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Hydrocarbon Pollution: Effects on Living Organisms, Remediation of Contaminated Environments, and Effects of Heavy Metals Co-Contamination on Bioremediation

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1. Introduction

Hydrocarbon contamination in the environment is a very serious problem whether it comes from petroleum, pesticides or other toxic organic matter. Environmental pollution caused by petroleum is of great concern because petroleum hydrocarbons are toxic to all forms of life. Environmental contamination by crude oil is relatively common because of its widespread use and its associated disposal operations and accidental spills. The term petroleum is referred to an extremely complex mixture of a wide variety of low and high molecular weight hydrocarbons. This complex mixture contains saturated alkanes, branched alkanes, alkenes, naphthenes (homo-cyclics and hetero-cyclics), aromatics (including aromatics containing hetero atoms like sulfur, oxygen, nitrogen, and other heavy metal complexes), naphtho-aromatics, large aromatic molecules like resins, asphaltenes, and hydrocarbon containing different functional groups like carboxylic acids, ethers, etc. Crude oil also contains heavy metals and much of the heavy metal content of crude oil is associated with pyrrolic structures known as porphyrins. Petroleum is refined into various fractions such as light oil, naphtha, kerosene, diesel, lube oil waxes, and asphaltenes, etc. The light fractions, which are distilled at atmospheric pressure, are commonly known as light ends and the heavy fractions like lube oil and asphaltenes are known as the heavy ends. The light and the heavy ends of petroleum have different hydrocarbons composition, the light ends contain low molecular weight saturated hydrocarbons, unsaturated hydrocarbons, naphthenes, and low percentage of aromatic compounds; while the heavier ends consist of high molecular weight alkanes, alkenes, organometallic compounds, and high molecular weight aromatic compounds. This portion is comparatively rich in metals and N,S,O containing compounds. Figure 1 shows some of the chemical structures of common hydrocarbons compounds. These hydrocarbon molecules are widespread in the environment due to the wide range of petroleum uses, which are presented elsewhere (http://www.dep.state.fl.us/waste/quick_topics/publications/pss/pcp/PetroleumProductDescriptions.pdf).

Heavy metals are naturally present in soils; however due to human activity, the concentration of heavy metals in soil is increasing. Some areas contain such a high concentrations of heavy metals and metalloids that they are affecting the natural ecosystem. It has been observed that hydrocarbon contaminated sites co-contaminated with heavy

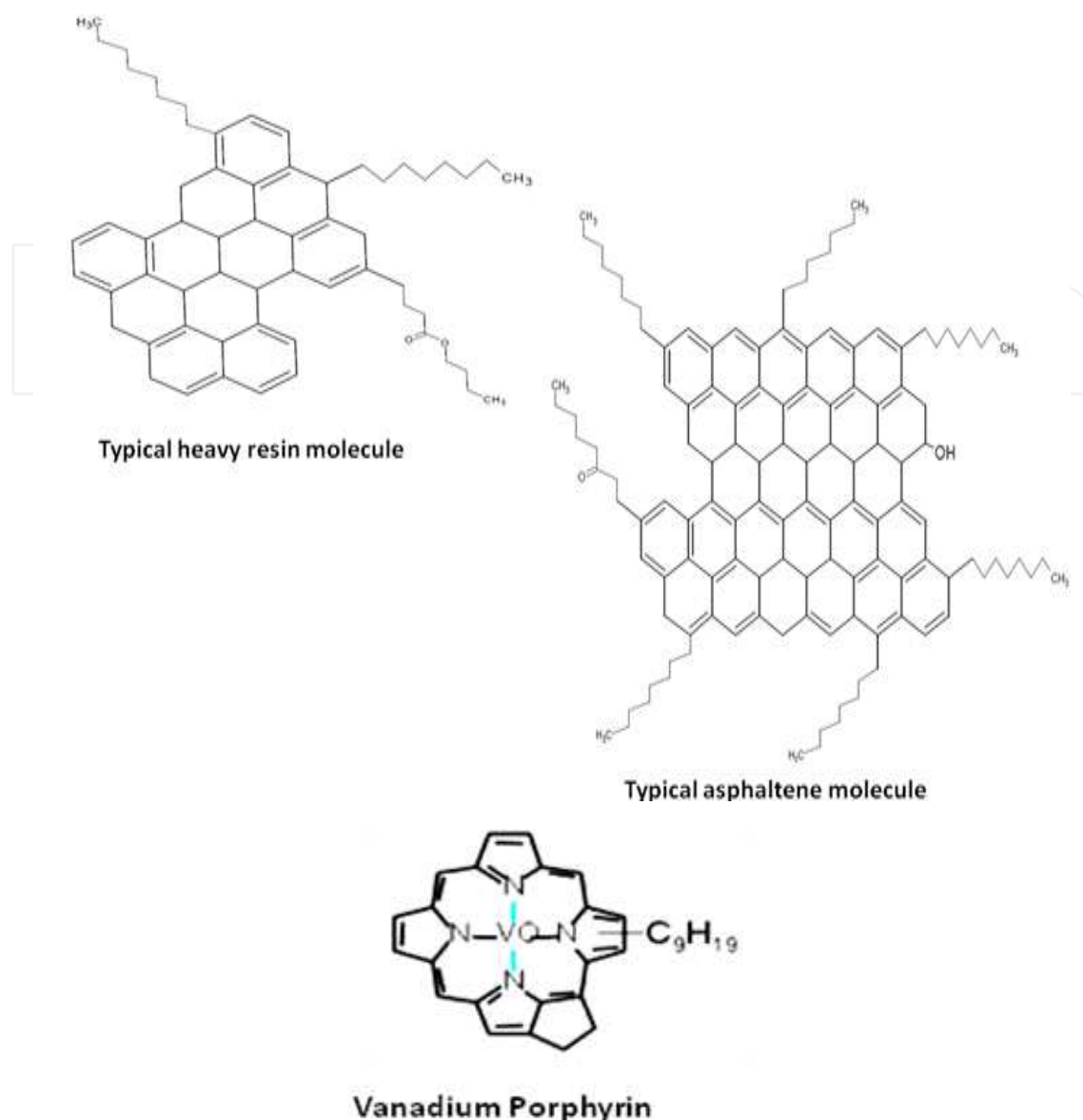


Fig. 1. Some of the representative molecules in the light and heavy fractions found in petroleum (<http://nepis.epa.gov/Adobe/PDF..P1000AE6.pdf>; Wenpo et al., 2011; Dmitriev and Golovko, 2010, Qian et al., 2008).

