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Influence of Bacterization of Biostimulant (Maize Chaff) on Hydrocarbon Degradation of Crude Oil-impacted Soil

Senyene I. Umana
Lecturer, Department of Biological Sciences, Akwa Ibom State University, Nigeria
Joseph P. Essien
Professor, Department of Microbiology, University of Uyo, Akwa Ibom State, Nigeria
Mfoniso P. Uko
Lecturer, Department of Biological Sciences, Akwa Ibom State University, Nigeria
Maria P. Bassey
Lecturer, Department of Biological Sciences, Akwa Ibom State University, Nigeria
Otobong D. Akan
Lecturer, Department of Biological Sciences, Akwa Ibom State University, Nigeria

Abstract:
The influence of bacterization of biostimulant (maize chaff) on hydrocarbon degradation of crude oil impacted soil was studied. The study revealed the presence of bacterial isolates with strong crude oil utilization potentials. Pseudomonas aeruginosa, and Bacillus subtilis were the strongest degraders, Micrococcus and Corynebacterium were moderate degraders, Alcaligenes sp, Enterobacter sp and Klebsiella sp exhibited very weak crude oil utilization while Nitrosomonas sp, Nitrobacter sp, Enterococcus sp and Staphylococcus aureus were unable to degrade hydrocarbons. However, results obtained when microbially (B. subtilis) augmented bio-stimulants was used in a Bacterization-Biostimulation (BB) remediation protocol revealed enhanced degradation of crude oil and it components. Viable cell measurement showed that the higher the biostimulants/contaminant (BC) - ratio employed the more the heterotrophic activity but less hydrocarbonoclastic activity. For soils remedied for 8 weeks with bacterized – maize chaff, the degradation rates were remarkably high, 98.25% as against 28.95 % recorded for the control (treatment with maize chaff alone). The best degradation rate (98.28 %) was attained when 10 % was applied at a BC ratio of 3.34: 1. Beyond these ratios the treatments created "diauxic influence", retarding the growth and activities of hydrocarbonoclastic bacteria while heterotrophic bacteria proliferate. Longer incubation would certainly have led to complete or higher hydrocarbon degradation when hydrocarbonoclastic degradation enters the second log phase. Enhanced remediation with maize chaff using BB protocol is strongly recommended.

Keywords: *Bacterization, Biostimulant, Hydrocarbon, Degradation and Bioremediation*

1. Introduction

The deleterious and degrading effects of pollution on the environment have led to increased awareness of its effects on the environment. Exposure of microbial habitats to pollutants has been known to cause substantial inhibition on certain members of microbiocoenosis, while increasing the relative abundance of those able to utilize the substance for growth and multiplication (Peresutti *et al*., 2003). Assemblages from such contaminated ecosystem can adapt to the presence of pollutant producing shifts in the physiologic and generic diversity of the community (Macnaughton *et al*., 1999; Hollaway *et al*, 1980; Chikere *et al*., 2011). Margesin and Schinner (2001) and Lakshmi (2013) have demonstrated that the density of hydrocarbon utilizers from a natural ecosystem can be increased by improving their natural capacity to degrade contaminants. These capabilities are currently being exploited in enrichment procedures for naturally selecting populations that could attack and detoxify environmental pollutants in bioremediation strategies.

Organic pollutants are presumed to undergo degradation as a result of the metabolic activities of the microbial communities in the environment (Okpokwasili and Olisa, 1991, Chikere *et al*., 2011) though some man-made compounds are relatively refractory to degradation creating special problems for environmental protection. The resulting accumulation of intermediate metabolites can be toxic and/ or refractory to further metabolism and have fueled the request for financial compensation to losses and damaged lands in the Niger Delta. These concerns have led to the development of various remediation technologies including bioremediation – which mainly depends on the ability of microorganisms to degrade, transform, detoxify or breakdown the contaminant. Bioremediation can be defined as the process where contaminants in the soil, sediments, sludge or groundwater are biologically degraded into innocuous substances such as carbon dioxide, water, fatty acids and biomass, through the action of microbial metabolism (Sharma 2012; UNIDO 2014).

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 (Rockne *et al.*, 2002; Essien *et al.*, 2012). 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 for biological sources. This increasing health hazard and soil pollution has compelled the scientists to look for non-chemical way of supplying the fertilizers to enhance crop productivity. As a result, the application of biofertilizers (biostimulants) in place of chemical fertilizers has been recommended. Biofertilizer means input of plant nutrients of biological origin for improved growth of plants. Biofertilizers are also known as 'microbial inoculants' or microbial preparations. They themselves do not increase the soil fertility directly, but instead they exercise their biological effect on improving nutritional conditions including enhanced microbial activities in soil (Subba-Rao, 1982; Singh and Dwivedi, 2004; Trabelsi and Mhamdi, 2013; Zhen *et al*., 2014).

Nitrogen-rich organic substrates are being used as biostimulants currently, and they have proven to be useful in enhancing the rate of bioremediation. Examples of nitrogen-containing organic substrates which are used as biostimulants, include sugarcane bagasse, banana peel, yam peel, rice husk, coconut shell, cow manure, pig manure, poultry manure and spent brewing grain (Nwogu *et al*., 2015; Agarry, *et al.*, 2015).

Recent studies have shown that hydrocarbons degrading microorganisms are widely distributed in contaminated ecosystems (Jain *et al*., 2011) and may adapt to interact with heterogeneous materials which serve as primary environmental sorbent for Polycyclic aromatic hydrocarbons and hydrophobic hydrocarbons in ways that facilitate these pollutants and their subsequent metabolism (Steinberg *et al*., 2008, Das and Chandran, 2011). Most studies carried out on bioremediation of soils impacted in the Niger Delta of Nigeria are majorly on enhanced remediation using biostimulation and bioaugmentation protocols. A combination of both protocols (Biostimulation - Bioaugmentation [BB] protocol) has not been attempted. This study is focused on the Influence of bacterization of biostimulants on the degradation of hydrocarbons in crude oil impacted garden soil.

