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Microbiological and Physicochemical Characteristics of Soil Contaminated With Used Petroleum Products in Umuahia, Abia State, Nigeria

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Abstract The discharge of used petroleum products from machines such as power generating sets, vehicles and motorcycles has become a major source of soil pollution around Umuahia metropolis. The objectives of this study were to assess the microbiological and physicochemical characteristics of soils contaminated with used petroleum products. A total of four (4) sampling sites (filling stations, vehicle mechanic workshop, motorcycle workshop and areas around power generating plants) were investigated. The total heterotrophic bacterial and fungal counts ranged from $1.6x10^7$ to $1.9x10^8$ CFU/g and $1.0x10^7$ to $1.7x10^7$ CFU/g respectively. The hydrocarbon utilizing bacterial and fungal counts ranged from $3.8x10^6$ to $7.8x10^6$ CFU/g and $1.0x10^6$ to $1.4x10^6$ CFU/g respectively. The bacterial isolates were *Pseudomonas aeruginosa, Escherichia coli, Klebsiella* spp, *Staphylococcus aureus, Micrococcus* spp, *Bacillus* spp, *Citrobacter* spp and *Streptococcus* spp. Fungal isolates included *Aspergillus* spp, *Cladosporium* spp, *Mucor* spp, *Rhizopus* spp and *Geotricum* spp. The total organic carbon concentration ranged from 5.20 to 16.74 mg/kg and 4.81 mg/kg for control. The contamination also affected other physicochemical parameters such as sulphate, nitrate and phosphate and ranged from 28.70 to 63.71 mg/kg, 21.73 to 53.79 mg/kg and 1.24 to 3.54 mg/kg respectively compared to the control sample, 11.93 mg/kg, 5.39 mg/kg and 0.64 mg/kg respectively. The contamination increased calcium, sodium, potassium, magnesium, iron and zinc concentrations compared to the control and caused a reduction in pH. These adverse conditions have impacted negatively on soils around the polluted site could affect nutrient cycles and consequently affect vegetation around the area.

Keywords: *microbiological, physicochemical, contaminated, petroleum, products, Umuahia, Nigeria*

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1. Introduction

With an ever increasing world's population, there is a concomitant increase in the demand for petroleum and petroleum products, which apparently constitutes a source of environmental pollution [1]. Oil pollution is a major environmental concern in many countries, and this has led to a concerted effort in studying the feasibility of using oil-degrading bacteria for bioremediation.

Pollution of the environment by petroleum products is an inevitable consequence of oil production, transportation and distribution activities. Large amounts of petroleum products handled on land every year create the possibility for land contamination. In addition, large volumes of crude oil and/or refined petroleum products are transported across the world's oceans from producing areas to consumer countries [2].

The toxicity of crude oil or petroleum products varies widely, depending on their composition, concentration, environmental factors and on the biological state of the organisms at the time of the contamination. The discharge of used oil from vehicles or motorcycles is a major source of oil pollution in mechanic workshop and its environs. Biodegradation of hydrocarbons by natural population of microorganisms represents one of the primary mechanisms of eliminating petroleum pollution from the environment [3]. The ability to degrade and/or utilize hydrocarbon substrates is exhibited by a wide range of bacteria and fungi [2].

Used petroleum products contribute immensely to land pollution as a result of the wrong channeling of used oil that is dispersed during their operation. These used oils if improperly channeled to a collecting container may find their way into surrounding soil environment thereby causing pollution on land. This form of land pollution has an adverse effect on the soil surrounding the generator and its house. Most human activities contribute large quantity of pollutants in the environment. Activities from oil industries, mechanic workshops automobile also play a role in land pollution by oil. The biodegradation of these pollutant by microorganism present in the soil as a result of their metabolic diversity occurs when there is pollution in areas where microorganisms are present [3].

Microbial degradation of hydrocarbon-contaminated site is performed with the help of a diverse group of microorganisms, particularly the indigenous bacteria present in soil. A large number of *Pseudomonas* strains capable of degrading Poly aromatic hydrocarbons have been isolated from soil and aquifers [4,5] Other petroleum hydrocarbon degrading bacteria include *Bacillus, Micrococcus, Alcaligenes* spp., *Flavobacterium, Corynebacterium* species and *Streptococcus* species [6,7]. Other organisms such as fungi are also capable of degrading the hydrocarbons in engine oil to a certain extent.

