KINETICS OF BIODEGRADATION OF TOTAL PETROLEUM HYDROCARBON IN DIESEL CONTAMINATED SOIL AS MEDIATED BY ORGANIC AND INORGANIC NUTRIENTS

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ABSTRACT

In this study, the kinetics of biodegradation of total petroleum hydrocarbon (TPH) in diesel contaminated soil as mediated by organic [cow dung (CD), poultry waste (PW), and pig dung (PD)] and inorganic (ammonium chloride, trypton X and sodium nitrate) nutrients was determined. Spread plating method was used to quantify the bacterial and fungal communities and their identification done using macroscopic, biochemical and microscopic techniques. The kinetics of biodegradation study involves TPH determination using gravimetric method and first order kinetic models. The results revealed the presence of bacterial and fungal species namely: Pseudomonas sp., Aeromonas spp., Corynebacterium sp., Serratia spp., Citrobacter spp., Rhizopus sp., Aspergillus spp., Fusarium spp., Geotrichium sp., Sacharomyces sp. and Mucor sp. Samples amended with organic wastes showed higher increase in all the microbiological parameters while the inorganic wastes has a lower count in all the parameters with only Trypton X trying to compare favourably with the organic waste (CD) in the treatment Groups. The result of the TPH showed significant percentage reductions of TPH content in all the treatments with PD and PD having the highest and lowest reductions of 92.56 % (3, 541.08 mg/kg) and 92.17 % (3, 729.95 mg/kg) in comparison with the unamended sample (control) which showed significantly reduction of 58.00 % (20, 000 mg/kg). Thus, the technology used in this study offers a promising, better, cheaper and more eco-friendly system that if adequately applied in diesel polluted soil will lead to non-toxic environment for plant, animal and general public.

Keywords: Bacteria, Biostimulation, Diesel pollution, Biodegradation kinetics, Organic and inorganic nutrients

INTRODUCTION

In Nigeria, the incidence of recorded environmental pollution due to high rate of petroleum related activities has been associated with frequent oil spills, especially through oil well blow outs, tanker accidents and accidental damage or vandalization of oil pipelines. These mishaps result in the release of crude oil and refined petroleum products into terrestrial and aquatic environments (Chikere *et al*., 2012). Decontamination and clean-up of hydrocarbon polluted sites is of paramount importance because of the environmental degradation and health risk associated with this form of pollutants (Chikere *et al*., 2012).

Bioremediation is the act of reduction, removal, transformation or degradation of pollutants or contaminants to less harmful substances through biological means (Lynch and Moffat, 2005; Calvo *et al.,* 2009). Bioremediation technology has been accepted as a cost-effective and environmental friendly means of clean-up of contaminated lands (Lynch and Moffat, 2005; Calvo *et al.,* 2009). The use of biological process to degrade contaminating substances was initially developed to treat contamination of petroleum hydrocarbons (Juwarkar *et al.,* 2010). Today, there are more than 70 known genera of oil-degrading microorganisms each capable of breaking down a specific group of molecules. They include bacteria such as *Achromobacter, Acinetobacter, Bacillus* and fungus like *Allescheria, Aspergillus, Candida* and many others which are widely distributed in the soil (Joo *et al.,* 2008; Rufino *et al.,* 2013). The oil-degrading microorganisms are either found naturally in the contaminated soils mostly due to situation of chronic contamination and may require only biostimulation to encourage active growth of degraders as the case with Exxon Valdez oil clean up (Lindstrom *et al.,* 1991). Others may need additional bioattenuation to enrich the overall capabilities of the degrading community of microorganisms as have been reported successful in several studies (Joo *et al*., 2008; Xu and Lu, 2010; Zhang *et al.,* 2010).

Biostimulation using inorganic fertilizer or nutrient has been extensively employed worldwide in reclaiming oil polluted soil (Emami *et al*., 2014; Ezekoye *et al*., 2015). Most laboratory studies have shown that the addition of limiting nutrients likes nitrogen and phosphorous has enhanced the rate of oil biodegradation (Agarry *et al*., 2010). The use of organic nutrients such as chicken droppings, periwinkle shells, pig manure, cow dung for the bioremediation of crude oil polluted environments other than mangrove swamps have been previously reported in Nigeria (Ijah and Antai, 2003; Obire *et al*., 2008). Extensive research is crucial in order to identify potential organic and inorganic fertilizers needful in the remediation of hydrocarbon-polluted soil ecosystem. Several studies have been conducted on the utilization of organic and inorganic nutrients for the bioremediation of diesel contaminated soil. There are dearth of literatures regarding the kinetics of biodegradation and reduction of diesel contaminated soil enhanced by organic and inorganic nutrients and thereby prompted the thrust of this research. This study was undertaken to determine the kinetics of biodegradation of TPH in diesel contaminated soil as mediated by organic and inorganic nutrients with a view of comparing the effectiveness of both nutrient supplements.

MATERIALS AND METHODS

Sample Collection: The composite soil sample used in this study was collected from a fallow plot close to the generator's house of Chukwuemeka Odumegwu Ojukwu University Uli, Ihiala Local Government Area, Anambra State, aseptically with a sterile hand trowel into clean plastic buckets (Eziuzor and Okpokwasili, 2009). The soil was collected at depths of 0 – 15 cm and transported to Microbiology Laboratory of Chukwuemeka Odumegwu Ojukwu University for the bioremediation studies. Co-ordinates of the soil sampled point was determined using handheld Global Positioning System (GPS) (GPSMAP 76SC). The diesel oil was obtained from a commercial petrol station at Uli, Anambra State, Nigeria.