2. Materials and Method

2.1. Sources of Materials

2.1.1.Biostimulating Agents

The Maize chaff was obtained from a pap seller at Itam market, Uyo Akwa Ibom State, Nigeria. The maize chaff was air dried and stored at room temperature, while Bonny light crude oil was obtained from Oil Company operating in the Niger Delta Region of Nigeria.

2.1.2. Crude Oil Degrading Bacteria

Bacteria with potentials to effectively degrade crude oil were isolated from the humic collected from Eniong River in Itu LGA of Akwa Ibom State, Nigeria.

2.1.3. Chemical Analysis of Maize Chaff

Chemical parameters of the maize chaff samples were determined using standard analytical procedures recommended by APHA (1992, 1998).

2.1.4. Microbiological Analysis

Standard microbiological techniques were employed in this study.

2.2. Determination of Microbiological Loads of the Garden Soil and Bio-stimulants

(a) Serial Dilution

This was done according to the method of Collins and Lyne (1976). Ten grams of the samples was measured and introduced into beaker containing 90ml of distilled water. It was shaken for even distribution; 1ml of the aliquot was aseptically transferred into sterile test tube containing 9ml of diluents to give a dilution of 10^{-1} (10-fold dilution). Further 10-fold serial dilution was carried out up to factor 10^{-9} dilution factor.

(b) Analytical Media

Nutrient Agar (NA) was used to determine total heterotrophic bacterial counts (THBC), Ammonium Carbonate Medium was used to determine *Nitrosomonas* counts, Nitrite carbonate Medium was used to determine *Nitrobacter* counts, Sabouraud Dextrose Agar was used to determine total Fungal counts while Mineral salt agar supplemented with crude oil was used to isolate hydrocarbon utilizing bacteria. The media were prepared according to manufacturer's instructions and sterilized by autoclaving at 121^{o}C at 15 minutes.

(c) Enumeration of Microbial Densities

The density of the total heterotrophic bacteria (THBC) in the biostimulants (maize chaff) and garden soil was assessed using the pour plate technique (Collins and Lyne, 1976) while the population of oil degrading bacteria (ODB) was estimated by the vapour phase transfer technique as described by Okpokwasili and Amanchukwu (1988). The inoculation of selected media was done with the desired diluent.

For THBC, Nutrients agar a general-purpose medium was used. The density was estimated by the pour plate technique of Collins and Lynes (1976). The inoculated plates were incubated incubated in an inverted positionat room temperature for 24 hours and colonies formed were counted and expressed as cfu/g of the sample.
On the other hand, the density of oil degrading bacteria in the soil and biostimulant was measured by vapour phase technique. 1 g of samples was serially diluted. The desired diluents were plated on Mineral salt medium (MSM) fortified with sterilized Bonny Light crude oil and was incubated at room temperature for 14 to 21 days depending on the growth rate on the plates. Colonies of bacteria on plates treated with crude oil were enumerated.

2.2.1. Isolation of Crude Oil Degrading Bacteria from Humic Sediment

(a) Enrichment and Isolation of Bacterial Isolates
The enrichment culture technique was employed. Precisely 1 g of humic sediment sample from Eniong River was inoculated into three sets of conical flasks containing 50 ml of sterile Mineral Salt Medium [K_2HPO_4 – 6g, NaCl – 12g, KH_2PO_4 – 6g, $(NH_4)_2SO_4$ – 6g, $MgSO_4.7H_2O$ – 2.6g, $CaCl_2.2H_2O$ – 0.16g, per liter (pH 7.0 $\pm$ 0.2)] (MSM) enriched with 1% crude oil as carbon source. The medium was incubated at 28 °C in shaker incubator (100 rpm) for 7 days. After 7 days of incubation, the samples were serially diluted using sterile water and plated on Nutrient Agar (NA) to obtain viable cells of bacteria. Discrete colonies obtained were sub-cultured using streak method as described by Cheesbrough (2006) to obtain pure cultures.

(b) Maintenance of Pure Cultures of Oil-degrading Bacterial Isolates
Distinct colonies of the oil degrading bacteria isolated from the humic sediment were sub-cultured into McCartney bottles containing freshly prepared Nutrient Agar slants and incubated at 30 $\pm$ 2 ^oC for 24 hours before storage at 4 ^oC for characterization.

(c) Screening for Crude Oil Utilizing Potential of the Bacterial Isolates
Crude oil utilizing potential of the bacteria isolates was determined using the hydrocarbon overlay method. Precisely 15 g of agar-agar was added to mineral salt medium, sterilized and allowed to set. The solidified plates were overlaid with 1% (v/v) sterile crude oil, allowed for about 15 to 30 minutes then the test isolates were streaked on the surface of the plate.
All inoculated plates were incubated at room temperature for 5-15 days with periodic observation. Colonies that eventually developed showing area of clearing were selected and rated. The utilization was rated based on the diameter and luxurious nature of the developed colonies, i.e., '+', '++' or '+++' indicating the magnitude of the oil degrading potentials as described by Ekundayo and Obire (1987).

(d) Characterization of Bacterial Isolate
The best crude oil utilizing bacterial isolates were characterized based on their cultural and morphological attributes as well as their responses to standard biochemical test as described by Cheesbrough (2006).

2.3. Ex situ Analysis of the Influence of Organic Amendments (Biostimulants) on the Remediation of Crude Oil Contaminated soil
The garden soil was carefully and manually sorted to remove debris. Thereafter 4kg of the soil contained in perforated 30 x 30 cm wooden boxes was contaminated with different concentrations of crude oil. Graded concentrations (1, 5, 10, 15 and 20 %) of the biostimulants was bacterized with 20ml broth culture of the oil degrading bacterium (isolated from humic fresh water sediments) and then used to amend the contaminated soil. Un-bacterized but contaminated soil and unpolluted soil served as controls.
The influence of the organic amendment on the physicochemical parameters of the amended and un-amended soil was assessed at the end of the degradation course (2 months after treatment) while microbial properties of the test soils and rate of crude oil biodegradation was carried out for a period of two months at 7days interval (i.e. 0, 7^{th}, 14^{th}, 21^{st}, 28^{th}, 35^{th}, 42^{nd}, 49^{th} and 56^{th} day after amendment).

