

Comprehensive Perspectives in Bioremediation of Crude Oil Contaminated Environments

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1. Introduction

Bioremediation is a biotechnological approach of rehabilitating areas degraded by pollutants or otherwise damaged through mismanagement of ecosystem. It is the ability of microorganisms to degrade or detoxify organic contaminated areas by transforming undesirable and harmful substances into non-toxic compound (Bioremediation overview, 2003).

Diverse components of crude oil and petroleum such as polycyclic aromatic hydrocarbons (PAHs) have been found in waterways as a result of pollution from industrial effluents and petrochemical products (Beckles, *et al.*, 1998). Petroleum hydrocarbon pollution of the environment may arise from oil well drilling production operations, transportation and storage in the upstream industry, and refining, transportation, and marketing in the downstream industry. Petroleum hydrocarbon pollution could also be from anthropogenic sources (Oberdorster and Cheek, 2000). Some non combusted hydrocarbons escape into the environment during the process of gas flaring. Until recently, the bulk of the associated gas produced during drilling in Nigeria, was flared. Sources of petroleum and its products in the environment will also include accidental spills and from ruptured oil pipelines. Today the international oil and gas-pipelines span several million kilometers and this is growing yearly due to inter-regional trade in petroleum products. Just like any other technical appliance, pipelines are subject to "tear and wear", thus can fail with time (Beller, *et al.*, 1996). Spilled petroleum hydrocarbons in the environment are usually drawn into the soil due to gravity until an impervious horizon is met, for example bedrock, watertight clay or an aquifer.

Poor miscibility of crude oil accounts for accumulation of free oil on the surface of groundwater and this may migrate laterally over a wide distance to pollute other zones very far away from the point of pollution. Industrial and municipal discharges as well as urban run-offs, atmospheric deposition and natural seeps also account for petroleum hydrocarbon pollution of the environment (Baker, 1983). It is worthy of note that groundwater is one of the many media by which human beings, plants and animals come into contact with petroleum hydrocarbon pollution. In the Niger delta area of Nigeria, extensive farm land

has been lost due to contamination with crude oil. Also sources of drinking water and traditional occupation such as fishing and water transportation are greatly affected by crude oil contamination.

Biotechnology is the major reliable method involved in bioremediation and is defined as a set of scientific techniques that utilize living organisms or parts of organisms to make, modify or improve products (which could be plants or animals). It is also the development of specific organisms for specific application or purpose and may include the use of novel technologies such as recombinant DNA, cell fusion and other new bioprocesses (Anon, 1991.) It is also that aspect of biotechnology, which specifically addresses issues in environmental pollution control and remediation (Onwurah, 2000). This goes to say that it involves several disciplines such as biology, agriculture, engineering, health care, economy, mathematics, chemistry and education. It is regarded today as fundamentally an engineering application of microbial ecology (Rittman *et al.*, 1990) and process design. The engineering aspect of environmental biotechnology involves the design/construction of special machines or equipment referred to as reactors or bioreactors. Environmental biotechnology also encompasses quantitative mathematical modeling whereby understanding and control of many inter-related processes become possible. Mathematical modeling technology transcends the boundary of single traditional scientific disciplines and technologies, whereby a logical framework resolves related problems (Ziegler 2005; Onwurah, 2002b). They are tools utilized economically for explaining the cost and effectiveness of different options of clean-up technology and control (Onwurah and Alumanah 2005; Ziegler 2005). One of the greatest challenges to humanity today is the endangering of biota as a result of environmental pollution from crude oil.

To estimate the biological danger of oil after a spill, knowledge of the harmful effects of the components is necessary. In order to obtain or ascertain the effects of such polluting substance, every living being and life function can be considered a potential biomarker or bio-indicator. A biomarker is an organism or part of it, which is used in soliciting the possible harmful effect of a pollutant on the environment or the biota. Biomonitoring or biological monitoring is a promising, reliable means of quantifying the negative effect of an environmental contaminant. In a broad sense, biological markers (biomarkers) are measurement in any biological specimens that will elucidate the relationship between exposure and effect such that adverse effects could be prevented (NRC, 1992). It should be instituted whenever a waste discharge or oil spill has a possible significant harm on the receiving ecosystem. It is preferred to chemical monitoring because the latter does not take into account factors of biological significance such as the combined effects of the contaminants on DNA, protein or membrane. Some of the advantages of biomonitoring include the provision of natural integrating functions in dynamic media such as water and air, possible bioaccumulation of pollutant from 10^3 to 10^6 over the ambient value and/or providing early warning signal to the human population over an impending danger due to a toxic substance. Microorganisms can be used as an indicator organism for toxicity assay or in risk assessment. Tests performed with bacteria are considered to be most reproducible, sensitive, simple, economical and rapid (Mathews, 1980). Some examples include the 'rec-assay' which utilizes *Bacillus subtilis* for detecting hydrophobic substances (hydrocarbons) that are toxic to DNA (Matsui, 1989), *Nitrobacter sp.* which is based on the effect of crude oil on oxidation of nitrite to nitrate (Okpokwasili and Odukuma, 1994), and *Azotobacter sp.* used in evaluating the effect of oil spill in aquatic environment (Onwurah, 1998). Multiple

bioassays that utilize a variety of species can be applied to gain a better understanding of toxicity at a given trophic level and under field condition. Several criteria exist for selecting biomarkers of plant and animal origin. Biomarkers, as fingerprints for identifying mystery oil spills, are now in use and they include steranes, phytanes, and hopanes. Normal hexadecane, an n-alkane found in crude oil is often used because of its low volatility and high hydrophobicity (Foght, *et al.*, 1990). These markers are integral part of crude oil and are not affected or degraded easily by any biological process. Hence they remain as “skeleton” of the crude oil even after a natural degradation has taken place. Steranes in crude oil are derived from the algae or the plant from which the source rock originated, while hopanes are derived from the hopenetrol present in bacteria (Peters and Moldowan, 1993). Hopanes can be used to determine the nature of the source rock that generated a crude oil. Biosensor is a technology that promises to be important in generating future standards regarding both bioavailability and toxicity of any pollutant being released into the environment. Biosensors are usually photo detector systems, which operate on the genes that control luminescence (King *et al.*, 1990). Most of the biosensor tests are not quantitative, but rather can detect the potential activity or presence of an environmental toxicant. Examples includes the Petro-Risk Soil Tests System (DTSC, 1996) used in detecting total petroleum hydrocarbons in a given soil after an oil spill. The test kit uses enzyme-linked immunosorbent assay (ELISA) technology. It involves an antibody with affinity to certain petroleum hydrocarbons. The antibody that does not react with the methanolic extract of the petroleum hydrocarbons or crude oil in the soil sample is detected by a color reaction. The color intensity developed decreases as the hydrocarbon concentration increases. A differential photometer is usually incorporated. Other examples include the Microtox, which utilizes the luminescent bacteria, *Vibrio fischeri* (*Photobacterium phosphoreum*), in monitoring toxicity of petroleum hydrocarbon. The bacterium *Vibrio fischeri* utilizes about 10% of its metabolic energy for bioluminescent activity. The luminescent pathways are linked to cellular respiration whose disruption will change the light output (Ross, 1993), or on the structure-activity relationship of the individual compounds in the crude oil or petroleum (Cronin, and Schultz, 1998).

The issue of crude oil contamination of the environment should be clearly examined and proper solution proffered with respect to the level of contamination and uniqueness of the environment. Thus, this review is set to x-ray how crude oil bioremediation and bio-monitoring will benefit bioremediation scientists and experts.

2. Contamination components in crude oil

Total petroleum hydrocarbons (TPH) comprise a diverse mixture of hydrocarbons that occur at petrochemical sites and storage areas, waste disposal pits, refineries and oil spill sites. According to McElroy *et al* (1989) TPHs are considered persistent hazardous pollutants, and include compounds that can bioconcentrate and bioaccumulate in food chains. Heitkamp and Cerniglia (1988) showed that THPs are acutely toxic. Some such as benzene and benzo[a]pyrene are recognized mutagens (IARC, 2000) and carcinogens Mortelmans *et al* (1986). Since this group includes chemicals that have physical and chemical characteristics that vary in magnitude, TPHs are divided into two categories:

1. Gasoline range organics (GRO) corresponds to small chain alkanes (C6-C10) with low boiling point (60-170°C) such as isopentane, 2,3-dimethyl butane, n-butane and n-

pentane, and volatile aromatic compounds such as the monoaromatic hydrocarbons benzene, toluene, ethylbenzene, and xylenes (BTEX).

2. Diesel range organics (DRO) includes longer chain alkanes (C10-C40) and hydrophobic chemicals such as polycyclic aromatic hydrocarbons (PAH).

Whereas most of these contaminants do have natural sources, concentration and release of contaminants through anthropogenic activities has led to significant contamination of soil and groundwater. Individual contaminants behave differently. Some contaminants such as BTEX compounds are highly mobile in the environment, while others such as PAHs tend to bind strongly to soil particles near the source or remain entrapped within an organic phase, thus immobilizing them and making them difficult to move out of their percolated environments. Since hydrocarbon spills at different sites represent different mixtures, it is very difficult to find a single, efficient method of cleanup. Current treatment techniques usually involve excavation and *ex situ* treatment of the source material and the contaminated soils. However, residual contamination often exceeds regulatory limits by a relatively small margin, and occurs over extensive areas (NRC, 1994). The large volume of soil affected precludes *ex-situ* treatment due to economical constraints and requires the use of relatively inexpensive remediation schemes, such as phytoremediation.

3. Chemistry of petroleum hydrocarbon

Petroleum has been known for several years to occur in surface seepage and was first obtained in pre-Christian times by the Chinese. The modern petroleum industry had its beginnings in Romania and in a well sunk in Pennsylvania by Colonel E. A. Drake in 1859 (Alloway and Ayres, 1993). The basic use of petroleum products was for the replacement of expensive whale oil for lighting. However, today apart from its use as fuel, it also serves as sources for the production of various chemicals.

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. It also contains elements such as nitrogen, sulphur and oxygen, all of which constitute less than 3% (v/v). There are also trace constituents, comprising less than 1% (v/v), including phosphorus and heavy metals such as vanadium and nickel. Crude oil could be classified according to their respective distillation residues as paraffin, naphthenes, or aromatics and based on the relative proportions of the heavy molecular weight constituents as light medium or heavy (<http://www.academicjournals.org/BMBR>). Also, the composition of crude may vary with the location and age of an oil field, and may vary with the depth 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 asphalt residue (Atlas, 1981).

On a structural basis, the hydrocarbons in crude oil are classified as alkanes (normal or iso), cyclo-alkanes, and aromatics (fig 1). 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., henyalkanes, account for the high numbers of hydrocarbons that occur in crude oil. In

addition, smaller amounts of oxygen - (phenols, naphthenic acids), nitrogen- (pyridine, pyrrole, indole), and sulphur -(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).

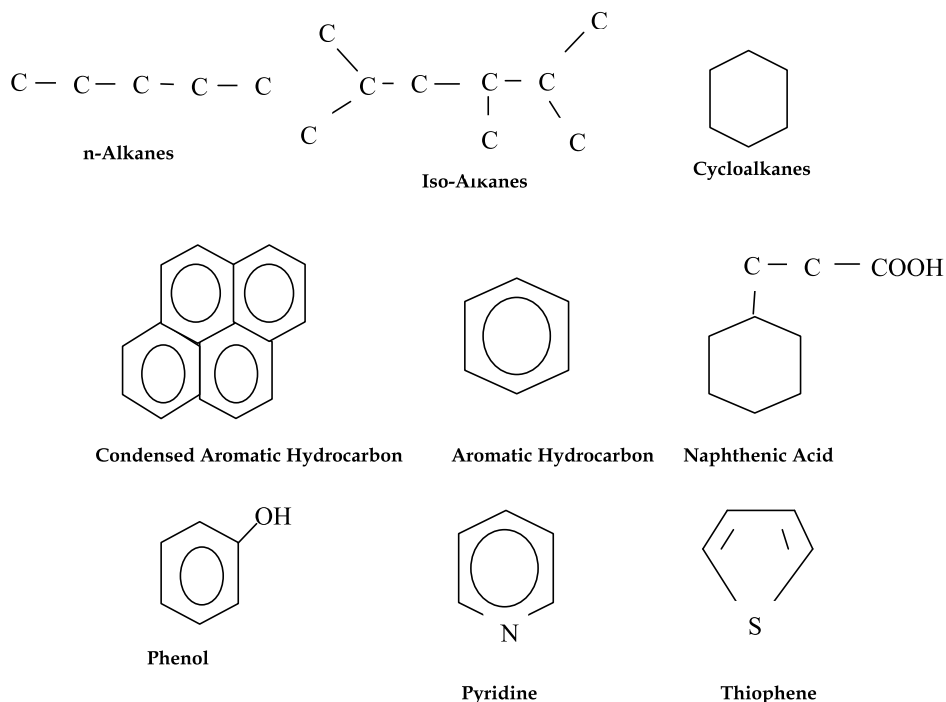


Fig. 1. Structural Classification of some Crude Oil components (Alloway and Ayres, 1993).

