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Evaluation of the Impact of Crude Oil Contamination on Soil's Physicochemical Characteristics, Micro-flora and Crop Yield

Ugochukwu Chukwuma Okafor

Applied Microbiology and Brewing Department, Faculty of Biosciences, Nnamdi Azikiwe University, Awka, Nigeria

Correspondence: uc.okafor@unizik.edu.ng

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ABSTRACT: The effects of crude oil pollution on soil physicochemical properties, microflora, and ecotoxicity were evaluated. Soil samples were contaminated with crude oil, and the effects of contamination on the physicochemical parameters, microflora, and growth index of bean (*Phaseolus vulgaris*) seeds were studied over a 6-month period. The heterotrophic bacteria isolated from the uncontaminated soil were *Micrococcus, Klebsiella, Flavobacterium, Bacillus, Pseudomonas*, and *Serratia* species, and the moulds included microbes such as *Aspergillus niger, Fusarium, and Mucor sp*. Petroleum contamination increased the pH of the soils to alkaline values while increasing the total nitrogen, organic carbon, and phosphorus contents. Electrical conductivity, nitrogen content, and phosphorus content were significantly reduced after petroleum contamination ($p < 0.05$). The heavy metal contents of the contaminated soils decreased with increasing remediation time. Zinc, total nitrogen, total organic carbon, and electrical conductivity contents were statistically significantly different among samples throughout the bioremediation period ($p < 0.05$). The ability of isolates to utilise hydrocarbons was highest for *Pseudomonas* and *Bacillus* species and lowest for *Klebsiella* and *Serratia* species. After a germination period of 12 days, a germination test showed that the bioattenuated polluted soil improved germination of bean seeds. Bioattenuation methods should be used and improved as a means of remediating petroleum-polluted sites because they are cost-effective and environmentally friendly.

KEYWORDS: Bioattenuation; crude oil; microorganisms; physicochemical properties; pollution

1. Introduction

The impact of crude oil and other petroleum wastes on marine and terrestrial life, settlement, and human health is of great concern. The toxicity of crude oil depends largely on the chemical and physical properties of the particular type of crude oil and the quality of the contaminated water or soil [1]. During migration, the released hydrocarbons penetrate soil capillaries that were previously filled with water or air and bind to soil particles or to minerals in the interstitial spaces of the soil. All these regions are ecologically damaged as both air and water are excluded

from the soil microenvironment [2]. The oil deposited on the leaves of the plants penetrates into the leaves and reduces transpiration and photosynthesis [3]. It was also observed that plant growth was affected when soil contamination was light, on the order of 1% or less. When oil spills occur on the soil and the oil is not removed quickly, cropland and marshes are severely affected, and the soils become unsuitable for agriculture [4]. Higher and lower molecular weight hydrocarbons evaporate, while medium and heavier volatiles penetrate the soil, the study found. Oil pollution causes oil-degrading bacteria such as Azotobacter and Beijiernickia to become more abundant, while nitrifying bacteria such as *Nitrosomonas* sp. decrease [5]. Microorganisms are the main agents responsible for the biodegradation of environmentally harmful molecules, including petroleum hydrocarbons [6-7]. Hydrocarbon-degrading bacteria, yeasts, and fungi are widely distributed in marine, freshwater, and soil habitats. In aquatic ecosystems, bacteria and yeasts appear to be the dominant degraders, while in soil environments, fungi and bacteria are the main degraders [8].

A consortium of microorganisms appears to be involved in the degradation of crude and refined oils, including both eukaryotic and prokaryotic forms. The most common genera responsible for oil degradation include mainly N*orcardia, Pseudomonas, Acinetobacter, Flavobacterium, Micrococcus, Arthrobacter, Corynebacterium, Achromobacter, Rhodococcus, Alcaligenes, Mycobacterium, Bacillus, Aspergillus, Mucor, Fusarium, Penicillium, Rhodotorula, Candida* and Sporobolomyces [9]. Crude oils have very complex physicochemical properties; therefore, there are a large number of species of microorganisms that degrade the oil. Most of them are indigenous to the environment where the oily sludge occurs, while others have been bioengineered to degrade the oil sludge [4]. Each strain of indigenous microorganisms involved in biodegradation is usually characterized by the ability to utilize only a few types of hydrocarbons: bacterial genera such as *Acinetobacter, Arthrobacter, Bacillus, Corynebacterium, Flavobacterium, Vibrio*, and *Pseudomonas* contain indigenous species that together can degrade most components of crude oil, including aliphatic, alicyclic, aromatic, and polycyclic aromatic hydrocarbons [10, 11].

