein DcmA, which is responsible for DCM dehalogenase production, is normally repressed by binding of the regulatory protein DcmR to the *dcmA* promoter. De-repression (i.e. activation) of *dcmA* occurs by putative substrate-induced release of DcmR. In addition, Bertoni et al. (3) showed that the XylR protein has both repressor and activator roles in the control of toluene biodegradation by *Pseudomonas putida*. Undoubtedly, some of these regulatory mechanisms are strictly intracellular in nature. However, we speculate that extracellular release of regulatory proteins acting as autoinducers such as those already identified in other types of quorum sensing and cell-cell communication would confer a selective advantage. For example, Visick and Ruby (30) suggest that quorum sensing may prevent unnecessary gene induction, allow a rapid sensing of changes in the environment, and serve to coordinate consortial metabolism among bacterial species. Such concentration-

dependent phenomena could result in observations of MTBE degradation similar to those documented in this study.

While several MTBE-degrading bacterial species were cultivated from the Ronan aquifer, MTBE biodegradation in the aquifer is difficult to assess in relation to other processes of natural attenuation. However, low dissolved oxygen (DO) measurements and low temperatures (4-12°C) suggest that biodegradation in the aquifer is relatively slow. DO measurements from the first to the fourth quarters of 1999 were consistently less than 0.5 mg L⁻¹ for MW-10. Over this time period for the same monitoring well, MTBE measurements first increased over an order of magnitude to 3,580 µg L⁻¹ and then gradually decreased again to < 300 µg L⁻¹. Likewise, total petroleum hydrocarbon (TPH) measurements increased almost two orders of magnitude to 10,900 µg L⁻¹ and then gradually decreased to < 900 µg L⁻¹. Background DO levels obtained from well MW-11 upgradient of the contaminant plume as well as from well MW-6 and MW-16 (adjacent to MW-12 but outside the contaminant plume) were consistently between 4-6 mg L⁻¹, indicating oxygen utilization coupled to the degradation of gasoline hydrocarbons inside the plume. Other studies have reported that low concentrations of DO and low temperatures severely slowed rates of MTBE degradation rate (21) and injection of pure oxygen into an impacted aquifer enhanced MTBE degradation (6). Because measured DO levels indicate that oxygen may be limiting biodegradation at this site, we recommend that any applied treatment technology should investigate oxygen addition.

This study demonstrates that bacteria capable of degrading MTBE were present in the gasoline-contaminated aquifer near Ronan, MT. To our knowledge, this is only the

second study of a gasoline-contaminated site in which indigenous MTBE-degrading bacteria have been reported (5). Other studies targeting the identification of native bacterial populations with the capacity to degrade MTBE in natural environments will help to establish the ubiquity of MTBE-degrading organisms and allow better prediction of the efficacy of biodegradation as a means to remediate MTBE-contaminated aquifers. In addition, further laboratory studies designed to elucidate factors that influence MTBE degradation, such as co-substrates or the growth factor proposed in this study, may provide insight into more efficient methods of stimulating MTBE degradation by naturally occurring bacteria.

Literature Cited

1. **Angle, J. S., S. P. McGrath, and R. L. Chaney.** 1991. New culture medium containing ionic concentrations of nutrients similar to concentrations found in the soil solution. *Appl. Environ. Microbiol.* **57**(12):3674-3676.
2. **Barker, J. F., M. Schirmer, and C. E. Hubbard.** 1996. Presented at the The Proceedings of the 1996 Petroleum Hydrocarbons & Organic Chemicals in Ground Water: *Prevention, Detection, and Remediation* Conference, The Westin Galleria, Houston, Texas.
3. **Bertoni, G., J. Perez-Martin, and V. DeLorenzo.** 1997. Genetic evidence of separate repressor and activator activities of the XylR regulator of the TOL plasmid, pWW0, of *Pseudomonas putida*. *Mol Microbiol* **23**:1221-1227.
4. **Borden, R. C., R. A. Daniel, L. E. L. IV, and C. W. Davis.** 1997. Intrinsic biodegradation of MTBE and BTEX in a gasoline-contaminated aquifer. *Water Res Res* **33**:1105-1115.
5. **Bradley, P. M., J. E. Landmeyer, and F. H. Chapelle.** 1999. Aerobic mineralization of MTBE and *tert*-butyl alcohol by streambed sediment microorganisms. *Environ Sci Technol* **33**:1877-1879.
6. **Carter, S. R., J. M. Bullock, and W. R. Morse.** 1997. Presented at the In Situ and On-Site Bioremediation, Columbus, OH.
7. **Church, C. D., L. M. Isabelle, J. F. Pankow, D. L. Rose, and P. G. Tratnyek.** 1997. Method for determination of methyl *tert*-butyl ether and its degradation products in water. *Environ Sci Technol* **31**:3723-3726.
8. **Cremonesi, L., S. Firpo, M. Ferrari, P. G. Righetti, and C. Gelfi.** 1997. Double-gradient DGGE for optimized detection of DNA point mutations. *Biotechniques* **22**(2):326-30.
9. **Ferris, M. J., G. Muyzer, and D. M. Ward.** 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. *Appl Environ Microbiol* **62**:340-346.
10. **Garnier, P. M., R. Auria, C. Augur, and S. Revah.** 1999. Cometabolic biodegradation of methyl *t*-butyl ether by *Pseudomonas aeruginosa* grown on pentane. *Appl Microbiol Biotechnol* **51**:498-503.

