ENHANCED REMEDIATION OF HYDROCARBON CONTAMINATED SOIL USING BIOSTIMULATION-BIOAUGMENTATION PROTOCOL.

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Opeyemi K. Fatunla*[1,2,3], Anthony A. Adegoke[1,2,3], Shaibu Solomon[3,4] Utibe Ofon[2], Olapeju, O. Omolehin[5], Rasheed O. Yusuf[6], Edinam-Abasi Udofia[1], Edu J. Inam[2,3] & Joseph P. Essien[1,2,3]

[1]Molecular &Biotechnology Laboratory, Department of Microbiology, University of Uyo

[2]Department of Microbiology, University of Uyo, P.M.B. 1017, Uyo, Akwa Ibom State.

[3]International Centre for Energy and Environmental Sustainability Research, University of Uyo, Akwa Ibom State

[4]Department of Chemistry, University of Uyo, P.M.B. 1017, Uyo, Akwa Ibom State.

[5]Department of Microbiology, Federal University Lokoja, Sule Oyidi Street, Lokoja, Kogi, 260101, Nigeria.

[6]Faculty of Public Health, University of Ibadan, Oyo State Nigeria

*Corresponding author's email: opeyemifatunla@uniuyo.edu.ng

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Abstract: An enhanced remediation of a contaminated garden soil in Niger Delta region of Nigeria was carried out using bioaugmentation-biostimulation (BB) protocols. The In-Vessel System of composting was employed for the production of biostimulants (biosolid and brewer's spent grain) from the feedstocks and a strong hydrocarbon-degrading strain of *Bacillus altitudinis* (GenBank accession number KY569499.1 was used for the bacterization of the treated biostimulants. The total residual petroleum hydrocarbons (TRPH) were extracted from the soil samples and quantified using Gas chromatograph HP 5890 while gas chromatography (GC, Hewlett-Packard HP 6890 series) coupled to a mass spectrometer MS, model 5971, Hewlett-Packard) was used to quantify extractable organic (Polycylic Aromatic Hydrocarbons) PAHs. Biosolid was significantly ($p < 0.05$) more effective at driving TPH reduction (85.39-94.76%) depending on contamination level) in the soil than BSG (71.88-89.16%). The reduction of Σ17 PAHs in the contaminated beds was 55±3% higher in the remedied beds compared to the bed that was left for natural attenuation. It was obvious from this study that enhanced degradation occurred in beds contaminated and remedied using the Biostimulation-Bioaugmentation (BB) protocol. This was achieved by increasing their respective soil nutrient and the addition of a potent allochthonous hydrocarbon degrader (*Bacillus altitudinis*). It may, therefore, be appropriate to recommend their use as a suitable biostimulant during the remediation of agricultural land previously contaminated with hydrocarbon.

Keywords: Hydrocarbon, contamination, remediation biostimulation, Bioaugmentation

Introduction

Land degradation and declining soil fertility are critical problems affecting agricultural productivity and human welfare in Sub-Saharan Africa. The main soil-environmental concerns in the region are nutrient depletion, loss of soil organic matter (SOM) and loss of soil functions. This problem of land degradation is especially rife in the Niger Delta region of Nigeria where crude oil exploration has impacted arable lands through oil spillage with its attendant devastating impacts on not only the environment but also on the residents. In Nigeria today crude oil pollution has been a major threat to the environment and well-being inhabitants of oil exploration zones. Oil exploration and pipeline sabotage has led to increased amounts of crude oil spills in the Niger Delta of Nigeria. Some past spills have led to a complete relocation of some communities, loss of ancestral homes, pollution of freshwater bodies, loss of forest and agricultural land, destruction of fishing grounds and reduction of fish population (Essien and Umana, 2016). Oil spill impacts soil fertility, affects germination, growth and yields of plants and therefore food productivity. It has carcinogenic, mutagenic, teratogenic and cytotoxic effects and may potentially lead to cardiac dysfunction and oedema if hydrocarbons contaminated foods and water are continuously consumed (Inam et al. 2014) as it is in Niger Delta of Nigeria. Despite the advantages of bioremediation, its efficiency is limited majorly by the limited bioavailability of hydrocarbons to microorganisms. This is attributed to the low solubility and strong and/or irreversible sorption to soil matrix (Essien et al. 2012 & 2016, Rockne et al. 2002). To solve this problem, several methods have been developed to enhance the bio-availability of hydrocarbons and the Remediation by Enhanced Natural Attenuation (RENA) is one, with preference to biological sources. Most studies on hydrocarbon remediation have primarily focused on the use of biostimulation and bioaugmentation protocols individually. Biostimulation involves the addition of nutrients to stimulate the activity of indigenous microorganisms, while bioaugmentation introduces specific microorganisms to enhance the degradation process. However, a more integrated approach, the Biostimulation-Bioaugmentation (BB) protocol, has been developed and successfully applied in the remediation of hydrocarbon-contaminated soils in Nigeria (Essien and Umana, 2016). This innovative protocol combines the strengths of both methods, leveraging nutrient-rich and highly bacterized organic amendments to accelerate the breakdown of hydrocarbons (Fatunla et al., 2024).

