Petroleum Biodegradation in Marine Environments

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Abstract

Petroleum-based products are the major source of energy for industry and daily life. Petroleum is also the raw material for many chemical products such as plastics, paints, and cosmetics. The transport of petroleum across the world is frequent, and the amounts of petroleum stocks in developed countries are enormous. Consequently, the potential for oil spills is significant, and research on the fate of petroleum in a marine environment is important to evaluate the environmental threat of oil spills, and to develop biotechnology to cope with them.

Crude oil is constituted from thousands of components which are separated into saturates, aromatics, resins and asphaltenes. Upon discharge into the sea, crude oil is subjected to weathering, the process caused by the combined effects of physical, chemical and biological modification. Saturates, especially those of smaller molecular weight, are readily biodegraded in marine environments. Aromatics with one, two or three aromatic rings are also efficiently biodegraded; however, those with four or more aromatic ring are quite resistant to biodegradation. The asphaltene and resin fractions contain higher molecular weight compounds whose chemical structures have not yet been resolved. The biodegradability of these compounds is not yet known.

It is known that the concentrations of available nitrogen and phosphorus in seawater limit the growth and activities of hydrocarbon-degrading microorganisms in a marine environment. In other words, the addition of nitrogen and phosphorus fertilizers to an oil-contaminated marine environment can stimulate the biodegradation of spilled oil. This notion was confirmed in the large-scale operation for bioremediation after the oil spill from the *Exxon Valdez* in Alaska.

Many microorganisms capable of degrading petroleum components have been isolated. However, few of them seem to be important for petroleum biodegradation in natural environments. One group of bacteria belonging to the genus *Alcanivorax* does become predominant in an oil-contaminated marine environment, especially when nitrogen and phosphorus fertilizers are added to stimulate the growth of endogenous microorganisms.

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Introduction

Petroleum is a viscous liquid mixture that contains thousands of compounds mainly consisting of carbon and hydrogen. Oil fields are not uniformly distributed around the globe, but being in limited areas such as the Persian Gulf region. The world production of crude oil is more than three billion tons per year, and about the half of this is transported by sea. Consequently, the international transport of petroleum by tankers is frequent. All tankers take on ballast water which contaminates the marine environment when it is subsequently discharged. More importantly, tanker accidents exemplified by that of the T/V *Exxon Valdez* in Prince William Sound, Alaska, severely affect the local marine environment. Off-shore drilling is now common to explore new oil resources and this constitutes another source of petroleum pollution. However, the largest source of marine contamination by petroleum seems to be the runoff from land. Annually, more than two million tons of petroleum are estimated to end up in the sea. Fortunately, petroleum introduced to the sea seems to be degraded either biologically or abiotically (Readman *et al.*, 1992). In this article, the fate of petroleum in a marine environment is reviewed, with special emphasis placed on its biodegradation.

Components of Petroleum

All petroleum products are derived from crude oil whose major constituents are hydrocarbons. Petroleum components can be separated into four fractions, the
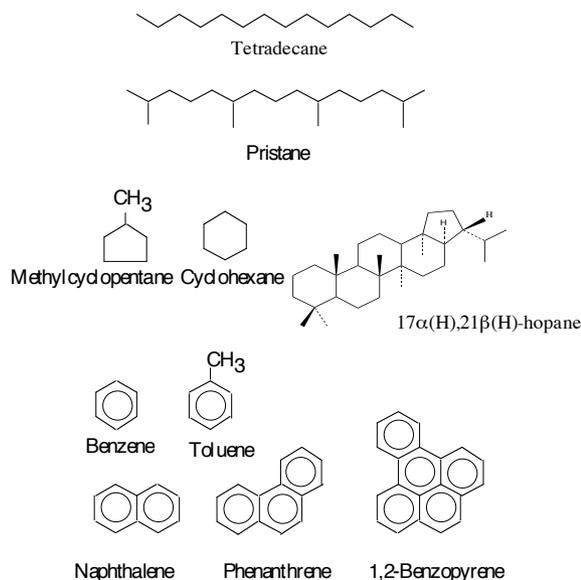

Figure 1. Representative Hydrocarbons
Tetradecane (an *n*-alkane), pristane (a branched alkane), and methylcyclopentane, cyclohexane and 17α[H],21ß[H]-hopane (cycloalkane compounds) are present in the saturated fraction of crude oil. The other compounds shown in this figure are present in the aromatic fraction.

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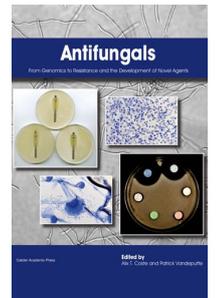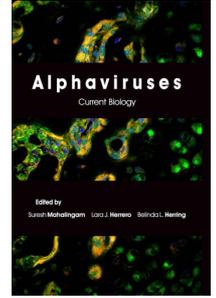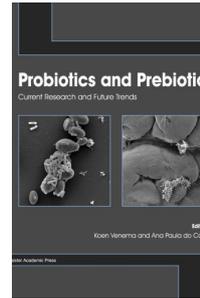
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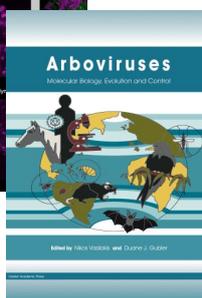
saturated, aromatic, resin and asphaltene fractions, by absorption chromatography. Each of these fractions contains a large number of compounds (Karlsen and Larter, 1991).

