FUDMA Journal of Sciences (FJS)
ISSN online: 2616-1370
ISSN print: 2645 - 2944
Vol. 5 No. 1, March, 2021, pp 278 – 287
DOI: https://doi.org/10.33003/fjs-2021-0501-566

EFFECT OF THE APPLICATION OF GOAT DUNG ON THE BIOREMEDIATION OF POLLUTED SOIL BY HYDROCARBON DEGRADING BACTERIA: A MICROCOSM STUDY

Ogujoifor, I. F., Machido, D. A. and *Atta, H. I.

Department of Microbiology, Faculty of Life Sciences, Ahmadu Bello University, Zaria

*Corresponding author's email address: hiatta@abu.edu.ng

ABSTRACT

Biostimulation is an effective means of enhancing bioremediation of toxic compounds. In this study, goat dung was used as an additional source of nutrients to improve the degradation of petroleum hydrocarbons in a heavily contaminated soil. Soil samples collected from a mechanic workshop in Zaria, Nigeria, was subjected to microcosm studies in a screen house and three proportions (2%w/w, 3%w/w, 4%w/w kg^{-1}.soil) of goat dung added to the soil samples. The effect of the addition of nutrients from the goat dung on the hydrocarbon degrading bacterial community in the soil was determined by measuring the hydrocarbon utilizing bacterial counts, percentage degradation of hydrocarbon and biostimulation efficiency. At the end of the seven-week experiment, it was observed that 4% w/w of goat dung had the highest hydrocarbon utilizing bacterial count but, the maximum percentage hydrocarbon degradation occurred in the treatment with 2% w/w goat dung and biostimulation efficiency was optimum with both 2% w/w and 4% w/w goat dung. Nutrient addition can be an effective tool during bioremediation, however, laboratory scale studies should be conducted prior to field studies in order to achieve maximum results.

Keywords: bacteria, biostimulation, hydrocarbon, soil, goat dung.

INTRODUCTION

With the ever-increasing human population, there is concomitant increase in demand for energy used for transportation, domestic and industrial consumption. Petroleum-based (fossil) fuels are the major sources of energy since 1950s (American Petroleum Institute, API, 2004). Increased utilization of petroleum and its derived products such as gasoline, diesel and engine oils has led to a marked increase in soil contamination worldwide (Alizera and Asli, 2011). The illegal dumping of used motor oil is an environmental hazard with global ramifications (Blodgett, 2001). Akoachere *et al*. (2008) reported the discharge of used crankcase oil from vehicles as a major cause of oil pollution in Nigeria. Various contaminants such as used engine oil and heavy metals have been found to alter soil biochemistry, including alteration in soil microbial properties, pH, Oxygen and nutrient availability (Odjegba and Sadiq, 2002).

Over the years, several approaches have been employed for the remediation of hydrocarbon polluted sites. Of the many approaches available, bioremediation appears to be the most favoured being highly promising, cost effective, environment friendly and therefore, the most sustainable approach (Al-mutaiei *et al*., 2008, Cerqueira *et al*., 2014; Wu *et al*., 2017). Several bioremediation strategies have been devised but, most of those in current application exploit the innate capacity of microorganisms to degrade the various components of the petroleum product introduced into the polluted sites as sources of energy, carbon and other essential nutrients for cellular growth (Chaillan *et a*l. 2006). Of these strategies, biostimulation is the most widely adopted, and it involves the activity of indigenous microorganisms being enhanced by the addition of essential organic nutrients such as carbohydrates and fiber to offset their shortage in the available pool. This usually results from the input of large quantities of carbon sources in form of hydrocarbon compounds. Biostimulation therefore, is essentially the addition of nutritional amendments to increase microbial metabolism and to encourage bioremediation (Kauppi *et al*., 2011; Sayara *et al*., 2011; Raji *et al*., 2012; Taccari *et al*., 2012; Abed *et al.*, 2014).

A significant proportion of water bodies and soil in Nigeria are exposed to intense pollution from hydrocarbons. This has a negative impact to ecosystem of the polluted area leading to loss of aquatic and terrestrial life, reduction of productivity of the polluted site and health of the organisms inhabiting these sites are in great jeopardy (Ebuehi *et al.*, 2005).