4. Effect of crude oil on soil nutrients

There is a direct proportionality between the quantity of oil spillage and accumulation of manganese and ferrous elements. Adam and Ellis (1960) pointed out that there was an accumulation of manganese and ferrous ions to levels which became very toxic to plants. Plant growth in crude oil contaminated soil is adversely affected due to changes in the nutrient status of the soil and disruption of microbial activities.

McGill (1976) noted that a lasting effect of crude oil in soil, eventually result in nutrient supply beneficial to crop production. According to Rowell (1977), the decomposition of spilled crude oil may result in an increased yield of soil nutrients. Plice (1948) also explained that soil polluted with crude oil remained barren for some years (i.e. seven years and above), after which the tested soil was shown to be richer in nutrients than the normal soils in the uncontaminated area. Previous research conducted by different scientists using different crops allow concluding that crude oil spillage on farm land has both positive and negative effect on soil. When a soil is polluted with crude oil, plant growth becomes adversely affected for some time and after a period the hydrocarbons become decomposed

and are converted to soil organic matter which improves the nutrient content of the soil. However, this could take a long period of time under which the soil may remain unproductive for agricultural purposes.

5. Effect of crude oil spillage on germination and growth of crop

The ability of crops to germinate or grow on crude oil polluted soil is dependent on the level of crude oil spillage on soil (Odu, 1972). This means that a high level of crude oil pollution of the soil impairs germination of seedlings.

Rowell (1977) stated that at low level of spillage (e.g. one percent of oil contamination) germination may be delayed due to lack of moisture and hardening of soil structure. Moreover, at high contamination of soil, there may be no germination. Hence seed rotting will take place due to seeping of crude oil into the seeds through the outer integument. The interference of oil to soil air and water is another means of inhibiting seed germination. McGill (1976) noted that the toxic effect of crude oil coupled with poor aeration and the altered wettability of the soil due to oil spillage results in poor seed germination.

According to Baker (1970), spillage on land (soil) causes oil to enter into the leaves of plants and other economic trees through their pores and hampers the process of photosynthesis and evapo-transpiration. The pores of leaves are penetrated by films of oil, which is evidenced by the darkening of leaves as the pore becomes filled with oil. A patch of dark oil cuts sunlight from the leaves and where the shielding of sunlight becomes too much the leaves experience necrosis and the plant eventually dies (Nelson-Smith, 1979).

6. Metal accumulation abilities of plants

Soils contaminated with heavy metals cause several environmental and human health problems, which calls for an effective technological solution. Many affected sites around the world remain contaminated, because it is expensive to clean them up by available technologies. Phytoremediation is considered to be an innovative, economical, and environmentally compatible solution for remediation of heavy metal contaminated sites (Wang *et al.*, 2003; O'Connor *et al.*, 2003). Heavy metals may be bound or accumulated by particular plants, which may increase or decrease the mobility and prevent the leaching of heavy metals into ground water. Growing plants can help to reduce heavy metal pollution. The advantage of this technique is evident as the cost of phytoremediation is much less than the traditional *in situ* and *ex situ* processes; plants can be easily monitored to ensure proper growth; and valuable metals can be reclaimed and reused through phytoremediation. The metals most commonly accumulated in plants are lead, cadmium, zinc, nickel, or radioactive isotopes such as uranium or cobalt (Lombi *et al.*, 2001).

Some plants accumulate heavy metals by transporting the metals and concentrating them into their shoots for harvesting. These groups of plants are known as heavy metal hyperaccumulators. In metal accumulation it is necessary to use plants as *Polygonum hydopiper L.*, *Rumex acetosa L.*, (Wang *et al.*, 2003), *Thlaspi caerulescenes J. Presl*, *Zea mays L.* (Lombi *et al.*, 2001). Hyperaccumulators are used in the removal of metal contaminants since they take up 100 times the concentration of metals over other plants (Cunningham *et al.*, 1995). They accumulate toxic metals through their roots and transport them to the stems.

Most hyperaccumulators could be grown in contaminated soils. The metal could then be recovered and recycled when burned and the ash collected.

According to Peculyte *et al.*, (2006), for a low metal contaminated soil, using maize and vetch plants as metal accumulator, after three weeks of growth, a negative effect on the length of shoots and roots was observed. The biomass of the plant seedlings was significantly smaller in the metal-contaminated soil in comparison to controls. This is due to metal toxicity of the plants by the metal contaminants. Metal accumulation in combination with crude oil in a contaminated site could pose a serious challenge than when contamination is by either the metal or crude oil alone.

7. Crude oil and human health

There is a direct correlation between environmental health and human health. Obviously, human health has been intensively studied for quite long, while environmental health is a recent field. Environmental health is the assessment of the health of individual organisms with a direct correlation of observable changes in the environment. Crude oil pollution has been linked to be the causative effect of many diagnosed diseases. The health problems associated with crude oil spill may be through any or combinations of the following routes: contaminated food and/or water, emission and/or vapours. Toxic components in oil may exert their effects on man through inhibition of protein synthesis, nerve synapse function, and disruption in membrane transport system and damage to plasma membrane (Prescott, *et al.*, 1996). Crude oil hydrocarbons can affect genetic integrity of mutagenesis and impairment of reproductive capacity (Short and Heintz 1997). The risk of drinking water contaminated by crude oil can be extrapolated from its effect on rats that developed hemorrhagic tendencies after exposure to water-soluble components of crude oil (Onwurah, 2002). Volatile components of crude oil after a spill have been implicated in the aggravation of asthma, bronchitis and accelerated aging of the lung (Kaladumo, 1996). Other possible health effects of oil spill can be extrapolated from rats exposed to contaminated sites and these include increased liver, kidney and spleen weights as well as lipid peroxidation and protein oxidation (Anozie and Onwurah, 2001).

8. Toxicity of Nigerian crude oils

Some of the Nigerian crude oils associated with high toxicity level are: Forcados Blend (FB), Bonny Light (BL) and Bonny Medium (BM). A work by Imevbore *et al.* (1987) aimed at determining the toxic effect of the above listed crude oil against *Desmicaris trispinosa* and *Palaemonetes africanus*, in fresh water and brackish water shrimps respectively. The work established that after 96 hours of contact between crude oil and bacteria, the concentration LC₅₀ (crude oil lethal dosage) contained in FB, BL, and BM crude oils ingested by *D. trispinosa* was 2.75, 16.22 and 38.02 µg/l respectively while it was 38.90 and 4.17 µg/l respectively for *P. africanus*. The toxicity could occur at even lower concentrations among species that are more sensitive than those tested such as earthworm. Another demonstration of the toxicity level of the crude oil spill was demonstrated by Ezeala (1987) with *Pistia stratoites* (a fresh water plant) abundant in most fresh water of the oil producing areas in Nigeria. In a simulated condition of very light pollution (0.5 ml m⁻²), *Pistia stratoites* was very susceptible to the crude oil pollution. The magnitude was a reduction in leaf number of about 63%, a loss in the photosynthetic chlorophylls of about 70%, 65% decreases in leaf area

and 80% loss in productivity after 7 weeks of growth period. There are several studies carried out by Nigerian scientists that support the fact that crude oil is indeed toxic. Crude oil toxicity in rats (Anozie and Onwurah, 2001), effects on chlorophyll contents (Ezeonu and Onwurah, 2009) are among many such facts.

9. Bioremediation process

Bioremediation is a natural process that can be harnessed or optimized to enhance the rate at which microbes' biodegrade organic chemicals that have been released into the environment. The rate at which biodegradation can take place may be favoured by providing an optimal living environment for the microbes. Most microbes that degrade petroleum hydrocarbon make use of appropriate levels of oxygen, water, acidity (pH), and nutrients such as nitrogen and phosphorus. When the microbial environment is optimized by having the right amounts of water, air and nutrients and by maintaining proper acidity, biodegradation rates will increase (DOE/PERF, 2002).

The rate-limiting factors that are typically assessed and which are important include oxygen, nutrients, salinity, temperature, pH, and soil moisture content or water content. In order to determine which of the rate-limiting factors may require modification, an initial assessment should be performed (DOE/PERF, 2002).

These rate-limiting factors may vary from medium to medium (i.e. soil, groundwater, fresh water, seawater, wastes, or wetlands) and they may vary from site to site depending upon climate, ecosystem, and human disturbance (DOE/PERF, 2002). Once the rate-limiting factors for biodegradation are assessed, it is important to develop a well thought out plan for correcting them in a manner that will enhance microbial degradation rates without causing more harm to the environment (DOE/PERF, 2002).

10. Degradation of petroleum hydrocarbon

The lower n-alkanes as a structural group are the most biodegradable petroleum hydrocarbons, the C₅ - C₁₀ 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. Similar alkanes in the C₂₀ -C₄₀ 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).

Zengler *et al.*, (1999) emphasized that petroleum biodegradation by bacteria can occur under both oxic and anoxic condition, albeit by the action of different consortia of organisms. According to Holba *et al.*, (1996) crude oil biodegradation at the subsurface occurs primarily under anoxic conditions, mediated by sulfate reducing bacteria or other anaerobes using a variety of other electron acceptor as the oxidant.

Most micro-organisms attack alkanes terminally whereas some perform sub-terminal oxidation. Primary attachment on intact hydrocarbons always requires the action of oxygen. In the case of alkanes, monooxygenase attack results in the production of alcohol. The alcohol product is oxidized 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 or other bypass mechanisms. Therefore, n-alkanes are degraded more readily than iso-alkanes.

Bartha (1986) states that monocyclic compounds such as cyclopentane, cyclohexane, and cycloheptane are degraded like alkanes, since they 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.

Prokaryotes convert aromatic hydrocarbons by an initial dioxygenase attack, to trans-dihydrodiols that are further oxidized to dihydroxy products, e.g., catechol in the case of benzene (Atlas and Bartha, 1998). Eucaryotic micro-organisms use monooxygenases, producing benzene 1,2-oxide from benzene, followed by the addition of water, yielding dihydroxydihydrobenzene (cis-dihydrodiol).

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 direction. 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).

11. Reduction of toxicity by biodegradation of oil

Various investigations (Stewart, 2002; Nakles and Ray, 2002 and Prince 2002), have been carried out to look at possible production of toxic substances as a result of biodegradation. There are several tests available to analyze ecosystem function analysis in terrestrial and aquatic environments such as microbial response (e.g. most probable number), Microtox™ Solid and Liquid phase. In the case of freshwater/marine sites, some of the analysis available include: algal solid phase bioassay; daphnia survival; amphipod survival; gastropod survival; and fish bioassays (Prince, 1993). Prince (1993) reiterated that these tests pointed out that biodegradation results in a decrease, not an increase, in soil toxicity.

In addition, studies performed in Prince William Sound, Svalbard and St. Lawrence indicated that there was no evidence for any significant toxicity associated with bioremediation in marine or freshwater spills (Prince, 2002).

