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EFFICACY OF POULTRY LITTER AS A SOURCE OF NUTRIENT FOR THE BIOREMEDIATION OF TOTAL PETROLEUM HYDROCARBON CONTAMINATED TROPICAL BUNKERING SITE

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ABSTRACT
Contamination of soils with total petroleum hydrocarbon (tph) is a wide spread environmental problem requiring urgent remedial action and controls. This study evaluates the effectiveness of poultry litter for ex-situ bioremediation of total petroleum hydrocarbon (tph) contaminated soils. Four treatment groups in replicates consisting of 1 kg of soils was amended with poultry litre. Minimal salt medium was also included to enhance the rate of bioremediation at a temperature of 25^0c to 30^0c over a six week period. The levels of tph concentration was monitored by gas chromatography (gc) after extraction with n-hexane. The result showed that at optimal conditions, the tph removal efficiency of poultry litter and minimal salt composites reached 60.9% of the initial value. Corresponding tph removals were obtained as 37%, 44% and 42.9% respectively for other treatment options, demonstrating potential efficiency of poultry litter as co-substrate and source of inorganic nutrients for ex-situ bioremediation of petroleum compounds. At 10% by volume of dry weight sample, poultry litter increased the bacterial biomass and reduced the time necessary for biodegradation significantly ($p>0.05$). It was also observed that a mixed culture of bacterial (bacillus and pseudomonas) and fungal (trichoderma, fusarium and aspergillus) strains demonstrated more stable growth behavior and degraded the contaminants to much lower concentrations. The results of gc profiles also revealed that soil samples unamended with nutrients were the least degraded. This finding emphasises the need for nutrient amendment and environmentally friendly intervention strategies in the bioremediation of soils adversely impacted by tph.

Keywords: *Biodegradation, Nutrients, Poultry Litter, Petroleum hydrocarbon*

INTRODUCTION

Total petroleum hydrocarbons (TPH), one of the most common groups of persistent organic contaminants (Huang *et al.*,2005) is a widespread and one of the world's most common environmental problems (USEPA, 2000) with significant social and environmental impacts. The hydrophobic nature, low volatility, potential to enter the food chain and seriously affect animal and human health as well as other unique environmental challenges posed by petroleum contamination when present in soils has been a major concern. Without proper and adequate mitigation measures these contaminants may have adverse or lethal effects on the entire ecosystem (Solomon *et al.*, 2016).

Over the past few decades, the Nigerian environment has witnessed varying degree of pollution with petroleum hydrocarbon from several anthropogenic perturbations including oil industry operations, bunkering, pipeline vandalization and sabotage. Of all sources of contamination, sabotage, crude theft and bunkering has been of major concern to pollution experts; the scenario is more challenging as unskilled personnel who tend to use crude methods are usually involved in the nefarious activities resulting in devastating consequences (Yakubu *et al.,* 2007) and gross pollution of all components of the environment. According to United Nations Environment Programme (UNEP, 2011), certain mutagenic and carcinogenic pollutants including benzene with concentrations up to 900 times above the World Health Organization (WHO) standards have been recorded in the Niger Delta environments, especially Ogoniland. Hence, a suitable solution for removal or control of these soil contaminants is urgently needed.

Bioremediation technology has gained wide acceptance as a means of clean-up of contaminated sites due to its cost-effectiveness, environmental friendly nature and simplicity (Yang *et al.,* 2009; Odokuma, 2012). Moreover, microorganisms involved in the degradation of petroleum hydrocarbons in the environment have been found to be economical, efficient, versatile and environmentally friendly (Margesin and Schinner, 1999; Yakubu, 2007). However, the process of biodegradation is most often limited by a number of factors including nutrients, pH, temperature, moisture, oxygen, soil properties and the level of contaminant (Bundy *et al.,* 2002; Atagana 2008, Al Sulaimani 2010). Furthermore, unavailability of nutrients to the microbial biomass is a major constraint to the natural biodegradation process. Consequently, the need for suitable organic or inorganic material that will serve as a source of nutrient for optimum microbial growth. Young *et al.* (2001) noted that, nutrient levels need to be present in sufficient concentrations throughout the entire bioremediation program to support maximal growth rates of hydrocarbon degrading microorganisms. Significantly more nitrogen and phosphorous than what is normally present in the soil is also needed for the indigenous microorganisms to be able to convert carbon, the basic structural component of living matter into more biomass(Wright *et al.,* 1997). In general, the optimum nutrient balance required for hydrocarbon remediation is shown to be Carbon: Nitrogen: Phosphorus in a ratio of 100:10:4 (Thapa *et al.,* 2012). Moreover, the success of bioremediation is often correlated to the addition of inorganic nutrients such as phosphorous (P) and nitrogen (N) and the incorporation of oxygen (O_2) into the contaminated soil medium (Williams *et al.,* 1999).

