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Full Length Research Paper

Crude oil polluted soil remediation using poultry dung (chicken manure)

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Remediating crude oil polluted soil in developing countries by environment-friendly approach has attracted scientific interest. Sampled crude oil and poultry dung were obtained from oil pit and farm in Warri, Delta State. 500 g/kg of crude oil polluted soil was weighed into four different containers labeled A, B, C and D; the soil was amended with the application of sundried poultry manure at various weights of 50, 75 and 100 g/kg respectively leaving sample D without amendment as control for a period of 42 days. Isolation and identification process of bacteria strains were carried out using standard spread plate method on nutrient agar. Ten bacteria isolates were obtained from the amended and control soil in this research. Genus from *pseudomonas* and *staphylococcus* has the highest representation of three species each. The rate of degradation in the sample was determined by gravimetric analysis. The total petroleum hydrocarbon (TPH) present in sample D (control) initially was 4550.08 mg/kg. Samples A, B and C after remediation period reduced to the value of 3410.61, 2664.90 and 1598.95 mg/kg, respectively. The total hydrocarbon utilizing bacteria (HUB) increased gradually in the sample A, B, C and D to 7×10^{-4}, $7.9\ 10^{-4}$, 8.0×10^{-4} and 5.0×10^{-4} respectively. The total heterotrophic bacteria count (THB) increased from 10×10^{-5} at zero week to 8.3×10^{-5}, $8.5\ 10^{-5}$, 8.6×10^{-5} and 7.3×10^{-5} in the samples respectively. Physiochemical analysis of nitrogen increased from 0.04 to 0.17 mg/kg and phosphorous also increased from 1.72 to 6.73 mg/kg after the period of 42 days. This approach showed increased remediating effect of poultry dung on crude oil polluted soil.

Key words: Crude oil pollution, remediation, chicken dung, microorganism.

INTRODUCTION

Several methods of oil degradation have been developed. These include the use of chemical, physical and biological methods (bio-remediation). Bio-remediation is a method that refers to the use of natural occurring microorganisms or genetically isolated microorganisms by man to detoxify man-made pollutants. Bioremediation relies on bacteria, plants and fungi to degrade, breakdown, transform or remove contaminants or impairments of quality from the contaminated soil and water (Ijiah and Antai, 2003). Bio-remediation techniques

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are non-invasive, cost effective, and environment friendly which does not produce toxic by-product compared to their counterparts (physical and chemical methods of remediation). In addition bioremediation conserves soil texture and characteristics (Perelo, 2010).

Environmental degradation caused by exploitation and exploration of petroleum products or hydrocarbon among communities in Nigeria has been quite problematic. Organic and inorganic compounds are largely released into the environment every year, with the implication of human activities. For instance the disposal of oil spills from blow out and pipelines rupture are the most common source of Petroleum contamination (Resinger, 1995). Crude oil is a complex mixture of diverse hydrocarbons including alkanes, aromatics, branched and non-branched hydrocarbons compound including heavy metals such as hetero-atom of nitrogen, sulfur and oxygen which are polar fractions and asphaltens. The high request or demand for petroleum products in form of gas oil, engine lubricating oil, cooking gas, aviation fuel increases its production and then eventually results in oil spills and hydrocarbon pollution of the environments (Resinger, 1995). In Nigeria, the Niger Delta has witnessed several instance of oil spills in some communities like Olomoro and Uzere in Isoko South Local Government Area, Owhe-Ologbo and Erumukohwarian in Ugehil North. Mostly mangrove swamp and marsh (Eboh, 1995) have been recorded that oil contamination causes slow rate of germination in plants and reduces soil fertility. Adam and Duncan (2002) reported that this effect could be due to oil which acts as physical blamer or reducing access of the seeds to water and oxygen. It has been report that 1.7 to 8.8 million metric tons of petroleum hydrocarbon escape into the soil and water bodies every year or annually. This has caused critical environmental and health defects and increasing alternation has been paid for developing and implementing innovative technology for cleaning up this contamination. Hydrocarbon contamination of soil and fresh water especially poly-aromatic hydrocarbons (PAHs) attract public attention because PAHs are toxic, mutagenic and carcinogenic (Clemente et al., 2001).These challenges are quest for environmental sustainability that motivated researchers to search for organic substrates, which would serve as alternatives to synthetics fertilizers to enhance bio-remediation. Therefore this research has been considered because chicken dung is readily available, transportable and affordable.

