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Original Research Article

Microbial community structure of an oil polluted site in Effurun, Nigeria

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This study was done to ascertain the microbial diversity of a hydrocarbon polluted soil in Effurun, Uvwie Local Government Area of Delta State, Nigeria. Physicochemical analyses were carried out on the polluted and unpolluted (control) soil samples. Total petroleum hydrocarbon contents (TPH), and concentrations of some extracted heavy metals (lead, copper and zinc) were measured and calculated, comparatively. Mean values of total heterotrophic bacteria (THB), hydrocarbon utilizing bacteria (HUB), total heterotrophic fungi (THF) and hydrocarbon utilizing fungi (HUF) were also recorded in control and polluted samples. A total of nine bacterial (*Acinetobacter, Bacillus, Enterobacter*, *Escherichia, Klebsiella, Micrococcus, Proteus, Pseudomonas* and *Staphylococcus)* and seven fungal (*Aspergillus, Candida, Fusarium, Geotrichum, Mucor, Penicillium* and *Trichosporon*) species were identified. The study showed that these species are resistant to hydrocarbon contents and are able to utilize them as a source of energy. Hence, these species play important roles in remediation of oil in the environment.

Key words: Crude oil pollution, microbial remediation, total heterotrophic bacteria (THB), hydrocarbon utilising bacteria (HUB), total heterotrophic fungi (THF).

INTRODUCTION

The economic benefits of crude oil cannot be over emphasized however, there are well-known hazardous effects of direct and/or indirect contamination on human health. There are great toxicological concerns of natural or incidental spill on the environment. Thus, oil spill affects the microbial community composition of the soil, alters biogeochemical cycles, and triggers the mechanisms affecting sustainable soil fertility and environmental quality (Thapa et al*.*, 2012).

Soil is inhabited by a variety of microorganisms, including bacteria, fungi, algae, viruses and protozoa (Tamames et al*.*, 2010). Soil microorganisms play vital roles in the ecosystem; they adjust energy flow, cycle nutrients, plant and oil residues, and play a pivotal role in growth and development of agricultural crops, balance of the soil ecosystem, organic matter transfer and bioremediation. Furthermore, the diversity of the microbial community in soil is closely related to the function and structure of its surrounding ecosystem, and is one of the components to maintain soil productivity (Thorsten et al*.*, 2015).

The toxicity of crude oil or petroleum products vary widely, depending on their composition, concentration, environmental factors and on the biological state of the organisms at the time of the contamination. Degradation of hydrocarbons by natural population of microorganisms is the main process acting in the clean-up of hydrocarbons (Jain et al*.*, 2011). Several microorganisms including fungi, bacteria and yeasts are involved in biodegradation process.

Although the presence of these microorganisms in an oil-polluted environment is an evidence that they may be active in degradation of hydrocarbons, the reports involving algae and protozoa are relatively scarce (Das

and Chandran, 2011).

Due to the abilities of certain microbes to mineralize hydrocarbon components into environmentally friendly substances such as carbon dioxide and water, the ability of bacteria and fungi in breaking down hydrocarbons has gained growing attention in modern day research (Kadali et al., 2012). Biodegradation by microbes is the key removal process of hydrocarbons which is controlled by hydrocarbon physico-chemistry, environmental conditions, bioavailability and the presence of catabolically active microbes (Chorom et al., 2010).

Soil microbial communities can be extremely diverse (Delmont et al., 2011) and alters with variable ecological factors. It is clear that, among the multiple species of microorganisms that share one ecological niche, one or a few population that best adapts to the new ecologic conditions becomes predominant, while others exist as minorities. Molecular ecological approaches may be able to detect the dominant species, where traditional culture-dependent methods may fail to do so (Christoserdova, 2010).

Hydrocarbon pollution surely changes the diversity, and this kind of alteration usually results in nutrient depletion and destruction of viable microorganisms. Studies have suggested that biodiversity of the bacterial communities may dramatically be reduced because of the presence of hydrocarbons in the environment, despite an increase in the hydrocarbon utilizing microbes' percentage. This often leads to selective enrichment of hydrocarbon degraders, to the relative detriment of biodiversity (Alonso-Gutierrez et al., 2011).

