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Framework for Monitoring Bioremediation Projects by Regulatory agencies

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Abstract

The major aim in bioremediation of oil spill site is the reduction of the contaminants to permissible level or the required standard that does not pose health hazards. Reduction of contaminants has direct effect on indigenous microorganisms and their activities. The characterization of microorganisms using molecular, biochemical and physiological tests provide direct relationship between microorganisms and their activities but do not provide an understanding of the process networks in-situ. Culture independent techniques have extended capabilities in this regard but a high through-put technology (Illumina Miseq) should be employed in studying bacterial diversity during bioremediation of polluted sites and has proven more reliable and accurate. The report of United Nations Environmental Programme (UNEP) on the impact of oil spill in Ogoni land lacks conclusive microbial indices. Therefore, this review aims at developing a framework for monitoring bioremediation progress using high through-put microbial detection technology. This is necessary since biostimulation (remediation by enhanced natural attenuation) of indigenous hydrocarbon degrading bacteria remains the remedial technology of choice according to Shell Petroleum Development Company (SPDC) and other oil industries in Nigeria.

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Introduction

The petroleum industry is one of the major sources of organic contamination to the natural environment, releasing hydrocarbon contaminants into the environment in a number of ways. Severe subsurface pollution of soils and water can occur via the leakage of underground storage tanks and pipelines, spills at production wells and distribution terminals and seepage from gasworks sites during coke production. Seepages of gasoline from underground storage tanks have caused widespread soil and aquifer contamination, threatening the safety of various potable water supplies (Philp., *et al*. 2005). The biggest concerns of petroleum pollution in the environment are damages to farmland, fisheries, and portable water supplies considering that most people depend on farming, fishing and water for domestic purposes (Duke., *et al*. 2000; Ezekoye., *et al*. 2017). Efforts to remediate the negative impact of hydrocarbon pollution on the water and soil has resulted in several devices such as Remediation by Enhanced Natural Attenuation (RENA) which involves many techniques including Land farming by biostimulation or bioaugmentation of soil biota with commercially available micro-flora (Ebuehi., *et al*. 2005; Ezekoye., *et al*. 2017).

Biostimulation is the process of providing microbial communities with favourable environment to effectively degrade contaminants and in most cases provides rate-limiting resources like nitrogen, phosphorus and oxygen (usually by tilling to aerate the soil) to speed up the bioremediation process (Rolling., *et al*. 2002, 2004a; Kaplan and Kitts, 2004; Ezekoye., *et al*. 2017). In cases where natural communities of degrading consortia are at low levels or not present at all, the addition of contaminant degrading microorganisms, a process referred to as bioaugmentation, may speed-up the process (Kaplan and Kitts, 2004; van Elsas., *et al*. 2007; Ezekoye., *et al*. 2017). Although research has been performed in this area, bioaugmentation is generally not practiced, since introduced microorganisms in most areas are unable to compete favourably with well-adapted autochthonous microbial communities because of the strange environmental conditions and therefore needing more time to acclimatize (Odokuma and Dickson, 2003; van Hamme., *et al*. 2003; Ezekoye., *et al*. 2017). In situ bioremediation offers cost effective means of pollutant cleanup. It enhances natural fate of biodegradable pollutants and represents green approach to the problem of environmental pollution with little or no ecological impact (Cappello., *et al*. 2007; Kumar and Khanna, 2010; Chikere., *et al*. 2011). Water and carbon dioxide which are end products of biodegradation are innocuous to man and the environment. During hydrocarbon bioremediation, a number of indices are monitored to score the success of the technology. Use of fundamental chemical analyses for pollutant identification and standard microbiological techniques for quantification of viable microbial populations are the starting points of monitoring. The monitoring process usually begins with determining the nature and concentrations of the contaminant matrix. This is followed by determining the nature of the contaminant and measuring the microbial populations involved in the biobiodegradation process. Finally, the environmental factors that influence the rate of microbial metabolism are determined (Chikere., *et al*. 2011)

Bioremediation

Bioremediation as defined by the U.S. office of Management and Budget involves techniques using biological processes to treat contaminated environments (soil/groundwater). It is a field that combines basic microbiology, advanced biotechnology, and environmental engineering and does so within the context of public demands for clean waters and soils, evolving risk – based regulatory frameworks that govern performance criteria, and public concerns about microorganisms especially the deliberate release of genetically modified microorganisms into the environment (Atlas and Philp, 2005). Thus, bioremediation is interdisciplinary (involving microbiology, geochemistry, hydrogeology, engineering etc.) and is still a developing field. It is an innovative technology; there is a great interest in research and development as well as in the actual applications.