MATERIALS AND METHODS

Samples collection

Crude oil contaminated soil

A known weight of 2500 g of crude oil contaminated was collected from chevron pit in Titan project Nigeria limited (TPNL) located at Ogula Forcados Terminal, Burutu L.G.A, Delta State. It was collected in a sterile polythene bag at the depth of 0 -12 cm, using a sterile spade and transported to Petroleum Training Institute Microbiology Laboratory. The chicken dung: The chicken dung was collected from poultry farm in Garki market found along Benin Sapele Road, Warri Delta State. It was collected into a Ziploc bag by packing with hand trowel and was transported to the microbiology laboratory in Petroleum Training Institute for further analysis.

Methods of sample analysis

Sample preparation, amendment and stimulation

The crude oil contaminated soil was sun dried for a period of one week and sieved through a 2 mm mesh sieve. 500 g/kg of crude oil contaminated soil was weighed using an electronic weighing balance into four clean experimental bowl respectively, labeled A, B, C and D. The chicken dung was also sun dried for a period of one week after which it was sieved to remove impurities from it. In different variations, 50, 75, and 100 g of poultry droppings were then added to the crude oil contaminated soil weighed in the experimental bowls A, B and C, respectively. While bowl D was left as the control without amendment. The set up was then left for a period of 6 weeks while the microbial analysis and the physiochemical analysis were carried out at the zero weeks and subsequently at one week intervals with proper supervision.

Microbial enumeration

A set up of six test tubes containing 9 mL of distilled water was arranged into two places in a test tubes rack then 1 g of poultry dung and the contaminated soil was weighed and introduced into the first beakers respectively. It was shaken for even distribution after which 1.0 ml of aliquot (the mixture) was aseptically transferred into the second test tube to give 10^{-1} (tenfold) dilution; further tenfold serial dilution was carried out to factor 10^{-6} dilution factor. After the serial dilution process, nutrient agar was prepared by measuring 7 g into 250 ml of volumetric flasks and shaken very well until the agar is readily dissolved. It was then autoclaved at 5000 read per minute (rpm), with temperature for some minutes when the agar was fully sterilized. It was left to cool for some minutes on a sterile working bench until when it was favorably to handle. The agar was poured into sterile Petri dishes (two plates for each sample) and was blended with Nystatin to suppress the growth of fungi growth. The plates were allowed to solidify within 24 h and also to check if it is contaminated while pouring or not. Meanwhile, after 24 h, 0.1 ml of the serial dilutions from both samples were measured using a micro pipette from factor 10^{-4} and 10^{-6} which were afterwards inoculated into the poured plate and gently swirled using the inoculums spreader. Then the plate was inverted inside the fume cupboard at room temperature (28°C) for 24 h, after which bacterial colonies that grew were counted using the standard plate counting techniques (International Pharmacopoeia, 2018).

Number of colonies population was calculated using the formula:

$$\frac{\text{No of colonies} \times \text{dilution factor}}{\text{Volume of culture plate}}$$

Isolation techniques

Discrete colonies from the primary plate were picked with the help

of a sterile wire loop. They were sub-cultured into a fresh agar plate and incubated for another 48 h inside the fume cupboard. The morphological characteristics of the isolates were observed and identified after 48 h of incubation; each organism with different morphological characteristics was further plated in a slant bottle and preserved in the refrigerator at 40°C for biochemical characterization.

