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Autochthonous Microbial Bioaugmented Remediation of Crude Oil Contaminated Soil in the Niger Delta

Monday Ubogu[*a], Lucky O. Odokuma[b] and Ejiro Akponah[c]
[*a] Department of Microbiology, Federal University of Agriculture Makurdi, Nigeria.
[b] Department of Microbiology, University of Port Harcourt, Nigeria.
[c] Department of Microbiology, Delta State University, Nigeria
[*a] Corresponding author: ubomon@yahoo.co.uk; +234-806-437-1271

Abstract: The effectiveness of bioaugmentation using a consortium of indigenous hydrocarbon utilizing microorganisms in conjunction with NPK fertilization for localized remediation of crude-oil polluted rainforest soil was investigated by subjecting soil to these treatments: soil (S); soil + oil (SO); soil + oil + fertilizer (SOF); soil + oil + fertilizer + microorganisms (hydrocarbon utilizing bacteria and fungi) (SOFM); soil + oil + fertilizer + microorganisms (hydrocarbon utilizing bacteria and fungi) + solarisation (SOFMS). Soil was monitored and evaluated for 120 days for culturable heterotrophic and hydrocarbon utilizing bacteria and fungi populations, and residual total petroleum hydrocarbon (TPH). Results indicated that while culturable heterotrophic populations rose continuously throughout the study, hydrocarbon utilizing bacterial and fungal populations increased up to day-90 before diminishing in contaminated soils. Bacterial populations were consistently higher than fungal for all applied treatments ($P < 0.05$). Residual TPH decreased in all contaminated soils with time. Treatment SOFM had the highest TPH reduction in soil with 66.81 % loss at degradation rate of 39.25 mg/kg/day; SO had the lowest loss of 24.82 % at the rate of 14.58 mg/kg/day within 120 days. Soil inoculation with constituted autochthonous microbial consortium in conjunction with NPK fertilization was effective for localized remediation of crude oil contaminated soil.
Keywords: Autochthonous; bioaugmentation; crude oil; hydrocarbon utilizing microorganisms; Niger Delta; remediation.

INTRODUCTION

Despite the enormous derivable benefits from crude oil, its deliberate or accidental releases into the environment remained a major source of global concern. There are numerous reports of spill incidences across the world totalling millions of barrels of crude oil. In the Niger delta alone, about 13.0 million barrels of crude oil have been emptied into its environment over the last 50 years (FME, 2006). Many of these crude oil spills were either never cleaned or improperly cleaned; rendering the impacted sites desolate lands (Kadafa, 2012). There are a number of documented evidences of negative impacts of crude oil on the environment and human health. In soil, increased toxicity of its biomagnified components, ground and surface water contamination, flora and fauna extermination, and bareness of previous cultivable lands (Obire and Anyanwu, 2009) are some of its well known detrimental effects.

Bioremediation is a viable technology for the treatment and removal of crude oil from contaminated soils. This technology which relies on the use of biological systems for environmental decontamination (Dua *et al.*, 2002; Kuiper, 2004; Barea *et al.*, 2005; Shukla *et al.*, 2010), comparatively have more public endorsement, cost-effectiveness, efficiency and environmentally friendliness than other well known intrusive and complex physicochemical and engineering anchored techniques, such as chemical oxidation, soil vaporization, excavation, thermal desorption, soil washing, incineration, photolysis and hydrolysis (Khan *et al.*, 2004; Zhou *et al.*, 2005; Do *et al.*, 2009; Tang *et al.*, 2010; Zawierucha and Malina, 2011; Niti *et al.*, 2013; Suja *et al.*, 2014; Gkorezis *et al.*, 2016). Biostimulation and bioaugmentation are among the most widely applied bioremediation techniques in the clean-up of crude oil contaminated soils. Biostimulation involves the modification of factors limiting microbial activities, particularly oxygen and nutrients addition to the polluted sites to stimulate autochthonous pollutant-degrading microbes to speed-up the remediation process (Mohan *et al.*, 2006; Malina and Zawierucha 2007; Perfumo *et al.,* 2007; Tyagi *et al.*, 2010; Adams *et al.*, 2015; Wu *et al.* 2017; Goswami *et al.*, 2018).

As a result of insufficient indigenous hydrocarbon utilizing microorganisms or elevated contaminants levels, biostimulation may fail to achieve the desired results (Ueno *et al*., 2007). Hence, bioaugmentation: the supplementary addition of autochthonous or allochthonous wild type or genetically engineered microbes well adapted to the contaminants to speed-up remediation process (Mrozik and Piotrowska-Segetb, 2009; Zawierucha and Malina, 2011; Adams *et al*., 2015; Goswami *et al*., 2018). Autochthonous soil microbiota, are microorganisms that are natural inhabitants of soil, existing in time of evolution, and typical of the habitat (Schaechter, 2004; Kolwzan *et al*., 2006; Maczulak, 2011).

Substantial numbers of bioremediation studies on oil contaminated soils, employing bioaugumentation techniques have been reported (Alvarez *et al*., 2011; Chang *et al*., 2011; Roy *et al*., 2012; Suja *et al*., 2014; Benyahia and Embaby, 2016; Biktasheva *et al*., 2017; Buraimoh *et al*., 2017; Chen *et al*., 2017; Ataikiru *et al*., 2018). However, many of these studies executed in soil relied on small number of microbes-degrading pollutants with pollutants requiring multilevel degradation process (Mrozik and Piotrowska-Segetb, 2009; Forsyth *et al*., 1995). To achieve complete and timely mineralization of crude oil in soil, a consortium of large number of well adapted hydrocarbon utilizing microorganisms is required. Unlike a single species which can only metabolize a narrow range of substrates, microbial consortium containing large and varied groups of microbial species, exude a more robust capacity to degrade and utilize the numerous components in this complex organic mixture and the resultant metabolic intermediates generated in the course of their breakdown (Goux *et al.* 2003; Ghazali *et al.* 2004; Bordenave *et al.*, 2007; Al-Saleh and Obuekwe , 2009).

Although, some measurable successes have been reported in the use of genetically modified microorganism for bioaugmented remediation of crude oil and its derivatives (Brown *et al*., 1988; King *et al*., 1990; Ford *et al*., 1999; Ezezika and Singer, 2010), most of the laboratory successes failed to show commensurate performance in field trials (Venosa *et al*., 2002; Cunningham *et al*., 2004). There are an abysmal low number of reports in literature where genetically engineered bacteria performed better than natural ones in the removal of recalcitrant pollutants in the natural environment (de Lorenzo, 2009). Furthermore, some of these products are not only costly (Suja *et al*., 2014), they fail to live up to manufacturer' claim of effectiveness (Mohammed *et al*., 2007). The deployment of genetically engineered organisms in the field is also bedevilled by a number of challenges such as regulation, ethical, human health and environmental concerns (Das and Chandran, 2011; Prakash, *et al*., 2011). Prakash *et al.* (2011) categorized some of these challenges to include: genetic contamination / interbreeding; competition with natural species; ecosystem impacts; impossibility of follow-up; horizontal transfer of recombinant genes to other microorganisms.

