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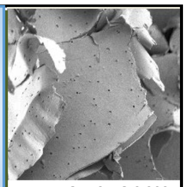

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Bioremediation of Petroleum-Impacted Soil Using Poultry Manure

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ABSTRACT

This 60-day study investigated the use of poultry manure (PM) at 10% w/w to drive crude oil elimination in impacted soil at a loading contaminant concentration of 5% v/w. Gas chromatography determined removal efficiency while the bacterial community was analysed using standard microbiological techniques. The presence of crude oil as a contaminant made the soil more alkaline and reduced its nutrient content across the board. The addition of PM improved soil nutrient properties and brought soil pH closer to neutral. PM amendment resulted in total petroleum hydrocarbon elimination levels of 96.83%. The total heterotrophic bacterial (THB) and hydrocarbon utilising bacterial (HUB) abundance in the PM-treated soils showed a steady rise till day 40 with values of 7.93 Log CFU/g and 8.83 Log CFU/g respectively, after which mean counts dropped. Statistically significant differences at 95% confidence interval ($p \leq 0.05$) in TPH elimination were seen between the control studies and the PM-treated soil. THB and HUB counts also differed significantly ($p \leq 0.05$) between the unpolluted control and the PM treatment study but no statistically significant differences were obtained between the PM-treated soil and the polluted control. Bacterial diversity analysis revealed a predominance of *Bacillus* spp., *Staphylococcus* spp., and *Micrococcus* on day 0. Dominance shifted to *Klebsiella pneumoniae*, *Bacillus,* and *Citrobacter* spp. by the end of the study, indicating that these genera were the drivers of crude oil biodegradation during the study. The application of poultry manure effectively enhanced the biodegradation of crude oil in impacted soil.

INTRODUCTION

Global concern is rising regarding environmental deterioration due to toxic pollutants [1]. Crude oil and its derivatives have a long history of environmental degradation. They get into soil and water systems mainly by accidents, storage failure and normal processing activities [2]. Spillage of petroleum into the environment is known to have long-term ecological impact. A report from the ITOPF [3] stated that an estimated 14,920 barrels (2000 metric tonnes) of oil found their way into environments across the globe in 2023 from oil tanker incidents while NOSDRA [4] places the net volume of spills into soil and water ecosystems across Nigeria in 2023 at 19,000 barrels. Environmental remediation following a spill event can be tasking and complicated and will typically entail physical, chemical and biological approaches like application of surfactants and dispersants, excavation, combustion and collection using adsorbents. While only partially effective, these methods tend to impact the already stressed environment negatively [5]. Studies have proven that chemical surfactants and dispersants are toxic to aquatic and terrestrial life forms and can accumulate in the environment, creating a long term challenge [6]. This has led to the global emphasis on biological approaches to contaminant management in the environment as this is considered the most sustainable and the least disruptive for the environment and environmental players at various trophic levels.

Several reports have highlighted the vital role of microorganisms in detoxification and ultimate biodegradation of contaminants in soil and water systems. Therefore, bioremediation efforts often target modifying the environment to encourage the rapid proliferation of indigenous microorganisms with the capacity to degrade the contaminant in question. This is achieved by adding limiting nutrients, mainly Nitrogen in the form of nitrates and phosphorus as phosphates and micronutrients like iron, required for the contaminant biodegradation process. Carbon and oxygen may also be included as required [5,7]. The key benefit of biostimulation is that biodegradation is driven by autochthonous bacterial groups already in the system without the introduction of new species that may upset the precarious ecological balance.

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Furthermore, these species are well-suited to the environmental conditions and would require only a limited acclimatisation period in response to the contaminant. Researchers consider the use of organic nutrient sources against inorganic ones more suitable. Organic stimulants are preferred in soil remediation interventions due to their availability, low cost and contribution to soil organic matter content and overall soil quality [5,8,9]. This is a 60-day bioremediation study designed to stimulate the autochthonous bacterial community and facilitate the biodegradation of crude oil in soil using poultry manure.

MATERIALS AND METHODS

Sample Collection

The soil used for the study was obtained from the agricultural farm at the University of Port Harcourt while the crude oil used was Bonny Light crude from the Nigerian National Petroleum Co-operation (NNPC) at Eleme, Rivers state, Nigeria. The soil was collected up to a 25 cm depth. The soil was sieved using a 2 mm sieve to remove large particles and debris. The poultry manure (PM) was supplied by the Halleluyah Farms, Port Harcourt, Rivers state, Nigeria. The PM was sun dried for about 10 – 15 days then pulverised before application.