Determination of culturable hydrocarbon utilizing bacterial (HUB)

In determining the hydrocarbon utilizing bacteria, serial dilution and pour plate technique was adopted. Mineral salt medium (MSM) was prepared and sterilized by autoclaving at 121°C, 15 psi for 15 min and dispensed into Petri dishes. The plates were inoculated and duplicated with 0.1ml aliquot of the sample serially diluted at 10^{-4} and 10^{-6} dilution factor. The plates were incubated at 280C+/-200C for 7days and the colonies were counted from triplets; mean values were recorded in colony forming units per gram (cfu/g).

Morphological characteristics

The isolates were characterized and identified based on their cultural characteristics and biochemical procedures and reaction as follows.

Gram reaction

This was carried out to differentiate gram positive from gram-negative organisms.

Procedure: A wire loop was sterilized in Bunsen burner and allowed to cool; then a loopful of growth was collected from the agar plate and applied on a clean grease-free slide. A drop of normal saline was added, emulsified and heat fixed by passing over a flame three times. The smear was flooded with crystal violet for 30-60 s and then covered with iodine (as mordant) for 30-60 s and then washed off. It was decolorized with acetone until no color runs off the slide and rinsed immediately. The slide was covered with Safranine dye for 1 min and then washed off with clean water. The slide was kept in a track to air dry after wiping the back with cotton wool. The stand smear was then examined microscopically under oil immersion at x100 objective lens. The gram-positive bacteria appeared dark purple while gram-negative bacteria appeared red.

Motility test

Motility test was aimed at identifying motile bacteria.

Procedure: A drop of normal saline was placed on a sterile slide and colony of test organism was suspended then covered with a cover slip. The slide was examined microscopically using x10 and x40 objective lenses. Movement in different directions gave a positive test while static position gave a negative test.

Catalase test

This was carried out to differentiate those bacteria that produce enzyme catalase such as Staphylococcus aureus and *Escherichia coli* which were also used as positive and negative controls respectively.

Procedure: Three milliliters of hydrogen peroxide solution was poured into a sterile test tube. Then a sterile glass rod was used to collect several colonies of the test organisms and inoculated into the hydrogen peroxide solution. It was observed for immediate active bubbling for positive test.

Oxidase test

This was carried out to identify bacteria species that will produce the *cytochromeoxidase* enzyme, *Pseudomonas aeruginosa* and *E. coli* employed as positive and negative controls respectively.

Procedure: A piece of filer paper was placed in a clean Petri dish and 2-3 drops of fresh or nascent oxidase reagent were added. A colony of test organism was collected using a glass rod and smeared on the filter paper and observed. Blue-purple color within few seconds showed a positive test.

Citrate test

This test is based on the ability of an organism to use citrates as its source of carbon. It was used to identify the Enterobacteria.

Procedure: Simon's citrate agar medium was prepared in a slant bijou bottle. A sterile wire loop was used to inoculate the test organism onto the slant medium and incubated at 30°C for 48 h after which it was examined for color formation. A bright blue color in the medium gave a positive citrate test. *Klebsiella pneumonia* and *E. coli* were employed as positive and negative controls respectively.

Methyl red test

This was carried out to identify Enterobacteria based on the ability to produce and maintain stable acid end -product from glucose fermentation. *E. coli* was used as positive control.

Procedure: Glucose phosphate peptone was used for the inoculations of test organisms and incubated for 48 h at 37°C after which few drops of methyl red solutions were added to the culture and read immediately. Formation of red color immediately showed a positive test.

Physiochemical characterisation

Determination of pH

The pH of the crude oil contaminated soil was determined using pH meter (Jenway 3015 UK). Ten grams of sun dried soil (passed through 2 mm sieve) was weighed into a 20 ml beaker and 5 ml of distilled water was added. The suspension was shaken with the use a mechanical shaker for 25-30 min, then allowed to stand for 50 min and stirred occasionally with a glass rod. The electrode was rinsed with water and dried with a piece of tissue. The electrode was inserted into the partly settled suspension to be analyzed and the pH range of the solution was measured. The pH meter was calibrated at pH 7.0 (Manas et al., 2007).

