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MICROBIAL DEGRADATION OF AN OIL POLLUTED SITE IN ABULE-EGBA, NIGERIA

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ABSTRACT

A pilot study was carried out on soil collected from Abule-Egba oil-pipeline in an area where pipeline vandalization was common to determine the effect of pig dung on microbiological composition and total petroleum hydrocarbon degradation. Top soil (0-15 cm depth) samples were randomly collected and one kilogram of the gasoline polluted-soil was measured into each of nine plastic containers. Pig dung was mixed with the soil at the rate of 0, 50 and 100 g kg^{-1} soil in triplicate and the containers were arranged in a completely randomized design. Soil samples were taken from each container at 21 and 42 days for hydrocarbon utilizing bacteria and total petroleum hydrocarbon determination using standard methods. Data were subjected to descriptive and inferential statistics. The species identified were *Bacillus, Staphylococcus, Escherichia, Pseudomonas and Enterobacter*. The total petroleum hydrocarbon (mg kg^{-1}) of the soil before pig dung application was 6.39 ± 0.11. After the amendments (at 0, 50 and 100 g kg^{-1}), the total petroleum hydrocarbon (mg kg^{-1}) values were 3.25 ± 0.17, 0.03 ± 0.01, 0.03 ± 0.01 and 1.58 ± 0.10, 0.03 ± 0.01, 0.03 ± 0.01 for 21 and 42 days respectively. Pig dung significantly enhanced the biodegradation process as an impressive 99% remediation efficiency was achieved 21 days after amendment.

Keywords: Degradation, Hydrocarbon, Pig dung, Polluted soil

INTRODUCTION

Environmental pollution with crude oil and petroleum products has become a serious problem in Nigeria (Alexander, 2000). Most components of oil are toxic to human and wildlife because it's not only incorporated into the food chain, but also have a direct impact on species and habitats, which may set off a cascade of perturbations that affect the entire food chain. For this reason, scientists have focused their interest on examining the distribution, fate and behaviour of oil and its derivatives in the environment (Alexander, 2000; Semple *et al*., 2001).

The uncontrolled release of petroleum and its derivative products into soils and groundwater has become a significant problem globally, especially in Nigeria. A number of technologies have been tested to remediate polluted sites. Commonly used methods for soil remediation include mechanical, burying, evaporation, dispersion, and soil washing (Das and Chandra, 2011). Research has shown that these methods are capital intensive and result to partial degradation of contaminants (Das and Chandra, 2011). It has been proposed that bioremediation of petroleum hydrocarbons is an effective, economic, and environmentally friendly technology (Mulligan *et al*., 2001; Bundy *et al*., 2002; Bento *et al*., 2005; Joo *et al*., 2008; Gallego *et al*., 2010). Amendment of soil with organic or inorganic nitrogen-rich nutrients in a process known as biostimulation is an effective strategy to enhance the biodegradation process (Margesin *et al*., 2007). The potential use of organic wastes derived from plants and animals have been investigated by few researchers. Such wastes include rice husk and coconut shell (Nyankanga *et al*., 2012), plantain peels and cocoa pod husk (Agbor *et al*., 2012), *Moringa oleifera* and soya beans (Danjuma *et al*., 2012) and animal organic wastes like cow dung, pig dung, poultry manure and goat dung (Yakubu, 2007; Adesodun and Mbagwu, 2008; Agarry *et al*., 2010; Agarry and Ogunleye, 2012) as biostimulation strategies for petroleum hydrocarbon biodegradation in polluted environments.

However, cost effective methods and environmentally friendly strategies of enhancing

petroleum hydrocarbon biodegradation in soil necessitated this study. Since oil pipeline network is widespread across almost all the agro-ecological zones in Nigeria, oil spillages are no longer restricted to the oil producing zones. Incidences of crude oil spills at non-oil producing zones, and the consequent contamination of terrestrial ecosystems abound (Jidere and Akamigbo, 2009). Oil spills have degraded most arable lands and have turned hitherto productive areas into wastelands. With increasing soil infertility due to the destruction of soil micro-organisms, and dwindling agricultural productivity, farmers have been forced to abandon their land, to seek non-existent alternative means of livelihood.

