CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Nature of Petroleum Hydrocarbon

Petroleum is a natural product, comprising a complex mixture of various hydrocarbons, created by the decomposition of plant remains from the carboniferous period under high temperature and pressure (Van Hamme et al., 2003). It is a mixture of aliphatic saturated compounds, including n-alkanes, branched n-alkanes and cyclo-alkanes; aromatics, including naphthalene, toluene, xylene and benzene; asphaltanes, including phenols, fatty acids ketones, esters and porphyrins; resins, waxes and high molecular weight tars, including pyridines, quinolines, cardaxoles, sulphonates and amides (Leahly and Colwell, 1990). Crude oils from different wells differ greatly in their composition. Distillation of the crude oil will yield different fractions which will vary in size, complexity and volatility from the petroleum gases with a boiling point of 30^0C to fuel oils residues with a boiling point of over 350^0C

Petroleum varies in colour, specific gravity, viscosity and other physical properties depending on the source. It is a complex mixture of hydrocarbons and non-hydrocarbons, particularly compounds containing nitrogen (N), sulphur (S) and oxygen (O). Trace amounts of metals are present in petroleum (Rainswell et al., 1992). The chemical composition of petroleum varies from different producing region and depth. Crude oil contains aliphatic, cycloaliphatic, aromatic, mixed cycloaliphatic/aromatic hydrocarbons and N, S and O compounds (Figure 2.1)

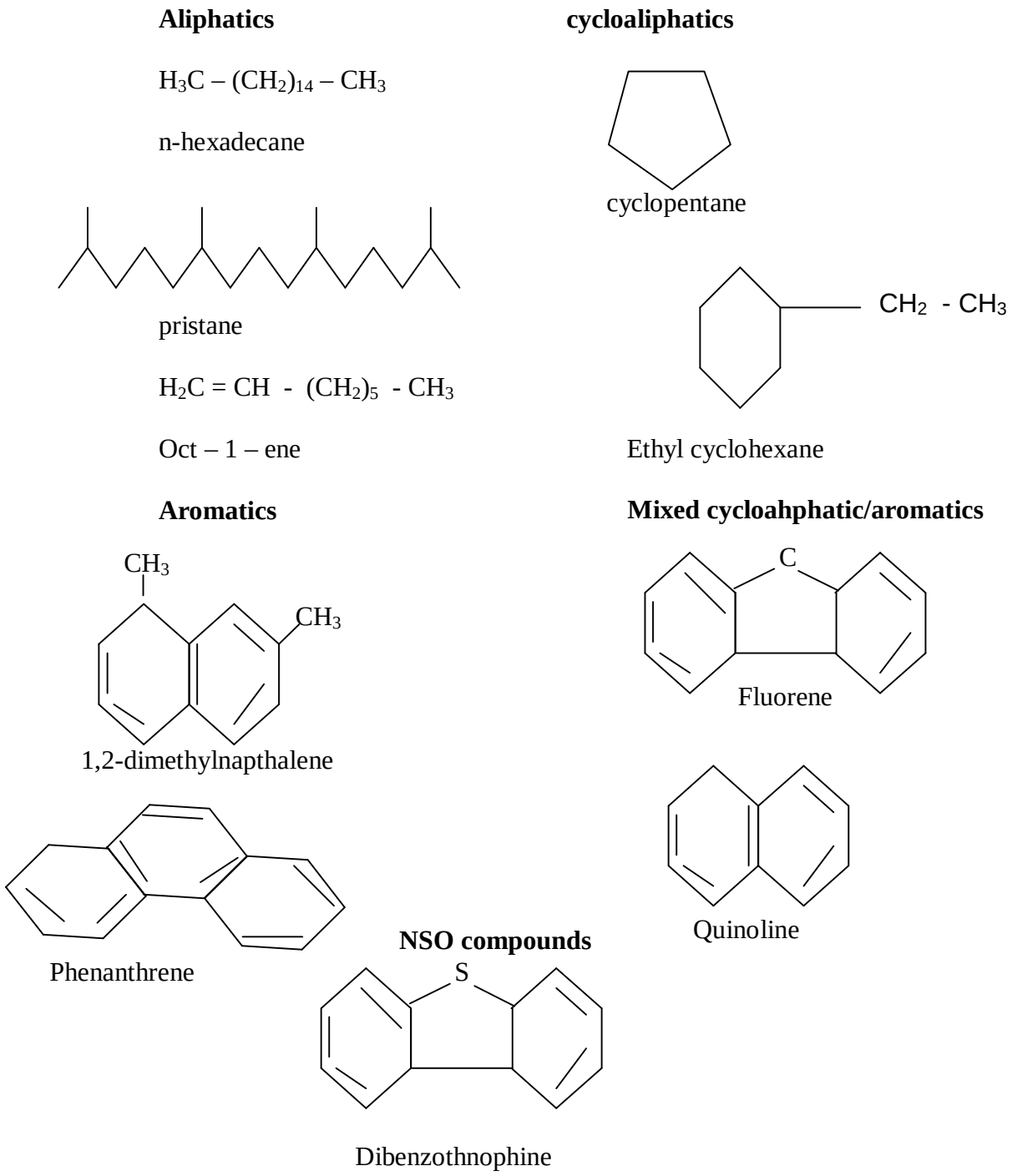

Figure 2.1: Hydrocarbons and non – hydrocarbon compounds found in crude oil (Jim et *al.,* 2005)

Petroleum hydrocarbons are organic compounds comprised of carbon and hydrogen atoms arranged in varying structural configurations. In the broadest sense, they are divided into two families, aliphatics (fatty) and aromatics (fragrant). The polarity of hydrocarbon structures governs the degree to which molecules interact with themselves and with water. Generally, as polarity increases, water solubility (i.e. interaction with water) and boiling points increase. It follows that aromatics are more water soluble and less volatile than alkanes with a corresponding number of carbons (Leahly and Colwell, 1990).

Another key feature of petroleum hydrocarbons is that they typically have a large number of *isomers.* Isomers are compounds that have the same elemental formula but have different structural configurations. In general, as the carbon number increases, the number of possible isomers increases rapidly. An alkane with six carbon atoms has five possible isomers. An increase in the number of carbons to ten increases the number of possible isomers to seventy-five. The large number of isomeric compounds in petroleum mixtures accounts for their high degree of complexity (Jim et *al.*, 2005). Petroleum mixtures with high boiling point constituents have high average carbon numbers; therefore, they have a large number of isomers and greater chemical complexity than petroleum products with low boiling point constituents.

2.2 Impact of Weathering on Petroleum Composition

When petroleum products are released into the environment, changes in product composition take place. Collectively, these changes are referred to as weathering (Jim et al., 2005). The main weathering processes are dissolution in water, volatilization and biodegradation. In the case of spills on land or water surfaces, photo degradation can also be significant. Each of the weathering processes affects hydrocarbon families differently. For example, aromatics tend to be more water soluble than aliphatics, whereas aliphatics tend to be more volatile. Thus when a fuel mixture is released into the environment, the principal water contaminants are likely to be aromatics while aliphatics will be the principal air contaminants. Solubility and volatility of all compounds generally decrease with an increase in molecular weight. In general, the more water soluble and volatile compounds are lost most rapidly from contaminated soil. These compounds have the lowest molecular weight, thus there is a general shift to higher molecular weight compounds in residual materials (Jim et al., 2005).

The rates of weathering by dissolution in water or volatilization of individual petroleum compounds are retarded by the fact that the fuels are mixtures. For example, the solubility of pure benzene in water is approximately 1800 mg/L. The equilibrium concentration of benzene in water in contact with gasoline containing 1% benzene will be approximately 20 mg/L (Pancirov et al., 1980). The solubility and volatility of individual compounds in petroleum hydrocarbon mixtures are proportional to the solubility or volatility of the compound in its pure state and its concentration in the mixture. Solubility and volatility of a compound decrease when the compound is present in a mixture.

If volatilization rather than dissolution were the dominant weathering process, lower molecular homologs within each series would be depleted first. The greater a compound's volatility, the more rapid it get loss from a hydrocarbon mixture. As indicated above, alkanes tend to be much more volatile than aromatics, thus alkanes would be lost preferentially. The trend in volatility by compound class is: alkenes = alkanes > aromatics = cycloalkanes (Jim et al., 2005).

Considering volatilization and dissolution trends together, one can predict the composition of fuel mixtures after release in the environment. Where volatilization is the dominant process, the loss of lower molecular weight alkanes will be the most significant change in the product. In situations where dissolution is the dominant weathering process (i.e. there is contact with water and limited potential for volatilization because soil pore spaces are filled with water), the aromatics will be depleted with benzene removed most rapidly.

A third process that is almost always operative when petroleum mixtures are released in the environment is biodegradation. It has been widely demonstrated that nearly all soils and sediments have populations of bacteria and other organisms that are capable of degrading petroleum hydrocarbons. Degradation occurs both in the presence and absence of oxygen. Two key factors that determine degradation rates are oxygen supply and molecular structure. In general, degradation is more rapid under aerobic conditions. Trends in degradation rates according to structure are: (1) *n*-alkanes, especially in the C_{10} to C_{25} range are degraded readily, (2) isoalkanes are degraded more slowly, (3) alkenes degrade more slowly than alkanes, (4) Benzene, toluene, ethylbenzene and xylenes (BTEXs) are metabolized when present in concentrations which are not toxic to the microorganisms, (5)

Polycyclic aromatic hydrocarbons (PAHs) degrade more slowly than monoaromatics, and (6) degradation of higher molecular weight cycloalkanes may be very slow. These trends typically result in the depletion of the more readily degradable compounds and the accumulation of the most resistant in residues.

It has been shown that biodegradation strongly affects the composition of diesel fuel after a spill in soil. At the initial stages of degradation, the *n*-alkanes are degraded selectively. Over time (weeks or months), they are completely biodegraded. The compounds most easily recognizable in the remaining diesel fuel mixture at this point are the isoprenoids, which include pristane (C_{19}) and phytane (C_{20}). These compounds are alkanes with highly branched structures. This branched structure greatly reduces the rate at which biodegradation occurs. Eventually these compounds are also degraded, leaving behind a complex residue. The limited composition data available for these complex mixtures indicate that there are no detectable BTEXs and the concentrations of carcinogenic PAHs are very low.

2.3 Environmental Pollutants and their Biodegradability

Environmental pollution is the disruption of the natural equilibrium between the living species, and their natural environment. The degradation of the environment has resulted in increase in disease, reduction of the average life spans and growth in infant mortality rates. Civilization appears to have gone frenzy and the future of planet earth has never been in greater jeopardy than it is today (Soeze, 2002).

The past century witnessed a vast increase in global pollution. Industrial development, population growth, urbanization, and disregard for the environmental consequences of releasing chemicals into the environment all contributed to the modern pollution situation (Jim et al., 2005). A huge range of industries, including most notably the oil and gas industry, contributed to the problem. There was a large increase in the diversity of organic compounds that are industrially produced and which are carelessly released into the environment. Consequently, in the natural environment today there are numerous chemical contaminants, which are toxic to biological systems, that have originated from both natural (biogenic and geochemical) and anthropogenic sources (Jim et al., 2005)

The broadest environmental pollutants classification is into two major categories: organic and inorganic pollutants (Jim et al., 2005). Quantitatively organic pollutants that are of most concern are the hydrocarbons in their various forms. The most common are petroleum hydrocarbons (mixtures of n-alkanes, mono-, di-, and polyaromatic compounds, heterocyclic aromatics and other minor constituents) and host of other compounds (Jim et al., 2005). The petroleum industry is a major contributor of organic contaminants to the natural environment, releasing hydrocarbon contaminants into the environment in a number of ways. Accidental and deliberate crude oil spills have been, and still continue to be, a significant source of environmental pollution, and poses a serious environmental problem, due to the possibility of air, water and soil contamination (Trindade et al., 2005). For example, approx. 6×10^7 barrels of oil was spread over 2×10^7 m^3 soil and 320 oil lakes were created across the desert during the first Gulf War in Kuwait (Al-Saleh and Obuekwe, 2005). A comprehensive encyclopedia (Irwin et al., 1997) provides information on toxicity, carcinogenicity and fate of the diversity of environmental pollutants. Some of the effects of

pollution are listed in Table 2.1. Many of these pollutants are biodegradable by microorganisms in soil and water (Alexander, 2001; Wackett and Hershberger, 2001).

Table 2.1 Effects of pollution and those affected

Effect	The affected
Cancer	Humans
Lung disease	Humans
Heart disease	Humans
Hypertension	Humans
Teratogenic effects	Humans
Reproductive effects	Humans
Allergy	Humans
Biomagnification	Humans, environment
Acute toxicity (immediate)	Humans, environment
Chronic toxicity (delayed)	Humans, environment
Neurotoxicity	Humans, environment
Biodiversity loss	Environment
Phytotoxicity	Environment
Zootoxicity	Environment
Eutrophication	Environment
Deoxygenation	Environment
Explosion	Humans, infrastructure
Fire	Humans, environment, infrastructure
Corrosion	Infrastructure
Radiation	Humans, environment

Jim et al., (2005)

The biodegradability of environmental pollutants and the degree of persistence of contaminants in natural environments is influenced by various factors, most important of which are the chemical structure of the contaminants, presence of viable microbial population able to degrade the contaminant(s), and the environmental condition suitable for microbial biodegradative activities (Jim et al., 2005). Persistent contaminants are especially problematic if they are lipophilic because they can be biomagnified as they move

through the food web; also some contaminants can be metabolized to more toxic or carcinogenic compounds (Jim et al., 2005).

2.4 Biodegradation of Petroleum Hydrocarbons

Hydrocarbons are diverse molecules that are extremely abundant in nature. However, the ability of plants and animals to degrade hydrocarbons is limited. It is within the bacteria, filamentous fungi and yeast that hydrocarbon biodegradation is most common. Several reasons for the limited ability of Eukaryotes to degrade hydrocarbons are: many hydrocarbons are virtually insoluble in water, and thus their bioavailability is limited, hydrocarbons are generally chemically inert but are subject to oxygen additions by various enzymes (Jim et al., 2005).

Biodegradation may be defined as the breakdown of organic compounds, in natural or man-made environments, by bacteria, Actinomycetes and fungi (Wilkinson et al., 2002). Microorganisms are equipped with metabolic machinery to use petroleum as a carbon and energy source (Van Hamme et al., 2003). Recent interest in biodegradation has focused on the possibility of using these natural processes for the decontamination of soil or water contaminated with complex hydrocarbon mixes. The relative contribution of bacteria and fungi to hydrocarbon mineralization in soil has been reported (Ijah and Antai, 2003). Similarly, many other investigators (Joo et al., 2008; Greenwood et al., 2009; Obuekwe, et al., 2009) have reported the involvement of bacteria and yeasts in petroleum degradation. The growth of microorganisms on hydrocarbons is often accompanied by the emulsification of the insoluble carbon source in the culture medium. In most cases this has been due to the

production of extracellular emulsifying agents during the breakdown of hydrocarbons. These processes aid microorganisms in growing on and metabolizing petroleum.

2.4.1 Microbial Degradation of Hydrocarbons

In recent years the oil industry has shown considerable interest in the use of microorganisms, especially for controlling and dispersing oil spills using surfactants, bioremediation and oil recovery. The term *"hydrocarbonoclastic"* has been used to describe hydrocarbon utilizing microorganisms. This specifically relates to microbes, that are capable of degrading hydrocarbons, and all of which share some of the following characteristics (Jim et al., 2005):

i. They are able to extensively degrade partially or fully petroleum based compounds.
ii. They have a capable and efficient hydrocarbon uptake system.
iii. They have receptor sites for binding hydrocarbons.
iv. They are capable of producing surfactants.
v. They are well adapted to the environment, genetically stable with rapid reproduction rates.
vi. They have been selected for their environment.
vii. They must not be pathogenic or produce toxic metabolic end-products.
Viii. They must have group-specific oxygenases to introduce molecular oxygen into the hydrocarbon and, with relatively few reactions, generate intermediates that subsequently enter common energy-yielding catabolic pathways.

The oil industry and other related industries, such as oil extraction and transport, pose a threat to the environment because they can cause a huge influx of petroleum hydrocarbons into the environment (Allard and Neilson, 1997; Atlas and Bartha, 1992; Jim et al., 2005).

For this reason, there is an increasing interest in finding ways to reverse degradation of ecosystems, which the introduction of petroleum hydrocarbon sources has adversely affected (Ding and Luo, 2001; Hu *et al.,* 2005; Liu *et al.,* 2004; Zheng *et al.,* 2004). Currently, microorganisms that have the ability to degrade, remove, or transform organic chemicals are used for the purpose of bioremediation of ecosystems that have been polluted by petroleum oil or its fractional compositions (Ding and Luo, 2001; Seklemova *et al.,* 2001). Many studies have used indigenous microorganisms to remediate polluted soils or wastewater (Cunningham *et al.,* 2000; Gallego *et al.,* 2001; Greenwood et al., 2009; Obuekwe et al., 2009). Also microbial strains, which have the ability to biodegrade crude oil *(Candida* sp. and *Rhodococcus* sp.) (Palittapongarnpim et al., 1998; Sharma and Pant, 2000) or fractional compositions of crude oil, such as aromatic hydrocarbon *(Aeromonas* sp., *Alcaligenes* sp., *Bacillus* sp., *Corynebacteria* sp., *Flavobacterium* sp., *Micrococcus* sp., *Mycobacterium* sp., *Nocardia* sp., *Pseudomonas* sp., and *Rhodococcus* sp.) (Bouchez et al., 1995; Bouchez et al., 1999; Raghavan and Vivekanandan, 1999; Wilson and Jones, 1993), diesel oil *(Rhodococcus* sp. and *Acinetobactersp.)* (Gallego et al., 2001; Guzev et al., 1997), and heavy oil *(Pseudomonas* sp.) (Setti et al., 1999), have been isolated from soils. The list of different genera of microorganisms with capabilities to degrade hydrocarbons is shown in Table 2.2 below.

Table 2.2 Genera of microorganisms reported to utilize petroleum fractions for growth

Bacteria	Yeast	Filamentous fungi	Actinomycetes	Algae
Acinetobacter	*Candida*	*Absidia*	*Actinomyces*	*Chlorella*
Achromobacter	*Cryptococcus*	*Acremonium*	*Endomyces*	*Prototheca*
Actinomyces	*Debaryom yces*	*Aspergillus*	*Nocardia*	
Alkaligenes	*Endom yces*	*Botrytis*		
Arthrobacter	*Hansenula*	*Cephalosporium*		
Bacillus	*Mycotorula*	*Chaetomium*		
Corynebacterium	*Pichia*	*Chloridium*		
Flavobacter	*Rhodotorula*	*Cladosporium*		
Methanomonas	*Saccharomyces*	*Colletotrichum*		
Micrococcus	*Torulopsis*	*Cunninghamella*		
Micromonospora	*Trichosporon*	*Fusarium*		
Mycobacterium		*Gliocladium*		
Nocardia		*Graphium*		
Pseudomonas		*Monilia*		
Streptomyces		*Helicostylum*		
		Helminthosporium		
		Mucor		
		Paecilomyces		
		Penicillium		
		Rhizopus		
		Scolecobasidium		
		Spicaria		
		Syncephalastra		
		Trichoderma		

Source: Miller and Litsky (1976).

Since many hydrocarbons are naturally occurring complex mixtures of organic compounds, derived by biosynthesis, it is not surprising that microorganisms have adapted and evolved the ability to utilize these compounds. The effects of natural selection mean that for every compound there is at least one microorganism able to at least partially degrade it, if the environmental conditions are favourable. Therefore, when oil is lost to the environment the indigenous population will most likely contain a small population of microorganisms capable of degrading the contaminating hydrocarbons (Jim et al., 2005). The microorganisms degrade oil and produce intermediate products including alcohols, phenols,

esters, aldehydes, ketones and fatty acids. These in turn are converted into CO_2, water and microbial biomass. This process results in complete mineralization of the pollutant and is clearly the ultimate goal of any bioremediation process.

