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BIOREMEDIATION OF OIL SPILLS: A REVIEW OF CHALLENGES FOR RESEARCH ADVANCEMENT

Babajide Milton Macaulay*[1], Deborah Rees*[2]

[1,2] Natural Resources Institute, University of Greenwich, Chatham Maritime, Kent ME4 4TB, United Kingdom

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ABSTRACT

As the demand for liquid petroleum increases, the need for reliable and efficient oil spill clean-up techniques is inevitable. Bioremediation is considered one of the most sustainable clean-up techniques but the potential has not been fully exploited in the field because it is too slow to meet the immediate demands of the environment. This study reviews the challenges to managing oil spills in terrestrial and marine environments to identify areas that require further research. Current challenges associated with bioremediation of spilled petroleum include resistance of asphalthenes to biodegradation; delay of heavy or high molar mass polycyclic aromatic hydrocarbon (PAH) biodegradation, eutrophication caused by biostimulation, unsustainability of bio-augmentation in the field, poor bioavailability of spilled petroleum, inefficiency of biodegradation in anoxic environments and failure of successful bioremediation laboratory studies in the field. Recommendations offered include encouraging asphalthene biodegradation by combining heat application (80°C), biosurfactant (thermophilic emulsifier) and bioagumentation (using a consortium containing *Bacillus lentus* and *Pleurotus tuberregium* as members) but as a temporary measure, adopting the use of 'booms and skimmers' and 'organic sorbents' for water and land clean-up, respectively. Heavy PAHs may be rapidly degraded by applying nutrients (biostimulation) and biosurfactants to sites that are oleophilic microbe-rich. Oleophilic nutrients may be the most effective strategy to reduce eutrophication in marine environments whilst on land, slow-release nutrient application or organic-inorganic nutrient rotation may help prevent soil hardening and infertility. The use of encapsulating agents and genetically-engineered microbes (GEMs) may increase the efficiency of bioaugmentation in the field, but temporarily, indigenous oleophilic microbes may be employed in the field. Poor bioavailability of crude oil may be eliminated by the use of biosurfactants. In terrestrial anoxic sites, bioslurping-biosparging technology could be used whilst the marine anoxic site requires more research on how to transport nutrients and biosurfactants to oleophilic anaerobes residing in the ocean beds. The involvement of both governmental and non-governmental environmental institutions in sponsoring field studies in order to improve the reliability of bioremediation research. Further studies to test the practicability and cost of these recommendations in the field are needed.

***Keywords*:** Crude oil bioremediation, land oil spill control, marine oil spill control, biostimulation, bioaugmentation, microbial remediation and hydrocarbon compounds.

1. INTRODUCTION

1.1. Background to the Study

Human activity has led to the release of liquid petroleum hydrocarbon (also known as *crude oil*) into the environment, causing the pollution of marine/coastal waters, shorelines and land as well. Liquid petroleum hydrocarbons are a naturally-occurring fossil fuel, formed from dead organic materials in the earth's crust [1]. They is used to synthesize plastics, fertilizers, pesticides and other petrochemical products. In addition, they are refined to form fuels to run internal combustion engines of cars and vehicles as well as heavy plants and machinery used by a wide range of industries around the world. The domestic utilization of petroleum products in households for heating, cooking, lighting and electricity generation, has increased the demand for these liquid hydrocarbons.

In 2008, the volume of petroleum demanded globally was 85.62 million barrels per day [2]. However, liquid petroleum has become one of the most prevalent pollutants in industrialised and developing countries [3]. Its transportation and global usage has increased the tendency to pollute the environment [4]. The source of the pollution is usually accidental spills, uncontrolled landfills, leaking underground storage

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* [1]Email: mb210@gre.ac.uk; Mobile: +447574885638,
[2]Email: D.rees@gre.ac.uk; Mobile: +441634883522

tanks or improper storage of crude oil [5]. Oil spills pose serious environmental challenges due to the possibility of air, water and soil pollution [6]. Large oil spills threaten both terrestrial and marine ecosystems; hence, attention has been drawn towards identifying eco-friendly and cost-effective clean-up methods [7].

1.2. Justification for the Study

It is imperative to note that the increasing demand for liquid petroleum may likely not reduce the number of oil spill occurrences. Therefore, oil spill accidents are prone to occur considering the enormous pressure on oil companies/drilling firms to make the petroleum product readily available for global consumption. Whenever there is an oil spill, shorelines, marine waters, groundwater, soils (including farmlands), lakes, rivers and creeks, stand the risk of being severely polluted, and if not controlled within a short time frame, may lead to long-term ecological devastation. Several oil spill remediation techniques for the clean-up of polluted terrestrial and marine environments have been established. However, most of them have been proven to be cost-ineffective and environmentally-unfriendly and hence unsustainable. As a result, a control measure that will be swift, efficient and sustainable is a necessity. This study is a review of one of the most sustainable methods of oil spill control.

One of the methods of cleaning up oil spills that has been explored is the use of “oil-eating” or “oil-loving” (oleophilic) microbes [8], a process known as *Bioremediation*. Bioremediation employs living organisms such as bacteria and fungi to degrade complex chemical compounds (which are often harmful to the environment) into simpler compounds (which are harmless to the environment). Bioremediation may be *in situ* – if the oil is treated at the contaminated site – or *ex situ* – if the oil is carried away from the contaminated site to a treatment facility.

Naturally-occurring bioremediation is also known as *bioattenuation* [9] or natural attenuation but it is usually too slow to meet the immediate need of the environment after an oil spill. Therefore, attempts have been made to increase the efficiency of the process through various enhanced techniques. These techniques, also referred to as *biorestoration* in some old publications [10-12] include landfarming, composting, use of bioreactors [13], bioventing/biosparging, pump and treat strategies, bioslurping, biostimulation, and bioaugmentation [14].

Many authors [4,6,15-17] consider bioremediation to be a cost-effective (involves the use of ubiquitous oleophilic microbes) and eco-friendly (which breaks down crude oil into non-toxic products and intermediates) clean-up method compared to other oil spill control techniques. However, other authors [14,18-20] have pointed out that bioremediation is ineffective in cleaning up heavy components of crude oil and is often limited by abiotic factors such as nutrient availability, temperature and oxygen concentration.

Therefore, this study is intended to identify the main challenges associated with the use of bioremediation to clean-up crude oil pollution in both terrestrial and marine environments, and also to develop recommendations, which are likely to form the basis for new lines of research on how to overcome these challenges.

2. SPILLED PETROLEUM HYDROCARBONS

2.1. The chemical Composition and Nature of Spilled Petroleum Hydrocarbons

The rate of biodegradation of petroleum hydrocarbons varies depending on the composition and chemical nature of the constituent parts. Crude oil is a liquid petroleum containing thousands of hydrocarbon components. Each component has a unique chemical behavior that makes it either easily biodegradable, quite difficult to digest or not degradable at all [21].

Petroleum hydrocarbon molecules can be grouped into four broad categories: saturates (branched, unbranched and cyclic alkanes), aromatics – ringed hydrocarbon molecules such as monocyclic aromatic hydrocarbons (MAHs) and polycyclic aromatic hydrocarbons (PAHs), resins (polar oil-surface structures dissolved in saturates and aromatics) [22], and asphalthenes (dark-brown amorphous solids colloidally dispersed in saturates and aromatics) [21-23]. In the structural arrangement of the four main hydrocarbon components of crude oil, saturates make up the outermost layer of the oil whilst asphalthenes constitute the innermost portion of the oil due to their greater molar masses.

According to van Hamme et al. [24], the susceptibility of crude oil components to microbial degradation are in the following order: alkanes > light aromatics (MAHs) > cycloalkanes > heavy aromatics (PAHs) > asphalthenes. Resins are easily degraded naturally because they are light polar molecules [25].

PAHs contain more than one benzene ring and those that are made up of two or three cyclic rings such as naphthalene (two-ringed), phenanthrene (three-ringed) and anthracene (three-ringed) with molecular weights of 128, 178 and 178 g/mol, respectively, are referred to as low molecular weight or light PAHs [26]. PAHs made up of four rings and above such as pyrene (four-ringed), chrysenes (four-ringed), fluorenthene (five-ringed), benzo[a]pyrene (five-ringed) and coronenes (seven-ringed) with molar masses of 202, 228, 202, 252 and 300 g/mol, respectively, are referred to as high molar masses or heavy PAHs [26-30].

PAHs are common petroleum contaminants in the environment that are considered to be potentially mutagenic and carcinogenic [31,32]. The Breast Cancer Fund [33] reported that heavy PAHs such as benzo[a]pyrene damage the DNA of living organisms (i.e. they are genotoxic) and are implicated in human breast cancer. This accounts for a large number of studies on the biodegradation of PAHs in order to safeguard the environment and biodiversity from severe long-term ecological and medical damage by oil spills. However, the focus has been on the biodegradation of light PAHs whilst very little research has been carried out on the biodegradation of heavy PAHs that have been found to be of medical importance.

Asphalthenes are considered to be highly resistant to biodegradation due to their heavy, viscous nature [21]. Asphalthenes are very complex chemical structures made up of sulfur (0.3 - 10.3%), nitrogen (0.6 - 3.3%), oxygen (0.3 - 4.8%) and trace amounts of metals such as iron, nickel and vanadium [34]. In addition, asphalthenes have the highest molar mass of all hydrocarbon compounds in crude oil with values ranging from 600 to 3×10^5 g/mol and from 1000 to 2×10^6 g/mol [26,35,36]. This chemical complexity has rendered asphalthenes resistant to microbial attack and unfortunately few studies have been carried out to enhance the potential of biodegradation of asphalthenes.

2.2. The Fate of Spilled Petroleum Hydrocarbons

Fundamental variation exists in the manner in which crude oil behaves when spilled on land and water. Oil spilled on the sea surface undergoes various weathering processes simultaneously, such as spreading – influenced by wind, turbulence and the presence of ice on the water surface [37], evaporation, emulsification, photo-oxidation, dispersion, sinking, resurfacing, tar ball formation, and biodegradation – which makes oil spill control very difficult [20]. Hence, the extent of the damage caused by the spill and the ease of clean-up depends on how quickly the clean-up response takes effect. The kinetics of these processes depends largely on sea conditions and the meteorological environment [38].

