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Microbial Activities in Fertilizer-Amended Contaminated Soils

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ABSTRACT: Bioremediation, an affordable and environmentally friendly technique, remains a viable strategy for tackling water and soil pollution. This study investigated microbial activities in crude oil-contaminated soils amended with fertilizer. Unpolluted soil samples obtained from the bed of Ovia River, Benin City, Nigeria, were contaminated with crude oil, and thereafter remediated with NPK/urea fertilizer. Textural classification of the soils showed that the clay soil contained 77 % clay, while that of the sandy-clay soil was 72 %. Crude oil-polluted soils (3 kg) were investigated in seven groups, each for the two soil types (clay and sandy-clay). Soil microbes were enumerated using standard procedures every 6 days for 36 days. The results of this study showed that total hydrocarbon utilizing fungi (THUF), total hydrocarbon fungi (THF), total hydrocarbon utilizing bacteria (THUB) and total hydrocarbon bacteria (THB) of clay and sandy-clay soils were significantly and concentration-dependently increased by crude oil contamination as well as fertilizer remediation at day 0 to 12, and then decreased from day 18 through 36 ($p < 0.05$). The results indicate that with appropriate management, NPK/urea fertilizer may constitute an organic supplement for remediating hydrocarbon-polluted soils, especially in places where crude oil pollution is a major environmental challenge.

Keywords: Bioremediation, Clay, Contamination, Crude oil, Fertilizers, Soil.

Introduction

Soil is a natural environment consisting of organic and inorganic components present in the gaseous, aqueous, and solid states. Differing significantly in their genetic and environmental characteristics soils generally function as reservoirs of water and nutrients that support the growth of plants and microorganisms (Gatiboni, 2022). Hydrocarbons resulting from crude oil contamination of soil can persist for years, leading to reduced fertility, decreased productivity, groundwater contamination, and loss of biodiversity (Megharaj *et al*., 2015). They can increase soil acidity, reduce microbial activity, and hinder nutrient uptake, thus aggravating land degradation. The hydrophobic nature of petroleum hydrocarbons affects soil structure, reducing water infiltration and air permeability, which further inhibits microbial activity and plant growth. The risks these pollutants in the environment pose to both ecological and human health calls for intervention (Rao *et al*., 2014; Chettri *et al.*, 2021). Remediation of hydrocarbon-polluted soils is essential for restoring the ecosystem and ensuring food security. Traditional methods, such as chemical treatments or excavation employed to address hydrocarbon contamination are often expensive (requiring intensive labour), and environmentally disruptive. Since chemical treatments may lead to secondary pollution, they are less sustainable (Sharma, 2020). Bioremediation using organic waste offers a cost-effective, eco-friendly alternative (Guo *et al.*, 2014). Organic waste, derived from sources like municipal solid waste, animal manure, and plant residues, introduces beneficial microorganisms, nutrients, and organic matter into the soil. This process enhances biodegradation of hydrocarbons through microbial metabolism, while simultaneously improving soil structure and chemical balance (Das and Chandran, 2011). This study investigated microbial activities in fertilizer-amended contaminated soils.

Materials and methods

Chemicals/reagents: The chemicals and reagents used in this study were of analytical grade, and they were products of Sigma-Aldrich Limited (UK).

Collection of crude oil and soil samples: The crude oil used in this study was obtained from Shell Petroleum Limited, Jala, Warri South Local Government Area, Delta state, Nigeria. The unpolluted soil samples were obtained from the bed of Ovia River, Unuamen, Ovia North-East Local Government Area, Edo state, Nigeria. The soil samples bagged in a clean container were transported to the Department of Soil Science, Faculty of Agriculture, University of Benin, Benin City, Edo State, for textural classification. Textural classification of the soils showed that the clay soil contained 77 % clay, while that of the sandy-clay soil was 72 %.

Experimental design: The unpolluted soil samples were contaminated with crude oil at varied concentrations (3000 – 8000 ppm), and thereafter remediated with NPK/urea fertilizer after 36 days.

Determination of residual hydrocarbon content (RHC): Crude oil-contaminated soil sample (5 g) mixed with n-hexane (25 mL) in a 100 mL conical flask was vigorously shaken for l0 min and then allowed to stand and covered before the filtrate was filtered and read at 460 nm. Exactly 1.18 mL blend crude oil was pipetted and then made up to 1 L with n-hexane. A working standard of varied concentration (0, 10, 20, 40, 60, 80 and 100 ppm) was obtained from the preparation.

$$\text{RHC (mg/kg)} = \frac{Instrument.Reading \times Slope\ Reciprocal \times 25}{5\ \text{g}}$$

*Determination of total microbial count (TMC)***:** Already prepared diluent was used to prepare dilution of the soil-water extract sample (10^{-1}, 10^{-3} and 10^{-5} dilutions were obtained). Using cover glass, the colony counting chamber was assembled. Few drops of methylene blue solution was added to the water sample and dilutions. On the ruled area of the counting chamber, a standard loop was used to place a loop full of water sample (including the various dilutions) and the chamber was allowed to rest for 5 min. Bacteria in 50 - 100 square selected at random was counted using 4 mm Lens (x 16 objective Lens), after examination under a microscope, so that the total number of bacteria was about 500. Triplicate count was performed by dividing the number of count by the number of squares and the result was multiplied by the dilution factor and a constant k. Using this method, the total number of bacteria in milliliter of a given water sample was determined.

Isolation, characterization and identification of crude oil degrading bacteria

Morphology: Morphology of bacterial isolates were examined, and individual colonies were characterized in terms of shape, appearance, colour, margin and spore (Cheesbrough, 2005).

Gram reaction: A thin smear of isolated bacteria was made on a sterile slide and stained with crystal violet after heat fixing it and allowed to stand for 60 s, and thereafter rinsed with clean water. Few drops of iodine was added to the surface of the thin bacterial specimen before incubation. There was also addition of a drop of decolourizer (ethanol) which was left for 3 min before it was then washed away with clean water. Furthermore, there was addition of counter stain safranine which was allowed to stand for 60 s and then cleaned away. The thin specimen was allowed to dry up and then viewed under the microscope.