Despite its success in hydrocarbon remediation, the broader implications of the BB protocol on soil health remain underexplored. Specifically, the effects of this combined approach on microbial community dynamics, soil enzyme activities, and key fertility indices in hydrocarbon-impacted soils have not yet been thoroughly investigated. These factors are crucial for understanding the long-term sustainability and ecological impacts of the remediation process. Therefore, the present study aims to address this gap by evaluating the effects of the BB protocol on these critical aspects of soil health, providing a more comprehensive understanding of its overall efficacy and potential environmental consequences.

Materials and Methods

The soil used for the experiment was the sandy-loamy soil of the Botany Department, University of Uyo Botanical Garden. Under strict safety precautions, municipal sludge samples (SS) were obtained from Lower Usuma Dam Water Treatment Plant (LUDWTPS) in Abuja, Nigeria while samples of brewers spent grain (BSG) were obtained from Champions Breweries located along Aka Rd, Uyo, Akwa Ibom State, Nigeria. The bulking agent (wood chips or sawdust) used for composting process were obtained from Uruan Timber Market at Idu -Uruan, Akwa Ibom State. The hydrocarbon source used for this study is crude oil (Bonny light) with specific gravity 0.818g/cm^3 sourced from an oil servicing company located in Eket, Akwa Ibom State, Nigeria. The In-Vessel System of composting was employed for the production of biostimulants from the feedstocks as described by Fatunla 2017 and Andreoli et al. 2007. Strong hydrocarbon-degrading strain of *Bacillus altitudinis* (GenBank accession number KY569499.1) was used for the bacterization of the treated biostimulants. The inoculum of the bacterizing agent was prepared by growing the isolate in a Yeast Extract-Mannitol Broth. The broth was then blended with the biostimulants (BSG and biosolid) to obtained bacterized biofertilizers. The number of cells of *B. siamensis* in 1ml of bacterization substrate was approximately 10^{8}CFU. Treatments were completely randomized. Six 2m x 2m plots prepared as crop beds were contaminated with 0.5%, 1.0% and 1.5% levels ($^V/_W$) of hydrocarbon source and allowed to condition for two weeks. Four uncontaminated beds served as control (**Fig. 1**). Bacterized biosolid and BSG were applied as basal dressings on the beds and monitored for 56 days.

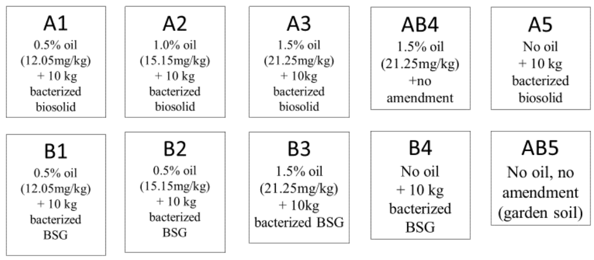

Fig. 1 Experimental Set-up

Soil samples were collected from the top (0-10 cm) of the beds, air-dried and sieved to <5 mm. Samples for total petroleum and PAH content analysis were collected with the aid of a sterile hand trowel, stored in an amber bottle and transported to the laboratory for immediate analysis. The hydrocarbon fractions remaining after degradation in the treated soil were determined using the methods of ASTDM 3921 and USEPA 8270B (TPI 2007). The total residual petroleum hydrocarbons (TRPH) were extracted from the soil samples and quantified using Gas chromatograph HP 5890. A gas chromatograph (GC, Hewlett-Packard HP 6890 series) coupled to a mass spectrometer MS, model 5971, Hewlett-Packard) was used to quantify extractable organic PAHs. Data obtained were subjected to descriptive statistics and multivariate analysis of variance (MANOVA). The LSD and Levene's homogeneity of variance were also investigated using IBM SPSS windows version 20 package (IBM Corp, USA), and the significance of the treatment means was tested at $p \leq 0.05$.