The natural function of abundant microbes in soil is to decompose nitrogenous and carbonaceous materials into inorganic materials. However, when oil or hydrocarbons remain the only carbon source, oil-degrading microbes survive in the soil and become dominant species [13]. Hydrocarbon-degrading microbes are commonly used in bioremediation. These include bacteria, fungi, yeasts, and some algae. They are normally found on land and in the sea. These microorganisms have been isolated from heavily oil-polluted reservoirs or a variety of soils and waters continuously exposed to hydrocarbons for several years [14, 15]. In this work, we aimed to investigate the effects of petroleum pollution on soil physicochemical properties, microflora and ecotoxicity assessment using germination index of bean seeds.

2. Materials and Methods

2.1. Study area.

T The survey was conducted in Onne, also known as Onne-Eleme, a town in Eleme, Rivers State, Nigeria. Onne is located next to Ogu Creek, near Bonny River in Eleme Local Government Area of Rivers State, Nigeria at latitude: 4°41'13.79 "N and Longitude: 7° 09' 16.80" E.

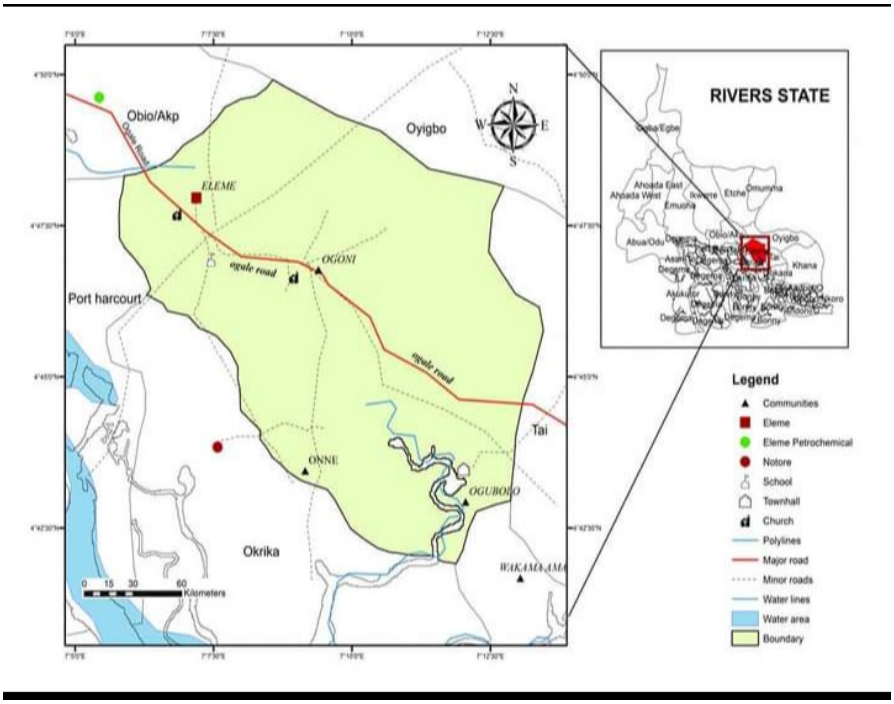

Figure 1. Map of study area at Eleme LGA.

2.2. Sample collection.

Three hundred and twenty (320) grams of soils were collected using a soil auger at a depth of 12 cm from four sites in Onne, Eleme LGA of Rivers State (80 g from each site). The samples were transferred to polythene containers, stored in cold packs and transported to the laboratory for analysis. The crude oil used for the assay wasobtained from Onne-Eleme oil field, Eleme L.G.A , Rivers State, Nigeria. Soil samples were collected in triplicate from each site and labeled A-D. Sampling was done according to the earlier methods of [13]. The microbiological and physicochemical properties of the soil were determined prior to mixing with the crude oil using the methods previously described by [16].

