Microbiological Composition of a Hydrocarbon Polluted Soil Amended with both Organic and Inorganic Materials

Chuku, O. S.
Department of Applied and Environmental Biology
Rivers State University, Port Harcourt.
ovundah4solomon@yahoo.com, ucheemiri@gmail.com

Abstract
Studies to investigate the microbiological composition of a petroleum hydrocarbon (crude oil) polluted soil following remediation with leaf litter from Terminalia catappa and hydrogen peroxide with garden egg, beans and cucumber as test crops were carried out in the Rivers State University research farm. A block design comprising of 14 treatments and 5 replicates was used. T1:100ml Crude Oil + 100ml H_2O_2, T2:100ml Crude Oil + 100g l/l, T3:200ml Crude Oil + 200ml H_2O_2, T4:200ml Crude Oil + 200g l/l, T5:300ml Crude Oil + 300ml H_2O_2, T6:300ml Crude Oil + 300g l/l, T7:400ml Crude Oil +300ml H_2O_2, T8:400ml Crude Oil + 400g l/l, T9:500ml Crude Oil + 500ml H_2O_2, T10:500ml Crude Oil + 500g l/l, T11:no pollution + 500ml H_2O_2, T12:no pollution + 500g l/l, T13:500ml Crude Oil + no amendment and T14:control. Garden egg, Beans and cucumber seeds were used as test crops. 10kg of soil was weighed into experimental bags and the pollutant was applied at different concentrations. A Viability test was carried out before planting. 420 experimental bags of 10kg capacity were filled with 10kg soil and polluted with Crude Oil at different levels. 140 experimental bags were used for each plant. Organic and Inorganic manure were applied at 1 month interval after pollution for 4 months, and planting was done afterwards. Soil samples were analyzed for the microbial load at 6 different times; before, after pollution, and 1 month after amendment for a period of 4 months. Result of microbial analysis on the Total Heterotrophic Bacteria (THB) which had low values after pollution drastically increased after organic amendment while the inorganic amended soils had no significant increases. Total Heterotrophic Fungi (THF), Hydrocarbon Utilizing Bacteria (HUB) and Hydrocarbon Utilizing Fungi (HUF) which were very low before and after pollution greatly increased after addition of amendment agent (Organic manure) while the hydrogen peroxide amended soils had little increases.

Keywords: *microbiological, pollution, crude oil, leaf litter, hydrogen peroxide.*

Introduction

Crude oil contamination and pollution produce toxic effect on living organisms in whichever form that it may appear (Adenipekun *et al.*, 2009). Odejimi, and Oghalu, (2006) described plants as "the first victims of oil spill on land ecosystem". (McGill, 1980) reported that " the lack of sufficient air in crude oil polluted soils leads to negative conditions for plant growth and as a result, delays photosynthesis and other plant physiological processes in plant".

Hydrogen peroxide is one of the most successfully used remedial chemical for contaminated soils. Oxidation with hydrogen peroxide can be direct and/or through the generation of free radicals (hydroxyl radicals OH). The latter relies on the decomposition of hydrogen peroxide catalysed by most ions of transition metals (Fe, Cu, Zn, etc.), and by natural minerals of those metals (hematite, goethite, etc.) present in soil. The basic reaction is:

$H_2O_2 + Fe^{2+} \rightarrow OH^- + OH + Fe^{3+}$

By reacting with the contaminant in place, the reagent serves to eliminate the possibility of contaminant vertical movement other than resulting from the act of vertical injection itself, which is often a concern in other remediation technologies (Watts. *et al.*, 2002).
Leaf litter contains considerable amount of nutrients and bound energy which are released during decomposition. The significance of ecology of litter-decomposing fungi has been emphasized by several workers (Garrett, 1962, 1966, 1975; Webster, 1956; Webster & Dix, 1960; Hudson, 1968; Sharma & Dwivedi, 1975).Nutrients return to the soil through litter fall help to maintain soil fertility by increasing the soil organic matter in the soil (Bernhard-Reversat, 1993).
The study therefore investigated the microbial load of crude oil polluted soil remediated with leaf litter and hydrogen peroxide using garden egg, beans and cucumber as test crops.