In a study on the effect of oil spill on crop production, Chindah and Braide (2000) reported that oil spill on crops caused great damage to the plant community due to high retention time of oil occasioned by limited flow. In fact, oiled shoots of crops like pepper and tomatoes may wilt and die off due to blockage of stomata thereby inhibiting photosynthesis, transpiration and respiration. Germination, growth performance and yield of these crops are stifled by oil spillage (Odjuvwuederhie *et al*, 2006). Therefore, the objectives of this study are to assess effect of pig dung on the soil chemical properties and degradation of total petroleum hydrocarbon in soil polluted with gasoline.

MATERIALS AND METHODS

Samples Collection, Preparation and Experimental Design

Pig dung was collected from Piggery Unit, Teaching and Research Farm, Federal University of Agriculture Abeokuta (FUNAAB), Nigeria. The manure was air dried, ground, mixed, sieved with a 2 mm sieve and stored in polythene bag.

Top soil (0-15 cm depth) was collected from Abule-Egba oil-pipeline in an area where pipeline vandalization was common (Latitude 6^{o} $39^{'}$ $4.6714^{''}$ N and Longitude 3^{o} $71^{'}$ $59.834^{''}$ E) in Lagos State, Nigeria using a soil auger. The soil was air dried in a clean, well ventilated laboratory, homogenized by crushing and sieved by passing through a 2 mm mesh sieve. One kilogram of soil was measured into nine clean dry containers of three litres each

Pig dung was applied at the rate of 0 (control), 50 and 100 g kg^{-1} soil in triplicate. The pig dung was thoroughly mixed with the soil and the nine containers were arranged in a Completely Randomized Design in a greenhouse. Soil samples were taken from each container at 0 (zero day refers to the day pig dung was added to the soil), 21 and 42 days for pH, organic carbon, nitrogen, phosphorus, potassium, hydrocarbon degrading bacteria count, hydrocarbon utilizing bacteria and total petroleum hydrocarbon determination.

Laboratory Analysis

Soil Chemical Properties

The pH, organic carbon, total nitrogen, potassium and available phosphorus were determined in the soil samples using the methods described in Chopra and Kanwar (2011).

Cultural Characterization of Bacteria

Pure cultures of representative bacteria colonies were randomly picked from inoculated plates and were grouped on the basis of their colonial characteristics such as colony elevation, colour, size, opacity, shape, consistency, and edge (Barnett and Hunter, 1985).

Morphological Characterization of bacteria

Cultural grouping was followed by microscopic examination of isolates for cellular morphology. Day-old cultures of the bacteria isolates were stained with cotton blue lacto-phenol blue and observed microscopically for cell shape, size and sporulation (Barnett and Hunter, 1985).

Biochemical Characterization of Bacteria

A modified method of Cheesbrough (2006) was used for Gram staining, catalase test, urease test, citrate utilization test, indole test, motility test, coagulase test and sugar fermentation test.

Determination of Total Hydrocarbon Utilizing Bacteria Count

Total hydrocarbon utilizing bacteria count was carried out on mineral salt medium (MSM) agar as described by Balogun and Fagade (2010); and the isolated microorganisms were identified using Bergey's manual of systemic bacteriology (Krieg and Holt, 1984).

Determination of Total Petroleum Hydrocarbon

Ten grams of the petroleum products-polluted soil sample was weighed into a clean bottle and 25 ml of dichloromethane was added, the mixture was allowed to stand on a mechanical shaker for a period of 3- 4 hours. The procedure was repeated twice and the aliquots were collected and mixed together in a beaker. The aliquots were concentrated on a steam bath reducing the extracts to about 5 ml. The concentrate was passed through a pipette packed with anhydrous sodium sulphate on top of a glass wool to remove moisture and other impurities. The final extract was analysed using a Hewlett-Packard 5890 series GC system coupled to a mass spectrophotometer (VG TRIO 2000) to determine the quantity of total petroleum hydrocarbons.