1.1 Effects of petroleum hydrocarbons on living matter

Living matter is exposed to petroleum in many ways directly or indirectly. Some byproducts, formed during petroleum refining and processing which are used for the manufacturing of other products are highly toxic. Constantly, these toxic compounds are inadvertently released into the environment and if this effect is connected to the effect of accidental crude oil spills worldwide, then these combined sources of unrestricted hydrocarbons constitute the major cause of environmental pollution. Despite the large number of hydrocarbons found in petroleum products, only a relatively small number of the compounds are well characterized for toxicity. Petroleum hydrocarbon molecules, which have a wide distribution of molecular weights and boiling points, cause diverse levels of toxicity to the environment. The toxicity of the hydrocarbon molecules and their availability for microbial metabolism depend on their

chemical and physical nature. Petroleum is toxic and can be lethal depending upon the nature of the petroleum fraction, the way of exposure to it, and the time of exposure. Chemicals and dispersants in crude oil can cause a wide range of health effects in people and wildlife, depending on the level of exposure and susceptibility. The highly toxic chemicals contained in crude oil can damage any organ system in the human body like the nervous system, respiratory system, circulatory system, immune system, reproductive system, sensory system, endocrine system, liver, kidney, etc. and consequently can cause a wide range of diseases and disorders (Costello, 1979).

Individuals more susceptible to harm by the toxic effects of crude oil are as follows.

1. Infants, children, and unborn babies.
2. Pregnant women.
3. People with pre-existing serious health problems.
4. People living in conditions that impose health stress.

The damage caused by the toxicity of crude oil to organ systems may be immediate or it may take months or years. Singh et al.(2004) studied the toxicity of fuels with different chemical composition on CD-1 mice (A swiss mice strain that is used as a general purpose stock and an oncological and pharmaceutical research. This is a vigorous outbred stock. These mice are fairly docile and easy to handle). The objective of the study was to establish a correlation between the physico- chemical properties of the fuel and their biologic effects on mice. The results of the study demonstrated that the automobile derived diesel exhaust particles were more toxic than the exhaust generated by forklift engines. It was also found that the diesel exhaust particles contain ten times more extractable organic matter than the standard exhaust material generated by forklift engines. A similar type of study conducted by Kinawy (2009) revealed that the inhalation of leaded or unleaded (containing aromatics and oxygenated compounds) gasoline vapors by rats impaired the levels of monoamine neurotransmitters and other biochemical parameters in different areas of the rats' brains. Likewise, several behavioral changes causing aggression in rats were observed. Menkes and Fawcett (1997) discussed the toxicities of lead and manganese added gasoline and the public health hazards due to aromatic and oxygenated compounds in gasoline. The extent of absorption of petroleum components by inhalation, oral, and dermal routes varies significantly because of the wide range of physicochemical properties of these components. The incorporation of crude oil into the body may affect the reproductive health of humans and to other lives. Obidike et al. (2007) observed that when the male rats were given an oral crude oil treatment using a drenching tube, degeneration and necrosis of interstitial cell occurred followed by the exudation into the interstices in the testes of rats. The study concluded that exposure of rats to crude oil induces reproductive cytotoxicity confined to the differentiating spermatogonia compartment, likewise it may also harm human reproductive cells. The extent of absorption through the various routes depends on the volatility, solubility, and other properties of the specific component or mixture. The more volatile and soluble the oil fractions (low molecular weight aliphatics and light aromatic compounds) are the faster they can leak into groundwater or vaporize into the air. Therefore, living matter may be easily exposed to these crude oil fractions by breathing the contaminated air and by drinking the contaminated water (Welch et al., 1999). As reported by Knox and Gilman (1997), petroleum derived volatiles are one of the causes for the geographically associated childhood cancers.

On the other hand, the non-volatile heavy fractions of crude oil tend to be absorbed by the soil and persist at the site of release, which may harm living beings by skin contact, by intake of contaminated water or food. The heavy fraction of crude oil consists mainly of naphthene-aromatics and poly-aromatic compounds that are carcinogenic and long exposure to these compounds often leads to tumors, cancer, and failure of the nervous system. The aromatic compounds found in petroleum are an important group of environmental pollutants. These aromatic compounds are introduced into the environment from various sources such as natural oil seeps, refinery waste products and emissions, oil storage wastes, accidental spills from oil tankers, petrochemical industrial effluents and emissions, and coal tar processing wastes, etc. Petroleum hydrocarbons can rapidly migrate from the site of contamination and adversely affect terrestrial and aquatic ecosystems and humans. Crude oils also contain polar organic compounds that contain N, S, and O atoms in various functional groups. The chemical properties of these NSO compounds, particularly their solubility and toxicity, are of environmental concern. It has been documented, that at the sites where oil spills have occurred a portion of the polar organic compounds present in the oil had partitioned into the groundwater rendering high concentration of total petroleum hydrocarbons in drinkable water sources (Mahatnirunkul et al. 2002, Delin et al., 1998, Oudot, 1990). Therefore, drinkable water sources turn out to be unsafe for the human population as well as for the aquatic animals, aquatic plants, and microbes (Griffin and Calder, 1976). Fortunately a diverse group of microorganisms like bacteria, fungi, and yeast may efficiently breakdown crude oil fractions into nontoxic components. There are a large number of studies available on the microbial degradation of crude oil or hydrocarbons.

2. Remediation

At present, it is widely recognized that contaminated land or water systems are a potential threat to human health. Awareness of this reality has led to international efforts to remediate many of these sites, either as a response to the health risks or to control the detrimental effects on the environment caused by contamination aiming the recovery of the contaminated sites. For decades efforts have been directed toward the evaluation of cost effective methods to cleanup oil contaminated soils. Over the years, many cleanup methods have been developed and applied. However, the remediation of oil contaminated environments is difficult because petroleum is a complex mixture of chemical compounds, and their degradation whether chemical or biological is not easy as different class of compounds needs different treatments. Furthermore, crude oil composition is reservoir dependent; therefore it is of great importance to know first the composition of the oil and the physico-chemical nature of the contaminated site before deciding the remediation strategies. Crude oil degradation in the natural environment depends on several factors such as pH, chemical composition, and physical properties of the contaminated soil and/or water, among others.