Biostimulating Agents: Cow dungs were collected from an abattoir at Odumodu Market, Umunya. Poultry droppings were collected from Okoye Farm located at Nkpor, while the pig dungs were collected from different animal farms in Uli all in Anambra State, Nigeria. Cow dungs, poultry droppings and pig dungs (200 g each) were air-dried for 7 days and grounded into semi-fine particles using a sterile mortar and pestle. The inorganic nutrients: ammonium chloride (NH_4Cl) and sodium nitrate ($NaNO_3$) were products of Guangdong, China while Triton X was product of Burgoyne, India. Physicochemical and microbiological analyses were carried out and recorded before the bioremediation study.

Baseline Study: 1,500 g of soil in a bucket was contaminated with 150 mL of diesel and homogenously mixed. The unpolluted and the polluted soils were analysed for phytochemical, microbial and petroleum hydrocarbon compositions to serve as the baseline (self-control) thus establishing a standard (Ezekoye *et al*. 2015).

Experimental Design: The experiment was laid in a complete randomized design of seven treatments replicated thrice. The design was adopted from Eziuzor and Okpokwasili (2009) as shown in Table 1.

Table 1: Experimental design[1] for bioremediation of diesel polluted soil amended with organic and inorganic nutrients

Experimental set	Test experiment
Group I	1,500 g of soil + 150 mL of diesel (control)
Group II	1,500 g of soil + 150 mL of diesel + 50 g of cow dung
Group III	1,500 g of soil + 150 mL of diesel + 50 g of poultry dropping
Group IV	1,500 g of soil + 150 mL of diesel + 50 g of pig dung
Group V	1,500 g of soil + 150 mL of diesel + 50 g of ammonium chloride
Group VI	1,500 g of soil + 150 mL of diesel + 50 g of trypton X
Group VII	1,500 g of soil + 150 mL of diesel + 50 g of sodium nitrate

[1]*each group was replicated thrice*

One thousand five hundred grams (1,500 g) of soil sample each was introduced into seven different plastic buckets labeled groups I to VII and contaminated with 150 mL of diesel oil, respectively. Group I served as control without any amendment. Diesel polluted soil in groups II, III and IV were amended each with 50 g of cow dung, poultry dropping and pig dung respectively, while diesel polluted soil in groups V, VI and VII were amended each with 50 g ammonium chloride (NH_4Cl), trypton X and sodium nitrate ($NaNO_3$) respectively (Ezekoye *et al*., 2015). The polluted soil samples containing nutrients and control were regularly turned using different sterile hand trowel as well as moistened with 20 mL of distilled water every 2 weeks. The assay for the phytochemical, microbial and petroleum hydrocarbon contents of each bucket was carried out at 14 days interval for 56 days (Romanus *et al.,* 2015).

Physiochemical Composition: Following the method stated by AOAC (2012), the pH, conductivity, temperature, moisture content, nitrate, phosphate and total organic carbon (TOC) were determined.

Microbiological Analysis

Total heterotrophic bacteria (THB) and total heterotrophic fungi (THF) counts: The spread plate technique on nutrient agar (NA) and potato dextrose agar (PDA) were used in the quantification of THB and THF at fourteen days (14 days) interval. One gram (1 g) each of the soil samples were used to carry out 10-fold serial dilutions in sterile test tubes containing 9 mL of distilled water and finally diluted to 10^{-5}. Zero point one milliliter (0.1 mL) of each dilution were pipetted, and inoculated onto sterile freshly prepared nutrient agar and potato dextrose agar in triplicates, respectively. A sterile glass rod was used to spread the inoculum over the media. The plates were incubated for 18 – 24 hours and 5 days for the bacteria and fungi at a temperature of 25 ± 2^{o}C after which the emerging colonies were counted. Colonies that formed during this incubation period were counted using this formula:

$$\frac{\text{Number of colonies x Dilution factor}}{\text{Amount used}}$$

Values were expressed as colony forming units per g (Cfu /g) (Chikere *et al*. 2009).

Total culturable hydrocarbon utilizing bacteria (TCHUB) and total culturable hydrocarbon utilizing fungi (TCHUF) counts: The modified method of Chikere and Chijioke-Osuji (2006) was used to determine the TCHUB and TCHUF on mineral salt agar containing: 0.04 g $MgSO_4$, $7H_2O$, 0.03 g KCl, 0.09 g KH_2PO_4, 0.04 g $NaNO_3$, 0.13 g K_2HPO_4

and 2.0 g NaCl in 15 g of agar powder, and 100 mL of distilled water amended with 0.01 g of Nystatin and 0.1 mL of tartaric acid to inhibit the growth of fungi and bacteria. After sterilization and solidification of the medium, at 121 °C and 15 psi for 15 minutes and 45 °C, 0.1 mL of the inocula was aseptically pipetted and spread onto the surface of the plates using spreading rod. A sterile filter paper (Whatman No. 1) was impregnated with diesel and was aseptically placed on the cover of the Petri-dishes and covered. The plates were incubated by inversion for 7 – 10 days at 25 ± 2^{o}C. After incubation, colonies were counted and expressed as Cfu/g (Chikere *et al*. 2009).

Characterization of the Hydrocarbon Utilizing Bacterial and Fungal Isolates

Microscopic characterization: The method of Cheesbrough (2006) was adopted for the Gram staining and lactophenol cotton blue staining of all the bacterial and fungal isolates.

Biochemical characterization: The test kit (Analytical Profile Index, API) was used for the biochemical characterization of all the isolates following the method of Cheesbrough (2006).

Identification of the hydrocarbon bacterial and fungal isolates: Bergey's manual for determinative bacteriology (Holt *et al.,* 1994) was used for the identification of hydrocarbon bacterial and fungal isolates. The fungal isolates were identified using the most representative and typical keys in fungal identification by matching their colonial and microscopic observation with those of recognized taxa as defined by Chukwura *et al.* (2005), Samson and Varga (2007) and Oyeleke and Manga (2008).