The degradation of petroleum products was expressed as the percentage of petroleum products degraded in relation to the amount of the remaining fractions in the appropriate abiotic control samples (Equation 1). The biodegradation efficiency (BE) based on the decrease in the total concentration of hydrocarbons, was calculated using Equation 1 (Mohan *et al*., 2006).

$$BE = 100 - \left(\frac{A_s \times 100}{A_{ac}}\right) \qquad \text{Equation 1}$$

Where A_s = total area of peaks in each sample, A_{ac} = total area of peaks in the appropriate abiotic control and efficiency BE (%) = biodegradation

Statistical Analysis

Data obtained were subjected to descriptive (mean and standard deviation) and inferential (ANOVA) statistics. Means were separated using Duncan Multiple Range Test (DMRT).

RESULTS

Chemical Properties of Soils and Pig Dung

The value of soil pH, total nitrogen (N), available phosphorous (P), exchangeable potassium (K), organic carbon (OC) and total petroleum hydrocarbon (TPH) determined before pig dung application were 7.2 ± 0.10, 1.80 ± 0.04 g kg^{-1}, 38.76 ± 1.20 mg kg^{-1}, 0.38 ± 0.11 Cmol kg^{-1}, 61.18 ± 0.32 g kg^{-1} and 6.39 ± 0.11 mg kg^{1} respectively (Table 1).

Table 1: Chemical Properties of Abule-Egba Soil without Pig Dung before Experiment

Parameters	Value
pH	7.2±0.10
Nitrogen (g kg^{-1})	1.80±0.04
Available phosphorus (mg kg^{-1})	38.76±1.20
Exchangeable potassium (Cmol kg^{-1})	0.38±0.11
Organic Carbon (g kg^{-1})	61.18±0.32
THDB (CFU g^{-1})	1.54 x 104±0.5 x 10^4
TPH (mg kg^{-1})	6.39±0.11

THDB = Total hydrocarbon degrading bacteria
TPH = Total petroleum hydrocarbon

The pig dung was high in organic carbon (51.00 ± 0.56 g kg^{-1}) and also contained total hydrocarbon-degrading bacteria of 8.0 × 10^3 ± 1.24 × 10^3 CFU g^{-1} while the total petroleum hydrocarbon was below detection limit (Table 2).

Table 2: Proximate Analysis of the Pig Dung

Parameters	Value
pH	7.9±1.23
Nitrogen (g kg^{-1})	16.4±3.21
Phosphorus (mg kg^{-1})	1.25±0.36
Potassium (Cmol kg^{-1})	0.28±0.03
Organic Carbon (g kg^{-1})	51.0±4.63
Moisture Content (%)	92.2±6.42
THDB (CFU g-^{1})	8.0 x 10^{3}±1.22x10^{3}
TPH (mg kg^{-1})	BDL

THDB = Total hydrocarbon degrading bacteria
TPH = Total petroleum hydrocarbon
BDL = Below detection limit

Effect of Pig Dung Application on the Soil Chemical Properties

Application of pig dung significantly ($p < 0.05$) increased pH of the contaminated soil compared to the control (without pig dung application) at 21 and 42 days (Table 3). The pH of soil before pig dung application was 7.90 ± 0.10 while control, 50 and 100 g of pig dung were 6.8 ± 0.06 and 6.7 ± 0.20; 7.3 ± 0.10 and 7.2 ± 0.06; 7.4 ± 0.15 and 7.2 ± 0.06 for day 21 and 42 respectively indicating a downward trend. Total N of the soil (g kg^{-1}) before pig dung application was 1.92 while control, 50 and 100 g of pig dung were 0.86 ± 0.03 and 0.72 ± 0.06, 1.41 ± 0.07 and 1.13 ± 0.08, 1.94 ± 0.08 and 1.57 ± 0.09 for 21 and 42 days respectively indicating a downward trend for the experimental. Significantly ($p < 0.05$) lower N, P, K and organic carbon were recorded in 50 and 100 g pig dung kg^{-1} soil at 21 and 42 days respectively.