2.3.1. Assessing the Influence of Biostimulants on the Physicochemistry of Amended Soil
Standard analytical techniques were employed to determine the changes in the physicochemistry of the amended soil. The total hydrocarbon content of Spectrophotometerthe amended soil was measured at 460nm after extraction with 50ml hexane with the aid of Mamotte 701 using n-hexane as blank (Valcarcel, 2000). On the other hand, the residual hydrocarbons load (hydrocarbon fractions) remaining after degradation in the treated soil were determined using the methods of ASTDM 3921 and UDEPA 8270B (TPI, 2007). The total residual petroleum hydrocarbons (TRPH) and polycyclic aromatic hydrocarbons (PAHs) were extracted from the soil samples and quantified using Gas chromatograph HP5890.

2.3.2. Assessing the Influence of Biostimulants on the Microbiological Attributes of Amended Soil
The mean total aerobic count for bacteria, fungi, hydrocarbon degraders, *Nitrosomonas* and *Nitrobacter* in treated soil were determined at 7 days interval for two months.

2.4. Data Analysis
The data collected were subjected to correlation matrix analysis to establish relationships between the microbial groups. Simple percentage was also to determine the degradation rate among treatment.

3. Results

3.1. Chemical Properties of the Bio-stimulants (Maize Chaff)

Table 1shows the chemical properties of the organic bio-stimulants used for the remediation study. The moisture content of maize chaff was 3.2±0.17%. The results have also revealed a high nutritive salt content of the bio-stimulant. The concentrations of sulphates, phosphate, nitrate and chloride recorded for the maize chaff were 0.09±0.01mg/kg, 0.38±0.03mg/kg, 1.34±0.04mg/kg and 26±0.60mg/kg respectively.

Parameter	Maize chaff			
	1	2	3	Mean±SD
Sulphates (mg/kg)	0.09	0.10	0.08	0.09±0.01
Phosphate (mg/kg)	0.35	0.39	0.40	0.38±0.03
Nitrate (mg/kg)	1.37	1.35	1.30	1.34±0.04
Chloride (mg/kg)	26.6	25.4	26	26±0.60
Moisture (%)	3.3	3.0	3.3	3.2±0.17
Ash (%)	1.7	1.9	1.8	1.8±0.10
Fibre (%)	1.6	1.9	1.6	1.7±0.17
Protein (%)	18.0	17.9	18.4	18.1±0.26
Lipid (%)	2.8	3.0	3.2	3.0±0.20
CHO (%)	75.2	75.6	75.7	75.5±0.26
Caloric value (kcal)	401.2	400.8	400.7	400.9±0.26
PAH (mg/kg)	0.37	0.37	0.37	0.37
TPH (ppm)	1.372e4	1.372e4	1.372e4	1.372e4
pH	5.30	5.30	5.30	5.30
Total organic carbon (%)	55.05	54.98	55.00	55.01±0.04
Nitrogen	1.89	1.90	1.85	1.88±0.03
Key: CHO = carbohydrates, PAH = Polycyclic aromatic hydrocarbon, TPH = total petroleum hydrocarbons				

Table 1: Chemical properties of biostimulants (maize chaff) used in the remediation of contaminated soils

3.2. Microbiological Properties of Test Soil, Biostimulant

The results presented in Table 2 revealed the microbiological properties recorded for the garden soil and organic amendment (maize chaff) used for the enhanced remediation study. The results have revealed the rich microbial assemblage and diversity of the garden (test) soil used. Table 3 shows the morphological, cultural and biochemical characteristics of isolates from humic sediment, soil and organic biostimulants. The result in Table 4 revealed a rich bacteriological diversity in soil than in organic biostimulant.

Microbial group	Soil	Maize chaff
Heterotrophic bacterial counts	2.0×10^7	1.27×10^3
Hydrocarbonoclastic bacterial counts	4.0×10^2	1.4×10^1
Nitrosomonas counts	3.3×10^3	-
Nitrobacter counts	1.7×10^3	-
Fungal Loads	1.3×10^5	3.4×10^3
Values are mean of three determinations Key: - = not detected ND = not done		

Table 2: Microbiological properties of test soil and biostimulants

Morphological characteristics					Citrate	Catalase	Coagulase	Indole	Oxidase	Methyl Red	Vorges Paskeur	Sugar fermentation				Spore	Motility	Probable Organism
	Surface	Colour	Shape	Gram's Reaction								Glucose	Manitol	Sucrose	Lactose			
1	Rough	Milky	Circular	- rod	-	+	-	-	+	-	+	AG	A	A	-	-	+	*P. aeruginosa*
2	Rough	Milky	Circular	+ rod	-	+	-	-	-	-	+	A	-	-	-	+	-	*Corynebacterium* sp
3	Smooth	Opaque	Circular	+ rod	-	+	-	-	+	-	-	-	-	-	-	-	+	*Alcaligenes* sp
4	Smooth	Milky	Spherical	+coci	-	+	-	-	-	+	-	AG	AG	A	A	-	-	*Staphylococcus albus*
5	Smooth	Milky	Spherical	+coci	-	+	+	-	-	+	-	AG	AG	A	A	-	-	*Staphylococcus aureus*
6	Rough	Milky	Irregular	+ rod	-	+	-	-	-	-	+	AG	A	AG	AG	+	+	*Bacillus subtilis*
7	Rough	Milky	Irregular	+ rod	-	+	-	-	-	-	+	AG	A	A	AG	+	+	*Bacillus cereus*
8	Dry	Milky	Circular	+ rod	+	+	-	-	+	+	-	A	-	A	A	-	-	*Micrococcus* sp
9			Straight	- rods	-	-	-	-	-	-	-	-	-	A	-	-	+	*Nitrosomonas* sp
10				- rods	-	+	-	-	-	-	-	-	A	-	-	-	+	*Nitrobacter* sp
11				- rods	-	+	-	+	-	+	+	AG	A	A	A	-	_	*Klebsiella* sp
12				- rods	+	+	+	-	-	-	+	AG	A	A	+	-	+	*Enterococcus* sp
Key: A=Acid production, G= Gas production, +=Positive, -=Negative reaction, VP =Voges Proskauer, sp =species																		