Biostimulation and Kinetic Study

Total petroleum hydrocarbon (TPH): The modified method of Adesodun and Mbagwu (2008) was adopted. Diesel polluted soil (5 g) was suspended in 25 mL of hexane and shaken for 20 minutes using a mechanical shaker. The solution was filtered using a Whatman filter paper and the filtrate diluted by taking 1 mL of the extract into 50 mL of hexane. The absorbance of this solution was read at 460 nm with spectrophotometer using n-hexane as blank. The TPH was determined at 14 days interval for 56 days. The actual TPH (mg/kg) was deduced as follows: TPH = Instrument reading (Conc. obtained from calibration) x Volume of extract (mL) x DF ÷ Weight of sample (kg), while biodegradation percentage was calculated using the following formula:

$$\% \text{ Biodegradation} = \frac{THC_i - THC_r}{THC} \times 100$$

Where THC_i and THC_r are the initial and residual total hydrocarbon contents (THC), respectively.

Biostimulant efficiency: The evaluation of the efficiency of each organic and inorganic nutrients added to the diesel contaminated soil was evaluated by determining the net percentage loss and biostimulant efficiency based on each group. The percentage (%) biostimulant efficiency was calculated using the equation: % Biostimulant efficiency (BE) = Percentage loss in THC of diesel polluted soil amended with nutrients - % loss in THC of unamended polluted soil (control) (Agarry, 2013).

First order kinetics model and half-life of diesel biodegradation: The TPH data obtained in this study were substituted into the model as described by Agarry (2013), Agarry *et al.* (2013) and Omoni *et al.* (2015) thus:

$$TPH_t = TPH_o e^{-kt}$$

Where TPH_o is the initial TPH content in soil (mg/kg), TPH_t is the residual TPH content in soil (mg/kg) at time t, k is the biodegradation rate constant (day^{-1}) and t is time (day). The model estimated the biological half-life ($t_{1/2}$) as the time taken for half of its original amount to be converted. Half-life was evaluated using the model described by Agarry (2013) and Agarry *et al.* (2013) as follow:

$$\text{Half-life } (t_{1/2}) = \frac{\ln 2}{k}$$

Where k is the biodegradation rate constant (day^{-1}).

Statistical Analysis: The data in this study were subjected to analysis of variance (ANOVA) followed by Tukey's multiple comparison of means. Significant differences between means of treatments was accepted at 95 % ($p<0.05$) connivance limit. The data analysis was performed using GraphPad Prism version 7.00.

RESULTS

Physicochemical Characteristics: The result of the physicochemical characteristics of the soil sample before diesel contamination is presented in Table 2.

Table 2: Physicochemical characteristics of soil sample before diesel contamination

Parameter	Value
pH	7.40 ± 0.06
Conductivity (µS/cm)	500.00 ± 28.87
Temperature (°C)	27.10 ± 0.26
Moisture content (%)	20.01 ± 0.10
Nitrate (NO_3) (mg/kg)	2.00 ± 0.03
Phosphate (PO_4) (mg/kg)	1.80 ± 0.01
Total organic carbon (TOC) (%)	4.95 ± 0.01

From the results, the pH, conductivity, temperature, moisture content, nitrate, phosphate and total organic carbon were 7.40 ± 0.06, 500 ± 28.87 µS/cm, 27.10 ± 0.26 °C, 20.01 ± 0.10 %, 2.00 ± 0.03 mg/kg, 1.80 ± 0.01 mg/kg and 4.95 ± 0.01 %, respectively.

Physicochemical and Microbiological Characteristics of Biostimulating Agents: The results of the physicochemical and microbiological characteristics of animal nutrients used for biostimulation study are presented in Table 3. From the result, the pH, conductivity, temperature, moisture content, nitrate, phosphate, and total organic carbon were: 7.50 ± 0.25, 340.00 ± 12.13 µS/cm, 28.20 ± 0.82 °C, 40.01 ± 0.21 %, 4.00 ± 0.07 mg/kg, 2.20 ± 0.07 mg/kg, 6.95 ± 0.03 %; 7.00 ± 0.09, 280.00 ± 2.89 µS/cm, 29.00 ± 0.42 °C, 50.54 ± 0.51 %, 5.00 ± 0.09 mg/kg, 2.10 ± 0.06 mg/kg, 5.25 ± 0.07 % and 8.90 ± 0.38, 290.00 ± 26.46 µS/cm, 30.50 ± 0.63 °C, 62.00 ± 0.88 %, 4.00 ± 0.35 mg/kg, 2.80 ± 0.25 mg/kg and 3.83 ± 0.04 % for cow dung, poultry dropping and pig dung, respectively.

Table 3: Physicochemical and microbiological characteristics of nutrients from animal waste used for biostimulation of diesel polluted soil

Parameter	Cow dung	Poultry dropping	Pig dung
pH	7.50 ± 0.25	7.00 ± 0.09	8.90 ± 0.38
Conductivity (µS/cm)	340.00 ± 12.13	280.00 ± 2.89	290.00 ± 26.46
Temperature (°C)	28.20 ± 0.82	29.00 ± 0.42	30.50 ± 0.63
Moisture content (%)	40.01 ± 0.21	50.54 ± 0.51	62.00 ± 0.88
Nitrate (mg/kg)	4.00 ± 0.07	5.00 ± 0.09	4.00 ± 0.35
Phosphate (mg/kg)	2.20 ± 0.07	2.10 ± 0.06	2.80 ± 0.25
Total organic carbon (%)	6.95 ± 0.03	5.25 ± 0.07	3.83 ± 0.04

Baseline Characteristics of Diesel Contaminated Soil: The result of the baseline physicochemical and microbiological characteristics of the diesel contaminated soil is presented in Table 4.