Because of the presence of hydrocarbon-degrading microorganisms, areas already heavily contaminated with oil are more likely to manage with a further input of hydrocarbon pollutants. Normal populations of hydrocarbon utilizing microorganisms account for 0.1% of the population but may reach 100% under selective pressure after a spill or prolonged chronic discharges, returning to background levels after the pollutant is removed. Higgins and Gilbert (1978) found that in unpolluted water hydrocarbon oxidizing bacteria were low in number but that their number increased by two orders of magnitude in polluted waters. Environmental factors were found to be important in influencing the prevalence of oil-degrading microorganisms. Baker (1995) and Leahy and Colwell (1990) found that hydrocarbon utilizers were more prevalent in summer, at times of high nitrogen and phosphorous, in well aerated water, and in shipping lanes where the autochthonous population had become acclimatized to chronic pollution. Filamentous fungi, because of their mycelia growth habit, can spread between food bases, penetrate oil droplets and are perhaps more adapted to environments where they grow as a film on a fixed surface. Bacteria are able to utilize a broader range of target structures and provide a crucial role in initiating hydrocarbon biodegradation. The limited available evidence does not suggest an ecologically significant role for algae and protozoan in the degradation of hydrocarbons. However, algae and protozoan populations may be directly affected by hydrocarbon concentration. They may suffer direct toxic effects, or, as hydrocarbon degrading bacteria flourish, protozoa population expands as they graze on the bacteria. However, protozoa

population explosions have also been shown to 'significantly reduce the number of bacteria available for hydrocarbon removal so their presence in a biodegradation system may not always be beneficial.

2.4.2 Causes of biodegradation

Once expelled from source rocks, petroleum is subject to a complex series of compositional modifications that may occur during migration and within reservoir (Larter and Aplin, 1995; Hunt, 1996). One of the most important alteration processes is biodegradation by subsurface microbial communities, predominantly bacteria and archea (Peters and Moldowan, 1993; Roling et al., 2003). Until recently, it was generally accepted that most surface and subsurface petroleum biodegradation was caused by aerobic degradation (Palmer, 1993; Whelan et al., 1994), with oxygen provided by meteoric water flushing of the reservoir. However, conservative mass balances of the volumes of water needed to transport sufficient oxygen present overwhelming problems geologically in most reservoirs, even if meteoric water saturated with free oxygen can reach a reservoir (Horstad et al., 1992). Studies have shown that anaerobic sulphate reducing and fermenting microbial consortia can also degrade petroleum (Caldwell *et al.*, 1998; Zengler *et al.*, 1999; Widdel and Rabus, 2001). A variety of metabolites which occur solely under conditions of anaerobic hydrocarbon degradation have now been found in reservoired oils, providing convincing evidence that oil biodegradation is a mainly anaerobic process in the subsurface (Wilkes et al., 2003).

2.5 Chemistry of Petroleum Hydrocarbon

Petroleum is defined as any mixture of natural gas, condensate, and crude oil. Crude oil which is a heterogeneous liquid consisting of hydrocarbons comprised almost entirely of the elements hydrogen and carbon in the ratio of about 2 hydrogen atoms to 1 carbon atom (Okoh, 2006). It also contains elements such as N, S, and O, all of which constitute less than 3% (v/v). There are also trace constituents, comprising less than 1% (v/v), including P and heavy metals such as Va and Ni. Crude oils could be classified according to their respective distillation residues as paraffins, naphthenes or aromatics and based on the relative proportions of the heavy molecular weight constituents as light, medium or heavy. Also, the composition of crudes may vary with the location and age of an oil field, and may even be depth dependent within an individual well. About 85% of the components of all types of crude oil can be classified as either asphalt base, paraffin base or mixed base. Asphalt base contain little paraffin wax and an asphaltic residue (Atlas, 1981). The S, O and N contents are often relatively higher in comparison with paraffin base crudes, which contain little or no asphaltic materials. Mixed crude oil contains considerable amount of oxides ofN and asphalt.

Petroleum oil biodegradation by bacteria can occur under both oxic and anoxic conditions (Zengler et al., 1999), albeit by the action of different consortia of organisms. In the subsurface, oil biodegradation occurs primarily under anoxic conditions, mediated by sulfate reducing bacteria (Holba et al., 1996) or other anaerobes using a variety of other electron acceptors as the oxidant. On a structural basis, the hydrocarbons in crude oil are classified as alkanes (*normal* or *iso*), cycloalkanes, and aromatics. Alkenes, which are the unsaturated analogs of alkanes, are rare in crude oil but occur in many refined petroleum

products as a consequence of the cracking process. Increasing carbon numbers of alkanes (homology), variations in carbon chain branching (*iso*-alkanes), ring condensations, and interclass combinations e.g., phenylalkanes, account for the high numbers of hydrocarbons that occur in crude oil. In addition, smaller amounts of O – (phenols, naphthenic acids), N - (pyridine, pyrrole, indole), and S - (alkylthiol, thiophene) containing compounds, collectively designated as "resins" and partially oxygenated, highly condensed asphaltic fraction occur also in crude but not in refined petroleum (Atlas and Bartha, 1973).

The inherent biodegradability of these individual components is a reflection of their chemical structure, but is also strongly influenced by the physical state and toxicity of the compounds. As an example, while *n*alkanes as a structural group are the most biodegradable petroleum hydrocarbons, the $C_5 - C_{10}$ homologues have been shown to be inhibitory to the majority of hydrocarbon degraders. As solvents, these homologues tend to disrupt lipid membrane structures of microorganisms. Similarly, alkanes in the $C_{20} - C_{40}$ range, often referred to as "waxes", are hydrophobic solids at physiological temperatures. Apparently, it is this physical state that strongly influences their biodegradation (Bartha and Atlas, 1977). Primary attack on intact hydrocarbons always requires the action of oxygenases and, therefore, requires the presence of free O. In the case of alkanes, monooxygenase attack results in the production of alcohol. Most microorganisms attack alkanes terminally whereas some perform sub-terminal oxidation (Figure 2.2).

The alcohol product is oxidised finally into an aldehyde and finally, to a fatty acid. The latter is degraded further by *beta*-oxidation. Extensive methyl branching interferes with the

beta oxidation process and necessitates di terminal attack (Figure 2.3) or other bypass mechanisms. Therefore, *n*alkanes are degraded more readily than iso alkanes.

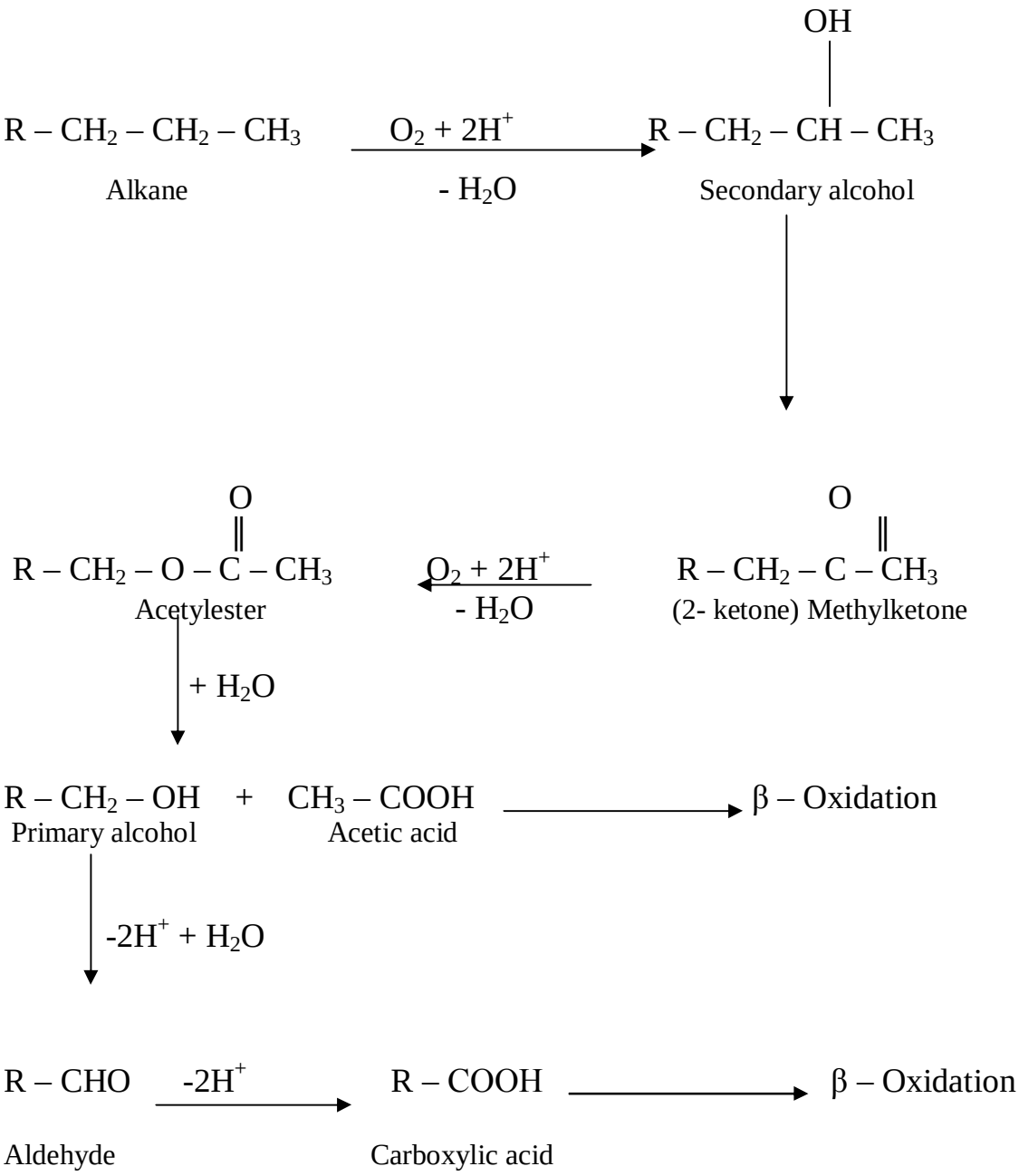

Figure 2.2 Pathways, through which sub terminal oxidation of alkanes yield two fatty acid moieties, which are metabolized further by beta-oxidation (Atlas and Bartha, 1998).

Cycloalkanes are transformed by a not fully characterized oxidase system to a corresponding cyclic alcohol, which is dehydrated to ketone. Then, a monooxygenase system lactonises the ring, which is subsequently opened by a lactone hydrolase. These two oxygenase systems usually never occur in the same organisms and hence, the frustrated attempts to isolate pure cultures that grow on cycloalkanes (Bartha 1986b). However, synergistic actions of microbial communities are capable of dealing with degradation of various cycloalkanes quite effectively. As in the case of alkanes, the monocyclic compounds, cyclopentane, cyclohexane, and cycloheptane have a strong solvent effect on lipid membranes, and are toxic to the majority of hydrocarbon degrading microorganisms. Highly condensed cycloalkane compounds resist biodegradation due to their structure and physical state (Bartha, 1986a).

Prokaryotes convert aromatic hydrocarbons by an initial dioxygenase attack, to *trans*-dihydrodiols that are further oxidised to dihydroxy products, e.g., catechol in the case of benzene (Atlas and Bartha, 1998). Eucaryotic microorganisms use monooxygenases, producing benzene 1, 2-oxide from benzene, followed by the addition of water, yielding dihydroxydihydrobenzene (*cis*-dihydrodiol). This is oxidised in turn to catechol, a key intermediate in biodegradation of aromatics, which is then opened by *ortho*- or *meta*-cleavage, yielding muconic acid or 2- hydroxymuconic semialdehyde, respectively.

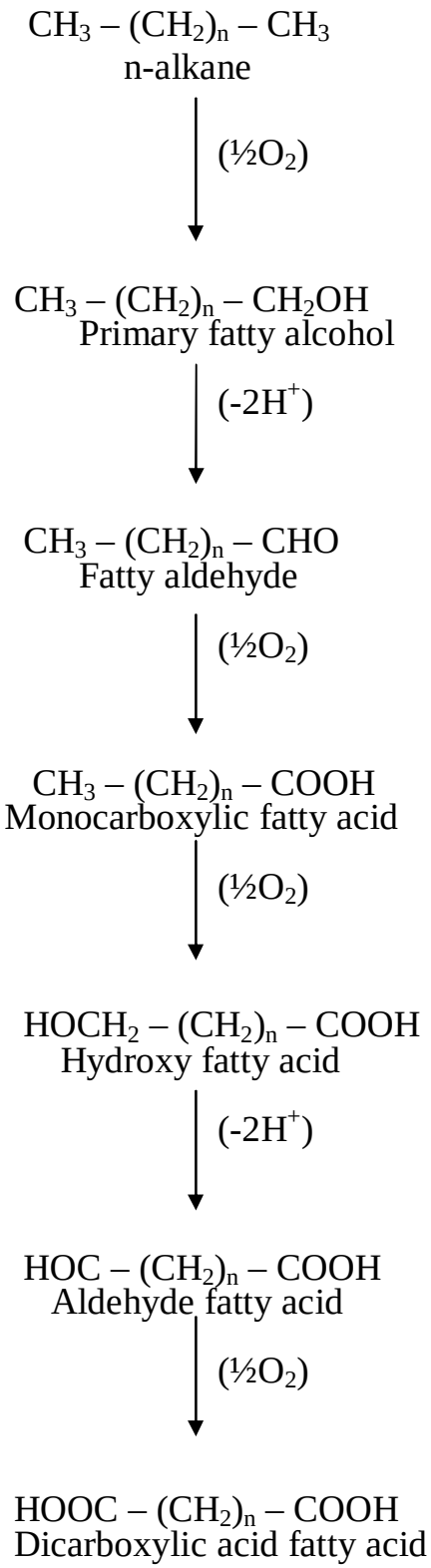

Figure 2.3 Pathway of diterminal alkane oxidation (Atlas, 1984)

Condensed polycyclic aromatics are degraded, one ring at a time, by a similar mechanism, but biodegradability tend to decline with the increasing number of rings and degree of condensation (Atlas and Bartha, 1998). Aromatics with more than four condensed rings are generally not suitable as substrates for microbial growth, though, they may undergo metabolic transformations. Biodegradation process also declines with the increasing number of alkyl substituents on the aromatic nucleus. Asphaltics tend to increase during biodegradation in relative and sometimes absolute amounts. This would suggest that they not only tend to resist biodegradation but may also be formed from beginning by condensation reactions of biodegradation and photodegradation intermediates.

In crude petroleum as well as in refined products, petroleum hydrocarbons occur in complex mixtures and influence each other's biodegradation. The effects may go in negative as well as positive directions. Some *iso*alkanes are apparently spared as long as *n*-alkanes are available as substrates, while some condensed aromatics are metabolized only in the presence of more easily utilizable petroleum hydrocarbons, a process referred to as co-metabolism (Wackett, 1996).

2.6 Biodegradation of different hydrocarbons fractions

Dry gases are dominated by methane relative to the higher homologs with a dryness coefficient [$C_1/\sum (C_1 - C_5)$] of >95%, while wet gases are rich in C_{2+} components with a dryness coefficient of <90%. Gaseous hydrocarbons are easily utilized by microorganisms. There is a general opinion that propane is attacked preferentially (James and Burns 1984; Horstard and Larter, 1997; Pallasser, 2000). Whiticar (1994) indicated that microorganisms can metabolize methane, and methanotrophs have been identified in heavily degraded oil

reservoirs (Head et al., 2003). In general, it is the wet gases that are preferentially attacked, with propane and n-butane reacting most rapidly. Biodegraded gases are usually compositionally dry with very few exceptions.

Gaseous-range n-alkane (butane and pentane) are more easily biodegraded than branched isomers (iso-butane and iso-pentane) (Pallasser, 2000; George et al., 2002; Wenger et al., 2002). Based on these findings, the extent of biodegradation can be deduced by several molecular parameters, i.e., C_2/C_3, n-C_4/C_3, i-C_4/n-C_4, i-C_4/C_3, and i-C_5/n-C_5. All these ratios increase with the extent of biodegradation.

2.6.1 Light Hydrocarbons

The C_{6-15}-n-alkane are among the most rapidly biodegraded components of oil, although they are also susceptible to removal by extensive water washing (Palmer, 1993). Empirically, the first sign of biodegradation are usually n-alkane in the C_{10} to C_{13} range, which probably reflects an optimal carbon number with increasing enthalpy of reaction and decreasing water solubility as the alkane carbon number increases. Aromatic hydrocarbons are typically more resistant than aliphatic hydrocarbons, although Holba et al., (1996) indicated that under putative iron-reducing conditions, alkylbenzenes react first. Benzene or toluene is less affected by biodegradation than n-heptane, 3-methylhexane, cyclohexane and methylcyclohexane (Masterson et al., 2001). Cyclic and branched-chain alkanes are more resistant to biodegradation than linear alkanes. There is a trend of reduced susceptibility to biodegradation with greater alkyl substitution for isoalkanes, alkylcyclohexanes, alkycyclopentanes and alkylbenzenes (George et al., 2002).

The position of methylation has a strong effect on susceptibility to biodegradation. Of the branched alkanes, 2-methylalkanes are more susceptible to degradation than 4-methylalkanes, which in turn are more susceptible than 3-methylalkanes (George et al., 2002). Adjacent methyl group will reduce the susceptibility of an isomer to biodegradation, for example, 1,1-dimethylcyclopentane and 1,1-dimethylcyclohexane are the most resistant of the alkylcyclohexanes and alkylcyclopentanes; 1,2,3-trimethylbenzene and 1,2,3,4-tetramethylbenzene are more resistant to biodegradation than other C_3- and C_4-alkylbenzenes (George et al., 2002). Molecular parameters such as 3-methylpentane/n-hexane, 1,1-dimethylcyclopentane/n-heptane, and 3-methylpentane/2-methylpentane thus increase with increasing degree of biodegradation.

2.6.2 Biodegradation Effects on C_{15+} Aliphatic Hydrocarbons

Different classes of compounds in petroleum have different susceptibilities to biodegradation. Among the C_{15+} aliphatic hydrocarbons, the normal alkanes are removed first, followed by loss of acyclic isoprenoid alkanes (e.g. norpristane, pristine[Pr], and phytane [Ph]). Compounds derived from natural products by molecular rearrangement are usually more resistant to biodegradation than equivalent non-rearranged hydrocarbons. For example, diasteranes and diahopanes are less readily biodegraded than regular steranes and hopanes (Peters and Moldowan, 1993).

Biodegradation proceed along a path of stepwise depletion of compound classes. Although slight differences exist among different studies, a rough order of removal for aliphatic hydrocarbons can be established as follows (from most susceptible to least susceptible compounds): n-alkanes > alkylcyclohexanes > cyclohexanes > acyclic isoprenoid alkanes >

bicyclic alkanes > C_{27-29} steranes > C_{30-35} hopanes > diasteranes > C_{27-29} hopanes > C_{21-22} steranes > tricyclic terpanes (Peters and Moldowan, 1993; Alberdi et al., 2001).

2.6.3 Microbial degradation of polycyclic aromatic hydrocarbons (PAHs) in soils

PAHs are aromatic hydrocarbons with two or more fused benzene rings with natural as well as anthropogenic sources (Haritash and Kaushik, 2009). Although PAH may undergo adsorption, volatilization, photolysis, and chemical degradation, microbial degradation is the major degradation process (Haritash and Kaushik, 2009). Many different bacteria and fungi are able to partially or completely metabolize PAH. Fungal degradation of PAH occurs through two different pathways. White rot fungi produce unspecific extracellular ligninolytic enzymes, peroxidases and laccases that initiate a free radical attack by a single electron transfer, leading to formation of quinones (Cerniglia, 1993). Degradation of PAH by non-ligninolytic fungi involves the activity of the cytochrome P-450 monooxygenases. These enzymes catalyze a ring epoxidation to form an unstable arene oxide, which is further transformed to *trans*-dihydrodiol by enzymatic hydration or rearranged to phenols by non-enzymatic reactions (Sutherland, 1992).

