11 Biodegradation of Oil Hydrocarbons and Its Implications for Source Identification

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11.1 Introduction

Hydrocarbons have been part of the biosphere from its inception, produced initially by prebiotic processes, and subsequently both by living organisms and during the generation of fossil fuels. As highly reduced forms of carbon, hydrocarbons provide a rich source of energy and carbon to those organisms, typically microorganisms, which are able to consume them. Indeed, almost all hydrocarbons are readily degraded under appropriate conditions. There is, nevertheless, a clear preference for the catabolism of some molecules before others; hence, the composition of a fuel or crude oil changes as biodegradation proceeds.

This chapter addresses the fundamentals of hydrocarbon biodegradation, especially of liquid fossil fuels, and attempts to bring together the conclusions from two rather disparate areas of research. One is from the community of microbiologists and environmental scientists studying biodegradation in the laboratory and the field. The other is from the community of geochemists studying petroleum in reservoirs. The potential timescales for these processes may be quite different. Laboratory and environmental studies of hydrocarbon biodegradation occur over days to years whereas deep subsurface reservoirs may be filled with oil for millions of years. Nevertheless, both groups agree that biodegradation leaves distinctive molecular and isotopic fingerprints in the residual material that reflect the initial nondegraded composition and the nature and extent of the microbial alteration. For environmental scientists, these molecular signatures can provide information on the type and source of contamination at spill sites, allow extrapolation of how much future biodegradation may be expected, and perhaps suggest ways of speeding up this process. For geochemists, the fingerprints can provide important information on the source rocks that matured to form the petroleum and the processes that may be altering oil quality along migration pathways or while contained in reservoir rocks.

11.2 Biochemistry of Petroleum Biodegradation

Hydrocarbons pervade the biosphere, albeit usually at low levels. They are made by many plants, animals, and microorganisms, and are a significant part of the great carbon cycle of our planet (Berner, 2003). They also are present in fossil fuels, especially petroleums (literally rock oils), which result from the combined effects of temperature and pressure on buried biomass (kerogen) over prolonged time (Tissot and Welte, 1984). Petroleum has

been leaking to the surface for a very long time. There is good evidence for active petroleum generation, and likely leakage, during the early Precambrian, 2.63 to 3.2 billion years ago (Rasmussen, 2005), even before earth's atmosphere had any appreciable oxygen (Kasting, 2004).

Biogenic and petrogenic hydrocarbons provide a rich source of energy and carbon for those organisms able to degrade them. Species in some 90 genera of bacteria grow on hydrocarbons (Prince, 2005), and many more degrade hydrocarbons while growing on other substrates. Species in more than 100 genera of fungi also have been shown to degrade hydrocarbons (Prince, 2005). Just which of these organisms, or indeed others yet to be characterized, are likely to be present during active petroleum biodegradation is probably determined by the availability of suitable terminal electron acceptors, the actual hydrocarbons present, and perhaps the history of the local environment. Some organisms have the ability to grow on a broad range of hydrocarbons, others on only a few, and the microbial ecology likely changes as the most readily degradable hydrocarbons are consumed from a petroleum mixture, and/or the availability of oxidants changes.

Before beginning a discussion of the biodegradation of petroleum hydrocarbons, it is necessary to have an overview of the composition of the various hydrocarbon fuels in commerce. Crude oils are produced all over the world, and are typically transported long distances through pipelines, by rail, and by sea before they are refined. These oils range in quality from clear, volatile fluids to near-solid, highly viscous asphalts. The density of a crude oil is the principal determinant of its value, and this is described by the API (American Petroleum Institute) gravity.

$$\text{API Gravity} = \frac{141.5}{\text{specific gravity}} - 131.5$$

expressed as degrees (°). Thus, water has an API gravity of 10°. Oils with API gravities greater than 40° are usually said to be light oils, while those with API gravities of less than about 17° are said to be heavy. Note that almost all transported oils float on water; only those with API gravities <10 will sink in fresh water.

Light oils and condensates range from orange-yellow to clear in color and are composed mostly of volatile ($<C_{10}$) saturated and aromatic hydrocarbons. These light fluids may contain minor to trace amounts of large polynuclear aromatic hydrocarbons (PAHs) and sulfur-containing compounds such as mercaptans. Crude oils are typically brown to black, principally due to large polycyclic aromatic hydrocarbons and light-absorbing heterocyclic molecules such as aliphatic and aromatic sulfides, nitrogen-containing pyrroles and pyridines, and oxygen-containing phenols, acids, and furans. These heteroatomic molecules, known variously as polars, NSO-compounds, resins, or asphaltenes, are still principally composed of carbon and hydrogen, but contain one or more sulfur, nitrogen, and/or oxygen atoms. Most are not amenable to gas chromatography and remain relatively uncharacterized (Sirota, 2005). The average composition of crude oils is ~57% saturated hydrocarbons, 29% aromatic hydrocarbon, and ~14% polars (Tissot and Welte, 1984). Aromatic hydrocarbons are defined as those containing one or more aromatic ring and include compounds with substantial alkyl substitution and/or the addition of saturated (alicyclic) rings. The saturated hydrocarbons are themselves approximately half acyclic (known as paraffins in the oil industry) and half alicyclic (known as naphthenes in the oil industry), so there is typically a rough parity between the concentration of paraffins, naphthenes, and aromatics in most crude oils, albeit with wide variation in unusual or altered examples.

Refineries convert crude oils into a range of valuable products, especially fuels. Refining starts with distillation, and the simplest distinction of the various refined products can be related to boiling-point distributions. The most volatile liquid fuel is aviation gasoline, followed by automobile gasoline, jet fuels, diesel and heating oils, and then the heavy oils used

for fueling ships and some electrical generation. All sizeable ships contain quite large volumes of heavy fuel oil, often known as Bunker C, which is barely liquid at ambient temperatures and must be kept warm to be pumped into the engines. Distillation cuts correspond roughly with carbon number distribution. Most of the molecules in gasoline have between 4 and 10 carbons, most in diesel fuel have between 9 and 20, and heavy fuel oils typically have very few molecules with less than 15 carbon atoms except for those added as diluent to achieve the appropriate viscosity. The lightest products, those with the lowest boiling points such as gasolines and diesels, are almost entirely hydrocarbons, while the heavy fuel oils are enriched in the polar constituents such as asphaltenes; this is reflected in the color of the products.

It is important to recognize that fuels are graded and sold based on their properties, such as octane- or cetane-rating or viscosity, and not on their molecular composition. There are many petroleum mixtures that meet specific product specifications. Refineries manipulate the chemical composition of the initial distillates to satisfy these requirements, and fuels with the same name can have very different chemical compositions even though all meet their specifications.

Despite the best efforts of producers, refiners, and consumers, petroleum and petroleum products invariably get spilled into the environment. Fortunately, the vast majority of the hydrocarbons in petroleum and refined products is biodegradable — the focus of this chapter. Understanding this process is important for effective cleanup of accidental oil spills, explaining the alteration that occurs in natural oil seeps, and predicting the quality of oil in potential new discoveries.

Since aerobes activate hydrocarbons by the introduction of free oxygen, whereas anaerobes must use alternative pathways, we will address aerobes separately. Environmental conditions, such as moisture, temperature, salinity, availability of trace nutrients, and composition of the fuel, further constrain and define the microbial ecology.

11.2.1 Aerobic Biodegradation of Hydrocarbons

Oxygen is both an essential reactant in the initial activation of hydrocarbons under aerobic conditions and the terminal electron acceptor for microbial growth. Hydrocarbons are initially activated through the addition of either one or both atoms of diatomic oxygen by enzymes known, respectively, as monooxygenases and dioxygenases (Figures 11-1 and 11-2) (Fritsche and Hofrichter, 2000; Kanaly

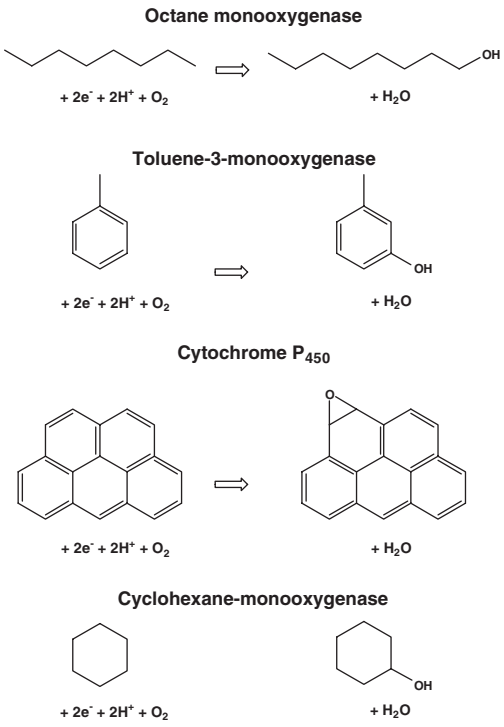


Figure 11-1 Typical reactions catalyzed by hydrocarbon monooxygenases. Octane monooxygenase of *Pseudomonas putida* catalyzes the oxidation of octane to octanol (van Beilen et al., 1994), toluene-3-monooxygenase of *Ralstonia pickettii* catalyzes the oxidation of toluene to 3-cresol (Tao et al., 2004), the cytochrome P450 of *Mycobacterium vanbaalenii* apparently catalyzes the oxidation of benzo[*a*]pyrene to benzo[*a*]pyrene-11,12-epoxide (Moody et al., 2004), and the cyclohexane monooxygenase of *Brachymonas petroleovorans* oxidizes cyclohexane to cyclohexanol (Brzostowicz et al., 2005). Other enzymes catalyze the subterminal oxidation of alkanes (Ludwig et al., 1995), and the oxidation of the 2- or 3-position of toluene (Yeager et al., 1999; Tao et al., 2004).

