

Bridging the Gap Between Basic Research and the Application of New Technology: Anaerobic Hydrocarbon Metabolism, Bioremediation and Future Energy Supplies

Joseph M. Suflita, Todd Townsend, Lisa Gieg, Irene Davidova, Mark Nanny, and Roger C. Prince[†]
Institute for Energy and the Environment and Department of Botany and Microbiology
The University of Oklahoma; Norman, Oklahoma 73019-0245 and [†]ExxonMobil Research and Engineering Co.
Annandale, NJ 08801 email: JSuflita@ou.edu

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Introduction The worldwide demand for oil is expected to grow unabated for the foreseeable future. It is estimated that global oil consumption will increase annually by an average of more than 4×10^7 barrels per day to eventually reach 4.3×10^{10} barrels per year by 2020 [1]. This reliance on oil as the dominant form of energy has many ecological consequences. Most obvious is by the release of hydrocarbons into the environment. Fortunately less than 0.02% of U.S. annual consumption enters the ultimate environmental repository – the world oceans, with approximately an equal amount coming from natural seeps. Nevertheless, localized spills and discharges can have a substantial impact on marine, freshwater, and soil environments.

Understanding the processes that control the fate of oil in the environment is paramount for determining the associated environmental risks and for designing appropriate remedial measures. It is well recognized that the susceptibility of hydrocarbons to microbial biodegradation governs the persistence of these chemicals in the environment. The metabolic diversity and patterns of hydrocarbon biodegradation exhibited by aerobic microorganisms have been well studied [2]. This fundamental knowledge base helps underpin biotechnological advances for aerobic biodegradation, biotransformation and biocatalysis processes as summarized in a report of a recent meeting [3].

However, a comparable knowledge base on the transformation of hydrocarbons by anaerobic microorganisms does not exist. This is because historically anaerobic hydrocarbon biodegradation was considered slow, metabolically limited and ecologically insignificant. This view has been completely altered in recent years. An appreciation for the metabolism of hydrocarbons coupled with the electron acceptors other than oxygen has grown substantially in the last two decades. There is no doubt that anaerobes are capable of catalyzing remarkable reactions that have far reaching biotechnological implications.

A detailed review of the mechanisms of anaerobic hydrocarbon decay by microorganisms is beyond the scope of this communication. Rather, our objective will be to offer an overview of some of the more generalizing metabolic features pertinent to the destruction of important classes of hydrocarbons. We will also attempt to illustrate how such bioconversions can be of practical significance for selected environmental problems that impact the energy industry. Lastly, we will speculate on the potential role of such

processes on the reduction of greenhouse gas emissions and future energy supplies.

Patterns of anaerobic hydrocarbon biodegradation

Crude oils are enormously complex mixtures containing tens of thousands of individual components [4,5]. Even condensates and refined products include a dizzying array of constituent hydrocarbons. Once released in the environment, the relative concentrations of these chemicals change over time reflecting individual susceptibilities to the various fate processes such as sorption, volatilization, dispersion and biodegradation. One way of assessing the susceptibility of such complex mixtures to anaerobic biodegradation is to consider individual chemical classes of hydrocarbons and to determine how metabolism varies with structural complexity.

For instance, the anaerobic biodegradation of the BTEX hydrocarbons (benzene, toluene, ethylbenzene and the xylene isomers) has been most extensively studied because of the relatively high water solubility of these compounds and their toxicological impact. There is no doubt that each of these hydrocarbons can be mineralized under a variety of anaerobic conditions. While generalizations are often difficult to make, toluene is the most readily metabolized hydrocarbon of this group while benzene is far more recalcitrant. Not only are the BTEX compounds metabolized, evidence from a variety of laboratories clearly show that other classes of chemicals including the *n*-alkanes, branched alkanes, olefins, alicyclic and polynuclear aromatic hydrocarbons (PAHs) are amenable to biodegradation under anaerobic conditions [for reviews see 68; 911]. The quantitatively most important group of hydrocarbons, the *n*-alkanes, are rather labile under a variety of electron accepting conditions. As the degree of structural complexity increases by methylation, biodegradation of the branched alkanes tends to slow, but is far from precluded. Even the highly branched pristine and phytane, long considered to be suitable biomarker chemicals in crude oils because of their relative persistence, can no longer be considered as such since their anaerobic biodegradation has been documented [12,13]. Similarly, the cyclic alkanes are readily metabolized as are most of the mono- and dimethylated and even ethylated derivatives. However, more complicated substitution patterns leads to a more limited attack on structural isomers and generally increased resistance to anaerobic biodegradation. The latter

is also observed among the naphthalene homologs. The preferred anaerobic metabolism of 2-substituted methylnaphthalenes over 1-substituted isomers has been reported in both a naphthalene-degrading enrichment culture [14] and primary enrichments [6]. Recent results extend this observation to the ethyl- and dimethyl-substituted homologs [10].

