Elevational Gradient Effects on Microbial Distribution in Crude Oil Polluted Sites

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Abstract

Crude oil is a major environmental pollutant in the Niger Delta region of Nigeria due to heightened operations of the petroleum industry in this location. This comparative study of two different petroleum hydrocarbon-polluted soils from the Bodo and Tombia communities in Rivers State was carried out to determine microbial abundance (distribution) in relation to the depth and soil texture of the polluted sites. Soil samples were collected at 15 cm and 30 cm from each site. Baseline physicochemical parameters and microbial counts were determined using standard methods. Hydrocarbon utilizing bacteria were screened with 2,6-dichlorophenol indophenol (DCPIP) for crude oil degradation efficiency. The average total petroleum hydrocarbons (TPH) collected from the areas values are higher than the intervention value of 5000 mg/kg as stipulated by the Department of Petroleum Resources (DPR) in the Environmental Guidelines and Standards for the Petroleum Industry in Nigeria (EGASPIN). Microbial distribution patterns were not significantly different (p=0.08) between the topsoil and subsoil in the sites investigated. There was no significant variance in the relationship between soil texture and microorganism distribution (p=0.998). An interaction of the effects between the two independent variables produced an insignificant variance (p=0.411). Average viable culturable heterotrophic and hydrocarbon utilizing microbial counts for the sites varied considerably between 105 and 106 colony forming units per gram of soil (cfu/g) at both depths. A consortium of *Pseudomonas spp.*, *Proteus spp.*, *Sphingobacterium spp.*, and *Bacillus spp*. isolated from the sites efficiently degraded crude oil, showing a 100% decolourization of DCPIP in Bushnell-Haas-crude oil broth. This study demonstrated that soil texture and depth affect microbial diversity and their function in crude oil contaminated sites.

Introduction

Crude oil, a hydrocarbon-containing material, is one of the most serious environmental contaminants. It contains n-alkanes, cycloalkanes, and polycyclic aromatic hydrocarbons (PAHs), which are harmful to the environment and human health [4]. Drilling, shipping, and processing of these products, among other operations, can cause crude oil pollution. Saturated and aromatic hydrocarbons, asphaltenes, and resins make up the most crude petroleum oil [19].

Oil spills are common occurrences because of manufacturing and transportation errors due to the widespread usage of oil and petroleum products in our everyday life [18]. Oil is a liquid fossil fuel extracted from below the ground, formed over millions of years due to high pressure and heat. Because of the time required for it to form, it is a nonrenewable energy source. The liquid components of oil accumulate in porous rock formations, and different types of crude, "sweet or sour ", have varying sulphur content [2]. Crude oil is refined and used as fuel for transportation or cooking in forms such as petrol, diesel, cooking gas, etc., with other derivatives such as bitumen used for binding asphalt, which is used in making roads [14, 2].

For over a century, crude oil has been extracted from subsurface reservoirs with devastating effects on communities and environments from which it is being extracted due to spillage. In Ogoniland, Nigeria, the contamination from spillage, vandalism and illegal refineries, killed large areas populous with mangroves. Levels found in the more polluted sites are high enough to cause severe impacts on the ecosystem and on human health [24].

Toxic effects may occur when the spill is new and has a substantial amount of light aromatic hydrocarbons. The oil can coat vegetation and water masses, suffocating them and causing death and affecting animal life in the area of contamination. Most of the toxic hydrocarbons evaporate or are degraded by microbes within the soil quite quickly [24]. Polycyclic aromatic hydrocarbons (PAHs) build up quicker in soil, being strongly adsorbed onto sediment, making soil a noteworthy PAH sink [26]. Crude oil pollution may have effects on physical properties of soil such as decreased soil aeration, decreased soil macroporosity/ permeability, hydraulic conductivity, and increased bulk density, which could affect vegetation. One research found soil sampling beyond the depth of 15 cm a constraint due to hard pan formation from crude oil and soil [1]. Crude oil contamination also inhibits the germination of food crops thus affecting the livelihoods of farmers in areas of spillage [23].

There is a need to recover soil and water polluted by crude oil, which is achieved by a process known as remediation; remediation can be physical, chemical, or biological (bioremediation).

Bioremediation is preferable due to its efficiency with both cost and safety. Furthermore, it does not require the addition of foreign or toxic chemicals, something not true of chemical remediation.

Fig 1: Scene of the oil spill in Ogoniland showing the extent of damage to land and water. Photo credit - George Osodi

Bioremediation mainly uses fungi and bacteria as agents for remediation and can be combined with mechanical treatment and dispersants application. Microorganisms in their metabolic activities produce energy that forms the key to the bioremediation process. The continued presence of desirable microbes with appropriate metabolic capacity is an important prerequisite for the bioremediation process [30, 12]. Subsurface microorganisms tend to be capable of degrading hydrocarbons found in crude oil. Bioremediation methods include bioaugmentation, which involves introducing microorganisms with known degradation ability for the contaminant to a polluted site followed by nutrient enrichment (biostimulation). Biostimulation is when nutrients like nitrogen and phosphorus are added to stimulate microbe growth [36, 30, 8] or organic fertilizers such as poultry droppings, sewage slurry, and cow dung are also used [21]. To determine which microorganisms are best suited for bioremediation at a specific site, microorganisms already present in the polluted site are isolated and their effectiveness tested *ex situ*. Any bacteria found in a contaminated site are likely to have hydrocarbon degrading abilities and the appropriate bacteria can be introduced in situ for remediation. Methods of choice for bioremediation depend on the physicochemical properties of the polluted matrix, on the degree, and the age of the spill. Some families of microorganisms found to be effective in bioremediation include *Proteobacteria, Actinobacteria, Firmicutes* and *Fungi* strains. For effective remediation, variable environmental conditions are controlled such as oxygen availability, hydrocarbon solubility, and nutrient balance [9].

Colony forming units (CFUs) are used to determine the abundance of various microbes in the soil. In order to identify the components of the crude oil, a Total Petroleum Hydrocarbon (TPH) test is needed. This is necessary for the bioremediation process.