Table 3: Morphological, cultural and biochemical characteristics of isolates from test samples

Isolate	Soil	Maize chaff
P. aeruginosa	+	+
Corynebacterium sp	+	-
Alcaligenes sp	+	-
Bacillus subtilis	+	+
Micrococcus sp	+	+
Bacillus cereus	-	+
Staphylococcus albus	-	+
Staphylococcus aureus	+	+
Nitrosomonas sp	+	-
Nitrobacter sp	+	-
Enterobacter sp	+	-
Klebsiella sp	+	-
Enterococcus	+	-
Key: - = not present, + = present		

Table 4: Diverse species of bacteria isolated from the test soil and biostimulants

3.2.1. Hydrocarbons Utilization Potential of Bacteria Isolated from Test Soil *In situ*

The capability of indigenous bacterial species isolated from test soil to utilize crude oil is presented in Table 5. The results revealed the presence of bacterial isolates with strong crude oil utilization potentials. *P. aeruginosa*, and *Bacillus subtilis* were the strongest degraders, *Micrococcus* and *Corynebacterium* were moderate degraders, *Alcaligenes* sp, *Enterobacter* sp and *Klebsiella* sp exhibited very weak crude oil utilization while *Nitrosomonas* sp, *Nitrobacter* sp, *Enterococcus* sp and *Staphylococcus aureus* were unable to degrade hydrocarbons.

Isolate code	Growth on crude oil after 7 days	Growth on crude oil after 14 days
P. aeruginosa	+++	+++
Corynebacterium sp	+	++
Alcaligenes sp	-	+
Bacillus subtilis	+++	+++
Micrococcus	++	++
Staphylococcus aureus	-	-
Nitrosomonas sp	-	-
Enterococcus sp	-	-
Klebsiella sp	-	+
Nitrobacter sp	-	-
Enterobacter sp	+	+
Key: - = no growth, + = 1-5mm (weak) + = 6-10 mm (moderate), ++ = 11-15 mm (strong), +++ =16-20 mm (strongest)		

Table 5: Hydrocarbonoclastic potential of bacteria isolated from garden soil

3.2.2. Influence of biostimulation with Maize Chaff and Augmentation with Oil Degrading Strain of *Bacillus subtilis* on the Activities of Bacteria in Crude Oil Contaminated Soil

The Influence of biostimulant (maize chaff) application on the activities of bacteria in garden soil contaminated with crude oil and augmented with oil degrading strain of *Bacillus subtilis* are presented in Figures 1-5. The influence of biostimulation with maize "cropped" with 20 ml (2.9 x 10^4cfu/ml) of broth culture of *B. subtilis* was determined by the level of contamination and varied over time and with the concentrations of stimulants. Amendments with 1% and concentrations as much as 10% and above encouraged higher heterotrophic than hydrocarbonoclastic activities.

3.2.3. Changes in Bacterial Activities of the Unpolluted Garden Soil (Control 1) and Polluted Soil Remedied with Un-bacterized Bio-stimulant (Control 2)

The changes in bacterial activities of the unpolluted soil over time are presented in Figure 6. The results obtained revealed fluctuation in activities of *Nitrosomonas* and *Nitrobacter* during the exposure time with decline or dead phase in the 7th week of exposure while the counts of total heterotrophic bacteria and oil degrading bacteria decreased over time. Figures 7 shows the changes in bacterial activities of polluted soil remedied with oil Un-bacterized Bio-stimulants. The result recorded variation in the activities of the microbial growth over time.

3.3. Total Petroleum Hydrocarbon Contents of Soil after Remediation with Organic Amendments "Cropped" with B. subtilis -HSC 1

The *in-situ* degradation study carried out for 8 weeks showed that, the degradation of crude oil and its components was faster when enhanced with biostimulant "cropped" with oil degrading strain of *B. subtilis* than when carried out with biostimulants alone (Table 6). Polycyclic aromatic hydrocarbons profile of the biostimulants (maize chaff) and unpolluted soil is presented in Table 7. The result reveals that the PAH content of the maize chaff and unpolluted soil was 0.37 and 0.09 respectively.

Figure 8 shows the efficiency of the adopted biostimulants/crude oil ratios on the remediation of oil contaminated soils. Crude oil degradation rates were found to decrease with increase in percentage of organic amendment applied. In soil remedied with microbially cropped maize chaff, the best degradation rate (98.28 %) recorded was attained when 10 % was applied with a BC ratio of 3.35

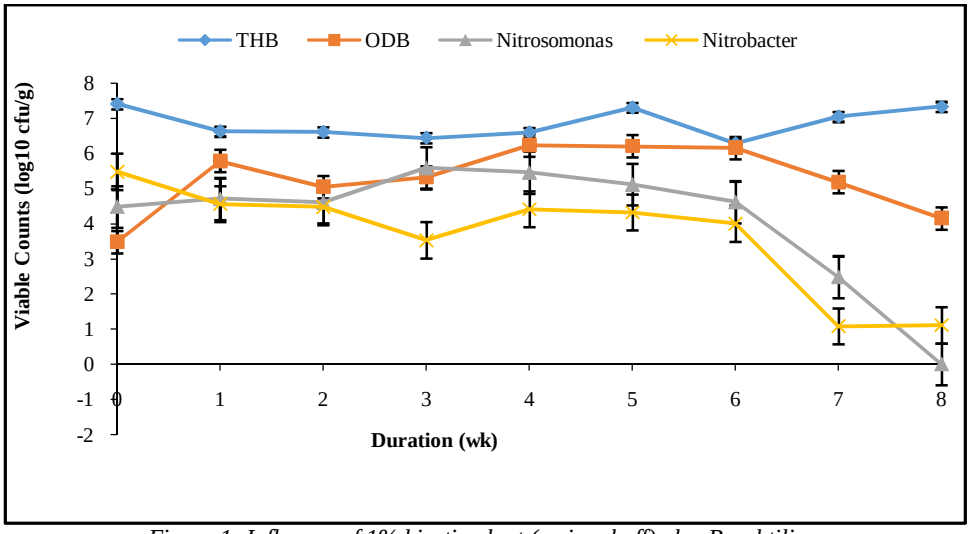

Figure 1: Influence of 1% biostimulant (maize chaff) plus B. subtilis – HSC 1 application on the activities of bacteria in garden soil contaminated with 2.08 % crude oil
Key: THBC = Total heterotrophic counts; ODB = Oil degrading bacteria

