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Effect of some stimulants on bioremediation of crude oil contaminated soil

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ABSTRACT

This study investigated the effect of some stimulants on the bioremediation of crude oil-contaminated soil. Uncontaminated soil sample was collected from the Rivers State University Demonstration farm. The baseline physicochemical and microbiological parameters were determined using standard methods before the soil sample was contaminated with 5% crude oil and divided into eight treatment options. The laboratory-based experimental set-ups were prepared in clay pots, designated as unpolluted and crude oil-polluted soil, which served as controls, while other pots contained various concentrations of cow dung, NPK and rhamnolipid. The microbial community dynamics, hydrocarbon degradation percentage, and changes in soil physicochemical properties were monitored for 70 days. The hydrocarbon utilizing bacterial and fungal isolates were identified based on biochemical characteristics. The concentrations of nitrogen, phosphate, pH, temperature, total organic carbon, soil organic matter, and total petroleum hydrocarbon content of the soil were 110.42±0.4 mg/kg, 4.03±0.3 mg/kg, 6.5±0.1, 31.0±0.5^{o}C, 2.01±0.2 mg/kg, 1.0±0.1 mg/kg, and 186±0.6 mg/kg, respectively. The total heterotrophic bacterial, fungal, and hydrocarbon utilizing bacterial and fungal counts were 4.9×10^{7}, 7.4×10^{4}, 5.9×10^{4} and 4.0×10^{3} CFU/g, respectively. *Bacillus, Pseudomonas, Serratia, Cronobacter, Micrococcus* and *Alcaligenes* species were the hydrocarbon-utilizing bacteria identified, while the hydrocarbon-utilizing fungi were *Rhizopus, Aspergillus, Rhodotorula*, *Penicillium, Geotrichum* and *Mucor* species. The NPK stimulated soil (40g and 20g) showed a significant ($P<0.05$) reduction (80.7 and 79.3%) of total petroleum hydrocarbon (TPH) followed by 40mL and 20mL rhamnolipid with a percentage TPH reduction of 73.4% and 54.4%. The control had the lowest % TPH reduction of 10.1%. However, while the microbial parameters increased due to nutrient utilization, the nitrate and phosphate concentrations decreased except for the decline in microbial parameters towards the end of the monitoring period. The stimulants were effective in enhancing the growth of these organisms, which in turn brought about a decrease in TPH. Hence, the use of rhamnolipid and cow dung is recommended for bioremediation of crude oil-contaminated soil due to the high percentage of total petroleum hydrocarbon reduction recorded in this study, and since these stimulants do not compete in food production.

Keywords: Biostimulants, crude oil, bioremediation, contaminated soil, rhamnolipids.

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INTRODUCTION

Petroleum is a naturally occurring, yellow-to-black liquid found in geological formations beneath the Earth's surface, commonly refined into various fuels. Components of petroleum are separated using a technique called fractional distillation. The name petroleum covers both naturally occurring unprocessed crude oil and petroleum products made up of refined crude oil (Okoye and Okunrobo, 2014). It consists of hydrocarbons of various molecular weights and other organic compounds. Crude oil is mainly composed of hundreds of different hydrocarbon molecules, which are mainly alkanes from C1 to C40 straight chain, C6–C8 branched-chain, cyclohexanes, aromatics and compounds containing sulphur, nitrogen and oxygen

(Romanus et al., 2015).

The physicochemical properties of Nigeria's crude oil vary from one oil field to another. This is attributed to the fluctuating quantity of hydrocarbons (alkanes, alkenes, alkynes, cyclo, and aromatics compounds) and their derivative forms (the presence of heteroatoms such as nitrogen, sulfur, and oxygen (Onyenekenwa, 2011) as well as organic compounds with carboxylic (-COOH) and alcohol (-OH) functionalities) (Onyema and Manilla, 2010). They also contain varying compositions of heavy metals confirmed as major pollutants in oil-producing regions (Madu et al., 2011).

Sites highly contaminated with oil cannot be conducive to plant crops, and those not highly contaminated could give room for growth but in the long run, with the possibility of stagnant or stunted growth (Okoye and Okunrobo, 2014). Biological treatment is an alternative pollutant removal method because this technique does not elicit deleterious effects on the environment. This treatment may also be less expensive than other techniques. In the soil environment, certain microbes have a distinctive ability to degrade or convert organic pollutants into harmless biological products. Bioremediation mainly relies on these talented microorganisms surviving in the soil (Esin and Ayten, 2011).

Biosurfactants are surfactants of biological origins, like microorganisms produced as metabolic products. They are a group of molecular compounds made up of surface active agents, made of both hydrophobic and hydrophilic components that reduce surface and interfacial tension and cause solubility of non-polar compounds (Fenibo et al., 2019; Sar et al., 2023). The most common biosurfactants are rhamnolipids, surfactin, sophorolipids and emulsans (Fenibo et al., 2019). These compounds are used for the bioremediation of hydrocarbons and crude oil, which makes them bioavailable for the microorganisms to degrade. Hence, the transfer of the hydrocarbons to the aqueous phase in bulk is an important process for its bioavailability (Adrion et al., 2016). Surfactants and nutrients (both organic and inorganic) have been adopted to increase the efficiency of bioremediation. Thus, this present study evaluated the effect of different concentrations of organic and inorganic nutrients, including biosurfactants in the bioremediation of crude oil-contaminated soil. These stimulants are used because when there is an oil spill, the carbon concentration increases in the soil, while the other essential nutrients become limiting (Douglas et al., 2020).

MATERIALS AND METHODS

Sample collection

Unpolluted soil samples and cow dung were collected from the Rivers State University demonstration farm. The soil was collected at a depth of 0 -15cm using a sterile hand trowel (disinfected by cleaning the surface with cotton wool immersed in 70% ethanol). This was transported to the lab for analysis. The nitrogen, phosphate and potassium (NPK 10:15:15) fertilizer was obtained from the Agricultural Development Programme (ADP) farm at Rumuodumanya, Port Harcourt. Rhamnolipid was donated by Stepan Company: 22 West Frontage Road, Nortfield, IL 60093, US.

Baseline analysis of sample

Physicochemical parameters

The physicochemical parameters carried out were nitrogen, phosphate, pH, total organic carbon (TOC), soil organic matter (SOM), and total hydrocarbon content (THC) of the soil. These parameters were analysed as described in APHA (2012).

Enumeration and isolation of total heterotrophic bacteria and fungi

This was done using the spread plate method. In this method, 1g of the soil sample was added to a test tube containing 9 ml of sterile normal saline and agitated well by shaking vigorously. This served as the stock. One millilitre (1ml) volume of the stock was withdrawn using a sterile 1mL pipette and transferred to a test tube containing sterile 9mL normal saline. Subsequent 10-fold serial dilution was carried out to achieve a dilution of 1:100, 000. The total heterotrophic bacterial and fungal counts were obtained by plating an aliquot (0.1ml) of 10^{-2} to 10^{-4} on nutrient agar and Sabouraud dextrose agar plates (containing chloramphenicol, 250mg), respectively. Inoculation was done in duplicate and incubated at 37 °C for 24 - 48 hours. After incubation, the colonies were counted and used for enumeration of THB and total fungal. While distinct colonies were sub-cultured on fresh nutrient agar and SDA plates, purified and used for identification of the isolates (Douglas and Cornelius, 2019).

Isolation of hydrocarbon utilizing bacteria (HUB) and fungi (HUF)

The Hydrocarbon utilizing microorganisms were isolated using a mineral salt medium, as described by Douglas and Cornelius (2019). The vapour phase transfer method was used. In this method, 1 ml of crude oil was poured on sterile Whatman filter paper that was placed on the lid of the Petri dish. An aliquot from 10^{-2} to 10^{-3} dilutions was

transferred into the centre of the prepared ketoconazole-fortified mineral salt agar plates and tetracycline-supplemented mineral salt agar for the enumeration and isolation of HUB and HUF, respectively. The inoculated plates were evenly spread using a sterile bent glass rod and incubated at 37°C for 5 to 7 days, while the HUF was incubated at 25°C for 7 days.

Characterization and identification of isolates

The bacterial isolates were characterized based on their Gram's reaction and response to biochemical tests such as catalase, citrate, oxidase, coagulase, methyl red, motility, indole, starch hydrolysis, Voges-Proskauer and sugar fermentation tests (Cheesbrough, 2006). Further confirmation was done by comparing their characteristics with those of known taxa as outlined in Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). The fungal isolates were identified using their morphological features such as colony colour, shape, texture and size of the colony followed by microscopic examination (conidial shape, arrangement of hyphae and type of spore) of their wet mounts prepared with lactophenol cotton blue and referenced to the Fungal Identification Manual (Sarah et al*.,* 2016).

Bioremediation setup

The unpolluted soil was contaminated with 5% crude oil and allowed to stand for 21 days to ensure even distribution and bonding of crude oil to the soil (Ogbonna et al*.,* 2019). After which, 1kg of the soil was weighed and put into each of the eight labeled clay pots. The experimental setup is presented in Table 1.

Table 1. Experimental setup.

Clay Pots	NPK	Cow dung	Rhamnolipids	Quality
Control 1	-	-	-	1000g
Control 2	-	-	-	1000g
20gNPK+Soil	20g	-	-	1000g
40gNPK+Soil	40g	-	-	1000g
20gCD+Soil	-	20g	-	1000g
40gCD+Soil	-	40g	-	1000g
20gRM+Soil	-	-	20mL	1000g
40gRM+Soil	-	-	40mL	1000g

Keys: CD = cow dung, RM = rhamnolipids, NPK = Nitrogen-Phosphate-Potassium fertilizer, - = not applied, + = applied.

Biodegradation monitoring

Changes in microbial counts

The total heterotrophic bacteria, fungi, and hydrocarbon utilizing bacterial and fungal counts of all the experimental set-ups were monitored during the period of the bioremediation. The methods adopted to monitor the changes in the microbial parameters are the same as those adopted in the baseline.

Determination of physicochemical parameters

Determination of pH

The pH of the soil sample was determined using APHA (2012). The meter was switched on and allowed for some time. It was then calibrated with buffer solutions of a high pH range between 8 and 9 as well as a lower pH range between 1 and 6 by dipping the electrode into the buffer solutions. Ten grams (10g) of soil was weighed into a 100 ml beaker; 50 ml of distilled water was then added to allow immersion of the electrode, mixing was done by stirring frequently for a few minutes. The beaker was allowed to stand for 15 minutes, and the electrode was immersed into the sample. The pH values for each sample were recorded accordingly.

Determination of temperature

Temperature was determined using a mercury-in-glass thermometer. The thermometer was immersed into the samples so that the mercury bulb was well covered by the samples and allowed the reading to stabilize before the temperature reading was finally taken (APHA, 2012).

Determination of total Nitrogen

This was done using the Kjeldahl method, where 1.0g of oven-dried soil sample was weighed into a labeled dry and clean digestion tube. Then 15ml digestion mixture

was added to each tube and digested for 2 hours. Twenty-five millilitres (25ml) of distilled water was added to the digested soil and properly mixed. It was allowed to cool before the addition of 50 ml of distilled water and left to stand to obtain a clear solution (APHA, 2012).