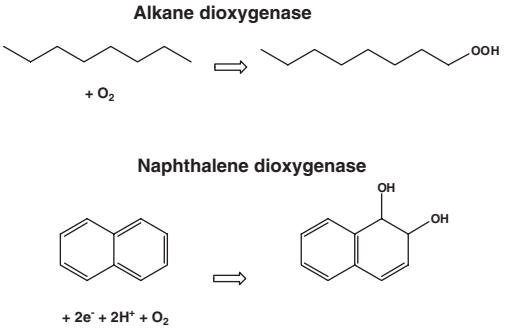


Figure 11-2 Typical reactions catalyzed by hydrocarbon dioxygenases. The alkane dioxygenase of an *Acinetobacter* apparently converts alkanes to alkanehydroperoxides (Maeng et al., 1996). Naphthalene dioxygenase of *Pseudomonas putida* oxidizes naphthalene to *cis*-naphthalene-1,2-dihydrodiol (Karlsson et al., 2003).

and Harayama, 2000; Arp et al., 2001; Parales et al., 2002; Prince, 2002; Karlsson et al., 2003; Leahy et al., 2003; Van Hamme et al., 2003; Hlavica, 2004; van Beilen and Witholt, 2005). The alkane dioxygenase of Figure 11-2 is enigmatic; it was initially proposed by Finnerty (1977) and received some support from the work of Maeng et al. (1996), but is otherwise unexplored. All other enzymes shown in Figures 11-1 and 11-2 require a source of reductant, in the form of NADH (reduced nicotinamide adenine dinucleotide), but subsequent oxidation of the oxygenated products returns this investment.

Once at least one oxygen atom has been added to a hydrocarbon, the molecule is generally amenable to manipulation by the central metabolism of the cell. Alkane alcohols (Figure 11-1) are oxidized to acids, attached to Coenzyme A, and directed to the lipid catabolic pathways. Saturated oxygenated rings, such as cyclohexanol (Figure 11-1) or cyclododecanol, are oxidized to the ketones and then by a monooxygenase to the lactone, which is hydrolyzed to the dicarboxylic acid (Cheng et al., 2002; Brzostowicz et al., 2003). These, too, are directed into the lipid catabolic pathways.

Complex branching hinders both the initial oxidation and the subsequent lipid catabolism, apparently because tertiary and quaternary carbon atoms interfere by steric hindrance with the oxidation enzymes. Thus, branched hydrocarbons are generally more resistant to degradation than linear ones, although they are eventually consumed. One way an organism can bypass tertiary and quaternary carbon atom blockage of β-oxidation is by degrading the chain from both ends, ω-oxidation. Another way is to produce different enzymes with the ability to degrade branched molecules, as elegantly demonstrated by Pirnik et al. (1974) with a *Brevibacterium.* When provided with both *n*-hexadecane and pristane (2,6,10,14-tetramethylpentadecane), the organism did not degrade pristane until the *n*-hexadecane had been degraded to less than 5% of the total hydrocarbon; afterwards, pristane was completely consumed.

This phenomenon has been used to identify the onset of biodegradation in the field. Crude oils, diesels, and heavy fuel oils usually contain both *n*-heptadecane and *n*-octadecane, together with pristane and phytane (2,6,10,14-tetramethylhexadecane), which elute just after the respective *n*-alkanes in typical gas chromatography. Miget et al. (1969) seem to have been the first to realize that a decrease in the ratio of *n*-heptadecane to pristane or *n*-octadecane to phytane presaged the onset of biodegradation.

Aromatic compounds pose the additional hurdle of opening the ring. Many pathways have been described that convert the products of initial monooxygenase activation (Figure 11-1) to dihydroxybenzoates or catechols (Ellis et al., 2006), and dioxygenases produce catechols directly (Figure 11-2). Catechols are then cleaved by extradiol or intradiol dioxygenases to linear acids that can enter classical catabolic pathways (Figure 11-3).

Most natural petroleums contain only trace amounts of alkenes. A few crude oils contain minor amounts from radiolysis (Frolov et al., 1998), but alkenes can be quite abundant in refined products such as gasoline. These are readily degraded (Solano-Serena et al., 1999, 2001). Small alkenes such as propylene and ethene are activated by specific monooxygenases to generate epoxides, which are then carboxylated with the aid of Coenzyme M and

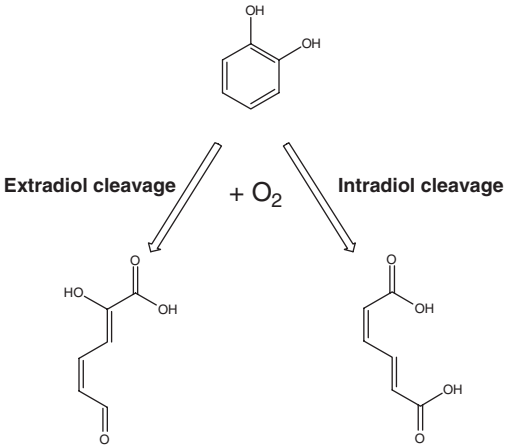


Figure 11-3 The opening of aromatic rings by extradiol and intradiol dioxygenases. Examples include the extradiol protocatechuate 4,5-dioxygenase of *Sphingomonas paucimobilis* (Sugimoto et al., 1999) and the intradiol protocatechuate 3, 4-dioxygenase of *Acinetobacter* (Vetting et al., 2000).

delivered to central metabolism (Hartmans et al., 1989; Allen et al., 1999; Ensign, 2001). Larger alkenes are dioxygenated by toluene dioxygenase (Lange and Wackett, 1997).

Further diversity in hydrocarbon catabolism is seen in the degradation of molecules that combine aromatic, alicyclic, and/or alkyl parts. For example, an *Alcanivorax* initially isolated by its ability to degrade *n*-alkanes starts degrading long-chain alkyl substituted cyclohexanes and benzenes with the oxidation of the terminal alkyl carbon (Dutta and Harayama, 2001). Whether such compounds also can be degraded by an initial oxidation of the ring is unresolved, but seems likely (Dutta, 2005). The initial oxidation of tetralin (1,2,3,4-tetrahydronaphthalene), which consists of an aromatic ring joined to a saturated cyclohexane, is by a monooxygenase on the alicyclic ring in a *Pseudomonas stutzeri*, but by a dioxygenase on the aromatic ring in *Sphingomonas macrogolitabida* (Martínez-Pérez et al., 2004).

An interesting caveat of the foregoing discussion is that many (although not all) of the enzymes capable of hydrocarbon oxidation have quite broad specificities. For example, the well-studied alkane monooxygenase (also known as alkane hydroxylase) of *Pseudomonas putida* (formerly *Ps. oleovorans*) of Figure 11-1 is capable of oxidizing *n*-alkanes with 5 to 12 carbons (*n*-pentane to *n*-dodecane), along with methyl- and, in many cases, dimethyl-forms (van Beilen et al., 1994). It is also capable of oxidizing cyclopentane, cyclohexane, methyl- and ethylcyclohexane, ethylbenzene, and several other aromatics. In fact, the enzyme oxidizes 1,3-diethylbenzene faster than it oxidizes *n*-nonane, its preferred alkane substrate. It is also a very good oxidizer of 1,4-diethylbenzene, but has very low activity with the 1,2-isomer (van Beilen et al., 1994).

Similarly, aromatic dioxygenases, such as those exemplified in Figure 11-2, often have a very broad specificity that typically extends to heterocyclic aromatics, saturated rings, and linear alkenes (Gülensoy and Alvarez, 1999; Gibson and Parales, 2000; Boyd et al., 2001; Kasai et al., 2003) although not, as far as is known, to alkanes. The potential evolutionary relationship of these enzymes to the alkane dioxygenase of Figure 11-2 is unknown. Many aromatic monooxygenases also have quite broad specificities (Sazinsky et al., 2004). For example, the appetites of four different organisms, all grown on toluene, for the C3-benzenes in gasoline (trimethyl, methyl-ethyl, propyl, and isopropyl benzenes) are distinctly different (Figure 11-4).

Among the most resistant resolvable saturated hydrocarbons under aerobic conditions are the hopanes and steranes, which are molecular fossils of the biomass that gave rise to the original kerogen (Ourisson and Albrecht, 1992; Peters et al., 2005). Some, such as the cholestanes, degrade at about the same rate as the methylated polycyclic aromatics (Prince et al., 2002). Others, such as C_{30}-hopane, are so sufficiently resistant to biodegradation that they have proved useful conserved internal markers for following biodegradation in the field. Nevertheless, several reports of hopane biodegradation have now appeared (Tritz et al., 1999; Bost et al., 2001; Frontera-Suau et al., 2002; Watson et al., 2002; Huesemann

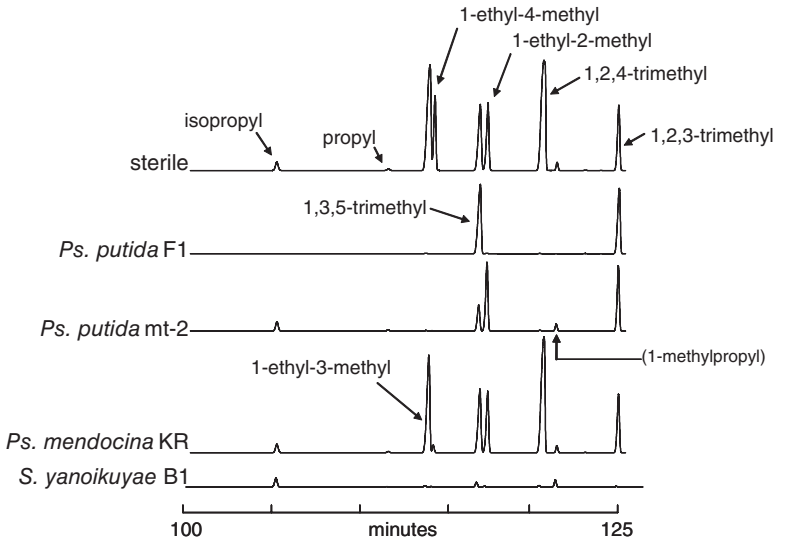


Figure 11-4 The biodegradation of trimethyl, methyl-ethyl, propyl, and isopropyl benzenes by four organisms. *Pseudomonas putida* F1 degrades toluene with a dioxygenase (Cho et al., 2000), while *Ps. putida* mt-2 (Bühler et al., 2000), and *Ps. mendocina* KR (Tao et al., 2004) use monooxygenases, directed at the methyl group and the *para*-position, respectively. *Sphingomonas yanoikuyae* B1 possesses a biphenyl dioxygenase and a xylene monooxygenase (Kim and Zylstra, 1999), and it is certainly possible that all strains contain other hydrocarbon-oxidizing enzymes. These experiments (Prince, V.L., Zylstra, G.J., and Prince, R.C., unpublished) used cells initially grown with vapor phase toluene that were washed and resuspended in minimal medium with 1 µl of gasoline in 10 ml of culture in a 40-ml vial and incubated for 90–113 hr. The traces are for the $m/z = 105$ ion.

et al., 2003). Interestingly, several of the papers report biodegradation products such as carboxylic acids (Watson et al., 2002) or olefins (Tritz et al., 1999), which are quite different from the putative biodegradation products seen in biodegraded crude oils from reservoirs that we discuss ahead.

