



The Effects of Enrichment Nutrients on the Growth of Indigenous Bacteria Species in Spent Lubricating Oil Contaminated Water

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Author's contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

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ABSTRACT

Aim: This study was conducted to compare the effects of enrichment nutrients, NPK (Nitrogen, Phosphorus, Potassium) and organic wastes on the growth of indigenous bacterial species in spent lubricating oil contaminated water. Six bacterial species which were isolated from spent lubricating oil impacted soils (*Pseudomonas sp.*, *Bacillus sp.*, *Actinomyces sp.*, *Acinetobacter sp.*, *Enterobacter sp.*, and *Micrococcus sp.*) and showed profuse utilization of spent lubricating oil on screening, were used for this study.

Place and Duration of Study: The study was conducted in the laboratory of the Department of Environmental Management and Toxicology, Federal University of Petroleum Resources, Effurun, between 2018 and 2019.

Methodology: The study and was conducted using Mineral Salts Medium broth, spent lubricating oil substrate and NPK (20:10:10), Chicken droppings and Cow dung as nutrient sources (biostimulants). The effect of the biostimulants on the growth of the bacterial isolates was assessed weekly for 14 days by measuring the turbidity, bacterial counts and pH.

Results: *Pseudomonas sp.* recorded the highest count of 1.16E+19 CFU/ml, 2.53E+17 CFU/ml and 1.74E+14 CFU/ml for biostimulation with NPK, Chicken droppings and Cow dungs respectively.

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The treatment with NPK enhanced the bacterial isolates most, of the three treatments used at the end of the test period. The pH values obtained for the test cultures at the end of the study, ranged from 6.52 ± 0.02 for *Enterobacter sp.* in Cow dung treated cultures to 7.85 ± 0.03 for *Pseudomonas sp.* in NPK treated cultures. The values were within the optimum biodegradation range of 6.50 -8.50. There was significant difference between the bacterial counts obtained with the cultures treated with NPK and Chicken droppings ($P=0.006$), between NPK and cow dungs ($P = 0.031$) and between NPK and the control ($P = 0.033$). The study affirms the benefits of using organic wastes in the bioremediation process of hydrocarbon contaminated sites; it enhances the nutrients required by the bacteria for the remediation process and it's a waste management strategy for disposing these organic wastes at very minimal costs and in an ecofriendly manner.

Keywords: Bacterial growth; enrichment nutrients; organic wastes; spent lubricating oil.

1. INTRODUCTION

Lubricating oil is a common contaminant in water and soils. Generally, lubricating oil comprises 80% of hydrocarbon lubricant, with the remainder being additives, which consists partly of zinc diaryl, molybdenum disulfide, zinc dithiophosphate, metal soaps and other organometallic compounds [1].

Large amounts of lubricating oil are liberated into the environment when the motor oil is changed and disposed into gutters, water drains, open vacant plots and farmlands, a common practice by motor mechanics and generator mechanics [2]. Spent lubricating oil is produced when new lubricating oil is subjected to high temperature and high mechanical strain [3].

Spent engine oil as a pollutant in the environment and causes damage to our ecosystem and it's a health hazard to human beings [4]. Spent Oil has contaminated soils used for agricultural lands and has not spared the aquatic and marine plants and animals in Nigeria. Ground water has also been contaminated hence polluting the crops and farm animals [5].

Huge increase of vehicles due to rapid increase of human population has led to presence of various kinds of informal and formal automobiles hence increased use of motor oil [6]. Used motor oil contains metals and heavy polycyclic aromatic hydrocarbons (PAHs) that could contribute to chronic hazards including mutagenicity and carcinogenicity [7].

Polycyclic Aromatic Hydrocarbons (PAHs) are important contaminant which are retained in the environment and could also disrupt the endocrine system of animals affecting reproduction [8].