Bioremediation is relevant to industrial engineers, site supervisors and managers who apply this technology today to remove pollutants from contaminated soils and waters but fail to acknowledge the work done by these microorganisms in removing pollutants from the contaminated environment and to academic researchers whose efforts will develop future bioremediation technologies that can be applied for cost-effective cleanup efforts. Approaches to bioremediation are varied and include in - situ or ex - situ methods and ones which may involve biostimulation, i.e. stimulating the microbial activities by optimizing environmental conditions such as adding nutrients or oxygen to increase the rates of biodegradation; bioaugmentation (i.e., adding microorganisms to increase the diversity of microorganisms capable of biodegrading the contaminants); or natural monitored attenuation (i.e. monitoring the natural biodegradative activities to see that removal of contaminants occurs at rates needed to meet target value set to reduce risk to human health and the environment). To be successful, bioremediation must be economical and technically competitive with other physical and chemical remediation technologies (Atlas and Philp, 2005).

The Emergence/Evolution of Bioremediation

Microorganisms, especially bacteria, have benefitted from time, evolving into many types with exceedingly diverse metabolic capabilities which can be utilized in bioremediation. Bacteria have existed on the planet for more than 3 billion years. Given their small size, large surface-to-volume ratio, very high rate of growth and division and genome plasticity, bacteria evolve quickly. Synthetic chemicals which have no natural counterparts have become common in the environment and constitute environmental pollutants such as pesticides, herbicides, biocides, detergents, and halogenated solvents. That the biosphere has not been catastrophically polluted by these chemicals is a testimony to the ability of the bacteria to emerge/evolve. These ideas were formalized by Alexander (1965) as the principle of

microbial infallibility which states that "no natural organic compound is totally resistant to biodegradation provided that environmental conditions are favourable.'' Given that most synthetic compounds are very similar to naturally occurring counterparts, it is not surprising that they can be biodegraded by microorganisms. Some xenobiotic (man-made) compounds have molecular structures that are not readily recognized by existing degradative enzymes; such compounds resist biodegradation or are metabolized incompletely, resulting in the accumulation of these xenobiotics in the environment. However, the finding that some naturally halogenated compounds do exist and indeed may be produced by microorganisms (Oberg, 2002) means that there is further potential scope for harnessing the ability of microbes to transform such xenobiotics. The major reasons for the control of environmental pollution and the consideration of bioremediation are public health concerns; environmental concerns and the cost of remediation decontamination (Atlas and Philp, 2005).

Bioremediation Monitoring

Monitoring is a critical part of remediation effort. The need for monitoring bioremediation projects start even before technology selection. Firstly, the nature of contamination must be determined in terms of the specific contaminants and their concentrations. Then the nature of the environmental matrix containing those contaminants must be considered thus determining whether or not the process can be considered as a possible remediation strategy.

In a bioremediation monitoring, one must be able to determine or establish the initial parameters and to show that in the end, the target goal/value of reducing the contaminant to a safe level has been achieved. In some cases, only the initial and end points need to be measured to demonstrate success but in other cases it is critical to follow the progress of the remediation and to monitor critical parameters, including those that may need to be modified to optimize the remediation process. For bioremediation, monitoring may involve measuring not only the contaminants concentrations but also the microbial populations involved in the degradation or transformation of those contaminants and the environmental parameters that influence rates of microbial metabolism (Philp., *et al*. 2005). Parameters such as total petroleum hydrocarbon (TPH), polyaromatic hydrocarbons (PAHs) and total organic carbon (TOC) are important in bioremediation monitoring of polluted soil.

Methods for monitoring microbial diversity in soil during bioremediation

For bioremediation to be considered a remediation technology, it is critical to establish that there is an adequate active microbial population that is capable of attacking the specific contaminants (Macnaughton., *et al*. 1999; Chaillan., *et al*. 2004; Maila., *et al*. 2006; Wolicka., *et al*. 2009). Some important questions to be considered before monitoring include a) Does the site have the requisite values for water content and pH? b) Is porosity within the desirable range? c) Is the site contaminated with only petroleum hydrocarbon? If the answer to these questions is yes then, there is a very good chance that there will be an active population of hydrocarbon utilizing bacteria in the soil and that bioremediation may be able to succeed (Atlas and Philip, 2005; Ollivier and Magot, 2005). Use of fundamental chemical analyses for pollutant identification and standard microbiological techniques for quantification of viable populations of microorganisms are the starting point for monitoring. The techniques for determining the presence of hydrocarbon utilising microorganisms are routine, inexpensive, and relatively rapid (Atlas and Bartha, 1998). Even when it is difficult to cultivate specific microbial populations, the technique of enrichment culture can still reveal the presence of important degrading microbes and establish that they have the natural propensity to degrade the pollutant at an acceptable rate (Williams., *et al*. 1999; Atlas and Philip, 2005). There are also emerging techniques in molecular microbial ecology that do not rely on cultivation because of the viable but non - culturable phenomenon and these have been found very useful for monitoring the progress of bioremediation (Kloos., *et al*. 2006; Brons and van Elsas, 2008; Malik., *et al*. 2008; Ruberto., *et al*. 2008; Zengler, 2008). During bioremediation, microbial population changes can be investigated, along with more detailed analytical work such as gas chromatograph (equipped with either of the detectors: flame ionization detector (FID) or electron capture detector ECD), high performance liquid chromatography, tests on the fate of 14C- radiolabeled substrate in order to identify specifically whether biodegradation and or mineralization of the substrate is taking place or a mere transformation to a more or less toxic or mobile metabolite (Okpokwasili., *et al*. 1986; Leahy and Colwell, 1990; Young and Cerniglia, 1995; Atlas and Philip, 2005). A number of laboratory- and field-scale bioremediation trials have employed these strategies and found them useful in monitoring the progress of bioremediation in different environmental media (Stroud., *et al*. 2007; Chikere., *et al*. 2008a, b; Chikere., *et al*. 2009; Popp., *et al*. 2009; Wolicka., *et al*. 2009).