2.3. Experimental design and remediation study.

Three hundred and twenty grams of virgin soil were mixed with distilled water and incubated for 7 days at room temperature in the laboratory. The samples were mixed with crude oil at a ratio of 1:5, i.e., 16 ml of crude oil was mixed with 80 g of soil samples in glass jars for 2 weeks to homogenize them. An exact amount of pristine soil without crude oil contamination was used as a control. This was done as in previous methods of [15]. Eighty grams (80 g) of the soil samples were mixed with 16 ml of crude oil and prepared in quadruplicate in a glass jar and left in the microbiology laboratory for 2 weeks to allow the crude oil to mix well with the soil. The experimental setups were observed every two weeks for 24 weeks after contamination to observe the natural attenuation and to study the changes in the physicochemical and microbiological properties of the samples. The total petroleum hydrocarbon content was determined by gas chromatographic methods.

2.4. Microbiological analyses.

2.4.1. Isolation of indigenous microorganisms from the pristine soil and the crude oil polluted soil.

Microbiological examination of uncontaminated soil and soil contaminated with petroleum was performed using previous methods [16]. Contaminated soils that were properly mixed with crude oil were labeled accordingly. Ten grams of the contaminated soil sample was placed in a sterile 100-ml bottle with 50-ml of sterile water, shaken, and allowed to stand for 10 minutes. This was done to allow all particles and microorganisms to dissolve from the soil before the second shaking. From each sample, 1 ml of the liquid portion was aseptically pipetted and serially diluted through five tubes. The second and fourth test tubes of each set were selected for inoculation on Sabaraud Dextrose Agar (SDA) and Nutrient Agar (NA) culture plates, the former for fungi and the latter for bacteria. Using the spreading method, 0.1 ml of each dilution (10-2 and 10-4) of both the effluent and supernatant from the uncontaminated and contaminated soil were added to the NA and SDA plates in duplicate. Then, 0.1 ml of the diluted samples were added to the agar media surface and spread with a sterile curved glass spreader. The NA plates were incubated at room temperature for 24 hours, while the Sabaraud dextrose agar plates were incubated at 281 °C for 72 hours. Subcultures were prepared using the streak method for bacteria and the stab inoculation method for fungi to obtain discrete colonies (pure cultures). The discrete colonies were again plated on NA and SDA plates and incubated at 4 °C for further characterization. The total number of microorganisms was then determined.

2.4.2. Isolation and characterization of hydrocarbon utilizing bacteria and fungi

This was done using the vapor phase transfer method as described in [17]. The mineral salt medium was sterilized by autoclaving at 121 °C for 15 minutes and placed in Petri dishes. Plates were inoculated in duplicate with 0.1 ml aliquots of the 10^{-4} and 10^{-6} tenfold serially diluted samples using the spread plate technique. Plates were inverted onto dishes using 9-cm Whatman No. 1 filter paper that had been previously soaked in crude oil. A 0.1 ml aliquot of 10^{-4} and 10^{-6} tenfold serially diluted samples was used for the fungal plates. To suppress bacterial growth on the fungal plates, 0.5 ml of streptomycin and to suppress fungal growth on the bacterial plates, nystatin were added to the mineral salt agar.

2.4.3. Biochemical identification of isolates.

The biochemical tests were performed using standard methods as previously described by [18]. The following tests were performed for the bacterial isolates: Gram stain, urease, citrate utilization, sugar fermentation tests, motility, indole, and catalase. Gram staining techniques for yeast cells and a lactophenol-cotton blue staining test for molds were used to identify fungal isolates.

2.5. Determination of the frequency of hydrocarbon utilization by the bacterial isolates.

The tendency of bacterial isolates to use crude oil as their sole source of carbon and energy was determined using earlier methods of [19]. The amount of crude oil degradation by the bacterial isolates was determined using the gravimetric analysis method as described in [20].

2.6. Physicochemical analyses of the unpolluted soil and the polluted soil.

Physicochemical parameters such as electrical conductivity, total phosphorus, total nitrogen, total organic carbon and pH of the uncontaminated and crude oil contaminated soils were determined using previous methods of [21], while the heavy metal concentration of nickel (Ni), copper (Cu), zinc (Zn), manganese (Mn), lead (Pb), chromium (Cr), cadmium (Cd) and iron (Fe) were determined using standard methods as described in [23]. Total nitrogen concentration was determined using the earlier method of [24].