The success of bioaugmentation is hinged on a number of biotic and abiotic factors. Influencing abiotic factors include, aeration, moisture, organic matter, nutrients availability, contaminant levels, soil type, temperature and pH conditions (Mrozik and Piotrowska-Segetb, 2009; Shukla *et al*., 2010). Important biotic factors that may affect the success of biogumentation include competition between the introduced (exogenous) and indigenous soil microbes for scarce nutrients, protozoan predation, antagonistic interactions and bacteriophage attack (Mrozik and Piotrowska-Segetb, 2009). These interactions are responsible for reduced inoculants populations (England *et al*.,1993; Sorensen et al., 1999) usually witnessed shortly after soil inoculation (Mrozik and Piotrowska-Segetb, 2009). Furthermore, successful bioaugmentation requires a careful selection of microorganisms in the consortium that can be cultured easily, well-adapted to local conditions, reproduce rapidly, and tolerate elevated pollutant levels with high survival

rate in the designated soil environment (Suja *et al*., 2014; Goswami *et al*., 2018).

Bioremediation therefore must be designed in line with site specific conditions (Boopathy, 2000). In this regard, autochthonous (indigenous) microbial strains are considered as best suited for the remediation of the contaminated site (Rahman *et al*., 2003; Suja *et al*., 2014).

It is on the above bases that this study was aimed at remediating crude oil contaminated rainforest soil in the Niger Delta employing a plethora of autochthonous hydrocarbon-utilizing microbial consortium.

MATERIALS AND METHODS

Evaluation of Soil Physicochemical Reference Data

Soil samples used for this study were collected from a rainforest agricultural soil at Okpaka, Udu local government area of Delta State, Nigeria, at 0 to 15 cm depth within an area of 100.00 m^2. Prior to crude oil contamination and soil treatment, soil was analyzed for total petroleum hydrocarbon (TPH) using USEPA Method-8015C (2007); total organic carbon in soil (TOC) (Skjemstand and Baldock, 2006); nitrogen (van Reeuwijk, 2002); phosphorus (van Reeuwijk, 2002); pH (Hendershot *et al*., 2006); porosity (Ezzati *et al*., 2012), and textural components (Aliyu and Oyeyiola, 2011) to ascertain its physicochemical baseline properties.

Isolation, Quantification and Characterization of Culturable Heterotrophic Soil Bacteria and Fungi

Soil culturable heterotrophic and hydrocarbon utilizing bacteria and fungi were isolated and quantified using the soil plate dilution method. One gram (1.0 g) of homogenized soil sample was added to 9.0 ml of diluents (physiological saline) in a test tube (sterile). From this, 5-fold serial dilutions (10^{-5}) was prepared. Aliquots of 0.1 ml of diluted sample of soil were then plated out aseptically on agar plates that have properly dried employing the spread plate technique. The respective dilutions were prepared in triplicate plates on potato dextrose agar (PDA) (Oxoid) for fungi; nutrient agar (NA) (Titan Biotech Ltd.) for bacteria; oil mineral salt agar (OMA) as constituted in Dutta and Singh (2016) for hydrocarbon- utilizing bacteria (HUB) and hydrocarbon utilizing fungi (HUF); with the incorporation of tetracycline for the selective isolation of fungi on fungal plates. Incubation of the entire plates was done at room temperature (28 ± 2 ºC). Counts of culturable heterotrophic bacteria and fungi were carried out after incubating for 48 hours and 5 days on NA and PDA respectively. On the other hand, HUB and HUF counts were performed after 72.0 hour and 5-7 days respectively on OMA. Thereafter, microbial colony counts on the respective agar plates were represented as colony forming unit per gram of soil (cfu/g). Microbial counts for the various soil treatments including controls were determined for 120 days at 0, 30, 60, 90 and 120 days intervals.

Bacteria and fungi isolated from soil samples prior to crude oil contamination were purified, stored in agar slants from where further studies were carried out. Pure isolates of bacteria were presumptively characterized on the basis of cultural, morphological and biochemical properties of isolates relying on Bergey's Manual of Systematic Bacteriology (Garrity *et al*., 2005), and identification schemes of Cheesebrough (2006); Vos *et al*. (2009) and Whiteman *et al*. (2012). Fungal isolates were also presumptively identified on morphological and cultural basis, using the system of identification outlined by Ellis et al. (2007); Humber, (2005); Watanabe, (2002) and Barnett and Hunter (1998). Ability of isolated bacteria and fungi to utilize petroleum hydrocarbon was confirmed through Vapour Phase Transfere (VPT) method (Chaudhry *et al*., 2014).

Soil Treatments

Bioremediation studies was carried out by subjecting soil in plastic pots to the following treatments: soil (S); soil + oil (SO); soil + oil + fertilizer (NPK: 15:15:15) (SOF); soil + oil + fertilizer (NPK: 15:15:15) + microorganisms (hydrocarbon utilizing bacteria and fungi) (SOFM); soil + oil + fertilizer (NPK: 15:15:15) + microorganisms (hydrocarbon utilizing bacteria and fungi) + solarisation (SOFMS). Each of the above treatments was prepared in triplicates. Contaminated soils as well as uncontaminated control were regularly watered on daily basis using distilled sterile water and tilled for aeration at intervals of 7.0 days within the 120 days period (Ubogu *et al*., 2019).

Soil Contamination

Soils contamination in pots were prepared by adding 240.0 g crude oil (0.818 g/cm3 specific gravity) to 4000.0 g of soil contained in each pot to attain a TPH concentration of 7050 mg/kg in soil (Ubogu *et al*., 2019). Soil and oil in pots were thoroughly mixed together before determining TPH concentration.

Soil Solarisation

Soil solarisation was carried out on crude oil contaminated soil in plastic pots using the method of Elmore *et al*. (1997) as adapted by Ubogu *et al*. (2018). The daily mean temperature recorded within the 14 days duration of solarisation was 43.0 ± 2.5 ºC.

Fertilizer Application

Twelve gram (12.0 g) of NPK fertilizer (15:15:15) were added to contaminated soil at the above sated rate to accomplish the suggested 1.0 – 5.0 % nitrogen by weight in crude oil contaminated soil for actual nutrient enhancement modification (Head and Swannell, 1999). Thereafter, fertilizer was thoroughly worked into soil and oil mixture with a hand trowel.

HUB and HUF Scale-up and Soil Inoculation

Confirmed HUB and HUF isolated from the uncontaminated rainforest agricultural soil were scaled-up for soil inoculation using the method outlined by Ubogu *at al.* (2018). Three hundred millilitres (300.0 ml) of HUB and HUF consortium in physiological saline suspension in a 500.0 ml capacity conical flak was gently swirled and poured into each contaminated pot at the rate of 4.5 x 10^{10} cfu/ml for HUB and 5.5 x 10^{8} cfu/ml for HUF. The microbial consortium inoculants mixture was then thoroughly worked into soil in pots.

Effect of Treatments on Soil Residual TPH

Soil residual TPH in treated and untreated contaminated soils were determined at day-0, day-30, day-60, day-90 and day-120. Triplicate soil samples were analyzed for all treatments at the designated intervals employing US EPA-Method 8015C for non-halogenated hydrocarbon (US EPA, 2007). Estimation of average daily degradation speed of TPH in soil was calculated thus: Degradation rate (mg/kg/day) $= \frac{\text{Total amount of TPH degraded}}{\text{Time taken (days)}}$.

