Microbiology Research Journal International

31(10): 36-44, 2021; Article no.MRJI.84253
ISSN: 2456-7043
(Past name: British Microbiology Research Journal, Past ISSN: 2231-0886, NLM ID: 101608140)

Effect of Pristine Soil Addition and Cow Dung Treatment on the Community Structure Post Bioremediation

Barisiale S. Baranu [a*], Chimezie J. Ogugbue [b] and Gideon C. Okpokwasili [b]

[a] Center for Oilfield and Chemicals Research, University of Port Harcourt, Rivers State, Nigeria.
[b] University of Port Harcourt, Rivers State, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/MRJI/2021/v31i1030349
Editor(s):
(1). Dr. Ana Cláudia Coelho, University of Trás-os-Montes and Alto Douro, Portugal.
Reviewers:
(1). Debora Conde Molina, National Technological University, Argentina.
(2). Veronica Fabian Nyoyoko, Topfaith University, Nigeria.
Complete Peer review History, details of the editor(s), Reviewers and additional Reviewers are available here: https://www.sdiarticle5.com/review-history/84253

Original Research Article

Received 20 October 2021
Accepted 25 December 2021
Published 27 December 2021

ABSTRACT

The pollution of crude oil on agricultural soil has become a menace in the world today. The ecologically friendly approach of restoring this polluted soil back to its original state is pertinent. In this study, 1000 g of crude oil polluted soil was amended with 12.5g of cow dung and 500 g of unpolluted soil, 1000 g of polluted soil with crude oil without cow dung amendment, and 1000 g of unpolluted soil without crude oil and cow dung amendment was the control. The setup was monitored for their physicochemical parameters such as total petroleum hydrocarbon (TPH), polyaromatic hydrocarbon (PAH), temperature, pH, electrical conductivity (Ec), and total nitrogen. The microbiological characterization for viable and culturable was done for total heterotrophic bacteria (THB) and hydrocarbon utilizing bacteria (HUB) while non-culturable but viable characterization was done using metagenomics-shotgun analysis to compare the changes in community structure before, during, and after remediation. The THB observed in the samples were 1.9×10^7, 1.3×10^3, 4.0×10^6, 3.58×10^8, 2.15×10^9 while HUB was 1.6×10^3, 1.0×10^1, 1.67×10^3, 2.5×10^4, and 3.55×10^4 respectively for A (unpolluted soil), B (polluted soil without 1.67×10^3, 2.5×10^4, and 3.55×10^4 respectively for A (unpolluted soil), B (polluted soil without

**Corresponding author: E-mail: barisiale.baranu@gmail.com;*

treatment), J1 (month 1), J2 (month 2), and J3(month 3). Over 90 % removal of the TPH was achieved by the treatment. The molecular characterization showed that Proteobacteria was the dominant phylum identified in the pristine soil, but Actinobacteria were the most dominant in the soil with treatment. Nitrogen-fixing *Bradirhizobium elkanii* was found to be 0.66%, 5.11%, 10.43% before pollution, during natural attenuation, and with unpolluted soil addition with cow dung amendment. Denitrifying *Nocardiodes daejeonensis* and *Nocardiodes terra* were identified at 0.1 and 2.0% respectively only three (3) months after the end of the bioremediation treatment.

Keywords: Bioremediation; pristine soil; biostimualtion; alpha diversity; cow dung; crude-oil pollution.

1. INTRODUCTION

In recent decades, contaminated land management has become a major concern in Nigeria and the world at large. Bioremediation is an internationally approved technology for cost-effective clean-up of oil-contaminated sites. These technologies involve enhancing the natural biological transformation of chemical petroleum-derived contaminants into less toxic and/or less mobile forms. These technologies are gaining increased attention due to their low cost and effectiveness [1]. Bio-stimulation involves the modification of the environment to stimulate existing bacteria capable of bioremediation. This can be done by the addition of various forms of rate-limiting nutrients and electron acceptors, such as phosphorus, nitrogen, oxygen, or carbon [1].

Microorganisms play a major role in bioremediation and their absolute number can determine the overall degradative ability [2]. Bio-stimulation emphasis is placed on identifying and adjusting certain physical and chemical factors (such as soil temperature, pH, moisture content, nutrient content, etc.) that may reduce biodegradation of the contaminants by the indigenous microorganism in the affected site [2] The primary advantage of bio-stimulation is that bioremediation will be undertaken by already present native microorganisms that are well-suited to the subsurface environment and are well distributed spatially within the subsurface while the primary disadvantage is that the delivery of additives in a manner that allows the additives to be readily available to subsurface microorganisms is based on the local geology of the subsurface [1].

