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Comparative Study on the Performance of Oil Palm Bunch Ash and Poultry Manure as Nutrient Supplements in Bioremediation of Crude Oil Polluted Soil

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A B S T R A C T

The biostimulation potentials of oil palm bunch ash (OPBA) and poultry manure (PM) for the remediation of crude oil polluted soil were comparatively investigated on a 70 Days study period. The hydrocarbon polluted soil was supplemented with different weights (200g and 250g) of OPBA and PM treatments singly and in combination. Analyses of composite samples for both physicochemical and microbiological parameters were carried out using standard methods. The results showed that addition of OPBA and PM significantly modified the soil physical, chemical and biological properties. The phenotypic and phylogenetic analyses of 16S and ITS rRNA gene sequence isolated showed that they are related to members of the species *Priestia megaterium*, *Bacillus aryabhattai* and *Penicillium javanicum*, *Aspergillus alabamensis* respectively. Of the treatments employed in this study, PS+125gOPBA+125gPM and PS+250gOPBA options presented the optimal levels with potentials as the cost effective biostimulants for remediation of crude oil impacted media.

Introduction

The soil is a primary recipient by design or accident of a myriad of waste products and chemicals used in modern society (Eneje *et al.*, 2012). Soil is the key component of natural ecosystem and environmental sustainability depends largely on sustainable ecosystem (Adenipekun, 2008; Onuh *et al.*, 2008a; Adedokun and Ataga, 2007). The deleterious effects of crude oil pollution on the properties of soil have been the subjects of many studies. Okolo *et al.*, (2005) reported that oil pollution increase carbon and reduces soil nitrates and phosphorus. Similarly, Adedokun and Ataga (2007) reported that any contact of soil with crude oil results in damage to the soil micro-organisms and plants. In Nigeria, in relatively recent time, there has been remarkable increase in population, urbanization and industrial activities, (Eze and Okpokwasili, 2010). Pollution caused by petroleum and its derivatives is the most

prevalent problem in the environment. The release of crude oil into the environment by oil spills is receiving worldwide attention (Millioli *et al.,* 2009). Crude oil contamination often retards the yield of plants and soil productivity owing to its effect on some soil elements essential for the growth of plants. Inorganic mineral nutrients in soil are fundamental for the blossoming growth and development of plants. Abnormal levels of these essential nutrients induce deleterious effects on the plants (Chukwuma *et al.,* 2018). The adverse effects of crude oil pollution on these arable agricultural lands have given rise to various soil treatment options such as the use of surfactants, alternate carbon substrates, organic and inorganic manures and bioremediation plants as bioremediation strategies. Organic manures have over time been used to improve soil fertility (Ijah *et al.,* 2008; Onuh *et al.,* 2008a, b; Okolo *et al.,* 2005). Their efficacy in promoting plant growth in crude oil polluted Nigerians soils has also been well documented (Ogboghodo *et al.,* 2005). Biostimulation involves the modification of the environment to stimulate existing bacteria capable of bioremediation. This can be done by addition of various forms of rate-limiting nutrients and electron acceptors, such as phosphorus, nitrogen, oxygen, or carbon (like oil palm bunch ash and poultry manure). The primary advantage of biostimulation is that bioremediation will be undertaken by already present native microorganisms that are well suited to the subsurface environment and are well distributed spatially within the subsurface (Ekanem and Nwachukwu, 2015). The use of oil palm bunch ash and poultry manure as an effective source of nutrient for growth of crops and biostimulants respectively has been effectively studied but its combined performance as well as optimal levels as limiting nutrient sources for crude oil pollution remediation in Niger Delta soils has not been well established.

Therefore, the objectives of this research study are to compare the performance effectiveness of single and combined application of oil palm bunch ash and poultry manure as limiting nutrients sources for remediation of crude oil polluted soil.

Materials and Methods

Experimental Site

Description of Study Area

This research was conducted at a fallow patch of land located at Rivers State Institute of Agricultural Research and Training (RIART), Lane F, Mgbuosimiri in Rivers State University, Nkpolu – Oroworukwo, Port Harcourt. The choice of this study area was based on the factors as follows: easy accessibility and has no record of oil spill over the years.

Collection of Soil Sample

The land for the experiment was cleared and allowed to stabilize for three days. Top soil (0 – 15cm depth) was randomly collected from four points, using soil auger, bulked to form a composite sample using procedures described in the Food and Agricultural Organization (FAO, 2007). This is because microbial influenced agricultural soil fertility is in the range of 0 – 15cm depth.

Five kilogram (5kg) each of the composite samples were weighed with a weighing balance and transferred into twelve labelled plastic buckets with drainage holes at the sides and base to enhance aeration. The plastic buckets were arranged in duplicates in a completely randomized design.

Collection of Crude Oil

Escravos crude oil (API gravity=34.09; viscosity @100^{o}F=4.2cSt; Specific gravity @60^{o}F=0.8545) was obtained from Nigerian National Petroleum Corporation (NNPC), Warri, Nigeria.

Collection of Oil Palm Bunch Ash

Empty Palm Bunches were collected from CHISA Oil Mill along Sunny Mill Road, off Ahoada Road, Elele Town. It was stored in a sterile container to prevent external contamination.

Collection of Poultry Manure

Poultry manure was aseptically collected from poultry farm situated in Rivers State University, Nkpolu-Oroworukwo area of Rivers State. It was stored in a sterile container to prevent external contamination.

Soil Contamination and Amendments

After land clearing, soil bulking and stabilization of the soil for three days, five kilogram (5kg) each of soil sample was experimentally polluted with 10% (500ml) concentration of crude oil in each bucket.

The objective of this pollution was to achieve severe pollution because, beyond 3% concentration, crude oil has been reported to be increasingly deleterious to soil biodata (Osuji *et al.,* 2005; Akpoveta *et al.,* 2011). The oil was thoroughly mixed with the soil in the plastic buckets and the set up was allowed to stand for seven days under natural condition for acclimatization between the soil and the oil (Onyelucheya *et al.,* 2013). This gave an insight of what happens to microbial activities after one week of pollution which should be enough time for contingency plans towards combating and abating the oil pollution (Ekpo and Nya, 2012).

Amendment of the polluted soils with oil palm bunch ash (OPBA) and poultry manure (PM), served as the nutrient supplements. Amendment with these biostimulating agents was carried out immediately after seven (7) days of pollution by incorporating 200g (4%W/W) and 250g (5%W/W) of each weight of the appropriate nutrient per bucket and stirring to ensure even distribution with the soil volume (Onuh *et al.,* 2008). Amendments showing experimental design layout, crude oil added, weight of soil/nutrients and % oil pollution are represented in Table 1. All experimental and control soils were tilled twice a week to make oxygen available to microorganisms, and watered with sterile distilled water to maintain 50% water holding capacity throughout the 70-Day period of the experiment (Ekwuabu and Chikere, 2016).

