



Bioremediation, Biostimulation and Bioaugmentation: A Review

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Abstract Bioremediation uses microbial metabolism in the presence of optimum environmental conditions and sufficient nutrients to breakdown contaminants notably petroleum hydrocarbons. We reviewed technologies for carrying out bioremediation and observed that biotechnological approaches that are designed to carry out remediation have received a great deal of attention in recent years. Biostimulation (meaning the addition of limiting nutrients to support microbial growth) and Bioaugmentation (meaning the addition of living cells capable of degradation) studies have enjoyed a heavy presence in literature and reviews of these technologies focusing on the technical aspects are very few if at all available. At times, nutrient application alone or augmenting with microbes is not sufficient enough for remediation leading to a simultaneous approach. Recent studies show that a combination of both approaches is equally feasible but not explicitly more beneficial. Evidently, selection of a technology hinges on site specific requirements such as availability of microorganisms capable of degradation in sufficient quantities, nutrient availability to support microbial growth and proliferation as well as environmental parameters such as temperature in combination with duration of exposure. This review focuses on these technologies and efforts are directed towards eventual manipulation of the processes of remediation all geared towards making bioremediation technically and economically viable for comprehensive treatment of petroleum hydrocarbon contaminated soils.

Keywords: *Bioremediation, Biostimulation, Bioaugmentation, combined technologies*

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1. Introduction

There is a growing concern about the rate of environmental degradation currently experienced throughout the world today, much of it arising from growing production and use of fossil fuels. In all continents, oil exploration and use threatens the health of the environment and living creatures including humans. An oil spill is the release of a petroleum hydrocarbon into the environment. Oil spills may be due to releases of crude oil from tankers, offshore platforms, drilling rigs and wells, as well as spills of refined petroleum products (such as gasoline, diesel) and their by-products, heavier fuels used by large ships such as bunker fuel, or the spill of any oily refuse or waste oil.

Crude oil and refined fuel spills have damaged natural ecosystems in Alaska, the Gulf of Mexico, the Galapagos Islands, France, the Niger Delta region in Nigeria and many other places worldwide. The quantity of oil spilled during accidents has ranged from a few hundred tons to several hundred thousand tons (e.g., Deepwater Horizon Oil Spill, Atlantic Empress, Amoco Cadiz) but it is a limited barometer of damage or impact. Smaller spills have also readily proven to have a great impact on

ecosystems, such as the spills experienced in the Niger Delta region in Nigeria because of the remoteness of the sites or the bottle necks hindering emergency environmental responses.

The effects of oil contamination are enormous. Oil penetrates into the structure of the plumage of birds and the fur of mammals, reducing their insulating ability, and making them more vulnerable to temperature fluctuations and much less buoyant in water. Animals that rely on scent to find their babies or mothers fade away due to the strong scent of the oil. This causes babies to be rejected or abandoned, leaving the babies to starve and eventually die (Hogan, 2008). Oil can impair a bird's ability to fly, preventing it from foraging or escaping from predators. As they preen, birds may ingest the oil coating their feathers, irritating the digestive tract, altering liver function, and causing kidney damage. Together with their diminished foraging capacity, this can rapidly result in dehydration and metabolic imbalance. Some birds exposed to petroleum also experience changes in their hormonal balance, including changes in their luteinizing protein. The majority of birds affected by oil spills die without human intervention. Some studies have suggested that less than one percent of oil-soaked birds survive, even after cleaning (Dunnet, *et al.*, 1982)

For humans an oil spill can represent an immediate fire hazard. The Kuwaiti oil fires produced air pollution that caused respiratory distress. The Deepwater Horizon explosion killed eleven oil rig workers. The fire resulting from the Lac-Mégantic derailment killed forty seven and destroyed half of the town's centre, In Ikarama, Bayelsa State, Nigeria, a spill resulted in fire and subsequent burning of at least fifty personnel on the work platform (SPDC, 2011).

Spilled oil can also contaminate drinking water supplies. For example, in 2013 two different oil spills contaminated water supplies for three hundred thousand people in Miri, Malaysia; Eighty thousand people in Coca, Ecuador. In 2000, springs were contaminated by an oil spill in Clark County, Kentucky (Campbell Robertson /Clifford Krauss, 2010).

Contamination can have an economic impact on tourism and marine resource extraction industries. For example, the Deepwater Horizon oil spill impacted beach tourism and fishing along the Gulf Coast, and the responsible parties were required to compensate economic victims (Yang 2009).

Based on available literature (Adams *et al*, 2014), oil contamination reduces the ability of soil to support the growth of plants, seeps into ground to contaminate ground water, and increases the presence of heavy metals which can bioaccumulate and biomagnify causing adverse health effects. It is well known that heavy metals can be extremely toxic as they damage nerves, liver and bones, and also block functional groups of vital enzymes (Moore, 1990; Ewan and Pamphlett, 1996). Some of the metals like Nickel are also listed as possible human carcinogens (group 2B) and associated with reproductive problems and birth defects. Besides, a range of detrimental effects on fauna and flora are also well documented. Often, these contaminants also inhibit biological remediation processes due to metal sensitivity of the strain and necessitate additional combat strategies for efficient operation (Malik, 2000; Malik *et al.*, 2001).

The challenge facing scientists and industrialists alike today is tackling this problem of environmental degradation in a safe, environmentally sound manner with rational cost implications. A technology that has been intensively studied is bioremediation. Although the technology has gained wide attention and studies, there is the need to investigate the trends that have evolved in studying bioremediation in the past decade; some aspects under focus are comparability of available data, the applicability of available technology, availability or unavailability of technology for laboratory investigations, geographical diversity, dearth of expertise in the field, regulatory bottlenecks associated with extensive trials and a general skepticism or acceptance of the effectiveness of the technology which may have interfered with further researches.

2. Bioremediation

The use of microbes in modern bioremediation is credited, in part, to George Robinson (US Microbics, 2003). He used microbes to consume an oil spill along the coast of Santa Barbara, California in the late 1960s. Since the 1980s bioremediation of oil spills and other hazardous wastes has received more consideration (Shannon and

Unterman, 1993). Bioremediation is a process which uses microorganisms and their products to remove contaminants from the soil (USEPA 2000, 2012; Leung 2004). In particular, native soil microorganisms play a key role in soil bioremediation as biogeochemical agents to transform complex organic compounds into simple inorganic compounds or into their constituent elements. This process is termed mineralization. The microorganisms are adsorbed to soil particles by the mechanism of ionic exchange. In general soil particles have a negative charge, and soil and bacteria can hold together by an ionic bond involving polyvalent cations (Killham, 1994).

