Chapter 2

REVIEW OF LITERATURE
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The role of microorganisms in the biodegradation of hydrocarbons has been well documented by now.

As early as in 1946, Zobell reported 100 species belonging to 30 genera, which were capable of utilizing hydrocarbons. Bartha and Atlas (1977) have listed 22 genera of bacteria and 14 of fungi which were known to be hydrocarbon utilizers. Floodgate (1984) lists 25 genera of hydrocarbon-degrading bacteria and 27 genera of hydrocarbon-degrading fungi which have been isolated from marine environment.

Biodegradation of hydrocarbons depends on various factors which include:
- Population size and microbial species capable of degrading the contaminant.
- Type of hydrocarbons.

2.1 Effect of Hydrocarbons on Microorganisms

Whenever there is an oil spill in the environment there is a change in the population size and microbial diversity of the contaminated site. Results of a study carried out
by Cohen (2002), indicate that degradation of oil was primarily done by aerobic heterotrophic bacteria. Strains of sulfate-reducing bacteria and aerobic heterotrophic bacteria were capable of degrading model compounds of aliphatic and aromatic hydrocarbons. Hydrocarbon degradation by microbial communities depends on the composition of the community and its adaptive response in the presence of hydrocarbons (Walker et al, 1976).

2.1.1 Impact on the population size

According to Atlas (1981), the hydrocarbon-degrading bacteria account for <1% of the total bacterial population in the natural uncontaminated environment. But when there is an oil spill, the proportion of hydrocarbon-degrading bacteria increases rapidly (Atlas, 1995). Perussitti et al (2003) observed a selective enrichment of hydrocarbon-degrading bacteria from 0.028% at the beginning of a bioremediation experiment to almost 100% two months after the spillage. The phenomenon, which results from the hydrocarbon-oxidizing potential of the community is called adaptation (Spain et al, 1980). Al-Hadhrami et al (1996) observed higher oxidation rates and greater reduction of n- alkanes for bacteria grown in mousse than those grown in nutrient broth indicating environmental adaptation of the test bacteria. Survey of petroleum-degrading bacteria in coastal water of Sunderbans Biosphere Reserve showed maximum number of bacteria in the waters of Haldia Port and its surrounding area where the water is highly contaminated by refinery discharges (Roy et al, 2002).

Experiments on the bioremediation of crude oil contaminated subantarctic intertidal sediment showed a two order of magnitude increase of saprophytic and hydrocarbon utilizing microorganisms during the first month of the experiment (Delille et al, 1998 and 2002).

Radwan et al (1997), reported an increase in number of hydrocarbon degraders in the soil sample immediately after the addition of crude oil. Song and Bartha (1990) observed that in subsurface soil, microbial numbers increased by 2-2.5 orders of magnitude as a result of jet fuel contamination. The number of phenanthrene and chrysene degrading populations increased after contamination
by polycyclic aromatic hydrocarbons (PAH) (Melcher et al, 2002). Similar results have been obtained by Azoulay et al (1983), Prince (1993) and Pritchard et al (1992). Diesel fuel contaminated soil and water contained $6 \times 10^6$ to $100 \times 10^6$ phenantherene degrading bacteria whereas uncontaminated sites like garden soil and reservoir water had no detectable or modest numbers of these organisms (Bogardt and Hemmingsen, 1992). Geiselbrecht and Staley (1996) reported that sediments from a creosote contaminated EPA superfund site (Eagle Harbour) contained $10^4$-$10^7$ polycyclic aromatic hydrocarbon degrading bacteria (dry weight) per gram of sediment whereas the concentration of an uncontaminated site ranged from $10^3$ - $10^4$ per gram of sediment. Vivekanand et al, (1999) observed degradation of crude oil in 24 hours with a corresponding increase in the bacterial population from $2 \times 10^3$ to $2 \times 10^6$ and $4 \times 10^8$ after 48 hours. They also observed a decline in the bacterial cell number after the fifth day. Increase in the bacterial population after contamination with hydrocarbons has also been observed by Patel and Ghosh (2002) and Bhupathiraju et al (1999).