Degradative capabilities of the communities are mainly dependent on physicochemical properties of hydrocarbons, and the presence of active hydrocarbon utilizing microorganisms. Under normal conditions, environmental breakdown of complex hydrocarbons is relatively slow, and needs some complex interactions of microbial communities. It has been observed that fast growing strains are the best adapted ones to specific culture media, and they grow preferentially than those which are not. Therefore, do not accurately represent the actual microbial community composition during aerobic biodegradation of crude oil (Chikere et al., 2012).

The aim of this study is to determine the microbial diversity of the crude oil polluted site in Effurun community, Delta State, Nigeria, and to investigate if the findings can be used for bioremediation of this study site, or not.

MATERIALS AND METHOD

Sample Collection

The soil samples were collected from a crude oil polluted soil in Effurun, Uvwie, Local Government Area of Delta State, Nigeria. Polluted and unpolluted samples were collected using a soil auger at 0-15cm depth randomly to form composite samples. A global positioning system (GPS) was used to determine the coordinates of the location; 5°34'49"N, 5°46'56"E (polluted soil) and 5°34'27"N, 5°46'38"E (control). The samples were aseptically collected inside sterile labelled polyethylene plastic bags, transported to the laboratory and stored inside an ice chest at 4 ±1°C, for further analyses.

Physicochemical Analysis

Analysis of physicochemical parameters (pH, moisture content, total organic carbon, TOC) and heavy metals of soil samples were performed according to APHA (2008) methods. The amounts of phosphates, sulphates and nitrates were measured by UV spectrometer (UV-VIS SPEC Model UV 9200) at 882nm, 420nm and 470nm, respectively. After readings of standards; the contents of magnesium, calcium, sodium and potassium of all of the samples were determined by specific lamps designed to emit light of specific wavelength at 285.2nm for magnesium, 422.7nm for calcium, 589nm for sodium and 766.5nm for potassium. Cationic exchange capacity (CEC) was calculated based on absorbance value of Mg^{2+}, Ca^{2+}, K^{+} and Na^{+}, by using the following formula for each:

$$\text{Exchangeable ion} = \frac{\text{absorbance} \times 100}{1000 \times \text{No of positive charges}}$$

Heavy Metals Analysis

Lead, copper and zinc concentration in polluted and control samples were quantified by using atomic absorption spectrophotometer (Buck Scientific Model 210 VGP).

Chromatographic Analysis of Total Petroleum Hydrocarbon and Polyaromatic Hydrocarbons

Total petroleum hydrocarbons (TPH) and polycyclic aromatic hydrocarbons (PAHs) were extracted and quantified with gas chromatograph-mass spectrometry (GC - MS).

Enumeration and Identification of Total Heterotrophic Bacteria (THB)

Total heterotrophic bacterial (THB) counts were determined by the methods of spread plate on nutrient agar (NA) and plate count agar (PCA), as adapted from Chikere and Ekwuabu (2014). From each sample, 1g or 1ml were homogenized in 9ml of 0.85% normal saline, using Stuart vortexing machine. Decimal dilutions (10-fold) of the suspensions were plated out on agar medium and after incubation at 30°C for 24 hours, colony forming units were counted. Discrete colony forming were randomly picked and sub-cultured onto nutrient agar (NA), for further purification. Purification was done by streaking twice and transferred onto agar slant for storage, and identified with the biochemical tests described in Bergey's Manual for