2.7. Residual oil content determination.

Residual oil content of crude oil contaminated soils was determined using methods described in [25].

2.8. Germination test.

Oil removal from the crude oil contaminated soils was also evaluated by observing the germination profile of Phaseolus vulgaris (bean) seeds using the method of [26]. Seeds were sterilized with 70% v/v ethanol for five minutes followed by repeated washing with sterilized distilled water. Four viable seeds of uniform size were used for the study. Seeds were planted in the center of each Petri dish, which contained 80 g of crude oil-loaded soil. The Petri dishes used for culturing the seeds were randomly shuffled each day to ensure a balance of parameters in the incubator. The germination test was performed in two phases, first immediately after crude oil contamination and second after 24 weeks of natural attenuation by authocthonic microorganisms. In addition, one Petri dish was cultured with each of the corresponding uncontaminated soil (soil without crude oil pollution), which served as a control to test the fertility of the soil. The cultured plates were irrigated with 5 ml of distilled water at intervals throughout the germination period to promote germination and then incubated at 28 ± 2 °C. Mean germination time (MGT), percentage of germination, and seedling length were recorded at regular intervals during the germination period. Recordings were made every 12 hours until the 12th day of germination. The germination index was determined as follows:

$$\text{ermination index, I (\%)} = \frac{(\%\ \text{Seed ermination , S}) \text{ X } (\%\ \text{rowth of root, R})}{100}$$

$$\text{Seed ermination , S (\%)} = \frac{(\%\ \text{ermination on contaminated soil, E}) \text{ X } 100}{(\%\ \text{ermination on control soil, C})}$$

$$\text{rowth of the root, R (\%)} = \frac{(\text{Elongation of root on contaminated soil, ERm}) \text{ X } 100}{(\text{Elongation of root on control soil, ERCm})}$$

2.8.1. Seedling length.

Seedling length was calculated by adding root length and shoot length and expressed in centimeters. Shoot length was determined by measuring from the base of the first leaf to the base of the hypocotyl, and the mean was expressed in centimeters. Root length was determined by measuring from the tip of the primary root to the base of the hypocotyl and the mean value was expressed in centimeters.

2.8.2. Seed germination and mean germination time (MGT)

The percentage seed germination and MGT was obtained by using the formula:

$$\text{ermination} \quad (\%) = \frac{\Sigma n}{N}$$

$$\text{MT} \quad = \frac{\Sigma n \,.\, D}{\Sigma n}$$

where n represents number of seeds geminated and N represents total number of seeds planted, D represents days from the beginning of the germination test, and Σn represents total number of germinated seeds.

2.9. Data analyses.

Data was analyzed using ANOVA and Statistical Package for Social Science (SPSS) version 21.0.

3. Results and Discussion

3.1. Microbial and biochemical characteristics of the pristine and crude oil impacted soil.

The total number of heterotrophs (THC) of bacterial and fungal isolates from the uncontaminated soils is shown in Table 1. Uncontaminated soils from sample A showed the highest bacterial count of 2.15±0.34 x 10^4 cfu/g while sample C showed the lowest count of 1.75±0.37 x 10^4 cfu/g. Fungal counts ranged from 1.22±0.08 x 10^4 cfu/g to 4.30±0.15 x 10^4 cfu/g.

Table 1. Enumeration of microorganisms from the pristine soils.

Soil Sample	Bacterial counts (10^4cfu/g)	Fungal counts (10^4cfu/g)
A	2.15±0.34	4.30±0.15
B	2.04±0.27	2.58±0.34
C	1.75±0.37	1.22±0.08
D	2.10±0.49	3.4±0.17

Data are mean of triplicate analyses ± SD

The mean bioload for all samples was statistically significant over the weeks up to week 24 ($p < 0.05$). Table 2 shows the cultural and biochemical characteristics of the bacterial isolates from the untouched soil. The heterotrophic bacteria isolated from the uncontaminated soil include: *Pseudomonas, Serratia, Flavobacterium, Bacillus, Micrococcus,* and *Klebsiella spp.* While the morphological characteristics of the indigenous molds from the uncontaminated soil, as shown in Table 3, include microbes such as *Mucor sp., Aspergillus niger*, and *Fusarium* sp.