More than 50% of the crude oil produced worldwide comes from the Arabian Gulf area (onshore and offshore oil reservoirs). In the period from 1995 to 1999, 550 oil spill incidents for a total volume of 14,000 barrels of oil were reported in the Arabian Gul; while 11,000 barrels of oil were spilled in the period from 2000 to 2003. Oil spills affect mostly water systems during oil production operations, as many oil production wells are located near sea shore.

There exists some chemical and biological methods to control oil spills, but most frequently applied are chemical methods because the chemical remediation of oil spills is faster if compared to bioremediation. However, bioremediation is getting worldwide attention. In general the remediation of oil contaminated sites can be performed by two basic processes: in-situ and ex-situ treatment using different cleaning technologies, such as biological treatment, thermal treatment, chemical extraction and soil washing, and through aerated accumulation techniques. However, at the present there is not a fully effective or universal remediation method for the cleaning up of oil contaminated sites. The subsequent paragraphs summarize some existing chemical and biological methods for the remediation of crude oil spills in soil and water systems such as bioventing, biosparging, in-situ biodegradation, and bioaugmentation.

2.1 Chemical and mechanical remediation methods

2.1.1 Oil spilled on the sea surface. There are different methods to remove oil from the sea surface and to prevent the oil from reaching the shoreline. Mechanical recovery and the application of dispersants are methods widely used. At sea, crude oil, which is usually lighter than water, spreads over the water surface and in a short time the thickness of the oil film on the water surface becomes very thin. The oil spreading velocity on the water surface depends on the type of oil, water temperature, atmospheric temperature, wind, and tide. The evaporation of the oil light components take place immediately and up to around 40 % of crude oil might evaporate during a short period of time. If accidental oil spills take place, booms (floating barriers placed around the oil or around the oil spill source) and skimmers (boats, vacuum machines, and oil-absorbent plastic ropes) are used to prevent the spreading of oil and to restrict the spill to a concise area, after which the oil is collected into a container by the skimmers. Chemical dispersants are frequently used to render the oil spill harmless for the aquatic life and other living organisms by, reducing the oil slick to droplets that can be degraded by naturally occurring bacteria. Dispersants can be applied to a large area but sometimes its hazards to the environment are of great concern.

Biosurfactants can also be applied to solubilise the oil, with the advantage that biosurfactants are generally not harmful to the environment. Sometimes in-situ burning of oil is also used as an optional method for oil spill remediation; however this method is applied only when the oil spill is fresh and the spilled oil layer is floating on the water surface or after the oil has been concentrated into a small area by the booms. This method has the downside that oil burning generates smoke and other by products, which pollutes the aquatic and atmospheric systems.

Every method has its advantages and disadvantages, and the effectiveness of any cleaning method depends on ocean currents and tides, as well as the weather. If the oil reaches the shoreline, then other methods are applied to clean up the sand and gravels. Sometimes sponge like oil sorbents are used to absorb oil. The oiled birds are washed manually with a diluted liquid soap solution, however sometimes in spite of an efficient manual cleaning the affected birds cannot be saved. Oiled vegetation can be flushed with water to remove the oil, but severely damaged plants will need to be destroyed and removed entirely.

The ex-situ remediation involves the pumping of contaminated water to a processing site; this process is applied only when the volume of contaminated water is low. The shore sand

and gravels are removed from the contaminated site and cleaned elsewhere. The remediated sand and gravel are then replaced on the shoreline.

2.1.2 Oil Spilled on Soil. Soil contamination occurs during offshore drilling and oil production operations due to leakages from well heads and pipelines, overflow from gathering stations, petroleum products, improper disposal of petroleum wastes and leakage from underground petroleum storage tanks. Conventional soil remediation techniques consist of the excavation and removal of the contaminated soil to a landfill, or to cap and contain the contaminated areas at the site. This method may create significant risks during the excavation, handling, and transport of contaminated material. Additionally, it is very difficult and increasingly expensive to find new landfill sites for the final disposal of the material. The cap and containment method is only an interim solution since the contamination remains on the site, requiring continuous monitoring and maintenance of the separation barriers long into the future, with all the associated costs and potential liability. Contaminated soil can be treated by two ways: in-situ or ex-situ. In-situ method of contaminated soil cleaning means that the treatment is applied to the soil at the site through physico-chemical processes such as ignition, air sparging, and soil air extraction or by combinations of these two processes, depending on the nature of the contaminants.

The application of in-situ treatments make use of technology and equipment from oil and gas drilling operations including vertical and horizontal drilling. In-situ techniques are more effective on sandy soils than in clay soils. Air sparging, also referred as soil venting (volatilisation) can be applied to take out the contaminants from soil and from groundwater-saturated soil by mobilization of the volatile compounds. This method also accelerates the growth of aerobic bacteria in the contaminated area by oxygen feeding. However, if oil contamination occurs in the groundwater, air sparging can be also conducted below the water table to extract the volatile compounds to unsaturated zones or to the surface by gravity segregation or through extraction wells. Another process known as slurping uses vacuum to extract oil from the boundary of the groundwater oil saturated or partially saturated soil. Steam injection into the contaminated oil is very effective in extracting volatile components trapped in the soil.

Ex-situ remediation of oil contaminated soil is usually applied to a small volume of soil. Ex-situ soil remediation involves the removal and transportation of the soil from the contaminated area to an off site remediation facility. The ex-situ remediation can perform through different processes as follows. Land farming in which the oil contaminated soil is excavated and spread over a bed where it is periodically tilled until the degradation of the contaminants takes place. This technique is usually limited to the treatment of 15 to 35 cm of surface soil. Composting which involves mixing of contaminated soil with nonhazardous organic matter to enhance the development of microbial species. Biopiles is a hybrid process that involves land farming and composting, in which engineered cells are constructed to stack the contaminated soil that can be aerated. The bio-processing of contaminated soil, sediment, and water is sometimes carried out in slurry or aqueous reactors known as bioreactors where the three phases solid (soil), liquid, and gas are continually mixed which enhances the biodegradation rate. The contaminated soil requires pre-treatment before loading into the bioreactors. The contaminants undergo chemical oxidation reactions converting the toxic compounds into less toxic or harmless compounds. The oxidation reactions can be catalyzed through dechlorination or UV. Generally, these methods present

some drawbacks such as elevated costs due to the complexity of the technology required; while bioremediation is an option that offers the possibility to degrade hydrocarbon contaminants using natural biological activity.