Bioremediation technology uses microorganisms to reduce, eliminate, contain, or transform to benign contaminants present in soils, sediments, water, and air.

Bioremediation is described as the use of microorganisms to destroy or immobilize waste materials (Shanahan, 2004). This process of detoxification targets the harmful chemicals by mineralization, transformation, or alteration (Shannon and Unterman, 1993). For centuries, civilizations have used natural bioremediation in wastewater treatment, but intentional use for the reduction of hazardous wastes is a more recent development.

Bioremediation involves the production of energy in a redox reaction within microbial cells. These reactions include respiration and other biological functions needed for cell maintenance and reproduction. A delivery system that provides one or more of the following is generally required: an energy source (electron donor), an electron acceptor, and nutrients. Different types of microbial electron acceptor classes can be involved in bioremediation, such as oxygen-, nitrate-, manganese-, iron (III)-, sulfate-, or carbon dioxide-reducing, and their corresponding redox potentials. Redox potentials provide an indication of the relative dominance of the electron acceptor classes.

The presence of microorganisms with the appropriate metabolic capabilities is the most important requirement for oil spill bioremediation according to Venosa *et al*, (2001). The communities which are exposed to hydrocarbons become adapted, exhibiting selective enrichment and genetic changes (Leahy and Colwell 1990; Atlas and Bartha, 1998). The adapted microbial communities can respond to the presence of hydrocarbon pollutants within hours (Atlas and Bartha, 1998) and exhibit higher biodegradation rates than communities with no history of hydrocarbon contamination (Leahy and Colwell, 1990) So, the ability to isolate high numbers of certain oil degrading microorganisms from an environment is commonly taken as evidence that those microorganisms are the most active oil degraders of that environment (Atlas, Bartha 1998) and can be used in the bioremediation of petroleum polluted sites. Since crude oil is made of a mixture of compounds, and since individual microorganisms metabolize only a limited range of hydrocarbon substrates, biodegradation of petroleum hydrocarbon requires mixture of different bacterial groups or consortia functioning to degrade a wider range of hydrocarbons (AL-Saleh, 2009, Bordenave, 2007). This process depends on nutrient availability and the optimum presence of other factors that support biological functions.

These are:

Contaminant concentrations: Directly influence microbial activity. When concentrations are too high, the

contaminants may have toxic effects on the present bacteria. In contrast, low contaminant concentration may prevent induction of bacterial degradation enzymes.

Contaminant bioavailability: Depends on the degree to which they sorb to solids or are sequestered by molecules in contaminated media, are diffused in macropores of soil or sediment, and other factors such as whether contaminants are present in Non-Aqueous Phase Liquid (NAPL) form. Bioavailability for microbial reactions is lower for contaminants that are more strongly sorbed to solids, enclosed in matrices of molecules in contaminated media, more widely diffused in macropores of soil and sediments, or are present in NAPL form (ICSS 2006).

Site characteristics: Have a significant impact on the effectiveness of any bioremediation strategy. Site environmental conditions important to consider for bioremediation applications include pH) with an optimum in the range of 6-8 (ICSS 2006), temperature, water content, nutrient availability, and redox potential.(ESTCP 2005

Redox Potential and oxygen content: typify oxidizing or reducing conditions. Redox potential is influenced by the presence of electron acceptors such as nitrate, manganese oxides, iron oxides and sulfate (ICSS 2006).

Nutrients: Are needed for microbial cell growth and division (ESTCP 2005). Suitable amounts of trace nutrients for microbial growth are usually present, but nutrients can be added in a useable form or via an organic substrate amendment (Parsons 2004), which also serves as an electron donor, to stimulate bioremediation.

Moisture content: Microbial growth requires an optimum presence of water in the environmental matrix. For optimum growth and proliferation, microorganisms require 12% to 25% of moisture (Mukherjee *et al.*, 2005).

Temperature: Directly affects the rate of microbial metabolism and consequently microbial activity in the environment. The biodegradation rate, to an extent rises with increasing temperature and slows with decreasing temperature (ESTCP 2005).

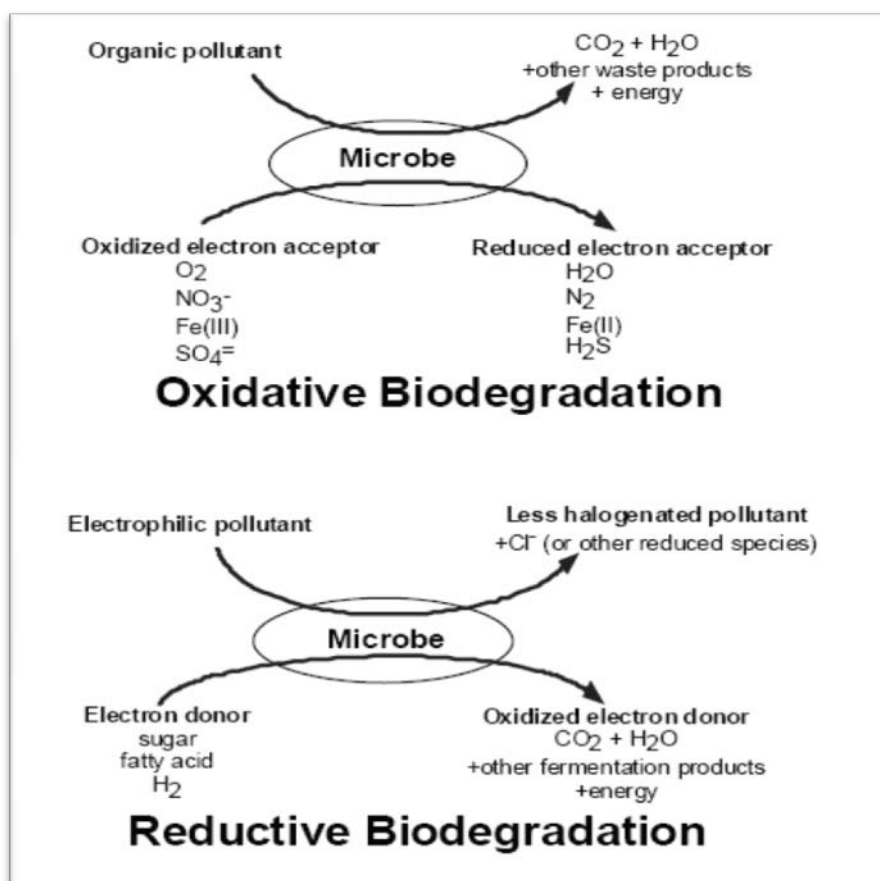


Figure 1. General Process of Organic Contaminant Degradation (Rockne and Reddy, 2003)

3. Types of Bioremediation

Feasibility of bioremediation depends on the location of contaminants. Approaches for implementation of bioremediation depend on whether the impacted soil to be treated is intact in the environment or it is to be excavated for treatment in an offsite facility. If on site, the term *In situ* remediation suffices and if offsite, it is described as *Ex situ*. Some authors (Kumar, *et al.*, 2011; Orji *et al.*, 2012; Hamzah *et al.*, 2013) have used this to describe the type of bioremediation. However, it is necessary to determine what exactly is done *in situ* and *ex situ* and use same to describe the types of bioremediation.

