



Effect of Woodchips on Bioremediation of Crude Oil-polluted Soil

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Authors' contributions

This work was carried out in collaboration between both authors. Author UCO managed literature, searches, designed the study and wrote the first draft of the manuscript. Author ASN performed the statistical analysis and wrote the protocol. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: To evaluate the effectiveness of woodchips in bioremediation of crude oil contaminated soil, phytotoxicity assay as an index of soil biological activities (germination index) using a selected agricultural seed (*Vicia faba*) was also evaluated.

Methodology: Soil sample collection from Aguleri and Nkwelle Ezunaka, both in Anambra State. Samples were stored in polythene bags and transported to the laboratory. The soil samples were air dried, sieved through 2 mm mesh and stored in polythene bags at room temperature.

Results: Results showed that relatively alkaline pH was observed in woodchips amended option while slight acidity was reported in the control soil. Using woodchips as biostimulant achieved 75% crude oil contaminant removal but only 50% in the control. The microorganisms isolated from the present study included *Klebsiella* spp *Pseudomonas* spp *Candida* spp *Fusarium*, *Penicillium* spp. Only 50% woodchips amended system produced growth of *Vicia faba* after 5 days incubation. There was no growth of *Vicia faba* at 10% and 30% of woodchips at the same incubation time.

Conclusion: Result of the present study showed that hydrocarbon removal from the lithosphere can occur either naturally or by strategy enhancement with amendments but posited that rate and extent of removal in each case always differ. Present study also proved that for recovery of polluted

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media such as soil, information on the concentration of the additional limiting factors is scientifically crucial. 50% woodchips supported high crude oil remediation in the polluted soil. Woodchips therefore, is a potential source of nutrients for microbial activity and it harbours microorganisms capable of utilizing hydrocarbons as source of carbon and energy thus, potentially useful in soil hydrocarbon spill response action.

Keywords: Bioremediation; woodchips; biostimulation; crude oil; pollution; soil.

1. INTRODUCTION

Bioremediation is the use of naturally occurring microorganisms or genetically engineered microorganisms by man to detoxify man-made pollutants [1,2]. Since bioremediation is a microbial process, it requires the provision of nutrients among other factors or requirements. The addition of organic waste materials such as woodchips and other organic biostimulants to the soil facilitates aeration through small pores and increases the water holding capacity of the soil, thus enhancing bioremediation [3,4]. It allows natural processes to clean up harmful chemicals in the environment. Microscopic “bugs” or microbes that live in soil and groundwater use certain harmful chemicals such as those found in gasoline and oil spills. Crude oil is a complex mixture of diverse hydrocarbons including alkanes, aromatics, alicyclics, branched hydrocarbons, and non-hydrocarbon compounds including polar fractions containing hetero-atoms of nitrogen, sulfur and oxygen (NSO fraction), and asphaltens [5,6].

The high demand for petroleum products in the form of cooking gas, aviation fuel, gas oil, engine lubricating oil, asphalt and coal tar results in increased production and this eventually leads to oil spills and hydrocarbon contamination of the environment especially through oil well blow out, tanker accidents, accidental rupture of pipelines and routine clean-up operations [7]. Current technologies for cleaning hydrocarbon contaminated soil include soil washing, solvent extraction, thermal treatment, composting, chemical oxidation (Fenton's reagent, permanganate, ozone etc.) and bioremediation (bioaugmentation, biostimulation and phytoremediation) [3,8,9]. Physical and chemical approaches are expensive and products may cause secondary contamination of soil and water resulting in the need for additional post-treatment. As such, there is a wide-spread interest in bioremediation for the complete mineralization of hydrocarbons to carbon dioxide and water which are environment friendly. In presence of abundant hydrocarbon spill,

Microorganisms cannot effectively breakdown the obnoxious contaminants into innocuous and eco-friendly intermediates unless a source of additional nutrients is provided [2]. Woodchips which are bulking agents apart from increasing the nutritional value of the polluted media also act as immobilizing materials which positively affect the physical characteristics of the soil (pH, porosity, water retention, permeability coefficient) as well as reduce competition by microorganisms for limited nutrients and consequently results to fast breakdown of hydrocarbon pollutants [2].