Biochemical screening: The different biochemical tests used for identification of bacterial isolates included: catalase, citrate, urease, and oxidase tests.

Isolation, characterization and identification of crude oil degrading fungi: Soil sample (1 g) was mixed with drop of Tween 80 uniformly to improve biodegradation and was then placed on Potato Dextrose Agar (PDA) media and incubated for 7 days at 30 ℃. Ampliclox (25 mg/L) was thereafter added to the media after autoclaving to avoid pollution by bacteria. Based on morphological characteristics, the pure colonies were selected (Aneja and Charles, 2005).

Cultural characteristics: For the purpose of purification of fungal isolates, the culture was cautiously and aseptically sub cultured on PDA. On the grounds of cultural and morphological characteristics, the fungal isolates were characterized based on spore type and mycelia.

Microscopic examination: Fungi of definite colony obtained from pure culture were coated on greasy free slide, stained with lactophenol cotton blue, and then viewed under light microscope.

Evaluation of crude oil utilization potential of microbial isolates: Evaluation of crude oil utilization potential of microbial isolates was carried out using mineral salt medium (MSM) which consisted of K_2HPO_4 (1.8 g), K_2HPO_4, (1.2 g), NH_4Cl (4 g), $MgSO4.7H_2O$ (0.2 g), NaCl (0.l g), $FeSO_4$, $.7H_2O$ (0.0l g) in 1 L of distilled water (Hamzah *et al.,* 2014).

Assessment of crude oil utilization potential of bacterial isolates: Each bacterial isolate was placed in sterilized MSM with the addition of crude oil (2 %, *v/v*) in a separating conical flask of 250 mL, which was incubated at 30 °C with vigorous mixing at 150 rpm for 12 days (D' Annibale *et al.,* 2012). The uninoculated conical flask medium of 250 mL in which 2 % crude oil was added served as the control. After a 2-day period, samples were

harvested for turbidity determination (Kumari *et al*., 2012).
Evaluation of crude oil utilization potential of fungal isolates: Fungal isolate was placed in sterilized MSM with the addition of crude oil (2 %, *v/v*) in a separate conical flask of 250 mL, which was incubated at 30 °C and vigorously mixed at 180 rpm for 12 days (D' Annibale *et al.,* 2006). Uninoculated conical flask medium of 250 mL which contained 2 % crude oil served as control. After two days, samples were harvested for turbidity test (absorbance read at 600 nm).
Determination of crude oil degrading potential of bacterial isolates: Bacterial isolate was placed in sterilized MSM with the addition of crude oil (2 %, *v/v*) in a separate conical flask of 250 mL, which was incubated at 30 °C and vigorously mixed at 150 rpm for 12 days (Kumari *et al.,* 2012). Uninoculated conical flask medium of 250 mL which contained 2 % crude oil served as control. After two days, samples were harvested for determination of residual total petroleum (USEPA, 2000; Mishra *et al.*, 2001).
Determination of the effect of CNP ratio on crude oil degradation in soil: Crude oil-polluted soils (3 kg) were weighed into seven different plastic buckets and subjected to treatment. Contaminated soils were investigated in seven groups, each for the two soil types (clay and sandy-clay). The clay soil groups were: Aa-3000 ppm, Ab-5000 ppm, Ac-8000 ppm, AA-3000 ppm, Ab-5000 ppm, Ac-8000 ppm and N served as experimental control for the clay soil. Groups for the sandy-clay soil were: Ba-3000 ppm, Bb-5000 ppm, Bc-8000 ppm, and F served as the experimental control for the sandy-clay soil. For the clay soil groups Aa, Ab, Ac were contaminated with crude oil, while groups AA, AB and AC were contaminated with crude oil and then treated with NPK/urea fertilizer. Group N which served as experimental control for the clay soil was not contaminated with crude oil. For sandy-clay soil, groups Ba, Bb and Bc were contaminated with crude oil and then treated with NPK/urea fertilizer. Group F which served as the experimental control for the sandy-clay soil was not contaminated with crude oil. Treatment using biostimulation of indigenous microbes commenced the same day. This entailed the use of NPK (15:15:15) and urea fertilizers. Varied concentrations of NPK and urea fertilizers were applied to all treated soils using C:N:P ratio of 100:2:0.2. Mechanical agitation was done every three days throughout the 36 days of remediation.
Determination of the effect of bioremediation enhancement treatment on crude oil degradation in soil: Crude oil-polluted soils (3 kg) were weighed into 7 plastic buckets (for each soil type). Contaminated soils were investigated in seven groups, each for the two soil types (clay and sandy-clay). The moisture content was adjusted to 65 – 85 % of water-holding capacity for all the groups. Vigorous mixing of soil samples in the container was performed every three days. The experiment lasted 36 days. Microbial growth analyses were carried out every 6 days for 36 days (Chijioke-Osuji *et al*., 2014; Shabir *et al*., 2008).
Microbial enumeration: Microbial enumeration was carried out by evaluation of colony forming units (CPU) with the dilution of sample suspension serially and with pour plate techniques on nutrient agar for bacteria (Chikere *el at*, 2009) and PDA for fungi (Cappuccino and Sherman, 1998). Enumeration of THUF, THF, THUB, and THB were done using standard methods (Hamamura, 2006; Chikere *et al.,* 2009).
Statistical analysis: Data are presented as mean ± standard error of mean (SEM). Student's *t* test was used to compare means between the two sample groups. The data analyses were performed using SPSS version 23. Values of $p < 0.05$ were considered statistically significant. Correlation analysis was performed to establish relationship between TPH and other parameters evaluated.

Results

Microbial activities in contaminated soils amended with fertilizer: Soil microorganisms were enumerated at different time points in the two types of soil contaminated with crude oil and amended with NPK/urea fertilizers. The results are presented in Figures 1 to 10.

The total hydrocarbon utilizing fungi (THUF) of the clay and sandy-clay soils contaminated with crude oil increased significantly and concentration-dependently at days 0, 6 and 12, but decreased markedly from day 18 through 36 ($p < 0.05$) (Fig. 1).

