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MICROBIAL COMMUNITY COMPOSITION OF TOP AND SUB SOIL LAYERS IN AN AGED OIL-SPILL SITE IN BOMU COMMUNITY, OGONILAND

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ABSTRACT

A holistic knowledge of the microbial community structure colonizing different soil depths is essential in designing effective microbial based remediation strategies. Top soil (TS) and sub soil (SS) samples at 0 - 15 cm and 0 - 35 cm depths were collected from an aged crude-oil spilled site in Bomu community, Rivers State characterized with microbiological and physicochemical analytical methods. Samples were enriched in Bushnell Haas broth and screened for the presence of oil-degrading bacteria and fungi. Total petroleum hydrocarbon (TPH) and polycyclic aromatic hydrocarbon (PAHs)constituents for TS and SS were 7439.59; 14.58 mg/kg and 8653.03; 1.21 mg/kg, respectively while mean values for hydrocarbon utilizing bacteria and fungi counts for TS and SS were 1.9×10^5; 0.5×10^3cfu/g; and 4.3×10^5; 0.4×10^3cfu/g, respectively. Bacterial and fungal community compositions were identified using phenotypic and microscopic techniques. A total of 24 bacterial species encompassing 11 genera and 10 fungal species from 7 genera were isolated and confirmed as oil degrading microorganisms using biodegradation assay. The bacterial genera for TS included Proteus, Salmonella, Citrobacter, Enterobacter, Klebsiella, Bacillus and Corynebacterium while SS were Escherichia, Flavobacterium, Corynebacterium, Pseudomonas, andBacillus. Gamma Proteobacteria were the dominant class across both soil layers. Fusarium spp. and Rhizopus spp. were the dominant fungal isolates for SS and TS, respectively. The different soil layers were variable in the microbial composition and abundance as well as physical and chemical soil characteristics.

Key words: Bioremediation, PAHs, TPH, Bomu Community, Ogoniland.

INTRODUCTION

Hydrocarbons are group of compounds that consist of hydrogen and carbon in their structure. The increase in the numbers of petrochemical industries worldwide has resulted to contamination due to increasing rate of oil spills. This is one of the major environmental problems faced globally (Chikere and Ekwuabu 2014). The environment is particularly being contaminated with accidental releases of petroleum products. This can be direct or indirect. Some of these chemicals are persistent organic pollutants (POPs). They are globally dispersed accumulating in

wildlife like seals and polar bears and in our bodies (Alexendra and Johanna, 2016).

The effect of pollution on the Niger delta people has been great. As a result of oil spills and industrial waste dumped into the Niger Delta River, fishing is no longer a viable occupation for the people who prior to the discovery of crude oil were predominantly fishermen.

A total of 116,459.67 barrels of crude oil was spilled in 3,663 incidents between 2010 and 2014 according to the 2016 report of the Department of Petroleum Resources (DPR), Nigeria's regulatory body for the oil and gas industry (DPR, 2016). A major spill incident in the Niger Delta was the December 2010 Bonga oil spill, where an estimated 40,000 barrels of crude oil was spilled across 950 square kilometers of water (SNEPCo, 2011).

Bacteria and fungi play a central role in hydrocarbon degradation. This is aided by the ability of the microorganism to utilize the hydrocarbons in other to satisfy their cell growth and energy needs. (Grupte and Sonawdekar, 2015). When there is low level hydrocarbon, the hydrocarbon degrading microorganisms present in such soil samples often thrive as minor members of the microbial communities (Hamamura *et al.*, 2006). The occurrence of crude oil contamination increases the microbial biomass of the crude oil degrading organisms. If these contaminants are of different compounds, there is a shift in organisms or a succession that leads to the proliferation of organisms with metabolic capability to utilize the different compound and become enriched with it (Powell *et al*., 2006). The light hydrocarbons are usually the first metabolized by these organisms before the more recalcitrant complex hydrocarbons (Baldwin *et al*., 2008).

The occurrence of an oil spill brings about the proliferation of the local communities of oleophilic microbes adapted to that environment. It takes a lot of time for the oleophilic microbes to increase in response to the influx of the new resources. When the normal chemical and physical environment is altered, there is often a lag period by which the microbial community adapts to the new conditions. Hydrocarbonoclastic bacteria usually help in the removal of alkanes which results in the formation of carbon dioxide and water, (Straud*et al*., 2007).