Determination of total organic carbon (TOC)

The method of Walkley-black chromic acid wet oxidation method was used, and 20g of soil sample was measured into a 500 ml conical flask. Ten 10 ml of 0.5M $K_2Cr_2O_7$ was added and swirled gently. Twenty millilitres (20 ml) of concentration H_2SO_4 were added rapidly and directly into the suspension but with care to avoid splashing. Immediately swirl gently for 1 minute until the reagents are mixed. The flask was allowed to stand for 30 minutes, and 200 ml of distilled water and 10 ml concentration of H_3PO_4 were added cautiously to avoid splashing. The mixture was cooled, and 3 drops of Ferroin indicator solution were added. The solution was titrated to a deep green end-point with 0.25M Ferroin Ammonium Sulphate (FAS) solution (APHA, 2012).

Determination of soil organic matter (SOM)

This was determined using the Gravimetric method. In this method, 1 gram of soil sample was transferred to a 10 ml beaker and incinerated in an electric muffle for 1 hour at 250, 300, 350, and 400 ^oC. Incineration times of 0.5, 1.0, 2.0, and 4.0 hours at 300 ^oC were also evaluated (APHA, 2012).

Determination of phosphate

Phosphomolybdenum blue (PMB) method was used. The phosphate levels in the samples were determined using an ultraviolet (UV) spectrophotometer. Twenty-five millilitres (25 ml) of 2.5% acetic acid were added to 1g of soil sample and shaken for 30 minutes. The suspension was filtered through a filter paper. Ten milliliter (10 ml) of the extract was transferred into a 50 ml volumetric flask. The extract was diluted with distilled water until the flask was about 2/3 full. Two millilitres (2 ml) of Ammonium Molybdate reagent was added and mixed with the extract. Two millilitres (2 ml) of Tin (II) Chloride were also added and mixed; the solution was diluted to the 50 ml mark with distilled water. The flask was allowed to stand for 30 minutes, and the absorbance was measured at a wavelength of 690nm (APHA, 2012).

Determination of nitrate

The nitrate levels for the samples were determined using an ultraviolet (UV) spectrophotometer. The spectrophotometric method was adopted. Five grams (5g) of samples were weighed into a shaking bottle. One hundred and twenty-five milliliter (125 ml) of distilled water was added and shaken for 10 minutes on a rotary shaker and then filtered to obtain the extract, 1 ml of the extract was transferred into 10 ml volumetric flask, 0.5 ml of Brucine reagent was added. Subsequently, 2 ml of concentrated sulphuric acid was rapidly added and mixed for about 30 seconds. The flasks were allowed to stand for 5 minutes. Two milliliters (2 ml) of distilled water were added and mixed for about 30 seconds. The flasks were allowed to stand in cold water for about 15 minutes, and the absorbance of the samples was measured using the spectrophotometer (APHA, 2012).

Total petroleum hydrocarbon (TPH)

Ten grams (10g) of soil samples were taken from each treatment setup and put into amber glass bottles respectively. Anhydrous sodium sulphate (Na_2SO_4) was added to the amber glass bottles containing the soil samples and mixed evenly. About 300 µg/ml of the surrogate (1-chlorooctadecane) standard was added to each soil sample and 30 ml of dichloromethane (DCM) was put into each sample as extracting solvent and then continuously mixing using a mechanical shaker for 5 to 6 hours. After agitation, the soil samples were allowed to settle for 1 hour and then filtered using 110 mm filter paper into clean beakers appropriately. The filtrates were allowed to concentrate to 1ml by evaporation overnight in a fume cupboard (Yang, 2013).

The separation and detection of compounds in soil samples were carried out using the Agilent 6890N Gas Chromatograph–Flame Ionization Detector (Agilent 6890 GC-FID) instrument. Three microlitres (3µl) each of concentrated samples eluted from the column were injected into GC vials separately. The blank DCM was injected into a micro-syringe of GC to clean the syringe (3 times) before taking the samples for analysis. The micro-syringe was further rinsed with the extracted soil sample individually; the samples were injected into the column for the separation of compounds in each sample. After separation, the compounds were passed through a flame ionization detector which detects the compounds in the sample. The amount of TPH was resolved at a particular chromatogram in mg/kg for the soil samples (Yang, 2013).

Percentage biodegradation

Percentage biodegradation was calculated as follows:

Step 1: Amount of pollutant remediated equals to initial pollutant concentration (Day 1) minus final pollutant

concentration at the end of experiment (Last day).
Step 2: Percentage (%) bioremediation equals to amount of pollutant remediated divided by initial pollutant concentration (Day 1) multiplied by 100.

$$\mathbf{BC = IC - FC}$$

Where;
BC = Amount of pollutant remediated
IC = Initial concentration of pollutant (Day 0 or 1)
FC = Final concentration of pollutant at end of experiment (Last day)

$$\%\ \mathbf{Bioremediation} = \frac{\mathbf{BC\ x\ 100}}{\mathbf{IC}}$$

Statistical analysis

The mean and standard deviation of the microbial counts, the physicochemical parameters and the TPH of the soil samples were determined using the statistical package for social science (SPSS version 27). ANOVA was carried out to check significant differences (P<0.05) and the Duncan Multiple range test was adopted in separation of means.

RESULTS

Baseline data of samples

The baseline physicochemical parameters of the contaminated and uncontaminated soil samples are presented in Table 2. Results revealed that the nitrogen, phosphate, pH, temperature, total organic carbon (TOC), soil organic matter (SOM), and total hydrocarbon content (THC) of the uncontaminated soil were 110.42±0.1, 4.03±0.1, 6.5±0.1, 31.0±0.0, 2.01±0.2, 1.0±0.1 and 186±0.6 mg/kg, respectively. Also, results of the concentrations of nitrogen, phosphate, pH, temperature, TOC, SOM, and total THC of the soil after contamination were 67.8±0.3, 1.3±0.2, 4.92±0.1, 30.3±0.0, 5.6±0.1, 3.3±0.0 and 5180±5.0 mg/kg, respectively.

Results of the microbial counts of the soil samples revealed that the total heterotrophic bacterial, fungal, and hydrocarbon utilizing bacterial and fungal counts for the uncontaminated soil were 4.9×10^7, 7.4×10^4, 5.9×10^4 and 4.0×10^3 CFU/g, respectively (Table 3).

Table 2. Physicochemical properties of the uncontaminated soil (Baseline) and soil after contamination.

Set up Code	Nitrogen (mg/kg)	Phosphate (mg/kg)	pH	Temp (^oC)	(TOC) (%)	(SOM) (%)	(TPH) (mg/kg)
Unpolluted soil	110.42±0.1 [b]	4.03±0.1 [b]	6.5±0.1 [a]	31.0±0.0 [a]	2.01±0.2 [a]	1.0±0.1 [a]	186±0.6 [a]
Contaminated soil	67.8±0.3 [a]	1.3±0.2 [a]	4.92±0.1 [a]	30.3±3.0 [a]	5.6±0.1 [b]	3.3±0.3 [a]	5180±5.0 [b]
P-value	0.03	<0.001	1.762	0.833	0.013	0.633	0.03

*Means with similar superscript revealed no significant difference (P<0.05),
Keys: Total Organic Carbon = TOC; Soil Organic Matter = SOM; Total Hydrocarbon Content = THC.

Table 3. Baseline data of microbial counts of soil sample before and after contamination.

Sample	THB ($\times10^7$) CFU/g	FC ($\times10^4$) CFU/g	HUB ($\times10^4$) CFU/g	HUF ($\times10^3$) CFU/g
Uncontaminated Soil	4.9±0.5 [b]	7.4±0.5 [b]	5.9±0.1 [a]	0.4±0.07 [a]
Contaminated soil	2.9±0.2 [a]	2.4±0.1 [a]	8.5±0.3 [b]	1.9±0.01 [b]
p-value	0.00	0.05	0.02	0.004

*Means with similar superscript down the group revealed no significant difference (P<0.05).
Keys: THB = total heterotrophic bacteria, FC = fungal counts, HUB = hydrocarbon utilizing bacteria, HUF = hydrocarbon utilizing fungi.

The changes in the total heterotrophic bacterial (THB) counts during the period of bioremediation showed that the THB for Days 1, 14, 28, 42, 56 and 70 ranged from 1.2×10^6 to 2.6×10^7, 4.1×10^6 to 2.6×10^7, 3.3×10^6 to 2.8×10^7, 3.4×10^6 to 9.2×10^6, 2.6×10^6 to 1.1×10^7 and 2.7×10^6 to 2.1×10^7 CFU/g, respectively (Figure 1). The fungal counts for Days 1, 14, 28, 42, 56 and 70 ranged from 3.5×10^3 to 1.9×10^4, 3.2×10^3 to 2.4×10^4, 7.0×10^3 to 3.5×10^4, 2.5×10^3 to 4.0×10^4, 1.6×10^4 to 1.5×10^5 and 3.1×10^4 to 1.1×10^5 CFU/g, respectively (Figure 2). Results of the HUB counts for Days 1, 14, 28, 42, 56 and 70 ranged from 1.4×10^4 to 7.6×10^4, 3.6×10^4 to 5.0×10^5, 1.0×10^4 to 5.4×10^5, 7.0×10^3 to 1.7×10^5, 5.0×10^3 to 6.5×10^4 and 2.0×10^3 to 8.5×10^4 CFU/g, respectively (Figure 3), while results of the HUF counts for Days 1, 14, 28, 42, 56 and 70 ranged from 1.40×10^4 to 7.6×10^4,

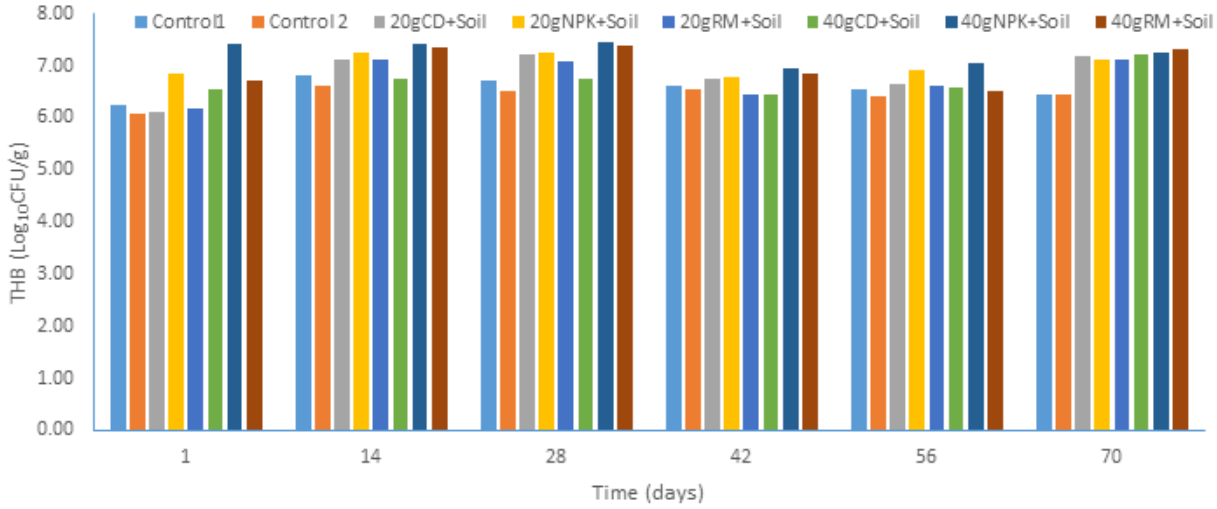

Figure 1. Changes in the total heterotrophic bacterial (THB) counts during the period of bioremediation.
Keys: Control 1 (uncontaminated soil), control 2 (contaminated soil).

