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ORIGINAL RESEARCH ARTICLE

Bioremediation of artificially crude oil polluted soil of veritas University Abuja using poultry manure

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ABSTRACT

The removal of hydrocarbon compounds from the environment has always been a difficult undertaking for people all over the world. As a result, remedial efforts are required to safeguard the environment as well as to restore agriculture. This study looked at how poultry manure (dung) could help in the bioremediation of Veritas University's intentionally crude oil polluted soil. The physical properties of the soil and total petroleum hydrocarbon content of the crude oil polluted soil were determined, followed by the isolation and identification of microorganisms present in the soil and poultry manure before and after pollution with crude oil. These analyses were done according to standard operating procedures. Within a six-month timeframe, the natural attenuation and poultry manure amendment option for remediating the crude oil-polluted soil were monitored and the residual hydrocarbon content of the polluted soil after remediation evaluated. There was an overall decrease in pH level during the experimental units containing 4 g amendment, 2 g amendment and the untreated polluted soil in the order 8.4 to 7.1, 8.4 to 7.2 and 8.1 to 6.7, respectively. Temperature was within 27°C and 31°C, the treated soil that contained 4 g dung had an initial moisture content of 58%, and 35% after remediation. The hydrocarbon utilizing bacteria isolated were *Pseudomonas* species, *Staphylococcus* species, and *Bacillus* species, while the hydrocarbon utilizing fungi isolated were *Aspergillus niger*, *Fusarium solani* and *Candida albicans*. There was more significant microbial increase in the sample containing 4 g poultry manure treatment than 2 g poultry manure treatment and natural attenuation. Total hydrocarbon quantity significantly decreased after six months with the complete removal of C4, C7, C19 and C37 from the treated sample containing 4 g poultry manure. This indicated that the negative effects of crude oil on the environment can be mitigated by adding poultry manure.

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INTRODUCTION

Environmental deterioration is rising at an alarming rate around the world. This issue could be linked to the rapid industrialization, population increase, and technological advancements that are occurring in almost every country today (Adams *et al.*, 2014). Numerous hazardous substances are released into the environment everyday which adds to the global pollution problem (Adams *et al.*, 2014). Oil spills are caused by the release of crude oil from tankers, offshore platforms, drilling rigs, and wells, as well as spills of refined petroleum products and by-products, and heavier fuels such as bunker fuel used by large ships, etc. (Godleads *et al.*, 2015). Oil spills have the greatest direct and economic consequences because they destroy the environment

(Onuorah *et al.*, 2014). According to Thapa *et al.* (2012), soil contamination due to oil spills is currently a serious global concern. Petroleum-contaminated soil poses a major threat to human health. Oil spills have wreaked havoc on coastal vegetation, contaminated drinking water, and sparked ethnic and regional strife, particularly in the Niger Delta (Okonokhua *et al.*, 2007).

The contaminated soil can be remedied in a variety of ways, including physicochemical and biological approaches. Biological approaches are less expensive and more effective than chemical and physical procedures (Thapa *et al.*, 2012). Bioremediation by microorganisms is more efficient than other biological methods, although the limited solubility and adsorption of large molecular weight hydrocarbons limits their accessibility to microorganisms (Thapa *et al.*, 2012). Some bacteria are mobile and have a chemotactic response, which means they detect the pollutant and move toward it, whereas fungi grow in a filamentous form near the contaminant (Modupe *et al.*, 2018a). A wide range of hydrocarbon utilizers (HCUs) established to be useful in the soil include the following species, *Pseudomonas*, *Rhodococcus*, *Enterobacter*, *Bacillus*, *Acinetobacter*, *Flavobacterium*, *Corynebacterium*, *Streptococcus* (Modupe *et al.*, 2018b). Other organisms such as fungi – *Trichoderma*, *Aspergillus*, Mucor are also capable of degrading the hydrocarbons but they take longer periods of time to grow when compared to their bacterial counterparts (Modupe *et al.*, 2018a). Studies have shown that poultry manure accelerates the biodegradation of atrazine and may be appropriate for the rehabilitation of crude oil-contaminated soil (Onuorah *et al.*, 2014). The aim of this study was to evaluate the bioremediation of artificially crude oil-polluted soil utilizing poultry manure as an amendment option at Veritas University. The specific objectives of this study are to; determine the physical properties of soil before pollution, determine the Total Petroleum Hydrocarbon content of the crude oil, determine the total heterotrophic bacterial and fungal counts of the soil, isolate and identify hydrocarbon-utilizing microorganisms present in the poultry manure and the soil, pollute the soil with crude oil and carryout remediation using poultry manure as an amendment option, determine the residual petroleum hydrocarbon content of the poultry manure-amended crude oil-polluted and un-amended polluted soil (natural attenuation) at the end of remediation period. The findings of this study will aid in the development of a process for cleaning-up paraffin crude oil-polluted environment.

MATERIALS AND METHODS

Study area

This research was carried out at Veritas University Abuja. Veritas University is located at Zuma II Bwari area council. It lies within the coordinates, 9.206°N and 7.4164°E.