One possible bioremediation strategy and most promising technology currently in use is biostimulation (Juwarkar *et al.,* 2010). Biostimulation involves the use of organic materials to increase the availability of nutrients necessary for optimum microbial growth and contaminants or pollutant removal in the environment (Erenee *et al.,* 2017). Nitrogen has been successfully introduced into the terrestrial subsurface for biostimulation using ammonia, nitrate, urea and nitrous oxide. Several inorganic and organic forms of phosphate have also been successfully used to biostimulate contaminated environments (USEPA, 1989).

Researchers all over the world have also begun to pay research attention to the use of organic waste as the source of limiting nutrients for effective bioremediation in order to achieve cost effectiveness (Ibiene *et al.,* 2011).Within the tropics, a wide range of organic materials has been applied in the bioremediation of hydrocarbon contaminated soil by different researchers (Okerentugba *et al.,* 2015). More recently, the effect of poultry droppings principally used for agricultural and horticultural purposes has been evaluated by various researchers for the bioremediation of crude oil-polluted soil (Williams *et al.,* 1999; Ugochukwu *et al.,* 2016).Williams *et al.* (1999) reported that the chemical, microbiological, and physical characteristics of poultry litter are suitable co-substrates and nutrient sources for potential applications in the soil bioremediation industry.Ugochukwu *et al.*(2016) also demonstrated that poultry litter as a potential source of nutrients for microbial activity habours microbes capable of utilizing hydrocarbons as source of carbon and energy. This study therefore evaluates the potential of poultry litter in enhancing ex-situ biodegradation of soils contaminated with total petroleum hydrocarbons.

MATERIALS AND METHODS

Sample Collection

Contaminated soils (0-10cm depth) were aseptically collected from four sites at an interval of 150cm using soil auger, to cover half the radius of the distributed area at a bunkering site, Orile Iganmu axis, Lagos State. Uncontaminated soils were also collected as control and the obtained samples mixed to form a composite for homogeneity. Samples for microbiological analyses and bioremediation studies were stored in sterile screw cap bottles and bags, and transported to Microbiology Research Laboratory, University of Lagos while those for physico-chemical analyses were transferred in polythene bags to the Physical and Chemical Oceanography Laboratory, Nigerian Institute of Oceanography and Marine Research (NIOMR), Victoria Island, Lagos within 24 hrs. Dried poultry droppings obtained from a local farmer were carefully sorted to remove wood shavings and

ground to semi-powder prior to the experiment. This was preserved in a dry place at the Laboratory located at the Department of Marine Sciences until required for analysis.

Laboratory procedure

Approximately 2kg of soils contaminated with about 639 to 910 mg/kg of TPH were spread to a depth of 2 cm and used to establish a range of four treatments with replicates. Treatment comprised of; uncontaminated soil only (control), contaminated soil only (Tray A), contaminated soil + 10% by volume minimal salt medium (Tray B), contaminated soil + 25g dry weight poultry litter (Tray C) and a composite of contaminated soil + poultry litter + 10% by volume minimal salts (Tray D). Soil moisture was maintained at approximately 15% through bi-weekly spray bottle misting while aeration was carried out daily by tilling with a stainless hand trowel to provide the necessary aeration and mixing of nutrients and microbes with the contaminated soil (Ayotamuno *et al.,* 2006). Throughout the six weeks (42 days) of the experiment, the soil pans were placed on a metal shelves in a constantly lighted room located at the Department of Marine Sciences, University of Lagos.