Lack of essential nutrients such as nitrogen and phosphorus is one of the major factors affecting biodegradation of hydrocarbon by microorganisms in soil and water environment. Therefore, the addition of inorganic or organic nitrogen-rich nutrients (bio stimulation) is an effective approach to enhance bioremediation process. Positive effects of nitrogen amendment on microbial activity and /or petroleum hydrocarbon degradation have been widely demonstrated [9]. Concentration of petroleum hydrocarbon determines to a great extent the rate of breakdown of the hydrocarbons from soil and water environment. High concentration of hydrocarbon can be inhibitory to microorganisms, and concentration at which inhibition occurs varied with the compound [10].

Bioremediation is one of the forms of biodegradation which involves the use of microorganisms to detoxify or remove organic and inorganic xenobiotic compounds from the environment. The process relies upon microbial enzymatic activities to transform or degrade the contaminants from the environment [11]. Hydrocarbon-degrading bacteria and fungi are widely distributed in marine, freshwater and soil habitats [12]. Many microorganisms have the ability to utilize hydrocarbons as sole sources of carbon as energy for metabolic activities and these microorganisms are widely distributed in nature. The microbial utilization of hydrocarbons depends on the chemical nature of the compounds within the petroleum mixture and on environmental determinant [13].

The ability to isolate high numbers of certain oil-degrading microorganisms from oil-polluted environment is commonly taken as evidence that these microorganisms are the active degraders of that environment [14]. Lack of essential nutrients such as nitrogen and phosphorus is one of the major factors affecting biodegradation of

hydrocarbon by microorganisms in soil and water environment.

A number of gram positive and negative microbes have been reported to be capable of utilizing a wide variety of hydrocarbons as carbon and energy. The microorganisms include bacteria of the genera *Klebsiella*, *Proteus*, *Bacillus*, *Escherichia*, *Pseudomonas*, *Streptomyces*, *Nocardia*, *Serratia*, *Xanthomonas*, *Micrococcus* [15] and fungi of the genera *Rhizopus*, *Fusarium*, *Penicillium*, *Cladosporium* and *Aspergillus* [16].

Poultry litter (chicken droppings) as reported by [17], contains hydrocarbon degrading microbes of the genera *Proteus*, *Micrococcus*, *Bacillus*, *Pseudomonas*, *Aspergillus* and *Rhizopus*. The authors also reported that poultry litter enhanced the degradation of crude oil by stimulating the organism present in the soil. Poultry litter and cow dung are Agro-based wastes rich in nutrient and microorganism, cheap and readily available.

This research is aimed at using organic wastes for the dual purpose; as a readily available and cheap source of enrichment nutrient for the remediation of spent lubricating oil contamination sites and also as a waste management strategy for the disposal for these organic wastes.

2. MATERIALS AND METHODS

2.1 Sample Collection and Preservation

2.1.1 Collection of hydrocarbon soils

Hydrocarbon contaminated soil samples were collected from automobile mechanic workshops and diesel generator site located in both Uvwie Local Government Area (Sample A) and Ughelli south LGA (Sample B) Delta state. Sample A was collected from FUPRE diesel generator site at Ugbomro (latitude 5°56'92", longitude 5°83'49") in Uvwie LGA, while sample B was taken from automobile mechanic workshop at Okwagbe town (latitude 6°23'58", longitude 5°55'11") in Ughelli South LGA, Delta state. Both sampling sites had operated for eight and seven years respectively. Composite soil samples were collected from each location. The samples were put into sterile polythene bags, labelled and transported to the laboratory where they were refrigerated at 4°C until required for use.

Spent engine oil was collected from the automobile workshop at PTI road, Effurun in a

clean glass bottle and kept at room temperature until required for use.

2.1.2 Collection of organic wastes (enrichment nutrients)

Partially dried chicken droppings was collected from a poultry farm along PTI road, Effurun. It was mixed and further dried. On drying, it was blended and stored in a closed container until required for use. The Cow dung was collected from an abattoir at PTI junction Effurun. It was mixed, air dried, grinded to powder and kept in a closed container until required for use. The NPK (20:10:10) was collected from Agricultural Development Programme office, Effurun, Delta State.