2.2 Bioremediation treatments

Bioremediation of oil contaminated sites is an efficient alternative to the conventional mechanical and chemical processes that is gaining worldwide attention. This technique utilizes the natural biological activity of microorganisms or enzymes to transform the toxic petroleum components into less toxic or harmless metabolites. Bioremediation requires the addition of nitrate or sulphate fertilizers to aid the decomposition of hydrocarbon compounds. Advantages of bioremediation are: it can be applied directly at the site, low cost, the degradation of pollutants do not produce side effects, and that it is a technology worldwide acceptable. Downsides of bioremediation are: (1) its application is restricted to petroleum components that are microbially degradable and (2) the bioremediation process take long times for completion. Hydrocarbon compounds having high aromaticity, chlorinated compounds, or having other resistant toxic functional groups are resistant to microbial attack and are almost impossible to degrade at aerobic conditions. However, these compounds can be degraded under anaerobic conditions. In this technique, the contaminated site is supplied with an oil degrading microorganism or a consortium of microorganisms that may be indigenous or isolated from elsewhere and inoculated into the contaminated site, which is known as bioaugmentation. For bioremediation to be effective, microorganisms must enzymatically attack the pollutants during their metabolic processes. Microorganisms synthesize surfactants known as biosurfactants, which have both hydrophobic and hydrophilic domains and are capable of lowering the surface tension and the interfacial tension of the growth medium. Biosurfactants are synthesized during the bacterial growth period on water-immiscible substrates, providing an alternative to chemically prepared conventional surfactants. In the case of the bacterial degradation of hydrocarbons, microbial cells synthesize biosurfactants that solubilizes oil droplets into the aqueous phase making easier the oil uptake by microbial cells as shown in Fig. 2 (Cameotra et al., 1983, Ganesh and Lin, 2009).

Biosurfactants show different chemical structures: lipopeptides, glycolipids, neutral lipids, and fatty acids (Cameotra et al. 2010, Muthusamy et al., 2008). Biosurfactants are nontoxic, biodegradable biomolecules that have gained importance in the fields of enhanced oil recovery, environmental bioremediation, food processing, and pharmaceutical due to their unique properties. The economics of biosurfactants production is a bottle neck because of the low production efficiency and the high costs associated to the recovery and purification of biosurfactants (Cameotra and Makkar, 1998). To overcome these problems, the use of unconventional substrates together with biosurfactant producing bacterial strains or genetically modified strains have been suggested, which could make possible for biosurfactants to compete with synthetic surfactants (Soumen et al., 1997, Makkar and Cameotra, 2002). Similarly, biosurfactants production costs could be reduced by using different production routes (development of economical engineering processes) aiming the increased biosurfactant production yield and the use of cost free or cost credit feed stock for microorganism growth and biosurfactant production (Gautam and Tyagi, 2006).

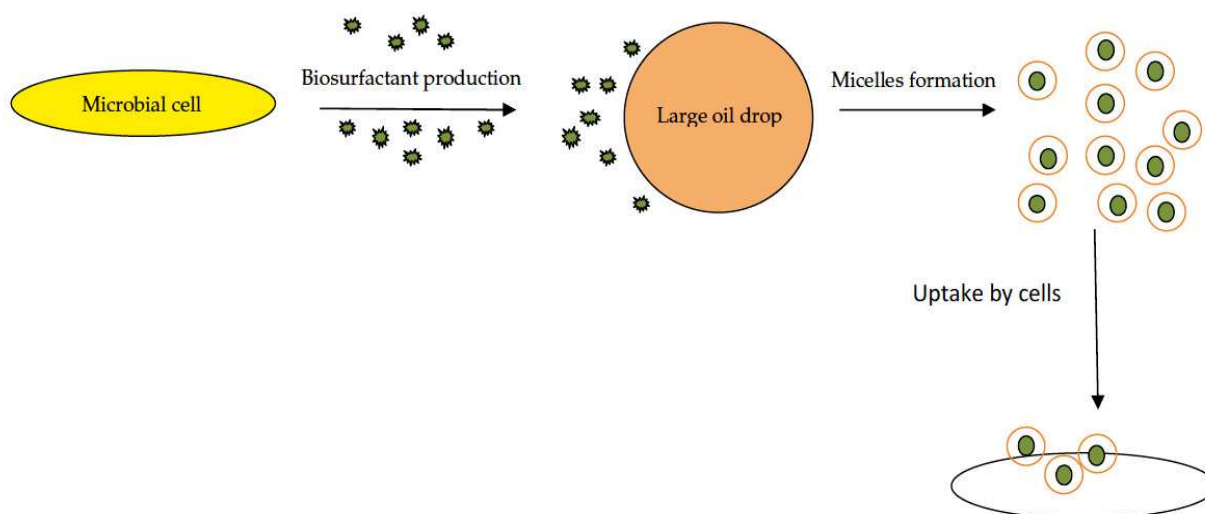


Fig. 2. Mechanism of oil solubilization by biosurfactants and uptake of solubilized oil by microbial cells.

The efficient biodegradation of petroleum hydrocarbon often involves the manipulation of the environmental parameters to allow microbial growth and degradation to proceed at a faster rate, which include the availability of sufficient amount of oxygen or other electron acceptors, essential nutrients, the penetration depth of the hydrocarbon pollutants into the soil, and the nature of the soil.

Usually, the application of bioremediation of hydrocarbon contaminated sites involves the injection of sufficient amounts of oxygen and nutrients to stimulate the oil degrading bacteria.

Figure 3 shows general pathways for the metabolism of different hydrocarbons. It is well known that alkanes (short or long chains) are oxidized mono-terminally to the corresponding alcohols, aldehydes, and mono basic fatty acids followed by beta oxidation (Nieder and Shapiro, 1975) however, further observations suggest di-terminal and sub-terminal n- alkane or fatty acid oxidation that is frequently found in branched alkanes (Forney and Markovetz, 1970, Cong et al., 2009). The enzymology of alkane oxidation is not clear yet, however common accepted mechanisms are dehydrogenation, hydroxylation, and hydroperoxidation. n-alkanes are the most easily degradable components of petroleum, followed by cyclo alkanes; while the aromatic fraction is the most resistant to microbial attack. A review of microbial petroleum hydrocarbon degradation is presented elsewhere (Atlas 1981).

