Original Research Article

Bioremediation of Illegally Refined Crude Oil Residue using Soybean Waste and Cow Dung.

ABSTRACT

Aims: This study was carried out to compare the effectiveness of soybean wastes and cow dung as an organic treatment for stimulating the hydrocarbon utilizing Bacteria.
Study design: In the laboratory, about 1000g of soil sample were weighed and transferred in to sterile pots labeled EP1, EP2, and EP3. The biostimulants were respectively transferred into the pots accordingly. The setup was watered and tilled once a week to provide moisture and oxygen for the organisms
Place and Duration of Study: Kpo fire contaminated soil samples were collected in plastic bag from four different points in marine base, Port Harcourt, Rivers State, Nigeria. The GPs of the location is given as N4o46'11.1252" E7o1'38.1324. the study was from March to April, 2018.
Methodology: The spread plate method was used for the enumeration of the total heterotrophic and hydrocarbon utilizing bacteria. One gram (1g) of soil sample was taken from each pot and aseptically transferred into test tubes containing 9ml sterile normal saline which was serially diluted to 10-6 and an appropriate dilution was inoculated on respective sterile media and incubated.
Results: The total heterotrophic bacterial loads revealed that the EP1 (control) ranged from 3.15 to 4.613 Log10Cfu/g. The EP2 ranged from 4.9 to 7.4 Log10Cfu/g while the EP3 ranged from 5.2 to 7.6 Log10Cfu/g. The hydrocarbon utilizing bacterial loads revealed that EP1 (control) ranged from 1.0 to 4.2 Log10Cfu/g. The EP2 ranged from 4.7 to 6.9 Log10Cfu/g while the EP3 ranged from 4.9 to 7.4 Log10Cfu/g. The HUB identified in this study were Citrobacter species, Bacillus species, Serratia species, Clostridium species, Micrococcus species Pseudomonas species, Proteus species and Enterobacter species.
Conclusion: From the investigation of the bioremediation potential of contaminated soil supplemented with soybean wastes and those supplemented cow dungs, it was observed that both stimulants yielded good results and can be used as organic stimulants. Furthermore, this study has shown that the cow dung stimulants yielded better results than soybean waste simulants.

Keywords: illegal refining deposit (kpo fire deposit), bacteria, biostimulation, cow dung, soybean waste.

1. INTRODUCTION (ARIAL, BOLD, 11 FONT, LEFT ALIGNED, CAPS)

A complex mixture of hydrocarbons compounds that can cause serious problems in the environment especially when it spills in to the environment is termed Crude oil [17, 30]. The crude oil industry no doubt has been of great benefit but the spillages of the product in the environment is detrimental thereby resulting to the alteration of the microbial communities in the soil as well as the biogeochemical cycles and have serious negative impacts on the fertility of the soil as well as the quality of the environment. Its effect is seen on the poor yield

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of plants and there are concerns on its effect on the health of human [3]. The Niger Delta environment is a highly sensitive ecological zone known for high biodiversity and the rural people of the oil rich Delta region depend on these resources for their livelihood. The Niger Delta vegetation consists mainly of rainforests, fresh water swamp, brackish swamp forests and mangrove forests [1].

The upsurge in exploration activities of oil companies in all areas of the Niger Delta area of Nigeria, including the success in drilling and transportation of the product to the refineries and its eventual storage have caused different form of discharge of the oil into the environment which is very rich for agriculture as well as sea foods [25]. It is worthy of note that not only regions which produce oil are liable for environmental pollution but also areas through which this product passes either via transportation through pipes or through tanks which convey the products. Over the years the Niger delta environment has suffered degradation due to oil and gas exploration and exploitation activities [16]. The people of the oil rich Niger Delta region of Nigeria have agitated against the degradation of their environment as a result of the environmental impact of oil and gas exploration and exploitation [2]. The agitation came to a climax with open arm confrontation against the Federal Government of Nigeria between 1999 and 2009 [26]. As a way out of the situation the government pacified the militants by granting amnesty to those who dropped their arms, while the non-violent and unemployed youths who were not rehabilitated resorted to boiling crude oil in metal containers (drums) to distil petroleum products; this is the origin of 'illegal refineries' in the Niger Delta. All known principles of environmental protection in refining crude petroleum are ignored as they empty the residue after boiling the crude, into the nearby rivers, creeks and other water bodies as well as on the soil [16]. The economic impact of oil theft associated with these illegal refineries are widely reported [24], however the impact of the operations of these refineries on the highly sensitive environment of the Niger Delta is scarcely reported [12]. Biological treatment methods have been found to be a less sophisticated natural method of clean-up of hydrocarbon polluted sites. The low solubility and adsorption of high molecular weight hydrocarbons limits their availability to microorganisms and thus tend to slow down the method of biological treatment [10, 15]. The driving force for petroleum hydrocarbon biodegradation depends on the ability of the microorganisms to utilize hydrocarbons to satisfy their cell growth and energy needs. One of the basic limitations to this practice of using isolated living cells microorganism is the problem of disposal. If not properly disposed, microorganism can cause outbreak of disease [6]. Recently, the introduction of exogenous microorganisms into the contaminated environment usually as a result of insufficient population of the indigenous microorganisms (bioaugmentation) and the use of organic and inorganic biostimulants (for biostimulation), is gradually becoming the centre of attraction owing to their low cost, ease of operation and availability [14]. Soybeans have not been given much consideration as a biostimulant. Thus, this research is aimed at comparing the effectiveness of soybean wastes and cow dung as an organic amendments for biostimulating hydrocarbon utilizing Bacteria.