This study seeks to find the correlation between elevation gradient (soil depth), soil texture, and microbial distribution (abundance) in polluted soils in response to an observed conflict in the literature reviewed. Some literature concluded that there is reduced microbial presence with soil depth in non-polluted soils, while others concluded that there is no variance in microbial distribution with soil depth in polluted soils.

Methodology

Sample Collection:

The polluted soil samples were collected from 15 cm and 30 cm depths from Bodo, Gokhana, Rivers state, and Tombia, Degema, Rivers state. The coordinates are 2.76NW, E90 and 4.79N, E6.90 respectively. Soil samples were immediately placed on ice before being transported to the laboratory where they were stored at 4°C before handling.

Experimental Setup:

The soil samples were labeled as surface soil and subsurface soil depth respectively. Four (4) for surface and four (4) for subsurface soils. For the Tombia site, the two topsoil samples were labeled TSP I and TSP II with the two subsurface soil samples from the same site labeled TSSP I and TSSP II. For the site at Bodo, topsoil samples were labeled BSP I and BSP II, with the subsurface samples labeled BSSP I and BSSP II.

Microbiological Analysis:

Enumeration of Total Heterotrophic Bacteria and Total Fungi

To enumerate the total heterotrophic bacteria and fungi in the samples, soil samples were given a 10-fold serial dilution after separation in normal saline. Samples were spread across Plate Count Agar plates and Potato Dextrose Agar (PDA) plates in duplicates. The plates were incubated for 24 hours at 37°C while potato dextrose plates were incubated for 72 hours at 28°C. Bacterial colonies and fungal spore forming units were thereafter counted and isolated.

Enumeration of Total Hydrocarbon Utilizing Bacteria and Fungi

To determine the hydrocarbon utilizing bacteria (HUB), samples were inoculated onto Bushnell Haas Agar in duplicates with crude oil placed in the plate cover via the vapour-phase transfer method. The duplicate samples were then incubated at 30 °C for 5 then plates with bacteria colonies between $30 \leq CFU \leq 300$ were counted [7].

For fungi, the Bushnell Haas Agar was supplemented with 0.05% chloramphenicol and incubated for 5-10 days at 30°C. Discreet colonies were sub-cultured on PDA.

Anaerobic plates were all incubated in a self-setup candle jar and incubated under the above stated conditions [17].

Identification of Isolates Using Biochemical and Microbiological Tests

The bacterial isolates recovered from this study were characterized using the following parameters: Gram Staining (GR)

and Morphology. This was done using microscopy. The morphology of bacteria could either be round (cocci), rod-shaped (Bacilli), spiral (spirilla), comma (vibrios) or pleomorphic etc. This is determined using the Gram stain, Catalase Test [34], Methyl Red and Voges-Proskauer [33], Indole Test [16], Motility Test [37], Citrate Test, Lactose Test, Sugar Utilization Test (Glucose, Sucrose & Lactose) [20], Triple Sugar Iron Agar (TSIA) Test [3], and Oxidase Test [29].

Fungal Isolate Identification Procedure

Agar with fungi to be identified was placed on a grease free slide. Lactophenol blue was applied to the cut agar. The slide was then covered with a coverslip and viewed under the 40X lens of a microscope [22].

Based on these results, a most probable microorganism was determined for the samples based on morphology and biochemical activity.

Determination of Total Petroleum Hydrocarbons (TPH):

Polluted soil samples were analyzed using a gas-chromatography flame ionization detector (GC-FID). This method is designed to resolve a broad range of hydrocarbons (i.e., n-C8 to approximately n-C44) [20]. Residual total petroleum hydrocarbon (TPH) and polycyclic aromatic hydrocarbon (PAH) contained in the polluted sample were extracted and then quantified using a gas chromatograph - mass spectrophotometer (GC-MS).

Hydrocarbon Degradation Screening Using Spectrophotometry:

Bacteria degradative abilities were screened using the redox dye, 2, 6-Dichlorophenol indophenol (DCPIP). 1 g of DCPIP was dissolved in 1 L of sterile water for preparation. Organisms to be screened were calibrated with a McFarland standard in normal saline to ensure parity in the amount of culture in all isolates. Each tube was vortexed to homogenize the mixture\. On achieving identical quantity of organism for each tube, 1 ml of organism in normal saline suspension was transferred into 9 ml nutrient broth and incubated for 24 hours at 35 ˚C. Bushnell-Haas broth was prepared according to the manufacturers guides and 9 ml were dispensed into test tubes and supplemented with 0.3 ml of crude oil prior to sterilization. To each test tube, 0.4 ml of the isolates were transferred into the Bushnell Haas broth with sterile medium. 0.3 ml of the dye DCPIP were then added. Preparations were done in duplicates with appropriate controls. The first control comprised DCPIP, BHB and crude oil. The second control contained only DCPIP and BHB while the last control comprised DCPIP, BHB and sterile water. The setup was monitored at an interval of 6 hours for 120 hours for colour and optical density changes using a spectrophotometer at AU600 [7].

Determination of Soil Texture: Sieve Method

The sieve method was used in combination with the soil texture triangle to determine the clay, silt or sand content of the soil. In this method, sieves of different fineness are used. The soil sample is first weighed. The various sieves are stacked according to their fineness from top to bottom in order of least fine to finest. The weighed soil sample was placed in the sieve and the setup was placed in a shaker [15].

The soil was separated into its components based on fineness. Each component was then weighed using a weighing machine accurate to the nearest 0.1 grams. Percentages were assigned to each soil segment and comparison in accordance to the World Reference Base for Soil Resources (WRB) was performed.

Method of Calculation:

% Fraction = [Mass of Fraction(g) / Total Mass of Soil Analyzed (g)] x 100% [31].

The table below shows the soil texture classification according to WRB and USDA:

Table 1: Soil Texture Classification. Table 1 shows the diameter of soil particles in millimeters for each soil texture type in the USDA and WRB measurements. It gives a range to use for measured weight for soil left in pan, giving the weightiest pan being the predominant texture for a soil.