Table 4: Baseline total hydrocarbon and microbiological characteristics of diesel contaminated soil

Parameter	Value
Total petroleum hydrocarbon (mg/kg)	47,619.05 ± 3.20
THB (LogCfu/g)	6.69 ± 0.01
THF (LogCfu/g)	6.53 ± 0.02
TCHUB (LogCfu/g)	6.51 ± 0.01
TCHUF (LogCfu/g)	6.49 ± 0.01

Key: THB = Total heterotrophic bacteria; THF = Total heterotrophic fungi; TCHUB = Total culturable hydrocarbon utilizing bacteria; TCHUF = Total culturable hydrocarbon utilizing fungi

From the results, the TPH content, THB, THF, TCHUB and TCHUF counts were 47, 619.05 ± 3.20 mg/kg, 6.69 ± 0.01 LogCfu/g, 6.53 ± 0.02 LogCfu/g, 6.51 ± 0.01 LogCfu/g and 6.49 ± 0.01 LogCfu/g, respectively.

Microbial Population of Diesel Contaminated Soil Treated with Organic and Inorganic Nutrients: The results of the THB count of the amended and unamended samples during the 56 days of study is shown in Figure 1. From the result, the pig dung had the highest count of 9.12 ± 0.03 LogCfu/g, while the ammonium chloride has the lowest count of 8.90 ± 0.06 LogCfu/g.

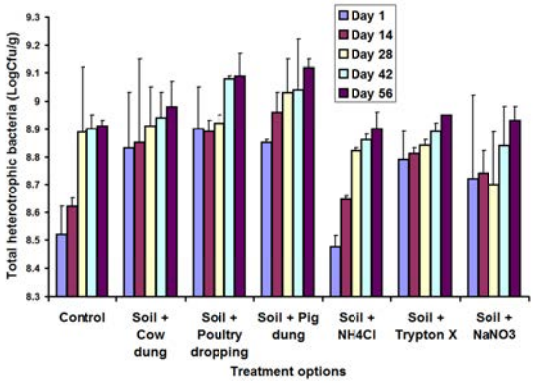

Figure 1: Total heterotrophic bacteria (THB) in amended and unamended soil samples during the 56 days bioremediation period of diesel contaminated soil

The result of the THF count of the amended and unamended samples during the 56 days of study is shown in Figure 2. From the result, the poultry dropping had the highest count of 8.93 ± 0.02 LogCfu/g, while the ammonium chloride had the lowest count of 8.78 ± 0.05 LogCfu/g.

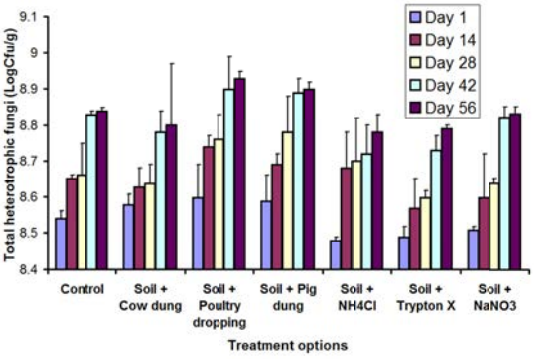

Figure 2: Total heterotrophic fungi (THF) in amended and unamended soil samples during the 56 days bioremediation period of diesel contaminated soil

The result of the TCHUB count of the amended and unamended samples during the 56 days of study is shown in Figure 3. From the result, the poultry dropping had highest count of 9.01 ± 0.08 LogCfu/g, while sodium nitrate ($NaNO_3$) had the lowest count of 8.85 ± 0.01 LogCfu/g compared with the control sample that ranged from 8.93 ± 0.09 LogCfu/g at day 1 to 8.87 ± 0.10 LogCfu/g at 56th day of the study.

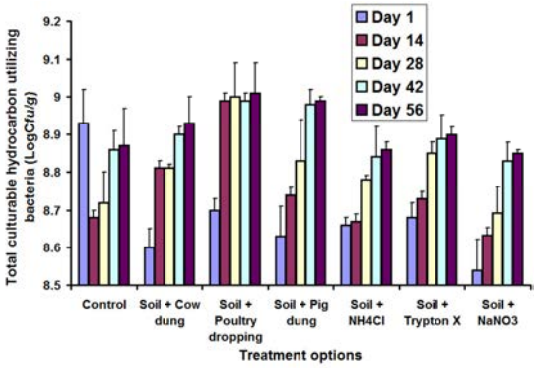

Figure 3: Total culturable hydrocarbon utilizing bacteria (TCHUB) in amended and unamended soil samples during the 56 days bioremediation period of diesel contaminated soil

The result of the TCHUF count of the amended and unamended samples during the 56 days of study is shown in Figure 4.

Figure 1: Total heterotrophic bacteria (THB) in amended

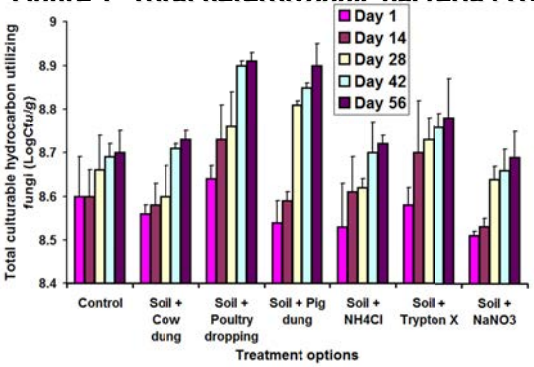

Figure 4: Total culturable hydrocarbon utilizing fungi (TCHUF) in amended and unamended samples during the 56 days bioremediation period of diesel contaminated soil

From the result, the sample amended with poultry dropping had the highest count of 8.91 ± 0.02 LogCfu/g, while the sample amended with sodium nitrate had the lowest count of 8.69 ± 0.06 LogCfu/g compared with the control sample that increased from 8.60 ± 0.09 LogCfu/g at day 1 to 8.70 ± 0.05 LogCfu/g at 56th day of the study. Statistically, there was significant difference ($p<0.05$) for all the microbiological populations in the six groups when compared with the control group.