Materials and Methods

Experimental site

The experiment was carried out in the Rivers State University Teaching and Research Farm, Port Harcourt situated on latitude 4.7923^0 and longitude 6.9825^0. The study site is characterized by tropical monsoon climate with mean annual temperature of 32.15^0C, 66% humidity and 0.9948 atmospheric pressure, while, the soil is usually sandy or sandy loam underlain by a layer of impervious pan.
The study site was situated at the Rivers State University Research Farm which functions under the Faculty of Agriculture, Rivers State University, Port Harcourt, Nigeria. An area of 20m x 10m was marked out with a measuring tape and then cleared to ground level. No covering was made so as to ensure sunlight had a direct focus on it, and rain to get to the plant. It represented a natural environment for proper and adequate growth.

Planting Materials

Treated seeds of garden egg (*Solanum melongena*), beans (*Phasseolus vulgaris*) and cucumber (*Cucumis sativus*) were the planting materials used for the experiment. They were obtained from ADP (Agricultural Development Programme) Rumuokoro, Port Harcourt, and Rivers State, Nigeria.

Experimental bags

A total of 420 experimental bags filled with soil were used for the whole experiment. 70 experimental bags were used for each plant which was replicated 2 times for the three (3) plants.
The experimental bags were purchased from mile 3 market, Port Harcourt, and were filled with 10kg soil. The experimental bags were punctured on all sides and beneath so as to prevent water logging of the experimental bags.

Crude oil

100 litres of crude oil was purchased from the Port Harcourt Refinery, Eleme, and Rivers State, which was then conveyed to the Rivers State University Research Farm and applied as a pollutant on the agricultural soil.

Measuring cylinder

A measuring cylinder of 1000ml capacity of different concentrations was used to measure crude oil used for polluting the soil.

Fertilizer

Organic and inorganic fertilizers were used to carry out this experiment. The organic fertilizer used was *Terminalia catappa* (leaf litter) while the inorganic fertilizer was Hydrogen peroxide (H_2O_2). The *Terminalia catappa* was obtained from a site in the Rivers State University while the hydrogen peroxide was obtained from a scientific supply shop in Alakahia, Port Harcourt. The leaf litters were gathered in very large quantities, dried in the University of Port Harcourt green house and analysed before use.

Experimental soil

There was a random collection of samples of soil with metal soil auger at the top layer of the soil (loamy soil) between the depths of 0 to 15cm from an agric farm in the school premises which was taken to the green house of the Rivers State University.

Soil Samples and Treatment

Samples of soil were collected by composite sampling using a metal soil auger. Samples of the soil were brought together, homogenized and 10kg measured into experimental bags with perforations (Onuh *et al*., 2008a) for both the first, second and third block. The soil parameter (microbiological analysis) was also determined in the Department of Plant Science and Biotechnology Laboratory, University of Port Harcourt. The process was carried out six (6) times; before pollution, after pollution, 1 months after amendment for a period of 4 months.

For the treatment, 10kg of soil was weighed into labelled punctured experimental bags (Onuh *et al.*, 2008a) both for the first, second and third replicates. It was mixed with carefully measured concentrations of crude oil and then put into the experimental bags.

In the first block, 100, 200, 300, 400 and 500ml of crude oil were introduced to the soil in the experimental bags except for the control. The same process was repeated on the second and third block respectively. The polluted and unpolluted soils were allowed to stand for 1 month (30 days) before amendment was applied. Thereafter, carefully weighed (in grams and mills) *Termiinalia catappa* (leaf litter) and Hydrogen peroxide (H_20_2) were introduced to the treatments except for the control. The soil samples were watered occasionally to avoid dehydration.