Thus, most petroleum hydrocarbons are biodegradable under aerobic conditions. Refined products such as gasoline, diesel, and jet fuel are essentially completely degradable (Eriksson et al., 1998; Solano-Serena et al., 1999, 2001; Marchal et al., 2003, Penet et al., 2004). Crude oils and heavy fuel oils, however, contain molecules that are very resistant to biodegradation, at least in the short term. For example, McMillen et al. (1995) compared the short-term biodegradability of 17 crude oils in soil microcosms and found that the API gravity was the most useful predictor of biodegradability, at least for the most degradable fraction of the oils. With 0.5 wt.% oil in a loam soil, with appropriate moisture, nutrients, and aeration, more than 61% of the most degradable oil (API = 46°) was lost in four weeks, while only 10% of the least degradable oil (API = 15°) was consumed under the same conditions. Further degradation occurs on a longer timescale; for example, some samples collected from the Baffin Island Oil Spill (BIOS) site (Sergy and Blackall, 1987) 20 years after the deliberate experimental spill of a medium crude oil (Lago Medio, API = 32°), had lost more than 87% of their initial hydrocarbons (Prince et al., 2002).

Aerobic biodegradation of crude oils and refined products seems to follow a clear progression. The most readily degraded compounds are the normal alkanes larger than hexane as well as aromatics, especially simply substituted benzenes and naphthalenes (up to at least four- or five-pendant carbons). The smaller normal and the branched alkanes, such as pristane and phytane, and the monocy-

cloalkanes are biodegraded at a slightly slower rate, as are the phenanthrenes and dibenzothiophenes. Slower still are the larger polycyclic aromatics, such as the chrysenes and pyrenes, and the larger heterocyclics. A clear pattern of biodegradation is seen within the alkylated three-ring polycyclic aromatics; the parent compound is degraded more rapidly than the various methyl forms, which in turn are degraded more rapidly than the dimethyl forms, in turn more rapidly than the trimethyl forms, etc. (Elmendorf et al., 1994; Douglas et al., 1996; Prince et al., 2003). While individual organisms show clear preferences for degrading particular benzene isomers (Figure 11-4), samples collected from the field or laboratory samples from experiments with enrichment cultures do not usually show such obvious preferences.

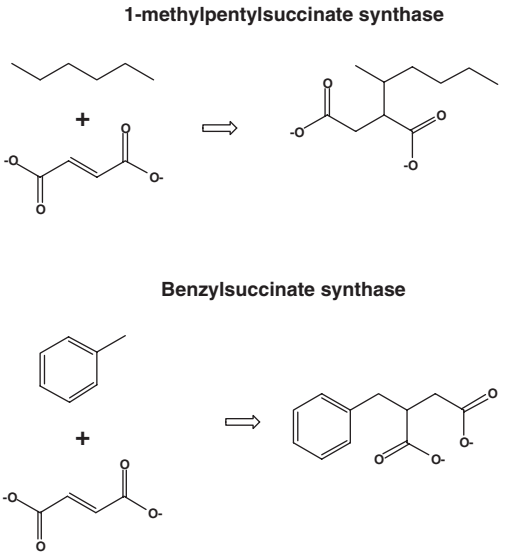


Figure 11-5 Anaerobic hydrocarbon activation by fumarate addition. The denitrifying bacterium HxN1 catalyzes the production of 1-methylpentylsuccinate from hexane (Rabus et al., 2001) and Biegert et al. (1996) were the first to demonstrate the production of benzylsuccinate under anaerobic conditions with *Thauera aromatica*. Similar reactions have been seen under a variety of conditions by a diverse array of organisms (Rabus, 2005).

11.2.2 Anaerobic Biodegradation of Hydrocarbons

Because no compounds can substitute for molecular oxygen in hydrocarbon-activating oxygenase enzymes, it was long thought that hydrocarbons could not be biodegraded under anoxic conditions. We now know that many simple hydrocarbons are consumed under a variety of oxygen-free conditions, including sulfate-reducing, nitrate-reducing, perchlorate-reducing, ferric ion-reducing, humic acid-reducing, and methanogenic conditions (Cervantes et al., 2000; Meckenstock et al., 2000; Spormann and Widdel, 2000; Zwolinski et al., 2000; Widdel and Rabus, 2001; Chakraborty and Coates, 2004; Meckenstock et al., 2004; Gieg and Suflita, 2005; Rabus, 2005). Despite this diversity of anaerobic metabolic conditions, the activation of hydrocarbons appears to follow certain themes. Most common seems to be the addition of fumarate, yielding a substituted succinate (Figure 11-5), which has been demonstrated for alkanes, aromatics, and naphthenes (Widdel and Rabus, 2001; Rios-Hernandez et al., 2003; Meckenstock et al., 2004; Cravo-Laureau et al., 2005). Subsequent metabolism and addition of Coenzyme A deliver the hydrocarbon to the lipid catabolic pathways of the cell. Apparently less common, but implicated for both alkanes and polycyclic aromatics, is the addition of inorganic bicarbonate/carbonate/CO_2 (Figure 11-6), perhaps after a transient initial hydroxylation of the substrate, as has been shown for benzene (Chakraborty and Coates, 2005; Ulrich et al., 2005). There are suggestions that methylation also can occur (Rabus, 2005; Ulrich et al., 2005; Safinowski and Meckenstock, 2006). *Azoarcus* and related organisms oxidize ethylbenzene, propylbenzene, and 3-methyl-2-pentene (but not 3-methyl-1-pentene) to ethanols, with the oxygen coming from water (Figure 11-6). So far, this activity seems restricted to these substrates, and the activation of these substrates by fumarate addition is more common (Figure 11-5).

Although anaerobic hydrocarbon microbiology is still in its infancy, a broad range of compounds have been found to be degraded,

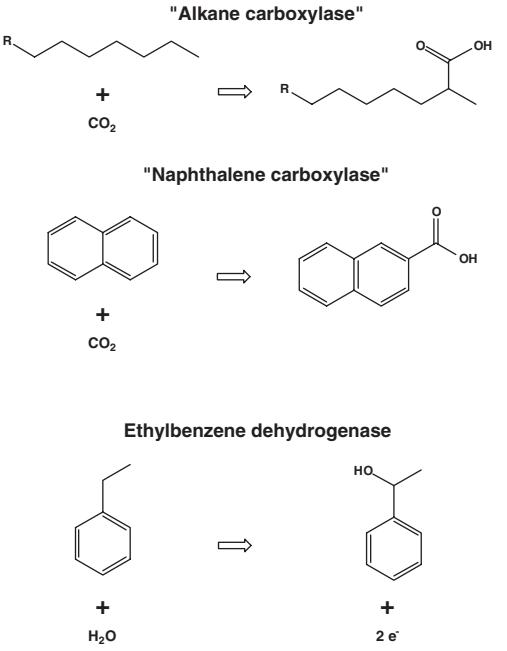


Figure 11-6 Anaerobic hydrocarbon activation by carboxylation and dehydration. Carboxylation at the 2-carbon of hexadecane by the sulfate-reducing strain AK-01 was reported by So and Young (1999). Carboxylation at the 3-position was subsequently reported for strain Hxd3 (So et al., 2003). Carboxylation of naphthalene by a sulfate-reducing enrichment culture was reported by Zhang and Young (1997). Recent work on benzene degradation (Chakraborty and Coates, 2005; Ulrich et al., 2005) suggests that a transient hydroxylation precedes carboxylation. While it seems clear that labeled bicarbonate is incorporated into the hydrocarbons, the enzymes responsible have not been characterized. In contrast, ethylbenzene dehydrogenase from *Azoarcus* has been well-characterized (Johnson et al., 2001; Kniemeyer and Heider, 2001); it contains the molybdopterin cofactor.

including alkanes up to *n*-C_{34} (Caldwell et al., 1998), phytane (Grossi et al., 2000), tetralin (Annweiler et al., 2002), polycyclic aromatic compounds with three and more rings (Chang and Shiung, 2002; Rothermich et al., 2002; Chang et al., 2003; Meckenstock et al., 2004), thiophenes and dibenzothiophenes (Annweiler et al., 2001; Onodera-Yamada et al., 2001; Marcelis et al., 2003), and cycloalkanes (Townsend et al., 2004). Interestingly, different strains seem to show clear steric preferences in their biodegradation; for example, various microorganisms have quite distinct preferences for the different xylene isomers (Suflita et al., 2004; Rabus, 2005).

One clear distinction from aerobic degradation is the apparent preference for degrading alicyclic compounds. These were among the most rapidly degraded compounds under sulfate-reducing and methanogenic conditions in the experiments of Townsend et al. (2004), yet they are among the last to go under aerobic conditions. Furthermore, Townsend et al. (2004) saw significant biodegradation of only *trans*-1,2-dimethylcyclopentane and *cis*-1,3-dimethylcyclohexane of all the dimethyl isomers of these alicyclic compounds in the gasoline they were using, and only under sulfate-reducing, not methanogenic conditions. The parent, methyl-, and ethyl-substituted compounds also were degraded. We know of no reports of such remarkable specificity in aerobic experiments with mixed microbial populations, but note that this may reflect a limited microbial population in the contaminated subsurface aquifer used as the source of inoculum in the anaerobic experiments compared to the potentially much broader diversity in well-mixed aerobic environments. We will return to this topic later.

