

# Microbial Degradation of Hydrocarbons in the Environment

JOSEPH G. LEAHY AND RITA R. COLWELL\*

*Department of Microbiology, University of Maryland, College Park, Maryland 20742*

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## INTRODUCTION

The recent spill of more than 200,000 barrels of crude oil from the oil tanker *Exxon Valdez* in Prince William Sound, Alaska (65), as well as smaller spills in Texas, Rhode Island, and the Delaware Bay (5), has refocused attention on the problem of hydrocarbon contamination in the environment. It is estimated that the annual global input of petroleum is between 1.7 and 8.8 million metric tons, the majority of which is derived from anthropogenic sources (95). Biodegradation of hydrocarbons by natural populations of microorganisms represents one of the primary mechanisms by which petroleum and other hydrocarbon pollutants are eliminated from the environment (95). The effects of environmental parameters on the microbial degradation of hydrocarbons, the elucidation of metabolic pathways and genetic bases for hydrocarbon dissimilation by microorganisms, and the effects of hydrocarbon contamination on microorganisms and microbial communities have been areas of intense interest and the subjects of several reviews (7, 9, 44, 95).

The intent of the present review is to present a broad and updated overview of the microbial ecology of hydrocarbon degradation, emphasizing both environmental and biological factors which are involved in determining the rate at which and extent to which hydrocarbons are removed from the environment by biodegradation. Aspects of biodegradation of petroleum and individual hydrocarbons in marine, freshwater, and soil ecosystems are presented. It should be noted that the majority of studies have been concerned with degradation of oil in the marine environment, and this is necessarily reflected, to a certain extent, in this review.

Applications of relatively recent advances in molecular biological techniques, such as the isolation of plasmid DNA and the construction of DNA probes, to the study of hydrocarbon degradation by microbial communities will be discussed, as well as the use of natural or genetically engineered microorganisms as seeds to increase rates of biodegradation of hydrocarbon pollutants in the environment.

## PHYSICAL AND CHEMICAL FACTORS AFFECTING THE BIODEGRADATION OF HYDROCARBONS

### Chemical Composition of the Oil or Hydrocarbons

Petroleum hydrocarbons can be divided into four classes: the saturates, the aromatics, the asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins), and the resins (pyridines, quinolines, carbazoles, sulfoxides, and amides) (44). Hydrocarbons differ in their susceptibility to microbial attack and, in the past, have generally been ranked in the following order of decreasing susceptibility: *n*-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes (104). Biodegradation rates have been shown to be highest for the saturates, followed by the light aromatics, with high-molecular-weight aromatics and polar compounds exhibiting extremely low rates of degradation (59, 76, 145). This pattern is not universal, however, as Cooney et al. (46) reported greater degradation losses of naphthalene than of hexadecane in water-sediment mixtures from a freshwater lake and Jones et al. (78) observed extensive biodegradation of alkylaromatics in marine sediments prior to detectable changes in the *n*-alkane profile of the crude oil tested. Fedorak and Westlake (53) also reported a more rapid attack of aromatic hydrocarbons during the degradation of crude oil

\* Corresponding author.

by marine microbial populations from a pristine site and a commercial harbor.

Horowitz and Atlas (71), using an in situ continuous-flow system in a study of biodegradation in Arctic coastal waters, and Bertrand et al. (26), using a continuous-culture fermentor and a mixed culture of marine bacteria, observed degradation of all fractions of crude oil at similar rates, in marked contrast to the results of most other studies. In the latter investigation, experimental conditions were optimized and extensive losses of resins (52%) and asphaltenes (74%) were observed. The microbial degradation of these fractions, which have previously been considered relatively recalcitrant to biodegradation (143), can be ascribed to cooxidation, in which non-growth hydrocarbons are oxidized in the presence of hydrocarbons which can serve as growth substrates (103). Evidence for cooxidation of asphaltenes was provided by Rontani et al. (114), who reported degradation of asphaltenic compounds in mixed bacterial cultures to be dependent upon the presence of *n*-alkanes 12 to 18 carbon atoms in length.

Compositional heterogeneity among different crude oils and refined products influences the overall rate of biodegradation both of the oil and of its component fractions. Walker et al. (147) compared the degradation of two crude and two fuel oils by a mixed culture of estuarine bacteria. Low-sulfur, high-saturate South Louisiana crude oil was the most susceptible to microbial degradation, and high-sulfur, high-aromatic Bunker C fuel oil was the least susceptible. Percent losses of saturated, aromatic, resinous, and asphaltenic hydrocarbons were highly variable among the four oils. Similarly, Jobson et al. (76) observed a greater degree of degradation of "high-quality" North Cantal crude oil than of Lost Horse Hill crude oil, which contained higher levels of sulfur, aromatics, asphaltenes, and resins, when a mixed culture enriched with the North Cantal oil was used. More extensive biodegradation of the Lost Horse Hill oil occurred when a mixed culture enriched with the same oil was used.