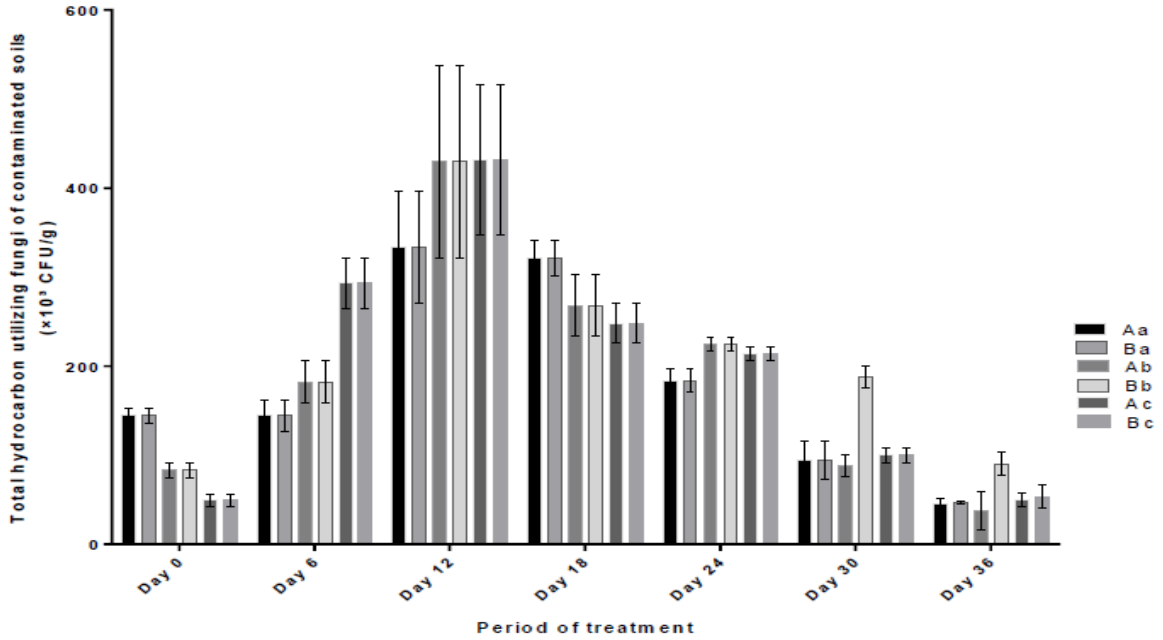

Figure 1: Total hydrocarbon utilizing fungi of contaminated clay and sandy-clay soils

As shown in Figure 2, total hydrocarbon utilizing fungi (THUF) of clay and sandy-clay soils increased significantly and concentration-dependently at days 0, 6, 12 and the 3000 ppm group at day 18 while the 5000 ppm and 8000 ppm groups decreased ($p < 0.05$). However, THUF were markedly decreased at day 24 (only 3000 ppm group experienced marked increment) through 36 ($p < 0.05$).

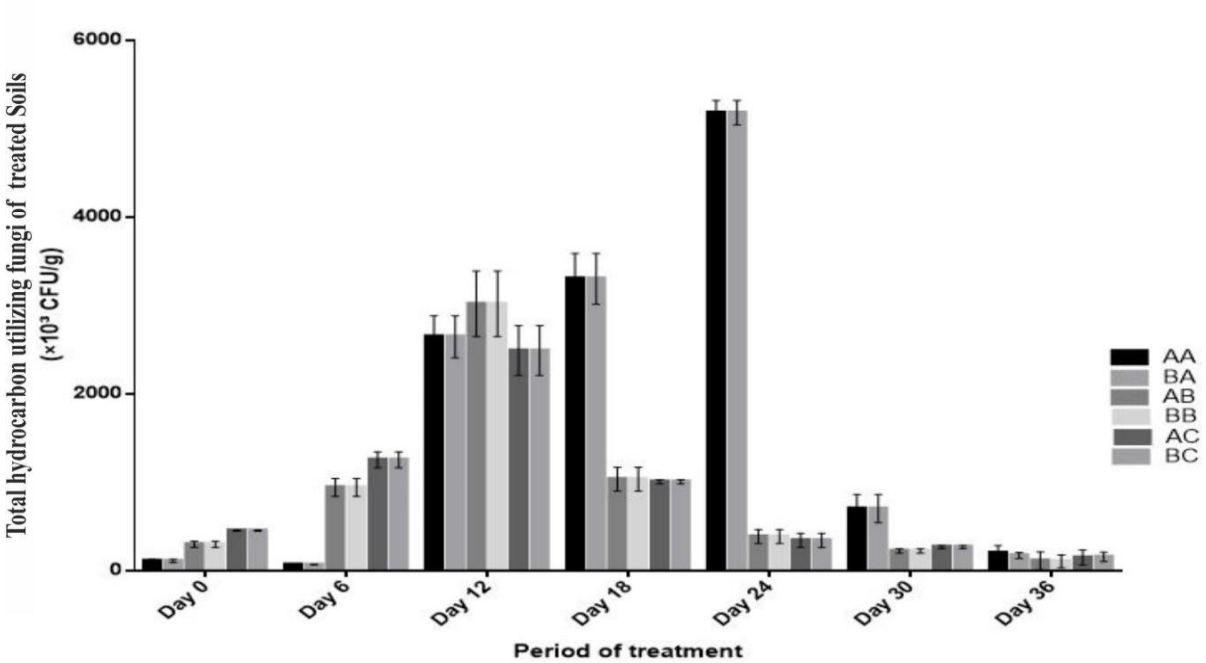

Figure 2: Total hydrocarbon utilizing fungi of treated clay and sandy-clay soils

The total hydrocarbon fungi of the clay and sandy-clay soils increased significantly and concentration-dependently at days 0, 6 and 12, but decreased markedly from day 18 through 36 ($p < 0.05$) (Fig. 3).