Table 1. Physicochemical parameters of soil samples used in the study

Parameters	Polluted soil	Unpolluted soil
Ph	6.80	7.3
Electrical Conductivity (EC) µs/cm	63	69
Total Organic Carbon, %	0.36	0.20
Moisture Content, %	10.17	11.25
Cation Exchange Capacity, meq/100g	24.56	24.44
Sulphate, mg/kg	4.85	1.61
Nitrate, mg/kg	<0.001	15.42
Phosphate, mg/kg	0.75	0.42
Potassium, mg/kg	76.83	76.13
Sodium, mg/kg	519.97	255.52
Calcium, mg/kg	4400	4352
Magnesium, mg/kg	235.97	374.42
Lead, mg/kg	12.47	7.53
Copper, mg/kg	16.08	12.07
Zinc, mg/kg	37.01	157.85
Total petroleum hydrocarbon (TPH), mg/kg	100.68	0.01
Poly aromatic hydrocarbons (PAHs), mg/kg	9.21	<0.0001

Determinative Bacteriology (Holt et al., 1994).

Isolation and Identification of Total Heterotrophic Fungi (THF)

Fungal isolates were counted using the spread plate method on potato dextrose agar (PDA) containing streptomycin to prevent bacterial growth in triplicates, according to the method of Benal et al. (2014). Appropriate dilutions of soil samples were inoculated on the agar medium, and incubated at 28±2°C for 3 days in the dark. Discrete fungal colonies forming on PDA were further purified by sub-culturing on PDA, by using the tweezing method. Pure fungal isolates were studied by lactophenol stain: a small portion of the fungal growth was picked with an inoculating loop and placed on a clean glass slides, it was flooded with lactophenol cotton blue (LPCB) and the preparation was covered with a cover glass slip. The slides were observed and examined by a light microscope.

Enumeration and Identification of Hydrocarbon Utilizing Bacteria (HUB) and Fungi (HUF)

Vapour phase method of Iheanacho et al. (2014) was adopted. Dilutions of samples were inoculated onto triplicate sterile petri dishes containing mineral salt agar (MSA). The MSA comprised of KH_2PO_4 (1g), K_2HPO_4 (1g), NH_4NO_3 (1g), $MgSO_4$ (0.2g), $FeCl_2$ (0.05g), CaCl (0.02g), agar agar (15g) and distilled water. The vapour phase method was adopted, after the medium was solidified, sterile filter paper (Whatman no.1) saturated with filter sterilized crude oil was placed inside the cover of the Petri dish, closed, inverted and incubated at 30°C for 7 days for bacterial isolates and 28±2°C in the dark for fungal isolates. The filter paper saturated with crude oil served as a sole source of carbon. Bacterial colonies from the MSA were further purified by subculturing on nutrient agar twice and transferred into agar slant for storage and identified using biochemical tests described in Bergey's Manual for Determinative Bacteriology (Holt et al., 1994). Fungal isolates from MSA were further purified by tweezing on PDA twice and transferred into agar slant for storage. Fungal morphology were studied macroscopically and microscopically using lactophenol cotton blue. The fungi were identified following the scheme of Barnett et al. (1990) and Malloch (1997).

Screening for degradative abilities

The method of Okerentugba et al. (2016) was used for the screening test. A loopful of a 24 hour culture of the isolates were inoculated into a sterile 100 ml of mineral salt broth (MSB) containing sterile crude oil (10% v/v) and redox indicator (2% v/v of 2,6-dichlorophenol indophenols). A control was also set up containing no microorganisms and left for 14 days.

RESULTS AND DISCUSSION

The physicochemical parameters of the soil samples (polluted and unpolluted) are shown in Table 1. The higher pH value (7.3) was recorded for the control sample which is neutral/slightly alkaline, while a lower soil pH (6.8) was recorded for the polluted sample. The decrease in pH value may be due to increase in degradation of crude oil by microorganisms in the soil resulting in accumulation of acidic metabolites (Ejileugha et al., 2015). As shown in Table 1, the electrical conductivity (EC) of the control sample was 69µs/cm and the polluted sample was 63µs/cm. According to Pathak et al. (2011), EC is a function of level of contamination at the polluted site, the higher the level of spill the lower the EC.