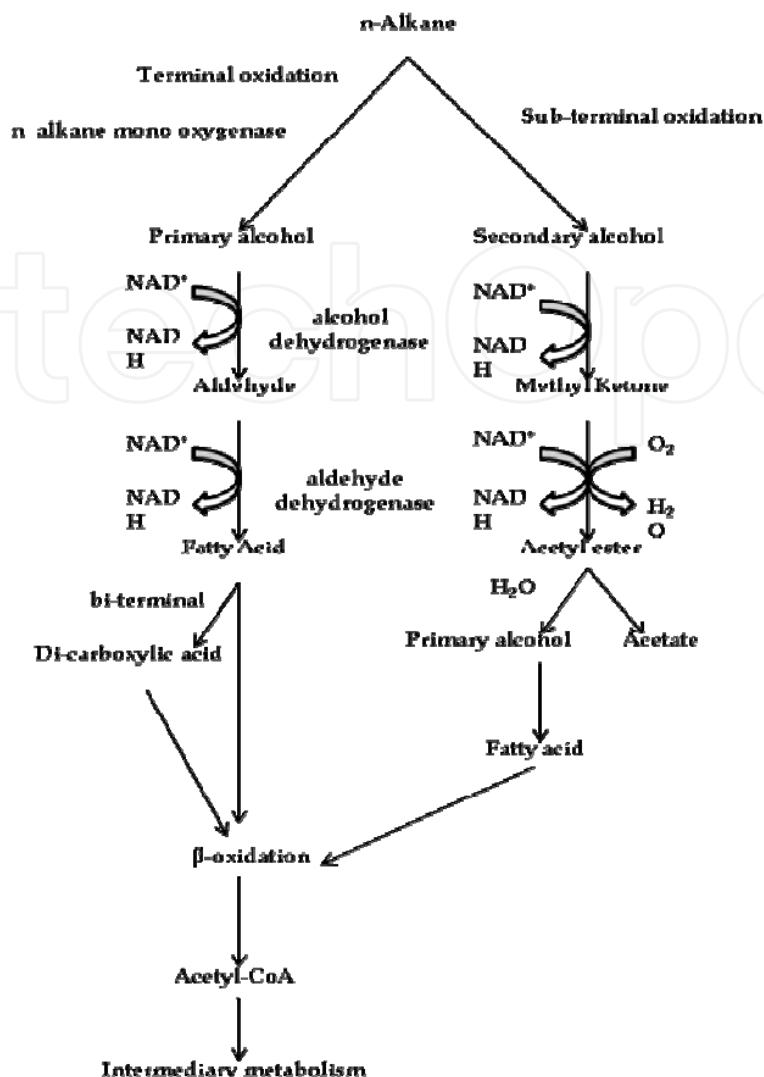


Fig. 3. General pathways for the metabolism of n-alkanes, cyclo alkanes, aromatics and other compounds (Fritsche & Hofrichter, http://www.wiley-vch.de/books/biotech/pdf/v11b_aero.pdf)

3. Effect of heavy metals on bioremediation

A number of microbial strains have been isolated which are very efficient in degrading petroleum hydrocarbons (Sandrin and Maier, 2003). The field application of bioremediation needs to consider several variables such as soil pH, temperature, salt concentration (salinity), pressure, presence of other co-contaminants as PCBs, pesticides, dyes, and heavy metal ions (Atlas, 1981). Likewise, the presence of recalcitrant hydrocarbons as poly aromatic/substituted aromatic can affect the degradation of other degradable hydrocarbons. These aromatic compounds can be toxic to individual microorganisms, thus inhibiting the microbial degradation capability. For instance, alkyl- and nitro-PAHs are highly toxic among common substituted PAHs. Substitution on aromatic rings produces several problems in their degradation. The ability of PAH dioxygenase to remove the substitutions is currently the subject of debate. The presence of alkyl branches may inhibit the proper orientation and accessibility of the PAHs into dioxygenases. Transition metals, some

metalloids, lanthanides and actinides can be referred as heavy metals. Heavy metal ions are naturally found in soil but human activities are increasing unexpectedly the concentration of heavy metals in the soil, which is of great concern. Heavy metals affect the ecosystem adversely for example mercury at low concentrations represents a major hazard to microorganisms and other biota. Inorganic mercury has been reported to produce harmful effects at concentrations of 5 µg/l in a culture medium (Boening, 1999, Orct et al., 2006). Similarly other metals also have an adverse effect on microorganisms and ecosystems (Babich and Stotzky, 1983; Sobolev and Begonia, 2008). Heavy metals not only contaminate the soil but also restrict the microbial activity at the polluted site consequently affecting the degradation of other organic pollutants (Mittal and Ratra, 2000).

Divalent heavy-metal cations are structurally very similar as is the case of the following divalent cations: manganese, ferrous, cobalt, nickel, copper and zinc. Oxyanions like chromate, arsenate, phosphate with four tetrahedrally arranged oxygen atoms and two negative charges, differ mostly in the size of the central ion, so the structure of chromate resembles that of sulfate. Thus, cells uptake systems for heavy-metal ions have to bind those ions tightly. Most cells solve this problem by using two types of uptake systems for heavy-metal ions: one is the fast uptake and the second uses high substrates specificity. These fast systems are usually driven only by the chemiosmotic gradient across the cytoplasmic membrane of bacteria. The second type of uptake system has high substrate specificity; it is slow and often uses ATP hydrolysis as the energy source. Microbes are very susceptible to metal toxic environments when exposed to high concentrations of heavy metals (Rajapaksha et al., 2004; Utgikar et al., 2001). Different microbes interact with metals in a different enzymatically or non-enzymatically way depending upon the microbial ability, gene regulation, protein expression, the type of metal, and the metal's toxicity (Ehrlich, 1997).

The expression of the gene for the fast and for the unspecific transporter may be diminished by mutation and the resulting mutants might be metal-tolerant. The protein families involved in heavy metal transport are as follows (Nies, 1999).

1. ABC transporters which are involved in both uptake and efflux process.
2. P-type proteins that are also involved in both functions.
3. A-type proteins involve in the efflux process.
4. RND family that is involved in the efflux process.
5. HoxN takes part in the uptake process driven by chemiosmotic energy.
6. CHR that is an integral protein membrane, which is involved in chromate transport.
7. MIT is also an integral protein membrane that is involved the uptake of most cations.
8. CDF is involved in the chemiosmotically efflux of zinc, cadmium, cobalt, and iron.

Table 1 summarizes a list of microbes capable of degrading petroleum hydrocarbon components and hydrocarbon derived components in the presence of metal contaminants (Sandrin and Maier, 2003).

Generally, heavy metals exert an inhibitory action on microorganisms by blocking essential functional groups, dislodging essential metal ions or transforming the active conformations of bio-molecules (Saurav and Kannabiran, 2011; Nies et al., 1999). After heavy metals ions entered into the cell, toxicity occurs through numerous biochemical pathways, which can be divided into five categories as follows (Harrison et al., 2007).