2.1.2 Effect of Hydrocarbon contamination on microbial diversity

Contamination by hydrocarbons affects the microbial diversity of the contaminated site and diverse microorganisms having capability to degrade hydrocarbons have been isolated from the contaminated site.

A contaminated site is dominated by those microorganisms which have the capability to degrade the contaminant. Therefore stressed systems are less diverse than the non stressed systems. Studies by Röling et al (2002) on the dynamics of bacterial communities during nutrient-enhanced oil spill bioremediation showed that bioremediation treatments decreased the biodiversity of the bacterial communities. Effect of crude oil on the metabolic activity of mixed microbial population showed that the presence of hydrocarbon increases the abundance of hydrocarbon-degrading bacteria but decreases microbial diversity (Nyman,1999).

Though many genera capable of hydrocarbon degradation have been studied,
Hanson et al, 1997; Eriksson et al, 2000). Ridway (1990) has reported that *Pseudomonas, Alcaligens, Nocardia and Micrococcus* accounted for 89.7% of bacteria isolated and identified from a gasoline contaminated aquifer. Nine out of eighteen isolates capable of using naphthalene and phenanthrene as sole carbon source obtained by Meyer et al (1999) belonged to genera *Pseudomonas* and *Sphingomonas*. Similar results have been obtained by Shen et al (1998). Microbial ecological studies of two districts of Gujarat indicated that *Pseudomonas* was the predominant genus amongst the hydrocarbon-degrading bacterial genera (Patel and Ghosh, 2003, Patel et al, 2001). Analyses of water and sediment samples from the Cuban shelf for hydrocarbon degrading bacteria showed that 6 genera viz. *Pseudomonas, Flavobacterium, Achromobacter, Bacillus, Alcaligenes and Micrococcus* were most prevalent (Montero et al 1996).

Isolates obtained by Melcher et al (2002) from the San Diego bay sediments contaminated with different polyaromatic hydrocarbons and hexadecane belonged to different marine genera like *Vibrio, Marinobacter or Cycloclasticus, Marinomonas, Pseudoalteromonas and Halomonas*. More than 20 genera of marine hydrocarbon degrading bacteria described over several (sub) phyla (α,β,γ proteobacteria) and Gram positives; *Flexibacter-Cytophaga-Bacterioids* have been described so far (Bruns and Corti, 1999; Engelhardt et al, 2001; Floodgate, 1995; Gauthier et al, 1992; Gieselbrecht et al, 1996; Yakimov et al, 1998).

During their study on polycyclic aromatic hydrocarbon degradation Kasai et al (2002) and Iwabuchi et al (2002) observed that members of genus *Cycloclasticus* were the most prominent degraders among marine bacteria. Harayama et al (1999) have reported that *Alcanivorax* group became more predominant in oil-contaminated sea water when nutrients were supplemented. Apart from bacteria, fungi and cyanobacteria capable of hydrocarbon degradation have been studied (Pickard et al, 1999 and Raghukumar et al, 2001). Natural and synthetic bacterial consortia have also been found to be effective degraders of hydrocarbons (Mohandass et al,1997; Foght et al, 1998; Vivekanandhan et al, 1999 and Thouand et al, 1999; Ward et al, 2003).
2.2 Biodegradation of various hydrocarbons

Biodegradation of crude oil depends on the inherent metabolic capacity and suitable environmental conditions. The composition of oil also plays a significant role in its degradation. The susceptibility of hydrocarbons is in the order: aliphatic compounds > branched chain aliphatic compounds > aromatic heterocyclic compounds > polycyclic compounds (Whyte et al, 1998, Perry, 1984, Fusey and Oudot, 1984, Walker et al, 1976). Resins and asphaltenes are thought to be recalcitrant or having very low degradation.