SOIL TEXTURE	DIAMETER (USDA) (mm)	DIAMETER (WRB) (mm)
Clay	<0.002	<0.002
Silt	0.002-0.05	0.002-0.063
Very Fine Sand	0.05-0.10	0.063-0.125
Fine Sand	0.10-0.25	0.125-0.20
Medium Sand	0.25-0.50	0.2-0.63
Coarse Sand	0.50-1.00	0.63-1.25
Very Coarse Sand	1.00-2.00	1.25-2.00

Results

Physicochemical Parameters

The results for the total petroleum hydrocarbons (TPH) and polycyclic aromatic hydrocarbons (PAH) and other physical and chemical parameters such as the pH, conductivity and soil texture analysis are tabulated below.

Table 2: Results of Physicochemical Analysis. These test measure different physical and chemical parameters that may affect microbial growth in the soil. pH as an example gives insight that the soil is more so acidified and flora may be adjusted to suit such environments. Total Petroleum Hydrocarbon (TPH) was above recommended amount by the DPR (5,000 mg/kg) which made a clear show of pollution in the studied areas.

Parameters	Soil Samples			
	Bodo 0-15 cm	15-30 cm	Tombia 0-15 cm	15-30 cm
pH	2.6	2.3	4.7	4.9
Temp (°C)	25.3	24.6	27	30
Conductivity	>2500	1850	550	370
HCO_3^- (mg/kg)	1000	2030	200	190
Phosphate	0.161	0.558	0.551	0.146
Nitrate	1.045	0.924	1.726	3.790
TPH (mg/kg)	15,642.5	11,342	14,684.9	9,647
PAH (mg/kg)	158	ND	500	164

TPH is above the recommended limits of 5,000 mg/kg as prescribed by the department of petroleum resources (DPR) as well as PAH for the Bodo topsoil and Tombia top and sub soils. pH for all soils were found to be more acidic and temperature was moderate within the soils.

Table 3: Soil Texture Analysis. Soil texture was classified using the FAO's relative method of classification. The Tombia soil going by analysis was found to be more clayey (9.56% clay) in comparison to the Bodo soil (0.13% clay) generally, more sandy.

		SOIL SITES			
	Sieve Size (mm)	Bodo Sample		Tombia Sample	
		Weight (g)	% of Total	Weight (g)	% of Total
Coarse Sand	**2.0**	5.8	1.47	8.8	1.57
	1.4	6.1	1.54	20.0	3.56
	0.71	42.4	10.73	126.2	22.45
Medium Sand	**0.5**	62.0	15.70	127.9	22.75
	0.355	99.2	25.11	95.6	17
Fine Sand	**0.25**	119.1	30.15	64.5	11.47
	0.18	43.7	11.06	23.6	4.2
Very Fine Sand	**0.125**	13.2	3.34	24.1	4.29
	0.09	3.0	0.76	17.7	3.15
	0.063	0.4	0.10	22.7	4.04
	Pan <0.063	0.1	0.04	31.0	5.52
	Total	395	100	562.1	100

In Table 3, the results for soil texture are shown. Bodo soil was determined to be sandy soil going by the percentage of sand (99.87% sand, 0.13% clay/silt) while Tombia soil was determined to be silt/clay (90.44% sand, 9.56% silt/clay) relative to Bodo soil using relative classification of soil for agriculture [13].

Microbiological and Biochemical Analysis

Heterotrophic bacteria were enumerated using nutrient agar while the fungi on Potato dextrose agar (PDA). Bushnell Haas agar was deployed for both bacterial and fungal enumeration (fungal enumeration differed by supplementing the agar with an antibiotic (chloramphenicol)).

Table 4: Heterotrophic count. All bacteria and fungi numbers within the soil were counted using Petri plates. This was to find the abundance of microbes at each strata in the soil sampled.

		Bacterial count (CFU/g)		**Fungal count (CFU/g)**	
Tombia		Topsoil	Subsoil	Topsoil	Subsoil
	Minimum	2.125×10^6	1.4×10^5	1.02×10^6	6.0×10^5
	Maximum	1.41×10^7	4.05×10^6	4.05×10^6	3.0×10^7
		Bacterial count (CFU/g)		**Fungal count (CFU/g)**	
Bodo		Topsoil	Subsoil	Topsoil	Subsoil
	Minimum	3.65×10^5	6.0×10^4	8.9×10^5	
	Maximum	5.95×10^6	1.2×10^7	7.15×10^6	

Table 5: Hydrocarbon utilizers. Bacteria and fungi that could utilize crude oil as a growth source were enumerated on a mineral medium.

		Bacterial count (CFU/g)		**Fungal count (CFU/g)**	
Tombia		Topsoil	Subsoil	Topsoil	Subsoil
	Minimum	3.55×10^5	1.055×10^6	2.0×10^4	4.85×10^5
	Maximum	4.7×10^6	4.95×10^7	5.2×10^6	1.85×10^6
		Bacterial count (CFU/g)		**Fungal count (CFU/g)**	
Bodo		Topsoil	Subsoil	Topsoil	Subsoil
	Minimum	4.0×10^5	2.4×10^7	2.1×10^5	4.2×10^5
	Maximum	3.45×10^6	2.71×10^7	1.8×10^6	3.85×10^6