Results

The microbiological and chemical properties of the amendment used (Biosolid and BSG) are presented in **Table 1**. Of the two amendments, biosolid had the highest value for nitrogen (10.05%) and sulphate (4.33 mg/kg), while BSG had the highest phosphorus (2.87%) and ammonium (11.2 mg/kg).

Table 1 Microbiological and chemical properties of Biostimulant used.

Properties	Biostimulant Type	
	Biosolid	**BSG**
HET (logCFU/g)	9.20	8.30
HDB(logCFU/g)	2.10	-
Fungi(logCFU/g)	3.6	2.80
pH	7.34	6.67
Organic Matter (%)	22.16	10.4
Nitrogen (%)	10.05	1.91
Phosphorus (%)	0.76	0.81
Potassium (mg/kg)	2.89	2.80
Phosphate (mg/kg)	1.13	2.87
Ammonium ion (mg/kg)	0.84	11.2
Sulphate (mg/kg)	4.33	1.29
Chlorides (mg/kg)	1.07	0.002

The results of the total and residual loads of TPH and PAH in contaminated and remedied garden soil are presented in **Table 2-6** while the TPH and PAH profile after the remediation period is presented in **Fig. 2-5**. The results revealed that the more volatile, semi-volatile and low molecular weight fractions were eliminated or degraded as lighter components including C_2- C_{11} was not detected during instrumentation even in the control samples. However, fractions from C_{12}- C_{38} were effectively eluted and detected in both contaminated (AB4) and amended beds. The eluted fractions included n- alkanes like hexacosane, Tetracosane, Octadecane, Peutacosane, n- Docosane and n- Elcosane. The rate of degradation varied with the level of contamination and type of amendment added to the soil. Degradation rates however ranged from 85.39-96.76% in beds amended with biosolid compared to ranges of 71.88-89.16% in beds amended with BSG. The contaminated but un-amended bed (AB4) had degradation rate of 58.31%. For PAH, Degradation rates ranged from 98.69-100% in beds amended with biosolid while all the beds amended with BSG recorded degradation rates of 100%. The contaminated but un-amended bed (AB4) had degradation rate of 45.1%.

Table 2 Effect of Treatment with Biosolid on the Total Petroleum Hydrocarbon (TPH) content of crude oil Impacted soil.

Treatment	Level of hydrocarbon after exposure		
	0.5% Exposure	**1.0% Exposure**	**1.5% Exposure**
TPH Level (mg/kg) after contamination	12.05	15.15	21.25
TPH level after 8 weeks of Amendment	1.76	0.79	1.19
Amount Degraded	10.29	14.36	20.06
% of Degradation	85.39	96.76	94.40

Table 3 Effect of Treatment with BSG on the Total Petroleum Hydrocarbon (TPH) content of hydrocarbon Impacted soil

Treatment		**Level of hydrocarbon after exposure**	
	0.5% Exposure	**1.0% Exposure**	**1.5% Exposure**
TPH Level (mg/kg) after contamination	12.05	15.15	21.25
TPH level after 8 weeks of Amendment	1.46	4.26	2.30
Amount Degraded	10.58	10.89	18.95
% of Degradation	87.82	71.88	89.16

Table 4 Effect of Treatment with Bacterized Biosolid on the Polycyclic Aromatic Hydrocarbon (PAH) content of crude oil Impacted soil

Treatment		**Level of hydrocarbon after exposure**	
	0.5% Exposure	**1.0% Exposure**	**1.5% Exposure**
PAH Level (mg/kg) after contamination	3.07	5.35	8.79
PAH level (mg/kg) after 8 weeks of Amendment	0.00	0.07	0.06
Concentration (mg/kg) Degraded	3.07	5.28	8.73
% of Degradation	100	98.69	99.32