While the overall percentage degradation rate of TPH within the study period was calculated thus: % TPH degradation $= \frac{\text{Total amount of TPH degraded at day 120}}{\text{Total TPH at day 0}} \times 100$

Data Analysis

Data garnered from this investigation were analysed using Microsoft Excel (Analysis Tool Pak). Resolution of replicate samples was carried out via the use of measure of central tendency and dispersion. Comparative assessments of the effect of corresponding treatments on culturable heterotrophic bacterial and fungal populations were analyzed using Student's *t*-Test. The effect of the various treatments on the populations of culturable heterotrophic bacteria and fungi, HUB, HUF, and residual TPH and speed of degradation was done using analysis of variance (ANOVA). Level of significance for all analysed data was fixed at $P < 0.05$ confident limits.

RESULTS

Soil Physicochemical Reference Data

Baseline physicochemical figures from the rainforest soil employed in this study revealed that there has not been any substantial crude oil impact on the soil as indicated by the relatively low TPH and TOC content (80.0 mg/kg and 0.02 % respectively). The soil had a pH that was acidic with a considerable porosity, reasonable nitrogen and phosphorus content. On the basis of its textural components the soil may be categorized as loamy sand (Table 1).

Culturable Bacteria and Fungi Isolated from Uncontaminated Soil

A total of 23 presumptively identified culturable microbial species were isolated from the uncontaminated rainforest soil. Ten of these species in the bacterial group belonged to seven genera while 13 of the fungal group belonged to 10 genera. Six of the bacterial species were identified as HUB and eight of the fungal species as HUF (Table 2). The rainforest soil naturally paraded substantial proportion of hydrocarbon utilizing microorganisms in terms of species diversity.

Effects of Treatments on Culturable Heterotrophic and Hydrocarbon-Utilizing Bacterial and Fungal Populations in Crude Oil Contaminated Soil.

With the exception of the uncontaminated rainforest soil, Culturable heterotrophic bacterial and fungal populations in the crude oil-contaminated soil increased steadily throughout the study period for all applied treatments (Fig. 1). While the bacterial populations were persistently higher than fungi for each of the corresponding treatments; the applied treatments produced significant effects on the respective populations of culturable heterotrophic bacteria and fungi with treatment SOFM recording the highest culturable heterotrophic bacterial and fungal populations among the various treatments ($P < 0.05$) (Fig. 1).

However, while there was no consistent increase in percentage populations of HUB and HUF in uncontaminated soil; a steady general increase occurred in the populations of HUB and HUF in contaminated soils up to day-90 for all the applied treatments before a slight decline in population (Fig. 2). Significantly, the applied treatments influenced the percentage populations of HUB and HUF with treatment SOFM having the highest populations of hydrocarbon utilizing bacteria and fungi ($P < 0.05$) (Fig. 2).

Effect of Treatments on Soil Residual TPH

Soil residual TPH in all contaminated soils diminished over time with treatment SOFM having the lowest residual TPH at day-120. TPH in soil was reduced from 7050 mg/kg at day-0 to 5300, 2660, 2340, and 2640 mg/kg at day-120 in soil for treatments SO, SOF, SOFM and SOFMS respectively (Fig. 3). Among the contaminated treated and untreated soils, oil degradation rates were in the order: SOFM > SOFMS > SOF > SO. At day-120, 66.81 % of TPH in soil were lost in treatment SOFM with an average degradation rate of 39.25 mg/kg/day (Table 3).

Table 1: Baseline physicochemical properties of soil

Characteristics	Values (Mean ± SE, n = 3)
TPH (mg/kg)	80.00 ± 1.5
TOC (%)	0.02 ± 0.0
Nitrogen (%)	0.15 ± 0.01
Phosphorus (mg/kg)	40.50 ± 1.5
Porosity (%)	61.0 ± 1.0
pH	6.2 ± 0.5
Sand (%)	88.0 ± 1.5
Clay (%)	7.0 ± 1.0
Silt (%)	5.0 ± 1.0

Table 2: Culturable heterotrophic bacteria and fungi isolated from uncontaminated soil

Bacteria	Fungi
Bacillus sp.[1]	*Aspergillus* sp.[1†]
Bacillus sp.[2]	*Aspergillus* sp.[2†]
Bacillus sp.[3†]	*Aspergillus* sp.[3†]
Corynebacterium sp.[†]	*Botryodiplodia* sp.
Micrococcus sp.[†]	*Candida* sp.
Pseudomonas sp[1]	*Curvularia* sp.
Pseudomonas sp.[2†]	*Geotrichum* sp.
Arthrobacter sp.	*Rhizopus* sp.
Nocardia sp.[†]	*Paecilomyces* sp.[†]
Actinobacillus sp.[†]	*Penicillium* sp.[1†]
	Penicillium sp.[2†]
	Trichoderma sp.[†]
	Verticillium sp.[†]

Isolates with superscript symbol (†) are hydrocarbon utilizing organisms.

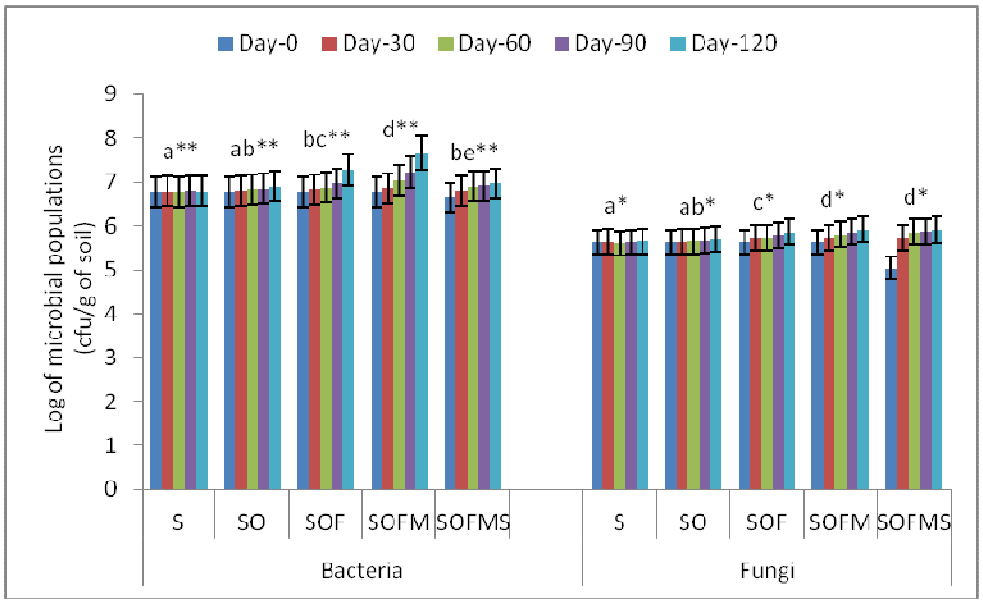

Figure 1: Culturable heterotrophic bacterial and fungal populations in crude oil contaminated soil receiving different treatments.