The occurrence of crude oil spillage affects the microbial diversity of the soil micro-flora. For this reason, there is the need to investigate the bacterial communities and diversities of a long term spilled site. (Adesina and Adelasoye, 2013). Chikere and Obieze (2018) investigated the diversity and fungal community shift after spiking and amending oil polluted soils with varying organic and inorganic fertilizers.

The aim of this research was to determine the microbial community structure and function at varying depths of an oil polluted soils in Bomu community, Ogoniland and to determine the amenability of the polluted soil to microbial based remediation.

MATERIALS AND METHODS

Study area

The hydrocarbon polluted soil was obtained from a polluted soil at Bomu in Ogoniland. The site was selected due to high level of pollution as a result of oil spill. Ogoniland is one of the many indigenous people in the region of the South South of Nigeria with 1.5 million people in a 404 - square - mile (1050 km^2) homeland.

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Sampling

The soil was collected with a soil auger at a depth of 0-15cm for top soil and 15-30cm for sub soil into a sterile polythene bags. The samples were transported to the Environmental Microbiology laboratory of the University of Port Harcourt, Nigeria. Samples were subsequently analysed in parallel for physicochemical and microbiological properties.

Determination of physicochemical parameters of sample

Physiochemical parameters such as pH, Electrical Conductivity (EC), Phosphate, Nitrate, Moisture Content (MC), Total Organic Carbon (TOC), Heavy metals (Zinc (Zn), Lead (Pb) and Nickel (Ni)), Total Petroleum Hydrocarbon (TPH) and Poly Aromatic Hydrocarbon (PAH) were determined according to methods of ASTM, (1999) and APHA, (1998).

Gas chromatographic analysis

This analysis was done to determine the residual total petroleum hydrocarbon and polycyclic aromatic hydrocarbon using Gas chromatogram ionization detector. The extraction of petroleum hydrocarbon was done with dichloromethane (DCM) using cold extraction method with ASTM D-3694 heavy machine for 1 hour.

Enumeration of total heterotrophic bacteria (THB) and fungi (TF)

THB counts were determined using plate count agar (PCA). From both the sub and top soil samples, 1g of soil was homogenized in 9ml of normal saline. Decimal dilutions (10 fold) of the suspensions was plated out on the PCA medium and incubated at 30^{o}C for 24 hours. The colony forming units were afterwards enumerated according to Chikere and Ekwuabu (2014). The medium of choice for fungi isolation and enumeration was Dichloran Rose Bengal Chloramphenicol (DRBC) agar. It involved the inoculation of DRBC with 0.1 ml of the sample suspension. The plates were then incubated at 30^{o}C for 48 hours after which the colony forming units were enumerated.

Enumeration and isolation of hydrocarbon utilizing bacteria (HUB) and fungi (HUF)

Hydrocarbon utilizing bacteria (HUB) was enumerated by a method adopted from Chikere and Ekwuabu, (2014) which involved a 10 fold dilutions of both samples and plating out differently on a mineral salt medium (Bushnell-Hass Agar) (Sigma-Aldrich, USA). Hydrocarbon was then supplied to putative hydrocarbon utilizes by placing sterile Whatmann No. 1 filter paper impregnated with 5ml crude oil on the lids of the inverted plates and incubated for 14days at 30^{o}C. Colony forming unit (cfu/g) was thereafter calculated.

Purification and characterization of hydrocarbon utilizing bacteria and fungi

Discreet colonies of the different HUB and HUF of both samples were randomly picked using a sterile inoculating wire loop and sub cultured for purification by streaking on nutrient agar plates in duplicates and incubated at 30^{o}C for 24 hours. Individual bacterial colonies of both samples were principally identified using biochemical tests as described in Bergey's Manual for Determinative Bacteriology. The fungi isolates were identified by both morphological and microscopic features using Atlas for fungi identification (Larone, 2011).