The total organic carbon (TOC) values were 0.20% and

Table 2. Mean values of microbial counts (cfu/g)

Microorganisms	Polluted soil	Unpolluted soil
Total heterotrophic bacteria (THB)	3.7×10^6	3.0×10^4
Hydrocarbon utilizing bacteria (HUB)	2.8×10^5	3.8×10^3
Total heterotrophic fungi (THF)	5.8×10^3	1.0×10^3
Hydrocarbon utilizing fungi (HUF)	4.0×10^3	0.5×10^2

0.36% for control and polluted samples, respectively. It was concluded that oil polluted soil had more carbon contents, when compared to that of the control sample. However, the moisture content of crude oil polluted soil was lower than that of control, with values of 11.25% for the control, and 10.17% for polluted samples. Snehal (2014) reported that either crude oil can coat the soil and consequently prevent the penetration of water, or the microorganisms utilize the water for their activities.

The amount of nitrate, phosphate, and sulphate were 15.42mg/kg, 0.42mg/kg, 1.61mg/kg and less than 0.001mg/kg, 0.75mg/kg, 4.85mg/kg for the control and polluted samples, respectively. Ibiene et al. (2011), reported the absence of nitrate in the polluted soil as an indicator that the limiting nutrients were released to the microorganisms involved. Also, crude oil pollution leads to the deterioration of soil mineral nutrients. Nutrients are essential for biodegradation to occur, hence the limiting nutrients (nitrogen, phosphate) should be added to speed up the process. This was similar to our observations.

The concentration of lead in the polluted sample was higher than the concentration in control. Lead concentration in polluted soil was 12.47mg/kg and the control soil sample was 7.53mg/kg. The concentration of copper in the two soils tested can be seen in Table 1. Higher concentration was measured in polluted sample, as 16.08mg/kg; while the lower concentration was in control as 12.07mg/kg. The polluted sample concentration for zinc was 37.01mg/kg, and was lower than that of the control which was measured as the higher value, 157.8 mg/kg. According to Tanee and Eshalomi-Mario (2015), increasing concentration of heavy metals in polluted soil may be due to the hydrocarbon pollution which altered the physico-chemical parameters of the soil, as well as increased concentration of heavy metals.This was in conformation with our findings.

As expected, concentrations of TPH (100.08mg/kg) and PAHs (9.21mg/kg) measured in polluted sample were extremely higher than that of control (0.01mg/kg and less than 0.0001mg/kg), surely due to hydrocarbon contents of crude oil that can lead to increased toxicity and destroy the ecosystem.

It is known that cfu counts are higher in polluted soil than unpolluted soil, and microbial countings of a contaminated site is the simplest method to monitoring microbial activities that can be used for bioremediation. The mean values of microbial counts obtained from polluted and unpolluted soil are shown in Table 2. In polluted site, THB had a mean value of 3.7×10^6 cfu/g, and HUB with a mean value of 2.8×10^5 cfu/g were measured. However, the values were recorded as 3.0×10^4 cfu/g and 3.8×10^3 cfu/g for the unpolluted samples, respectively. A similar observation was reported by Chikere and Ekwuabu (2014). The mean values of THF and HUF counts in the fresh soil were 1.0×10^3 cfu/g and 0.5×10^2 cfu/g; however, they were 5.8×10^3 cfu/g and 4.0×10^3 cfu/g, respectively, in polluted sample. Presence of low proportion of hydrocarbon utilizers in the estimated total heterotrophic population indicates that, the sample area probably had not been exposed to crude oil.

In this study, a total of 45 pure cultures of bacteria were able to grow on both nutrient agar and mineral salt medium, with crude oil as carbon source. These isolates were characterized by using biochemical tests and phenotypic characteristics as shown in Table 3 and Table 4. In confirming with the report of Eze et al. (2013), the populations of Gram-negative and Gram-positive bacteria that are hydrocarbon degraders, were isolated.