Table 5 Effect of Treatment with Bacterized BSG on the Polycyclic Aromatic Hydrocarbon (PAH) content of crude oil Impacted soil

Treatment		**Level of hydrocarbon after exposure**	
	0.5% Exposure	**1.0% Exposure**	**1.5% Exposure**
PAH Level (mg/kg) after contamination	3.07	5.35	8.79
PAH level (mg/kg) after 8 weeks of Amendment	0.00	0.00	0.00
Concentration (mg/kg) Degraded	3.07	5.35	8.79
% of Degradation	100	100	100

Table 6 Natural attenuation rates of TPH and PAHs in Untreated soil exposed to 1.5% crude oil Contamination

Treatment	**TPH**	**PAH**
Level (mg/kg) after contamination	22.15	8.79
level after 8 weeks of Amendment	8.86	4.83
Amount Degraded	12.39	3.96
% of Degradation	58.31	45.1

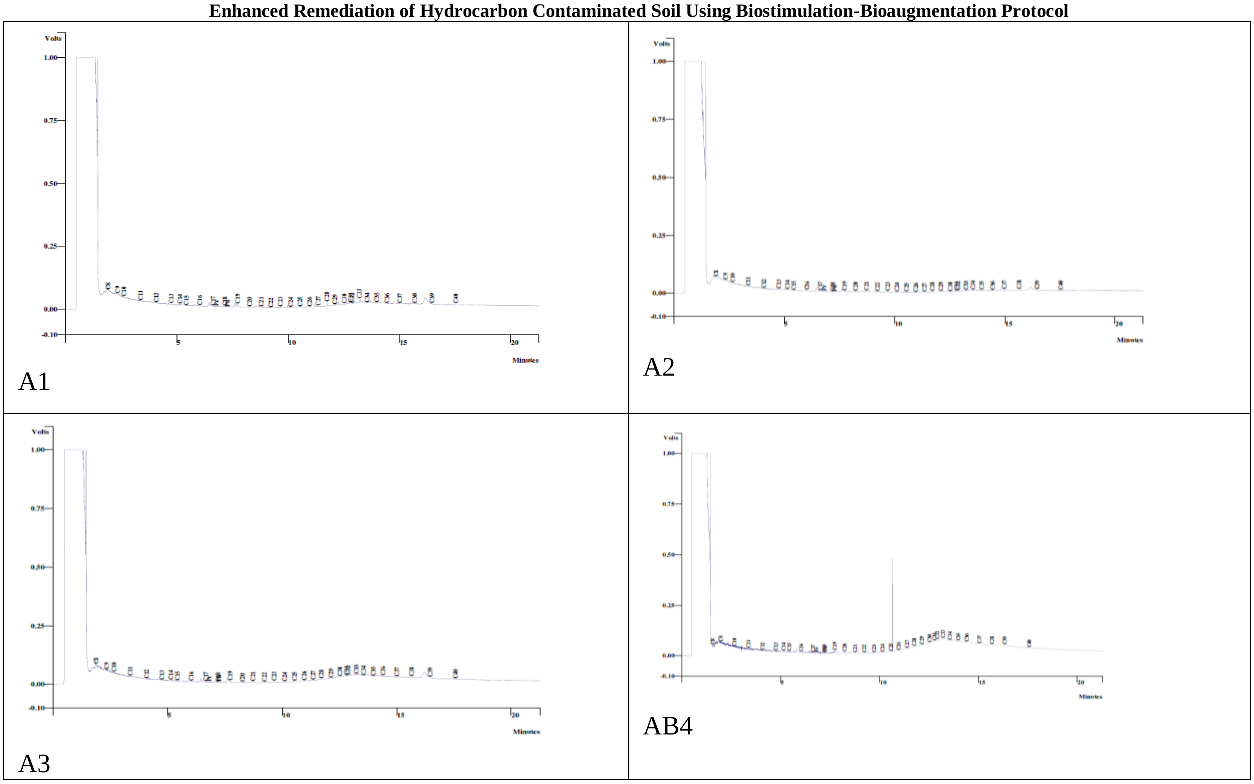

Fig. 2 Petroleum hydrocarbon profile of contaminated beds (A1-A3) amended with bacterized biosolid and the contaminated but un-amended bed (AB4) after 56 days.