Table 5: Characteristics of the hydrocarbon utilizing bacteria isolated from diesel contaminated soil amended with nutrients

Test	*Aeromonas* sp.	*Serratia fonticola*	*Pseudomonas* sp.	*Citrobacter freundii*	*Citrobacter diversus*	*Corynebacterium* sp.	*Serratia* sp.
Gram reaction	- Rods	- Rods	- Rods	- Rods	- Rods	+ Rods	- Rods
ONPG	**+**	**-**	+	**-**	**+**	**+**	+
ADH	+	+	-	-	+	-	-
LDC	+	+	+	+	+	+	+
ODC	+	+	-	+	+	+	+
Citrate	+	+	+	+	+	+	+
H_2S	-	-	-	+	+	-	-
Urease	-	-	-	-	-	-	-
TDA	+	+	-	+	-	+	+
Indole	-	-	-	-	+	-	-
VP	-	-	-	-	-	-	-
Gelatinase	+	+	+	+	-	+	+
Glucose	+	+	+	-	+	+	+
Mannitol	+	+	+	+	+	+	+
Inositol	-	-	+	-	+	-	-
Sorbitol	+	+	+	-	-	+	+
Rhamnose	+	+	+	-	+	+	+
Saccharose	+	+	+	-	+	+	-
Melbiose	+	+	+	-	+	+	+
Amigdalina	+	+	+	-	-	+	+
Arabinose	-	-	-	-	-	-	-
Nitrite	-	-	-	+	+	-	-
Nitrogen	+	+	+	+	+	+	+
Oxidase	+	-	+	-	-	-	-
Catalase	+	+	+	+	+	+	+

Key: + = Positive; - = Negative, ONPG = 2-nitrophenyl-B-D-galactopyranoside; ADH = Arginine dehydrolase; LDC =Lysine decarboxylase; ODC = Ornithine decarboxylase; H_2S = Hydrogen sulfide production; TDA= Tryptophan deaminase; VP = Voges-Proskauer Test

Characteristics of Bacterial and Fungal Isolates: The result of the phenotypic properties of the hydrocarbon utilizing bacterial isolates is presented in Table 5. From the results, most bacteria isolated were Gram negative rods, positive O-nitrophenyl, D-galactopyranoside arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate, tryptophan deaminase, gelatinase, glucose, mannitol, sorbitol, rhamnose, saccharose, melbiose, amigdalina, nitrogen but negative to urease, indole, Voges-Proskauer, inositol, arabinose, nitrite and oxidase to Gram reaction and biochemical tests.

The result of the identities of the hydrocarbon utilizing fungi isolates is presented in Table 6. From the result, most isolates were powdery, dark brown, flatty spread on the surface of the solid medium and brown reverse with septate and branched hyphae with conidia in chains as well as whitish and cottony vegetative mycelium with multi-segmented canoe-like spores with branched and segmented conidiophores.

Degradation and Kinetics: The result of the TPH content of the amended and unamended diesel polluted soil samples as well as percentage degradation of TPH in the amended and unamended diesel contaminated soil samples during the 56 days of study are shown in Figures 5 and 6. At the end of 56 days, the amended soil showed significant percentage reductions of TPH content in all the treatments with poultry dropping and pig dung (PD) having the highest and lowest reductions of 92.56 % (3,541.08 mg/kg) and 92.17 % (3,729.95 mg/kg) in comparison with the unamended

Table 6: Identities of hydrocarbon utilizing fungi isolated from diesel contaminated soil amended with nutrients

Isolates	Culture characteristics	Microscopic characteristics	Probable genera
HUF1	Pure white, thick and abundant cottony mycelium. Reverse was white	Non-septate sporangiophores, rhizoid, spongisphore and black sporangium containing hemispherical collumela	*Rhizopus* spp.
HUF2	Powdery, dark brown, flatty spread on the surface of the solid medium and brown reverse.	Septate and branched hyphae with conidia in chains	*Aspergillus* spp.
HUF3	Powdery, dark brown, flaty spread on the surface of the solid medium with brownish reverse	Septate and branched hyphae and conida in chains	*Aspergillus* spp.
HUF4	Whitish and cottony vegetative mycelium	Segmented canoe-like spores, with branched and segmented conidiophores	*Fusarium* spp.
HUF5	White colonial mycelium which developed within 4 days with extensive sub-surface	Coarse hyphae that segment into rectangular arthrospores varying in sizes	*Geotrichium* spp.
HUF6	White and cottony vegetative mycelium	Multi-segmented canoe-like spores with branched and segmented conidiophores	*Fusarium* spp.
HUF7	Creamy ovoid colonies which were easily picked	Spherical cells in clusters with buds	*Saccharomyces* spp.

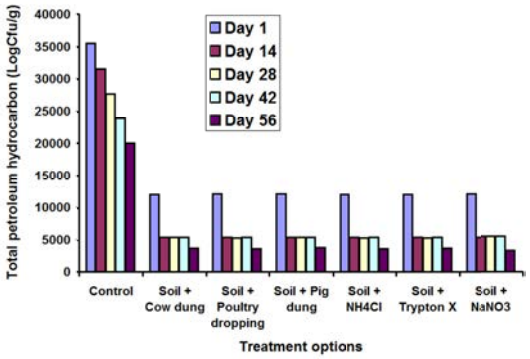

Figure 5: Total petroleum hydrocarbon (TPH) content of the amended and unamended samples during the 56 days bioremediation period of diesel contaminated soil

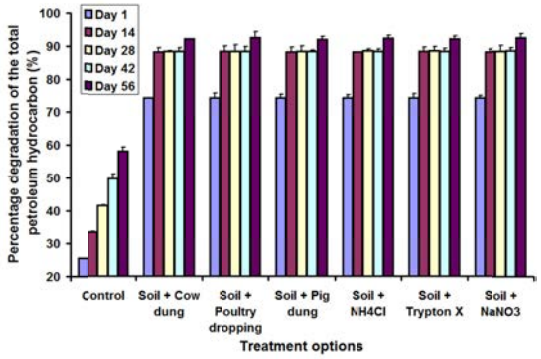

Figure 6: Percentage degradation of the total petroleum hydrocarbon (TPH) in amended and unamended samples during the 56 days bioremediation period of diesel contaminated soil

polluted soil sample (control) which had significantly reduction of 58.00 % (20,000 mg/kg). The result of the percentage net loss of TPH of the amended and unamended samples during the 56 days of study as well as the percentage (%) biostimulation efficiency of the amended and unamended samples during the 56 days of study are shown and presented in Figure 7.