At present, there is paucity of scientific information on the use of woodchips in the bio restoration of crude oil impacted soil, therefore, the aims of the study were to evaluate the effectiveness of the woodchips in bioremediation of crude oil contaminated soil, phytotoxicity assay as an index of soil biological activities (germination index) using a selected agricultural seeds (*Vicia faba*).

2. MATERIALS AND METHODS

2.1 Sample Collection

Soil sample was collected from four different locations for composite sample preparation in Aguleri and Nkwelle Ezunaka, both in Anambra State. The co-ordinates of the sampling points were: 453'53.990"N; 650'43.745"E and 45353.874"N; 650'43.564"E first the first location and 454'59.990"N; 620'43.745"E and 454'56.886"N 657'43.775"E for the second location. Composite samples were mixed and stored in polythene bags and transported to the Department of Applied Microbiology and Brewing's laboratory, Nnamdi Azikiwe University, PMB 5025, Awka, Anambra state, Nigeria between January, 2015 to March, 2016. The soil samples were air dried, sieved through 2 mm mesh and stored in polythene bags at room temperature [10].

The crude oil (black and slurry) was collected from Eleme oil field, Rivers state, Nigeria. The soil amendment material (woodchips) was

collected from community forest in Okpuno village Awka, in Anambra State, Nigeria. The woodchips were air dried, further ground and stored in the laboratory at room temperature ($28\pm 2^\circ\text{C}$).

2.2 Incorporation of Amendment Material into the Soil Sample

Three hundred and twenty grams of soil was moistened and kept at room temperature in the Microbiology laboratory for one week. The soil sample was polluted with the crude oil in the ratio of 5:1 i.e. 80 g of soil was mixed with 16 ml of crude oil and kept for 2 weeks. The woodchips were applied at 10%, 30% and 50% respectively to the oil polluted soil. The experimental samples were set up as shown in Table 1. Both the amended soil and the control (polluted soil without amendment) were incubated at room temperature and observed after every two weeks for 24 weeks after pollution and the effect of the amendment on the samples studied.

Table 1. Experimental design

S/No	Woodchips	Description
GCl	10%	80 g of polluted soil + 8 g of woodchips
GC2	30%	80 g of polluted soil + 24 g of woodchips
GC3	50%	80 g of polluted soil + 40 g of woodchips
GC4	nil	80 g of polluted soil + no woodchips

*GC= Glass Container

2.3 Bioremediation Study

This was carried out in the Microbiology laboratory. 80 g of the soil samples was mixed with 16 ml of crude oil and was prepared in four places using a glass crucible and was left in the microbiology laboratory for 2 weeks. The woodchips were then added to the crude oil polluted soil at the various concentrations for 10%, 30%, 50% and the control was left without amendment. The set up was then incubated for a period of 24 weeks [10]. The total petroleum hydrocarbon (TPH) was determined using Gas chromatographic methods. After 24 weeks of remediation, ecological status of the pollution was tested using seeds of *Vicia faba*.

2.4 Physicochemical Analyses of Soil Sample and Woodchips

Physicochemical analyses were carried out on both the woodchips before amendment and on

the polluted soil after amendment with woodchips and the analysis were carried out at the start of the research, repeated after the 12th week and then concluded after the 24th week. pH, Total nitrogen, phosphorus, and calcium were analyzed [11].

2.5 Microbial Enumeration

Mineral salts medium was sterilized by autoclaving at 121°C for 15 minutes and dispensed into Petri dishes. The plates were inoculated in duplicates with 0.1 ml aliquots of the 10^{-4} of 10-fold serially diluted samples using spread plate technique. The plates were inverted over the dish covers containing 9 cm Whatman No. 1 filter papers earlier impregnated with crude oil. Preparations were made differently for bacteria and fungi. 0.5 ml of nystatin was added to the bacterial plate (petri dishes) to stop fungal growth while equal concentration of streptomycin was added to fungal plate to stop bacterial growth.