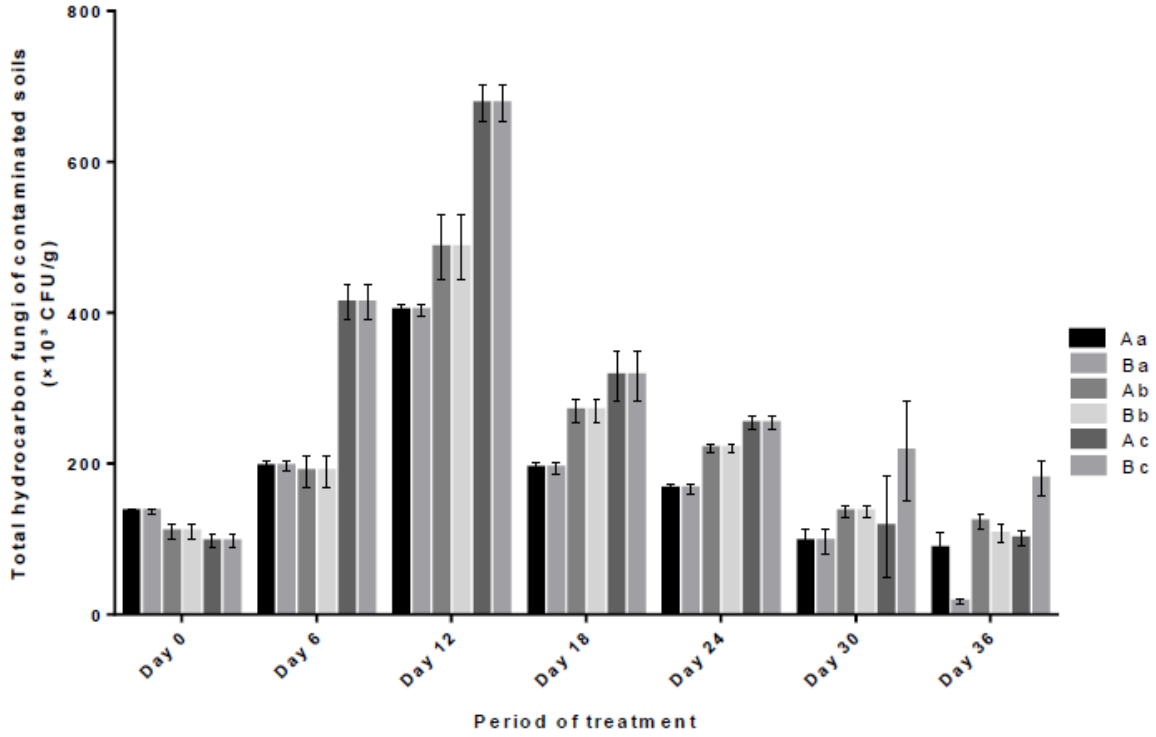

Figure 3: Total hydrocarbon fungi of contaminated clay and sandy-clay soils.

As shown in Figure 4, the total hydrocarbon fungi of treated clay and sandy-clay soils demonstrated a significant and concentration-dependent increase up to day 12 followed by a decline from day 18 to day 36 ($p < 0.05$).

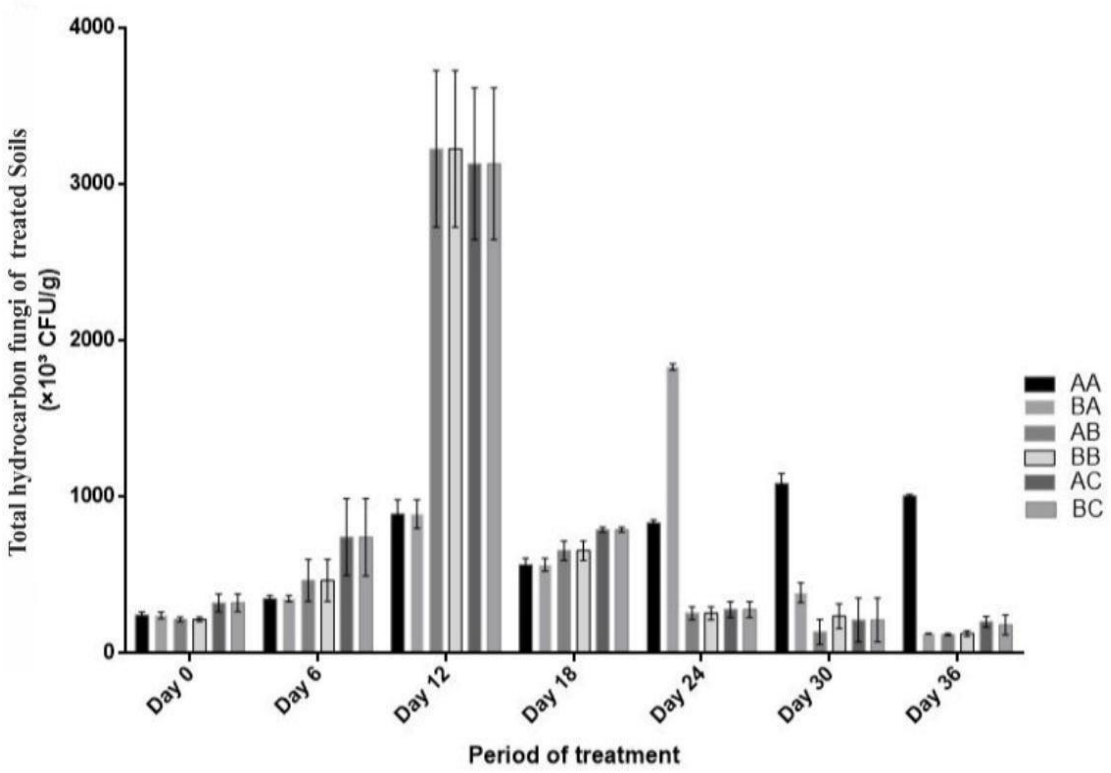

Figure 4: Total hydrocarbon fungi of treated clay and sandy clay-soils.