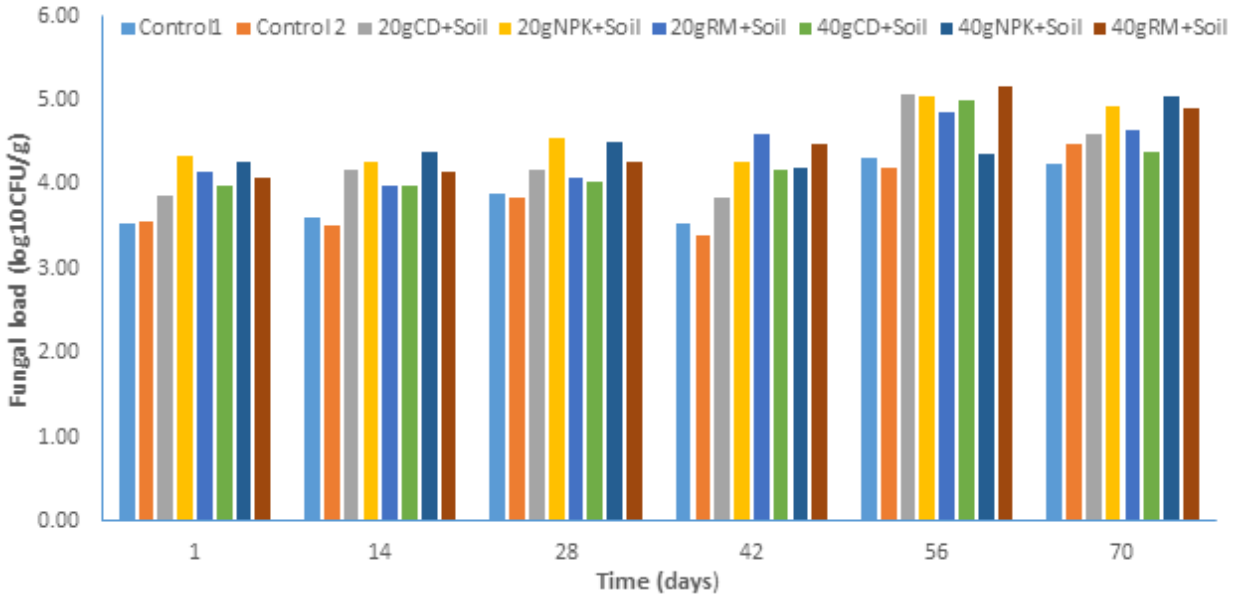

Figure 2. Change in the fungal counts during the period of bioremediation.
Keys: Control 1 (uncontaminated soil), control 2 (contaminated soil), CD: cow dung, NPK: nitrogen phosphate potassium fertilizer, RM: rhamnolipids.

3.6×10^4 to 5.0×10^5, 1.0×10^4 to 5.4×10^5, 7.0×10^3 to 1.7×10^5, 5.0×10^3 to 1.05×10^5 and 2.0×10^3 to 8.5×10^4 CFU/g, respectively (Figure 4).

Results of the phenotypic and biochemical characteristics of the bacterial isolates revealed that *Bacillus, Pseudomonas, Serratia, Cronobacter, Micrococcus* and *Alcaligenes* species were the hydrocarbon-utilizing bacterial isolates while the hydrocarbon-utilizing fungi were *Rhizopus, Aspergillus, Rhodotorula*, *Penicillium, Geotrichum* and *Mucor* species.

Results for the change in pH during the bioremediation process revealed that the pH varied across the treatment and in the period of the remediation. The highest pH (7.7 and 7.36) in day one was recorded in treatment 40g NPK

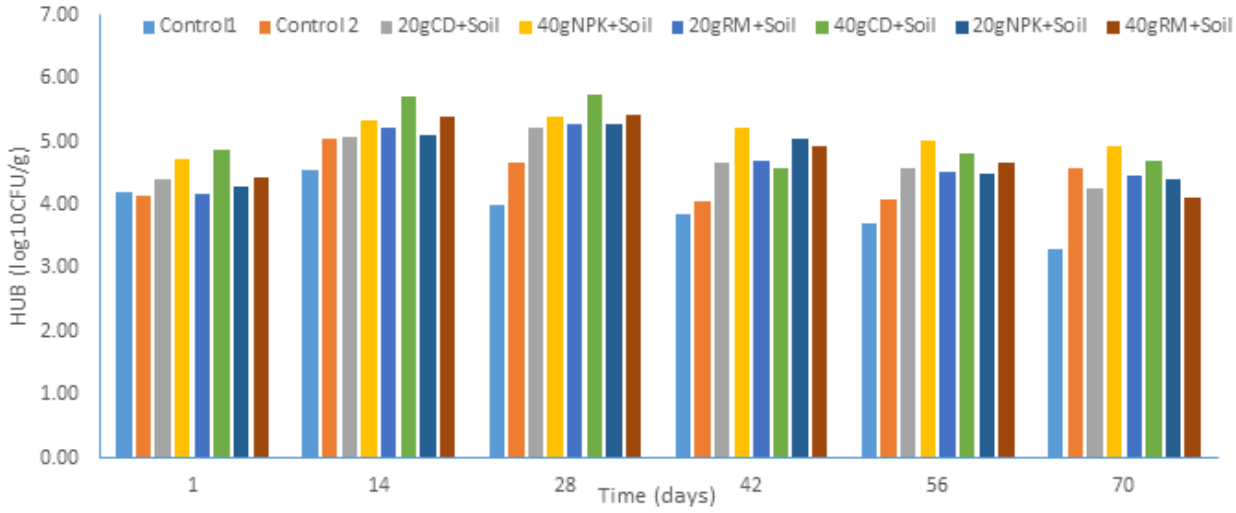

Figure 3. Change in the hydrocarbon utilizing bacterial counts during the period of bioremediation.
Keys: Control 1 (uncontaminated soil), control 2 (contaminated soil), CD: cow dung, NPK: nitrogen phosphate potassium fertilizer, RM: rhamnolipids.

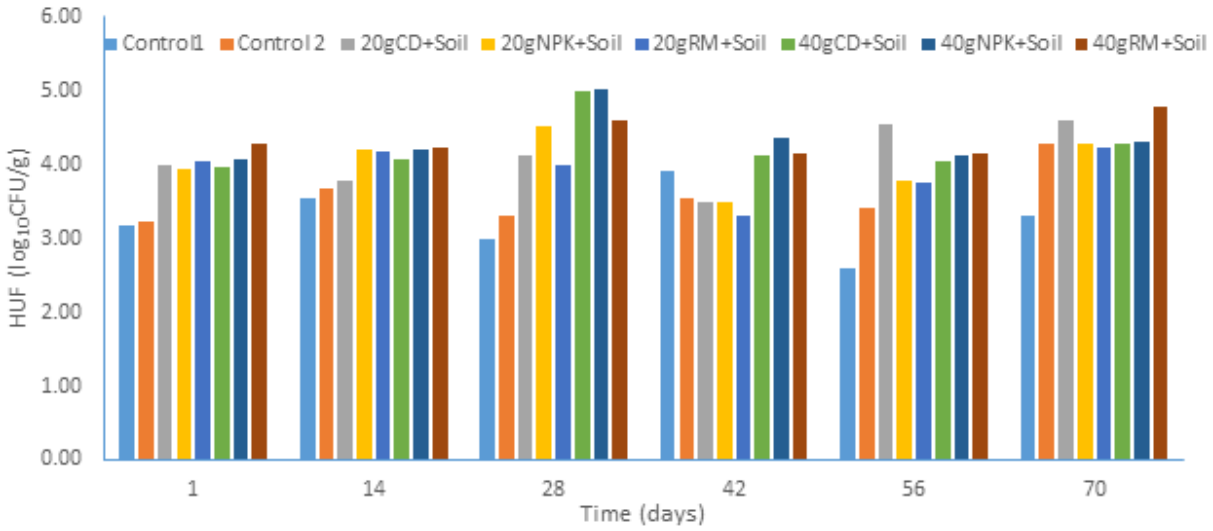

Figure 4. Change in the hydrocarbon utilizing fungal counts during the bioremediation.
Keys: Control 1 (uncontaminated soil), control 2 (contaminated soil), CD: cow dung, NPK: nitrogen phosphate potassium fertilizer, RM: rhamnolipids.

and the control samples, while a pH range of 6.5 - 6.81 was recorded for other treatments on day 1 (Figure 5).

Results of the changes in nitrate and phosphate concentrations of the treatments during the remediation period are presented in Figures 6 and 7, respectively. Results revealed that the nitrate and phosphate concentrations were reduced in all treatment options from day 1 to the last day.

The results of the total organic carbon and other parameters declined with time in the various treatment options during the duration of the remediation process (Figure 8).

Results of the chromatogram of the TPH for the various samples are presented in Supplementary figures 1 to 16. The changes in TPH of the setups and the 5.0 reduction of TPH presented in Table 4, revealed that the 40g NPK experimental set-up reduced the TPH values from 5180±mg/kg on the first day to 933.1±0.00mg/kg while

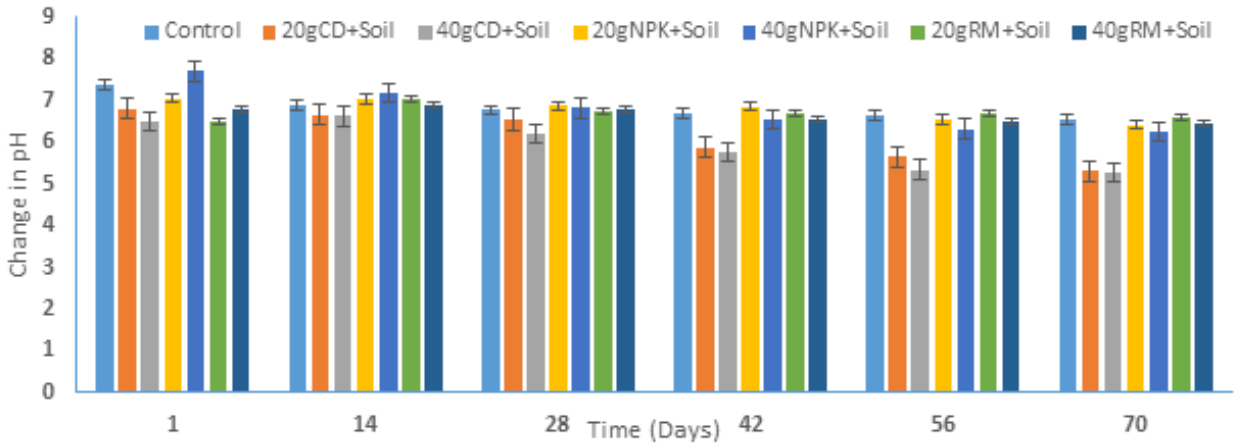

Figure 5. Changes in pH during bioremediation.
Keys: control (contaminated soil), CD: cow dung, NPK: nitrogen phosphate potassium fertilizer, RM: rhamnolipids.