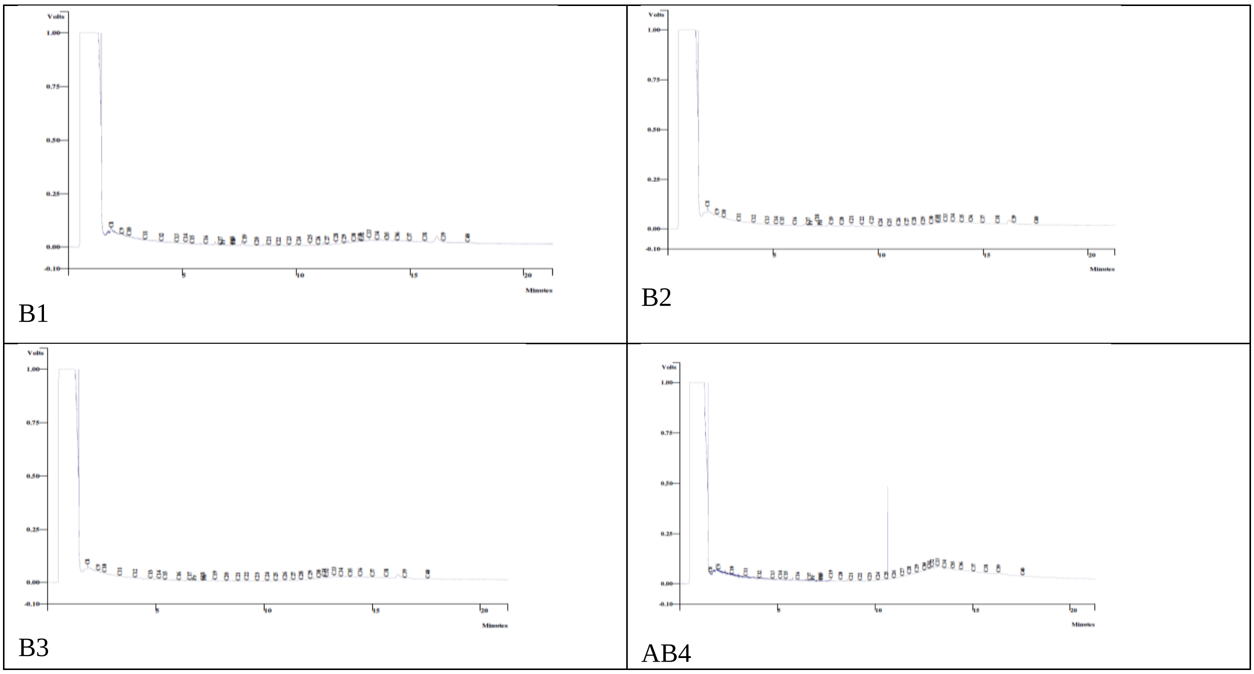

Fig. 3 Petroleum hydrocarbon profile of contaminated beds (B1-B3) amended with bacterized BSG and the contaminated but un-amended bed (AB4) after 56 days