#### Physical State of the Oil or Hydrocarbons

Oil spilled in water tends to spread and form a slick (25). As a result of wind and wave action, oil-in-water or water-in-oil ("mousse") emulsions may form (45). Dispersion of hydrocarbons in the water column in the form of oil-in-water emulsions increases the surface area of the oil and thus its availability for microbial attack. However, large masses (or plates) of mousse establish unfavorably low surface-to-volume ratios, inhibiting biodegradation (49). Tarballs, which are large aggregates of weathered and undegraded oil, also restrict access by microorganisms because of their limited surface area (43).

The formation of emulsions through the microbial production and release of biosurfactants is an important process in the uptake of hydrocarbons by bacteria and fungi (125). Broderick and Cooney (32) reported that 96% of hydrocarbon-utilizing bacteria isolated from freshwater lakes were able to emulsify kerosene, and it has been observed that mixed cultures of marine (108) and soil (77) bacteria which effectively degrade crude oil also exhibit strong emulsifying activity.

Artificial dispersants have been studied as a means of increasing the surface area and hence the biodegradability of oil slicks. Dispersant formulations used in the 1960s were highly toxic, and their application to oiled intertidal areas following the *Torrey Canyon* spill resulted in widespread mortality of flora and fauna (48, 126). More recently devel-

oped dispersants, such as Corexit, are considerably less toxic (52), but still have been shown to inhibit microbial processes (63). The effectiveness of dispersants in enhancing the biodegradation of oil has been shown to be extremely variable and to be dependent on the chemical formulation of the dispersant, its concentration, and the dispersant/oil application ratio. Studies with different dispersants have reported increases (112), decreases (56, 93), and transitory or slight increases (56, 93, 136) in the rates of microbial degradation of crude oil and individual hydrocarbons.

The key differences between petroleum biodegradation in soil and aquatic ecosystems following an oil spill, discussed by Bossert and Bartha (29), are related to the movement and distribution of the oil and the presence of particulate matter, each of which affects the physical and chemical nature of the oil and hence its susceptibility to microbial degradation. Terrestrial oil spills are characterized primarily by vertical movement of the oil into the soil, rather than the horizontal spreading associated with slick formation. Infiltration of oil into the soil prevents evaporative losses of volatile hydrocarbons, which can be toxic to microorganisms. Particulate matter can reduce, by absorption, the effective toxicity of the components of petroleum, but absorption and adsorption of hydrocarbons to humic substances probably contribute to the formation of persistent residues.

#### Concentration of the Oil or Hydrocarbons

The rates of uptake and mineralization of many organic compounds by microbial populations in the aquatic environment are proportional to the concentration of the compound, generally conforming to Michaelis-Menten kinetics (28, 105). Michaelian kinetics have been demonstrated for the microbial uptake and oxidation of toluene (35, 111), a low-molecular-weight aromatic hydrocarbon of relatively high water solubility, but may not apply to the more insoluble hydrocarbons. The rates of mineralization of the higher-molecular-weight aromatic hydrocarbons, such as naphthalene and phenanthrene, are related to aqueous solubilities rather than total substrate concentrations (135, 152, 153). The microbial degradation of long ( $\geq C_{12}$ ) alkanes, for which solubilities are less than 0.01 mg/liter (23), occurs at rates which exceed the rates of hydrocarbon dissolution (135, 156) and are a function of the hydrocarbon surface area available for emulsification or physical attachment by cells (55, 94, 148). Biodegradation rates for many hydrocarbons, therefore, will not display the dependence on concentration which is typically observed with more soluble organic substrates.

High concentrations of hydrocarbons can be associated with heavy, undispersed oil slicks in water, causing inhibition of biodegradation by nutrient or oxygen limitation or through toxic effects exerted by volatile hydrocarbons (see below). Fusey and Oudot (59) reported that contamination of seashore sediments with crude oil above a threshold concentration prevented biodegradation of the oil because of oxygen and/or nutrient limitation. It is likely that high concentrations of oil have similarly negative effects on biodegradation rates following oil spills in other quiescent, low-energy environments such as beaches, harbors, and small lakes or ponds, in which the oil is relatively protected from dispersion by wind and wave action. Rashid (109), for example, observed that the lowest rates of degradation of crude oil spilled from an oil tanker occurred in protected bays and the highest rates occurred in the areas of greatest wave energy.

The concept of a maximum or threshold concentration for

microbial degradation of hydrocarbons may apply also to soil ecosystems. Dibble and Bartha (51) reported increases in CO<sub>2</sub> evolution over the range of 1.25 to 5% hydrocarbon mass per dry weight of soil, when oil sludge was applied to soil. No increase was observed at a level of 10%, and the rates declined at 15%. Decreases in activity at high oil loading concentrations were ascribed to inhibition of microbial activity by toxic components of the oil sludge.

### Temperature

Temperature influences petroleum biodegradation by its effect on the physical nature and chemical composition of the oil, rate of hydrocarbon metabolism by microorganisms, and composition of the microbial community (7). At low temperatures, the viscosity of the oil increases, the volatilization of toxic short-chain alkanes is reduced, and their water solubility is increased, delaying the onset of biodegradation (10). Rates of degradation are generally observed to decrease with decreasing temperature; this is believed to be a result primarily of decreased rates of enzymatic activity, or the "Q<sub>10</sub>" effect (10, 60). Higher temperatures increase the rates of hydrocarbon metabolism to a maximum, typically in the range of 30 to 40°C, above which the membrane toxicity of hydrocarbons is increased (29). Thermophilic alkane-utilizing bacteria do exist, however (85, 89).