11. **Grosjean, E., D. Grosjean, R. Gunawardena, and R. A. Rasmussen.** 1998. Ambient concentrations of ethanol and methyl *tert*-butyl ether in Porto Alegre, Brazil, March 1996-April 1997. *Environ Sci Technol* **32**:736-742.
12. **Hanson, J. R., C. E. Ackerman, and K. M. Scow.** 1999. Biodegradation of methyl *tert*-butyl ether by a bacterial pure culture. *Appl Environ Microbiol* **65**:4788-4792.
13. **Hardison, L. K., S. S. Curry, L. M. Ciuffetti, and M. R. Hyman.** 1997. Metabolism of diethyl ether and cometabolism of methyl *tert*-butyl ether by a filamentous fungus, a *Graphium* sp. *Appl Environ Microbiol* **63**:3059-3067.
14. **Landmeyer, J. E., F. H. Chapelle, P. M. Bradley, J. F. Pankow, C. D. Church, and P. G. Tramyek.** 1998. Fate of MTBE relative to benzene in a gasoline-contaminated aquifer (1993-98). *Ground Water Mon Rem* **18**:93-102.
15. **Leisinger, T., R. Bader, R. Hermann, M. Schmid-Appert, and S. Vuilleumier.** 1994. Microbes, enzymes and genes involved in dichloromethane utilization. *Biodegradation* **5**:237-248.
16. **Mackay, D., W. Y. Shin, and K. C. Ma.** 1993. Illustrated Handbook of Physical-Chemical Properties and Environmental Fate for Organic Chemicals, vol. III. Lewis Publishers, Ann Arbor, MI.
17. **Mo, K., C. O. Lora, A. E. Wanken, M. Javanmardian, X. Yang, and C. F. Kulpa.** 1997. Biodegradation of methyl *t*-butyl ether by pure bacterial cultures. *Appl Microbiol Biotechnol* **47**:69-72.
18. **More, M. I., J. B. Herrick, M. C. Silva, W. C. Ghiorse, and E. L. Madsen.** 1994. Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment. *Appl Environ Microbiol* **60**:1572-1580.
19. **Pankow, J. F., N. R. Thomson, R. L. Johnson, A. L. Baehr, and J. S. Zogorski.** 1997. The urban atmosphere as a non-point source for the transport of MTBE and other volatile organic compounds (VOCs) to shallow groundwater. *Environ Sci Technol* **31**:2821-2828.
20. **Park, K., and R. M. Cowan.** 1997. Presented at the Fourth International In Situ and On Site Bioremediation Symposium, New Orleans, LA.
21. **Park, K., and R. M. Cowan.** 1997. Presented at the 213th ACS National Meeting, Division of Environmental Chemistry, San Fransisco, CA.

22. **Reuter, J. E., B. C. Allen, R. C. Richards, J. F. Pankow, C. R. Goldman, R. L. Scholl, and J. S. Seyfried.** 1998. Concentrations, sources, and fate of the gasoline oxygenate methyl *tert*-butyl ether (MTBE) in a multiple-use lake. *Environ Sci Technol* **32**:3666-3672.
23. **Robbins, G. A., S. Wang, and J. D. Stuart.** 1993. Using the static headspace method to determine Henry's Law constants. *Anal Chem* **65**:3113-3118.
24. **Salanitro, J. P., L. A. Diaz, M. P. Williams, and H. L. Wisniewski.** 1994. Isolation of bacterial culture that degrades methyl *t*-butyl ether. *Appl Environ Microbiol* **60**:2593-2596.
25. **Schirmer, M., and J. F. Barker.** 1998. A study of long-term MTBE attenuation in the Borden Aquifer, Ontario, Canada. *Ground Water Mon Rem* **18**:113-122.
26. **Shaffer, K. L. a. C. G. U.** 1997. Uptake of methyl tertiary butyl ether (MTBE) by groundwater solids. *Bull Environ Contam Toxicol* **59**:744-749.
27. **Skerman, V. B. D.** 1967. *A Guide to the Identification of the Genera of Bacteria*, 2 ed. The Williams and Wilkins Co, Baltimore, MD.
28. **Squillace, P. J., J. S. Zogorski, W. G. Wilber, and C. V. Price.** 1996. Preliminary assessment of the occurrence and possible sources of MTBE in groundwater in the United States, 1993-1994. *Environ Sci Technol* **30**:1721-1730.
29. **Steffan, R. J., K. McClay, S. Vainberg, C. W. Condee, and D. Zhang.** 1997. Biodegradation of the gasoline oxygenates methyl *tert*-butyl ether, ethyl *tert*-butyl ether, and *tert*-amyl methyl ether by propane-oxidizing bacteria. *Appl Environ Microbiol* **63**:4216-4222.
30. **Visick, K. L., and E. G. Ruby.** 1999. The emergent properties of quorum sensing: consequences to bacteria of autoinducer signaling in their natural environment, p. Chapter 21. *In* G. M. Dunny and S. C. Winans (ed.), *Cell-Cell Signaling in Bacteria*. ASM Press, Washington, D.C.
31. **Wilson, E.** 1997. Scientists wrangle over MTBE controversy. *Chem Eng News(May)*:54-56.