Table 3: Effects of Pig Dung Amendment on the Soil Chemical Properties

Pig dung level (g)	Days after amendment	pH	Nitrogen (g kg^{-1})	Phosphorus (mg kg^{-1})	Potassium (Cmol kg^{-1})	Organic carbon (g kg^{-1})
0	21	6.8 ± 0.06^{c}	0.86 ± 0.03^{f}	77.32 ± 1.17^{e}	0.36 ± 0.02^{d}	48.07 ± 0.05^{c}
	42	6.7 ± 0.20^{c}	0.72 ± 0.06^{f}	64.92 ± 2.46^{f}	0.14 ± 0.02^{e}	40.34 ± 0.63^{d}
50	21	7.3 ± 0.10^{ab}	1.41 ± 0.07^{d}	151.15 ± 2.55^{c}	1.01 ± 0.23^{b}	57.10 ± 0.16^{b}
	42	7.2 ± 0.06^{b}	1.13 ± 0.08^{e}	129.93 ± 3.32^{d}	0.80 ± 0.05^{c}	41.41 ± 2.23^{d}
100	21	7.4 ± 0.15^{a}	1.94 ± 0.08^{b}	202.30 ± 4.23^{a}	1.56 ± 0.03^{a}	61.24 ± 0.68^{a}
	42	7.2 ± 0.06^{b}	1.57 ± 0.09^{c}	189.81 ± 4.20^{b}	1.43 ± 0.20^{b}	48.41 ± 1.61^{c}

Values are means ± SD of three replicates. Different superscript in the same column indicate significant difference at $p < 0.05$ (Duncan's Multiple Range Test)

Effect of Pig Dung on the Hydrocarbon Degrading Bacteria Counts and Identification

The values of total hydrocarbon degrading bacteria decreased from 21 to 42 days in 0 (control), 50 and 100 g pig dung kg^{-1} soil (Table 4). The total hydrocarbon degrading bacteria were found to be higher in soil amended with pig dung than the control soil. Morphological characteristics of bacteria isolated from the polluted soil amended with pig dung at 42 days are presented in table 5. The size of the bacteria ranged between 1 – 5 mm. Most of the bacteria were irregular in shape, grey-white in colour, wet consistency, smooth edges, flat elevation and opaque. The types and relative abundance of microbial communities in microcosms due to natural attenuation and biostimulation treatment methods recorded in the contaminated soil are presented in table 6. Five hydrocarbon utilizing bacteria were identified from the polluted soil. The hydrocarbon degrading bacteria identified belong to the genera *Bacillus, Staphylococcus, Escherichia, Pseudomonas and Enterobacter*. *Bacillus* species were the most predominant isolated bacterial species across the treatments.

Table 4: Total Hydrocarbon Degrading Bacteria Count of the Polluted Soil Amended with Pig Dung

Pig dung level (g)	Day	THDB (CFU g^{-1})
0	21	$1.12 \times 10^4 \pm 1.35 \times 10^{3c}$
	42	$.63 \times 10^3 \pm 1.99 \times 10^{3c}$
50	21	$.55 \times 10^4 \pm 3.59 \times 10^{3ab}$
	42	$2.40 \times 10^4 \pm 1.44 \times 10^{3bc}$
100	21	$1.97 \times 10^4 \pm 7.94 \times 10^{3a}$
	42	$1.44 \times 10^4 \pm 3.03 \times 10^{3a}$

THDB = Total Hydrocarbon Degrading Bacteria
Values are means ± SD of three replicates. Different superscript in the same column indicate significant difference at $p < 0.05$ (Duncan's Multiple Range Test)

Table 5: Morphological Characteristics of Bacteria Isolated from the Polluted Soil

