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Role of Plant-Microbe Interactions in Rhizoremediation of Petroleum Product-Polluted Nigerian Soils

Umeaku Chinyelu Nkiru[1], Okpokwasili Gideon Chijioke[2], Iwuala Moses[3], Nwigwe Harriet Chinyelu[3], Chris-Umeaku Chiamaka Ijeoma[4]

[1]Department of Microbiology, Chukwuemeka Odumegwu Ojukwu University, Uli, Anambra, Nigeria

[2]Department of Microbiology, University of Port Haccourt, Choba, Rivers, Nigeria

[3]Department of Biotechnology, Federal University of Technology, Owerri, Nigeria

[4]Department of Biochemistry, Chukwuemeka Odumegwu Ojukwu University, Uli, Anambra, Nigeria

Email address:
chimeaku@yahoo.com (U. C. Nkiru)

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Abstract: Plant-microbe interactions in petroleum product-contaminated soils were examined under field conditions, to analyze the effects of environmental factors on rhizoremediation. Different plant types (elephant grass, cassava, carpet grass, Indian bamboo, fern, palm and a mixture of these plants) were considered. The abundance of effective indigenous microorganisms (EIM) and remediation time were also considered. Eight soil samples were collected from crude oil-contaminated sites of Ogoni, Rivers State, Nigeria. Total viable counts were taken using spread plate methods. Hydrocarbon utilizing microorganisms using mineral salt agar with crude oil vapor phase gave counts in the range of 10^2 – 10^8. Gas Chromatographic – FID methods gave results of 9 field samples for TPH to range from 0.96 mg/kg for control to 4,650 mg/kg for oil well 3 without vegetation. Polyaromatic hydrocarbon (PAH) of 9 field samples ranged from 0.26 mg/kg for control to 30.066 mg/kg depending on the extent of pollution of the site. Mixed plant population showed abundance of EIM (10^8) and greater TPH and PAH removal. The effect of time on the concentration of TPH and PAH in the different samples show that significant difference exist, P – value = 0.420. Microbial counts are significantly different over time, also microbial counts are significantly different in the different samples (F-value = 3.15, 2.76, 2.53, 2.1, 1.92 and 1.7) for rows, columns and layers. Local Nigerian plants could promote rhizoremediation of crude oil polluted soils.

Keywords: Rhizoremediation, Microorganisms, TPH, PAH, Microbial Counts, Gas Chromatographic, Remediation

1. Introduction

Background Information

Rhizoremediation is an elegant form of bioremediation that seeks to harness light energy via plants to biostimulate pollutant degradation by indigenous soil microbial community. Exploration and production of crude oil in Nigeria are carried out in the oil-rich Niger Delta region (Bayelsa, Rivers, and Delta) and Imo states. Over 80% of the country's oil comes from this ecological zone and its surrounding offshore areas [1]. Within the Delta, the numerous national deposits (oil fields), tank farms, flow stations, pipelines, tankers and loading jetties constantly provide potential sources of oil pollution [8]. Large scale pollution of both the terrestrial and aquatic environments as a result of activities of the oil industries have been documented ([23]; [8]). In addition to seepage and human activities, oil spills in Nigeria occur due to a number of other causes such as corrosion of pipelines and tankers. Infrastructure may be old and lack regular inspection and maintenance. Apart from ecological and health problems, there are significant financial repercussions associated with the release of these compounds into the soil. Contaminated soil generally remains toxic until suitable clean-up has occurred, a process that may take