2.1.3 Enrichment technique for the isolation of oil-degrading bacteria from spent engine oil contaminated soils

This was done according to the method described by [18] Ten gram of the soil was added to 100 ml of sterile water and it was properly homogenized by shaking. 2 ml of the suspension was then added to 250 ml of sterilized Mineral salts medium in a 500 ml Cornical flask. To the solution was added 0.1% v/v of spent engine oil and 0.5 ml trace metal solution. The mixture was stirred and incubated for 48 hours at 30 ± 2°C.

2.1.4 Enumeration and isolation of spent oil - degrading bacteria from spent oil contaminated soils

The procedure of [19] was adopted for this study. Bushnell-Haas (BH) media with the following composition (g/L): K₂HPO₄ (1.0 g), KH₂PO₄ (1.0 g), NH₄NO₃ (1.0 g), MgSO₄•7H₂O (0.2 g), FeCl₃•6H₂O (0.05 g), CaCl₂•2H₂O (0.02 g), was used as enrichment medium with spent engine oil - 2 % (v/v) added as the sole carbon source to isolate hydrocarbon- degrading bacteria from the hydrocarbon contaminated soils. Soil samples (10 g) was added to 50 mL BH media in 250 mL Erlenmeyer culture flasks. It was then incubated at 28 ± 2°C at for 7 days. After 7 days incubation, the bacteria cultures were isolated as single colonies on to nutrient agar (NA) media by streak-plate method. The pure bacteria isolates were maintained in slant cultures by preserving at 4°C and sub cultured at 2 weeks interval to maintain its viability. Isolates were identified on the basis of their cultural, morphological and biochemical characteristics based on the criteria

of Bergey's manual of Determinative bacteriology [20,21].

2.1.5 Screening test of bacterial isolates for the utilization of spent engine oil

A screening test was conducted for the utilization of the spent lubricating oil on the bacteria isolated according to the method of [22] and modified by [23]. Mineral salts medium of [24] was constituted and 99 ml was put into test tubes. To each of the test tube was added 1 ml spent engine oil and sterilized at 121°C for 15 minutes. Upon cooling each tube was inoculated with 0.1 ml of cell suspension of an isolate in sterile mineral salts broth and incubated for 14 days at 30 ± 2°C. The isolates were scored for turbidity on day 0, 7 and 14 using a UV Spectrophotometer at absorbance of 560 nm. Control sets of tubes without the isolates were used to standardize the instrument.

2.1.6 Preparation of standard cultures of bacterial isolates

Standard cultures were prepared for the isolates adapting the methods of [25] and [26]. One hundred (100) ml of mineral salt broth was dispensed into each of three different conical flasks and inoculated with each purified isolate from each stock culture and incubated at 28°C for 24 hr. After incubation, the cultures were serially diluted up to 10⁻² and 0.1 ml of each was added into sterile plates. Cool molten nutrient agar was added to the inoculated plates and incubated at 37°C for 24 hr. The plate counts were recorded and the values obtained were expressed as standard number of cells present in ml of the broth. This was used as the standardized culture.

2.1.7 Comparison of the effect of enrichment nutrients on the biodegradation of spent lubricating oil by bacteria isolates

The comparison of the effects of enrichment nutrients on the biodegradation of spent lubricating oil contaminated water by the six bacteria species isolated from the hydrocarbon contaminated soils was carried out using inorganic fertilizer (NPK 20:10:10), chicken droppings and cow dungs (organic nutrients) as enrichment agents. The six bacteria isolates used, exhibited profuse utilization of the spent oil as observed by the turbidity values obtained in the screening test.