Sample collection and preparation

Garden soil was collected from Veritas University Garden using a trowel at 10 cm depth. The soil was transported to the Microbiology laboratory in an unused polyethylene bag, it was air dried for seven days, sorted and sieved with a sieve size of 0.3 cm. poultry manure (dung) was collected from a poultry farm at Sabon Gari, Bwari Abuja. It was transported in a polyethylene bag to the microbiology laboratory, air dried for seven days, sorted, crushed using a ceramic mortar and pestle and sieved with a sieve size of 0.3 cm.

Determination of the physical properties of Soil

Determination of moisture content: The soil moisture content was determined using the oven-drying method (Blystone *et al.*, 2001). A clean crucible with lid was obtained, the mass of the crucible with lid was taken in gram using digital Metler weighing balance and the weight recorded as m_1. 1 g of soil sample was moistened with 0.5 ml distilled water. The wet soil was placed in the crucible and covered with the lid. The mass of the wet soil in the covered crucible was taken in gram and recorded as m_2. The wet sample was dried in the oven without the lid at 100°C until no change in mass was recorded. Mass of the dried sample in the crucible plus lid was taken in gram after cooling and recorded as m_3. The moisture content was calculated using the formula below;

Moisture content, w = mass of moisture / mass of dry soil × 100%

= (mass of container + wet soil) – (mass of container + dry soil) / (mass of container + dry soil) – (mass of container) × 100%

Moisture content of the soil was determined before pollution and after remediation. The experiment set-up was regularly moistened at 4 days interval during which it was mixed using a sterile spatula for circulation of oxygen.

Total organic carbon: The oven-drying method was used to determine the amount of organic matter in the soil (Schumacher, 2002). In a crucible, 10 gram of soil was weighed (ashing vessel). The soil-filled crucible was dried for 24 hours in a drying oven set at 200°C. It was taken out of the drying oven and set aside to cool on the bench. The difference between the initial and end sample weights was divided by the initial sample weight multiplied by 100 percent to compute organic matter content.

Percentage of organic matter (OM) = W_1-W_2 / W_1 × 100

Total organic carbon was determined before pollution and after remediation.

pH: The level of acidity or alkalinity of the soil and experiment set-up were determined using a hand-held digital pH meter. 1 g of the soil sample was weighed and dissolved in 20 ml distilled water in a sterile 50 ml beaker. The pH meter was calibrated using standardized buffer before immersion in the solution and reading taken and recorded. This procedure was repeated at 7 days intervals (Schumacher, 2002).

Temperature: The temperature of the soil was measured regularly using a manual thermometer. The thermometer was dipped into the soil and allowed for 10 minutes before reading was taken (Schumacher, 2002).

Soil texture: The soil texture was determined by feeling the texture of the soil as described by Thien (1979). Two tablespoons of the soil were placed on the left palm, water was added drop by drop while working the soil until it reached a sticky consistency. The wet soil was squeezed between the thumb and fore finger to form a flat ribbon. The flat ribbon was measured using a ruler to determine the length.

Analysis of total hydrocarbon

The component and total hydrocarbon of the crude oil used was determined by Gas chromatography-mass spectrometry (GC-MS) according to the method of Blystone *et al.* (2001). The oil was extracted from the polluted soil samples before remediation using the Soxhlet extractor and N-hexane as the extracting solvent. 10 g of the soil sample was weighed, placed in a filter paper and stapled. The sample was placed in the Soxhlet chamber, 20 ml of N-hexane was dropped in same chamber. 150 ml of the N-hexane was put in the round bottom flask placed in the heating mantle. The heating mantle was turned on and temperature set at 50°C which is below the boiling point of N-hexane (69°C). An inlet pipe was connected to a chiller through which water passes into the condenser and is removed through an outlet pipe. The chilled water was to cool the chamber and prevent the gas from escaping. This set-up was allowed to run for 2 hours after which it was stopped and the collected extract in the round bottom flask was transferred to a beaker and concentrated using the water bath at 50°C. The concentrated product was analyzed. This was referred to as the initial hydrocarbon analysis before remediation.

Determination of total heterotrophic bacterial count

The total heterotrophic bacterial count was determined by ten-fold serial dilutions of the soil sample and plated using the spread plate technique on nutrient agar. Fifty (50) mg of nystatin was added to prevent fungal growth. One (1) g of sample was weighed and dissolved in 10 ml sterile water in a 50 ml sterile beaker. The soil was allowed to sediment and the liquid decanted into another sterile beaker. 1 ml of the liquid (inoculum) was taken using a pipette and introduced into the first tube on the tube rack. The tubes were properly labelled and each contained 9 ml of sterile water. One (1) ml was taken from the first tube and introduced into the second, same was done serially to the ninth tube where 1ml was taken and discarded. 0.1 ml was taken from 10^{-4} dilution tubes and dropped on already gelled nutrient agar, the liquid was dispersed on the surface of the agar using a glass spreader, after inoculation, the plate was incubated for 24 hours, after which the plates were observed for appearance of colonies and it was counted using a colony counter (Cheesbrough, 2010). This was done in duplicates. This step was used to determine the total bacterial count of the unpolluted soil, poultry manure, untreated and treated experiment set-up before and after remediation.