The aim of this research work was to determine the effect of used generating plant oil on the microbiological and physiochemical characteristics of the soil around Umuahia metropolis, Abia State, Nigeria.

2. Materials and Methods

2.1. Study Area

Soil samples analyzed in this research were sourced from mechanic workshops, filling stations, motorcycle workshops and the surroundings of power generating plants.

2.2. Sample Collection

Soil samples within the aforementioned areas were aseptically collected separately using sterile sample containers with a spatula. The samples were transported to the laboratory within 6 hrs after collection and analyzed within 24 hrs.

2.3. Chemical Reagents

The chemical reagents used in the study were of analytical grade. They were products of BDH Chemicals, Poole's, England and Sigma Chemical Company, St. Louis Missouri, USA. The microbiological media used were products of Oxoid and Difco Laboratories, England. They included nutrient agar used for the estimation of total heterotrophic aerobic bacteria, purification of hydrocarbon utilizers and for pure culture; Sabouraud dextrose agar (SDA) used for the isolation of fungi. The modified mineral salt agar without and with antibiotic was used for the isolation of hydrocarbon utilizing bacteria and fungi respectively.

2.4. Enumeration of Total Heterotrophic Bacteria and Fungi

Samples of the contaminated soil were serially diluted in ten folds. Total aerobic plate counts were determined using pour plate technique. Then the molten nutrient agar, MacConkey agar and Sabouraud dextrose agar at 45 ^{0}C were poured into the petri dishes containing 1mL of the appropriate dilution for the isolation of the total aerobic bacteria and fungi and coliforms respectively. They were swirled to mix and colony counts were taken after incubating the plates at 30°C for 48 hrs for the bacterial count and 5 days for the fungal count. The bacterial isolates were preserved by sub-culturing them into nutrient agar slants which were used for biochemical tests.

2.5. Enumeration of Hydrocarbon-Utilizing Bacteria and Fungi

The hydrocarbon-utilizing bacteria and fungi were enumerated. The mineral salt agar of [8] as modified by [9] comprising per liter of distilled water NaCl, l0g; $MgSO_4.7H_2O$, 0.42g; KCl, 0.29g; K_2HPO_4, Na_2HPO_4, 0.83; $NaNO_3$, 0.42g; agar,15g; pH 7.2 was used. To 990mL of the mineral salt medium in conical flasks was added 10mL of the lubricating oil which served as source of carbon. However, for the hydrocarbon-utilizing fungi, the medium was supplemented with an antibiotic chloramphenicol. The hydrocarbon-utilizers were then enumerated after plating in duplicate using pour plate technique, 1mL of the appropriate dilutions of the samples on petri dishes. The molten mineral salt agar medium without antibiotic and the one containing antibiotic at 45°C were poured into the Petri dishes for the isolation of hydrocarbon-utilizing bacteria and fungi respectively. These were swirled to mix, allowed to solidify and incubated. Enumeration of the hydrocarbon-utilizers was performed after incubation at 30°C for 7 days. Colonies of the hydrocarbon-utilizing bacteria growing on the agar plates were counted, isolated, purified by streaking on nutrient agar plates and kept on nutrient agar slants as stock cultures for characterization and identifications. In the case of hydrocarbon-utilizing fungi, the isolates were streaked to purify onto Sabouraud dextrose agar plates and kept on Sabouraud agar slants as stock cultures for characterization and identification.

2.6. Characterization and Identification of Hydrocarbon utilizing Isolates

Bacterial isolates were characterized and identified after studying their Gram reaction as well as cell micro morphology. The other tests carried out were spore formation, motility, catalase production, citrate utilization, oxidative/fermentative utilization of glucose, indole production, methyl red - Voges Proskauer reaction, urease and coagulase production, starch hydrolysis, production of H_2S from triple sugar iron (TSI) agar and sugar fermentation. The tests were carried according to the methods described by [10,11,12,13]. Microbial identification was performed using the keys provided in the [14]. Fungal isolates were examined macroscopically and microscopically using the needle mounts technique. Their identification was performed according to the scheme of [15, 16].