In general, the first step in the aerobic bacterial degradation is the hydroxylation of an aromatic ring via a dioxygenase, with the formation of a *cis*-dihydrodiol. The *cis*-dihydrodiol is then dehydrogenated to give a cathecol, which undergoes further ring cleavage and is transformed into intermediates that enter the central pathways of metabolism and are used for energy production and biosynthesis (Mueller et al., 1996).

Bacteria can also degrade PAH via the cytochrome P-450 pathway, with production of *trans*-dihydrodiols (Moody et al., 2004). PAH have also been shown to be biodegradable anaerobically, *e.g.* under nitrate-reducing conditions (Eriksson et al., 2003).

PAH may be degraded in the rhizosphere of some plants and direct plant uptake of pyrene and phenanthrene has been observed (Gao and Zhu, 2004). However, the most common reason for the degradation of PAH in the presence of plants is the enhancement of the activity of PAH-degrading microorganism in the vicinity of the roots, where they find an environment rich in root exudates and nutrients (Chaudhry et al., 2005)

2.6.4 Biodegradation of Aromatic Hydrocarbons

Biodegradation can achieve complete and cost effective elimination of aromatic pollutants through harnessing diverse microbial metabolic processes (Cao et al., 2009). Aromatic compounds can be defined as organic molecules containing one or more aromatic rings, specifically benzene rings, for example. Different aromatic compounds co-exist as complex mixtures in petroleum refinery and distillation sites (Seo et al., 2009). Benzene and substituted benzenes constitute the naturally occurring aromatic hydrocarbons. Among the most important aromatic petroleum hydrocarbons are benzene, toluene (methylbenzene), ethylbenzene, xylenes (dimethylbenzenes), and the polycyclic aromatic hydrocarbons, of which naphthalene is the simplest representative.

Since all aromatic hydrocarbons are derivatives of benzene, its properties are important. Benzene has the elemental composition C_6H_6 and is a flat, six-membered ring with three carbon-carbon double bonds. Because it is cyclic and unsaturated, benzene is structurally

similar to the cyclic alkenes. It is, however, unusually stable and does not readily participate in reactions that are characteristic of alkenes.

The differences between aliphatic and aromatic hydrocarbons are real and provide a useful method for categorizing these compounds. With respect to biodegradability, however, similarities also occur. For example, although anaerobic biodegradation of aromatic hydrocarbons has been reported (Chakraborty and Coates, 2004; Foght, 2002), it is uncommon and slow relative to aerobic biodegradation. As is the case with aliphatic hydrocarbons, aerobic biodegradation of aromatic hydrocarbons involves the participation of molecular oxygen as a direct reactant and as the terminal electron acceptor. Finally, many important aromatic hydrocarbons can support the growth of bacteria when they are present as the sole source of carbon and energy. Although aromatic hydrocarbons are not as readily biodegradable as are normal and branched alkanes, they are somewhat more easily degradable than are the alicyclic hydrocarbons (Leahy and Colwell, 1990; Cao et al., 2009).

2.6.4.1 Biodegradation of Benzene

Because all of the important aromatic hydrocarbons that occur in petroleum are derivatives of benzene, a reaction that is common to all pathways that lead to mineralization of aromatic substrates is cleavage of the benzene ring. Therefore, biodegradation pathways for aromatic hydrocarbons begin with a description of the pathways used for mineralization of benzene itself.

Molecular oxygen serves a reactant in two steps in the pathways for benzene catabolism. In each of these reactions, both atoms from molecular oxygen become incorporated into the substrate. Enzymes that catalyze such reactions are called dioxygenases (Sheldon and Kochi, 1981). The stoichiometry of dioxygenase-catalyzed reactions can be written as:

$S + O_2$ ------> SO_2

Ring cleavage and subsequent bacterial metabolism of benzene requires that the aromatic ring be destabilized, that is, it must be made more reactive. This is accomplished by a dioxygenase-catalyzed reaction between benzene and molecular oxygen, resulting in production of benzene dihydrodiol (i.e., *cis* -1,2-dihydroxycyclohexa-3,5-diene) (Ribbons and Eaton, 1982; Gottschalk, 1986). Aromaticity is restored by a dehydrogenase-catalyzed conversion of benzene dihydrodiol to catechol (i.e., 1,2-dihydroxybenzene), which is the ring cleavage substrate. The reactions leading to catechol are shown in Figure 2.4.

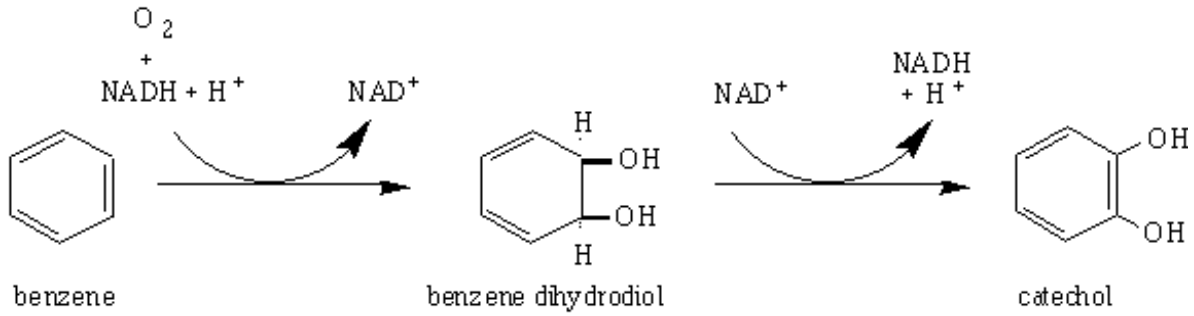

Figure 2.4 Oxidation of benzene to catechol (Wrenn, 1998)

Catechol can further be catabolized by ring cleavage, in which the aromatic ring is broken. Ring cleavage can occur by either of two pathways: the ortho-cleavage pathway, in which the aromatic ring is split between the two carbon atoms bearing hydroxyl groups, or the meta-cleavage pathway, in which the ring is broken between a hydroxylated carbon atom and an adjacent unsubstituted carbon atom (Gottschalk, 1986). Each of these ring-cleavage reactions is catalyzed by a dioxygenase. The subsequent metabolic pathways are quite different, but they both lead to TCA cycle intermediates (acetate and succinate) or to substrates that can be easily converted to TCA cycle intermediates (pyruvate and

acetaldehyde). The ortho-cleavage pathway (also called the b-ketoadipate pathway) is shown in Figure 2.5, and the meta-cleavage pathway is presented in Figure 2.6.

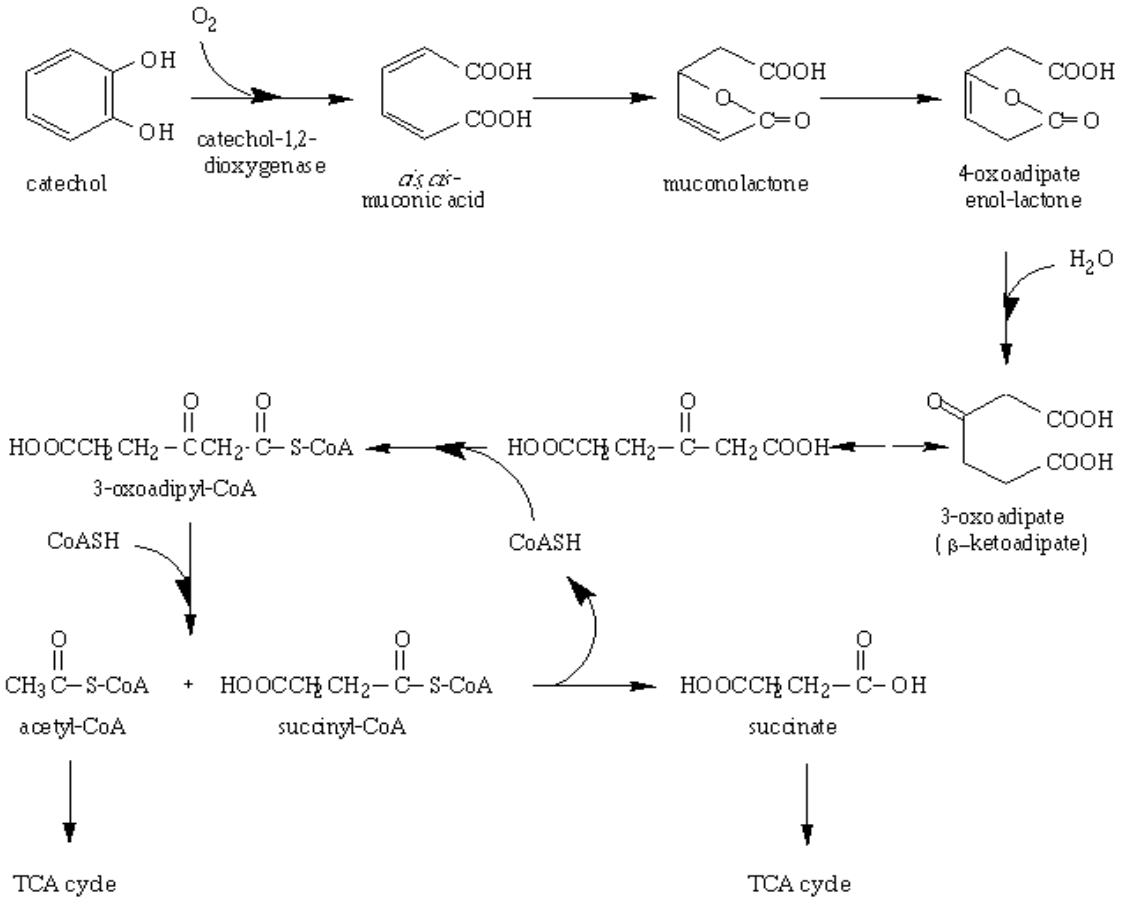

Figure. 2.5. *Ortho*- cleavage pathway for catabolism of catechol (Wrenn, 1998).

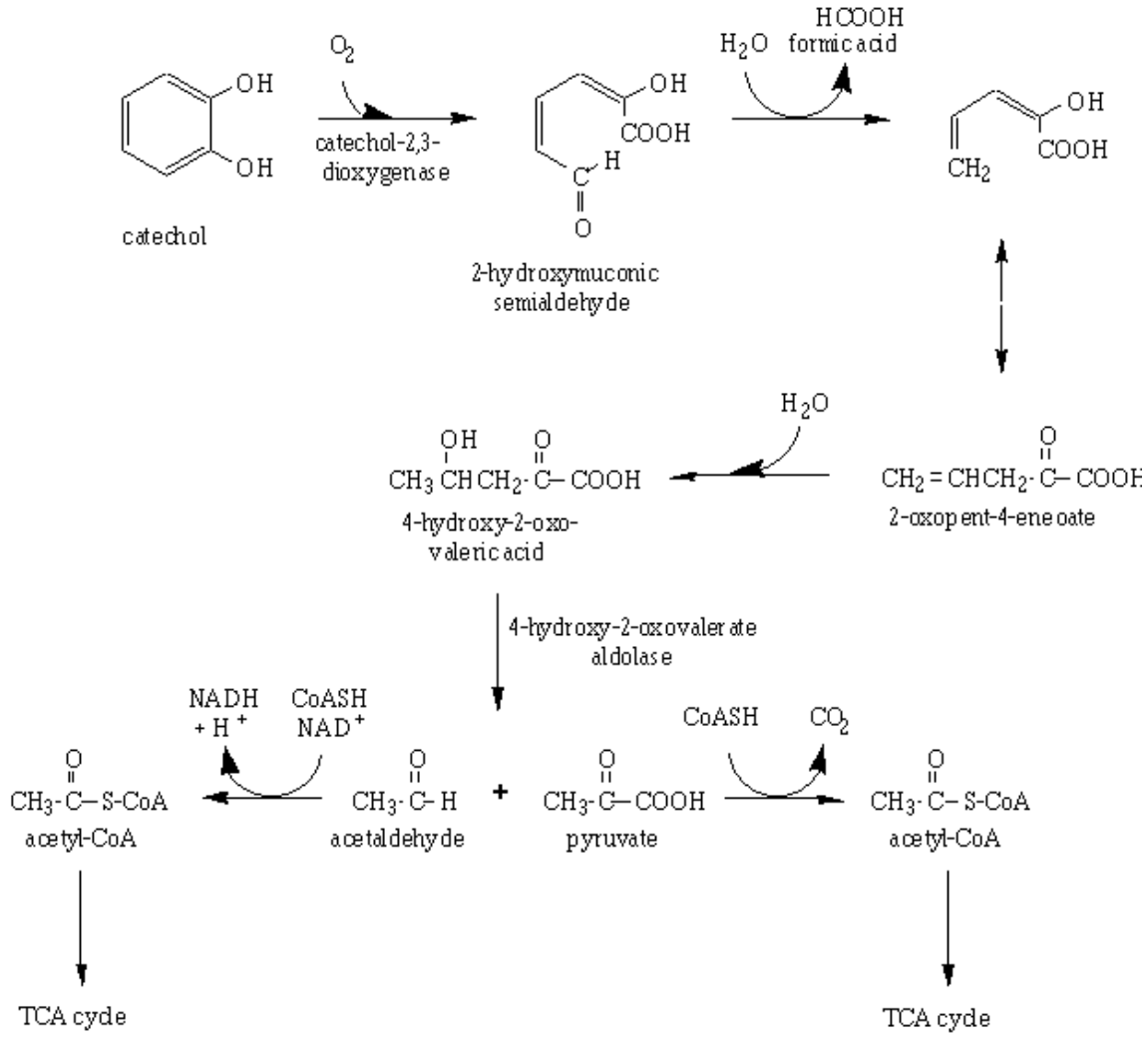

Figure 2.6 *Meta-* cleavage pathway for catechol catabolism (Wrenn, 1998).

2.6.4.2 **Biodegradation of Alkylbenzenes**

Alkyl-substituted benzenes, such as toluene, ethylbenzene, and the xylenes, are common environmental contaminants, because they are important contaminants of gasoline and are widely used as solvents and intermediates in chemical synthesis. These compounds can serve as the sole sources of carbon and energy for a variety of bacteria, including members of the *Pseudomonas*, *Achromobacter*, and *Nocardia* genera (Gibson and Subramanian,

1984). Metabolism of alkylbenzenes may be initiated by oxidation of either the alkyl side chain or the aromatic ring.

Growth of *Pseudomonas aeruginosa* on toluene is an example of a catabolic pathway that is initiated by side-chain oxidation (Ribbons and Eaton, 1982; Gibson and Subramanian, 1984). In a monooxygenase-catalyzed reaction, toluene is converted to benzyl alcohol, which is further oxidized to benzoic acid by dehydrogenation. Benzoic acid is the substrate for insertion of oxygen into the aromatic ring, leading to production of catechol. The reactions leading to catechol are shown in Figure 2.7. The reaction may be catalyzed by a non-specific alkane monooxygenase. The n-alkane monooxygenases of *P. aeruginosa* and of several *Nocardia* sp. are examples of this (Gibson and Subramanian, 1984).

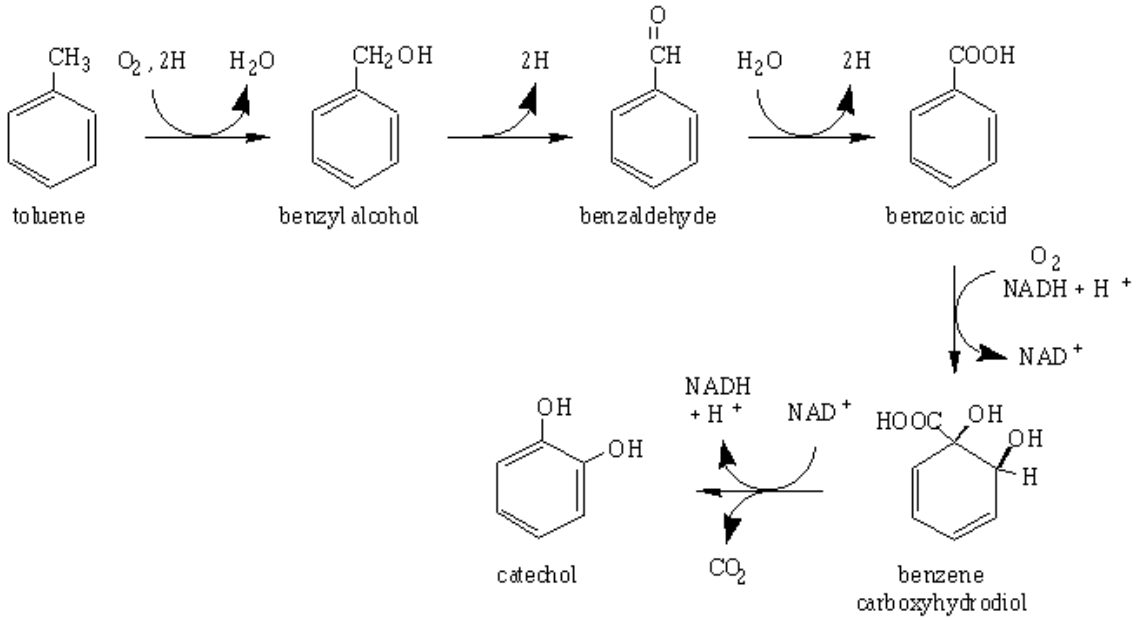

Figure 2.7 Oxidation of toluene to catechol by *Psuedomonas aeruginosa* (Wrenn, 1998).

2.7 Factors influencing microbial degradation of hydrocarbon

Microbial degradation of hydrocarbons in the environment largely depends on number of biotic and abiotic factors, some of these factors which will be discussed in detail in this section includes: (1) Bioavailability of hydrocarbon contaminant (2) Chemical composition of hydrocarbon (3) Physical state of the oil/hydrocarbons (4) Concentration of petroleum hydrocarbon (5) Temperature (6) Oxygen (7) Nutrients (8) pH

2.7.1 Bioavailability of the hydrocarbon contaminant

Bioavailability is extremely important to biodegradation of organic pollutants. It is frequently observed that the rate of removal of compounds from soil is very low even though the compounds are biodegradable, the substrates in these instance may not be in a form that is readily available to the microorganisms (Jim et al., 2005). Biodegradation of hydrophobic pollutants may take place only in the aqueous phase, e.g, naphthalene is utilized by pure cultures of bacteria only in the dissolved state (Wodzinski and Bertolini, 1972). Bouchez et al., (1995) similarly showed that phenanthrene biodegradation occurs only in the aqueous phase.

The three main classes of hydrocarbons (aliphatic, alicyclic and aromatic hydrocarbons) vary in their biodegradability according to size and solubility. It is believed that only molecules of hydrocarbons that are dissolved in the aqueous phase are available for intracellular metabolism (Sikkema et al., 1995). The rate at which a particular organic compound dissolves in water is critical to its biodegradability because this governs the rate of transfer to the organism (Jim et al. 2005). The rate of transfer is determined by the equilibrium and actual concentration in the bulk phase and aqueous phase. This central to the concept of bioavailability as it relates to biodegradation.

2.7.2 Chemical composition of hydrocarbon

The composition and inherent biodegradability of the petroleum hydrocarbon pollutant is the first and most important consideration when the suitability of a cleanup approach is to be evaluated (Okoh, 2006). Compositional heterogeneity among different crude oils and refined products influences the overall rate of biodegradation both of the oil and of its component fractions (Leahy and Colwell, 1990).

Biodegradability is inherently influenced by the composition of the oil pollutant. For example, kerosene, which consists almost exclusively of medium chain alkanes, is under suitable conditions, totally biodegradable. Similarly, crude oil is biodegradable quantitatively, but for heavy asphaltic-naphthenic crude oils, only about 11% may be biodegradable within a reasonable time period, even if the conditions are favourable (Bartha, 1986b). Okoh et al., (2002) reported that between 8.8 and 29% of the heavy crude oil *Maya* was biodegraded in soil microcosm by mixed bacterial consortium in 15 days, although major peak components of the oil was reduced by between 6.5 and 70% (Okoh, 2003). Also, about 89% of the same crude oil was biodegraded by axenic culture of *Burkholderia cepacia* RQ1 in shake flask (Okoh et al., 2001) within similar time frame. Fedorak and Westlake (1981) also reported a more rapid attack of aromatic hydrocarbons during the degradation of crude oil by marine microbial populations from a pristine site and a commercial harbor. Okoh, (2003) noted that heavier crude oils are generally much more difficult to biodegrade than lighter ones, just as heavier crude oils could be suitable for inducing increased selection pressure for the isolation of petroleum hydrocarbon degraders with enhanced efficiency. Also, Okoh et al., (2002) noted that the amount of heavy crude

oil metabolized by some bacterial species increased with increasing concentration of starter oil up to 0.6% (w/v), while degradation rates appeared to be more pronounced between the concentrations of 0.4 and 0.6% (w/v) oil.