The number of specific organisms known to degrade hydrocarbons under anaerobic conditions (Rabus, 2005) is so far much smaller than the number known to degrade hydrocarbons by aerobic respiration (Prince, 2005). All are Proteobacteria, a super-phylum of gram-negative bacteria whose members grow with a broad diversity of catabolic pathways and terminal electron acceptors, not to mention by anaerobic photosynthesis (Gupta, 2000). No isolated archaeal culture, grown under oxic or anoxic conditions, has been shown to degrade hydrocarbons (see Prince, 2005), but methanogenic archaea must be members of hydrocarbon-degrading consortia that operate under methanogenic conditions (Caldwell et al., 1998; Zengler et al., 1999; Townsend et al., 2003). Three groups of syntrophic microorganisms may be required: hydrocarbon-degrading bacteria; acetogenic bacteria; and methanogens (Figure 11-7). Although it has not been clearly demonstrated to date, it

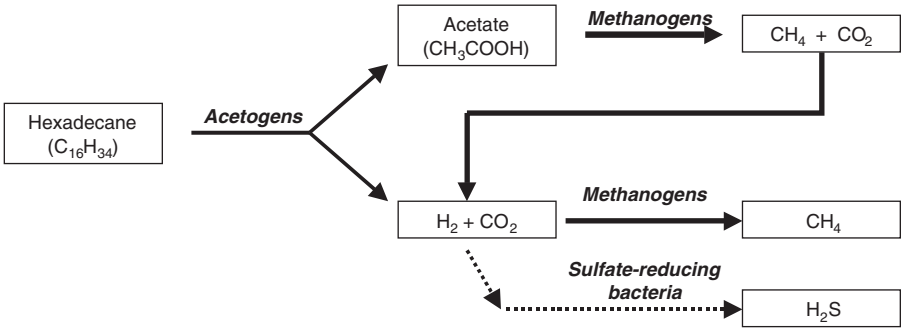


Figure 11-7 Anaerobic degradation of hexadecane under methanogenic conditions. A consortium of syntrophic eubacteria and archaea can degrade hexadecane to CH_4, CO_2, and H_2S. [Figure adapted from Parkes (1999) and Zengler et al. (1999)].

is likely that methanogenesis, including CO_2 reduction, only occurs in the absence of any other terminal electron acceptor, where the methanogen is acting as the "electron acceptor" of the otherwise anaerobically respiring hydrocarbon degrader.

The apparent paucity of anaerobic hydrocarbon degraders may reflect the bias of laboratory culture experiments. The identified Proteobacteria that grow in pure cultures are capable of utilizing specific hydrocarbons as their sole carbon source, but prefer other organic substrates, and growth on hydrocarbons is slow and considered stressed. Other bacteria are capable of degrading hydrocarbons under anaerobic conditions, but not as their sole carbon source. For example, Grishchenkov et al. (2002) reported degradation of biphenyl by *Citrobacter freundii* (a nitrate-reducing facultative anaerobe of the Proteobacteria) when grown along with culture media. Several recent studies have shown that anaerobic enrichment cultures are capable of growing on hydrocarbon mixtures (e.g., Rothermich et al., 2002; Eriksson et al., 2003; Noh et al., 2003; Rios-Hernandez et al., 2003) or whole oils (Townsend et al., 2003), and it may turn out that these cultures rely on interactions between organisms, and, thus, not be amenable to growth as pure cultures. The other potential explanation, that we will discuss in more detail ahead, is that almost by definition, anaerobic environments are not readily accessible to pelagic and air-borne invasion, so each site may have only a limited subset of the total biodiversity for this ecotype; perhaps there are far more types of anaerobes than we currently expect (Fraser, 2004), but they are geographically isolated.

11.3 Subsurface Biodegradation of Petroleum

The amount of biodegraded oil, worldwide, exceeds that of nondegraded, conventional oil. For example, the Orinoco Heavy Oil Belt in eastern Venezuela and the collective tar sands of Western Canada (e.g., Athabasca, Cold Lake, Wabasca, and Peace River) are estimated to contain ~1.2×10^{12} barrels of degraded oil each, while the supergiant oil field of Ghawar in Saudi Arabia contains ~1.9×10^{11} barrels of nondegraded oil (Roadifer, 1987). How is this biodegradation related to our understanding of biodegradation in the laboratory and the field that was discussed above?

The first thing to note is that the scales of the processes are vastly different, both in time and in volume. The vast majority of even large oil spills disappears from marine spill sites in a few years even without human help (Kingston, 2002), and although some of that oil may still be in the biosphere, its concentration is so low that it is rarely found. In contrast, biodegradation in reservoirs may occur over many thousands if not millions of years.

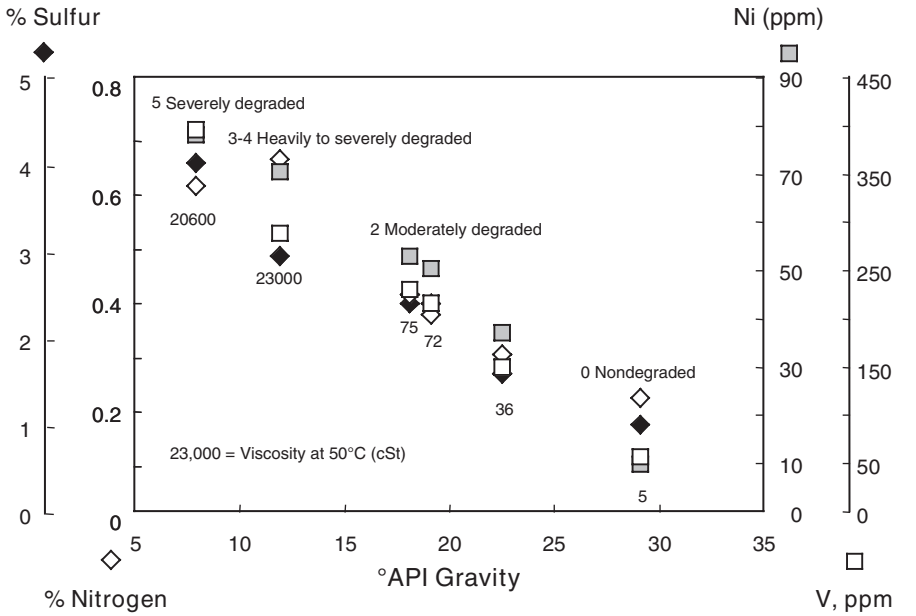


Figure 11-8 Bulk oil properties for a suite of oils (La Luna source) from Eastern Venezuela. Sulfur, nitrogen, nickel, and vanadium increase proportionally with decreasing API gravity. The degree of biodegradation is indicated by the numerical ranking of the Biomarker Biodegradation Scale (Peters et al., 2005) and by descriptive terms used by Wenger et al. (2002).

Economic reservoirs contain millions to billions of barrels of oil that even if severely biodegraded are worthwhile resources. Only the largest oil releases, such as occurred during the first Persian Gulf War or during the Ixtoc-1 blowout, are comparable in volume to minor subsurface reserves.

The second difference between laboratory, field, and reservoir biodegradation is that our understanding of the types of biodegradation becomes less clear as we move from laboratory biodegradation experiments, through monitoring biodegradation of subsurface spills in the field, to assessing biodegradation in oil reservoirs. We usually know the initial chemical composition of most surface spills and can be fairly confident in assigning biodegradative losses (Prince et al., 2002). In contrast, oil in reservoirs is usually fairly homogeneous, and while there are sometimes differences in composition from different levels or wells within a given play, it is uncommon to find equivalent, undegraded oil to establish the initial composition (although see Figures 11-8 and 11-9). Obviously, the redox conditions, whether aerobic or anaerobic, and available terminal electron acceptors are well-controlled in laboratory studies and can often be inferred from subsurface spills into near-surface aquifers. Redox conditions and the availability of electron acceptors are generally not known in deep subsurface reservoirs and, furthermore, may have varied over time. Thus, there is not yet a clear view of the relative contributions of aerobic and anaerobic biodegradation to the total biodegradative process in reservoirs, although forensic geochemistry is beginning to be applied to unravel these effects.

In any case, the oil exploration industry has considerable interest in biodegradation, as it can greatly impact the economic value and producibility of petroleum. Just as in laboratory experiments, saturated and aromatic hydrocarbons are consumed preferentially, and heterotomic compounds increase in relative abundance. Hence, petroleum quality

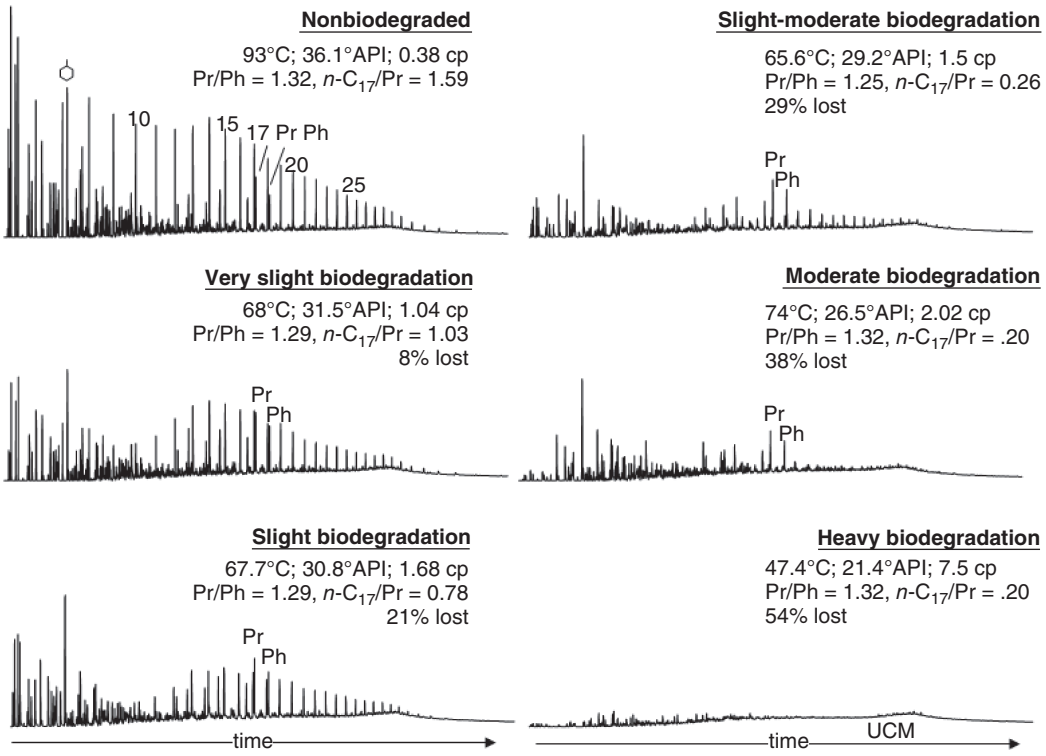


Figure 11-9 Gas chromatograms for a suite of selected crude oils from Africa. The oils were generated and expelled from the same source rock under comparable thermal conditions, but indicate quite different amounts of *in situ* biodegradation. Shown are reservoir temperature, API gravity, viscosity, pristane/phytane (Pr/Ph) and nC$_{17}$/ pristane (nC$_{17}$/Pr) ratios, and mass percentage lost calculated using hopane as a conserved internal standard. Number labels indicate *n*-alkane carbon number [modified from Wenger et al. (2002)].

decreases (i.e., the residuum has higher specific gravity, viscosity, and concentrations of heteroatoms and trace metals), and the volume of producible liquids decreases as gases and nonproducible solid bitumens increase.