2.7. Determination of the Physicochemical Parameters

A number of physicochemical parameters of the contaminated soil samples were determined. They included pH, conductivity, nitrate, phosphate, sulphate. Others included oil and grease, total petroleum hydrocarbon, total organic carbon heavy metals and exchangeable cations. The pH was measured using Hach pH meter (Model EC10); conductivity was measured using Hach conductivity meter (Model CO150). Sulphates, nitrates and phosphates were determined using Barium chloride (Turbidimetric method), Cadmium reduction and Ascorbic acid methods respectively. All analyses were in accordance with American Public Health Association (APHA) [17].

2.7.1. Heavy Metal Analysis

The heavy metals were determined using Unicam atomic absorption spectrophotometer (Model 969, Unicam) after digesting the samples with 10mL of water, 5mL of HCl (S.G. 1.19) and 1mL of HNO_3 (S.G. 1.42) in digestion containers. The digest was then analyzed using the atomic absorption spectrophotometer [18].

2.7.2. Determination of Oil and Grease and Total Petroleum Hydrocarbon (TPH)

The soil samples were air dried and sieved. Ten grams of the air dried sieved samples were weighed into 60ml glass bottles and 20ml of tetrachloroethylene was poured into the glass bottles. These bottles were placed into a shaker maintained at room temperature. The system was allowed to shake for about 30 min after they were allowed to settle. The extracts were filtered out into a 20ml glass bottle using a glass funnel stuffed with cotton wool on which anhydrous sodium sulphate was placed. The samples were analyzed using Hach DR4000 spectrophotometer. The TPH was determined by treating the extracts with silica gel before analyzing with the spectrophotometer [18].

2.7.3. Determination of Total Organic Carbon

One gram each of the air-dried samples was weighed out in duplicate and transferred to 250ml Erlenmeyer flask. Ten millimeters of 1N potassium dichromate solution and 20ml concentrated sulphuric acid was added and the flasks swirled until the soil and reagents were mixed. The flasks were allowed to stand on the sheet of asbestos for about 30 min after 100ml of distilled water was added. Three drops of indicator was added and then titrated with 0.5N ferrous sulphate solution. The endpoint was observed when the colour changed sharply from blue to red (maroon colour) in reflected light against a white background [18].

2.7.4. Determination of Exchangeable Cations

The soil samples were first extracted using 1N ammonium acetate solution. This was done by weighing 5g of sieved air-dried samples and adding to 30ml of the extracting solution in a tube. This was shaken on a mechanical shaker for two hours. They were then centrifuged for five minutes and the supernatant carefully decanted into a 100ml volumetric flask. This was then made up to the mark with the extracting solution. The exchangeable cations (Na, K, Ca^{2+}, and Mg^{2+}) of the extract were determined using Unicam atomic absorption spectrophotometer (Model 969) [17].

3. Results

The results of the microbiological and physicochemical analysis are shown in Table 1 - Table 6. Table 1 shows the microbial counts from the various samples.

The total heterotrophic bacterial counts ranged from $1.6x10^7$ CFU/g to $1.9x10^8$ CFU/g. The mechanic workshop had the highest count of $1.9x10^8$ CFU/g while the least count of $1.6x10^7$ CFU/g was recorded. The total heterotrophic fungal count ranged from $1.0x10^7$ CFU/g to $1.7x10^7$ CFU/g. The highest count of $1.7x10^7$ CFU/g was from mechanic workshop while the motorcycle workshop had the least count of $1.0x10^7$ CFU/g. The hydrocarbon-utilizing bacterial count ranged from $3.8x10^6$ CFU/g to $7.8x10^6$ CFU/g, while the hydrocarbon-utilizing fungal count ranged from $1.0x10^6$ CFU/g to $1.4x10^6$ CFU/g.

The motorcycle workshop had the highest hydrocarbon utilizing bacterial count of $7.8x10^6$ CFU/g while the least count of $3.8x10^6$ CFU/g was from the filling station. The mechanic workshop had the highest hydrocarbon-fungal count of $1.4x10^6$ CFU/g while the generating plant had the least count of $1.0x10^6$ CFU/g.