Isolate Code	Size (mm)	Shape	Colour	Consistency	Edges	Elevation	Opacity
0 g PD	3-5	Irregular	Grey-white	Dry	Rough	Flat	Opaque
0 g PD	1-2	Smooth	Yellow	Wet	Smooth	Slightly raised	Opaque
0 g PD	3-4	Round	Grey-white	Wet	Smooth	Raised	Opaque
50 g PD	2-3	Round	White	Wet	Smooth	Flat	Opaque
50 g PD	3-4	Irregular	Green	Wet	Rough	Flat	Opaque
50 g PD	3-4	Round	Grey-white	Wet	Smooth	Raised	Opaque
100 g PD	2-3	Round	White	Wet	Smooth	Flat	Opaque
100 g PD	1-2	Smooth	Yellow	Wet	Smooth	Slightly raised	Opaque
100 g PD	3-5	Irregular	Grey-white	Dry	Rough	Flat	Opaque

Table 6: Types and Relative Abundance of Micro-organisms in the Polluted Soil

Isolate code	GR	SP	CP	CA	CO	MO	IN	OX	CI	UR	MR	VP	G	L	M	Probable organism
100 g PD	GPB	+	+	+	-	+	-	-	-	-	+	-	A	-	-	*Bacillus subtilis*
100 g PD	GPC	-	-	+	+	-	-	-	-	-	-	+	A	A	A	*Staph. Aureus*
100 g PD	GNB	-	-	+	-	+	-	-	+	-	+	-	A	A	-	*Enterobactersp*
50 g PD	GNB	-	-	+	-	+	+	-	-	-	+	-	A	A	-	*Escherichia coli*
50 g PD	GNB	-	-	+	-	+	-	+	+	-	+	-	-	-	-	*Pseudomonas aeruginosa*
50 g PD	GNB	-	-	+	-	+	-	-	+	-	+	-	A	A	-	*Enterobactersp*
0 g PD	GNB	-	-	+	-	+	+	-	-	-	+	-	A	A	-	*Escherichia coli*
0 g PD	GPC	-	-	+	+	-	-	-	-	-	-	+	A	A	A	*Staph. aureus*
0 g PD	GPB	+	+	+	-	+	-	-	-	-	+	-	A	-	-	*Bacillussubtilis*

Keys: GR-Gram staining, SP- Spore staining, CA- Capsule staining, CT- Catalase, MO-Motility, IN- Indole, OX- Oxidase, CI- Citrate, IN- Indole, OX- Oxidase, CI- Citrate, UR- Urea, MR-
Methyl-red, VP- Vogesproskeur, G- Glucose, L- lactose, S- Sucrose, M- Mannitol, A-Acid production, PD = Pig dung, g = Gram, - =Absent, + = Present, A = Abundant

Biodegradation and Kinetics of Total Petroleum Hydrocarbon in the Contaminated Soil

Significantly ($p < 0.05$) higher concentration of total petroleum hydrocarbon was observed in 0 g pig dung kg^{-1} soil (control) at 21 days while the value that was recorded in 100 g pig dung kg^{-1} soil at 42 days was significantly ($p < 0.05$) lower (Table 7). Total petroleum hydrocarbon of the soil (mg kg^{-1}) before pig dung application was 6.39 ± 0.11 while control, 50 and 100 g of pig dung were 3.25 ± 0.17 and 1.58 ± 0.10; 0.03 ± 0.00 and 0.03 ± 0.00; 0.03 ± 0.00 and 0.03 ± 0.00. At 21 and 42 days after the amendment, highest total petroleum hydrocarbon reduction of 6.36 mg kg^{-1} (99.99 %) was observed at 50 and 100 g pig dung kg^{-1} soil (Table 7) compared with an initial concentration of 6.39 mg kg^{-1} (Table 1). Concentration of total petroleum hydrocarbon in unamended soil (control) and their natural logarithm were plotted against time as shown in figures 1 and 2 in order to analyze the kinetics for the biodegradation process. The biodegradation process followed first order kinetics since the graph of total petroleum hydrocarbon concentration in soil against time had an exponential curve (Figure 1) and that of ln (natural logarithm) of total petroleum hydrocarbon concentration against time was linear (Figure 2). Rate constant was found to be 0.033 day^{-1}. Correlation analysis (r) for the petroleum products biodegradation kinetics process was 0.999, indicating linearity and positive correlations for the decrease in concentration as a function of time.