12. Factors affecting degradation of crude oil

12.1 Soil Type: Nakles and Ray (2002), emphatically stated that biodegradation will occur in all soil types even though some may need additives or special care and equipment. Clay soils may need to be amended with bulking agents, in order to improve oxygen transport. Sandy soils may need to be amended with organic matter to improve the soil water holding capacity. A similar study was performed for refined products (jet fuel and heating oil), and the result also indicated that biodegradation rates are lower for sandy soils than for clay and loam soils. The reasons for this may be low water holding capacity, low total organic carbon content, and/or low surface area available for microbial growth in sandy soils. Biodegradation rates for the clay soils were similar or better than those for the loam; however in both studies moisture content was maintained at optimum levels to improve soil tilt. If moisture content in clay soils is not optimized, tilt and thus aeration will be impacted, leading to slower biodegradation rates in these soils (McMillen, 2002).

12.2 Effect of brine on biodegradation: According to Sublette (2001), elevated concentrations of salt may be inhibitory or lethal to many classes of microorganisms because salts can disrupt the osmotic balance of microorganisms in the soil and interfere with their enzyme activity. As a result, soils with high electrical conductivity (EC) values could retard biodegradation rates. McMillen (1994; 1995) stated that if the soil salinity is extremely high, usually, 40mmhos, microbial activity will cease altogether. However, the same basic methodology used for in-situ bioremediation for oil spill, works well to remediate a brine spill if the salt has a pathway out of the site. It has been observed that when combined spills of oil and brine are treated for oil remediation, additional treatments are required.

12.3 Moisture Content /Metal Salt Effect: A correlation between crude oil contamination and metal salt impact on water retention ability of contaminated soil has been established, which consistently affect the activity of microorganisms in the soil microcosm (Ezeonu, 2010). Thus, a soil devoid of moisture may not tolerate the survival and growth of microorganisms. Thus, heavily contaminated soil will not permit the retention of moisture. Only lightly polluted soil will have capillarity for the movement of water within the soil and hence provide moisture for the purpose of degrading the crude oil by the microorganism. Large quantities of metal salt may also become toxic to the biotic components of the contaminated environment and become recalcitrant inhibiting biodegradation of the crude oil contaminated soil. It may also affect the survivability of the microorganisms in the soil. In such a situation additional treatment may be necessary. Combination of phytoremediation and bioremediation may be effective in the treatment of metal salt and crude oil combined contamination of soil.

12.4 Oxygen Content: Biodegradation is effective in an environment with adequate oxygen. Only on rare occasion does biodegradation take place in anoxic environments. Open marine environments have highly oxygenated surface waters offering an excellent condition for biodegradation. The presence of stagnant water along coasts decreases the amount of oxygen in the sand which decreases the rates of degradation. Biodegrading bacteria need oxygen in order to break down oil. Coincidentally, oxygen content is prevalent along marshes and beaches where oil has contaminated the soil.

12.5 pH: Mostly, life thrives at neutrality. As such if the pH is right for the microorganisms in the crude oil spill environment, it will enable them to survive and make use of the carbon source for their growth and metabolism. Thus most heterotrophic bacteria prefer a more neutral pH-value living conditions. Fungi that can also biodegrade hydrocarbons are significantly less sensitive to pH levels and are able to withstand more acidic conditions but do not contribute as much biodegradation as bacteria. As the micro-organism makes use of the carbon source of the crude oil, the quantities of crude oil spilled in such environments are drastically reduced. In order to increase the rate of biodegradation in a given environment the microorganisms must be isolated to determine the optimum pH for their survival and growth.

Sublette (2001) stated that pH is one of several environmental conditions that can serve to inactivate enzymes when levels are not optimal. This has the effect of slowing microbial metabolism (the growth rate), which in turn has a detrimental effect on biodegradation rates.

Most bacteria survive better in the pH range of 6.5 to 8.5, and yeasts and molds thrive better at pH range of 4.5 – 5.3. The optimal pH range for biodegradation is considered to be 6.0 to

8.5. Biodegradation processes may cause the soil pH to drop over time and therefore frequent monitoring of pH is important (Nackles and Ray, 2002).

Lime or limestone when added to soil can increase pH to neutral values; while aluminium sulfate and ferrous sulfate can be added to decrease high pHs (Nakles and Ray, 2002). Lime and sulfur requirements are soil type dependent. Both acidifying and neutralizing amendments should be added to topsoil gradually and thoroughly tilled in. Agricultural experts should be consulted before pH amendment is carried out.

12.6 Duration of biodegradation. The findings of a DOE/PERF (2002) study revealed that the rate at which biodegradation will occur depends on the following variables:

- The type and concentration/mass of petroleum hydrocarbons present,
- The depth of the impacted area,
- Optimization of the biodegradation environmental conditions
- The type of technology used.

12.7 Nitrogen and Phosphorus: These two elements are limiting factors for biodegradation, and their availability to bacteria can affect their ability to consume oil products. The addition of nitrogen and phosphorous increases the proliferation of biodegrading bacteria, resulting in an increase in degradation rates (Rosenberg *et al.* 1996).

12.8 Temperature: Extreme temperatures, too high or too low will naturally not allow the thriving of most microorganisms. Such temperatures will also reduce the action of enzymes in a crude oil spilled environment. As temperature decreases, the rates of degradation decrease probably because of decreased rates of enzymatic activity. However, bacteria populations in colder climates are more adapted to cold temperatures thereby increasing their capability of degradation at near freezing temperatures (Rowland *et al.* 2000). Temperature is perhaps one of the most important factors, affecting both biodegradation and the consistency of the oil spilled. Temperature is a crucial factor in the beginning stages of biodegradation. Approximately 3 months after an oil spill, the rates of degradation at different temperatures are very similar since the remaining compounds in the weathered oil are so difficult to break down. At this point, temperature becomes obsolete (Gibb *et al.* 2001).

Also, as temperature decreases, viscosity of oil increases, becoming thicker. This increase in viscosity leads to the clumping of oil, which facilitates the use of mechanical methods for its removal out of the water, but hinders the use of suction mechanisms. Also, as temperature decreases volatility decreases, making the oil less likely to evaporate (Rowland 2000). Therefore, biodegradation is significantly more successful at warmer temperatures as found in the tropics.

12.9 Seasonal Effects: Nakles and Ray (2002) stated that bioremediation can be initiated at any time of the year, but consideration should be given to the potential effect of climate on the results. According to Nakles and Ray (2002), rainy seasons may cause excessive runoff, while drought or seasonally dry conditions may result in the need for irrigation. In addition, temperature can have an effect, since excessively cold or hot temperatures can slow biodegradation rates. For this reason, bioremediation projects are often initiated during the "growing season" for agricultural crops.

12.10 Effect of nutrient on bioremediation-treatment: Fertilizer should be added gradually to the impacted soil zone in order to avoid excessively high pH and high concentrations of nitrogen that might be toxic to soil microbes (Prince and McMillen, 2002). Most of the time, nutrients have to be added to the impacted soil in order to enhance microbial growth. The two most important nutrients that need to be added are nitrogen and phosphorus. In slightly alkaline soils, organic nitrogen source (such as manure) can cause accumulation of nitrites that are toxic to microbes.

Research has shown that the use of specialty fertilizers such as oil soluble and slow-release fertilizers yield similar biodegradation results (Nakles and Loehr, 2002).

Prince and McMillen (2002) explained that manure can be used as a fertilizer in bioremediation, but consideration in regards to odour and nitrite accumulation should not be overlooked.

Another consideration in applying manure is that its nitrogen and phosphorus levels are not known. It is known however, that manure does supply trace nutrients and improves the soil structure, thereby stimulating microbial growth (Sublette, 2001). Manure is usually added as a bulking agent, not as the main source of fertilizer.

12.11 Molecular Weight of Oil Components: Components that are low in molecular weight such as the aliphatic hydrocarbons tend to be degraded first, leaving behind the much larger molecules (aromatic hydrocarbon) which take much longer to break down. The lighter carbon components of the crude oil are also less viscous and can easily degrade and become volatile when acted upon by weather and environmental elements. This trend indicates the presence of biodegradation by microbial bacteria who cannot break down the larger oil compounds left after the initial phases of degradation (Ezra *et al.*, 2000).

12.12 Enzymes and surfactants in bioremediation: Sublette (2001) pointed out that enzymes are protein catalysts that are responsible for driving almost all of the chemical reactions within a cell. Enzymes act as efficient catalysts and alter the reaction mechanism of a cell in such a way as to lower the energy of activation for the overall reaction (Sublette, 2001). The quantity of enzymes produced by the bacterial or fungi in a crude oil contaminated environment will determine the duration of bioremediation in such an environment. Enzymes are subject to inactivation by a variety of environmental conditions including: heat, adverse pH, salt, strong oxidizing or reducing agents, organic chemicals and detergents or surfactants (Sublette, 2001). More to this is the fact that there are commercial 'enzyme' products in the market that can be used effectively for biodegradation, but there are little of any data that prove their cost-effectiveness in bioremediation projects (Sublette, 2001).

Surfactants have a micelle action such as observed in detergents when washing out dirt and stains from fabrics. Surfactants dislodge the oil from the soil particle making it possible for the oil to be washed off by rain or acted upon easily by microorganisms. Sublette (2001) further explained that the use of surfactants in soil to break up oil is not generally recommended because they can potentially interfere with cell membranes and enzymes, which in turn can reduce biodegradation rates. Surfactants should be pre-screened for toxicity to indigenous microbes. Dispersants, a type of surfactant, has been used successfully in breaking up marine oil spills (Sublette, 2001).

12.13 Frequency of Oil Exposure: Bacterial populations that are frequently exposed to oil spills are very likely to display higher degradation rates than populations being exposed for the first time. Repetitively exposed bacterial populations become acclimated to the presence of oil and are capable of degrading it more successfully (Rowland *et al.* 2000). Exposures of the crude oil help also to increase the surface area to enable weathering and activities of microorganism. As a result of these many weathering processes, oil spilled in marine environments rapidly loses its original properties and breaks down into different hydrocarbon fractions. These fractions have different chemical compositions and exist in different forms that can be further dispersed and degraded. After their initial, rapid transformation into these fractions, the degradation rates of the oil products slow down. In the final phase of the transformation, the compounds are completely converted into carbon dioxide and water. It is possible then for marine environments to naturally cleanse themselves in time, as long as the amount of toxic chemicals does not exceed a certain threshold (Patin, 1999). Figure 2 presents a summary of the routes of crude oil degradation in marine environments.

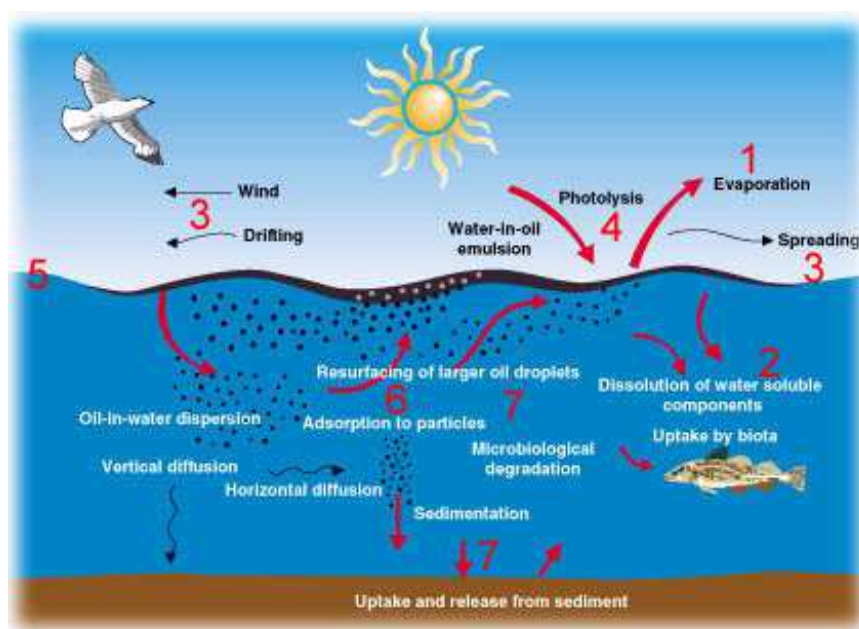


Fig. 2. Routes of Crude oil degradation. Adapted from Patin, 1999.