The soil is a dynamic natural body having properties derived from the combined effects of climate and abiotic activities [3]. The deliberate addition of microorganisms increases biodiversity at the site of the spill and helps in the study of the relationship between diversity and ecosystem service functions. It is imperative to understand the dynamics of the population of bacterial communities, what bacteria are present, in what numbers, and how these numbers change as a function of time in the soil [4]. Crude oil pollution affects not just the fertility of the soil, but it affects food production and leads to diseases and death of human beings and animals alike [5].

According to Dhaliwal et al., [6], different techniques have been proffered for remediating soils such as physical, chemical, and biological methods. These methods mentioned above are very expensive, not safe, labor-intensive, and require keen monitoring [7]. However, bioremediation is an easier, cheaper, and more eco-friendly approach and has shown to be efficient in degrading, immobilizing, or mineralizing hydrocarbon pollutants [8,9].

A more recent technique for monitoring pollutant degradation is metagenomics, which helps to analyze and decode the community DNAs [10,11] and the possible functional outcomes in that community [12]. An understanding of the community dynamics during bioremediation using metagenomics will give a clearer indication of what is responsible for the pollutant reduction during bioremediation and the restoration of biodiversity [13].

Attention is mostly given to TPH reduction without much reference to functions. This informed the need to achieve both hydrocarbon reduction and ecosystem function restoration via metagenomics in this study. Metagenomics is used to analyze microbial functions, structure, and detect functional genes [14,15].

This study investigated the extant hydrocarbonoclastic microbes in petroleum-contaminated soil during a bioremediation protocol and the biodiversity of the soil three (3) months post bioremediation using

metagenomics to determine the effect of total petroleum hydrocarbon (TPH) dissipation on the community structure and restoration of ecosystem function in the soil.

2. MATERIALS AND METHODS

2.1 Sample Collection

The soil samples were collected from an agricultural farm using an auger from three different points at depths of 0 – 30 cm, mixed to form a composite sample, and kept in a sterile bag. The soil was artificially polluted with crude oil. 1000 g of crude oil polluted soil was weighed and placed in earthen pots containing 500g of unpolluted soil to which 12.5g of cow dung (CD) was added. In another pot was 1000 g of polluted soil without amendment or unpolluted soil and in another 1000 g of unpolluted soil. The content was mixed thoroughly for homogeneity and monitored for physicochemical parameters and microbiological characteristics. Physicochemical characteristics

Determination of total petroleum hydrocarbon (TPH) content and polyaromatic hydrocarbon (PAH) content of soils Ten (10) grams of a composite soil sample from each treatment pot was weighed into a solvent rinsed beaker. Thereafter, 20–50 ml of dichloromethane (DCM) was added to the samples. Samples were spiked with ortho-terphenyl and shaken in a vortex mixer for 1–5 minutes. The mixture was placed in an orbital shaker for 10–30 minutes and the extract were filtered through a glass funnel with glass wool and anhydrous sodium sulfate. The extract was transferred to a Teflon-lined screw-cap vial ready for TPH and PAH analysis. TPH and PAH contents determination were done according to the method stipulated by USEPA 8015C and USEPA 8270D respectively. Other physicochemical parameters monitored were pH (EPA 9045D), electrical conductivity (ASTM D1125), total organic nitrogen (ASTME258), and temperature [16].

2.2 Culture Dependent Microbial Identification

The culture-dependent hydrocarbon utilizing bacteria (HUB) was carried out by enriching the samples in a 250ml Erlenmeyer flask containing 1g of the soil sample. One hundred (100ml) of sterilized Bussnell Hass Broth (BHB) having 1% weight by volume of crude oil and naphthalene as the sole carbon source. The Erlenmeyer flask containing the mixture was kept at room temperature for seven days on a rotary shaker. After seven days, 0.1 ml of the sample was inoculated on BHB mineral salt agar using the vapor transfer method and incubated for 7 days. Bacterial enumeration, isolation, and identification were done. The total heterotrophic bacteria (THB) were carried out using the spread plate method. One gram (1 g) of soil sample was weighed into 9.0 ml of sterile normal saline to give a 10-fold dilution 10^1 from which 0.1 ml was taken and put in a sterile test tube containing 9.0 ml of normal saline to give 10^2 diluents until 10^4 and 10^5 was achieved. 0.1ml of the dilution was then aseptically spread into sterile Petri dishes containing nutrient agar, the petri dish was inverted, incubated at 28^oC for 24 hours, and observed for the total viable count [17].