Determination of total heterotrophic fungal count

The total heterotrophic fungal count was determined by tenfold serial dilutions of soil sample and plated using spread plate method on potato dextrose agar, and amended by adding 50 mg of chloramphenicol to avoid bacterial growth. One (1) g of sample was weighed and dissolved in 10 ml sterile water in a 50 ml sterile beaker. The soil was allowed to sediment and the liquid decanted into another sterile beaker. 1 ml of the liquid was taken using a pipette and introduced into the first tube on the tube rack. The tubes were properly labelled and each contained 9 ml of sterile water. 1ml was taken from the first tube and introduced into the second, same was done serially to the ninth tube where 1 ml was taken and discarded. 0.1 ml was taken from tubes 10^{-4} dilution and dropped on already gelled potato dextrose agar; the liquid was dispersed on the surface of the agar using a glass spreader. The plate was incubated on the bench for 72 hours after which the plates were observed for appearance of colonies and it was counted using a colony counter (Cheesbrough, 2010). This was done in duplicates. This step was used to determine the total fungi count of the unpolluted soil, poultry manure, untreated and treated experiment set-up before and after remediation.

Isolation and identification of total hydrocarbon-utilizing bacteria

The bacteria capable of utilizing hydrocarbon was isolated using mineral salt medium as composed by Onuorah *et al.* (2014) as follows; 10 g NaCl, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 1 g KH_2PO_4, 1.5 g K_2HPO_4, 0.5 g $NaNO_3$, 25 g agar-agar in 1 L distilled water. The substances were mixed in a sterile conical flask and sterilized in the autoclave at 121 °C for 15 minutes. The sterilized medium was cooled on the bench to 45 °C and evenly distributed into sterile petri dishes and allowed to solidify. The plates were dried in the oven at 40 °C for 20 minutes to remove moisture from the surface of the medium before inoculation. The unpolluted soil sample, poultry manure, untreated, and treated soil samples were serially diluted in various test tubes and inoculation sample taken from 10^{-1} dilution. This was inoculated onto the dried mineral salt medium. Filter paper soaked in crude oil was placed over each inoculated medium, this served as the source of carbon and energy to the organisms through vapor phase transfer. The plates were incubated at 37 °C for 72 hours. After incubation, the plates were observed for growth. The colonies seen were purified further by sub-culturing on nutrient agar. Gram stain and biochemical tests (catalase, coagulase, motility, indole, oxidase, methyl-red, Voges Proskauer) was carried out to identify the isolates (Cheesbrough, 2010).

Isolation and identification of total hydrocarbon-utilizing fungi

The fungi capable of utilizing hydrocarbon was isolated using mineral salt medium as composed by Onuorah *et al.* (2014) as follows; 10 g NaCl, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 1 g KH_2PO_4, 1.5 g K_2HPO_4, 0.5 g $NaNO_3$, 25 g agar-agar in 1 L distilled water. The substances were mixed in a sterile conical flask and sterilized in the autoclave at 121 °C for 15 minutes. The sterilized medium was cooled on the bench to 45 °C and evenly distributed into sterile petri dishes and allowed to solidify. The plates

were dried in the oven at 40 °C for 20 minutes to remove moisture from the surface of the medium before inoculation. The unpolluted soil sample, poultry manure, untreated, and treated soil samples were serially diluted in various test tubes and inoculation sample taken from 10^{-1} dilution. This was inoculated onto the dried mineral salt medium. Filter paper soaked in crude oil was placed over each inoculated medium, this served as the source of carbon and energy to the organisms through vapor phase transfer. The plates were incubated at 25°C for 14 days. After incubation, the plates were observed for growth and were further purified by sub-culturing on potato dextrose agar slant. The isolates were identified macroscopically and microscopically.

Colony identification: The colonies of both bacteria and fungi that appeared on the cultured media were studied for morphological characteristics according to Cheesbrough (2010).

Gram staining technique: Gram staining technique was used to distinguish Gram negative from Gram positive bacteria isolates. A grease free slide was obtained, and a loopful of saline was picked using a sterile wire loop. The saline was placed on the slide. A colony was picked from the cultured medium and emulsified in saline to make a smear. The smear was allowed to air dry and passed through flame to heat-fix. The slide was placed on the staining rack and the tap was turned on. A good quantity of crystal violet was poured on the slide and allowed to act for one minute after which it was washed off. Lugol's iodine was generously poured on the slide and allowed to act for one minute after which it was washed off. Acetone alcohol was mildly dropped on the slide and rinsed off immediately to avoid over differentiation. Safranin was poured on the slide and allowed to act for one minute after which it was washed off. The slide was blotted dry using a filter paper and allowed to dry completely on the bench. A drop of immersion oil was placed on the slide before it was viewed under the microscope using ×100 objective lens.