Determination of Physicochemical characteristics of samples

1 g of soil samples each were collected on day 0, 14, 21, 28 and 42 for the determination of pH, nitrate, phosphate, moisture content and organic matter content. The temperature was measured using a digital thermometer. Soil pH was determined using the method of Bates, (1954) and moisture content determined in accordance with ASTM D2216-92. Available phosphorus (Phosphate) in soils was determined in accordance with stannous chloride reduction method described in American Public Health Association (APHA, 1998) and Nitrate was determined by procedures described in methods of soil analysis (American Society of Agronomy, 1996). Organic matter content was determined by placing an oven dried soil sample in a fume cupboard to burn off the organic matter. It was maintained in that position until no more soot was given off and a constant weight was obtained. The difference in weight of the soil samples was taken as the organic content.

Extraction and Analysis of Residual Oil Concentration (ROC)

Prior to extraction, soil samples (0.5g wet weight) were properly mixed with anhydrous sodium sulphate to remove excess water. Extraction of residual oil (TPH) in contaminated soils was done by adding 30ml of n-hexane according to USEPA method 3500 reported by Williams *et al.* (1999). The mixture of n-hexane and soil was shaken vigorously after which the contents were allowed to settle and

then filtered through a filter paper in a funnel. The solvent system was measured colorimetrically using a spectrophotometer at 410nm.

Gas Chromatography Analysis

Approximately 0.5g (wet weight) of soil samples was weighed and mixed with equivalent weight of anhydrous sodium sulfate for dehydration before Gas Chromatography analysis for TPH. The homogenized sample of soil and anhydrous sodium sulfate was placed in cold solvent extraction cell and extracted by shaking with 30ml of dichloromethane. This procedure was repeated twice. The extracted sample was reduced to 1ml and clean-up was carried out in a chromatographic column using silica gel and eluted with 30ml hexane. The extracted sample was reduced to 1ml by evaporation; thereafter, the one microlitre (1µl) of the test sample was introduced into the gas chromatograph which had been initially calibrated with a hydrocarbon standard to generate a chromatogram (Williams *et al.,* 1999).

Microbiological Analysis

Serially diluted samples were plated onto duplicate nutrient agar (NA) plate for bacteria and potato dextrose agar (PDA) plate for fungi resulting in a total of four plates per dilution. Colonies were enumerated following incubation at 37^0C for 24hrs (bacteria) and $28 + 2^0$C (fungi) for 7 days. Total heterotrophic microorganism (bacteria and fungi) was enumerated in accordance with the American public Health Association (APHA, 1998). Hydrocarbon utilizing bacteria and fungi were enumerated by the same serial dilution process and dispersed onto Minimal salts agar plates containing the inorganic nutrients.

0.5ml of streptomycin was added to the minimal salts agar to suppress bacterial growth for enumeration of hydrocarbon utilizing fungi. Diesel fuel hydrocarbons were incorporated into the agar media by vapor phase transfer during the incubation period and petroleum degrading microorganisms were enumerated following incubation at 27^0C for 14 to 21 days. The pure isolates were characterized and identified to their species level using conventional microbiological and biochemical tests (Carpenter, 1977; Cruickshank *et al.,* 1980; Murray *et al.,* 1981; Cheesebrough, 1998).

Statistical Analysis

All the data obtained were subjected to Statistical Analysis of Variance (ANOVA) using computer aided SPSS statistical program. Microbiological enumeration of data was transformed to logarithms prior to analysis and significant differences for all comparisons were determined at 5% confidence level ($p < 0.05$).

RESULTS

Physicochemical Properties of Soil samples

The results of examined physicochemical parameters of soils are presented in Table 1. The pH of total petroleum hydrocarbon (TPH) contaminated soils recorded in the ranged of 7.0 to 7.8 were lower compared to the values (8.5 to 8.8) recorded for uncontaminated soil throughout the study duration. Significant variations ($p < 0.05$) were observed in pH levels across all treatments. Nitrate level ranged from 6.0 to 9.1 mg/L in contaminated soils and 5.5 to 5.9 mg/L in control soils. The highest mean concentration of nitrate (7.62 ± 0.07) was recorded in Tray D while the lowest mean concentration (5.68 ± 0.25) was recorded in the control. The available phosphorus determined as phosphate in soils was relatively higher (1.2 to 1.82 mg/L) in contaminated soil when compared to the levels recorded in control soils (1.1 to 1.18mg/L). The percentage total organic content (TOC) of oil contaminated soils (10.10 – 29 .80%) was relatively higher than that of oil free soil (12.31 – 15.35%). Similarly, moisture content of oil polluted soils in the range of 11.30 to 15.09% was higher than uncontaminated soil (9.59 to 11.40%). Significant differences ($p < 0.05$) were recorded in mean levels of nitrate, phosphate, TOC and moisture content of control sample and other treatments.