Amendment Materials and Treatment

Taminalium cattapa (leaf litter) and hydrogen peroxide were used for the soil remediation. The experiment was in 3 blocks for each plant. Block 1; 10kg of soil was used with 100, 200, 300, 400 and 500ml of crude oil amended with leaf litter (100,200,300,400,500g) and hydrogen peroxide (100,200,300,400 and 500ml) with 3 bags of unpolluted soils that served as the control. The same experiment was replicated for block 2 and 3 respectively.

Garden Egg, Beans and Cucumber Experiment Using Organic and Inorganic Manure as Fertilizer

T1	100ml crude oil + 100ml H_20_2
T2	100ml crude oil + 100g leaf litter
T3	200ml crude oil + 200ml H_20_2
T4	200ml crude oil + 200g leaf litter
T5	300ml crude oil + 300ml H_20_2
T6	300ml crude oil + 300g leaf litter
T7	400ml crude oil + 400ml H_20_2
T8	400ml crude oil + 400g leaf litter
T9	500ml crude oil + 500ml H_20_2
T10	500ml crude oil + 500g leaf litter

T11	NO POLLUTION + 500ml H_20_2
T12	NO POLLUTION + 500ml leaf litter
T13	500ml crude oil + NO AMENDMENT
T14	CONTROL

Amendments were properly upturned in each experimental bag containing the soil and were left for 4 months under natural environmental condition during which amendment was carried out monthly prior to planting. Each treatment had a total of three (3) replicates and the containers were arranged accordingly using a block design for *solanum melongena, phaseolus vulgaris and cucumis sativus.*

Microbial Analysis
Samples of soil required for treatment were collected and placed on sterile containers and taken for analysis.

Determination of Total Fungi (TF)
Reagents
1. 0.85%NaCl
2. Potato Dextrose Agar (PDA)

Procedure
1 g of soil sample was weighed into 9 ml sterile diluents (0.85 % NaCl) under aseptic conditions. It was then shaken vigorously and serially diluted. 0.1ml Aliquot of inoculums were inoculated on Potato Dextrose Agar (PDA) acidified on 0.1 % Lactic acid to inhibit growth of bacteria and allowed for only the growth of fungi. The inoculated plates were incubated at a temperature of 32 ^{0}C for 5-7 days. Thereafter the numbers of visible colonies were enumerated to obtain the colony forming unit per gram (cfu/g) of samples. Cultural characteristics of isolates were observed and subcultured for purification. Microscopic examination was done using lactophenol cotton blue stained with x400 magnification.

Determination of Total Heterotrophic Bacteria (THB)
Reagents:
1. 0.85% NaCl
2. Nutrient Agar (NA).

Procedure:
One gram (1g) of soil sample was weighed into 9ml sterile diluents (0.85% Nacl) under aseptic conditions. It was mixed thoroughly to homogenize and diluted serially diluted. Then 0.1ml aliquot of the inoculums was collected using a sterile pipette, inoculated on Nutrient Agar (NA) medium. The inoculum was spread evenly with a sterile hockey stick. Plates were incubated at 37^{0}C for 24 hours. Thereafter, colonies were counted to obtain colony forming unit (cfu) value per gram of the soil sample. Distinct colonies with different morphological patterns were picked and streaked and sub-cultured on freshly prepared nutrient agar medium to obtain pure culture after 34 hours incubation at 37^{0}C. The pure cultures were gram stained for microscopic examination. Biochemical tests for characterization and identification of the isolates were carried out

Determination of Hydrocarbon Utilizing Bacteria (HUB)
Reagents:
1. 0.85% Nacl

2. Mineral Salt Agar (MSA).

Procedure:
One gram (1g) of soil sample was weighed into 9ml sterile diluents (0.85% Nacl) under aseptic conditions, shaken vigorously and serially diluted. Aliquot (0.1ml) of inoculums was inoculated using the spread plate technique. A sterile blotter paper was immersed in crude oil and placed in a Petri-dish lid and incubated for 3-5days. Visible colonies were enumerated to obtain a colony forming unit per gram (cfu/g) of samples.