3.1. Biostimulation

Hydrocarbon biodegradation in soil can be limited by many factors, including nutrients, pH, temperature, moisture, oxygen, soil properties and contaminant presence (Atagana 2008, Al Sulaimani 2010; Bundy *et al.*, 2002). Biostimulation involves the modification of the environment to stimulate existing bacteria capable of bioremediation. This can be done by addition of various forms of limiting nutrients and electron acceptors, such as phosphorus, nitrogen, oxygen, or carbon (e.g. in the form of molasses), which are otherwise available in quantities

low enough to constrain microbial activity (Elekrowicz, 1994; Piehler *et al.*, 1999; Rhykerd *et al.*, 1999).

It was described by Perfumo *et al.*, (2007) as the addition of nutrients, oxygen or other electron donors and acceptors to the coordinated site in order to increase the population or activity of naturally occurring microorganisms available for bioremediation.

Margesin, *et al.*, (2000) defined biostimulation is a type of natural remediation that can improve pollutant degradation by optimizing conditions such as aeration, addition of nutrients, pH and temperature control. They opined that biostimulation can be considered as an appropriate remediation technique for petroleum pollutants removal in soil and requires the evaluation of both the intrinsic degradation capacities of the autochthonous microflora and the environmental parameters involved in the kinetics of the in situ process.

The primary advantage of biostimulation is that bioremediation will be undertaken by already present native microorganisms that are well-suited to the subsurface environment, and are well distributed spatially within the subsurface. The primary challenge is that the delivery of additives in a manner that allows the additives to be readily available to subsurface microorganisms is based on the local geology of the subsurface. Tight, impermeable subsurface lithology (tight clays or other fine-grained material) make it difficult to spread additives throughout the affected area. Fractures in the subsurface create preferential pathways in the subsurface which additives preferentially follow, preventing even distribution of additives. Addition of nutrients might also promote the growth of heterotrophic microorganisms which are not innate degraders of Total Petroleum Hydrocarbon thereby creating a competition between the resident micro flora (Adams, 2014).

3.2. Bioaugmentation

Since the 1970s, bioaugmentation, or the addition of oil-degrading microorganisms to supplement the indigenous populations, has been proposed as an alternate strategy for the bioremediation of oil contaminated environments. The rationale for this approach is that indigenous microbial populations may not be capable of degrading the wide range of potential substrates present in complex mixtures such as petroleum (Leahy and Colwell, 1990) or that they may be in a stressed state as a result of the recent exposure to the spill. Other conditions under which bioaugmentation may be considered are when the indigenous hydrocarbon-degrading population is low, the speed of decontamination is the primary factor, and when seeding may reduce the lag period to start the bioremediation process (Forsyth *et al.*, 1995). For this approach to be successful in the field, the seed microorganisms must be able to degrade most petroleum components, maintain genetic stability and viability during storage, survive in foreign and hostile environments, effectively compete with indigenous microorganisms, and move through the pores of the sediment to the contaminants (Atlas, 1977; Goldstein *et al.*, 1985).

Different microbial species have different enzymatic abilities and preferences for the degradation of oil compounds. Some microorganisms degrade linear,

branched, or cyclic alkanes. Others prefer mono- or polynuclear aromatics, and others jointly degrade both alkanes and aromatics.

The study of microbes in bioremediation systems makes possible the selection of microorganisms with potential for the degradation and production of compounds with biotechnological applications in the oil and petrochemical industry.

Successful bioaugmentation treatments depend on the use of inocula consisting of microbial strains or microbial consortia that have been well adapted to the site to be decontaminated. Foreign microorganisms (those in inocula) have been applied successfully but their efficiency depends on ability to compete with indigenous microorganisms, predators and various abiotic factors. Factors affecting proliferation of microorganisms used for bioaugmentation including the chemical structure and concentration of pollutants, the availability of the contaminant to the microorganisms, the size and nature of the microbial population and the physical environment should be taken into consideration when screening for microorganisms to be applied.

Bioaugmentation involves the introduction of microorganisms isolated from the contaminated site, from a historical site or carefully selected and genetically modified to support the remediation of petroleum hydrocarbon contaminated sites based on the assumption and/or confirmation that indigenous organisms within the impacted site cannot biodegrade petroleum hydrocarbon.

4. Types of Bioremediation

Various studies have focused on bioremediation by addition of nutrients or introduction of microorganisms to petroleum contaminated sites. Nutrient additives can be natural or synthetic as well as organic or inorganic, in some cases enzymes can be added to stimulate the remediation processes which again can be naturally occurring or synthetic enzymes. Bioaugmentation can involve isolation of native organisms and 'mass-cultivating' same for reintroduction to contaminated sites, simply procuring preserved microbial cells and inoculation to impacted sites or better still, using genetically modified cells with specificity for the contaminants in question for bioaugmentation processes. There is an array of technology and experimental setup/methodology employed in these studies.

4.1. Biostimulation Using Organic Nutrients

Some reports on the use of organic nutrients for stimulation of petroleum hydrocarbon contaminated sites are shown in Table 1.

Based on these studies, organic nutrients are potentially useful as stimulating nutrients for bioremediation.

Contaminated soil containing more than 38,000mg/kg TPH was remediated using sewage sludge and wood chips compost by Atagana (2008). Pertinent to this research was the temperature regimes in the compost systems, nutrient composition and moisture content. Temperature fluctuations were observed in the control experiment ranging between 12°C and 30°C. In the sewage sludge compost treatment, temperature rose to 58°C in the second month of the experiment which was attributed to high

initial microbial loads. He reported that nitrogen content in the sewage sludge compost decreased faster than the control as the experiment progressed and attributed this microbial activity and higher rate of breakdown in the

compost system. The research noted that within two months, 68.8% of TPH had been degraded by the sewage sludge compost as against 10% in the control and at the end of the 19 month period.