Jobson et al (1972) studied the utilization of crude oil by pure and mixed culture at 4°C and 30°C and found that the n-saturate fraction was preferentially used. Phenanthrene-utilizing bacteria AR-3 could grow on saturated hydrocarbons of Malaysian crude oil without the addition of yeast extract but yeast extract was required for the biodegradation of the aromatic fraction. No significant degradation of asphaltene fraction was observed even with addition of high concentration of yeast extract (Law and Teo, 1997). Dutta and Harayama (2000) also observed a rapid decrease in the saturated fraction compared to the aromatic, asphaltene and resin fraction.

In a study carried out by Vinas et al (2002), three different microbial consortia were incubated with crude oil to elucidate their metabolic capability. Consortia F1AA and TD removed 100% n-alkanes and branched chain alkanes whereas with consortium AM 91% branched alkanes remained. Efficiency on polycyclic aromatic hydrocarbons was 19% (AM), 11% (TD) and 7% (F1AA). Low molecular weight aromatic hydrocarbons are toxic to microorganisms but can be metabolized when present in low concentration. N-Alkanes of chain length between C_{10} and C_{25} are most amenable to biodegradation as compared to isoalkanes with extensive branching. Reddy et al (2002) studied the persistence of petroleum hydrocarbons in marsh sediments, thirty years after the West Falmouth Oil Spill and found that only n-alkanes underwent complete degradation. Biodegradation of other compound classes occurred but did not progress to completion. Studies by Kropp et al (1997) on the biodegradation of organosulfur
compounds in the aromatic fraction shows that the recalcitrance increases with alkyl substitution.

The age of spill, the composition of hydrocarbons and the availability of the hydrocarbons to microbial attack influences biodegradation. Aged spills are more likely to have a higher proportion of recalcitrant hydrocarbons and low bioavailability (Margesin and Schinner, 1999). Studies by Ijah and Antai (2003) on the removal of Nigerian Light Crude Oil in soil over a 12 month period showed that the quantity of crude oil spilled and age of the contamination influence the rate and total extent of disappearance of oil in the environment. Sugiura et al., (1997) studied the degradation of four crude oil samples: Arabian light, Dubai, Maya and Shengli by Acenitobacter sp. T4 and microbial consortium SM 8. The degree of biodegradation of crude oil components differed according to the crude oil, the saturated-fraction being more susceptible to biodegradation than the aromatic fraction in all crude oil samples. They also found that crude oil samples with higher API gravity were more susceptible to biodegradation. This observation was also made by Walker et al, 1976. Though Acenitobacter could not degrade polycyclic aromatic hydrocarbons, 10% of the aromatic fraction was degraded in Arabian light crude oil.

In a study conducted by Mc Millen et al (1995) 60% of oil having API 46 was lost in four weeks whereas only 10% of oil having API 15 was consumed under the same conditions. Prince (1993) has reported 97% biodegradation potential for light crude oils. Biodegradation of three different crude oil samples, Bonny Light crude oil (API gravity 35.3), Alaskan North Slope 521 (API 27.8) and Venezuelan oil (API 11.8) showed that biodegradation of Bonny light crude oil > ANS 521 > Venezuelan oil (Frontera Sau et al, 2002).

2.2.1 Biodegradation of Saturates

Saturated hydrocarbons are those with only single carbon-carbon bonds and usually constitute the largest group. Of these, the straight chain alkane series is most abundant and readily degraded. Compounds up to C_{44} can be metabolized but those having 10 - 24 carbon atoms are the easiest to metabolize. Light oils contain
10% - 40% straight chain alkanes but weathered and heavier oils have less percentage of the same.