Corresponding treatments having different number of (*) for bacteria and fungi differ significantly (n = 5, Student's *t*-Test, $P < 0.05$). Different treatments for culturable heterotrophic bacteria and fungi respectively, having same alphabet are statistically the same (n = 25, ANOVA, $P < 0.05$). S = soil; SO = soil + oil; SOF = soil + oil + fertilizer; SOFM = soil + oil + fertilizer + microorganisms (hydrocarbon utilizing bacteria and fungi); SOFMS = soil + oil + fertilizer + microorganisms (hydrocarbon utilizing bacteria and fungi) + solarisation.

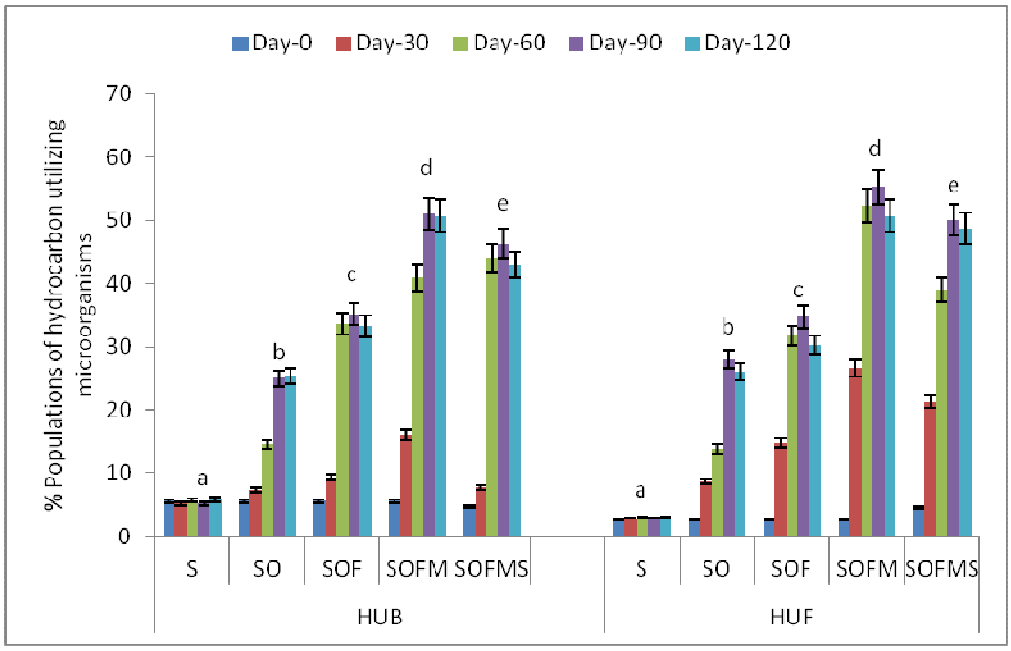

Figure 2: Populations of culturable hydrocarbon utilizing bacteria and fungi in crude oil contaminated soil under different treatment.

Treatments having different alphabet for HUB and HUF respectively are statistically different (n = 25, ANOVA, $P < 0.05$). HUB = Hydrocarbon utilizing bacteria; HUF = Hydrocarbon utilizing fungi. S = soil; SO = soil + oil; SOF = soil + oil + fertilizer; SOFM = soil + oil + fertilizer + microorganisms (hydrocarbon utilizing bacteria and fungi); SOFMS = soil + oil + fertilizer + microorganisms (hydrocarbon utilizing bacteria and fungi) + solarisation.

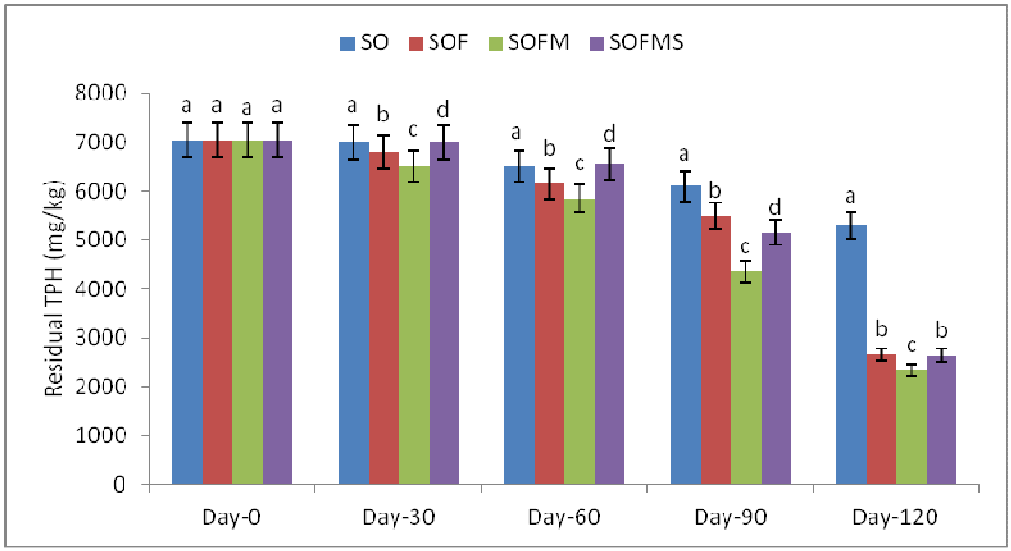

Figure 3**:** Residual TPH in crude oil contaminated soil receiving different treatment.

*Plotted values are the averages of triplicate samples. Treatments having different alphabet differs significantly (n = 12, ANOVA, $P < 0.05$). S = soil; SO = soil + oil; SOF = soil + oil + fertilizer; SOFM = soil + oil + fertilizer + microorganisms (hydrocarbon utilizing bacteria and fungi); SOFMS = soil + oil + fertilizer + microorganisms (hydrocarbon utilizing bacteria and fungi) + solarisation.

Table 3: Crude oil degradation rates in contaminated soils receiving different treatments

TPH Degradation		
Treatment	Daily degradation rate (mg/kg/day)	% total loss (in 120 days)
SO	14.58 ± 2.0^{a}	24.82
SOF	36.58 ± 2.2^{b}	62.27
SOFM	39.25 ± 2.0^{c}	66.81
SOFMS	36.75 ± 1.5^{b}	62.55

Values having same superscript alphabet in the same column are statistically the same (n = 12, ANOVA, $P < 0.05$). S = soil; SO = soil + oil; SOF = soil + oil + fertilizer; SOFM = soil + oil + fertilizer + microorganisms (hydrocarbon utilizing bacteria and fungi); SOFMS = soil + oil + fertilizer + microorganisms (hydrocarbon utilizing bacteria and fungi) + solarisation.