Chemical methods of bioremediation monitoring

Total hydrocarbon content (THC) could be determined as described by UNEP (2006). Five grammes of soil sample were weighed into a beaker and 10ml of xylene was added under the cork cover for 30 min. Aliquot of the extract was placed in an infrared spectrophotometer analyzer. The total hydrocarbon (THC) value could be determined by comparison to a calibration curve constructed from dilutions of a stock solution of 1:1 Bonny light crude, and Bonny medium. The spectrophotometric measurement was done at 420 nm using HACH DR 2400 spectrophotometer.

Total petroleum hydrocarbon (TPH) could also be determined using the method of Saari., *et al*. (2007). Five grammes portion of soil sample was weighed out, and 0.1 mg was weighed out and transferred into extraction vessels. Fifty millilitres of acetone was added and the vessel was closed. The vessel was heated at temperature of 150°C for 15 min. The vessel was allowed to cool to room temperature and 5g of sodium sulphate was added. The extraction mixture was filtered through ashless filter paper, and the sediment was thereafter rinsed and washed with acetone into a bigger container. The extracted volume was adjusted to 20 mL with 20 mL hexane solution before GC-FID measurement. Polar compounds such as vegetable oils, animal fats were removed by solid phase extraction under silica gel (florisil). Calibration was carried out using Bonny light crude oil, acetone, and mixture of Bonny light crude and acetone.

Gas chromatography flame- ionization detection system

The extracts were analysed by gas chromatography, using HP Agilent 6890 gas chromatography (Agilent technologies, Berkshire, United Kingdom) equipped with FID detector, an Agilent 7673 auto sampler and 5 capillary column (15m x 0.25nm) with a nominal film thickness of 0.25 µm, splitless injection method (all in batch). Injection volume was 1µl and injection temperature, 330°C. Helium was used as a carrier gas (2 mL min^{-1}). The column was held at 35°C for 1.50 min. The temperature was increased from 15°C/min to 310°C/min and held for 10 min. This enabled complete run within 27 min. The amount of total petroleum hydrocarbon (TPH) was then determined as a sum total of resolved and unresolved components eluted from the GC capillary column between retention times of 5 min to 35 min. This method called peak sum calculates TPH by summing up all components of crude oil from C_{10} and upwards. Real values of TPH were calculated as product of raw data on FID table or graph and dilution factor used for each sample.

Microbial methods of bioremediation monitoring

For bioremediation to be considered as a remediation technology, it is important to establish that there is an adequate active microbial population that is capable of attacking the specific contaminants. If the polluted site, has the requisite values for water content, pH, porosity and petroleum hydrocarbons contamination, then there is a very good chance that there will be an active population of hydrocarbon utilizing microorganisms in the soil and that bioremediation may be able to succeed. There are also emerging molecular microbial ecology techniques that do not rely on cultivation (Ilumnia Miseq) which are useful for determining whether bioremediation is feasible.

Once a bioremediation project has begun, quantification of bacterial populations generally will not give much additional useful information. The concern is on the activities and the rate of disappearance of the pollutants. Exceptions may be in cases of bioaugmentation, where the interest may include ensuring that the added microorganisms persist and remain viable. Yet even when enumeration of microbial populations is not critical for monitoring the progress of a bioremediation effort, measurement of microbial oxygen consumption and CO_2 production in aerobic bioremediation can serve as a health check to establish that the process is proceeding according to plan. Considering that gas respirometry can be done by relatively simple techniques on-site, these data can be acquired more often than the analytical chemistry data that inform only about pollutant removal (Philp., *et al*. 2005).

Monitoring Hydrocarbon Utilizing Bacteria

Hydrocarbons are chemically heterogeneous and almost ubiquitous in the environment. Not only are they found at polluted sites but chemical analysis has revealed their presence (both aliphatic and aromatic), in most soils and sediments. However, they are present in unpolluted sites in low concentrations. The probable origins of these low concentrations of hydrocarbons are seepages from natural

deposits, especially gaseous hydrocarbons and ongoing synthesis of some hydrocarbons by plants and microorganisms. It is therefore not surprising that hydrocarbon utilizing bacteria are widely distributed in nature (Philp., *et al*. 2005).