Table 1. BIOSTIMULATION USING ORGANIC NUTRIENTS

Nutrient Added	Type of contaminant	Initial TPH concentration	Percentage Removed	Duration	Comment	References
Compost made from wood chips and sewage sludge	Petroleum Hydrocarbon-crude oil	38,000mg/kg	100% removal of 2-3 ringed PAHs within the first 3 months	570 days	Showed 100% removal over a 19 month period with removal linked to the native microbial population and improved growth in the system	Atagana (2008)
Brewery spent grains, banana skin and spent mushroom compost	Petroleum hydrocarbons-spent lubricating oil	Not indicated	79% and 92% for 5% oil contamination linked to the presence of organic waste and low contamination while reduction was between 17% and 24% in 15% w/w oil contamination initially and 36% to 55% after 84 days linked to high initial concentration	84 days	Showed significant removal of TPH using the organic nutrient sources	Abioye <i>et al.</i> (2012)
Poultry droppings	Petroleum hydrocarbons-contaminated marine sediments	106.4ppm-116ppm TPH and 96.6 – 104ppm PAH	95.35% for TPH and 98.92% for PAH	56 days	Significant degradation of PAH and TPH in a bioreactor using 20g poultry litter and 1 liter seawater	Chikere <i>et al.</i> (2012)
Cowdung	Hydrocarbon polluted mangrove swamp	14,103.02mg/kg	62.96%	70 days	Significant reduction observed when compared with control using cowdung as Biostimulation agent	Orji <i>et al.</i> (2012)
Tea leaf, soy cake and potato skin	Petroleum hydrocarbon-diesel fuel	100000mg/kg and 200000mg/kg variation in concentration	Between 25% and 82%	126 days	Showed significant degradation of TPH for the treatment with soy cake	Dadrasnia and Agamuthu (2013)
Oil palm empty fruit bunch and sugar cane bargasses	Petroleum hydrocarbon crude oil	Not indicated	100% for sugarcane bargasse and up to 97% for empty palm fruit bunch	20 days	Showed significant biodegradation using these supplements for stimulating microbial growth	Hamzah <i>et al.</i> (2014).

Various organic nutrient sources were employed in bioremediation of used motor oil in soil by Abioye *et al.* (2012). Bioremediation of soil contaminated with 5% and 15% w/w of spent oil and amended with 15% of Brewery Spent Grain (BSG), Banana Skin (BS), and Spent Mushroom Compost (SMC) was studied for a period of 84 days *ex situ*. Up to 92% of biodegradation was recorded in soil contaminated with 5% used lubricating oil and amended with Brewery Spent Grain. The same substrate only recorded 55% biodegradation in 15% used lubricating oil contaminated soil. Notably, in this study, the addition of the nutrient sources markedly improved the moisture content of the soil-nutrient-oil matrix as the nutrients (especially BSG) had high moisture content of up to 71.84%.

The findings of this research revealed higher degradation percentage than that recorded by Adesodun and Mbagwu (2008) who reported 42% Total Petroleum Hydrocarbon Degradation after three months. However, in the study category with higher spent lubricating oil composition (15%), exploitation of a mixture of nutrient sources might have proven advantageous since the researchers concluded that BSG improved the moisture content, nutrient content as well as microbial population. This is important more so that Nduka *et al.* (2012) reported that a mixture of nutrient sources to form an improved 'diet' source for microorganisms and induced optimum microbial proliferation conferred high biodegradation

potential in soils with high pollutant concentration and also reduced the duration of degradation-their study revealed high percentage degradation during the first 15 days. Conclusively, the presence of nutrients in the right quantities is necessary in TPH degradation in contaminated soil, the rate of degradation depended on the concentration of contaminants and the nutrient availability; this can further be improved by exploiting consortia of nutrient sources.

In a study similar to that carried out by Abioye *et al.* (2012), Dadrasnia and Agamuthu (2013) observed that biowastes (soy cake, potato skin and tea leaf) had potential to remediate diesel contaminated soil. Their study which lasted 126 days indicated that percentage oil pollution played a part in the rate of remediation as the test sample impacted with 10% oil had up to 82% TPH reduction while the test sample impacted with 20% oil returned just 25% reduction. Both test categories had a colony forming unit count range of between 150×10^6 and 176×10^6 CFUg⁻¹. Soil enzyme (dehydrogenase) activity in their study was markedly enhanced by the application of organic wastes. Specifically, diesel range carbons were degraded by the application of the biowastes with soy cake having the highest biodegradation rate constant of 0.153 day^{-1} at 10% oil pollution. Further, the control soil had a low Nitrogen (0.8%) and Phosphorus (0.6%) content compared to the treatment categories with organic wastes. The rapid decrease in TPH in all the treatment categories

amended with organic wastes as compared with soil without amendment was attributed to the presence of nutrients from the organic wastes especially in 10% oil contamination. This corroborated Bartha's (1986) finding that indicated rapid degradation within three months when oil at rates of 0.5 to 10% based on weight was added to soil.

Fifty six hydrocarbon utilizing bacterial isolates including *Staphylococcus spp.*, *Pseudomonas spp.*, *Citrobacter spp.*, *Klebsiella spp.*, *Micrococcus spp.*, *Corynebacterium spp.*, *Bacillus spp.*, *Rhodococcus spp.*, *Alcanivorax spp.*, *Alcaligenes spp.*, *Serratia spp.*, *Arthrobacter spp.*, *Nocardia spp.*, *Flavobacterium spp.*, *Escherichia spp.*, *Acinetobacter spp.*, *Proteus spp.* were identified from poultry litter and contaminated soil in a study that used poultry dropping as nutrient source for *ex situ* remediation studies carried out by Chikere *et al* (2012). A bioreactor vessel was loaded with 1 kg wet weight of sediments, 1 L of seawater, 20ml of crude oil, 20mg of anthracene and 20g of poultry droppings, another bioreactor vessel with sediment without nutrient served as control 1 while autoclaved sediment with no nutrients in a bioreactor served as control 2. The primary objective of this research was to determine whether the indigenous bacteria in sediments and seawater had the natural propensity to degrade petroleum hydrocarbons while also measuring the role of abiotic factors in the loss of petroleum hydrocarbons. Their study revealed that total heterotrophic and hydrocarbon utilizing bacteria were within the range of 10^5 cfu/g indicating that bacterial makeup was capable of utilizing petroleum hydrocarbons. Baseline concentrations of TPH and PAH was between 106 and 124.2ppm in all treatments and control while it had between 96 and 104.4ppm PAH content in treatment categories and control initially. On completion of the research poultry droppings amended soil had a decreased content of up to 95.35% and 98.92 % for TPH and PAH respectively. Although the rate of degradation was significantly high, the authors reported that autochthonous microorganisms particularly isolated gram-negative bacteria identified in the sediments were peculiar in their ability to grow without using carbohydrates and amino acids as growth substrates. Head *et al.* (2008); Yakmov *et al* (2008) and Pery *et al.* (2008) have all reported that ability of gram negative bacteria to produce gluco-lipids biosurfactant confers an ability to use hydrocarbons almost exclusively as carbon source. Interestingly other researchers (Adams *et al.*, 2014; Dadrasnia and Agamuthu, 2013; Abioye *et al.*, 2012) have suggested that organic nutrients notably nitrogen and phosphorus used as amendment optimized natural degradative ability of microorganisms including gram negative bacteria. This study (Chikere *et al.*, 2011) also revealed the importance of abiotic factors such as agitation and homogenization received during stirring in reduction of hydrocarbon contents.