Despite these problems associated with hydrocarbon contamination, there are no strategies and no effort is invested on the side of the authorities to combat the problems associated with hydrocarbon pollutions. The physical and chemical approaches to cleaning up crude oil polluted sites are hoaxed with various short-comings (Ijah *et al.*, 2008). Unless properly managed, hydrocarbon polluted environments can remain impacted for long periods of time. However, with active biodegradation process and remediation programme using biostimulation, significant higher rates of oil removal could be achieved (Cerqueira *et al*., 2014).

This study was conducted to assess the impact of using goat dung on petroleum hydrocarbon degradation efficiency and the population dynamics of indigenous hydrocarbon degrading bacteria during bioremediation of spent engine oil contaminated soil.

MATERIALS AND METHOD

Collection of samples

Goat dung

One kilogram (1kg) of goat dung was collected from a goat pen located in Kaduna state, Nigeria. It was collected using a clean hand-trowel and the composite was placed into a clean polythene bag and transported to the Environmental Microbiology laboratory, Department of Microbiology, Ahmadu Bello University Zaria, for further studies. The sample was air dried and ground using a mortar and pestle to fine particle size and then stored in the screen house at ambient condition until further use. The proximate composition of the goat dung was determined using standard methods (AOAC,2010).

Soil

Soil sample with long history of petroleum contamination was collected from a mechanic workshop in Zaria, Kaduna State, Nigeria. Using a clean hand trowel, samples were collected from the top 20cm layer from several spots, and placed into clean polythene bags and transported to the Environmental Microbiology laboratory, Department of Microbiology Ahmadu Bello University Zaria. The samples were bulked together and mixed thoroughly to obtain a single sample representing the entire workshop premises. The bulk sample was then crushed and passed through a sieve of a 2mm sized mesh. Two subsamples of hundred grams each of the soil were measured into a clean polythene bag for the determination of hydrocarbon utilizing bacterial counts and physicochemical properties, respectively. The remaining bulk sample was kept at the screen house at ambient condition for the bioremediation studies.

Determination of physicochemical properties of the soil sample

Physico-chemical properties including soil textural class, water holding capacity, pH, organic carbon content, total nitrogen, phosphorous and exchangeable base contents of the soil samples were determined following standard procedures (APHA, 1995). The C:N ratio was also calculated.

Proximate analysis of the goat dung

The components that were analyzed includes, the moisture content, ash content, crude fat, crude protein and carbohydrate content as described by Association of Official Analytical Chemists (AOAC) (2010).

Determination of petroleum hydrocarbon content of the soil

This was determined following standard solvent extraction procedure as described by Ofoegbu *et al*. (2015). A gram of the soil from each set-up was dissolved in chloroform and the clear upper layer was collected with a clean test tube upon which, it was dehydrated by the addition of 2mL of anhydrous sodium sulphate. The absorbance of the clear extracted solution was determined at 420nm using spectrophotometer (HACH DR/2010). The total hydrocarbon content was extrapolated from standard curve prepared using engine oil.

Determination of hydrocarbon-utilizing bacterial count (HUB)

Preparation of mineral salt medium (MSM)

The mineral salt medium (MSM) had the following composition per litre of distilled water: KH_2PO_4 (2.0g), $NaNO_3$ (2.0g), NaCl (0.8g), KCl (0.8g), $Na_2HPO_4.12H_2O$ (2.0g), $MgSO_4$ (0.2g), $FeSO_4.7H_2O$ (0.001g). The preparation was enriched with 2% (v/v) of engine oil as sole carbon source and 20g of agar agar was added for solidification. It was then sterilized by autoclaving at 121^oC for 15 minutes. The preparation was allowed to cool to about 50^oC and dispensed into sterile petri dishes and allowed to solidify (Eniola *et al.*, 2014).

Hydrocarbon-utilizing bacterial count (HUB)

The abundance of hydrocarbon utilizing bacteria (HUB) indigenous to the soil was determined following the procedure described by Eniola *et al*. (2014). One gram of the soil sample was suspended in 9mL of sterile distilled water and a ten-fold dilution was prepared up to dilution 10^{-5}. An aliquot of 0.1ml from the 10^{-5} dilutions was inoculated onto the surface of freshly prepared mineral salt agar medium supplemented with 2% (v/v) engine oil as the sole source of carbon. The inoculated plates were incubated at 37^oC for 72 hours. The resulting bacterial colonies were enumerated using a colony counter and the number of HUB was calculated and expressed in colony forming units (CFU/g) per gram of soil.