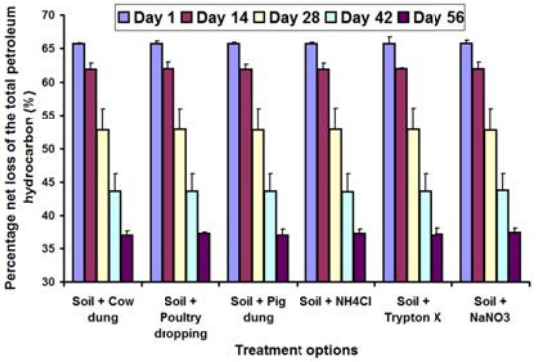

Figure 7: Percentage net loss of the total petroleum hydrocarbon in amended and unamended samples during the 56 days bioremediation period of diesel contaminated soil

From the results, PD had the highest net percentage loss of TPH (37.07 %), while the lowest net percentage loss of TPH (37.34 %) was observed in amendment with PW at the end of day 56. The result revealed higher

biostimulation efficiency (BE) in amendment with PD (20.93 %), while the lowest in PW (20.66 %) for the organic nutrients. The highest BE for the inorganic nutrient was observed in trypton X (20.87 %), and the lowest in NH_4Cl (20.68 %) and $NaNO_3$ (20.68 %) (Table 7).

Table 7: Percentage biostimulation efficiency of amended and unamended soil samples during the 56 days bioremediation period of diesel contaminated soil

Experimental Group	Percentage degradation (%)	Biostimulation Efficiency (%)
Control	58.00	66.89
Soil + Cow dung	92.26	20.87
Soil + Poultry dropping	92.56	20.66
Soil + Pig dung	92.17	20.93
Soil + NH_4Cl	92.53	20.68
Soil + Trypton X	92.26	20.87
Soil + $NaNO_3$	92.53	20.68

The result of the biodegradation rate constant (k) and half-life ($t_{1/2}$) of TPHs in amended and unamended polluted soil samples during the 56 days of study is presented in Table 8.

Table 8: Biodegradation rate constant (*k*) and half-life ($t_{1/2}$) of petroleum hydrocarbons in amended and unamended samples during the 56 days bioremediation period of diesel contaminated soil

Experimental Group	Biodegradation constant (*k*) (Day-1)	R2	Half-life ($t_{1/2}$) (Day)
Control	0.0067	0.9999	103.434
Soil + Cow dung	0.0198	0.7990	34.929
Soil + Poultry dropping	0.0202	0.6009	34.375
Soil + Pig dung	0.0198	0.9310	35.089
Soil + NH_4Cl	0.0201	0.8900	34.426
Soil + Trypton X	0.0198	0.7990	34.929
Soil + $NaNO_3$	0.0207	0.8700	33.527

From the results, $NaNO_3$ had the highest biodegradation constant of 0.0207 day^{-1} and the lowest half-life of 33.527 days, while the unamended soil (control) had the lowest biodegradation constant of 0.0067 day^{-1} and the highest half-life of 103.434 days.

DISCUSSION

The increase in the exploration and usage of petroleum products have resulted in wide spread contamination of the environment. This has led to concerted efforts in studying the possibility of detoxifying oil contaminants using organic and inorganic wastes (Romanus *et al.* 2015). In this study, the kinetics of biodegradation of TPH in diesel contaminated soil as mediated by organic and inorganic nutrients was evaluated. The study revealed that the soil is neutral to alkaline in pH, moderate in conductivity, low in nitrate, phosphate and total organic carbon contents, high in moisture content with moderate/mesophilic temperature. The amended nutrients were neutral to alkaline in pH, moderate to high in conductivities, high in moisture contents with moderate/mesophilic temperatures, low in nitrates, phosphates and total organic carbon contents. Several authors have implicated nitrogen and phosphorus content of these wastes in accelerating the bioremediation of oil contaminated soil ecosystem (Orji *et al.*, 2012; Ezekoye *et al.*, 2015; 2017). The result of the study also showed slightly alkaline in pH, high in conductivities, low in moisture contents with moderate/ mesophilic temperatures, low in nitrates, phosphates and total organic carbon contents, high quantities of TPH contents. There were significant counts of TCHUB and fungi than counts of total heterotrophic bacteria and fungi revealing the probable negative impact of the diesel on the natural soil microflora. The significant count of these microflora also revealed that they are moderately suitable for bioremediation and is in line with previous studies (Ebuehi *et al.,* 2005; Orji *et al.*, 2012; Ezekoye *et al.*, 2017).

The heterotrophic bacterial and fungal counts were found to be higher than the crude oil utilizing bacterial and fungal counts in all the samples at the 56th day of study which could be due to nutrient limitation in the culturing media for the diesel utilizers. The

responses of native hydrocarbon utilizing bacteria and fungi to the bioremediation treatments were largely positive with increasingly higher counts as time elapsed. The bacterial and fungal species exhibited ability to either degrade or utilize the petroleum hydrocarbon components as sole carbon sources; this was in agreement with the findings of Okolo *et al.* (2005) in their study on bioremediation of crude oil polluted sandy-loamy soil, in which they reported that poultry dropping was able to significantly sustain increase in the population of fungal organisms utilizing crude oil. The hydrocarbon utilizing bacterial and fungal isolates identified in this study were predominantly Gram negative bacteria and moulds. Gram negative bacteria and moulds have been widely implicated in the bioremediation of oil contaminated soils and the result of this study corroborated with findings of earlier studies (Chikere *et al.*, 2012; Akpe *et al*., 2013; Omoni *et al.*, 2015; Romanus *et al*, 2015).