The isolates were activated by aseptically introducing a scoop of the isolates from the stock culture into 20 ml of sterile nutrient broth in MacCartney bottles. The cultures were incubated at 28 ± 2°C for 24 hours and were used as inoculum for this experiment.

Mineral salts medium of [27] was prepared and 99ml was dispensed into sets of 250 ml Cornical flasks. To each of the flask was added 1 ml of filter-sterilized spent lubricating oil and 1 ml of activated bacterial isolates. Each isolate was exposed to the three enrichment nutrients (NPK, chicken droppings and cow dungs). A set of flasks was not inoculated with any isolate and was used as a control. The experimental set up was conducted in replicates as follows:

- 1) 99 ml of mineral salts medium (MSM) + 1 ml of sterile spent lubricating oil + 1 ml of each isolate (in broth) + 1 g chicken droppings
- 2) 99 ml of MSM + 1 ml of sterile spent lubricating oil + 1 ml of each isolate in broth + 1 g cow dungs
- 3) 99 ml of MSM + 1 ml of sterile spent lubricating oil + 1 ml of each isolate in broth + 1 g NPK fertilizer (20:10:10)
- 4) 99 ml of MSM + 1 ml of sterile spent lubricating oil + 1 ml of all six isolate in broth
- 5) 99 ml of MSM + 1 ml of spent oil without bacterial isolate (control).

The flasks were incubated at 28 ± 2°C for 14 days. The turbidity was read as optical density (OD) at 560 nm, total viable count (TVC) of the isolate and pH of the culture in each flask were monitored at 2 days intervals for 14 days.

2.1.8 Statistical analysis

Analysis of variance (ANOVA) was used to test for significant differences between the treatment means and the control, using the SPSS software.

3. RESULTS AND DISCUSSION

3.1 Enumeration of Total Heterotrophic and Hydrocarbon Degrading Bacteria in Spent Oil Contaminated Soils

The bacterial isolates were identified using cultural, Morphological and biochemical methods and were identified as *Pseudomonas sp.* (C1), *Bacillus sp.* (C2), *Actinomyces sp.* (C3),

Acinetobacter sp. (C4), *Enterobacter sp.* (C5) and *Micrococcus sp.* (C6), using the method of [20].

The mean population densities of total heterotrophic bacteria in the hydrocarbon contaminated soils from site A (FUPRE Generator house) and B (mechanic workshop from Okwagbe, Delta State) were $1.18E+05$ and $7.60E.04CFU/g$ respectively. Percentage (%) total spent lubricating oil –utilizers to total heterotrophic bacteria in soil samples A and B were 29.32 ± 0.02 and $27.63 \pm 0.04\%$ respectively. The percentages obtained were relatively low. This could be attributed to the prolonged disposal of the hydrocarbons on the surrounding soils, which have compacted the soil and affected the soil texture and by extension the oxygen and water holding capacity of the soil [28].

3.2 Results of Screening Test for the Utilization of Spent Lubricating Oil by Bacterial Isolates

The results obtained from this test showed all six isolates were capable of utilizing spent lubricating oil as Carbon source though to varying degrees. The ability to utilize the oil was assessed by the optical density values obtained as shown in the following decreasing order: *Pseudomonas sp.* (1.765 ± 0.04) > *Actinomyces sp.* (0.670 ± 0.02) > *Acinetobacter sp.* (0.524 ± 0.04) > *Micrococcus sp.* (0.354 ± 0.03) > *Bacillus sp.* (0.224 ± 0.04) > *Enterobacter sp.* (0.124 ± 0.03), (Fig. 1).

3.3 Comparison of the Effects of Enrichment Nutrients on the Growth of Indigenous Bacteria Isolates In spent Lubricating Oil Contaminated Water

The effects of the enhancing nutrients, (N.P.K 20:10:10, chicken droppings and cow dungs) on the growth of the bacterial isolates in spent oil contaminated water was monitored by assessing the bacterial counts and pH of the culture over a period of 14 days in Mineral Salts Medium [27].