2.3 Non-culture-dependent Identification

The viable but nonculturable bacteria were determined using metagenomic sequencing by Zymo Research Corp, Irvine, California, U.S.A. The DNA extraction was done using the ZymoBIOMICS-96 MagBead DNA kit. The library preparation was done using Nextera DNA Flex Library Prep Kit. The final library was sequenced on NovaSeq. The community dynamic profiling was done using shotgun analysis. Raw sequence beads were trimmed to remove low-quality fractions and adapters with Trimmomatic-0.33 (Bolger et al, 2015). Microbial composition was profiled using Centrifuge (Kim et al., 2016). Alpha and beta diversity

3. RESULTS AND DISCUSSION

3.1 Enumeration of Bacterial Isolates in Samples

The THB observed in the samples were 1.9 x 10^7, 1.3 x 10^3, 4.0 x 10^6, 3.58 x 10^8, 2.15 x 10^9 while HUB was 1.6 x 10^3, 1.0 x 10^1, 1.67 x 10^3, 2.5 x 10^4, and 3.55 x 10^4 respectively for A, B, J1, J2, and J3 (Table 1). The treatments increased the microbial structure of the soil, this may be due to the rich nutrient in the cow dung amendment.

Table 1. THB and HUB of samples

Samples	THB (cfu/g)	HUB (cfu/g)
A	1.9×10^7	1.6×10^3
B	1.3×10^3	1.0×10^1
J1	4.0×10^6	1.67×10^3
J2	3.58×10^8	2.5×10^4
J3	2.15×10^9	3.55×10^4

Where 1 (month 1), 2 (month 2), and 3 (month 3).

Physicochemical characterization of treatments.

The pH range of the setup was 6.0-6.3 with the highest range being in J3. The Ec ranged between

110-210 with J2 having the highest value, this may be due to the loss in the nutrient in soil due to pollution. The temperature was fairly constant amongst replicates during the course of the bioremediation. The study investigated the effectiveness of unpolluted soil addition in the restoration of community structure and ecosystem functions during bioremediation of petroleum-contaminated soil. Treatments employed were able to efficiently degrade hydrocarbon and reduce the total petroleum hydrocarbons as seen in Fig.1.

This agrees with Baranu et al, 2021. Physicochemical characteristics of the treatments indicate the presence of high organic matter in the treated soil and, soil acidity. Organic matter helps the soil's ability to degrade pollutants while the acidity of soil indicates the microbial flora that will be found there. The Ec indicates the salinity of the soil and high amounts affect the fertility of the soil. The TPH of the replicates was fairly constant during the course of bioremediation ranging between 47708 to 47771 initial concentration and 3425 to 4516 final concentrations (Fig 2). The % Bioremediation as proposed by Bento et al, 2005 was between 90-93. The polyaromatic hydrocarbon percentage reduction was between 87 % to 88.9 % (Fig 3).