Microbiological Analysis of Samples

Soil samples were prepared for microbial analysis according to the method described by Prescott *et al.*, (2005). A portion (10g) of the homogenously mixed soil samples were aseptically transferred into 90mL of 1% peptone water and properly mixed. Then, 1mL of the aliquots was again diluted up to 10^{-6} using tenfold serial dilution. Microbial counts which included total heterotrophic bacterial counts, fungal counts, hydrocarbon utilizing bacterial counts and hydrocarbon utilizing fungal counts were carried out on control and experimental soil samples.

Enumeration of Total Heterotrophic Bacteria and Total fungi

Total heterotrophic bacteria (THB) for each sample were enumerated from the soil sample using spread plate technique as described by Prescott *et al.*, (2005). Samples were serially diluted and an aliquot from each sample was placed on nutrient agar medium (Oxoid) for isolation of THB with the addition of 50µg/ml nystatin to suppress the growth of fungi (Williams and Madise, 2018). Plates were incubated at 30°C for 24 hours before the colonies were counted. The bacterial isolates were characterized using microscopic techniques (Gram staining) and ABIS tools for biochemical tests (Williams and Money, 2017).

Acidified potato dextrose agar plates containing streptomycin (1 mg/100 ml) were used to obtain fungal isolates. The plates were incubated at 28 – 30^{o}C and observed after 3days, after this, isolation of pure isolates was done (Williams and Money, 2017).

Enumeration of Hydrocarbon Utilizing Bacteria and Fungi

The vapor phase transfer method used by Hamamura *et al.,* (2006) was adopted in isolating and enumerating the population of hydrocarbon utilizing bacteria and fungi. The modified mineral salt agar medium was sterilized by autoclaving at 121^{o}C for

15mins at 15psi. Fungusol and lactic acid were added to suppress the growth of fungi and bacteria respectively (Odokuma, 2003; Orji *et al.*, 2012 and Obire *et al.*, 2008). The plates were inverted and incubated at 37^{o}C for 2 - 5days (HUB) and 28 – 30^{o}C for 5days (HUF). The filter paper saturated with sterile crude oil served as the sole source of carbon in the mineral salt agar. After incubation, the colonies that developed on the plates were counted and recorded as counts of total heterotrophic and hydrocarbon utilizing fungi, expressed as colony forming unit per gram.

Identification of Microbial Isolates

Discrete bacterial and fungal colonies were further purified by sub-culturing on freshly prepared nutrient agar and potato dextrose agar plates respectively. The bacterial isolates were identified based on the Gram's reaction, morphological and biochemical test using the ABIS online identification tools. References were made to Bergy's Manual of Determinative Bacteriology for identification of bacterial isolates (Cheesbrough, 2006). The fungal isolates were identified macroscopically and microscopically. Macroscopic identification involved observing the colonial characteristics such as colony growth pattern, size, and texture, color and colony pigmentation. The technique described by Obire *et al.*, (2008) was adopted for microscopic identification of fungal isolates using lactophenol stain in a wet preparation. A small portion (inoculum) of the fungal growth was picked with a sterilized needle and placed on a clean and grease free slide. A drop of lactophenol stain was added and the preparation was covered with a cover slip. The slide was mounted and observed under light microscope with x10 and x40 objective lenses.

Molecular Identification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer (Chikere and Fenibo, 2018). The PCR amplicons from the soil isolates were sequenced using a 3500 genetic analyzer. The obtained sequence was edited using the bioinformatics algorithm Trace edit. Similar sequences were downloaded from the National Centre for Biotechnology Information (NCBI) database using BLASTN. These sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0. The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analysed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969).

Physicochemical Analyses

Physicochemical analyses carried out on control and experimental soil samples include pH (electrometric method as described by Mylavarapu and Kennelley (2002), moisture content (gravimetric method as described by Bahuguna *et al.*, (2011), water holding capacity, total nitrogen (Kjeldahl method as described by Onyeonwu (2000), phosphorus (Bray method), total organic carbon (titrimetric method), C:N ratio (quantitative method) and exchangeable cations (APHA method 3030E). The extraction of petroleum hydrocarbon was done with dichloromethane (DCM) using cold extraction method with ASTM D-3694 heavy machine for 1 hour and procedure as reported by TPI (2007).

Data Analysis

An unpaired t-test in which a two-tailed *P*-value was calculated and results were presented as Mean±SD where necessary. Statistical significance was defined as a *P*-value of less than 0.05 at 95% confidence interval. Chromas-Lite, version 2.1.1/ Geeious version 9.0.5 was used to analyze the sequence data generated and the phylogenetic trees using Neighbor-Joining method at the Gene Bank.

Results and Discussion

Results of microbiological and physicochemical analyses of control and experimental soils are

presented in tables, graphs and charts. The pH values of the unpolluted soil, polluted soil, poultry manure and oil palm bunch ash ranged from 5.79±0.07 to 9.56±0.11. The moisture content (%) and Water holding capacity (%) values of the unpolluted soil, polluted soil, oil palm bunch ash, poultry manure samples ranged from 16.44±2.12 to 61.65±1.15 and from 21.10±1.01 to 47.40±0.80 respectively. The available phosphorus (mg/kg) values of unpolluted soil, polluted soil, poultry manure, unpolluted soil and oil palm bunch ash ranged from 1.47±0.09 to 5.41±0.08. The values of total organic carbon (%) and total nitrogen ranged from 1.14±0.11 to 4.71±0.01 and 0.04±0.00 to 0.11±0.01 respectively. The values of the C/N ratio unpolluted soil, polluted soil, PM, and OPBA ranged from 15.30 ±2.42 to 134.58±0.29. The values of exchangeable cations (mEq/g) which included calcium, magnesium, potassium and sodium respectively ranged from 1.54±0.09 to 2.47±0.23, 1.17±0.01 to 1.53±0.04, 0.12±0.01 to 4.30±0.08 and 1.02±0.01 to 1.11±0.01.

Table 4 shows the changes in the concentrations of total petroleum hydrocarbons (mg/kg) for all the treatments during bioremediation studies. The unpolluted soil sample recorded a TPH value of 391mg/kg at the beginning of the experiment and a TPH value of 188mg/kg at the end of experiment. The polluted soil (PS) sample recorded a TPH value of 20,193mg/kg at the beginning of experiment and a TPH value of 11,427mg/kg at the last day of experiment. In the biostimulation treatment setup; PS + 200g PM had the lowest TPH removal of 76.44% whereas, PS + 250gOPBA and PS+125gOPBA+125gPM had the highest TPH removal of 93.09% and 93.27% respectively. The difference in the values of TPH for each treatment was calculated by subtracting the TPH concentration at the end of the experiment from the TPH concentration at the beginning of the experiment, from which the percentage TPH removal for the various treatments was estimated. Effects of OPBA and PM single and combined treatments on TPH concentrations during bioremediation studies are shown in figure 8.