2.7.3 Physical state of the oil or hydrocarbons

The physical and chemical nature of the oil pollution is a critical factor in determining rates of biodegradation. Access to the pollutant is a major consideration when trying to degrade hydrocarbons which are, to a greater extent, hydrophobic. The dispersion of crude oil as an oil-in-water emulsion will increase the surface area available for microbial attack and thus increasing the rate of biodegradation. However, water-in-oil, or *"mousse"*, emulsions can form, creating a low surface area to volume ratio, inhibiting biodegradation (Wilkinson et al., 2002). The degree of spreading of oil in water or soil environment determines in part the surface area of oil available for microbial colonization by hydrocarbon-degrading microorganisms; in aquatic systems, the oil normally spreads, forming a thin slick (Atlas, 1981). The degree of spreading is reduced at low temperatures because of the viscosity of the oil. In soils, petroleum hydrocarbons are absorbed by plant matter and soil particles, limiting its spreading.

Similarly *'tar balls'*, which are large aggregates of weathered un-degraded oil, restrict access by microorganisms because of their limited surface area. Auto-oxidation, photo-oxidation and the removal of low molecular weight hydrocarbons by microbes all aid their formation and tar balls may take thousands of years to degrade (Wilkinson et al., 2002).

Hydrocarbon-degrading microorganisms act mainly at the oil-water interface. Hydrocarbon-degrading microorganisms can be observed growing over the entire surface of

an oil droplet; growth does not appear to occur within oil droplets in the absence of entrained water. Availability of increased surface area will under normal circumstance accelerate biodegradation (Atlas, 1981; Ijah and Antai, 2003b). Not only is the oil made more readily available to microorganisms, but movement of emulsion droplets through a water column makes oxygen and nutrients more readily available to microorganisms.

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. Ijah (1998) reported that *Candida tropicalis* and *Acinectobacter calcoaceticus* isolated from oil polluted soil were able to emulsify crude oil at a greater extent, also Pruthi and Cameotra (1997) reported the production of surface active agent by *Serratia marcescens* which formed stable emulsion with a wide variety of hydrocarbons. Broderick and Cooney (1982) 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 and soil bacteria which effectively degrade crude oil also exhibit strong emulsifying activity (Leahy and Colwell, 1990).

Clearly, the physical state of the polluting hydrocarbon in soil or water will affect the uptake and utilization of the substrate, and ultimately the speed of *breakdown* of the pollutant. The oil may be dissolved in the aqueous phase and therefore be available for uptake. Alternatively, microbes may directly adhere to large oil drops at aqueous-hydrocarbon interface. There may also be direct contact with pseudosolubilized oil as fine or sub-micron size droplets. There may also be enhanced uptake of oil as a result of natural

microbial production of *biosurfactants* or emulsifiers that increase the apparent aqueous solubility of the hydrocarbon (Wilkinson et al., 2002).

2.7.4 Concentration of Petroleum hydrocarbon

Concentration of Petroleum hydrocarbon determines to a greater extent the rate of breakdown of the hydrocarbons from the environment. Concentration of hydrocarbon can affect its biodegradability and toxicity to the degrading organisms. High concentration of hydrocarbon can be inhibitory to microorganisms, and concentration with which inhibition occurs will vary with the compound. Concentrations of 1 to 100µg/ml of water or 1 to 100µg/g of soil or sediment (on dry weight basis) are not generally considered to be toxic to common heterotrophic bacteria and fungi. Ijah and Antai, (2003b) reported high degradation of hydrocarbons in soil contaminated with 10% and 20% crude oil compared to those contaminated with 30 and 40% crude oil which experienced partial degradation of hydrocarbons within a period of 12 months. Another authors reported that percentage of degradation by mixed bacterial consortium decreased from 78% to 52% as the concentration of crude oil was increased from 1 to 10% (Rahman et al., 2002). 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. Fusey and Oudot (1984) 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.

2.7.5 Temperature

Temperature plays very important roles in biodegradation of petroleum hydrocarbons, firstly by its direct effect on the chemistry of the pollutants, and secondly on its effect on the physiology and diversity of the microorganisms (Okoh, 2006). Ambient temperature of an environment affects both the properties of spilled oil and the activity or population of microorganisms (Venosa and Zhu, 2003).

Temperature plays a significant role in controlling the nature and extent of microbial hydrocarbon metabolism (Nedwell, 1999; Frederic et al., 2005). Temperature affects the rate of biodegradation, as well as the physical nature and chemical composition of hydrocarbons (Whyte et al., 1998; Rowland et al., 2000). Although microbial activity is generally reduced at low temperatures, many of the components in crude oil and diesel can actually be degraded by psychrophilic and psychrotrophic microorganisms (Margesin and Schinner, 1999a; Delille, 2000; Gibb et al., 2001; Baraniecki et al., 2002; Eckford et al., 2002). The bioavailability of soluble hydrophobic substances, such as aliphatic and polyaromatic hydrocarbons, is temperature dependent. A temperature increase leads to an increase in diffusion rates of organic compounds notably by a decrease of their viscosity (Northcott and Jones, 2000). Thus, higher molecular reaction rates due to smaller boundary layers are expected at elevated temperatures. In counterpart, the increased volatilization and solubility of some hydrocarbons at elevated temperature may enhance their toxicity (Whyte et al., 1998; Niehaus et al., 1999). Such an increase in toxicity may delay the onset of degradation (Leahy and Colwell, 1990; Ita¨vaara et al., 2000).

Temperature influences the rate of abiotic weathering process notably evaporation. Temperature can also affect hydrocarbon utilization; bacteria relatively metabolize isoprenoids at 30^0C but have difficulty doing so at 40^0C. Although many species can withstand freezing and thawing, bacteria cease growth and metabolism altogether at temperature below 12^0C due to the formation of intracellular ice (Margesin and Schinner, 1999b). It is essential that contaminated sites be at the optimum temperature for bioremediation to progress successfully, since excessively high or low temperatures sometimes inhibit microbial metabolism. Although hydrocarbon biodegradation can occur over a wide range of temperatures, the rate of biodegradation generally decreases with decreasing temperature. Highest degradation rates generally occur in the range of 30 – 40^oC in soil environments, 20 – 30^oC in some freshwater environments, and 15 – 20^oC in marine environments (Bartha and Bossert, 1984). In addition, the solubility and bioavailability of a contaminant will increase as temperature increases, and oxygen solubility will be reduced, which will leave less oxygen available for microbial metabolism (Margesin and Schinner, 1999a). For example, a selection of *Rhodococcus* species that were isolated from an Antarctic soil were able to successfully degrade a number of n-alkane at -2^0 C but were severely inhibited at a higher temperature (Bej et al., 2001). In addition, the PAHs naphthalene and phenanthrene were successfully degraded from crude oil in seawater at temperatures as low as 0^0 C (Siron et al., 1995).

Atlas and Bartha (1972) found that the effects of temperature differ, depending on the hydrocarbon composition of a petroleum mixture. Low temperatures retard the rates of volatilization of low-molecular-weight hydrocarbons, some of which are toxic to microorganisms. The presence of such toxic components was found to delay the onset of oil biodegradation at low temperatures (Atlas, 1981). In a subsequent study, Atlas (1975)

examined the biodegradability of seven different crude oils and found biodegradation to be highly dependent on the composition and on incubation temperature. At 20^0C, lighter oils had greater abiotic losses and were more susceptible to biodegradation than heavier oils; rates of oil mineralization for the heavier oils were significantly lower at 20^0C than for the lighter ones. The light crude oils, however, had toxic volatile components which evaporated only slowly, inhibiting microbial degradation of these oils at 10^0C.

2.7.6 Oxygen

Often the most important factor limiting rates of biodegradation in the environment is the availability of molecular oxygen (Jim et al., 2005). The initial step in the catabolism of hydrocarbons by bacteria and fungi involves the oxidation of the substrate by oxygenase to which molecular oxygen is required. Aerobic condition is therefore necessary for this route of microbial oxidation of hydrocarbons in the soil environment. The availability of oxygen in the soil is dependent on rates of microbial oxygen consumption, the type of soil, and the presence of utilizable substrate, which can lead to oxygen depletion (Bartha and Bossert, 1984). Delivering air or oxygen to contaminated soils may be difficult for a number of reasons: the soil porosity may not be favourable and therefore mass transfer from the gas phase to the aqueous phase will be limited. Also relatively low solubility of oxygen in water is a primary limiting factor (Jim, et al. 2005). Most contaminated soil may contain large populations of the appropriate microorganisms but can remain contaminated for decades or longer as a result of conditions that do not favour rapid biodegradation of complex pollutants.

The complete oxidation of aromatic compounds and hydrocarbons to carbondioxide is difficult in the absence of molecular oxygen due to the great stability of C – H and C – C bonds (Boll et al., 2002). Virtually all hydrocarbons are biodegradable under aerobic conditions. Bacteria and fungi have mono and dioxygenases, which incorporate hydroxyl groups derived from molecular oxygen into the aliphatic chain or the aromatic ring (Heider et al., 1999). Aerobic biodegradation of many aromatic compounds proceeds through formation of catechol both for eukaryotic and prokaryotic organisms (Bouwer and Zehnder, 1993). Dioxygenases oxidize PAHs to *cis*-hydrodiols, and PAHs can be oxidized to cytochrome P-450 to arene oxides (Mueller et al., 1996). White-rot fungi (Bogan and Lamar, 1996) and litter-decomposing fungi (Steffen et al., 2002) produce lignolytic enzymes that oxidize PAHs to PAH-quinones.

Although anaerobic microorganisms have the potential to metabolize organic contaminants and do so in many field situations, oxygen is often an integral part in the oxidation of many organic pollutants including hydrocarbons, because molecular oxygen is required to oxidize the carbon moiety (Evans et al., 1996).

2.7.7 Nutrients

Nutrients are very important ingredients for successful biodegradation of hydrocarbon pollutants, especially N, P and in some cases Fe (Okoh, 2006). Inadequate mineral nutrient, especially N, and P, often limits the growth of hydrocarbon utilizers in water and soils. Addition of N and P to an oil polluted soil has been shown to accelerate the biodegradation of the petroleum in soil (Ijah and Safiyanu, 1997; Ijah and Antai, 1995). Abioye et al.,

(2009a), reported that degradation of crude oil in soil amended with melon shells as source of nutrients was 30% higher than those of unamended polluted soil within the period of 28 days.

Addition of a carbon source as a nutrient in contaminated soil is known to enhance the rate of pollutant degradation by stimulating the growth of microorganisms responsible for biodegradation of the pollutant. It has been suggested that the addition of carbon in the form of pyruvate stimulates the microbial growth and enhances the rate of PAH degradation (Lee et al., 2003). Mushroom compost and spent mushroom compost (SMC) are also applied in treating organopollutant contaminated sites (Eggen 1999; Trejo-Hernandez et al., 2001). Addition of SMC results in enhanced PAH-degrading efficiency (82%) as compared to the removal by sorption on immobilized SMC (46%). It is observed that the addition of SMC to the contaminated medium reduced the toxicity, added enzymes, microorganisms, and nutrients for the microorganisms involved in degradation of PAHs (Lau et al., 2003).

Depending on the nature of the impacted environment, some of these nutrients could become limiting, hence the additions of nutrients are necessary to enhance the biodegradation of oil pollutants (Choi et al., 2002; Kim et al., 2005). Pelletier et al., (2004) assessed the effectiveness of fertilizers for crude oil bioremediation in sub-Antarctic intertidal sediments over a one year and observed that chemical, microbial and toxicological parameters demonstrated the effectiveness of various fertilizers in a pristine environment. Frederic et al., (2005), observed that addition of commercial oleophilic fertilizers containing N and P to hydrocarbon contaminated soil increased the hydrocarbon-

degrading microbial abundance and total petroleum hydrocarbon degradation, and also reported 77 – 95% loss of total alkanes and 80% of PAHs in hydrocarbons contaminated soil within the period of 180 days. In another study using poultry manure as an organic fertilizer in contaminated soil, biodegradation was reported to be enhanced in the presence of poultry manure alone, but the extent of biodegradation was influenced by the incorporation of alternate carbon substrates or surfactants (Okolo et al., 2005). However, excessive nutrient concentrations can inhibit the biodegradation activity (Challain et al., 2006), and several authors have reported the negative effect of high NPK levels on the biodegradation of hydrocarbons (Oudot et al., 1998; Chaıneau et al., 2005) and more especially on the aromatics (Carmichael and Pfaender, 1997).

2.7.8 pH

Soil pH can be highly variable, ranging from 2.5 in mine spoils to 11.0 in alkaline deserts (Bossert and Bartha, 1984). Most heterotrophic bacteria and fungi favor a pH near neutrality, with fungi being more tolerant of acidic conditions (Atlas, 1988). Extremes in pH, as can be observed in some soils, would have a negative influence on the ability of microbial populations to degrade hydrocarbons. Verstraete et al., (1976) 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 (1979) 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. Hambrick et al., (1980) found that the rates of microbial mineralization of octadecane and napthalene 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.

2.8 Remediation strategies

Internationally, petroleum contamination is widespread, posing serious environmental risks including surface and groundwater contamination (Balasubramaniam et al., 2007). The environment can potentially be affected by numerous operations in petroleum exploration, production and transportation (Amro, 2004), with common sources of contamination being leaking underground storage tanks (Nadim et al., 2000). Contamination poses serious environmental risks, including surface and groundwater contamination, and risks to human health and safety (Balasubramaniam et al., 2007). Thus, the remediation of contaminated soil is an essential practice (Amro, 2004). Some of the different techniques used in remediating contaminated soil are discussed below.

2.8.1 In situ soil vapour extraction

Volatile and some semi-volatile organic compounds (VOCs and Semi-VOCs) can be removed from unsaturated soils by a process known as soil vapour extraction (SVE). SVE been an in situ clean-up process allows contaminated soil to be remediated without disturbance or excavation (Nadim et al., 2000).

Soil vapor extraction (SVE) is an in situ unsaturated (vadose) zone soil remediation technology in which a vacuum is applied to the soil to induce the controlled flow of air and

remove volatile and some semi-volatile contaminants from the soil. The gas leaving the soil may be treated to recover or destroy the contaminants. The drawback in the use of SVE for remediation of contaminated site is that SVE can not remove heavy oils, metals, PCBs, or dioxins from contaminated soil; it is only effective for remediation of soil contaminated with VOCs and Semi-VOCs. Because the process involves the continuous flow of air through the soil, however, it often promotes the in situ biodegradation of low volatility organic compounds that may be present.

2.8.2 In situ steam injection vapour extraction

Cold soil vapour extraction is a common technique for remediating volatile organic compounds from the unsaturated subsurface. Limitations in efficiency can be overcome by using thermal enhancement, e. g. steam as a fluid heat transport medium to speed up the process (Sleep and Ma, 1997).

In situ steam extraction is a new technology and has had limited use across the globe. Steam extraction can be used in two different systems; mobile and stationary. The mobile system has a unit that volatilizes contaminants in small areas in a sequential manner by injecting steam and hot air through rotating cutter blades that pass through the contaminated medium. The stationary system uses steam injection as a means to volatilize and displace contaminants from the undisturbed subsurface soil. In both systems, steam (at 200^0C) and compressed air (at 135^0C) is forced through the soil medium and the mixture of air, vapor and chemicals are collected by extraction wells (Nadim et al., 2000).

2.8.3 Air sparging

Air sparging is an in situ technology in which air is injected through a contaminated aquifer. Injected air traverses horizontally and vertically in channels through the soil column, creating an underground stripper that removes contaminants by volatilization (EPA, 2001a). Air sparging can also be explained as a method of site remediation that introduces air (or other gases) into the saturated zone contaminated with VOCs. In addition to volatilization of VOCs, air sparging promotes the growth of aerobic bacteria in saturated zones and may oxidize reduced chemical species (Nadim et al., 2000). Air sparging has been shown to be effective in removing several types of contaminants such as the lighter petroleum compounds (C_3–C_{10}) and chlorinated solvents (Marley et al., 1992; Reddy et al., 1995).

2.8.4 Excavation

Excavation (and removal) is a fundamental remediation method involving the removal of contaminated soil/media, which can be shipped off-site for treatment and/or disposal, or treated on-site when contaminants are amenable to reliable remediation techniques. Excavation is generally utilized for localized contamination and point source and is also used for the removal of underground structures that are out of compliance or have been identified as a potential or actual point source of contamination. The limiting factor for the use of excavation is often represented by the high unit cost for transportation and final off-site disposal. EPA (1991) further stated some limiting factors that may limit the applicability and effectiveness of the process to include:

i. Generation of fugitive emissions may be a problem during operations.

ii. The distance from the contaminated site to the nearest disposal facility will affect cost.

iii. Depth and composition of the media requiring excavation must be considered.

iv. Transportation of the soil through populated areas may affect community acceptability.

In this respect, the on-site removal and treatment can often yield significant savings and, in addition, the treated soil may have beneficial secondary use (e.g. as construction fill or road base material) at the same site.

2.8.5 Monitored natural attenuation

The term monitored natural attenuation (MNA) refers to the reliance on natural processes to achieve site-specific remedial objectives (Pope and Jones, 1999). Monitored natural attenuation is always used in combination with source control; that is, removal of the source of the contamination as far as practicable. Natural attenuation processes include a variety of physical, chemical, or biological processes that, under favorable conditions, act without human intervention to reduce the mass, toxicity, mobility, volume, or concentration of contaminants in soil or ground water. These processes include biodegradation; dispersion; dilution; sorption; volatilization; and chemical or biological stabilization, transformation, or destruction of contaminants (Figure 2.8) (Pope and Jones, 1999). MNA is less expensive compared to other treatment methods but takes a longer time for the contaminated soil or water to be remediated. MNA is used when other methods will not work or are expected to take almost as long. Sometimes MNA is used as a final cleanup step after another method cleans up most of the pollution (EPA, 2001b).

Limitation of MNA is that it has not gain public acceptance as an active remediation technique. The time frame in MNA is very long and results cannot be guaranteed on laboratory experiment. The cost of MNA can also increase to exceed other treatment

methods due to intensive monitoring required, and if potential risks are detected, the cost can increase dramatically.

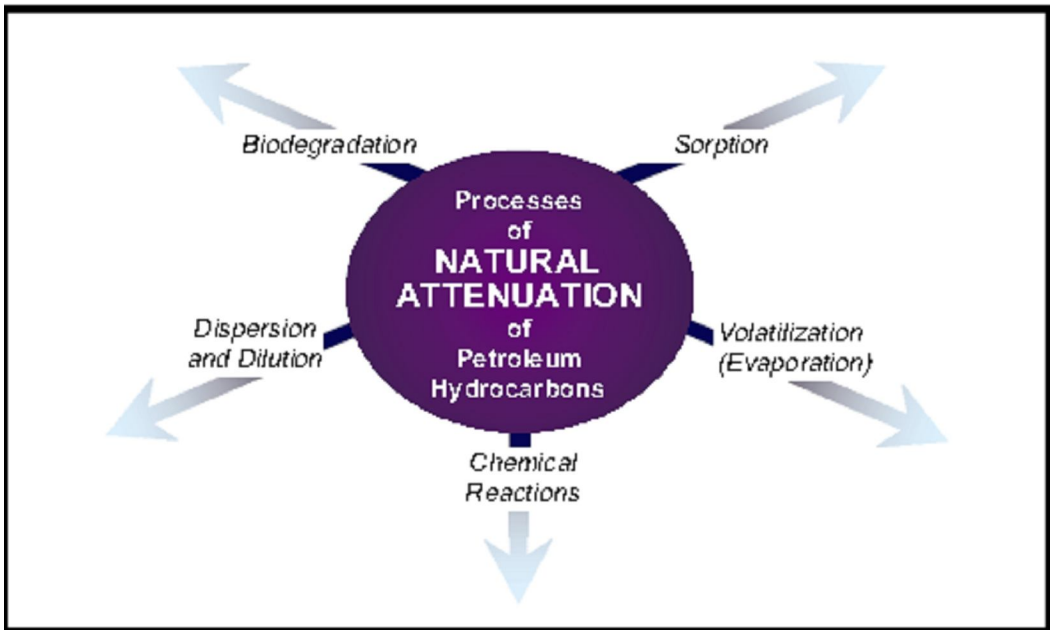

Figure 2.8 Processes of monitored natural attenuation of petroleum hydrocarbons (Pope and Jones, 1999).