Table 4 shows the enumeration of hydrocarbon-utilizing microorganisms in the soil contaminated with crude oil over a 24-week (6-month) period of natural attenuation. The count of microorganisms in the soil contaminated with crude oil over a remediation period of 24 weeks showed that the number of bacteria in all samples was low at the beginning of remediation, between the 8th and 12th week of remediation. This reduction in the number of microbes could be due to the effect of the degradation processes of the organisms eliminating

microorganisms and thus reducing the microbial population, as noted by [28]. The mean bioload for all samples was statistically significant across weeks until week 24 ($p < 0.05$).

Table 2. Microscopic and biochemical properties of bacterial isolates from the pristine soil.

	Cell morphology	Gram reaction	Spore formation	Motility	Catalase	Oxidase	Citrate	Indole	MR	VP	Coagulase	Mannitol	Lactose	Glucose	Maltose	sucrose	Organisms isolated
A	Straight rods	-	-	+	+	-	+	-	-	+	-	-	A	AG	+	ND	*Serratia spp.*
B	Rods	-	-	+	+	+	-	-	+	+	ND	-	AG	AG	AG	A	*Pseudomonas spp.*
C	Short rods	+	+	+	+	-	+	+	-	+	-	AG	A	A	-	-	*Bacillus spp.*
D	Rods	-	ND	-	+	-	-	+	+	+	-	+	+	A	+	+	*Klebsiella spp.*

Key: + = Positive; AG = Acid + Gas production; VP = Voges Proskaeuer; – = Negative; MR = Methyl Red; ND= Not Done; A = Acid production; G = Gas production

Table 3. Morphological characteristics of indigenous molds from the pristine Soil.

Sample	Nature of hyphae	Type Of Spores	Growth	Front view	Back view	Probable Organism
A	Ellipsoidal	Conidiospore	Rapid	White	Whitish	*Mucor sp.*
B	Non-Septate	Conidiospores	Rapid	Grey black	Dark brown	*Aspergillus niger*
C	Septate	Conidiospore	Abundant	Pale brown	Dark zonation	Fusarium sp.
D	Non-Septate	Conidiospores	Rapid	Grey black	Dark brown	*Aspergillus niger*

Table 4. Enumeration of the hydrocarbon utilizing microorganisms in the crude oil impacted soil over a 6 months bioattenuation period.

	Bacterial count (10^4cfu/g)				Fungi count (10^4cfu/g)			
Month	A	B	C	D	A	B	C	D
0	2.80 ±0.20	2.70±0.20	2.40±0.20	2.20±0.30	2.50 ±0.36	2.60±0.36	2.50±0.31	1.80±0.43
1	2.40±0.25	2.90±0.02	2.70±0.08	2.56±0.21	2.20±0.38	1.60±0.36	2.60±0.34	1.80±0.32
2	2.2±0.46	2.9±0.31	3.20±0.14	2.62 ±0.42	2.30±0.39	3.10±0.34	2.90±0.31	2.20±0.44
3	1.20±0.47	3.00±0.28	2.30±0.25	2.70±0.41	3.20±0.35	2.30±0.38	3.20±0.28	2.40±0.54
4	1.30±0.26	1.80±0.20	1.00±0.27	1.40±0.40	1.57±0.45	4.58±0.32	4.21±0.20	3.26±0.29
5	0.20±0.43	0.80±0.19	0.70±0.14	1.02±0.23	2.10±0.74	2.40±0.34	2.20±0.46	1.30±0.37
6	0.10±0.44	0.70±0.19	0.50±0.17	0.33±0.43	1.10±0.34	1.30±0.35	1.02±0.31	1.10±0.31