2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY (ARIAL, BOLD, 11 FONT, LEFT ALIGNED, CAPS)

2.1 Study Area

The study site was the marine base, Port Harcourt, Rivers State, Nigeria. The GPs of the location is given as N4o46'11.1252" E7o1'38.1324.

2.2 Collection of Soil samples

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Kpo fire contaminated soil samples were collected in plastic bag from four different points in the study area. The samples were collected using soil auger and were transferred to the Laboratory immediately after collection for analysis. In the laboratory, about 1000g of soil sample were weighed and transferred in to sterile pots labeled EP1, EP2, and EP3. The biostimulants were respectively transferred into the pots accordingly. The setup was watered and tilled once a week to provide moisture and oxygen for the organisms [22]

2.3 Experimental design

Table1. Experimental design

EXPERIMENTAL SET	TEST EXPERIMENT
EP 1	1000 grams of contaminated soil sample
EP 2	1000 grams of contaminated soil + 100 grams of soybeans waste
EP 3	1000 grams of contaminated soil + 100 grams of cow dung

2.4 Total Heterotrophic Bacteria Load

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The spread plate method was used for the enumeration of the total heterotrophic bacteria load. One gram (1g) of soil sample was taken from each pot and aseptically transferred into test tubes containing 9ml sterile normal saline. This was serially diluted to 10-6. This was done for all the samples. About 0.1 ml aliquot with the aid of a sterile 1 ml pipette was dropped into the surface of the sterile dried nutrient agar plates in duplicates. This was later spread evenly with the aid of a sterile bent glass rod and was incubated at 370C for 24 hours. Thereafter, counts were taken to calculate the colony forming unit and colonies that developed were further purified using the freshly prepared nutrient agar plates. Pure isolates were stored frozen in glycerol [31].

2.5 Hydrocarbon utilizing Bacteria Counts

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The vapour phase transfer method using mineral salt medium composition of [32] was used as modified by [33]. The plates after inoculation of the 0.1 ml aliquot of 10-2 were also spread evenly using a sterile glass rod and incubation at 37 0C for 5-8 days followed. Thereafter, colonies were counted and used in enumerating the hydrocarbon utilizing bacteria and pure isolates that were purified by continuous spreading on sterile dried nutrient agar plates. This was stored frozen in 5% glycerol in the refrigerator.

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2.6 Identification of Bacterial Isolates

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Characterization and identification of the bacterial isolates were done both macroscopically, biochemically and microscopically [7]. Macroscopic examination of bacterial growth was done by observing their shape, size, texture, and colour. Bacterial isolates were later stained using the Grams reagent and were viewed under the light microscope at x100 with emersion oil. Biochemical tests: fermentation of some sugars, catalase, motility, citrate and oxidase tests were carried out to as described in the Bergy's manual of determinative bacteriology [13] confirm the identity of the isolates.

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2.7 Biodegradation of Crude Oil in the Soil

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About ten grams of the soil was weighed and allowed to stand in an Erlenmeyer flask containing 25ml diethyl Ether. This was later shaken to facilitate extraction of the oil. Complete evaporation was achieved by allowing the mixture to stand open over night at 22 0C. The weight of the beaker which contained the residual oil and the percentage of oil degraded was recorded [19].

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2.8 Determination of pH

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The pH meter (Crison micro pH 2000 Model) was used in determining the pH of the treated soil and control. Ten grams of the sample was allowed to stand in a 250 ml beaker containing 25 ml distilled water. Thereafter, the mixture was stirred continuously to enhance homogeneity.