Biodegradation screening

Axenic bacterial cultures from both soil samples were subjected to further biodegradation screening using okono medium crude oil. Test tubes containing bushnell Haas broth was amended with sterile crude oil and inoculated with individual isolates to determine the potential of the isolates to utilise hydrocarbons as sole carbon source. One test tube containing Bushnell Haas broth and crude oil served as the control. Samples were drawn on days 0, 3, 9 and 12 from both the inoculated and control test tubes to determine changes in turbidity of the media using spectrophotometer at AU_{600}. Increase in turbidity of the media is directly proportional to hydrocarbons utilisation (Chikere *et al*., 2015)

RESULTS

Top soil and Sub soil physicochemical parameters

Results of the physicochemical parameters that affects microbial diversity and hydrocarbons degradation that were analysed during this study is shown in Table 1. The pH of both soils were at 5.8 and 5.5. Moisture content, phosphate, nitrate, heavy metals and TPH concentrations between the top and sub soils were also not largely different. C30 hydrocarbon with concentration of 7439.59 mg/kg was detected in the Top soil while the Sub soil sample contained 8653.03 mg/kg of C40 hydrocarbon. Fig 3. and 4 shows the gas chromatographic chromatograms of TPH for the Top and Sub soils.

Table 1 Soil physicochemical parameters

Parameter	Method	Top soil	Sub-soil
pH	APHA 4500H^+B	5.8	5.5
Electrical conductivity, µs/cm	Cond. Meter	120	10
Phosphate (P_2O_5), mg/kg	APHA 4500-P	<1.0	<1.0
Nitrate (NO_3), mg/kg	APHA 4500- NO^{3-}	0.1	0.1
Moisture content, %	BS 1377-2:1990 clause 1	12.5	9.4
Total Organic Carbon, %	BS 1377-3:1990 clause 3	9.15	3.09
Zinc (Zn), mg/kg	ASTM D 1691	0.09	0.95
Lead (Pb), mg/kg	ASTM D 3559	<0.001	<0.001
Nickel (Ni), mg/kg	ASTM D 1886	<0.001	0.23
Total Petroleum Hydrocarbon (TPH), mg/kg	EPA 8015	7439	8653
Poly Aromatic Hydrocarbon (PAH), mg/kg	EPA 8260	14.5	1.21

Analysis of poly-aromatic hydrocarbons

Floureanthene, Benz (a) anthracene, Chrysene, Benzo (b) flouranthene, Benzo (a) pyrene, Benzo (g, h, i) perylene, Dibenz (a, h) anthracene and Indeno (1,2,3-cd) pyrene were the PAHs detected in the top soil. Only Benzo (a) pyrene and Benzo (g, h, i) perylene were detected in the sub soil. Their concentration is shown in table 2 while Fig. 1. and Fig. 2. shows the chromatograms and peaks of the detected poly-aromatics.

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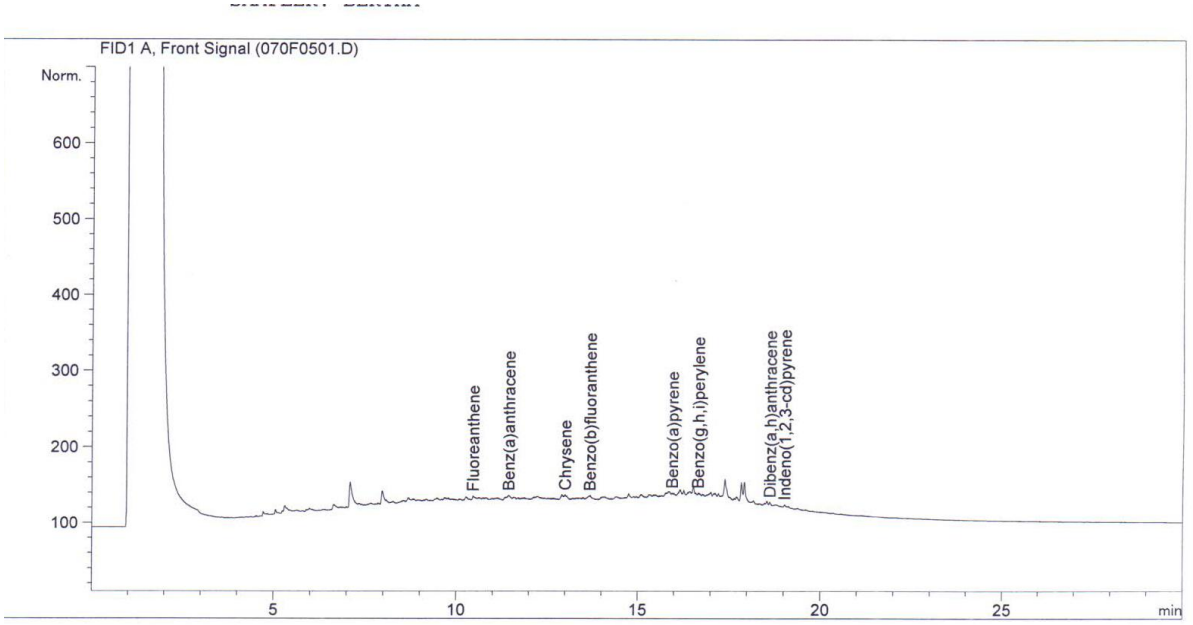