Experimental Design

The bioremediation study was carried out in replicated sets, each consisting of 2000 g of soil. Crude oil was applied at 5 % v/w while the PM was applied at 10 % w/w. the study was set up as follows: **Control 1**: Soil alone (unpolluted control); **Control II**: Soil + 5 % v/w crude oil (polluted control); **Set III**: Soil + 5 % v/w crude oil + 10 % w/w poultry manure.

The soil was aerated via regular tilling and moisture was maintained at 60 %. Following a 7-day acclimatisation period, the soil nutrient levels, total petroleum hydrocarbon (TPH), microbial diversity, and abundance were monitored at regular intervals for 60 days. These parameters served as indicators of the bioremediation process.

Soil pH and Nutrient Properties

pH levels

The pH values of the water samples were determined by suspending about 10 g of soil in deionised water at 1:5 solid/solution ratio. The suspension was left to stand for roughly 30 minutes before the pH reading was taken using a combined glass calomel electrode and a pH meter (Jenway, UK). The pH meter was first calibrated using buffer solutions then the electrode was dipped in the soil suspension to take the reading. This was done thrice for accuracy. Mean values were noted.

Total nitrogen, Phosphate and Potassium content

The total nitrogen content was established using the Kjeldahl digestion method of Brenmer [10] while the phosphate and potassium content in the soil samples were ascertained by the flame photometric technique after extraction using ammonium acetate [11].

Total Petroleum Hydrocarbon (TPH)

The liquid-liquid extraction method was employed to determine the TPH content in the samples. This was done using a gas chromatograph fitted with a flame ionisation detector (GC-FID) (Agilent 6890N, USA). The samples were extracted with dichloromethane and eluted using pentane in a capillary column [12].

Enumeration, Isolation and Characterisation of Soil Bacterial Community

The diversity and abundance of the total cultivable heterotrophic bacteria (THB) and cultivable hydrocarbon-utilising bacteria (HUB) were monitored at 20-day intervals.

For THB, isolation was carried out on nutrient agar while plate count agar was employed for enumeration via the dilution plate technique. The plates were incubated at 30 °C ± 2 ºC for 48 h. Only plates with counts of 30 – 300 colonies were selected for enumeration. Counts were expressed as mean colony forming units (CFU) per gram of soil. The isolates obtained were purified by streaking onto fresh nutrient media.

The HUB was isolated and enumerated using the vapour phase technique on the Bushnell-Haas medium. The soil samples were serially diluted and 1 mL aliquots of suitable dilutions were spread on the Bushnell-Haas medium in agar plates. Sterile Whatmann's No. 1 filter papers saturated with crude oil were then aseptically positioned on the lids of the inverted agar plates. The plates were incubated in the inverted position at 30°C ± 2 ºC for up to 7 days. Discrete colonies that developed on the agar plates were purified by sub-culturing twice via the streaking plate technique and preserved on slants. Representative isolates were identified on the basis of their macroscopic, microscopic, and biochemical characteristics [13].

Data analysis

The relationships existing in TPH removal levels and cultivable bacterial abundance between the set-ups treated with PM and the Control experiments were ascertained via analysis of variance within groups and between groups at 95 % confidence interval using SPSS software (version 23.0).

RESULTS AND DISCUSSION

The physicochemical properties of the soil samples are outlined in **Table 1**. The presence of crude oil as a contaminant made the soil more alkaline and reduced its nutrient content across the board. The physicochemical properties of the unpolluted control did not vary considerably during the study. This is indicative of a balanced, undisturbed system. The addition of PM boosted soil nutrient properties and raised soil pH levels, bringing them closer to neutral. Nutrient levels and pH decreased in the soils with PM amendment as the study progressed. It can be concluded that treatment with poultry manure effectively remediated petroleum hydrocarbons in soil. There was a marked reduction in petroleum hydrocarbon concentration in soil following the application of PM.

Based on **Fig. 1**, TPH elimination levels of 96.83 % and 3.99 % were obtained for the PM-treated soil and the polluted control study, respectively. The chromatograms from the GC-FID, as seen in **Fig. 2**, revealed C_8, C_{11}, C_{15}, and C_{18} to be the recalcitrant aliphatic fractions at the end of the study. The mean abundance of THB (6.45 – 6.47 Log CFU/g) and HUB (4.38 – 4.46 Log CFU/g) remained relatively constant in the unpolluted control throughout the study; however, marked increases in THB and HUB were seen in the polluted control and the PM-treated soil. For the polluted control, THB abundance peaked on day 40 at 8.62 Log CFU/g, and HUB peaked at 7.81 Log CFU/g on day 20. The PM treatment study showed a steady rise in both counts up to day 40 with values of 7.93 Log CFU/g and 8.83 Log CFU/g for THB and HUB, respectively, after which mean counts dropped (**Fig. 3A** and **3B**).