As presented in Table 6, twenty of the bacterial isolates belonging to the genera *Acinetobacter, Bacillus, Enterobacter*, *Klebsiella, Micrococcus, Proteus* and *Pseudomonas,* had the ability to utilize crude oil. The predominant genera recorded; *Bacillus*, *Micrococcus*, *Proteus* and *Pseudomonas* have been previously reported as oil and hydrocarbon degrading microorganisms (Chikere and Ekwuabu, 2014). In the manner of THB, *Escherichia* and *Staphylococcus* are known not able to utilize the hydrocarbons of crude oil.

As shown in Table 5, fungal species belonging to a total of seven known genera, and one unidentified fungus were also isolated; and listed as moulds (*Aspergillus*, *Fusarium*, *Mucor*, *Penicillium* and *Trichosporon)* and yeasts *(Candida* and *Geotrichum). Aspergillus*, *Candida* and *Penicillium* were the predominant fungi. All of the moulds and yeasts were known petroleum-utilizers, as seen in Table 6. Similar observations were reported by Obire and Anyanwu (2009) and Akpoveta et al. (2011). In accordance with the findings of Benal et al. (2014), majority of the population of hydrocarbon degrading fungi isolated in this investigation belonged to deuteromycetous fungi.

During the biodegradation screening, all of the bacterial isolates except *Escherichia* and *Staphylococcus,* and fungal isolates had the ability to grow on crude oil, by utilizing it as a carbon and energy source. Their abilities were scored by turbidity, emulsification and colour change from deep blue to colourless. Table 6 shows the degradative abilities of microbial isolates based on degradation of 10ml mineral salt broth containing 0.1ml crude oil and redox indicator.

Table 3. Biochemical identification of bacterial isolates

Isolate	Starch hydrolysis	Casein hydrolysis	Gelatin hydrolysis	Methyl red	Vogue Proskeur	Indole	Citrate	Catalase	Oxidase	Urease	H_2S	Phenylalanine deamination	Motility	Spore formation	Triple sugar iron (TSI)			Dnase	Probable isolate
															Slant	Butt	Gas		
HUB1	-	-	+	-	-	-	+	+	+	-	-	+	-	-	K	A	-	-	*Acinetobacter*
HUB2	+	+	+	+	-	-	-	+	-	-	-	-	+	+	K	A	-	+	*Bacillus*
HUB3	-	-	-	-	+	-	+	+	-	+	-	-	+	-	A	A	+	-	*Enterobacter*
HUB4	+	+	-	-	+	-	+	+	-	+	-	-	-	-	A	A	+	-	*Klebsiella*
HUB5	-	+	+	-	-	-	+	+	+	-	-	+	-	-	K	K	-	-	*Micrococcus*
HUB6	-	+	+	+	-	-	-	+	-	+	+	+	+	-	A	A	+	-	*Proteus*
HUB7	-	+	-	-	-	-	+	+	+	-	-	-	+	-	K	K	-	-	*Pseudomonas*
THB1	-	-	+	-	-	-	+	+	-	-	-	-	-	-	K	K	-	+	*Staphylococcus*
THB2	_	_	_	+	_	+	_	+	_	_	_	_	+	_	A	A	+	_	*Escherichia*

Key: HUB = Hydrocarbon utilizing bacteria, THB = Total heterotrophic bacteria, + = Positive result, - = Negative result A = Acid (Yellow), K = Alkaline (Red)

Table 4. Morphological characteristics of bacterial isolates

Isolate	Identified isolate	Macroscopic Characteristics	Microscopic Characteristics
HUB1	*Acinetobacter*	Small, creamy irregular raised and transparent colonies	Gram negative coccobacilli
HUB2	*Bacillus*	White slimy irregular convex colonies with lobate margin	Gram positive rods
HUB3	*Enterobacter*	Creamy, smooth, mucoid, convex colonies with entire margin	Gram negative rods
HUB4	*Klebsiella*	Small non motile colonies	Gram negative rod
HUB5	*Micrococcus*	Yellowish small round and smooth opaque colonies	Gram positive cocci
HUB6	*Proteus*	Moist swarming colonies	Gram negative rods
HUB7	*Pseudomonas*	Small, creamy, round smooth mucoid, flat and opaque colonies	Gram positive rods
THB1	*Staphylococcus*	Small golden brown, round, smooth, irregular, flat, convex and translucent colonies	Gram positive cocci
THB2	*Escherichia*	Shiny mucoid, slightly raised colonies with entire margin	Gram positive rods