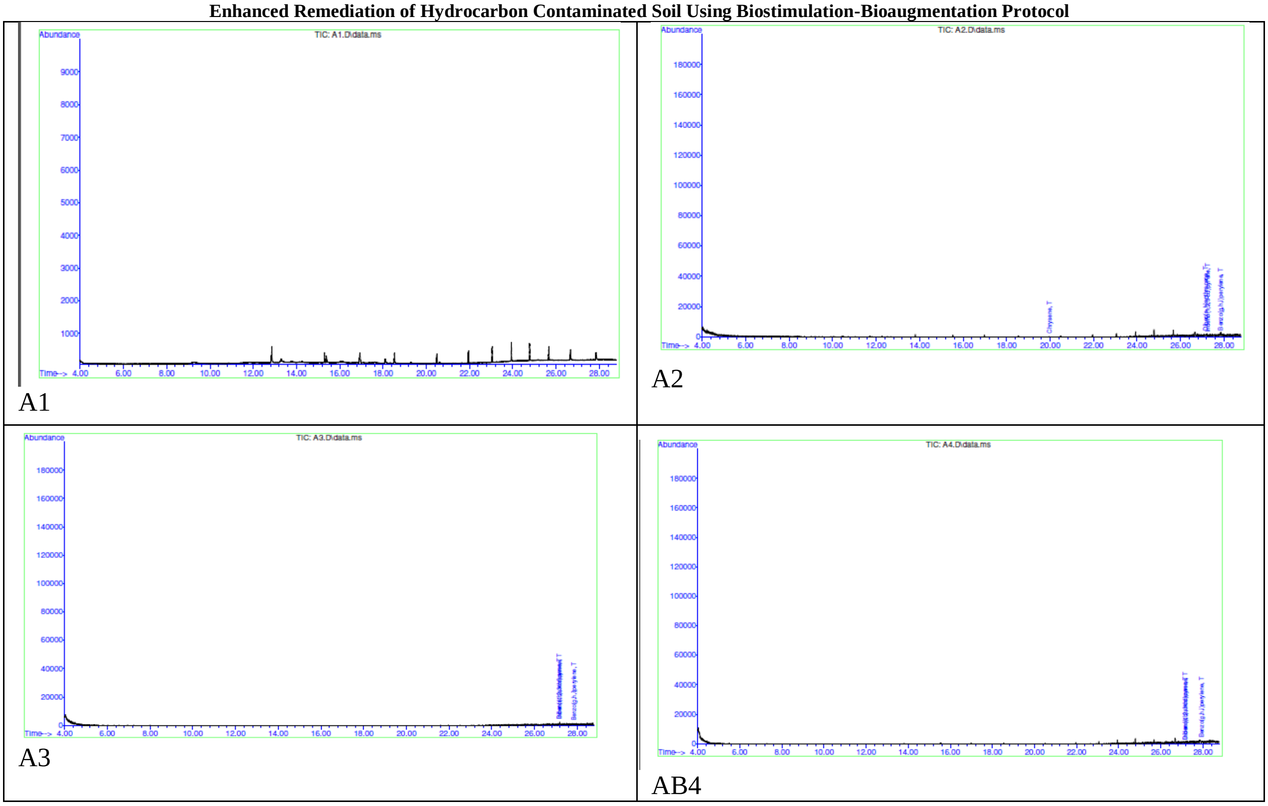

Fig. 4 PAH profile of contaminated beds (A1-A3) amended with bacterized biosolid and the contaminated but un-amended bed (AB4) after 56 days.