Determination of Hydrocarbon Utilizing Fungi (HUF)
Reagents:
1. 0.85% Nacl
2. Mineral Salt Agar (MSA)
3. 0.1% Lactic acid.

Procedure:
One gram (1g) of soil sample was weighed into 9ml sterile diluents (0.85% Nacl) under aseptic conditions and shaken vigorously and serially diluted using sterile pipette. 0.1ml aliquot of inoculum was inoculated on Mineral Salt Agar (MSA) acidified with 0.1% lactic acid. This inhibits the bacteria, and permits only the growth of hydrocarbon utilizing fungi. Sterile filter paper was soaked in crude oil and placed in Petri-dish. Plates were incubated in inverted position at ambient temperature for 5-7days. Colonies were counted to obtain colony forming unit per gram of sample. Thereafter, cultural characteristics of isolates were observed and purified by sub-culturing on a freshly prepared medium and incubated again for 3-5days. Microscopic examination of the pure cultures were done using lactophenol stained on cotton blue and observed with x400 magnification.

Results
Soil Microbial Determination
Total Heterotrophic Bacterial (THB) Count in Polluted and Amended Soils
The results for the total heterotrophic bacteria (THB) counts of garden egg, beans and cucumber grown in crude oil polluted soil amended with hydrogen peroxide (H_2O_2) and leaf litter (LL) from *Terminalia catappa* is shown in Fig 1 below. The result shows an increase in THB with increase in time from the first month to the final month of amendment (1.5×10^7–9.7×10^8). It also shows that the highest THB counts were observed in the treatments with leaf litter as amendments especially in T2 ($6.1 \times 10^6 – 9.1 \times 10^8$), T4 ($6.7x10^6$ - $9.7x10^8$) and T6 ($5.9x10^6$ - $8.9x10^8$) and while the soils amended with hydrogen peroxide had lower THB counts for all the treatments with values within the ranges of ($1.5x10^7$- $5.5x10^7$) as shown in the chart below. The control soils (T14) had high values but not up to the values recorded for leaf litter amended soils in THB with values within the ranges of (4.5×10^6– 8.4×10^8).
The results in fig.1 below indicated that leaf litter greatly increased the total heterotrophic bacterial count of the soil better than that of hydrogen peroxide both in the polluted and amended soils and unpolluted and amended soils respectively.