Table 1: Physicochemical properties of soil samples

Parameters	Treatment options				
	Control	Tray A	Tray B	Tray C	Tray D
pH	8.63 ± 0.05	7.72 ± 0.03	7.57± 0.09	7.12 ± 0.05	7.30 ± 0.04
	* (8.50-8.80)	(7.60-7.80)	(7.30-7.80)	(7.00-7.20)	(7.20-7.40)
Nitrate mg/L	5.68 ± 0.07	6.60 ± 0.26	7.24 ± 0.46	7.34 ±0.47	7.62± 0.47
	(5.50-5.90)	(6.00-7.25)	(6.25- 8.75)	(6.25-8.85)	(6.50- 9.10)
Phosphate mg/L	1.14 ± 0.02	1.32 ± 0.05	1.45± 0.09	1.47 ± 0.09	1.52 ± 0.09
	(1.10 -1.18)	(1.20-1.45)	(1.25-1.75)	(1.25-1.77)	(1.30-1.82)
Total Organic Carbon	13.85 ± 0.49	13.77 ± 2.25	17.03 ± 1.58	24.77± 0.24	27.57 ± 1.32
(TOC) %	(12.31-15.35)	(10.10-21.93)	(13.64-22.57)	(23.88-25.15)	(22.95-29.80)
Moisture Content	10.79 ± 0.33	12.77 ± 0.45	12.86 ± 0.36	12.42 ± 0.35	12.23 ± 0.72
(MC)%	(9.59-11.40)	(12.10-14.54)	(12.30-14.30)	(11.70-13.70)	(11.30-15.09)

*Mean ± SE (range in parenthesis)
Control- uncontaminated soil;
Tray A - contaminated soil only;
Tray B- contaminated soil + minimal salt medium (10% by volume)
Tray C- contaminated soil + poultry litter;
Tray D- contaminated soil + minimal salt medium +poultry litter

Total Heterotrophic Bacteria, Fungi and TPH Degraders in poultry litter and Soil samples

The results of the total heterotrophic bacteria, fungi and total petroleum degraders in poultry litter and soil samples are presented in Fig. 1 to 4**.** The population density of bacteria recorded in poultry litter used for the present study was 4.0×10^5 $cfug^{-1}$ while that of fungi was found to be 7.0×10^2 $sfug^{-1}$. The counts of hydrocarbon degraders in poultry litter was found to be 4.0×10^3 $cfug^{-1}$ for bacteria and 4.0×10^2 $sfug^{-1}$ for fungi. On the other hand, the count of Total Heterotrophic Bacteria (THB) recorded in TPH contaminated soils ranged from 3.3. x 10^6 cfu g^{-1} to 1.8×10^7 cfu g^{-1}, while in the control, the counts ranged from 1.0×10^7 cfu g^{-1} to 1.1×10^7 cfu g^{-1}. The counts of total heterotrophic fungi (THF) ranged from 5.0×10^3 $sfug^{-1}$ to 1.2×10^4 $sfug^{-1}$ in contaminated soils and from 2.0×10^2 $cfug^{-1}$ to 2.4×10^3 $sfug^{-1}$ in the control soil samples.

Relatively higher counts of Hydrocarbon Utilizing Bacteria (HUB) were recorded in TPH contaminated soils (1.3×10^7 $cfug^{-1}$ to 8.0×10^7 $cfug^{-1}$) when compared to the control samples (1.0×10^7 $cfug^{-1}$to 8.0×10^6 $cfug^{-1}$) as shown in Fig 3. However,

there was no significant difference ($P > 0.05$) between the control and amended contaminated soils. The results also showed that Hydrocarbon Utilizing fungi (HUF) ranged from 1.0×10^2 $sfug^{-1}$ to 1.1×10^4 $sfug^{-1}$ in contaminated soil and from 1.0×10^2 $sfug^{-1}$ to 2.0×10^3 $sfug^{-1}$ in the control (Fig 4). However, there was no significant difference ($P > 0.05$) in Total hydrocarbon utilizing fungi for all examined samples.