13. Factors limiting petroleum hydrocarbon biodegradation

Most crude oil polluted sites are not successfully treated at a given period of time due to environmental as well as inherent negative quality of the crude oil itself. The toxicity of the crude oil is an inherent factor that limits its breakdown since it inhibits the thriving of biodegraders. The aggregation of crude oil in piles, blocks or slurry also limits the action of environmental elements in its biodegradation. Successful application of bioremediation technology to contaminated systems requires knowledge of the characteristics of the site and

the parameters that affect the microbial biodegradation of pollutants (Sabate *et al.*, 2004). A number of limiting factors however, have been identified to affect the biodegradation of petroleum hydrocarbons. Some of these factors are listed in Table 1.

Limiting Factor	Explanations or Examples
Petroleum hydrocarbon composition (PHC)	Structure, amount, toxicity
Physical State	Aggregation, Spreading, dispersion, adsorption
Weathering	Evaporation, Photo-oxidation
Water Potential	Osmotic and matrix forces, exclusion of water from hydrophobic aggregates.
Temperature	Influence on evaporation and degradation rate.
Oxidant	O ₂ required to initiate oxidation, NO ₃ or SO ₄ ²⁻ to sustain, PHC biodegradation.
Mineral nutrients	N, P, Fe may be limiting.
Reaction	Low pH may be limiting.
Microorganisms	PHC degraders may be absent or low in numbers.

Source: Bartha 1986.

Table 1. Some common factors limiting petroleum hydrocarbon biodegradation.

The composition of the oil pollutant to a great extent influences the rate of its biodegradation. Kerosene, for example, consists mainly of medium chain alkanes which under suitable condition, achieves total biodegradation. 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, 1986). Okoh (2002) explained that the composition and inherent biodegradability of the petroleum hydrocarbon pollutant, therefore, is the first and most important to be evaluated. Hence the heavier crude oils generally contain more degraders; therefore more petroleum hydrocarbon degraders could be isolated in heavier crude oils than lighter ones. 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 concentration of 0.4 and 0.6% (w/v) oil. In another report, Rahman *et al.* (2002) noted that the percentage of degradation by the mixed bacterial consortium decreased from 78% to 52% as the concentration of crude oil was increased from 1 to 10%. Thouand *et al.*, (1999) also stated that in some situations microbial biomass will be required only to a particular threshold, enough to produce the appropriate enzyme system that carry through the degradation process even when biomass production had ceased.

Recent studies have reported that photo-oxidation increases the biodegradability of petroleum hydrocarbon by increasing its bioavailability and thus enhancing microbial activities (Maki *et al.*, 2005). In a related study as reported by Trindade *et al.*, (2005) on assessing the bioremediation efficiency of a weathered and recently contaminated soil in Brazil, the authors reported low biodegradation efficiencies in the weathered soil contaminated with a high crude oil concentration compared to recently contaminated soil. Also, both soils (weathered and recently contaminated) submitted to bioaugmentation and

biostimulation techniques presented biodegradation efficiencies approximately twice higher than the ones without natural attenuation.

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 microbial milieu. Ambient temperature of an environment affects both the properties of the spilled oil and the activity or population of microorganisms (Venosa and Zhu, 2003). According to Atlas (1981), at low temperatures, the viscosity of the oil increases, while the volatility of toxic low-molecular-weight hydrocarbons is reduced, delaying the onset of biodegradation. Temperature also affects the solubility of hydrocarbons (Foght *et al.*, 1996). Although hydrocarbon biodegradation can occur over a wide range of temperatures, the rate of biodegradation generally decreases with decreasing temperature. Bossert and Bartha (1984), Cooney (1984), variously observed that the highest degradation rates generally occur in the range of 30-40°C in soil environments, 20-30°C in some freshwater environments, and 15-20°C in marine environments.

Inadequate mineral nutrient, especially nitrogen, and phosphorus, often limits the growth of hydrocarbon utilisers in water and soils. Iron was reported to be limiting only in clean, offshore seawater (Swannell *et al.*, 1996). Cooney (1984) observed that nutrients are very important ingredients for successful, biodegradation of hydrocarbon pollutants, especially nitrogen, phosphorus and in some cases iron. Depending on the nature of the impacted environment, some of these nutrients could become limiting thus affecting the biodegradation processes. When a major oil spill occurs in marine and freshwater environments, the supply of carbon is dramatically increased and the availability of nitrogen and phosphorus generally becomes the limiting factor for oil degradation (Atlas, 1984). According to Okolo *et al.* (2005), using poultry manure as organic fertilizer in contaminated soil increases biodegradation. Biodegradation was enhanced in the presence of poultry manure alone, but the extent of biodegradation was influenced by the incorporation of alternate carbon substrates or surfactants. Hence the addition of nutrients is necessary to enhance the biodegradation of oil pollutants (Choi *et al.*, 2002; Kim *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 Nitrogen, Phosphorus and Potassium (NPK) on the biodegradation of hydrocarbons (Oudot *et al.*, 1998; Chaineau *et al.*, 2005) and more especially the degradation of the aromatic compounds.

14. Fate of contaminants in soil systems

Organic contaminants can reach the groundwater zone in dissolved form mixed with water or as organic liquid phases that may be immiscible in water. According to Hall and Quam (1976), contaminants travel with the soil moisture and are retarded in their migration by various factors. One of the most important factors in contamination of water by petroleum products is the extremely low concentration of the product that can give rise to objectionable tastes and odour. The major aspects of contamination can be broadly classified into:

1. The formation of surface films and emulsions and
2. The solubility in water of certain petroleum products.

The problems associated with surface films are minimized by the ability of aquifers to absorb much of the product. However, this phenomenon magnifies the problems associated

with the solubility components of the product, since hydrocarbons held in this manner are subject to leaching as water passes over them. Surface films may affect the aesthetics and interfere with treatment or industrial processes. They may also be toxic to animal or plant life if they emerge into surface waters.

Hall and Quam (1976) further stated that, the water soluble components of petroleum products that give rise to taste and odour problems are the aromatic and aliphatic hydrocarbons. Phenols and cresols are examples of these compounds and generate taste and odours at concentrations as low as 0.01 mg/L. Hence, when chlorine is added to drinking water, as in most municipal water supplies, it reacts with phenols to form chlorophenols, which have objectionable taste and odours at concentrations as low as 0.001mg/L. Therefore, very small quantities of hydrocarbons can cause widespread contamination of water resources.

15. Transport and distribution of soil contaminants

The properties that enhance the transport and transformation process for organic dissolved contaminants such as crude oil can be classified into:

- a. **Physical Processes** e.g. advection, dispersion and volatilization, adsorption and ion-cation exchange.
- b. **Chemical Processes** e.g. ionization, hydrolysis, oxidation-reduction and complexation.
- c. **Biological processes** e.g. bioaccumulation and biodegradation (Mackay and Roberts, 1985).

However, Mackay and Roberts (1985) explained that the migration of an immiscible organic liquid phase is governed largely by its density, viscosity, and surface-wetting properties. Density differences of about 1% are known to influence fluid movement significantly. Organic liquids less dense than water floats and spreads across the water table, and organic liquids more dense than water (e.g. halogenated hydrocarbon) sink through water and plummet through sand and gravel aquifers to the underlying aquitard (relatively impermeable layer) if present. Method of movements of crude oil and other liquid contaminants are as follows:

15.1 Advection, dispersion, and volatilization

According to Mackay and Roberts (1985), in sand and gravel aquifers, the dominant factor in the migration of a dissolved contaminant is advection, the process by which solutes are transported by the bulk motion of flowing groundwater. Groundwater velocity ranges between 1 and 1000 m/year. So that dissolved contaminants spread as they move with the ground water. Dispersion results from two basic processes: molecular diffusion in solution and mechanical mixing. The process results in an overall net flux of solutes from zone of high concentration to a zone of lower concentration. Diffusion in solution is the process whereby ionic or molecular constituents move under the influence of their kinetic activity in the direction of their concentration gradient.

Volatilization refers to the process of pollution transfer from soil to air. It is a form of diffusion that takes place by the movement of molecules or ions from a region of high concentration to a region of low concentration. Volatilization is an extremely important

pathway for many organic chemicals; while most ionic substances are usually considered to be non volatile.

15.2 Adsorption and non-cation exchange

Adsorption is a common phenomenon in all viscous substances. Crude oil is highly viscous with a little percentage of volatiles. Adsorption of crude oil has to do with adhesion of pollutant ions or molecules of crude oil to the surface or soil solids, causing an increase in the crude oil concentrations on the soil surface over the concentration present in the soil moisture. Adsorption occurs as a result of a variety of processes with a variety of mechanisms, and some processes may cause an increase of pollutant concentration within the soil solids not merely on the soil surface. Mackay and Roberts (1985) illustrated that adsorption can drastically retard the migration of pollutants in soils; therefore, knowledge of this process is of importance when dealing with contaminant transport in soil and groundwater. For organic compounds, it appears that partitioning between water and the organic compound content of soil is the most important adsorption mechanism.

16. Biomonitoring in crude oil pollution

In a broad sense, biological monitoring involve any component that make use of living organisms, whole or part as well as biological systems to detect any harmful, toxic or deleterious change in the environment especially with the aim of detecting deleterious, harmful or toxic substances in that environment. There are various components employed in biomonitoring of contaminants in the environment. They include biomarkers (biological markers) biosensors and many others. Biomonitoring or biological monitoring is a promising, reliable means of quantifying the negative effect of an environmental contaminant.

16.1 Biological Markers: A biomarker is an organism or part of it, which is used to establish the possible harmful effect of a pollutant on the environment or the biota (Onwurah, *et al.*, 2007). Biological markers (biomarkers) are measurements taken from any biological specimen that will elucidate the relationship between exposure and effect such that adverse effects could be prevented (NRC, 1992). The use of chlorophyll production in *Zea mays* to estimate deleterious effect of crude oil contaminants on soils is a typical plant biomarker of crude oil pollution (Ezeonu and Onwurah, 2009). When a contaminant interacts with an organism, substances like enzymes are generated as a response. Thus, measuring such substances in fluids and tissue can provide an indication or "marker" of contaminant exposure and biological effects resulting from the exposure.

The term biomarker includes any such measurement that indicates an interaction between an environmental hazard and biological system (NRC, 1989). It should be instituted whenever a waste discharge has a possible significant harm on the receiving ecosystem. It is preferred to chemical monitoring because the latter does not take into account factors of biological significance such as combined effects of the contaminants on DNA, protein or membrane. Onwurah *et al.* (2007) stated that some of the advantages of biomonitoring include the provision of natural integrating functions in dynamic media such as water and air, possible bioaccumulation of pollutant from values of 10^3 to 10^6 over the ambient value and/or providing early warning signal to the human population over an impending danger

due to a toxic substance. Microorganisms can be used as an indicator organism for toxicity assay or in risk assessment. Tests performed with bacteria are considered to be most reproducible, sensitive, simple, economic and rapid (Matthew, 1980). Table 2 provides an outline of common biomarkers and their application.