Table 7: Rate of Change of Total Petroleum Hydrocarbon during Biodegradation of the Polluted Soil

Pig dung level (g)	Time (days)	TPH (mg kg^{-1})	In TPH	TPH Degraded (mg kg^{-1})	Degradation (%)
0	21	3.25 ± 0.17^{a}	1.17	3.14	49.14
	42	1.58 ± 0.10^{a}	0.45	4.81	75.27
50	21	0.03 ± 0.01^{bc}	-3.51	6.36	99.99
	42	0.03 ± 0.01^{bc}	-3.51	6.36	99.99
100	21	0.03 ± 0.01^{bc}	-3.51	6.36	99.99
	42	0.03 ± 0.01^{bc}	-3.51	6.36	99.99

TPH = Total Petroleum Hydrocarbon

Values are means ± SD of three replicates. Different superscript in the same column indicate significant difference at $p < 0.05$ (Duncan's Multiple Range Test)

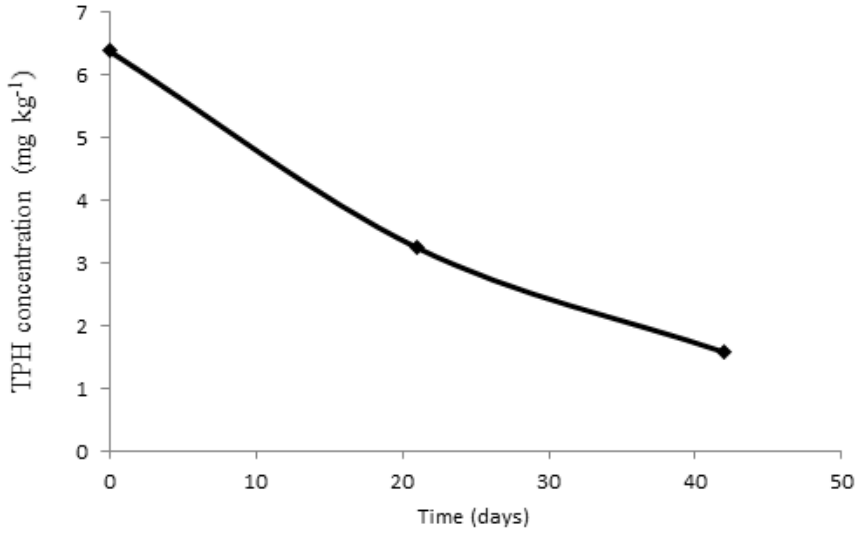

Figure 1: First Order Profile for the Biodegradation of the Unamended Polluted Soil

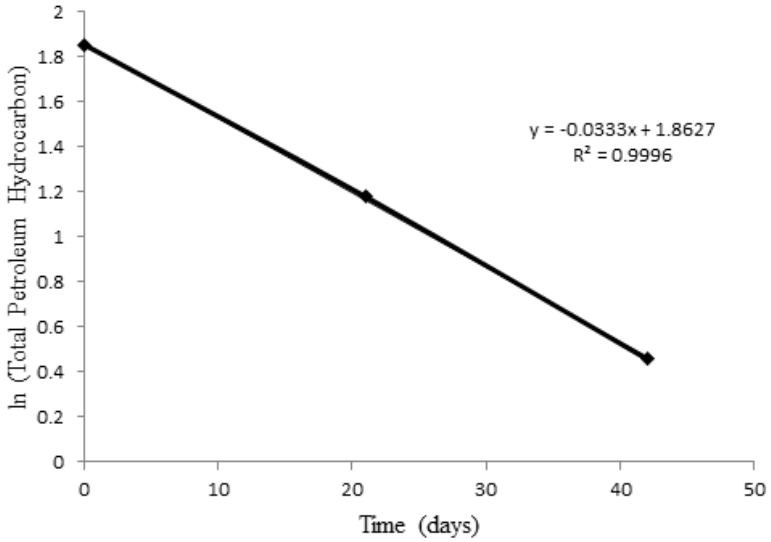

Figure 2: ln (Total Petroleum Hydrocarbon) against Time for the Biodegradation of the Unamended Polluted Soil

DISCUSSIONS

In this study, natural attenuation and biostimulation treatment method of Abule–Egba gasoline polluted soil was monitored with a view to gaining an insight into conditions necessary for petroleum hydrocarbon degradation. Application of pig dung significantly ($p < 0.05$) increased pH of the polluted soil compared to the control at 21 and 42 days. Soil pH range between 6.9 and 7.5 is good for most hydrocarbon utilizing bacteria (Vidali, 2001; Yakubu, 2007).