In the marine environment, evaporation generally accounts for 30-50% of spilled petroleum. About 100% of volatile hydrocarbons (e.g., alkanes), 75% of heavier hydrocarbons (e.g., aromatic hydrocarbons) and 10% of the non-volatile hydrocarbons (e.g., asphalthenes) are lost due to evaporation [37, 39]. The remainder after most of the evaporation and solubilisation of lighter fractions have taken place form a stable and gelatinous water-in-oil emulsion (contains 70-80% of water) known as 'mousse'. It is in the mousse form that most oil that has been spilled offshore actually impacts shorelines and beaches. Further weathering of mousse by biological and photo-oxidation produces lumps of a very dense, semi-solid, asphaltic residuum, known as 'tar balls' [40]. As a result of the various natural weathering processes that crude oil spilled in water is subjected to, the fate and behavior of the spill remains largely unpredictable [41,42]. On the other hand, the fate and behaviour of oil spills on land have a higher level of predictability mainly because the terrestrial habitat is more confined; therefore, spilled oil travel is limited. Land surface is rarely smooth so the thickness of oil layers varies considerably and the oil may collect, forming pools in depressions [43]. There is a high tendency of spilled oil on land to flow downhill and empty into ditches, streams, creeks and rivers. The rate of downhill movement depends on oil viscosity, topography and atmospheric/ground temperature.

In addition, the rate of natural weathering of the spilled oil on land largely depends on the exposed surface area of the spill and slows down over time compared to water oil spills where crude oil thins to a thickness of a few millimetres [43] as it spreads extensively. Light crude oil components (such as alkanes) leach into the soil depending on the soil porosity and permeability or may evaporate rapidly based on favorable atmospheric conditions [43]. Heavy crude oil components (such as asphalthenes), on the other hand, tend to be retained at the surface due to their higher viscosity and thickness [43]. Oil spill stability is usually a land phenomenon and it occurs within a short period of time with the gradual stoppage of further weathering. This rarely occurs in marine oil spills [43], probably as a result of the dynamic nature of the marine environment.

2.3. Ecological Impacts of Spilled Petroleum Hydrocarbons

Both land and marine ecosystems suffer from the impact of oil spills in similar ways. The impact on living organisms could either be direct, indirect or acute (short-term) and chronic (long-term). Direct impacts include suffocation (clogging of the lungs, nasal passages or oxygen-exchange sites), anoxia (thick oil slicks on the surface inhibit oxygen from dissolving in ocean waters), and inhibition of movement of animals within the soil, river or ocean due to the viscous nature of crude oil. Indirect impacts, on the other hand, include stunted growth (in both plant and animal forms), reproductive and morphological deformities and trophic cascades [44].

The short-term impacts include acute narcosis mortality (a state of unconsciousness that leads to death), acute exposure of feathers and fur causing hypothermia (a condition in which the core body temperature drops below the optimum required for normal metabolism), smothering, drowning and ingestion of toxic compounds during preening, whilst the long-term impacts include exposure of embryos to weathered oil, ingestion of contaminated prey or foraging in polluted sedimentary pools, and the disruption of important social functions (such as care-giving) in gregarious species [44]. Marine environments suffer a greater ecological damage compared to terrestrial environments mainly as a result of the greater difficulty of controlling the spill.

It is vital to note that the size of a spill does not necessarily tell much about its potential to cause damage, because a small spill can wreak havoc in an ecologically-sensitive environment [40]. A few tons of petroleum spilled in the wrong place at the wrong time may have the potential to kill most living species at the spill site [1]. For example, a small operational discharge of oily bilge washings from a tanker killed an estimated 30,000 seabirds near Norway in 1981 because it occurred at a location where they were seasonally abundant [37].

Similarly, the Exxon Valdez oil spill in 1989 released about 245,000 barrels of crude oil which led to the death of 250,000 birds, mostly black guillemots, which are also known as murres [45]; but the Braer oil spill in 1993 released about 595,000 barrels of crude oil, which killed only 1,500 birds despite its much larger spill size [46]. According to Kingston [1], this variation in the response of biodiversity to oil spills may have been caused by factors, such as breeding season, which either reduces or increases the resultant effect of the spill in the polluted area. In other words, a few barrels of oil spilled during the reproductive season of living organisms could lead to the complete extinction of already threatened species.

Oil spills in marine habitats affect mostly marine birds (diving birds in particular) and fish, rendering them vulnerable to the adverse effect of petroleum pollutants [20]. However, the chemical dispersants used to control the spill have been found to be very harmful and, in some cases, kill shellfish [20]. The environment near a petroleum refinery or a tanker terminal can be freely exposed to chronic oil pollution from frequent spills, and from the continuous discharge of contaminated process water [40]. In some cases, a high frequency of both cancerous and non-cancerous disease of fish and shellfish has been detected in severely polluted sites [47].

Experimental studies by Freedman [40] showed that vegetation with meristematic tissues that were not totally damaged by an oil spill had a remarkable post-pollution regeneration. However, lower plants such as lichens and bryophytes that were predisposed to the experimental oiling of tundra and boreal forest vegetation had no resistance during the first few post-oiling years and almost died completely [40]. There are cases when recovery appeared to be almost impossible or may take decades to be achieved. For example, the Amoco Cadiz oil spill killed the population of small sand hopper (amphipod, *Ampelisca*), which dominated the polluted community. The high sensitivity of the amphipod species to oil pollutants prolonged the recovery of the species, as it took over 10 years for the species to re-populate the area [1].

Oil spills in third-world countries often have a much larger impact on the environment and human lives compared to oil spills in the developed countries, probably due to the insufficient infrastructure or technologies for controlling oil spills. Other factors include the higher number of uneducated citizens who are not enlightened about the potential impacts of oil spills and the weak environmental laws in third-world countries, which if strengthened, would control the activities of oil drilling firms. For example, in the Bodo community and the Ogoni land of the Niger Delta region of Nigeria, Shell Petroleum massively spilled several million barrels of oil in 2009, polluting farmlands and groundwater via land pits or wells [48]. The crude oil in groundwater ended up in nearby rivers, killing aquatic life and disrupting fishing activities, the only means of livelihood in the rural area.

This massive spill also prevented the fetching of clean water from streams for domestic use by the inhabitants of the rural community. Some of the villagers still go to the polluted rivers to fetch water, as they are ignorant of the consequences of their actions, thereby increasing the impact on human health [49] via ingestion, inhalation and contact with the skin [50].

Post-spill ecological impacts are more severe when oil spill clean-up measures are not executed on time, compared to the impact during a spill. Ba-Akdah [51] and Kingston [1] expressed concern about the possibility of the concentration of pollutants in living tissues, which is known as *bioaccumulation*. This could be taken up by other animals through ingestion, leading to the transfer of toxic hydrocarbon compounds up the food chain. It is a consequence of long-term pollution from crude oil [52-53]. For example, in the marine environment the pollutant could be carried by a mussel (a bivalve gastropod), to an amphipod (sand hopper) and finally to a fish. Bioaccumulation can also occur in plants when they are grown on oil polluted farmlands, which leads to the translocation of hydrocarbon contaminants from the root system to the plant tissues [50] as observed in lichens and mosses in Alaska [53,54].

More recent reports by Achenbach [55] and Thomas and Rahman [56] highlight the dangers of long-term oil spills in marine environments using the consequences of ecological damage of the Gulf of Mexico as a reference. The authors discovered that the continued pollution of the Gulf of Mexico for over two decades has led to the creation of a hypoxic dead zone characterised by insufficient oxygen, which is detrimental to aerobic organisms resident in that area. This dead zone runs east-west along the Texas-Louisiana coastline and keeps expanding in size as oil spill incidents increase. The hypoxic Gulf sites have been found to have Atlantic croakers suffering from impairment of reproductive output. The Atlantic croakers at the site had a disproportionate sex ratio of 61% males to 39% females compared to the sex ratio (52% males to 48% females) of Atlantic croakers in non-polluted sites.

Also, a report by NSF [57] revealed that the corals that live at a depth of 4,300 feet in the Gulf of Mexico were observed to possess brown spots on their body surface, which were shown to be hydrocarbon residues from the deepwater Macondo oil well. Further research on the possible medical effect of the brown spots to the corals was suggested. However, Al-Dahash and Mahmoud [58] reported that two species of corals (*Acropora clathrata* and *Porites compressa*) in the Arabian Gulf (Qaro and Umm Al-Maradim Islands south of Kuwait) appeared healthy despite living in an oil-polluted environment. The authors discovered that the corals invulnerability to petroleum pollutant was as a result of some oil-degrading bacteria groups (*Gammaproteobacteria, Actinobacteria* and *Firmicutes*) living in the tissues and mucus of the corals.

3. CRUDE OIL BIOREMEDIATION STRATEGIES

Several bioremediation strategies have been explored but most of them are designed for land oil spill control. The bioremediation strategies include landfarming, composting, use of bioreactors, bioventing/biosparging, bioslurping, pump and treat strategy, biostimulation and bioaugmentation. These strategies were briefly described in the introduction, but the details on how they are applied and their potential success will be discussed in detail below.

3.1. Landfarming

Landfarming is a soil bioremediation technique that involves mixing of the hydrocarbon-contaminated soil. It also involves relying on the biological, physical and chemical processes within the soil for biodegradation. The technology has been employed since the 1980s due to its simplicity and cost-effectiveness [59]. It is a ‘low-tech’ oil spill control method specialized for the clean-up of oil-polluted uppermost soil surfaces [60]. It can be carried out *in situ* or *ex situ,* but the latter method is more common.

The contaminated soil is often transferred to a treatment site where it is spread over a prepared soil surface and tilled periodically for aerobic microbial degradation to occur [61]. However, this simple technology is riddled with inherent challenges such as the inhalation of hydrocarbon volatiles by humans and the risk of other hydrocarbon contaminants leaching through the soil profile to the groundwater region [62]. This challenge has been managed in recent times by providing the treatment site with a layered polythene material about 250 μm in thickness, which is laid at the bottom of the topsoil in order to prevent the leachates from seeping to the groundwater zone [62]. Also, the dispersed hydrocarbon volatiles have been controlled by building a greenhouse to confine the extent of diffusion [61,62].

Land farming has been successful in degrading a number of hydrocarbon compounds since most oleophilic microbes are confined to the superficial layer of soils, 15-30cm deep [61]. The major challenges associated with landfarming are that it is a very slow biodegradation process and has been unsuccessful in degrading high molar mass PAHs. However, a number of successes have been recorded as regards the biodegradation of light PAHs. For example, Picado et al. [63] reported a 63% reduction of mostly low molar mass PAHs after three months of landfarming; Bossert and Bartha [64] recorded an 80-90% reduction of low molar mass PAHs after three years. Although the latter report is quite dated compared to the former, it still reveals the fact that landfarming is indeed a very slow bioremediation process.

The slow nature of landfarming has been attributed to the unavailability of petroleum hydrocarbons to the oleophilic soil biota [62]. Therefore, it has been suggested that the use of surfactants such as detergents can help improve bioavailability. On the other hand, adsorbents such as straws can help mop up the non-biodegradable heavy hydrocarbon residues from the soil [62]. However, surfactants are chemical-based substances and therefore potentially toxic to the environment. Maila and Cleote [62] also suggested that incorporating biostimulation (i.e., supplying the oleophilic soil biota with nutrients) with landfarming may reduce the hydrocarbon-contaminated soil biodegradation time.