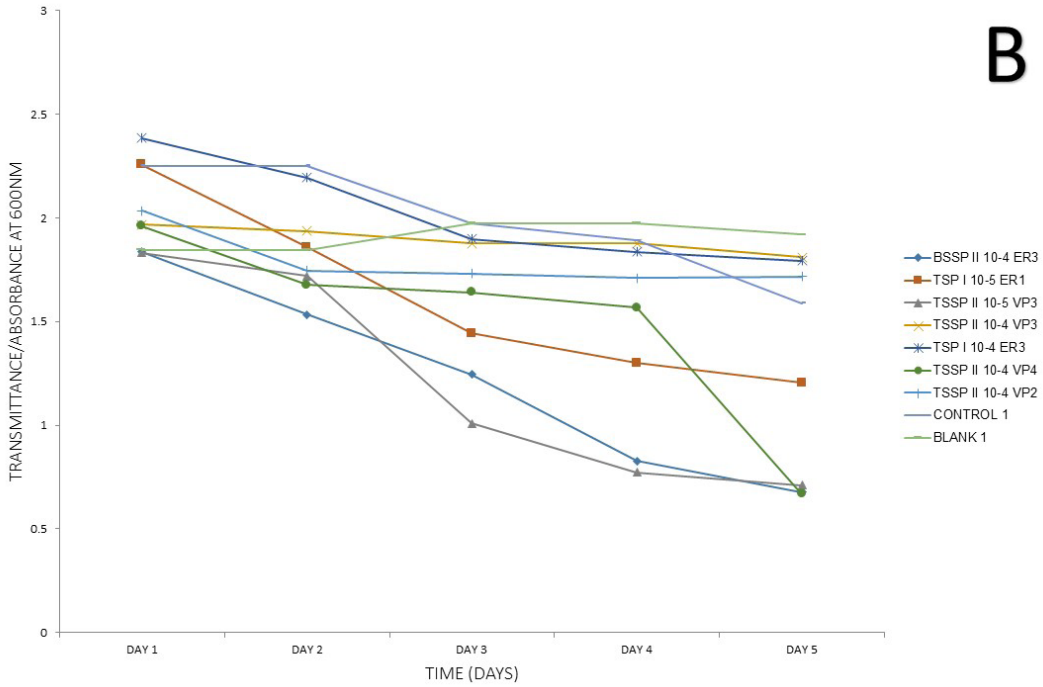
Table 6: Organisms identified and codes. Bacteria discovered from samples identified through biochemical test and microscopic examination are listed out below. *Pseudomonas* was the most abundant species found within all the soils. It is a well-known hydrocarbon utilizer.

Isolate	Probable Identity
TSSP II 10^{-5} VP3	*Sphingobacterium* sp.
TSP I 10^{-4} ER3	*Pseudomonas* sp.
TSSP I 10^{-4} ER1	*Pseudomonas* sp.
BSP II 10^{-4} ER4	*Proteus* sp.
TSSP I 10^{-4} VP3	*Pseudomonas* sp.
TSSP II 10^{-4} VP2	*Bacillus* sp.
BSSP II 10^{-4} ER3	*Staphylococcus* sp.
TSSP I 10^{-4} ER5 V.S.	*Pseudomonas* sp.
TSP 1 10^{-5} ER1	*Pseudomonas* sp.
TSSP II 10^{-4} VP3	*Pseudomonas* sp.
TSP II 10^{-4} ER1	*Bacillus* sp.
TSSP II 10^{-4} VP4	*Pseudomonas* sp.

Biodegradation Screening

Twelve bacterial isolates and four fungal isolates were screened for their degradative abilities on crude oil. This was done using the redox dye 2,6 –dichlorophenol indophenol based on the microorganism's ability to decolourize the dye within a 120-hour period. This was observed using spectrophotometry at optical density AU 600nm taking readings at intervals.

Figure 2 A and B: Degradation Rates of the Isolates for Crude oil. Microorganisms ability to degrade crude oil based on the discolouration of the redox dye 2,6 –dichlorophenol indophenol was performed every 12 hours using spectrophotometry to measure. The organism for TSSP II 10-4, Pseudomonas had one of the greatest powers and was identifiable from the greenish colour growing on Nutrient agar.



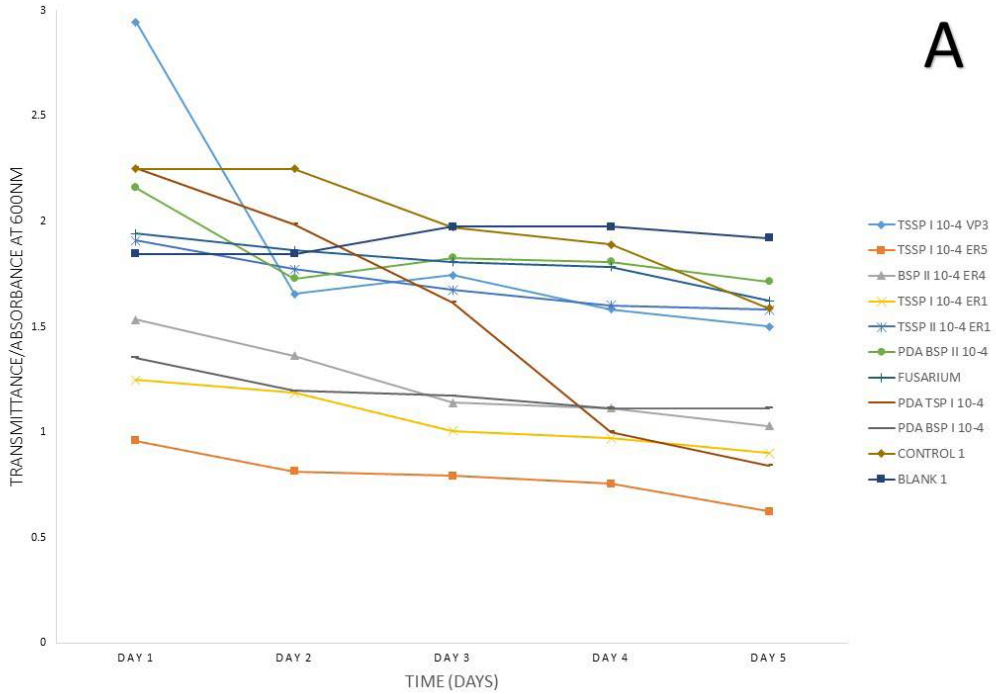


Table 7: Two-way ANOVA Analysis with Replication was carried out to determine the effects of soil texture and soil depth on how microorganisms were distributed throughout samples taken. This was done independently and dependently under each factor under consideration.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Soil Texture (Tombia/Bodo)	1.5E-06	1	1.5E-06	4.92E-06	0.998252
Soil Depth (Topsoil/Subsoil)	1.030033	1	1.030033	3.380498	0.08087
Interaction between factors	0.215083	1	0.215083	0.705887	0.410744
Within groups	6.09397	20	0.304698		
Total	7.339087	23			