The emerging patterns associated with anaerobic hydrocarbon biodegradation are at least as complex as those that are observed under aerobic conditions. However, this complexity is compounded by the fact that the patterns may not be the same under all anaerobic conditions. Diverse modes of anaerobic respiration frequently occur in anoxic environments. The most studied electron acceptors are carbon dioxide, sulfate, nitrate, and iron, serving as the ultimate electron acceptors in methanogenic, sulfate-reducing, nitrate-reducing, and iron-reducing environments, respectively. A variety of other metals, oxyanions, and even large molecular weight organic compounds have can also serve as electron acceptors in the absence of oxygen. Although some microorganisms are capable of multiple forms of anaerobic respiration, the availability of the individual electron acceptors largely dictates the physiological types of bacteria that can proliferate in an environment and thus the types of metabolic activities that can be reasonably anticipated. Even a cursory review of the literature will reveal that totally different pathways of hydrocarbon degradation can be observed depending on the availability of electron acceptors.

Initial Bioconversions Studies of anaerobic alkylbenzene decay often employ toluene as a model substrate. Denitrifying strains of *Thauera* and *Azoarcus* [15,16] as well as the sulfate reducing bacteria strain PRTOL1 [17] and *Desulfobacula toluolica* [17a] catalyze the first step of toluene decay via the addition of the aryl methyl carbon to the double bond of fumarate to form benzylsuccinic acid. This remarkable reaction is catalyzed by a glycol radical containing enzyme - benzylsuccinate synthase [18,19]. The same mechanism was reported for toluene degradation under methanogenic conditions [20] and anoxic phototrophic conditions [21]. Biodegradation of *m*-xylene and *o*-xylene was observed in enrichments under sulfate-reducing [22,23], nitrate-reducing [24] and methanogenic conditions [25]. There are also a few reports on the biodegradation of *p*-xylene by sulfate- and nitrate-reducing enrichments [22,26]. Several pure cultures of denitrifying and sulfate-reducing bacteria capable of *m*-xylene and *o*-xylene metabolism have been obtained [27-30]. Under these conditions, *m*- and *o*-xylene are also activated by a fumarate addition reaction [15,31]. Three pure denitrifying cultures were isolated for their ability to completely mineralize ethylbenzene [28,32]. In these organisms, ethylbenzene was initially activated via dehydrogenation to form 1-phenylethanol. While this observation attests to the diversity of anaerobic metabolism, the same parent substrate was converted to the corresponding ethylbenzylsuccinic acid under sulfate-reducing conditions

[33]. In fact, the accumulation of the corresponding fumarate addition derivatives in cultures metabolizing ethylbenzene, the *o*-, *m*-, or *p*-xylene isomers, toluene and even *m*-toluic acid was reported. Recent studies with a sulfate-reducing bacterium confirmed the activation of ethylbenzene via the fumarate addition mechanism [34].

As mentioned above, benzene is the least reactive of all the BTEX hydrocarbons but there is no doubt that it can be metabolized in the absence of oxygen. Benzene can be degraded under nitrate- [35-37], sulfate- [38-41], and Fe(III)-reducing [42-44] as well as methanogenic conditions [45-47]. Till now, only a single pure benzene-degrading anaerobe has been described and an initial methylation reaction to form toluene has been suggested [48]. However, alkylation reactions are not entirely consistent with previous information on anaerobic benzene decay. In a study using ¹³C-benzene, the presence of ¹³C-phenol and ¹³C-benzoate as intermediates in various enrichment cultures were confirmed [49]. Phenol and benzoate have been consistently detected as intermediates of anaerobic benzene decay, suggesting that hydroxylation is a key initial reaction. The conversion of the resulting phenol to benzoate could then occur by carboxylation to form *p*-hydroxybenzoate, followed by the reductive removal of the hydroxyl group. ¹³C-carbon and deuterium labeling studies confirm that the carboxyl carbon of the benzoate intermediate is derived from one of the carbon atoms of benzene [49,50]. Therefore, it seems plausible that multiple mechanisms for anaerobic benzene decay must exist in anaerobes – a conclusion that contradicts the dogma of benzene recalcitrance under anaerobic conditions of only a few years ago.