Metal	Organic	Lowest metal concentration reported to reduce biodegradation	Microbe studied	Environment	pH	Reference
Cd ²⁺	2,4-DME	0.100 mg/L ^a	Indigenous community	Sediment (microcosm)	6.5	Said and Lewis 1991
Cd ²⁺	2,4-DME	0.629 mg/L ^a	Indigenous community	Aufwuchs ^c (microcosm)	5.6	Said and Lewis 1991
Cd ²⁺	4CP, 3CB, 2,4-D, XYL, IPB, NAPH, BP	< 25.3-50.6 mg/L ^{a,b}	Alcaligenes spp., Pseudomonas spp., Moraxella sp.	Tris-buffered minimal medium plates	7.0	Springael et al. 1993
Cd ²⁺	2,4-D	> 3 mg/L ^a	Alcaligenes eutrophus JMP134	Mineral salts medium	6.0	Roane et al. 2001
Cd ²⁺	2,4-D	24 mg/L ^a	Alcaligenes eutrophus JMP134	Mineral salts medium containing cadmium-resistant isolate	6.0	Roane et al. 2001
Cd ²⁺	2,4-D	0.060 mg/g ^a	Alcaligenes eutrophus JMP134	Soil microcosms	8.2	Roane et al. 2001
Cd ²⁺	2,4-D	0.060 mg/g ^a	Alcaligenes eutrophus JMP134	Field- scale bioreactors	8.2	Roane et al. 2001
Cd ²⁺	PHEN	1 mg/L ^d	Indigenous community	Soil microcosms	7.6	Maslin and Maier 2000
Cd ²⁺	NAPH	1 mg/L ^d	Burkholderia sp.	Dilute mineral salts medium containing 1.4 mM phosphate	6.5	Sandrin et al. 2000
Cd ²⁺	TOL	37 mg/L ^a	Bacillus sp.	Mineral salts medium containing 36 mM phosphate	5.9	Amor et al. 2001
Cd ²⁺	Naphthalene	500 µM	Comamonas testosteroni	Tris buffered MSM	6.5	Douglas et al.
		100µM		PIPES buffered MSM	6.5	
		500 µM(not complete inhibition)		Bushnell-Hass medium	6.5	
Co ²⁺	4CP, 3CB, 2,4-D, XYL, IPB, NAPH, BP	< 13.3-1,330 mg/L ^{a,b}	Alcaligenes spp., Pseudomonas spp., Moraxella sp.	Tris-buffered minimal medium plates	7.0	Springael et al. 1993
Cr ³⁺	2,4-DME	0.177 mg/L ^a	Indigenous community	Aufwuchs ^c (microcosm)	6.1	Said and Lewis 1991
Cr ⁶⁺	4CP, 3CB, 2,4-D, XYL, IPB, NAPH, BP	< 131 mg/L ^{a,b}	Alcaligenes spp., Pseudomonas spp., Moraxella sp.	Tris-buffered minimal medium plates	7.0	Springael et al. 1993
Cr ⁶⁺	Phenol	200mg/ L ^a	Burkholderia cepacia	Minimal medium	7.0	Silva et al.2007

Cu ²⁺	2,4-DME	0.076 mg/L ^a	Indigenous community	Sediment (microcosm)	6.1	Said and Lewis 1991
Cu ²⁺	2,4-DME	0.027 mg/L ^a	Indigenous community	Aufwuchs ^c (microcosm)	5.0	Said and Lewis 1991
Cu ²⁺	4CP, 3CB, 2,4-D, XYL, IPB, NAPH, BP	< 14.3-71.6 mg/L ^{a,b}	Alcaligenes spp., Pseudomonas spp., Moraxella sp.	Tris-buffered minimal medium plates	7.0	Springael et al. 1993
Cu ²⁺	PHB	8 mg/L ^d	Acidovorax delafieldii	Agar plates containing 4.70 mM phosphate	6.9	Birch and Brandl 1996
Cu ²⁺	Crude oil	6.30 mg/L ^a	Pseudomonas sp.	Mineral salts medium containing 31 mM phosphate	7.2	Benka- Coker and Ekundayo 1998
Cu ²⁺	Crude oil	11.25 mg/L ^a	Micrococcus sp.	Mineral salts medium containing 31 mM phosphate	7.2	Benka- Coker and Ekundayo 1998
Cu ²⁺	PH	0.01 mg/L ^a	Acinetobacter calcoaceticus, AH strain	Bioreactor medium containing 0.15 mM phosphate	7.8	Nakamura and Sawada 2000
Hg ²⁺	2,4- DME	0.002 mg/L ^a	Indigenous community	Aufwuchs ^c (microcosm)	6.8	Said and Lewis 1991
Hg ²⁺	4CP, 3CB, 2,4-D, XYL, IPB, NAPH, BP	< 45.2-226 mg/L ^{a,b}	Alcaligenes spp., Pseudomonas spp., Moraxella sp.	Tris-buffered minimal medium plates	7.0	Springael et al. 1993
Mn ²⁺	Crude oil	317.0 mg/L ^a	Pseudomonas sp.	Mineral salts medium containing 31 mM phosphate	7.2	Benka- Coker and Ekundayo 1998
Mn ²⁺	Crude oil	28.2 mg/L ^a	Micrococcus sp.	Mineral salts medium containing 31 mM phosphate	7.2	Benka- Coker and Ekundayo 1998
Ni ²⁺	4CP, 3CB, 2,4-D, XYL, IPB, NAPH, BP	5.18- 10.3 mg/L ^{a,b}	Alcaligenes spp., Pseudomonas spp., Moraxella sp.	Tris-buffered minimal medium plates	7.0	Springael et al. 1993
Ni ²⁺	TOL	20 mg/L ^a	Bacillus sp.	Mineral salts medium containing 36 mM phosphate	5.9	Amor et al. 2001
Pb ²⁺	Crude oil	2.80 mg/L ^a	Pseudomonas sp.	Mineral salts medium containing 31 mM phosphate	7.2	Benka- Coker and Ekundayo 1998
Pb ²⁺	Crude oil	1.41 mg/L ^a	Micrococcus sp.	Mineral salts medium containing 31 mM phosphate	7.2	Benka- Coker and Ekundayo 1998