In the treatment with spent lubricating oil only, bacteria counts obtained ranged from $3.48E+07$ CFU/ml (C5) to $2.56E+12$ CFU/ml (C1) at the end of the test period (Fig. 2) while the pH values at the end of the test period were between 6.80 ± 0.04 (C1) and 7.35 ± 0.03 (C4) (Fig. 3). The growth of *Enterobacter sp.* (C5) was least, while,

Pseudomonas sp. (C1) obtained the highest count. For the treatment with spent lubricating oil and NPK (20:10:10), at the end of the test period (14 days), the growth of *Pseudomonas sp.* (C1) was enhanced most with a count of $1.16E+19$ CFU/ml while *Bacillus sp.* (C2) was least enhanced with a count of $1.84E+13$ CFU/ml (Fig. 2). The pH values at the end of the test period were between 6.92 for *Micrococcus sp.* (C6) and 7.85 for *Pseudomonas sp.* (C1) (Fig. 3). For the treatment with Chicken droppings, at the end of the test period (14 days), the growth of *Pseudomonas sp.* (C1) was enhanced most with a count of $2.53E+17$ CFU/ml while *Bacillus sp.* (C2) was least enhanced with a count of $2.16E+10$ CFU/ml (Fig. 2). The pH values at the end of the test period were between 6.85 ± 0.04 for *Enterobacter sp.* (C5) and 7.24 ± 0.04 for *Bacillus sp.* (C2) (Fig. 3). For the treatment with cow dungs, at the end of the test period (14 days), the growth of *Pseudomonas sp.* (C1) was enhanced most with a count of $1.74E+14$ CFU/ml while *Enterobacter sp.* (C5) was least enhanced with a count of $2.84E+09$ CFU/ml (Fig. 2). The pH values at the end of the test period were between 6.52 ± 0.04 for *Enterobacter sp.* (C5) and 7.23 ± 0.04 for *Bacillus sp.* (C2) (Fig. 3).

In all the treatments, the biodegradability potentials of the bacteria was enhanced most by the treatment with NPK as corroborated by the highest growth of the bacteria isolates, followed by Chicken droppings, Cow dungs, while the treatment with spent lubricating oil only (control) enhanced growth of the bacterial the least. *Pseudomonas sp.* (C1) exhibited the highest growth and the highest biodegradability potential while *Enterobacter sp.* (C5) exhibiting the lowest growth and least biodegradability potential (Fig. 2). These observations are in line with similar findings by [29]. Nduka et al. [30] who observed that NPK stimulates microbes to degrade hydrocarbon better than organic nutrients. They also observed that Poultry litter (Chicken droppings) enhances the degradation of hydrocarbons better than cow dung or the combination of both. In the control, it is noticed that there is initial rise in population count of the microbes due to utilization of the hydrocarbons for carbon and energy, but they begin to die due to lack of fertilizer stimulation. Research has shown that such wastes are useful material to modify the physical and chemical properties of the soil or water and also, to release nutrients for a longer period of time. The biostimulants thus provided, maintains the favourable conditions for growth of the microorganisms [31].