Bioremediation studies

Experimental design

The microcosm experiment was designed to assess the effect of goat dung as a biostimulant on the growth and activity of hydrocarbon degrading bacteria indigenous to engine oil contaminated soil. The experimental treatments consisted of three concentrations of goat dung: 2% (w/w), 3% (w/w) and 4% (w/w), respectively; and an untreated control with two replicates for each treatment. One kilogram (1kg) of the soil sample were weighed and placed in twelve plastic containers (15cm diameter x 8cm depth) with the bottom perforated to allow aeration. The eight experimental soil samples were divided into four groups; three groups were treated with 20, 30 and 40g of goat droppings kg^{-1} of soil, respectively, while the fourth group was devoid of goat dung to serve as the control.

Microcosm experiment under screen house conditions

To each of the experimental microcosm prepared earlier, distilled water equivalent to the predetermined water holding capacity of the soil was added and they were incubated under ambient conditions in a screen house following a complete randomized arrangement for a period of seven (7) weeks. Soil from each microcosm was sampled at weekly intervals for the determination of hydrocarbon utilizing bacterial (HUB) count and the percentage of hydrocarbons degraded following standard procedures.

Enumeration of hydrocarbon-utilizing bacteria (HUB) in soil during bioremediation

This was carried out following the method described by Eniola *et al*. (2014). A tenfold dilution of each experimental soil was prepared by measuring one gram of soil and suspended in 9mL of sterile distilled water. The suspension was diluted to obtain a dilution of 10^{-5}. An aliquot of 0.1ml from the dilutions were inoculated into Petri dishes containing freshly mineral salt agar medium supplemented with 2%v/v engine oil using the spread plate method. The plates were incubated at 35℃ for 72 hours. The resulting colonies were enumerated using a colony counter and the counts of HUB expressed in colony forming units (CFU) per gram of soil.

Assessment of performance of Goat dung as Biostimulant

The performance of organic fertilizer as a bio-stimulant was assessed by calculating the degree of hydrocarbon degradation (D) achieved during the bioremediation process and the Biostimulation Efficiency (BE) of the organic fertilizer treatments.

The degree of degradation (D) was calculated using the formula:

$$\%D = \frac{THC0_0 - THCx}{THC_0} * 100$$

Where, THC_o = Initial hydrocarbon content of the soil, THC_r = Residual hydrocarbon content of soil after each week of the bioremediation process.

The Biostimulation efficiency (BE) of the organic fertilizer treatments was calculated using the formula:

$$BE = \frac{\%THCr - \%THCu}{\%THCr} * 100\%$$

Where %THCr = percentage removal of hydrocarbon in amended soil, %THCu = percentage removal of hydrocarbon in unamended soil.

Determination of physicochemical properties of soil during bioremediation

The texture, pH and moisture as well as total hydrocarbon, organic carbon and nitrogen content of the experimental soil during bioremediation were determined following procedures described by APHA (1995). The mean C: N ratio in the remediated soils was also calculated.

RESULTS

Physicochemical properties of soil

The pH of the soil increased from 5.91 to 6.61 while the N and P content of the soil were increased from 0.32% and 0.38% to 1.05% and 0.85% respectively (Table 1). An increase in the organic carbon content of the soil was also observed. This resulted in drastic decrease in the C: N ratio from 41 to 16.4 (Table 1).

Table 1 Physicochemical properties of experimental soil before and after bioremediation

	*Sand (%)	*Silt (%)	*Clay (%)	*Moisture content (%)	pH	THC (g/kg)	**Organic Carbon (%)	**Total Nitrogen (%)	**Total Phosphorus (%)	**C:N
Before	84 ± 0.00	3 ± 0.00	13 ± 0.00	2.10 ± 0.00	5.91± 0.00	10.00± 0.00	13.22 ± 0.00	0.32 ± 0.00	0.38 ± 0.00	41.00 ± 0.00
After	84 ± 0.00	3 ± 0.00	13 ± 0.00	2.54 ± 0.00	6.61 ± 0.00	8.70 ± 0.00	17.20 ± 0.00	11.05 ± 0.00	0.85 ± 0.00	16.40 ± 0.00

*Mean of two replicates **Mean of three replicates

Proximate composition of goat dung used in bioremediation

The goat dung used as amendment was found to contain high content of dry matter (89.20%) and considerable amounts of moisture (10.8%). Crude proteins, fibre, carbohydrates were found to be present in significant amounts as shown in Table 2.