Determination of total nitrates (NO_3)

Total Nitrogen of the soil samples was determined by the macro Kjedahl digestion method. 100 g of potassium chloride was weighed into 1000 mL volumetric flask; 800 mL of deionized water was added to it and stored thoroughly until it dissolved. Distilled water was added to make it up to 1000 ml, the volumetric flask was

Table 1. Physiochemical and microbial baseline data of crude oil contaminated soil and amended soil at zero weeks.

Parameter	Value
pH	7.5
Nitrate (%)	0.04
Phosphate (mg/kg)	1.72
TPH (mg/kg)	4550.08
THBC (cfu/g)	7.0×10^5
THUBC (cfu/g)	4.5×10^4

Table 2. Physiochemical values of total nitrogen (mg/kg) on amendment soil within 6 weeks (42days) of remediation.

Duration (days)	Sample A (mg/kg)	Sample B (mg/kg)	Sample C (mg/kg)
0	0.04	0.04	0.04
14	0.06	0.07	0.09
28	0.08	0.09	0.12
42	0.10	0.13	0.17

capped with paraffin and inverted several times to mix. 4 g of the amended soil sample was weighed and placed in a conical flask. 20 mL of the extraction solution was added to the soil sample and the flask was played in a mechanical shaker box and shaken for 1 h at a given speed. The content was filtered using a line filter paper. Filtrate was analyzed using Atomic Absorption Spectrophotometer (Manas et al., 2007).

Determination of phosphorus

50 ml of 0.5 M Hydrochloric acid solution was added to a weighed 5 g of soil sample in a conical flask. The mixture was shaken and allowed for a 50 min digestion. A filtration apparatus was set up and the mixture was filtered. The filtrate was then collected in beaker. 1.0 g of phosphate mixture was weighed and dissolved in 100 ml of 2 M Sulfuric acid which was left to stand for about 20 min. The content in the beaker was left to stand for about 30 min for full color formation. UV-Visible Spectrometer was used to determine the absorbance of the phosphate concentration of the soil sample (Carter and Gregorich, 2008).

Estimation of total petroleum hydrocarbon (TPH)

Using soil sample extraction method (ASTM D575^6-97), 10 g of each of the amended and contaminated soil was weighed into an organic free amber glass container and 10 ml of the extractant (N-hexane, dichloromethane and acetone in ratio 2:1:1) was added, using a mechanical shaker. The mixture was gently shaken for 30 min. The sample was extracted from the solutions using a sonicator and was filtered. The final volume of the extract was stored in a dried organic free chromic acid pre-clean vial; 1.00 µl was withdrawn using an automated gas-tight syringe of the auto sampler and analyzed by direct injection into the GC-FID preset at specific condition. The analysis was allowed to run and quantify data; and at various temperatures, the hydrocarbon peaks were shown on the screen and the analysis of the results was obtained. The extract remaining was refrigerated at about 4°C for further analysis (Osuji et al., 2005).

Data processing and reporting for soil and sediment

The total petroleum hydrocarbon (TPH) concentration (mg/kg) of samples (R)

$$\frac{\text{Instrument reading (total conc. in mg/L)} \times \text{Volume of the extract}}{\text{Weight of the samples (in kg)}} \times \text{DF}$$

The actual TPH (mg/kg) = R X DF; Where DF: Dilution factor; GF: Calibration Graph Factor.

RESULTS

Tables 1 to 3 show the initial values of the physiochemical and microbial characteristics of the crude oil contaminated soil and the soil amended with chicken dung at the beginning of the practical process. The TPH available in the crude oil contaminated soil was 4550.08 mg/kg, the total hydrocarbons utilizing bacteria count (THUBC) was obtained as 4.5×10^4 cfu/g and the total heterotrophic bacterial count (THBC) was obtained to be 7.0×10^5 cfu/g; while the nitrate, phosphate and the pH values were 0.04, 1.72 and 7.5 respectively (Figure 1). Microbiological characteristics of amended soil within 42 days of bioremediation (Table 8)

DISCUSSION

The initial/baseline physiochemical and microbiological characteristics of the crude oil polluted soil and the amended soil are analyzed in Table 1. The pH, Nitrate, and Phosphate were 7.5, 0.04 and 1.72 mg/kg respectively. The result in this table shows a low value of 0.04 mg/kg for nitrogen and a higher value of 1.72 mg/kg

Table 3. Physiochemical values of total phosphorous in the amended soil within the six weeks of bioremediation.