Table 5 shows that the total heterotrophic bacterial counts (THBC) of the unpolluted soil (US) and polluted soil (PS) samples were $7.6\pm0.07x10^7$ and $4.2\pm0.21x10^7$ CFU/g; total fungal counts (TFC) of US and PS samples were $6.8\pm0.00x10^5$ and $4.0\pm0.28x10^5$ CFU/g; hydrocarbon utilizing bacterial counts (HUBC) of US and PS samples were $4.3\pm0.28x10^5$ and $1.8\pm0.21x10^5$ CFU/g; hydrocarbon utilizing fungal counts (HUFC) of US and PS samples were $1.8\pm0.28x10^3$ and $1.1\pm0.00x10^3$ CFU/g.

Results showing the trends of the effects of the unpolluted soil samples, polluted soil samples and the different amended soil samples on the microbial counts from the beginning to the end of experiment are graphically presented in Figures 9, 10, 11 and 12 for the microbial counts of total heterotrophic bacteria, total fungi, hydrocarbon utilizing bacteria and hydrocarbon utilizing fungi respectively.

The computed evolutionary distances using Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolate A5 within the *Klebsiella* genus and revealed a close relatedness to *Klebsiella grimonti* (CP056150) than other *Klebsiella* sp., A2 within the *Myroides* genus (Flavobacteria class) was closely related to *Myroides odoratimimus* (MT465451) than other *Myroides* sp., A1 within the *Priestia* genus was closely related to *Priestia megaterium* (MW578437) than other *Priestia* sp., and A4 within the *Bacillus* genus was closely related to *Bacillus aryabhattai* (MH196977) than other *Bacillus* sp., as shown in phylogenetic tree in Figure 13 for bacteria. The internal transcribed space (ITS) of the isolates obtained showed a percentage similarity to other species at 100%.

The computed evolutionary distances using Jukes-Cantor method were in agreement with the phylogenetic placement of ITS of A8 within the *Aspergillus* sp., and revealed a close relatedness to *Aspergillus alabamensis* (MW513987), A6 within the *Penicillium* sp., was closely related to *Penicillium javanicum* (MK450698) and *Penicillium*

oxalicum (MW485755) than other *Penicillium* sp., as shown in the phylogenetic tree in Figure 14 for fungi.

ABIS tools was used to identify bacteria isolates which showed that *Enterobacter* sp., *Citrobacter* sp., *Myroides* sp., *Acinetobacter* sp., *Escherichia coli*, *Proteus* sp., *Pseudomonas* sp., *Staphylococcus* sp., *Klebsiella* sp., *Aeromonas* sp., *Micrococcus* sp., and *Serratia* sp. were isolated from both US and PS samples, and in addition, the US sample had *Chromobacterium* sp., *Actinomycetes* sp., and *Nocardia* sp. Also, similar fungi including *Fusarium* sp., *Trichodema* sp., *Geotrichum* sp., *Aspergillus* sp., *Mucor* sp., *Penicillium* sp., and *Candida* sp. were isolated from both soil samples, and in addition, the US sample only had *Rhizopus* sp.

Pollution of the natural environment such as soil by petroleum is a universal problem because of their effect on soil ecosystem (Akoto *et al.,* 2008). The individual parameters analysed were monitored for seventy days to compare their effects on control samples and treated soil samples.

The baseline mean values of the physicochemical compositions of US, PS, OPBA and PM (Table 2) showed that: pH value of oil palm bunch ash was highest compared to the unpolluted soil, polluted soil and PM. This has been shown in the work of Hasnol *et al.,* (2005); total organic carbon concentrations of PS was higher compared to US, OPBA and PM which is in agreement with the work of Ekundayo and Obuekwe (2000) who reported that hydrocarbon contamination can increase soil total organic carbon; cation exchangeable capacity (Ca^{2+}, Mg^{2+}, K^{+} and Na^{+}), phosphorus and total nitrogen values were generally lower in polluted soil than in unpolluted soil. Nutrient deficiencies observed in crude oil polluted soil could be attributed to microbial immobilization of soil nutrients as fast as they are made available (Anya and Ekpo, 2006).

Moreover, Arocena and Rutherford (2005) and Kisic *et al.,* (2009) reported that crude oil contamination results in changes in other soil chemical properties.

The pH values of polluted soil, and polluted soil amended with oil palm bunch ash and poultry manure were higher than that of unpolluted soil (Figure 2). Increase in pH of the polluted soil amended with PM, OPBA and OPBA+PM is an indication of increased metabolic activities during the process of decomposition and buffering effect organic manure has on crude oil polluted soil (Ijah *et al.,* 2008). Increase in soil pH after pollution with crude oil is in line with the works of Amadi *et al.*, (2005) and Njoku *et al.,* (2008) who reported that crude oil pollution increased the pH of the soil. Polluted treated soil samples with different weights of OPBA showed higher pH values, followed by different weights of OPBA+PM, and then different weights of PM. This result agrees with the works of Hansol *et al.,* (2005) and Amajuoyi and Wemedo (2015) who reported that OPBA is highly basic.

Available phosphorus concentration in unpolluted soil samples were higher compared to those of the polluted soil throughout the period of experiment. This is in agreement with the work done by Godwin and Bassey (2013) who reported that presence of hydrocarbon significantly decreased the available phosphorus in the soil, which could be due to immobilization of nutrients by microbial degrading crude oil (Agarry and Jimoda, 2013). Addition of nutrients (OPBA, PM and OPBA+PM) resulted in corresponding increase in the values of available phosphorus for all the treatments. This increase in the percentage phosphorus may be as a result of anthropogenic inputs of these nutrients from the organic manures because organic manures have been reported as being capable of increasing soil nutrients by supplementing the limiting nutrients (Mbah *et al.,* 2009:2006; Tanee and Kinako, 2008).

A gradual decrease in the concentration of available phosphorus for all the treated soil samples from Day 14 to Day 70 was observed (Figure 3). Decrease in phosphorus level from Day 14 to Day 70 in all the soils could be due to microbial activities which utilized it as there were no additional nutrients as the experimental period increased. Baran *et al.,* (2002) reported that degrading effect of petroleum derived

compounds on soil led to severe phosphorus depletion.