Table 2: Proximate composition of goat dung used to enhance bioremediation of contaminated soil

Composition	**Amount (%)**
Dry Matter Content	89.20
Crude Protein	11.31
Moisture Content	10.8
Fibre Content	4.88
Ash	6.35
Carbohydrates	64.74

Effect on growth of Hydrocarbon-Utilizing Bacteria (HUB)

Application of goat dung had a stimulatory effect on the growth of HUB. This was evidenced by the consistently slower increase of bacterial counts and lower HUB counts in the untreated soil compared to the soils treated with organic fertilizer, as observed on week 6 of the experiment (Figure 1). It was also observed that, growth of HUB in the amended soil exhibited an almost exponential increase between weeks 2 - 5, for soils treated with 20, 30 and 40g/kg of goat dung respectively. On the other hand, only a gradual increase in the population of HUB was recorded in the unamended soil during the corresponding period with peak population of 10^5CFU/g on the 4th week of the study (Figure 1). The HUB count in the amended soil was also found to be much greater than that recorded in the unamended soil between weeks 2 and 5. However, by the 7th week, the population of HUB in the amended soils dropped to 10^4 CFU/g as recorded for the unamended soil (Figure1).

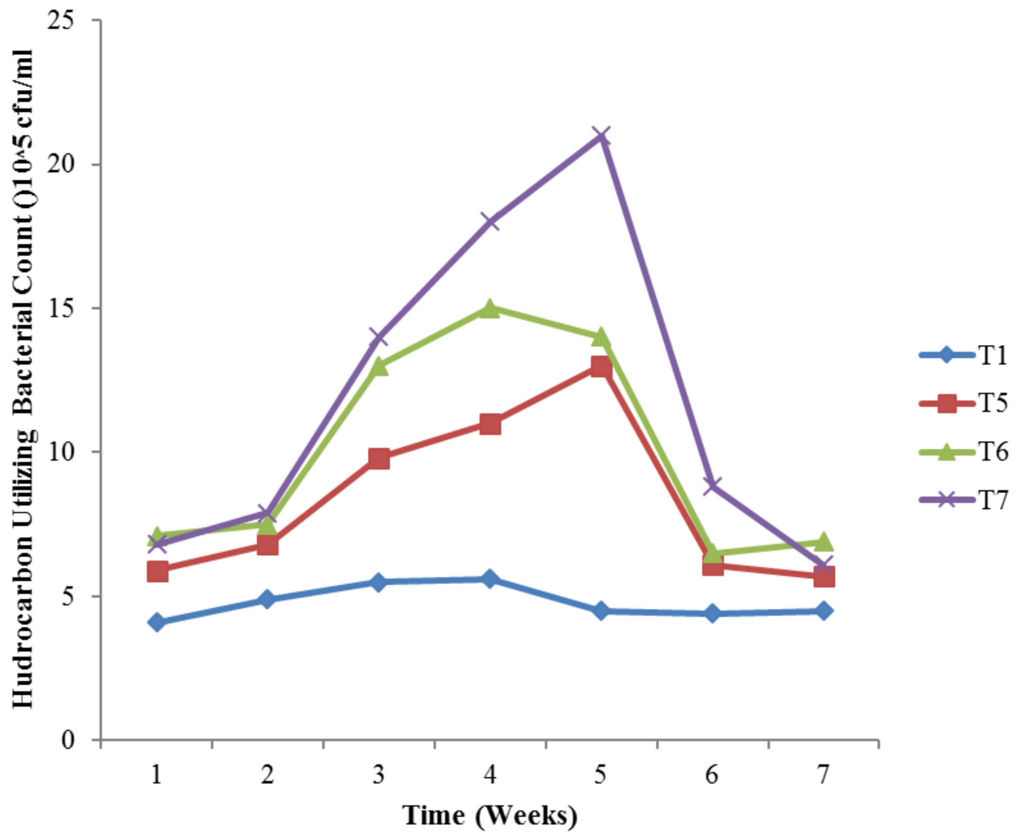

Fig. 1: Effects of Goat dung on growth of Hydrocarbon-Utilizing Bacteria During bioremediation of engine oil polluted soil
Key: **T1**; Unamended Soil, **T5**: Soil amended with 2%w/w of goat dung, **T6**; Soil amended with 3%w/w of goat dung, **T7;** Soil amended with 4%w/w of goat dung

Effect of goat dung on rate of biodegradation of hydrocarbons

In general, the fertilizer treatment proved to be stimulatory to biodegradation. It was observed that, while the mean percentage of hydrocarbon removed in the untreated soil remained below 0.5% per week during the first 4 weeks of incubation, it steadily increased from 0 - 24% in soils treated with the experimental levels of organic fertilizer (Figure 2).