The total hydrocarbon utilizing bacteria of clay and sandy-clay soils increased significantly and concentration-dependently at days 0, 6, and 12 and only the 3000 ppm group at day 18 while the 5000 ppm and 8000 ppm groups decreased ($p < 0.05$). However, total hydrocarbon utilizing fungi were markedly decreased at day 24 (only 3000 ppm experienced marked increment) through 36 ($p < 0.05$). The highest increment was observed in 3000 ppm group of day 12 (Fig. 5).

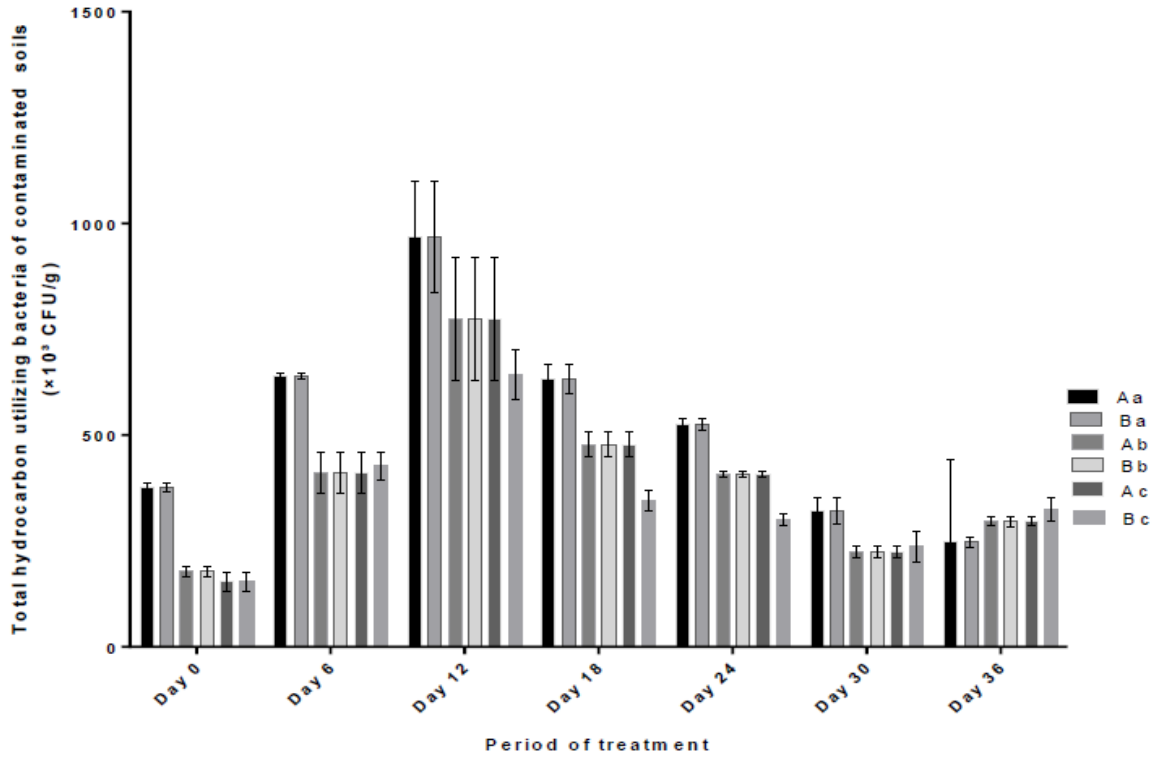

Figure 5: Total hydrocarbon utilizing bacteria of contaminated clay and sandy-clay soils.

Figure 6 shows that the total hydrocarbon utilizing bacteria of treated clay and sandy-clay soils demonstrated a progressive and concentration-dependent increment day 0 to 12 followed by a sharp decline from day 18 through day 36 ($p < 0.05$).

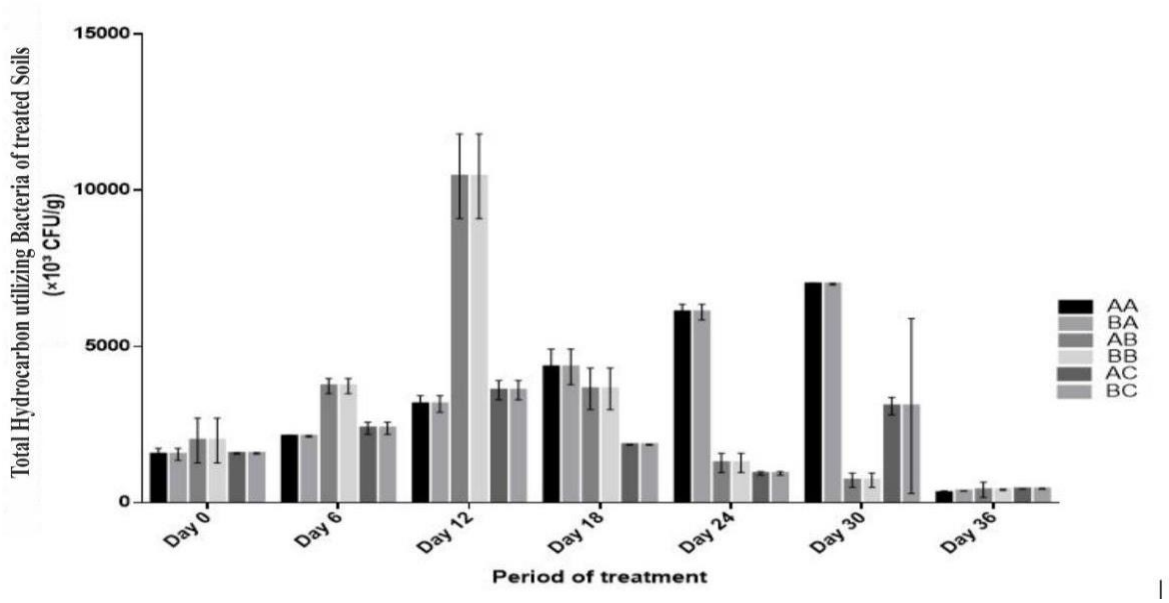

Figure 6: Total hydrocarbon utilizing bacteria of treated clay and sandy-clay soils.