Climate and season would be expected to select for different populations of hydrocarbon-utilizing microorganisms which are adapted to ambient temperatures. Colwell et al. (43) reported extensive degradation of Metula crude oil by mixed cultures of marine bacteria at 3°C, and Huddleston and Cresswell (73) observed petroleum biodegradation in soil at -1.1°C. By contrast, only negligible degradation of oil was exhibited in Arctic marine ice (14) and in frozen tundra soil (15). Low winter temperatures were the limiting factor for the biodegradation of polyaromatic hydrocarbons in estuarine sediment (124) and of a variety of hydrocarbons in freshwater lakes (46).

### Oxygen

The initial steps in the catabolism of aliphatic (125), cyclic (104), and aromatic (37) hydrocarbons by bacteria and fungi involve the oxidation of the substrate by oxygenases, for which molecular oxygen is required. Aerobic conditions are therefore necessary for this route of microbial oxidation of hydrocarbons in the environment. Conditions of oxygen limitation normally do not exist in the upper levels of the water column in marine (54) and freshwater (45) environments. Aquatic sediments, however, are generally anoxic except for a thin layer at the surface of the sediment (45, 66). The availability of oxygen in soils is dependent on rates of microbial oxygen consumption, the type of soil, whether the soil is waterlogged, and the presence of utilizable substrates which can lead to oxygen depletion (29). The concentration of oxygen has been identified as the rate-limiting variable in the biodegradation of petroleum in soil (139) and of gasoline in groundwater (74).

Anaerobic degradation of petroleum hydrocarbons by microorganisms has been shown in some studies to occur only at negligible rates (18, 74, 150), and its ecological significance has been generally considered to be minor (7, 29, 45, 54). However, the microbial degradation of oxidized aromatic compounds such as benzoate (134) and of halogenated aromatic compounds such as the halobenzoates (131), chlorophenols (30), and polychlorinated biphenyls (40) has

been shown to occur under anaerobic conditions. Recent evidence also indicates that microbial consortia from soil and sludge are capable of metabolizing unsubstituted and alkyl-substituted aromatics, including benzene, toluene, xylene, 1,3-dimethylbenzene, acenaphthene, and naphthalene, in the absence of molecular oxygen (62, 90, 91, 157). Hydroxylation of the aromatic ring of toluene and benzene is believed to depend on water as a source of oxygen (62). Nitrate can act as the final electron acceptor under denitrifying conditions (91, 157). The anaerobic transformation of benzene and toluene under methanogenic conditions has been tentatively characterized as a fermentation, in which the substrate is partially oxidized and partially reduced, yielding carbon dioxide and methane as end products (62). The amount of substrate removed by anaerobic biodegradation can be significant; at least 50% of benzene and toluene were mineralized in 60 days under methanogenic conditions (62), and naphthalene and acenaphthene were degraded to nondetectable levels in 45 and 40 days, respectively, under denitrifying conditions (91). The importance of anaerobic biodegradation of aromatic hydrocarbons in the environment is unknown, and further studies are required to elucidate anaerobic pathways, as well as determine whether other hydrocarbons, such as alkanes, and hydrocarbon mixtures, such as crude oil, can be fully degraded under denitrifying or methanogenic conditions.

### Nutrients

The release of hydrocarbons into aquatic environments which contain low concentrations of inorganic nutrients often produces excessively high carbon/nitrogen or carbon/phosphorus ratios, or both, which are unfavorable for microbial growth (7, 45). It is well established that the availability of nitrogen and phosphorus limits the microbial degradation of hydrocarbons in estuarine water and sediment (140), seawater (11), marine sediment (27), freshwater lakes (149), Arctic ponds (24), freshwater sediments (46), and groundwater (74). Adjustment of carbon/nitrogen/phosphorus ratios by the addition of nitrogen and phosphorus in the form of oleophilic fertilizers, including paraffinized urea, octylphosphate, ferric octoate, paraffin-supported MgNH<sub>4</sub>PO<sub>4</sub>, and 2-ethylhexyldipolyethylene oxide phosphate, stimulates the biodegradation of crude oil and individual hydrocarbons in seawater and in Arctic ponds and lakes (12, 13, 24, 50, 71, 72, 100). Inorganic salts of nitrogen and phosphorus are effective in enclosed systems (12, 13, 50, 72) but tend to wash out in simulated field experiments (12, 13).

Nitrogen and phosphorus may also be limiting in soils, and the acceleration of the biodegradation of crude oil or gasoline in soil and groundwater by the addition of urea-phosphate, N-P-K fertilizers, and ammonium and phosphate salts has been demonstrated in several studies (51, 74, 77, 138). Other investigators observed no increase in biodegradation rates (87) or an increase only after a delay of several months to a year (97, 110) when fertilizer amendments were used. These seemingly contradictory results have been attributed by Bossert and Bartha (29) to the variable and complex composition of soils and to other factors such as nitrogen reserves and the presence of nitrogen-fixing bacteria.