3.2. Composting

Composting is a simple *ex situ* bioremediation technology that utilizes biological agents in organic amendments (such as manure, plant residue, sewage sludge and other biowastes) to aerobically degrade spilled pollutants [65]. Composting is also referred to as the solid-phase treatment [17], which involves the mixing of hydrocarbon contaminants with fresh organic amendments to produce a rich microbial consortia that are heat-loving (mainly thermophiles). The process is characterised by an increased temperature of up to 50°C, high nutrient content, optimum oxygen concentration and a neutral pH [61]. Organic amendments are also known as a bulking agents [66].

The elevated temperatures of compost stimulates hydrocarbon degradation and enhances the bioavailability of the hydrocarbon pollutant [67]. If the organic amendment is not fresh, the thermophilic phase will not develop and this will reduce the efficiency of the process. For example, van Gestel et al. [65] reported that 85% of diesel fuel was degraded under thermophilic composting generated by fresh biowaste but recorded only 35% reduction when mature compost was utilized. The type of organic amendment used may influence the rate of biodegradation as Namkoong et al. [68] reported that a biopile (a pile of biowaste ready for composting) of sewage sludge degraded more hydrocarbon compared to that of plant residues, probably as a result of the microbial richness of sewage sludge; and van Gentel et al. [65] buttressed this view by stating that the number of oelophilic microbes in a biopile depends mainly on the total number of microbes present in the biopile.

The higher oleophilic microbial population (derived from the organic amendments) and elevated temperature makes composting a more promising bioremediation process compared to landfarming, which relies solely on oleophilic soil biota. In addition, composting also produces an end-product of mature compost that is useful for agricultural purposes [65]. The only disadvantage of this method is the longer treatment time when compared to other *ex situ* bioremediation processes [61]. If the process could be accelerated it would be more cost-effective.

3.3. Use of bioreactors

A bioreactor comprises a reaction chamber equipped with a mixing mechanism, a system that supplies oxygen and nutrients and influent and effluent pumps. It is an *ex situ* bioremediation technology that offers the direct control of environmental/nutritional factors (such as oxygen, moisture, nutrients, pH and even microbial population) that influence biodegradation [61]. Hydrocarbon-polluted soils are added to the bioreactor chamber and mixed together with the periodic input of oxygen and nutrients to accelerate biodegradation. This makes the technology more reliable than most *in situ* bioremediation technologies in which the factors that influence bioremediation at the spill site are not easy to control.

There are about six types of bioreactors, namely fluidized bed, plug flow, submerged fixed-film, sequencing batch, slurry phase and vapor phase bioreactor [61]. The last two are the most commonest types.

In slurry phase bioreactors the reaction chamber is filled with excavated hydrocarbon-polluted soil mixed with liquid waste saturated with microbes to form a slurry and then mechanically agitated to encourage aerobic biodegradation [66]. The manipulation of environmental/nutritional parameters is also possible with this type of bioreactor system. The vapor phase bioreactor, also known as biofiltration [61], is specially designed for the containment of volatile organic compounds (VOCs) or polluted air emissions.

The reactor chamber is packaged with the biogenic material (such as compost) containing the microbial population. The gas to be treated is released into the chamber containing the biogenic material where it comes in contact with the micro-organisms causing the hydrocarbon pollutant to be “stripped off” the gas, hence, the term air stripping [14]. There are two main types of biofilters: the treatment bed biofilters and the soil filters [61]. The mechanism of operation is the same for the two biofilter types.

Zhang et al. [69] reported that the hydraulic loading rate (HLR, also referred to as the substrate addition rate by Daugulis [70]), of the polluted material to be treated has an effect on the pollutant-removal efficiency of biofilters. Daugulis [70] revealed that if the substrate concentration is too high in the bioreactor chamber, it may kill the micro-organisms, whereas if added too slowly it may lead to the starvation of the microbes, consequently reducing the biofilter's optimum efficiency. This view was corroborated by Zhang et al. [69] that at an optimum HLR of 3.0 m/h, the semi-volatile organic compounds (SVOCs) such as di-n-butyl phthalate and bis-2-ethylhexyl phthalate, were reduced by 71.2% and 84.4%, respectively, while at an increased HLR a remarkable decrease in SVOCs-removal efficiency was observed.

The prospects of bioreactors (biofilter or air stripping) as a highly efficient bioremediation strategy have been proven in a number of studies. For example, Liu and Liu [71] reported the efficiency of a sequencing batch bioreactor (aerated and pH-regulated) in decontaminating diesel-polluted soils. After two weeks it was observed that 90% of 35,000 ppm/volume of diesel oil was degraded in a bioreactor enriched with the oleophilic microbe *Rhodococcus erythropolis* (NTU-1 strain).

In addition, PAHs such as phenanthrene and naphthalene were degraded successfully in a bioreactor with the addition of organic solvents (such as decane, silicone oil and oleyl alcohol), to facilitate the bioavailability of the hydrocarbon substrate [70, 72, 73]. However, the use of bioreactors has its underlying challenges, such as the excavation of polluted soils or pumping of contaminated groundwater to the treatment site that is cost-ineffective and the production of toxic sludge as a by-product of the bioreactor and therefore requires further treatment, consequently increasing the operational cost [61].

3.4. Bioventing/Biosparging

Bioventing is an *in situ* bioremediation technology designed for the decontamination of soils at the vadose or unsaturated zone. The technology relies solely on the indigenous oleophilic microbes occupying the unsaturated zone to aerobically break down the spilled petroleum in the soil. These microbes are supplied with nutrients (if required) and oxygen (under low pressure) via an injection well [74]. Bulman et al. [75] reported that after six months the total hydrocarbon concentration treated with bioventing technology reduced by 10 to 30% in 3 m deep soil. After adding nutrients, a further 30% reduction in total hydrocarbon concentration was observed after another six months at a depth of 3.5 m.

Bioventing is of two types: active and passive. In passive bioventing, the oxygen in the injection wells is supplied through atmospheric pressure but in active bioventing, the oxygen is forced through by a blower or pump [61]. Bioventing technology does not decontaminate the capillary fringe and groundwater that are located in the saturated zone; this is where biosparging becomes of importance.

Biosparging is also an *in situ* bioremediation strategy but it is targeted directly at the saturated zone (the region below the water table). The indigenous oleophilic microbes in the saturated zone are similarly provided with nutrients (if required) and oxygen (under high pressure so that it can get to the saturated zone) [61] to facilitate aerobic biodegradation below the water table. Kao et al. [76] reported that 70% of BTEX (benzene, toluene, ethylbenzene and xylene) were biodegraded in 10 months using biosparging technology at an average groundwater temperature of 18°C.

The two bioremediation technologies are sometimes combined with the SVE if volatile hydrocarbons are present in the soil [74]. The main advantage of these strategies is that they are easy to set up and cost-effective [61]; however, they have shown to be too slow in degrading heavy petroleum hydrocarbons such as heavy PAHs even when oxygen and nutrients are supplied [74]. This is probably as a result of the absence of other natural processes of oil-degradation (such as evaporation) in the vadose and saturated zones that could have offered support.

3.5. Bioslurping

Bioslurping is a relatively new *in situ* bioremediation strategy that combines bioventing with a free-product recovery system. This method of remediation achieves two aims simultaneously – aerobic microbial biodegradation of the vadose zone through air injection and SVE and the removal of the light non-aqueous phase liquid saturates (NAPLS –free-phase petroleum pollutants) from the capillary fringe and water table via dual-pumps (through gravity-gradient, the first pump forces the flow of petroleum from the vadose zone into the well and the second pump skims off the petroleum to the surface) [77]. Kittel et al. [77] suggested the use of vacuum-enhanced pumps to speed up the petroleum recovery rate.

A modernized bioslurping technology described by Khan et al. [78] replaced the dual pumps with a vacuum-enhanced pump (known as a slurp tube) as well as the incorporation of the bioventing and SVE systems that helped to increase the recovery rate of both vapor-phase and free-phase petroleum products from the vadose zone and the water table. The pumped mixture (vapor-phase oil product, free-phase oil product and some groundwater) in the slurp tube is separated into oil-water and vapor-liquid compartments by an above-ground bioslurping system. Gidarakos and Aivalioti [79] reported that the application of the bioslurping technology in cleaning up petroleum spills at a Greek petroleum site was relatively successful. The recovered petroleum hydrocarbons include toluene, xylene, paraffins, naphthalenes and olefins. However, the authors noted that some petroleum residues in groundwater were not recovered due to the fact that bioslurping technology does not directly treat the saturated zone.

3.6. Pump and Treat Strategy

This is an *ex situ* bioremediation strategy that is specially designed to address groundwater pollution. It involves the pumping of polluted groundwater to the surface, treatment at a remediation facility and the injection of the treated groundwater back to the initially polluted site [14]. It is a challenging process that entails location of the groundwater contaminant plume, designing a capture mechanism and installing extraction and injection wells [80]. The construction of the extraction well or trench equipped with pumps helps to lower the water table, thereby improving the suction of water through the pump and also aerating the enlarged vadose zone, encouraging biodegradation of contaminants in the unsaturated zone [81].

The extracted groundwater is cleaned through aerobic biodegradation, although other non-microbial cleaning processes could also be used, including: phase separation, air stripping and liquid-phase granular activated carbon adsorption [82]. Despite the effective groundwater clean-up offered by the pump and treat bioremediation method, building of the withdrawal and injection wells and the treatment of the groundwater have been found to be very expensive [17] when compared to other bioremediation strategies.

3.7. Biostimulation

Biostimulation is an *in situ* bioremediation strategy that involves the supply of nutrients (mainly nitrogen and phosphorus) to hydrocarbon-polluted sites in order to "stimulate" the indigenous micro-organisms to break down more crude oil [84]**.** The justification for the use of this bioremediation strategy is that hydrocarbon metabolism is limited by nutrient availability; therefore, by supplying the required nutrients microbial degradation of hydrocarbon is expected to increase. This strategy is compatible with land [85] and the aquatic environments [86]. The nutrients supplied could be from organic or inorganic sources. Joshi and Pandey [3] reported the success of organic nutrient (cow dung) application, while the success of inorganic nutrient (sodium nitrate and dihydrogen phosphate) was recorded by Roling et al. [87].

Biostimulation has been widely accepted to degrade alkanes [88], BTEX [3] and PAHs [89,90] and also is regarded as cost-effective [4] because it does not require the excavation of polluted soils or the transfer of polluted water to a treatment facility. However, high concentrations of nutrients applied to the environment may lead to eutrophication (the response of the ecosystem to the introduction of foreign substances) [88,91] usually in aquatic environments. Eutrophication has been reported to cause algal bloom, oxygen depletion or may even induce toxic responses in humans and the marine ecosystem [88,92]. This has led to the need to test the safety levels of nutrients applied in bioremediation.