substantial time and money. Although the physical and chemical processes such as dispersion, dilution, sorption, volatilization and abiotic transformation are important means of hydrocarbon elimination, biodegradation is most often the primary mechanism for contaminant clean-up. Plants and their associated microorganisms are efficient for decontamination of crude oil-polluted soils [15]. Rhizoremediation utilizes the absorption, stabilization and rhizosphere effect of the plant to remediate polluted soils. Oil spills result in an imbalance in the carbon-nitrogen ratio at the spill site, because crude oil is essentially a mixture of carbon and hydrogen. This causes the nitrogen deficiency in an oil soaked soil, which retards the growth of bacteria and utilization of carbon source [11]. Furthermore, large concentration of biodegradable organics in the top layer of agricultural soils deplete oxygen reserves in the soil and slow down the rate of oxygen diffusion to deeper layers [1]. Rhizoremediation draws upon the fields of rhizosphere ecology and microbial biodegradation. Although accumulating studies show that rhizoremediation is a viable treatment option for petroleum hydrocarbon [1], this technology is still under-valued and under-utilized. The primary reason is the lack of consensus between study findings. Plant species vary greatly in their ability to increase the hydrocarbon degradation capacity of soil microbial communities ([16]; [30]; [7]). The goal of this research is to evaluate the degradation potential of rhizosphere microorganisms of some local Nigerian plants in the remediation of crude oil-polluted soils with a view of using them in rhizoremediation of crude oil-polluted soils.

2. Materials and Methods

2.1. Designation of Field Samples from Ogoni

8 samples were collected from the crude oil-polluted sites as described above, and designated;

A	-	Non rhizosphere oil well site at Kpopie
B	-	Palm rhizosphere
C	-	Indian bamboo rhizosophere
D	-	Mixed population
E	-	Oil well 3 korokoro - no vegetation
F	-	Oil well 3 korokoro - with elephant grass
G	-	Grass rhizosphere
H	-	Fern rhizosphere
Control	-	Control

All samples were collected on same day. They were collected at 15cm depth from the soil surface with sterile cellophane bags, the cellophane bags were sterilized by washing with 70% ethanol, followed with a minimum of five rinses with sterile water. These were carefully labeled and transported to the laboratories for analysis.

2.2. Collection of Soil Samples

Rhizosphere samples were collected from the root zones of plants in the plots at 15cm depth. The soil samples were sieved and immediately taken to the laboratory for physicochemical analysis. Roots were shaken to dislodge loose soil. Rhizosphere soil was also sent to the laboratory for hydrocarbon analysis. Rhizosphere soil from all sites were archived at -4°C as standby or soil sample bank. Washed roots were dried at room temperature. Microbiological analysis commenced 24hrs – 36hrs after collection.

2.3. Physico-chemical Analysis

The following physico-chemical parameters were analyzed, pH, total organic carbon, percentage organic matter, total nitrogen, magnesium/kg, microbial respiration, nitrate, phosphate, moisture content, Pb, Cu^{2+}, Al^{3+}, Fe^{2+}, Cd, and Hg according to Methods of APHA [3].

2.3.1. Determination of pH

pH of saturated paste of soil samples were analyzed using the calibrated pH meter, scientific water quality multimeter-850081 model was used.

2.3.2. Nitrate Determination

Nitrate in the samples was determined by the cadmium reduction method (APHA 4500-NO3E) using cecil-ce 1021(1000 series) ultraviolet visible spectrophotometer at wavelength of 543nm.

2.3.3. Phosphate Determination

Phosphate in the samples was determined by the 4500-PD test method. A colorimeter method based on a blue complex induced by the addition of stannous chloride. The samples were analyzed at a wavelength of 690nm with cecil-ce 102(1000 series) ultra violet visible spectrophotometer.