CHAPTER 5

SUMMARY

Three studies were conducted to develop a better understanding of the importance of microbial population biology in the degradation of organic contaminant mixtures. The first study investigated the selection of contaminant-degrading microbial populations during the degradation of crude oil and a synthetic hydrocarbon mixture. In this study I determined that functionally unique populations were responsible for the degradation of n-alkanes and isoprenoids, two of the primary contaminant classes in crude oil. While previous studies had reported physiological evidence suggesting the specialization of isolates to individual hydrocarbon classes, this is the first study that uses molecular analyses to link microbial population selection with the sequential degradation of individual contaminants in the crude oil mixture. However, the hypothesis posed earlier regarding the selection of either generalist or specialist species during the degradation of contaminant mixtures is likely an oversimplification of the ecology of bioremediation of crude oil. My findings suggest that while generalist species may have broad metabolic capacities that would increase their fundamental niche breadth, the realized niches of these populations are governed through competition. By demonstrating that different populations are responsible for the degradation of individual components of a mixture, future bioremediation efforts may involve a more integrated approach of stimulating the activity of individual populations to control the degradation of complex mixtures. In

addition, these results may guide us in developing testable hypotheses regarding specific mechanisms controlling the sequential degradation of individual contaminants in a complex mixture, further moving us toward the goals of prediction and control. For example, future studies may seek to determine mechanisms of competition for n-alkanes between the *A. calcoaceticus*- and *R. koreensis*-like populations selected in my enrichments. Factors such as surface chemistry (based on biosurfactant production) may be important in governing the population biology in these enrichments and increasing our knowledge about such mechanisms may lead to the development of a better predictive knowledge of contaminant mixture degradation.

The second study addressed the influence of different NAPLs on the kinetics of phenanthrene degradation and microbial population selection. In this study I found that different NAPLs affected the kinetics of phenanthrene degradation as well as the selection of contaminant-degrading populations. Heptadecane had the most significant effect on the kinetics of phenanthrene degradation and the stability of microbial communities selected in the enrichments. Previous reports have established that NAPLs are important in governing the degradation of nonpolar contaminants and this study reveals their influence on microbial community structure and function. The effect of heptadecane and pristane on the selection of phenanthrene-degrading populations was difficult to understand, due to the inability to directly link phylogeny with function in my enrichments. However, the results of the first two chapters of this thesis may aid in the development of new technologies for linking phylogeny and function. The observation that phylogenetically distinct populations (e.g. *A. calcoaceticus* and *R. koreensis*) are

responsible for the degradation of different components of a mixture, suggests that this system may be valuable for the development of new techniques based on retrieving and separating molecular biomarkers synthesized from labeled substrates. For example, the construction of parallel enrichments on phenanthrene and heptadecane with a different ^{14}C -labelled substrate in each enrichment followed by the separate specific capturing of ^{14}C -labelled 16S rRNA molecules of each population may allow the elucidation of which populations degrade which hydrocarbons. Furthermore, the label could be tracked through nucleic acids extracted from the enrichments over time to study possible consortial interactions in a contaminant mixture-based food web.

The results of the third study indicated the presence of a phylogenetically diverse group of MTBE-degrading bacteria at a gasoline-contaminated aquifer. These results suggest the possibility for natural attenuation of MTBE following the release of reformulated gasoline in soils and aquifers. Another significant finding of this study was the observation of a quorum-sensing-like response to the amendment of spent culture medium to the MTBE-degrading consortium. Additional study of this phenomenon will be necessary to determine the mechanistic basis and any application to stimulating the activity of naturally-occurring populations in gasoline-contaminated aquifers. In addition, future studies may investigate the influence of gasoline hydrocarbons on the kinetics of MTBE-degradation and the selection of MTBE-degrading bacteria.

In conclusion, these studies provide evidence of the importance of microbial population biology in bioremediation ecology. Results from these studies may guide the development of more comprehensive bioremediation strategies and promote additional

research toward understanding ecological factors that control the selection of contaminant-degrading microorganisms most fit for bioremediation of natural systems.

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