A number of general nutritional requirements are needed to achieve hydrocarbon utilization in bacteria. Hydrocarbons, as their name implies, are composed of hydrocarbon and carbon; there is a need to supply all other elements required for growth in the medium. These include molecular oxygen, nitrogen, phosphorus etc. The satisfaction of these requirements can be monitored by enumeration of total culturable hydrocarbon utilizing bacteria (HUB) using vapour phase method as reported by Hamamura., *et al*. (2006) and Ezekoye., *et al*. (2017). In this method appropriate dilutions of the samples are inoculated into mineral salt agar (MSA). Filter papers (Whatman No 1), saturated with bonny light crude oil are placed aseptically onto the covers of Petri dishes and inverted. The Hydrocarbon saturated filter papers supply hydrocarbon by vapour phase transfer to the inocula (Atuanya and Ibeh, 2004; Abu and Chikere, 2006; Ezekoye., *et al*. 2017). The plates (in triplicates) are incubated at 28°C for 7 days; colonies counted, and mean values recorded in colony forming units per gramme (Cfu/g). However, a more reliable method involves enumeration by a method adopted by Hamamura., *et al*. (2006) and Ezekoye., *et al*. (2017). This method involves dilutions of appropriate sample suspensions and plating out on Bushnell-Haas agar. Bushnell-Haas agar is known to contain most limiting nutrients that is required for growth of microorganisms. It is composed of the following salts: Magnesium sulphate (0.20 g/l), Calcium chloride (0.02 g/l), Monopotassium phosphate (1.00 g/l), Dipotassium phosphate (1.00 g/l), Ammoniun nitrate (1.00 g/l), Ferric chloride (0.05 g/l), and Agar – agar (15.00 g/l) (Sigma – Aldrich, USA). Studies have shown that, Bushnell-Haas is the best known medium for the isolation of hydrocarbon utilizers (Evans., *et al*. 2004; Quatrini., *et al*. 2008), with the only source of carbon and energy supplied by the hydrocarbon through vapour phase transfer in the case of vapour-phase methodology.

Aerobic hydrocarbon utilizing microorganisms have been found in almost all ecosystems that have been diligently screened (Magesin and Schinner, 2001; Prince, 2002). This includes Arctic and Antarctic marine sediments, terrestrial soils, and essentially all locations. Organisms able to use hydrocarbons as sole source of carbon and energy have been found in the domains: bacteria, archae, and fungi (Prince, 1998). The taxonomy of microorganisms that utilize hydrocarbons, remains poorly characterized, but so far, more than sixty genera of aerobic bacteria, five genera of anaerobic bacteria, two genera of aerobic archal bacteria, nine genera of algae, and ninety five genera of fungi have been shown to contain species that can degrade at least some hydrocarbons (Prince, 1998). The distinguishing feature of oil degraders or utilizers is their ability to somehow activate the hydrocarbon, either aerobically by inserting one or two oxygen atoms or anaerobically by adding some other moiety such as fumarate or carbon dioxide (Biegert., *et al*. 1996; Beller and Spormann, 1997; Zhang and Young, 1997; Prince 1998; Heider., *et al*. 1999; Spormann and Widdel, 2000; Widdel and Rabus, 2001). Such activation, and perhaps a few metabolisms, allows the hydrocarbon to enter the standard cellular pathways of metabolism.

Hydrocarbon utilizers may be ubiquitous, but they are typically only a small fraction of the biota of unpolluted sites, probably because they are substrate limited. An oil spill removes this limitation and there is generally a bloom of hydrocarbon-degrading microorganisms so that they become a major fraction of the microbial population. Modern molecular tools allow us to monitor these changes at the species level (Stephen., *et al*. 1999; vanHamme., *et al*. 2000; Chang., *et al*. 2000; Grossman., *et al*. 2000; Juck., *et al*. 2000; Iwamoto and Nasu 2001; Kasai., *et al*. 2001; Tay., *et al*. 2001; Kasai., *et al*. 2002). It is reasonable to imagine that this approach may be developed to provide indications of when the environmental impact of a spill has diminished to presplit conditions.

Genetically modified organisms are being useful for monitoring hydrocarbon contamination and its remediation (Atlas., *et al*. 1992; Sticher., *et al*. 1997; Sousa., *et al*. 1998; Macnaughton., *et al*. 1999; Xing., *et al*. 2000; Bundy., *et al*. 2001). Typically, a light-emitting system, such as the luc or lux system is inserted immediately after a gene in the early metabolism of a hydrocarbon. Then when the organism activates its hydrocarbon degradation system, it emits light. In fact, a bioluminescent genetically engineered Pseudomonas fluorescens strain had been approved for field testing as a reporter of naphthalene biodegradation (Ripp., *et al*. 2000) and was employed in semi-contained soil bioreactors. Whether genetically engineered microorganism will ever play a role in bioremediation in the marine environment or not, perhaps organisms engineered to degrade particularly recalcitrant chemical species, awaits a resolution

to this conflict (Zilinskas and Balint, 1998). Given the ubiquity of hydrocarbon utilizing microorganisms, however, genetically modified organisms will need to have the requisite properties to enable them survive and compete with the indigenous microorganisms.