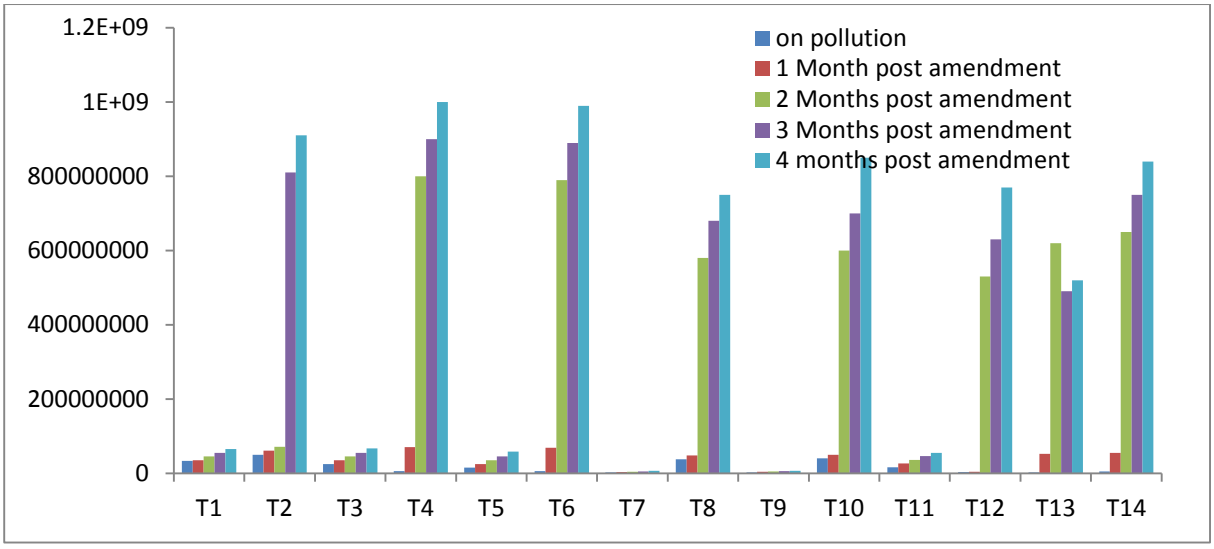

Fig 1: Effects of Organic and Inorganic Manure on the Total Heterotrophic Bacteria (THB) Count in Crude Oil Polluted Soil. T1 = (100ml crude oil + 100ml H_20_2), T2 = (100ml crude oil + 100g leaf litter), T3 = (200ml crude oil + 200ml H_20_2), T4 = (200ml crude oil + 200g leaf litter), T5 = (300ml crude oil + 300ml H_20_2), T6 = (300ml crude oil + 300g leaf litter), T7 = (400ml crude oil + 400ml H_20_2), T8 = (400ml crude oil + 400g leaf litter), T9 = (500ml crude oil + 500ml H_20_2), T10 = (500ml crude oil + 500g leaf litter), T11 = (no pollution + 500ml H_20_2), T12 = (no pollution + 500g leaf litter), T13 = (500ml crude oil + no amendment), T14 = (control).

Total Fungal Count in Polluted and Amended Soils

The results for the total fungi (TF) counts of garden egg, beans and cucumber grown in crude oil polluted soil amended with hydrogen peroxide (H_2O_2) and leaf litter (LL) are shown in Fig 2. The result shows an increase in TF with increase in time (months) in all the treatments. It also shows that the highest TF counts were observed in the treatments with leaf litter as amendments: T8 ($5.2x10^4$- $8.2x10^4$) and T10 ($4.0x10^7$ -$9.2x10^4$). The control soil (T14) also had high values ($3.4x10^4$ - $7.6x10^4$) which were lower than the other treatments with amendments. But generally; there was an increase in total fungi in all the treatments according to the months of amendment (T1-T14).

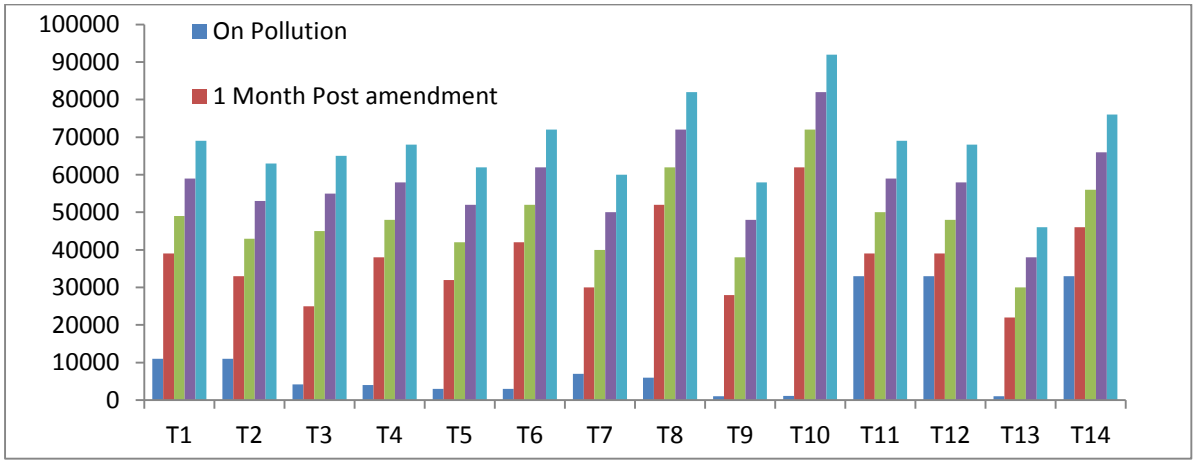

Fig 2: Effects Of Organic And Inorganic Manure On The Total Fungi (TF) Of Soil Polluted With Crude Oil. T1 = (100ml crude oil + 100ml H_20_2), T2 = (100ml crude oil + 100g leaf litter), T3 = (200ml crude oil + 200ml H_20_2), T4 = (200ml crude oil + 200g leaf litter), T5