Recall, that the initial concentration of hydrocarbons plays important role in biodegradation. As revealed by the baseline data, concentration of TPH and PAH were relatively low and within microbial tolerable limits which suggests that the high success recorded in the study under review might not be unconnected with the low concentration, high initial microbial load and further enhanced by nutrient availability. This underlines a part of the conclusion drawn by the researchers insinuating that

contaminated marine sediments (as standalone) had the natural propensity to fully biodegrade Total Petroleum Hydrocarbons and PAH. When concentrations of hydrocarbons overwhelm native microbial population, biodegradative rates are slow and take longer times within which enough ecological damages might have taken place. It then justifies the postulation that for effective bioremediation of petroleum impacted sediments, poultry droppings as well as other nutrient sources could be utilized as further recognized by the researchers in their concluding remarks.

Using cow dung as organic nutrient source has shown good promises in the bioremediation of crude oil impacted mangrove swamps in the Nigeria delta part of Nigeria as reported by Orji *et al* (2012). In their study which lasted 70 days, 500g of contaminated mangrove soil was further spiked with 50ml of Bonny light crude oil simulating the condition of a major spill, and mixed with 50g of dried cow dung. The research was undertaken primarily to determine the effectiveness of cow dung as a source of limiting nutrients in bioremediation of polluted mangrove. The sampling results revealed a minimum of 3.6×10^4 cfu/g and 2.4×10^4 cfu/g of hydrocarbon utilizing bacteria and fungi respectively. The addition of cow dung markedly increased bacterial population to 2.8×10^7 cfu/g on the final sampling day.

THC percentage loss was 62.08% on the 70th day compared to 20% in the control, the reduction in the treatment category was significantly different from the control ($P < 0.05$).

4.1.1. Biostimulation Using Inorganic Nutrients

Inorganic fertilizer sources have also been utilized as Biostimulation agents throughout the world. A study carried out by Chorom *et al* (2010) investigated the efficacy of inorganic fertilizer (NPK) in enhancing microbial degradation of petroleum hydrocarbons in soil. Gas Chromatography results showed that normal paraffin and isoperoid (Phitane and Pristane) decreased in the range 40-60% in all the treatment categories in less than 10 weeks. Addition of inorganic fertilizer improved the CNP ratio of the test setup ultimately promoting microbial degradation.

Agarry and Ogunleye (2012) studied enhanced bioremediation of soil artificially contaminated with spent engine oil *ex situ*. Inorganic NPK fertilizer and non ionic surfactant concentration were used as independent biostimulation variables with the primary objective evaluating TPH reduction as dependent variables. After 42 days, there was a 67.20% reduction in TPH concentration. Using numerical optimization technique based on desirability function, the authors revealed the optimum values for Biostimulation agent studied to achieve 67.20% degradation of TPH was 4.22g and 10.69ug/g for NPK and nonionic surfactant respectively.

Furthermore, Agarry *et al* (2012) using kerosene as source of TPH and inorganic NPK (4.30g) as source of nutrients, obtained total petroleum hydrocarbon degradation of 75.06%. The better performance of NPK in reducing TPH in kerosene contaminated soil when compared to spent engine oil contaminated soil was probably due to the presence of lighter chains of hydrocarbons in the latter as revealed by chromatographic results. This confirmed earlier studies (Venosa *et al.*, 2002)

reporting that microorganisms more readily degraded light end hydrocarbons than heavy end hydrocarbons.

Using modified Fenton and NPK fertilizer, Silva-Castro *et al* (2013) achieved 58%, 57% and 32% TPH reduction in the surface layer, non saturated and saturated layer of diesel contaminated soil (20,000mg/kg). They revealed

that immediately after soil contamination, a specialization and differentiation of the bacterial community occurred, stating that there was a post stimulation enhancement of the degrading microbiota and improvement in degrading biological activity.

Table 2. Bioaugmentation carried out in different countries

COUNTRY	BIOLOGICAL SYSTEMS USED
USA	Pure or mixed cultures of <i>Bacillus</i> , <i>Clostridium</i> , <i>Pseudomonas</i> , and Gram-negative rods; mixed cultures of hydrocarbon degrading bacteria; mixed cultures of marine source bacteria; spore suspension of <i>Clostridium</i> ; indigenous stratal microflora; slime-forming bacteria; ultramicrobacteria
Russia	Pure cultures of <i>C. tyrobutiricum</i> ; bacteria mixed cultures; indigenous microflora of water injection and water formation; activated sludge bacteria; naturally occurring microbiota of industrial (food) wastes
China	Mixed enriched bacterial cultures of <i>Bacillus</i> , <i>Bacteroides</i> , <i>Eubacterium</i> , <i>Fusobacterium</i> , <i>Pseudomonas</i> ; slime-forming bacteria: <i>Brevibacterium viscosogenes</i> , <i>Corynebacterium gumiform</i> , <i>Xanthomonas campestris</i>
Australia	Ultramicrobacteria with surface active Properties
Bulgaria	Indigenous oil-oxidizing bacteria from water injection and water formation
Canada	Pure culture of <i>Leuconostoc mesenteroides</i>
Former Czechoslovakia	Hydrocarbon oxidizing bacteria (predominant <i>Pseudomonas sp.</i>); sulfate-reducing bacteria
England	Naturally occurring anaerobic strain, high generator of acids; special starved bacteria, good producers of exopolymers
Former East Germany	Mixed cultures of thermophilic <i>Bacillus</i> and <i>Clostridium</i> from indigenous brine microflora
Hungary	Mixed sewage-sludge bacteria cultures (predominant: <i>Clostridium</i> , <i>Desulfovibrio</i> , <i>Pseudomonas</i>)
Norway	Nitrate-reducing bacteria naturally occurring in North Sea water
Oman	Autochthonous spore-forming bacteria from oil wells and oil contaminated soil
Poland	Mixed bacteria cultures (<i>Arthrobacter</i> , <i>Clostridium</i> , <i>Mycobacterium</i> , <i>Peptococcus</i> , <i>Pseudomonas</i>)
Romania	Adapted mixed enrichment cultures (predominant: <i>Bacillus</i> , <i>Clostridium</i> , <i>Pseudomonas</i> , and other Gram-negative rods)
Saudi Arabia	Adequate bacterial inoculum according to requirements of each technology
The Netherlands	Slime-forming bacteria (<i>Betacoccus dextranicus</i>)
Trinidad-Tobago	Facultative anaerobic bacteria high producers of gases
Venezuela	Adapted mixed enrichment cultures