The bacterial and fungal spp isolated and their percentage occurrences are shown in Table 2 and Table 3.

Table 1. Total Microbial Counts of various samples

Sample	THBC (CFU/g)	HUBC (CFU/g)	HFC (CFU/g)	HUFC (CFU/g)
Filling station	$9.1x10^7$	$3.8x10^6$	$1.4x10^7$	$1.2x10^6$
Mechanic workshop	$1.9x10^8$	$7.4x10^6$	$1.7x10^7$	$1.4x10^6$
Motorcycle workshop	$1.8x10^8$	$7.8x10^6$	$1.0x10^7$	$1.1x10^6$
Generating plant	$1.6x10^7$	$6.4x10^6$	$1.2x10^7$	$1.0x10^6$
Control	$2.2x10^7$	$1.4x10^6$	$8.0x10^6$	$4.0x10^5$

THBC: Total heterotrophic bacterial count. **HUBC**: Hydrocarbon-utilizing bacterial count. **HFC**: Heterotrophic fungal count. **HUFC**: Hydrocarbon-utilizing Fungal Count.

Table 2. Percentage occurrence of bacterial isolates

Bacteria	Number of Isolates	Percentage Occurrence
***Bacillus* spp**	4	20
***Pseudomonas* spp**	4	20
Escherichia coli	4	20
***Staphylococcus* spp**	3	15
***Klebsiella* spp**	3	15
***Citrobacter* spp**	1	5
***Micrococcus* spp**	1	5
Total	20	100

Escherichia coli, *Bacillus*, *Pseudomonas* spp had the highest occurrence of 20.0% while the *Micrococcus* and *Citrobacter* spp had the least occurrence of 5.0% for the bacterial isolates.

Table 3. Percentage occurrence of fungal isolates

Fungi	Number of Isolates	Percentage Occurrence
***Penicillium* species**	2	33.3
***Rhizopus* species**	1	16.7
***Aspergillus* species**	1	16.7
***Cephalosporium* species**	1	16.7
***Mucor* species**	1	16.7
Total	6	100

For the fungal isolates, *Penicillium* spp had the highest occurrence of 33.3% while *Mucor*, *Rhizopus*, *Aspergillus* and *Cephalosporium* spp had the least occurrence of 16.7%.

The result of the physiochemical parameters are shown in the Table 4. The result ranged as follows: pH, 5.60 – 5.85; total organic carbon, 5.20 to 6.94%; oil and grease, 12.76 to 16.74 mg/kg; phosphate 1.24 to 3.54 mg/kg; sulphate, 28.70 to 63.71 mg/kg; nitrate,16.29 to 34.66 mg/kg; total iron, 1.03 to 2.15 mg/kg; sodium, 154.74 to 206.76 mg/kg; potassium, 73.45 to 86.63 mg/kg and calcium, 43.81 to 64.29 mg/kg.

Table 4. Isolation and identification of bacterial species from soil samples

S/N	Colony Morphology	Cell arrangement	Gram Reaction	Motility	Spore Formation	Catalase	Oxidase	Coagulase	Citrate	Indole	Methyl Red	Vogue Proskauer	Urease	Starch Hydrolysis	TKSA	Sugar fermentation				Organism Suspected
																Lactose	Glucose	Mannitol	Sucrose	
1	Smooth colonies with grey tint on nutrient agar	Short rods	-	+	-	+	+	-	+	+	+	-	+	+	H_2S	-	A	AG	-	*Pseudomonas aeruginosa*
2	Yellow colonies on nutrient agar	Clusters of small uniform cocci in pairs	+	-	-	+	-	-	-	-	+	-	-	+	A	A	A	AG	G	*Micrococcus* spp
3	Smooth and circular translucent colonies on nutrient agar	Short rods singles and separate	-	-	-	+	-	-	-	+	+	-	-	+	AG	A	AG	AG	G	*Escherichia coli*
4	Large circular smooth slightly raised colonies	Short rods	-	-	-	+	-	-	+	-	+	-	+	+	H_2S	AG	AG	AG	-	*Klebsiella* spp
5	Raised long rods	Straight long rods	+	-	+	+	-	-	+	+	-	-	-	+	AG	A	A	A	-	*Bacillus* species
6	Milky to white colonies	Cocci in clusters	+	-	-	+	-	+	-	-	+	-	-	+	A	A	AG	G	A	*Staphylococcus aureus*
7	White to cream medium sized colonies	Short rods	-	+	-	+	-	-	-	-	+	+	+	+	A	A	G	AG	-	*Citrobacter* spp
8	Translucent smooth colonies mucoid in appearance	Cocci in chains	+	-	-	-	+	+	-	-	-	-	+	+	AG	AG	AG	A	A	*Streptococcus* spp