DISCUSSION

The baseline microbial feature of the uncontaminated rainforest soil employed in this study showed that the soil did not only harbour diverse microbial populations but also possess substantial number of autochthonous hydrocarbon utilizing microbial species. About 60.0 % of its culturable populations have intrinsic ability to utilize petroleum hydrocarbon. The low levels of soil physicochemical reference data on TPH and TOC indicated that the soil have not been impacted significantly by crude oil, this suggests that the relatively high proportion of microbial species diversity observed able to utilize petroleum hydrocarbon in soil was not an induced trait but a naturally acquired one. A vast array of microorganisms in soil are intrinsically equipped with the appropriate genetic tools to degrade environmental pollutants such as long chain alkane, cycloalkane, aromatics and polyaromatic hydrocarbons to their basic constituent elements (Cerniglia, 1993; Kastner and Mahro, 1996; Heidelberg *et al*., 2002).

The following genera of bacteria and fungi: *Corynebacteria*, *Micrococcus*, *Pseudomonas*, *Nocardia*, *Bacillus*, *Actinobacillus*, *Aspergillus*, *Paecilomyces*, *Penicillium*, *Trichoderma*, and *Verticillium* identified as hydrocarbon utilizing microorganisms in this study have previously been reported (Ollivier and Magot, 2005; Narajo *et al*., 2007; Obire and Anyanwu, 2009; Chikere *et al*., 2009; Chuma, 2010; Behera *et al* 2012). Therefore, the constitution of the above culturable autochthonous hydrocarbon degrading microbial consortium is likely to provide the requisite viable assortment for a localized crude oil remediation work.

Within the study period, the uncontaminated soil did not show any consistent increase or decrease in the populations of culturable heterotrophic and hydrocarbon utilizing bacteria and fungi. Similar findings were reported by Obire and Anyanwu (2009), Oyeyiola (2010), Oyeyiola *et al*. (2013). On the hand, a general steady increase occurred with time in the contaminated soils for culturable heterotrophic and hydrocarbon utilizing bacterial and fungal populations. This rise in population is attributable partly to the increased carbon input from the contaminating oil. Numerous reports lend credence to this observed trend (Obayori *et al.*, 2008; Chikere *et al*., 2009; Obire and Anyanwu, 2009; Abbasian *et al.* 2016). Crude oil addition to soil triggers general microbial population rise and particularly that of microbial unit capable of utilizing hydrocarbons in soil (Coulon *et al*., 2006; Hamamura, 2006).

It was also observed in this study that the populations of hydrocarbon utilizing bacteria and fungi went on a downward trend after 90 days of soil contamination and treatments.

This reduction in hydrocarbon utilizing microbial populations may be due to the depletion of crude oil in soil and toxic effects of some components of oil and degradation products (Obayori *et al.* 2008; Ziołkowska and Wyszkowski, 2010; Ikueasn, 2017).

Although there was higher fungal species diversity than bacterial in this study, the overall bacterial populations were consistently more than that of fungi for all the corresponding treatments applied. Most cultivable soils have been reported to have higher populations of bacteria than fungi (Tortora *et al*., 2002). Bacteria and fungi are primarily responsible for petroleum hydrocarbon breakdown in soil (Atlas, 1984; Das and Chandran, 2011) while bacteria tend to respond more rapidly to oil contamination in soil, fungi experience an initial repression (Pinholt *et al*., 1979). Conversely, the activity of fungi tends to persist long after bacterial activity has tapered off (Jensen, 1975).

The highest populations of culturable heterotrophic and hydrocarbon utilizing bacteria and fungi were observed in treatment SOFM over the other treated and untreated contaminated soils. The addition of NPK fertilizer along with hydrocarbon utilizing bacteria to crude oil contaminated soil have been observed to trigger a rise in soil microbial populations (Odokuma and Dickson, 2003; Chikere *et al*., 2009; Liu *et al*., 2009; Ibiene *et al*., 2011); this possibly explains the observed elevated microbial populations in SOFM. However, the application of solarisation treatment in SOFMS may have caused a reduction in microbial populations in comparison to treatment SOFM. Soil solarisation generally reduces soil microbial populations over a period of time before re-colonization (Elmore *et al*., 1997).

Among the treated and untreated soils, treatment SOFM produced the highest degradation rate with 66.81 % percentage reduction in TPH in soil at day-120 with a degradation rate of 39.25 mg/kg/day. The efficiency of treatment of SOFM over the others in the reduction of soil TPH may be ascribed to the inoculation of a plethora of well-adapted consortium of autochthonous hydrocarbon utilizing bacteria and fungi in conjunction with NPK fertilization, which concomitantly resulted in the yield of that critical microbial biomass required for an accelerated crude oil breakdown. The inoculation of hydrocarbon degrading organisms and fertilizer application have been reported to cause accelerated removal of oil from soil (Odokuma and Dickson, 2003; Obayori *et al*., 2008; Chikere *et al*., 2009; Tang *et al*., 2010; Chorom *et al*., 2010; Zand *et al*., 2011).

CONCLUSION

Results from this study showed that soil indigenous hydrocarbon utilizing microorganisms can be constituted into a viable consortium for its own remediation. Furthermore, soil inoculation with autochthonous hydrocarbon utilizing microorganisms along with NPK fertilization is an efficient and viable technique for a localized treatment and removal of oil from contaminated soil.

RECOMMENDATION

The combined application of autochthonous hydrocarbon utilizing microbial consortium along with NPK fertilization is recommended as an efficient means of cleaning-up crude-oil contaminated rainforest soil in the Niger Delta.

REFERENCES

Abbasian, F., Lockington, R., Megharaj, M., Naidu, R. (2016). The biodiversity changes in the microbial population of soils contaminated with crude oil. *Current Microbiology* 72: 663 - 670.

Adams, G.O., Fufeyin, P.T., Okoro, S.E., Ehinomen, I. (2015). Bioremediation, Biostimulation and Bioaugmention: A Review. *International Journal of Environmental Bioremediation and Biodegradation* 3 (1): 28 - 39.

AL-Saleh, H.D. and Obuekwe, C. (2009). Predominant culturable crude oil-degrading bacteria in the coast of Kuwait. *International Biodeterioration and Biodegradation* 63 (4): 400 - 406.

Aliyu, M. B. and Oyeyiola, G.P. (2011). Rhizosphere bacterial flora of groundnut (*Arachis hypogeae*). *Advances in Environmental Biol*ogy 5(10): 3196 - 3202.

Alvarez, V.M., Marques, J.M., Korenblum, E. and Seldin, L. (2011). Comparative Bioremediation of Crude Oil-Amended Tropical Soil Microcosms by Natural Attenuation, Bioaugmentation, or Bioenrichment. *Applied and Environmental Soil Science* Volume 2011, Article ID 156320, 10 pages. doi:10.1155/2011/156320.

Ataikiru, T. L., Okerentugba, P. O. and Iheanacho, C. C. (2018). Bioremediation of Bonny light crude oil polluted soil by bioaugumentation using yeast isolates (*Candida adriatica* ZIM 2468 and *Candida taoyuanica* MYA-4700). *International Journal of Public and Environmental Health* 5(4): 52 - 61.

Atlas, R. M. (1984). *Petroleum Microbiology*. Macmillian, New York P.692.

Barnett, H. L. and Hunter, B. B. (1998). *Illustrated genera of imperfect fungi*, 4th edn. American Phytopathological Society Press, St. Paul.