Biochemical tests: Further bacteria characterization using biochemical tests was performed to identify the isolates. The tests carried out were as follows:

Catalase test: This is used to identify the presence of the enzyme catalase in an organism. A colony was picked and placed on a dry slide, a drop of 3% H_2O_2 (hydrogen peroxide) was applied on the slide and observed for bubbles. Presence of strong bubbles indicates positive reaction while absence of bubbles indicates negative reaction.

Coagulase test: This is used to differentiate coagulase producing organisms from the organisms that do not produce coagulase. Two (2) grease free slides were obtained and a drop of saline was placed on each of the slides. A colony was picked and emulsified on each of the slides to make thick suspension. Undiluted human plasma was dropped on one of the slides and mixed thoroughly. The slide was observed for the presence of clumps. The other slide without plasma served as a negative control. Presence of clumps indicate positive reaction while absence of clumps indicates negative reaction.

Motility test: This is used to test the ability of an organism to move with the aid of flagella or fibrils. It differentiates motile bacteria from the non-motile ones. A grease free slide was obtained and a loop full of the organism was picked from 24 hours nutrient broth culture, it was placed on the slide and covered with a cover slip. This was viewed under the microscope using x10 then x40 objective lens. Motile organisms show directional movement.

Oxidase test: This is used to determine the ability of an organism to produce the cytochrome oxidase enzyme. A filter paper was obtained and impregnated with tetramethyl-p-phenylenediamine dihydrochloride, moistened the filter paper with sterile distilled water, a colony was picked and smeared on the filter paper. The inoculated zone was observed within 30 seconds for color change. The appearance of purple colour indicates positive reaction. No color change indicates negative reaction.

Indole test: This is used to determine the ability of some bacteria to deaminate the amino acid tryptophan to generate indole using tryptophanase enzyme. A sterilized test tube containing 4ml of tryptophan broth was aseptically inoculated with a colony from a 24 hrs culture plate. The tube was properly covered with a cotton wool and incubated at 37 °C for 24 hours. After incubation, 0.5 ml Kovac's reagent was dropped in the broth culture and observed for the presence of ring. Appearance of red ring at the top layer indicates positive while no colour change indicates a negative reaction.

Methyl-red: This test is used to determine the ability of an organism to produce acid during the fermentation of glucose. A broth culture containing methyl red reagents was prepared in test tubes, the test organism was inoculated into the medium and incubated at 37 °C for 24 hours. 2 drops of methyl red indicator were introduced into the medium after incubation and observed for change in colour. Appearance of red colour at the top layer of the medium indicates positive reaction while yellow colour indicates a negative reaction.

Voges Proskauer: This is used to determine the ability of an organism to produce acetyl methyl carbinol from glucose fermentation. The Voges Proskauer reagent was prepared and allowed to cool. The test organism was inoculated into the medium and incubated at 37 °C for 24 hrs. After incubation, six drops of 5% alpha naphthol was added and mixed properly. Two drops of potassium hydroxide were also added and mixed properly. The medium was observed within 30 minutes for colour change. Appearance of pink/red colour indicates positive reaction while light brown colour indicates a negative reaction. Microscopic

examination of the isolated fungi. The isolated fungi were examined microscopically by putting a drop of 70% alcohol on a grease free slide, the organism was picked using a sterile wire loop and teased in the alcohol. A drop of lacto-phenol cotton blue was added on the slide and carefully covered with a cover slip. The preparation was viewed under the microscope for identification of fungal spores and other structures using x10 and x40 objective lens (Cheesbrough, 2010).

Assessing the remediation of the crude oil polluted soil

The experiment was set-up by contaminating 100 g of soil with 50ml of crude oil in a perforated bowl in triplicates for the untreated sample. Same measurement of soil and oil was also set up in two units as experimental unit A, B and C. Unit A was treated with 2 g of poultry manure, unit B was treated with 4 g of the poultry manure, while unit C was without treatment. Moisture content, total organic carbon, temperature and pH of each unit of the experiment set-up was recorded as well as the microbial count. On day 28 (first month), after the experiment set-up, oil was extracted from the polluted soil samples using the Soxhlet extractor and N-hexane as the extracting solvent. This was done for the treated and untreated experiment set-up to check the level of degradation of the hydrocarbon present (Shabir *et al*., 2008).

Determination of the residual hydrocarbon content after remediation

Six (6) months after the experiment set-up, the oil was extracted from the polluted soil samples using the Soxhlet extractor and N-hexane as the extracting gas. 10 g of the soil sample was weighed, placed in a filter paper and stapled. The sample was placed in the Soxhlet chamber, 20 ml of N-hexane was dropped in same chamber. 150 ml of the N-hexane was put in the round bottom flask placed in the heating mantle. The heating mantle was turned on and temperature set at 50°C which is below the boiling point of N-hexane (69 °C). An inlet pipe was connected to a chiller through which water passes into the condenser and is removed through an outlet pipe. The chilled water was to cool the chamber and prevent the gas from escaping. This set-up was allowed to run until the hexane in the thimble was clear after which it was stopped and the collected extract in the round bottom flask was transferred to a beaker and concentrated using the water bath at 50 °C. The concentrated product was sent to an analytical laboratory for GC-MS analysis (Shabir *et al*., 2008). This was done for the treated and untreated experiment set-up.