2.8.6 Bioremediation strategies

The term bioremediation describes the process of contaminant degradation in the environment by biological methods using the metabolic potential of microorganisms to degrade a wide variety of organic compounds (Scragg, 2005). The main advantage of bioremediation is its reduced cost compared to conventional techniques. Besides cost-effectiveness, it is a permanent solution, which may lead to complete mineralization of the pollutant. Furthermore, it is a non-invasive technique, leaving the ecosystem intact (Perelo, 2010). Bioremediation can deal with lower concentration of contaminants where the cleanup by physical or chemical methods would not be feasible.

The goal of bioremediation is to degrade organic pollutants to concentrations that are undetectable, or if detectable, to concentrations below the limits established as safe or acceptable by regulatory agencies. Bioremediation has been used for the degradation of chemicals in soils, groundwater, wastewater, sludge, industrial wastewater systems, and gases (Okoh and Trejo-Hernandez, 2006). For bioremediation to be effective, microorganisms must enzymatically attack the pollutants and convert them to harmless products. As bioremediation can be effective only where environmental conditions permit microbial growth and activity, its application often involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate (Vidali, 2001). Potential advantages of bioremediation compared to other treatment methods include destruction rather than transfer of the contaminant to another medium; minimal exposure of the on-site workers to the contaminant; longtime protection of public health; and possible reduction in the duration of the remedial process (Okoh and Trejo-Hernandez, 2006). These advantages of the bioremediation systems over the other technologies have been summarized (Leavin and Gealt, 1993) as follows: can be done on site i.e. *in situ* application; keeps site destruction to a minimum; eliminates transportation costs and liabilities; eliminates long-term liability; biological systems are involved, hence often less expensive; and can be coupled with other treatment techniques to form a treatment train. There are three classifications of bioremediation according to Hornung, (1997):

Biotransformation - the alteration of contaminant molecules into less or nonhazardous molecules

Biodegradation - the breakdown of organic substances in smaller organic or inorganic molecules

Mineralization - is the complete biodegradation of organic materials into inorganic constituents such as CO_2 or H_2O.

There are three general approaches to cleaning up contaminated soils:(i) Soil can be excavated from the ground and be either treated or disposed of (Ex-situ treatment), (ii) Soil can be left in the ground and treated in place (in-situ treatment), or (iii) soil can be left in the ground and contained to prevent the contamination from becoming more widespread and reaching plants, animals, or humans (containment and intrinsic remediation), (Jim et al., 2005).

2.8.6.1 In-situ bioremediation technologies

In situ bioremediation (ISB) is the use of microorganisms to degrade contaminants in place with the goal of obtaining harmless chemicals as end products (Jim et al., 2005). Most often *in situ* bioremediation is applied to the degradation of contaminants in saturated soils and groundwater. Examples of different ISB technologies are shown in Table 2.3. The technology was developed as a less costly, more effective alternative to the standard pump-and-treat methods used to clean up aquifers and soils contaminated with chlorinated solvents, fuel hydrocarbons, explosives, nitrates, and toxic metals. ISB has the potential to provide advantages such as complete destruction of the contaminant(s), lower risk to site workers, and lower equipment/operating costs (US EPA, 1997).

In situ bioremediation technology is highly dependent upon external conditions, which is the key to determining whether bioremediation can be performed in situ. The conditions of greatest importance are the physicochemical and chemical conditions that exist in the contaminated soil. These conditions includes dissolved oxygen for aerobic processes, moisture content, pH, nutrient availability especially with regard to nitrogen and

phosphorus, temperature, soil composition and concentration of contaminants (Jim et al., 2005).

These techniques are generally the most desirable options due to lower cost and fewer disturbances since they provide the treatment in place by avoiding excavation and transport of contaminants. *In situ* treatment is limited by the depth of the soil that can be effectively treated. In many soils effective oxygen diffusion for desirable rates of bioremediation extend to a range of only a few centimeters to about 30 cm into the soil, although depths of 60 cm and greater have been effectively treated in some cases (Vidali, 2001).

Accelerated *in situ* bioremediation is where substrate or nutrients are added to an aquifer to stimulate the growth of a target consortium of bacteria. Usually the target bacteria are indigenous; however enriched cultures of bacteria (from other sites) that are highly efficient at degrading a particular contaminant can be introduced into the aquifer (bioaugmentation). Accelerated ISB is used where it is desired to increase the rate of contaminant biotransformation, which may be limited by lack of required nutrients, electron donor or electron acceptor. The type of amendment required depends on the target metabolism for the contaminant of interest. Aerobic ISB may only require the addition of oxygen, while anaerobic ISB often requires the addition of both an electron donor (e.g., lactate, benzoate) as well as an electron acceptor (e.g., nitrate, sulfate). Chlorinated solvents, in particular, often require the addition of a carbon substrate to stimulate reductive dechlorination. The goal of accelerated ISB is to increase the biomass throughout the contaminated volume of aquifer, thereby achieving effective biodegradation of dissolved and sorbed contaminant

(Wiedemeier et al., 1998). Accelerated in situ bioremediation can be carried out in two ways: biostimulation and bioaugmentation.

Table 2.3 Examples of in situ bioremediation technologies for treating contaminated soil

Method	Description	Cost	Contaminants treated	Reference
Intrinsic Mulligan remediation/ 2004; Monitored natural 2004 attenuation	Relies on natural subsurface processes, it includes monitoring of the site.	Depends on Duration of monitoring	Benzene, toluene,ethyl benzene & xylene (BTEX), chlorinated and petroleum hydrocarbons	Renner, 1998; and Yong, Salminen et al.,
Biosparging Breedveld,	Oxygen/air is added below groundwater surface to stimulate microbial activity and degradation	50 – 110€/ton	Organic contaminants	Doelman and 1999
Bioventing EPA, 2005	Oxygen/air is added to soil vapour phase to sti--mulate aerobic degradation	25 - 120€/ton	Petroleum hydrocarbons, nonchlorinated solvent.	FRTR, 2005;
Enhanced Breedveld Bioremediation/ Bioresaturation	Carbon sources and/or nutrients and/or electron acceptors and/or fungi/bacteria (bioaugmentation) are added through injection wells or by spraying, depending on required soil depth.	15-160 €/ton	Petroleum hydrocarbons, solvents, pesticides, wood preservatives & other organic chemicals as well as munition	Doelman and 1999;
Phytoremediation 2000	Plants are used to remove, transfer, stabilize and destroy contaminants in soil and sediments	depends on method	Organic or inorganic contaminants	Adams et al.,
Chemical oxidation and EPA, 2005	Hazardous contaminants are oxidized to non-hazardous or less toxic compounds	70-400€/ton	Many toxic organic chemicals	FRTR,2005
Chemical reduction	Reduction of contaminants by zero-valent iron Powder or sodium polythiocarbonate		Chlorinated solvents and metals	EPA, 2005

Table 2.4 Cont'd

Soil flushing EPA 2005	Contaminants are extracted from soil with water or other suitable aqueous solutions.	19-190$/m^3	Volatile organic compounds (VOCs) and semivolatile (SVOCs)	FRTR, 2005;
Soil vapor EPA 2005 Extraction/Dual	Vacuum is applied to unsaturated soil to induce the controlled flow of air & to remove contaminants	15-160€/ton	VOCs and some SVOCs, and some fuels	FRTR, 2005;
Thermal treatment	Soil is heated with warmed gas, with electric current or electromagnet	30-130$/m^3	VOCs and some SVOCs, some pesticides and fuels	FRTR, 2005
Solidification	Physically bounding or enclosing contaminants within stabilized mass	50-130€/ton	Inorganic and some organic contaminants	FRTR, 2005
Stabilisation EPA 2005	Added stabilizing agent reacts chemically with Contaminants and reduces their mobility	50-130€/ton	Inorganic and some organic contaminants	FRTR, 2005;

2.8.6.2 Biostimulation

The most widely used bioremediation procedure is the biostimulation of indigenous microorganisms by the addition of nutrients because the input of large quantities of carbon sources tends to result in a rapid depletion of the available pools of major inorganic nutrients such as N and P. Levels of N and P added to stimulate biodegradation at contaminated sites are often estimated from C/N ratios (Sang-Hwan et al., 2007).

Biostimulation aims at enhancing the activities of indigenous microorganisms that are capable of degrading pollutant from soil environment, it is often been applied to the bioremediation of oil-contaminated soil. Addition of inorganic nutrients do act as fertilizer to stimulate biodegradation by autochthonous microorganisms in some cases (Avakian, 2004); in other cases, it is the intentional stimulation of resident xenobiotic-degrading bacteria by use of electron acceptors, water, nutrient addition, or electron donors (Widada, et al., 2002). Combinations of inorganic nutrients often are more effective than single nutrients (Sutherland, et al., 2000). Laboratory-based respiration experiments by Liebeg and Cutright (1995) showed that a low level of macronutrients and a high level of micronutrients were required to stimulate the activities of indigenous microbes. The greatest stimulation was recorded with a solution consisting of 75% sulphur, 3% N and 11% P.

Nitrogen is the most commonly used nutrient in bioremediation project Liebeg and Cutright (1995). It is used primarily to support biosynthesis (NH_4^+ and NO_3^-) or as an alternative electron acceptor to oxygen (NO_3^-). Activated sludge has been suggested to be a useful

source of N for PAH biodegradation in soils (Juteau et al., 2003). Dried blood acts as a slow release agent of nitrogen (Straube et al., 2003), as well as a range of natural materials such as peat, compost and manure (Moorman et al., 2001).

2.8.6.3 Composting bioremediation

Biostimulation can also be achieved by the use of composting bioremediation technologies. Composting bioremediation strategy relies on mixing the primary ingredients of composting with the contaminated soil, wherein as the compost matures, the pollutants are degraded by the active microflora within the mixture (Semple et al., 2001). A large variety of organic amendments has been used in composting bioremediation. Many are based on the application of manure, from cows, pigs or chickens (WS Atkins Environment, 2002; Ijah and Antai, 2003a; Atagana et al., 2003; Sasek, 2003; Adesodun and Mbagwu, 2008). Adriana et al., (2007) recorded 63% TPH removal in soil contaminated with petroleum hydrocarbon and amended with raw coffee beans. Sewage sludge is abundant globally, and it has been successfully used as an amendment in composting bioremediation (Hur and Park, 2003). Virtually any putrescible material available can be used, such as vegetable (Atagana et al., 2003), spent mushroom compost (SMC) (Eggen, 1999; Lau et al., 2003), and even garden waste (Michel et al., 2001; Guerin, 2001a; Guerin, 2001b). The use of composting approaches to bioremediation of organic pollutants generally (Semple et al., 2001) and specifically the use of composting to treat PAHs (Antizar-Ladislao et al., 2004) have been reviewed. The use of SMC is an interesting case. A fascinating feature of SMC is that it may contain relatively abundance of extracellular ligninolytic fungal enzymes (Lau et al., 2003), which are relatively nonspecific in their substrate preference. Hence they

assist in the biodegradation of aromatic molecules such as PAHs, giving SMC an additional role in composting bioremediation.

Composting is an efficient method that relies on added matrix material and on mixing/aeration, but not on addition of microbial inoculum (Jørgensen et al., 2000). 70% mineral oil biodegradation was recorded by Jørgensen et al., (2000), when they use bark chips as a bulking agent for composting lubricating oil-contaminated oil in a field scale study within the period of five months. Abioye et al., (2009a) recorded 75% loss of oil in soil contaminated with crude oil and composted with melon shells within the period of 28 days.

Organic wastes like BS, SMC and BSG in earlier studies were found to enhance the biodegradation of used lubricating oil up to 90% loss of oil within the period of 3 months (Abioye et al., 2009b, 2010).

Composting has been demonstrated to be effective in biodegradation of PAHs at both laboratory and field scales using different types of compost bulking agents such as spent mushroom (Lau et al., 2003), soot waste (Moretto et al., 2005), green wastes (Antizar-Ladislao et al., 2005a) and maple leaves and alfalfa (Haderlein et al., 2006). Lau et al., (2003) used SMC (waste by-product of the mushroom industry) as a bulking agent (5%) to bioremediate PAH-contaminated soil. Complete degradation of individual naphthalene, phenanthrene, benzo[a]pyrene and benzo[g,h,i]perylene was observed in 48 h at 80^0C. Also, Siu-Wai et al., (2009) reported the removal of spilled petroleum in industrial soil amended with SMC of *Pleurotus pulmonarius,* they observed that removal of petroleum by 3% SMC amendment applied twice accounted for 56-64%, 31-33% and 51-54%

disappearance of the TPH, oil and grease and di(2-ethylhexyl) phthalate contaminants respectively, in 22 days.

Haderlein et al., (2006) studied the effects of composting or simple addition of compost to soil during the mineralization of pyrene and benzo[a]pyrene by addition of maple leaves and alfalfa. It was reported that neither composting nor the addition of compost had any effect on benzo[a]pyrene mineralization. In contrast, the pyrene mineralization rate increased dramatically with the amount of time that the soil had been composted (more than 60% mineralization after 20 days). Antizar-Ladislao et al., (2005b, 2006) used in-vessel composting technology for the remediation of coal tar contaminated soil and optimized the soil composting temperature at 38^0C for the most effective degradation. In a related study, solid culture with small amount of low-quality raw coffee beans was used for total petroleum hydrocarbon removal from a weathered and polluted soil (Adriana et al., 2007). The author recorded 63% TPH removal in soil amended with coffee bean within 15 days. Amendment of soil contaminated by heavy mineral oil using sawdust, hay and compost was reported by Sang-Hwan et al., (2008) that after 105 days of experiment the heavy mineral oil were reduced by 18 - 40% from the initial level of contamination of 7490 mg hydrocarbon kg^{-1}, whereas the level of hydrocarbon reduction in non-amended soil was just 9%. The author also observed significantly higher microbial activities in compost amended contaminated soil. Corn and sugar cane residues were reported to stimulate the biodegradation of diesel oil in diesel-contaminated soil by 67% (Molina et al., 2004). Ijah et al., (2008) also observed that increase in biodegradation of crude oil in crude oil contaminated soil amended with chicken droppings. They reported 75% of crude oil degradation in soil amended with chicken droppings while only 56.3% degradation was recorded in unamended polluted soil within the 10 weeks of the experiment.

2.8.6.4 Bioaugmentation

Bioaugmentation is the introduction of microorganisms with specific catabolic abilities into the contaminated environment in order to supplement the indigenous population and to speed up or enable the degradation of pollutants (Perelo, 2010). It was described by Alexander, (2001) as the inoculation of contaminated soil or water with specific strains or consortia of microorganisms to improve the biodegradation capacity of the system for a specific pollutant organic compound(s). Bioaugmentation strategies may prove successful especially in the remediation of man-made contaminants, where specialized bacteria with the appropriate catabolic pathways may not be present in the contaminated habitat (Perelo, 2010).

Bioaugmentation is a promising and low-cost bioremediation method in which an effective bacterial isolate(s) or microbial consortium capable of degrading xenobiotics is administered to contaminated sites (Gentry et al., 2004). The number of petroleum-degrading microbial isolates available for bioaugmentation is increasing (Van Hamme et al., 2003; Singer et al., 2005). However, the soil environment is very complicated and the degrading ability of exogenously added microorganisms tends to be affected by the physicochemical and biological features of the soil environment. Sometimes, the administration of petroleum degrading microorganisms leads to a failure of bioaugmentation (Vogel 1996; Gentry et al., 2004).

There are three fundamental approaches to bioaugmentation of a contaminated site. The first is to increase the genetic diversity by inoculation with allochthonous microorganisms (Jim and Atlas, 2005). By increasing the genetic diversity of the soil or water, it is assumed

that this increases the catabolic potential and thereby the rate of removal of the contaminant(s) by biodegradation will increase (Dejonghe, et al., 2001). The second is to take samples from the site and use them as initial inocula for serial enrichments with the contaminant(s) in question as the sole source of carbon, this inoculums is then returned to the site in large numbers in order to increase the rate of biodegradation. (Figure 2.9). The third approach involves the addition of uncharacterized consortia present in materials such as sewage sludge, garden waste and compost (Jim and Atlas, 2005).

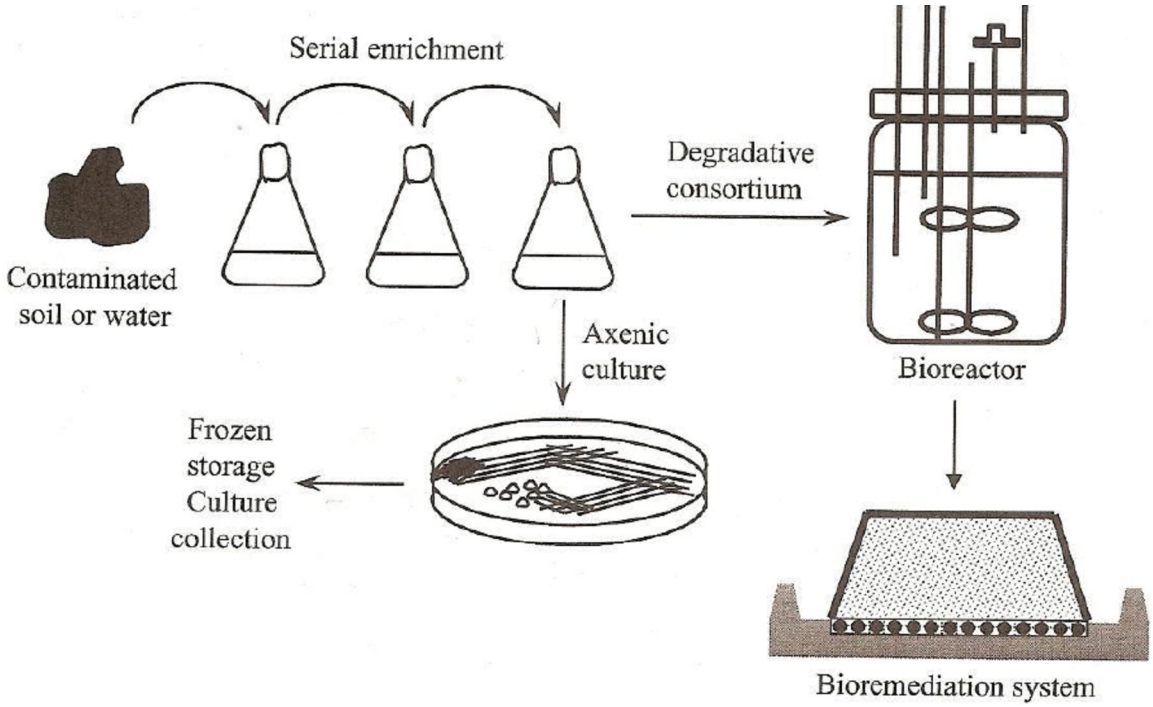

Figure 2.9 Typical serial enrichment procedures for bioaugmentation (Jim and Atlas, 2005)

According to literature, bioaugmentation technology has mostly been used for the degradation of pure compounds (Gray et al., 2000). The mineralization of high concentrations of phenanthrene has been reported when successive inoculations were tested (Schwartz and Scow, 2001). According to the success of these results, it has been reported that the knowledge of new strains could be of interest to accelerate the remediation of zones

polluted with high concentrations of hydrocarbons. Most bioaugmentation studies have been carried out using filamentous fungi inoculated into model soil systems and using contaminants of low molecular weight PAHs with up to four rings (D'Annibale et al., 2006). The interest of these microorganisms is their ability to synthesize relatively unspecific enzymes involved in cellulose and lignin decay that can degrade high molecular weight, complex and more recalcitrant toxic compounds, including aromatic structures (Colombo et al., 1996). For the breakdown of complex aromatic structures, fungi–bacteria consortia are preferred due to the successful results reported. For example, the consortium comprising *S. maltophilia* - *P. janthinellum* degraded 44–80% of a chrysene, benzo[a]anthracene, benz[a]- pyrene and dibenz[a,h]anthracene mixture, in 100 days (Boonchan et al., 2000).