+: Positive, -: Negative, A: Acid production, AG: Acid and Gas production.

Table 5. Isolation and identification of fungal isolates

Colony Morphology	Microscopic Morphology	Identification
Dark grey surface, reverse velverty or cottony	Non septate, conidiophores terminating in a globus or clarate swelling bearing phialides at the apex	*Aspergillus* spp
Pink to violet surface and yellow reverse	Have branched slender conidiophores, slightly enlarged at the apex conidia arise one at a time	*Cladosporium* spp
Colony grayish green or olive green, reverse orange brown	Sporengiospore either tall with many spores	*Mucor* spp
Colony pale brownish grey, reverse brownish black	Branched rhizoids sporangiospore irregular in shape, globolose and interwoven mass of hyphae	*Rhizopus* spp
Colonies white, reverse brown	Septate hyphae, conidia globolose, thin and smooth wall. Hyaline, phialide borne directly on the vesicle	*Geotricum* spp

Table 6. Values Physicochemical parameters analyzed

Parameter	Filling station	Mechanic workshop	Motorcycle workshop	Generating plant	Control
pH	5.85	5.70	5.60	5.78	6.94
Temperature (^{0}C)	29.0	28.5	28.0	30.0	29.0
Oil/Grease (mg/kg)	13.65	16.74	12.76	14.56	6.76
Organic Carbon (%)	6.48	6.94	7.47	5.20	4.81
SO_4 (mg/kg)	37.46	63.71	47.71	28.70	11.93
NO_2 (mg/kg)	16.29	34.66	29.78	26.17	9.56
NO_3 (mg/kg)	25.66	53.79	49.29	21.73	5.39
PO_4 (mg/kg)	1.83	2.90	3.54	1.24	0.64
Calcium (mg/kg)	53.19	64.29	55.17	43.81	17.33
Sodium (mg/kg)	154.74	206.76	173.35	163.86	85.81
Potassium (mg/kg)	73.45	86.63	82.16	78.22	56.52
Magnesium (mg/kg)	2.87	2.64	2.66	2.84	0.69
Iron (mg/kg)	2.05	1.06	1.03	2.15	0.82
Zinc (mg/kg)	4.94	5.74	4.57	2.77	1.74

4. Discussion

Oil pollution on land is usually very difficult to clean up and it has steadily increased. In this study, similar bacteria were recovered from both the control and contaminated samples apart from few species. Studies have shown that oil degrading microbes are abundant and are not limited to oil producing areas [19]. Soils have been a favorable habitat for the proliferation of microorganisms, but the addition of refractory humic substances slows down the activities of these microorganisms, thus giving room to diverse bacterial and fungal species that have evolved the metabolic capacity to degrade hydrocarbons [20,21].

The most predominant bacterial species were *Pseudomonas* and *Bacillus* spp. Other prevalent bacteria

that were isolated include *Klebsiella, Citrobacter, Micrococcus* spp and *Staphylococcus aureus*. [21], reported that the most prevalent bacteria that degrade hydrocarbons include *Pseudomonas, Bacillus* and *Micrococcus* species. The total heterotrophic count (CFU/g) ranged from $1.59x10^7$ to $1.9x10^8$. The increased bacterial count which was observed in the sample contaminated with used petroleum products was due to increased hydrocarbon content which in agreement with the findings of Okerentugba and Ezeronye [22]. Gradual increase in bacterial population in contaminated soil was also reported by [23]. There was an increase in the total fungal count compared to the control, which is in agreement with what [24] had reported.