Biomarker type	Uses	Reference
'Rec-assay' utilizes <i>Bacillus subtilis</i>	Detection of hydrophobic substances (hydrocarbons) toxic to DNA	Matsui, 1989
Chlorophyll content <i>Zea mays</i> L	Detection of level of hydrocarbon contamination of agricultural soil	Ezeonu and Onwurah, 2009
Sensitivity of <i>Nitrobacter sp</i>	Based on the effect of crude oil on oxidation of nitrite to nitrate	Okpokwasili and Odukuma, 1994
<i>Azotobacter sp</i>	Used in evaluating the effect of oil spill in aquatic environment	Onwurah, 1998
Algae/ plant steranes and Bacteria hopanes	Steranes formed as components of crude oil and hopanes used to determine the source rock that generated a crude oil	Peters and Moldown, 1993
Ethoxyresorufin-O-deethylase (EROD) in fish in-vivo	Indicates exposure of fish to planar halogenated hydrocarbons (PAHs) by receptor-mediated induction of cytochrome P-450 dependent monooxygenase exposed to PAHs and similar contaminants	Bucheli and Fent, 1995 Stegeman and Hahn, 1994

Table 2. Biomarkers and their applications

16.2 Biosensor

A biosensor is an analytical device consisting of a biocatalyst (enzyme, cell or tissue) and a transducer, which can convert a biological or biochemical signal or response into a quantifiable electrical signal (Wilson and Walker, 1994). A biosensor could be divided into two component analytical devices comprising of a biological recognition element that outputs a measurable signal to an interfaced transducer (Ripp *et al.*, 2010). Biorecognition typically relies on enzymes, whole cells, antibodies, or nucleic acids, whereas signal transduction exploits electrochemical (amperometric, chronoamperometric, potentiometric, field-effect transistors, conductometric, capacitative), optical (absorbance, reflectance, luminescence, chemiluminiscense, bioluminescence, fluorescence, refractive index, light scattering), piezoelectric (mass sensitive quartz crystal microbalance), magnetic, or thermal (thermistor, pyroelectric) interfaces (Ripp *et al.*, 2010). The biocatalyst component of most biosensors is immobilized on to a membrane or within a gel, such that the biocatalyst is held in intimate contact with the transducer, and may be reused. Biosensors are already of major commercial importance and their significance is likely to increase as the technology develops (Wilson and Walker, 1994). Biosensors are still emerging biotechnology for the future in environmental biomonitoring since they have specific limitations. Biosensors on a general sense are often employed for continuous monitoring of environmental contamination or as bioremediation process monitoring and biocontrol tools to provide informational data on what contaminants are present, where they are located, and a very sensitive and accurate evaluation of their concentrations in terms of bioavailability. Ripp *et*

al. (2010) explained that bioavailability measurements are central to environmental monitoring as well as risk assessment because they indicate the biological effect of the chemical, whether toxic, cytotoxic, genotoxic, mutagenic, carcinogenic, or endocrine disrupting, rather than mere chemical presence as is achieved with analytical instruments. As the name suggest they are biological instruments that detect and signal the presence of harmful contaminants in the environment. There are different types based on the biological components on which their sensitivities are based. Some of them, though not exhaustive are presented in Figure 3.

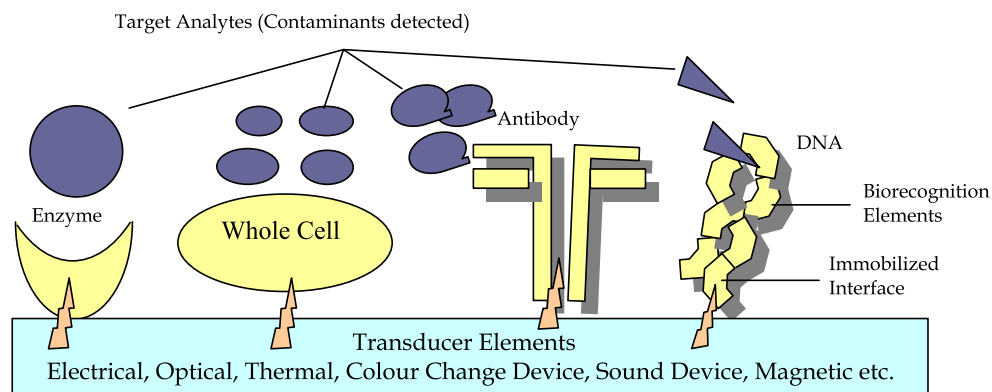


Fig. 3. Anatomy of a Biosensor. The interaction between the target analyte and the biorecognition element creates a signaling event detectable by the interfaced transducer element. Modified from source: Ripp *et al.* (2010).

16.3 Enzyme-based biosensors

Leyland Clark in the 1960s used an enzyme biosensor which consists of glucose oxidase enzyme immobilized on an oxygen electrode for blood glucose sensing. This historical application of enzyme based biosensor has found a world wide lucrative application in especially medical diagnosis. Nevertheless, enzymes based biosensor gradually gained application in environmental monitoring. According to Ripp *et al.* (2010) enzymes act as organic catalysts, mediating the reactions that convert substrate into product. Since enzymes are highly specific for their particular substrate, the simplest and most selective enzyme based biosensors merely monitor enzyme activity directly in the presence of the substrate. A good example of enzyme base biosensor in the oil industry is Ethoxyresorufin-O-deethylase (EROD) in fish in-vivo. This enzyme based biosensor (Bucheli and Fent, 1995, Stegeman and Hahn, 1994) indicates exposure of fish to planar halogenated hydrocarbons (PAHs) by receptor-mediated induction of cytochrome P-450 dependent monoxygenase exposed to PAHs and similar contaminants.

Various immobilization techniques are adopted in the attachment of the enzyme to the transducing element (Lojou and Bianco, 2006) they include: adsorption, covalent attachment, entrapment in polymeric matrices such as sol-gels or Langmuir-Blodgett films, or direct cross-linking using polymer networks or antibody/enzyme conjugates. Immobilization provides the biosensor longevity and with recent integration of redox active carbon-based nanomaterials (nanofibers, nanotubes, nanowires, and nanoparticles) as

transducers and their unique ability to interact with biological material, a promising advancement in enzyme biosensor design and sensitivity is in sight. When devices such as these are developed specifically for crude oil detection, it will be easier to monitor crude oil pollutants in drinking water and agricultural products.

Optical transducers (absorption, reflectance, luminescence, chemiluminescence, evanescent wave, surface plasma resonance) are also commonly employed in enzyme based biosensor (Ripp *et al.*, 2010).

16.4 Antibody-based biosensors (immunosensors)

These types of biosensors make use of antibodies as recognition elements (immunosensors). They are used widely as environmental monitors because antibodies are highly specific, versatile, and bind stably and strongly to target analytes (antigens) (Ripp *et al.*, 2010). Antibodies can be highly effective detectors for environmental contaminants, and advancements in techniques such as phage display for the preparation and selection of recombinant antibodies with novel binding properties assures their continued environmental application. Perhaps the best introduction to antibody-based biosensing is the Automated Water Analyzer Computer Supported System (AWACSS) which is an environmental monitoring system developed for remote, unattended, and continuous detection of organic pollutants for water quality control (Tschmelak *et al.*, 2005). AWACSS uses an optical evanescent wave transducer and fluorescently labelled polyclonal antibodies for multiplexed detection of targeted groups of contaminants, including endocrine disruptors, pesticides, industrial chemicals, pharmaceuticals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and other priority pollutants, without requiring sample pre-processing. Antibody binding to a target sample analyte occurs in a short 5 minute preincubation step, followed by microfluidic pumping of the sample over the transducer element, which consists of an optical waveguide chip impregnated with 32 separate wells of immobilized antigen derivatives (Ripp *et al.*, 2010). As the antibody/analyte complexes flow through these wells, only antibodies with free binding sites can attach to the well surface (in what is referred to as a binding inhibition assay). Thus, antibodies with both of their binding sites bound with analyte will not attach to the surface and will pass through the detector. A semiconductor laser then excites the fluorophore label of bound antibodies, allowing for their quantification, with high fluorescence signals indicating high analyte concentrations. A fibre optic array tied to each well permits separation and identification of signals by the well, thereby yielding a simultaneous measurement of up to 32 different sample contaminants. The instrument has been used for groundwater, wastewater, surface water, and sediment sample testing with detection limits for most analyte in the ng/L range within assay times of approximately 18 minutes (Ripp *et al.*, 2010). Another design by Glass *et al.*, (2004), similar to the above but less refined benchtop flow-through immunosensor (KinExA) demonstrated to detect analytes successively based on a replaceable flow cell containing fluorescently labeled antibody. Their time of assay was approximately 26 minutes, with detection limits at picomolar concentrations.

Although not as elaborate as the AWACSS, a multitude of other antibody-based biosensors have been applied as environmental monitors, traditionally serving as biosensors for pesticides and herbicides, but their target analytes have broadened considerably over the

past several years to include heavy metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), explosives (TNT and RDX), phenols, toxins such as microcystin, pharmaceutical compounds, and endocrine disruptors (Ferre *et al.*, 2007).

16.5 DNA-based biosensors

The principle underlying the DNA based biosensor is the ability of a transducer to monitor a change in the nucleic acid's structure occurring after exposure to a target chemical. These structural changes are brought on either by the mutagenic nature of the chemical, resulting in mutations, intercalations, and/or strand breaks, or by the chemical's ability to covalently or non-covalently attach to the nucleic acid (Ripp *et al.*, 2010). Immobilizing the nucleic acid as a recognition layer on the transducer surface forms the biosensor, and detection of the chemically induced nucleic acid conformational change is then typically achieved electrochemically (i.e., a change in the current) or less so through optical or other means (Fojta, 2002).

Nucleic acid biosensors are generally nonselective and provide an overall indication of a potentially harmful (genotoxic, carcinogenic, cytotoxic) chemical or chemical mix in the test environment and, depending on the biosensor format, an estimate of concentration. Bagni *et al.* (2005) illustrated a conventional DNA biosensor which was used to screen soil samples for genotoxic compounds, using benzene, naphthalene, and anthracene derivatives as model targets. Double-stranded DNA was immobilized on a single-use disposable screen-printed electrochemical cell operating off a handheld battery-powered potentiostat (Sassolas *et al.*, 2008). A 10 μL drop of a preprocessed and preextracted contaminated soil sample was placed onto the working electrode for 2 minutes, and resulting electrochemical scans, based on the chemical's propensity to oxidize DNA guanine residues, were measured. The magnitude of these "guanine peaks" in relation to a reference electrode was linearly related to their concentration in solution (i.e., the higher the concentration of the target chemical, the more damage is imposed on the DNA, and the lower the electrochemical measurement of the oxidation signal). In a very discrete application of this DNA biosensor, the authors also applied it to the detection of this DNA biosensor and also to the detection of PAHs in fish bile, using the accumulation of PAH compounds in live fish to monitor for water contamination events (Lucarelli *et al.*, 2003). Nucleic acid can be manipulated similarly to create target specific aptamers using a process called SELEX (systematic evolution of ligands by exponential enrichment) (Ripp, 2010). By iteratively incubating nucleic acid with the desired target, one can select for oligonucleotide sequences (or aptamers) with the greatest affinity for the target.

Selectivity, though, has been demonstrated by several groups using deoxyribozymes (DNAzymes) or ribozymes (RNAzymes). These engineered catalytic oligonucleotides can mediate nucleic acid cleavages or ligation, phosphorylation, or other reactions. For example, DNAzyme biosensor for lead uses a single stranded DNAzyme absorbed to a gold electrode (Xiao *et al.*, 2007). The DNAzyme incorporates a methylene blue tag at concentrations as low as 62 ppb, the DNAzyme strand is cleaved, allowing the methylene blue tag to approach the transducer and transfer electrons, thereby instigating an electrochemical signal (Ripp *et al.*, 2010). Advantages of this technique are promptness (only a few minutes to detect sample processing is often necessary), sensitivity (typically down to low part-per billion levels), ease of use, and cost-effectiveness screening environmental sites for toxic chemical intrusions or

monitoring operational endpoints of bioremediation efforts. A colourimetric DNAzyme-based biosensor for lead has also been demonstrated (Wei *et al.*, 2008).

Currently, perhaps, one of the greatest applications of DNA for the detection of crude oil pollutants is the use of 'Rec-assay' which utilizes *Bacillus subtilis* to detect hydrophobic substances (hydrocarbons) (Matsui, 1989) toxic to DNA.