Figure 1 Chromatogram of top soil sample for PAH

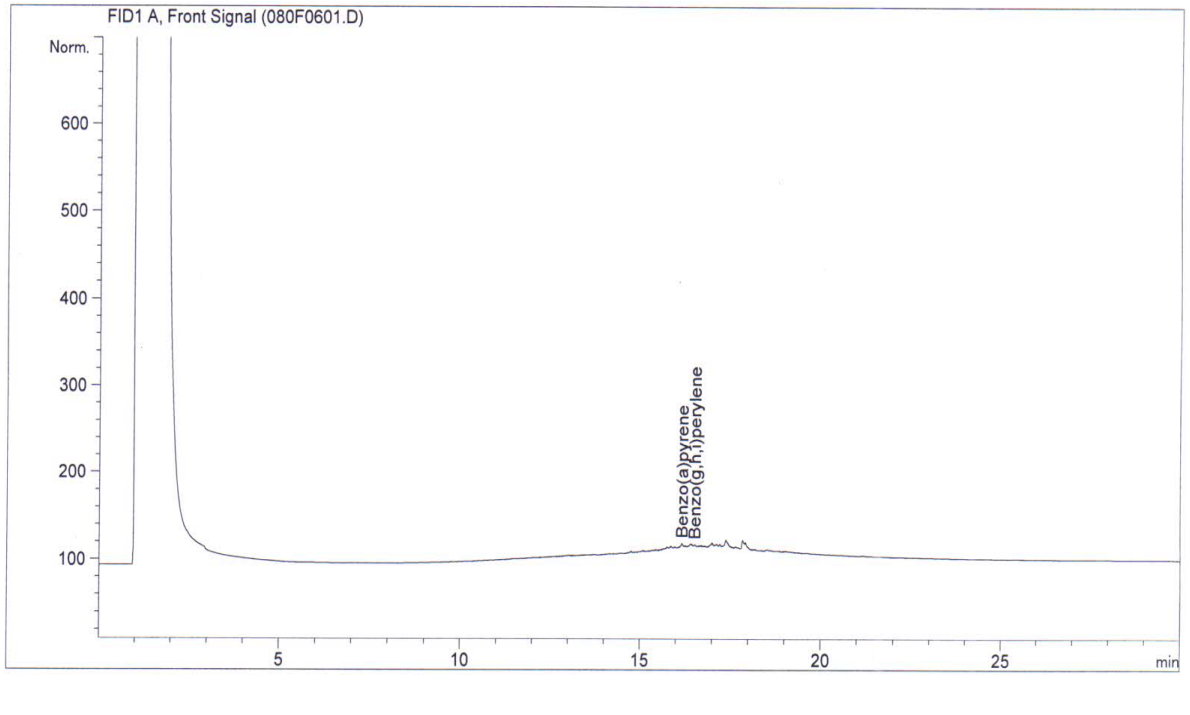

Figure 2 Chromatogram of sub soil polluted sample for PAH

Table 2 Polycyclic aromatic hydrocarbon constituents of both soil layers

S/N	PAH	Top soil (mg/L)	Sub soil (mg/L)
1	Naphthalene	-	-
2	Acenaphthylene	-	-
3	Acenaphthene	-	-
4	Fluorine	-	-
5	Phenanthrene	-	-
6	Anthracene	-	-
7	Floureanthene	1.48671	-
8	Pyrene	-	-
9	Benz (a) anthracene	4.20662	-
10	Chrysene	3.26903	-
11	Benzo (b) flouranthene	9.50658^{-1}	-
12	Benzo (k) flouranthene	-	-
13	Benzo (a) pyrene	1.42038	7.72767^{-1}
14	Benzo (g, h, i) perylene	1.56567	4.39288^{-1}
15	Dibenz (a, h) anthracene	9.26930^{-1}	-
16	Indeno (1,2,3-cd) pyrene	7.22472^{-1}	-
Total		14.58448	1.21206