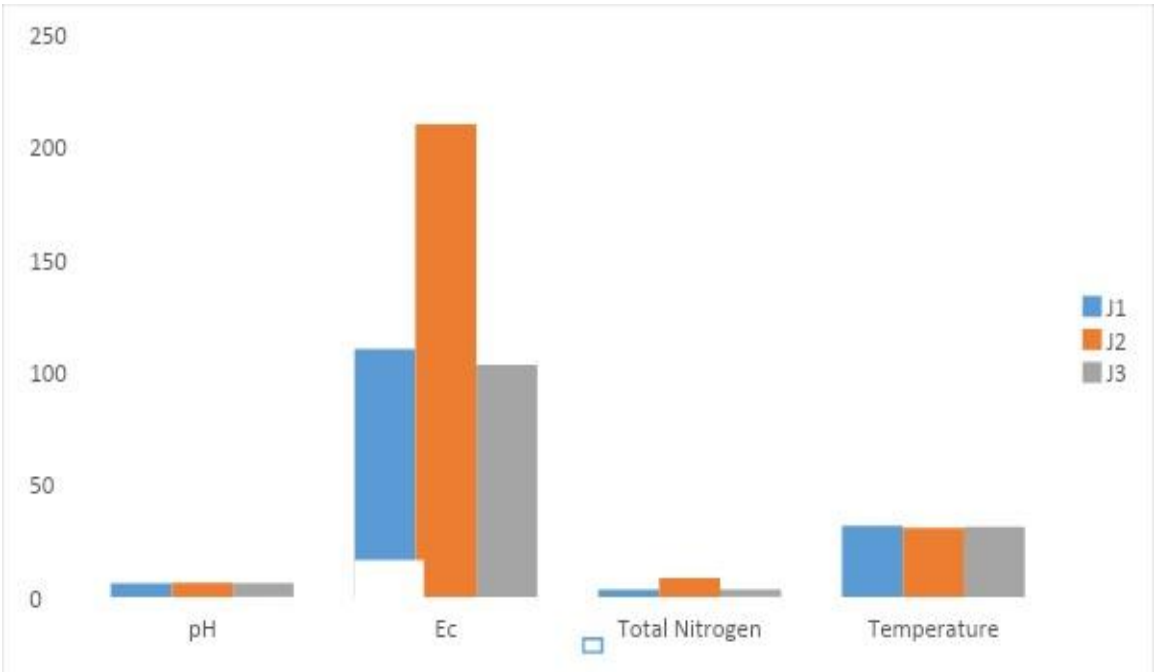

Fig. 1. Physicochemical characteristics of treatments. Ec (µS/cm), Total Nitrogen (%), Temperature (^{O}C)

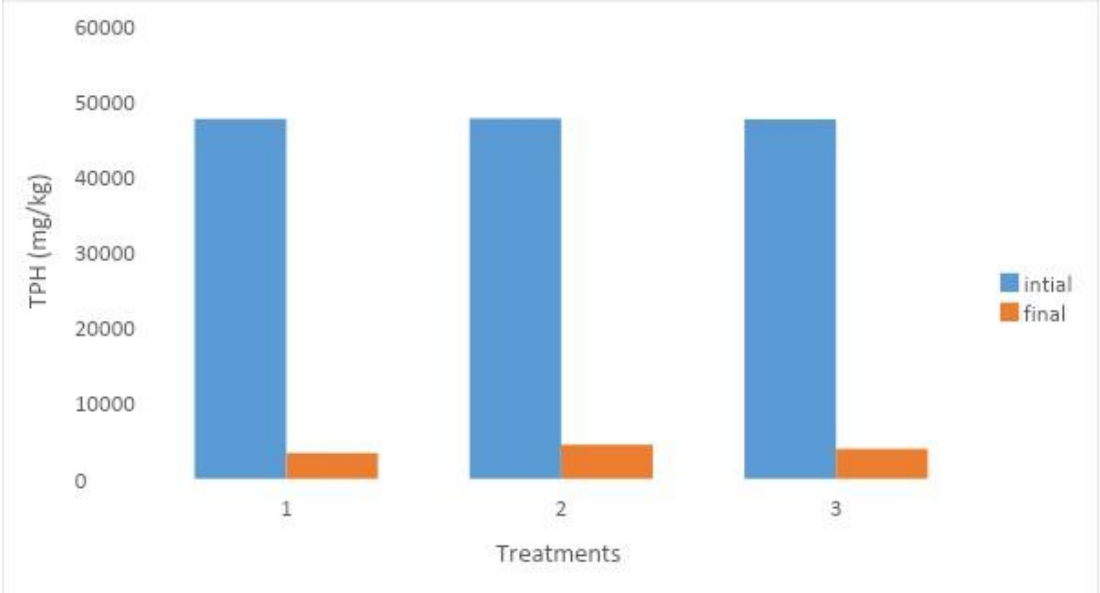

Fig. 2. Total petroleum hydrocarbon reduction in samples. Where 1 (month 1), 2 (month 2), and 3 (month 3)