The hydrocarbon utilizing microbial isolates were identified to be species of *Bacillus*, *Pseudomonas*, *Aspergillus*, *Fusarium*, *Trichoderma* and Yeast. *Bacillus* and *Pseudomonas* were more frequently isolated among the bacteria while yeast, *Trichoderma* and *Aspergillus* were the most frequently isolated fungal species.

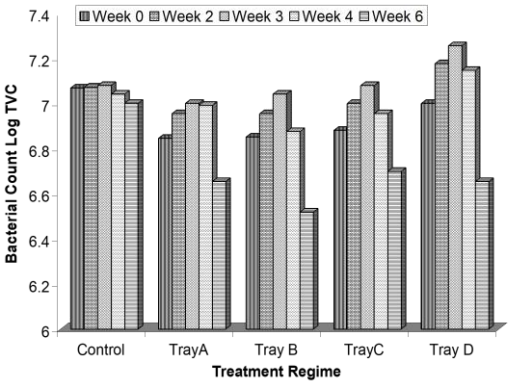

Fig. 1: Total Heterotrophic Bacterial Population

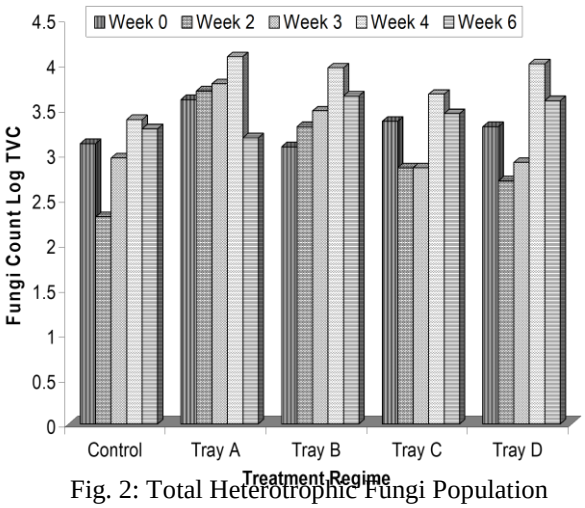

Fig. 2: Total Heterotrophic Fungi Population

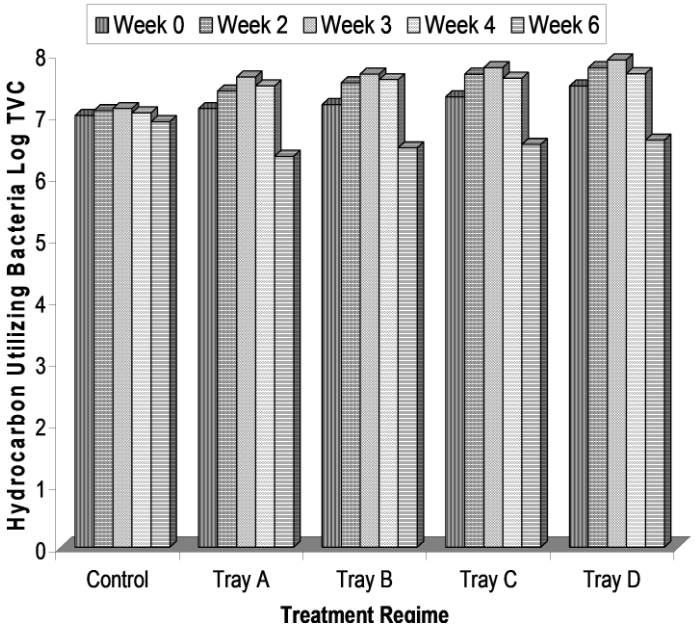

Fig. 3: Total Hydrocarbon Utilizing Bacterial

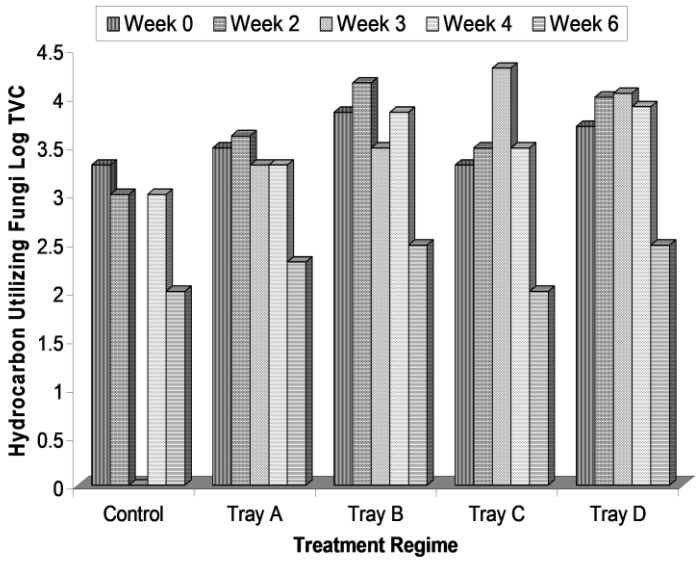

Fig. 4: Total Hydrocarbon Utilizing Fungal

Percentage total petroleum hydrocarbon removal from the unit without added nutrient was measured to be 37% (Tray A) during the 6th week of the study as compared to 44%, 42.9% and 60.9% in the unit with minimal salts (Tray B), poultry litters (Tray C) and composite of both minimal salts and poultry litter (Tray D) respectively. The reduction of total petroleum hydrocarbon in soil supplemented with poultry