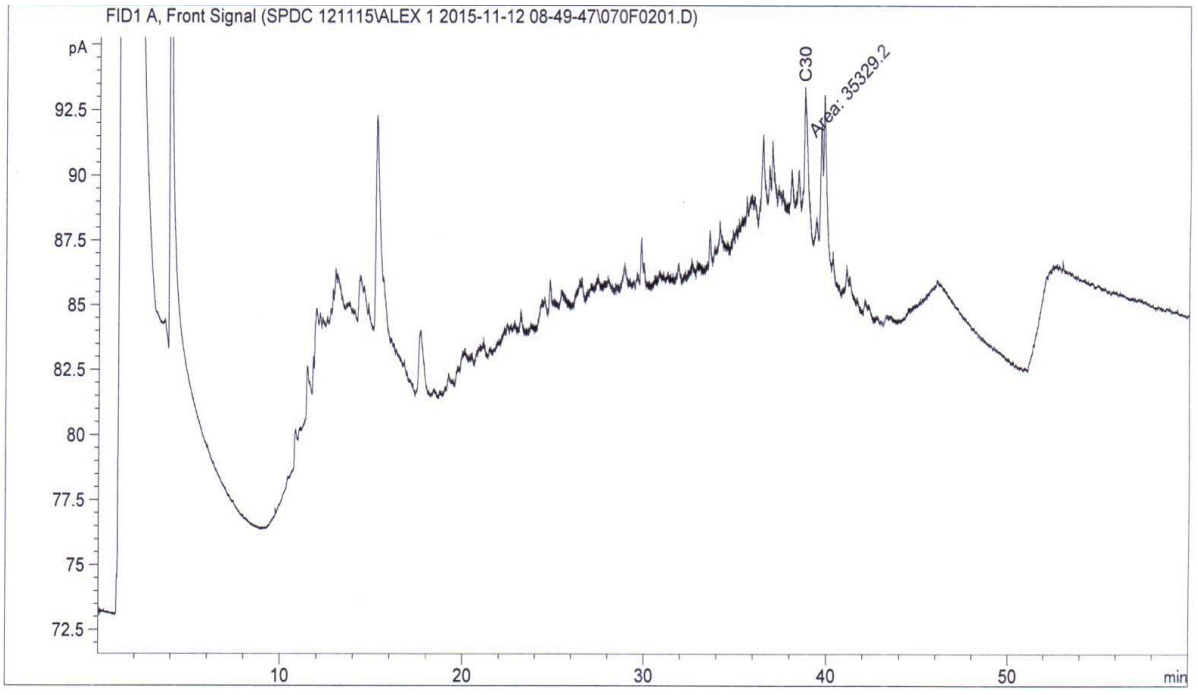

Figure 3 Chromatogram of top soil polluted sample for TPH

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Analysis of total petroleum hydrocarbons
Analysis of TPH on both top and sub soil samples to determine the concentration and class of hydrocarbons revealed the presence of heavy fractions of saturated hydrocarbons. Table 3. Shows the concentration of TPH and the class of hydrocarbons detected for both soils. Fig. 4. and 5. are the chromatograms showing the detected hydrocarbons.

Table 3: Total petroleum hydrocarbon constituents of both soil layers

S/N	TPH	TOP SOIL (mg/kg)	SUB SOIL (mg/kg)
1	C8	-	-
2	C9	-	-
3	C10	-	-
4	C11	-	-
5	C12	-	-
6	C13	-	-
7	C14	-	-
8	C15	-	-
9	C16	-s	-
10	C17	-	-
11	Pr	-	-
12	C18	-	-
13	Ph	-	-
14	C19	-	-
15	C20	-	-
16	C21	-	-
17	C22	-	-
18	C23	-	-
19	C24	-	-
20	C25	-	-
21	C26	-	-
22	C27	-	-
23	C28	-	-
24	C29	-	-
25	C30	7439.58507	-
26	C31	-	-
27	C32	-	-
28	C33	-	-
29	C34	-	-
30	C35	-	-
31	C36	-	-
32	C37	-	-
33	C38	-	-
34	C39	-	-
35	C40	-	8653.03041
	TOTAL	7439.58507	8653.03041