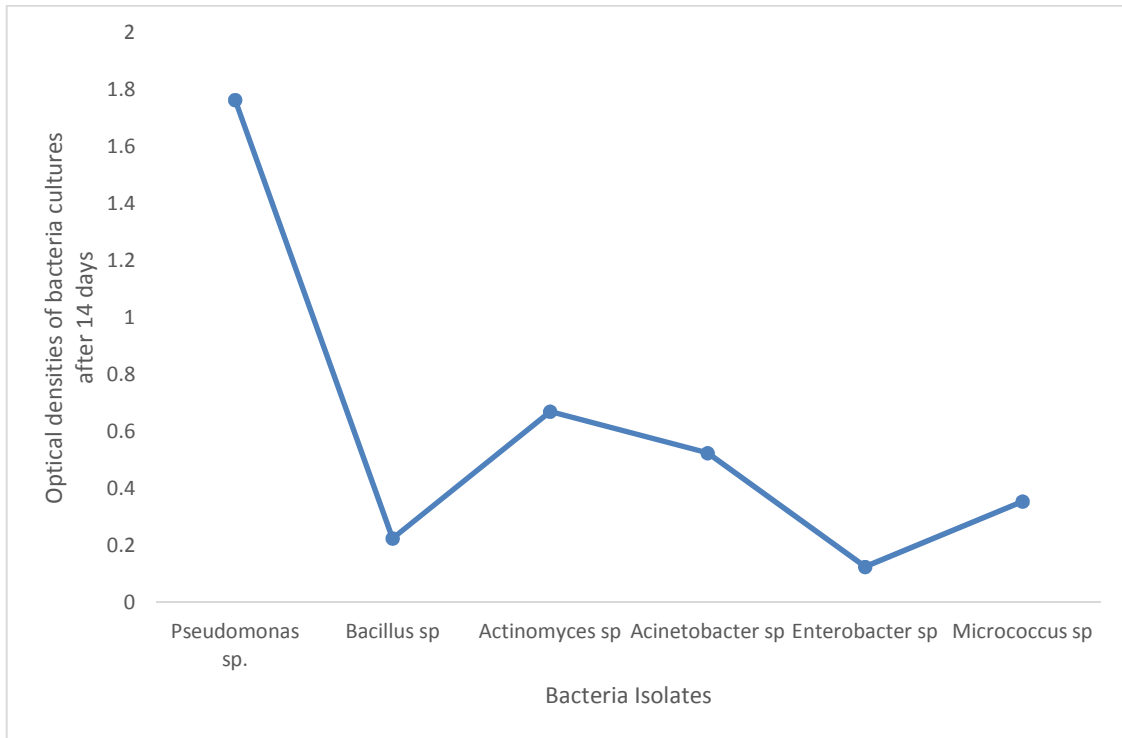


Fig. 1. Growth profile of bacteria isolates utilizing spent lubricating oil on day 14