Values are mean of triplicate determinations ± SD

The highest number of hydrocarbon-utilizing bacteria, as shown in Table 4, was obtained in the 2nd month of sample C with a value of $3.20 \pm 0.14 \times 10^4$ cfu/g, while the lowest number of hydrocarbon-utilizing bacteria was obtained in sample A in the 6th month. The highest number of hydrocarbon-utilizing fungi was reached in sample B at month 4 with a value of $4.58 \pm 0.32 \times 10^4$ cfu/g, while the lowest number of hydrocarbon-utilizing fungi was reached in sample C at month 6 with a value of $1.02 \pm 0.31 \times 10^4$ cfu/g. All bacterial isolates from the pristine soils, as shown in Table 2, were able to utilize hydrocarbons. The hydrocarbon utilizing (hydrocarbonoclastic) bacteria isolated from the crude oil contaminated soils were *Serratia, Pseudomonas, Bacillus* and *Klebsiella* species. Similar hydrocarbon utilizing microorganisms were found from petroleum contaminated sites remediated with chicken manure [13, 15]. The crude oil-utilizing bacteria recovered in this study have been previously implicated to varying degrees in the biodegradation of crude oil from various sources [20, 13]. Stimulated biodegradation of crude oil is currently being promoted because it ensures rapid remediation of oil-contaminated ecosystems [28]. The degree of utilization of crude oil by bacterial isolates is shown in Table 5. Strong growth in the mineral salt medium was observed in Pseudomonas and *Bacillus* species, while *Serratia* and *Klebsiella spp.* showed the lowest growth levels. *Bacillus* species was the predominant bacterial species during crude oil recovery. Its prevalence

could be due to the fact that it forms spores, which helps the microorganisms to withstand harsh conditions such as sun drying. The isolation of *Bacillus* species from crude oil polluted soils could also be due to the ubiquity of the microorganisms. The isolation of *Bacillus, Pseudomonas, Flavobacterium, Serratia, Bacillus* and *Klebsiella* among other bacteria from crude oil polluted lithospheric environment was reported [13].

Table 5. Utilization of crude oil by the bacterial isolates.

Isolate	Frequency of growth in crude oil (MSM)
Bacillus spp.	+++
Pseudomonas aeruginosa	+++
Serralia spp.	+
Klebsiella spp.	+

Key: + small growth; +++ heavy growth.

3.3. Residual oil content of the impacted soil.

Table 6 shows that the residual oil content was highest in sample B with a value of 2941.47±1.71 mg/kg, while the least TPH value was obtained in the sample A with a value of 1887.50±1.35 mg/kg. This shows a significant oil content reduction. Previous studies also reported similar findings while working on the use of chicken droppings in the clean-up of crude oil polluted sites [13, 15].

Table 6. Residual oil estimation.

Polluted Samples (%)	Initial Oil content (mg/kg)	Oil content after 6 months (mg/kg)	Residual Oil content (mg/kg)
A	6105.53±0.01	4218.03±1.22	1887.50±1.35
B	6105.53±0.01	3164.06 ±1.07	2941.47±1.71
C	6105.53±0.01	3652.46±1.04	2453.07±1.07
D	6105.53±0.01	3192.35±1.11	2913.18±1.03

Results are expressed in mean ± standard deviation of triplicate determinations.

3.4. Physicochemical parameters of the pristine soil and the crude oil impacted soil.

The mean physicochemical properties of the crude oil-impacted soils and the pristine soils are shown in Tables 7-10. The levels of zinc, total nitrogen, total organic carbon, and electrical conductivity were statistically significantly different among the samples throughout the biodegradation period (< 0.05). This means that the heavy metal contents in the crude oil contaminated soils significantly decreased with increasing remediation period. Heavy metals decreased significantly with increasing remediation duration after six months of biodegradation, similar to the results of [28, 29]. Petroleum contamination increased soil pH to alkaline and also increased total nitrogen, organic carbon content, and a relative amount of phosphorus. Electrical conductivity, nitrogen content, and phosphorus content were significantly reduced after crude oil contamination [13, 29].

At the beginning of remediation, the pH of the crude oil contaminated soil varied from 7.83± 0.36 in sample D to 8.84± 0.06 in sample B, while the control had a much lower reduced acidic pH of 6.20±0.04. In the oil sampples contaminated with crude oil, the concentration of lead ranged from 0.08± 0.01 to 0.82±0.05, while that of copper ranged from 0.00±0.00 to 0.67±0.04 ppm. Zinc concentration ranges from 1.17±0.12 to 3.22±0.77 ppm. Cobalt content ranges from 0.00±0.00 to 1.28±0.03. Electrical conductivity ranges from 80.79±0.83 to 137.00±0.50 us/cm. Phosphorus concentration ranges from 3.81± 0.03 to 7.45 ± 0.03 mg/l. The

concentration of total nitrogen ranges from 4.14±0.02 to 5.84±0.05 %, while total organic carbon ranges from 1.82±0.60 to 4.93±0.02. This is consistent with previous studies [13, 29].