The total hydrocarbon utilizing bacterial count ranged from $3.8x10^6$ to $7.8x10^6$ CFU/g. This increase is due to the presence of hydrocarbon availability in the soil. This is in agreement with the findings of [24]. Total heterotrophic fungal count ranged from $1.0x10^7$ to $1.7x10^7$ CFU/g, while the total hydrocarbon utilizing fungal count ranged from $1.0x10^6$ to $1.4x10^7$ CFU/g. The increases in the hydrocarbon-utilizing bacterial and fungal counts should have resulted in the removal of hydrocarbons resulting in the decrease in the value. But because the oil is discharged continuously into the surrounding environment the level of the hydrocarbons was not affected hence the difference in the value between the contaminated soil and the control. Other factors that can affect hydrocarbon removal in the soil include runoffs, flood and leaching, evaporation and photo-oxidation [25]. The high values of the total organic carbon show how richly the soil is enriched with organic matter. The presence of organic matter shows the presence of other nutrients in the soil. This also encouraged the growth of the microbial population.

Bartha and Atlas [26] reported that when natural environments are contaminated with pollutants the indigenous microbial communalities are likely to contain microbial populations of different taxonomic characteristics which are capable of degrading the contaminating waste. Degradation of macromolecules in waste to smaller molecules is enhanced by soil microorganisms which produce a tremendous range of potentially useful enzymes that help in breaking down or decomposition of these macromolecules.

The result of the physicochemical composition of the different soil samples showed varying temperature readings ranging from 28.0 to 30.0°C while pH readings of soil samples were slightly acidic. These results are similar to those obtained by Atlas [2] who showed that slightly acidic to neutral pH encourages biodegradation by bacterial species in soils. The type of microorganisms that participate in hydrocarbon degradation is determined by the pH of the soil [28]. Bacteria have limited tolerance for acid conditions and fungi are more tolerant [29]. Since the pH in this study was at low pH, it could be assumed that fungi were more involved in the degradation of the oil.

The nitrate, phosphate and sulphate were higher in value than the control. The increase in the sulphate content can be attributed to the fact that as sulphates are being removed from the soil, they are being replaced. It has been shown that some bacteria particularly, species of *Thiobacillus* (*T. thioparus, T. novellas*) oxidizes H_2S and other sulphur compounds and because they have a low acid tolerance, deposit elemental sulphur rather than generate sulphuric acid by further oxidation. Other members of the genus *Thiobacillus* produce sulphate from the oxidation of elemental sulphur and other organic sulphur compounds [30,31].

Phosphorus is not an abundant component of the ecosphere [32]. It had been shown that its availability is further restricted by its tendency to precipitate in the presence of bivalent metals (Ca_2^+, Mg_2^+) and ferric (Fe_3^+) ion at neutral to alkaline pH. Phosphates are combined with calcium within many habitats rendering them insoluble and unavailable to plants and many microorganisms are capable of solubilizing phosphates from such sources, assimilate and release them for use by other organisms [32]. Nitrogen is an important constituent of protein and nucleic acid. In most microorganisms and plants, inorganic nitrogen is taken up as nitrate (NO_3^-) or ammonium (NH_4^+) ions. It has been shown that nitrogen can be lost from the soil because some species of bacteria convert nitrate to gaseous nitrogen by using nitrate as a metabolic electron acceptor in place of oxygen [33]. Nitrates are important nutrient in the soil and can cause eutrophication in aquatic environment.

5. Conclusion

There is a general increase in the fertility of the soil after oil pollution, but since oil is continually dumped around the study areas, the soil remain polluted and infertile which means that it is rare to find good vegetation around it. The changes in the physicochemical parameters were as a consequence of the build up in the soil of the products of oil degradation.

The result of this study revealed that, the indigenous microbial populations in soils of oil contaminated areas are capable of mineralizing these pollutants in the environment to safe and acceptable levels. We conclude that oil-degrading bacteria are abundant in soils contaminated with used petroleum products. This can be exploited for large oil-spill clean-up campaigns. This study also provides information on the physicochemical requirements for optimum degradation by these bacteria.

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