2.6 Screening test for Hydrocarbon Utilization

The ability of the bacterial isolates to utilize crude oil as the only source of carbon and energy was determined [12]. 0.1 ml of 24 hours old nutrient broth culture was inoculated onto each test tube containing 10 ml of sterile mineral salt medium (MSM) of Bushnell and Haas (1941) and 1% crude oil. Control test tubes were set up containing 10 ml of MSM with 1% crude oil without bacterial seeding. The tubes were incubated at 28°C for 10 days. At the end of the incubation period, the growth of the isolates was determined by visual observation of the oil medium for turbidity, as compared to the control tubes [10]. The extent of degradation of the crude oil by the bacterial isolates was gas chromatographically determined [13]. The amount of crude oil left after the incubation time was determined by extracting the residual oil with 50 ml of toluene from the 100 ml culture. The mixture was separated using a separator/ funnel and then filtered off with Whatman No 1 filter paper. The optical density was read on a spectrophotometer at 550 nm wavelength. Using a previously prepared standard curve, the weight of the crude oil was determined. The amount of crude oil degraded was calculated by subtracting the weight of residual crude oil from weight of the added (initial) crude oil, divided by the weight of the initial crude oil and then multiplied by 100 [14].

Amount degraded= (Weight of initial crude oil - Weight of residual of crude oil / Weight of initial crude oil) x (100/1)

Crude oil utilization test was carried out for the confirmatory identification of actual petroleum-utilizing fungi using isolates obtained from the oil agar preliminary isolation medium. The vapour phase transfer method was used [15].

Putative petroleum-utilizing fungi isolates were streaked on plates of agar medium (one Isolate per plate). In the inside of the Fein-dish cover was placed a sterile filter paper (Whatman No 1) saturated with filter-sterilized crude oil used in the study. This was aimed at supplying hydrocarbons as sole sources of carbon and energy for the growth of the micro-organisms on the mineral salts agar medium surface through vapour phase transfer. All the plates were inverted and incubated at 28°C for 7 to 14 days [9]. Uninoculated plates served as control. Colonies which appeared on the mineral salts agar medium plates were picked and purified on plates of potatoes dextrose agar transferred onto Sabouraud dextrose agar slants. These were then considered confirmed petroleum-utilizing fungi.

2.7 Total petroleum Hydrocarbon (TPH) Determination

Total hydrocarbon concentrations were determined using Gas Chromatographic methods according to the toluene extraction method [1] and Sonication water bath methods. Fifteen grams (15 g) of each of the sample was weighed into 50 ml conical flasks, and then 1 ml of 60 µg/ml of 1-chlorooctadecane surrogate standard was added. Then 30 ml of dichloromethane (extraction solvent) was added to extract oil in the soil. After shaking vigorously in water bath for 5 hr, the mixture was allowed to stand for 60 minutes and then filtered through Whatman No. 1 filter paper fitted with cotton wool and sodium sulphate into a clean beaker washed with methylene chloride. The residue was then washed with 20 ml extracting solvent and then filtered through funnel. The extracted oil was transferred to vial and placed on a GC for analysis. The amount of crude oil degraded was calculated by subtracting the weight of residual crude oil from weight of the initial crude oil, divided by the weight of the initial crude oil and then multiplied by 100 [15,16].

TPH for Soil (mg/kg) = (Instrument reading x Total weight of extract / Weight of sample)

2.8 Crude Oil Plant Toxicity Assay

Ecotoxicity is the subject of study of the field of ecotoxicology, which refers to the potential for biological, chemical or physical stress to affect ecosystem. Aside the normal laboratory tests, the remediated soil samples were further subjected to ecotoxicity tests to show the success of the remediation process and to determine the relationship between the growth rate of plants and treated soil at different amendment concentrations. Bean seed (*Vicia faba*) was first cultivated in 1 kg soil sample without pollution to determine its suitability for germination purposes. When this was ascertained, seeds were planted both In-situ and Ex-situ environments of 1 kg crude oil polluted soil amended with the woodchips at varying concentrations.