Saturates are hydrocarbons containing no double bonds. They are further classified according to their chemical structures into alkanes (paraffins) and cycloalkanes (naphthenes). Alkanes have either a branched or unbranched (normal) carbon chain(s), and have the general formula C_nH_{2n+2}. Cycloalkanes have one or more rings of carbon atoms (mainly cyclopentanes and cyclohexanes), and have the general formula C_nH_{2n}. The majority of cycloalkanes in crude oil have an alkyl substituent(s) (Figure 1). Aromatics have one or more aromatic rings with or without an alkyl substituent(s). Benzene is the simplest one (Figure 1), but alkyl-substituted aromatics generally exceed the non-substituted types in crude oil (Mater and Hatch, 1994). In contrast to the saturated and aromatic fractions, both the resin and asphaltene fractions contain non-hydrocarbon polar compounds. Their elements contain, in addition to carbon and hydrogen, trace amounts of nitrogen, sulfur and/or oxygen. These compounds often form complexes with heavy metals. Asphaltenes consist of high-molecular-weight compounds which are not soluble in a solvent such as *n*-heptane, while resins are *n*-heptane-soluble polar molecules. Resins contain heterocyclic compounds, acids and sulfoxides.

The components of petroleum in crude oil have been analyzed mainly by using gas chromatography in combination with mass spectrometry (GC/MS). Consequently, the chemical structures of the higher-molecular-weight components (the heavy fractions) that cannot be identified by GC are mostly unknown. Furthermore, the compositions of many branched alkanes and alkyl cyclo-alkanes have not been determined because their isomers are numerous and cannot be resolved by GC (Killops and Al-Juboori, 1990; Gough and Rowland, 1990). Therefore, a multitude of analytical techniques such as flame ionization detection, IR- and UV-absorption spectrometry, NMR and elemental analysis in combination with appropriate separation techniques such as various chromatographic methods and/or chemical conversion is necessary to characterize petroleum, and especially its heavy fractions.

Various petroleum products are produced by refining crude oil. Refining is essentially a fractional distillation process by which different fractions or cuts are produced. Alkenes, a series of unsaturated hydrocarbons including ethylene, are not found in crude oil, but are produced during the cracking of crude oil.

Behavior of Petroleum in Marine Environments

When petroleum is spilled into the sea, it spreads over the surface of the water. It is subjected to many modifications, and the composition of the petroleum changes with time. This process is called weathering, and is mainly due to evaporation of the low-molecular-weight fractions, dissolution of the water-soluble components, mixing of the oil droplets with seawater, photochemical oxidation, and biodegradation.

Those petroleum components with a boiling point below 250 °C are subjected to evaporation. Therefore, the content of *n*-alkanes, whose chain length is shorter than C14, is reduced by weathering. The content of aromatic hydrocarbons within the same boiling point range is also reduced as they are subjected to both evaporation and dissolution. The mixing of oil with seawater occurs in several forms. Dispersion of the oil droplets into a water column is induced by the action of waves, while water-in-oil emulsification occurs when the petroleum contains polar components that act as emulsifiers. A water-in-oil emulsion containing more than 70% of seawater becomes quite viscous; it is called chocolate mousse from its appearance. After the light fractions have evaporated, heavy residues of petroleum can aggregate to form tar balls whose diameter ranges from microscopic size to several tenths of a centimeter.

After a large oil spill, the oil slick is sometimes treated with a dispersant. Dispersants emulsify petroleum by reducing the interfacial tension between petroleum and water. The small droplets that are formed are dispersed into a water column to a depth of several meters, preventing wind-induced drift of the oil slick. It is claimed that treatment by a dispersant enhances the biodegradation of petroleum. However, the results of such tests are controversial (Tjessem *et al.*, 1984). The original dispersants used were highly toxic; however, less toxic dispersants have subsequently been developed.

Under sunlight, petroleum discharged at sea is subjected to photochemical modification. Some reports have suggested the light-induced polymerization of petroleum components, while others have suggested their photodegradation. An increase in the polar fraction and a decrease in the aromatic fraction have also been observed. Aliphatic components do not significantly absorb solar light, and are by themselves photochemically inert. However, they can be degraded by photosensitized oxidation. The aromatic or polar components in petroleum and anthraquinone that is present in seawater can provoke the degradation of *n*-alkanes into terminal *n*-alkenes (a carbon-carbon double bond at position 1) and low-molecular-weight carbonyl compounds (Ehrhardt and Weber, 1991).

The water-soluble components of petroleum exert a toxic effect on marine organisms. In general, aromatic compounds are more toxic than aliphatic compounds, and smaller molecules are more toxic than larger ones in the same series. Solar irradiation affects oil toxicity: Surface films become less toxic due to the loss of polycyclic aromatic hydrocarbons, but the toxicity of the water-soluble fraction increases as its concentration increases (Nicodem *et al.*, 1997).