Total nitrogen content of unpolluted soil was low and decreased with time throughout the experiment (Figure 4). This indicated that the soil was low in plant nutrients and amendment with different weights of the OPBA, PM and OPBA+PM were ideal to boost the soil nutrient. Total nitrogen contents in the unpolluted soil and polluted soil was very low. However, total nitrogen content of the unpolluted soil was higher than that of the polluted soil. This is because petroleum-products such as crude oil are known to reduce nitrogen availability as observed by Agbogidi *et al.,* (2007). Addition of different weights of the nutrients (OPBA, PM and OPBA+PM) to crude oil polluted soils led to an increase in total nitrogen on Day 1 compared to the unpolluted soil and the polluted soil without nutrient amendments. There was significant reduction of total nitrogen in soils treated with different weights of nutrient amendments (OPBA, PM and OPBA+PM) from Day 14 to Day 70 indicating that aerobic conditions were achieved or rather the use of high quantity of the nutrients resulted in high microbial activity leading to equilibrium in the mineralization of this nutrient. The decrease in nitrogen content of the amended soil could be that it served as a source of nutrient for oil degraders (Benson *et al.,* 2016). Generally, the decrease in total nitrogen over time in all the groups may be due to the utilization of nitrogen by soil microorganisms (Chukwuma *et al.,* (2018).

The significant increase in the organic carbon content of the amended soil observed in this study may have beneficial effect on the soil chemical and physical properties. This is in line with earlier reports (Mbah *et al.,* 2006, 2009) which stated that organic carbon from wastes can influence the ability of microorganisms to degrade pollutants. Higher increase in total organic carbon was observed in all the soils treated with OPBA, PM and OPBA+PM at different weights. The combined (OPBA+PM) treatments had highest total organic carbon contents compared to single (OPBA and PM) treatments. Percentage organic carbon content of the soil samples increased with increase in the concentration of crude oil pollution (Figure 5). The increase in the percentage organic carbon observed in this study had been observed earlier (Amadi *et al.,* 2005; Ogboghodo *et al.,* 2005; Osuji and Onojake, 2006) and may be attributed to the microbial mineralization of the crude oil.

Generally, the C:N ratio was low in unpolluted soil compared to both the polluted soil and treated soil samples during the dry and wet seasons. Watson *et al.,* (2002) stated that the lower the C:N ratio, the more rapidly nitrogen will be released into the soil for immediate crop use. Notably, the C:N ratio of polluted soil samples had higher carbon to nitrogen ratio, throughout the investigation (Figure 6), which could lead to immobilization of nitrogen.

There is increase in C:N ratio for all treated soil samples which is an indication of a healthy soil as they were maintained at a certain level throughout the investigation. Sequential reductions in C:N ratio with increase in treated soil with different weights of organic nutrients (OPBA, PM and OPBA+PM) was observed, which could be attributed to microbial fixation of nitrogen from the atmosphere into the amended soil samples (Oyedele *et al.,* 2015).

Exchangeable cations (calcium, magnesium, potassium and sodium) were observed to have decreased with increased levels of crude oil pollution. This may be attributed to the use of these exchangeable bases by the microbes present in the experimental soil samples. The results showed that there were slight increases in the levels of magnesium, calcium, potassium and sodium in the polluted soil amended with OPBA and PM (Figure 7). Mbah *et al.,* (2006, 2009) observed similar results.

Table.1 Treatment Showing the Volume and Percentage of crude oil applied, weight of soil and different weights of the Stimulants

Treatments	Wt. of soil (g)	Wt.(%) of stimulants (g)	Vol.(%) of crude oil (mL)
US_1	5000	0 (0)	0 (0)
US_2	5000	0 (0)	0 (0)
PS_1	5000	0 (0)	500 (10)
PS_2	5000	0 (0)	500 (10)
PS_1 + OPBA	5000	200 (4)	500 (10)
PS_2 + OPBA	5000	200 (4)	500 (10)
PS_1 + OPBA	5000	250 (5)	500 (10)
PS_2 + OPBA	5000	250 (5)	500 (10)
PS_1 + PM	5000	200 (4)	500 (10)
PS_2 + PM	5000	200 (4)	500 (10)
PS_1 + PM	5000	250 (5)	500 (10)
PS_2 + PM	5000	250 (5)	500 (10)
PS_1 + OPBA+PM	5000	100+100 (4)	500 (10)
PS_2 + OPBA+PM	5000	100+100 (4)	500 (10)
PS_1 + OPBA+PM	5000	125+125 (5)	500 (10)
PS_2 + OPBA+PM	5000	125+125 (5)	500 (10)

Sources: Akpoveta *et al*., 2011; Ekpo and Nya, 2012; Onyelucheya *et al*., 2013; Amajuoyi and Wemedo, 2015

Table.2 Baseline Mean Values of Physicochemical Compositions of the Unpolluted Soil (US), Polluted Soil (PS), Oil Palm Bunch Ash (OPBA) and Poultry Manure (PM).

Parameters	US	PS	PM	OPBA	*P-Values*
pH	5.79±0.07	5.99±0.06	6.82±0.04	9.56±0.11	<0.0001
Moisture content (%)	61.65±1.15	47.55±3.25	20.55±0.45	16.44±2.12	0.0003
Water holding capacity (%)	47.40±0.80	25.9±3.80	26.2±1.40	21.10±1.01	0.0032
Phosphorus (mg/kg)	4.27±0.06	1.47±0.09	2.07±0.02	5.41±0.08	<0.0001
Total organic carbon (%)	1.14±0.11	4.71±0.01	1.60±0.02	1.66±0.01	<0.0001
Total nitrogen (%)	0.08±0.01	0.04±0.0	0.10±0.0	0.11±0.01	0.0005
C/N ratio	15.30±2.42	134.58±0.29	16.0±0.20	15.85±0.85	<0.0001
Ca2+ (mEq/g)	2.07±0.03	1.82±0.06	1.54±0.09	2.47±0.23	0.0256
Mg2+ (mEq/g)	1.31±0.07	1.25±0.03	1.17±0.01	1.53±0.04	0.0158
K+ (mEq/g)	0.17±0.05	0.10±0.01	0.48±0.09	4.30±0.08	<0.0001
Na+ (mEq/g)	1.08±0.03	1.04±0.03	1.02±0.01	1.11±0.01	0.0724

Table.3 Mean±SD values of exchangeable cations of all treatments during remediation period

Treatments	Exchangeable cations (mEq/g)			
	Calcium ion	Magnesium ion	Sodium ion	Potassium ion
US	3.29±0.09	1.48±0.08	1.05±0.02	0.07±0.01
PS	2.79±0.07	1.28±0.03	0.16±0.02	0.07±0.01
PS + 200g OPBA	6.59±0.61	2.28±0.61	1.10±0.11	0.53±0.11
PS + 250g OPBA	6.85±0.60	2.39±0.58	1.10±0.11	0.55±0.13
PS + 200g PM	5.96±0.63	3.02±0.31	1.56±0.28	1.06±0.13
PS + 250g PM	6.00±0.64	3.04±0.31	1.62±0.23	1.09±0.12
PS + 100g OPBA + 100g PM	7.62±0.43	2.98±0.34	1.65±0.35	1.01±0.11
PS + 125g OPBA + 125g PM	7.62±0.44	2.99±0.34	1.68±0.35	1.02±0.11