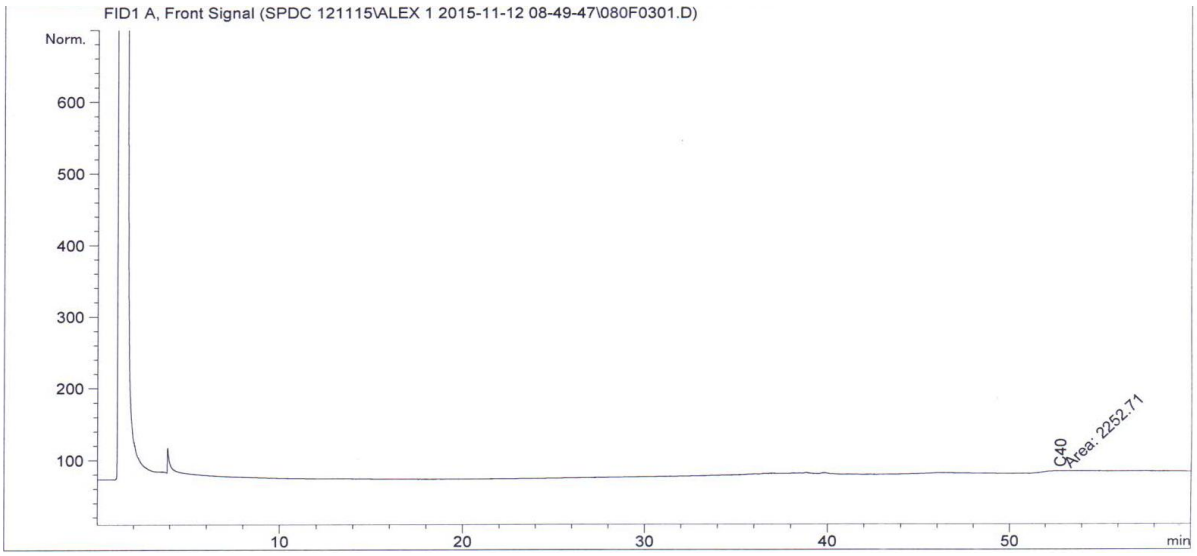
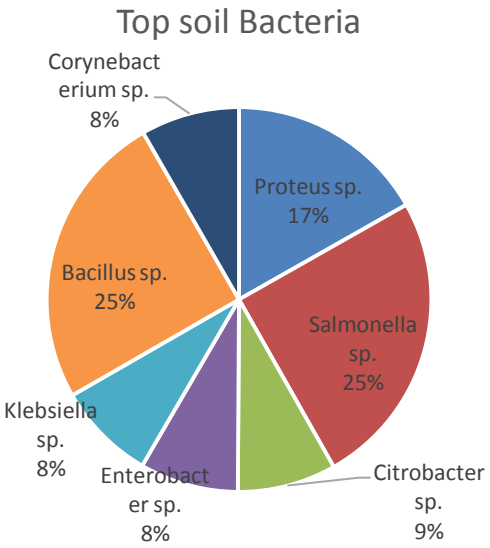
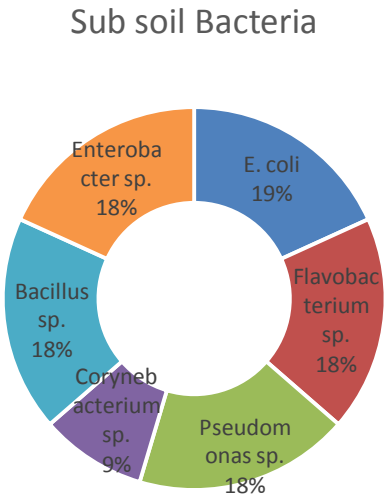


Figure 4: Chromatogram of top soil sample for TPH

Microbial diversity and enumeration

The mean heterotrophic bacterial and hydrocarbon utilizing bacterial count is shown in Table 4. The percentage hydrocarbon degrading bacterial species in the top soil was 75% while the hydrocarbon degrading fungi was 23%. The fraction of hydrocarbon utilizers in the Sub soil for bacteria and fungi was 35.8% and 40% respectively. The percentages and diversity of the organisms recovered from this study is shown in Fig. 5.