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2.9 Determination of Nitrate, Phosphate and Total Organic Carbon

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The nitrate concentration was determined using the Kjeldahl method. While the method described by Black [4] was used in determining the phosphate concentration. Total organic carbon was determined using the Bossert and Bartha [5] method.

3. RESULTS AND DISCUSSION

The total heterotrophic bacterial loads as illustrated in Fig1 revealed that the EP1 (control) ranged from 3.15 to 4.613 Log10Cfu/g. The EP2 ranged from 4.9 to 7.4 Log10Cfu/g while the EP3 ranged from 5.2 to 7.6 Log10Cfu/g. This result revealed an increase in population of the total heterotrophic bacteria of the contaminated soil supplemented with soybean waste and cow dung as the bioremediation process was taken place. Also, the day 28 has the highest total heterotrophic bacteria in the treated soil. While the EP1 which had no form of

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biostimulant decreased across the days with day 28 having the least total heterotrophic bacteria unlike the increase observed in the EP2 and EP3.

The hydrocarbon utilizing bacterial loads as revealed in fig2 shows that EP1 (control) ranged from 1.0 to 4.2 Log10Cfu/g. The EP2 ranged from 4.7 to 6.9 Log10Cfu/g while the EP3 ranged from 4.9 to 7.4 Log10Cfu/g. Similar to the total heterotrophic bacteria loads, a gradual or continuous increase was observed in the hydrocarbon utilizing bacteria of the EP2 and EP4 whereas there was a decline in the hydrocarbon utilizing bacteria of the EP1. Furthermore, the soil treated with cow dung had higher bacterial populations (both total heterotrophic and hydrocarbon utilizing bacteria) than those treated with soybean waste. Despite this notable increase, there was no significant difference between the two treatments using the student T-Test and Two-way ANOVA ($P>0.05$).

The increase in the total heterotrophic bacteria as well as the hydrocarbon utilizing bacteria of soil treated with soybean waste and cow dung (i.e. EP2 and EP3) during the bioremediation process could be attributed to the increased nutrient available to support the rapid multiplication and division of the bacteria population. Also, the decline in microbial populations (fig1 and fig2) of the EP1 could be attributed to the dearth of the required nutrient needed to support the growth of the bacteria population. Perhaps, a decline phase in the growth phase has resulted. It has been reported that when the required nutrients are supplied to microorganisms, they grow and multiply continually whereas if the available nutrient is used up, population drops [27]. *Citrobacter* species, *Bacillus* species, *Serratia* species, *Clostridium* species, *Micrococcus* species *Pseudomonas* species, *Proteus* species and *Enterobacter* species are the hydrocarbon utilizing bacteria identified in this study. Previous studies have implicated most of the bacteria genera in this study as hydrocarbon degraders [8, 9, 20, 28]. *Bacillus* species and *Psedomonas* species have been documented by previous studies to be cosmopolitan [11, 21, 29] and are considered to be of importance in the environment as well as in biotechnology due to their wide catabolic abilities, resilience in harsh environmental conditions and ability to produce bio-surfactant [11, 20, 29].

The nitrate concentration, Total organic carbon (TOC) and the phosphate concentration of the samples is presented in fig3, fig 4 and fig5. The results revealed a gradual increase in their concentration as compared to the decreasing concentration of the nutrients as seen in the control (EP1) which was not supplemented with any stimulant. Also, the cow dung biostimulant yielded more of the nutrient as compared to those yielded by the soybean waste. Thus, this could be the reason why higher bacteria loads were recorded in the total heterotrophic and HUB of the sample treated with cow dung (Fig 1 & 2). Also, the total petroleum hydrocarbon which was very high in the beginning of the bioremediation process was observed to decline as the bioremediation process progressed. The TPH of EP2 declined from 386.28 in day zero to 189. 9 mg/kg in day 14 and finally to 86.95 in day 28 respectively. Similarly, the TPH of the EP3 sample declined from 386.28 in day zero to 120.3 in day 14 and finally 60.04mg/kg in day 28 (fig 6). Furthermore, there was a slight decline in the TPH of the control (EP1) indicating that bioremediation process was taken place but in a very slow pace. Also, the result revealed that cow dung biostimulant supported bioremediation better than the soybean waste stimulants. It could also be said that supplementing the normal flora with stimulants helped to facilitate the bioremediation process. The efficiency of cow dung as an organic stimulant is well documented [23].