Duration (days)	Sample A (mg/kg)	Sample B (mg/kg)	Sample C (mg/kg)
0	1.72	1.72	1.72
14	2.91	3.72	4.86
28	3.42	4.48	5.71
42	4.11	5.26	6.73

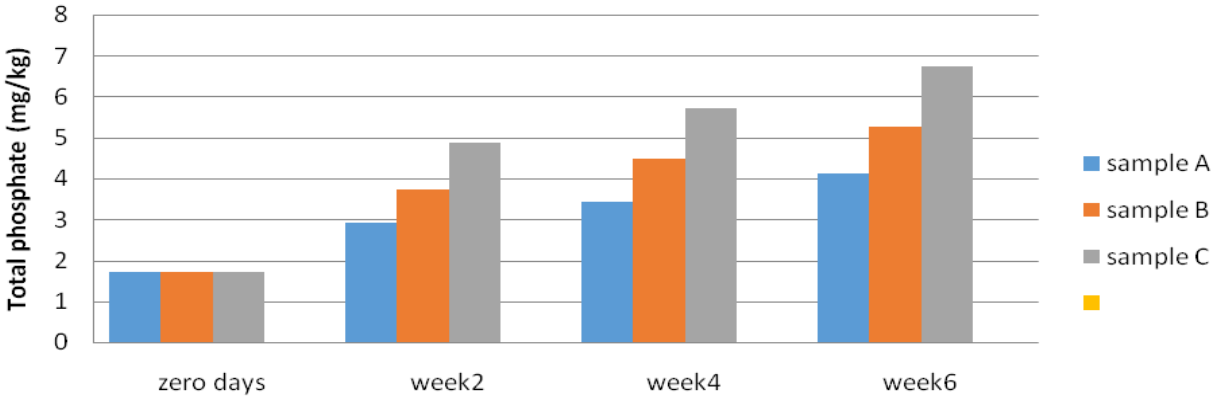


Figure 1. Effect of amendment concentration on total phosphorous. A graph showing the increase phosphorous.

for total phosphorous content with a higher content of hydrogen ion concentration (pH) of 7.5 due to the presence of hydrocarbon present in the soil. Tables 2 and 3 show the physiochemical properties of total nitrogen and phosphorous content in the amended soil for a period of six weeks (42 days). There is a progressive increase from the zero weeks to the sixth weeks of remediation in the properties of the soil with the value of 0.04-0.12 (mg/kg) in nitrogen and 1.72-6.73 (mg/kg) in phosphorous. This has been possible because the microbes present in the chicken dung found food (hydrocarbons) to feed on in the soil thereby increasing in population.

The total counts of heterotrophic bacteria populations (HBP's) are presented in Table 4 and Figures 2 to 4. The results showed that there was observed increased in the amended soil and control sample from 0 to 14days and decreased from 28 to 42 days. There are values of 8.3×10^5, 8.5×10^5 and 8.6×10^5 in sample A, B, C respectively. While a slow increase value of 7.3×10^5 was observed in sample D (control). However, the bacteria population from soil treated with chicken manure at 100 g/kg was significantly higher while that of control was reversed. This agreed with the position of Njoku and Obi (2009) that nutrient deficiencies which arise due to petroleum hydrocarbon contamination of soil may however be offset by addition of chicken dung to the soil. The low results obtained might be attributed probably to initial inhibition of water and nutrient uptake due to the hydrophobic character of crude oil.