Method of [17,18] was used. The Germination index was determined as follows:

$$\text{Germination Index, GI(\%)} = [(\% \text{ Seed Germination, SG}) \times (\% \text{ Growth of root, GR}) / 100]$$

Where % Seed Germination, SG =

$$[(\% \text{ Germination on contaminated soil, EG}) \times 100 / (\% \text{ Germination on control soil, CG})]$$

And % Growth of the root, GR=
$$[(\text{Elongation of root on contaminated soil, GERm}) \times 100 / (\text{Elongation of root on control soil, GERcm})]$$

3. RESULTS AND DISCUSSION

3.1 Soils Physicochemical and Microbiological Status

The physicochemical parameters of the woodchips are summarized in Table 2.

3.2 Bioload Status

The total heterotrophic microorganisms in the woodchips enumerated on nutrient agar and sabaroud dextrose agar for bacteria and fungi respectively. Woodchips was found to contain considerably high level of microorganisms and this was added to the polluted soil autochthonous microorganisms. This resulted in higher microbial loads (bacteria and fungi) in the woodchips amended option throughout the study.

The TPH of the crude oil polluted soil for 24 week incubated quantified gas chromatographically is

summarized in Table 4. The 10% woodchips amendment showed little loss of hydrocarbon contaminant thus, a low removal efficiency. Both 30% and 50% woodchips portion produced greater than 50% hydrocarbon removal efficiencies.

Hydrocarbon introduction into the soil resulted to loss of soil germination action. No germination results were obtained in the control, 10% and 30% woodchips amended microcosms except the 50% woodchips amended system which was greater than 90% (Table 7).

Table 2. Physicochemical properties of woodchips

pH	8.1
Total Nitrogen (%)	1.8
Total Phosphorus (%)	0.9
Total Calcium (%)	1.8
Total Magnesium (%)	0.5
Total Potassium (%)	2.7

Table 3. Total heterotrophic microbial counts of the woodchips and the soil before amendment

Woodchips (10⁴ cfu/g)	Soil (10⁴ cfu/g)
Heterotrophic bacterial count	Heterotrophic bacterial count
3.40	2.58
Fungal count	Fungal count
1.77	1.74

3.3 Enumeration of Hydrocarbon Degraders

Table 5 and 6 shows the weekly report of crude oil contaminated soil enumerated on mineral salt medium via vapour phase technique. Microbial dynamics indicated initial growth lag and a steady rate of growth between week 1 and week 2 then followed an observable cell decline. Population bacterial degraders were relatively higher than the fungi degraders throughout the study [19,20].

4. DISCUSSION

The physicochemical parameters of the woodchips revealed an alkaline pH of 8.1 and a low value of 0.5% magnesium content as shown in Table 2 [18,21,22].

The result of the ecotoxicity assay among different concentrations of the woodchips (10%, 30%, 50%), control and soil sample collected from the same site without pollution or amendment are presented in Table 7. Seed germination on the free soil (soil not polluted) showed 100% germination performance indicating the arable stability of the soil.

The isolation of microbial genera from the woodchips in this work was in agreement with earlier report by [15,16]. The microbiological assessment of the woodchips in this research revealed the bioload level of the woodchips (Table 3). 50% woodchips added to soil removed about 55% of the crude oil whereas additive-free soil was naturally remediated to just over 36%. The microbial population of the woodchips before being used for amendment shows the mean count of 3.4 x10⁴ Cfu/g and 1.77 x10⁴ Cfu/g for bacteria and fungi respectively.