2.3.4. Determination of Total Organic Carbon (TOC)

The TOC of the sample was determined using the Walkey and Black wet oxidation method of APHA [3]. About 0.5g of each of the samples was weighed into a 250ml conical flask. 10ml of 1N potassium dichromate solution and 20ml of concentrated sulfuric acid were added. The mixtures were shaken thoroughly for about 2 minutes and were allowed to stand for 10 minutes on an asbesto mat. 200ml of distilled water and 10ml of phosphoric acid were then added and mixed gently. The resultant mixtures were titrated with 0.5M ferrous ammonium sulphate solution using 1M of diphenylamine solution as indicator. A blank was set up, manual titration was used in the experimental sample. The percentage carbon in the samples were calculated thus;

$$M\ X\ \frac{\mathrm{V1}-\mathrm{V2}}{\mathrm{W}}\ X\ 0.30\ \mathrm{X\ CF}$$

Where:

M is the molarity of the $FeSO_4$ solution (from blank titration),

V1 is the volume (mL) of $FeSO_4$ required in blank titration,

V2 is the volume (mL) of $FeSO_4$ required in actual titration,

W is the weight (g) of the oven-dried soil sample and

CF is the correction factor. The CF is a compensation for the incomplete oxidation and is the inverse of the recovery. This CF was set by Walkley and Black as 1.32 (recovery of 76 %).

Automated methods were used for the field samples. The automated method made use of the gas chromatograph (GC) attached to a flame ionization detector. The CO_2 was first converted to CH_4 by passing the evolved gas through the heated aluminum coated with nickel in a hydrogen enriched atmosphere. The results were read from the chromatogram using USEPA [33] methods. The percentage carbon in the samples were calculated thus;

2.3.5. Determination of Moisture Content

10.0g + 0.001g of each of the samples was weighed into a pre-tarred crucible. The crucible and the soil sample were then heated in an oven at 105^0C (degree Celsius) for 2 hours. The crucible was then transferred into a desiccator and weighed. This later process was continued until a constant weight was obtained. The percentage moisture was calculated.

$$\text{Moisture content \%} = \frac{\text{W2} - \text{W3}}{\text{W2} - \text{W1}} X \frac{100}{1}$$

Where %W = Percentage of Moisture in the sample.

W1 = Weight of container with lid.

W2 = Weight of container with lid and sample before Drying.

W3 = Weight of container with lid and sample after Drying.

2.3.6. Total Nitrogen

Methods of Kjeldahl which involves digestion of the samples to convert all nitrogenous compounds into ammonium was used. Soil for analysis was finely ground to pass through 60-80mm mesh-sieve. 25g of soil was weighed into a dry micro Kjeldahl flask and 20ml of distilled water was added. The flask was swirled for few minutes. A mixture of 10g of K_2SO_4, 1g of $CuSO_4$, 1g of selenium and 30ml of conc. H_2SO_4 was added to the flask. The flask was gently heated continuously, then more strongly until the acid was boiled. Heating was continued until the organic acid was destroyed (the solution was clear light yellow to gray). The flask was allowed to cool until crystals appeared. The content of the flask was transferred into a 200ml volumetric flask washing 4 times with NH_3 free water. Distillation:10ml of the solution was pipetted into a Kjeldahl flask, 25ml of 4% boric acid and 4 drops of bromocrysol green methyl red indicator (M and Zuazaga indicator) was added into a 100ml Erlenmeyer flask. The outlet was connected to the condenser so that the tip of the outlet (glass-tube) was just beneath the boric acid, 10ml of 40 % NaOH was allowed to slide slowly down the side of the distillation flask. 50ml of the distillate was collected for 5mins.

Observed N_2 volume was calculated thus:

$$\text{Vo.} = R_2 - R_1$$

where Vo = Observed N_2 Volume (ml)

R_1 = Initial count reacting

R_2 = Final count reacting

Corrected N_2 volume (ml) = Vc

Automated methods using GC were also utilized in this study.