Source: Lazar *et al* 2007; Al-Wahaibi *et al* 2012; Bahry *et al* 2013, Al Sulaimani 2010, Alsulaimani, 2011, Al-Wahaibi, 2013).

Table 3. Specific Bioaugmentation Researches

Type of contaminant	Initial concentration	TPH	Microorganisms added	Source of Microorganisms	Duration of research	TPH reduction (%)	Comment	References
TPH (Crude oil)	4200mg/kg		Acinetobacter baumannii T30C	Tapis crude oil contaminated soil of oil refinery plant	35 days	43%	TPH induction was not entirely induced by the introduction of <i>A. baumannii</i> T30C as the reduction observed was not significantly different from the control	Chang <i>et al</i> , (2011)
TPH (Crude oil)	>100mg/kg		Serratia Sp BF40	Crude oil contaminated saline soils	Unknown	>60%	BF40 Strain of Serratia showed high utilized potential for biodegradation of crude oil contaminated saline soils due to its high surface activity and salt tolerance	Wu <i>et al</i> (2012)
TPH, contaminated by parking trucks, human activities and diesel	10,000mg/kg		<i>Bacillus cereus</i> , <i>Gordoni rubripertincta</i> , <i>Kocirria rosea</i> , <i>Bacillus subtilis</i> (Strains 7A and 9A), <i>Aspergillus terreus</i> , <i>Aspergillus carneus</i>	Isolated from pre contaminated sites and assayed for ability to degrade TPH	90days	52.0%	Soil TPH degradation was not significantly different from reduction observed in control, although there was a significant reduction ($P < 0.05$) relative to initial TPH concentration	Diaz-Ramirez <i>et al</i> (2013)
TPH (diesel)	16,300mg/kg		<i>Candida tropicalis</i> SK 21	Petroleum contaminated soil	120days	83%	Inoculation of yeast resulted in 83% TPH removal as against 61% using indigenous microorganisms	Fan <i>et al</i> , 2013
TPH (Diesel from Tank top accident)	>5,000mg/kg		<i>Pseudomonas fluorescens</i>	Isolated from previously contaminated soil	270days	>60%	Phospholipid Fatty Acids (PLFA) released was measured as an indication of living microbial biomass. Augmentation with <i>P. fluorescens</i> markedly improved the PLFA and degradation as compared to samples relying on autochthonous microbes	Kuran <i>et al</i> (2014)
TPH (Biodiesel Diesel)	Unknown		<i>Pseudomonas aeruginosa</i> , <i>Arthrobacter xylosoxidans</i> and <i>Ochrobacterium intermedium</i>	Exogenous and identified through sequencing of 16s rRNA gene	32 days	32.2%	Successive bioaugmentation displayed positive effects on biodegradation with substantial reduction in TPH levels	Colla <i>et al</i> 2014

4.2. Bioaugmentation

Application of bioaugmentation in different countries around the world is presented on Table 2. Also, some recent reports on the application of microbial strains for

the remediation of crude oil contaminated soils are presented in Table 3.

Microbial degradation of Tapis crude oil contaminated soil by *Acinetobacter baumannii* T30C was studied by Chang *et al* (2011) to evaluate the efficiency of the selected potential hydrocarbon degraders in stimulating bioremediation of crude oil contaminated soil. The microcosm study lasted for 35 days and revealed a low level of degradation and depletion of available nutrients. Initial microbial counts were within the prescribed range of 10^5 CFU/g for optimum bioremediation (Mishra *et al* 2001), further justified by the final degradation results which were not significantly different from the control samples (with no augmentation). The authors concluded that addition of nutrients was necessary for enhancing bacterial growth and degradation activity except in cases where the indigenous identifiable petroleum degraders was too small and necessitates introduction of biodegrading strains.

Surface activity of salt tolerant *Serratia Spp.* and crude oil degradation in saline soil has been demonstrated. (Wu *et al.*, 2012). A novel strain of *Serratia Spp* BF40 was isolated from crude oil contaminated soils and evaluated for its salt tolerance, surface activity and ability to degrade crude oil in saline soils. The authors suggested that BF40 could decrease surface tension of oily soil surfaces, induce hydrocarbon breakdown and concluded that using organisms with biosurfactant producing ability was efficient but deserved further insight into growth requirements for the organisms to achieve continuous biosurfactant supply.

Diaz-Ramirez *et al* (2013) have studied hydrocarbon biodegradation potential of native and exogenous microbial inocula in Mexican tropical soils. A laboratory experiment using artificially contaminated soil (with Olmeca crude) revealed that the number of viable microorganisms was higher in cultures with standardized mixture with bioaugmentation strains. The results of the

research under spotlight indicated that the application of microorganisms (bacteria and fungi) was favorable in terms of overall growth, stimulating a synergistic effect between both populations with no significant difference found between the two treatments for biodegradation of oil. Further analysis of the findings showed that in the first 30 days of treatment, there was a rapid decline in the concentration of hydrocarbons in both bioaugmented and non bioaugmented soils, with the rate of biodegradation slightly decreased in the latter. Colony-morphology observations further revealed that microorganisms inoculated in the treated soil were prevailing at the end of the assay thus conferring a slight advantage. On the overall, non bioaugmented soils showed a good potential when compared to bioaugmented soils as the rate of degradation was not of significant difference at the initial phase of the research.

Candida tropicalis (SK 21 strain) has been studied for bioaugmentation ability. (Fan, *et al* 2013). The initial TPH concentration of 16,300mg/kg (51% saturated hydrocarbons and 31% aromatic hydrocarbons). At optimum pH 96% and 42% of TPH were degraded by the strain at the initial diesel oil concentrations of 0.5 and 5% (v/v) respectively while the control setup yielded 61% reduction. It was also found that yeast inoculation remarkably enhanced dehydrogenase and polyphenoloxidase activities in soil.