As a result, licensing was developed in the United Kingdom and methodology testing is being used in Canada [93] and the United States [94] to limit the indiscriminate use of nutrients. In reality, the establishment of these laws might not be sufficient enough to curtail the excessive use of nutrients; therefore, it is important for researchers to strategise novel nutrient application methods that would help to limit eutrophication.

In addition, the success of biostimulation has been suggested to depend largely on the geography, water-body, habitation and other environmental-specificity of the contaminated site [53]. As a result, biostimulation investigations in the laboratory are not as reliable as in situ investigations carried out at the contaminated site. For example, a study by Gallego et al. [95] demonstrated that biostimulation when carried out in the laboratory degraded 90% of diesel oil. In contrast, Seklemova et al. [96] and Bento et al. [97] both reported the failure of biostimulation in managing diesel spill at a contaminated site. Bento et al. [97] suggested that the poor performance could have been caused by low bioavailability of the nutrients and oil (i.e., the poor solubility of the nutrient and oil in soil or water, which makes them inaccessible to oleophilic microbes). Consequently, "one of the difficulties of developing bioremediation strategies lies in achieving as good or better results in the field as in the laboratory" [98].

3.8. Bioaugmentation

Biostimulation is an *in situ* bioremediation technology that involves the introduction of indigenous (obtained from the contaminated site) or exogenous (obtained elsewhere) oleophilic microbial cultures to a polluted site in order to "augment" microbial degradation at the site [61]. It is a technology used for both soil and aquatic oil spill clean-up. To facilitate the breakdown of a wider range of hydrocarbon compounds, Ledin [99] proposed that a multi-component strategy be employed (which entails the introduction of a microbial consortium) rather than the single-component approach. Since individual oleophilic microbes are hydrocarbon-specific, a microbial consortium will provide the metabolic diversity needed in the field [100].

However, there is an ongoing debate over the efficiency of bioaugmentation, as few successes have been recorded. This is because the introduced exogenous microbes often fail to compete favorably with the indigenous microbes at the polluted site [53, 61] probably due to the site condition and ecological-specificity of the polluted area. For example, Bento et al. [97] reported that the addition of a microbial consortium to diesel-contaminated soil (Long Beach soil sample), degraded 58% of the pollutant weekly, but in the Hong Kong soil sample, natural attenuation was more successful as a result of the poor adaptability of the introduced microbes, probably due to the nature of the soil.

An alternative approach is to harvest oil-degrading indigenous microbes from the polluted site and culture them for re-introduction. The success of this method in degrading a wide-range of hydrocarbon compounds has been reported by Devinny and Chang [101], Alisi et al. [102] and Li et al. [103], but the sustainability of this method has been questioned by many authors.

4. THE CHALLENGES OF OIL SPILL BIOREMEDIATION

4.1. Understanding How Oleophilic Microbes Can Be Supported in Breaking down Recalcitrant Asphalthenes Both on Land and Water

It has been established in the literature that crude oil components such as saturates (*n*-alkanes), MAHs (BTEX) and PAHs are generally biodegradable whilst asphalthenes have been reported by many authors to be highly resistant to biodegradation.

There are different models of asphalthene structure; however, Speight and Moschopedis [35] modelled the asphalthene structure as a system of 6 to 20 or more condensed aromatic structures linked by alkyl chains. The chemical complexity of asphalthenes have rendered them resistant to microbial attacks [104, 105], leading to their accumulation in the environment [26]. As a result of the complexity, the metabolic route for asphalthene biodegradation is yet to be fully understood [34, 106,].

Although Flores and Mestahoward [26] reported the natural biodegradation of asphalthenes, they added that it is an extremely slow process, achieving an estimated maximum reduction of 5-35% (duration not estimated). The authors also suggested that asphalthene breakdown can be relatively faster if a physical process, such as photo-oxidation, is first applied to degrade the asphalthene into three parts – alkanes, light PAHs and heavy PAHs – each of which is susceptible to microbial degradation after the maximum of seven days (for 100% degradation), 200 days (for 100% degradation) and 990 days (for 70-91% degradation), respectively. However, this is still too slow to meet the immediate demand of the environment in the face of asphalthene contamination.

In the event of an oil spill, resin, which solubilizes asphalthene in crude oil, is lost through natural processes (such as evaporation). This leads to precipitation of asphalthene molecules in saturates and aromatics. The free asphalthenes in terrestrial environments may block the interstitial spaces of soils, thereby, inhibiting water and nutrient uptake by plant roots. In marine environments on the other hand, free asphalthenes may clog the nasal cavity or breathing apparatus of aquatic animals, causing suffocation. Also, the body of aquatic animals may be smeared with asphalthene preventing their free movement in water. The aforementioned ecological impacts of asphalthenes make a fast and efficient clean-up strategy to be of immediate concern.

As a result, a combination of physical and chemical methods has been employed in order to achieve faster results in cleaning-up asphalthenes. However, these methods are expensive and challenging [34], while some others are potentially toxic. The need for a cost-effective and ecologically-safe technique for the clean-up of asphalthenes has made researchers and oil-companies initiate the move for a biological approach.

Ogbo and Okhuoya [107] reported that after 17 days of incubation, asphalthenes had a low biodegradation rate (39%) using a white rot fungus, *Pleurotus tuberregium.* However, a recent study by Tavassoli et al. [34] involving 25 species of microbes isolated from oil-polluted soil and oil samples obtained from the Dorood oil field in the south of Iran, showed that five pure culture isolates (*Pseudomonas*, *Bacillus licheniformis*, *B. lentus*, *B. cereus* and *B. firmus*) and a mixed culture (of the five isolates) degraded asphalthenes to a higher significant level than earlier recorded. Of the five pure culture isolates investigated, *B. lentus* degraded the highest amount of asphalthene (46%) incubated for 60 days at 28°C but in the mixed culture, the record amount of 48% asphalthene reduction was observed when incubated for 60 days at 40°C. This result is in agreement with Flores et al. [108], who reported that a bacterial consortium (containing isolates such as *Corynebacterium*, *Bacillus*, *Brevibacillus* and *Staphylococcus* species) is capable of utilizing asphalthene as their sole carbon source.

4.1.1. Discussion

The level of success recorded by Tavassoli et al. [34] is relatively low compared to the biodegradation rates of other hydrocarbon compounds and too slow to meet the demands of the environment. Microbial consortia have more of a promising potential to breakdown asphalthenes than microbial isolates. Therefore, there is a need to carry out further research into identifying the functions of the individual microbes in the asphalthene-degrading consortium and finding out if new microbes can be added to facilitate greater asphalthene biodegradation. This knowledge may be helpful in determining the factors or elements that can be manipulated in order to increase asphalthene reduction levels to about 60% in the nearest future. Also, the record amount of 48% reduction of asphalthene was achieved at a temperature (40°C) higher than the average ambient temperature in most parts of the world. Therefore, a heating system may be required to achieve in the field a similar success recorded in the laboratory. It appears that high temperature plays a significant role in asphalthene susceptibility to microbial degradation.

The white rot fungus *Pleurotus tuberregium* showed a promising potential to degrade asphalthene (39% degraded in 17 days). The white rot fungus is known to be the agent of wood decay. Therefore, its promising potential for recalcitrant asphalthene biodegradation may be attributed to its metabolic affinity for complex polysaccharides such as lignin (a hydrocarbon compound in wood). However, the combination of *B. lentus* and *P. Tuberregium* may produce a more rapid biodegradation following the proposed thermal decomposition of asphalthenes.

4.2. Investigating How the Delay in Heavy PAH Biodegradation Can Be Reduced

Light PAHs have two to three benzene rings whilst heavy PAHs have four rings and above. The biodegradation of light PAHs (such as naphthalene and phenanthrene) is easier and faster than heavy PAHs (such as pyrene and fluoranthrene) [109]. However, a lot of research has been carried out to speed up the rate of biodegradation of light PAHs. Up to about 80% reduction level in seven days is reported by Othman et al. [110], but very little has been done to achieve a similar feat with heavy PAHs [24].

Heitkamp et al. [111] described the aerobic biochemical pathway involved in pyrene (four-ringed hydrocarbon) biodegradation. The authors added that the enzymes di-oxygenase and mono-oxygenase released by oleophilic microbes help to open up the first benzene ring in the pyrene structure at the *ortho* position. The enzymes go further to break off other rings until a monocyclic compound known as cathecol is left, which then enters the Kreb's cycle [111] to complete pyrene metabolism. Similarly, Schneider et al. [112] reported that benzo[a]pyrene (five-ringed hydrocarbon) is biodegraded following the same metabolic route but the first benzene ring in the structure is opened up at the *meta* position.

Pyrene was reported to be biodegradable up to a level of 60% reduction achieved by soil-polluted microbes in about four days at 24°C [111]. Mueller et al. [113] also reported that a seven-member bacterial consortium (the identity of the isolates used was withheld) degraded fluoranthrene (5-ringed hydrocarbon) to levels below detection after three days of incubation. They also added that other PAHs were broken down after prolonged incubation. This result is in agreement with the findings of Tam et al. [114], who stated that a microbial consortium will degrade heavy PAHs more efficiently than individual isolates or pure cultures. In addition, Kazunga and Aitken [115] reported that some bacteria such as *Mycobacterium*, *Sphingomonas yanoikuyae* and *Pseudomonas saccharophila*, have the potential to mineralise pyrene into intermediate products (metabolites) that are easily biodegradable. For example, Mohandass et al. [116] reported that 58.98% of benzo[a]pyrene was degraded by a mixed culture of *Bacillus cereus* and *B. vireti* after 35 days of incubation with the production of cis-4-(7-

hydroxypyren-8-yl)-2-oxobut-3-enoic acid, which can be easily biodegraded.

A more recent study by Mao et al. [32] involving the bioremediation of PAH-contaminated soils (containing 90.6% four- and five-ringed PAHs) using a microbial consortium, showed that bioaugmentation – the introduction of 10% and 20% exogenous bacterial suspension – helped removing 20.2% and 35.8% of total PAHs from the soil, respectively, after 56 days of incubation. However, it was observed that the introduced exogenous microbes later declined in growth probably as a result of the difficulty to adapt to the new environment [117]. This is in agreement with Vinas et al. [118] who observed that inoculated exogenous microbes could not compete favourably with indigenous microbes at the polluted site. It has also been suggested that the soil chemical, physical and biological specificity could have prevented the inoculum from further establishing a niche [119]. Therefore, it was proposed by Gogoi et al. [120] that the application of sufficient nutrients (biostimulation) may improve the bioremediation of heavy PAH-contaminated sites.