Statistical Analysis

A two-way Analysis of variance was performed, shown in Table 7, to determine the first objective of this study – the effect of soil depth and soil texture on the distribution of microorganisms. It tested 3 null hypotheses:

H01: That Soil depth has no significant effect on microorganism distribution

H02: That Soil texture has no significant effect on microorganism distribution

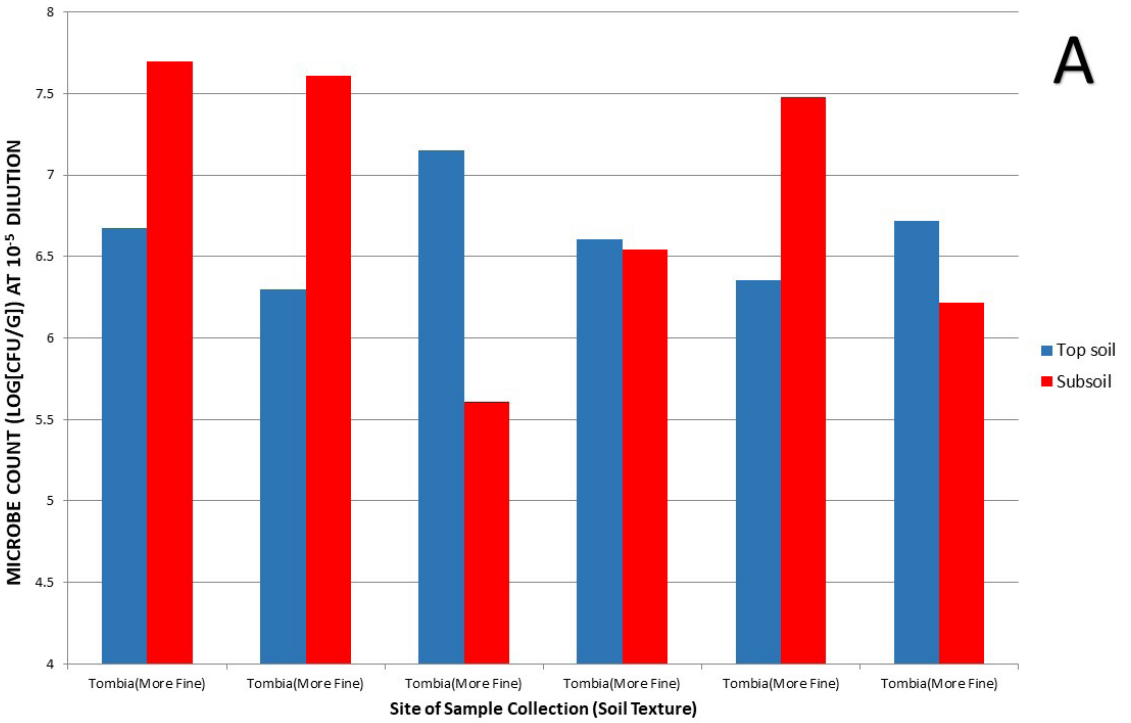
H03: That the interaction between soil texture and soil depth has no effect on microorganism distribution The two-way ANOVA revealed no significant difference (p=0.08) in the relationship between soil depth and microorganism distribution and no significant difference (p=0.998) in the relationship between soil texture and microorganism distribution. Equally, there was no significant difference (p=0.411) when the effect of both factors (soil depth and soil texture) was combined in relationship to microorganism distribution.

Discussion

Two sites of known crude oil contamination, Bodo and Tombia, were selected for sample collection. Two samples of topsoil and two samples of sub soil were collected from both sites and subjected to various analysis.

The change in transmittance/absorbance in colorimetric tests [seen in Figure 2] on inoculation with the various isolates, shows action of crude oil/hydrocarbon degrading microorganisms (bacteria) and proves that there was pollution by crude oil at the above-mentioned sites. One such proof of crude oil degrading activity observed was the change in absorbance/transmittance values at 600nm, for sample BSSP II 10-4 ER3, from 1.837 to 0.677 over a period of five days, which indicates degradation of crude oil.

Table 6 shows which biochemical and microbiological tests, combining microscopy and morphology, were used in the identification of some of the microorganisms present at the sites of crude oil pollution. From these tests, five (5) species of bacteria were identified – three were from the Tombia site (*Sphingobacterium* sp., *Pseudomonas* sp. and *Bacillus* sp.) and the remaining two (*Proteus* sp. and *Staphylococcus* sp.) were from the Bodo site. *Pseudomonas* sp. made up 70% of the species and *Sphingobacterium* sp. made up 10%. At Bodo, Proteus sp. and *Staphylococcus* sp. made up 50% each of the species identified. It is noteworthy that the Bodo soil was determined to be sandy soil going by the percentage of sand (99.87% sand, 0.13% clay/silt) while Tombia soil was determined to be silt/clay (90.44% sand, 9.56% silt/clay) relative to Bodo soil using relative classification of soil for agriculture. This did not prevent cultures of Bodo soil samples from yielding similar levels of viable counts for hydrocarbon degrading bacteria and fungi as seen in the results. A closer examination of the count figures in the mentioned tables, using Figure 3A and 3B for rough analysis, shows there is an insignificant difference in prevalence of microorganisms between the two sites. This is confirmed by the statistical analysis.