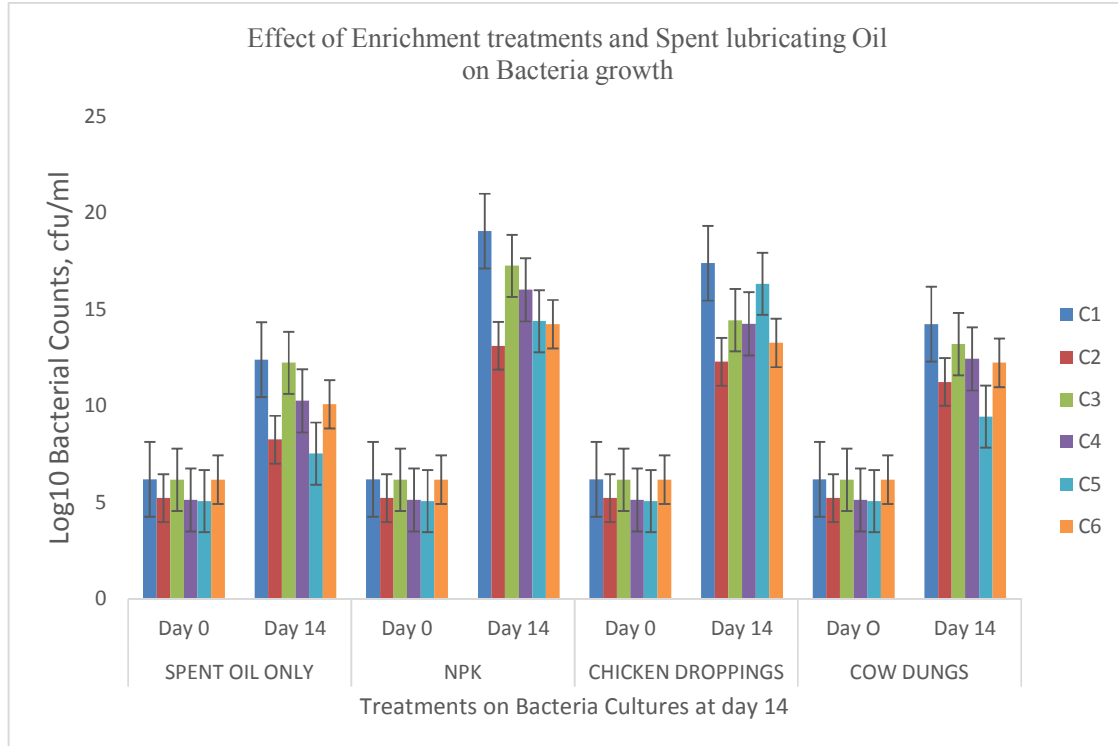


Fig. 2. Effect of enrichment treatments and spent lubricating oil on bacteria growth
 Legend: *Pseudomonas sp.* (C1), *Bacillus sp.* (C2), *Actinomyces sp.* (C3), *Acinetobacter sp.* (C4), *Enterobacter sp.* (C5) and *Micrococcus sp.* (C6)