4.2.1. Discussion

Biostimulation may help hasten the biodegradation of heavy PAHs if applied to the polluted sites that are oleophilic microbe-rich. In addition, the possibility of harvesting indigenous microbes from the contaminated site, culturing and re-introducing them to the site may probably solve the problem of environmental intolerance by exogenous microbes. Based on the literature reports, an indigenous microbial consortium containing mixed isolates that can mineralise and utilize heavy PAHs is expected to achieve higher biodegradation rates of heavy PAHs within a shorter time frame due to their synergistic metabolism. For example, *Alcanivorax borkumensis* cannot directly degrade PAHs but produces PAH-mineralizing biosurfactant, but can be combined with a PAH-degrading microbe such as *Cycloclasticus* to breakdown recalcitrant PAHs. This is in agreement with the suggestion made by McKew *et al.* [86].

4.3. The Method(s) that Can be Employed to Reduce the Effect of Eutrophication Caused by Biostimulation

It has been established in the literature that biostimulation, the application of nutrients to an oil-polluted site in order to increase the metabolic rate of oleophilic microbes, may cause eutrophication. Eutrophication is the ecological response to the excessive inputs of foreign substances into the environment, thereby causing a population imbalance in the ecosystem and it is common in aquatic environments. According to Roling et al. [1], when nutrients are added in low quantity it will result in sub-optimal biodegradation of oil, and when added in high concentrations may lead to eutrophication. Therefore, there is a need to regulate the rate of nutrient application. Although laws have been established to curtail the excessive use of nutrients in some developed countries, it is expected that novel methods of nutrient application should be developed in order to limit the excessive release of nutrients.

The source of nutrient (usually nitrogen and phosphorus) could be organic or inorganic. Inorganic nutrient sources such as NPK, $(NH_4)_2SO_4$, K_2HPO_4, NO_3-N, have been reported to be successful in cleaning-up crude oil both on land and water by stimulating the growth of remediating microbes [1,85,121,122]. However, in the treatment of oil-contaminated soils, inorganic nutrients have been found to cause soil hardening (i.e., the hardening of soil layers, disallowing the free movement of nutrients, water and oxygen within the soil) and decline in soil fertility [107]. Inorganic nutrients have the tendency to be released rapidly into the environment (probably as a result of their availability in the free state), and therefore, have a higher potential to cause eutrophication. Therefore, some scientists have considered using organic nutrients as a result of their slow-release rate and consequently, lower potential to cause eutrophication.

Joshi and Pandey [3], Amadi and De Bari [123], Obire and Akinde [124] and Akiakwo et al. [125] have all investigated the potential of organic fertilizers as an ecologically-safe source of nutrient since the release of nutrient is slower, although may be too slow to accelerate biodegradation. However, Ogbo and Okhuoya [107] stated that an organic nutrient source (poultry litter) was more effective in accelerating biodegradation in oil-contaminated soils than the application of inorganic fertilizers (NPK).

The right amount of nutrient needed for crude oil biodegradation to occur at an optimum rate without causing eutrophication depends on a number of factors such as the geology of the polluted site, type of crude oil and the type of nutrient applied [126]. In addition is the action of tides and waves which makes the stable release of nutrients in aquatic environments (mainly marine environments) very difficult [127,128]. Therefore, the authors suggested that adding the nutrient to the oil-water interface may be a reasonable means of reducing nutrient washouts due to tidal and wave actions, improving nutrient bioavailability and consequently reducing eutrophication.

However, a recent study by Nikolopoulou and Kalogerakis [126] reported three nutrient application strategies (water-soluble inorganic nutrients, slow-release fertilizers and oleophilic biostimulants) designed for the clean-up of oil-polluted marine environments. Water-soluble inorganic nutrients such as KNO_3 and $NaNO_3$ are applied in the field by spraying aqueous formulations or spreading dry granules. Successful field trials have been recorded [129] but there is still the problem of nutrient washouts by tides and waves which could lead to eutrophication. There is also the problem of quick dilution which might make the nutrients far from the reach of microbes and potentially causing eutrophication.

The slow-release fertilizers such as customblen (composed of ammonium nitrate, calcium phosphate and ammonium phosphate) were developed to overcome the washout problems of water-soluble inorganic nutrients. These are inorganic nutrients such as calcium phosphate and ammonium nitrate coated with a hydrophobic layer such as paraffin or vegetable oil [88]. The slow-release fertilizers helped to solve the problem of multiple nutrient application in the field [126] and release the nutrients slowly such that it becomes unaffected by tidal and wave actions. Despite the promising potential of this strategy, the nutrient release rate has shown to be too slow to meet the demands of optimum biodegradation. If released too quickly, it will not be sustained over a long period, and if released too slowly, will be insufficient to achieve optimum biodegradation rates. Therefore, maintaining the release rate at prolonged optimum levels is a problem that is yet to be solved [126].

Oleophilic biostimulants such as Inipol EAP 22 (composed of oleic acid, trilaureth-4-phosphate, 2-butoxyethanol, urea and water) are fertilizers containing oleic acid, which is the reason for their hydrophobic nature. These oleophilic fertilizers are considered to be the most effective of all nutrient types because they are available in the oil-water interphase, thereby reducing wastage, enhancing biodegradation rates [130] and consequently limiting eutrophication. This nutrient application strategy has been noted to be successful in cleaning-up the oil spill at the shorelines of Prince William Sound in Canada [129,131]. However, oleophilic fertilizers appear to be very expensive to employ [126].

4.3.1. Discussion

The literature shows that each nutrient application strategy has its pros and cons. The choice of nutrient application method employed may be site-specific. For example, in marine environments where tidal and wave action is very low, the water-soluble inorganic nutrients can be applied. If the release rates of water-soluble inorganic nutrients are maintained at an optimum level, the probability of causing eutrophication will be reduced. On the other hand, in marine environments where the tides are high, oleophilic fertilizers can be employed but they are expensive. The slow-release fertilizers may also serve as viable alternatives if there is the possibility of maintaining a prolonged optimum nutrient release. These measures may help to solve the problem of eutrophication.

Although eutrophication is not common on land, the problem of soil hardening and loss of soil fertility caused by the application of inorganic nutrients may be solved by strictly using organic nutrients (manures) as nutrient sources for land oil spill clean-up (this has been proven to be more productive as it will not only provide nutrients to microbes, but will enrich the soil as well). It has been found that inorganic nutrients are more expensive than organic nutrients; however, it may be challenging to produce organic nutrients in commercial quantities, which may explain why the use of inorganic nutrients has persisted.

4.4. Suggesting Novel Methods by Which Bioaugmentation Can be Improved

Many authors [132-134] have argued that bioaugmentation, the introduction of hydrocarbon-degrading microbes to oil-polluted sites, is ineffective in cleaning-up oil spills on land and water. However, more recent reports have shown that bioaugmentation is effective in degrading petroleum hydrocarbons For example, Mckew et al. [135] reported the bio-degradation of *n*-alkanes, branched alkanes and PAHs using *A. borkumensis*. However, Thouand et al. [136] reported that natural inocula (indigenous microbes) degraded more crude oil (25% reduction) than commercial inocula (exogenous microbes – 18% reduction), which is in agreement with the findings of Alisi et al. [102] and Li et al. [103].

A bioremediation experiment by Trindade et al. [6] on the biodegradation of recently contaminated soil (RCS) and weathered contaminated soils (WCS, which had been pre-exposed to oil for four years) showed that there was approximately 15 days adaptation period for the native microbes in the RCS before biodegradation began. This was not observed in the native microbes in the WCS. The authors suggested that the reason is because of the previous exposure of the soil to crude oil which had made the native microbes well-adapted to hydrocarbon pollutants, in agreement with Alexander [137] and Zouboulis and Moussas [61]. However, when exogenous microbes and nutrients (phosphorus) were introduced to the RCS, the adaptation phase was eliminated. The reason for the loss of the adaptation phase was attributed to the introduction of exogenous microbes in agreement with Davis and Madsen [137,138].

However, Tyagi et al. [4] and Gentry et al. [139] pointed out that, in reality, the introduction of exogenous microbes to a contaminated site initially causes a decrease in the population due to one or more of the following: fluctuating temperatures, water content, pH, nutrient depletion and toxic levels of pollutants. Bouchez et al. [140] reported that the challenge of using bioaugmentation technology in the field lies in the potential competition that may take place between exogenous and indigenous oleophilic microbes (which often does not benefit exogenous microbes) or the predation-tendency of protozoans living in the oil-polluted site. However, the use of carrier materials such as agar, agarose, alginate, polyurethrane and polyvinyl alcohol gel [141] has provide protection and physical support for introduced exogenous microbes – a process known as encapsulation [142] or immobilization [143]. This process extends the survival rate of introduced exogenous microbes by making accessible the following: nutrients, moisture, oxygen [144] and protecting the microbial cells from the toxicity of petroleum pollutants and from predation and competition [4]. The success of this process has been recorded by Liu et al. [145].

A recent study by Nikolopoulou and Kalogerakis [126] reports that it is important to carry out a feasibility study at the oil-polluted site before deciding whether to apply the bioaugmentation technique because the addition of oleophilic microbes to a nutrient-limited site has been shown to have no effect on biodegradation. The authors added that native oleophilic microbes in a contaminated site are capable of degrading hydrocarbon pollutants as long as limiting-nutrients are provided.

Microbial geneticists and biotechnologists have considered employing genetic engineering in settling the dispute over the potential of bioaugmentation. As a result, attempts have been made to genetically-engineer non-oleophilic microbes by developing hydrocarbon-degrading metabolic pathways for them (incorporating genes that express such a metabolic ability) and then releasing them to the polluted site [146]. This hydrocarbon-degrading ability is then transferred to other non-oleophilic microbes at the polluted site via a reproductive process known as conjugation that occurs through horizontal gene transfer (HGT) demonstrated in the laboratory by Ma et al. [147].

Sayler and Ripp [148] reported the success of genetically-engineered microbes (GEMs) (using *Pseudomonas fluorescens*) in the field which is in contrast with the report of Lenski [149] who stated that GEMs suffered from competitive-fitness in the field due to the energy-demand imposed on them by the new genetic material in their genome.

Paul et al. [146] and Giddings [150] reported that the use of GEMs is likely to cause proliferation. However, Keasling and Bang [151] suggested the need to design a self-destructive system in GEMs such as a suicide gene mechanism which can be activated when required to control their population. Ensley and DeFlaun [152] pointed out that the use of suicide genes might lead to greater ecological risks if the genes are transferred to non-target organisms via HGT. The cost implication and the stringent laws in place for the use of GEMs makes their application in crude oil bioremediation very challenging [148].

4.4.1. Discussion

A feasibility study may be required to find out whether bioaugmentation is needed at a contaminated site in order not to waste resources and time. Oil-polluted sites containing sufficient amounts of oleophilic native microbes may require other bioremediation techniques such as biostimulation to encourage biodegradation, rather than introducing more microbes to the site, which has been reported to have no effect on crude oil remediation. Exogenous microbes that have been pre-exposed to crude oil may gain competitive-fitness in the field.