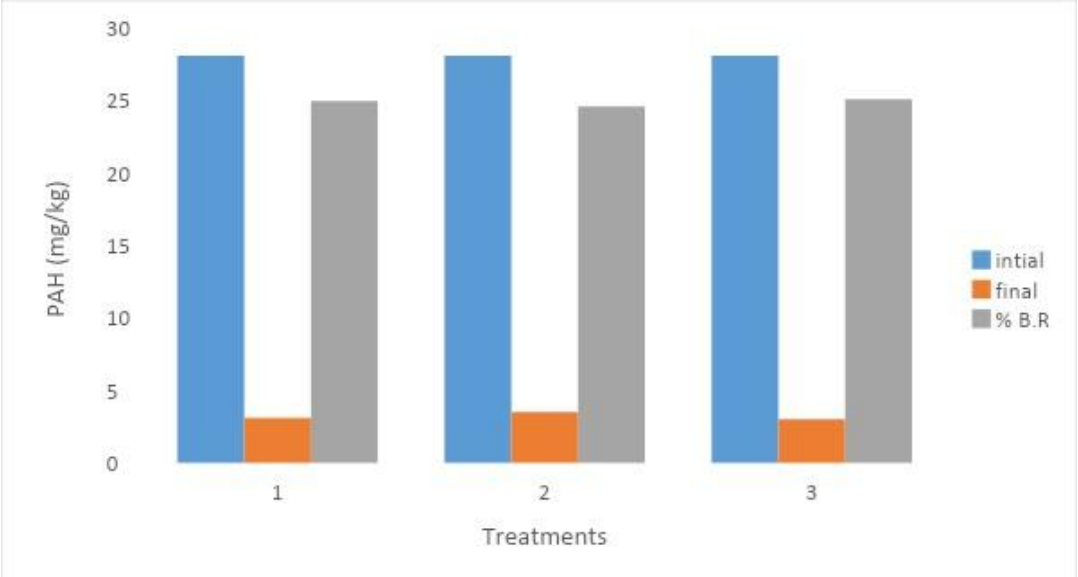

Fig. 3. Polyaromatic hydrocarbon reduction and % bioremediation during the study. Where 1 (month 1), 2 (month 2), and 3 (month 3)

3.2 Molecular Characterization of Samples

The dominant phylum observed across the various treatments was Proteobacteria with 69.7% in 5B (Fig 4), 89.1% in J3 at the end of the three-month bioremediation protocol, and 86.5% six months after bioremediation. However, the dominant phylum observed in the pristine soil was Actinobacteria at 76.3%, followed by Proteobacteria at 16.5% and the least was Firmicutes at 0.9%.

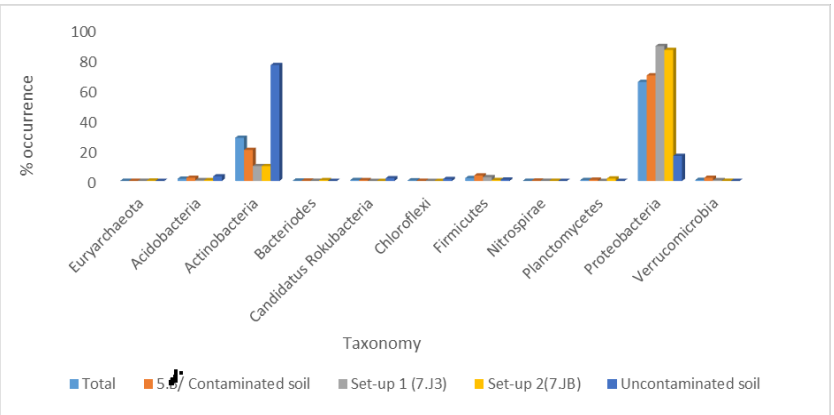

Fig. 4. Dominant phylum identified and their percentage occurrence. B (contaminated soil), Set-up 1 ((J3 treatment after three months), Set-up 2 (JB after 6months of bioremediation)

The observed genus in this group [pristine soil (A), polluted soil without amendment (B), polluted soil with 12.5 g of cow dung, and 500 g of pristine soil] at the end of 3-month bioremediation (J3) and 6 months into bioremediation (JB) are shown in Fig 5. The yellow arrow with [a] indicates the genus present while the black arrow with [ab] indicates the absent genus in the samples. The organisms observed in this group are *Bradyrhizobium, Paenibacillus, Microbispora, Sorangium, Minicystis, Nonomuraea, Bacillus, Rhodovarius, Azospira, Pseudolabrys, Azorhizobium, Actinoallomarus, Verrucomicrobia, Geothrix, Micromonospora, Novosphingobium, Dyella, Aquisphaera, Pandoraea, Anaeromyxobacter, Immunodisolibacter, Proteobacteria (Unknown), Burkholderia, Afipia, Aquabacterium, Pseudomonas, Cupriavidus, Extensimonas, Cellulomonas, Paraburkholderia, Caulobacter, Sphingomonas, Xanthobacter, Streptomyces, Actinobacteria, Oryzihumus, Solirubrobacter, Phycicoccus, Nocardia, Acidobacteria, Actinomadura, Candidatus Rokubacteria, Terrabacter, Nocardiodes, Streptacidiphilus, Marmoricola, Geodermatophilus, Mycobacterium, Pseudonocardia, and Sinomonas. Out of all these organisms, those present in the pristine soil are Streptomyces, Actinobacteria, Oryzihumus, Solirubrobacter, Phcicoccus, Nocardia, Acidobacteria, Actinomadura, Candidatus Rokubacteria, Terrabacter, Nocardiodes, Streptacidiphilus, Marmoricola, Geodermatophilus, Mycobacterium, Pseudonocardia and Sinomonas, Bradyrhizobium, Micromonospora* and an unknown genus of Proteobacteria. All these organisms that were present in the pristine soil (A) are absent in the polluted soil without amendment (B) except Streptomyces, which is present, however, none of these are present in the polluted soils with amendment (J3 & JB). The genera Dyella, Aquisphaera, Pandoraea, Anaeromyxobacter are absent in A, B & J3 but Pandoraea, Anaeromyxobacter is moderately present in 1. JB. Anaeromyxobacter is a deltaproteobacterium usually found in large numbers in rice paddy soils, a large number in river/sediments but found in small amounts in upland soils (Masuda et al, 2017), this explains why it is in such a small amount here (less than 0.05) a diazotrophic bacteria capable of increasing nitrogen-fixing capabilities in paddy soil environments (Masuda et al, 2020), and its resurgence 3 months post bioremediation suggests that the soil will soon become fertile for plant growth. Although this agrees with Masuda et al, 2020, *Anaeromyxobacter dehalogens* have been described as an organism with the ability to reduce Fe (III) to Fe (II) and reduce NO^- to NH^+ with NO^- as the intermediate through the pathway of respiratory ammonification. Although Anaeromyxobacter has not been classified as a denitrifier due to its ability to reduce NO^- to NH^+ and its absence of *nirK* and *nirS*, it has been shown to influence chemodenitrification through Fe(III) and NO^- reduction and ultimately denitrifies to N_2 via coupled biotic-abiotic reactions [18]. Two species of Pandoraea were identified in this study, pnomenusa and an unknown species. According to Liz et al, [19], *Pandoraea pnomenusa* has been implicated in a consortium of polychlorinated biphenyl (PCB) from transformer oil-polluted soils.