The anaerobic biodegradation of *n*-alkanes has also been demonstrated under various anaerobic conditions. Several sulfate-reducing and denitrifying bacterial strains [23, 51-54] as well as sulfate-reducing or nitrate-reducing enrichment cultures [24,55] are capable of the complete conversion of *n*-alkanes to carbon dioxide. All of the sulfate-reducing isolates are short oval rods belonging to ?-subclass of the *Proteobacteria*. None of them has been fully characterized and the complete metabolic pathway for anaerobic alkane decay remains speculative. When deuterated or protonated dodecane was incubated with an alkane-degrading sulfate-reducing culture, it could be shown that the primary attack on the parent substrate was also by addition to the double bond of fumarate. The reaction formed alkylsuccinic acid derivatives with complete deuterium retention [55]. The same mechanism was also demonstrated for a denitrifying *Azoarcus*-like strain [56]. This bioconversion represents a remarkable reaction that superficially resembles the anaerobic biodegradation of toluene. However, alkanes are not activated at a methyl position like toluene (or the xylenes). The succinyl moiety is attached subterminally at the C₂ (and less frequently the C₃) position of alkanes [55,56]. In this way, the reaction may be more like the attack of fumarate on the methylene carbon of ethylbenzene [33,34]. The enzymology of alkane metabolism is still under investigation, though EPR spectroscopy suggests

a comparable radical mechanism [56]. The major point is that the most quantitatively important fraction of petroleum, the alkanes, are indeed amenable to complete anaerobic destruction.

Polynuclear aromatic hydrocarbons are also of great environmental and regulatory concern. Recent studies have shown that naphthalene can be completely mineralized by pure cultures of sulfate-reducing and denitrifying bacteria [57,58]. Anaerobic degradation of phenanthrene was demonstrated in sediments [59,60] and in a sulfate-reducing enrichment culture [61]. Recent studies of marine sediments also indicate the loss of 25 ring PAHs under anaerobic conditions, with the smaller PAHs degrading more rapidly than the heavier molecular weight counterparts [60]. It is known that unsubstituted PAHs, such as naphthalene and phenanthrene, are initially attacked by carboxylation [61, 62]. The carbon in both cases arises from inorganic CO₂. Some alkylated PAHs, notably 2-methylnaphthalene, are also converted to naphthoic acid during the course of their metabolism [63], and the mechanism for the conversion is by addition to fumarate [62,64].

Alicyclic hydrocarbons can also comprise a substantial fraction (often ~12% wt/wt) of the organic molecules in petroleum mixtures. Despite this importance, little is known about the metabolic fate of these hydrocarbons. Recently, a study of the anaerobic metabolism of ethylcyclopentane revealed that it too was initially activated by a fumarate addition reaction to form ethylcyclopentylsuccinic acid [11]. Thus, it is increasingly clear that fumarate addition reactions represent an important mechanism for the initial anaerobic microbial attack on a wide variety of petroleum hydrocarbons.

Transitioning basic findings to the marketplace The recognition of the importance of anaerobic hydrocarbon decay has led to the development of goods and services of potential interest to the private sector. For instance, recent changes in U.S. regulatory policy precludes the disposal of offshore drilling-fluids (proprietary hydrocarbon mixtures) at sea unless the components are shown to be amenable to anaerobic biodegradation [65]. Anaerobic decay of drilling fluids allows for cheaper on-site disposal and/or the avoidance of costly barging and on-shore disposal. The basic assay procedures for determining the susceptibility of various materials to anaerobic biodegradation have been published [66,67] and such services are commercially available.

Similarly, assays of novel anaerobic metabolites can be particularly useful for evaluating the realistic prospects for the intrinsic bioremediation of petroleum-contaminated environments. Given the large number of sites known to be impacted by hydrocarbons, relying on natural biodegradation processes can be an attractive, low-cost approach to site management and restoration. Advances in knowledge on the anaerobic metabolic pathways for petroleum hydrocarbons and the detection of signature metabolites *in situ* have proven particularly useful for

confirming the importance of intrinsic biodegradation activities [33, 68-71]. The procedures for detecting these metabolites have also been transitioned to the private sector.

Just as basic information on the predominant pathways for aerobic petroleum decay lead to the practice of aerobic bioremediation, so too does fundamental information on hydrocarbon metabolism by anaerobes lead to anaerobic bioremediation attempts. In the former case, the overwhelming importance of hydrocarbon-oxidizing microorganisms with oxygenases capable of catalyzing the initial attack on a wide variety of hydrocarbon substrates is well recognized. Such organisms use oxygen as both a co-reactant for such reactions and as a terminal electron acceptor. Not surprisingly, the demand for oxygen is very high in aerobic bioremediation scenarios and a variety of strategies have been devised to provide this and other essential nutrients to the requisite microorganisms.