Apart from sites of oil pollution where petroleum hydrocarbons are found, chemical analysis has revealed the presence of both aliphatic and aromatic hydrocarbons, in most pristine soils and sediments (Heiss-Blanquet., *et al*. 2005; Ollivier and Magot, 2005; Philp., *et al*. 2005; Kloss., *et al*. 2006; Quatrini., *et al*. 2008). The probable origins of these low concentrations of hydrocarbons in pristine environmental media are seepage from natural deposits and biosynthesis by plants and microorganisms (Atlas and Philip, 2005; Ollivier and Magot, 2005). It is therefore not surprising that HUB is widely distributed in nature. Several investigations have demonstrated an increase in numbers of HUB in oil-polluted habitats undergoing bioremediation (Chikere and Chijioke-Osuji, 2006; Hamamura., *et al*. 2006; Rojas-Avelizapa., *et al*. 2007; Quatrini., *et al*. 2008; Ruberto., *et al*. 2009). However, previous and recent works have suggested that despite an increase in the HUB percentage, the biodiversity of the bacterial community may be dramatically reduced since the presence of hydrocarbons in the environment often leads to select enrichment of HUB, to the relative detriment of biodiversity (Maila., *et al*. 2005; Hamamura., *et al*. 2006; Popp., *et al*. 2006; Quatrini., *et al*. 2008; Rodrigues., *et al*. 2009). In other to achieve hydrocarbon utilization by bacteria, a number of rate limiting nutritional requirements need to be provided. Hydrocarbons as their name implies are composed of hydrogen and carbon; therefore there is a need to supply all other elements essential for growth in the growth medium (Philp., *et al*. 2005).

The technique chosen for isolation of HUB depends on the type of hydrocarbons. Isolation techniques have in common their need for a solid surface upon which discreet colonies of bacteria can grow. This is important in order to obtain axenic cultures. Therefore, the starting point is the need for an agar-based mineral salt medium for isolation (Amouric., *et al*. 2006; Hamamura., *et al*. 2006; Rodrigues., *et al*. 2009; Ruberto., *et al*. 2009; Wolicka., *et al*. 2009). In order to specifically isolate HUB, the hydrocarbons must be provided in the growth medium as the sole source of carbon and energy (Leahy and Colwell, 1990; Odokuma and Dickson, 2003; Atlas and Philp, 2005; Chikere and Chijioke-Osuji, 2006).

As a result of the limitations of the traditional solid agar-based isolation methods, liquid culture methods were developed by using the most probable-number (MPN) procedure (Mills., *et al*. 1978). MPN is a statistical method based upon dilution of a sample to extinction i.e. multiple replicates of a sample are analysed and the results compared with statistical tables to determine the MPN of microorganisms in the original sample. The development of 96-well microliter plates miniaturised this method. The sheen screen method introduced by Brown and Braddock (1990), marked the beginning of the miniaturized MPN method for oil degraders. This method was specific for crude oil as a substrate. Wrenn and Venosa (1996) developed a 96-well microtiter plate MPN procedure to separately enumerate aliphatic and aromatic hydrocarbon degraders in separate plates. The alkane degrader MPN method uses n-hexadecane as the carbon source while growth is scored by turbidity and the reduction of iodonitrotetrazolium violet to iodonitrotetrazolium formazan (red precipitate) is used as an indicator of electron transport activity. An inherent limitation in the 96-well microtiter plate MPN method is lack of bioavailability of the hydrocarbon. Because of the small confined nature of the wells, both oxygen mass transfer and mixing of the oil and the aqueous phase are limited. This means that the incubation periods are long (usually 2 weeks) and there may be a high risk of wells drying out or the medium may become very saline through evaporation. Hydrocarbon utilizing bacteria may also be isolated based on enzymatic activity. The enzymes of greatest interest in hydrocarbon degradation are the dioxygenases because of their important roles in substrate activation and aromatic ring cleavage (Atlas and Bartha, 1998). Dioxygenases convert indole to indigo and the presence of blue colonies is the selection criterion (Philp., *et al*. 2005). A more specific enzyme screening is for catechol 2, 3-dioxygenase. Catechol is an extremely important and common intermediate in aromatic hydrocarbon catabolism (van Elsas., *et al*. 2007). Colonies can be sampled by filter lift from plates sprayed with catechol. The appearance of yellow pigment within 10 min of incubation at room temperature implies catechol 2, 3-dioxygenase activity. Alquati., *et al*. (2005) used the catechol colour assay to isolate naphthalene degrading bacteria belonging to the genera *Rhodococcus, Arthrobacter, Nocardia* and *Pseudomonas* from a petroleum contaminated soil. In more recent times, primers specific for hydrocarbon degrading enzymes are used in PCR and other fingerprinting methods to elucidate the degradative genes in putative hydrocarbon degraders from petroleum contaminated soil (Zucchi., *et al*. 2003; Alquati., *et al*. 2005; Higashioka., *et al*. 2009).