The first indications of oil biodegradation typically involve the selective removal of C_6–C_{12} normal alkanes and small aromatics (Figure 11-9), although the latter may also be lost by water washing. As biodegradation proceeds, saturated hydrocarbons outside the initial range are selectively removed, with normal alkanes removed more rapidly than mono- and multimethylated alkanes. As the major resolved compounds diminish, the chromatographic baseline hump becomes more prominent. This hump is called the "unresolved complex mixture" (UCM) and consists of bioresistant compounds, including highly branched and cyclic saturated, aromatic, naphthenoaromatic, and polar compounds (Sutton et al., 2005). Depending on the amount of high-molecular-weight polar compounds and asphaltenes that are initially present in nondegraded petroleum, the UCM may account for nearly all to less than half of the total mass of a highly degraded oil. We reiterate that most of the polar and asphaltene compounds do not volatilize and are not detected by whole-oil gas chromatographic analysis.

Since normal and branched paraffins typically constitute ~35–50% of the hydrocarbons of a nonbiodegraded oil, their removal greatly alters the physical properties and economic value of a crude oil. Wenger et al. (2002) illustrated the impact of biodegradation on oil

quality by analyzing a suite of petroleum samples that were generated from the same marine shale source facies and expelled at about the same level of maturity (Figure 11-9). Correlation of the oils was made using bioresistant cyclic saturated and aromatic hydrocarbons, including many biomarkers (Peters et al., 2005), that are unaltered at this level of biodegradation. The differences in physical properties between the nondegraded and heavily degraded oils are considerable; API gravity changes from 36.1–21.4° and viscosity increases from 0.38–7.5 centiPoise, respectively. Even mild to moderate levels of microbial alteration can have a strong influence on whether reservoir accumulations are deemed suitable for economic development.

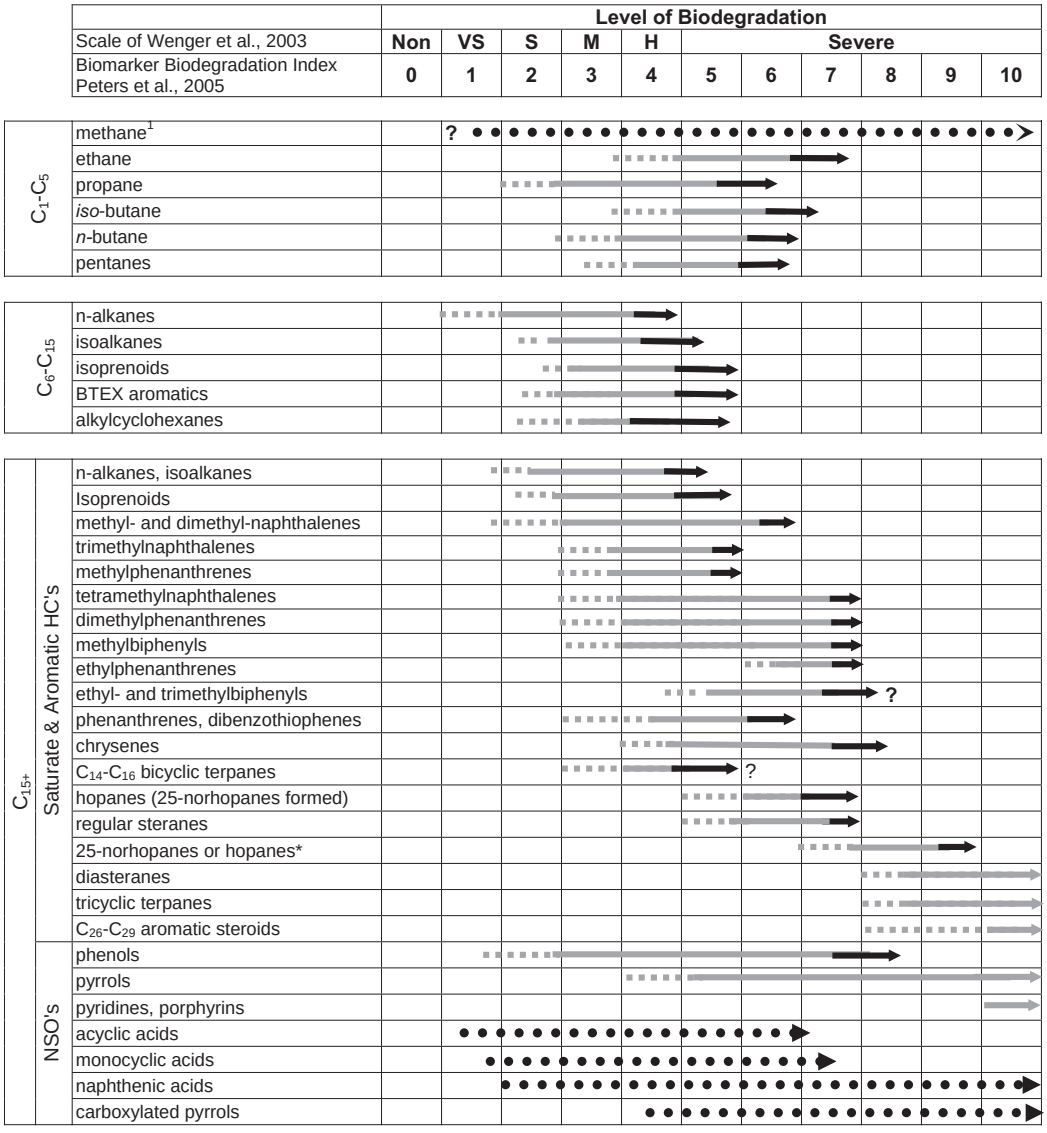
Saturated and aromatic biomarkers are biodegraded only after consumption of *n*-alkanes, most simple branched alkanes, and some of the alkylated aromatics (Seifert and Moldowan, 1979; Seifert et al., 1984; Moldowan et al., 1992; Peters et al., 2005). Laboratory and empirical observations indicate that these compounds also are consumed in a preferential order. Regular steranes and alkylated aromatics are the most susceptible to biodegradation, followed by hopanes, aromatic steroidal hydrocarbons, diasteranes, and tricyclic terpanes. At advanced stages of alteration, certain biomarkers, such as 25-norhopanes and secohopanes, appear to be created.

Because the polar (NSO) fraction is enriched by biodegradation, these molecules were long considered to be very resistant to microbial alteration. In fact, while some species, such as porphyrins, are highly conserved, others such as phenols are removed at rates comparable to aromatic hydrocarbons, while pyrroles may persist with altered alkyl sidechains. Total acid abundance increases with increasing biodegradation, but some acyclic and monocyclic acids are consumed (Kim et al., 2005). Because of their differential resistance to biodegradation, comparisons of the relative amounts of compound classes can be used to rank extent of biodegradation of petroleum on a scale of 1–10 (Peters et al., 2005; Figure 11-10). Different degrees of bioresistance noted within chemical classes (Figure 11-11) are attributed to conformational selectivity of specific enzymes involved in the degradation process.

11.3.1 The Biodegradation of Hopanes and the Formation of 25-Norhopanes

The biodegradation of hopanes, a homologous series of C_{27}–C_{35} pentacyclic triterpanes, remains something of a conundrum. We previously discussed the evidence from laboratory experiments, where degradation to hopanoic acids is sometimes reported. Here we address the evidence from reservoir oils. Some severely biodegraded oils contain 25-norhopanes, and hopanes were apparently removed prior to steranes, while other severely biodegraded oils contain no 25-norhopanes, and steranes were apparently removed prior to hopanes. From such studies, Brooks et al. (1988) concluded that two pathways for hopane biodegradation occur in the subsurface: one where 25-norhopanes are generated from microbial alteration of hopanes and another where hopanes are more completely catabolized.

The presence of 25-norhopanes is enigmatic as they appear to be generated from the selective removal of the methyl group at the C-10 position (Rullkötter and Wendisch, 1982; Trendel et al., 1990) (Figure 11-12). This produces a homologous series of 25-norhopanes with a distribution similar to the parent hopanes (Figure 11-13), and their presence is thought to be indicative of severe biodegradation even though no laboratory culture has yet shown such activity, nor have they been reported to be produced at seeps or at oil spill sites. Somewhat puzzling is that some oils contain these compounds yet appear to be relatively nonbiodegraded as indicated by the predominance of *n*-alkanes and acyclic isoprenoids; Volkman et al. (1983) proposed that such oils were mixtures of biodegraded oil residues that were subsequently dissolved by nondegraded oil during accumulation in the reservoir. Such mixed oils are common in



[1] Aerobic and anaerobic oxidation of methane is well established for the near surface but not in the deep subsurface.

* hopanes degraded without the formation of 25-norhopanes

Figure 11-10 The extent of biodegradation of mature crude oil can be ranked on a scale of 1–10 based on differing resistance of compound classes to microbial attack. Biodegradation is quasi-sequential because some of the more labile compounds in the more resistant compound classes can be attacked prior to complete destruction of less resistant classes [from Peters et al. (2005)]. Arrows indicate where compound classes are first altered (dashed lines), substantially depleted (solid gray), and completely eliminated (black). Sequence of alteration of alkylated PAHs is based on work by Fisher et al. (1998) and Trolio et al. (1999). Qualitative descriptions of the degree of biodegradation from Wenger et al. (2002) reflect changes in oil quality.