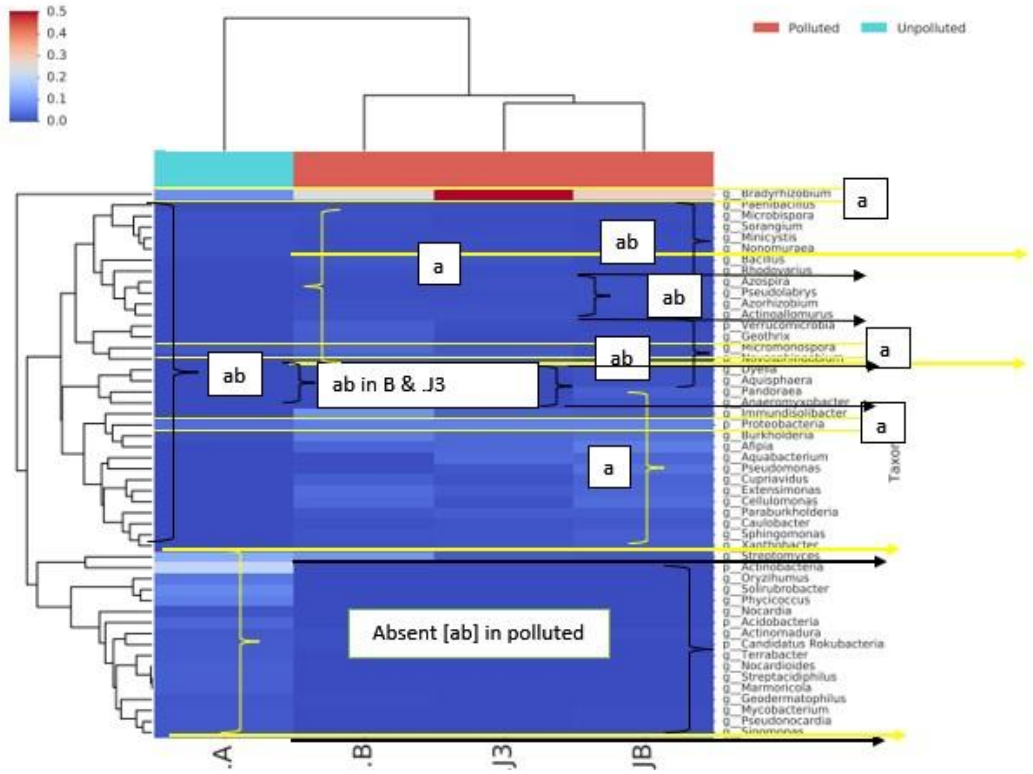

Fig 5. Gene pool between the unpolluted, polluted without amendments, and polluted with amendments

.