The microbial catabolic pathway of the degradation of linear alkanes or paraffins by a variety of bacteria and fungi has been well studied. The initial steps in the terminal pathway is catalyzed by a monooxygenase to give the corresponding primary alcohol which is further oxidized to corresponding aldehyde by alcohol dehydrogenase and then to the corresponding fatty acid by aldehyde dehydrogenase. Certain bacteria catabolyze alkane through sub terminal pathways in which the first intermediate of alkane oxidation is a secondary alcohol oxidized by a monooxygenase, which is converted to ketone and eventually to a fatty acid. Whyte et al (1998) reported that shorter chain alkanes (C_{12}-C_{16}) are more readily degraded than the longer chain alkanes (C_{28}-C_{32}) and the longer chain alkane reduction was greater than shorter chain alkanes particularly dodecane. Alkanes with more complicated branching are generally considered much more recalcitrant than pristane and phytane. The degradation involves oxidation of beta carbon to the ketones, further oxidation to the esters and subsequent hydrolysis before beta-oxidation.

2.2.2 Naphthenes (Cycloalkanes)

These are readily degraded by microorganisms. Perry et al (1984), studied the degradation of cyclohexane by Acenitobacter sp. It possesses enzyme cyclohexane monooxygenase that converts cyclohexane to cyclohexanol, which is converted to ketone, cyclic acid lactone and ring cleavage to di-terminal carboxylic acid. The enzyme involved in conversion of ketone to lactone is another monooxygenase and yet another monooxygenase is used in hydrolytic cleavage of lactone resulting in the conversion to adipate. Thus cyclic alkane conversion requires larger energy, because each monooxygenase turn over requires an NADH. Though hopanes are considered to be relatively recalcitrant, they can be degraded by a dual substrate system known as co-oxidation. Studies of biodegradation of decaline by Pseudomonas aerugenosa K 1, in the presence of hexadecane resulted in the complete degradation of decaline.
2.2.3 Aromatic hydrocarbons

Amongst all classes of hydrocarbons, the low molecular weight compounds evaporate readily and are toxic to marine life while polyaromatic hydrocarbons are of greatest concern because of their toxicity and carcinogenicity (Dewitt et al., 1989 and Harvey, 1996). As the molecular weight and the complexity increase, the aromatic compounds are less readily degraded. Biodegradation of polyaromatic hydrocarbons has been studied by many groups (Milcher et al., 2002; Kasai et al., 2002).

Aerobic microbial attack on polycyclic aromatic hydrocarbons can be summarized as follows: dioxygenase attack on a single (usually terminal) ring leading to catechol like structure, oxygenolytic cleavage of the catechol (either meta or ortho) to dihydroxyl group by the action of another dioxygenase, followed by further ring cleavage to metabolically utilizable substrates (Rockne and Strand, 1998).

The genes that encode the enzyme for the naphthalene degradation pathway in *Pseudomonas sp.* are generally plasmid borne. The degradation of larger polyaromatics may follow a similar route. The plasmid encoded naphthalene catabolic genes are expressed constitutively at low level and a high degree of homology exists among different degradative plasmids of different sizes. *P. putida mt* 2 chromosomal genes encode the ortho pathway and the TOL plasmid encodes the meta pathway.

2.3 Biodegradation of Vegetable Oils

Vegetable oil spills occur commonly and their frequency is expected to increase as the production, use and transportation of these materials continue. Despite the perception that they are non-toxic, and therefore not harmful, the potential of vegetable oils to damage sensitive ecosystems is similar to that of crude oil and other petroleum products (Crump-Weiser and Jennings, 1975; Rigger, 1997; Calanog et al., 1999). They may cause intestinal lesions and dehydration from strong laxative effects (Wrenn,1999).
Studies on mussels exposed to four different oils caused 19% mortality in four days of exposure (Mudge, 1993 and 1996). Microorganisms can convert oil into harmless products like carbon dioxide and methane. Periera et al (1996) studied the aerobic and anaerobic degradation of sunflower and linseed oil. Three days after the addition of linseed oil the number of heterotrophic bacteria increased five fold. In a stimulated spill in salt marsh, linseed oil penetrated into the sediments and caused decreased sediment permeability and abundance of plant roots. There was an increase in the aerobic, anaerobic and sulfate reducing bacteria suggesting that these bacteria degraded the oil (Pereira et al, 2002).