Common order of susceptibility to Biodegradation: Most → Least

Class	Biodegradation Susceptibility
n-Alkanes	C_3 ~ C_8-C_{12} > C_6-C_8 ~ C_{12}-C_{15} > C_{6-} ~ C_{15+}
Branched alkanes	Monomethyl > polymethyl > highly branched (e.g., pristane>>2,3,4-trimethylpentane)
Acyclic Isoprenoids	Lower molecular weight (e.g., C_{10}) > higher molecular weight (e.g., C_{20}). Acyclic isoprenoids degraded before major alteration of polycyclic biomarkers.
Alkylated benzenes and PAHs	1-Ring > 2-Ring > 3-Ring > 4-Ring. Methyl and dimethyl > trimethyl or extended alkylated species
Alkylbiphenyls and Alkyldiphenylmethanes	Alkylation at C-4 > C-2 or C-3
Hopanes (25-norhopanes present)	17α-hopanes: C_{27}-C_{32} > C_{33} > C_{34} > C_{35} , C_{31}-C_{35} 22R > 22S (Peters and Moldowan, 1991). Exceptions do occur (e.g., Rullkötter and Wendisch, 1982).
Steranes (25-norhopanes present)	$\alpha\alpha\alpha$ 20R and $\alpha\beta\beta$ 20R > $\alpha\alpha\alpha$ 20S and $\alpha\beta\beta$ 20S C_{27} Cholestane > C_{28} > C_{29} > C_{30} Alkylcholestanes
Steranes (25-norhopanes absent)	$\alpha\alpha\alpha$20R(C_{27}-C_{29}) > $\alpha\alpha\alpha$20S(C_{27}) > $\alpha\alpha\alpha$20S (C_{28}) > $\alpha\alpha\alpha$20S(C_{29}) ≥ $\alpha\beta\beta$(20S+20R)(C_{27}-C_{29})
Hopanes (25-norhopanes absent)	17α-hopanes: C_{35} > C_{34} > C_{33} > C_{32} > C_{31} > C_{30} > C_{29} > C_{27} and 22R > 22S.
Diasteranes	C_{27} > C_{28} > C_{29}
Non-hopanoid triterpanes	Gammacerance and oleanane are more resistant to biodegradation than the 17α-hopanes.
Aromatic steroids	C_{20}-C_{21} TA > C_{27}-C_{29} 20R MA ~ C_{26}-C_{28} 20R TA > C_{21}, C_{22} MA. TA = triaromatic MA = monoaromatic
Porphyrins	No evidence for significant biodegradation of porphyrins (e.g., Sundararaman and Hwang, 1993)

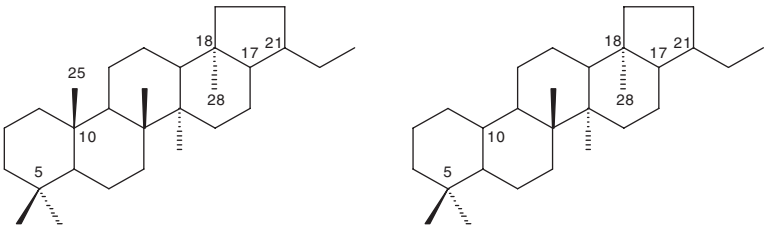
Figure 11-11 Differential biodegradation within compound classes. Modified from Peters et al. (2005).

basins with shallow (<80°C) reservoirs and may develop either as a continuous process, where the rates of biodegradation are comparable to the rates of reservoir charging, or as episodic events, where a reservoir is charged and biodegraded, and then recharged with nondegraded oil.

11.4 Factors Limiting Biodegradation

Many different factors control the extent and nature of petroleum biodegradation. Furthermore, biodegradation is likely a dynamic process with changing conditions, microbiota, and petroleum components as the process proceeds. When conditions are appropriate, large volumes of oil can be degraded in a relatively short time compared to geologic and geochemical processes. In order for petroleum biodegradation to occur

1. Terminal electron acceptors (e.g., molecular oxygen, nitrates, sulfates, ferric iron, or carbon dioxide) must be present. As we shall discuss, contaminated near-surface aquifers exhibit clear progressions as the more favorable oxidants are exhausted (Bekins et al., 1999, 2001; Haack and Bekins, 2000; Lovley and Anderson, 2000; Chapelle et al., 2002a; Kleikemper et al.,



C_{29} 5α,17α,21α-30-norhopane C_{28} 5α,17α,21α-25,30-bisnorhopane

Figure 11-12 25-norhopanes have the same optical configuration as hopanes. They differ only by the removal of the methyl group attached to the C-10 carbon.

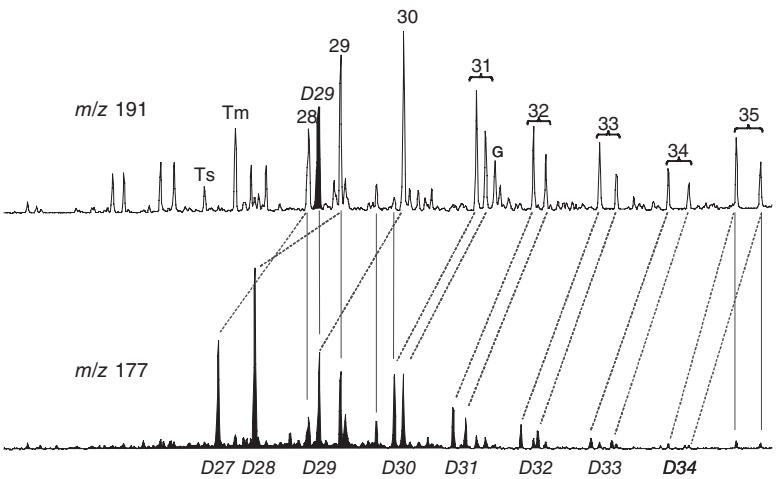


Figure 11-13 Mass chromatograms of hopanes (*m/z* 191) and norhopanes (*m/z* 177) from Eastern Venezuela. Ion traces are scaled proportional to their relative response. The 25-norhopanes (indicated by D-carbon number) are believed to originate by loss of a methyl group from C-10 in hopanes. Thus, the single epimer of C_{30} 17α,21β (H)-hopane (top) has been partially altered to C_{29} 25-nor-17α-hopane (bottom), while each of the C_{31}–C_{35} 17α-hopane (22S + 22R) epimers correspond to two C_{30} to C_{34} 25-norhopane epimers. Vertical lines indicate some peaks that yield both *m/z* 191 and 177 ions. Modified from Peters et al. (2005).

2002). Adding terminal acceptors such as sulfate, nitrate, or chelated iron can stimulate anaerobic biodegradation (Da Silva et al., 2005; Tang et al., 2005).

2. Essential nutrients must be available. Hydrocarbons provide a rich source of carbon and energy, but in general they do not provide the other essential elements of life, such as useful nitrogen, phosphorus, and trace metals, and these can limit biodegradation. Bioremediation by at least partially overcoming these limitations is a useful tool in responding to oil spills at sea and on land (Prince and Clark, 2004).
3. Microbes must be able to access the oil. Soluble components such as benzene, toluene, ethylbenzene, and the xylenes (BTEX) are washed out of petroleum and fuels, but the very low aqueous solubility of most hydrocarbons mandates that most biodegradation takes place at the oil–water interface. This implies that *in situ* biodegradation is at least partially dependent on the geometry of the reservoir (Larter et al.,

1999), and the rate at which the most biodegradable hydrocarbons can diffuse to the oil–water interface (Huang et al., 2004). Increasing a spilled oil's surface area with dispersants is an important tool in responding to oil spills at sea (Prince and Clark, 2004). When the oil is in soil or a reservoir, the matrix must have sufficient porosity and permeability to allow the diffusion of nutrients, electron acceptors, and bacteria. For example, Brooks et al. (1988) found greater biodegradation in coarse- than in fine-grained reservoir lithologies in heavy oil accumulations in western Canada.

4. Water must be present (Holden et al., 2001), and it must not be too saline for the indigenous microbes. Biodegradation in the soil experiments of McMillen et al. (1995) was severely inhibited at a soil conductivity of 41.7 mSiemens/cm, about 35 ppt ionic solids, although this was not a salt-acclimated sample. Halophilic bacteria that can degrade hydrocarbons in the presence of several molar salts have been described (Gauthier et al., 1992), but it seems that salinity should generally be less than ~100–150 parts per thousand for optimal biodegradation.
5. Temperatures must remain within limits that support life. Although no hyperthermophilic oil-degraders have yet been found, extreme thermophiles such as *Thermus* and *Bacillus* species degrade polycyclic aromatic hydrocarbons and long-chain alkanes at up to 83°C (Kato et al., 2001; Hao et al., 2004), and empirical evidence suggests that this is an upper limit for biodegradation in reservoirs (Shi Ji-Yang et al., 1982; Connan, 1984). Under typical geothermal gradients, such temperatures correspond to depths <2000 m. Significant biodegradation occurs below 0°C (Rike et al., 2003).
6. Microorganisms capable of degrading hydrocarbon must be present. Exposure to temperatures >80°C may have "sterilized" subsurface reservoirs that have subsequently cooled to more moderate temperatures (Wilhelms et al., 2001).
7. The environment must lack bacteriocides. For example, H_2S is highly toxic to aerobic microbes, and concentrations above ~5% H_2S inhibit anaerobic sulfate reducers. Indeed, sulfate-reducers are known to poison themselves with H_2S if there is no sink for this toxic waste, such as iron that can be precipitated as pyrite, or zinc that can be precipitated as sphalerite (Labrenz et al., 2000).

Microbiologists also recognize two distinct ways in which the chemical composition of a hydrocarbon mixture can affect its biodegradation. One is related to gene regulation, where the synthesis of a biodegradation enzyme or pathway is controlled by the presence of one or more of the substrates. Toluene is a prime example of a molecule that upregulates systems for its biodegradation (Leuthner and Heider, 1998; Diaz and Prieto, 2000; Tropel and van der Meer, 2004), and as we saw in Figure 11-4 and discussed above, some toluene-degrading systems have a broad substrate range. Thus, it is reasonable to expect that the biodegradation of a broad range of compounds will be enhanced in the presence of toluene, and indeed this is seen. Gülensoy and Alvarez (1999) reported enhanced aerobic biodegradation of benzene, *p*-xylene, and naphthalene by several pseudomonads when toluene was present, and a similar phenomenon occurred under anaerobic conditions (Prince and Suflita, 2006).

The other microbiological complication is the phenomenon of co-metabolism, a process usually defined as "any oxidation of substances without utilization of the energy derived from the oxidation to support microbial growth" (Horvath, 1972). Many fungi degrade hydrocarbons in this way, oxidizing large polycyclic aromatic hydrocarbons with their lignin-degrading systems (Gramss et al., 1999), but the phenomenon is well known for smaller substrates as well, such as xylenes (Tsao et al., 1998; Prenafeta-Boldu et al., 2002), thiophene, and benzothiophene (Dyreborg et al., 1996; Rivas et al., 2003). In pure cultures, such co-metabolism tends to lead to

oxidized byproducts rather than complete mineralization to CO_2, but these products are usually biodegradable by other organisms and are rarely found in mixed cultures or in the field. Unraveling the complexities of cometabolism will require much work, but it seems likely that it plays an important role in hydrocarbon degradation in the biosphere, implying that the extent of biodegradation of some hydrocarbons will depend on the presence of others.