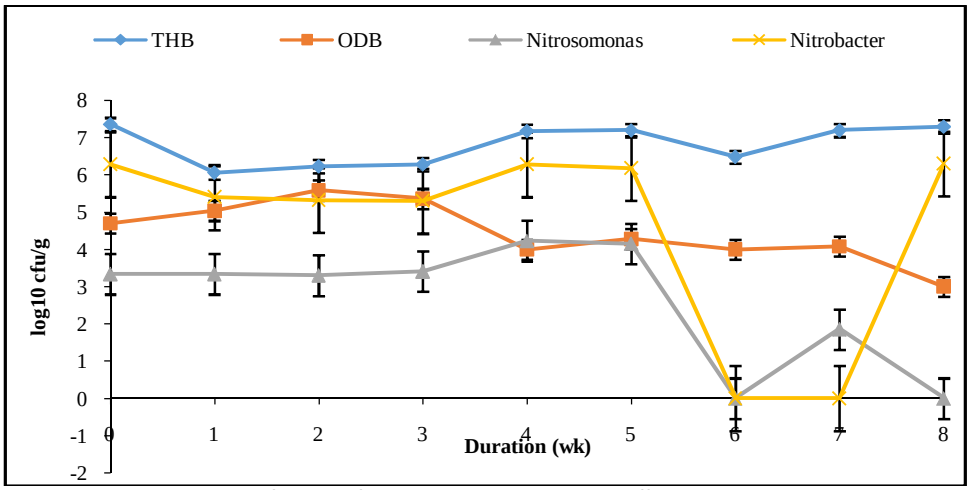

Figure 2: Influence of 5% biostimulant (maize chaff) plus B. subtilis – HSC 1 application on the activities of bacteria in garden soil contaminated with 2.28 % crude oil
Key: THBC = Total heterotrophic counts; ODB = Oil degrading bacteria

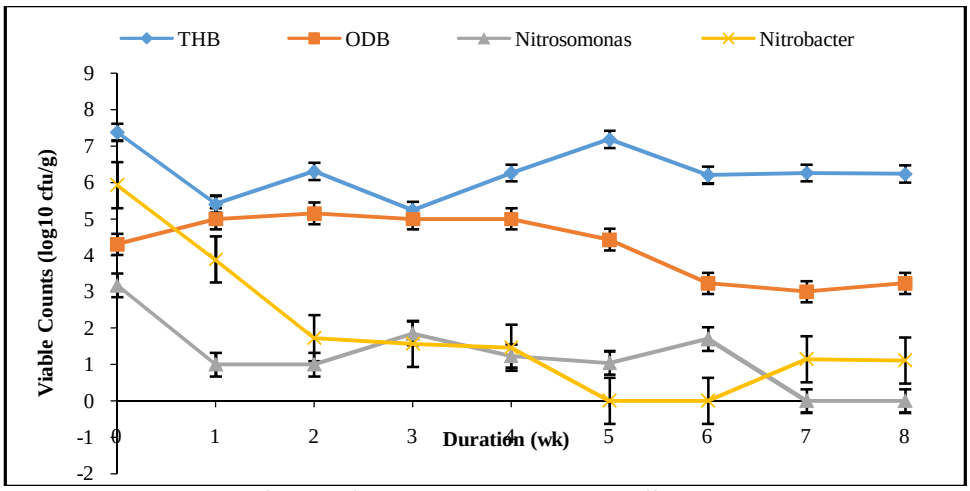

Figure 3: Influence of 10% biostimulant (maize chaff) plus B. subtilis – HSC 1 application on the activities of bacteria in garden soil contaminated with 2.6 % crude oil
Key: THBC = Total heterotrophic counts; ODB = Oil degrading bacteria

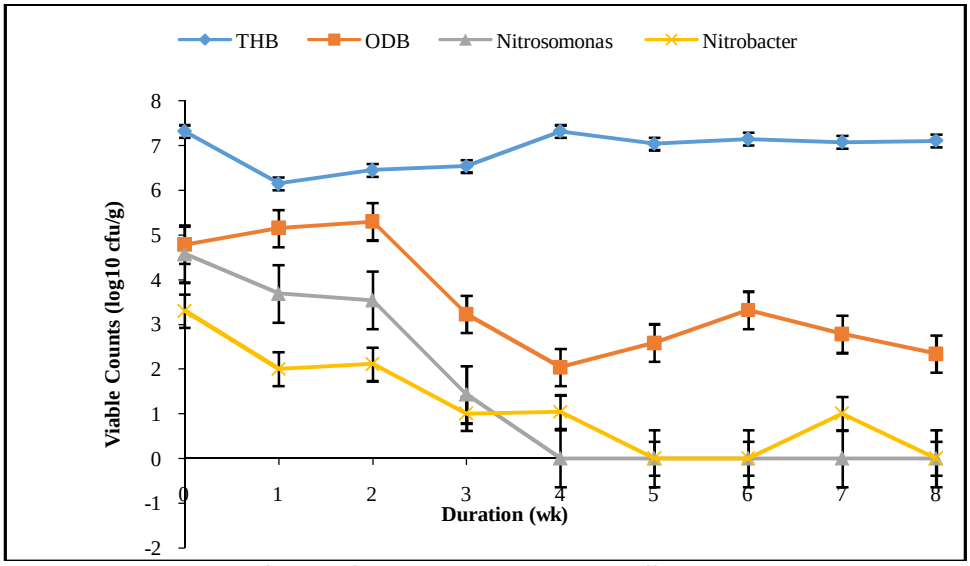

Figure 4: Influence of 15% biostimulant (maize chaff) plus B. subtilis – HSC 1 application on the activities of bacteria in garden soil contaminated with 3.12 % crude oil
Key: THBC = Total heterotrophic counts; ODB = Oil degrading bacteria