Li and Wrenn (2003) used anaerobic organisms for the biodegradation of vegetable oil. Kavitha et al (1997) studied the biodegradation of various oils by Acremonium alternata. Studies by Li et al, (2001) on vegetable oil spills in freshwater showed that sedimentation followed by anaerobic biodegradation could be a feasible response to such spills. Biodegradation was completed within several weeks. Also higher loading resulted in longer lag phase and time required for complete degradation was also longer. They also showed that addition of ferric hydroxide stimulated vegetable oil degradation (Li et al, 2003).

### 2.4 Biodegradation of Lubricating Oils

Lubricating oils are a complex mixture of hydrocarbons having linear and branched chain alkanes, cyclic alkanes and aromatic hydrocarbons (Westlake et al, 1974 and Goto et al, 1994). According to Hoffman (1985) 207 tons of lubricating oil enters the Narrangansett Bay watershed. Biodegradation of both used and unused lubricating oil was studied by Jirasripongpun (2002). Of the 26 isolates obtained and belonging to various genera like Nocardia, Acinetobacter, Ralstonia, Pseudomonas, Gordono, Rhodococcus, Agrobacterium and Debaryomyces, Nocardia yielded the best results. Studies by Shirai et al (1995) on the biodegradation of heavy oil suggested Rhodococcus strain to be the best. Xie et al (1995) studied the biodegradation of lubricating oils in aquatic environment and found that lubricating oils were difficult to degrade as only 23% removal was achieved in 67 days (Xie et al, 1995).
2.5 Frequency and Distribution of Plasmids

Bacteria isolated from oil contaminated environments are shown to be more effective in degrading crude oil and its components as compared to those isolated from pristine environments. Plasmid DNA plays an important role in the genetic adaptation which provides the bacteria with hydrocarbon-oxidizing ability. Plasmids encode enzymes for hydrocarbon degradation and thus impart selective advantage to the strains possessing them (Leahy and Colwell, 1990). Higher degradation rates have been linked to the higher incidence of plasmid DNA as a consequence of adaptation by Barkay et al (1988) and Spain et al (1980).

A study conducted by Hada and Sizemore (1981) in the northwestern Gulf of Mexico have shown that higher proportion of Vibrio spp. isolated from an oil field site carried plasmids as compared to those isolated from control sites. Out of the five strains of Pseudomonas isolated from the oil fields of Mehsana and Ahmedabad region, three were found to be bearing high molecular weight plasmid (Alaknanda Patel, 2000).

2.6 Effect of Hydrocarbons on vegetation and Bioremediation

Bioremediation is one of the most important technologies for environment restoration. It uses microorganisms to reduce the concentration and toxicity of pollutants like petroleum hydrocarbons, polycyclic aromatic hydrocarbons and other hydrocarbons.

As discussed earlier, bioremediation can be carried out by either seeding or fertilization or both. Though fertilization is effective and widely studied, another technique is to inoculate the hydrocarbon contaminated site with hydrocarbon-degrading microorganisms. Such microorganisms are found everywhere but sometimes bioaugmentation may increase pollutant removal rates (Alexander, 1999; Atlas, 1981; Colwell and Walker, 1977). On the other hand there are reports of inoculation of contaminated site failing to reduce either the total hydrocarbons or enhance the biodegradation rates.
In field studies (Mohn et al, 2001) and laboratory studies (Mohn and Stewart, 2000) have found that inoculation of fuel contaminated Arctic tundra soils did enhance fuel biodegradation in addition to enhancement due to nutrient addition. Studies on the effectiveness of bioremediation of crude oil contaminated subantarctic intertidal segment by Delille et al (2002) showed a rapid and efficient microbial response in spite of the severe weather conditions and the rate of biodegradation was improved in presence of bioremediation agents. Radwan et al (2000) found that fertilizing oily desert soil sample with a mixture of glucose and peptone resulted in enhancing hydrocarbon disappearance in that soil. Margesin (2000) studied the role of cold adapted microorganisms for the bioremediation of experimentally and chronically oil contaminated Alpine soils and found that oil degradation can be significantly enhanced by biostimulation.