Statistically significant differences at 95 % confidence interval (p≤0.05) in TPH elimination were seen between the control studies and the PM-treated soil. THB and HUB counts also differed significantly (p≤0.05) between the unpolluted control and the PM treatment study, but no statistically significant differences were obtained between the PM-treated soil and the polluted control.

Table 1. pH and inorganic nutrient characteristics of the soil samples.

Time (days)	pH	Total Nitrogen (%)	Phosphorus content (mg/kg)	Potassium content (mg/kg)
Unpolluted Control				
Day 0	6.52	5.73	0.674	38.23
Day 20	6.59	5.58	0.672	37.47
Day 40	6.51	5.78	0.653	37.26
Day 60	6.53	5.52	0.655	37.42
Polluted Control				
Day 0	7.29	0.84	0.158	48.31
Day 20	7.43	0.81	0.149	41.31
Day 40	7.73	0.72	0.133	37.19
Day 60	7.66	0.70	0.124	33.01
Treated soil (10 % PM)				
Day 0	6.93	8.92	0.826	510.65
Day 20	6.84	5.03	0713	318.91
Day 40	6.69	5.69	0.520	186.03
Day 60	6.62	2.18	0.201	97.34

PM – poultry manure; values are means of triplicates

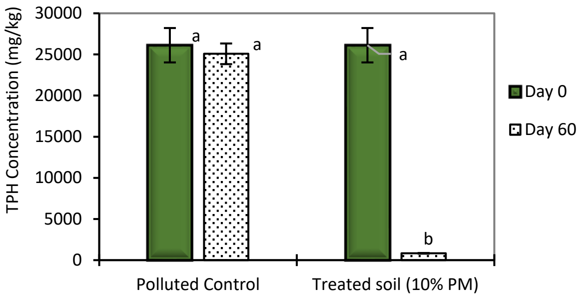

Fig. 1. Extent of primary biodegradation of total petroleum hydrocarbon (TPH) following 60-day treatment with poultry manure. Different letters indicate significant differences (p≤0.05). Bars represent the standard deviation from the mean. PM – poultry manure.

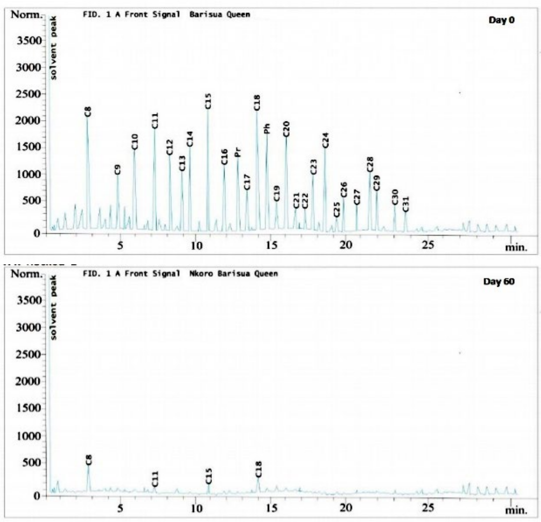

Fig. 2. Initial and residual aliphatic hydrocarbon fractions of crude oil after soil treatment with poultry manure. Compounds are represented by their carbon chain lengths.