Table.4 TPH Values of Different Treatments during Bioremediation Monitoring from First Day – Last Day, and Percentage TPH Removal

Treatments	First Day TPH Values (mg/kg)	Last Day TPH Values (mg/kg)	*P-Value*	TPH Removal (%)
US	391.0±7.1	188.0 ±12.7	0.0026	51.92
PS	20193.0±9.9	11427.0±603.9	0.0024	43.41
PS + 200g OPBA	17263.0±3.5	2209.0±149.9	<0.0001	87.2
PS + 250g OPBA	16899.0±3.5	1168.0±19.8	<0.0001	93.09
PS + 200g PM	18099.0±24.0	4265.0±35.4	<0.0001	76.44
PS + 250g PM	17991.0±3.5	2992.0±19.8	<0.0001	83.37
PS + 100g OPBA+100gPM	16981.0±15.6	1899.0±4.2	<0.0001	88.82
PS + 125g OPBA+125gPM	16400.0±70.7	1105.0±22.6	<0.0001	93.27
P-Value	**<0.0001**	**<0.0001**	-	-

Table.5 Baseline microbial counts (M±SD) of unpolluted and polluted soil samples

Parameters	Unpolluted soil (US)	Polluted soil (PS)
THBC (CFU/g)	$7.6\pm0.07 \times 10^7$	$4.2\pm0.21 \times 10^7$
TFC (CFU/g)	$6.8\pm0.00 \times 10^5$	$4.0\pm0.28 \times 10^5$
HUBC (CFU/g)	$4.3\pm0.28 \times 10^5$	$1.8\pm0.21 \times 10^5$
HUFC (CFU/g)	$1.8\pm0.28 \times 10^3$	$1.1\pm0.00 \times 10^3$