11.5 Microbial Ecology of Petroleum Biodegradation

Microbial ecology may be defined as the study of interrelationships between microorganisms and their environment, both living and inert. For about a century, indeed since the days of the great 19th-century microbial ecologist Beijerinck, it has been widely accepted that "Microbes are everywhere, the environment selects" (Papke et al., 2003). In other words, the environment is an open system and microbes possessing traits making them suited for a particular environment will inhabit those niches in abundance while populations of unsuited organisms will be minimal. The apparent ubiquity of bacteria and archaea in many environments can be attributed to their immense population size and to their ability to disperse through the atmosphere, hydrosphere, and possibly even the lithosphere. Griffin et al. (2002) estimated that ~10^{18} bacterial cells are transported annually from one continent to the next through the air. Transport in the ocean is slower, but still open to the entire microbial biota. Because of this high dispersion, the probability of local extinction of any microbial species is low and even if the environment is totally unsuitable, some cells will be present. For example, thermophilic bacteria are found in cold sea water (Isaksen et al., 1994) and newly emerging, deep-sea hydrothermal vents are quickly colonized by thermophilic organisms (Tunnicliffe et al., 1997).

While the ubiquitous dispersion of microbial species appears true for environments on or near the surface of the earth, this may not be true for deep subsurface environments that are essentially closed systems, or for particularly unusual environments. Hyperthermophilic organisms, in particular, may not be widely dispersed. Papke et al. (2003) and Whitaker et al. (2003) studied, respectively, the genetic diversity of thermophilic cyanobacteria (*Synechococcus* and *Oscillatoria*) and archaea (*Sulfolobus solfataricus*) in hot springs from around the world and discovered genetic differences that increased with geographic distance. The genetic drift suggests that cellular exchange is not rapid between these isolated habitats. The biotic diversity and transport within the lithosphere is much lower than on the surface. Thus, microbes inhabiting contaminated aquifers and oil reservoirs are likely to be a limited subset of the surface microbes able to survive burial during deposition or transport by basinal fluid flow. Unlike the surface, the microbial population is probably not constantly renewed, and ecological principles suggest that the microbial community in a reservoir will change over time by a succession process. Similarly, the microbial ecology of a reservoir rock will likely undergo a succession of population changes as the reservoir fills with hydrocarbons.

11.5.1 The Succession of Microbial Communities

Typically, microorganisms are limited by available carbon sources or other nutrients needed for growth, such as available nitrogen or terminal electron acceptors. In the former case, incipient starvation is immediately relieved by the introduction of spilled or migrating oil or other organic pollutants. Under such conditions, microbial growth would likely be limited mostly by the availability of an oxidant unless the organisms were in a very porous environment, such as an oiled gravel shore. Nitrate, sulfate, perchlorate, carbon dioxide, organic substrates such as quinones, and oxidized metal ions, such as Mn^{IV} and Fe^{III} can all serve as oxidants in the absence of molecular oxygen. Common oxi-

Table 11-1 Comparison of Aerobic and Anaerobic Respiration Reactions

e-*Acceptor*	*Reaction (Toluene)*	*ΔG°, kJ/mol Toluene*	*Molar Ratio*	*Mass Ratio*
	Aerobic respiration			
O_2	$C_7H_8 + 9O_2 \rightarrow 7CO_2 + 4H_2O$	−3913	9	3.1
	Denitrification			
NO_3^-	$C_7H_8 + 7.2NO_3^- + 0.2H^+ \rightarrow 3.6N_2 + 7HCO_3^- + 0.6H_2O$	−3554	7.2	4.8
	Manganese reduction			
Mn(IV)	$C_7H_8 + 21MnO_2 + 14H^+ \rightarrow 7MnCO_3 + 14MnO + 7H_2O$	−3502	21	27
	Iron reduction			
Fe(III)	$C_7H_8 + 94Fe(OH)_3 \rightarrow 7FeCO_3 + 29Fe_3O_4 + 145H_2O$	−3398	94	109
	Sulfate reduction			
$SO_4^=$	$C_7H_8 + 4.5SO_4^{2-} + 3H_2O \rightarrow 7HCO_3^- + 2.5H^+ + 4.5HS^-$	−205	4.3	4.5
H_2O (CO_2)	*Methanogenesis*			
	$C_7H + 7.5H_2O \rightarrow 2.5HCO_3^- + 2.5H^+ + 4.5CH_4$	−131	7.5	1.5

Source: Modified from Heider et al. (1998) and Zwolinski et al. (2000). Note that these values reflect the overall free energy available, which may be harnessed by consortia of organisms, each capable of catalyzing only part of the process. For example, some reducing nitrate to nitrite, others reducing nitrite to nitrogen. Methanogenesis involves intermediate production and reduction of HCO_3^-.

dants, also referred to as terminal electron acceptors, used in microbial metabolisms are listed in Table 11-1 by order of decreasing energy yield. The amount of energy obtained varies appreciably. Microorganisms able to use terminal electron acceptors that yield the greatest amount of energy are likely to be most competitive and will persist until that terminal electron acceptor is exhausted. The microbial community is then replaced with organisms that can use a different terminal electron acceptor, presumably the one yielding the next-greatest energy yield. This process continues as oxidants are depleted, and the microbial community will develop as a succession of populations dictated by the availability of terminal electron acceptors.

The best example of the succession of different microbial populations driven by the availability of terminal electron acceptors is in aquifers contaminated with oil, especially the water-soluble BTEX (benzene, toluene, ethylbenzene, and the xylenes) (Bekins et al., 1999, 2001; Haack and Bekins, 2000; Lovley and Anderson, 2000; Chapelle et al., 2002a; Kleikemper et al., 2002, 2005). Distinct zones of microbial processes are formed as hydrocarbons move through the aquifer and various electron acceptors are consumed, usually in order of decreasing redox potential. At the leading edge of the flow path, oxygen is quickly consumed by aerobic organisms. Oxygen solubility in water limits its concentration to a range of 300 to 400 μM depending on temperature. After the oxygen is exhausted, nitrate-reducing bacteria become dominant if there is any nitrate. Some nitrate-reducers are facultative aerobes and switch their metabolism as oxygen is depleted; other nitrate-reducers are obligate anaerobes. With the exhaustion of nitrate, manganese- and iron-reducing bacteria become predominant and, when these metal ions are depleted, sulfate-reducing bacteria thrive if sulfate is available. Murphy and Schramke (1998) showed that sulfate may be produced through the oxidation of reduced sulfur species in the aquifer and need not be derived from gypsum/anhydrite dissolution. Sulfate-reducing bacteria outcompete methanogens in environments with even trace amounts of available sulfate, such as freshwater lakes (Lovley and Klug, 1983). The last surviving microbial population, called the "climax community," is methanogenic with carbon dioxide serving as the terminal electron acceptor being reduced to methane.

Beyond the fundamental energetics (Table 11-1), physiological controls enforce the seg-

regation. Obligate and facultative aerobes regulate their metabolism to utilize oxygen when available. The segregation of iron reduction, sulfate reduction, and methane production into discrete zones seems to be controlled by the levels of acetate and hydrogen, which are used by sulfate-reducers and methanogens. Iron-reducers keep these chemical species so low in concentration that sulfate-reducers cannot use them, but when iron-reducers start to fail for lack of ferric ion, the levels of these substrates rise and the sulfate-reducers thrive. Sulfate-reducers, in turn, keep the levels too low for the methanogens, and only when the available sulfate runs out can the methanogens emerge. Measurements of the concentrations of sulfate, iron, hydrogen, and acetate in subsurface environments have been used to predict which anaerobic microbial process is taking place (Chapelle et al., 1996; Cozzarelli et al., 2000; Kleikemper et al., 2002, 2005).

11.5.2 Deep Subsurface Ecology

The microbial ecology of a contaminated aquifer suggests that an analogous scenario occurs as oil fills a reservoir formation, creating a succession of different groups of bacteria utilizing the available terminal electron acceptors in preferential order. The aquifer model also suggests that if microbial activity persists in a reservoir with no external inputs of terminal electron acceptors (such as sulfate), it will eventually become methanogenic.

11.5.2.1 Aerobic Respiration

Aerobic biodegradation certainly takes place in seeps and spills where oil is in direct contact with the atmosphere or oxic waters. Near-subsurface reservoirs that are in contact with abundant quantities of oxygen-bearing meteoric waters also are likely to be degraded by aerobic bacteria. Transporting large amounts of dissolved oxygen into deep subsurface reservoirs is more problematic. Typical near-surface groundwaters contain less than 5 ppm dissolved oxygen and most (all?) of the oxygen is likely to be consumed before reaching a deep reservoir, either biotically or abiotically, as the water passes through soils and aquifers with reactive organic matter and minerals such as pyrite. The stripping process is very efficient as studies of shallow contaminated aquifers show that even small plumes of organic contaminants are sufficient to remove free oxygen from near-surface groundwater (e.g., Baedecker et al., 1993; Chapelle et al., 2002a). On the other hand, if meteoric water is constrained to flowing into deep reservoirs along fractures, unconformities, or highly permeable rocks, oxygen-bearing water could be transported to deep strata once all reducing agents are consumed along the pathway.

Many petroleum systems with active aquifers have flushed completely, or partially — all connate brines. Hanor et al. (2004), for example, showed that the entire sedimentary section of the National Petroleum Reserve, Alaska (NPRA), east of the Meade Ridge has been flushed with meteoric water to a depth of 2 km or more. Few studies, however, have attempted to determine the volume of water encountered per volume of oil. Horstad et al. (1992) estimated that several thousand reservoir volumes of meteoric water would be required to supply the oxygen demand required for aerobic biodegradation of oil in the Gullfaks field. Such volumes, while staggering, are not inconceivable. Mauk and Burruss (2002) estimated water : oil ratios of 300 : 1 to over 9000 : 1 in the Midcontinent rift system based on the hydrocarbon compositions of fluid inclusions. The water : oil ratio is believed to be typically <300 in sedimentary basins with commercial accumulations (Ballentine et al., 1996), and the current consensus is that nearly all deep-reservoir strata can only support anaerobic communities.