Table 5 showed a progressive rise in hydrocarbon utilizing bacteria population (HUBP's) observed over the course of experiment in all the contaminated soils. There was an enhanced increased growth in the HUBP's given the values of 7.9×10^4, 8.0×10^4 and 9.0×10^4 (cfu/g) and a lower decreased value of 5.0×10^4 (cfu/g) in the un-amended soil. This finding is in consistency with the report of Thieman and Palladino (2009) that addition of nutrients in the soil leads to increase in number of microorganisms, enhances their growth and increases the rate of biodegradation. It was also observed that the population increased with the increase in the weight of poultry manure. The increase in the effect of manure on population's growth of soil microorganism, according to Brandi et al. (2013), may not be unconnected with the composition of the poultry manure.

Table 6 were obtained from Gas Chromatographic (GC) that showed a decrease in the TPH from week zero to 6 weeks (42 days) in all the samples A, B and C. The TPH content was greater in the control system (Sample D) with a value of 4550.08 mg/kg at the beginning of the experiment. This value increased gradually from week one to week six (42 days) of remediation with different rate in the samples A, B and C.

The values obtained were 2406.55, 2076.74 and 1598.95 m/kg respectively. Therefore, the rapid decrease observed was as a result of the microbes present in the chicken dung serving as organic supplement for the

Table 4. Total heterotrophic bacteria population amended non-amended crude contaminated soil with different variations of chicken dung.

Durations (days)	Sample A	Sample B	Sample C	Sample D (Control)
0	7.0	7.0	7.0	7.0
14	7.2	7.4	7.8	7.0
28	7.8	8.2	8.4	7.1
42	8.3	8.5	8.6	7.3

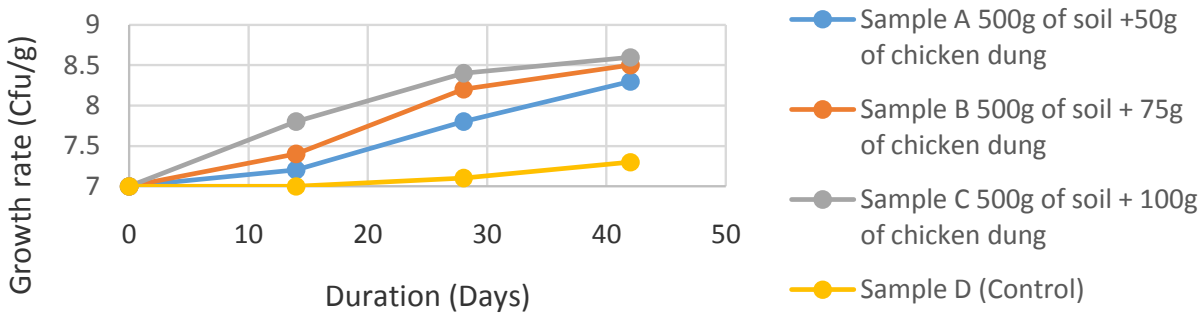


Figure 2. Total heterotrophic bacteria count.

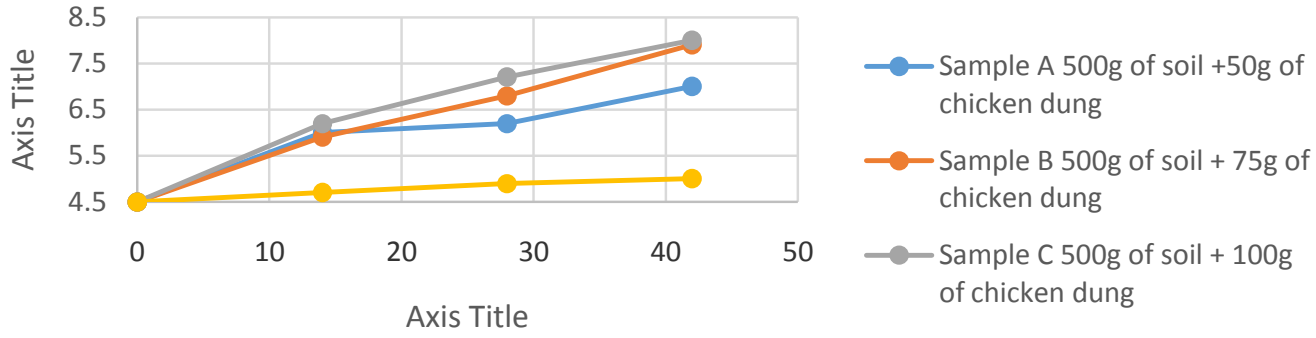


Figure 3. Total hydrocarbon utilizing bacteria. Chart showing total hydrocarbon utilizing bacteria growth.