Table 5. Fungal macroscopic and microscopic characteristics

Isolate	Macroscopic	Microscopic	Probable isolate
HUF1	Creamy to white, circular, mucoid, raised and opaque colonies with entire margin.	Round to oval shaped ellipsoidal budding cells with blastoconidia and pseudohyphae. Some cells appeared singly and others in pairs.	*Candida*
HUF2	Black, circular with vesicle, downy to powdery, entire, umbonate colonies.	Hyphae are septate and hyaline. Conidiophores are long, smooth and hyaline. Spores are in chain from the phialides arising from the central vesicle.	*Aspergillus*
HUF3	White to creamy, globose to ellipsoidal, woolly to cotton, spreading flat colonies.	Phaliades are short, bean shaped, macroconidia not in chains and are fusiform.	*Fusarium*
HUF4	White to greyish brown, wooly to cotton spreading colonies and darkens with time.	Broad non septate hyphae. Sporangiophores are long and terminates in a round spore filled sporangium. Absence of rhizoids.	*Mucor*
HUF5	Greeny with white edge, velvety to powdery colonies.	Hyphae are septate, hyaline, conidiophores are branched. Phaliades appear like brushlike clusters at ends of conidiophores. Conidia are round and in chains.	*Penicillium*
HUF6	Colonies are white to creamy with flat shiny mucoid appearance.	Mycelia has septate hyphae, hyaline. Arthroconidia are slightly barreled shaped in chains and budding cells.	*Trichosporon*
HUF7	Off -white to creamy velvety textured colonies without mycelium.	Hyphae are septate and hyaline and branched. Aeriel hyphae produce arthroconidia in chains.	*Geotrichum*

HUF = Hydrocarbon utilizing fungi

Table 6. Screening for degradative abilities

Isolate	Tentative identity	Degradative ability
HUB1	*Acinetobacter*	P
HUB2	*Bacillus*	P
HUB3	*Enterobacter*	P
HUB4	*Klebsiella*	P
HUB5	*Micrococcus*	P
HUB6	*Proteus*	P
HUB7	*Pseudomonas*	P
THB1	*Staphylococcus*	N
THB2	*Escherichia*	N
HUF1	*Candida*	P
HUF2	*Aspergillus*	P
HUF3	*Fusarium*	P
HUF4	*Mucor*	P
HUF5	*Penicillium*	P
HUF6	*Trichosporon*	P
HUF7	*Geotrichum*	P

P = positive (degrades hydrocarbon), N = negative

The change in colour from deep blue to colourless, was due to the reduction of the redox dye by the oxidized product of the degradation process. This was also reported by Iheanacho et al. (2014) and Okerentugba et al. (2016).

The exposure of microorganisms to hydrocarbons makes them better suited to degrade the pollutants through higher growth and replication, and more efficient metabolism. Thus, they can be used to maximize the rate of hydrocarbons removal from the soil.

CONCLUSION

This study has revealed that, the autochthonous microbial community of an oil spill site can be used for bioremediation. However, simultaneous action of several metabolically versatile microorganisms, with favourable environmental conditions such as pH, temperature and availability of nutrients, are required for an effective degradation of crude oil. In the case of terrestrial oil spill, it is better to manage the appropriate conditions for remediation that depends on diversity and functional soil microbial communities. However, further investigations based on molecular or culture-independent methods are necessary, in order to profile microbial biomass, diversity or activity of novel organisms, and enzymes associated with hydrocarbons degradation.

Conflict of interest

No conflict of interest exits in the submission of this manuscript.

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