= (300ml crude oil + 300ml H_2O_2), T6 = (300ml crude oil + 300g leaf litter), T7 = (400ml crude oil + 400ml H_2O_2), T8 = (400ml crude oil + 400g leaf litter), T9 = (500ml crude oil + 500ml H_2O_2), T10 = (500ml crude oil + 500g leaf litter), T11 = (no pollution + 500ml H_2O_2), T12 = (no pollution + 500g leaf litter), T13 = (500ml crude oil + no amendment), T14 = (control).

Hydrocarbon Utilizing Bacteria in Polluted and Amended Soils

The results for the Hydrocarbon utilizing bacteria (HUB) counts of the three crops grown in crude oil polluted soil amended with hydrogen peroxide (H_2O_2) and leaf litter (LL) are shown in Fig 3. There was an increase in HUB in the leaf litter amended soils in all the months after amendment as compared to the hydrogen peroxide amended soils and control soils except treatment 1 which gave the highest HUB in the 4^{th} month (2.5×10^5). This is an indication that leaf litter (organic manure) greatly increases the hydrocarbon utilizing bacteria of the soil upon its introduction whereas hydrogen peroxide reduces soil HUB. The soils with pollution (T13), amended without pollution(T11 and T12) and control (T14) had low HUB values on all the months of sampling that were below 1.0×10^6 an indication that soils require the presence of petroleum hydrocarbons to enable this bacteria present in the soil utilize them.