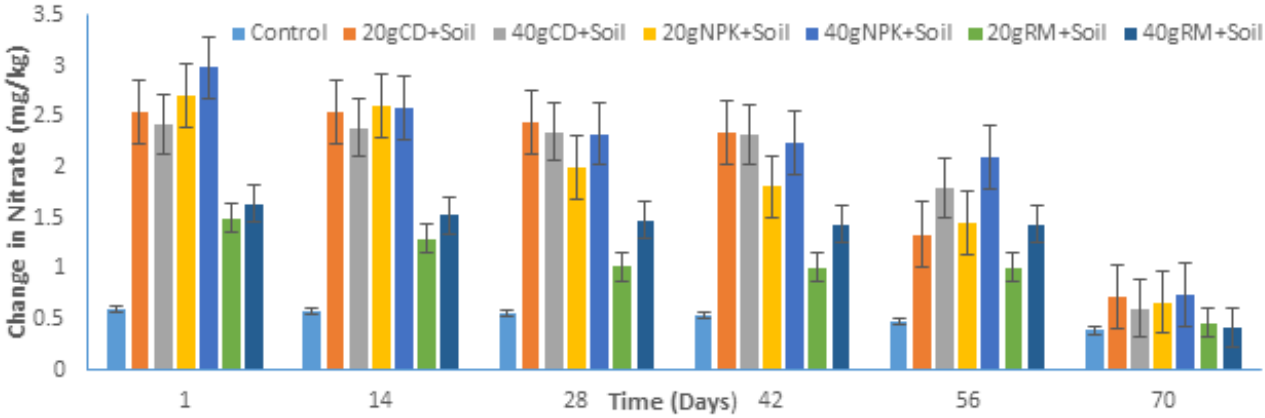

Figure 6. Change in nitrate concentration (mg/kg) during bioremediation
Keys: Control (contaminated soil), CD: cow dung, NPK: nitrogen phosphate potassium fertilizer, RM: rhamnolipids.

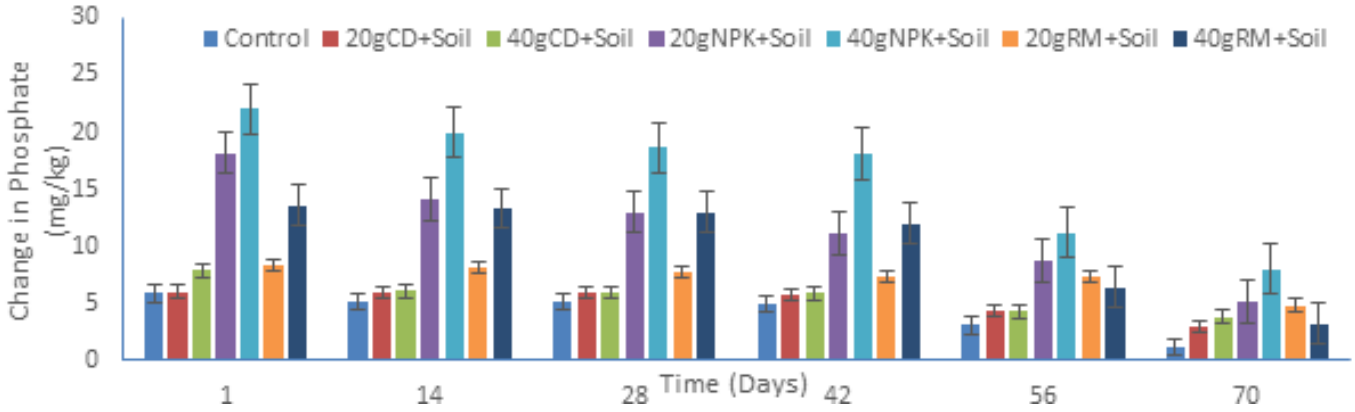

Figure 7. Change in phosphate concentration (mg/kg) during bioremediation
Keys: Control (contaminated soil), CD: cow dung, NPK: nitrogen phosphate potassium fertilizer, RM: rhamnolipids.

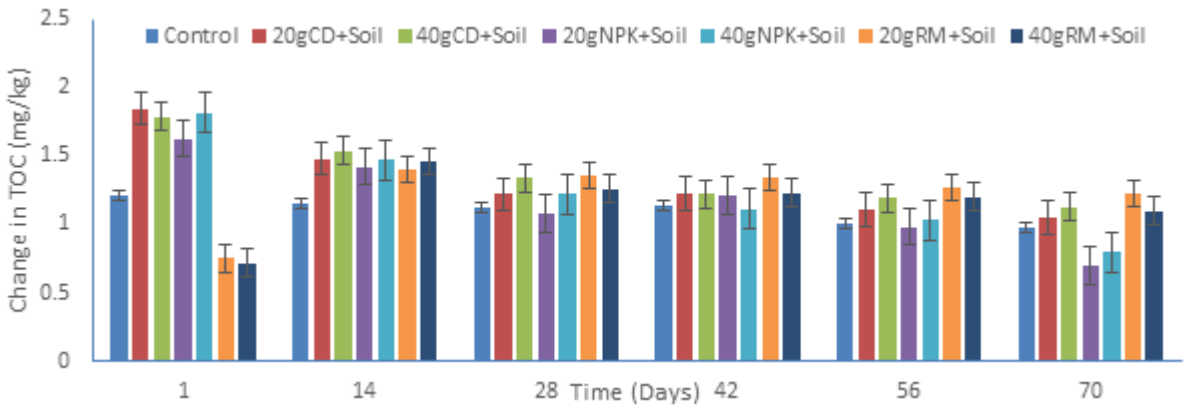

Figure 8. Change in total organic carbon (mg/kg) during bioremediation
Keys: Control (contaminated soil), CD: cow dung, NPK: nitrogen phosphate potassium fertilizer, RM: rhamnolipids.

Table 4. Change in TPH of the samples and the % reduction of TPH.

Clay Pots	Day 1	Day 14	Day 28	Day 42	Day 56	Day 70	% Remediated
Control 1	5180.24±34.2	4811±0.01	4683±0.00	4538±0.00	4512±0.00	4338±0.00	10.1
20gCD+Soil	5180.24±34.2	40200±0.01	3812.8±0.00	2994.3±0.00	2642.7±0.00	2443.5±0.00	49.3
40gCD+Soil	5180.24±34.2	3965.4±0.02	3552.9±0.00	2982.1±0.00	2572.5±0.00	1542.4±0.00	68.0
20gNPK+Soil	5180.24±34.2	3001.8±0.01	2119.8±0.00	1087.5±0.00	1022±0.00	1000.8±0.00	79.3
40gNPK+Soil	5180.24±34.2	2998.6±0.00	2008.9±0.00	1036.8±0.00	997.19±0.00	933.1±0.00	80.7
20gRM+Soil	5180.24±34.2	4002±0.00	3881±0.00	3481.8±0.00	3006.6±0.00	2200±0.00	54.4
40gRM+Soil	5180.24±34.2	3009±0.00	2372.6±0.00	2120±0.00	2042.3±0.00	1280.9±0.00	73.4

Percentage Bioremediation of Crude Oil Polluted Soil
Keys: Control (contaminated soil), CD: cow dung, NPK: nitrogen phosphate potassium fertilizer, RM: rhamnolipids.

the 20g NPK set-up reduced the TPH from 5180±5.0 to 1000.8±0.00 mg/kg. The soil sample supplemented with 40g cow dung reduced the TPH from 5180±5.0mg/kg to 1542.4±0.00 mg/kg, 20g CD reduced the TPH from 5180±5.0mg/kg to 2443.5±0.00 mg/kg while the 40mL and 20mL rhamnolipids treated soils reduced the TPH from 5180±5.0 to 1280.9±0.00 and 2200±0.00 mg/kg, respectively. The percentage (%) reduction of TPH presented in the control was 10.1%, while the highest TPH reduction of 80.7% was recorded in soil supplemented with 40g NPK, followed by the 20g NPK (79.3%) supplemented sample (Table 4).

DISCUSSION

It was observed from the baseline results that the soil sample has an adequate microbial population, which if enhanced would use up the hydrocarbon components and bring about remediation. The physicochemical properties of the soil such as pH, nitrate, phosphate, TOC and SOM would also support microbial activities in the soil. It was observed that the introduction of the crude oil led to a decrease in the concentrations of some parameters like nitrogen, phosphate, and pH, while it led to an increase in concentrations of some parameters such as TOC and SOM, indicating an increase in organic contaminants.

Physicochemical parameters such as the pH, nitrate, and phosphate of the experimental setup decreased gradually across the setups during the period of bioremediation. The total organic carbon had no regular pattern as it fluctuated across the sample and period of the bioremediation. The pH values were slightly or weakly acidic and optimal for microbial activities. The pH is a vital factor that influences the activities of living organisms (Prescott et al., 2011). Thus, pH values exceeding the optimum limit of an organism could have a detrimental effect on the organism by affecting enzymatic activities. This may have resulted in the fluctuations observed in the microbial populations in the present study. Fungal isolates are known to grow better in acidic pH, while bacterial isolates are slightly acidic to alkaline (Chikere et al., 2016). Moreover, the pH values of the setups were within the optimum pH for rapid decomposition of waste, which is usually in the range of 6.5 to 8.5 (Akinbile et al., 2014). The observed decrease in the pH of the treated samples could mean that the crude oil increased the acidity of the samples, and this would be a reflection of the fluctuations in microbial

populations observed during the bioremediation process (Douglas and Penu, 2019). It corroborates with a previous study which reported that a decrease in the pH of polluted samples is indicative that petroleum pollutants make habitats more acidic, which could lead to changes in microbial structure (Nweze and Aniebonam, 2009).

The depletion of the nitrate and phosphate concentrations in the polluted soil with treatments during the period of remediation could be attributed to the vigorous microbial activities, which were reflected in the increased bacterial counts, especially for Days 1 to 28. Similar studies have reported depletion of phosphate and nitrate concentrations during bioremediation (Albert and Anyanwu, 2012; Douglas et al., 2020). The findings revealed that the NPK fertilizer resulted in higher microbial growth, while the setup without supplements had the least growth of organisms. The high microbial growth in the NPK-treated soil could be responsible for the high bioremediation efficiency recorded during the period. Previous studies have reported that NPK fertilizers have higher nitrate and phosphate contents than most organic wastes (Akinbile et al., 2014). The nitrate and phosphate concentrations of the treatments were significantly higher in Days 1, 14 and 28 than the concentrations observed in Days 42 to 70. It was also reflected in the microbial load which increased during the bioremediation study, and the nutrients used up by the organisms for their growth.

The total organic carbon (TOC) was slightly constant in the control (contaminated soil without supplement) throughout remediation but was depleted in the treated samples during the period of remediation. Constant TOC in the control corroborates with Albert and Anyanwu, (2012). The TOC is an indicator used to measure the compounds that contain carbon. It can be used to gauge how much organic pollution is present in a specific environment (Owhonka and Obire, 2019, 2020). Although the TOC fluctuated across the samples, the findings showed a slight reduction and this could be attributed to the microbial degradation of organic contaminants in the polluted soil, which is also reflected in the increase in hydrocarbon utilizing bacterial and fungal counts. Heterotrophic microorganisms are known to be responsible for the utilization of organic carbon, thus making it available to the different food webs (Owhonka and Obire, 2020). It has been reported that microorganisms involved in bioremediation metabolize organic contaminants as their carbon and energy source, which leads to the breakdown of complex organic compounds into simpler, often less harmful substances thereby decreasing the overall organic carbon content in the environment (Atlas and Philip, 2005).