Table 4. Residual TPH at different amendment concentration between week 1 and week 24

Woodchips (%)	Initial TPH (mg/kg)	TPH (mg/kg)24 weeks	total removed (ml/g)	% removal
10	6609.83	3788.03	2821.80	42
30	6609.83	3144.06	3465.77	52
50	6609.83	2951.37	3658.46	55
Control	6609.83	4192.35	2417.48	36

Table 5. Hydrocarbonoclastic bacterial counts (CFU/g)

S/ No	Woodchips (%)	Months							Mean
		0 (start)	1 (4 wk)	2 (8 wk)	3 (12 wk)	4 (16 wk)	5 (20 wk)	6 (24 wk)	
1	10	1.2	1.3	1.8	1.7	1.6	0.9	0.5	1.29
2	30	1.4	1.7	2.0	2.3	2.0	0.7	0.5	1.51
3	50	1.7	1.9	1.9	2.0	1.9	0.9	0.7	1.57
4	Non-amended	0.8	0.4	0.2	0.2	0.3	0.1	0.1	0.20

Table 6. Hydrocarbonoclastic fungal counts (CFU/g)

S/No	Woodchips	Months								Mean
		0	1	2	3	4	5	6		
1	10%	0.8	0.8	1.2	1.4	1.1	0.3	0.1	0.59	
2	30%	0.5	0.6	0.9	1.2	0.8	0.2	0.1	0.61	
3	50%	0.6	0.6	1.1	1.3	0.5	0.4	0.3	0.69	
4	Non-amended	0.5	0.2	0.3	0.2	0.2	0.1	0.1	0.22	

Table 7. Germination index of *Vicia faba*

Woodchips (%)	In situ (%)	Ex situ (%)
Control	0.00	0.00
10	0.00	0.00
30	0.00	0.00
50	95.00	95.00
SWP (%)	100.00	100.0

SWP = Soil without pollution and amendment collected from the same site

Analysis by gas chromatography revealed that the highest TPH residual of 4192.35 mg/kg was obtained in the control (system without woodchips amendment), while the least TPH value was obtained in the sample with 50% amendment with a value of 2951.37 mg/kg (Table 4). This however depicted a significant reduction of crude oil contaminant in 50% amendment system. This was attributed to the additional biodegradative activities performed by the allochthonous microbial diversity from the woodchips at 50 % concentration.

The hydrocarbon utilizing microorganisms isolated in the present study included: *Klebsiella* spp *Pseudomonas* spp *Candida* spp *Fusarium*, *Penicillium* spp [9]. The mean hydrocarbon utilizing microbial counts of the crude oil polluted soil after 24 weeks of amendment showed the highest count of hydrocarbon utilizing bacteria at 50% amendment with a value of 1.57×10^4 Cfu/g, while the least hydrocarbon utilizing bacteria count was obtained in the control sample without amendment with a value of 2.0×10^5 Cfu/g.

The highest count of hydrocarbon utilizing fungi was obtained at 50% amendment with a value of 6.9×10^5 Cfu/g, while the least hydrocarbon utilizing fungi count was obtained in the control sample without amendment with a value of 2.2×10^5 Cfu/g (Tables 5 and 6).

Ecotoxicity test carried out on the crude oil polluted soil amended with poultry droppings (Table 7) revealed growth of the bean seed after 5 days of incubation in the 50% chicken dropping amended system. There was no growth in the

other treated options thus indicated that the added amendment was not enough to restore the lost soil biological activities (seed germination).

5. CONCLUSION

The results in this study showed that hydrocarbon removal from the lithosphere can occur either naturally or by strategy enhancement with amendments but posited that rate and extent of removal in each case always differ. Present study also proved that for recovery of polluted media such as soil, information on the concentration of the additional limiting factors is scientifically crucial. 50% woodchips supported high crude oil remediation in the polluted soil. Woodchips therefore, is a potential source of nutrients for microbial activity and it harbours microorganisms capable of utilizing hydrocarbons as source of carbon and energy thus, potentially useful in soil hydrocarbon spill response action.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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