2.4. Microbiology

2.4.1. Culturable Microbiological Analysis

Entire plants were placed on ice pack for transport back to the laboratory and were stored at the temperature of -4 degree Celsius until process and analysis (within 36hrs of sampling for soil samples and 72hr for endophytic samples). Soil from each plant and from each site was serially diluted in distilled water. Total heterotrophic bacteria were enumerated using the spread plate method on nutrient agar according to methods of Cheesbrough [6]. One gram of each of the soil sample was weighed and transferred into 250ml flask containing 9ml of sterile distilled water. The suspensions were shaken intermittently for about 30 minutes. Each solution was allowed to stand for 1 hour after which the suspension was decanted into another 250ml flask. Ten-fold serial dilution was set up from the soil suspension. 0.1ml of the one in10^2, 10^3, 10^4, 10^5, 10^6, 10^7, and 10^8 were spread in duplicate. Freshly prepared nutrient agar was poured and allowed to set. This was used as control. Plates were incubated at room temperature for 24 hrs. Total plate counts were carried out using the colony counter. Organisms were subcultured in sterile nutrient agar for total heterotrophs. Mineral salt agar was used for hydrocarbon utilizing organism (HUO) according to methods of Okpokwasili, and Odokuma [22]. Anaerobic organisms were cultivated using petri plates sealed with vaseline and incubating under anaerobic condition (methods of Kirk *et al.,* [12]). Sabaraud dextrose agar (SDA) was used for fungi culture. These were incubated at room temperature for 3-5 days. Fungal atlas was used in identifying the organisms.

2.4.2. Enumeration of Hydrocarbon Utilizing Bacteria

Culturable hydrocarbon degraders, also known as hydrocarbon degrading bacteria (HUB) were assayed for. Here crude oil was used as the sole source of energy and carbon. Sterilized crude oil was used to soak sterilized filter paper. This was placed in the lid of inverted plates. The plates were incubated at room temperature for 6 days and HUB were counted ([21]; [24]). Bushnell Haas (BH) mineral salt agar (per litre: 0.29g $MgSO_2.7H_2O$, 0.02g $CaCl_2.2H_2O$, 1.0g kH_2PO_4, 1.0g (NH_4), HPO_4, 1.0g KNO_3, 0.05 $FeCl.6H_2O$., pH 7) was used to culture the serially diluted samples. Plates were incubated in the dark at room temperature [25]. Nystatin and Streptomycin were added for selectivity for bacteria and fungi respectively.

2.4.3. Characterization and Biochemical Identification of Isolates

Pure isolates were examined for their cultural appearance. Endospore, acid fast and Grams staining techniques were

performed to establish the cell morphology of the isolates. Motility, citrate utilization, oxidase, catalase, indole and coagulase tests were carried out according to methods of (Umeaku[34]; Talaro, and Talaro, [32]; Cheesbrough [6]). Microbial identification was performed using the keys provided by Bergey's Manual of Determinative Bacteriology (1994).

2.4.4. Endophytic Microbial Community

Rinced roots were surface-disinfected by sequential washing with 95% ethanol and 5.25% sodium hypochlorite, followed by a minimum of 5 rinses with sterile water. To assess surface sterility 100µ aliquot of the samples of final rinse water were spread on 1/10th TSA plates. Endophytic extracts were produced by macerating 2.5g surface-sterile root from each treatment plot in replicate 22.5ml monopotasium phosphate (MPP) buffer (0.65g K_2HPO, 0.10g $MgSO_4$ per litre of water) using a sterile mortar and pestle. Root extracts were serially diluted in MPP. Serial 10-fold dilutions of these were used for culturable microbial enumeration.

2.5. Gas Chromatographic Analysis

Gas chromatographic analysis was used for TPH and PAH tests. Residual total petroleum hydrocarbons (TPHs) and polycylic aromatic hydrocarbons PAHs) were extracted from the soil samples collected from Ogoni and quantified using gas chromatograph-flame ionization detector (GC- FID) according to the methods of ASTDM 3912(2013) and USEPA [33]. The collected samples were air dried and extracted using hexane (5µg). The samples were injected into the gas chromatograph (using a microsyringe) through a rubber septum into a flash vaporizing port at the head of the column. The temperature of the sample port is usually about 50^oC higher than the boiling point of the least volatile component of the sample. Inside the GC the carriers' gas is allowed to move each analyte through the column. Each analyte will require a different amount of time to pass through the column. The sample's outlet stream was monitored with a detector.