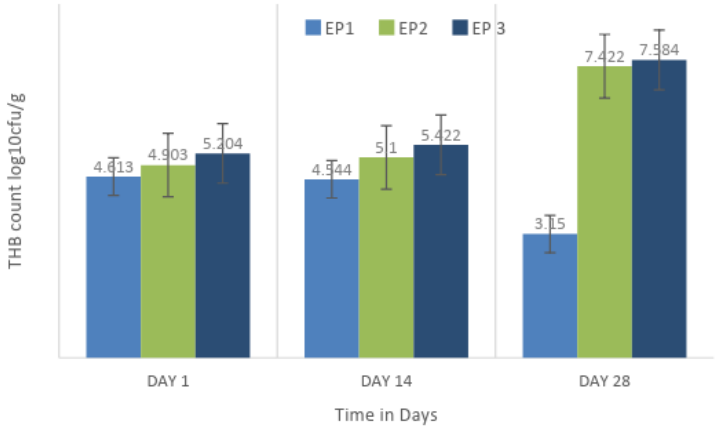

Figure 1: the flow in the total heterotrophic bacteria of the control and soil treated with the biostimulants.

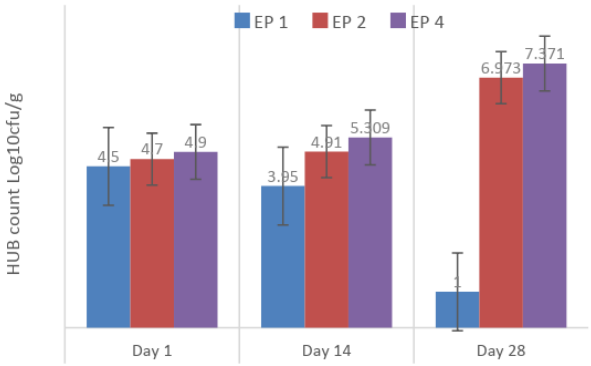

Figure2. The flow in the total hydrocarbon utilizing bacteria of the control and soil treated with the biostimulants.

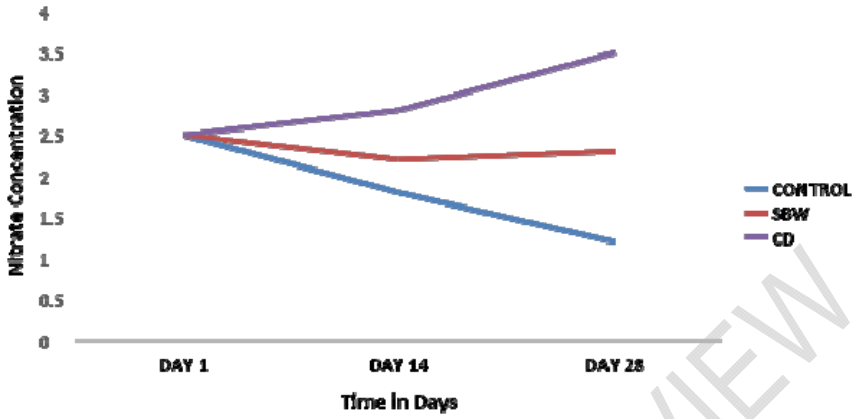

Figure 3: showing the effect of the biostimulants on the nitrate concentration

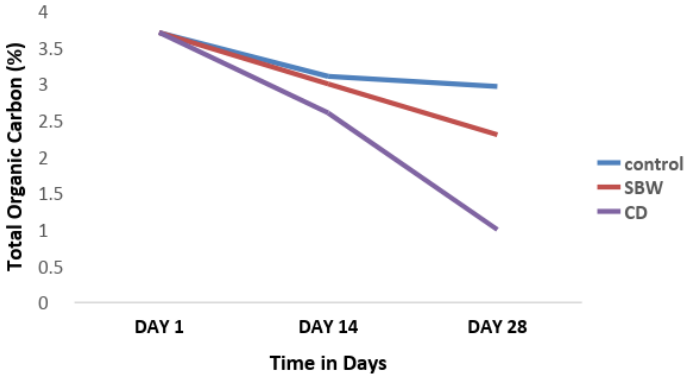

Figure 4: showing the effect of the biostimulants on Total Organic Carbon concentration of the various soil samples in the various sample.