### Salinity

There are few published studies which deal with effects of salinity on the microbial degradation of hydrocarbons. Shiaris (124) reported a generally positive correlation between

salinity and rates of mineralization of phenanthrene and naphthalene in estuarine sediments. Kerr and Capone (82) observed a relationship between the naphthalene mineralization rate and salinity in sediments of the Hudson river that was dependent upon the ambient salinity regime, with estuarine sites exhibiting a lack of inhibition of mineralization over a wider range of salinities than was the case for the less saline upstream site. In a study of hypersaline salt evaporation ponds, Ward and Brock (151) showed that rates of hydrocarbon metabolism decreased with increasing salinity in the range 3.3 to 28.4‰ and attributed the results to a general reduction in microbial metabolic rates.

### Pressure

The importance of pressure as a variable in the biodegradation of hydrocarbons is most probably confined to the deep-sea environment. Reports of the effects of pressure have been limited to the studies conducted by Schwarz et al. (120–122) in which the degradation of tetradecane, hexadecane, and a mixed hydrocarbon substrate by a mixed culture of deep-sea sediment bacteria was monitored at 1 atm (ca. 101 kPa) and 495 or 500 atm (ca. 50,140 or 50,650 kPa). At 4°C, 94% of the hexadecane was utilized only after a 40-week incubation under conditions of high pressure, compared with 8 weeks at 1 atm (122). Colwell and Walker (44) have suggested that oil which reaches the deep-ocean environment will be degraded very slowly by microbial populations and, consequently, that certain recalcitrant fractions of the oil could persist for years or decades.

### Water Activity

The water activity or water potential ( $a_w$ ) of soils can range from 0.0 to 0.99, in contrast to aquatic environments, in which water activity is stable at a value near 0.98 (29). Hydrocarbon biodegradation in terrestrial ecosystems may therefore be limited by the available water for microbial growth and metabolism. Dibble and Bartha (51), in a study of oil sludge degradation in soil, reported optimal rates of biodegradation at 30 to 90% water saturation. The failure to observe inhibition of degradation at the lower values was ascribed to a hydrocarbon-mediated reduction in the water-holding capacity of the soil.

Atlas (7) has suggested that tarballs deposited on beaches may represent another situation in which available water limits hydrocarbon biodegradation.

### pH

In contrast to most aquatic ecosystems, soil pH can be highly variable, ranging from 2.5 in mine spoils to 11.0 in alkaline deserts (29). Most heterotrophic bacteria and fungi favor a pH near neutrality, with fungi being more tolerant of acidic conditions (8). Extremes in pH, as can be observed in some soils, would therefore be expected to have a negative influence on the ability of microbial populations to degrade hydrocarbons. Verstraete et al. (138) reported a near doubling of rates of biodegradation of gasoline in an acidic (pH 4.5) soil by adjusting the pH to 7.4. Rates dropped significantly, however, when the pH was further raised to 8.5. Similarly, Dibble and Bartha (51) observed an optimal pH of 7.8, in the range 5.0 to 7.8, for the mineralization of oily sludge in soil.

The pH of sediments in special environments such as salt marshes may be as low as 5.0 in some cases (102). Hambrick

et al. (66) found the rates of microbial mineralization of octadecane and naphthalene to be depressed at this pH compared with pH 6.5. Octadecane mineralization rates increased further when the pH was raised from 6.5 to 8.0, whereas naphthalene mineralization rates did not.

## BIOLOGICAL FACTORS AFFECTING THE BIODEGRADATION OF HYDROCARBONS

### Hydrocarbon Degradation by Bacteria, Fungi, and Other Microorganisms

Hydrocarbons in the environment are biodegraded primarily by the bacteria and fungi. Although ubiquitous in terrestrial (16, 79) and aquatic (33, 92, 149) ecosystems, the fraction of the total heterotrophic community represented by the hydrocarbon-utilizing bacteria and fungi is highly variable, with reported frequencies ranging from 6% (80) to 82% (106) for soil fungi, 0.13% (80) to 50% (106) for soil bacteria, and 0.003% (69) to 100% (92) for marine bacteria. Individual organisms can metabolize only a limited range of hydrocarbon substrates (31), so that assemblages of mixed populations with overall broad enzymatic capacities are required to degrade complex mixtures of hydrocarbons such as crude oil in soil (29), freshwater (45), and marine (7, 54) environments.

The ability to degrade and/or utilize hydrocarbon substrates is exhibited by a wide variety of bacterial and fungal genera. Floodgate (54) lists 25 genera of hydrocarbon-degrading bacteria and 27 genera of hydrocarbon-degrading fungi which have been isolated from the marine environment; a similar compilation by Bossert and Bartha (29) for soil isolates includes 22 genera of bacteria and 31 genera of fungi. Based on the number of published reports, the most important hydrocarbon-degrading bacteria in both marine and soil environments are *Achromobacter*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Flavobacterium*, *Nocardia*, and *Pseudomonas* spp. and the coryneforms; the importance of hydrocarbon-degrading strains of the salt-requiring *Vibrio* spp. is limited to the marine environment. Austin et al. (17), in a numerical taxonomy study of petroleum-degrading bacteria from Chesapeake Bay water and sediment, found that *Pseudomonas*, *Micrococcus*, and *Nocardia* spp., members of the family *Enterobacteriaceae*, actinomycetes, and coryneforms made up 95% of the isolates. Among the fungi, *Aureobasidium*, *Candida*, *Rhodotorula*, and *Sporobolomyces* spp. are the most common marine isolates and *Trichoderma* and *Mortierella* spp. are the most common soil isolates. Hydrocarbon-degrading *Aspergillus* and *Penicillium* spp. have been frequently isolated from both environments. Based on the work of Kirk and Gordon (84), the truly marine, beach-adapted genera *Corollospora*, *Dendryphiella*, *Lulworthia*, and *Varicosporina* should be added to the list.