Total hydrocarbon bacteria of contaminated clay and sandy-clay soils increased significantly and concentration-dependently at days 0, 6, and 12, but decreased markedly at day 18 through 36 ($p < 0.05$). The highest increment was observed in 8000 ppm group of day 12 (Fig. 7).

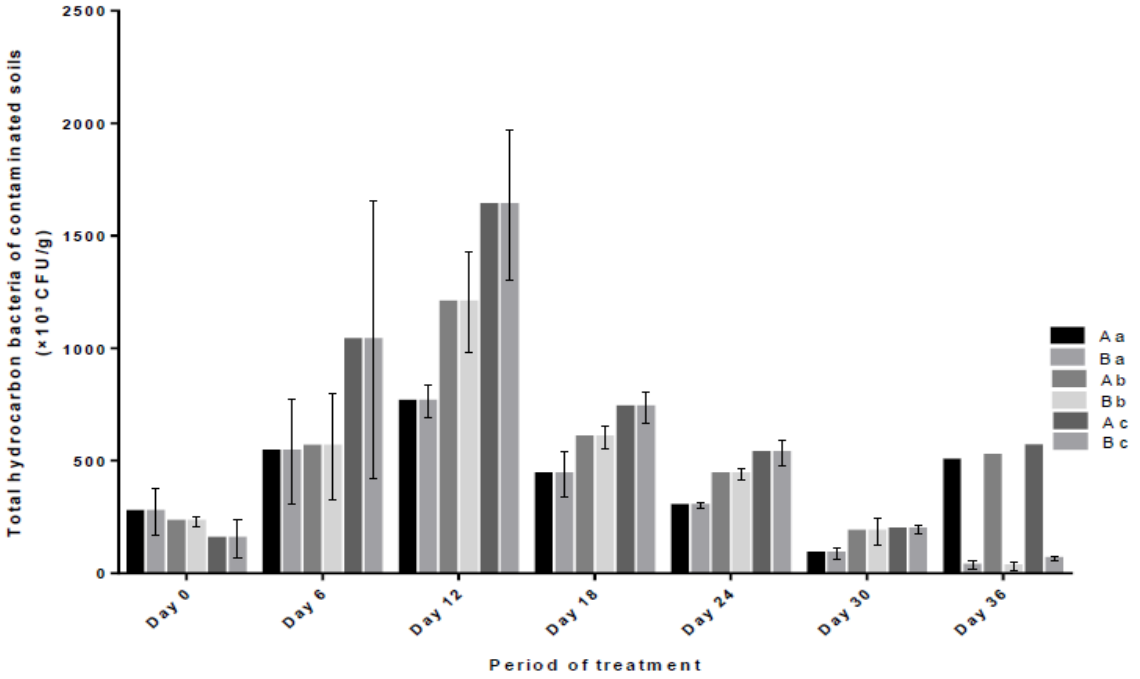

Figure 7: Total hydrocarbon bacteria of contaminated clay and sandy-clay soils.

Figure 8 shows that the total hydrocarbon bacteria of treated clay and sandy-clay soils increased significantly and concentration-dependently at days 0, 6, and 12, but decreased markedly at day 18 through 36 ($p < 0.05$). The highest increment were observed in 5000 and 8000 ppm group of day 12.

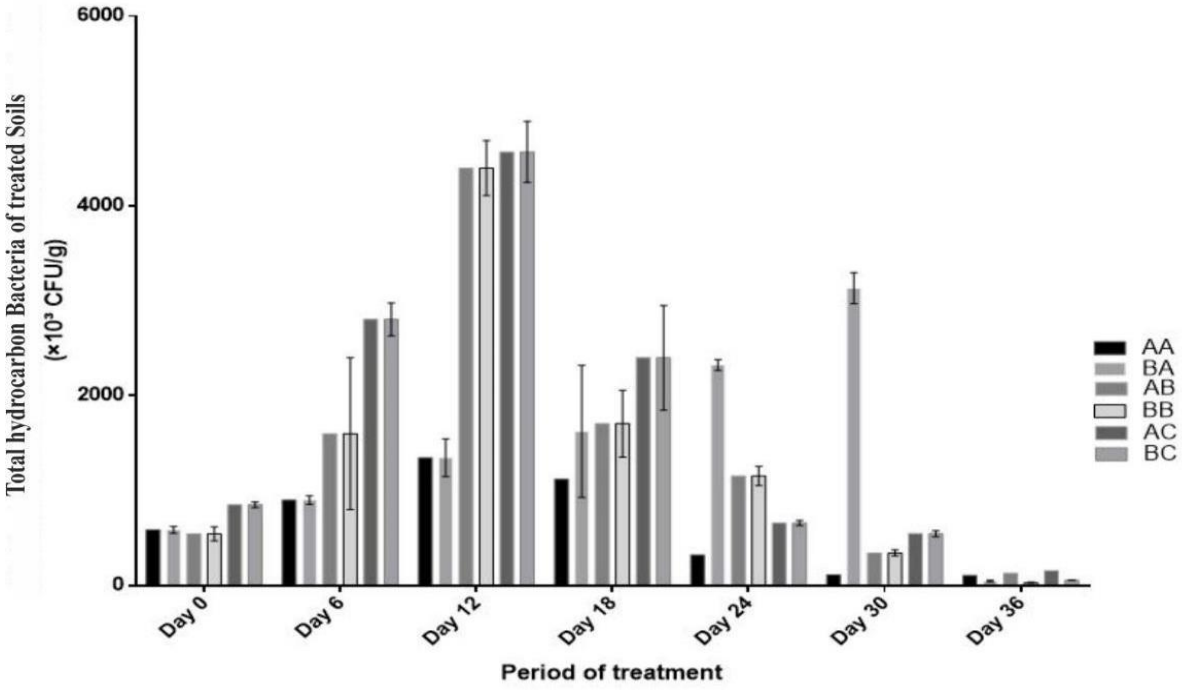

Figure 8: Total hydrocarbon bacteria of treated clay and sandy-clay soils**.**

Figure 9 shows that the total petroleum hydrocarbon (TPH) of contaminated clay and sandy-clay soils demonstrated a progressive decreased from day 0 through 36 ($p < 0.05$).