Fig.1 Experimental Setup for Bioremediation Monitoring

Fig.2 Trends of pH during bioremediation monitoring

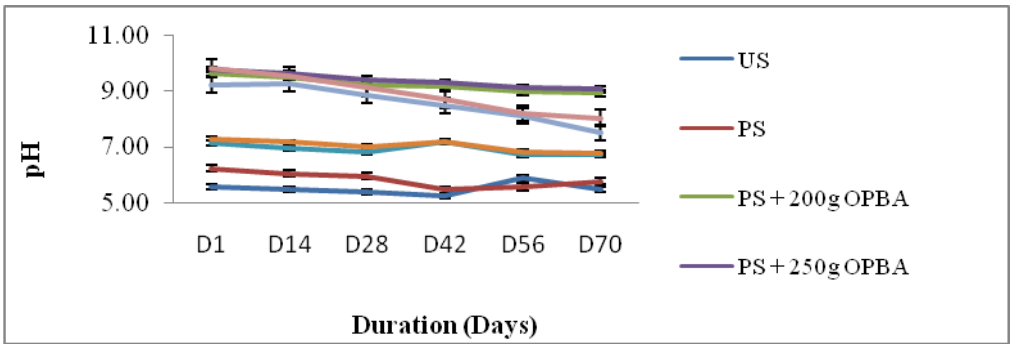


Fig.3 Trends of available phosphorus during bioremediation monitoring

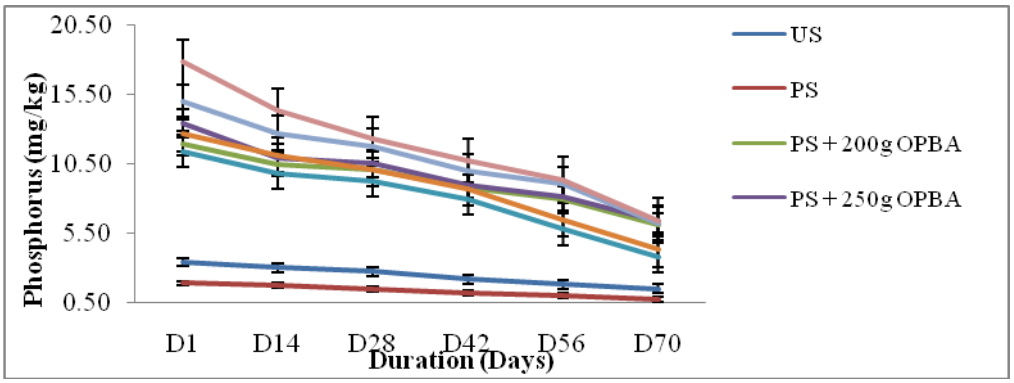


Fig.4 Trends of total nitrogen during bioremediation monitoring

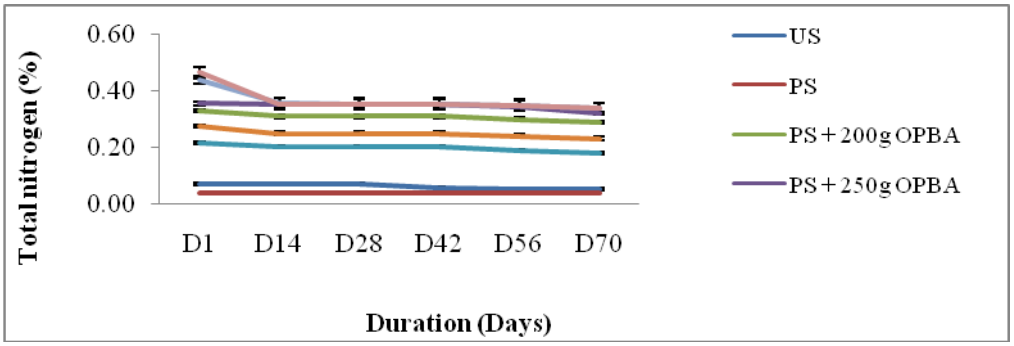


Fig.5 Trends of total organic carbon during bioremediation monitoring

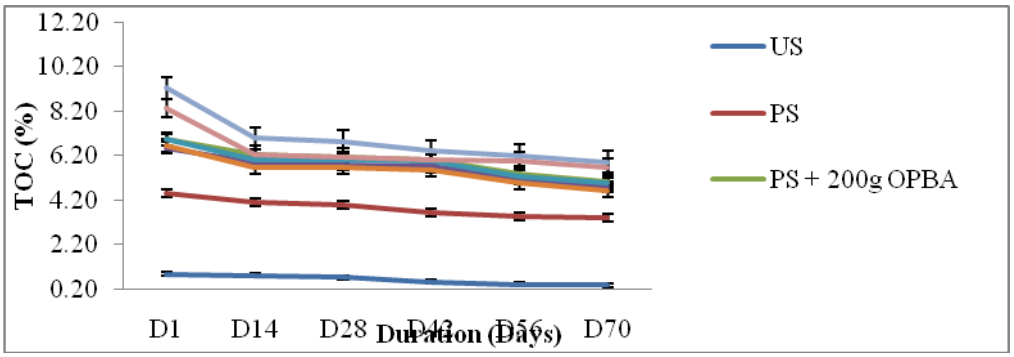


Fig.6 Trends of carbon:nitrogen ration during bioremediation monitoring

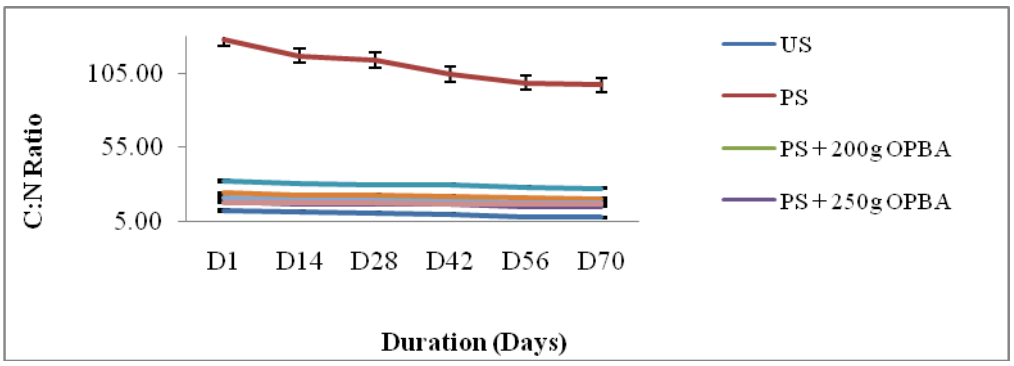


Fig.7 Trends of exchangeable cations (Ca^{2+}, Mg^{2+}, K^{+}, and Na^{+}) during bioremediation monitoring

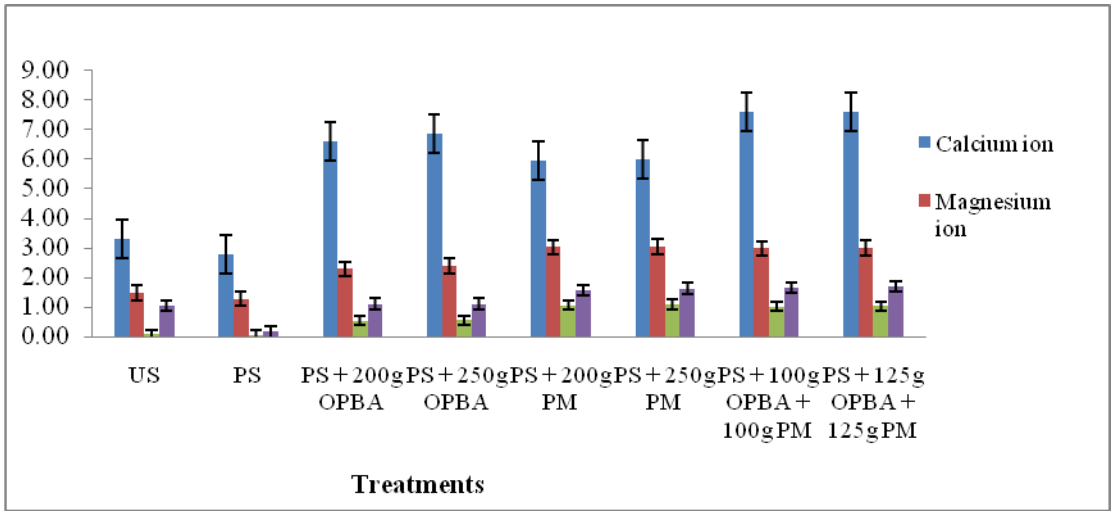


Fig.8 Effect of OPBA and PM single and combined treatments on TPH concentration during bioremediation monitoring

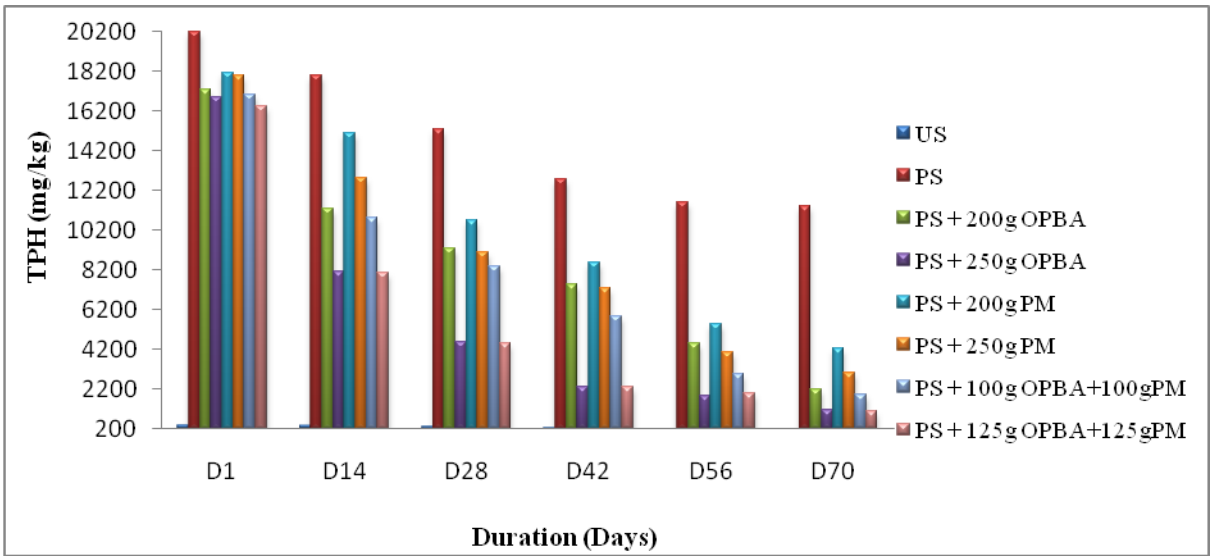


Fig.9 Microbial Counts of Total Heterotrophic Bacteria during Bioremediation Monitoring