Also, the multi-component microbial consortium selected for bioaugmentation should not have antagonistic oleophilic microbes, such as *Thalassolituus*, as part of the consortium as this would threaten the microbial diversity of the system and this may affect the potency of the approach. Encapsulation or immobilization agents have had limited field applications, therefore more field trials are required to confirm their practicability.

GEM application has been controversial partly as a result of the negative perception from the public. This may have led to the limited research carried out on GEM application in bioremediation. The success of GEMs in the field is unpredictable, which may be attributed to the geology of the contaminated site, type of GEM applied and the petroleum type. The problem of HGT which may cause the death of non-target organisms can be solved by designing a near-perfect containment system in the field which is currently a more theoretical concept than a practical one. If the challenges of HGT, regulation and cost can be effectively managed, GEMs may have the potential of achieving higher biodegradation rates compared to naturally-occurring oleophilic microbes.

4.5. How the Problem of Bioavailability of Liquid Hydrocarbon both on Land and Water Can be Addressed

Spilled petroleum has been found to have low bio-availability, the inaccessibility of oleophilic microbes to spilled crude oil, thus delaying biodegradation. This is because crude oil is a hydrophobic material and therefore has low water solubility [135]. Nikolopoulou and Kalogerakis [7] reported that microbial degradation of spilled petroleum takes place at the oil-water interphase; therefore, the dispersion or solubility of the oil at the interface either by chemical or biological means is expected to enhance biodegradation due to the increased surface area of the oil available for microbial degradation. The chemical agents used to achieve oil spill bioremediation mostly in marine environments are surfactants but are potentially toxic to the environment; therefore, attention has been diverted to the use of biological products because they are less toxic, cheaper [153] and more effective [154,155].

The biological products are amphiphilic compounds (having both hydrophilic and hydrophobic terminals) of microbial origin [156] and are referred to as biosurfactants [153] or bioemulsifiers [157]. The most common biosurfactant, rhamnolipid, is produced by the bacterium *Pseudomonas aeruginosa* [158]. The laboratory success of rhamnolipid in assisting marine oil spill bioremediation was reported by Nikolopoulou and Kalogerakis [7]. Heat-tolerant biosurfactants such as thermophilic emulsifiers (produced by *Bacillus stearothermophilus*) [158], have been recommended in case bioremediation is to be achieved under high temperature conditions [159].

The problem of bioavailability of crude oil is not confined to the marine environment as it also occurs during land oil spills where spilled petroleum becomes unavailable to native oleophilic soil microbes [160, 161] The laboratory success of a biosurfactant, alasan (produced by *Acinetobacter radioresistens*), in assisting terrestrial oil spill bioremediation was reported by Barkay et al. [162]. Biosurfactants have been shown to increase the water-retention capacity of oil-polluted sandy soil, which is expected to enhance the bioavailability of the spilled petroleum to the native oleophilic soil organisms [157] and have also been suggested to displace the oil contaminant from sticky soil particles such as clay [163]. Calvo et al. [157] also stated that the use of biosurfactants may help reduce the adaptation time of microbes at a contaminated site.

However, it has been reported that the commercial production of biosurfactants is very expensive to achieve [126]. Moreover, their potency in the field needs to be tested beyond reasonable doubt. This has led to the continuous study of the potentials of biosurfactants in crude oil bioremediation [164]. Calvo et al. [157] proposed the genetic development of hyper-producer microbial strains capable of multiplying biosurfactant production yields.

4.5.1. Discussion

The compatibility of biosurfactant with land and water oil spill bioremediation technologies makes it a promising approaches capable of enhancing the biodegradation of even recalcitrant hydrocarbon compounds in the environment. For example, Nikolopoulou and Kalogerakis [7] reported the enhanced effectiveness of biodegradation by combining oleophilic fertilizers with biosurfactants. However, the limited trials of biosurfactants in the field may have been caused by the high cost of synthesizing the amphiphilic compounds commercially. Also, the promising potential of hyper-producer strains is likely to be faced with the problem of regulation and field-constraints.

4.6. Suggestions for Employing Bioremediation Strategies Where Anoxic Conditions Exist

Bioremediation is well-suited for fresh oil spills [165]. If the process is not enhanced on time, prolonged crude oil exposure in the environment may cause the hydrocarbon compounds to accumulate as fragmented oil particles below land or water surfaces where biodegradation rates are very low. The literature has shown that oxygen concentration is a limiting factor for the microbial degradation of crude oil. Therefore, the rate of biodegradation in anoxic environments (oxygen-deficient sites) such as the soil subsurface (unsaturated and saturated zones), permafrost in the polar region (such as the Arctic) and ocean and marsh sediments/substrata is expected to be very slow.

In terrestrial anoxic environments, injection wells (through which oxygen is transferred to the soil subsurface and groundwater) are constructed to enhance biodegradation as seen in land bioremediation technologies such as bioventing, biosparging and bioslurping, although the efficiency of these methods can still be improved upon. On the other hand, the construction of oxygen-delivering technologies in marine anoxic environments has been considered to be very difficult [126] mostly due to the enormous depth of marine waters.

Atlas [165] reported the existence of subsurface oil residues at Prince William Sound in Canada, buried in the beach substratum containing fine sediments and the efficiency of biodegradation was questioned due to the anaerobic condition of the site. Venosa et al. [166] showed through a laboratory experiment that by agitating a polluted marine site, the oil may be displaced from the subsurface. They proposed that the addition of NO_3^- might help increase the porosity of the sediments which might help oxygenated water reach the sequestered oil. However, Atlas and Braggs [167] argued that mere agitation or chemical addition to polluted marine sites may not be feasible in the field. Nikolopoulou and Kalogerakis [126] suggested that substratum tilling may be useful in aerating marine areas of shallow-depth (for example the shorelines) whilst the provision of oxygen to deep marine waters remains a problem to be solved.

Wolicka and Borkowski [168] reported the possibility of PAH breakdown via carboxylation (an anaerobic process) using an anaerobic bacterium *Dechloromonas,* which utilize senergy acceptors other than oxygen. Earlier reports by Zhang and Young [169] stated that naphthalene and phenanthrene were broken down completely by indigenous sulfidogenic microbial consortia (sulphate-reducing microbes) after 150 days through the process of carboxylation, a key step in the anaerobic biodegradation of hydrocarbons which leads to the final production of CO_2. However, the research on oleophilic anaerobes in the literature appears to be sparse.

Yang et al. [53] stated that oxygen-deficiency in permafrost soils is a limiting factor of biodegradation in the cold region, however methods by which oxygen (and other limiting factors) can be safely delivered to the native oleophilic aerobes in permafrost soils in order to increase the efficiency of biodegradation is still under investigation.

4.6.1. Discussion

Most hydrocarbon-degrading microbes are aerobes and this may be the reason they are the biological agents employed by most researchers. As a result, little is known about the biochemical pathways for hydrocarbon metabolism by oleophilic anaerobes. Not all anaerobes possess the metabolic pathway to break down hydrocarbon compounds; therefore, the possibility of genetically-engineering non-oleophilic anaerobes may need to be investigated as this may be helpful in cleaning-up oil spills in anoxic sites. However, the safety levels of this process need to be considered as well in order to gain public acceptance.

In permafrost soils, the use of an oxygen-delivering system may be impracticable due to the thick layer of ice covering the soil. According to Yang et al. [53], in permafrost soils anaerobes are higher in population than aerobes; therefore, the use of an oxygen-delivering system may not be necessary. These anaerobes may require certain nutrients to degrade oil; the oleophilic formulation of such nutrients may be more effective in such an environment where the availability of liquid water is very low. However, the freeze-thaw seasonal cycles might interfere with the bioavailability of the nutrient and the spilled oil. In the case of a low number of native oleophilic anaerobes at the contaminated-permafrost site, the native microbes can be harvested, cultured (and pre-exposed to petroleum hydrocarbon) and re-introduced. However, harvesting oleophilic anaerobes from the contaminated-permafrost site may be very difficult.

In deep marine waters anaerobes are expected to be present, yet a lot of questions remain unanswered. For example, if nutrients are applied at the water surface, what mechanism will be in place to ensure that the nutrient gets to the ocean substratum without dispersing? Also, what proportion of oleophilic anaerobes inhabit the ocean beds and how can they be harvested? These questions make the biodegradation of oil-polluted ocean beds to be very difficult and in need of further research.

In terrestrial anoxic environments, a number of underground strategies and oxygen-delivering technologies have been designed; however, there is the need for better bioremediation technology that would enhance biodegradation and lower the clean-up cost.

4.7. Reasons Why Field Experiments Rather Than Laboratory-Based Experiments Need to be Encouraged

Many researchers [17,86,170] discovered that bioremediation strategies (whether for terrestrial or marine environments) have been more successful in the laboratory than in the field. For example, Head et al. [177] reported the failure of bioaugmentation in the field whilst Bento et al. [96] also reported the failure of biostimulation in the field.

Rosenberg et al. [172] understood the discrepancies that occur between laboratory studies and field trials and therefore carried out bioaugmentation and biostimulation trials both in the laboratory and in the field so as to obtain a more reliable result. In addition, Gallego et al. [173] carried out an *in situ* bioremediation of spilled petroleum so as to predict correctly the behavior of nutrients, surfactants and other bio-remediation amendments in the field.

However, the reason for the failure of laboratory studies in the field has been highlighted as follows: nutrient dilution and dispersion; introduced microbe not adapting to the new site; procedure used in releasing the bioremediation agent to the polluted site; predation by protozoa; type and quantity of spilled petroleum and geology/ecological-specificity of the polluted site [4,86,170]

4.7.1. Discussion

Real-life conditions cannot be perfectly mimicked in the laboratory. For example, most laboratory experiments on bioremediation do not put into consideration the presence of predators and antagonistic microbes that are capable of limiting the effectiveness of such processes in the field. Moreover, the quantity and rate of dispersion of spilled petroleum in the field may be very difficult to simulate in the laboratory. In addition is the application of GEM in bioremediation, which cannot be reliably achieved without successful field trials. Also, the effect of low production yield of biosurfactants could not have been discovered in the laboratory without field trials.

The dynamic nature of the environment may have discouraged researchers from setting up field experiments and also the challenge of monitoring individual environmental indices in the midst of external factors that tend to influence the environmental parameter under study and the high cost of sponsoring field studies. However, relying on laboratory studies due to the ease of factor-manipulation does not translate to a successful result in the field; thus, there is a need to encourage *in situ* bioremediation experiments in order to close the gap that exists between laboratory studies and field trials.