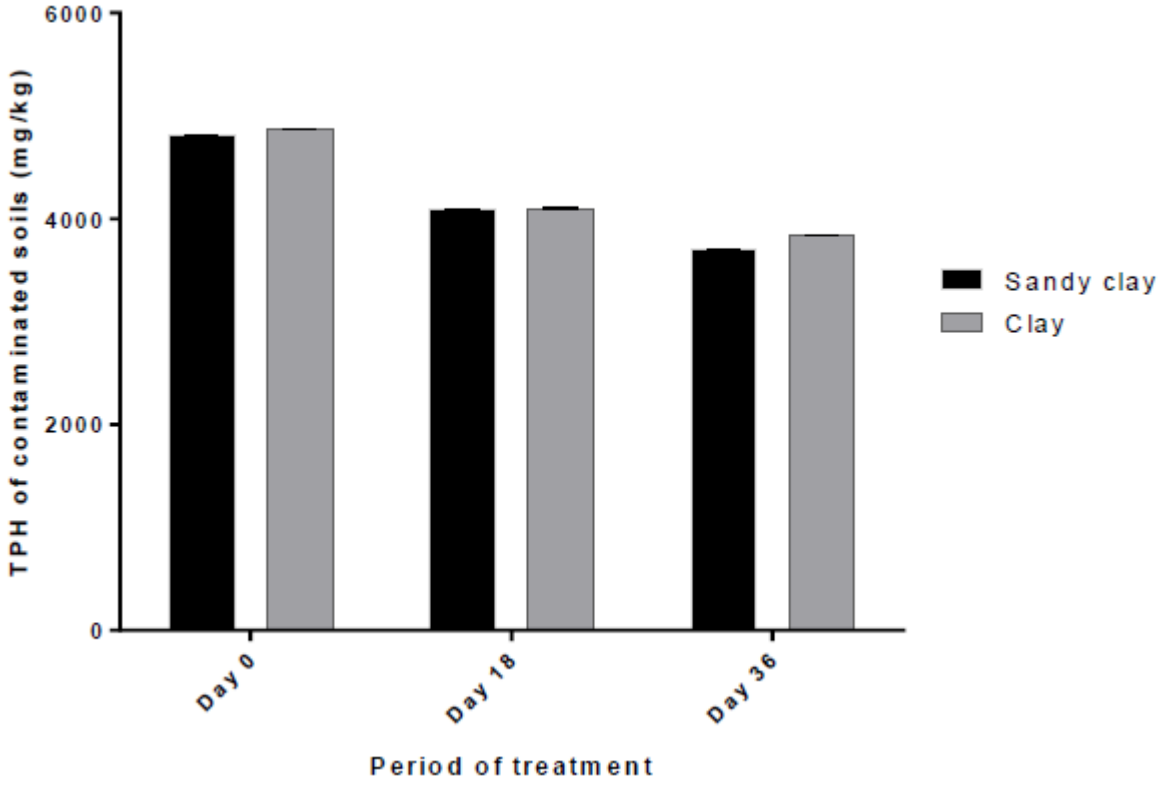

Figure 9: Total petroleum hydrocarbon (TPH) of contaminated clay and sandy-clay soils

The total petroleum Hydrocarbon (TPH) of treated clay and sandy-clay soils demonstrated a progressive and concentration-dependent decrease from day 0 through 36 ($p < 0.05$). The TPH of day 0 was significantly higher than those of the other days ($p < 0.05$) (Fig. 10).

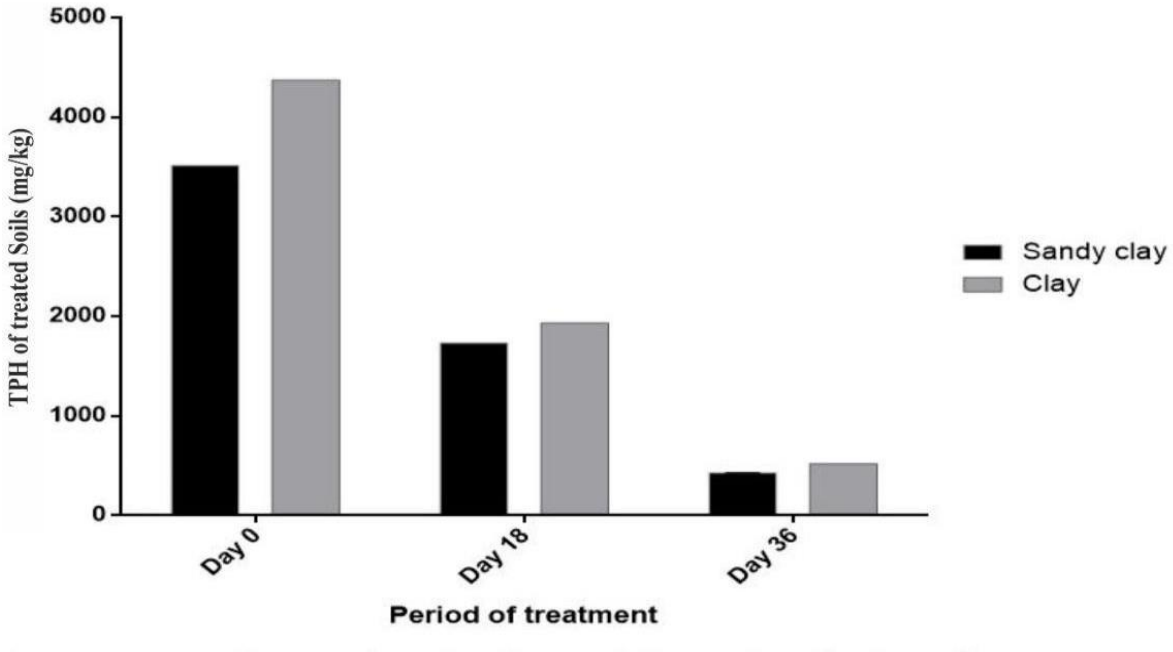

Figure 10: Total petroleum hydrocarbon (TPH) of treated clay and sandy-clay soils

Results of correlation analysis: The relationship between TPH and other parameters investigated in this study including microbial load (THUF, THF, THUB and THB) and enzyme activity (Lacase, peroxidase, catalase and lipase) was evaluated using Pearson Correlation Analysis. The Correlation Coefficient (R) values obtained indicated a significant negative linear relationship between TPH and other parameters investigated at 0.01 probability level. The negative relationship was weak with peroxidase activity, moderate with THUF and very strong for laccase, lipase and catalase. The negative relationship implied that increase in the parameter THB, THUB, THF, THUF, laccase, lipase, catalase, peroxidase) resulted in decrease in TPH (Table 1).