2.6. Molecular Biologic Analysis

2.6.1. Plasmid Extraction: TENS - Mini Prep

1.5ml of overnight culture from each sample was spinned for one min in a microcentrifuge to pellet the cells. The supernatants were gently decanted, leaving 50 - 100µl together with cell pellet, these were vortexed at high speed to re-suspend cells completely. 300µl of TENS (a mixture of Tris 25mM, EDTA 10mM, NaOH 0. IN and SDS 0.5%) was added to each tube. The tubes were inverted 3-5 times until the mixture became sticky. Samples were set on ice to prevent degradation of chromosomal DNA. 150µl of 3.0 sodium acetate pH 5.2 was added to each tube and vortexed to mix completely. The content of each tube was spinned for 5mins in micro centrifuge to pellet cell debris. The supernatants were transferred into fresh tubes, properly mixed with 900µl of ice-cold absolute ethanol. These were spinned for 10mins to pellet plasmid DNA -white pellets. The supernatants were discarded, and pellets were rinsed twice in 1ml of 70% ethanol. The pellets were re-suspended in 20-40 µl of TE buffer or distilled water for further use.

2.6.2. Preparation of Agarose Electrophoresis

For 100ml of Tris borate EDTA (TBE), 0.80g of agarose was weighed. This was melted in the microwave oven for 2mins, 10sec. The electrophoretic tank was set up. Then, the agarose was brought out of the oven and allowed to cool. Few drops of etidium bromide were added after preparing the gel. 10ml of the extracted DNA was mixed with 5ml of bromothymol blue and kept aside. The TBE/Gel was poured onto the electrophoretic tank. Micropipette was used to load the DNA mixture onto the wells of the electrophoretic tank.

2.7. Statistical Analysis

The major statistical technique used in this study for the analysis of the collected data is Analysis of Variance (ANOVA). The ANOVA being basically an arithmetic method for breaking down the total variation of the collected data into components representing the sources of variations recognized in the study. The sources of variation were determined by the criteria used to classify the observations. In this study, only one-way, two-way and three-way Analysis of Variance were used. Tukey's pairwise comparison was utilized. Minitabs were used to present the data.

3. Results

Table 1 – Presents the physicochemical parameter in the various soil samples obtained from the crude oil-polluted environment of Ogoni, Nigeria.

Table 2 – Describes the mean value of TPH in the various sites.

Figure 1 Presents the mean value of TPH in the various study sites.

Figure 2 – Presents the mean value of PAH in the various sites.

Table 1. *Physiochemical parameters of the various soil samples from crude oil – polluted environment, Ogoni, Niger Delta*

S/N	Sample code	pH	moisture content%	N_2 (%)	Cu^{2+} (mg/Kg)	Pb^{2+} (m/kg)	Al^{3+} (mg/kg)	Fe^{2+} (mgkg)	Cd (mgkg)	Hg (mg/kg)	Cumulative Co_2 -21 days
1	A	5.78	16	0.210	Trace	78.27	4.29	210.26	0.022	<0.001	183.9
2	B	4.93	26	0.455	Trace	78.27	10.72	430.18	0.074	<0.001	658.9
3	C	5.52	38	0.315	49.85	326.15	26.44	514.77	0.048	<0.001	766.7