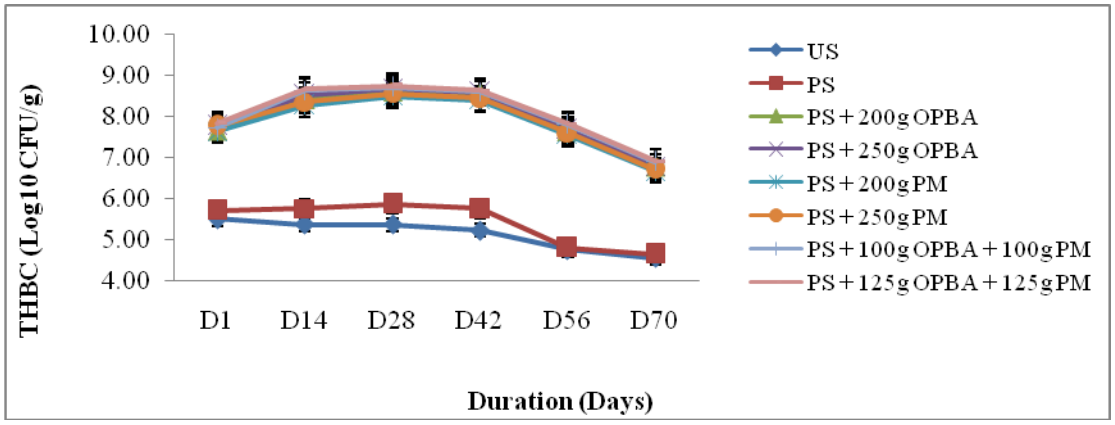


Fig.10 Microbial Counts of Total Fungi during Bioremediation Monitoring

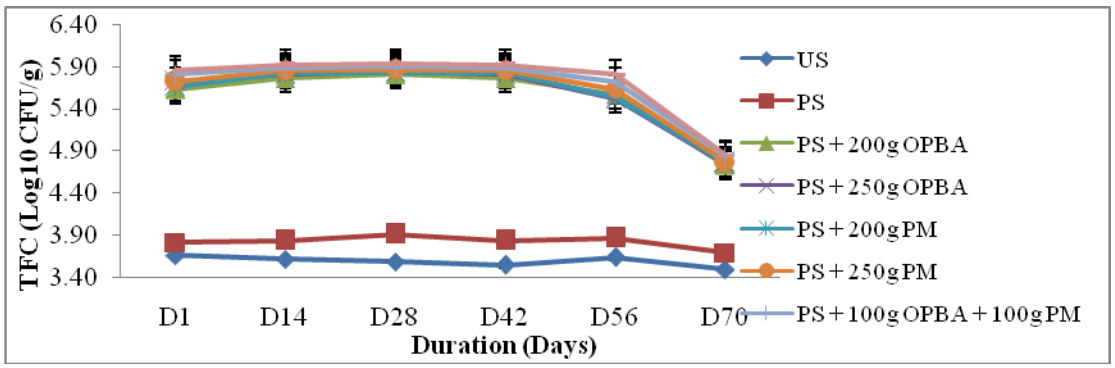


Fig.11 Microbial Counts of Hydrocarbon Utilizing Bacteria during Bioremediation Monitoring

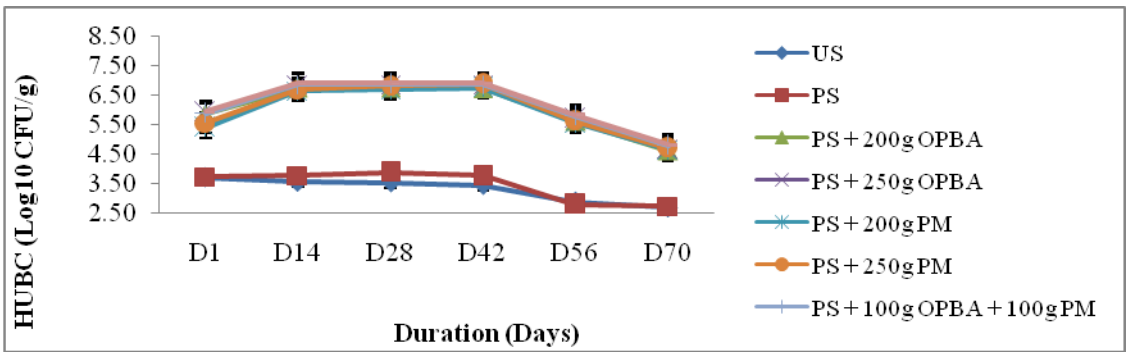


Fig.12 Microbial Counts of Hydrocarbon Utilizing Fungi during Bioremediation Monitoring

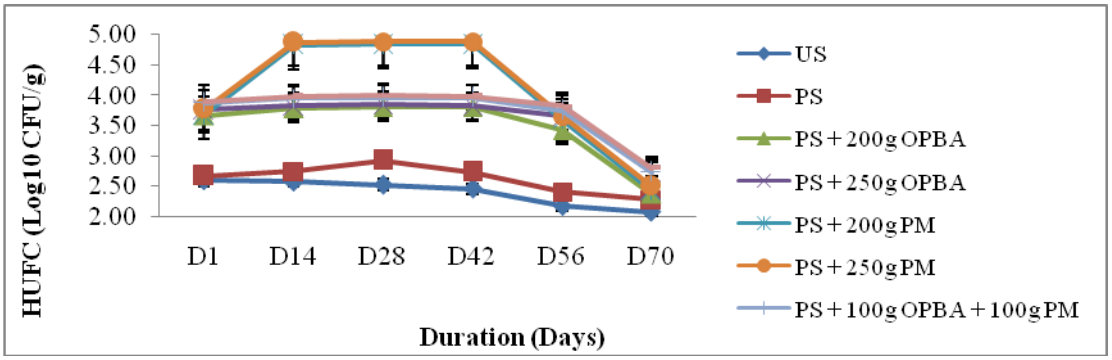


Fig.13 Phylogenetic Tree Showing the Evolutionary Distance between the Bacterial Isolates with their Accession Numbers

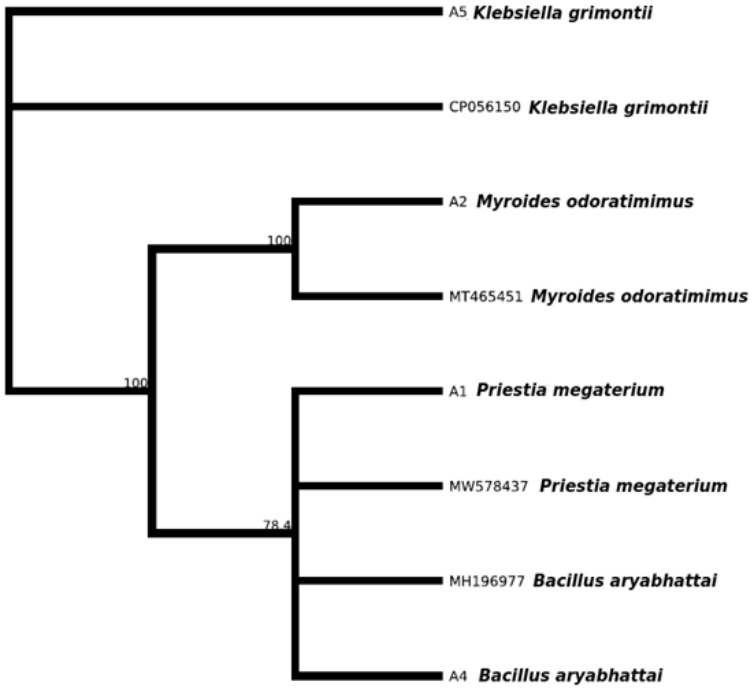
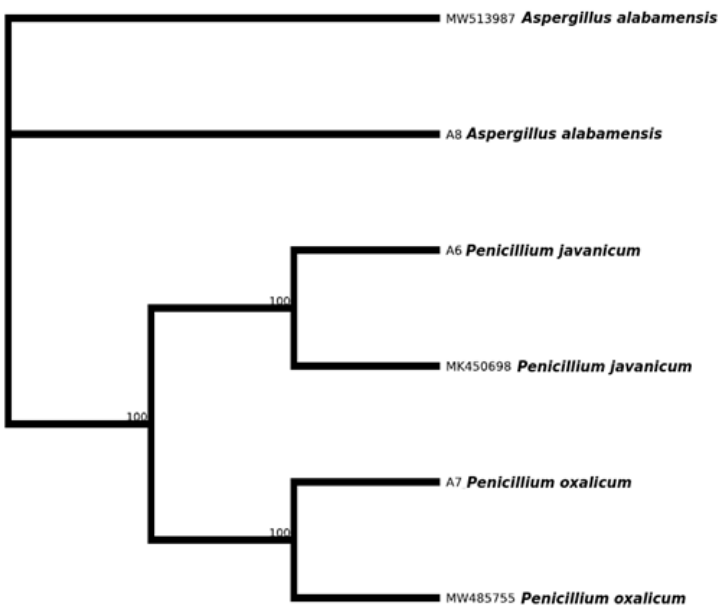