Biodegradation of Petroleum Components

n-Alkanes, a major group in crude oil, are readily biodegraded in the marine environment. *n*-Alkanes are aerobically biodegraded by several pathways. The degradation of *n*-alkanes of medium chain length by *Pseudomonas putida* containing the OCT plasmid is initiated by alkane hydroxylase. This enzyme consists of three components: the membrane-bound oxygenase component, and two soluble components called rubredoxin and rubredoxin reductase. The catalytic center of the oxygenase component contains a dinuclear iron cluster
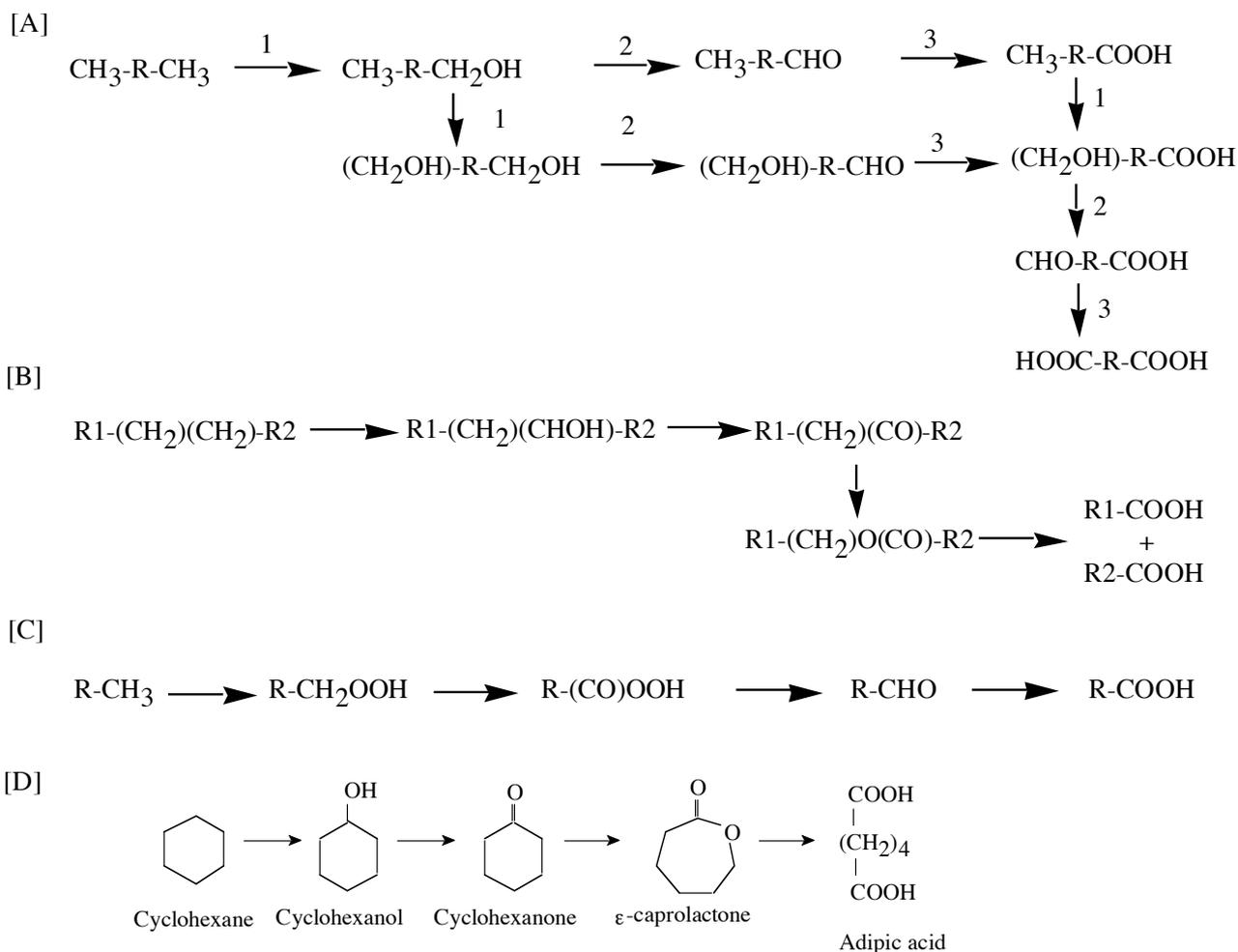
[A]

$$CH_3\text{-}R\text{-}CH_3 \xrightarrow{\ 1\ } CH_3\text{-}R\text{-}CH_2OH \xrightarrow{\ 2\ } CH_3\text{-}R\text{-}CHO \xrightarrow{\ 3\ } CH_3\text{-}R\text{-}COOH$$

$$CH_3\text{-}R\text{-}CH_2OH \xrightarrow{\ 1\ } (CH_2OH)\text{-}R\text{-}CH_2OH \xrightarrow{\ 2\ } (CH_2OH)\text{-}R\text{-}CHO \xrightarrow{\ 3\ } (CH_2OH)\text{-}R\text{-}COOH$$

$$CH_3\text{-}R\text{-}COOH \xrightarrow{\ 1\ } (CH_2OH)\text{-}R\text{-}COOH \xrightarrow{\ 2\ } CHO\text{-}R\text{-}COOH \xrightarrow{\ 3\ } HOOC\text{-}R\text{-}COOH$$

[B]

$$R1\text{-}(CH_2)(CH_2)\text{-}R2 \longrightarrow R1\text{-}(CH_2)(CHOH)\text{-}R2 \longrightarrow R1\text{-}(CH_2)(CO)\text{-}R2$$

$$R1\text{-}(CH_2)(CO)\text{-}R2 \longrightarrow R1\text{-}(CH_2)O(CO)\text{-}R2 \longrightarrow R1\text{-}COOH + R2\text{-}COOH$$