S/N	Sample code	pH	moisture content%	N_2 (%)	Cu^{2+} (mg/Kg)	Pb^{2+} (m/kg)	Al^{3+} (mg/kg)	Fe^{2+} (mgkg)	Cd (mgkg)	Hg (mg/kg)	Cumulative Co_2 -21 days
4.	D	5.26	12	0.455	72.21	208.73	37.88	554.6	0.062	<0.001	185.9
5.	E	6.14		0.420	43.51	295.71	58.60	882.11	0.086	<0.001	183.7
6.	F	5.23	28	0.490	37.77	513.14	59.32	827.73	0.138	<-.001	___
7.	G	5.51	22	0.595	12.69	143.50	14.29	362.51	0.010	<0.001	542.3
8.	H	5.62	32	0.560	16.92	200.04	22.15	561.89	0.096	<0.001	566.5
9.	CO	5.14		0.525	25.98	173.94	34.30	604.19	<0.001	<0.001	210.1
10	Normal Value	6.75			15-30	7-42		50			

A: Non-rhizosphere soil,
B: Palm rhizophere
C: Indian bamboo rhizophere,
D: Mix plant rhizophere,
E: Oil well 3 korokoro- no vegetation,
F: Oil well 3 with elephant grass,
G: Grass rhizophere,
H: Fern rhizophere,
CO: Control.

Table 2. *Microbial population changes during biodegradation of crude oil polluted farmlands in Ogoni, Niger Delta.*

Sampling site/ code	TPH mg /kg	PAH mg /kg	THB	HUB	FC	HUF
A. Non Rhizosphere	313.25	2.949	$2.3x\ 10^5$	$1.9\ x10^4$	$1.0\ x\ 10^4$	$2.6\ x10^3$
B. Palm Rhizosphere	1551.67	16.176	$4.7\ x10^6$	$5.6\ x\ 10^5$	$7.0\ x\ 10^5$	$4.9\ x\ 10^3$
C. Indian Bamboo Rhizosphere	1,301.41	14.560	$3.3\ x10^6$	$4.2\ x\ 10^5$	$9.0\ x10^5$	$3.6\ x10^3$
D. Mixed plant population	56	0.314	$1.6\ x10^7$	$4.5\ x10^8$	$1.6\ x\ 10^6$	$1.35\ x\ 10^5$
E. Oil well 3 korokoro No weight	4, 650.13	30.066	$1.0\ x10^5$	$1.0\ x\ 10^4$	$1.0\ x\ 10^5$	$2.4\ x\ 10^3$
F. Oil well 3 korokoro oil- elephant grass	3,538.13	24.593	$5.2\ x\ 10^6$	$2.5\ x10^6$	$6.0x\ 10^5$	$2.80\ x10^3$
G. Grass Rhizosphere	1,520.92	15.623	$1.3\ x10^6$	$2.9\ x10^7$	$6.0\ x10^5$	$1.8\ x10^3$
H. Fern Rhizosphere	415.92	3.839	Confluent	$1.58x10^8$	$1.0\ x10^6$	$1.56\ x\ 10^2$
Control	53.96	0.26 5	$2.5\ x10^4$	$7.0\ x\ 10^4$	$30.0\ x\ 10^2$	$1.4\ x10^2$

TPH: Total petroleum hydrocarbon
PAH: Polyaromatic hydrocarbon
THB: Total heterotrophic bacteria
HUB: Hydrocarbon utilizing bacteria
FC: Fungal Count