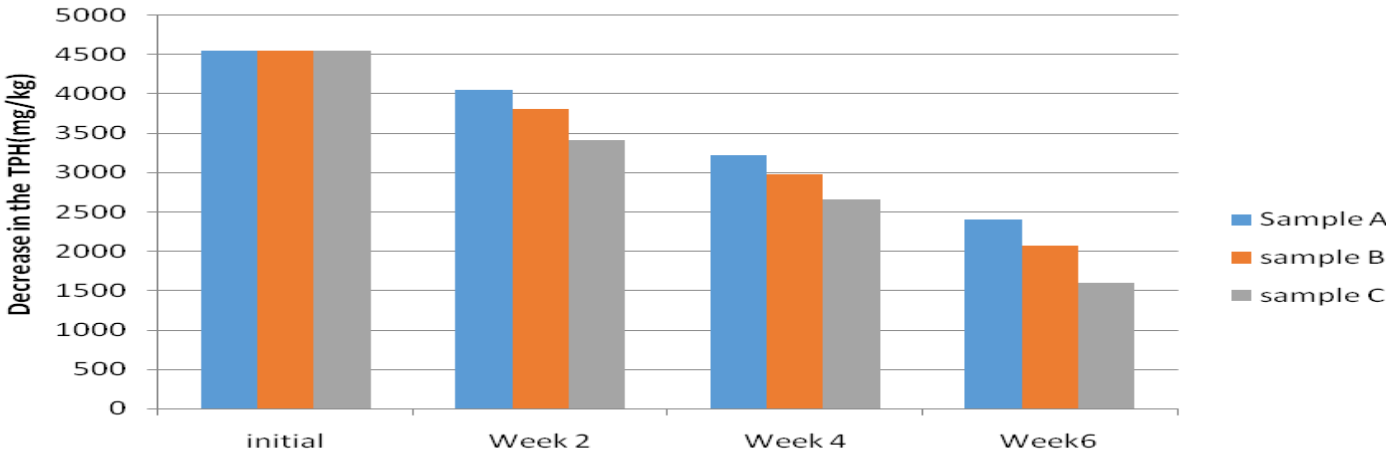


Figure 4. Effect of amendment concentration on TPH. A graph showing the decrease in total petroleum hydrocarbon.

Table 5. Total hydrocarbon utilizing bacteria count in Amended and Non-amended crude oil contaminated soil.

Duration (days)	Sample A	Sample B	Sample C	Sample D with no amendment
0	4.5	4.5	4.5	4.5
14	6.0	5.9	6.2	4.7
28	6.2	6.8	7.2	4.9
42	7.0	7.9	8.0	5.0

Table 6. The concentration of TPH after 42 days of remediation with different variations of chicken dung.

Sample g/g (%)	Initial TPH mg/kg (0 day)	TPH mg/kg after (14 days)	TPH mg/kg after (28 days)	TPH mg/kg after (42 days)	Percentage of TPH removed
10	4550.08	4053.16	3221.91	2406.55	47
15	4550.08	3811.78	29984.45	2076.74	54
20	4550.08	3410.61	2664.90	1598.95	64

Table 7. Biochemical characterization of bacteria isolated from crude oil contaminated soil.