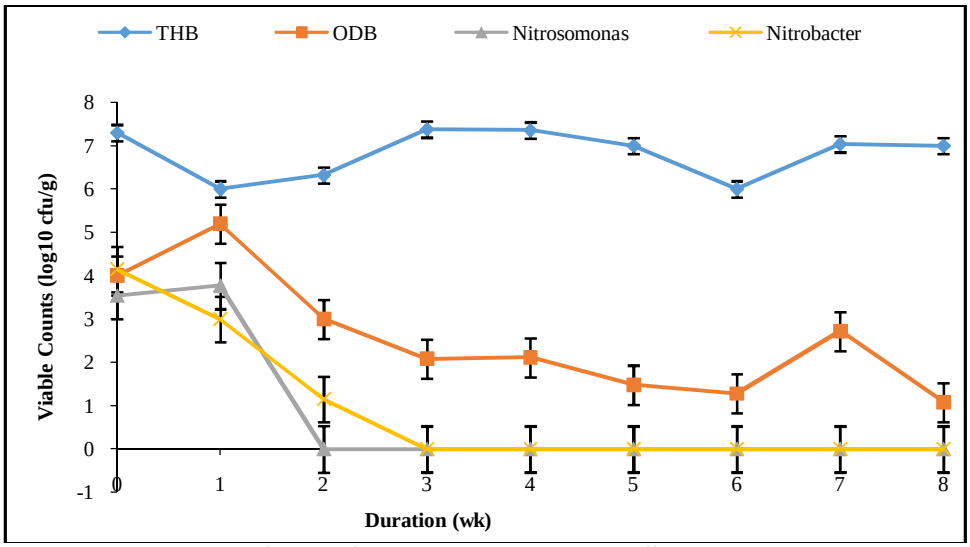

Figure 5: Influence of 20% biostimulant (maize chaff) plus B. subtilis – HSC 1 application on the activities of bacteria in garden soil contaminated with 4.16 % crude oil

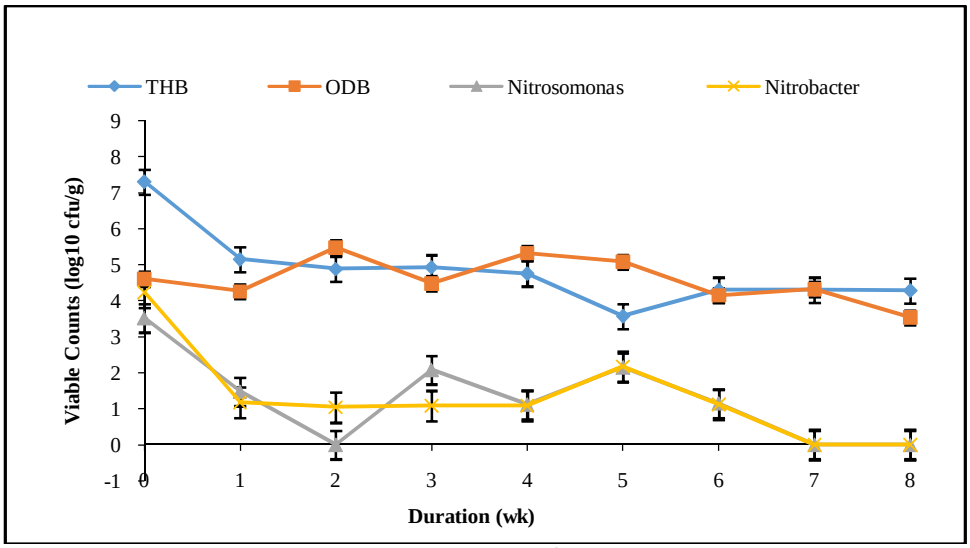

Figure 6: Changes in microbial counts of unpolluted soil (control 1)
Key: THBC = Total heterotrophic counts; ODB = Oil degrading bacteria

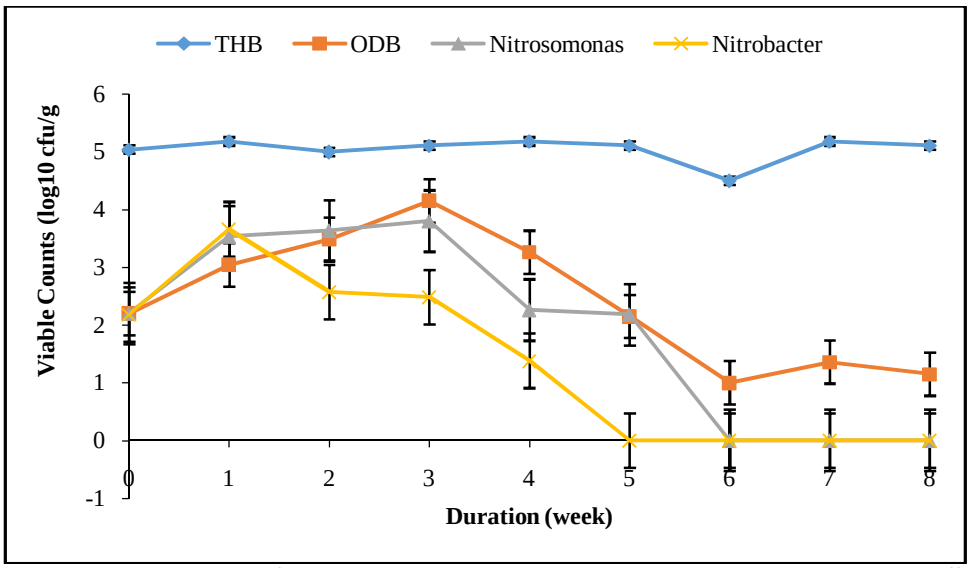

Figure 7: Changes in bacterial count of polluted soil remedied with un- bacterized bio-stimulants (maize chaff) (control 2)