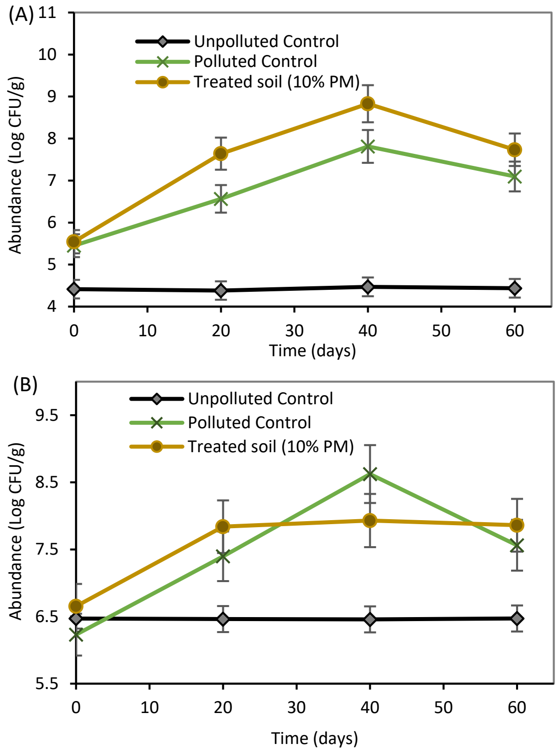

Fig. 3. Growth profile of (A) hydrocarbon-utilising bacteria and (B) total heterotrophic bacteria in the soil during the study. Bars represent the standard deviation from the mean. PM – poultry manure.

Bacterial diversity analysis revealed a predominance of *Bacillus* spp., *Staphylococcus* spp. and *Micrococcus* on day 0. Dominance shifted to *Klebsiella pneumoniae*, *Bacillus* and *Citrobacter* spp. by the end of the study, indicating that these genera were the drivers of crude oil biodegradation during the study (**Fig. 4**). These findings show that poultry manure better supported the proliferation of both total heterotrophic bacteria and hydrocarbon-utilising bacteria than control studies.

The pH levels in the polluted control in the present study were seen to become more alkaline as the study progressed, but the pH levels in the PM-amended soils became more acidic. Changes in pH noted during biodegradation often indicate a trend towards acidity, as was the case in the current study after the addition of PM, and has been attributed to the production of organic acids as by-products of the biodegradation process [14,15].

The decrease in pH in the current study after amendment with PM does not support the conclusion that incorporating agricultural wastes into hydrocarbon-impacted soil will trigger a rise in pH [16]. The pH increase in the polluted control (without PM addition) could indicate enhanced concentrations of soil dehydrogenase (DHA) enzyme as highlighted by Li *et al.* [17]. The degrading microorganisms in soil may respond to the presence of the hydrocarbon pollutant and the absence of a suitable nutrient ratio by synthesising more DHA. The observed increase in pH following the introduction of crude oil in the polluted control does not correspond with the findings of Muhammad *et al.* [18], who stated that TPH contaminants tend to release hydrogen ions into the polluted system, causing pH levels to tend towards acidity.

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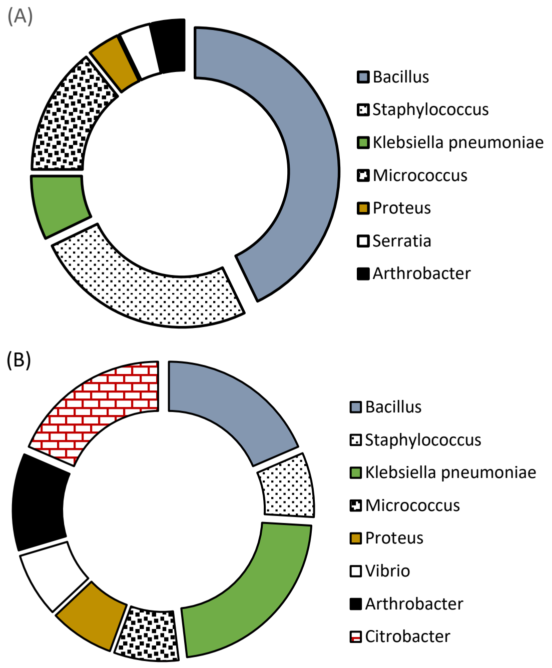

Fig. 4. Cultivable bacterial isolates obtained from the PM-treated soil at the (A) onset (day 0) and (B) end (day 60) of the study.

As found in the current study, NPK levels will typically increase upon the addition of organic amendments to soil [19]. The steady drop in NPK levels obtained during bioremediation using PM (**Table 1**) could result from increased microbial abundance and increased metabolic activity, requiring more nutrients for energy production. Hanson-Akpan *et al*. [20], in a bioremediation study using organic fertilizers against spent engine oil, found a pH range of 5.98 – 6.02 in the polluted control and 7.22 – 8.00 in the contaminated soils treated with PM. These values are greater than those obtained in the current study, but the nitrogen and phosphorus content found in the current study is much higher than the 0.26 % – 0.7 % and 32.70 mg/kg – 35.21 mg/kg for total Nitrogen and phosphorus reported by Hanson-Akpan *et al*. [20]. These differences may be attributed to contaminant type, soil type, geographical location and the poultry feed used on the farm from where the PM was procured.