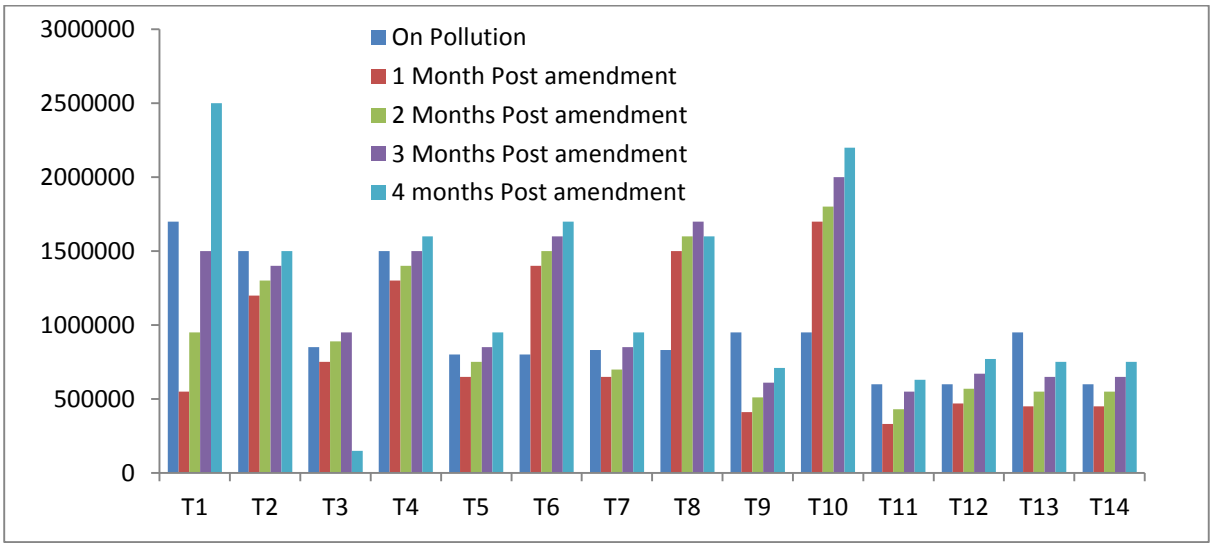

Fig 3: Effects Of Organic And Inorganic Manure On The Hydrocarbon Utilizing Bacteria (HUB) Of Soil Polluted With Crude Oil. T1 = (100ml crude oil + 100ml H_2O_2), T2 = (100ml crude oil + 100g leaf litter), T3 = (200ml crude oil + 200ml H_2O_2), T4 = (200ml crude oil + 200g leaf litter), T5 = (300ml crude oil + 300ml H_2O_2), T6 = (300ml crude oil + 300g leaf litter), T7 = (400ml crude oil + 400ml H_2O_2), T8 = (400ml crude oil + 400g leaf litter), T9 = (500ml crude oil + 500ml H_2O_2), T10 = (500ml crude oil + 500g leaf litter), T11 = (no pollution + 500ml H_2O_2), T12 = (no pollution + 500g leaf litter), T13 = (500ml crude oil + no amendment), T14 = (control).

Hydrocarbon Utilizing Fungi in Polluted and Amended Soils

The results for the Hydrocarbon utilizing fungi (HUF) counts of garden egg, beans and cucumber grown in crude oil polluted soil amended with hydrogen peroxide (H_2O_2) and leaf litter (LL) are shown in Fig 4. HUF increased in values as the months increased in all the treatments with greater values recorded in leaf litter amended soils (T2, T4, T6 and T8). The organic amended soils; T2 had value of $4.9x10^4$ - $7.9x10^4$, T4 = $5.0x10^4$- $8.0x10^4$, T6= $5.3x10^4$ - 8.3×10^4, and T8= $5.6x10^4$ - $8.8x10^4$ which were higher than those of in organic amended soils; T1= $4.3x10^4$-$7.3x10^4$, T3=$3.3x10^4$ - $5.3x10^4$, T5=$2.3x10^4$ - $5.3x10^4$, T7=$1.3x10^4$ - $4.6x10^4$and T9= $3x10^4$- $3.3x10^4$.

T13 and T14 had the highest values of HUF in the 1st, 2nd, 3rd and 4th month after amendment on all the treatments with values within the ranges of $10\times 10^6 - 18 \times 10^6$.
From the result below, the introduction of leaf litter increased the HUF of polluted soil than that of hydrogen peroxide, and the control soil without any pollutant contains appreciable load of hydrocarbon utilizing fungi.