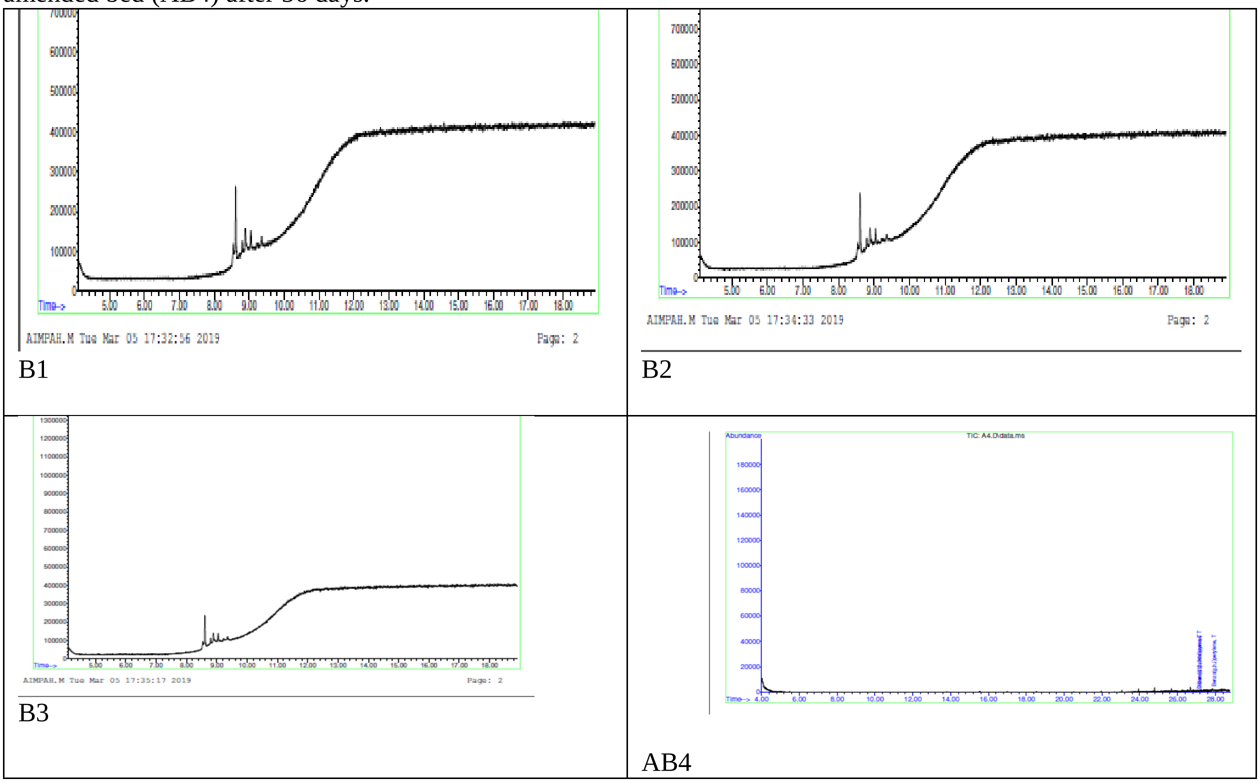

Fig. 5 PAH profile of contaminated beds (B1-B3) amended with bacterized BSG and the contaminated but un-amended bed (AB4) after 56 days

Discussion

Hydrocarbons are usually adsorbed by organic matter in soils. Contaminants desorption becomes very slow and as a consequence, slows the rate of biodegradation. Addition of organic amendment (biosolid and BSG as in this study) solves this problem and enhances degradation process by increasing microbial population of the soil (Oruru, 2014; Raimi and Sabinus, 2018). Hydrocarbon biodegradation in soil can be limited by factors like microorganism type, nutrients, pH, temperature, oxygen, moisture, soil properties, contaminant concentration levels etc. (Bradi *et al*. 2000; Semple et al. 2001; Sabate et al. 2004; Ghazali et al. 2004; Walter *et al.* 2005; Atlas and Bartha 2006; Raimi and Sabinus 2018). Increase in soil temperature and moisture content during the degradation of organics in the soil promotes biological activities (Hou and Al-Tabbas, 2014). Therefore, an increase in nutrient properties of the amended soils can be attributed to the addition of organic amendment which on decomposition supplied conditions necessary for microbial activity for the release of nutrients (Adesua 2014; Raimi and Sabinus 2018). Crude oil in the environment is biodegraded primarily through the activities of bacteria and fungi. The reported efficiency of biodegradation ranged from 6.0% to 82.0% for soil fungi, 0.13 to 50.0% for soil bacteria and 0.003 to 100% for marine bacteria. Several reports reveal that mixed consortia of crude oil utilizing microorganisms with broad overall enzymatic capabilities are necessary to degrade complex mixtures of hydrocarbon in soil, freshwater and marine environment (Das and Mukherjee 2007). The results obtained in this study have shown that depending on the level of contamination and amount of amendment added, both biosolid and BSG improve the soil heterotrophic bacteria properties and indirectly, the hydrocarbonoclastic bacteria activities of soil. The activities of the oil degraders may have been enhanced by the improved levels of nitrogen and phosphorous in the amended soil. In addition to a readily utilizable source of carbon, microbes require nutrients such as nitrogen, phosphorus, and potassium (NPK) for cellular metabolism and successful growth (Sihag and Pathak 2014). In a contaminated site where organic carbon levels are always high, available nutrients can rapidly be depleted during metabolism (Okerentugba *et al.* 2003). Therefore, it is highly necessary to supplement contaminated soil with nutrients to complement the utilization of carbon by the microorganisms. Hydrocarbon degradation can occur under aerobic and anaerobic conditions (Okoh, 2006: Adesua, 2014). In general, aerobic metabolism of hydrocarbons initially requires oxygenase enzymes that incorporate molecular oxygen into the reduced substrate. Most microorganisms attack alkanes terminally whereas some perform sub-terminal oxidation. The alcohol product is finally oxidized into an aldehyde and to a fatty acid. The latter is degraded further by beta-oxidation (Abioye et al. 2012). Generally, the mechanisms involved in the degradation of petroleum hydrocarbons include mediation by specific enzyme system (e.g oxygenase, hydroxylases), attachment of microbial cells to the substrates and production of biosurfactants by hydrocarbon-degrading microorganisms (Walter et al. 2005; Ghazali et al. 2004; Das and Murkherjee 2007; Kaczorek et. al; 2013). Soil microbial degradation capacity is dependent on many factors including soil type and environmental micro and macro conditions (Margesin 2000). The soils used in this current study were predominantly sandy with pH range typical of acidic soil characterized by low levels of organic carbon and phosphorus. Despite this, the natural degradation capacity was apparent in beds contaminated but not amended as a decrease in contaminant levels occurred even without any amendment. However enhanced degradation occurred in beds contaminated and remedied using the Biostimulation-Bioaugmentation (BB) protocol. This was achieved by increasing their respective soil nutrient and the addition of a potent allochthonous hydrocarbon degrader (*Bacillus subtilis*). The present study revealed little variability within each treatment(beds) for the initial TPH data before the addition of amendments (12.05 to 21.25 mg kg^{-1}) indicating a homogeneous initial distribution of the hydrocarbon used in the soil. Biosolid was significantly ($p < 0.05$) more effective at driving TPH reduction depending on contamination level) in the soil than BSG depending on contamination level).