The availability of electron acceptors for anaerobic bioremediation activity is equally important. For instance, aerobic heterotrophic respiratory activity in a shallow aquifer contaminated by gas condensate hydrocarbons effectively reduces oxygen to undetectable levels such that anaerobic conditions prevail [69]. The same thing is true of the ferric iron and sulfate status at this site. Sulfate is quantitatively the more important electron acceptor and the consumption of many hydrocarbons linked to sulfate reduction has been reported [9-11,33,69]. It is not unusual for anaerobic hydrocarbon decay to proceed to the point where the endogenous electron acceptors are depleted and the predominant flow of carbon and energy shifts toward methanogenesis (see below). It follows then that the provision of electron acceptors to stimulate desirable bioconversions is a pertinent basis for anaerobic bioremediation practices. This approach has been successful [e.g. 72] and additional case studies will be evident in the future. Commercial companies that possess the expertise in anaerobic microbiology to implement anaerobic bioremediation are known.

Speculations on the importance of methanogenesis from oil. Recent findings have clearly demonstrated that, despite some differences in biodegradation rate and substrate specificity, organisms from a gas condensate-contaminated aquifer can biodegrade comparable amounts of oil under both sulfate-reducing and methanogenic conditions [9,10]. This activity was far more extensive and rapid than previous observations of the microbial production of methane from hexadecane [73,74]. The recognition that hydrocarbons can be converted to methane (and CO₂) prompts our speculation along several lines. First, climate change concerns may force worldwide reductions in atmospheric CO₂ concentrations. However, the CO₂ emission trend associated with fossil fuels consumption in the U.S. has steadily increased relative to the 1990 Kyoto protocol reference year (Figure 1). It is well known that natural gas consumption produces a fraction of the CO₂ per BTU

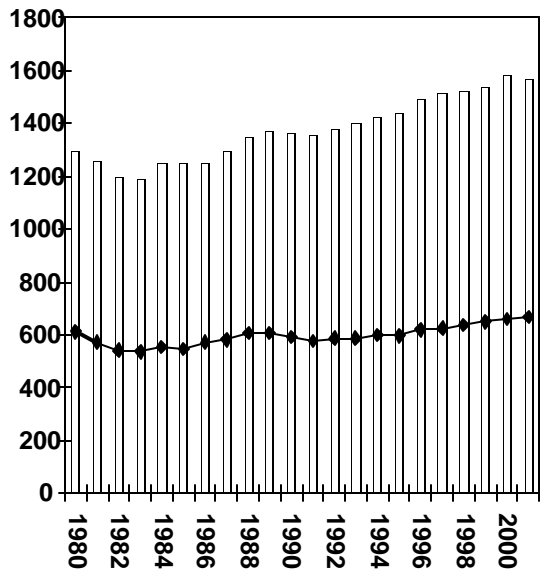


Figure 1. Total U.S. carbon dioxide emissions 1980-2001 from the consumption of fossil fuels (bar) and the fraction derived from petroleum (line) (units: million metric tons of carbon equivalent)

generated relative to that released by oil or coal utilization [75]. In fact, natural gas produces only about 56% and 71% of the CO₂ associated with the equivalent amount of energy produced from coal or oil consumption, respectively. Moreover, methane consumption results in less NO_x, SO₂ and particulates produced per amount of energy generated relative to other energy sources. Thus, the consumption of methane has many desirable environmental characteristics and it is projected to be an increasingly important fraction of the overall energy profile in many countries.

If methane, rather than petroleum, were used to generate energy in the U.S. it would obviously result in less CO₂ production. But how much of a reduction in petroleum use would be required in order to approach the 1990 emission reference year? The difference in the total amount of CO₂ produced in 2001 vs. 1990, requires a reduction of approximately 200 million metric tons of carbon equivalent (Figure 1). Considering the amount of CO₂ emitted due to petroleum utilization alone (Figure 1), the need to reduce total overall quantities and yet produce an equivalent amount of energy by natural gas consumption, a decrease in the amount of U.S. petroleum consumption of almost 30% would be required. Obviously, this dramatic shift in energy usage patterns is entirely unlikely. However, the increased use of methane instead of petroleum could certainly help reduce the rate of increase in CO₂ emissions for many countries.