A decrease in the peaks of the hydrocarbon components was observed to decrease with time on the chromatogram. However, the findings showed that pristane, phytane and other carbon compounds which ranged from C8 to C40 were among the components of the crude oil. The initial concentration of 5180±5.0 mg/kg of the total petroleum hydrocarbon in the crude oil-contaminated soil was higher than the target value of 50 mg/kg recommended by the Department of Petroleum Resources (DPR) as the target value for soil and sediment (EGASPIN, 2018). With the above value obtained for the TPH, there is a need for intervention because the 5,000 mg/kg intervention value set by the DPR was exceeded. The bioremediation of the TPH revealed that the level of TPH in the crude oil-polluted soil with amendments reduced drastically during the period of bioremediation, unlike the slight reduction in TPH values observed in the un-amended crude oil-contaminated soil (control). The findings further revealed that the reduction of the TPH values was as follows: 40gNPK+Soil > 20gNPK+Soil > 40gRM+Soil > 40gCD+Soil > 20gRM+Soil > 20gCD+Soil > Control as presented in Table 4. The level of TPH remediated in the present study by biostimulation agreed with Abioye et al. (2010), who observed that biostimulation was more effective than bioaugmentation in removing crude oil pollutants from the soil.

However, the effect of the different treatments revealed that those with higher concentrations of the supplement (nutrient) had higher bioremediation percentages; for instance, the 40g NPK set-up had the highest percentage bioremediation of 80.7%, while the 20g NPK had a bioremediation percent of 79.3%. It was observed that there was no significant difference between the 40g and 20g NPK remediation efficiency (80.7% and 79.3%). The set-up containing 40g CD had a bioremediation percent of 68.0% as against the 49.3% recorded for the 20g CD. Similar observations were made for the rhamnolipids-supplemented soil, where the 40 ml rhamnolipids had a higher percentage than the 20 ml. The bioremediation efficiency recorded in this present study for all treatments was higher than the bioremediation efficiency of 31.55-67.58% by Ekwuabu et al. (2016). The difference in percentage bioremediation between the present study and theirs could be attributed to the higher TPH value of 8695.77 mg/kg compared to 5180±5.0 mg/kg of this present study. The treatments with NPK fertilizer showed higher bioremediation efficiency than the 40 ml rhamnolipids treated than the cow dung (organic nutrient) supplemented soil. Douglas and Ikirikoba (2018) reported that cow dung gave higher TPH reduction than soybean waste for the bioremediation of illegally refined crude oil residue. Ibiene et al. (2011) also reported that when cow dung, poultry dropping and spent mushrooms were used for bioremediation of hydrocarbon-contaminated soil, cow dung also gave the highest TPH loss.

To successfully replenish nutritional deficiencies, inorganic fertilizer has been used extensively in agriculture for better crop yield and bioremediation. The extensive use of inorganic fertilizers however has been

linked to several problems, including increased soil acidity, mineral imbalance, soil degradation, and gaseous emissions (Gupta et al., 2016). Among the setbacks of applying inorganic fertilizer are soil hardening, soil degradation, and eutrophication (Adams et al., 2015). Since fertilizer must be reapplied frequently, this system is also expensive to maintain (Ekwuabu et al., 2016). Inorganic fertilizers can readily leach out of the soil when applied, run off into neighbouring streams, and have negative effects on both humans and animals, according to Macaulay and Rees, (2014). However, crop residues and animal wastes made into organic stimulants are more environmentally friendly and sustainable as they also possess the potential to enhance microbial degradation (Nduka et al*.,* 2012; Chikere et al., 2016). The impact of sewage sludge (SS) and cow dung (CD) on the bioremediation of spent lubricating oil in contaminated soil was examined by Agamuthu et al. (2013). They observed that both organic amendments, CD and SS, could function as biostimulants in hydrocarbon degradation because they decreased the amount of lubricating oil in the soil by 94% and 82%, respectively, which corroborates the present study.

Biosurfactants are known to enhance the efficiency of bioremediation by increasing their cell membrane hydrophobicity and changing the membrane permeability, enhancing their ability to take up the hydrocarbon compounds and utilize them, thereby leading to their biodegradation (Patowary et al., 2018). Sar et al. (2023) reported that biosurfactants can lower the surface and interfacial tension between the soil-water and air-water systems. Lowering these forces enhances the contact of the biosurfactant with the contaminant, mobilizing the pollutant. The application of an adequate quantity of the biosurfactant leads to micellization, which encapsulates the organic matter, leading to rapid breakdown. The present study showed that 40 ml of rhamnolipids decreased the TPH level in the soil by more than 20%. The presence of rhamnolipids makes the crude oil more bioavailable making it available to the organisms in the soil and leading to the reduction of the TPH (Sar et al., 2023). Shu-Wen et al. (2020) in their study reported that rhamnolipid supplementation increased the rate and extent of total petroleum hydrocarbon biodegradation to a maximum of 81% within 35 days, which was higher compared to the present study, which lasted for 70 days, with a percentage bioremediation of 73.4% and 54.4% for 40ml RM and 20ml RM, respectively. Lai et al. (2009) and Sar et al. (2023) reported that when rhamnolipid and surfactin were used in comparison with synthetic surfactant (Tween 80 and Triton X-100) for petroleum hydrocarbon removal from contaminated soil. The TPH was removed from the contaminated soil by 23%, 14%, 6% and 4%, respectively. This shows that the biosurfactant was more effective than the synthetic surfactant.

CONCLUSION

This research has shown that the addition of stimulants like rhamnolipid, cow dung and NPK fertilizers enhanced the bioremediation of crude oil-contaminated soil. The effects of the supplements were concentration-dependent implying that the stimulants with higher concentrations were more effective than those with lower concentrations. NPK fertilizer was more effective in the reduction of TPH, followed by the rhamnolipids and cow dung. Cow dung and rhamnolipids are recommended for use since they are not competing for food production and are environmentally friendly.

Conflicts of interest

The authors declare that there is no competing interest.

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Supplementary figures

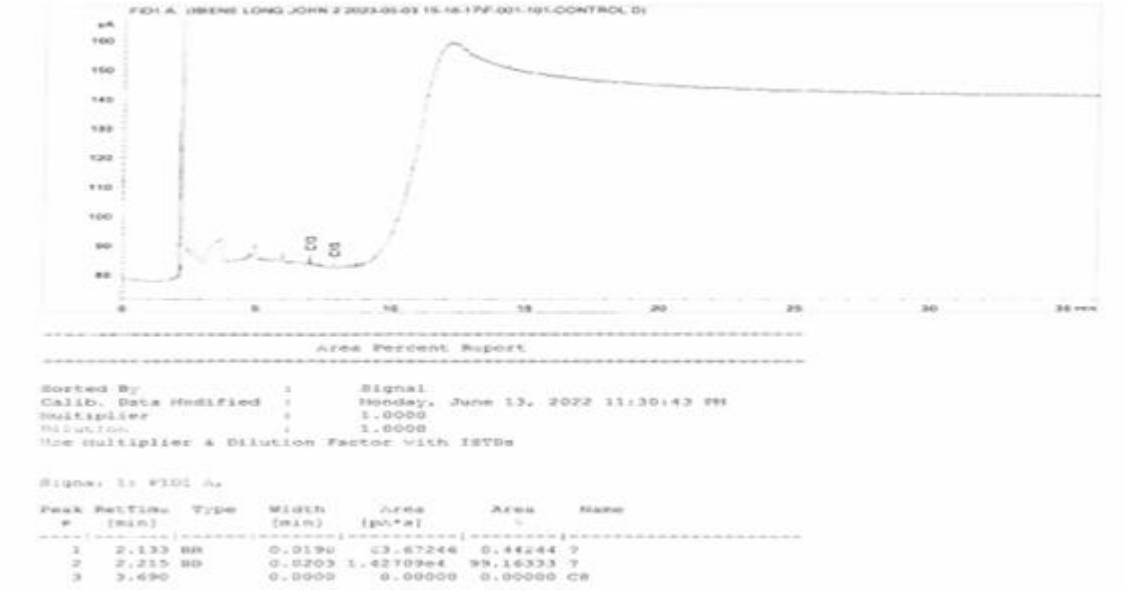

Figure 1. Chromatogram of TPH in day 1 of uncontaminated soil.

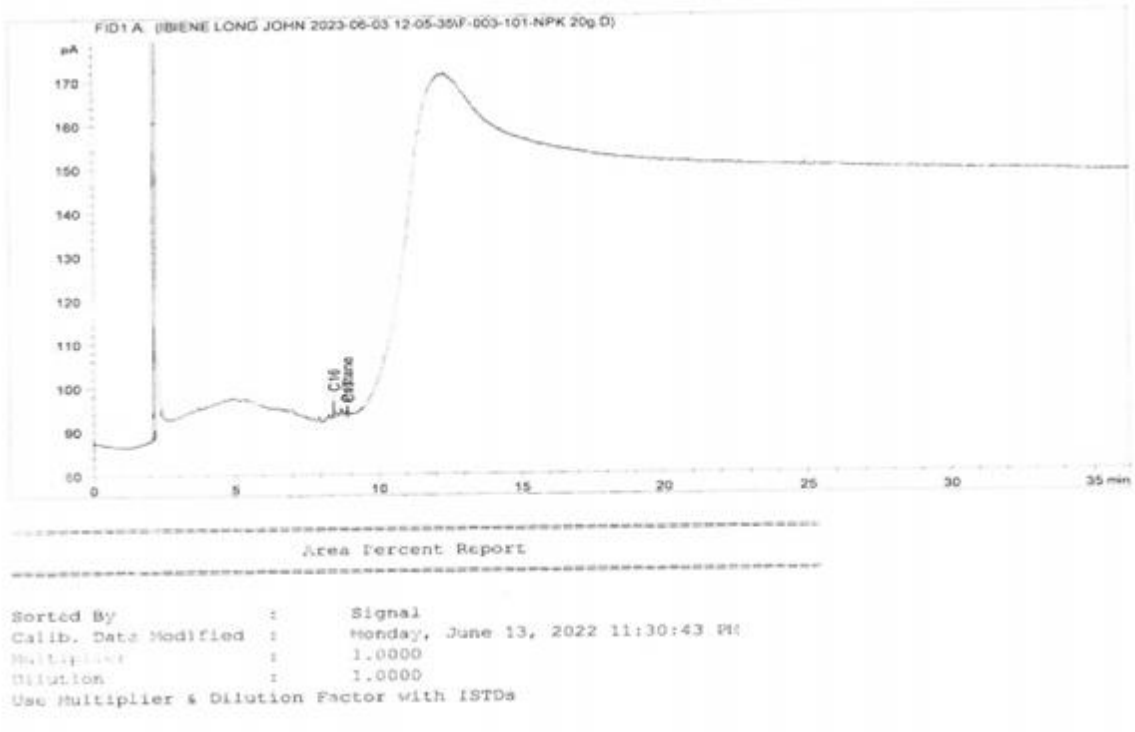

Figure 2. TPH for day 1 contaminated soil (TPH = 5180.236 mg/kg).