The extent to which bacteria, yeasts, and filamentous fungi participate in the biodegradation of hydrocarbons has been the subject of only limited study, but appears to be a function of the ecosystem and local environmental conditions.

In the marine environment, bacteria are generally considered to represent the predominant hydrocarbon-degrading element of the microbial community. Floodgate (54) suggested that fungi were relatively minor components of the marine microflora, increasing in numbers in nearshore regions (3, 4), the intertidal zone (84, 107), and salt marshes and mangrove areas (3). Fungi have also been reported to be important inhabitants of specialized niches such as sub-

merged wood (84), the surface film of water, decomposing algae, and the surface of tarballs (2). Few studies, though, have directly compared the degrees of hydrocarbon degradation accomplished by bacteria and fungi in the marine environment. Walker and Colwell (140) reported that Chesapeake Bay bacteria accounted for all of the utilization of a model petroleum at 0 and 5°C; at 10°C, yeasts but not filamentous fungi contributed to the utilization of the petroleum. A hydrocarbon-degrading fungus, *Cladosporium resinae*, was responsible for 20 to 40% of the degradation of petroleum when added to the inoculum. Ahearn and Meyers (3) found that populations of yeasts increased in oil-contaminated estuarine sediments over a 4-month period, but declined in open-ocean waters despite the presence of oil. Ahearn and Crow (1) observed relatively small numbers of yeasts associated with crude oil after the *Amoco Cadiz* spill in the North Sea.

Even less is known of the comparative roles of bacteria and fungi in degrading hydrocarbons in freshwater ecosystems. Yeasts are at least an order of magnitude more abundant in rivers and lakes than in the open ocean, and the frequency of molds is generally higher in freshwater than seawater samples (3). Cooney and Summers (47) reported 100-fold-greater numbers of hydrocarbon-utilizing bacteria than yeasts and filamentous fungi in sediments from freshwater lakes, but the relative amounts of biomass of the two groups were considered to be nearly equal.

Both bacteria and fungi are relatively plentiful in soil, and members of both groups contribute to the biodegradation of hydrocarbons (29). Hydrocarbon-utilizing bacteria (16, 75, 106) and fungi (16, 88, 106) are readily isolated from soil, and the application of oil or oily wastes to soil results in increased numbers of bacteria and fungi (75, 88, 106). In the only published comparative study of hydrocarbon degradation by bacteria and fungi in soil, Song et al. (127) observed that 82% of *n*-hexadecane mineralization in a sandy loam was attributed to bacteria and only 13% was attributed to fungi.

Algae and protozoa are important members of the microbial community in both aquatic and terrestrial ecosystems, but the extent of their involvement in hydrocarbon biodegradation is largely unknown. Walker et al. (146) isolated an alga, *Prototheca zopfi*, which was capable of utilizing crude oil and a mixed-hydrocarbon substrate and exhibited extensive degradation of *n*- and isoalkanes, as well as aromatic hydrocarbons. Cerniglia et al. (38) observed that nine cyanobacteria, five green algae, one red alga, one brown alga, and two diatoms could oxidize naphthalene. Protozoa, by contrast, have not been shown to utilize hydrocarbons. Rogerson and Berger (113) found no direct utilization of crude oil by protozoa cultured on hydrocarbon-utilizing yeasts and bacteria. Overall, the limited available evidence does not appear to suggest an ecologically significant role for algae and protozoa in the degradation of hydrocarbons in the environment (29, 96).

#### Adaptation—Effect of Prior Exposure

Prior exposure of a microbial community to hydrocarbons, either from anthropogenic sources such as accidental oil spills, petroleum exploration and transportation activities, and waste oil disposal, or from natural sources such as seeps and plant-derived hydrocarbons (21, 95), is important in determining how rapidly subsequent hydrocarbon inputs can be biodegraded. This phenomenon, which results from increases in the hydrocarbon-oxidizing potential of the com-

munity, is known as adaptation (128). The three interrelated mechanisms by which adaptation can occur are (i) induction and/or depression of specific enzymes, (ii) genetic changes which result in new metabolic capabilities, and (iii) selective enrichment of organisms able to transform the compound or compounds of interest (128, 129). Selective enrichment has been widely observed in studies of hydrocarbon and petroleum degradation in the environment. A large number of reports, reviewed by Colwell and Walker (44), Atlas (7), Floodgate (54), Cooney (45), and Bossert and Bartha (29), have shown that the numbers of hydrocarbon-utilizing microorganisms and their proportion in the heterotrophic community increase upon exposure to petroleum or other hydrocarbon pollutants and that the levels of hydrocarbon-utilizing microorganisms generally reflect the degree of contamination of the ecosystem. In some cases, this phenomenon has been associated with the predominance of a few hydrocarbon-degrading genera (88), whereas in other studies the diversity of heterotrophic populations was shown to be unchanged (42, 69, 101, 106) or even to increase (70). The specific effect of hydrocarbon contamination on the generic composition of the microbial community would be expected to exhibit a strong dependence on the environment and local conditions, which may explain these apparently conflicting observations.