[C]

$$R\text{-}CH_3 \longrightarrow R\text{-}CH_2OOH \longrightarrow R\text{-}(CO)OOH \longrightarrow R\text{-}CHO \longrightarrow R\text{-}COOH$$

[D]

Cyclohexane → Cyclohexanol → Cyclohexanone → ε-caprolactone → Adipic acid

Figure 2. Alkane Degradative Pathways
[A] Terminal oxidation of *n*-alkanes. α- and ω-hydroxylation is catalyzed by the same set of enzymes. With bacteria, steps 1, 2 and 3 are catalyzed by alkane monooxygenase, fatty alcohol dehydrogenase and fatty aldehyde dehydrogenase, respectively. With yeast, step 1 is catalyzed by P450 monooxygenase, while steps 2 and 3 are catalyzed either by fatty alcohol oxidase and fatty aldehyde dehydrogenase, respectively, or by the P450 monooxygenase involved in step 1; [B] Subterminal oxidation of *n*-alkanes; [C] *n*-Alkane degradation via alkyl hydroperoxides; [D] Degradation of cyclohexane.

which is also found in other enzymes such as methane monooxygenase and ribonucleotide reductase (Shanklin *et al.*, 1997). In *P. putida* (OCT), oxidation of the methyl group of *n*-alkanes by alkane hydroxylase yields *n*-alcanols that are further oxidized by a membrane-bound alcohol dehydrogenase to *n*-alkanals. The *n*-alcanals are subsequently transformed to fatty acids and then to acyl CoA by aldehyde dehydrogenase and acyl-CoA synthetase, respectively (Figure 2; van Beilen *et al.*, 1994). An *n*-alkane-degrading pathway yielding secondary alcohols has also been reported. In this pathway, *n*-alkanes are oxidized by monooxygenase to secondary alcohols, then to ketones, and finally to fatty acids (Figure 2; Markovetz and Kallio, 1971; Whyte *et al.*, 1998). In *Acinetobacter* strain M-1, *n*-alkanes are transformed to *n*-alkyl peroxides, and these molecules would be further metabolized to the corresponding aldehyde. The first enzyme involved in this pathway contains FAD$^+$ and Cu^{2+} as prosthetic groups (Figure 2; Maeng *et al.*, 1996).

In the 1950s and 1960s, the microbial degradation of petroleum attracted particular attention as petroleum hydrocarbons are considered to be non-expensive substrates for producing biomass. Many yeast species, e.g. *Candida maltosa*, *Candida tropicalis* and *Candida apicola*, were investigated for use with *n*-alkanes. The first step of alkane degradation (terminal hydroxylation) and of ω-hydroxylation is catalyzed by P450 monooxygenase. The alcohols thus formed are processed by fatty alcohol oxidase and fatty aldehyde dehydrogenase. The P450 enzyme from some yeast strains can catalyze not only the terminal hydroxylation of long-chain *n*-alkanes and the ω-hydroxylation of fatty acids, but also the subsequent two steps to yield fatty acids and α,ω-dioic acids (Figure 2; Scheller *et al.*, 1998). Catabolic pathways for the degradation of branched alkanes have been elucidated for a few bacteria; for example, *Rhodococcus* strain BPM 1613 degraded phytane (2,6,10,14-tetramethylhexadecane), norpristane (2,6,10-trimethylpentadecane) and farnesane (2,6,10-trimethyldodecane) via ß-oxidation (Nakajima *et al.*, 1985).

Cycloalkanes, including condensed cycloalkanes are degraded by a co-oxidation mechanism. The formation of

a cyclic alcohol and a ketone has been observed. A monooxygenase introduces an oxygen into the cyclic ketone, and the cyclic ring is cleaved (Figure 2). The degradation of substituted cycloalkanes seems to be less difficult than that of unsubstituted cycloalkanes (Morgan and Watkinson, 1994).

A multitude of catabolic pathways for the degradation of aromatic compounds have been elucidated; for example, toluene is degraded by bacteria along five different pathways. On the pathway encoded by the TOL plasmid, toluene is successively degraded to benzyl alcohol, benzaldehyde and benzoate, which is further transformed to the TCA cycle intermediates. The first step of toluene degradation with *P. putida* F1 is the introduction of two hydroxyl groups to toluene, forming *cis*-toluene dihydrodiol. This intermediate is then converted to 3-methylcatechol. With *Pseudomonas mendocina* KR1, toluene is converted by toluene 4-monooxygenase to *p*-cresol, this being followed by *p*-hydroxybenzoate formation through oxidation of the methyl side chain. With *Pseudomonas pickettii* PKO1, toluene is oxidized by toluene 3-monooxygease to *m*-cresol, which is further oxidized to 3-methylcatechol by another monooxygenase. With *Bukholderia cepacia* G4, toluene is metabolized to *o*-cresol by toluene 2-monooxygenase, this intermediate being transformed by another monooxygenase to 3-methylcatechol. *Burkholderia* sp. strain JS150 is unique in using multiple pathways for the metabolism of toluene (Figure 3; Johnson and Olsen, 1997).