Laboratory based bioremediation and extrapolation to *in situ* bioremediation

Laboratory studies have shown that the overall bacterial community response to hydrocarbon contamination is variable, but certain hydrocarbon-degrading taxa do become prevalent in oil-impacted environments. It has also been reported that archaea do not have an important role in hydrocarbon degradation on contaminated beaches and because of their sensitivity to oil pollution, could be useful markers of ecosystem recovery (Roling., *et al*. 2004). Before drawing wider conclusions, however, it is important to know whether results that are obtained in laboratory microcosms can be extrapolated to the field (Roling., *et al*. 2004). Field studies have shown that many of the features observed in laboratory microcosms are also observed under field conditions. In beach sediment studies in the field, bacterial communities have limited diversity and are homogeneous in composition in space and time.

The overall response of the bacterial community in the field to oiling and bioremediation treatments is variable, similar to the response in the laboratory microcosms, although in field studies using oil treatment alone or in situations in which added nutrients were not retained in interstitial waters, the bacterial communities did not seem to respond and were similar to those in uncontaminated beach sediment (Roling., *et al*. 2004). Commonly, in laboratory experiments, specific hydrocarbon degrading-organisms (for example, *Alcanivorax* sp.) were selected in a field under conditions in which nutrient addition resulted in an increase in nutrient concentrations in interstitial waters. Despite these important common features of laboratory and field experiments, important differences have been observed. In the field, the rate and the extent of oil degradation have been found to be lower, probably as a result of the lower mean temperature in these experiments, which ranged from more than 20°C to less than 50°C.

In laboratory microcosms, phenanthrene and dibenzothiophenes were degraded, but such degradation was not observed in the above field experiment (Roling., *et al*. 2004). In addition, a marked increase in members of the proteobacteria has been observed during the later stages of oil-spill bioremediation treatments on a beach in Delaware. This increase has also been observed in laboratory-microcosm experiments but, curiously, not in other field trials, in United Kingdom and Japan (Ogino., *et al*. 2001). In the Japanese study, a strong dominance by bacteria related to *Pseudomonas putida* was observed after 12 weeks, and in the UK field trial, *Pseudomonas* sp. most closely related to *Pseudomonas stutzeri*, which was not found in corresponding laboratory studies, was detected only transiently early in the experiment. One of the greatest contrasts observed between field and laboratory experiments has been the effect of oil on archaeal communities. In laboratory microcosms, oil was observed to have an unequivocal negative effect on Archaea (Roling., *et al*. 2004). If this were the case in the field, then Archaea could be excellent markers of ecosystem recovery. However, the data from field experiments are more variable, and it is evident that regular tidal inundation returned sufficient numbers of Archaea to the beach sediments for them to be detected readily in oil-contaminated samples (Roling., *et al*. 2004).

Although some broad similarities are observed in the dynamics of microbial communities in response to spilled oil and bioremediation (for example, selection of Alcanivorax sp. and Cycloclasticus sp.), the specific response may vary from beach to beach, and bacterial-community composition does not converge after oil contamination and nutrient amendment. A similar observation has been made for diesel-contaminated soil (Bundy., *et al*. 2002). Even at the same beach location, similar treatments can result in heterogeneity in the dynamics of the bacterial communities as a whole (Bundy., *et al*. 2002). Because bacterial-community composition is consistent across a beach before any induced perturbation through oil contamination, it seems that the lack of convergence in bacterial communities does not reflect the initial composition of the bacterial communities present. However, laboratory-scale studies of bioremediation of heavy metals and hydrocarbon polluted sites are indispensable before a field study or field work. In the laboratory-scale bioremediation studies, factors such as pH, temperature, aeration, and effect of nutrient are studied. The real field conditions are simulated to a laboratory microcosm. However, a few conditions such as tidal influence may not be easily simulated (Singh., *et al*. 2005) especially during a laboratory-based bioremediation of hydrocarbon polluted mangrove swamp soil (Ezekoye., *et al*. 2015; Ezekoye., *et al*. 2017). Interestingly, laboratory-scale bioremediation of tetrachloroethene contaminated groundwater in the United States co-related with field scale situation (Ibinni., *et al*. 2010). The practice of carrying out laboratory-scale remediation before field-scale is indispensable in order to avoid waste of resources through employing an unpromising method of bioremediation in field-scale (Ibinni., *et al*. 2010). Laboratory-scale bioremediation which is a bio-treatability study helps to identify whether a pollutant is degradable. It has also been useful in the identification of new microbial players in pollutants degradation (Ibinni., *et al*. 2010).