litter and the minimal salts is shown in Fig. 5. From statistical analysis, there was no significant difference ($p > 0.05$) in the rate of total petroleum hydrocarbon removal measured for all treatment groups when compared to the control.

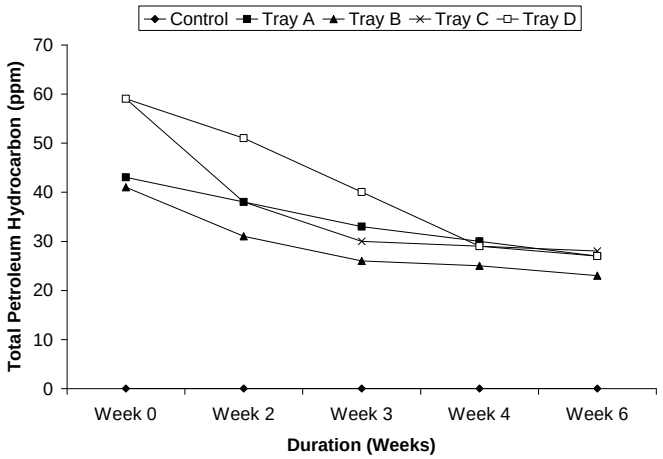

Fig. 5: Rate of Change in the concentration of Total Petroleum Hydrocarbon in Soil Samples during the period of investigation

Examination of Gas Chromatography sample runs on untreated and treated soils showed that most of the petroleum contamination was a complex mixture of hydrocarbons. The compounds that are present in high concentrations produce individual peaks on the hump which are likely to consist of multiple overlapping compounds. Most of the complex mixture of peaks did not appear again in the chromatogram by week 6 because of its removal through microbial degradation. Untreated unit A was the least degraded as shown in the chromatogram on plate 1a to plate 4b.

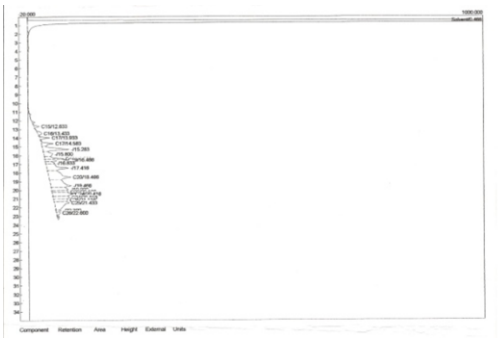

Plate 1a: Tray A (Day 0)

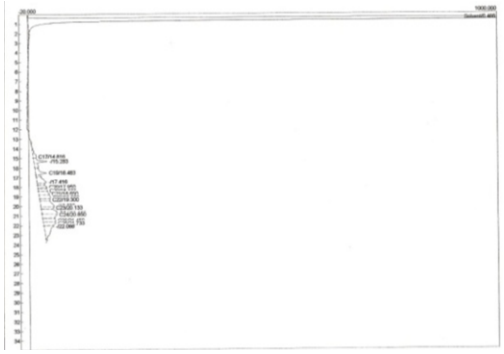

Plate 1b: Tray A (Day 0)