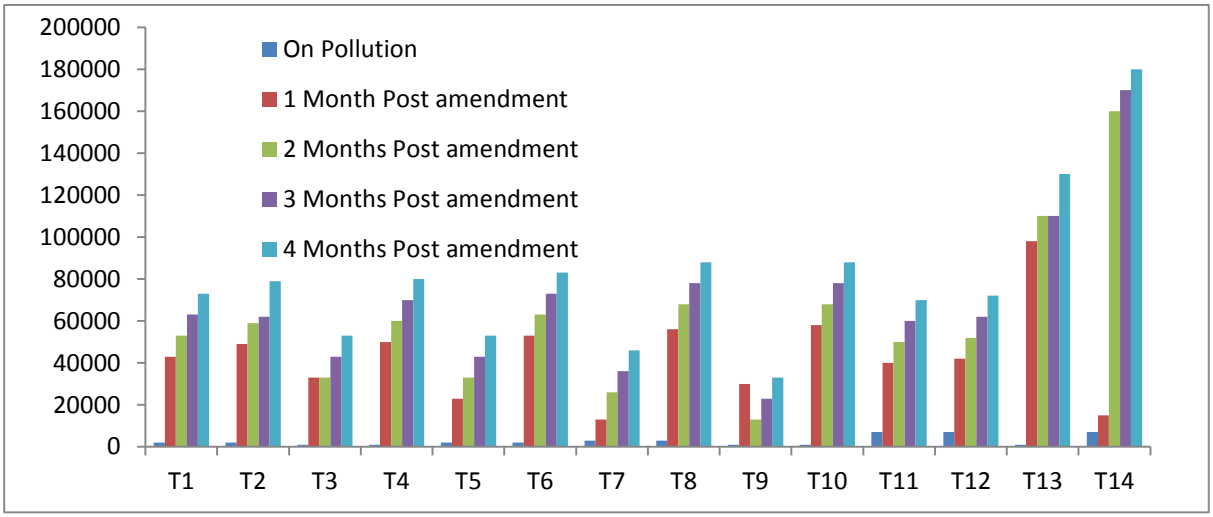

Fig 4: Effects Of Organic And Inorganic Manure On The Hydrocarbon Utilizing Fungi (HUF) Of Soil Polluted With Crude Oil. T1 = (100ml crude oil + 100ml H_20_2), T2 = (100ml crude oil + 100g leaf litter), T3 = (200ml crude oil + 200ml H_20_2), T4 = (200ml crude oil + 200g leaf litter), T5 = (300ml crude oil + 300ml H_20_2), T6 = (300ml crude oil + 300g leaf litter), T7 = (400ml crude oil + 400ml H_20_2), T8 = (400ml crude oil + 400g leaf litter), T9 = (500ml crude oil + 500ml H_20_2), T10 = (500ml crude oil + 500g leaf litter), T11 = (no pollution + 500ml H_20_2), T12 = (no pollution + 500g leaf litter), T13 = (500ml crude oil + no amendment), T14 = (control).

Discussion

Soil Microbiological Analysis

Results of the soil microbial analysis on the experimental soils at the beginning and the end of the experiment showed that upon addition of amendment agents, the soil microbial content greatly increased, this agrees with the work of Nwoko *et al.,* (2007)
The total heterotrophic bacteria (THB) greatly increased after 2 months of amendment with leaf litter while that of hydrogen peroxide had little increases when compared to the leaf litter amended soils.

The result also showed that upon introduction of remediation agents, the soil total fungi (TF) increased in the whole experiment and were obvious in the leaf litter amended soils (T8 and T10). This agrees with the work of Akaochere *et al.,* (2008)
Result for HUB showed that upon introduction of remediation agents, the hydrocarbon utilizing bacteria (HUB) greatly increased in the leaf litter amended soils more than the other treatments including the control (T14). The highest HUB increase was seen in T1 and T10 which may be due to light contamination (T1) and high quantity of amendment (T10). Hydrogen peroxide amended soils also had slight increases according to the months. The increase in HUB number can be linked to the rise in the availability of readily metabolizable components of hydrocarbon for the organisms.
Result for HUF revealed increases in the whole experiment (both organic and inorganic manure treatments) upon introduction of both pollutant and amendments. Leaf litter amended soils

recorded the highest HUF values in the whole experiment. Treatment 13 and Treatment 14 had the highest values when compared to the other treatments which imply that polluted soils and agricultural soils contain large amount of hydrocarbon utilizing fungi.

Nwoko *et al.,* (2007) Akaochere *et al.,* (2008) and Atlas (1981) gave reports that both the population with fungi and bacteria noted in this study confirmed the toxic impacts of hydrocarbons which exhibit a microbial flora of indigenous value.

Conclusion

This study has given an insight on how crude oil affects the soil microbial load. The introduction of Crude oil interfered with microorganisms in the soil but upon the introduction of the remediation agents, the petroleum hydrocarbon was utilized.

Leaf litter is a potential source of nutrient for microbial activity and harbours microorganisms capable of utilizing hydrocarbons as source of carbon and energy thus, potentially useful in soil hydrocarbon response action. Hydrogen peroxide on the other hand, is a potential source of hydrogen and oxygen to the soil which degrades petroleum hydrocarbons present in the soil; purifies the soil thereby making the soil favourable for plant growth.

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