Framework for Bioremediation (clean-up) of oil spill sites

Site assessment: Firstly, you identify whether the site was contaminated or not and if so whether the contamination had migrated laterally and/or vertically. Also, you investigate the source, history and impact of the spill. In this regard, the impact of contamination on aquatic organism, vegetation and public health is evaluated.

When oil spill occurs on soils, various scenarios can arise, among them include (a) no remedial action is taken, leaving the contamination in place and exposed to the elements, (b) fire break out, killing vegetation and creating a crust over the soil, making remediation or vegetation difficult, and (c) remediation by natural attenuation is attempted at the site before fires occurs.

When spills occur on soils but no remedial action is taken, the oil seeps to the ground and flows to low lying areas. This spread is exacerbated by rainfall, which enables oil to run off into nearby farms, ponds, swamps or creeks. When the oil reaches the root zone, plants begin to experience stress and in extreme cases, death follows. However, in due course, even when no remedial action is initiated, thick layers of oil will eventually wash off from the soil, making it possible for more tolerant plant species to re-establish, giving the area an appearance of having returned to healthy stage. When farming commences, plants generally show signs of stress and yields are reportedly lower than in non-impacted areas. This naturally has an impact on the livelihood of the community. Also farming in soil which is contaminated also exposes the community to dermal contact with hydrocarbons (UNEP, 2011).

The Environmental guidelines and standards for the petroleum industries in Nigeria (EGASPIN), issued in 1992, set out the standards which are currently the minimum operating requirement for the soil industry in Nigeria (EGASPIN, 1992).

EGASPIN proposes two possible options for pollution incidents:

1. Application of the Standard Guide for Risk-Based Corrective Action Applied at Petroleum sites, prepared by the American Society for Testing of Materials (E1739–95, reapproved 2010);
2. An approach based on intervention value and target values. Even though the EGASPIN document itself was reissued in 2002, no further guidance has been produced in the last 20 years, such that the approaches suggested in 1992 still form the operational basis for the oil industry in Nigeria.

EGASPIN defines intervention values as those values that indicate the quality for which the functionality of the soil for human, animal and plant life are threatened with being seriously impaired.

Concentration in excess of the intervention values means a serious contamination. Target values are defined as those values which indicate the soil quality required for sustainability or expressed in terms of remedial policy or the soil quality required for the full restoration of soils functionality for human, animal and plant life. The target values therefore indicate the soil quality levels ultimately aimed for" (UNEP, 2011).

Site clearing and baseline study: The polluted sites are cleared and the affected plants and vegetation burned (Figure 1), using Ikarama community in Bayelsa State as a case study. Afterwards, the samples of the polluted soil are collected from different points and homogenous sample are sent to laboratory for physiochemical, microbiology, and molecular analyses.

Bioremediation proper (monitoring): The gravity of the spill will determine the period of remediation by enhanced natural attenuation (RENA). Farmland (Pristine soil) 70 to 80m away from the polluted soil serve as a control soil. Control should be employed in every remediation projects.

During bioremediation, adequate care must be taken to observe the following protocols that enhance the disappearance of hydrocarbon in the polluted soil (i.e. enhances bioremediation):

Figure 1: *Burning of affected vegetation's after site clearing during bioremediation of polluted Soil in Ikarama community, Bayelsa State.*

Aeration: The polluted site should be adequately tilled with tractor to expose it to bacterial activity in order to break the carbon chain. Studies have shown that most microorganisms that enhance remediation are aerobic, therefore, needs oxygen for growth and survive. Recall that after spill, the soil nutrients such as oxygen, nitrate, phosphate etc. are limited and the soil saturated with hydrocarbon.

Excavation/Bulking: For a major spill, bulking is very important. Bulking entails excavating polluted soil as deep as 2 m below the pipeline right of way and filling it with unpolluted soil.

Nutrient amendment/Biostimulation: There is also a need to add limiting nutrients to the polluted soil to enhance the growth of indigenous hydrocarbon utilizers as to metabolize the hydrocarbon.

Creation of windrows: The creation of ridges/windrows after amendment is crucial as this will help to break up the lump soil. Consistent turning of the ridges for a week will also help to expose the hydrocarbon to microbial attack, after which the soil will be leveled to its original state.

Post bioremediation assessment: The remediation site must conform to EGASPIN intervention target value (500 mg/kg for TPH) or conform to Nigerian standards as per the EGASPIN legislation which indicate satisfactory completion of remediation of impacted potable water shall be undertaken in conformity to the EGASPIN recommended target level of 10 ppm of dissolved TPH (UNEP, 2011).

One of the most important criteria for oil spill and contaminated site management – specifically is any criterion that triggers remediation (called intervention values) or indicates its closure (known as target values) (UNEP, 2011).