While these environmental considerations are not likely to justify major changes in the energy arena, the prospect of methanogenesis from hydrocarbons may find

more serious consideration for the enhanced recovery of energy. This is because the U.S. consumes more energy than it produces with the difference made up by foreign imports. Yet, the U.S. has estimates of over 649 billion barrels of oil in place of which only 183 barrels account for cumulative oil production [76]. In effect, there are vast reserves of oil (377 billion barrels) that remain unexploited for various reasons. In an energy producing state like Oklahoma, over two-thirds of total oil reserves remains in reservoirs because recovery is not economically defensible. What if these reservoirs were inoculated with the appropriate microbial communities and at least some fraction of the available energy recovered as methane gas?

Initial laboratory experimentation on this topic has been promising (Figure 2). Samples (10g) taken from a field in Nowata, OK that had undergone secondary oil recovery procedures (water flooding) were used to test the importance of a methane-producing oil-degrading inoculum enriched from a gas-condensate contaminated aquifer [9,10]. When

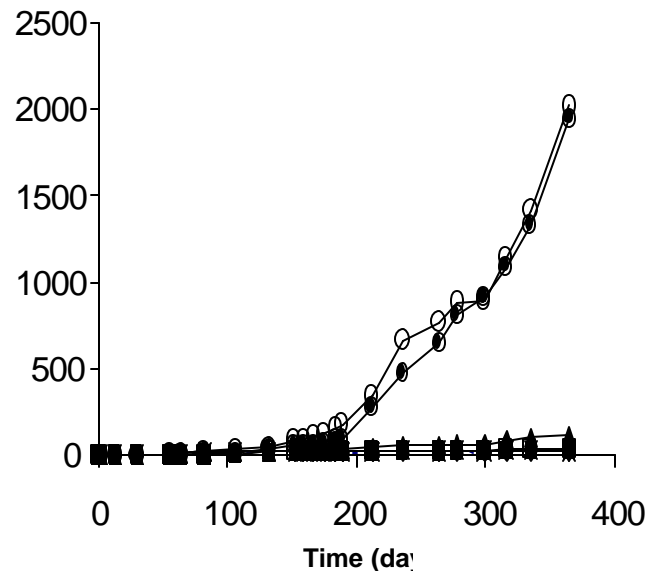


Figure 2. Methane production from residual oil in core samples inoculated with a methanogenic bacterial enrichment capable of anaerobic hydrocarbon metabolism. Symbols: Oil unamended control (○); Nowata crude oil (●); Production water (▲); An artificially weathered Alaska north slope oil standard (□); Crushed core (△); Pebbled core (◇). Heat inactivated and uninoculated controls are not depicted, but were negative.

core samples were ground or broken into small portions, the inoculum was effective in stimulating methanogenesis relative to a variety of controls. The latter included a heat-inactivated preparation, an oil-unamended control, and production water from the same field that received the inoculum (Figure 2). Interestingly, the rate of methanogenesis was much greater with the core samples

than that observed with the inoculum and a standard oil or even the formation (Nowata) crude (Figure 2). No significant methanogenesis was evident in comparable incubations that did not receive the inoculum. While the reasons for these results are under investigation, it is clear that such inocula may play a potential role for the enhanced recovery of methane in mature reservoirs.

However, increases in methanogenesis from oil may also have an important influence on the actual recovery of oil. This effect may ultimately prove more important than the actual recovery of methane. Techniques for the enhanced recovery of oil are often designed to make oil flow that would otherwise not move. This frequently involves the solubilization of oil and the reduction of interfacial tension to release oil from the subterranean surfaces. The processes involved are complex and have been reviewed [77]. It has long been recognized that gases dissolve in oil, lowering its viscosity and causing swelling. This is a major driving force for oil mobilization. The viscosity lowering of a crude by dissolved methane and its relevance for enhanced oil recovery has been considered previously [78]. Therefore, the production of methane and carbon dioxide from the microbial consumption of some of the oil may dissolve into the remainder, and reduce its viscosity to the point where it is more easily recovered. At the moment this is pure speculation, but it is estimated that enhanced oil recovery techniques can potentially add up to 60 billion barrels of oil in the near term though the increased use of existing domestic fields [76].

These few examples of progress on the biodegradation of hydrocarbons in the absence of oxygen will hopefully serve as a harbinger of future progress in the more general area of anaerobic biotechnology. While much remains to be learned, there hopefully is a narrowing gap between basic research discoveries and the extrapolation of findings to new technology.

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