It was also noted that, even when the process eventually started between weeks 4 and 5, the rate was noticeably slow reaching a low peak level of 4% on the 7th week representing a 1.35% mean removal per week. On the other hand, an average rate of 17.6% mean removal per week was recorded in the treated soils. Also, most of the biodegradation process occurred during the first 4 weeks of the study. However, it was observed that, the degree of hydrocarbon degradation in soils treated with the three tested levels of goat dung application was low during the first 21 days and highest during the last 28 days of the study (Table 2). On the whole, the biostimulation efficiency of the organic fertilizer treatment on the bioremediation process ranges between 96.9 to 99% for all tested levels (Figure 3 and Table 3).

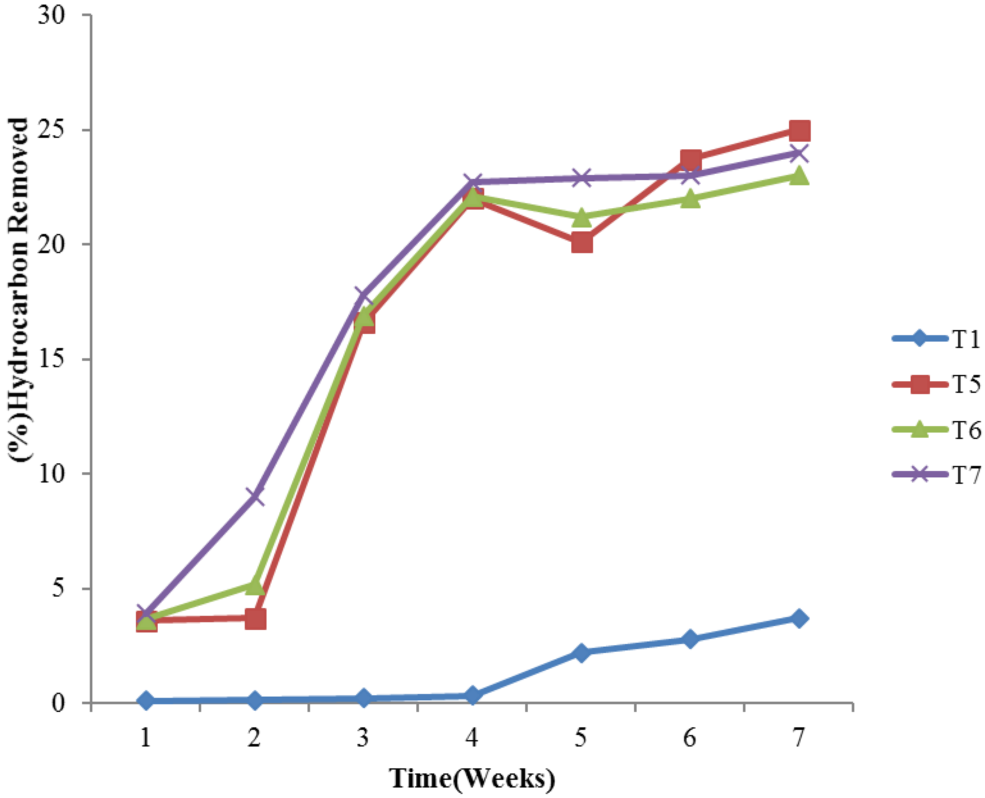

Fig. 2: Effect of Goat dung on degradation of hydrocarbons by bacteria in polluted soil
Key: **T1**; Unamended Soil, **T5**: Soil amended with 2%w/w of goat dung, **T6**; Soil amended with 3%w/w of goat dung, **T7;** Soil amended with 4%w/w of goat dung