Less certain is whether large near-surface accumulations of tar sands have been biodegraded by aerobic or anaerobic processes. Connan et al. (1997) concluded that aerobic biodegradation is a dominant process in shallow petroleum reservoirs and tar sands as the degree of alteration exceeds that observed in deeper, anaerobic reservoirs. Nonetheless,

some have questioned how accumulations the size of the Orinoco or Alberta tar sands could be degraded solely by aerobic microbes, when small plumes of organic contaminants are effective at removing all dissolved oxygen. Trace metabolites consistent with an anaerobic biodegradation pathway for naphthalene (Aitken et al., 2004) and biogenic methane associated with the tar sands provide indirect evidence that these large accumulations were at least partially biodegraded by anaerobic microbial processes (Head et al., 2003).

Also unresolved is the effect that low to negligible oxygen levels may have on the subsurface microbial ecology. Facultative aerobes and obligate anaerobes would use only anaerobic pathways. Less certain is the role of microaerophilic bacteria that are capable of maintaining aerobic pathways under a low O_2 condition via biochemical pathways termed *oxidative metabolic gearing* (Ludwig, 2004). This adaptation allows microaerophilic bacteria to quickly respond to changing O_2 levels with minimal energy costs and provides a selective advantage that allows them to exploit an ecological niche not favorable for either normal aerobic or anaerobic respirers. However, little work has yet been done on hydrocarbon-degrading microaerophiles (Holden et al., 2001; Yerushalmi et al., 2001) or on the capabilities of facultative aerobes to function under suboxic conditions (Berthe-Corti and Fetzner, 2002).

11.5.2.2 Anaerobic Respiration

Although almost any oxidized pair of a redox couple, inorganic or organic, may serve as a terminal electron acceptor for microbial growth, few are readily available in reservoir rocks to support large-scale biodegradation. Of the inorganic oxidizers, only sulfate may occur in significant amounts in reservoir fluids. Although it is one of the least energetic of the inorganic terminal electron acceptors, sulfate-reducing bacteria capable of catabolizing hydrocarbons are common in oil-field formation waters (Magot, 2005). It is reasonable to conclude that sulfate-reducing bacteria are responsible for petroleum biodegradation, where sulfate is available from connate waters or gypsum/anhydrite dissolution.

Of the metals, only iron is likely to be present in any appreciable abundance in the deep subsurface. The significance of iron-reducing bacteria is just beginning to be appreciated (e.g., Coleman et al., 1993; Schmitt et al., 1996; Prommer et al., 1999; Lovley et al., 2000), and these microorganisms may play a significant role in degrading oil. Iron-reducing bacteria seem to be particularly adapted to life in the deep subsurface. All hyperthermophilic microorganisms appear capable of iron reduction, suggesting that this may have been the earliest form of anaerobic respiration (Vargas et al., 1998). Iron-reducing bacteria have been identified in enrichment cultures of oil field brines (Nazina et al., 1995; Slobodkin et al., 1999; Magot, 2005), but the extent of oil biodegradation in the deep subsurface may be severely limited by the availability of iron oxide surfaces. The abundance of Fe^{III} in sandstones is in the order of 0.4 to 4.0 oxide wt.%, found on grain coatings or pore filling material (Pettijohn, 1963). While the energy derived from reduction of Fe^{III} is comparable to that obtained from aerobic respiration or the reduction of NO_3^-, the amount of oxidant needed is appreciably more (Table 11-1). On a volume bases, ~10 L of hematite would be needed to biodegrade ~1 L of hydrocarbon. Given the very low bioavailability of ferric iron in hematite, such a process would likely be very slow and eventually hampered further by the fact that hematite tends to be removed during diagenesis and much of the iron in sedimentary reservoir rocks is bound in even less reactive silicates and sulfides.

Geobacter, the most common iron-reducer in the subsurface, must adhere to Fe^{III} oxides to reduce them (Nevin and Lovley, 2000a). Interestingly, the electron transfer may occur *via* pili that act as biological nanowires (Reguera et al., 2005). However, other bacteria have evolved several adaptions to utilize iron minerals while not in intimate contact (Nevin and Lovley, 2000b; Rosso et al., 2003). Lovley et al. (1994) showed that

anaerobic degradation of benzene in sediments was greatly stimulated by the addition of humic substances, which they reasoned shuttled electrons from the bacteria to Fe^{III} oxides (Lovley et al., 1996, 1998). Quinones (Scott et al., 1994) and humic matter are widespread (Coates et al., 1998), although not all iron-reducing bacteria can use them (Nevin and Lovley, 2000b). Some methanogenic archaea have been shown to use electron shuttles to oxidize Fe^{III} (Bond and Lovley, 2002).

Another intriguing possibility is that various oil components may serve as electron shuttles, electron donors, or terminal electron acceptors. For example, species with humic-like properties, such as asphaltenes and resins, could serve as the electron acceptors when hydrocarbons are oxidized. Phenazines are present in oil and have been proposed to act in this way (Hernandez and Newman, 2001).

Methanogens utilize CO_2 as a terminal electron acceptor. While the archaea do not appear to be capable of degrading hydrocarbons directly (Prince, 2005), methanogens do participate in bacterial consortia that collectively degrade hydrocarbons. Known consortia consist of at least three interacting groups: (1) various fermentative bacteria that consume complex compounds and excrete volatile fatty acids, H_2, and CO_2; (2) acetogenic and other bacteria that oxidize the higher acids to acetate or formate and H_2; and (3) methanogens that use several enzymatic pathways to form microbial methane, one of which is carbon dioxide reduction, where CO_2 or CO_3^{2-} is converted to CH_4 using electrons from H_2 or formate.

Since CO_2 is present as a buffered species in all reservoir brines, CO_2-reducing methanogens would not be limited by the availability of a terminal electron acceptor. Methanogenic archaea are certainly present in the deep subsurface (Obraztsova et al., 1987; Orphan et al., 2000), and biogenic methane is a component of reservoir gases that are in association with biodegraded oils (e.g., Troll field; Horstad and Larter, 1997). Such methane has been termed *secondary biogenic methane* (Scott et al., 1994) and has been proposed as a way of harnessing productive hydrocarbons in cases involving advanced biodegradation or in reservoirs that have come to the end of their productive life (Larter et al., 1999; Suflita et al., 2004).

The presence of biogenic methane with biodegraded oils, however, is not necessarily coupled as there are alternative sources of hydrogen for reducing CO_2. Nonbiotic hydrogen may form through mineral reactions, radiolysis of hydrocarbons or water, or thermal conversion of organic compounds (Kotelnikova, 2002). In fact, the deep subsurface may be dominated by hydrogen-based ecosystems where the hydrogen is derived from inorganic processes. For example, Chapelle et al. (2002b) described such an ecosystem in deep hydrothermal waters (200 m below ground) from Lidy Hot Springs in Idaho, where methanogens dominate the ecosystem and appear to thrive exclusively on geothermal hydrogen and carbon dioxide. No reduced form of carbon is available. The existence of pure subsurface lithoautotrophic microbial ecosystems, or SLiMEs, is likely but not proven (Nealson et al., 2005).

11.6 Conclusions; Implications of Biodegradation on Identification

We have seen that biodegradation can have a major impact on the composition of petroleum products and crude oils. Indeed, very few hydrocarbons are totally resistant to the process in the long term. The only other significant routes for hydrocarbons to leave the biosphere are combustion and photochemical oxidation. Photooxidation is a very important process in the atmosphere (Griffin et al., 1999; Spaulding et al., 2003), and while it does convert aromatic compounds in slicks to oxygenated species (Garrett et al., 1998), it accounts for relatively little loss of nonvolatile hydrocarbons. Burning seeps, such as the Fire Mountains on the Absheron Peninsula, have been known for millennia, and deliberate ignition is sometimes an appropriate oil spill response tool (Buist, 2003), Nevertheless, combustion pales in comparison to microbial

degradation as the principal fate of hydrocarbons in the biosphere.

Biodegradation seems to follow clear patterns that can be exploited in identifying spills and reservoir oils from similar sources, determining how far biodegradation has proceeded in a particular sample and predicting how much further biodegradation can be expected at a spill site. Linear alkanes and simple one- and two-ring aromatic compounds are degraded first under all conditions studied to date. Branched alkanes are degraded somewhat less rapidly, so ratios of linear to simply branched alkanes (e.g., octadecane to phytane) change rapidly during the early stages of biodegradation, making such ratios good indicators of the initiation of biodegradation, and poor ones for identifying sources. Multiple branched alkanes are degraded more slowly, especially those with tertiary carbons, so ratios of these molecules to each other (e.g., 2,2-dimethylbutane to 2,2-dimethylpentane) have the potential to serve as hydrocarbon fingerprints of different gasolines. 2,2,4-trimethylpentane has proven a good conserved internal marker for following the biodegradation of gasoline, but even this is eventually biodegraded completely.

Alicyclic molecules are degraded rapidly under some anaerobic conditions (Townsend et al., 2004), but apparently rather more slowly in most aerobic systems. Thus, changes in the ratios of simple alicyclic compounds to more stable ones (e.g., 2,2,4-trimethylpentane), especially if they are more rapid than the biodegradation of, for example, multiple substituted benzenes that are degraded rapidly under aerobic conditions (Solano-Serena et al., 1999), may imply that anaerobic biodegradation is the dominant process.

Three-ring aromatic hydrocarbons (phenanthrene, acenaphthene, anthracene, dibenzothiophene, etc.) are next on the seriatim, and within these compounds there is a clear pattern that increasing alkylation, at least of multiple methyl groups, slows degradation. Douglas et al. (1996) showed that the degradation of alkyl phenanthrenes and dibenzothiophenes in field samples occurs at similar rates such that the ratios of these compounds (e.g., C_3-phenanthrenes to C_3-dibenzothiophenes, where C_3 implies trimethyl-, methylethyl-, and propyl-substituents) stayed constant during substantial biodegradation, allowing these compounds to act as reliable fingerprints for discriminating different oils in the environment. Chrysenes and larger polycyclic aromatic hydrocarbons are degraded more slowly than the three-ringed aromatics.

Some steranes are degraded contemporaneously with the alkylated three-ring aromatics but are still among the least degradable components of most diesel fuels and can act as conserved markers for following biodegradation. Hopanes are more resistant than steranes in surface conditions and may be equal or more resistant under reservoir conditions. Since hopanes and oleananes are among the last saturated molecules to be biodegraded in oil spills, the distributions of these compounds can act as fingerprints for identifying oils from the same source, and they can serve as conserved internal markers for following biodegradation, and indeed photooxidation, evaporation, and partial combustion (Prince and Douglas, 2005).

Further useful forensic indicators will likely emerge as our understanding of the various different pathways of biodegradation of hydrocarbons in the environment continues to grow.

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