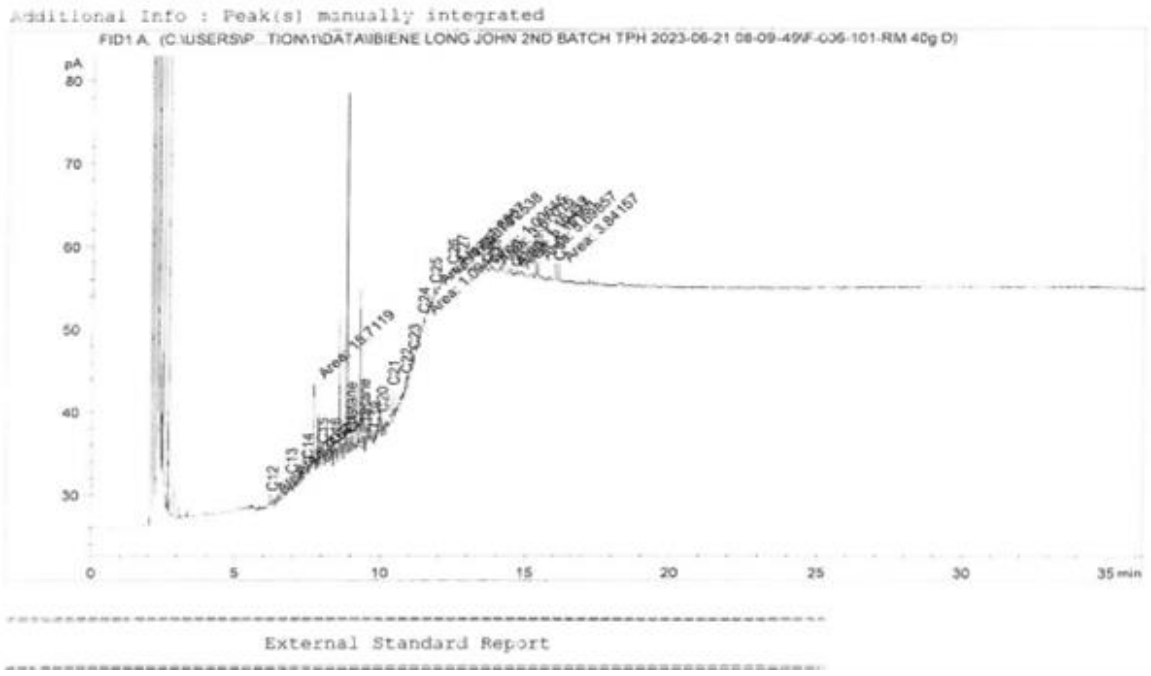

Figure 3. TPH for day 14 (40g RM).

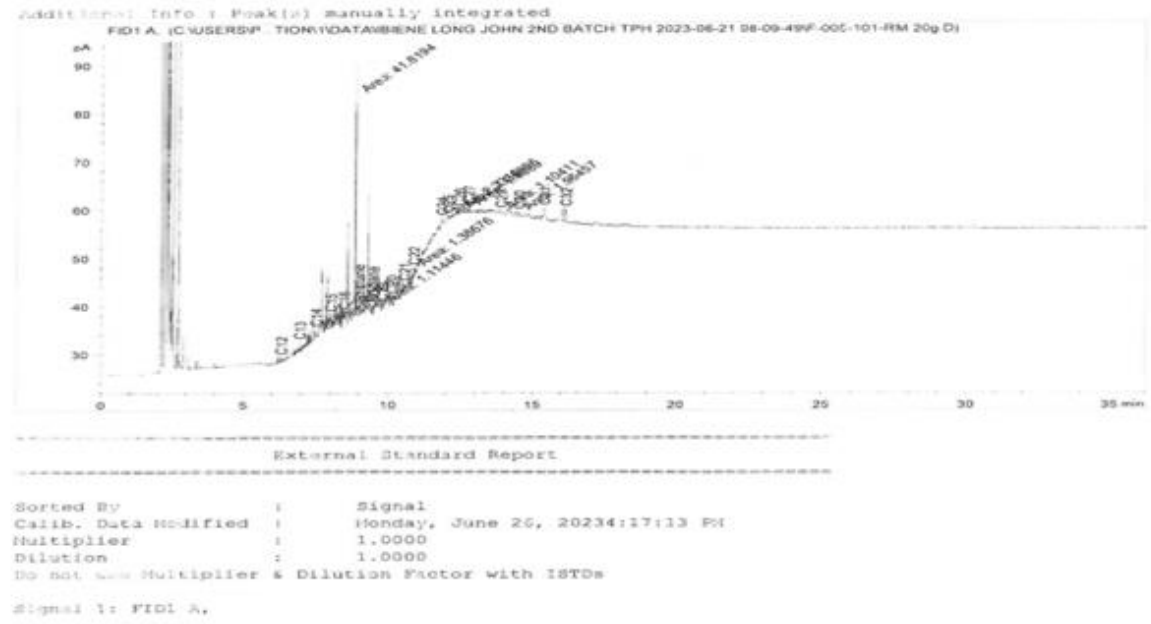

Figure 4. Chromatogram of TPH for day 14 (20g RM).

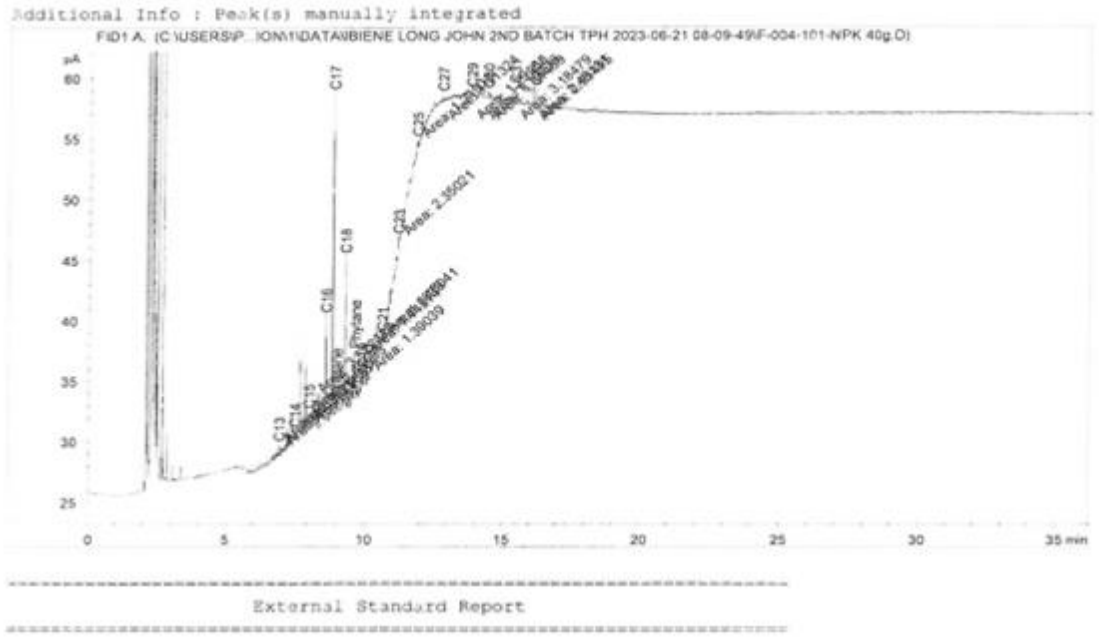

Figure 5. Chromatogram of TPH for day 14 (40g NPK).

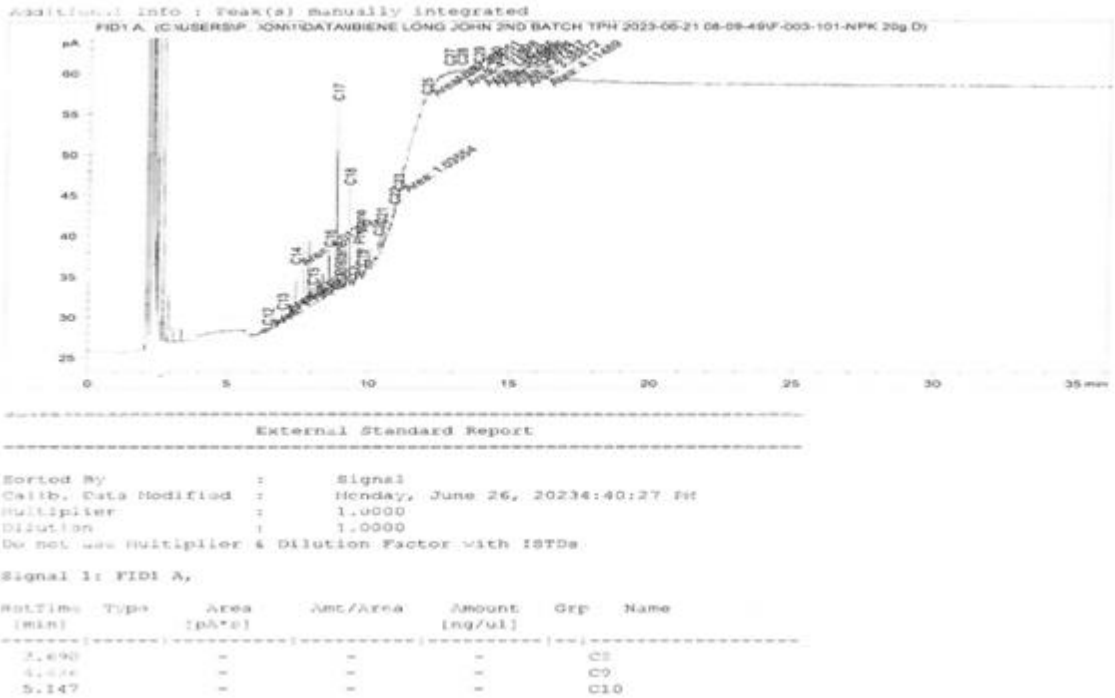

Figure 6. Chromatogram of TPH for day 14 (20g NPK).

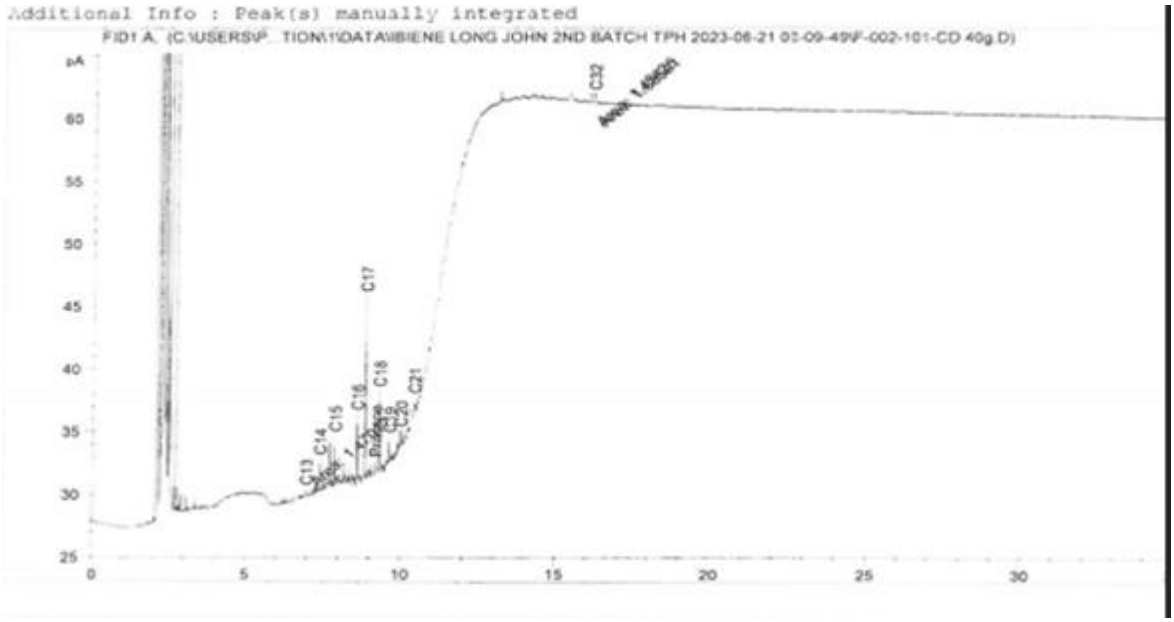

Figure 7. Chromatogram of TPH for day 14 (40g cow dung).