Statistical analysis

Statistical analysis was performed using SPSS package (IBM SPSS Statistics 23) with two-way ANOVA. Least significant difference (LSD) was applied to test for significance at $P<0.05$ between the means.

RESULTS AND DISCUSSION

Physical properties of the soil

The physical properties of the soil are summarized in Table 1 and 1b below. pH of the untreated soil decreased from 8.1 to 6.7, while the polluted soil with 2 g manure treatment decreased from 8.4 to 7.2, also the p^H of the polluted soil with 4 g manure treatment decreased from 8.4 to 7.1. The moisture content of the unpolluted soil was 66%, the untreated polluted soil moisture content decreased from 54% to 45%, the polluted soil with 2 g manure treatment, it also decreased from 56% to 38%, the polluted soil with 4 g manure treatment decreased from 58% to 35%. Total organic carbon (TOC) of the soil before pollution was 47%, the untreated polluted soil TOC reduced from 47% to 30%, the polluted soil with 2 g manure treatment TOC decreased from 47% to 26%, while the polluted soil with 4 g manure treatment TOC decreased from 47% to 22%. Temperature was within the range of 27°C to 31°C. Hence, loamy soil.

Total hydrocarbon content

There was a total of 16 alkane hydrocarbons with total quantity of 189.4796 ug/ml. The hydrocarbons identified are C8, C9, C10, C11, C12, C13, C14, C16, C19, C20, C26, C28, C30, C33, C34, C35. C26 had the least value of 1.6452 ug/ml while C16 had the highest value of 27.0548 ug/ml. The physical analysis of the soil showed that the soil type is loamy, the initial moisture content of the treated polluted soil with 2 g amendment was 56%, which decreased to 44% after experiment period, while the treated polluted soil with 4 g amendment was initially 58% and decreased to 35% the reduction in moisture could be because microorganisms require water to accomplish their growth as documented by Abatenh *et al.* (2017). The total organic carbon (TOC) of the treated soil containing 2 g poultry manure decreased from 47% to 26%, the TOC of the treated soil containing 4 g decreased from 47% to 22%, TOC of the untreated soil decreased from 47% to 30%, while that of the unpolluted soil was 47%. TOC of the treated soil sample with 4 g manure showed the most decrease because it directly relates to the increase in microbial population and it could have used up the

Table 1. Summary of physical properties of the soil.

Sample	pH		Moisture content		Total organic carbon		Temperature	Soil type
	Initial	Final	Initial	Final	Initial	Final		
Unpolluted soil	ND		66%	ND	47%	ND	27°C – 31°C	Loamy
Untreated polluted soil	8.1	6.7	54%	45%	47%	30%	27°C – 31°C	Loamy
Treated polluted soil 2 g	8.4	7.2	56%	44%	47%	26%	27°C – 31°C	Loamy
Treated polluted soil 4 g	8.4	7.1	58%	35%	47%	22%	27°C – 31°C	Loamy

Table 2. Summary of physical properties of the soil.

S/N	Component (Hydrocarbon)	Name of hydrocarbon	Quantity ug/ml
1	C8	Octane	7.1257
2	C9	Nonane	12.14
3	C10	Decane	9.6073
4	C11	n-undecane	22.7636
5	C12	n-dodecane	23.6822
6	C13	n-tridecane	10.4425
7	C14	n-tetradecane	12.5783
8	C16	n-hexadecane	27.0548
9	C19	n-nonadecane	6.0465
10	C20	n-icosane	17.1479
11	C26	n-hexacosane	1.6452
12	C28	n-octacosane	7.9305
13	C30	n-triacontane	8.7814
14	C33	n-tritriacontane	5.5538
15	C35	n-pentatriacontane	10.0523
16	C36	n-hexatriacontane	6.9276
Total			**189.4796**

carbon present and it agrees with the work of Abatenh *et al.* (2017), who stated that temperature is the most determinant factor for the survival of microorganisms and compositions of the hydrocarbons. Very low temperatures can cause the transport channels within the microbial cells to shut down rendering it metabolically inactive. The rate of microbial activities rises with temperature, and reaches to its maximum level at an ideal temperature. Therefore, during this study, temperature was monitored and it was within the range 27°C to 31°C (Table 2).