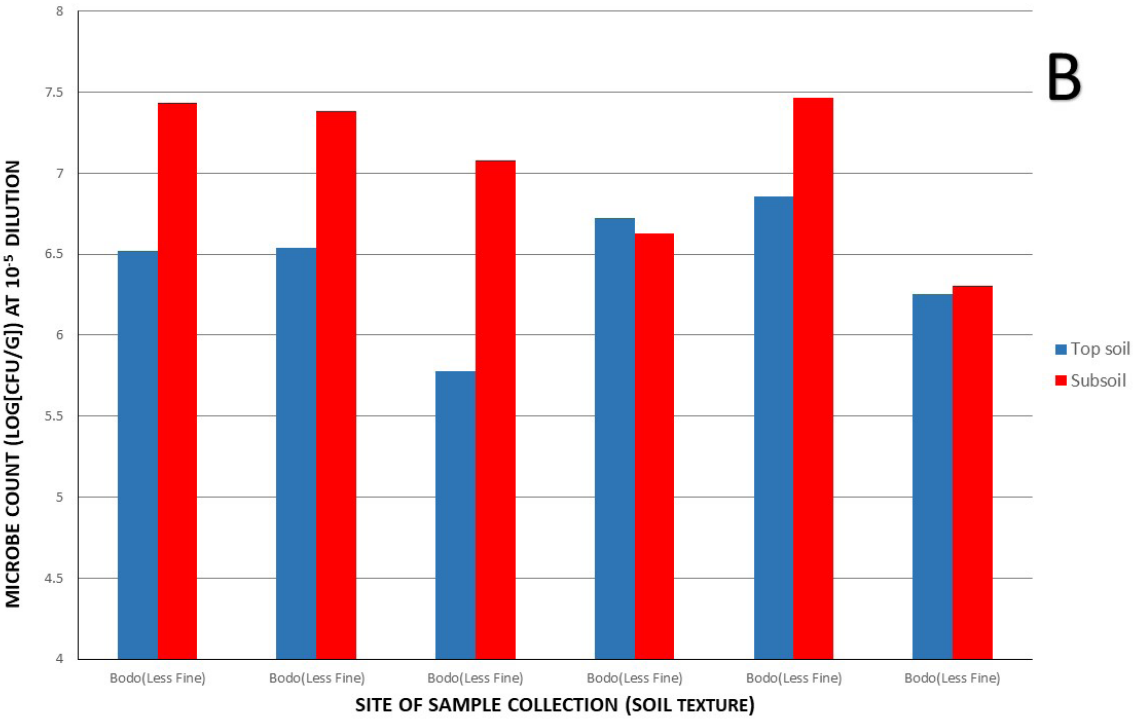


Figure 3A and 3B: Effect of Soil Depth and Soil Texture on Microbe Abundance. The chart shows the abundance of all microorganisms found in Bodo (fig. 3B) and Tombia (fig. 3A) areas in log (CFU/g). The subsoil markedly in both soils has a greater abundance of microbial life. This shows the role texture and depth have on the presence of microorganisms to begin degradation intrinsically in the soil.

A two-way ANOVA analysis, however, found the effect of the said factors (soil depth and soil texture) on the distribution of microorganisms in polluted soil to be insignificant. As shown in Table 7, the relationship between elevation gradient and microorganism distribution was insignificant (p=0.08). The relationship between soil textures of samples, which differed based on the source site, and microorganism distribution was found to be insignificant (p=0.998) as well. The effect of a combination of the independent variables was equally found to be of no significant effect (p=0.411). Thus, the null hypotheses were accepted. This result tallied with the conflicting outlier study of Spina *et al.* [32], which stated the studied independent factors had no significant relationship with microbial abundance thus provoking this study.

The soil texture was determined using the sieve method with guidance from the WRB soil texture classification found in Table 1 [31, 5] and the Texture Triangle by the USDA, which classifies all particles mm>0.063 as sand. Still, looking at the varied structure of the two soils, the results in Table 3 revealed that the Bodo site samples had significantly less coarse sand than the Tombia site, and significantly more medium sand than the Tombia site, with a possibility existing that medium sand could hold sufficient moisture and organic matter. The higher percentage of silt/clay in Tombia could have compensated in moisture and organic matter content, which has been indicated as necessary to support microorganism growth [11, 35]. Seaton *et al.* [28] analyzed a sample size of over 330 soils from varying habitats and found a relationship between soil textural heterogeneity (evenness of mixing of different fractions of the soil) and microbe diversity, and highlighted the affinity of certain classes of microbes to certain fractions of soil, which was dependent on the mineral constitution of those fractions of soil. This allows a possibility that the Bodo soil was more heterogeneous or that its fractions supported a different taxon of microbes in relatively equal abundance with the Tombia soil and could explain why the microbe distribution in our results showed no significant variance with soil texture between the two sites. Heterogeneity and microbe composition were not explored in the Tombia and Bodo soil samples, which leaves room for this to be incorporated for study in further research.

The average total petroleum hydrocarbons (TPH) in each site were 15,642.5 mg/kg in the topsoil and 11,342 mg/kg in the subsoil collected from the Bodo community whereas polluted soil from the Tombia site had an average of 14,684.9 mg/kg and 9,647 mg/kg of TPH in the top and sub soils respectively. These values are higher than the intervention value of 5000 mg/kg of soil as stipulated by the Department of Petroleum Resources (DPR) in the Environmental Guidelines and Standards for the Petroleum Industry in Nigeria (EGASPIN), indicating pollution [21].