17. Bioremediation of crude oil polluted environments

Crude oil pollutants introduction into the environment occurs through three major routes: land, aquatic environments and the atmosphere. Each of these environmental components is a habitat for biotic existence. In the refining of crude oil most components are broken down into highly volatile fragments and are disposed in gaseous form. Thus gas flaring is a major source of environmental pollution that is introduced into the atmosphere and has direct effect in Ozone layer perforation as well as increase in green house gases emission. Acid rain and petrochemical smog are other environmental hazards resulting from crude oil processing. Both the land and the aquatic environments are affected due to the impact of the non-volatile petroleum hydrocarbon (polycyclic aromatic hydrocarbons). In the course of petroleum exploration, drilling fluids are also other sources of pollution normally neglected as emphasis is shifted to crude oil pollutants. Aquatic and terrestrial environments polluted by crude oil results from man's carelessness and negligence of his environment. Crude spills are the major source of contamination of land and marine bodies. During drilling, uncontrolled spill occurs in offshore environments thereby damaging the aquatic environment. In Nigeria, over 20 percent of crude oil spill results from vandalism of pipelines. Transportation spill accounts for over 10 percent pollution in both marine and soil pollution. Automobile spent engine oil and exhaust release of gases such as carbon dioxide and carbon monoxide are other ways of polluting the environment in cities. Some of the bioremediation methods used in Nigeria and elsewhere are clean up procedures which use bio-microbs. Some of these clean up procedures include: introduction of organic manure, phytoremediation, and optimizing the factors that enhance the thriving of microorganisms.

Some of these standard eco-friendly methods used for crude oil bioremediation will be emphasized in this chapter.

18. Natural attenuation

Natural attenuation refers to decrease in concentrations of chemicals in the environment due to natural phenomena such as microbial degradation, evaporation, and adsorption (where chemicals adsorb unto solids). Prince (2002) explained that as the age of an oil spill increases, there will be more opportunity for the oil to weather and for its constituents to attenuate in the environment. Generally, the more weathering that take place consequently reduces the biodegradation that occurred.

19. Bioremediation of soil

Bioremediation can be a cost effective technology often used to treat oil spills in all types of environmental media including: soils, ground-water, and surface water (both fresh and marine) (DOE/PERF, 2002). Oily wastes are also treated using bioremediation processes.

Spill oil is subjected to a wide range of physical, biological and chemical processes that actually “weather” the oil, and attenuate it in the environment (DOE/PERF, 2002). Biodegradation is one of the weathering processes that are unique because it is the primary process by which the oil is actually removed from the environment. Most of the other weathering processes transfer the oil from one medium to another (as in the case of volatilization where certain oil constituents evaporate into the air), or dilute it (such as from wave action, which may disperse oil throughout the water column in a marine environment) (DOE/PERF, 2002).

Methodologies for bioremediation of terrestrial oil spills are well developed. Research on bioremediation in surface soils is currently focused on optimizing bioremediation rates, developing appropriate treatment goals, and site restoration following remediation (DOE/PERF, 2002). Crude oil and refined oil products are frequently stored and transported on or over land, and as a result, oil spills that impact soil and groundwater tend to be quite common although usually smaller in volume than marine or freshwater spills (DOE/PERF,2002).

Bioremediation uses microscopic organisms (primarily bacteria) that live on soil and ‘eat’ chemicals, such as petroleum hydrocarbons. They use certain components of the petroleum hydrocarbons as their food source; leaving other chemicals behind. The waste products of the process are generated in form of water and carbon dioxide a process known as mineralization that characterizes the complete removal of crude oil from the environment.

20. Use of bulking agents/tilling for bioremediation

Nackles and Ray (2002), clearly elaborated that in composting applications, organic bulking agents allow for successful treatment of higher oil concentrations, increase the biodegradation rate and also increase the temperature. Locally available bulking agents can be cost-effective, and can include palm husks, wood chips, saw dust, rice hulls, manure, straw and hay. Of these, wood chips, palm husks, straw and hay can serve as structural bulking agents. The source of bulking agents should always be carefully checked to ensure that there is no potential for residual substance (like pesticides) to be present that could be toxic to microbes. Bulking agents are added to soil or wastes to improve permeability and water holding capacity, which in turn increases biodegradation rates (Sublette, 2001). Bulking agents fall into two general categories: structural and organic (Sublette, 2001). Structural bulking agents improve the porosity and permeability of the soil by creating larger and more numerous pore spaces. Organic bulking agents initially act as structural bulking agents but also biodegrade themselves, producing degradation products that build soil structure on a long term basis. Sublette (2001), insisted that bulking agents improve the water holding capacity which is especially important in sandy soils, but they can also increase fertilizer demand.

For soil treatment, organic bulking agents should be blended into the soil until a porous soil structure is obtained with no visual evidence of oil. The amount of bulking agent to be used in composting depends upon the original texture of the soil or waste (DOE/PERF, 2002).

Prince and McMillen (2002) stated that tilling the active biological zone should be performed regularly to overcome any oxygen deficiencies and to mix the soil with the nutrients and bulking agent. Prince and McMillen (2002) further stated that mixing also helps to optimize

contact among the microorganisms, hydrocarbons, moisture and nutrients to enable maximum degradation rates. Furthermore, according to Nakles and Ray (2002), in farming operations, tilling is recommended at an interval of twice per month. When composting is used, tilling can be performed or a force or passive aeration system can be used to supply oxygen. Generally, the aeration method is determined by equipment availability and engineering considerations such as the compost pile configuration. Rocky soils may not be ideal for bioremediation because of lower microbial populations, the inability to retain moisture, and difficulty in tilling. For rocky soils, one needs to consult with a bioremediation expert.

21. Microbial bioremediation

Bioremediation is the use of biological systems for the reduction of pollution from air, aquatic or terrestrial systems (EFB, 1999), it also involve extracting a microbe from the environment and exposing it to a target contaminant so as to lessen the toxic component (Vallero, 2010). Thus, the goal of bioremediation is the employment of bio-systems such as microbes, higher organisms like plants (phytoremediation), and animals to reduce the potential toxicity of chemical contaminants in the environment by degrading, transforming, and immobilizing these undesirable compounds.

Biodegradation is the use of living organisms to enzymatically and otherwise attack numerous organic chemicals and break them down to lesser toxic chemical species. Biotechnologists and bio-engineers classify pollutants with respect to the ease of degradation and types of processes that are responsible for their degradation, sometimes referred to as treatability (Vallero, 2010).

Biodegradation with micro-organisms is the most frequently occurring bioremediation option. Micro-organisms can break down most compounds for their growth and/or energy needs. These biodegradation processes may or may not need air. In some cases, metabolic pathways where organisms normally use for growth and energy supply may also be used to break down pollutant molecules. In these cases known as co-metabolisms, the micro-organism does not benefit directly. Researchers have taken advantage of this phenomenon and used it for bioremediation purposes (EFB, 1999).

A complete biodegradation results in detoxification by mineralising pollutants to carbondioxide (CO_2), water (H_2O) and harmless inorganic salts (EFB, 1999). Incomplete biodegradation (i.e mineralization) will produce compounds that are usually simpler (e.g cleared rings, removal of halogens), but with physical and chemical characteristics different from the parent compound. In addition, side reactions can produce compounds with varying levels of toxicity and mobility in the environment (Vallero, 2010).

Biodegradation may occur spontaneously, in which case the expressions "intrinsic bioremediation" or "natural attenuation" are often used (EFB, 1999). In many cases the natural circumstances may not be favourable enough for natural attenuation to take place due to inadequate nutrients, oxygen or suitable bacteria. Such situations may be improved by supplying one or more of the missing/inadequate environmental factors. For instance extra nutrients (EFB, 1999) were disseminated to speed up the break down of the oil spilled on 1000 miles of Alaskan shoreline by the super tanker Exxon Valdez in 1989.

According to Vallero (2010), there are millions of indigenous species of microbes living at any given time within many soil environments. The bioengineer simply needs to create an environment where those microbes are able to use a particular compound as their energy source. Biodegradation processes had been observed empirically for centuries, but putting them to use as a distinct field of bioremediation began with the work of Raymond *et al.* (1975). This seminal study found that the addition of nutrients to soil increases the abundance of bacteria that was associated with a proportional degradation of hydrocarbons, in this case petroleum by-products (Raymond *et al.*, 1975).

Vallero (2010) indicates that the success of bioremediation depends on the following factors:

1. The growth and survival of microbial populations
2. The ability of these organisms to come into contact with the substances that needs to be degraded into less toxic compounds.
3. The size of the micro-organisms population.
4. The appropriate microbial environment that must be habitable for the microbes to thrive.

Sometimes, concentrations of compounds can be so high that the environment is toxic to microbial populations. Therefore, the bioengineer must either use a method other than bioremediation or modify the environment (e.g dilution, change of pH, oxygen pump, adding organic matter, etc) to make it habitable. An important modification is the removal of non-aqueous phase liquids (NAPLs) since the microbes' biofilm and other mechanisms usually work best when the microbe is attached to a particle, thus, most of the NAPLs need to be removed, by vapour extraction (Vallero, 2010). Thus, low permeability soils, like clays, are difficult to treat, since liquids (water, solutes and nutrients) are difficult to pump through these systems. Usually bioremediation works best in soils that are relatively sandy, allowing mobility and greater probability of contact between the microbes and the contaminant (Vallero, 2010). Therefore, an understanding of the environmental conditions sets the stage for problem formulation (i.e identification of the factors at work and the resulting threats to health and environmental quality) and risk management (i.e what are the various options available to address these factors and how difficult it will be to overcome obstacles or to enhance those factors that make remediation successful). In other words, bioremediation is a process of optimization that involves the selection options among a number of biological, chemical and physical factors. This process includes the correct match of the degrading microbes to the conditions of the contaminated soil, the understanding and controlling of the movement of the contaminant (microbial food) so as to come into contact with microbes, and the characterization of the abiotic conditions controlling both of these factors (Vallero, 2010). Optimization can vary among options, such as artificially adding microbial populations known to break down the compounds of concern. Only a few species can break down certain organic compounds (Vallero, 2010). Two major limiting factors of any biodegradation process are toxicity to the microbial population and inherent biodegradability of the compound. Numerous bioremediation projects include in-situ (field treatment) and ex-situ (sample/laboratory treatment) waste treatment using bio-systems (Vallero, 2010).

21.1 A practical application of microorganism in crude oil bioremediation: According to (Onwurah, 2003) many microorganisms can adapt their catabolic machinery to utilize certain environmental pollutants as growth substrates, thereby bioremediating the

environment. Some microorganisms in carrying out their normal metabolic function may fortuitously degrade certain pollutants as well. This process termed cometabolism obviously requires adequate growth substrates. Diazotrophs, such as *Azotobacter vinelandii*, beyond their ability to fix atmospheric nitrogen also have the capacity in some case, to cometabolise petroleum hydrocarbons (Onwurah, 1999).

Onwurah (2003) carried out a bioremediation study that involved two bacteria, a hydrocarbonoclastic and diazotrophic bacteria. The hydrocarbonoclastic was tentatively identified as *Pseudomonas sp* and designated as NS₅₀C₁₀ by the Department of Microbiology, University of Nigeria, Nsukka. The diazotrophic bacteria were *Azotobacter vinelandii*, which was isolated from previously crude oil contaminated soil (Onwurah, 1999). This study describes the mineral media and procedure for isolation and multiplication of the bacteria to the required cell density. Crude oil spill was simulated by thoroughly mixing 50, 100, 150 mg fraction of crude oil with 100g batches of a composite soil sample in beakers. The soil samples were taken from a depth of 0 - 50cm from the Zoological garden, University of Nigeria, Nsukka. The mixing was conducted using a horizontal arm shaker adjusted to a speed of 120rpm for 30 minutes. The contaminated soil samples, in beakers, were inoculated with optimal combinations (cell density) of NS₅₀C₁₀ and *A. vinelandii*. Water was added to the crude oil-contaminated soil samples (both inoculated and those not inoculated to a saturation point but not in excess) and then the samples were left to stand undisturbed for seven days. NS₅₀C₁₀ was applied first, followed by *A. vinelandii*, 12 hours later. At the seventh day of soil treatment, 20 sorghum grains (previously soaked overnight in distilled water) were planted in each soil sample followed by irrigation to aid germination. Seven days after the planting of the sorghum grains, the soil from each beaker was carefully removed. The number of germinated seed per batch of soil sample was noted and the length of radicle was measured and the mean length was taken from each batch.