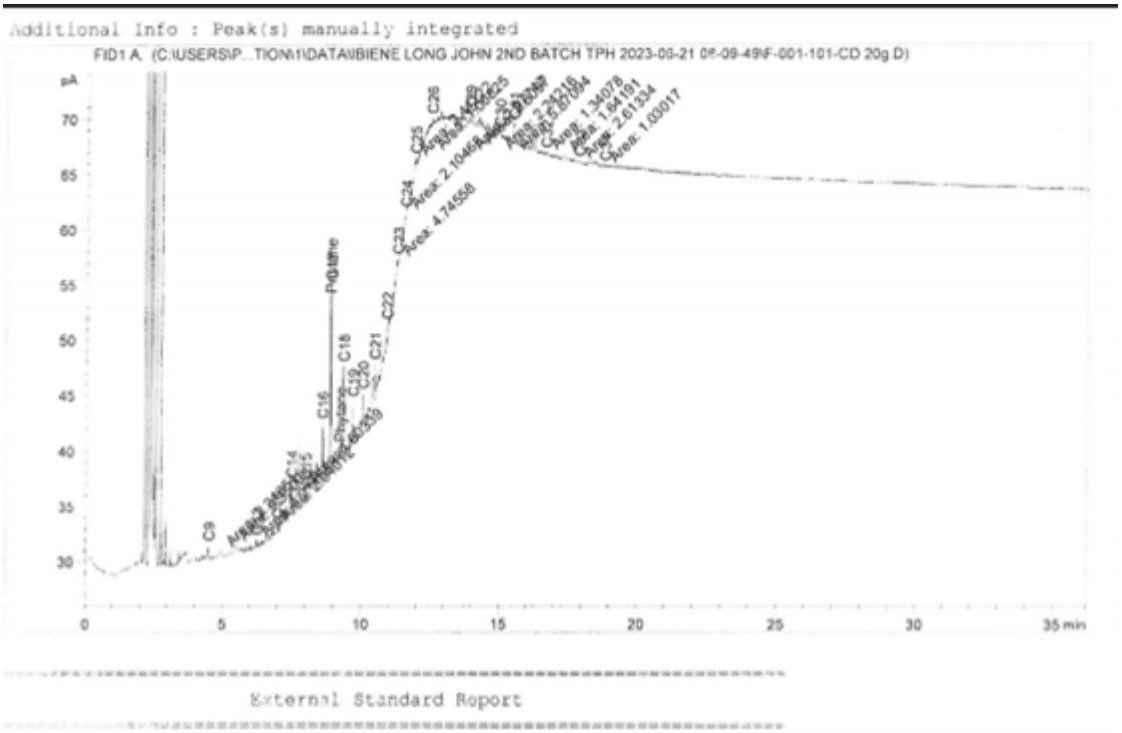

Figure 8. Chromatogram report of TPH for day 14 (20g cow dung).

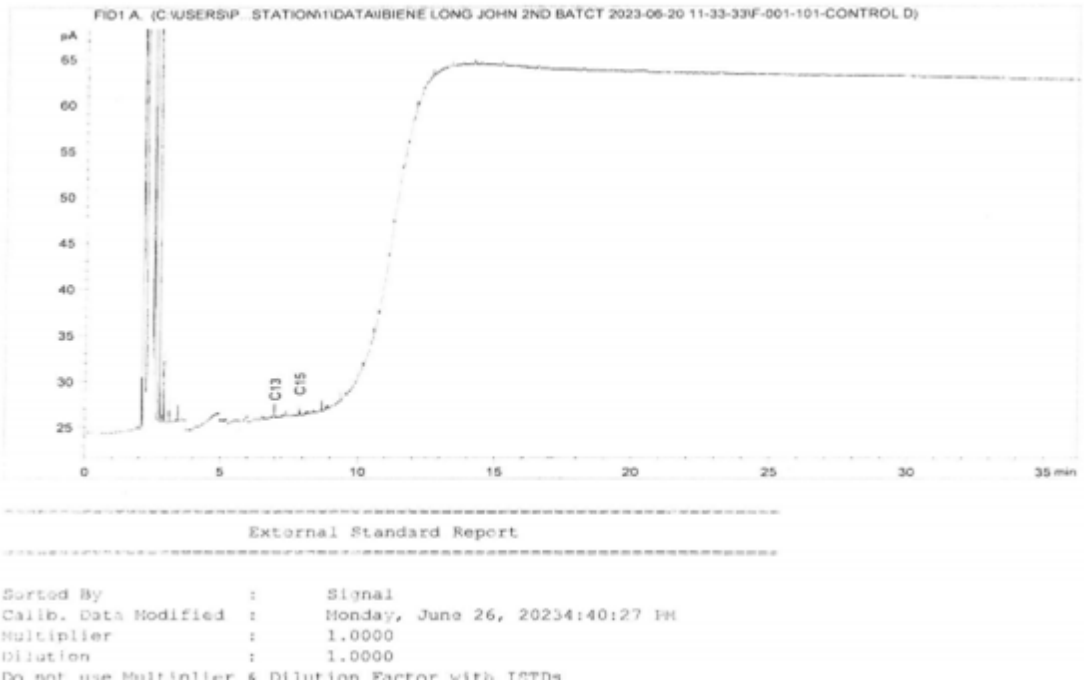

Figure 9. Chromatogram of TPH for day 14 (control).

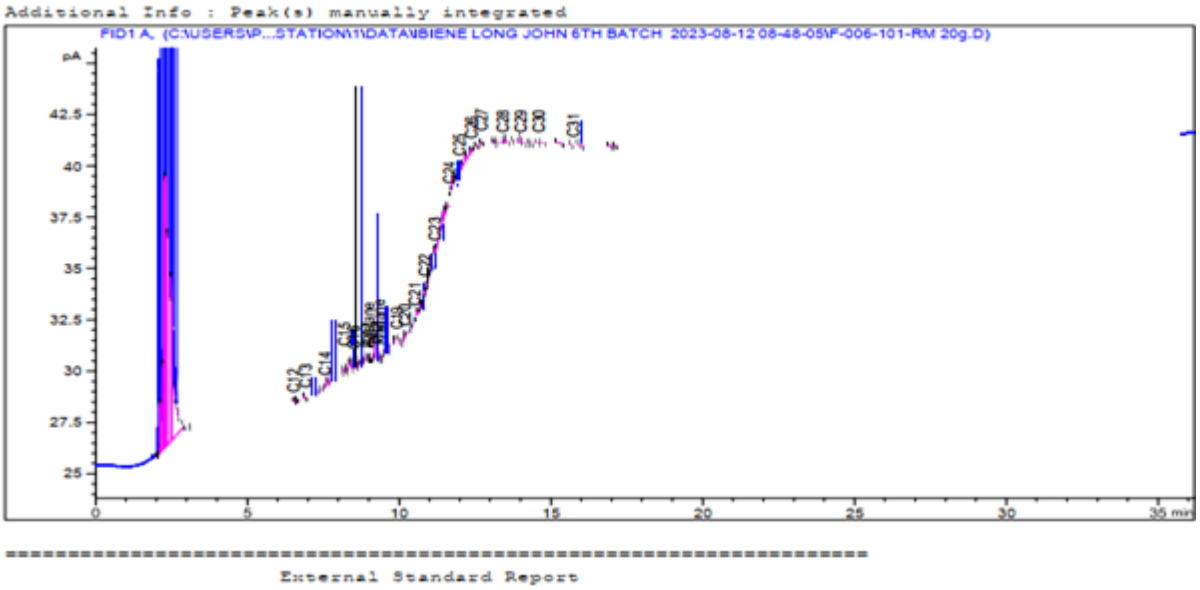

Figure 10. Chromatogram report of TPH for day 70 (20ml RM).

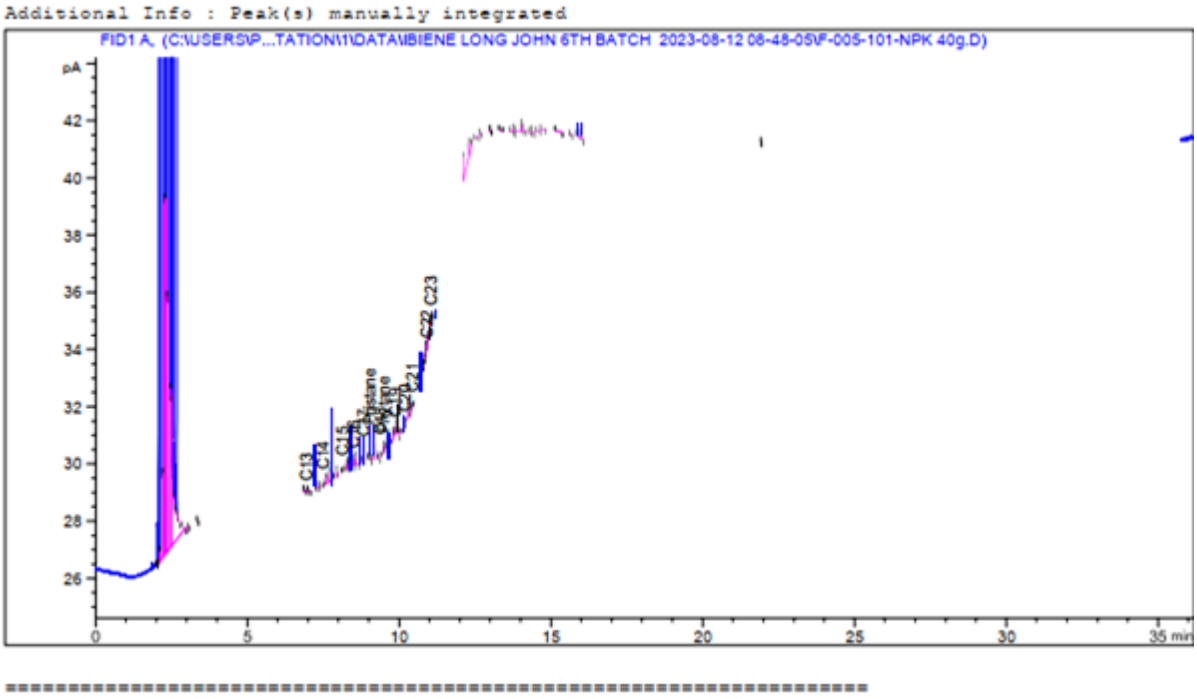

Figure 11. Chromatogram of TPH for day 70 (40g NPK).