To run the two-way ANOVA, the serial dilution values of 10-5 were the only values used and were converted to log(CFU/ml) before use. In cases where one serial dilution showed TNTC (Too Numerous to Count) and another dilution had a valid value within the range $0 \leq CFU \leq 300$, a value of 300 CFUs was adopted for the dilution that was TNTC, in accordance with practices

in research done by Global Alliance for TB Development for tuberculosis (TB) patients under cohort study [6]. Where the values of all serial dilutions for a sample were NA, the results were omitted from the statistical computation as reported by Parshionikar *et al.* [25] in a revised document prepared to serve as an internal guide to personnel of the U.S. Environmental Protection Agency on microbiological methods of analysis. Where all dilutions were TNTC, a count value of 300 was adopted for the final dilution [25]. Where both plates, at the same dilution, gave a count of TNTC and a non-zero count, a value of 300 was assigned to the TNTC plate and it was averaged with the count of the non-zero plate [6]. Where plates on equal serial dilution levels had one non-zero count and a zero count, the counts were averaged to find the log (CFU/ml) [6]. Where a plate of the same dilution level had an NA count and the other a non-NA count, the log(CFU/ml) of the non-NA plate was used [6]. To ensure equal number of rows for both independent variables in the analysis, data rows were deleted in order to match rows with unusable data (NU) in either of the groups. For instance, if TSP1 had an unusable dilution value at 10-5, TSSP 1, BSP1 and BSSP1 would also be deleted, and this resulted in a deletion of 8 out of 32 entries [6]. Plates with NA and 0 counts on the final serial dilution were marked NU and excluded from computations [6. 10].

Conclusion

Soil depth and soil texture, when considered independently or concurrently, have no significant effect on the microorganism distribution/abundance in petroleum hydrocarbon polluted soil based on this study.

Soil depth appears not to be a determinant of microorganism's abundance from this study, which could be attributed to the fact that different taxa of microbes thrive in different mineral conditions, that is, there are relatively equals number of microbes in top strata as there are at lower strata with the only difference being in their diversity.

Soil texture also proved to not be a determinant of microorganism abundance evidenced by the difference in soil texture between the Tombia and Bodo sites, with a surprisingly insignificant difference in microbe numbers. The force of mineral based selection or heterogeneity in soil texture seems a probable force here as well. Another reason for the non-significant difference in texture could be because the Tombia and Bodo sites have relatively similar textures where sand to silt/clay content is concerned.

From the presence of hydrocarbon degrading activity of the isolates observed, Proteus sp. and Staphylococcus sp. are suitable organisms for the bioremediation of the Bodo site. Sphingobacterium sp., Pseudomonas sp. and Bacillus sp. are potential microorganisms for bioremediation judging from their presence in soil samples from the Tombia site of which the same samples have shown activity in degrading crude oil in vitro as observed using colorimetric studies. Of course, Pseudomonas sp. seemed to thrive best with a presence in both topsoil and subsoil and a seemingly high prevalence considering frequency of identification in the isolates. This may be attributed to the bacteria being a facultative anaerobe [27] and hence could have the best suitability for remediation at Tombia.

References

1. Abosede, E. E. (2013). Effect of Crude Oil Pollution on some Soil Physical Properties. *IOSR Journal of Agriculture and Veterinary Science, 6(3), 14–17. https://doi.org/10.9790/2380-0631417.*

2. Amadeo K. (2019). Crude Oil, Its Types, Uses, and Impact *https://www.thebalance.com/crude-oil-prices-trends-and-impact-on-the-economy-and-you-3305738.*

3. Aryal, S. (2018). Triple Sugar Iron Agar (TSIA) Test. *Biochemical Test: Microbe Notes.* https://microbenotes.com/triple-sugar-iron-agar-tsia-test/#objectives-of-tsia-test.

4. Bao, M. T., Wang, L. N., Sun, P. Y., Cao, L. X., Zou, J., & Li, Y. M. (2012). Biodegradation of crude oil using an efficient microbial consortium in a simulated marine environment. *Marine pollution bulletin, 64*(6), 1177-1185.

5. Benham E., R. J. Ahrens, W.D. Nettleton. (2009). Clarification of Soil Texture Class Boundaries. *USDA Natural Resources Conservation Service,* 1, 1.

6. Burger, D. and Mans, L. (2016). A phase 2 open-label partially randomized trial to evaluate the efficacy, safety and tolerability of combinations of bedaquiline, moxifloxacin, pa-824 and pyrazinamide during 8 weeks of treatment in adult subjects with newly diagnosed drug-sensitive or multi drug-resistant, smear-positive pulmonary tuberculosis. *Statistical Analysis Plan. Global Alliance for TB Drug Development,* (3), 1-72. https://clinicaltrials.gov/ProvidedDocs/76/NCT02193776/SAP_001.pdf.

7. Chikere, C. B., Tekere, M., & Adeleke, R. (2019). Enhanced microbial hydrocarbon biodegradation as stimulated during field-scale landfarming of crude oil-impacted soil. *Sustainable Chemistry and Pharmacy,* 14, 100177. https://doi.org/10.1016/j.scp.2019.100177.

8. Chikere, C. B., Tekere, M., & Adeleke, R. (2021). Microbial communities in field-scale oil-polluted soil remediation using 16S rRNA amplicon sequencing. *International Journal of Environmental Studies,* 78(3), 410–426. https://doi.org/10.1080/00207233.2020.1817276.

9. Fuentes, S., Méndez, V., Aguila, P., & Seeger, M. (2014). Bioremediation of petroleum hydrocarbons: Catabolic genes, microbial communities, and applications. *Applied Microbiology and Biotechnology, 98*(11), 4781–4794. https://doi.org/10.1007/s00253-014-5684-9.

10. Grace-martin, K. (n.d.). Outliers: To Drop or Not to Drop. *The Analysis Factor.* Retrieved August 9, 2021, from https://www.theanalysisfactor.com/outliers-to-drop-or-not-to-drop/

11. Griffiths R. I., Whiteley A. S. (2003). Influence of depth and sampling time on bacterial community structure in an upland grassland soil. *FEMS Microbiology Ecology,* 43(1), 35-43.

12. Gupta, K., Biswas, R., & Sarkar, A. (2020). Advancement of omics: prospects for bioremediation of contaminated soils. *Microbial Bioremediation and Biodegradation,* 113-142.

13. Jackson, R. S. (2008). 5 - Site Selection and Climate. In R. S. B. T.-W. S. (Third E. Jackson (Ed.), Food Science and Technology (pp. 239–269). Academic Press. https://doi.org/https://doi.org/10.1016/B978-012373646-8.50008-1.