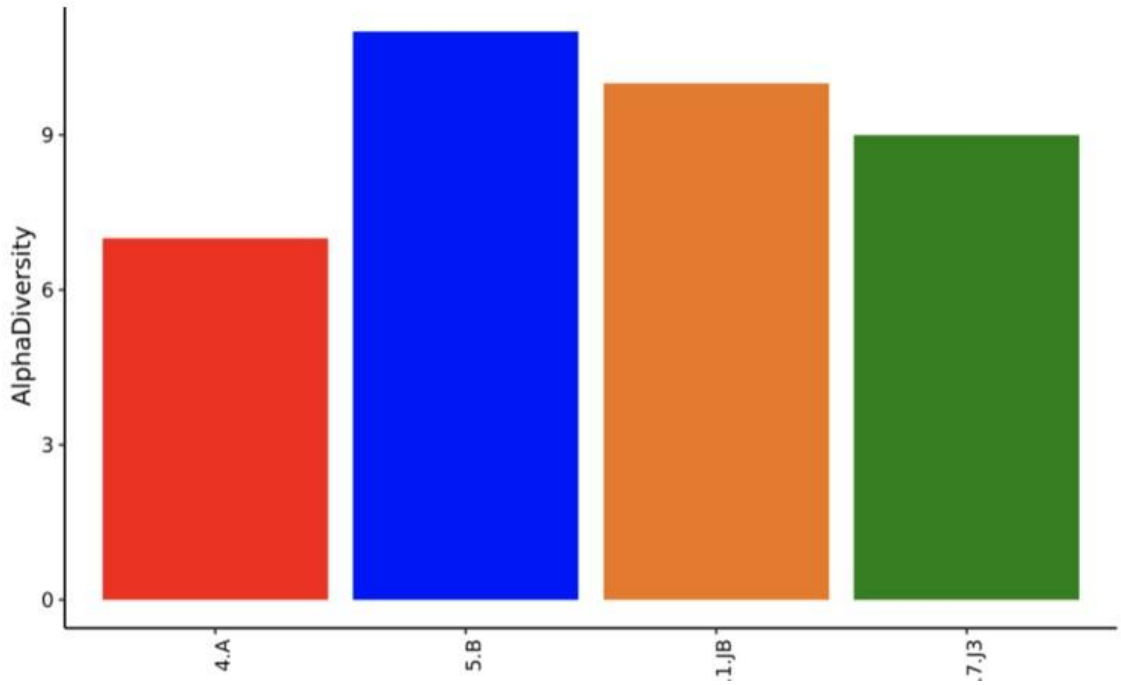

Fig 6. Alpha diversity showing the measurement of microbial diversity within samples.

The clustering in Fig 5 is within polluted samples indicating that the microbial communities of polluted soils are similar while those observed in the pristine soil are not genetically similar. This difference is important in using pristine soil to stimulate the degradation of hydrocarbons during bioremediation because it introduces gene families that would not have been there ordinarily due to the adverse effects of crude oil on the soil community [20]. Interestingly of all the genera observed in these four samples, Bradyrhizobium and *Proteobacteria spp* (unknown) is present in all four (although they are not present in the same numbers). Bradyrhizobium is highest in J3 and least in JB. A trend where the genus Paenibacillus, Microbispora, Sorangium, Minicystis, Nonomuraea, Bacillus, Rhodovarius, Azospira, Pseudolabrys, Azorhizobium, Actinoallomarus, Verrucomicrobia, Geothrix, Micromonospora, Novosphingobium, Dyella, and Aquisphaera were absent in JB just like A was observed. This indicates the soil genus in JB was returning closer to those observed in pristine soil (A) than the other polluted soils (B, and J3). This suggests that the addition of pristine soil is effective in restoring the microbial community after bioremediation.

The alpha diversity shows the measurement of the microbial diversity in samples. The unpolluted soil had the lowest diversity while the polluted soil without amendment had the highest microbial diversity. Sample J3 (500g of pristine soil and 12.5g of cow dung at the end of the bioremediation protocol) showed an increase in microbial diversity three months post bioremediation (J.B) (Fig 6). This further suggests that the pristine soil addition during bioremediation is more effective in restoring microbial diversity and possible ecosystem functions than natural attenuation.