Quantity of soil treated	Amount of oil added	Level of Contamination	Amount of maize chaff added	Biostimulant/Crude Oil Ratio	Residual Load of TPH in remedied soil(mg/kg)	Rate of biodegradation	% Degradation
4 kg Bio-stimulated & Bacterized	83.2 g (20.8 g/kg)	2.08 %	40 g (1 %)	0.480	2149.49	18650.51	89.66
4 kg Bio-stimulated & Bacterized	91.52 g (22.88 g/kg)	2.28 %	200 g (5 %)	2.185	819.39	22060.61	96.42
4 kg Bio-stimulated & Bacterized	104 g (26 g/kg)	2.6 %	400 g (10 %)	3.346	445.51	25554.49	98.28
4 kg Bio-stimulated & Bacterized	124.8 g (31.2 g/kg)	3.12 %	600 g (15 %)	4.807	1535.66	29664.34	95.07
4 kg Bio-stimulated & Bacterized	166.4 g (41.6 g/kg)	4.16 %	800 g (20 %)	4.807	3673.34	37926.66	91.16
4 kg Con. 2 Bio-stimulated No Bacterization	83.2 g (20.8 g/kg)	2.08 %	40 g (1 %)	0.480	14776.33	6023.67	28.95

Table 6: The rate of degradation obtained from the various remediation treatments using maize chaff

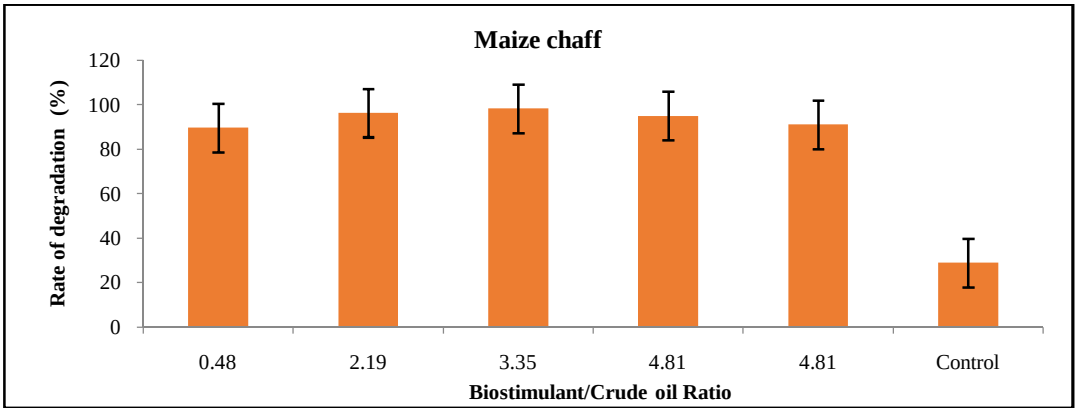

Figure 8: Influence of biostimulant/crude oil ratio on the rate of hydrocarbons degradation in garden soils

Parameter		Results (mg/kg)
	Maize chaff	Unpolluted soil
Naphthalene	0.02	0.02
2-Methylnaphthalene	0.02	0.02
Acenapthylene	0.02	0.02
Acenaphthene	0.02	0.02
Fluorene	0.03	0.03
Phananthrene	0.03	0.03
Anthracene	0.02	0.02
Flouranthene	0.01	0.01
Pyrene	0.02	0.02
Benzo(a)anthracene	0.04	0.02
Chrysene	0.03	0.03
Benzo(b)flouranthene	0.03	0.02
Benzo(k)flouranthene	0.04	0.03
Benzo(a)pyrene	0.04	0.03
Dibenzo (a, h) anthracene	0.05	0.02
Benzo (g, h, i) perylene	0.03	0.02
Indeno(1,2,3-d) pyrene	0.11	0.05
Total	0.37	0.09

Table7: Polycyclic aromatic hydrocarbons profile of the biostimulants (maize chaff) and unpolluted soil

4. Discussion

The total hydrocarbon content (THC) of the soil is a measure of the hydrocarbon content of the soil. Sources of hydrocarbon accumulation in an environment include natural sources (e.g. plant and animal matter, oil seeps); the atmosphere; accidents during transportation, storage, or use of petroleum products, inland oil exploration and exploitation, as well as municipal/industrial wastes. The THC content of the garden soil used for the study was below detectablelimit of 10 mg/kg, indicating the absence of hydrocarbon contamination. Hence the level (0.09 mg/kg) of polycyclic aromatic hydrocarbons (PAHs) was negligible.

The composition maize chaff used as bio-amendment for the remediation of contaminated soil revealed waste substrates of nutritional significance. The presence of resident microflora initiates these processes within the shortest time, in an attempt to utilize it as sole carbon source (Robertson *et al*., 2010). The lipid contents observed were 3 ± 0.2 % which was low and much lower than 11.6 % reported for banana peels (Essien *et al*., 2005). Lipids are vital to the structure and biological functions of cells and are used as alternative energy source. The fibre contents of the biostimulants were high but Ajanakua *et al*., (2010) reported a higher value of fibre and ash in spent grains. The crude fibre content (1.7 ± 0.17 %) was observed for the maize chaff. Fibre contain appreciable amount of nutrients which are released slowly in further enzymatic action. High fibre content reduces the rate of glucose and fat absorption in biological cells (Mottram, 1979). It is implied here that the low fibre content of the biostimulants may likely favour the growth of fastidious microorganism.