Fig.14 Phylogenetic Tree Showing the Evolutionary Distance between the Fungal Isolates with their Accession Numbers.



Increase in the concentration of Ca^{2+}, Mg^{2+}, K^{+}, and Na^{+} upon addition of OPBA, PM and OPBA+PM could be attributed to their properties which contain high level of these nutrients as shown from the baseline analysis (Tables 2). This result is also consistent with the report from Obasi *et al.*, (2013), who reported that Mg^{2+}, Ca^{2+} and K^{+} were all influenced by the addition of various organic manures to the unpolluted and crude oil polluted soils.

Results of polluted soil showed drastic increase in TPH value on Day 1. The reason for high concentrations of TPH observed on the Day 1 was due to pollution of soil with crude oil in such a quantity as to simulate pollution. The release of petroleum hydrocarbon into the environment is the leading and most reported form of environmental pollution (Singh and Chandra, 2014). Crude oil pollution reduces the soil fertility such that most of the essential nutrients are no longer available for microbial utilization (Abii and Nwosu, 2009).

The results showed that there was a marked significant decrease in the total petroleum hydrocarbon concentration of crude oil polluted soil amended with different weights of OPBA, PM and OPBA+PM relative to both the unpolluted and polluted soils (Table 4). A marked reduction in the TPH concentration was observed between Day 14 and Day 42 of treatment with different weights of OPBA, PM, and OPBA+PM (Figure 8), and this could be due to the ability of microorganisms to use the OPBA, PM and OPBA+PM as both carbon and nitrogen sources to degrade the hydrocarbon compounds. More so, Tanee and Kinako (2008) report showed significant loss in TPH contents amended with poultry manure and NPK, which they attributed to the stimulating effect of organic manure such as increasing the total heterotrophic microbial growth and activity. Highest loss of total petroleum hydrocarbon content was evident in the PS+125gPM + PS+125gOPBA followed by 250gOPBA treatments.

Results of baseline analysis of unpolluted and polluted soil samples showed that microbial counts of THB, TF, HUB, and HUF were higher in unpolluted soil than in crude oil polluted soil samples (Table 5), which implied that the unpolluted soils were rich in nutrient contents. The trend of microbial counts enumerated is similar to the study

carried out by Ra *et al.*, (2019) who recorded higher counts of both the THB and TF present in unpolluted soil than in polluted soil. Chukwuma *et al.*, (2018) reported that crude oil contamination of the environment awfully impacted soil ecosystem, through adsorption and surface assimilation of soil particles, contributing to excess carbon which might be unfeasible for use by the microbial populace, thereby bringing about constraints in the soil nutrients.

The mean microbial counts were used to compare the various treatment samples for the different microbial parameters. Generally, results showed that lower microbial counts were observed in the control samples (US and PS) than in the treated samples for all the microbial counts obtained throughout the period of experiment. This could reveal depletion of nutrient which influenced microbial growth. This is in line with the work carried out by Menkit and Amechi (2019) who reported lower heterotrophic microbial counts in the control samples compared to the counts in the treated soil samples. The bacterial counts in both the polluted and unpolluted soil samples were higher than the fungal counts in both soil samples. The higher counts of bacteria compared to the fungi may be as a result of the nutrient status of the soil and the presence of some toxic components which do not favor fungal growth (Onifade and Abubakar, 2007). The fungal counts obtained from polluted soil could be attributed to the fact that fungi are notably aerobic and can also grow under environmentally stressed conditions such as low pH and poor nutrients' status (Ameh and Kawo, 2017). Results of polluted soils amended with different weights of OPBA, PM and OPBA+PM showed higher microbial counts compared to that of both unpolluted and polluted soil samples. It is obvious from the trends of THB, TF, HUB and HUF counts that increase in the weights of each treatment (OPBA, PM, OPBA+PM) resulted in a corresponding increase in the microbial counts (Figures 9 – 12). A consistent observations was reported by Ayotamuno *et al.*, (2007); Ogbonna *et al.*, (2007); Shabir *et al.*, (2008); Chikere *et al.*, (2009) and Nrior and Mene (2017) who revealed that both total heterotrophic bacterial and hydrocarbon utilizing bacterial counts increased to the maximum with increase in the remediation period upon addition of poultry manure, piggery manure, goat manure and NPK fertilizer treatments. High bacterial loads were observed with increase in the remediation period as compared to Day 1 for the different treatments. Sarand *et al.*, (2001) suggested that soil bacteria adapted to degrading organic contaminants was probably because of concentration of natural organic compounds. Polluted amended soils on Day 42 showed gradual decrease in both bacteria and fungi loads compared to Day 14 and 28 which could be due to nutrient depletion (Figures 9 – 12).

The study revealed that the deleterious effects of crude oil pollution on soils can be remedied by addition of organic manure (OPBA, PM and OPBA+PM) and by maintaining other environmental factors. The results suggested that OPBA+PM can be used as an amendment material to improve pH of highly acidic soils as well as a nutrient supplement in soils with leached nutrients. Generally, the results in this study showed that both individual and combined treatments modified the soil's physical, chemical and biological properties for enhanced biodegradation and bioremediation of a crude impacted soil ecosystem. Of all the treatments employed, PS+125gOPBA+125gPM and PS+250gOPBA showed optimal levels with potentials as the cost effective biostimulants for remediation of crude oil impacted soil in the Niger Delta region of Nigeria.

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