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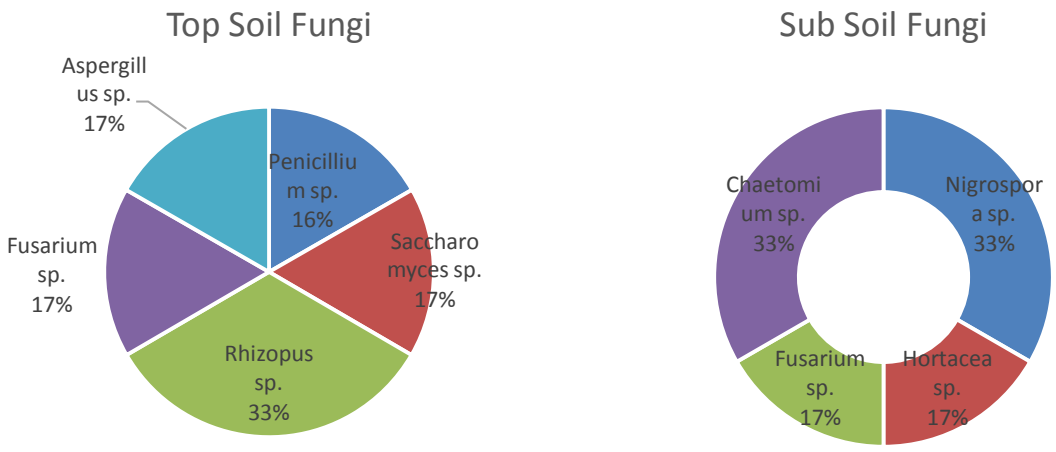

Figure 5 Percentage occurrence and diversity of the top and sub soil microbes

Table 4: Mean count of recovered microbial species from the Top and Sub soil samples

Sample	Mean of THB	Mean of HUB	Mean of TF	Mean of HUF
Sub soil (cfu/g)	5.3×10^5	1.9×10^5	1.0×10^3	4.0×10^2
Top soil (cfu/g)	5.7×10^5	4.3×10^5	2.1×10^3	5.0×10^2

Degradation screening

A total of 24 bacterial (11 genera) isolates was obtained from the two samples as putative hydrocarbon utilizing bacteria and fungi using Bushnell Hass broth with crude oil supplied as carbon source which were further screened for their degradation potential using spectrophotometer. From the result, 24 bacterial isolates were significant for crude degradability assay evidenced by increase in media turbidity (biomass increase). The results of the degradation screening and tentative identities of each of the screened bacterial cultures is shown in Table 5. and 6.

Table 5: Characterization of bacterial isolates from top soil sample

Isolate code	Microscopy/Gram Reaction	Morphology (Macroscopy)	Tentative Identity	Degradation screening/rate
HUB 1	Rod (-)	Large, circular, grey, smooth colonies	*Proteus* sp.	(++)
HUB 2	Rod (-)	Large, circular, grey, smooth colonies	*Proteus* sp.	(++)
HUB 3	Rod (-)	Small, moist, grey, raised colonies	*Salmonella* sp.	(++)
HUB 4	Rod (-)	Small, moist, grey, raised colonies	*Salmonella* sp.	(++)
HUB 5	Rod (-)	Small, moist, grey, raised colonies	*Salmonella* sp.	(++)
HUB 6	Rod (-)	Small, moist, grey, flat, shiny colonies	*Citrobacter* sp.	(+++)

HUB 7	Rod (-)	Small, irregular, smooth colonies	*Enterobacter* sp.	(++)
HUB 8	Rod (-)	Small, circular, convex colonies	*Klebsiella* sp.	(+++)
HUB 9	Rod (+)	Small, cream, flat, circular colonies	*Bacillus* sp.	(+++)
HUB 10	Rod (+)	Small, cream, flat, circular colonies	*Bacillus* sp.	(+++)
HUB 11	Rod (+)	Small, cream, flat, circular colonies	*Bacillus* sp.	(+++)
HUB 12	Rod (+)	Irregular, greyish-white raised colonies	*Corynebacterium* sp.	(+++)

* +, ++, +++ = Degradation rate
Rod (+) =Gram Positive Rod
Rod (--) =Gram Negative Rod