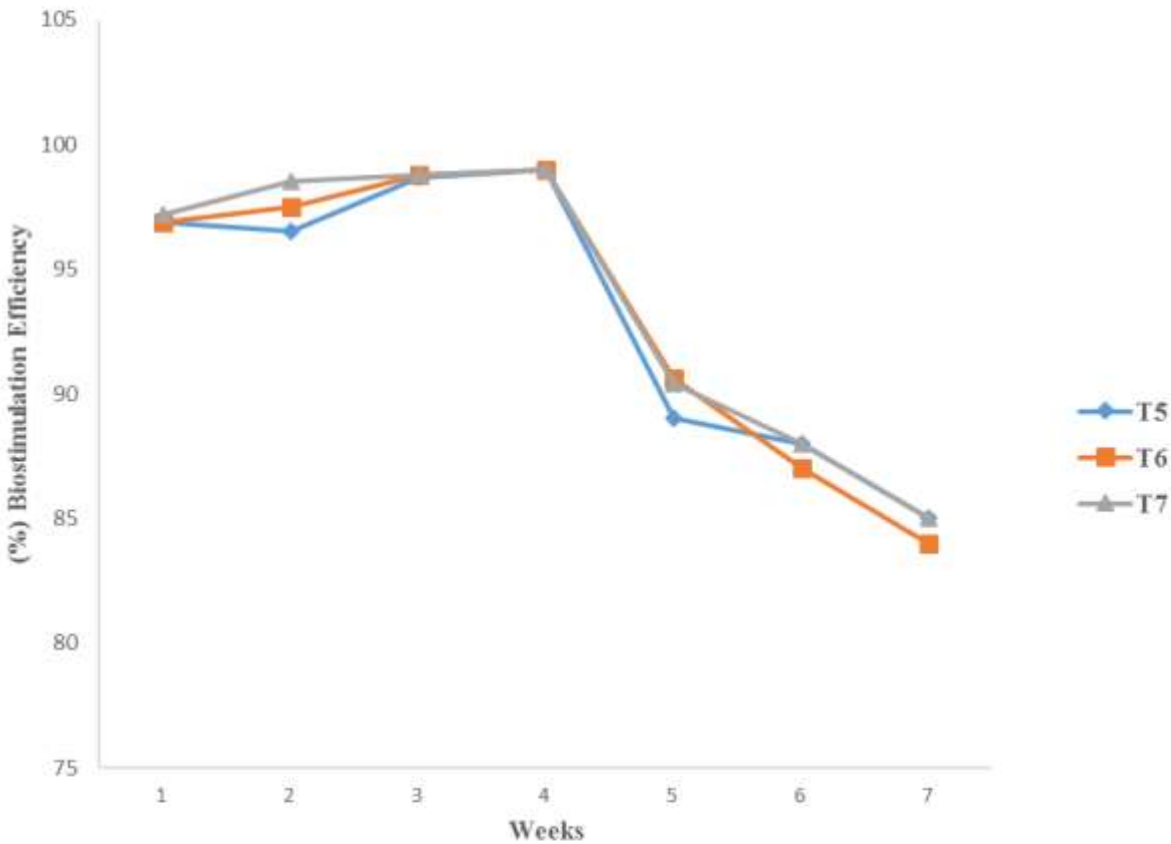

Fig. 3: Biostimulation efficiency in treatments containing only organic treatments

Key: **T5**: Soil amended with 2%w/w of goat dung, **T6**; Soil amended with 3%w/w of goat dung, **T7;** Soil amended with 4%w/w of goat dung

Table 3 Percentage of Biodegradation of hydrocarbons in soils amended with goat dung

Conc. of Organic	**Degree of Hydrocarbon degradation (%)**						
Treatments	Wk1	Wk2	Wk3	Wk4	Wk5	Wk6	Wk7
2%w/w	3.6	3.7	16.6	22	20.	24	25
3%w/w	3.7	5.15	16.9	22.1	21.2	22	23
4%w/w	3.9	9	18	23	22.9	23	24

Table 4 Percentage of Biostimulation efficiency of three levels of Organic fertilizer in biodegradation of hydrocarbons

Conc. of Organic	**Biostimulation efficiency**						
Treatments	Wk1	Wk2	Wk3	Wk4	Wk5	WK6	WK7
2%w/w	96.9	96.5	98.7	99	89	88	85
3%w/w	96.9	97.5	98.8	99	91	87	84
40%w/w	97.2	98.5	98.8	99	90.4	88	85

DISCUSSION

The outcome of the physicochemical analysis indicates that the soil is heavily contaminated with petroleum hydrocarbons. The concentration of petroleum hydrocarbons in the contaminated soil is 1%w/w. Decrease in availability of nutrients such as Nitrogen and Phosphorous which occurs with increase in hydrocarbon content has been reported by Okoli *et al.* (2015). The decrease in growth limiting nutrients could have adverse effect on the growth of the hydrocarbon utilizing population. Addition of goat dung which results in increased bioremediation has been reported by other workers (Benchouk *et al.,* 2017; Ugochukwu *et al.,* 2017). The present study supports the above observation of enhanced effects of growth of hydrocarbon utilizing bacteria with addition of goat dung.