Isolates	Gram reaction	Cellular arrangement	IND	C-T	V-P	MR	MOT	OX	UR	MAN	CAT	LAC	GLU	SUC	Probable organism
S_1	+VE	**	NR	NR	NR	-VE	-VE	-VE	-VE	D	+VE	+VE	+VE	A/G	*Staphylococcus*
S_2	+VE	Cocci in Chains	NR	NR	NR	NR	-VE	-VE	D	NR	-VE	NR	NR	NR	*Streptococcus*
S_3	-VE	*	+VE	-VE	+VE	-VE	+VE	-VE	-VE	+VE	-VE	+VE	A/G	D	*E. coli*
S_4	-VE	Rods	-VE	+VE	NR	NR	-VE	+VE	D	-VE	+VE	-VE	D	-VE	*Pseudomonas*
S_5	-VE	*	+VE	-VE	+VE	-VE	-VE	-VE	-VE	+VE	-VE	+VE	A/G	D	*E. coli*
S_6	+VE	**	NR	NR	NR	-VE	-VE	-VE	-VE	D	+VE	+VE	+VE	A/G	*Staphylococcus*
S_7	-VE	Rods	-VE	-VE	NR	NR	-VE	+VE	D	-VE	+VE	-VE	D	-VE	*Pseudomonas*
S_8	+VE	**	NR	NR	NR	-VE	-VE	-VE	-VE	D	+VE	+VE	+VE	A/G	*Staphylococcus*
S_9	-VE	Rods	+VE	+VE	NR	NR	-VE	+VE	D	-VE	+VE	-VE	D	-VE	*Pseudomonas*
S_{10}	+VE	Cocci in Chains	-VE	NR	NR	NR	-VE	-VE	D	NR	-VE	-VE	NR	NR	*Streptococcus*

IND, Indole; C-T, Citrate; V-P, Vogues Proskauer; MR, Methyl red; MOT, Motility; OX, Oxidase; UR, Urease; CAT, Catalese; LAC, Lactose; GLU, Glucose; SUC, Sucrose;+VE, Positive;-VE, Negative; NR, no reaction.

petroleum oil degrading; it aided their fast metabolic rate thereby consuming the crude oil present in the contaminated soil. Reduction was observed to increase with increased quantity of chicken dung (poultry manure) as earlier reported by Osazie et al. (2015). He reported that 90 g of cow dung fertilizer proved the best treatment option with the removal of 52.59% of crude oil from amended soil sample and this was also due the nutrient present in the chicken dung.

The isolation of diverse genera and species of bacteria from the poultry dung in this work is represented in Table 7 and is in agreement with the earlier report by Dowd et al., (2008). The hydrocarbon utilizing microorganisms isolated

Table 8. Total heterotrophic bacteria population Amended Non-amended crude contaminated soil with different variations of chicken dung.

Durations (days)	Sample A	Sample B	Sample C	SampleD (Control)
0	7.0	7.0	7.0	7.0
14	7.2	7.4	7.8	7.0
28	7.8	8.2	8.4	7.1
42	8.3	8.5	8.6	7.3

in the present study include: *Bacillus spp. Pseudomonas* spp., *Micrococcus* spp. *E. coli* spp., *Staphylococcus* spp. *and Streptococcus* spp. While the predominant genus bacteria is *Staphylococcus* spp.

Conclusion

The results in this study showed that 20% of chicken droppings supported high crude oil remediation in the polluted soil. The crude oil contaminated at the initial stage was highly degraded and a total removal of 64% of hydrocarbons was obtained. Chicken dung apart from being cost effective is also an environment friendly approach and a potential source of nutrients for microbial activity; it harbors microorganisms capable of utilizing hydrocarbons as source of carbon and energy. Thus, it is potentially useful in soil pollution response action. Therefore, crude oil polluted soil was remediated by chicken manure at 100 g/mg as bio-stimulating agent.

Recommendation

Poultry dung serves as a potential source of nutrients for microbial activity and it harbors microorganisms capable of utilizing hydrocarbons as source of carbon and energy which is potentially useful in soil pollution control. The research work has proved that chicken dung to a larger extent can biodegrade crude oil contaminated soil. It is recommended that further research should be carried out on other animal dung that are readily available in our immediate environment such as goat dung, cow dung, dog dung, pig dung etc. To know if the bacteria found in their dung would be as effective as chicken dung to degrade crude oil spilled in soil.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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