Barea, J.M. Pozo, M.J. Azcon, R. and Azcon-Aguilar, C. (2005). Microbial co-operation in the rhizosphere. *Journal of Experimental Botany* 56: 1761 - 1778.

Behera, B.C., Mishra, R. R. and Thatoi, H. N (2012). Diversity of soil fungi from mangroves of Mahanadi delta, Orissa, India. *Journal of Microbiology and Biotechnology Research* 2 (3): 375 - 378.

Benyahia, F. and Embaby, A. S. (2016). Bioremediation of Crude Oil Contaminated Desert Soil: Effect of Biostimulation, Bioaugmentation and Bioavailability in Biopile Treatment Systems. *Int. J. Environ. Res. Public Health* 2016, 13, 219; doi:10.3390/ijerph13020219.

Biktasheva, L., Galitskaya, P., Selivanovskaya, S. (2017). Bioaugmentation for oily waste remediation: harm or benefit? Proceedings of the Sixteenth International Waste Management and Landfill Symposium/ 2 - 6 october 2017. S. Margherita di Pula, Cagliari, Italy / © 2017 by CISA Publisher, Ital.

Boopathy, R. (2000). Factors limiting bioremediation technology. *Bioresour Technol*., 74: 63 - 67.

Bordenave, M. S., Goni-Urriza, P. C. and Duran, R. (2007). Effects of heavy fuel oil on the bacterial community structure of a pristine microbial mat. *Applied and Environmental Microbiology* 73(19): 6089 - 6097.

Brown, C. M., Campbell, I and Priest, F.G. (1988). *Introduction to Biotechnology*. Billing and Sons Ltd., Uk P.169.

Buraimoh, O. M., Ogunyemi, A. K., Ibrahim, N. H., Adebusoye, A. S., Ilori, M. O. and

Amund, O. O. (2017). Efficacy of Intervention Strategies for Bioremediation of Crude Oil in

Polluted Soil Microcosm. *Ife Journal of Science* 19(2): 303 - 313.

Cerniglia, C. E. (1993). Bioremediation of polycyclic aromatic hydrocarbons. *Current Opinion in Biotechnology* 4: 331 - 338.

Chang, L. K., Ibrahim, D. and Omar, I.C. (2011). A laboratory scale bioremediation of Tapis crude oil contaminated soil by bioaugmentation of *Acinetobacter baumannii* T30C. *African Journal of Microbiology Research* 5(18): 2609 - 2615.

Chaudhry, S., Luhach, J., Sharma, V. and Sharma, C. (2014). Assessment of diesel degrading potential of fungal isolates from sludge contaminated soil of petroleum refinery, Haryana. *Research Journal of Microbiology* 2014: 7 (3): 182 - 190.

Chen, Q, Li, J., Liu, M., Sun, H. and Bao, M. (2017). Study on the biodegradation of crude oil by free and immobilized bacterial consortium in marine environment. *PLOS ONE* https://doi.org/10.1371/journal.pone.0174445.

Cheesebrough, M. (2006). *District laboratory practice in tropical countries Part 2*. Cambridge University Press, UK.

Chikere, C. B., Okpokwasili, G. C. and Chikere, B. O. (2009). Bacteria diversity in a tropical crude oil polluted soil undergoing bioremediation. *African Journal of Biotechnology* 8(11): 2535 - 2540.

Chorom, M., Sharifi, H.S., Motamedi, H. (2010). Bioremediation of a crude oil-polluted soil by application of fertilizers. *Iranian Journal of Environmental Health, Science and Engineering* 7(4): 319 - 326.

Chuma, C. O. (2010). Enhanced bioremediation of hydrocarbon contaminated mangrove swamp in the Nigerian oil rich Niger Delta using seawater microbial inocula amended with crude biosurfactants and micronutrients. *Nature and Science* 8(8):195 - 206.

Coulon F., Mckew, B.A., Osborn, A. M., McGenity, T. J., Timmis, K. N. (2006). Effects of temperature and biostimulation on oil-degrading microbial communities in temperate estuarine waters. *Environmental Microbiology* 9: 177 - 186.

Cunningham, C. J., Ivshina, I. B., Lozinsky, V. I., Kuyukina, M. S. and Philp, J. C. (2004). Bioremediation of dieselcontaminated soil by microorganisms immobilised in polyvinyl alcohol. *International Biodeterioration and Biodegradation* 54 (2-3): 167–174.

Das, N. and Chandran, P. (2011). Microbial degradation of petroleum hydrocarbon contaminants: An overview. *Biotechnology Research International* Volume 2011, Article ID 941810, 13 pages .Doi:10.4061/2011/941810.

de Lorenzo, V. (2009). Recombinant bacteria for environmental release: what went wrong and what we have learnt from it. *Clinical Microbiology and Infection* 15 (Suppl. 1): 63 - 65.

Do, S. H., Jo, J. H., Jo, Y. H., Lee, H. K. and Kong, S. H. (2009). Application of a peroxymonosulfate/cobalt (PMS/Co(II) system to treat diesel-contaminated soil. *Chemosphere* 77: 1127 - 1131.

Dua, M., Sethunathan, N. and Johri, A. K. (2002). Biotechnology and bioremediation: successes and limitations. *Applied Microbiology and Biotechnology* 59: 143 - 152.

Dutta, S. and Singh, P. (2016). Hydrocarbon degradation potential of indigenous fungal isolates from Indian oil refinery, Haldia, (W.B) India. *Science Research Reporter* 6(1): 4-11.

Ellis, D., Davis, S., Alexiou, H., Handke, R. and Bartley, R. (2007). *Descriptions of Medical Fungi.* Mycology Unit

Women's and Children's Hospital, North Adelaide 5006, Australia.

Elmore, C. L,, Stapleton, J., J., Bell, C. E. and Devay, J. E. (1997). *Soil solarization, a nonpesticidal method for controlling diseases, nematodes, and weeds*. University of California, Division of Agriculture and Natural Resources Publication.

England, L. S., Lee, H., Trevors, J. T. (1993). Bacterial survival in soil: effect of clay sand protozoa. *Soil Biology and Biochemistry* 25: 525 - 31.

Ezzati, S., Najafi, A., Rab, M. A. and Zenner, E. K. (2012). Recovery of soil bulk density, porosity and rutting from ground skidding over a 20-year period after timber harvesting in Iran. *Silva Fennica* 64 (4): 521 - 538.

Ezezika, O. C., Singer, P. A. (2010). Genetically engineered oil-eating microbes for bioremediation: prospects and regulatory challenges. *Technol Soc* 32:331 - 335

Federal Ministry of Environment (FME). (2006). *Niger Delta Resource Damage Assessment and Restoration Project*. Conservation Foundation Lagos, WWF UK and CEESP-IUCN Commission on Environmental, Economic, and Social Policy.

Ford, C. Z., Sayler, G. S., Burlage, R. S. (1999). Containment of a genetically engineered microorganism during a field bioremediation application. *Applied Microbiology and Biotechnology* 51(3): 397 - 400.

Forsyth, J.V., Tsao, Y.M., Blem, R.D. (1995). Bioremediation: when is augmentation needed? In: Hinchee, R.E. et al. (eds) Bioaugmentation for Site Remediation. Battelle Press, Columbus, OH, pp1-14.