Simple polynuclear aromatic hydrocarbons (PAHs) such as naphthalene, biphenyl and phenanthrene are readily degraded aerobically. The degradation of these compounds is generally initiated by dihydroxylation of one of the polynuclear aromatic rings, this being followed by cleavage of the dihydroxylated ring. Ring hydroxylation is catalyzed by a multi-component dioxygenase which consists of a reductase, a ferredoxin, and an iron sulfur protein, while ring cleavage is generally catalyzed by a iron-containing *meta*-cleavage enzyme. The carbon skeleton produced by the ring-cleavage reaction is then dismantled, before cleavage of the second aromatic ring (Saito *et al.*, 1999; Harayama *et al.*, 1992; Figure 4).

PAHs possessing four or more fused aromatic rings have very low water solubility and tend to be adsorbed to a solid surface. These characteristics constitute a major constraint for biodegradation. *Mycobacteria* and *Sphingomonas* have been isolated as bacteria that are able to degrade PAHs possessing four or more fused aromatic rings. Some *mycobacteria* mineralize (degrade into CO_2 and H_2O) pyrene, fluoranthene and benzo[*a*]pyrene (Harayama, 1997).

Asphaltenes and resins are considered to be recalcitrant to biodegradation. Rontani *et al.* (1985) have reported that about half of the asphaltene components were co-metabolically oxidized by a microbial consortium in the presence of C12 to C18 *n*-alkanes. These observations should be reinvestigated as the preparation of pure fractions of resins and asphaltenes is difficult and these
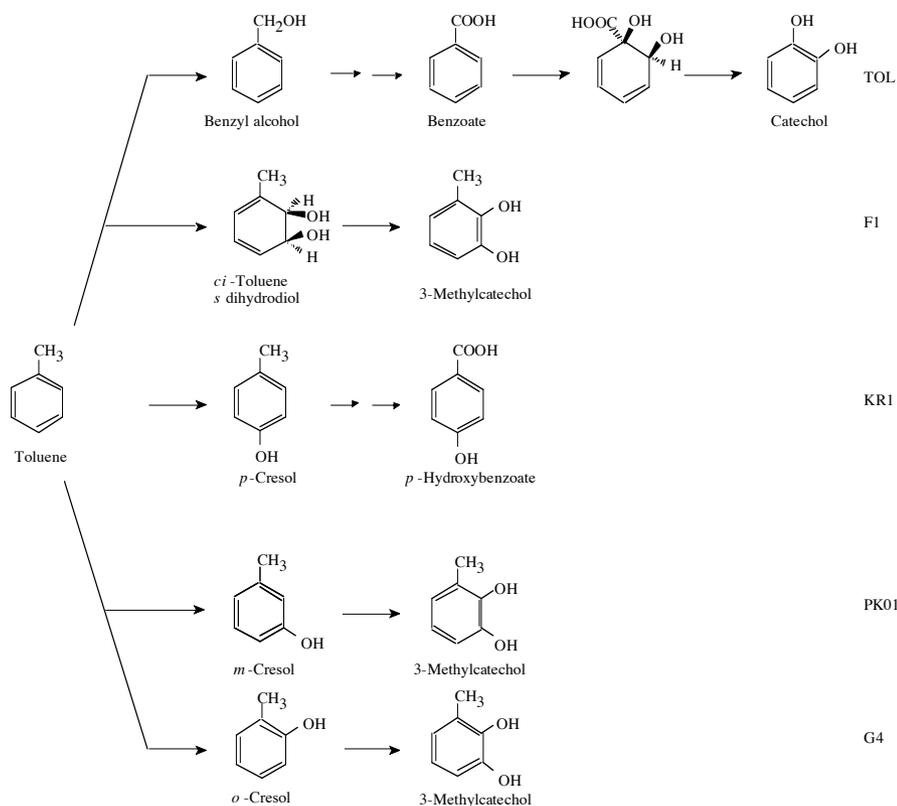