Table 7. initial physicochemical properties of the pristine soils.

Parameters	A	B	C	D
pH	6.65±0.10	6.65± 0.05	6.40± 0.05	7.44± 0.10
Lead	0.20± 0.00	0.00± 0.00	0.18± 0.00	0.07 ± 0.00
Copper (ppm)	0.12 ± 0.00	0.08± 0.00	0.07± 0.00	0.04 ± 0.00
Zinc (ppm)	4.58± 0.22	1.99± 0.00	0.77± 0.02	1.37 ± 0.05
Cobalt (ppm)	0.00± 0.00	0.46± 0.03	0.03± 0.00	0.01 ± 0.00
Conductivity (us/cm)	191.12± 0.95	188.40± 1.60	183.77± 0.89	172.21 ± 0.94
Phosphorus (mg/l)	4.92± 0.06	5.90 ± 0.02	5.38± 0.05	5.34± 0.07
Total nitrogen %	5.15± 0.06	5.91 ± 0.15	5.98± 0.04	5.11 ± 0.01
Total organic carbon	0.58± 0.00	0.60± 0.00	0.69± 0.06	0.52± 0.02

Results are expressed in Mean ± Standard deviation of triplicate determinations; *WHO limit, 2006 - pH 6.5-8.5, Total nitrogen 10 [30]; *NESREA limit, 2009 - pH 6.0-9.0, Total nitrogen 20 [31].

Table 8. Initial physicochemical characteristics of the crude oil impacted soils.

Parameters	A	B	C	D
pH	8.54± 0.08	8.84± 0.06	8.47± 0.04	7.83± 0.36
Lead	0.28± 0.03	0.18± 0.01	0.12± 0.05	0.15± 0.01
Copper (ppm)	0.14± 0.03	0.00± 0.00	0.03± 0.00	0.67± 0.04
Zinc (ppm)	3.20± 0.05	2.09± 0.01	1.85± 0.57	1.27± 0.12
Cobalt (ppm)	0.00± 0.00	0.17± 0.00	0.01± 0.00	0.00± 0.00
Conductivity (us/cm)	84.32± 1.51	80.79± 0.83	81.17± 1.02	80.96± 0.90
Phosphorus (mg/l)	3.81± 0.03	4.78 ±0.25	4.77± 0.04	4.00± 0.01
Total nitrogen %	4.92± 0.05	5.01± 0.03	5.00± 0.06	4.14± 0.02
Total organic carbon	3.76± 0.29	4.93± 0.02	3.95± 0.01	3.82± 0.60

Results are expressed in Mean ± Standard deviation of triplicate determinations; *WHO limit, 2006 - pH 6.5-8.5, Total nitrogen 10 [30]; *NESREA limit, 2009 - pH 6.0-9.0, Total nitrogen 20 [31].

Table 9. Physicochemical characteristics of the crude oil impacted soils after 3 months bioattenuation period.

Parameters	A	B	C	D
pH	7.83± 0.06	7.83± 0.05	7.48± 0.01	7.66± 0.54
Lead	0.18± 0.00	0.15± 0.02	0.10± 0.03	0.08± 0.05
Copper (ppm)	0.10± 0.00	0.00± 0.00	0.02± 0.00	0.34± 0.04
Zinc (ppm)	3.04± 0.03	2.20± 0.04	1.51± 0.01	1.05± 0.02
Cobalt (ppm)	0.00± 0.01	0.10± 0.00	0.00± 0.00	0.00± 0.00
Conductivity (us/cm)	108.04± 0.78	93.95± 0.11	98.73± 0.30	108.25± 0.89
Phosphorus (mg/l)	3.97± 0.01	4.91 ± 0.01	4.49± 0.01	4.31± 0.02
Total nitrogen %	4.96± 0.19	5.75± 0.05	5.94± 0.04	4.84± 0.12
Total organic carbon	2.94± 0.05	2.97± 0.09	2.37± 0.01	1.82± 0.60

Results are expressed in Mean ± Standard deviation of triplicate determinations; *WHO limit, 2006 - pH 6.5-8.5, Total nitrogen 10 [30]; *NESREA limit, 2009 - pH 6.0-9.0, Total nitrogen 20 [31].