4. CONCLUSION

The study showed that the addition of unpolluted soil with cow dung amendment was effective in remediating crude oil polluted agricultural soils using TPH as an index for successful bioremediation, however, the indigenous microorganisms in the unpolluted soil were able to enhance the biodegradation of the pollutants and improve the soil's biodiversity. Interestingly, the treatment with 500 g of unpolluted soil with 12.5g of cow dung proved to be very effective in encouraging the growth of hydrocarbon utilizing bacteria and resurgence of nitrogen fixers like *B. elkanii* and overall biodiversity barely six months into bioremediation. The use of as low as 12.5g of organic nutrient (cow dung) enriched the bacterial structure of the soil which in turn will improve the ecosystem function of the soil barely three months after remediation. Reference

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCE

1. Kanissery RG, Sims GK. Biostimulation for the enhanced degradation of herbicides in soil. Applied and Environmental Soil Science; 2011.
2. Adeyinka JS, Urum KCN. Assessment and determination of bioremediation efficiency in a post crude oil pollution treatment. Tropical Journal of Environmental Research. 2001;3:181-189.
3. Nõges P, Argillier C, Borja Á, Garmendia JM, Hanganu J, Kodeš V, Birk S. Quantified biotic and abiotic responses to multiple stress in freshwater, marine, and groundwaters. Science of the Total Environment. 2016;540:43-52.
4. Hirano SS, Upper CD. Bacterial community dynamics. In Microbial Ecology of Leaves (pp.). Springer, New York, NY;1991:271-294.
5. Muzaffar T, Laisram N, Kothari SY. Correlation of gross motor function with topographical diagnosis in children with cerebral palsy. Int J Pharm Med Res. 2012;23:10-4.
6. Dhaliwal SS, Singh J, Taneja PK, Mandal A. Remediation techniques for removal of heavy metals from the soil contaminated through different sources: a review. Environmental Science and Pollution Research. 2020;27(2):1319-1333.
7. Salleh AB, Ghazali FM, Rahman RNZA, Basri M. Bioremediation of Petroleum Hydrocarbon Pollution; 2003.
8. Abatenh E, Gizaw B, Tsegaye Z, Wassie M.. The role of microorganisms in bioremediation-A review. *Open Journal of Environmental Biology*. 2017'*2*(1):038-046.
9. Azubuike CC, Chikere CB, Okpokwasili GC. Bioremediation techniques–classification based on site of application: principles, advantages, limitations, and prospects. World Journal of Microbiology and Biotechnology, 2016;32(11):1-18.

10. Bharagava RN, Purchase D, Saxena G, Mulla SI. Applications of metagenomics in microbial bioremediation of pollutants: from genomics to environmental cleanup. In Microbial Diversity in the Genomic Era. Academic Press. 2019:459-477
11. Aydin S. Enhanced biodegradation of antibiotic combinations via the sequential treatment of the sludge resulting from pharmaceutical wastewater treatment using white-rot fungi Trametes versicolor and Bjerkandera adusta. Applied Microbiology and Biotechnology,. 2016;100(14): 6491-6499.
12. Pinu FR, Beale DJ, Paten AM, Kouremenos K, Swarup S, Schirra HJ, Wishart D. Systems biology and multi-omics integration: viewpoints from the metabolomics research community. Metabolites. 2019;9(4): 76. 20.
13. Mani D, Kumar C. Biotechnological advances in bioremediation of heavy metals contaminated ecosystems: an overview with special reference to phytoremediation. International Journal of Environmental Science and Technology. 2014;11(3), 843-872.
14. Mustapha MU, Halimoon N. Microorganisms and biosorption of heavy metals in the environment: a review paper. J. Microb. Biochem. Technol. 2015;7(5): 253-256.
15. Alkorta I, Epelde L, Garbisu C.. Environmental parameters altered by climate change affect the activity of soil microorganisms involved in bioremediation. FEMS Microbiology Letters. 2017;364(19).
16. APHA. Standard Methods for the Examination of Water and Wastewater 20th ed. Advances in Microbiology. 2005; 6(11).
17. Chikere CB, Tekere M, Adeleke R. Enhanced microbial hydrocarbon biodegradation as stimulated during field-scale landfarming of crude oil-impacted soil. Sustainable Chemistry and Pharmacy. 2019;14:100177. 10.
18. Onley JR, Ahsan S, Sanford RA, Löffler FE. Denitrification by Anaeromyxobacter dehalogenans, a common soil bacterium lacking the nitrite reductase genes nir S and nirK. Applied and Environ-mental Microbiology. 2018;*84*(4): e01985-17.
19. Liz JA, Jan-Roblero ZE, J de la Serna, JZD, de León AVP, Hernández-Rodríguez C. Degradation of polychlorinated biphenyl (PCB) by a consortium obtained from contaminated soil composed of Brevibacterium, Pandoraea, and Ochrobactrum. World Journal of Microbiology and Biotechnology. 2009;25(1):165-170.
20. Atlas RB. Bioremediation of marine oil spills: when and when not - the Exxon Valdez experience. Journal of Microbiology and Biotechnology., 2009; 2(2):213-21.

Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/84253