Bagherzadeh et al., (2008) evaluated the efficiency of pollutant removal by selected microorganisms and reported thus: Five mixed cultures and 3 single bacteria strains, *Pseudomonas* sp., *Arthrobacter* sp. and *Mycobacterium* sp. were isolated from hydrocarbon-contaminated soils by enrichment on either crude oil or individual hydrocarbons, as the sole carbon sources. The strains were selected based on their ability to grow in medium containing crude oil, used engine oil or both. Their ability to degrade hydrocarbon contamination in the environment was investigated using soil samples contaminated with used engine oil. The mixed starter culture #1 degraded 66 % of aliphatic compounds in the engine oil, after 60 days of incubation. The mixed starter culture #5 removed 47 % of aromatic compounds during 60 days of incubation. Bento et al. (2005) reported 72.7% light TPH fraction and 75.2% heavy TPH fraction degradation in diesel

contaminated soil bioaugmented with bacterial consortium of *Bacillus cereus, Bacillus sphaericus, Bacillus fusiformis, Bacillus pumilus Acinetobacter junii* and *Pseudomonas* sp. Ying et al. (2010) augmented a PAH-contaminated soil with *Paracoccus* sp. strain HPD-2 and observed 23.2% decrease in soil total PAH concentrations after 28 days, with a decline in average concentration from 9942 to 7638 μg kg^{-1} dry soil. They discovered percentage degradation of 3-, 4- and 5(+6)-ring PAHs was 35.1%, 20.7% and 24.3%, respectively.

2.10 Kinetic of Biodegradation Process

The kinetics for modelling the bioremediation of contaminated soils can be extremely complicated. This is largely due to the fact that the primary function of microbial metabolism is not for the remediation of environmental contaminants (Maletic et al., 2009). Instead the primary metabolic function, whether bacterial or fungal in nature, is to grow and sustain more of the microorganism. Therefore, the formulation of a kinetic model must start with the active biomass and factors, such as supplemental nutrients, oxygen source, that are necessary for subsequent biomass growth (Cutright, 1995; Rončević et al., 2005; Pala et al., 2006).

Studies of the kinetics of the bioremediation process proceed in two directions: (1) the first is concerned with the factors influencing the amount of transformed compounds with time and (2) the other approach seeks the types of curves describing the transformation and determines which of them fits the degradation of the given compounds by the microbiological culture in the laboratory microcosm and sometimes, in the field (Maletic et al., 2009). A literature survey has shown that studies of biodegradation kinetics in the natural environment are often empiric, reflecting only a basic level of knowledge about the

microbiological population and its activity in a given environment. One of such kinetic model is

$y = ae^{-kt}$ (Yeung et al., 1997)

This kinetic model was used in this present study. Where y is the residual hydrocarbon content in soil (g kg^{-1}), a is the initial hydrocarbon content in soil (g kg^{-1}), k is the biodegradation rate constant (d^{-1}) and t is time (d). The model estimated the biodegradation rate and half-life of hydrocarbons in soil relative to treatments applied. Half-life was then calculated from the model of Yeung et al., (1997) as

$$\text{Half life} = \ln(2)/k$$

This model was based on the assumption that the degradation rate of hydrocarbons positively correlated with the hydrocarbon pool size in soil (Yeung et al., 1997). Another kinetic model often used in to determine rate of biodegradation of contaminants from soil is

$$\frac{dC}{dt} = kC^n$$

where C is the concentration of the substrate, t is time, k is the rate constant of the compound degradation and n is a fitting parameter (mostly taken to be unity) (Hamaker, 1972). Using this model, one can fit the curve of substrate removal by varying n and k until a satisfactory fit is obtained. It is evident from this equation that the rate is proportional to the exponent of substrate concentration. Researchers involved in kinetic studies do not always report whether the model they used was based on theory or experience and whether the constants in the equation have a physical meaning or if they just serve as fitting parameters (Bazin et al., 1976).

In the simple model, depending on the nature of the substrate and experimental conditions, various investigators obtain different values for the rate constant of substrate degradation: for n-alkanes, 0.14 to 0.61 day^{-1} (Holder et al., 1999); for crude oil, 0.0051 to 0.0074 day^{-1} (Seabra et al., 1999); and for PAHs, 0.01 to 0.14 day^{-1} (Hinga, 2003; Holder et al., 1999; Winningham et al., 1999). Reported rates for the degradation of hydrocarbon compounds under field or field-simulated conditions differ by up to two orders of magnitude. Selection of the appropriate kinetics and rate constants is essential for accurate predictions or reconstructions of the concentrations of hydrocarbons with time in soil after a spill (Hinga, 2003).

Kinetic constants are important design parameters to determine the degradation of a substrate in biological treatment systems. The rate of petroleum hydrocarbons degradation depends on numerous factors. Remediation time can be roughly determined from the degradation step of hydrocarbons in the contaminated soil samples. A number of experimental studies have shown that biodegradation kinetics can be approximated with first order kinetics (Heitkamp et al., 1987; Heitkamp and Cerniglia, 1987; Venosa et al., 1996; Seabra et al., 1999; Holder et al., 1999; Winningham et al., 1999; Namkoonga et al., 2002; Grossi et al., 2002; Hohener et al., 2003; Collina et al., 2005; Rončević et al., 2005). First order kinetics, such as the well known Michaelis–Menten kinetic model, are the most often used equation for representation of the degradation kinetics (Namkoonga et al., 2002; Grossi et al., 2002; Hohener et al., 2003; Collina et al., 2005; Rončević et al., 2005; Pala et al., 2006).

Few works have been dedicated to investigate the kinetics of soil bioremediation (Hutchins et al., 1991; Alexande,r 1999; Greene et al., 2000; Reardon et al., 2002; Hwang et al., 2001; Antizar-Ladislao et al., 2005b; Li et al., 2006). Information on kinetics is extremely important because it characterizes the concentration of the chemical remaining at any time and permits prediction of the levels likely to be present at some future time. First-order kinetics is commonly used to describe biodegradation in environmental fate models because mathematically the expression can be incorporated easily into the models (Greene et al., 2000). In different environments, first-order constants and the number of cells able to metabolize the substrate will differ (Alexander, 1999; Greene et al., 2000).

Hwang et al., (2001) investigated the bioremediation of diesel-contaminated using composting techniques. The results of the applied first order kinetics model agreed to a great extent with the experimental results. They found that the average first order kinetic rate constant of diesel oil was 0.099/day. Antizar- Ladislao et al., (2005b) have studied the biodegradation of 16 polycyclic aromatic hydrocarbons using laboratory scale in-vessel composting at different temperatures. The degradation took place in mixed culture of bacteria, fungi, and actinomycetes. They found out that the first order kinetics can satisfactorily describe bioremediation process and the first order kinetic constant for all contaminates ranged between 0.009/ day at 70°C and 0.013/day at 38°C. Li et al., (2006) studied the biodegradation of diesel contaminated soil by an isolated bacterial genus Planococcus. They used a Luong model to describe the bioreaction kinetics. The kinetic model was solved to obtain a maximum growth rate $\mu max=0.34/h$ and saturation concentration Ks=0.041 mM/l.

2.11 Toxicity in contaminated soil

Petroleum hydrocarbons released into the environment can pose risk to ecosystems and human health. Some compounds in petroleum products are known to be mutagenic and carcinogenic. Extensive chemical extraction and analysis of petroleum contaminated soil can provide detailed information about the total contaminant concentration. However, the potential impact on the ecosystem may not be easily predicted using only concentration data (Banks and Schultz, 2005). The use of bioassays for ecotoxicity evaluation of contaminated soil has gained widespread attention over the past twenty years. Bioassays have clearly demonstrated that chemical analysis alone is not adequate to assess the potential ecological impact of contaminated soil. These tests have been shown to be useful particularly when predicting the effect of a complex mixture of compounds, such as petroleum (Banks and Schultz, 2005).

Due to the complexity of soil ecosystems, the impacts of pollutants vary and range from direct toxicity symptoms to effects on reproduction of organisms and indirect effects to predator-prey relationships as well as changes in landscape. Pollutants impact on all levels: organisms, population, community, ecosystem and landscape level (Edwards, 2002). Soil ecotoxicity tests were developed in order to determine the toxicological impacts of chemicals on ecological receptors, such as bacteria, earthworm and plants (Saterbak et al., 1999). Toxicity of soil can be determined directly from the soil, from the leachate produced in a soil leaching test or from soil extracts.

Since 1980s, phytotoxicity tests have been required by environmental legislations and included as parts of the guidelines developed by different authorities for environmental monitoring and assessment (European Chemicals Bureau 1992; US EPA 1996; Organization for Economic Cooperation and Development 2002). Several solid-phase or

terrestrial bioassays with plants and soil animals have been used for evaluating soil site contamination. Widely used soil animal tests for assessing soil quality are earthworms and enchytraeid worm assays; as well as plants test, e.g. seed germination, root elongation and early seedling growth bioassay (Dorn and Salanitro, 2000).

Lettuce is an important agricultural crop and is fairly sensitive to toxic chemicals, which led to the widespread use of *Lactuca sativa* L. for toxicity tests (US EPA, 1994). Other plants have been used, but there is no consensus of the most effective plant for germination testing in petroleum contaminated soil. Plant species usually recommended for this assessment have been chosen based on ease of seed handling (larger seeds are preferred) and germination rate (Banks and Schultz, 2005).

2.12 Phytoremediation of hydrocarbon-contaminated soil

Phytoremediation is remediation method which utilizes plants to remove, contain or detoxify environmental contaminants (Palmroth, 2006). Phytoremediation of contaminated soils offers an environmentally friendly, cost effective, and carbon neutral approach for the cleanup of toxic pollutants in the environment (Dowling and Doty, 2009). Phytoremediation appears attractive because in contrast to most other remediation technologies, it is not invasive and, in principle, delivers intact, biologically active soil (Wenzel, 2009). It has now emerged as a promising strategy for in situ removal of many contaminants (Susarla et al., 2002; Pulford and Watson, 2003; Greenberg, 2006; Pilon-Smits, 2005). Some major advantages and disadvantages of phytoremediation are shown in Table 2.4.

Microbe-assisted phytoremediation, including rhizoremediation, appears to be particularly effective for removal and/or degradation of organic contaminants from impacted soils,

particularly when used in conjunction with appropriate agronomic techniques (Zhuang et al., 2007; Gerhardt et al., 2006). Variety of pollutant attenuation mechanisms possessed by plants makes their use in remediating contaminated land and water more feasible than physical and chemical remediation (Greenberg, 2006; Gerhardt et al., 2009). An estimated 350 species of plants naturally take up toxic materials from the environment (Thieman and Palladino, 2009). The most common plant species used in phytoremediation of organic compounds includes willows, poplar and different types of grasses. Comprehensive list of plants that has proved positive in phytoremediation of organic compounds are listed in Table 2.5.

Major drawbacks of phytoremediation include the fact that the detoxification of organic pollutants is often slow and if decomposition is not complete, toxic compounds may accumulate in plant tissue and can be released to the environment or enter food-chains (Aken, 2008).

Table 2.4 Advantages and disadvantages of phytoremediation over traditional technologies

Advantages	Disadvantages
Relatively low cost	Longer remediation time
Easily implemented and maintained	Climate dependent
Several mechanisms for removal	Effects to food web might be unknown
Environmentally friendly	Ultimate contaminant fate might be unknown
Aesthetically pleasing	Results are variable
Reduces landfilled wastes	
Harvestable plant materials	
Costs 10 – 20% of mechanical treatments	Slower than mechanical treatments
Faster than natural attenuation	Only effective for moderately hydrophobic compounds
High public acceptance	Toxicity and bioavailability of biodegradation products is not known.
Fewer air and water emissions	Contaminants may be mobilized into the ground water
Conserves natural resources	Influenced by soil and climate conditions of the site.

(Susarla et al., 2002; Kamath, et al., 2004)

Establishment of a vegetative cover on contaminated sites can retain contaminants in place thereby reducing losses via erosion and percolation through soil profile (Pulford and Watson, 2003). Coupling of phytoremediation of contaminated soil with soil amendments such as organic matter, compost, phosphate, fertilizers, Fe, Mn oxyhydroxides and clay minerals usually reduce the mobility of contaminants in soil (Mench et al., 2000; Madejon et al., 2006).

2.12.1 Methods of phytoremediation application

Phytoremediation application can be carried out by three different methods which are: i. *In situ phytoremediation* ii. *In-vivo phytoremediation with relocated contaminants* iii. *In-vitro phytoremediation*

i. **In situ phytoremediation**

In situ phytoremediation involves placement of live plants in contaminated surface water, soil or sediment that is contaminated, or in soil or sediment that is in contact with contaminated ground water for the purpose of remediation. In this approach, the contaminated material is not removed prior to phytoremediation. If the phyto-mechanism consists of only uptake and accumulation as opposed to transformation of a contaminant, the plants may be harvested and removed from the site after remediation for disposal or recovery of the contaminants. A requirement of the in-situ approach is that the contaminant must be physically accessible to the roots. This approach generally is the least expensive phytoremediation strategy (Susarla et al., 2002).

ii. **In-vivo phytoremediation with relocated contaminants**

For sites where the contaminant is not accessible to the plants, such as contaminants in deep aquifers, an alternate method of applying phytoremediation is possible. In this approach the contaminant is extracted using mechanical means, then it is transferred to a temporary treatment area where it can be exposed to plants selected for optimal phytoremediation. After treatment, the cleansed water or soil can be returned to its original location and the plants may be harvested for disposal if necessary. This approach generally is usually more

expensive than the in-situ phytoremediation. Treatment could occur either at the site of contamination or at another location (Susarla et al., 2002).

iii. **In-vitro phytoremediation**

This third method of phytoremediation application is usually via components of live plants, such as extracted enzymes. In theory, this approach could be applied in situ under some situations, e.g. applying plant extracts to a contaminated pond or wetland, or through use of an enzyme impregnated porous barrier in a contaminated ground water plume. This approach could also be applied to contaminated material that has been relocated to a temporary treatment area. Theoretically, this approach is the most expensive method of phytoremediation because of the costs of preparing/extracting the plant enzymes; however, in some plants, such as tarragon, (*Artemisia dracunculas* var *satiya*), exudates are released under stress that could result in reduced production costs (Susarla et al., 2002).

Table 2.5 Examples of plants used for phytoremediation of organic contaminants

Plant used	Contaminants	Results	Reference
Jatropha curcas	Coal fly ash, lead, cadmium, arsenic and chromium	Enhanced heavy metals uptake by 117% in root, 62% in stem and 86% in leaves when EDTA was applied at 0.3g/kg to fly ash. Jatropha accumulated Cd and Pb in the shoot. It shows increase bioaccumulation potential of As and Cr with increase in metal concentration in soil system.	Santosh et al., (2009) Jamil et al., (2009) Mangkoedihardjo and Surahmaida (2008)
Carex exigua, Panicum virgatum Tripsacum dactyloides	Petroleum hydrocarbons	70% loss of total petroleum hydrocarbons was recorded after one year growth of these plants in contaminated soil.	Euliss et al., (2008)
Vicia faba	Crude petroleum oil	47% of total petroleum hydrocarbon was degraded in 60 days.	Diab (2008)
Populus tremula	Cadmium and Zinc	Both Cd and Zn accumulated in the leaves with maximum foliar concentration of 35 and 2400mg/kg	Hassinen et al., (2009)
Ditch reed and Alfalfa	Liquid bitumen agar (mainly paraffins & naphtenes) 70.9g/kg and soil containing PAHs 80mg/kg	82% removal was achieved in 27 months with both plants.	Muratova et al., (2003)
Tall fescue	PAHs in creosote contaminated soil.	Removal of acenaphtene and fluorine in 36 months was slightly higher in the presence of tall fescue than in unvegetated soil.	Robinson et al., (2003)
Rye grass and Sweet clover	Aged PAHs from manufacture gas plant.	PAHs removal in 12 months was higher in the presence of plants, 9% to 24% compared to 5% without plant.	Parish et al., (2004)

2.12.2 Enhancement of Phytoremediation

On-site phytoremediation of petroleum hydrocarbons can be enhanced by employing a combination of common agronomic practices (e.g. fertilizer application, tillage and irrigation), this is because available nutrient reserves can be quickly depleted as the microbial community begins to degrade the contaminants (Farrell and Germida, 2002). Therefore, fertilizer applications may enhance the degradation of petroleum hydrocarbons in soil by reducing competition for limited nutrients. Cutright (1995) found that increasing the amount of N and P in soil under aerobic conditions increased the degradation of PAHs by the soil fungus *Cunninghamella echinulata* var. *elegans.* Loss of 2- and 3- ring from soil contaminated with weathered petroleum compounds also was more rapid when the soil was amended with sludge compost high in nitrogen compared to no amendment or low nitrogen amendment. Palmroth et al., (2002) recorded 60% loss of diesel fuel in 30 days in diesel-contaminated soil planted with pine tree and amended with NPK fertilizer. Also, Vouillamoz and Milke (2009) observed that compost addition combined with phytoremediation, increases the rate of removal of diesel fuel in soil.

In another related experiment, Lin and Mendelssohn (1998) discovered that fertilizer applications enhanced both the establishment and growth of *Spartina alterniflora* and *S. patens* transplanted into crude oil contaminated soil and degradation of the crude oil was more pronounced in the fertilized soil compared to unfertilized control soil. Amadi et al., (1993) reported that the addition of poultry manure to soil contaminated with crude oil had a positive effect on the growth of maize compared to contaminated soil without manure supplements. Green manure crops typically nitrogen fixing, legumes incorporated into soil

to improve soil fertility can also be used to provide nitrogen at contaminated sites and in doing so may enhance phytoremediation efforts (Biederbeck et al., 1996).

Tillage is another agricultural practice that provides proper aeration for the soil microflora. Incorporation of readily decomposed organic matter into the soil do improve aeration of the soil. The beneficial effects of tillage may then lead to enhanced biological activity and biodegradation efficiency in the soil. Thus, proper tillage practices may play an important role in maximizing the phytoremediation potential of plant systems in contaminated soils (Loehr and Webster, 1996; Atlas and Bartha, 1998).

2.12.3 Factors affecting phytoremediation

Different factors normally affect phytoremediation process, some of these factors includes:

1. **Bioavailability**

Bioavailability of contaminants to the plant root is one of the important factors affecting phytoremediation of contaminated soil. For plants and their associated microbes to remediate pollutants, they must be in contact with them and able to act on them. Pollutant bioavailability depends on the chemical properties of the pollutant, soil properties, environmental conditions, and biological activity (Pilon-Smits, 2005). Sand does not bind molecules as readily as silt or clay, so the bioavailability of hydrocarbons is higher in sandy soils (Edwards et al., 1982). The higher hydrocarbon bioavailability and hydraulic conductivity of sand means that spills on sandy soils are more likely to result in ground water contamination than spills on heavier textured soils. Organic matter and clay tend to bind lipophilic compounds, decreasing bioavailability of this material to plants, although not necessarily to soil microorganisms (Leahy and Colwell, 1990; Otten et al., 1997).