Table 10. Physicochemical characteristics of the crude oil impacted soils after 6 months bioattenuation period.

Parameters	A	B	C	D
pH	7.06± 0.01	6.80± 0.10	6.97± 0.30	7.04± 0.09
Lead	0.00± 0.00	0.06± 0.05	0.00± 0.00	0.00± 0.00
Copper (ppm)	0.05± 0.00	0.00± 0.01	0.06± 0.00	0.03± 0.00
Zinc (ppm)	2.20± 0.03	1.16± 0.07	3.14± 0.01	1.99± 0.04
Cobalt (ppm)	0.13± 0.00	0.00± 0.00	0.00± 0.00	0.26± 0.04
Conductivity (us/cm)	117.41± 4.09	128.48± 0.77	127.86± 1.00	137.00± 0.50
Phosphorus (mg/l)	4.69± 0.48	7.45 ± 0.03	4.92± 0.05	5.68± 0.15
Total nitrogen %	5.03± 0.01	5.84± 0.05	5.96± 0.01	5.65± 0.17
Total organic carbon	0.80± 0.01	0.94± 0.00	0.94± 0.00	0.85± 0.04

Results are expressed in Mean ± Standard deviation of triplicate determinations; *WHO limit, 2006 - pH 6.5-8.5, Total nitrogen 10 [30]; *NESREA limit, 2009 - pH 6.0-9.0, Total nitrogen 20 [31].

3.5. Germination test.

In the germination test, the bioattenuated contaminated soils improved the germination of bean (*Phaseolus vulgaris*) seeds compared to the control after 12 days of germination, but more

effective growth was observed in the control option of the samples without crude oil contamination. The average germination time of the samples at the end of the 6-month natural decay period showed 67.5% growth in sample A, 75% in sample B, 85% in sample C and 82.3% in sample D. Seeds planted in the crude oil contaminated soil showed no growth during the first 14 days after contamination, as shown in Figure 1. The comparison of percentage germination, seedling length (cm) and mean germination time (MGT) of bean (*Phaseolus vulgaris*) seeds is shown in Table 11 for the different samples. The percentage of germination of bean (*Phaseolus vulgaris*) seeds ranged from 67.5% to 100%. This is in agreement with the work of [15, 28]. The results showed that the germination percentage of the seeds planted on the plates containing the soil contaminated with crude oil after natural attenuation (bioattenuation) was significantly lower than that of the seeds planted in the untreated soil without additive, as shown in Table 12. However, this differs significantly from the results of [31], who found growth in two of the three seeds grown. This could be due to the differences in the topography of the soil, the degree of crude oil contamination, and the differences in the type of microorganisms involved in the remediation.

Table 11. Germination index of *Phaseolus vulgaris.*

Sample	Germination (%)	MGT (days)	Seedling length (cm)
A	67.5	12.04	5.01
B	75.5	12.91	5.07
C	85.5	12.10	6.03
D	82.3	12.00	6.00
Pristine soil (Control)	100.0	8.00	8.05

4. Conclusions

The investigation showed that the contamination by crude oil affected the physicochemical properties of the soil. Contamination by crude oil increased the pH of soils to alkaline and also increased total nitrogen, organic carbon content and a relative amount of phosphorus. Electrical conductivity, nitrogen content, and phosphorus content were significantly reduced after crude oil contamination. Heavy metals decreased significantly with increasing remediation time after six months of biodegradation. The bioload of the crude oil contaminated soil over a remediation period of 6 months showed that the microbial counts for all samples were low at the beginning of remediation, between the 8th and 12th month. The germination test showed that the longer the crude oil contaminated areas were left to bioattenuation, the more the soils increased the crop yield, as shown by the growth of *Phaseolus vulgaris* seeds. Bioattenuation methods should be studied, applied and improved to remediate sites polluted by crude oil as they are cost effective and environmentally friendly compared to other methods.

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Competing Interest

I hereby declare that no competing interests exist regarding this publication.

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