5. Combination Strategies

Scientists have tried to exploit a combination basis for both mechanisms for achievement of higher degradation rates. As long as the nutrients are not toxic and detrimental to microbial survival, then a combination technology is feasible.

The table below presents summary of some combination strategies.

Table 4. COMBINATION THERAPY

Type of contaminant	Initial TPH concentration	Microorganisms and nutrients added	Source of Microorganisms	Duration of research	TPH reduction (%)	Comment	References
TPH from diesel oil	Not stated	A consortia isolated from previously contaminated soil and $(\text{NH}_4)_2 \text{SO}_4$ and K_2HPO_4	Previously contaminated soil	84 days	45% and 72.7% for biostimulation and bioaugmentation respectively	Bioaugmentation using microbial load from a pre-contaminated soil enhanced the autochthonous strain and overall degradation as opposed to biostimulation as stated by the authors	Bento <i>et al</i> , 2004
TPH from motor oil	40,000mg/kg	<i>Bacillus Sp.</i> , <i>Pseudomonas Sp.</i> and <i>Proteus</i> , NPK fertilizer	Isolated from automobile workshops	42 days	65% reduction	Nutrient addition improved the rate of remediation as control option amended with water only returned 42% reduction	Abdusalam <i>et al</i> , 2009
TPH from crude artificially spiked	50,000ppm	Halotolerant actinobacterial strains and sodium chloride. <i>Rhodococcus Sp.</i> , <i>Gordonia rubripincta</i> , <i>Rhodococcus Sp.</i> , <i>G. Alkanivorans</i> , <i>R. equi</i> , and <i>Rhodococcus Sp.</i>	Previously contaminated soil	Not stated	Up to 36.2% degradation for first soil sample and 51% of second soil sample	This research indicated a higher degradation for n-alkanes than total petroleum hydrocarbons; 67.7% and 77.3% for sample A and B as against 36.2%, 51% respectively	Alvarez <i>et al</i> , 2011
TPH from diesel contaminated site	30,000ppm	<i>Rhodococcus Sp.</i> EH 831 and Tween 80 surfactant	Isolated from previously contaminated soil	46 days	>50% reduction	The addition of surfactant seemed to enhance the availability of the diesel for microbes subsequently improving TPH degradation	Eun Hee <i>et al</i> , 2011
TPH from spent motor oil	14,100mg/kg	<i>Pseudomonas aeruginosa</i> , <i>Bacillus subtilis</i> and fertilizer; NPK 20:10:10	Stock culture obtained from a research institute	70 days	75% reduction	Nutrient availability was optimum for the entire duration of the research as observed in the Nitrogen, Phosphorus and Potassium content measured throughout the research	Abdusalam <i>et al</i> , 2011

Soils contaminated in the field were collected and comparatively studied for the effect of microbes and nutrients on the overall degradation of diesel TPH over a 12week study period by Bento *et al* (2004). The research design was done to compare the process of combination therapy with bioaugmentation. The researchers studied the degradation of diesel oil in the light (C₁₂-C₂₃) and heavy (C₂₃-C₄₀) range. They reported a reduction of 63-84% of the light fraction using microbial consortium and 72% in the light fraction using a combination treatment (Biostimulation and bioaugmentation). A degradation of 19% and 31% was reported for heavy fractions upon combination treatment and bioaugmentation respectively. Notably, the authors stated that the addition of nutrients did not significantly impact the number of diesel degrading microorganisms and heterotrophic population and acknowledged a major limitation in a lack of detailed site specification and characterization. This they claim was very necessary prior to deciding the technique to be adopted for bioremediation. Conclusively, the researchers advocated that nutrient provision for remediation was more or less not as important as inoculation of proven microbial strains with biodegradative qualities; although the difference in these rates seemed not to be statistically significant.

Employing a combination therapy is also useful in TPH degradation with spent motor oil being the contaminant (Abdusalam and Omale, 2009). In a study that lasted 42 days, biostimulation had the highest TPH removal rate of 69.2% followed by biostimulation and bioaugmentation combined which had 65.2% reduction. Other categories (water amendment and control) had 58.4% and 43% reduction respectively as reported by the researchers. Inoculation with a consortium of *Bacillus Sp*, *Pseudomonas Sp*, and *Proteus Sp* (all known degraders) and NPK fertilizer did not improve the degradation rates as only nutrient amended (NPK) recorded a better overall percentage reduction.

Using a combination of experimental techniques, Alvarez *et al* (2011) studied bioremediation of tropical soil microcosms by bioenrichment, bioaugmentation and natural attenuation. The authors studied the degradation rate of n-alkanes as well as TPH. Crude oil contaminated soil was augmented with actinomycetes strain, combination with sodium chloride and a final category monitored closely. Their results indicated that degradation of n-alkanes was uniform in all categories nullifying a dependence on amendment or augmentation (since the monitored category showed degradation as well). Interestingly, they reported that total bacterial community in the soils was mainly affected by the experimental period of time and concluded that monitored attenuation was the best strategy for remediation with or without addition of sodium chloride.

Eun-Hee *et al* (2011) employed a combination of *Rhodococcus Sp*. EH831 and a surfactant for the bioremediation of diesel contaminated soils and compared this treatment with bioaugmentation using *Rhodococcus* species only. As observed by the authors, the addition of the surfactant had no significant effect on the remediation performance. Even though surfactants alone achieved significant remediation rates as reported by Reznik *et al* (2010), their presence in the research under focus affected

neither TPH removability nor the physiological properties of the microorganisms.

A study demonstrated the feasibility of using microorganisms and nutrients for bioremediation (Abdusalam *et al* 2011). Using aerobic fixed bed reactors to comparatively study bioremediation using bacteria, bacteria and inorganic fertilizer (and potassium dihydrogen orthophosphate) the microbes achieved a degradation rate of 75% as opposed to 66% and 50% in bioaugmentation and control categories respectively. They conclusively indicated that a combination technique could be used to develop a realistic treatment technology for soils contaminated with spent motor oil.

6. Recent Strategies for Bioremediation

The use of certain genetically engineered microorganisms to influence their ability to utilize specific contaminants such as hydrocarbons and pesticides is gaining grounds. This technique had an early mention in the late 1980s and early 1990s. The ability to 'engineer' microorganisms to improve degradative properties is based on a possibility to explore genetic diversity and metabolic versatility of microorganisms (Fulekar, 2009).