Table 6: Characterization of bacterial isolates from sub-soil sample

Isolate code	Gram reaction	Morphology	Tentative identity	Degradation screening
HUB 1	Rod (-)	Small, circular, cream, slightly raised colonies	*Escherichia* sp.	(++)
HUB 2	Rod (-)	Small, circular, cream, slightly raised colonies	*Escherichia* sp.	(++)
HUB 3	Rod (-)	Smooth, shiny, small, convex colonies	*Flavobacterium* sp.	(++)
HUB 4	Rod (-)	Smooth, shiny, small, convex colonies	*Flavobacterium* sp.	(++)
HUB 5	Rod (-)	Circular, raised, cream, shiny colonies	*Pseudomonas* sp.	(++)
HUB 6	Rod (-)	Circular, raised, cream, shiny colonies	*Pseudomonas* sp.	(++)
HUB 7	Rod (+)	Irregular, greyish-white raised colonies	*Corynebacterium* sp.	(++)
HUB 8	Rod (+)	Small, cream, flat, circular colonies	*Bacillus* sp.	(++)
HUB 9	Rod (+)	Small, cream, flat, circular colonies	*Bacillus* sp.	(++)
HUB 10	Rod (-)	Small, irregular, smooth colonies	*Enterobacter* sp.	(++)
HUB 11	Rod (-)	Small, irregular, smooth colonies	*Enterobacter* sp.	(++)
HUB 12	Rod (-)	Small, irregular, smooth colonies	*Enterobacter* sp.	(++)

* +, ++, +++ = Degradation rate
Rod (+) =Gram Positive Rod
Rod (--) =Gram Negative Rod

DISCUSSION

This study investigated the physicochemistry, microbial diversity and function in two layers of oil polluted soil. A comparative analysis of this nature, detailing the microbial community of different soil layers, will be crucial in developing effective remediation strategies.

Physicochemical parameters analysed during this study is shown in Table 1. The values shown by the result indicated both samples had been exposed to hydrocarbon pollution. The sub soil and top soil comprised aromatic hydrocarbons as well as C30 and C40 saturated hydrocarbon compounds. The result suggest lighter

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fractions of the crude oil have been possibly removed by both physical and biological processes that occur naturally due to the age of the spill. Soil pH is important because it can affect the availability of nutrients and the survival of certain microbial species. Vidali (2001) and Chawla *et al*. (2013) studies revealed that for biodegradation to occur, the pH should have an optimum value 6.5-8.0 while for microbial activity to occur, the pH value should be between 5.8 - 8.8. The almost acidic pH (Topsoil: 5.8 and Subsoil: 5.5) did not result in a higher presence of fungal species, neither did it affect microbial activity or their ability to utilize hydrocarbons in any of the investigated soils.

Microbial activity was determined by the enumeration of the culturable THB and TF. Mean values for hydrocarbon utilizing bacterial and fungi counts for Topsoil and Subsoil were 1.9×10^5 cfu/g; 5.0×10^2 cfu/g; and 4.3×10^5; 0.4×10^3cfu/g respectively. The Topsoil sample had a higher microbial count compared to the Subsoil, this result is similar to that of Nunan *et al*. (2003) where microbial counts of Cropped Topsoil, Fallow Topsoil and Subsoil were compared. Their study revealed that the Subsoil had the least microbial count. The presence of more nutrients and oxygen in topsoil could be responsible for the always higher microbial number. The high percentage of hydrocarbon utilizing microorganisms, particularly in the Topsoil is an indication that the indigenous microorganisms have adapted to the environmental stress posed by the oil spill. Chikere *et al*. (2015) suggested that long term exposure of bacterial species to hydrocarbon pollution increases their potential to develop the necessary catabolic genes for hydrocarbon degradation.

Biodegradation screening using turbidometric method showed a higher degradation rate by isolates recovered from the Topsoil. Similar finding was made by Chikere and Ekwaubu (2014). The fungal and bacterial species screened as hydrocarbon utilizers have severally been implicated as dominant species in crude oil polluted environments (Gesinde *et al*., 2008; Ibiene *et al*., 2011; Ferrari *et al*., 2011; Chikere *et al*., 2012; Obiukwu and Otokunefor 2014; Chikere and Obieze, 2018).

The diversity of organisms in the Topsoil was different from that of the Subsoil (Fig. 5). This could have been influenced by nutrient availability, difference in hydrocarbons constituent composition (C40-Subsoil; C30-Topsoil) and the differences in pH and conductivity.

The proliferation of hydrocarbon degrading bacterial species in both the Topsoil and Subsoils, suggest the amenability of the study site to microbial based remediation. This is supported more by the disappearance of the lighter fractions of hydrocarbons from the polluted site, suggesting natural hydrocarbon sequestration which could be linked to either biological or physical processes.

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