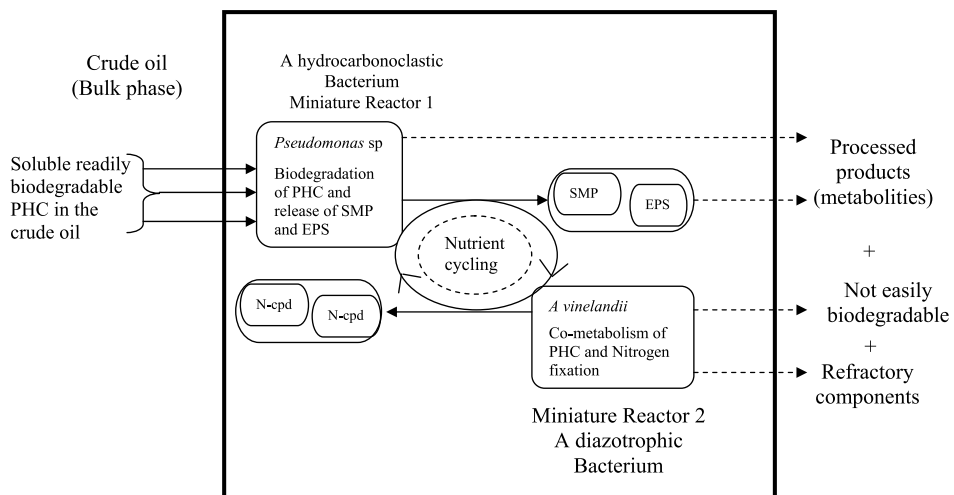


Fig. 4. Simplified bioremediation conceptual model of *Pseudomonas sp* and *A. vinelandii* operating as a unit of two miniature sequencing bioreactors, in situ, (SMP= Soluble microbial products; N-cpd= fixed nitrogen compounds; EPS= Exopolysaccharide; PHC=petroleum hydrocarbons) (Onwurah, 2003).

The results of this experiment showed that *Pseudomonas sp.* grew well on agar plates containing a thin film of crude oil as the only carbon source while *A. vinelandii* did not. However, cell free extract of *Azotobacter vinelandii*, fixed atmospheric nitrogen as ammonium ion (NH_4^+) under appropriate condition. The specific growth rate values in contaminated soil samples inoculated with both normal $\text{NS}_{50}\text{C}_{10}$ and *A.vinelandii* (consortium) were highest in all cases. By adding an aerobic, free living diazotroph *A.vinelandii* with the *Pseudomonas sp.* ($\text{NS}_{50}\text{C}_{10}$), an improvement on bioremediation of soil over that of the pure $\text{NS}_{50}\text{C}_{10}$ alone was achieved to the order of 51.96 to 82.55%. This innovative application that uses the synergetic action of several microorganisms to clean up oil polluted soil has potential application for the bioremediation of oil contaminated soil in the Niger delta region.

The method described above is the biotechnological application known as **Bioaugmentation** which is the addition of selected organisms to contaminated soils (sites) in order to supplement the indigenous microbial population and speed up degradation. Figure 4 presents a model of the process involved in this bioremediation technique.

22. Phytoremediation techniques

Phytoremediation is a biological technology process that utilizes natural plant processes to enhance degradation and removal of contaminants in contaminated soil or groundwater (Schooner, *et al.*, 1995). Phytoremediation utilizes physical, chemical, and biological processes to remove, degrade, transform, or stabilize contaminants within soil and groundwater. Hydraulic control, uptake, transformation, volatilization, and rhizodegradation are important processes used during phytoremediation. These processes are briefly in the subsequent paragraphs.

22.1 Hydraulic Control: Phytoremediation applications can be designed to capture contaminated groundwater plumes to prevent off-site migration and/or decrease downward migration of contaminants. Trees and grasses act as a solar “pump” removing water from soils and aquifers through transpiration. Contaminant plume capture relies on the formation of a cone of depression within an aquifer due to uptake of water by plants and subsequent transpiration. Downward migration of contaminants due to percolation of rainwater can also be controlled with phytoremediation. Within the upper region of an aquifer, grasses with dense, fibrous root systems are used to transpire water and limit percolation of contaminants through the vadose zone and to intercept rainwater that may discourage tree root penetration through the water table. However, plume capture is not limited to shallow aquifers, as poplar trees planted in well casings have been used to tap water tables at a depth of 10-m (Gatliff, 1994).

22.2 Phytovolatilization: Volatile pollutants diffuse from the plant into the atmosphere through open stomata in leaves. Radial diffusion through stem tissues has also been reported (Zhang, *et al.*, 2001; Narayanan, *et al.*, 1999; Davis *et al.*, 1999). For example, methyl-tert-butyl ether (MTBE) can escape through leaves, stems, and the bark to the atmosphere (Hong, *et al.*, 2001; Trapp and McFarlane, 1994).

The natural ability of a plant to volatilize a contaminant that has been taken up through its roots can be exploited as a natural air-stripping pump system. Phytovolatilization is most applicable to those contaminants that are treated by conventional air-stripping.

Tree core samples of hybrid poplars exposed to TCE (Trichloroethylene) also showed radial diffusion from the stem (Ma, and Burken, 2003) rather than transpiration from leaves (Ma, and Burken, 2003; Newman *et al.*, 1998) as the main dissipation mechanism. Generally, the concentration of volatile organic components (VOCs) in the xylem decreases with increasing distance from the roots (Ma, and Burken, 2003). Once released into the atmosphere, compounds with double-bonds such as TCE and perchloroethylene (PCE) could be rapidly oxidized in the atmosphere by hydroxyl radicals. However, under certain circumstances (e.g., poor air circulation) phytovolatilization may not provide a terminal solution. For example, MTBE is long lived in the atmosphere and can pose a risk to shallow groundwater during precipitation (Penkow *et al.*, 1997). In such cases, simple mass balance models can be utilized to determine if phytovolatilization poses a significant risk to humans and/or the environment (Narayan *et al.*, 1999; Ma, and Burken, 2003; Aitchison, *et al.*, 2000).

Nevertheless, the rate of release of VOCs from plant tissues is generally small relative to other emissions (Aitchison, *et al.*, 2000). Thus, phytovolatilization is a potentially viable remediation strategy for many volatile organic chemicals.

22.3 Rhizodegradation: Microbial degradation in the rhizosphere might be the most significant mechanism for removal of diesel range organics in vegetated contaminated soils (Aprill, *et al.*, 1990; Banks, *et al.*, 1999; Binet, *et al.*, 2000; Liste and Alexander, 2000; Reilley, *et al.*, 1996; Miya and Firestone, 2000; Miya and Firestone, 2001) Rhizodegradation occurs because contaminants such as PAHs are highly hydrophobic and their sorption to soil decreases their bioavailability for plant uptake and phytotransformation. Briggs *et al.* (1982) first demonstrated that the lipophilicity of a pesticide determines its fate in a barley plant. High Kow values (an indicator of hydrophobicity) corresponds to a greater possibility that the compound would be retained in the roots. Burken and Schnoor (1998) published similar results for the sorption of a wide range of organic contaminants to roots of hybrid poplar plants grown hydroponically. Where the Root Concentration Factor (RCF) (L/kg dry roots) is the ratio of organic chemical sorbed on the root (mg/kg of fresh root tissue) to that in hydroponic solution (mg/L). This equilibrium partitioning coefficient has generally proved to be a good indicator of whether a plant retains a contaminant in the root, which increases the probability of microbial degradation (not withstanding significant bioavailability limitations). However, a few exceptions exist such as phenol and aniline, which bind irreversibly to the root (especially aniline) and are chemically transformed.

23. Factors enhancing phytoremediation site treatability

23.1 Source removal: For phytoremediation to succeed, it is very important to physically remove the source of contamination (e.g., excavation of highly-contaminated soil and/or extraction of free phase). The presence of a continuous source can be detrimental to the health of the plants and can extend the life of the phytoremediation project indefinitely. Perhaps pretreatment methods that reduces the bulk quantity of crude oil such as application of cow dung, poultry wastes as well as extended time between contamination and phytoremediation can also help to reduce the bulk accumulation of crude oil in surface soil so that the plant is able to grow in such environments. The crude oil source could also be dispersed over a large surface area by mixing with bulk substances such a rice husk and other lignocelluloses and exposed to weathering and microbial degradation before being made available to the plants as source of nutrient.

23.2 Depth of Contamination: Dept of crude oil contamination of soil is also monitored before application of phytoremediation. 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. 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 (Gatliff, 1994). On the other hand, roots of some grasses (alfalfa, switchgrass, and tall fescue) can reach soil depths of only 0.25- 0.4 m. Buffel-grass 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 were used to remediate saline soils contaminated with TPHs (Total Petroleum Hydrocarbons), had roots which grew to a depth of more than 1.5 m by growing through the brackish water table into a zone of concentrated contaminants (USACE, 2003). Optimizing irrigation patterns can also facilitate biodegradation of contaminants by creating an "expanded rhizosphere" due to translocation of organic root exudates and inorganic nutrients to relatively deep soil layers. Phytoremediation can therefore influence soils to the depth where irrigation water reaches, even if the roots are sparse in the contaminated zone.

23.3 Soil composition and quality: Soil quality is another important factor for determining successful germination, growth and health of plants. Heavily contaminated soils have a tendency towards poor physical conditioning which is unsuitable for vigorous growth of vegetation and rhizosphere bacteria. It is therefore critical to use amendments to improve the quality of soil before planting. Common limitations are poor moisture-holding capacity, insufficient aeration, low permeability and nutrient deficiencies. Agronomic soil analysis and preliminary greenhouse or pilot scale experiments can help identify these constraints. For example, nutrient analysis of contaminated soils from a site at the Unocal Bulk Storage Terminal at Superior, Wisconsin (Rentz, *et al.*, 2004) indicated general deficiencies in nitrogen, phosphorus, potassium, and zinc. To decrease the soil pH addition of sulfur was recommended. This information was subsequently used in greenhouse treatability studies, from which a formula of 50 lb/ac phosphorus, 225 lb/ac zinc, and 50 lb/ac potassium was identified as optimum for growth of native grasses. Organic amendments such as aged manure, sewage sludge, compost, straw, or mulch can be used to increase the water-holding capacity of a contaminated soil. Soil pH can be increased and decreased by the addition of lime and sulphur respectively.

23.4 Weather: Phytoremediation might be best suited for tropical countries such as Nigeria, Africa and most part of Asia where plant growth occurs all year round. Plants are known to take up numerous inorganic and organic contaminants and store them in various plant organs when they can utilize them effectively for their benefits. 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 due to continues humid nature of the environment. However, a combination of grasses can be used to prolong the growing period.

23.5 Plant Selection Criteria: Plants should be selected according to the needs of the application, the contaminants of concern, and their potential to thrive on contaminated soil. Design requirements should include the use of native plants, to avoid introduction of invasive species. Apart from this, vegetation should be fast growing, hardy, easy to plant

and maintain. The main aim is to ensure that roots expand throughout the entire contaminated zone. In temperate climates with shallow contaminated aquifers, phreatophytes, such as *Populus* sp. (hybrid poplar, cottonwood, aspen) and *Salix* sp. (willow) are often selected because of fast growth, deep rooting ability down to the surface of groundwater, large transpiration rates, and the fact that they are native throughout most of the country. Among tropical plants tested for use in the Pacific Islands, three coastal trees, kou (*Cordia subcordata*), milo (*Thespesia populnea*), and kiawe (*Prosopis pallida*) and the native shrub beach naupaka (*Scaevola serica*) tolerated field conditions and facilitated clean-up of soils contaminated with diesel fuel (Hetch and Badiene, 1998). Wang and Meresz (1981) assessed onions, beets, tomatoes, and soil for 17 PAHs including Barium Phosphate. They found most of the PAH contamination localized in the onion 'peels'. Other factors influencing the localization of PAH in plants include: the rate of PAH uptake by plant species, the nature of the substrate that the plant is growing in, PAH solubility, PAH phase (vapor or particulate), and molecular weight (Edwards, 1983). These latter findings are of potential significance since in an oil spill the PAH compounds would be present along with benzene. Grasses are often planted in tandem with trees at sites with organic contaminants as the primary remediation method. They provide a tremendous amount of fine roots in the surface soil, which is effective at binding and transforming hydrophobic contaminants such as TPH, BTEX, and PAHs. Grasses are often planted between rows of trees to provide for soil stabilization and protection against wind-blown dust that can move contaminants off-site. Legumes such as alfalfa (*Medicago sativa*), alsike clover (*Trifolium hybridum*), and peas (can be used to restore nitrogen to poor soils. Fescue (*Vulpia myuros*), rye (*Elymus* sp.), clover (*Trifolium* sp.) and reed canary grass (*Phalaris arundinacea*) have been used successfully at several sites, especially petrochemical wastes. Once harvested, the grasses can be disposed off as compost or burned. Plant tolerance to high contaminant concentrations is also a very important factor to keep in mind. The phytotoxicity of petroleum hydrocarbons is a function of the specific contaminant composition, its concentration, and the plant species used. Major adverse effects typically include reduced germination and growth if contaminant concentrations are sufficiently high. In general, TPH values of 15 percent or greater can result in significant reductions in plant growth and in some cases mortality. Compared with uncontaminated soil, soils with 2% TPH reduced alfalfa yields by 32 percent (Wiltse *et al.*, 1998). Production of biomass by ryegrass was reduced 46 percent at a soil concentration of 0.5 percent (5000 mg/kg) hydrocarbons (Gunther *et al.*, 1996). It was found that plants pre-grown in clean soil and subsequently transplanted to the contaminated soil grew nearly as well as the control, showing that toxicity was associated with germination and/or early plant growth. Similarly, poor rooting of ryegrass compared to legumes appeared to adversely affect the removal of TPH from Gulf War-contaminated soils (Yateem, 1999).