Studies by Hozumi et al (2000) on the biodegradation of heavy oil spilled by Russian tanker, Nakhodka on the Japan coast by commercially available microbial cultures for bioremediation, TerraZyme TM showed extensive degradation of oil. Fayad et al (1992) studied the effectiveness of bioremediation product in degrading the oil spilled in the 1991 Gulf War and found that addition of both nutrients and bacteria had a more pronounced effect than that of nutrients only. Raghavan and Vivekanandan (1999) conducted a field study with naturally adapted Pseudomonas putida isolated from oil contaminated site for biodegradation in open environment. Replicate field trials comprising oil, oil plus bacteria and oil plus bacteria plus fertilizer were monitored for total viable count and rate of biodegradation of crude oil. There was an increase in number of bacteria and an increased biodegradation in the plot with oil plus bacteria plus fertilizer.

An investigation into enhanced bioremediation through the addition of macro and micro nutrients in PAH/petroleum contaminated soil by Ward Liebeg and Cutright (1999) showed the bioactivity of foreign consortium was greatest when a high level of micronutrients was used. The bioremediation of light Arabian oil in sandy sediment by a mixed culture N_p associated to indigenous microorganisms resulted in 42.9% reduction of the heavy fraction of oil in 28 days when N and P sources were provided. Test performed from the native flora showed only 11.9% removal of
these compounds. Thus usage of cultures adapted to oil enhances the productivity and efficiency of the bioremediation process (Del'Arco and De Franca, 1999).

Several groups have studied the effect of hydrocarbons on vegetation. Atuanya (1987) has reported that waste oil causes breakdown of soil texture. Gill and Nyawuane in their study showed that crude oil binds the soil particles into a water impregnable soil block which seriously impairs water drainage and oxygen diffusion. Atuanya also found that seeds failed to germinate in oil polluted soil (Atuanya, 1987). Crude oil contamination was found to affect the growth and anatomical features of *Chromolaena odorata* (L.) K & R, in a study conducted by Gill and Nyawuane (1996). Udo and Fayumi (1975) have also noted the adverse effect of crude oil on plant growth.

Salanitro *et al.* (1997) studied crude oil hydrocarbon bioremediation and carried out soil ecotoxicity assessment. They found that after bioremediation, hydrocarbons in oily soils decreased from 70-90%, from 40-60% and from 35-60% for those carbon number species in the range of C\textsubscript{11}-C\textsubscript{22}, C\textsubscript{23}-C\textsubscript{32} and C\textsubscript{35}-C\textsubscript{44}, respectively. Seed germination and plant growth were also significantly reduced (0-25% of controls) in untreated soils and bioremediated soils were neither toxic to earthworms nor inhibited seed germination. Maila and Cloete, (2002) have reported level of germination of *Lepidium sativum* decreased with increasing concentration of PAH in the artificially contaminated soil while no germination occurred in historically contaminated soil. At concentration of 1000 ppm and 50 ppm the germination levels were < 16% and > 75% respectively. Brown alga *F. visculosus* is reported to have dosage related inhibition of growth with No.2 Fuel oil. Bioremediation by fertilizers could reduce the deleterious affects of oiling on *F. visculosus* (Wrabel and Peckol, 2000). Proffitt *et al* studied the effects of oil treatment on survival and growth of *Rhizophora mangle* and *Avicennia germinans* and found that oiling depressed survival, stem growth, leaf production and maximum leaf size in *R. mangle* and none of the oiled *A. germinans* survived longer than a few weeks (Proffitt *et al*, 1995).
In studies carried out by Hanson et al (1997), *Acinetobacter* sp. A3–treated soil permitted better germination of Mung beans (80% seed germination in bioaugmented soil as compared to 35% in the untreated soil) and growth which was attributed to the reduction of phytotoxicity of the crude oil due to degradation. Treatment for shorter time also supported better germination than the untreated soil incubated for the same period. They have also suggested the crude oil degradative capability of *Acinetobacter* sp. A3 could be used for bioremediation purpose.