The pH level reduced from 8.4 to 7.2 in the treated soil containing 2 g manure and reduced from 8.4 to 7.1 in the treated soil containing 4 g manure, the untreated soil decreased from 8.1 to 6.7. This agrees with the findings of Abatenh *et al.* (2017), who confirmed that a pH of 6.5-8.5 is optimal for biodegradation. Similar studies also confirmed that higher pH range between 6 and 9 provides better conditions for degradation of hydrocarbons since most microorganisms especially bacteria capable of degrading hydrocarbons perform best at pH conditions close to neutrality (Tanee and Kinako, 2008). The decrease in pH observed could have been as a result of the release of more exchangeable bases during microbial activity that reduced pH of the soil towards neutrality. Pawar (2015) documented that it may be that microbial community particularly bacteria were predominant and functional in degradation at pH 7.5. Conversely, fungal population were greatest at acidic soil pH and with some evident at alkaline soil implies that degradation at lower pH might be set off by fungal population.

Total heterotrophic bacterial count

The Total heterotrophic bacterial (THB) count is shown in Table 3. Pre-experiment, the unpolluted soil had 3.4 x 10^5 cfu/ml and 3.3 x 10^4 post-experiment, this showed decrease in growth rate. Pre-experiment, poultry manure had 4.2 x 10^5 cfu/ml before it was introduced into the soil as treatment. Pre-experiment, the untreated polluted soil had 3.9 x 10^5 cfu/ml and 2.4 x 10^5 post-experiment. Pre-experiment, the treated polluted soil containing 2 g poultry manure had 2.9 x10^5 cfu/ml and 3.9 x 10^6cfu/ml post-experiment. Pre-experiment, the treated polluted soil containing 4 g poultry manure had 3.0 x 10^5 cfu/ml and 4.1 x 10^6 post-experiment. THB count of the treated polluted soil containing 4 g poultry manure had the highest growth rate of 4.1 x 10^6 cfu/ml while the unpolluted had the least of 3.3 x 10^4 cfu/ml after six months.

Total heterotrophic fungal count

Total heterotrophic fungi (THF) count result is shown in Table 4. The unpolluted soil had 1.2 x 10^5 cfu/ml and 1.6 x 10^5cfu/ml post -experiment, this showed low growth rate. Pre-experiment, poultry manure had 8.0 x 10^4 cfu/ml before it was introduced into the soil as treatment. Pre-experiment, the untreated polluted soil had 6.0 x 10^4 cfu/ml and 1.8 x 10^5 post-experiment. Pre-experiment, the treated polluted soil containing 2 g poultry manure had 1.1 x 10^5 cfu/ml and 2.8 x 10^5 post-experiment. Pre-experiment, the treated polluted soil containing 4g poultry manure had 1.3 x 10^5 cfu/ml and 3.1 x 10^5 cfu/ml post-

Table 3. Total heterotrophic bacterial count.

Sample	THBC (cfu/ml) Initial	THBC (cfu/ml) Final
Unpolluted soil	3.4 x 10^5	3.3 x 10^4
Poultry manure	4.2 x 10^5	ND
Untreated polluted soil	3.9 x 10^5	2.4 x 10^5
Treated polluted soil 2g	2.9 x 10^5	3.9 x 10^6
Treated polluted soil 4g	3.0 x 10^5	4.1 x 10^6

Table 4. Total heterotrophic fungal count.

Sample	THF (cfu/ml) Initial	THF (cfu/ml) Final
Unpolluted soil	1.2 x 10^5	1.6 x 10^5
Poultry manure	8.0 x 10^4	ND
Untreated polluted soil	6.0 x 10^4	1.8 x 10^5
Treated polluted soil 2 g	1.1 x 10^5	2.8 x 10^5
Treated polluted soil 4 g	1.3 x 10^5	3.1 x 10^5

Table 5. Morphological and biochemical properties of hydrocarbon-utilizing bacterial isolates.

Sample	Isolates	Colony	Gram reaction	Motility	Catalase	Coagulase	Indole test	VogesProskauer	Methyl red test	Oxidase	Organism isolated
Unpolluted soil	1	small, round, creamy	Gram negative rod	+ve	+ve	-ve	-ve	-ve	-ve	+ve	*Pseudomonas species*
	2	off-white, rough edge, circular, opaque	Gram positive rod	+ve	+ve	-ve	-ve	+ve	-ve	-ve	*Bacillus species*
Poultry manure	3	small, round, creamy	Gram negative rod	+ve	+ve	-ve	-ve	-ve	-ve	+ve	*Pseudomonas species*
	4	golden yellow, round, smooth, entire edge	Gram positive cocci	-ve	+ve	+ve	-ve	+ve	+ve	-ve	*Staphylococcus species*
Polluted soil	5	small, round, creamy	Gram negative rod	+ve	+ve	-ve	-ve	-ve	-ve	+ve	*Pseudomonas species*
	6	off-white, rough edge, circular, opaque	Gram positive rod	+ve	+ve	-ve	-ve	+ve	-ve	-ve	*Bacillus species*
	7	golden yellow, round, smooth, entire edge	Gram positive cocci	-ve	+ve	+ve	-ve	+ve	+ve	-ve	*Staphylococcus species*

Table 6. Macroscopic and microscopic properties of hydrocarbon-utilizing fungi.