Zn ²⁺	2,4- DME	0.006 mg/L ^a	Indigenous community	Sediment (microcosm)	6.4	Said and Lewis 1991
Zn ²⁺	2,4- DME	0.041 mg/L ^a	Indigenous community	Aufwuchsc (microcosm)	5.6	Said and Lewis 1991
Zn ²⁺	4CP, 3CB, 2,4-D, XYL, IPB, NAPH, BP	< 29.5- 736 mg/L ^{a,b}	Alcaligenes spp., Pseudomonas spp., Moraxella sp.	Tris-buffered minimal medium plates	7.0	Springael et al. 1993
Zn ²⁺	PH	10 mg/L ^a	Acinetobacter calcoaceticus, AH strain	Bioreactor medium containing 0.15 mM phosphate	7.8	Nakamura and Sawada 2000
Zn ²⁺	Crude oil	0.43 mg/L ^a	Pseudomonas sp.	Mineral salts medium containing 31 mM phosphate	7.2	Benka- Coker and Ekundayo 1998
Zn ²⁺	Crude oil	0.46 mg/L ^a	Micrococcus sp.	Mineral salts medium containing 31 mM phosphate	7.2	Benka- Coker and Ekundayo 1998
Zn ²⁺	TOL	2.8 mg/L ^a	Bacillus sp.	Mineral salts medium containing 36 mM phosphate	5.9	Amor et al. 2001

BP:biphenyl; IPB: isopropylbenzene; MTC: maximum tolerated concentration; TOL: toluene; XYL: xylene. ^aValue represents total metal added to system. ^bValue represents minimum inhibitory concentration (MIC) calculated by multiplying MTC by a factor of 2.25. MIC = MTC × 2.25. ^cFloating algal mats. ^dValue represents solution phase concentration of metal present in system.

Table 1. Microbes capable of degrading petroleum hydrocarbon components in the presence of metal contaminants (Sandrin and Maier, 2003).

1. Toxic metal species can bind to proteins in lieu of essential inorganic ions, thereby altering the biological function of the target molecule.
2. Heavy metals tend to react with thiol (SH) or disulfide groups destroying the biological function of proteins that contain sensitive S groups. By binding to these groups, metals can inhibit the functions or activity of many sensitive enzymes.
3. Certain transition metals can participate in catalytic reactions, known as Fenton-type reactions that produce reactive oxygen species (ROS). Collectively, these reactions place the cell in a state of oxidative stress, and increased levels of ROS damage DNA, lipids, and proteins through a range of biochemical routes,
4. The transporter-mediated uptake of toxic metal species might interfere with the normal transport of essential substrates owing to competitive inhibition.
5. Some metal oxyanions are reduced by the oxidoreductase DsbB, which draws electrons from the bacterial transport chain through the quinone pool. In fact, certain toxic metal species starve microbial cells by indirectly siphoning electrons from the respiratory chain.

It has been found that heavy metals greatly affect the biotransformation or biodegradation of organic pollutants by interacting with microbial enzymes or their cell walls, by interfering

with the microbial general metabolism or by interrupting the functioning of the enzymes participating in the degradation of hydrocarbons. (Beveridge and Murray, 1976; Sobolev and Begonia, 2008; Merroun, 2007; Haferburg and Kothe, 2007; Kim, 1985; Sandri and Maier, 2003; Wataha et al., 1994). Mergeay et al. (1985) found a plasmid bound metal resistance in *Alcaligenes eutrophus* that is a gram negative bacterium, which was considered to be the first gram negative bacterium showing plasmid bound resistance against cadmium and zinc. Said and Lewis (1991) claimed that heavy metal ions exert a stronger inhibitory effect on biodegradation than the inhibitory effect caused by high concentrations of toxic organic compounds. Therefore, it is very important to include the effect of heavy metal concentration in bioremediation studies. For instance, during the evaluation of metal impacted biological systems, it is critical to monitor the metal speciation to understand the microbial response to heavy metal stress (Sandrin and Maier, 2003).

The extent of the metal stress on the microbes can only be determined if chemical speciation analysis is performed during the degradation studies. Values of the minimum inhibitory concentration (MIC) can be misinterpreted for a metal if the reported metal concentration is not in the solution phase. In most standard media, it is found that the metal ions chelate or bind with organic entities of the media leading to the false interpretation of the MICs (Angle and Chaney, 1989). Silva et al. (2007) reported the high metal tolerance of the strain *B. cepacia*-JT50 for chromium and mercury, however a reduction in the degradation efficiency was observed at high metal concentrations. The study was carried out using minimal medium at pH 7.0 in which different chromium and mercury concentrations were incorporated; however it is not clear from this study the actual metal speciation of the mercury and chromium ions during testing. Kuo and Genthner (1996) showed that the anaerobic degradation or biotransformation of phenol, benzoate and their chloro derivatives 2-chlorophenol and 3-chlorobenzoate respectively slowed down in the presence of added heavy metal ions Cd(II), Cu(II), Cr(VI), Hg(II), however different metals showed different toxicity patterns to the organisms. The consortia showed susceptibility towards some specific metals for example 3-chloro benzoate degradation was most sensitive to Cd(II) and Cr(VI), degradation of phenol and benzoate was sensitive to Cu(II) and Hg(II), however enhanced biodegradation of phenol and benzoate was observed on the addition of small amount of Cr(VI). Cu(II) was found to enhance the degradation rate of 2-chlorophenol and interestingly Hg(II) 1.0 to 2.0 ppm was found to enhance the degradation rate of 2-chlorophenol and 3-chlorobenzoate after an extended acclimation period indicating the adaptation of the consortia to mercury.

Heavy metals are increasingly found in microbial habitats due to natural and industrial processes. Thus, microorganisms have been exposed to metal polluted environments for long time, which has forced microorganisms to develop several mechanisms to tolerate, resist or detoxify these metal ions by efflux, complexation, or reduction of metal ions, to use them as terminal electron acceptors in anaerobic respiration (Dhanjal and Cameotra, 2010; Jaysankar et al., 2008; Sobolev and Begonia 2008; El-Deeb, B., 2009; Johncy et al., 2010; Chaalal et al., 2005; Patel et al., 2006).

Silver (1996) proposed four mechanisms for bacterial metal resistance as follows.

1. Reduced uptake to keep the toxic metal ion out of the cell.
2. Highly specific efflux pumping (i.e. removing toxic ions out of cell)

3. Intra and extracellular sequestration
4. Enzymatic detoxification such as converting a toxic metal ions into less toxic forms

Toxicity of metals depends upon the extent to which it penetrates the microorganism cells. There are several variables influencing the microbial resistance to heavy metals. These variables are briefly discussed in the subsequent paragraphs.