The blueprint necessary for gene encoding for biodegradative enzymes is present in chromosomal and extra chromosomal DNA of such microbes. Recombinant DNA techniques explore the ability of an organism to metabolize a xenobiotic by detecting the presence of degradative genes and transforming them into appropriate hosts through a suitable vector within a controlled setting. This technology explores Polymerase Chain Reaction (PCR), anti-sense RNA technique, site directed mutagenesis, electroporation and particle bombardment techniques.

The first step in Genetically Modified Microorganism (GMM) construction is selection of suitable gene(s), next, the DNA fragment to be cloned is inserted into a vector and introduced into host cells. The modified bacteria are called recombinant cells. The next step is production of multiple gene copies and selection of cells containing recombinant DNA. The final step includes screening for clones with desired DNA inserts and biological properties.

Since the possibility of conferring new properties into existing organisms abound, researchers have studied the ability of modified organisms to degrade petroleum hydrocarbons or hydrocarbon based compounds. For example, Newby *et al*. (2000) studied transfer of plasmid pJP4 from two introduced donors, natural host *R. eutropha JMP134* and laboratory-constructed strain *Escherichia coli D11*, to indigenous microbes in soil contaminated with 2,4-D. A key difference between donors was their ability (*R. eutropha*) or inability (*E. coli*) to mineralize 2,4-D. Additionally, they studied transconjugant occurrence, their identification and plasmid persistence. Both inoculated donors were detectable and they transferred plasmid pJP4 to indigenous recipients to different extents. In the first experiment 2,4-D was degraded significantly faster (28 days) in soil inoculated with *R. eutropha JMP134* as compared with soil inoculated with *E. coli D11* (49 days). Interestingly, a greater number of transconjugants was detected in *E. coli D11*-inoculated soil and they were members of the

Burkholderia and *Ralstonia* genera. After reamendment 2,4-D was degraded more rapidly in the soil with *E. coli* *D11* inoculants than in *R. eutropha* *JMP134*-inoculated treatments. These results indicated that choice of donor microorganism is a crucial factor to be considered for bioaugmentation approach.

In an initial study, The University of Tennessee in collaboration with Oak Ridge National Laboratory performed field based bioremediation using Genetically Modified Microorganisms. The organism involved was *Pseudomonas fluorescens* strain designated HK44, released into a hydrocarbon contaminated environment. The original parental strain from which the strain HK44 was derived was isolated from a manufactured gas plant heavily contaminated with polyaromatic hydrocarbons (PAHs). The naphthalene catabolic plasmid (PUTK21) was introduced into the strain to form *P. fluorescens* *HK44*. Upon introduction of the modified organism to naphthalene (or the intermediate metabolite salicylate) there was an increased catabolic gene expression, naphthalene degradation and a coincident bioluminescent response.

Because of well-established tools from metabolic engineering and biochemistry it is possible to infuse different pathways into a 'designer' microbe. This technique is a very powerful approach to enhance petroleum hydrocarbon biodegradation. Very often, these pathways are combined with existing pathways to enable complete biodegradation. The construction of a hybrid strain which is capable of mineralizing components of a mixture of benzene, toluene and p-xylene simultaneously was attempted by redesigning the metabolic pathway of *Pseudomonas putida*. A hybrid strain expressing both the toluene and the p-xylene pathways was constructed and was found to mineralize a benzene, toluene and p-xylene mixture without accumulation of any metabolic intermediate. (Chen *et al.*, 2005).

Irrespective of the glowing prospects of genetic engineering to confer new properties on microbes and subsequently improve their abilities on the field, the practice is faced with some constraints. Sayler *et al.* (2002) report that there is difficulty in deducing the exact extent the microbe under modification actually contributes to the degradation process, recognizing that factors such as volatilization and chemical transformation simultaneously occur within a reactor system. In using GMM, it can be problematic distinguishing between GMM specific degradation and biodegradation due to the presence of indigenous microbial consortia. Another obstacle is an inability to statistically conclude on bioremediation efficiency because of the highly heterogeneous distribution of the contaminants. Sample-sample chemical analyses can typically vary by up to 200% making valid conclusions blurred. To buttress this, in an experiment using *P. fluorescens* *HK44* lysimeter release, soil PAH concentrations were dispersed heterogeneously ranging from 0.04 to 192ppm spatially. Consequently, a precise evaluation of the effectiveness of *P. fluorescens* *HK44* in the overall process could not be adequately determined. Statistical models that can incorporate chemical heterogeneity kinetics into the entire design are required before valid efficacy assessments can be obtained.

Another impediment to actualizing field release studies is the securing of the required governmental permission, which is often a difficult and lengthy endeavor. Although

necessary to ensure environmental and public health safety, the process often leads to an overall aversion to GEM implementation in environmental systems, with researchers concentrating rather on the optimization and commercial development of naturally occurring (intrinsic) microbial degradation [Cha *et al.*, 2000]. Also, during the approval process the GEM might undergo significant refinement and genetic restructuring while in the hands of researchers, making the originally proposed release microorganism somewhat obsolete. This unfortunately prevents the integration of state-of-the-art engineered microbes into field release studies.

7. Concluding Remarks

We have elucidated the fact that biodegradation of petroleum hydrocarbon in soils is achievable with microbial metabolism. The examples above have shown that relying on autochthonous microorganisms alone was not as reliable as other techniques since usually, the remediation percentages after supplement application (microbes or nutrients) were generally higher than control. It was evident that nutrients (organic and inorganic) could also promote microbial growth and degradation of hydrocarbons. There seemed to be a general agreement that indigenous microorganisms needed to be "promoted" and "nourished" when it is observed that nutrients notably nitrogen, phosphorus and potassium were deficient. Some authors postulated that the presence of hydrocarbons within tolerable ranges for microbial survival was enough nutrient for microbial growth and advocated that a supply of more microbial population tested and trusted (most likely isolated from previously contaminated sites) could serve as ready-to-use boost instead of nutrient addition. Worthy of note is the fact that nutrient addition if not done scientifically could be detrimental in the sense that it could aid heterotrophic population and inadvertently trigger an antagonistic situation thereby limiting the degradation process. In addition, some authors investigated a combination approach to further enhance the potential of a more robust technology. This approach seemed to fuse the advantages of each of the previously described technologies while trying to eliminate the hurdle of nutrient unavailability and petroleum degrading microbes' insufficiency. It proved an interesting deviation as exploitation of locally available support material and microbes isolated from previous contamination promise an economically viable and scientifically favorable process. Further, a successful implementation of a remediation regime required a consideration of the indigenous biota, nutrient availability as well as other environmental parameters necessary to achieve optimum results. Finally, a combination of technologies regulated within stringent conditions and allowed enough time will prove tremendously important in returning contaminated soils to fit-for-purpose states.

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