Plants require different soil textures and organic matter contents for optimal germination and growth. When screening plants for phytoremediation, those species naturally adapted to the soil texture at the contaminated site will likely be more successful than those adapted to different soil textures. Clay and organic matter content also affects microbial populations via their ability to form soil aggregates (Paul and Clark, 1989).

The oxidation state of an element may affect its bioavailability (e.g., its solubility), its ability to be taken up by plants, as well as its toxicity. Other physical conditions that affect pollutant migration and bioavailability are temperature and moisture. Higher temperatures accelerate physical, chemical, and biological processes in general. Precipitation will stimulate general plant growth, and higher soil moisture will increase migration of water soluble pollutants (Pilon-Smits, 2005). In polluted soils the more bioavailable (fraction of) pollutants tend to decrease in concentration over time due to physical, chemical, and biological processes, leaving the less or nonbioavailable (fraction of) pollutants. Consequently, pollutants in aged polluted soils tend to be less bioavailable and more recalcitrant than pollutants in soil that is newly contaminated, making aged soils more difficult to phytoremediate (Olson et al., 2003).

2. **Weather**

Phytoremediation might be best suited for tropical countries where plant growth occurs all year round. In temperate climates, the active contribution of phytoremediation is restricted to the growing period only. Winter operations may pose problems for phytoremediation when deciduous vegetation loses its leaves, transformation and uptake cease, and soil water

is no longer transpired. However, a combination of grasses can be used to prolong the growing period.

3. **Depth of Contamination**

Phytoremediation is most effective at sites with shallow (i.e., root accessible) contaminated soils where contaminants can be treated in the rhizosphere and/or by plant uptake (Kamath, 2004). Roots of phreatophytic trees can be expected to grow at least 3 meters into a soil profile, and it is possible to encourage rooting to a depth of 5 meters or more using the tree-in-a-well concept (Kamath, 2004). On the other hand, roots of some grasses (alfalfa, switchgrass, tall fescue) can reach soil depths of only 0.25-0.4 m. Buffelgrass roots to a depth of 0.75 m but has been observed to have dense rooting pattern within 0.3 m from the topsoil layer. Hawaiian plants, Milo and Kou which were used to remediate saline soils contaminated with TPHs, rooted to a depth of more than 1.5 m by growing through the brackish water table into a zone of concentrated contaminants (US Army corps of Engineer, 2003)

4. **Chelation and Compartmentation in Roots**

Plants can release compounds from their roots that affect pollutant solubility and uptake by the plant. Inside plant tissues such chelator compounds also play a role in tolerance, sequestration, and transport of inorganics and organics (Ross, 1994). Phytosiderophores are chelators that facilitate uptake of Fe and perhaps other metals in grasses; they are biosynthesized from nicotianamine, which is composed of three methionines coupled via nonpeptide bonds (Higuchi et al., 1999). Chelation in roots can affect phytoremediation efficiency as it may facilitate root sequestration, translocation, and/or tolerance. Root

sequestration may be desirable for phytostabilization whereas export to xylem is desirable for phytoextraction.

2.13 Mechanisms of Phytoremediation

Variety of pollutant attenuation mechanisms possessed by plants makes their use in remediating contaminated land and water more feasible than physical and chemical remediation (Glick, 2003; Huang et al., 2004, 2005; Greenberg, 2006; Gerhardt et al., 2009). As a result of their sedentary nature, plants have evolved diverse abilities for dealing with toxic compounds in their environment. Plants act as solar-driven pumping and filtering systems as they take up contaminants (mainly water soluble) through their roots and transport/translocate them through various plant tissues where they can be metabolized, sequestered, or volatilized (Greenberg et al., 2006; Abhilash, 2009). Plants utilizes different types of mechanisms for dealing with environmental pollutants in soil, some of this mechanisms/strategies are shown in Figure 2.10 as described by Abhilash et al., (2009). The mechanisms of phytoremediation include biophysical and biochemical processes like adsorption, transport and translocation, as well as transformation and mineralization by plant enzymes (Meagher, 2000). Plants have been shown to be able to degrade halogenated compounds like trichloroethylene (TCE) by oxidative degradation pathways, including plant specific dehalogenases (Nzengung et al., 1999). Dehalogenase activity was observed to be maintained after the plants death. Enzymes can become bound to the organic matrix of the sediment as plants die, they decay and they are buried in the sediment, thus contributing to the dehalogenase activity observed in organic-rich sediments (Nzengung, et al., 1999).

Variety of contaminant-degrading enzymes can be found in plants. These include peroxidases, dioxygenases, P450 monooxygenases, laccases, phosphatases, dehalogenases, nitrilases, and nitroreductases (Susarla et al., 2002; Singer et al., 2004 Chaudhry et al., 2005).

Phytoremediation is based upon the basic physiological mechanisms taking place in higher plants and associated microorganisms, such as transpiration, photosynthesis, metabolism, and mineral nutrition. Plants dig their roots in soils, sediments and water, and roots can take up organic compounds and inorganic substances; roots can stabilize and bind substances on their external surfaces, and when they interact with microorganisms in the rhizosphere (Marmiroli et al., 2006). Uptaken substances may be transported, stored, converted, and accumulated in the different cells and tissues of the plant. Finally, aerial parts of the plant may exchange gases with the atmosphere allowing uptake or release of molecules (Marmiroli et al., 2006). A series of six phytotechnologies have been identified (ITRC, 2001) which may address different contaminants in different substrates, and which rely on one or more of the plant properties.

1. Phytotransformation, ideal for organic contaminants in all substrates
2. Rhizosphere bioremediation, applied to organic contaminants in soil
3. Phytostabilisation, for organic and inorganic contaminants in soil
4. Phytoextraction, useful for inorganic contaminants in all substrates
5. Phytovolatilisation, which concerns volatile substances
6. Evapotranspiration, to control hydraulic flow in the contaminated environment

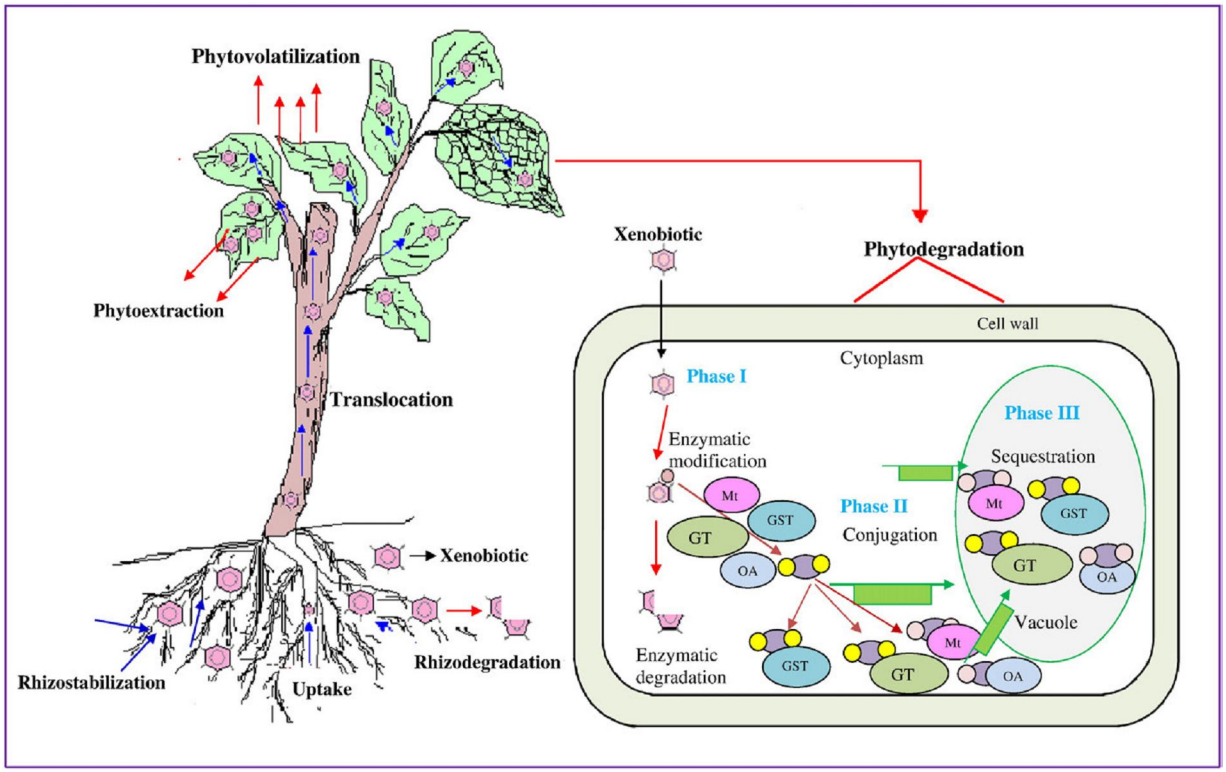

Figure 2.10 Typical attenuation mechanism possessed by plants against xenobiotics. The xenobiotics can be stabilized or degraded in the rhizosphere, adsorbed or accumulated in to the roots and transported to the aerial parts, volatilized or degraded inside the plant tissue. Plant detoxification generally involves conversion or enzymatic modification (phase I) followed by conjugation (phase II) followed by active sequestration (phase III). Active transporters are marked in green boxes (GST = glutathione S-transferases; GT = glucosyltransferases; Mt = Malonyltransferases; OA = organic acids (Newman and Reynolds, 2004; Pilon-Smits, 2005).

2.13.1 Phytodegradation

Phytodegradation can be explained as series of processes that plants utilizes to metabolize the contaminants they take up. Components of this mechanism are often utilized by plants exposed to herbicides and thus have been researched extensively (Abhilash et al., 2009). The metabolic processes involved in phytodegradation have strong similarities to those used by animals for modification and degradation of drugs and other toxins. Xenobiotic metabolism in human, animals and higher plants usually happen through three main biochemical processes; conversion or transformation (phase I), conjugation (phase II), and compartmentalization (phase III) (Schmidt et al., 2006). During phase I, hydrophobic

pollutants are converted to less hydrophobic metabolites through N-, O-, and S-dealkylation, aromatic and aliphatic hydroxylation, epoxidation, peroxidation, oxidative desulfuration, sulfoxidation or reduction by cytochrome P450s. Reactions catalyzed by cytochrome P450s are initial vital steps leading to detoxification, inactivation and excretion (Schmidt et al., 2006). This conversion usually produces less toxic metabolites. In phase II, organic pollutants or their phase I metabolites are directly conjugated with glutathione, sugars, or amino acids to produce hydrophilic compounds. Finally, in phase III, conjugated metabolites are deposited in vacuoles or cell walls (Hatzioz, 1997). Recently, the last phase of metabolism has been categorized into two independent phases, one confined to transport and storage in the vacuole, and a second one taking final reactions (cell wall bindings or excretion) (Theodoulou, 2000; Schroder, 2007).

2.13.2 Rhizodegradation

Rhizodegradation can be described as the transformation of contaminants by resident microbes in the plant rhizosphere (i.e., the microbe-rich zone in intimate contact with the root vascular system) (Abhilash et al., 2009). The presence of plants on contaminated sites can drastically affect soil redox conditions and organic content (often through the secretion of organic acids from roots), as well as soil moisture. Rhizodegradation is the dominant mechanism in the removal of total petroleum hydrocarbons from soil by deep-rooted trees (Carman et al., 1998) as well as annual species (Schwab and Banks, 1994).

Rhizodegradation is also referred to as microbe-assisted phytoremediation or rhizoremediation (Gerhardt et al., 2009). One type of microbe-assisted phytoremediation is rhizoremediation defined as degradation of contaminants in the rhizosphere.

Rhizoremediation is emerging as one of the most effective means by which plants can enhance the remediation of organic contaminants, particularly large recalcitrant compounds. Complex interactions involving roots, root exudates, rhizosphere soil and microbes do result in degradation of organic contaminants to non-toxic, or less-toxic, compounds. As much as 40% of a plant's photosynthate can be deposited in the soil as sugars, organic acids, and larger organic compounds (Kumar et al., 2006). These compounds are commonly used as carbon and energy sources by soil microbes (Singer et al., 2004; Chaudhry et al., 2005). On a per gram basis, rhizosphere soil has 10–100 times more microbes than non-vegetated soil (Lynch, 1990). In soil containing large volumes of roots, microbial populations can reach 10^{12} cells/g of soil (Whipps, 1990).

Plant roots can also release degradative enzymes into the rhizosphere (Schnoor et al., 1995). Reports are available on the degradation of nitro aromatic compounds (e.g. trinitrotoluene) by plant-derived nitro reductases and laccases at the laboratory scale (Boyajian and Carreira, 1997) and in field tests (Wolfe et al., 1993). Other plant-derived enzymes with the potential to contribute to the degradation of organic pollutants in the rhizosphere include dehalogenase involved in dehalogenising chlorinated solvents such as hexachloroethane and trichloroethylene, peroxidases degrading phenols, and phosphatases cleaving phosphate groups from large organophosphate pesticides (Susarla et al., 2002). The relationship between the plant root enzymatic and microbial interactions in degrading organic contaminants is shown in Figure 2.11 as described by Abhilash et al., (2009).

Apart from the direct release of degradative enzymes, plants are able to stimulate the activities of microbial degrader organisms/communities (Wenzel, 2009). Plant–degrader

interactions that are thought to be most relevant for the success of rhizodegradation are shown in Figure 2.12.

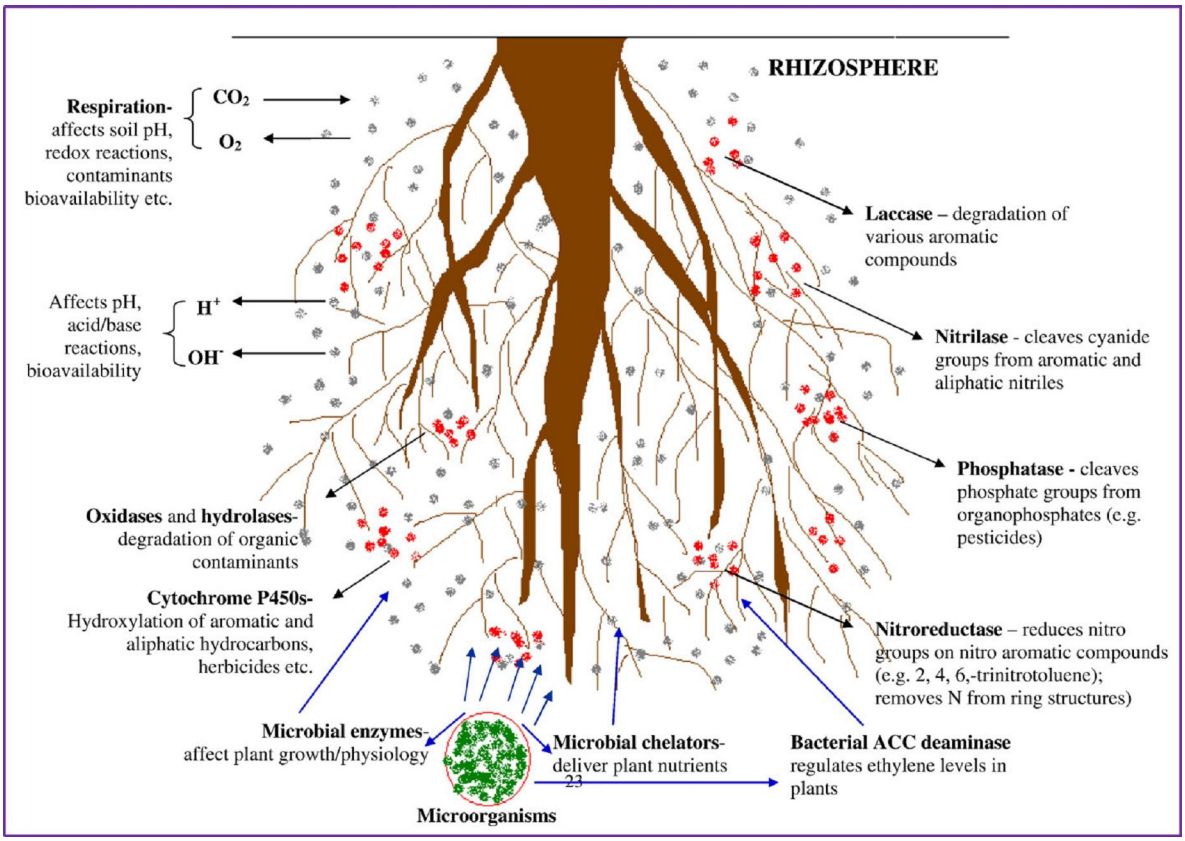

Figure 2.11 Schematic representation of the enzymatic and microbial activities responsible for the enhanced remediation in rhizospheric zone (Abhilash et al., 2009).

In rhizodegradation, plant roots do exude compounds that can serve as co-metabolites in microbial pollutant degradation (Hedge and Fletcher, 1996). This is important especially where microorganisms cannot utilize the pollutant as a sole carbon source for instance in the aerobic degradation of trichloroethylene (Hyman et al., 1995). Enhanced degradation of the polycyclic aromatic hydrocarbon benzo[a]pyrene by the rhizobacterium *Sphingomonas yanoikuyae* JAR02 was demonstrated in vitro in the presence of root extracts or exudates obtained from several plant species, including mulberry (*Morus alba*) and hybrid willow

(*Salix alba x matsudana*; Rentz et al., 2005). Some of the main processes involved in rhizodegradation of PAH are shown in Figure 2.13 as described by Gerhardt et al., (2009).

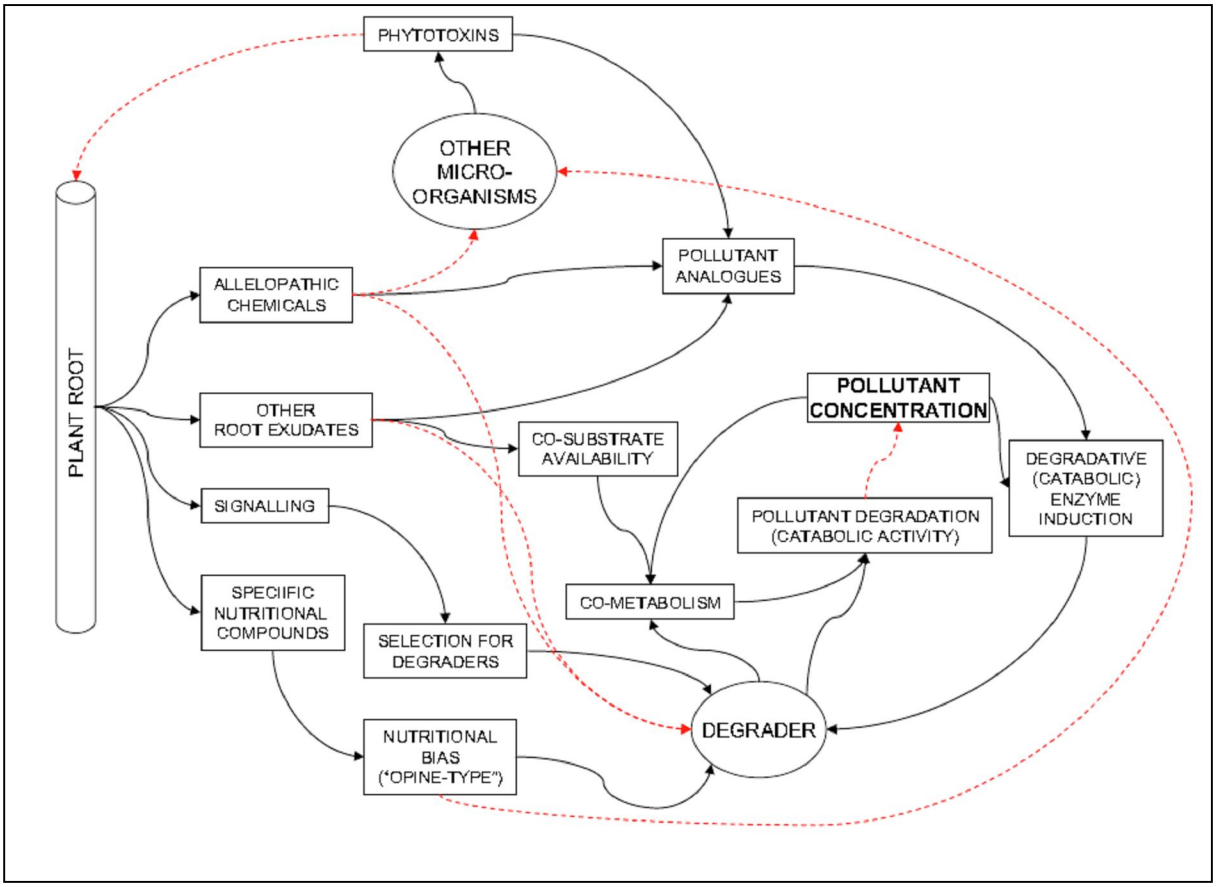

Figure 2.12 Plant-degrader interactions potentially involved in rhizodegradation (solid line arrows indicate positive, dashed line arrows indicate negative influence on the tested targeted process or component). (Wenzel, 2009).