Adaptation of microbial communities to hydrocarbons, i.e., increases in rates of transformation of hydrocarbons associated with oil-contaminated environments, has been reported in several studies. Walker et al. observed greater degradation of a mixed-hydrocarbon substrate (141) and South Louisiana crude oil (144) by sediment bacteria from an oil-polluted harbor than by bacteria from a relatively unpolluted environment. Uptake and mineralization rates of [<sup>14</sup>C]hexadecane were also higher for planktonic bacteria from the polluted site (142). Similarly, Caparello and La-Rock (36), in a study of mineralization of [<sup>14</sup>C]hexadecane and degradation of *n*-alkane mixtures by bacteria in several surface water and sand samples, concluded that areas with greater hydrocarbon burdens had higher hydrocarbon-oxidizing activity. Herbes and Schwall (67) reported that turnover times for naphthalene, anthracene, benzo[*a*]anthracene, and benzo[*a*]pyrene were 10 to 400 times longer in sediments of a pristine freshwater stream than in petroleum-contaminated sediments. Sherrill and Sayler (123) observed increased phenanthrene-biodegradative capacity in water samples from two reservoirs receiving industrial and domestic wastes compared with a reservoir not receiving wastes. Wyndham and Costerton (155) concluded that sediment microbial populations within the Athabasca oil sands were more capable of oxidizing hydrocarbons than were populations from control sites, based on respiration rates of radio-labeled hexadecane and naphthalene. Sayler et al. (117) showed that exposure of freshwater sediments to a synthetic oil accelerated the rate of polyaromatic hydrocarbon (PAH) mineralization. Cooney et al. (46) found that water-sediment mixtures from an oil-contaminated area of a freshwater lake exhibited higher rates of degradation of marker hydrocarbons in kerosene than did samples from the non-oiled area of the lake.

Bauer and Capone (22) and Kerr and Capone (82) provided evidence for "cross-acclimation" of sediment microbial communities to PAHs, in which exposure to one compound, such as phenanthrene, effects an increase in metabolism rates of a compound of similar structure, such as naphthalene. The occurrence of this phenomenon was attributed to the broad specificity of selected microbial populations for

PAHs and/or the existence of common pathways for PAH catabolism (22). The increase in transformation rates of naphthalene, phenanthrene, and benzo[*a*]pyrene with increasing ambient concentrations of PAHs, reported by Shiaris (124) for sediments of a polluted estuary, probably represents another example of cross-acclimation.

#### Adaptation by Alteration of the Genetic Composition of the Microbial Community

Of the three mechanisms for adaptation of microbial communities to chemical contaminants, induction and derepression of enzymes, genetic changes, and selective enrichment, only the third has been examined in detail, as discussed in the previous section. This has been primarily a result of limitations imposed by available methods, which have, until recently, restricted the study of adaptation of microbial communities to the phenomenon of selective enrichment, in which the numbers or proportion of microorganisms that can utilize the compound of interest increase within the community and can be enumerated by their ability to grow on a medium containing the compound as the sole carbon source.

The primary genetic mechanism for the adaptation of the microbial community is the amplification, by means of selective enrichment and gene transfer and mutation, of genes which are involved in the metabolism of the chemical contaminant (19, 129). Direct monitoring of this process with respect to adaptation to hydrocarbons has recently been made possible by the development of DNA probes specific for the genes encoding hydrocarbon-catabolic pathways (137). Sayler et al. (118), for example, using the colony hybridization technique, showed a correlation between the enhanced rates of PAH mineralization in oil-contaminated sediments and an increase in the number of colonies containing DNA sequences which hybridized to TOL (toluene oxidation) and NAH (naphthalene oxidation) plasmid probes. The colony hybridization procedure, however, has the disadvantage of requiring the growth of organisms on laboratory media, which limits sensitivity and does not allow detection of DNA sequences in viable but nonculturable microorganisms (116). Dot blot hybridization, in which DNA is extracted from environmental samples and then probed (68, 98), can be used to detect specific DNA sequences in the environment without the need for isolation and culture of microorganisms. The newly described polymerase chain reaction technique can improve the sensitivity of the dot blot method by 3 orders of magnitude, permitting the detection of 1 cell per g of sediment sample (130).

The use of these methods in conjunction with nucleic acid probes for genes involved in hydrocarbon metabolism will allow measurement of the frequency of those genes within the microbial community (137). This will permit assessment of the relative degree of adaptation of the community as well as a more detailed analysis of the dynamics of gene amplification associated with adaptation.