Figure 3. Divergent Pathways for the Aerobic Degradation of Toluene
The pathways from the top to the bottom are found with *P. putida* (TOL), *P. putida* F1, *P. mendocina* KR1, *P. pickettii* PKO1, and *B. cepacia* G4, respectively.
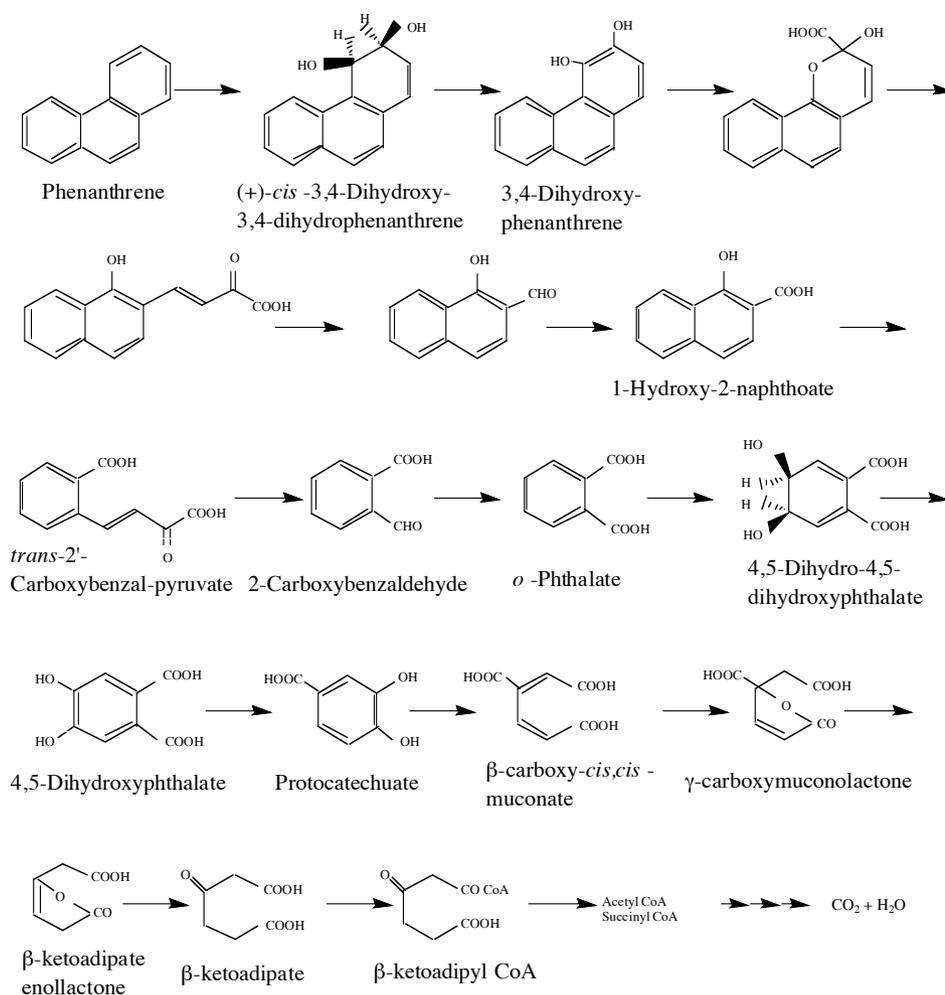
Figure 4. Catabolic Pathway for the Degradation of Phenanthrene with *Nocardioides* sp. KP7

fractions almost always contain saturates and aromatics (Myhr *et al.*, 1989).

Petroleum components which are trapped in marine sediment tend to persist under anaerobic conditions. Nevertheless, ecological studies have demonstrated that certain hydrocarbons can be oxidized under anaerobic conditions when either nitrate reduction, sulfate reduction, methane production, Fe(III) reduction or photosynthesis is coupled to the hydrocarbon oxidation (Evans and Fuchs, 1988). Many hydrocarbons, such as alkanes, alkenes and aromatic hydrocarbons including benzene, toluene, xylenes, ethyl- and propylbenzenes, trimethylbenzenes, naphthalene, phenanthrene and acenaphthene, are known to be anaerobically degraded (Bregnard *et al.*, 1997). The pathways for the degradation of alkanes and alkenes are not yet clear. Anaerobic bacterium strain HD-1 grows on CO_2 in the presence of H_2 or tetradecane. In the absence of H_2, tetradecane is degraded, and the major metabolic intermediate is 1-dodecene (Morikawa *et al.* 1996).

Many pathways for the anaerobic degradation of toluene have recently been proposed. All of these pathways transform the initial substrate into the common intermediate, benzoyl-coenzyme A (CoA). With *Thauera*

sp. strain T1, the oxidation of toluene is initiated by the formation of benzylsuccinate from toluene and fumarate. The genes for the pathway enzymes and the regulatory proteins have been characterized (Coschigano *et al.*, 1998). After the formation of benzyl-CoA, this intermediated is reduced by benzoyl-CoA reductase to yield cyclohex-1,5-diene-1-carboxyl-CoA. The subsequent steps, however, are controversial. With *Rhodospeudomonas palustris*, the diene intermediate is reduced to cyclohex-1-ene-1-carboxyl-CoA, while with *Thauera aromatica*, it is hydrated to 6-hydroxycyclohex-1-ene-1-carboxyl-CoA (Figure 5; Egland *et al.*, 1997; Breese *et al.*, 1998).