The results clearly showed that natural attenuation occurs in the control at day 56 leading to more 50 % loss as a result of abiotic factors such as sorption and volatilization based on the nature of the soil. There was significant differences ($p<0.05$) detected between the treated soil groups and the untreated contaminated soil group revealing the progressive influence of the organic and inorganic nutrients to the biodegradation of diesel contaminated soil. The findings was similar to previous studies in which higher degradation percentage of petroleum hydrocarbons in oil contaminated soil were reported (Agamuthu *et al.*, 2013; Omoni *et al.*, 2015; Romanus *et al.*, 2015).

It is remarkable to note that both organic and inorganic nutrients were active in biostimulating the petroleum hydrocarbon degraders which eventually led to a decrease in petroleum hydrocarbon in the soil polluted with diesel. Previous studies by Abioye *et al*. (2009), Omoni *et al.* (2015) and Ezekoye *et al.* (2015; 2017) that examined the effects of these nutrients on oil contaminated soil had similar findings to the ones in this study. However, the observed decline in petroleum hydrocarbons in diesel polluted soil may not necessarily stem from nutrients enhanced biodegradation, but may be by other abiotic processes or factors such as dispersion, evaporation, adsorption among others (Agamuthu *et al.*, 2013; Onuoha, 2013).

The estimated data obtained from this study were fitted with the first order kinetic model in order to determine the rate and half-life of biodegradation. Higher rate of degradation with concomitant least half-life observed in contaminated soil treated with sodium nitrate may be as result of its high contents of nitrogenous source which were made available to the native microbial flora in the oil contaminated soil when compared to other treatment options. The result contradicts the findings of several researchers who reported that organic nutrients had the highest rate of biodegradation with least half-life in oil contaminated soil (Adesodun and Mbagwu, 2008; Agamuthu *et al.* 2013; Omoni *et al.* 2015).

Conclusion: The findings from this study revealed that bioremediation of diesel contaminated soil with the use of organic nutrients (cow dung, poultry dropping and pig dung) and inorganic nutrients (ammonium chloride, trypton X, sodium nitrate) as biostimulating agents led to faster elimination of petroleum hydrocarbon. Therefore, the addition of organic and inorganic nutrients efficiently and significantly boosted the degradation and loss of petroleum hydrocarbon in diesel contaminated soil with higher biodegradation constant and lower half-life. The technology used in this study offers a promising, better, cheaper and more eco-friendly system that if adequately applied in diesel polluted soil will lead to a harmless and non-toxic environment for plant, animal and general public.

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REFERENCES

ABIOYE, O. P., ABDUL AZIZ, A. and AGAMUTHU, P. (2009). Stimulated biodegradation of used lubricating oil in soil using organic wastes. *Malaysian Journal of Science,* 28(2): 127 – 133.

ADESODUN, J. K. and MBAGWU, J. S. C. (2008). Biodegradation of waste lubricating petroleum oil in a tropical alfisol as mediated by animal droppings. *Bioresource and Technology,* 99: 5659 – 5665.

AGAMUTHU, P., TAN, Y. S. and FAUZIAH, S. H. (2013). Bioremediation of hydrocarbon contaminated soil using selected organic wastes. *In:* 2013 International Symposium on Environmental Science and Technology (2013 ISEST). *Procedia Environmental Sciences*, 18: 694 – 702.

AGARRY, S. E. (2013). Application of carbon-nitrogen supplementation from plant and animal sources in in-situ soil bioremediation of diesel oil. *Journal of Environmental Earth Sciences*, 3(7): 1 – 13.

AGARRY, S. E., AREMU, M. O. and AWORANTI, O. A. (2013). Kinetic modeling and half-life study on enhanced soil bioremediation of bonny light crude oil amended with crop and animal-derived organic wastes. *Petroleum and Environmental Biotechnology,* 4(137): 1 – 11.

AGARRY, S. E., OWABOR, C. N. and YUSUF, R. O. (2010). Bioremediation of soil artificially contaminated with petroleum hydrocarbon oil mixtures: evaluation of the use of animal manure and chemical fertilizer. *Bioremediation Journal*, 14(4): 189 – 195.

AKPE, A. R.**,** EKUNDAYO, A. O. and ESUMEH, F. I. (2013). Degradation of crude oil by bacteria: a role for plasmid-borne genes. *Global Journal of Science Frontier Research in Biological Science,* 13(6): 21 – 26.

AOAC (2012). *Official Method of Analysis*. 19th Edition. Association of Official Analytical Chemists (AOAC), Washington DC, USA.

CALVO, C., MANZANERA, M., SILVA-CASTRO, G. A., UAD, I. and GONZÁLEZ-LÓPEZ, J. (2009). Application of bioemulsifiers in soil oil bioremediation processes: future prospects. *Science of the Total Environment,* 407(12): 3634 – 3640.

CHEESBROUGH, M. (2006). *District Laboratory Practice in Tropical Countries, Part 2.* 2nd Edition, Cambridge University Press, New York, USA.

CHIKERE, B. O. and CHIJIOKE-OSUJI, C. C. (2006). Microbial diversity and physiochemical properties of a crude oil-polluted soil. *Nigerian Journal of Microbiology* 20: 1039 – 1046.

CHIKERE, C. B., CHIKERE, B. O. and OKPOKWASILI, G. C. (2012). Bioreactor-based bioremediation of hydrocarbon polluted Niger Delta marine sediment. *Niger Biotechnology,* 2 (1): 53–66.