- a. Effect of contact time - Contact time between microorganisms-pollutant influences the adaptation process of micro-organisms. Depending on the residence time of microorganisms in the metal environment, it has been found that 10 to 90% of the microorganisms become metal resistant. Doelman and Haanstra (1979) reported that a strain became lead tolerant after 2 years of contact with the metal, Kuo and Genthner (1996) also reported the adaptation of bacterial consortia to Cd(II), Cu(II), Cr(VI) and Hg(II) after a comparative extended adaptation period. Yeom and Yoo (2002) evaluated the enzymatic adaptation of *Alcaligenes Xylosoxidans* Y234 to heavy metal during degradation studies of benzene and toluene in the presence of heavy metal ions. They established that co-culturing is an effective method to reduce the metal inhibition effect. The reasoning is that, if one inhibitory metal ion to enzyme A is present in a system, then the inoculation of a microorganism which already has enough enzyme A through microbial adaptation or the addition of a compound inducing enzyme A production will offset the inhibition effect.
- b. Effect of cell cycle - Howlett and Avery (1999) investigated the relationship between the cell volume (as the volume of the cells vary during the cell cycle) and the susceptibility to copper toxicity in *Saccharomyces cerevisiae*. The correlation between the cell volume (by examining the forward angle light scatter -FSC) and the cell cycle stage (by examining the DNA content) indicated that the largest (the cells about to undergo mitosis) were most resistant to Cu and the smallest (newly divided cells) cells were also relatively resistant to copper. As the percent cell volume increased beyond 0-2% Cu resistance started decreasing and approach to minimal when the percent cell volume reached to 38-40% of the maximum cell volume. Then the resistance towards copper started increasing with further increase in cell volume and reached to maximum at the stage when cells having 98-100% of the maximum cell volume.
- c. Effect of temperature - Tynecka et al. (1981) observed that the uptake of cadmium (Cd^{2+}) was highly reduced at low temperatures (4°C), while the degradation of hydrocarbons is favored at higher temperatures. However, there are a number of cold adapted microorganisms reported that can efficiently degrade petroleum hydrocarbons at low temperatures (Braddock et al. 1997).
- d. Effect of pH - pH affects metal toxicity to a great extent because metal ions could form complexes with the medium or buffered components or metal ions could precipitate as phosphates or sulphates, especially at pHs ≥ 7.0 . Mergeay et al. (1985) replaced phosphate buffer by tris-HCl to avoid the precipitation of the metal ions. Tynecka et al. (1981) reported that the sensitive and resistant cells do not react in the same way to pH increase. Both types of cells accumulate more cadmium as the pH increases but the pattern of cadmium uptaking was different. Kelly et al. (1999) reported that the pH of the treatment influences the microbial population size, structure, activity, and that a decrease in pH exerts a stress on the soil microbial communities (Pennanen et al. 1996).
- e. Metal tolerant strains - Microorganisms can naturally exhibit the multifunctional properties for hydrocarbon degradation and metal resistance properties or

microorganisms with hydrocarbon degradation capability can be genetically modified for metal resistance or vice-versa. Ueki et al. (2003) identified two vanadium binding proteins and expressed them in *E. coli*, which showed the capability of accumulating twenty times more copper than their control strain. Marine bacteria are also known to have metal resistance capabilities (De et al., 2003). Keramati et al. (2011) isolated multi metal resistant bacteria highly resistant to mercury. El-Deeb (2009) reported a natural strain exhibiting the properties of organic pollutant degradation and tolerance toward heavy metals. Yoon and Pyo (2003) reported the development and characterization of a strain highly effective in degrading phenol and resistant to the effect of heavy metals ions. In order to restricted or minimized the effect of heavy metals on the hydrocarbon degradation capability of microorganisms the following actions are recommended (Riis et al., 2002; Malakul et al., 1998; Babich et al., 1977).

- Application of phosphate or sulfate compounds at the contaminated site to induce the precipitation of heavy metal ions.
- Addition of metal chelators.
- Increasing the pH of the soil. Addition of organic humus forming material

4. Conclusions and recommendations

Although extensive literature is available on petroleum hydrocarbon biodegradation, more research specifically oriented towards the effect of heavy metals on hydrocarbon biodegradation is required, particularly in the areas outline below:

1. Investigation of the nature of microbe interaction with metal ions in a variety of subsurface and sedimentary environments and under a range of terminal electron accepting metabolisms.
2. Development of technologies to evaluate the physical, chemical, and biological interactions of microorganisms with metal ions in the presence of petroleum hydrocarbons.
3. Evaluation of the effect of metal ions on the hydrocarbon degrading microbial physiological activities and responses.
4. Integration of experimental and more advanced computational approaches to better predict and interpret biochemical processes during hydrocarbon degradation in the presence of heavy metal ions.
5. Establishment of the relevant parameters needed for kinetic and thermodynamic computational models focused on microbe/metal interaction during hydrocarbon bioremediation processes.
6. Development of technology to investigate the gene expressed by the microorganism in the presence of heavy metal ions. Development of laboratory bioreactor systems that more accurately reproduce field conditions.

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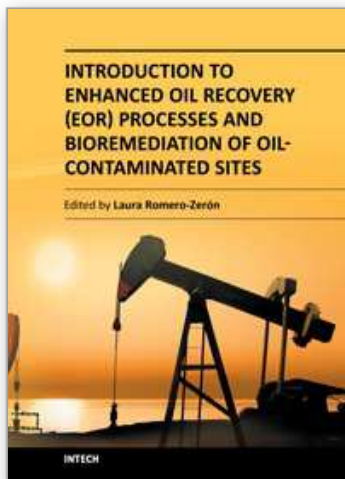
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Introduction to Enhanced Oil Recovery (EOR) Processes and Bioremediation of Oil-Contaminated Sites

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This book offers practical concepts of EOR processes and summarizes the fundamentals of bioremediation of oil-contaminated sites. The first section presents a simplified description of EOR processes to boost the recovery of oil or to displace and produce the significant amounts of oil left behind in the reservoir during or after the course of any primary and secondary recovery process; it highlights the emerging EOR technological trends and the areas that need research and development; while the second section focuses on the use of biotechnology to remediate the inevitable environmental footprint of crude oil production; such is the case of accidental oil spills in marine, river, and land environments. The readers will gain useful and practical insights in these fields.

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