The results from the current study buttress the findings of other studies that show that effective biostimulation increases contaminant elimination efficiency several fold. Studies have recognised that biostimulation using organic fertilisers, effectively eliminates petroleum contaminants from soil ecosystems. These biostimulants support the proliferation and metabolic activities of hydrocarbon-degrading microorganisms, thus, enhancing the rate of contaminant mineralisation [21,22]. The high crude oil elimination levels (about 96.8 %) obtained in the current study buttresses the conclusion of a parallel study that compared engine oil removal efficiency of cow and poultry manure with and without bulking using sawdust [20]. They determined that poultry manure was the more efficient organic stimulant. In tandem with the conclusions of the present study, Uwadiae and Obasi [14], in their 7-week bioremediation investigation, confirmed that the addition of poultry manure enhanced crude oil removal efficiency in soil.

They further determined that contaminant removal levels increased with increasing concentration of the PM applied. TPH removal efficiency has been established to improve with increasing PM application concentration in soil [20,23–24]. Results from a study that treated 5 % v/w engine oil-impacted soil showed that the highest TPH elimination efficiency of over 60 % was achieved following the application of poultry manure after 42 days [21]. Behera *et al*. [25] established TPH degradation levels of up to 90% in soil after 21 days by employing poultry litter and a bacterial consortium. A comparable study by Okafor *et al*. [24] showed lower crude oil elimination efficiency using PM than obtained in the current study. They recorded only a 57.31 % drop in crude oil concentration after about 90 days in a bioremediation study using poultry manure at 10 % w/w (same amendment level in the current study). The loading concentration of the contaminant (crude oil) could play a vital role here as the study by Okafor *et al*. [24] treated a 20 % v/w crude oil concentration, which is relatively high compared to the 5 % v/w used in the current study.

Research has indicated a correlation between microbial abundance and biodegradation levels, particularly following the introduction of nutrients [17]. This agrees with the findings of the present study as viable bacterial counts showed an initial rapid increase and then a gradual decrease as TPH levels dropped. A bioremediation study on crude oil in soil using pig dung obtained an initial drop in bacterial abundance followed by rapid increases and then a slow drop in counts simultaneous with a decrease in the concentration of crude oil in the system [15]. The impact of poultry droppings on the bioremediation of crude oil-polluted soil was evaluated in Anambra state, Nigeria [24]. The researchers determined that, akin to the present study, THB and HUB abundance rose steadily until a certain point and then gradually declined after treatment with PM. They obtained initial HUB counts of 4.08 Log CFU/g. This increased to 4.26 Log CFU/g and then dropped to 3.70 Log CFU/g by the end of the study. A similar trend in HUB counts was seen in the current study.

Citrobacter, K. pneumoniae and *Bacillus* spp. were shown to be the primary genera for hydrocarbon biodegradation as they had the greatest relative abundance compared to other species. The results from other comparable bioremediation and biodegradation studies substantiate this finding. Species of *Citrobacter* like *Citrobacter koseri*, *Citrobacter sedlakii* and *Citrobacter freundii* have been associated with the biodegradation of hydrocarbon complexes such as engine oil, diesel oil, and crude oil, particularly the long straight chain fractions [26–29]. *K. pneumoniae, Bacillus subtilis*, *Bacillus cereus, Bacillus licheniformis* and *Bacillus pumilus,* amongst other species of *Bacillus*, have also been established as efficient degraders of petroleum-derived compounds by a number of researchers as also noted in the current study [30–35].

The dexterity with which bacterial species degrade complex hydrocarbon compounds has been linked to their biosurfactant synthesis capacity. Scores of known hydrocarbon degraders produce intracellular and/or extracellular biosurfactants that function to emulsify hydrocarbon contaminants, rendering them more bioavailable and, consequently, more readily biodegradable. The dominant petroleum degraders obtained in the present study, *Klebsiella pneumoniae*, *Bacillus* and *Citrobacter,* are among the proven biosurfactant producers [36–39].

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CONCLUSION

The study substantiated the efficiency of poultry manure for the enhanced biodegradation of aliphatic fractions of crude oil from impacted soil. A crude oil elimination level of 96.8 % was seen after 60 days, with aeration. The dominant microbial species involved in the degradation process were *Bacillus*, *Citrobacter* and *Klebsiella pneumoniae*. Further research regarding process optimisation and analysis of the microbial community using omics techniques is recommended.

CONFLICT OF INTEREST STATEMENT

The authors declare that no known conflicts of interest are associated with this study.

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