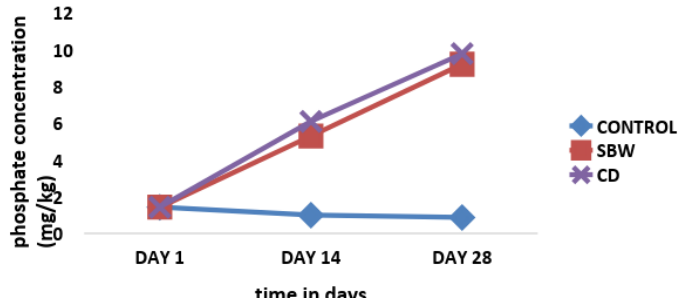

Figure 5: showing the effect of the biostimulants on phosphate concentration of the various soil samples in the various sample.

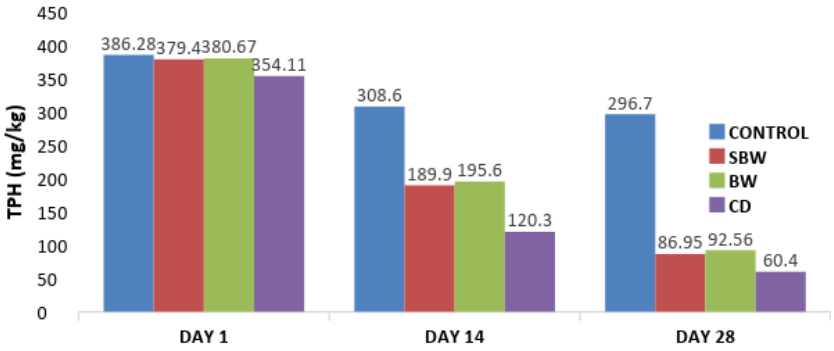

Figure 6: showing the effect of the biostimulants in the Total Petroleum Hydrocarbon concentration of the various soil samples in the various sample.

4. CONCLUSION

From the investigation of the bioremediation potential of contaminated soil supplemented with soybean wastes and those supplemented with cow dungs, it was observed that both stimulants yielded good results and can be used as organic stimulants. Furthermore, this study has shown that the cow dung stimulants yielded better results than soybean waste simulants.

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COMPETING INTERESTS

Authors have declared that no competing interest exists.

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REFERENCES

1. Aminayanaba A, Godwin O. Environmental impact of illegal refineries on the vegetation of the Niger Delta, Nigeria. Journal of Agriculture and Social Research. 2013; 13(2): 121

2. Asimiea A. O. Economic Impact of Developmental Project in the Oil Industry- A Paradigm Shift. Centre for Sustainable Development University of Ibadan. 2010

3. Atlas R.M. Microbial degradation of petroleum hydrocarbons: An environmental perspective. Microbiol. Rev. 1981; 45: 180–209.

4. Black C.A. Method of soil analysis II, American Society of Agronomy, Madison. 1965; 573-590.

5. Bossert I, Bartha R. The fate of petroleum in soil ecosystem. Petroleum Micrbiology, New York. 1984; 435-473.

6. Burland S.M, Edwards E.A. Anaerobic benzene biodegradation linked to nitrate reduction. Applied and Environmental Microbiology. 1999; 65(2): 529-533.

7. Cheesebrough M. District Laboratory Practice in Tropical Countries. Part 2,Cambridge University Press, London, UK. 2000; 143 – 156.

8. Chikere C.B, Okpokwasili G.C, Chikere O.B. Bacterial diversity in a tropical crude oil-polluted soil undergoing bioremediation. African Journal of Biotechnology. 2009b; 8 (11): 2535-2540.

9. Ekhaise F.O, Nkwelle J. Microbiological and Physicochemical Analyses of Oil Contaminated Soil from Major Motor Mechanic Workshops in Benin City Metropolis, Edo State, Nigeria. Journal of Applied Science, Environment and Management. 2011; 15 (4): 597 – 600.

10. Grossi V, Massias D, Stora, G. Bertrand J.C. Exportation and degradation of acyclic petroleum hydrocarbons following simulated oil spill in bioturbated Mediterranean coastal sediments. Chemosphere 2002; 48(9): 947–954.

11. Hamamura N, Olson S.H, Ward D.M, Inskeep W.P. Microbial population dynamics associated with crude oil biodegradation in diverse soils. Applied and Environmental Microbiology. 2006; (72): 6316-6324.

12. Hammadina M. K, Anyanwu D. I. A Cursory Review of the Consequences of Human Activities and Land-use Changes in the Niger delta. Research Journal of Environmental and Earth Sciences. 2012; 4(5): 597-604, 2012 © Maxwell Scientific Organization, 2012.