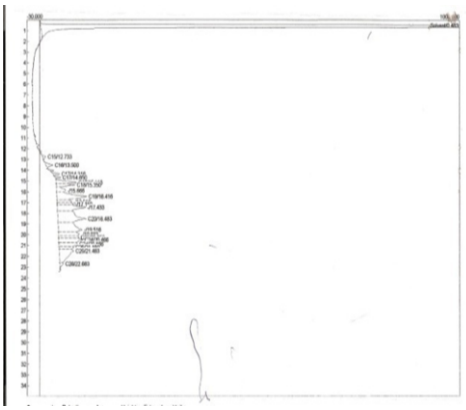

Plate 2a: Tray B (Day 0)

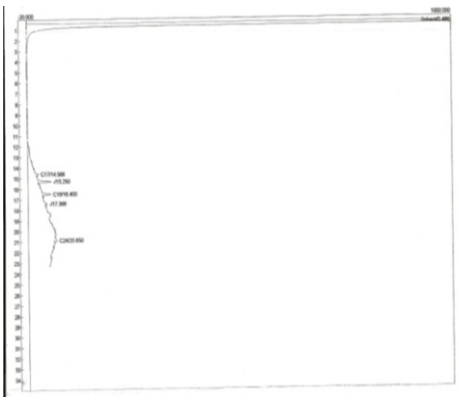

Plate 2b: Tray B (Day 0)

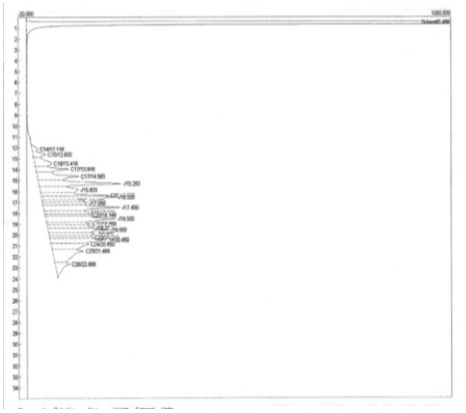

Plate 3a: Tray C (Day 0)

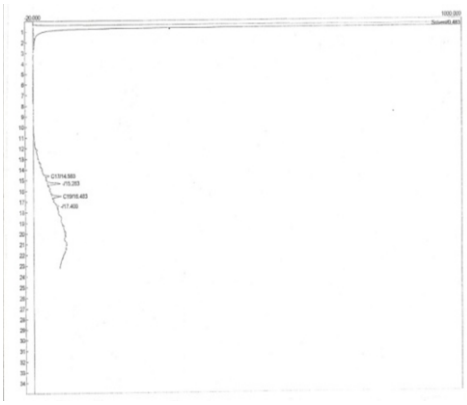

Plate 3b: Tray C (Day 0)

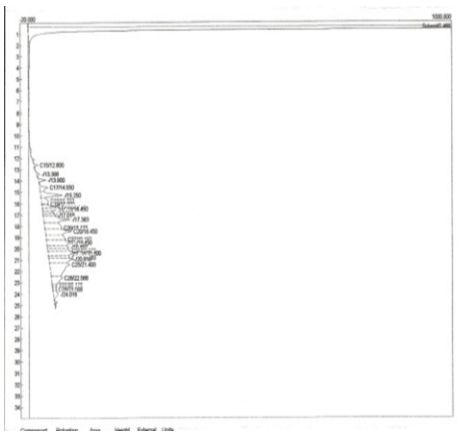

Plate 4a: Tray D (Day 0)

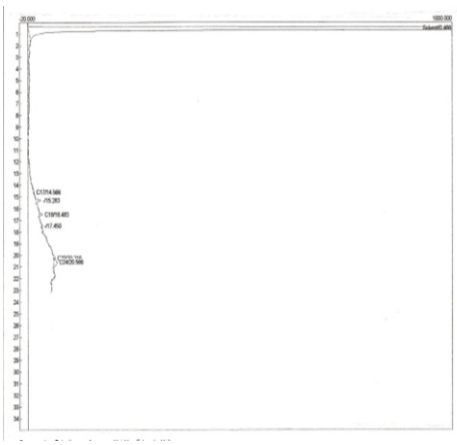

Plate 4b: Tray D (Day 0)

DISCUSSION

The biodegradation of hydrocarbons by natural population of microorganisms represents one of the primary mechanisms of eliminating petroleum pollution from the environment (Leahy and Colwell, 1990). However, the success of bioremediation as reported by other researchers depends on a number of complex factors including the nature of pollutant (degree of pollution, aggregate, and oxidation state of crude oil) and environmental conditions (Bamforth and Singleton, 2005; Gavrilescu, 2006; Agarryand Jimoda, 2013).