#### Role of Plasmids in Adaptation

Plasmid DNA may play a particularly important role in genetic adaptation in that it represents a highly mobile form of DNA which can be transferred via conjugation or transformation and can impart novel phenotypes, including hydrocarbon-oxidizing ability, to recipient organisms. The pathways for the metabolism of naphthalene, salicylate, camphor, octane, xylene, and toluene have been shown to

be encoded on plasmids in *Pseudomonas* spp. (39). Exposure of natural microbial populations to oil or other hydrocarbons may impose a selective advantage to strains possessing plasmids encoding enzymes for hydrocarbon catabolism, resulting in an overall increase in the plasmid frequency in the community.

Indirect evidence for the role of plasmids in adaptation to hydrocarbons has been provided by some studies in which a greater frequency and/or multiplicity of plasmids has been observed among bacterial isolates from hydrocarbon-contaminated environments than among isolates from uncontaminated sites. Hada and Sizemore (64) screened 440 *Vibrio* isolates from an active oil field and a control site in the northwestern Gulf of Mexico for the presence of plasmid DNA and found a higher proportion of plasmid carriage (35 versus 23%) and multiple plasmid carriage (58 versus 29%) for oil field isolates than for isolates from the control site. The average number of plasmids per plasmid-carrying strain was 2.5 and 1.5 for oil field and control site strains, respectively. By contrast, Leahy et al. (86), in a study of bacteria isolated from sediments in the Campeche Bank region of the Gulf of Mexico, concluded that there was no clear association between plasmid frequency or multiplicity and proximity to an oil field, but, rather, a positive correlation with the depth of the site from which the sediment was sampled. The failure to observe a higher incidence of plasmid-bearing bacteria within the oil field was attributed to the relatively low degree of oil contamination in the region at the time of the study. Burton et al. (34) reported that 15% of the aerobic, heterotrophic bacteria isolated from the sediment of a river receiving domestic and industrial effluents carried plasmids, compared with 10% of the bacteria from an unpolluted area upstream. Ogunseitani et al. (99) observed a higher frequency (19.4%) of plasmid-bearing bacterial isolates from an aquifer with a history of aromatic hydrocarbon contamination than from two pristine aquifers (1.8 and 7.7%). Fredrickson et al. (57) found that bacterial isolates from the deeper layers (29 to 260 m) of terrestrial sediments exhibited a higher incidence of plasmids (31 to 39%) than those from the surface soil (14%) or the shallow aquifer (16%). They hypothesized that the large plasmids associated with bacteria from the deeper strata, some of which hybridized to a TOL plasmid probe, contained genes encoding aromatic compound catabolism. Schutt (119) observed a similar proportion of plasmid carriage (34%) among the bacterial isolates from a dystrophic lake containing high concentrations of humic compounds. To confirm the participation of plasmid DNA in the genetic changes of the microbial community during adaptation, further studies are necessary to identify the functions of plasmids associated with bacteria indigenous to hydrocarbon-containing environments.

#### Seeding

Seeding involves the introduction of allochthonous microorganisms into the natural environment for the purpose of increasing the rate or extent, or both, of biodegradation of pollutants. The rationale for this approach is that the autochthonous microbial populations may not be capable of degrading the wide range of potential substrates present in complex mixtures such as petroleum. The criteria to be met by effective seed organisms have been reviewed by Atlas (6) and include the ability to degrade most petroleum components, genetic stability, viability during storage, rapid growth following storage, a high degree of enzymatic activity and growth in the environment, the ability to compete with

indigenous microorganisms, nonpathogenicity, and the inability to produce toxic metabolites.

Mixed cultures have been most commonly used as inocula for seeding because of the relative ease with which microorganisms with different and complementary hydrocarbon-degrading capabilities can be isolated. The potential for creating, through genetic manipulation, microbial strains able to degrade a variety of different types of hydrocarbons has been demonstrated by Friello et al. (58). They successfully produced a multiplasmid-containing *Pseudomonas* strain capable of oxidizing aliphatic, aromatic, terpenic, and polyaromatic hydrocarbons. The use of such a strain as an inoculum during seeding would preclude the problems associated with competition between strains in a mixed culture. However, there is considerable controversy surrounding the release of such genetically engineered microorganisms into the environment, and field testing of these organisms must therefore be delayed until the issues of safety, containment, and the potential for ecological damage are resolved (132).

Seeding of petroleum-contaminated aquatic environments has been attempted, with mixed results. Tagger et al. (133) observed no increase in petroleum degradation in seawater inoculated with a mixed culture of hydrocarbon-degrading bacteria. Atlas and Busdosh (13) reported increased degradation of oil in a saline Arctic pond after inoculation with an oil-degrading *Pseudomonas* sp., but no improvement in a freshwater pond. Horowitz and Atlas (72) found that greater losses of oil in seawater in an open flow-through system occurred when octadecane-coated bacteria were applied 2 weeks after the addition of an oleophilic fertilizer to the system than when the fertilizer alone was added. In the same study, no significant increases in the loss of gasoline from freshwater sediment were produced by seeding. Rosenberg et al. (115) were able to demonstrate removal of residual oil from the ballast tanks of oil tankers by microbial seeding, although removal appeared to result primarily from bioemulsification rather than biodegradation.