The gradual decrease in pH as biodegradation progressed recorded in this study was not unconnected to the biodegradation process which removed the contaminant and introduced some salts and ions from pig dung (Akpoveta *et al*., 2011). The decrease in soil N, P, K and organic carbon content from 21 to 42 days at every pig dung level might be due to their high demand by microorganisms for sugar phosphorylation, nucleic acid synthesis and other cellular processes (Andrew and Jackson, 1996). It has also been reported that petroleum hydrocarbon contaminants could destroy inorganic nutrient sources by reacting with them along with other substances present in soil (Teal *et al*., 1992; Andrew and Jackson, 1996). The reduction in population of total hydrocarbon degrading bacteria from 21 to 42 days in 0 (control), 50 and 100 g pig dung kg^{-1} soil recorded in this study might be due to the fact that mineralization of hydrocarbons could have possibly resulted in the production of toxic metabolites which on introduction into the system reduces the growth phase of the microbes (Akpoveta *et al*., 2011).

Microorganisms generally require mineral nutrients sources for growth (Andrew and Jackson, 1996). If any of the required nutrients is lacking or becomes limiting, particularly the macro-mineral elements, microbial population will decrease (Giordani *et al*., 1998; Lehtola *et al*., 1998; Vidali, 2001). Akpoveta *et al*. (2011) also reported a decline in bacterial population as the biodegradation progressed. *Bacillus* species were the most predominant isolated bacterial species, its prevalence could be attributed to the fact that it forms spores, which help microorganisms to withstand harsh conditions. Isolation of *Bacillus* species from hydrocarbon contaminated soil amended with pig dung could also be attributed to its ubiquitous distribution in nature.

Mansour *et al*. (1999) reported the isolation of *Bacillus, Acinetobacter, Staphylococcus* and *Enterobacter* among other bacteria from hydrocarbon contaminated soil. The oil utilizing bacteria isolated from this study have previously been implicated in hydrocarbon biodegradation, though from different sources (Ijah and Antai, 2003; Yakubu, 2007). Degradation of total petroleum hydrocarbon in the contaminated soil amended with pig dung might be due to the

bacterial consortium in the pig dung that attacked and degraded the components of the hydrocarbon (Yakubu, 2007; Adesodun and Mbagwu 2008). Higher significant concentration of total petroleum hydrocarbon was recorded in the soil without pig dung applications compared with the least significant ($p < 0.05$) value observed in 100 g pig dung kg^{-1} soil. Biostimulation has been reported as an important factor that enhances soil bioremediation (Cardona and Iturbe, 2003; Gallego *et al*., 2010).

Gallego *et al*. (2010) in their study of in situ bioremediation techniques reported that it is possible to degrade up to 90 % of hydrocarbon pollutant, during biostimulation. The decrease in total petroleum hydrocarbon concentrations from 21 to 42 days could be that mineral elements in pig dung contributed to the enhanced biodegradation. The first order kinetics observed in the degradation process in this study could be attributed to the fact that as concentration of the contaminant in the soil was decreasing with time, the concentration degraded by the microbes was also increasing for the biodegradation study (Peijun *et al*., 1996; Akpoveta *et al*., 2011).

CONCLUSION

In this study, microcosm experiments were conducted to evaluate the effectiveness of pig dung on the bioremediation of fuel-oil contaminated soils. After 21 days of incubation, approximately 99 % remediation efficiency was achieved at 50 and 100 g pig dung kg^{-1} soil. The results indicate that biostimulation of petroleum-products contaminated soil resulted in the enhancement of petroleum hydrocarbon degradation. Pig dung is a reliable and powerful tool for fuel-oil contaminated soils bioremediation processes.

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