Also, although the germination of sunflower seeds and beans was greater than that of maize, vegetative growth was greater for maize than beans, demonstrating that germination and later plant growth may be affected differently (Chaineau *et al.*, 1997). Aged spills tend to be much less phytotoxic than fresh ones, possibly because of the lower bioavailability of toxic compounds in the aged spills. However, the speciation of petroleum hydrocarbons is also very important in determining phytotoxicity. A fuel oil with 30 percent aromatics resulted in LC50 germination (oil concentration lethal to 50 percent of test plants) values of 7 percent (70,000 mg/kg) for sunflower seeds. The volatile fraction can prove most toxic to plants. Aromatic volatile petroleum hydrocarbons such as benzene have been used as

herbicides in the past years, illustrating their phytotoxicity when applied to plant leaves (Baker, 1970). In contrast, no phytotoxic effects were observed in hybrid poplar trees exposed to a simulated groundwater containing a mixture of VOCs including BTEX, chlorinated aliphatics, and alcohols at a total concentration of 169 mg/L (Ferro *et al.*, 1999). Reduction of the volatile fraction may be accomplished through management, such as tillage of the soil. If initial efforts at plant establishment at a site fail, replanting the area may ultimately lead to success as concentrations or bioavailability of the more phytotoxic components decline. Solution-phase concentrations of hydrocarbons are also important, particularly for aquifer remediation applications of phytoremediation. Additional components with phytotoxic effects include various unsaturated hydrocarbons and acidic hydrocarbons such as alicyclics with carboxylic acid groups (naphthenic acids) (Baker, 1970). A screening test and knowledge from the literature of plant attributes is essential for selection of plants. Most experts recommend a mixture of grasses or legumes to address surface soils contaminated with petroleum hydrocarbons. However, design engineers should work in interdisciplinary teams that include a botanist and/or agricultural specialist to identify and select plants that will grow well at the site. Preliminary greenhouse studies should also be used to identify plants that can thrive and enhance transformation of contaminants of concern to non-toxic or less toxic products.

23.6 Time scale of clean-up: Degradation of organics may be limited by mass transfer, i.e., desorption, and mass transport of chemicals from soil particles to the aqueous phase may become the rate determining step. Therefore, phytoremediation may require more time to achieve clean-up standards than other more costly alternatives such as excavation or ex-situ treatment, especially for hydrophobic pollutants that are tightly bound to soil particles. In many cases, phytoremediation may serve as a final "polishing step" to close sites after more aggressive clean-up technologies have been used to treat the hot spots.

24. Agronomic inputs of phytoremediation

24.1 Irrigation: Irrigation is a common practice in Nigeria and other tropical region especially during the dry seasons of the year. The dry season period is between November and April. Exposure of crude oil to elements such as sunshine, aeration and organic fertilizer followed by tillage and irrigation is bound to give the best result. For terrestrial phytoremediation applications, it is often desirable to include irrigation costs on the order of 10-20 inches of water per year, in the design. Spray irrigation is less efficient than drip irrigation as it encourages the growth of weeds that compete for nutrients with plants and hinder their delivery to the contaminated zone. Results suggest that irrigation can enhance bioremediation of certain diesel components. Irrigation of the plants is especially important during the start of the project. However, after the first year, hydrologic modeling can be used to estimate the rate of percolation to groundwater under irrigation conditions. Over time, irrigation can be withdrawn from the site, provided the area receives sufficient rainfall to sustain the plants.

24.2 Fertilizer Requirements: Contaminated soils are usually deficient in macro- and micro-nutrients necessary for establishing healthy vigorously growing plants and stimulating microbial contaminant degradation. Nitrogen fertilization of motor oil-contaminated soils was found to increase the growth of corn and reduce what appeared to be nitrogen-deficient

yellowing of the leaves (Giddens, 1970). The source of nutrients also appeared to affect the germination and growth of plants. Organic sources of nitrogen are better than inorganic sources. This is probably because organic nitrogen sources provide a slow release source of nitrogen, and also help to improve soil structure and soil water relationships for plant growth. It was found that poultry manure increased the growth of corn in a soil containing 3 percent weight per volume crude oil more than an inorganic fertilizer containing nitrogen, phosphorus, and potassium (Amadi *et al.*, 1993). The addition of sawdust alone improved germination by decreasing oil contact with seeds, but accentuated the adverse effect of the oil on later growth, apparently by further widening the carbon-to-nitrogen ratio (Amadi *et al.*, 1993). With respect to TPH degradation, nutrient addition during phytoremediation has yielded mixed results. Hutchinson *et al.* (2001) observed better degradation of TPH using grasses with N/P amendments than without inorganic amendments. Joner *et al.* (2002) reported improved degradation of 3 and 4 ringed PAHs with the addition of N/P, but diminished degradation of 5 and 6 ringed PAHs. Finally, Palmroth *et al.* (2002) observed no improved degradation of diesel fuel with nutrient amendments during phytoremediation with pine, poplar, or grasses. Microbial bioremediation of TPH contaminants with nutrient addition also produced widely varying results. Diesel fuel degradation was stimulated with the addition of N/P using cold region soils (Walworth *et al.*, 2003) and potassium amendments stimulated creosote degradation (Phillips *et al.*, 2000). There was an observed improved degradation of 4 ringed PAHs with N/P addition, but no increased degradation of 3 ringed PAHs (Breedveld and Sparrevik, 2000). However, Graham *et al.* (1999) assessed an array of N/P amendments for hexadecane biodegradation and suggested that amendments above stoichiometric requirements can lead to diminished rates of degradation. This potentially occurs because addition of excessive nitrogen results in an increase in soil salinity and this increases the osmotic stress and suppresses the activity of hydrocarbon-degrading organisms (Walworth *et al.*, 2003). Carmichael and Pfander (1997) observed slower degradation of 3 and 4 ringed PAHs with N addition and no effects for P addition. Johnson and Scow (1999) reported similar results indicating N/P addition inhibited or did not change phenanthrene degradation (3 ringed PAH). Their results showed that soil with initial low concentrations of nitrogen or phosphorus is more likely to show decreased degradation with N/P addition. Many PAH-degrading organisms are adapted to low nutrient conditions and activity may decrease with the addition of soil amendments. Thus, addition of nutrients should be considered on a site-by-site basis and a balance should be considered between biodegradation and plant growth. Application of amendments exclusively for plant growth may result in diminished contaminant degradation, the ultimate goal of phytoremediation.

24.3 Oxygen requirements: Soil oxygen is required for optimal aerobic microbial degradation of petroleum hydrocarbon contaminants. Similar to nutrient deficiencies, oxygen depletion is caused by natural microbial respiration of contaminants. Within phytoremediation, plants may provide a net positive or negative oxygen source (Lee, 2000).

Plants may improve soil oxygen through two mechanisms. First, specially adapted plants use parenchyma, channels of reduced air resistance, to transport oxygen to the root zone, enhancing aerobic biological degradation (Erickson *et al.*, 1993). Second, soil dewatering and fracturing increases soil porosity, allowing increased diffusion of atmospheric oxygen (EPA, 2001). Plant roots can also be a net oxygen sink within petroleum-contaminated soils. Rentz

et al., (2003) observed stimulation of hybrid poplar growth and increased poplar root density with the addition of Oxygen Release Compound (ORC) when plants were grown in petroleum smear zone soils (high biochemical oxygen demand). Flux of oxygen into soil by plants could be offset by root turnover and root exudation that provides microbial populations with simple carbon sources that could deplete soil oxygen when metabolized (Lynch, 1990). Furthermore, plant roots are known to require oxygen (Neuman *et al.*, 1996). For soils with a high biochemical oxygen demand, oxygen addition may be required to promote plant growth and stimulate microbial degradation. Passive methods of oxygen delivery are suggested to keep costs of phytoremediation low. These methods include the use of include the use of perforated aeration tubes placed next to cuttings that can supply oxygen to roots along a vertical axis (Ferro *et al.*, 2001). Perforated ADS (Adsorption) tubing that are placed at depth prior backfilling the planting trench provides oxygen on a horizontal plane. Gravel used to backfill planting trenches allows permeation of oxygen on vertical and horizontal axis. Finally, the use of solid peroxides (e.g. Oxygen Release Compounds) can provide oxygen to soils when in contact with water (Koenigsberg and Norris, 1999).

25. Biodegradation end points

It is important to monitor the rate at which biodegradation takes place so as to adjust the important parameters that will enhance the biodegrading process. The endpoint is however used to measure the completion of the biodegradation.

Endpoint criteria are typically concentrations of specific components or chemicals that are measured in the impacted soil. Accordingly, endpoints can be the bulk hydrocarbon content or the contraction of specific petroleum hydrocarbons (DOE/PERF, 2002). Desired end points should be considered when selecting a treatment technology, since certain technologies may be capable of achieving the end points in less time and with less money spent. The end-points that can be achieved by bioremediation are related to how much oil you start with, the composition of the oil, and the age of the spill (Nakles and Loehr, 2002).

If regulatory treatment goals have been established prior to initiating bioremediation treatment, then bioremediation treatment will be complete when the goal has been achieved. There may be regulatory constraints concerning whether bioremediation is permissible, and which biotreatment methods are approved. Therefore, experts should always consult with all the local and state regulatory agencies prior to the application of biotreatment (DOE/PERF, 2002). Life cycle assessment can also be used to investigate the viability of the biodegradation process by looking at the crude oil remaining at each stage of the bioremediation processes as well as gasses evolved and toxic level at each phase of the bioremediation project.

26. Conclusion

Crude oil spill in our environments have been a consistent challenge and as long as crude oil exploitation takes place spillage is bound to occur. This chapter is review of bioremediation techniques for the reclamation of oil contaminated lands and water bodies. Some of these bioremediation techniques include: bio-monitoring, microbial bioremediation and phytoremediation methods. Important factors that limit bioremediation were also described.

It is the wish of the authors that this review would be useful to those who are looking at practicable ways of bioremediating crude oil polluted environments.

27. Acknowledgement

To all who gave encouragement to go ahead with this work! All the authors whose works are hereby cited are duly acknowledged for their contributions to the body of knowledge to solve the crude oil pollution challenges in our respective environments. The greatest acknowledgement of all is to God the source of wisdom, strength, and knowledge.

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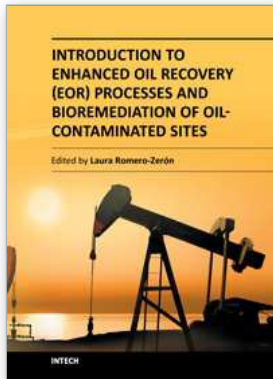
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Introduction to Enhanced Oil Recovery (EOR) Processes and Bioremediation of Oil-Contaminated Sites

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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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