Terrestrial ecosystems differ from aquatic ecosystems in that soils contain higher concentrations of organic and inorganic matter and, generally, larger numbers of microorganisms and are more variable in terms of physical and chemical conditions (29). The microbial community of soils usually includes a significant hydrocarbon-utilizing component, which readily increases in response to hydrocarbon contamination (16, 75, 88, 106). The presence of indigenous microbial populations which are highly adapted to a particular soil environment would be expected to influence negatively the ability of seed microorganisms to compete successfully and survive; for this reason, soils are not widely considered to be amenable to improvements in rates of biodegradation though seeding alone (6, 29). Other potential problems associated with the inoculation of soils, reviewed by Goldstein et al. (61), include inadequate (i.e., extremely low) concentrations of the chemical of interest, the presence of inhibitory substances, predation, preferential metabolism of competing organic substrates, and insufficient movement of the seed organisms within the soil. Addition of selected pure cultures of bacteria to soil has been found to increase the rate of degradation of pesticides such as isopropyl *N*-phenylcarbamate (41), 2,4,5-trichlorophenoxyacetic acid (83), DDT (dichlorodiphenyltrichloroethane) (81), and parathion (20). Similar attempts by Jobson et al. (77), Lehtomaki and Niemela (87), and Verstraete et al. (138) to enhance the biodegradation of hydrocarbons in soils were unsuccessful.

The best results from seeding experiments have been

reported in studies in which the environment is controlled to some extent, such as in fermentors and chemostats. The advantages of such arrangements are clear: competition with autochthonous microflora is reduced or nonexistent, and system parameters can be optimized to achieve the highest rates of biodegradation. The disadvantages are economic costs associated with equipment and equipment transport, energy input, and the impracticality of treating spills in some environments (e.g., uncontained oil slicks) in a closed or semicontained system (6). This approach has been used with some success in the biodegradation of crude oil in seawater (26) and in the in situ bioremediation of hydrocarbon-contaminated soil and groundwater (139). Microbial seeding from a chemostat has also been used in conjunction with conventional activated-sludge treatment to improve the ability of a refinery wastewater treatment plant to absorb intermittently high loads of hydrocarbons (154). The combination of microbial seeding with conventional large-scale treatment methods to improve the removal of hydrocarbons is therefore feasible and merits additional study.

## CONCLUSIONS

The biodegradation of petroleum and other hydrocarbons in the environment is a complex process, whose quantitative and qualitative aspects depend on the nature and amount of the oil or hydrocarbons present, the ambient and seasonal environmental conditions, and the composition of the autochthonous microbial community. Microbial degradation of oil has been shown to occur by attack on aliphatic or light aromatic fractions of the oil, with high-molecular-weight aromatics, resins, and asphaltenes considered to be recalcitrant or exhibiting only very low rates of biodegradation, although some studies have reported their removal at high rates under optimal conditions. In aquatic ecosystems, dispersion and emulsification of oil in oil slicks appear to be prerequisites for rapid biodegradation; large masses of mousse, tarballs, or high concentrations of oil in quiescent environments tend to persist because of low surface areas available for microbial activity. Petroleum spilled on or applied to soil is largely adsorbed to particulate matter, decreasing its toxicity but possibly also contributing to its persistence. Biodegradation rates generally increase with increasing temperature; ecosystems exposed to extremely low temperatures degrade hydrocarbons very slowly. The microbial degradation of petroleum in aquatic environments is limited primarily by nutrients such as nitrogen and phosphorus; salinity and pressure may be important in estuarine and deep-sea regions, respectively. Oxygen, nutrient concentrations, moisture, and pH are predominant factors in determining biodegradation rates in soil.

Hydrocarbon degradation by microbial communities depends on the composition of the community and its adaptive response to the presence of hydrocarbons. Bacteria and fungi are the key agents of degradation, with bacteria assuming the dominant role in marine ecosystems and fungi becoming more important in freshwater and terrestrial environments. Adapted communities, i.e., those which have been previously exposed to hydrocarbons, exhibit higher biodegradation rates than communities with no history of hydrocarbon contamination. The mechanisms of adaptation include both selective enrichment and genetic changes, resulting in a net increase in the number of hydrocarbon-utilizing organisms and in the pool of hydrocarbon-catabolizing genes within the community. The association of such genes with plasmid DNA may also lead to an increased

frequency of plasmid-bearing microorganisms. Seeding of petroleum-contaminated water or soils with hydrocarbon-utilizing bacteria has met with some success, particularly in situations in which chemostats or fermentors have been used to control conditions and reduce competition from indigenous microflora.

Probably the most significant recent developments in hydrocarbon biodegradation ecology involve the demonstration of anaerobic metabolism of aromatic hydrocarbons and application of DNA probes to the analysis of genetic adaptation of microbial communities upon exposure to aromatic hydrocarbons. The expansion of the DNA probe method to the detection of genes encoding the catabolism of other classes of hydrocarbons, as well as to the detection of specific RNAs, should revolutionize the study of the microbial degradation of hydrocarbons in the environment and remove many of the biases and limitations imposed by conventional culture techniques. Recombinant DNA technology may also allow the construction of bacterial and fungal strains exhibiting improved capability for hydrocarbon metabolism and suitability as seed organisms for the elimination of hydrocarbon pollutants, but field studies must await the resolution of the issue of release of genetically engineered microorganisms into the environment.

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