Table 1. Pearson correlation analysis for parameters measured

			TPH	THB	THUB	THF	THU F	Laccase	Peroxidase	Catalase	Lipase
TPH	Pearson	Correlation	1	.706**	-.650**	-688**	-	-.814**	-.384**	-.981**	-.986**
	Sig (2-		105	.000	.000	.000	.574*	.000	.000	.000	.000
	tailed) N			105	105	105	*000105	105	105	105	105
THB	Pearson	Correlation	-.706**	1	.983**	.710**	.655*	.848**	.544**	.635**	.715**
	Sig (2-		.000	105	.000	.000	*	.000	.000	.000	.000
	tailed) N		105		105	105	.000105	105	105	105	105
THUB	Pearson	Correlation	-.650**	.983**	1	.700**	.664*	.821**	.582**	.571**	.655**
	Sig (2-		-.000	.000	105	.000	*.000	.000	.000	.000	.000
	tailed) N		105	105		105	105	105	105	105	105
THF	Pearson	Correlation	-.688**	.710**	.700**	1	.961*	746**	.711**	.633**	.764**
	Sig (2-		.000	.000	.000	105	*.000	.000	000	000	.000
	tailed) N		105	105	105		105	105	105	105	105
THUF	Pearson	Correlation	-.574**	.655**	.664**	.961**	1	.681**	.718**	.493**	.663**
	Sig (2-		.000	.000	.000	.000		.000	.000	.000	.000
	tailed) N		105	105	105	105	105	105	105	105	105
Laccase	Pearson	Correlation	-.814**	.848**	.821**	.746**	.681*	1	.463**	.741**	.815**
	Sig (2-		.000	.000	.000	.000	*.000	105	.000	.000	.000
	tailed) N		105	105	105	105	105		105	105	105
Peroxi	Pearson	Correlation	-.384**	.544**	.562**	.711**	.718*	.463**	1	.327**	.448**
dase	Sig (2-		.000	.000	.000	.000	*.000	.000		.00	.000
	tailed) N		105	105	105	105	105	105	105	105	105
Catalase	Pearson	Correlation	-.981**	.635**	.571**	.633**	.493*	.741**	.327**	1	.967**
	Sig (2-		.000	.000	.000	.000	*	.000	.000		.000
	tailed) N		105	105	105	105	.000105	105	105	105	105
Lipase	Pearson	Correlation	-.966**	.715**	.655**	.764**	.663*	.815**	.446**	.967**	1
	Sig (2-		.000	.000	.000	.000	*	.000	.000	.000	
	tailed) N		105	105	105	105	.000105	105	105	105	105

Correlation was significant at the 0.05 level (2-tailed)

Discussion

Soil is a vital natural resource essential for the flow of matter and energy. It supports plant growth, directly influencing food production (Schoonover and Crim, 2015*).* Soil pollution is defined as the presence of a chemical or substance in an unintended location and/or at concentrations exceeding normal levels, resulting in harmful effects on non-target organisms (FAO and ITPS, 2015). An effective method for improving bioremediation is the application of organic waste materials to enhance microbial activity in contaminated soils (Amechi *et al*., 2017). Fertilizer is rich in biodegradable organic compounds, which have the potential to stimulate microbial growth, providing essential nutrients that enhance the degradation of petroleum hydrocarbons (Jude *et al.*, 2022). Studies have shown that organic amendments such as fertilizer can significantly improve microbial populations responsible for breaking down hydrocarbons (Bhatia and Sindhu, 2024). One type of persistent organic pollution is hydrocarbon pollution. They cause significant and often irreversible damage to ecosystems because of their bio-magnification (Chandra *et al*., 2013). Surface soil, groundwater, and the ocean are contaminated as a result of the widespread release of hydrocarbon pollutants from underground tanks, steamers, oil wells that have been left unplugged, including oil refinery sites that have been abandoned (Souza *et al*., 2014). The aim of this study was to investigate microbial activities in fertilizer-amended contaminated soils. The results of this study showed that THUF, THF, THUB and THB of clay and sandy-clay soils were significantly and concentration-dependently increased by crude oil contamination as well as fertilizer remediation at day 0 to 12, and then decreased from day 18 through 36. These results are consistent

with the findings of Jafari *et al*. (2023) that organic amendments enriched microbial communities, improving their ability to decompose petroleum hydrocarbons and demonstrated the effectiveness of organic waste-based strategies for soil bioremediation and waste management. These results appear to suggest that the presence of crude oil hydrocarbons and fertilizer in the soils may have stimulated microbial growth and activity, and that nutrient depletion later on could have been responsible to the later decline. Soil microbes play a pivotal role in the mineralization and decomposition of soil organic matter by producing various hydrolytic enzymes (Cardelli *et al*., 2019). The initial rise may be due to microbial adaptation and effluent nutrients enhancing fungal and bacterial growth. The later decline suggests competition or nutrient depletion, limiting further proliferation. A rise in fungal growth suggests active degradation processes, but the decline indicates the remediation process is likely unstable, which could affect long-term recovery. The results of this study showed that with appropriate management, NPK/urea fertilizer may constitute an organic supplement for remediating hydrocarbon-polluted soils, especially in places where crude oil pollution is a major environmental challenge.

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