Garrity, G., Brenner, D. J., Krieg, N. R. and Staley, J. R. (2005). *Bergey's Manual of Systematic Bacteriology: The Proteobacteria, Part B: The Gammaproteobacteria*. 2nd edn., Volume 2. Springer, New York, USA.

Ghazali, F, M., Rahman, R. N. Z. A., Salleh, A. B., Basri, M. (2004) Biodegradation of hydrocarbons in soil by microbial consortium. *Int. Biodeterior. Biodegrad.* 54:61–7.

Gkorezis, P., Daghio, M., Franzetti, A., Van Hamme, J. D., Sillen, W. and Vangronsveld, J. (2016). The Interaction between Plants and Bacteria in the Remediation of Petroleum Hydrocarbons: An Environmental Perspective. *Frontiers of Microbiology* 7:1836. doi: 10.3389/fmicb.2016.01836.

Goswami, M., Chakraborty, P., Mukherjee, K., Mitra, G., Bhattacharyya, P., Dey, S., Tribedi, P. (2018). Bioaugmentation and biostimulation: a potential strategy for environmental remediation. *J of Microbiol and Exp.* 6(5):223 - 231.

Goux, S., Shapir, N., El Fantroussi, S., Lelong, S., Agathos, S. N., Pussemier, L. (2003). Long term maintenance of rapid atrazine degradation in soils inoculated with atrazine degraders. *Water Air Soil Pollut Focus* 3:131 - 42.

Head, I. M., Swanell, P. J. R. (1999). Bioremediation of petroleum hydrocarbon contaminants in marine habitat. *Current Opinion in Biotechnology* 10: 234 - 239.

Hamamura, N., Oslon, S. H., Ward, D. M., Inskeep, W. P. (2006). Microbial population dynamics associated with crude oil biodegradation in diverse soils. *Applied Environmental Microbiology* 72:6316 – 6324.

Heidelberg, J. F., Paulsen, I. T., Nelson, K. I., Gaidos, E. J., Nelson, W. C., Read, T. D. and Eisen, J. A. (2002). Genome sequence of the dissimilatory metal ion-reducing bacterium *Shewanella oneidensis*. *Nature Biotechnology* 1:1 - 6.

Hendershot, W. H., Lalande, H. and Duquette, M. (2006). Soil reaction

and exchangeable acidity. In Soon Y. K., and Hendershot, W. H., (eds.), *Soil chemical analysis*. Taylor and Francis Groups, LLC, New York P.3 – 8.

Humber, R. A. (2005). *Entomopathogenic Fungal Identification*. USDA-ARS Plant Protection Research Unit US Plant, Soil & Nutrition Laboratory. Tower Road Ithaca, NY 14853-2901, USA.

Ibiene, A. A., Orji, F. A., Ezidi, C. O., Ngwobi, C. L. (2011). Bioremediation of hydrocarbon contaminated soil in the Niger Delta using spent mushroom compost and other organic waste. *Nigerian Journal of Agriculture, Food and Environment* 7(3):1-7.

Ikuesan, F. A. (2017). Microbial response to varying crude oil concentrations of crude oil pollution of agricultural soils in Ondo State, Nigeria. *Microbiol Res J Int*. 22(4):1 - 8.

Jensen, V. (1975). Bacterial flora of soil after application of oily waste. *Oikos* 26: 152-158.

Kadafa, A. A. (2012). Environmental impacts of oil exploration and exploitation in the Niger Delta of Nigeria. *Global Journal of Science Frontier Research Environment and Earth Sciences* 12(3): 1-11.

Kastner, M. and Mahro, B. (1996). Microbial degradation of polycyclic aromatic hydrocarbon. In soil affected by the organic matrix of of compost. *Applied Microbiology and Biotechnology* 44: 668 - 675.

Khan, F. I., Husain, T. and Hejazi, R. (2004). An overview and analysis of site remediation technologies. *Journal of Environmental Management* 71: 95 - 122.

King, J. M. H., Digrazia, P. M., Applegate, B., Larima, F. and Sayer, G. S. (1990). Rapid, sensitive bioluminescent reporter technology for naphthalene exposure and biodegradation. *Science* 240: 778 - 781.

Kołwzan, B., Adamiak,W., Grabas, K. and Pawełczyk, A. (2006) *Introduction to Environmental Microbiology***.** Oficyna Wydawnicza Politechniki Wrocławskiej, Wrocław. P.112.

Kuiper, I, Lagendijk, E. L., Bloemberg, G. V. and Lugtenberg, J. J. (2004). Rhizoremediation: A beneficial plant-microbe interaction. *Molecular Plant-microbe Interactions* 17 (1): 6-15.

Liu, J.C., Cui, Y. S., Zhang, Y.P. and Zou, S.Z. (2009). Effect of plants and microorganisms on remediation of petroleum contaminated soil. *Journal of Ecology and Rural Environment* 25: 80 - 83.

Maczulak, A. (2011). *Encyclopedia of Microbiology*. Facts On File, Inc. An imprint of Infobase Learning 132 West 31st Street New York NY 10001. P. 881.

Malina, G., Zawierucha, I. (2007). Potential of bioaugmentation and biostimulation for enhancing intrinsic biodegradation in oil hydrocarbon-contaminated soil. *Bioremediation Journal* 11:141 - 147.

Mohammed, D., Ramsubhag, A., Beckles, D. M. (2007). An assessment of the biodegradation of petroleum hydrocarbons in contaminated soil using nonindigenous commercial microbes. *Water Air Soil and Pollution* 182: 349-356.

Mohan, S.V., Kisa, T., Ohkuma, T., Kanaly, R. A., Shimizu, Y. (2006). Bioremediation technologies for treatment of PAH-contaminated soil and strategies to enhance process efficiency. *Rev Environ Sci Biotechnol* 5:347 - 374.

Mrozik, A., Piotrowska-Segetb, Z. (2009). Bioaugmentation as a strategy for cleaning up of soils contaminated with aromatic compounds. *Microbiology Research* 165(5):363 – 375

Naranjo, L., Urbina, H., De Sisto, A. and Leon, V. (2007). Isolation of autochthonus non-white rot fungi with potential for enzymatic degrading of Venezuelan extra-heavy crude oil. *Biocatalysts and Biotransformation* 25 (2):341-349.

Niti, C., Sunita, S., Kamlesh, K. and Rakesh, K. (2013). Bioremediation: An emerging technology for remediation of pesticides. *Research Journal of Chemistry and Environment* 17(4): 88-105.

Obayori, O.S., Ilori, M.O., Adebusoye, S.A., Amund, O.O. and Oyetibo, G.O. (2008). Microbial population changes in tropical agricultural soil experimentally contaminated with crude petroleum. *African Journal of Biotechnology*. 7(24):4512-4520.

Obire, O. and Anyanwu, E. C. (2009). Impact of various concentrations of crude oil on fungal populations of soil. *International Journal of Environmental Science and Technology* 6(2): 211-218.