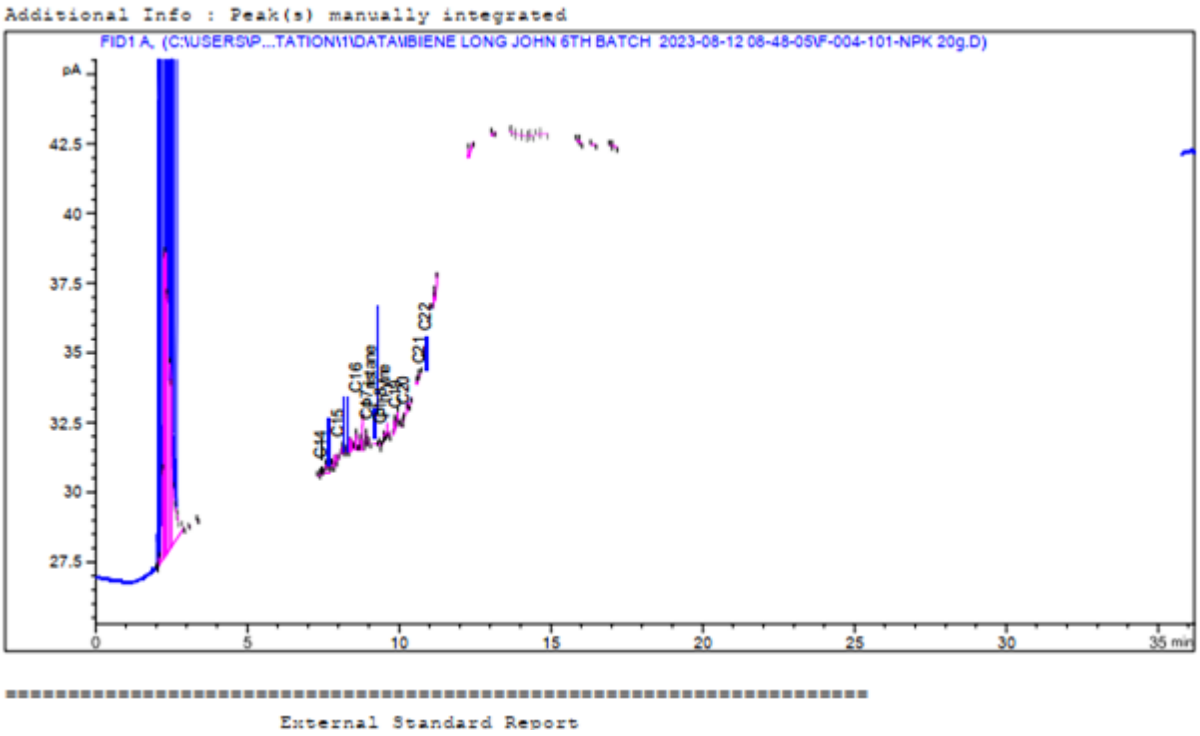

Figure 12. Chromatogram of TPH for day 70 (20g NPK).

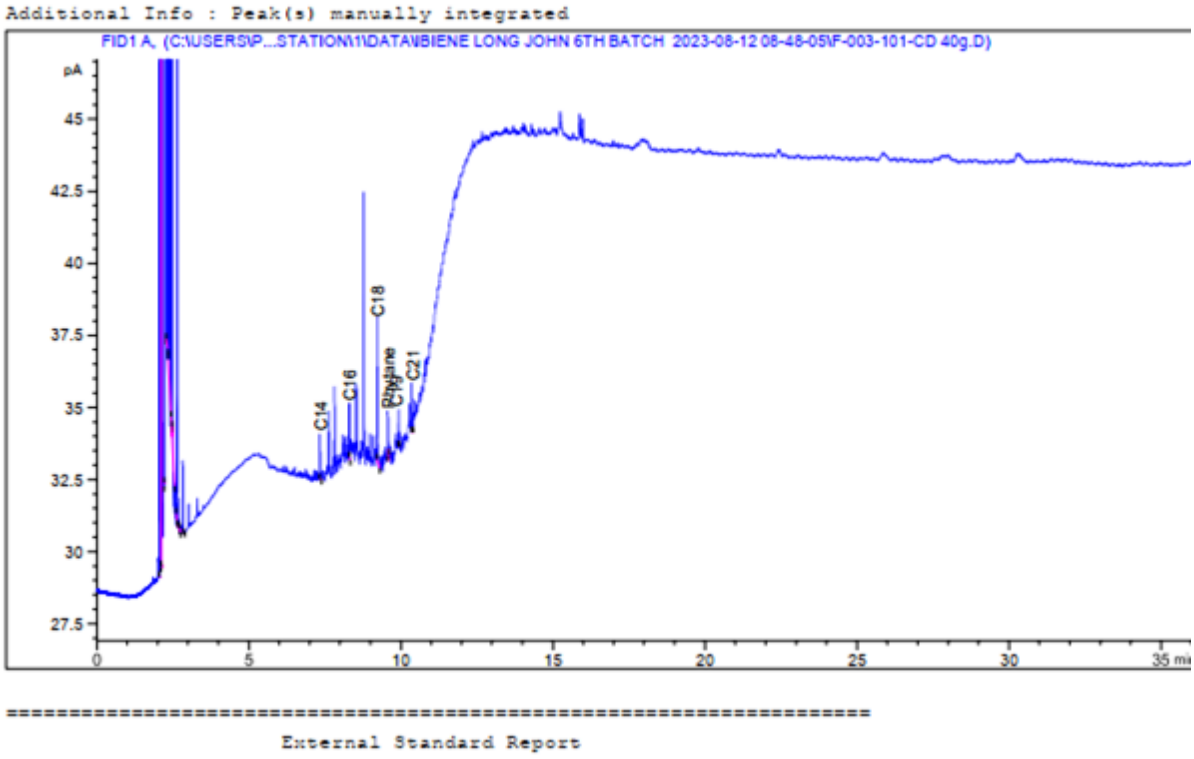

Figure 13. Chromatogram of TPH for day 70 (40g cow dung).

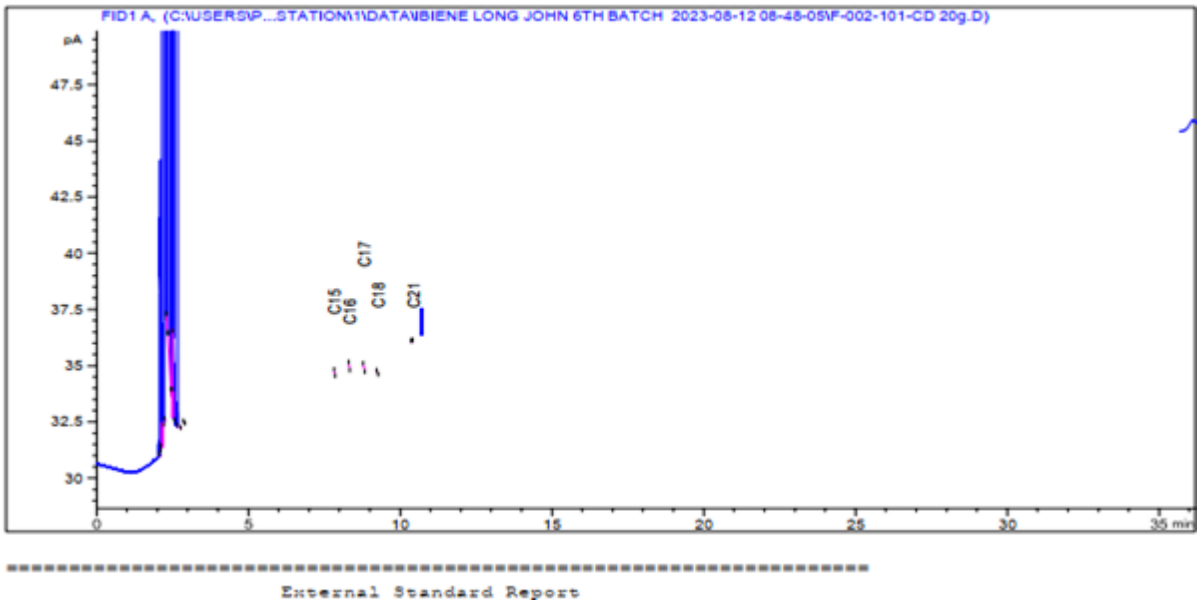

Figure 14. Chromatogram of TPH for day 70 (20g cow dung).

Figure 15. Chromatogram of TPH for day 70 (control).

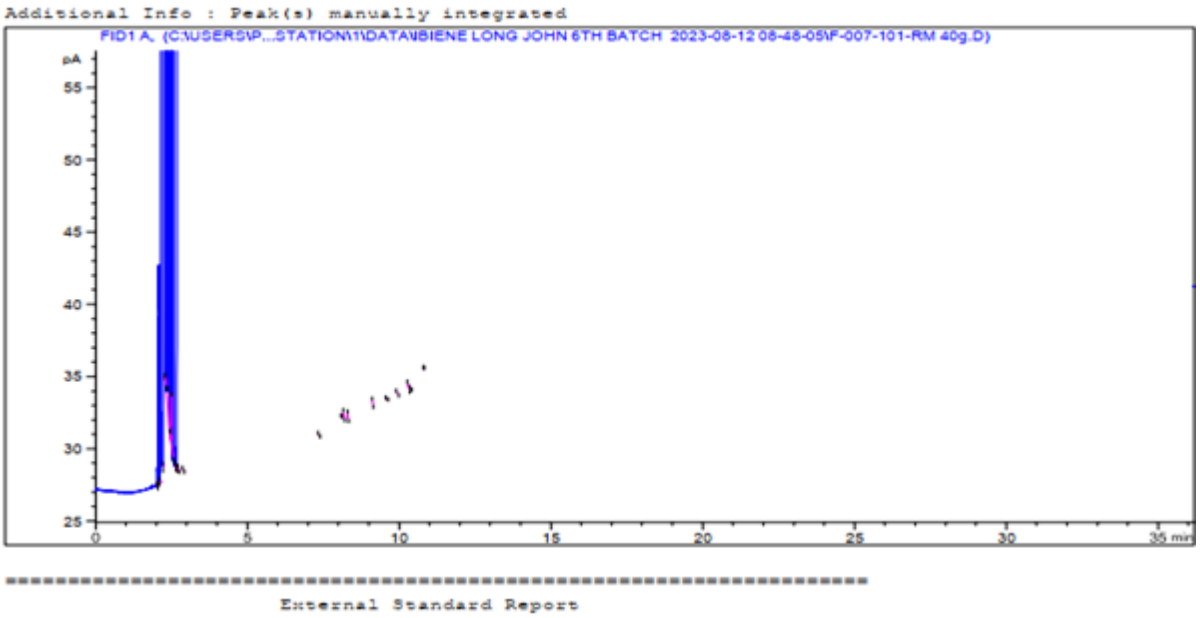

Figure 16. Chromatogram of TPH for day 70 (40ml RM).