5. RECOMMENDATIONS AND CONCLUSIONS

5.1. Recommendations

5.1.1. Due to the complexity of asphalthenes, it may be very difficult for microbes to degrade the recalcitrant compounds within a short time frame unaided. A considerable level (48%) of asphathene biodegradation was achieved at a temperature of 40°C. Therefore, given the availability of thermo-tolerant microbes, it might be valuable to investigate if further increase in temperature will accelerate asphalthene biodegradation. A heat supply system will be needed to achieve this in the field.

Combining heat application with thermophilic emulsifiers may further enhance microbial degradation of asphalthenes. The large surface slicks of asphalthenes when spilled on land and water may likely be fragmented under the application of heat through the process of cracking or thermal decomposition as demonstrated by Zhao and Yu [174], thereby increasing the surface area available to microbial attack. The thermophilic emulsifier, a heat-tolerant biosurfactant, may help to eliminate the asphalthenic oil-water interface, thereby rendering the asphalthene more susceptible to rapid microbial degradation. However, the setting up of a heat supply system in the field (especially in aquatic environments) may be expensive and challenging. Also, the maximum temperature required to achieve asphalthene fragmentation needs to be investigated. Wolicka and Borkowski [168] stated that most microbes cannot survive temperatures higher than 90°C; therefore, a field trial of 80°C is recommended since *Thermus* [175], *Geobacillus* and *Bacillus* [176] species have been found to survive the temperatures of 83°C, 80°C and 80°C, respectively. However, more research needs to be carried out to determine the distribution and thermal-threshold of oleophilic thermophiles (heat-loving hydrocarbon-utilizing microbes).

5.1.2. However, there is the immediate need to provide a temporary means of meeting the challenge of asphalthene biodegradation as further research is carried out. It might be necessary to adopt physical remediation methods on water and land in the meantime. Due to their non-volatile nature asphalthenes, float on the surface of water as mousse during water oil spills and settle on the soil surface (due to their thickness) during land oil spills. Therefore, the use of physical techniques such as 'booms and skimmers' and 'organic sorbents' (such as date palms), may be a temporary option for cleaning-up asphalthenes on water and land, respectively. These physical techniques are suggested to be expensive, which makes further studies on asphalthene biodegradation imperative.

5.1.3. A microbial consortium with *Bacillus lentus* and *Pleurotus tuberregium* as members may be potent for asphalthene biodegradation. However, more research needs to be carried out to aid our understanding of the microbial biochemical pathway utiltized for asphalthene metabolism.

5.1.4. The rapid biodegradation of heavy PAHs may be achieved by applying biosurfactants and nutrients (biostimulation) to oil-polluted sites that are oleophilic microbe-rich. The biosurfactants will increase the bioavailability of the hydrocarbon and the nutrient will encourage aggressive biodegradation.

5.1.5. It has been suggested in the literature that oleophilic fertilizers may be the most effective nutrient type to be applied in marine environments in order to prevent eutrophication. However, the ways in which the production cost of oleophilic nutrients can be reduced should be investigated. In terrestrial environments, soil hardening and loss of soil fertility caused by the excessive use of inorganic nutrients may be managed effectively by using the slow-release nutrient application strategy. Therefore, a regulatory system that will maintain the prolonged nutrient supply at optimum levels should be in place in order to prevent the possible under-supply of nutrients to oil-polluted soils. As an alternative, the combination of both organic and inorganic nutrients can be applied using a nutrient-rotation strategy. The rotation strategy will help reduce the effect of the toxicity and high cost of inorganic nutrients as well as augment the low commercial production of organic nutrients.

5.1.6 Microbial encapsulation is relatively new with limited field applications, but has been suggested to be effective in protecting introduced oleophilic microbial consortia from the limiting factors in the field. However, the safety levels of encapsulating agents as well as the practicability of the process in the field should be investigated as this would mean that both exogenous and indigenous oleophilic microbes can be freely employed as bioaugmentation agents. As a temporary option until more research is carried out on encapsulation, native oleophilic microbes should be employed as they are better adapted to the polluted site than exogenous oleophilic microbes. In addition, more genetic research needs to be carried out to determine how suicide genes can be confined within the genome of target microbes and carefully monitored to prevent horizontal gene transfer to non-target organisms. The public also needs to be convinced with reliable evidence that the process can be carried out safely in the field.

5.1.7. Biosurfactants are suggested to be very efficient in solubilising crude oil hydrocarbons and also appear to be compatible with most bioremediation strategies. Therefore, more field trials should be carried out as well as investigating ways in which they can be produced in industrial quantities at minimum cost. One method of producing biosurfactants in commercial quantities that should be investigated is the biotechnology of hyper-producer microbial strains. The cost, practicability and safety of this genetic approach should be researched.

5.1.8 It has been suggested that oleophilic anaerobes are abundant in permafrost soils. However, oleophilic nutrients will be most effective in stimulating anaerobic biodegradation of crude oil in permafrost soils. It is paramount to first identify the location of the oil plume in the permafrost before applying the nutrients as there is no mobile medium (free-water) available to circulate the nutrients.

In terrestrial anoxic environments, a novel approach can be applied for a more effective clean-up of the soil subsurface. Bioslurping and biosparging are bioremediation technologies specially designed to clean-up the saturated (groundwater) and unsaturated (vadose) zones independently. However, a novel approach is to combine both strategies, a method referred to as, 'bioslurping-biosparging technology' (see Figure 1) in order to obtain enhanced biodegradation of the unsaturated and saturated zones of permeable soils simultaneously.

This proposed combined technology has the potential to extract insoluble light hydrocarbons (NAPLS) through a slurp tube from the water table to be separated above-ground and transporting vapor-phase hydrocarbons (also known as VOCs) to an SVE chamber for treatment, as well as the release of a three-component product (oxygen, biosurfactant and nutrient) through an injection tube to the groundwater zone to facilitate the aerobic biodegradation of heavy hydrocarbon compounds in the saturated zone.

Water-soluble nutrient is the preferred nutrient choice for this technology because it will dissolve in the groundwater and encourage the biodegradation of soluble hydrocarbons found in the saturated zone. The problem of nutrient dispersion is expected to be low since groundwater is quite stable.