Increase in pH observed in this study was favorable to the growth of the bacteria and hence the degradation of hydrocarbon. This could be due to the modifications of the chemical compounds towards alkalinity which has been reported to favor the proliferation of hydrocarbon degrading bacteria (Sharma and Pathak, 2014). The proximate composition of the goat manure shows that it could have biostimulatory effect as most of key components analyzed were within the standard sti'pulated by AOAC (2010).

The higher population of bacteria observed in soils amended with goat dung (Figure 1) suggests that, amending petroleum hydrocarbon polluted soil with goat dung has beneficial effects on the growth of hydrocarbon utilizing bacteria owing to the additional nutrients provided by the amendments. The beneficial effects of application of goat dung on the growth of hydrocarbon utilizing bacteria observed in this study could be attributed to increase availability of nitrogen and phosphorous and other nutrients required by bacteria dependent on hydrocarbon as the sole source of carbon and energy. These observations indicate that proliferation of hydrocarbon utilizing bacteria in hydrocarbon polluted soils could be stimulated through the application of goat dung to the soil provided that other growth requirements are met. With the exception of the unamended soil which showed that the HUB had peak growth on week 4, the soils treated with goat dung all had their peak growth of HUB on week 5. This indicates that biostimulation of petroleum hydrocarbons using goat dung sustains the HUB population for longer duration thus ensuring increased rates of hydrocarbon degradation.

The significant ($p<0.05$) increase in the degree of degradation of hydrocarbons in the oil contaminated soils following the application of goat dung observed in this study could be attributed to several reasons. The most important amongst these is the possible of direct addition of nutrients such as nitrogen (N) and phosphorous (P) into the soil which required for the growth of the hydrocarbon utilizing bacteria which are usually available in limiting amounts in soils that are heavily contaminated with hydrocarbons. Such increase in the levels of available N and P in turn lowers the C:N ratio as a result of which the rate of the degradation of hydrocarbon compounds is speeded up as a natural consequence of increase in the populations and activities of hydrocarbon utilizing bacteria indigenous to the soil (Adams *et.al.,* 2015; Ugochukwu, 2017). Thus, the explosive increase in the population of hydrocarbon utilizing bacteria observed in this study could probably explain the increases in the degree of degradation of hydrocarbon observed in this study. This observation could be attributed to improvement in the C: N: P ratio in the treated soil as a result of addition of the goat dung. The beneficial effects of increased availability of N and P on bacterial growth and degradation of hydrocarbon in the water and soil environments have been widely reported (Atlas, 1981; Ayotamuno *et al*., 2006; Zawierucha and Malina, 2011; Ofoegbu *et al.,* 2015). According to these authorities, increased availability of N and P following application of organic fertilizers enhance the rate of growth of the bacteria indigenous to polluted soils thereby increasing the rate of hydrocarbon degradation.

Given the fact that, typical engine oil consists of aliphatic hydrocarbons with between 18 and 34 carbon atoms (Odjegba and Sadiq, 2000) as well as aromatic compounds of varying molecular complexities (Bamforth and Singleton, 2005), it is conceivable that, a complex bacterial community would be required for effective bioremediation of polluted sites. This is because, beside bacteria capable of producing the two key enzymes namely; Monooxygenase and Dioxygenase required for breakdown of aliphatic and aromatic hydrocarbons (Peng *et al*., 2008), the role of others, which produce biosurfactants such as surfactin, rhamnolipids and sophorolipids for effective biodegradation of hydrocarbons have been stressed (Cottin and Merlin, 2007).

The observations made in this study suggest that soil sites polluted with spent engine oil contain indigenous bacteria capable of remediating the polluted sites provided that sufficient nitrogen and phosphorous are made available in the polluted sites.

CONCLUSION

Based on the observations made in this study, it was concluded that application of goat dung at the tested rate could be a strategy for stimulating the growth of indigenous hydrocarbon utilizing bacteria and biodegradation of hydrocarbon components of spent engine oil in polluted soil. The concentration of 4% (w/w) of goat dung performed best in this study.

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