Sample	Isolate	Colony characteristics	Microscopic observation	Organism isolated
Unpolluted soil	1	Greenish yellow	Unbranched conidiophores, aseptate hyphae	*Aspergillus niger*
	2	Whitish, woolly	Branched conidiophores, smooth and rough conidia in chain	*Fusarium solani*
Poultry manure	3	creamy circular and mucoid	Circular spores, no hyphae	*Candida albicans*
	4	Whitish, woolly	Branched conidiophores, smooth and rough conidia in chain	*Fusarium solani*
Polluted soil	5	Greenish yellow	Unbranched conidiophores, aseptate hyphae	*Aspergillus niger*
	6	Whitish, woolly	Branched conidiophores, smooth and rough conidia in chain	*Fusarium solani*
	7	creamy circular and mucoid	Circular spores, no hyphae	*Candida albicans*

Table 7. Total hydrocarbon after first month

S.N.	Content	Name of hydrocarbon	Untreated soil (ug/ml)	Treated soil 2 g (ug/ml)	Treated soil 4 g (ug/ml)
1	C6	Hexane	-	2.3341	0.2235
2	C7	Heptane	-	0.48435	-
3	C8	Octane	21.77773333	3.0438	13.58115
4	C9	Nonane	13.30193333	19.3947	17.88885
5	C10	Decane	10.0155	8.2193	7.671
6	C11	n-undecane	20.0648	28.6951	19.65595
7	C12	n-dodecane	26.38273333	11.0136	12.95335
8	C13	n-tridecane	14.92826667	23.5074	20.5429
9	C14	n-tetradecane	13.116	7.1137	7.28785
10	C15	n-pentadecane	-	6.38485	27.26065
11	C16	n-hexadecane	27.02576667	18.9228	7.6346
12	C17	n-heptadecane	-	13.8963	6.7577
13	C18	n-octadecane	-	8.43015	10.58025
14	C19	n-nonadecane	6.6736	-	10.59885
15	C20	n-icosane	12.84103333	14.0543	14.12095
16	C26	n-hexacosane	1.77985	3.96745	4.7609
17	C27	n-heptacosane	-	5.96695	8.2471
18	C28	n-octacosane	8.56245	-	-
19	C30	n-triacontane	8.35075	17.2124	12.9435
20	C31	n-hentriacontane	-	10.0473	7.78895
21	C32	n-dotriacontane	-	10.958	10.66655
22	C33	n-tritriacontane	4.85255	10.9737	8.3616
23	C34	n-tetratriacontane	-	3.40712	3.3597
24	C35	n-pentatriacontane	15.9679	17.4387	11.8601
Total			**205.6409**	**245.4661**	**244.7460**

experiment. THF count of the treated polluted soil containing 4 g poultry manure had the highest growth rate of 3.1 x 10^5cfu/ml while the unpolluted had the least of 1.6 x 10^5cfu/ml after six months. The total heterotrophic count of bacteria increased in the treated soil samples compared to the untreated soil that decreased after the six months study period (Table 3) the indigenous bacteria in the untreated soil may have had limited nutrient to survive. The unpolluted soil recorded the least growth, this could also be as a result of lack of nutrient. Also, there was increase in growth rate of the total heterotrophic fungal count (Table 4) where the treated soil samples recorded the highest growth and the colony forming unit of the untreated soil decreased. The unpolluted soil had the least growth.

Isolation and characterization of hydrocarbon-utilizing bacteria

Hydrocarbon-utilizing bacteria were isolated from the unpolluted soil sample, poultry manure and polluted soil samples. *Pseudomonas* species and *Bacillus* species were isolated from the unpolluted soil, *Staphylococcus* species and *Pseudomonas* species were isolated from the poultry manure, while *Bacillus* species, *Pseudomonas* species and *Staphylococcus* species were isolated from the polluted and treated soil sample. The morphological and biochemical properties of the hydrocarbon-utilizing bacteria isolated are presented in Table 5.

Table 8. Residual hydrocarbon after six months.

S.N.	Content	Name of hydrocarbon	Untreated soil (ug/ml)	Treated soil 2 g (ug/ml)	Treated soil 4 g (ug/ml)
1	C4	Butane	-	0.55925	-
2	C7	Heptane	3.80935	-	-
3	C8	Octane	3.1069	2.14475	0.70485
4	C9	Nonane	4.14335	4.3889	3.1134
5	C10	Decane	5.06385	5.5647	2.2223
6	C11	n-undecane	7.36555	4.19495	1.4539
7	C12	n-dodecane	1.74975	1.1072	0.5433
8	C13	n-tridecane	10.7466	6.97175	5.5576
9	C14	n-tetradecane	2.1486	0.64285	0.9275
10	C16	n-hexadecane	5.68085	3.59435	6.98865
11	C19	n-nonadecane	5.18355	1.5084	-
12	C20	n-icosane	6.26935	1.5259	1.7189
13	C26	n-hexacosane	6.59745	1.1946	1.12005
14	C28	n-octacosane	5.18275	1.4255	0.9158
15	C30	n-triacontane	5.3537	1.098	2.3089
16	C33	n-tritriacontane	12.414	6.5121	3.50475
17	C34	n-tetratriacontane	10.202	4.29825	4.4254
18	C35	n-pentatriacontane	5.6809	5.25555	2.19755
19	C36	n-hexatriacontane	5.6918	4.2056	3.5626
20	C37	n-heptatriacontane	0.7179	-	-
Total			**107.1082**	**56.1926**	**41.2655**