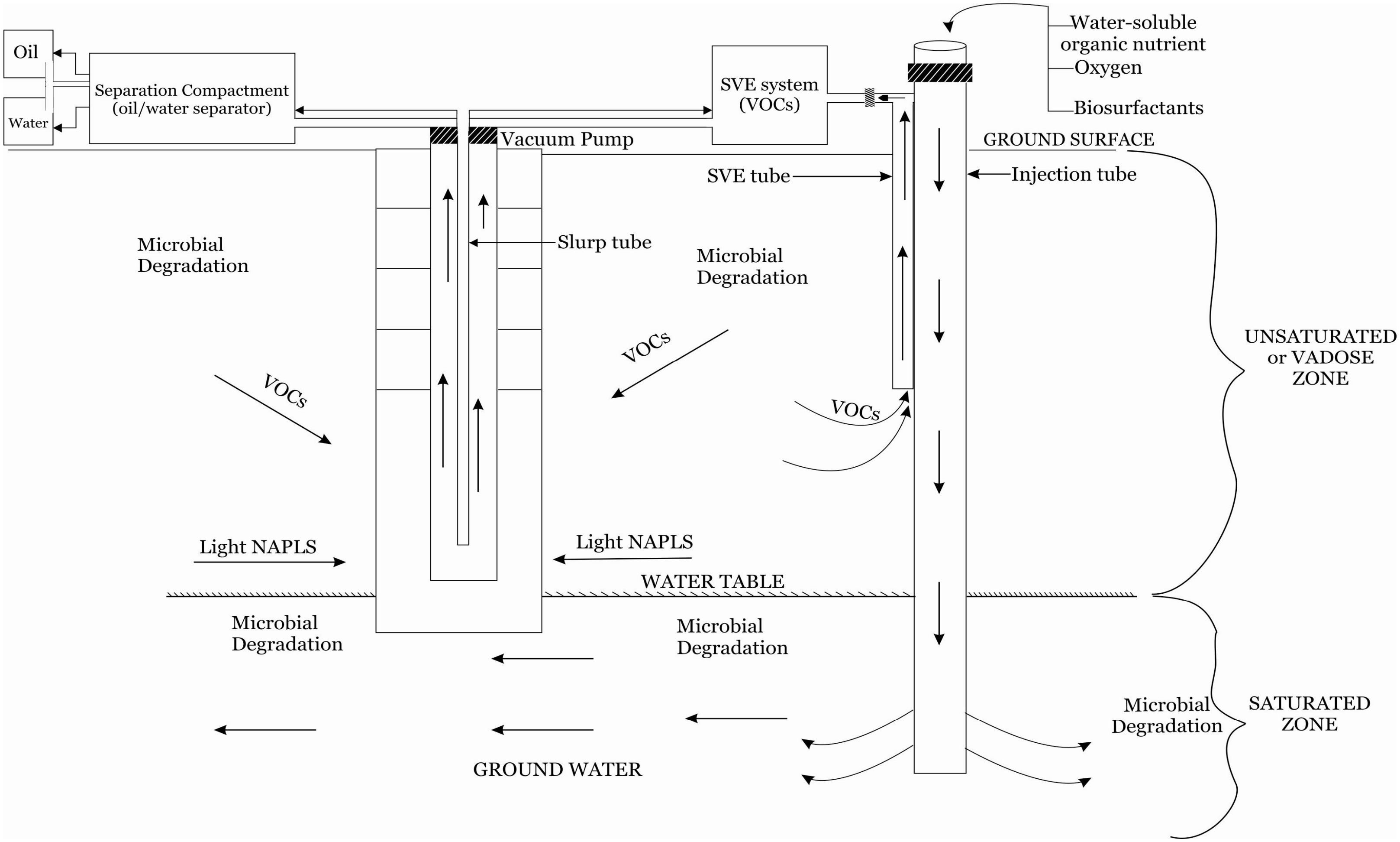

Figure 1 The proposed bioslurping-biosparging technology

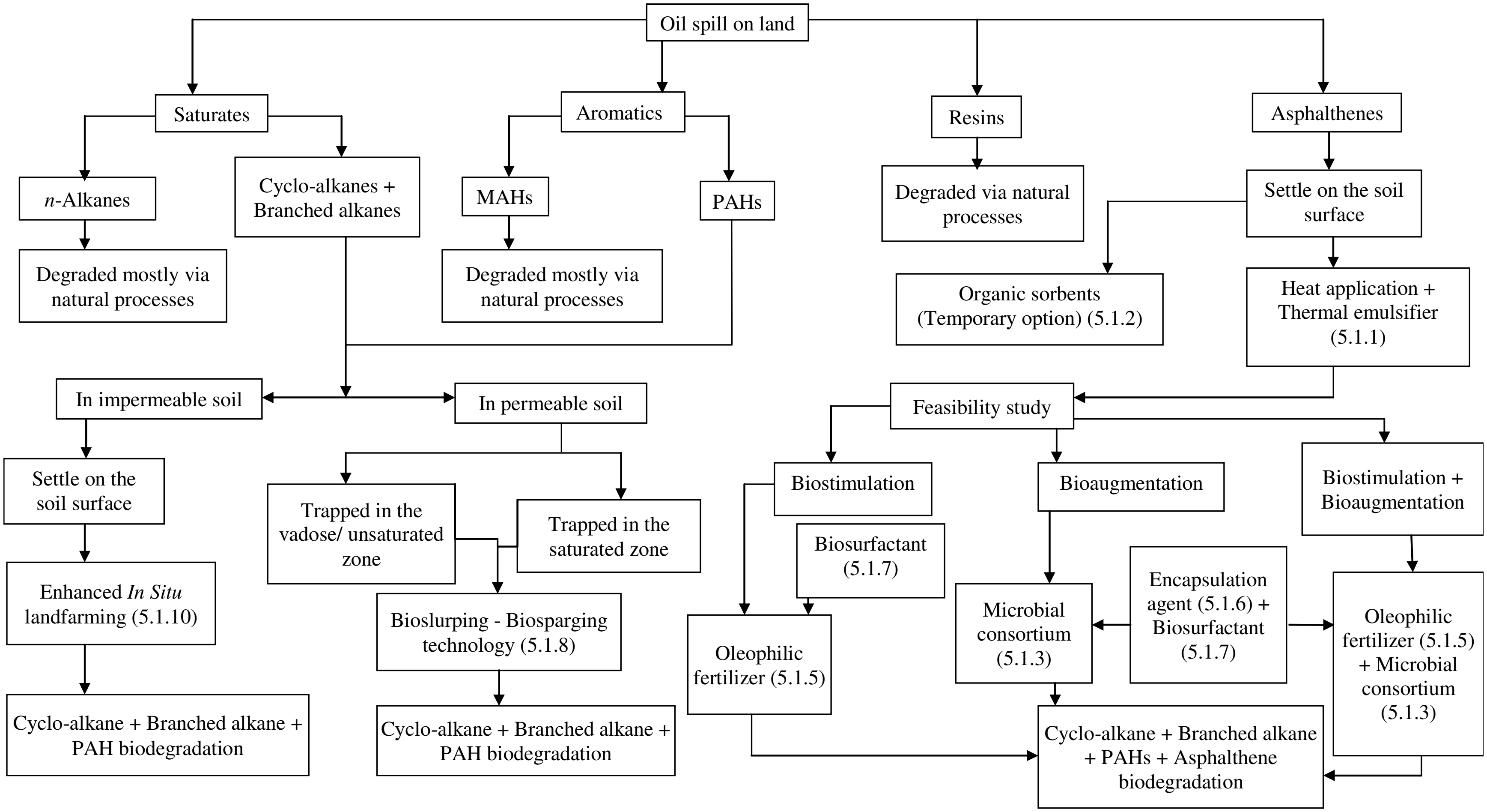

Figure 2: A simplified flowchart depicting the recommended clean-up pathway for land oil spill control

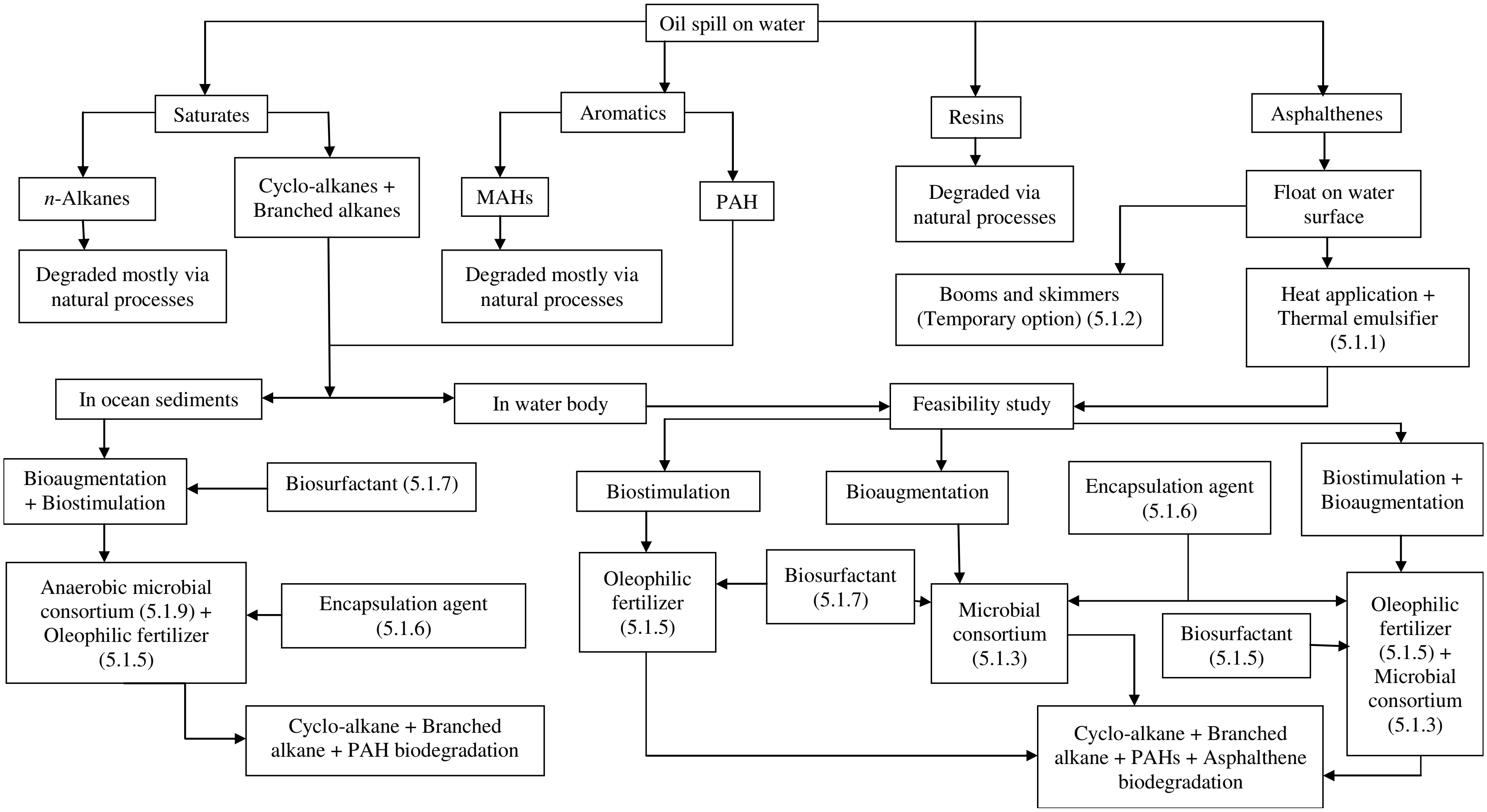

Figure 3: A simplified flowchart depicting the recommended clean-up pathway for water oil spill control

The bioslurping-biosparging system is a less sophisticated strategy and may be relatively cheap to set up when compared to the use of bioreactors and the pump and treat strategy. However, the bioslurping-biosparging technology is theoretical and thus requires cost analysis and field trials to prove its efficiency and sustainability

5.1.9. More research needs to be carried out to determine the distribution and process of harvesting oleophilic anaerobes. Also, the means by which nutrients can be transported safely to deep marine soluble hydrocarbons found in the saturated zone. The problem of nutrient dispersion is expected to be low since groundwater is quite stable. The bioslurping-biosparging system is a less sophisticated strategy and may be relatively cheap to set up when compared to the use of bioreactors and the pump and treat strategy. However, the bioslurping-biosparging technology is theoretical and thus requires cost analysis and field trials to prove its efficiency and sustainability anoxic sites such as the ocean beds without being dispersed should be investigated.

5.1.10. Impermeable soils such as clay soils retain petroleum pollutants at the soil surface. However, 'enhanced *in situ* landfarming' may be an effective way to clean-up land surface spills. This novel strategy involves the mechanical agitation of the polluted land surface followed by the addition of oleophilic nutrients and biosurfactants in order to improve crude oil bioavailability and encourage rapid biodegradation of all crude oil components except asphalthenes. Although impermeable soils may likely prevent the leaching of the pollutant to the groundwater zone, a greenhouse may be constructed over the polluted site in order to prevent VOCs from contaminating the atmosphere.

5.1.11. Oil spill bioremediation researchers should be encouraged to carry out field bioremediation trials. Research institutes, oil firms, government/environmental agencies and NGOs may help provide research funds for field studies as this would produce more reliable scientific reports on the potentials of the proposed bioremediation strategies and consequently improve oil spill management techniques for terrestrial and aquatic environments.

The above recommendations have been incorporated into simplified flowcharts (showing the relevant sections) combining the expected fate of spilled crude oil on land and water with the proposed bioremediation strategies that may be feasible for cleaning up oil spills, including the temporary measures that can be adopted until further research is carried out (see Figures 2 and 3).

5.2. Conclusions

The challenges associated with the bioremediation of oil spills have been investigated. The recent strategies developed to meet the challenges are also generally limited by one or more of the following: inadequate field trials (in the case of encapsulation, HGT, biosurfactants and oleophilic anaerobes), stringent regulations and negative public perceptions (in the case of GEM application), high cost of production (in the case of oleophilic nutrient and biosurfactant production). Therefore, when both governmental and non-governmental institutions sponsor field studies, it will enable researchers to compare the efficiency of the existing bioremediation technologies and devise eco-friendly ways in which they can be improved/enhanced with minimum cost.

Safety is the major concern with GEM application; therefore, until all necessary checks have been carried out to ensure that environmentally-damaging elements can be managed efficiently in the field, it might be important to limit GEM application to laboratory trials. Governments can register genetic research institutes such that the members of registered institutes will have the legal permit to utilize GEMs or genetically-related elements in their research work. Such institutes may need to renew their licenses yearly in order for their activities to be properly checked. If the institutes are registered, it might help eliminate the tortuous process currently experienced by GEM researchers to secure a legal permit. This is important because the application of GEM to oil spill bioremediation has the potential to greatly accelerate the biodegradation of spilled petroleum if it can be safely applied.

A single bioremediation approach may be incapable of breaking down all crude oil components (saturates, aromatics, resins and asphalthenes) within a reasonable time-frame at a minimum cost. Therefore, it may be necessary to employ integrated remediation technologies (such as the proposed bioslurping-biosparging technology, enhanced *in situ* landfarming, organic-inorganic nutrient rotation strategy and the heat application preceding asphalthene biodegradation) in order to create an aggressive synergistic approach which has the potential to be more effective. However, integrated remediation technologies may attract a high cost of utilization, therefore, the means by which the cost can be reduced to the barest minimum needs to be investigated as this would go a long way to improving oil spill management globally.

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