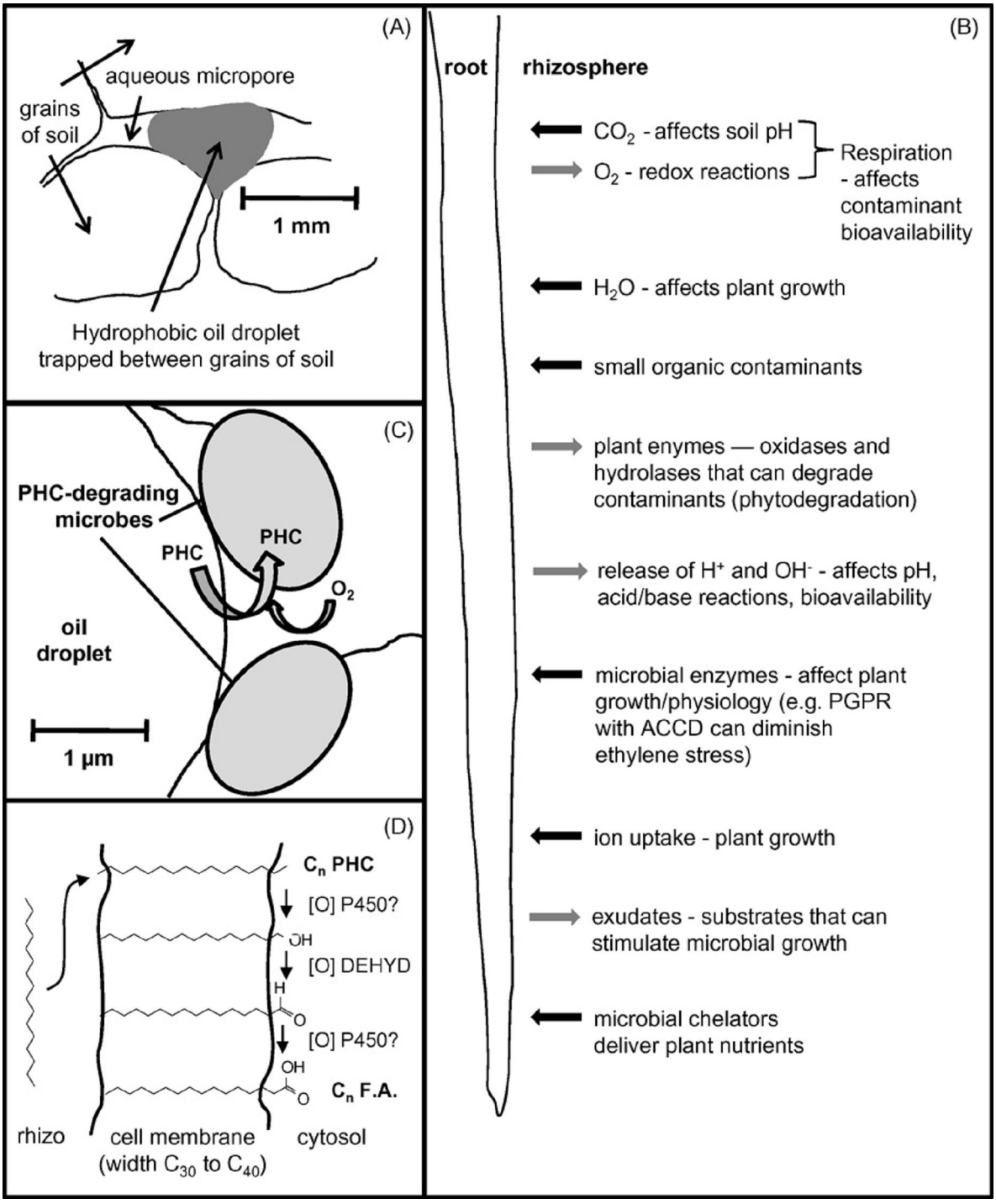

Figure 2.13 Rhizoremediation of petroleum hydrocarbon (PHC) (Gerhardt et al., 2009)

Alphabets in Figure 2.13 are described as follows: (A) Bioavailability of PHC: hydrophobic oil droplets are bound to soil particles or physically trapped in micropores and are not always easily bioavailable in bulk soil. Bioavailability depends on complex

interactions between chemical, biochemical, physical, and environmental parameters in the microenvironment (Doucette, 2003; Pilon-Smits, 2005). (B) General processes affecting rhizoremediation: plant roots support microbial growth at the root surface and in the rhizosphere. Roots create channels in soil that allow for movement of O_2 and H_2O, and that are wide enough for ‘‘trapped’’ contaminants to become accessible to microbes (Ferro, et al., 1999) PGPR, plant growth promoting rhizobacteria; ACCD, 1-aminocyclopropane-1-carboxylate deaminase. (C) Aerobic PHC degradation: at the PHC–water interface, microbes use adhesion methods and/or biosurfactants (Kuiper et al., 2004; Song et al., 2006). The microbial surface has membrane-bound oxygenases used for the first step in degradation. The first steps of the degradative pathway incorporate two O atoms into the PHC to form fatty acid analogues. These microbes then grow and multiply at the surface. Any given microbe can only degrade part of the petroleum (i.e., some PHC components of the complex mixture) and theoretically, it takes 150 mg N and 30 mg P for microbes to convert 1 g PHC to microbial biomass (Rosenberg, 1992). As the petroleum droplet is degraded, different microbes continue the degradation process. (D) Possible microbial oxygenation pathway of PHC to form a fatty acid: rhizo, rhizosphere; P450, cytochrome P450; DEHYD, dehydrogenase; F.A., fatty acid.

2.13.3 Phytoextraction

Phytoextraction involves the removal and subsequent storage of contaminants by the plant and is often applied to the exclusion and storage of metals that may undergo speciation in plants, but cannot be metabolized (Abhilash et al., 2009). It can also be explain to mean the ability of plants to take up contaminants into the roots and translocate them into the aboveground shoots or leaves (ITRC, 2009). For contaminants to be extracted by plants the constituent must be dissolved in the soil water and come into contact with the plant roots

through the transpiration stream. Alternatively, the uptake may occur through vapor adsorption onto the organic root membrane in the vadose zone. Once adsorbed, the contaminant may dissolve into the transpiration water or be actively taken up through plant transport mechanisms. Figure 2.14 shows the picture of phytoextraction mechanism. Once a chemical is taken up, the plant may store the chemical and/or its by-products in the plant biomass via lignification (covalent bonding of the chemical or its by-products into the lignin of the plant), sequester it into the cell vacuoles of aboveground tissues (as opposed to in root cells as part of phytosequestration). Alternatively, the contaminant may be metabolized through phytodegradation mechanisms and/or phytovolatilized in the transpiration stream exiting the plant (ITRC, 2009).

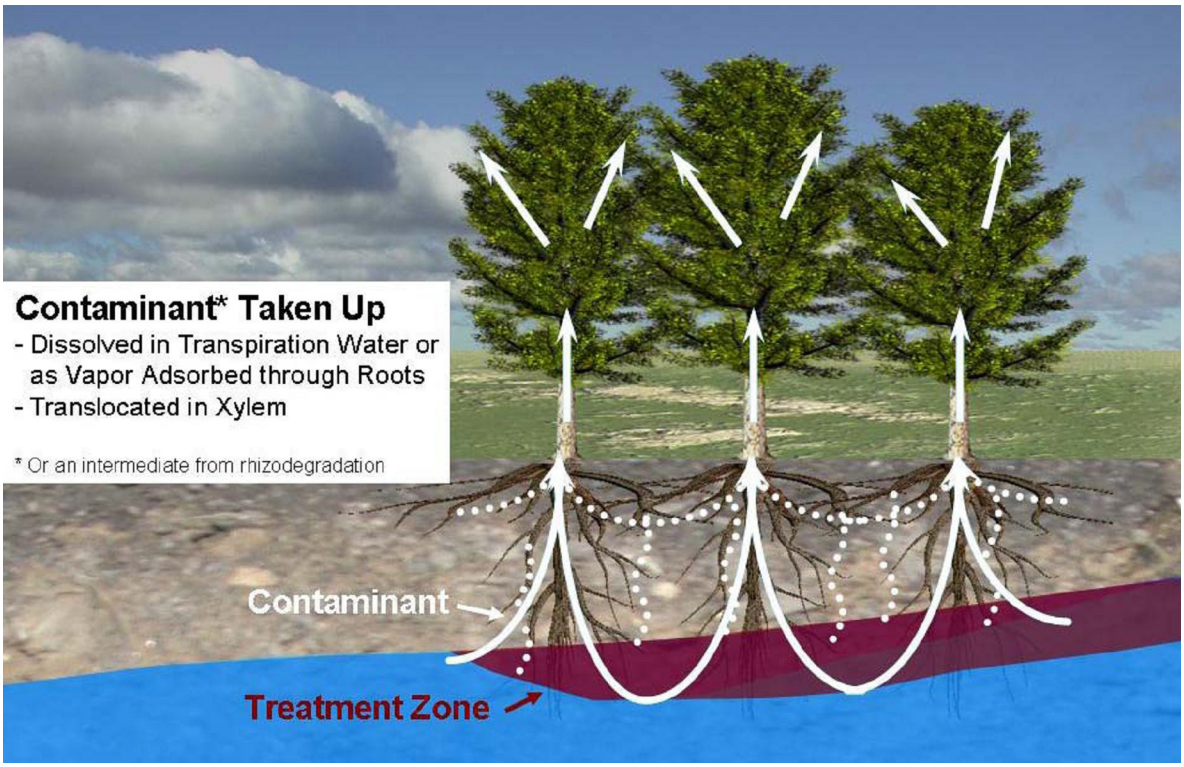

Figure 2.14 Phytoextraction mechanisms (ITRC, 2009)

2.13.4 Phytovolatilization

Phytovolatilization is the volatilization of contaminants from the plant either from the leaf stomata or from plant stems (Ma and Burken 2002), as shown in Figure 2.15. In some cases, a breakdown product derived from the rhizodegradation and/or phytodegradation of the parent contaminant along the transpiration pathway may be the phytovolatilized constituent.

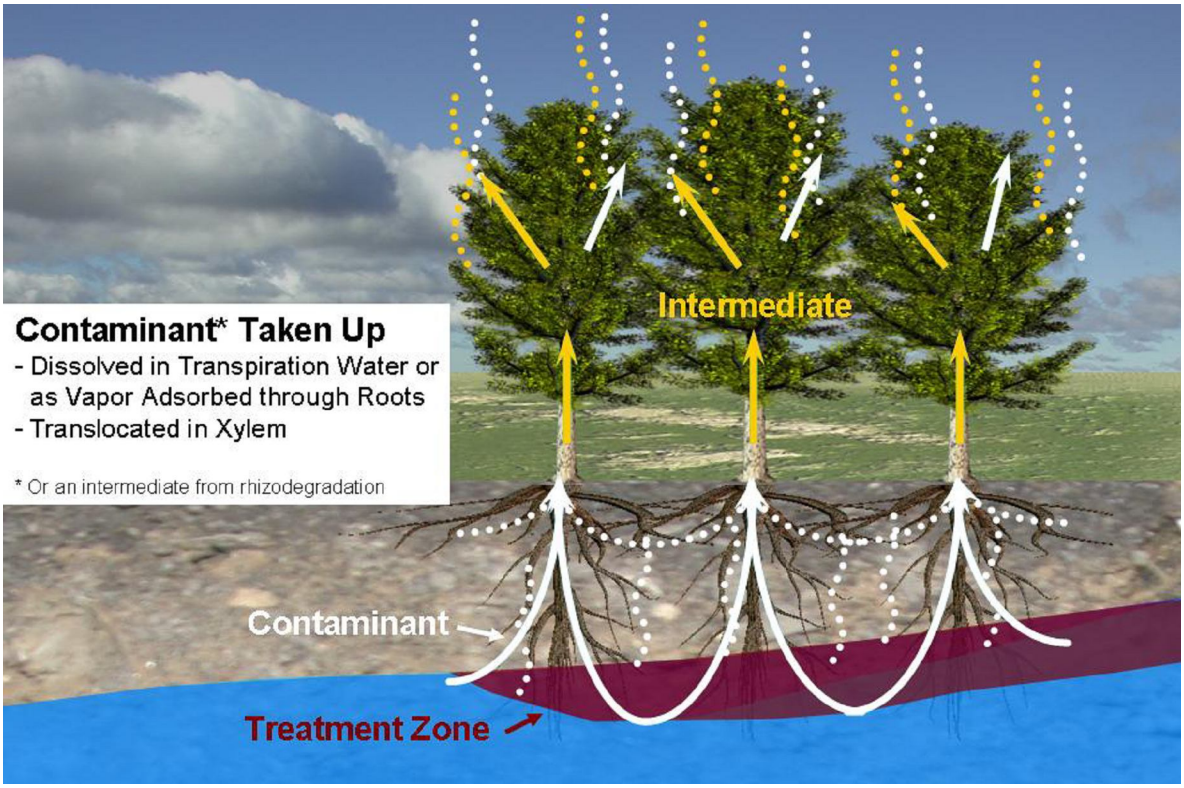

Figure 2.15 Phytovolatilization mechanisms (ITRC, 2009)

2.13.5 Phytostabilization

Phytostabilization is another mechanism that can be used to minimize migration of contaminants in soils. This process takes advantage of plant roots ability to alter soil environment conditions, such as pH and soil moisture content (Susarla et al., 2002). Many root exudates cause metals to precipitate, thus reducing bioavailability. One advantage of

this strategy over phytoaccumulation is the disposal of the metal-laden plant material is not required. By choosing and maintaining an appropriate cover of plant species, coupled with appropriate soil amendments, it may be possible to stabilize certain contaminants (particularly metals) in the soil (Cunningham et al., 2000), and reduce the interaction of these contaminants with associated biota.

2.14 *Jatropha curcas*

Jatropha curcas is a multipurpose plant with many attributes and considerable potential. It is a tropical plant that can be grown in low to high rainfall areas and can be used to reclaim land, as a hedge and/or as a commercial crop (Openshaw, 2000). *Jatropha curcas* is a perennial, deciduous, stem-succulent shrub (Foidl et al., 1996), which produces seeds rich in oil easily convertible into biodiesel meeting international standards (Azam et al., 2005). With its ability to reclaim degraded and/or dry lands with potentially positive impact on biodiversity and soil resources (Francis et al., 2005), *Jatropha* became a very important plant of high commercial value.

J. curcas is a native of tropical America, but is now found abundantly in many tropical and sub-tropical regions throughout Africa and Asia because of likely distribution by Portuguese ships via the Cape Verde islands and Guinea Bissau (Heller, 1996). *J. curcas* has spread beyond its original distribution because of its hardiness, easy propagation, drought endurance, high oil content, low seed cost, short gestation period, rapid growth, adoption to wide agro-climatic condition, bushy/shrubby nature and multiple uses of different plant parts (Divakara et al., 2010). The plant can be used to prevent and/or control erosion, to reclaim land, grown as a live fence, especially to contain or exclude farm animals and be planted as a commercial crop (Openshaw, 2000). Jatropha Curcas has the

advantage that not only is it capable of growing on marginal land, but it can also help to reclaim problematic lands and restore eroded areas (Fact foundation, 2006).

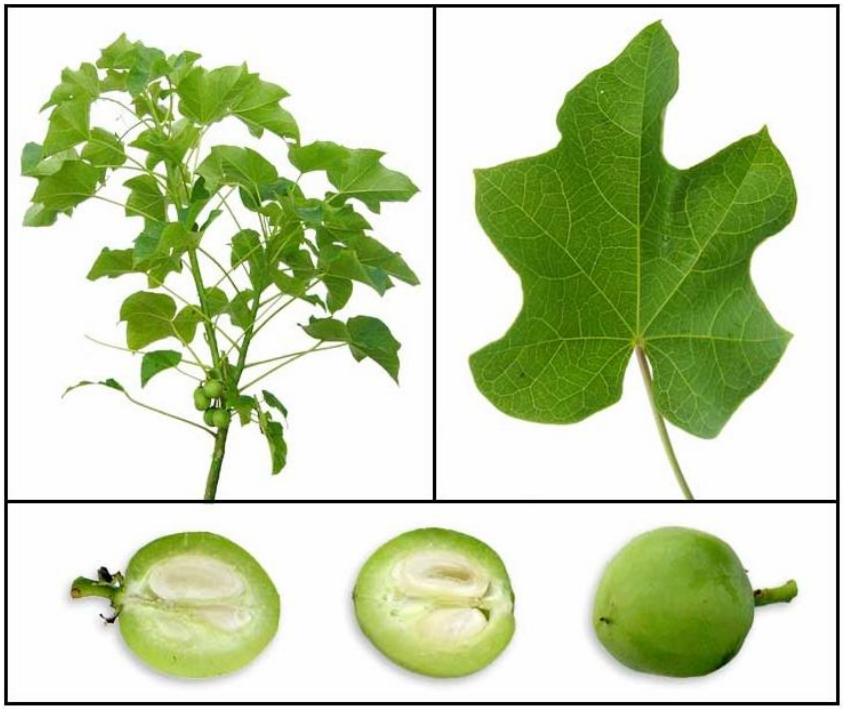

Plate 2.1 Picture of Jatropha curcas plant and seed

2.15 Hibiscus cannabinus L. (Kenaf)

Kenaf (*Hibiscus cannabinus* L.) is an annual herbaceous plant from the Malvaceae family. It is one of the world's leading sources of bast fibre in the cottage industry (Kuchinda, 2001). Research and development works have demonstrated the suitability of kenaf for use in building materials, adsorbents, textiles and, fibers in new and recycled plastics (Webber and Bledsoe, 1993). Its fiber was also recommended as reinforcement fiber of high performance biodegradable polymer composites (Nishino et al., 2003). Kenaf also makes good animal forage with high crude protein in leaves (Webber et al., 2002).

Plate 2.2 Picture of *Hibiscus cannabinus*

Kenaf (*Hibiscus cannabinus L.*) grow quickly, rising to height of 1.5 to 3.5 m tall and the stems are 1 - 3 cm diameter within 3 – 4 months (Bada and Raji, 2010). Its uses includes medicine (Cheng, 2001), food additive (Hosomi, 2000), medium for mushroom cultivation (Cheng, 2001), environmental cleaning (Lam, 2000), oil and chemical absorbents (Sameshima, 2000).

Kenaf, an annual herbaceous plant, indigenous to West Africa, apart from use in production of pulp and paper products, has proved very effective as an oil absorbent in cleaning oil spills. Kenaf has been reportedly used for the remediation of eutrophic water by Ikeda et al., (1999), nitrate was removed from eutrophic water by kenaf. Bada and Raji (2010)

reported significant positive correlation between Cd applied to soil and Cd absorbed by kenaf in Cd-contaminated soil. In another related studies Hiroyuki et al., (2005), conducted field trial using kenaf for three years (2001-2003) at a Cd-contaminated paddy field in south west Japan. The kenaf showed large amount of Cd uptake each year because the fallen leaves contained large amount of Cd. Ho et al., (2008) used kenaf for remediation of Pb contaminated soil and discovered that kenaf root was able to accumulate more than 85% of total plant Pb.