14. Lotha, G., Curley, R., Chopra, S., Singh, S. (2018). Crude Oil. *Encyclopedia Britannica.* https://www.britannica.com/science/crude-oil.

15. Lundberg, C. (n.d.). Soil Texture Using a Soil Sieve. *Terrestrial Ecology Extension Activity.* Retrieved August 9, 2021, from http://mathinscience.info.

16. MacWilliams, M. P. (2016). Indole Test Protocol. *American Journal for Microbiology,* 1(1), 1–9.

17. Maiti, P., Haldar, J., Mukherjee, P., & Dey, R. (2013). Anaerobic culture on growth efficient bi-layered culture plate in a modified candle jar using a rapid and slow combustion system. *Indian Journal of Medical Microbiology,* 31, 173–176. https://doi.org/10.4103/0255-0857.115218.

18. Michel, J., & Fingas, M. (2015). Oil spills: Causes, consequences, prevention, and countermeasures. *Columbia: Research Planning, Inclined. Fossil Fuels,* 159-201.

19. Moubasher, H. A., Hegazy, A. K., Mohamed, N. H., Moustafa, Y. M., Kabiel, H. F., & Hamad, A. A. (2015). Phytoremediation of soils polluted with crude petroleum oil using Bassia scoparia and its associated rhizosphere microorganisms. *International Biodeterioration & Biodegradation, 98*, 113–120. https://doi.org/10.1016/J.IBIOD.2014.11.019.

20. Murphy, B. L., & Morrison, R. D. (2014). Source Identification and Age Dating of Chlorinated Solvents. *Introduction to Environmental Forensics: Third Edition*, 311–345. https://doi.org/10.1016/B978-0-12-404696-2.00009-6.

21. Obieze, C. C., Chikere, C. B., Selvarajan, R., Adeleke, R., Ntushelo, K., & Akaranta, O. (2020). Functional attributes and response of bacterial communities to nature-based fertilization during hydrocarbon remediation. *International Biodeterioration and Biodegradation, 154*, 105084. https://doi.org/10.1016/j.ibiod.2020.105084.

22. Ofume, Vivian S. (2019). Microbiological air quality of gladys cookey international school facilities. *Department of Microbiology, University of Port Harcourt, Nigeria.* 14 – 19.

23. Onofeghara F. A., Amakiri J. O. (1984). Effects of crude oil pollution on the germination of Zea mays and Capsicum frutescens. *Ecological and Biological, 35*(2), 159-167

24. Olof Lindén and Jonas Pålsson. (2013). Oil Contamination in Ogoniland, Niger Delta. AMBIO 42, 685–701 https://doi.org/10.1007/s13280-013-0412-8.

25. Parshionikar, S., Hunt, M. E., Fred Genthner, D., Lincoff, A., Haugland, R. A., Cottrill, M., Harris, S., Pfaller, S., Shanks, O., Grimm, A., Oshiro, R. K., & Mano Sivaganesan, D. (2009). Method Validation of U.S. Environmental Protection Agency (EPA) Microbiological Methods of Analysis. *The EPA Forum on Environmental Measurements (FEM) , 2009*(1), 1–78. https://www.epa.gov/sites/production/files/2017-04/documents/final_edited_revision_microbiology_method_guidance_122116.pdf.

26. Patel, P. D., (2020). Polycyclic Aromatic Hydrocarbons: Sources, Toxicity, and Remediation Approaches. *Frontiers in Microbiology 11, 562813.* doi:10.3389/fmicb.2020.562813

27. Sajib Islam MS, T. Rahman. (2017). Assessment of Hydrocarbon Degradability of theBacterial Species Isolated from Different Oil Contaminated Sites of Bangladesh. *Environmental Science Indian Journal,* 13 (4), 141.

28. Seaton, F. M., George, P. B. L., Lebron, I., Jones, D. L., Creer, S., & Robinson, D. A. (2020). Soil textural heterogeneity impacts bacterial but not fungal diversity. *Soil Biology and Biochemistry, 144*, 107766. https://doi.org/10.1016/j.soilbio.2020.107766.

29. Shields, P. & Cathcart, L. (2010). Oxidase Test Protocol. *American Journal for Microbiology, 3*, 1-9. www.asmscience.org.

30. Singh, R., Safiyanu I, Abdulwahid Isah, A., (2015). Review on Comparative Study on Bioremediation for Oil Spills Using Microbes. *Research Journal of Pharmaceutical, Biological and Chemical Sciences.* 6(6), 783-790.

31. Soil texture - Wikipedia. (n.d.). Retrieved July 26, 2021, from https://en.m.wikipedia.org/wiki/Soil_texture#cite_ref-6.

32. Spina F., Spini G., Poli A., Blieux A., Regnier T., Gramellini C., Varese G. C., Puglisi E. (2018). Molecular and Microbiological Insights on the Enrichment Procedures for the Isolation of Petroleum Degrading Bacteria and Fungi. *Frontiers in Microbiology 9*, 2543.

33. Sylvia, M. (2009). Methyl Red and Voges-Proskauer Test Protocols. *American Society for Microbiology*, 1, 1–9. www.asmscience.org.

34. Tankeshwar, A. (2021). Gram Staining: Principle, Procedure, Results. *Microbe Online*. https://microbeonline.com/gram-staining-principle-procedure-results/.

35. Turner, S., Mikattla, R. (2017). Microbial Community Dynamics in Soil Depth Profiles Over 120,000 years of Ecosystem Development. *Frontiers Microbiology 2017*, 8, 874.

36. Ulrich A. C., Brown L. D., Cologgi D. L., Gee K. F. (2017). Bioremediation of Oil Spills on Land. In *Oil Spill Science and Technology: Second Edition,* 699–729. https://doi.org/10.1016/B978-0-12-809413-6.00012-6.

37. Watson R. (n.d). Glucose Broth and Durham tubes or Motility Agar. General & medical microbiology Molb/Micr 2021. https://www.uwyo.edu/molb2210_lab/info/biochemical_tests.htm#Glucose.