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

CHUKWURA, E. I., NWOKOLO, C. I. and NWACHUKWU, S. C. U. (2005). Bioremediation of crude oil polluted Escravos River using *Candida utilis*. *Nigerian Journal of Microbiology,* 19(1-2): 623 – 630.

EBUEHI, O. A. T., ABIBO, I. B., SHEKWOLO, P. D., SIGISMUND, K. I., ADOKI, A. and OKORO, I. C. (2005). Remediation of crude oil contaminated soil by enhanced natural attenuation technique. *Journal of Applied Environmental Management,* 9(1): 103 – 106.

EMAMI, S., POURBABAEI, A. A. and ALIKHANI, H. A. (2014). Interactive effect of nitrogen fertilizer and hydrocarbon pollution on soil biological indicators. *Springer Environmental Earth Science,* 72(9): 3513 – 3519.

EZEKOYE, C. C., AMAKOROMO, E. R. and IBIENE, A. A. (2015). Bioremediation of hydrocarbon polluted mangrove swamp soil from the Niger Delta using organic

and inorganic nutrients. *British Journal of Biotechnology,* 6(2): 62 – 78.

EZEKOYE, C. C., AMAKOROMO, E. R. and IBIENE, A. A. (2017). Laboratory-based bioremediation of hydrocarbon polluted mangrove swamp soil in the Niger Delta using poultry wastes. *Microbiology Research Journal International*, 19(2): 1 – 14.

EZIUZOR, C. S. and OKPOKWASILI, G. C. (2009). Bioremediation of hydrocarbon contaminated mangrove soil in a bioreactor. *Nigerian Journal of Microbiology,* 23(1): 1777 – 1791.

HOLT, J. G., KREIG, N. R., SNEATH, P. H. A., STALEY, J. T. and WILLIAMS, S. T. (1994). *Bergey's Manual of Determinative Bacteriology*. 9th Edition, Williams and Wilkins, Baltimore, Maryland, USA.

IJAH, U. J. J. and ANTAI, S. P. (2003). The potential use of chicken-drop micro-organisms for oil spill remediation. *The Environmentalist,* 23(1): 89 – 95.

JOO, H., NDEGWA, P. M., SHODA, M. and PHAE, C. (2008). Bioremediation of oil-contaminated soil using *Candida catenulata* and food waste. *Environmental Pollution,* 156(3): 891 – 896.

JUWARKAR, A. A., SINGH, S. K. and MUDHOO, A. (2010). A comprehensive overview of elements in bioremediation. *Reviews in Environmental Science and Biotechnology,* 9(3):215 – 288.

LINDSTROM, J. E., PRINCE, R. C., CLARK, J. C., GROSSMAN, M. J., YEAGER, T. R., BRADDOCK, J. F. and BROWN, E. J. (1991). Microbial populations and hydrocarbon biodegradation potentials in fertilized shoreline sediments affected by the T/V Exxon Valdez oil spill. *Applied and Environmental Microbiology,* 57(9): 2514 – 2522.

LYNCH, J. M. and MOFFAT, A. J. (2005). Bioremediation prospects for the future application of innovative applied biological research. *Annals of Applied Biology,* 146(2): 217 – 221.

OBIRE, O., ANYANWU, E. E. and OKIGBO, R. N. (2008). Saprophytic and crude oil degrading fungi from cow dung and poultry droppings as bioremediation agents. *Journal of Agricultural Technology*, 4(92): 81 – 89.

OKOLO, J. C., AMADI, E. N. and ODU, C. T. I. (2005). Effects of soil treatments containing poultry manure in crude oil degradation in a sandy loam soil. *Applied Ecology and Environment,* 3(1): 4753.

OMONI, V. T., AGUORU, C. U., EDOH, E. O. and MAKINDE, O. (2015). Biostimulation of hydrocarbon utilizing bacteria in soil contaminated with spent engine oil using banana and plantain agro-wastes. *Journal of Soil Science and Environmental Management,* 6(8): 225 – 233.

ONUOHA, S. C. (2013). Stimulated biodegradation of spent lubricating motor oil in soil amended with animal droppings. *Journal of National Integrated Sciences*, 3(12): 106 – 116.

ORJI, F. A., ABIYE, A. I. and EKAETTE, N. D. (2012). Laboratory scale bioremediation of petroleum hydrocarbon-polluted mangrove swamps in the Niger Delta using cow dung. *Malaysian Journal of Microbiology*, 8 (4): 219 – 228.

OYELEKE, S. B. and MANGA, S. B. (2008). *Essentials of Laboratory Practicals in Microbiology*. Tobest Publisher, Minna, Nigeria.

ROMANUS, A. A., OMOLOLA, E. A., PATRICK, A. S. and IFEOMA, O. G. (2015). Bacterial degradation of petroleum hydrocarbons in crude oil polluted soil amended with cassava peels. *American Journal of Research Communication*, 3(7): 99 – 118.

RUFINO, R. D., LUNA, J. M., MARINHO, P. H. C., FARIAS, C. B. B., FERREIRA, S. R. M. and SARUBBO, L. A. (2013). Removal of petroleum derivative adsorbed to soil by biosurfactant Rufisan produced by *Candida lipolytica*. *Journal of Petroleum Science and Engineering,* 109: 117 – 122.

SAMSON, R. A. and VARGA, J. (2007). *Aspergillus Systematics in the Genomic Era*. Commission for Biological Studies

(CBS), Fungal Biodiversity Centre, Utrecht.

XU, Y. and LU, M. (2010). Bioremediation of crude oil-contaminated soil: comparison of different biostimulation and bioaugmentation treatments. *Journal of Hazardous Materials*, 183(1–3): 395 – 401.

ZHANG, Z., GAI, L., HOU, Z., YANG, C., MA, C., WANG, Z., SUN, B., HE, X., TANG, H and XU, P. (2010). Characterization and biotechnological potential of petroleum-degrading bacteria isolated from oil-contaminated soils. *Bioresources and Technology*, 101: 8452 – 8456.