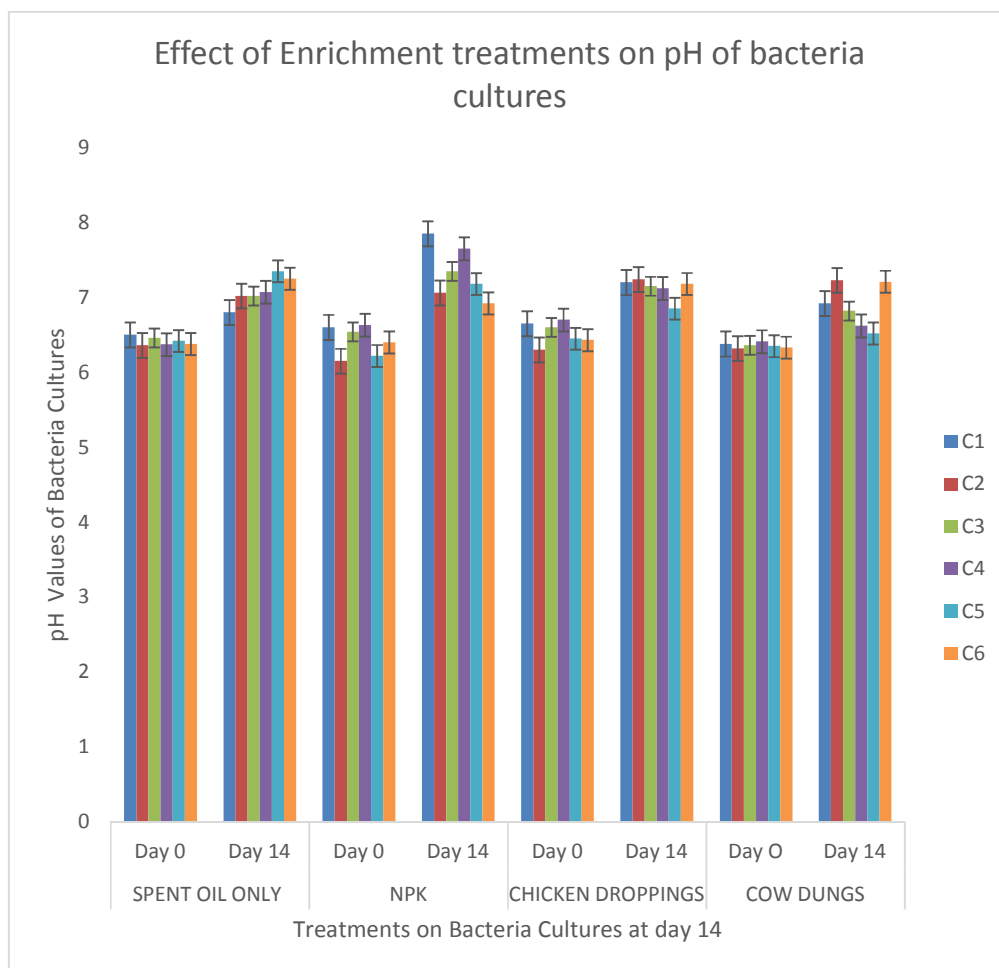


Fig. 3. Effect of spent lubricating oil and enrichment treatments on the pH of bacteria cultures
 Legend: *Pseudomonas sp.* (C1), *Bacillus sp.* (C2), *Actinomyces sp.* (C3), *Acinetobacter sp.* (C4),
Enterobacter sp. (C5) and *Micrococcus sp.* (C6)

The effect of oil on microbial populations depends upon the chemical composition of the oil and on the species of microorganisms present. Populations of some microbes increase; typically, such microbes use the petroleum hydrocarbons as nutrients. The same hydrocarbon can favor different genera at different temperatures [32].

All the pH values obtained were around approximately neutral, ranging from slightly acidic (6.52 ± 0.04) to slightly alkaline (7.85 ± 0.04). According to Desai and Vyas [33], pH is not of significant importance in marine environments owing to the fact that it is well buffered at about pH 8.5, and pH range of 7 to 8 has been reportedly detected to support optimum microbial degradation in such environment.

Hydrocarbon contamination of the air, soil, freshwater especially by polycyclic aromatic hydrocarbon (PAHs) attracts public attention because many PAHs are toxic, mutagenic, and carcinogenic [34]. Prolong exposure of high oil concentration may cause the development of liver or kidney diseases, possible damage to the bone marrow and increased risk of cancer [35,36].

Counts obtained between NPK and chicken dropping were significantly different ($P= 0.006$). Between NPK and cow dungs, counts obtained were also significantly different ($P= 0.031$). In the treatment between NPK and the control, counts were significantly different as well ($P= 0.033$). There was however, much more significant different between the NPK and Cow dungs and unenhanced (control) cultures. However, there

was no significant difference between bacteria growth in the cultures treated with cow dungs and the control ($P= 0.0001$).

4. CONCLUSION

The study revealed that several indigenous hydrocarbon –utilizing bacterial species exist in our environment especially in chronic hydrocarbon contaminated sites like the soils around mechanic workshops. Six indigenous bacterial species (*Pseudomonas sp.*, *Bacillus sp.*, *Actinomyces sp.*, *Acinetobacter sp.*, *Enterobacter sp.* and *Micrococcus sp.*), were isolated and identified. These bacteria species possessed biodegradative potentials for spent lubricating oil, that were further enhanced with the biostimulants (NPK, Chicken droppings and Cow dungs) though to varying degrees. It is important to point out that hydrocarbonoclastic microorganisms can readily utilize both organic and inorganic nutrients in accomplishing microbial degradation of oil pollutants. Since results obtained for the growth of the bacteria isolates were slightly significantly different between NPK and chicken droppings, preferably, the utilization of these organic nutrients tagged as waste products that are cheap, readily available and ecofriendly can be adopted as biostimulants in accomplishing bioremediation process. This will be a win-win situation, the wastes are properly managed, while the growth of the hydrocarbonoclastic bacteria are enhanced to degrade the spent oil and prevent it from impacting the environment.

These findings could be used by regulators of the Nigerian Oil and Gas industry to review her Guidelines and Standards, with the view to approving the use of indigenous bacteria species, which currently are prohibited, for the bioremediation of hydrocarbon contaminated sites. Further research could be done to mass produce, dehydrate and preserve indigenous hydrocarbonoclastic microorganisms, which could be promptly deployed in case of a spill.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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