Isolation and identification of hydrocarbon-utilizing fungi

Hydrocarbon utilizing fungi were isolated from the unpolluted soil, poultry manure and polluted soil samples. The isolates are *Aspergillus niger*, *Fusarium solani* and *Candida albicans*. The microscopic and macroscopic properties of the fungal isolates are presented in Table 6.

The organisms isolated and characterized that played roles in the remediation include the *Pseudomonas* species, *Bacillus* species, *Staphylococcus* species, *Aspergillus* species, *Fusarium* and *Candida* species as presented in Table 5 and 6. The isolation of *Fusarium* species confirms its role in biodegradation of aliphatic hydrocarbons as reported by Hiyadat and Tachibana (2012). Burghal *et al.* (2016) reported on the isolation of *Aspergillus* species and Candida species from Petroleum polluted soil. Abha *et al.* (2013) reported on the participation of *Bacillus* species and *Pseudomonas* on Bioremediation of oil and phenol contents in refinery waste water. This current research supports the claim that these organisms possess hydrocarbon degradative abilities. The bacteria and fungi synergistically degraded the pollutant. There have been reports that fungi and bacteria in degrading pollutants by attacking the component breaking it down, thereby exposing more surface areas where bacteria could adhere to easily.

Bioremediation of crude oil polluted soil

The level of remediation of the polluted soil amended with the poultry manure and the unamended polluted soil (natural attenuation) for the first one month of the remediation experiment is presented in Table 7, while the residual hydrocarbon content of the polluted soil amended with poultry manure and the unamended soil after six months is presented in Table 8. Table 8 shows that the quantity of the hydrocarbon decreased after the remediation period. The reduction was higher in the polluted soil amended with 4 g poultry manure with the value of 41.26545 ug/ml followed by the polluted soil amended with 2 g poultry manure with the value of 56.1926 ug/ml and least in the unamended soil that underwent natural attenuation with the value of 107.1082 ug/ml.

The evaluation of the total petroleum hydrocarbon showed the degree of efficiency of bioaugmentation over natural attenuation. Kensa (2011) had reported that bioaugmentation is a better alternative to natural attenuation because it speeds up degradation rate and saves time. This research supports that claim. There was significant decrease as seen in the residual hydrocarbon analysis (Table 8), the treated soil had the least quantity of total hydrocarbon after remediation compared to the untreated soil, this implies that it will take a longer time for the soil to clean itself. With the introduction of poultry manure to the polluted soil, nutrient was provided and biodegrading organisms added to aid the indigenous organisms in utilizing the pollutant. The indigenous organisms present in the untreated soil attempted to break down the pollutant but it was not so effective compared to the treated soil and some of the compounds may have been lost through volatility. The attempt could be as confirmed by Abatenh *et al.* (2017) that the presence of small quantity of contaminant can act as stimulant by switching on the operons for bioremediation enzymes.

From the analysis, the treated soil that contained 4 g of poultry manure performed better by 22% than that which contained 2 g with 30%, the untreated polluted reduced by 57%. The addition of poultry manure may have enhanced the growth of microorganisms which significantly reduced the quantity of the hydrocarbon from initial quantity of 189.4796 ug/ml to 41.26545 ug/ml in the treated soil with 4 g poultry manure. Hydrocarbon C14 was originally higher in quantity of about 27.88 ug/ml and was significantly reduced to 0.928 ug/ml after remediation period in

the treated soil containing 4 g manure. Also, C4, C7, C19, and C37 were completely removed from the treated soil containing 4 g manure as shown in Table 8. From the statistical data, the standard deviation value of the treated soil with 4 g of poultry manure was more significant post-experiment. Analysis of Variance (ANOVA) also shows statistical significance at 5% confidence interval, with the p-value less than 0.05 this implies that the result from this research is against the null hypothesis of this study therefore the alternative hypothesis should be accepted. More so, the effect level of the 4 g treatment from the regression model analysis is 0.982 and can be considered high.

Conclusion

The under ideal environmental conditions, the undesirable impacts of oil pollution on soils can be mitigated by adding poultry manure. The amendment material increased soil microbial activity, which resulted in a significant decline in total hydrocarbon intensity in the modified soils. Poultry manure is reasonably inexpensive, and a widely available raw material, that if carefully managed, has the potential to be an efficient bioaugmentation agent for the clean-up of oil polluted soil.

Conflict of interests

Authors declare that no conflict of interest exists.

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