In the present study, the pH of contaminated soils was observed to be lower when compared to the control; a finding which is in line with the report of Ijah and Abioye (2003). However, the pH of the soils was able to support degradation activity of the microorganisms which is optimal at about 6.5 to 8.0. Both nitrogen and phosphorus levels were higher in oil polluted soil than oil free soils. This agreed with the findings of Odu (1972) who reported increase in nitrogen and phosphorus contents of a crude oil polluted soil.

The moisture content of the oil polluted soil (especially the "amended soil samples") was higher than that of uncontaminated soil. This is in line with the work of Ijah and Abioye (2003) that used kerosene in their study. This observation might also be due to the fact that the polluted soil consisted of petroleum hydrocarbon components that did not actually coat the soil and permitted the penetration of water as well as higher organic content of the soil and the added nutrients.

Microorganisms capable of catabolizing petroleum hydrocarbons were shown to be present with varying counts in the various soil samples. These indigenous microorganisms were challenged over six weeks at optimal conditions with varying concentration of hydrocarbon present in the polluted soil. Increase in counts of THB and THF between the contaminated soil and control was significant after the baseline date (wk 0). This may be due to the changes in the physiochemical properties of the soil.

Generally, total heterotrophic bacteria (THB) and fungi (THF) counts were higher in the control soil at week 0 than TPH contaminated soil. Although there was no significant difference in microbial count recorded in all treated groups, a gradual and significant increase was observed after weeks of treatment with the highest microbial count recorded in week 3 (21 days). Additionally, this might have been due to biostimulation of the indigenous microorganism (Mishra *et al.,* 2001).This suggests increase in nutrient levels and the potential effect of poultry litter and

minimal salt medium (MSM). The present findings agreed with the report of Genovese *et al.* (2008) who noted that bioaugmentation or biostimulation is useful in improving degradation rate and efficiency depending on site requirements.

Hydrocarbon utilizing bacteria (HUB) in contaminated soil were higher than those of the control. According to previous reports, the presence of crude oil and related products in soils potentially boost the carbon supply, hence favors the growth of hydrocarbon utilizing bacteria as compared to uncontaminated soils (Ijah and Abioye, 2003; Ijah and Antai, 2003; Onifade *et al.*, 2007).

The rate of TPH biodegradation in the amended soil samples was observed to be more rapid than the un-amended soil samples. According to Nordar *et al.* (1990), poultry litter contains a diverse population of microorganisms including high numbers of the biodegrading bacteria, *Pseudomonas*. Furthermore, the rate of degradation by a combination of minimal salts medium and poultry litter amendment soils almost doubled the rate of degradation of the un-amended soil samples. This observation suggest that the added poultry litter was possibly composting thereby resulting in increased soil temperatures which enhanced volatilization of the higher petroleum fractions during the soil mixing for oxygen. This finding is consistent with the report by Gupta and Baummer (1996), which showed that poultry litter nearly doubled the rate of atrazine biodegradation.

The chromatograph profiles before and after the treatment period showed that degradation was much more enhanced in treatment with a combination of poultry liter and minimal salt medium. The result of the present study therefore emphasizes the efficacy of intervention strategies such as biostimulation and bioaugmentation.

CONCLUSION

Environmental contamination with total petroleum hydrocarbon and other petroleum by- products is a widespread environmental problem that requires urgent attention. Biostimulation with both organic and inorganic nutrient sources have proved to enhance the multiplication of indigenous microbes for the rapid biodegradation of the TPH contaminants in soils. The study has also demonstrated the efficacy of poultry litter as a co- substrate, source of inorganic nutrients and microorganisms for the bioremediation industry. However, the recovery was incomplete due to the time interval and huge quantity of TPH in soil samples

examined. Furthermore, the ultimate fate of these nutrients must be address to avoid Land-based and ground water pollution.

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