The reduction of Σ17 PAHs in the contaminated beds was 55±3% higher in the remedied beds compared to the bed that was left for natural attenuation. whereas in contaminated beds, the (BB) protocol improved the C10-C40 reduction by 11.9-56.3% compared to natural attenuation. One reason for a lower contaminant (C10-C40) reduction with (BB) protocol in the contaminated beds was probably the short ageing time of the contaminants: i.e. oil compounds have not had sufficient time to adsorbed strongly onto the soil particles and a significant part of it escaped during volatilization. Many bacteria strains, which can degrade petroleum, aromatic as well as polyaromatic hydrocarbons have been found, and most studied are species of *Rhodococcus, Pseudomonas, Paenibacillus*, and *Ralstonia* (Tyagi *et al*. 2011). Contaminant structure is one of the factors that can affect the efficiency of microbial degradation. The biodegradability of contaminants decreases the more complex the molecular structure of a contaminant is. For example, the biodegradability of oil components decreases in the following order: n-alkanes > branched-chain alkanes > branched alkenes > low-molecular-weight n-alkyl aromatics > monoaromatics > cyclic alkanes > polycyclic aromatic hydrocarbons > asphaltenes (van Hamme et al. 2003). In this study, the connection between the contaminant structure and its biodegradability was observed with PAH compounds found in the contaminated soil. The low molecular 2-3 ringed PAHs [Acenaphthene, Napthalene, Fluorene etc.) were biodegraded more efficiently than the more complex, 4-5 ringed PAHs (Chrysene, Diben(a,h)anthracene, Indeno(1,2,3-cd)pyrene, Benzo(g,h,i)perylene etc.]. The reduction percentages for 2-3 ringed PAHs were 100% in all treatment levels, while that for the 4-5 ringed PAHs was between 50-87.5% for all the treatment levels. On the other hand, the toxicity of the low molecular- weight compounds is usually higher, because of their higher volatility, solubility and bioavailability compared to the long-chained hydrocarbons. These characteristics are also believed to aid their biodegradability (Dorn and Salanitro 2000). Non-biodegradability and toxicity of C5-C10 hydrocarbons are usually caused by their tendency to disrupt membrane lipid structures of microorganisms (Bartha 1986). It was therefore interesting that the BB protocol led to complete removal of C5-C10 hydrocarbons in the amended beds. The reason might be the addition of a potent allochthonous hydrocarbon biodegrader (*Bacillus subtilis*) incorporated into the amendment used and the stimulation of other indigenous hydrocarbon-degrading microorganisms widely distributed in the soil.

Conclusion

It was obvious from this study that enhanced degradation occurred in beds contaminated and remedied using the Biostimulation-Bioaugmentation (BB) protocol. This was achieved by increasing their respective soil nutrient and the addition of a potent allochthonous hydrocarbon degrader (*Bacillus altitudinis*). It may, therefore, be appropriate to recommend their use as a suitable biostimulant during the remediation of agricultural land previously contaminated with hydrocarbon.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

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