The current approach by SPDC to clean-up contaminated sites through remediation by enhanced natural attenuation (RENA) should be discontinued instead, procedures should be put in place for any new spills to be assessed within the shortest possible time and heavily contaminated soil excavated and sent to the centralized facility for treatment and disposal. The final clean-up standards and ongoing monitoring plans should be discussed and agreed with relevant government agencies (UNEP, 2011).

More importantly, the opinion of the authors of this review paper is not to discontinue RENA approach but to acknowledge the activity of microorganisms in making remediation project successful. Hence, there is need for SPDC and other agencies to incorporate microbial indices in all remediation projects.

The RENA approach to remediation

Hydrocarbons, once released to land, can be transferred and degraded through a number of natural processes, including: evaporation to the atmosphere (volatilization), combustion, infiltration, alone or along with rainwater, to soil and eventually to groundwater, overflow into swamps and water bodies, and runoff with rainwater to swamps, water or ground water.

The principle of enhanced natural attenuation from clean-up of contaminated land is to augment one or more of the above processes so that the concentration of the contaminants can be reduced (UNEP, 2011). After reviewing contaminated land clean-up issues in Nigeria, Shell Global Solutions endorsed the RENA approach. Hence, it is SPDC's preferred procedure and 100 percent of oil spill remediation in Ogonil and has been undertaken using the RENA approach.

Under RENA, contaminated land (topsoil) is initially ploughed over, either mechanically or manually (labour), to increase aeration. Fertilizer is added to supplement the nutrient requirements of the bacteria as they break down the pollutants. The ploughed soil is then piled into neat windrows (Figure 2) to further enhance the aeration process. Samples are taken from the windrows every quarter and once the EGASPIN specification of 5,000 mg/kg of TPH is reached, the windrows are levelled (Figure 3)

Figure 2: *Making of windrows during bioremediation of polluted soil in Ikarama community, Bayelsa State.*

Figure 3: *Levelling of windrows after bioremediation has been completed and emergence of green grasses as evidence of a successful bioremediation.*

The implicit assumption in RENA approach applied by SPDC is that the natural process being enhanced is the bioremediation and still no inclusion of microbial indices. All enhancing actions whether ploughing, adding nutrients or windrowing, are applied to further natural biodegrading processes. In an ideal situation, this approach is scientifically defendable. However, the reality on the ground in Ogoniland suggests otherwise. The RENA process is failing to achieve either environmental clean-up or legislative compliance. As seen in the analyses and case studies presented in UNEP report (UNEP, 2011), it is also failing to achieve compliance with SPDC's own procedures.

SPDC Clean-up specifications

The second most important element of SPDC procedures, after RENA, is the recommended values for clean-up. SPDC uses 5,000 mg/kg TPH as its remediation criterion for soil. While no specific reason has been given for choosing this value, it was the assumption of NOSDRA that the value was taken from EGASPIN intervention value of 5,000 mg/kg.

There was disparity from EGASPIN document which forms the basis for the SPDC procedure. In one section the legislation defines a target value of 50 mg/kg TPH as the desired end point for restoration after oil spill, while in a section on remediation of contaminated land an "intervention value of 5,000 mg/kg TPH is given for remediation closure (UNEP, 2011). Meanwhile, Remediation Management System (RMS) sets a new remediation intervention value of 3,000 mg/kg for TPH to demonstrate commitment to remediation excellence.

During the early phase of discussions with SPDC, UNEP was informed that the remediation closeout value of 5,000 mg/kg TPH set by SPDC was not drawn from the EGASPIN but was based on a risk assessment. If this was a corporate decision, it is not stated as such in the SPDC documentation, nor is it communicated to the authorities as required by EGASPIN. Expert-level discussions are needed between Department of Petroleum Resources (DPR), National Oil Spill Detection and Regulatory Agencies (NOSDRA) and the oil companies to arrive at a technologically feasible target value.

It is recommended that SPDC works with the Nigerian regulators to clarify the paradox of remedial intervention and target values being the same. They should also agree on a consultative approach to setting site-specific clean values.

Conclusion

In as much as the success of bioremediation depends on the reduction of the pollutants by microbial activities to safe levels stipulated by the regulatory bodies, and the meeting of some specific performance criteria, monitoring of bioremediation should be a critical aspect of bioremediation projects. Bioremediation monitoring should employ both traditional, chemical and 16S bacterial metagenomics to demonstrate the presence of specific bacteria and their metabolic activities in the reduction of pollutants in contaminated sites. This will help to manage and design a more effective bioremediation technology and in monitoring bioremediation projects. Therefore regulatory agencies such as department of petroleum resources (DPR) and Ministry of Environment should acknowledge and adopt the use of high-through put microbial detection technologies (Illumina Miseq) as a framework in monitoring the progress of a bioremediation, thus, acknowledging input microbial indices in all bioremediation projects.

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