13. Holt J. G, Krieg N. R, Sneath P. H. A, Staley J. T, Williams S. T. Bergey's Manual of Determinative Bacteriology, Williams and Wilkins, Baltimore, Maryland, USA. 1994; 151 – 157.

14. Isitekhale H.H.E, Aboh S, Edion R. I, Abhanzioya M. I. Remediation of crude oil contaminated soil with inorganic and organic fertilizer using sweet potato as a test crop. Journal of Environment and Earth Science. 2013; 3(7):116– 121.

15. Jorgensen K.S, Puustinen J, Suortti A.M. Bioremediation of petroleum hydrocarbon-contaminated soil by composting in biopiles. Environmental Pollution. 2000; 107(2):245–254.

16. Kadafa A. A. Environmental Impacts of Oil Exploration and Exploitation in the Niger Delta of Nigeria. Global Journal of Science Frontier Research Environment & Earth Sciences. 2012Volume 12 Issue 3 Version 1.0.

17. Kato T, Haruki M, Imanaka T, Morikawa M, Kanaya S. Isolation and characterization of long-chain-alkane degrading *Bacillus thermoleovorans* from deep subterranean petroleum reservoirs. J. Biosci. Bioeng. 2001; 91: 64–70.

18. Keay R. W. J. Trees of Nigeria. Claredon Press Oxford-Nielsen. 1989.

19. Kokub O, Shafeeq M, Khalid Z.M, Malik K.A. Isolation screening and Characterization of biosurfactant producing bacteria. Proceedings of international symposium of Biotechnology or Energy. 1989; 221-232.

20. Lawson I.Y.D, Afenu J.K, Nartey E.K, Quaye J. Diesel oil utilizing bacteria associated with four Ghanaian soils. Agriculture and Biology Journal of North America. 2013; 2151-7525.

21. Margesin R, Labbe D, Schinner F, Greer C.W, Whyte L.G. Characterization of hydrocarbon degrading microbial populations in contaminated and pristine Alpine soils. Applied and Environmental Microbiology. 2003; 69: 3085-3092.

22. Obire O, Anyanwu E. C. Impact of various concentrations of Crude oil on Fungal Populations of Soil. International Journal of Environmental Science Technology. 2009; 6(2):211 – 218.

23. Ofoegbu RU, Momoh YOL, Nwaogazie IL. Bioremediation of Crude Oil Contaminated Soil Using Organic and Inorganic Fertilizers. J Pet Environ Biotechnology. 2015; 5: 198

24. Oredein O. Shell Sees Rise in Crude Theft in Nigeria. Dow Jones News wire. 2013. www.businessfox.com/news/2013/3/4.

25. Osuji LC, Egbuson EJ, Ojinnaka CM. Chem. Ecol. 2005; 21 (1): 1-10.

26. Peel M. A Swamp Full of Dollars: Pipelines and Paramilitaries at Nigeria's Oil Frontier. I.B.Tauris. 2011.

27. Prescott L.M, Harley J.P, Klein D.A. Microbiology, (9th Edition), London: WMC Brown Publishers. 2011.

28. Stanley H. O, Amakiri M.A, Okerentugba P.O. characterization of hydrocarbon utilizing bacteria in soil samples collected from various sites in port harcourt (Niger-Delta, Nigeria). Global journal of bioscience and technology. 2015; 4(1): 6-11.

29. Van Beilen, J.B, Funhoff E.G. Alkane hydroxylases involved in microbial alkane degradation. Applied and Microbiology Biotechnology. 2007; 74: 13-21

30. Zhang Z, Gai, L, Hou Z, Yang C, Ma C, Wang Z, et al. Characterization and biotechnological potential of petroleum-degrading bacteria isolated from oil-contaminated soils. Bioresour. Technol. 2010; 101, 8452.
31. Amadi E.N, Kiin-Kabari D.B, Kpormon, L.B, Robinson V.K.K. Microbial flora and nutritional composition of adult Palm - Wine Beetle (Rhychophorus phoenicus). International journal of Current Microbiology and Applied Science. 2014; 3(11) 189-192.
32. Mills A. L, Breuil C, Colwell R. R. Enumeration of Petroleum-degrading marine and estuarine microorganisms by the most probable number. Canadian Journal of microbiology. 1978; 24(5): 552 – 557
33. Okpokwasili G. C Okorie B. B. Biodegradation potentials of Microorganisms isolated from car engine lubricating oil. Tribol. Int. 1988; 21 (4): 215 – 220.