Biodegradation of Emulsified Petroleum

Most of the petroleum-degrading bacteria (bacteria capable of growing on Arabian crude oil as the sole source of carbon and energy) produce surfactants or emulsifiers (our unpublished observations). Therefore, cultures of these bacteria become brown and turbid as an oil slick is transformed into many small oil droplets. Bacterial cells are associated on the surface of the droplets, and such contact may facilitate the assimilation of petroleum
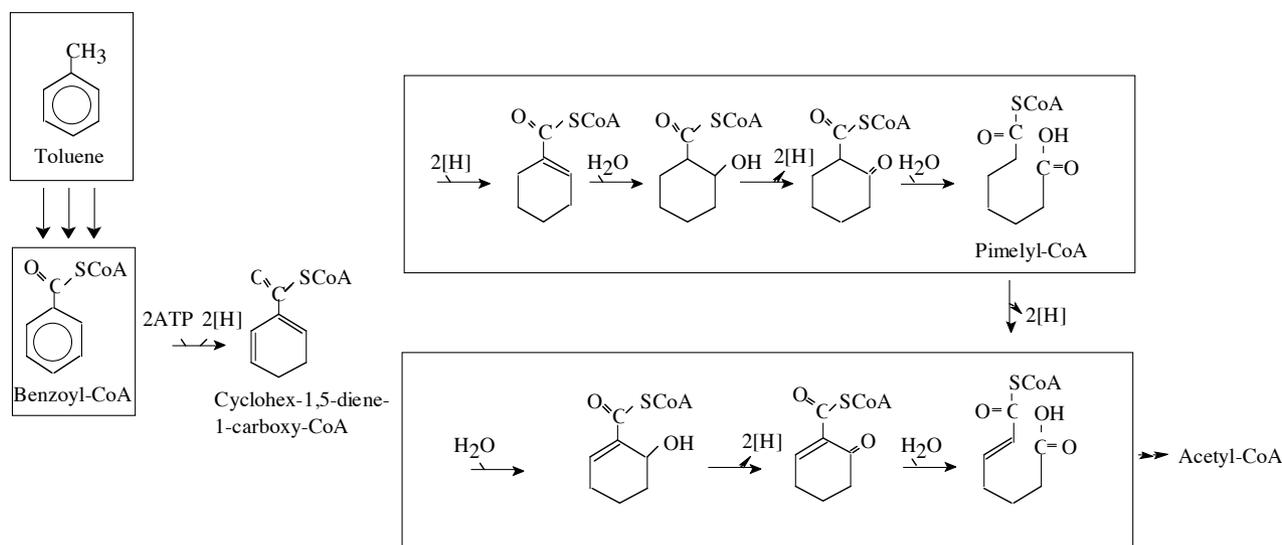
Figure 5. Anaerobic Degradation of Toluene
Several routes are proposed for the transformation of toluene to benzoyl-CoA. After the conversion of benzoyl-CoA into cyclohex-1,5-diene-1-carboxyl-CoA, this product is processed differently with two different bacteria, *R. palustris* (upper box) and *Thauera aromatica*. (lower box).

components into the cells. This observation suggests that surfactants/emulsifiers promote the biodegradation of petroleum components. However, conflicting results have been reported concerning the effect of surfactants/emulsifiers on the biodegradation of hydrocarbons (Rosenberg and Ron, 1997). Emulsan produced by *Acinetobacter calcoaceticus* RAG-1 inhibited the biodegradation of crude oil (Foght *et al.*, 1989) while rhamnolipide produced by *Pseudomonas aeruginosa* enhanced the biodegradation of octadecane (Zhang and Miller, 1992).

Petroleum Biodegradation in Marine Environments

Many catastrophic oil spills from large tanker accidents have attracted public attention to the fate of petroleum hydrocarbons in marine environments. In response to this concern, research into the biodegradation of petroleum in natural environments has been intensified. The pioneering studies by Atlas, Bartha and their colleagues (Atlas and Bartha, 1993; Prince, 1993) have demonstrated that the available concentrations of nitrogen and phosphorus in seawater are limiting factors for the growth of hydrocarbon-degrading microorganisms. Thus, the addition of nitrogen and phophorus fertilizers stimulates the biodegradation of petroleum. In general, small hydrocarbon molecules are more easily biodegraded than larger ones, and aromatics are degraded at a much slower rate than that of alkanes in marine environments (Oudot, 1984; Kennicut, 1988; Ishihara *et al.*, 1995; Sugiura *et al.*, 1997; Sasaki *et al.*, 1998; Wang *et al.*, 1998).

Many oil spills in the sea cause shoreline pollution, despite efforts to prevent the drift of a spill toward the coastline. Cleaning up a polluted coastline by enhancing microbial activities was first attempted in 1989 after the spill from the *Exxon Valdez*. The initial measure taken after this accident was physical washing with high-pressure water. Subsequently, fertilizers were applied to the polluted beaches to accelerate the growth and activities of

petroleum-degrading microorganisms. Two to three weeks later, pebbles on the beaches that had been treated with fertilizers had become significantly cleaner than those in the control area (Pritchard and Costa, 1991). Nevertheless, it was difficult to evaluate the effect of the treatment due to heterogeneity in the oil contamination of samples. Five-ring cycloalkanes, hopanes (Figure 1), are frequently used as a conserved quantitative internal standard because they are resistant to biodegradation. Using hopanes as an internal standard, it has been demonstrated that the fertilizer application significantly increased the rate of petroleum biodegradation, and that about half of the petroleum had been removed within three months after applying fertilizers in sufficient amounts (Bragg *et al.*, 1994).

Although hydrocarbons have usually been found to persist under strict anaerobic conditions, these compounds are degraded in some types of marine harbor sediment under sulfate-reducing conditions. When hydrocarbon-degrading sediment is used to inoculate a type of sediment that shows no hydrocarbon-degrading activity under anaerobic conditions, such activity is generated. This observation indicates that hydrocarbon contamination could be treated under sulfate-reducing conditions, and that the seeding (introduction of foreign microorganisms or equivalent samples) of anaerobic microbial consortia adapted to specific hydrocarbons would be effective to enhance the anaerobic biodegradation of these hydrocarbons (Coates *et al.*, 1997; Weiner and Lovley, 1998).