Microorganisms play an important role in the degradation of environmental pollutants. The science of bioremediation is greatly enhanced by the presence of a diversity of microbes which strive even in extreme conditions and concentrations of pollutants. However, microbial growth and metabolism in impacted areas can be mitigated by a number of factors such as pH, temperature, concentration of pollutants, moisture content, conductivity, oxygen content, nutrient availability and bioavailability, and the properties of the impacted soil medium (Rahman *et al*., 2002). The test soil had rich microbial loads with remarkable populations of heterotrophic bacteria and but low numbers of oil degrading bacteria. Precisely diverse species of bacteria were isolated from the test soil and most of the bacteria encountered have previously been isolated by Aliyu and Bala (2011). Amongst the 12 isolates only 5 exhibited the ability to degrade hydrocarbons. These include *Alcaligenes, Enterobacter, Micrococcus, Pseudomonas, Bacillus* and *Corynebacterium* species and may have contributed to the low count of oil degrading bacteria in the garden soil. On the other hand, the organic amendment was laden with heterotrophic bacteria but very poor in densities of oil degraders.

There are volumes of literatures on bacterial degradation of crude oil in the ecosystem (Ijah and Antai, 2003; Al-Wasify and Hamed, 2014). It is interesting to know that such wastes like maize chaff is rich in nitrogen necessary for crude oil degradation but contain relatively low numbers of oil degraders.

Stimulated biodegradation of crude oil is at present encouraged because it ensures rapid remediation of oil polluted ecosystems (Ijah and Antai, 2003; Malik and Ahmed, 2012). Much study has been conducted on the remediation of crude oil contaminated ecosystems where organic amendments are commonly used to enhance remediation process (Abioye *et al*., 2012; Adams *et al*., 2014). Studies have also been conducted on the bio-augmentation of remediation processes with microbes with strong hydrocarbon degrading potentials. In this study, both approaches were technically employed to enhance crude oil degradation in soil. The study used microbially augmented bio-stimulants (maize chaffs) for the remediation process. In this case, 20 ml (2.9 cfu/ml) of a strong crude oil degrading strain, *B. subtilis* isolated from a humic ecosystem, was "cropped" or introduced into the biostimulants.

The present study has shown that soil contamination with crude oil drastically reduced the population of denitrifying bacteria but increased the population of oil degrading bacteria in soil but has concentration-dependent effects on the densities of heterotrophic bacteria. Our findings revealed that the higher the biostimulants/contaminant ratio added, the more the heterotrophic activities but less hydrocarbonoclastic activities. This observation correlates with the report of Atlas and Bartha (1972) who reported that the application of crude oil to Arctic tundra soil caused overall increase in microbial numbers compared to un-oiled reference (control) soil. However, this study reveals that heterotrophic bacterial isolates were dominant in both polluted and unpolluted soil compared to other physiological groups of microorganisms, probably because heterotrophic bacteria are more numerous in the soil. Some of them are fast-growing and capable of utilizing a wide variety of organic compounds for survival (John *et al*., 2012).

The population of oil degrading microorganisms was higher in polluted soil than in the unpolluted soil. Similarly, large populations of hydrocarbon degrading bacteria were observed for oil polluted environments (Popp *et al*., 2006; Iiori *et al*., 2015). Population levels of hydrocarbon degraders within the microbial community appear to be an indication of environmental exposure to hydrocarbons. The nature of microbial population usually reflects the extent of exposure of a particular environmental hydrocarbon contamination (Chikere *et al*., 2009). Nevertheless, the reverse was the case in nitrifying bacterial isolates (*Nitrosomonas* and *Nitrobacter*). It was also observed that the polluted soil had a lower population than the unpolluted soil. This could be attributed to a rapid multiplication of some microorganisms which used up the available inorganic nitrogen for growth leaving nitrifiers at disadvantage. Among the nitrifiers, it was also observed that oil adversely affected the *Nitrobacter* more than *Nitrosomonas*. It was apparent that when the quantity of oil was increased, a smaller number of *Nitrobacter* was found than that of *Nitrosomonas*. This may be ascribed to the fact that *Nitrosomonas* oxidizes ammonia first to nitrite making use of the available oxygen leaving small amount for *Nitrobacter* to use in the oxidation of nitrite to nitrate.

The bacterial population dynamics observed during crude oil degradation could also be associated with the degradation patterns of the crude oil hydrocarbons in the different treatment options when compared with the non-amended crude oil. The activities of hydrocarbonoclastic microorganisms clearly influenced the hydrocarbons degradation rates in the remedied substrates. The research results have revealed that the rate of the degradation of crude oil and it components was faster when enhanced with biostimulant (Maize chaff) "cropped" with oil degrading strain of *B. subtilis* -HSC 1 than when carried out alone with biostimulants. The influence of 5% level of biostimulant application on the activities of heterotrophic bacteria in garden soil contaminated with 2.28% crude oil augmented

with oil degrading *Bacillus subtilis*, revealed a significant relationship (r=0.687) (Appendix I) between total heterotrophic bacteria and oil degrading bacteria. Crude oil degradation rates were found to decrease with increase in percentage of organic amendment applied. In soil remedied with microbially cropped maize chaff, the best degradation rate (98.28 %) was attained when 10 % was applied with a BC ratio of 3.35.

The present study has revealed a general increase in the population of hydrocarbonoclastic bacteria in oil polluted than unpolluted soil. This could be attributed to the stimulation of the hydrocarbon degraders in the soil and adaptation of the bacterial population to the oil. The enhanced remediation using microbially "cropped" or activated on the properties of the test soil revealed improvement in the levels of plants essential components.

5. Conclusion

The study has shown that bacterization of organic amendment with potent hydrocarbons utilizing species of bacteria would readily enhance the potency of biostimulation and the degradation of hydrocarbons in contaminated soils. The key to increasing the rate of biodegradation of hydrocarbon contaminant is to optimize the growth rate of indigenous terrestrial oil degrading microflora. Cropping of maize chaff with 2.9 x 10^4cfu/ml of broth culture of crude oil degrading strain of *B. subtilis* has proved to be effective in this regard as 10% level at a BC ratio of 3.35 of the amendment promoted extensive hydrocarbon activity and biodegradation of crude oil in soils.

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