Odokuma L.O. and Dickson A.A. (2003). Bioremediation of a crude oil polluted tropical mangrove environment. *Journal of Applied Sciences and Environmental Management.* 7(2): 23-29

Ollivier, B. and Magot, M. (2005). *Petroleum Microbiology*. American Society for Microbilogy. Washington D.C. USA P.364.

Oyeyiola, G.P. (2010). Rhizosphere bacteria of Amaranthus hybridus. *Research Journal of Microbiol*ogy. 5:137-143.

Oyeyiola, G.P., Arekemase, M.O., Sule I. and Agbabiaka, T.O. (2013). Rhizosphere bacterial flora of Okro (*Hibiscus Esculentus*). *Science International (Lahore)*. 25(2):273-276.

Pinholt, Y., Struwe, S. and Kjoller, A. (1979) Microbial change during decomposition in soil. *Holartic Ecology* 2:195-200.

Prakash, D., Verma, S., Bhatia, R. and Tiwar,y B. N. (2011). Risks and Precautions of GeneticallyModified Organisms. *International Scholarly Research Network Ecology* Volume 2011, Article ID 369573, 13 pages doi:10.5402/2011/369573

Perfumo, A., Banat, I. M., Marchant, R. and Vezzulli, L. (2007). Thermally enhanced approaches for bioremediation of hydrocarbon-contaminated soils. *Chemosphere* 66 (1): 179-184.

Rahman, K. M., Kourkoutas, T. J., Petsas, I., Marchant, R., Banat, I.M. (2003). Enhanced bioremediation of n-alkane in petroleum sludge using bacterial consortium amended with rhamnolipid and micronutrients. *Bioresour. Technol*. 90: 159-168.

Roy, A. S, Yenn, R., Singh, A. K., Boruah, H. P. D., Saikia, N., and Deka, M. (2012). Bioremediation of crude oil contaminated tea plantation soil using two *Pseudomonas aeruginosa* strains AS 03 and NA 108. *African Journal of Biotechnology* 12(19): 2600-2610.

Schaechter, M. (2004). *The Desk Encyclopedia of Microbiology*. Elsevier Academic Press 525 B Street, Suite 1900, San Diego, California 92101-4495, USA P.1169.

Shukla, K., P., Singh, N. K. and Sharma, S. (2010). Bioremediation: Development, current practices and perspectives. *Genetic Engineering and Biotechnology Journal* 3: 1-20.

Skjemstad, J. O. and Baldock, J. A. (2006). Total and organic carbon. In Soon, Y. K., and Hendershot, W. H., (eds.), *Soil chemical analysis*. Taylor and Francis Groups, LLC, New York pp. 3-8.

Sorensen, S. J., Schyberg, T., Ronn, R. (1999) Predation by protozoa on Escherichia coli K12 in soil and transfer of resistance plasmid RP4 to indigenous bacteria in soil. *Appl Soil Ecol* 1999:11:79–90.

Tang, J. C., Wang, R. G., Niu, X. W., Wang, M., Chu, H. R., and Zhou, Q. X. (2010). Characterization of petroleum-contaminated soil: effect of different influencing factors. *Biogeoscience* 7: 3961-73969.

Totora, G. J., Funke, B. R. and Case, C. L. (2002). *Microbiology, an introduction to Media Update*. Daryl Fox, USA.

Suja, F., Rahim, F., Taha, M. R., Hambali, N., Razali, M. R., Khalid, A., Hamzah, A. (2014). Effects of local microbial bioaugmentation and biostimulation on the bioremediation of total petroleum hydrocarbons (TPH) in crude oil contaminated soil based on laboratory and field observations. *International Biodeterioration and Biodegradation* 90(2014):115-122. http://dx.doi.org/10.1016/j.ibiod.2014.03.006.

Tyagi, M., da Fronseca, M. M., de Carvalho, C.C. (2010). Bioaugmentation and biostimulation strategies to improve the effectiveness of bioremediation processes. *Biodegradation* 22(2): 231–241.

Ubogu, M., Odokuma, L. O. and Akponah, E. (2018). Growth enhancement of *Phragmites australis*, *Eichhornia crassipes* and *Saccharum officinarum* for rhizoremediation of crude oil contaminated soils. *International Journal Environment* 7(1): 60-84.

Ubogu, M., Odokuma, L.O and Akponah, E. (2019). Enhanced rhizoremediation of crude oil contaminated mangrove swamp soil using two aquatic macrophytes (*Phragmitis australis* and *Eichornia crassipes*). *Brazilian Journal of Microbiology*. DOI 10.1007/s42770-019-00077-3. ISSN 1517-8382.

Ueno, A., Ito, Y., Yumoto, I., Okuyama, H. (2007). Isolation and characterization of bacteria from soil contaminated with diesel oil and the possible use of these in autochthonous bioaugmentation. *World J Microbiol Biotechnol* 23:1739 - 1745.

United State Environmental Protection Agency (US EPA) (2007). Method 8015C: Nonhalogenated organics using GC/FID. Washington, US EPA.

van Reeuwijk, L. P. (2002). *Procedures for soil analysis: Technical paper 9.* International Soil Reference and Information Centre, Netherlands.

Venosa, A. D., King, D. W. and Sorial, G. A. (2002). The baffled flask test for dispersant effectiveness: a round Robin evaluation of reproducibility and repeatability. *Spill Science and Technology Bulletin* 7(5-6):299 - 308.

Vos, P. D., Garrity, G. M., Jones, D., Krieg, N. R, Ludwig, W., Rainey, F. A., Schleifer, K. H., and Whitman, W. B. (2009). *Bergey's Manual of Systematic Bacteriology: The Firmicutes*. 2nd edn., Volume 3. Sringer, New York, USA.

Whiteman, W., Goodfellow, M., Kampfer, P., Busse, H.-J., Trujillo, M., Ludwig, W., Suzuki, K. I., Parte, A. (2012). The Actinobacteria. *Bergey's Manual of Systematic Bacteriology*, Volume 5. Sringer, New York, USA.

Wu, M., Li, W., Dick, W. A. and Ye, X. (2017). Bioremediation of hydrocarbon degradation in a petroleum contaminated soil and microbial population and activity determination. *Chemosphere* 169:124–130.DOI:10.1016/j.chemosphere.2016.11.059

Zand, A. D., Khodaei, H. R., Nabibidhend, G. R., Mehrdadi, N. (2011). Rhizoremediation of total petroleum hydrocarbons (TPHS) under the effect of plant species in Iran. Proceedings of the 12th International Conference of Enviromental Science and Technology. Rhodes, Greece, 8-10 September 2011.

Zawierucha, I. and Malina, G. (2011). Bioremediation of Contaminated Soils: Effects of Bioaugmentation and Biostimulation on Enhancing Biodegradation of Oil Hydrocarbons. In A. Singh et al. (eds.), Bioaugmentation, Biostimulation and Biocontrol. *Soil Biology* 28: 187-201.

Zhou, Q. X., Sun, F. H. and Liu, R. (2005). Joint chemical flushing of soils contaminated with petroleum hydrocarbons. *Environmental International* 31: 835–839.

Ziołkowska, A., Wyszkowski, M. (2010). Toxicity of petroleum substances to microorganisms and plants. *Ecological Chemistry and Engineering* 17(1): 73-82.