***Alcanivorax* as a Group of Bacteria Predominant in Oil-Contaminated Seawater**

Hydrocarbon-degrading bacteria and fungi are widely distributed in marine habitats (Floogate, 1984). However, their roles in a natural marine environment or in oil-contaminated soil with or without treatment by bioremediation (fertilizer application to enhance microbial activities) are largely unknown. Our recent research has
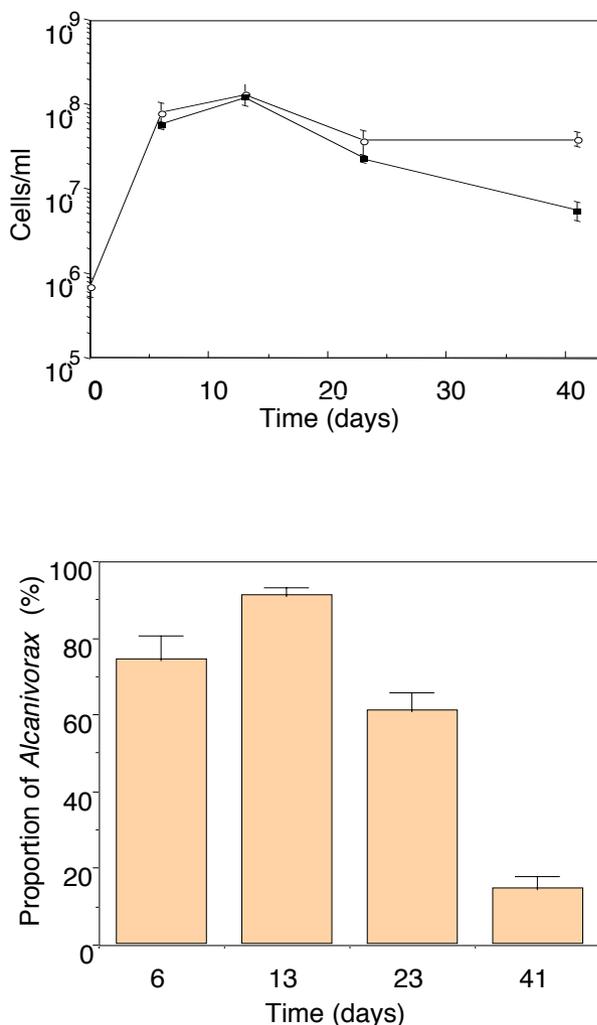
Figure 6. Population Analysis of Crude-oil Degrading Microorganisms
Natural seawater was sampled from Kamaishi Bay (Japan) to which NH_4NO_3 (1 g/l), K_2HPO_4 (0.2 g/l), ferric citrate (20 mg/l) and Arabian light crude oil (2 g/l) were supplemented. The culture was grown at 20 °C while shaking.[A] Time-course plot of the total number of bacteria determined by DAPI staining and that of the *Alcanivorax* population determined by the FISH method. [B] Time-course plot of the *Alcanivorax* population ratio.

made it clear that bacteria belonging to the genus *Alcanivorax* are important for the biodegradation of petroleum, especially under bioremediation conditions.

The *Alkanivorax* group was first isolated from the North Sea as biosurfactant-producing and *n*-alkane-degrading marine bacteria. These Gram-negative bacteria are peculiar as they cannot use carbohydrates and amino acids as growth substrates (Yakimov *et al.*, 1998). When grown on *n*-alkanes, however, they produce biosurfactants which have been shown to be glucose lipids (Abraham *et al.*, 1998). These bacteria become predominant in the microbial community in seawater containing crude oil. Figure 6 shows the result from a batch culture of natural seawater (without sterilization and without inoculation of exogenous microorganisms). At an early stage of cultivation, the *Alcanivorax* population exceeded more than 90% of the total population. Similar results were obtained when

petroleum-degrading populations were examined in the marine-beach-simulating mesocosms described by Ishihara *et al.* (1995). Furthermore, outgrowth of an *Alcanivorax* population in a water column from the Sea of Japan was observed after the oil spill from the Russian tanker, *Nakhodka,* on January 1997 (Kishira, Kasai and Shutsubo, unpublished results).

Future Prospects

The bioremediation studies after the oil spill from the *Exxon Valdez* demonstrated the promise of this technology for cleaning up a petroleum-contaminated shoreline. It has been estimated that a beach damaged by oil spill could be returned to its natural condition in as little as two to five years with bioremediation treatment, otherwise it would take ten years or more to reach this condition. Ten years after the *Exxon Valdez* accident, oil residues still remain subsurface in the spill-affected bays where contamination problems persist and affect fishery activities. This fact clearly indicates the necessity for further development of bioremediation technologies to manage marine oil pollution. The microorganisms which play the major roles in the bioremediation of offshore sediment, and their nutrient requirements (carbon, nitrogen, phosphorous, etc.) and environmental requirements (oxygen or an alternative electron acceptor, temperature, redox potential, salinity, pH, etc.) should be determined. Such knowledge would allow the manipulation of environmental factors that may limit or prevent the biodegradation of petroleum in marine sediment. As mentioned earlier, bioaugmentation or seeding would be useful to enhance the cleanup process of oil-contaminated sediment.

Undertaking bioremediation cannot be done without the agreement of local communities. Their concerns about bioremediation, a relatively new technology, should be mitigated by scientists who can explain to the local people the results of hydrocarbon contamination tests and microorganism tests, especially in regard to risk assessment. The identification of the *Alcanivorax* group as a major population that arose during a bioremediation treatment would be useful information to help convince people that the propagation of harmful microorganisms would not occur during bioremediation.

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