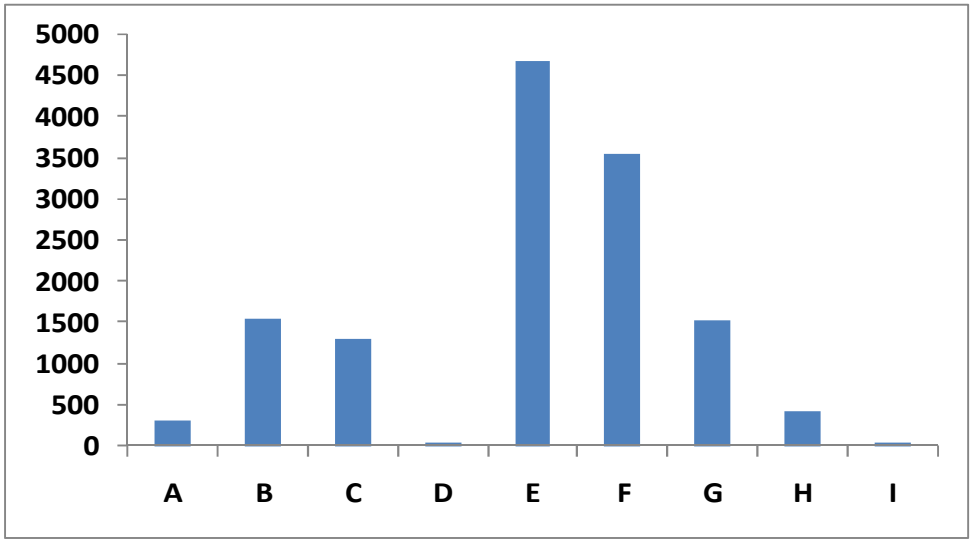

Figure 1. *Mean values of TPH in the various sites.*

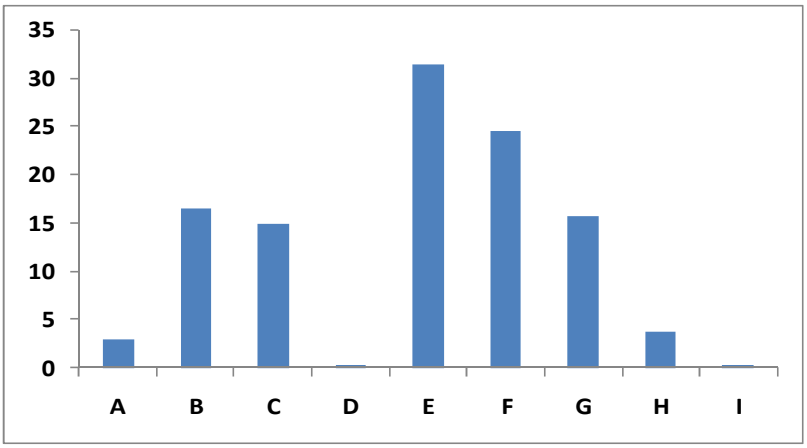

Figure 2. *Mean values of PAH in the various sites.*

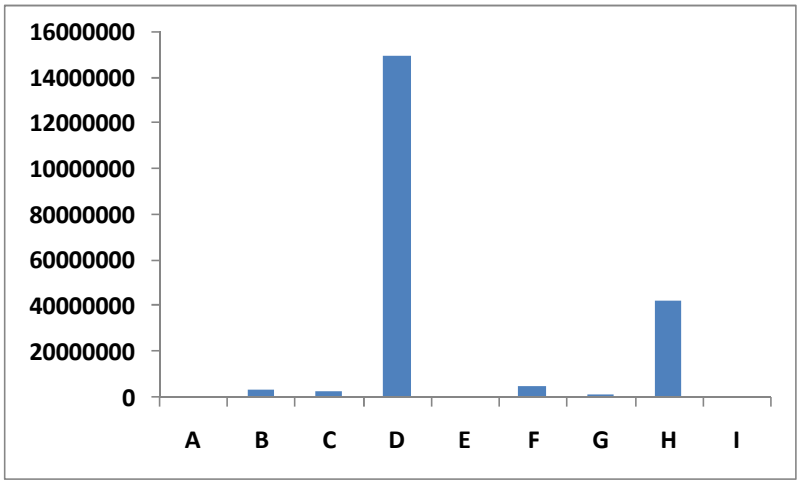

Figure 3. *Mean counts of THB in the various sites.*

Table 3 – Describes the biochemical characterization of the isolates while table 4 is the biochemical characterization of the crude oil degraders.

Table 5 – Demonstrates the succession observed in the study.

Figure 4 – Plasmid profile of some isolates.

Table 3. *Biochemical characteristics of heterotrophic bacterial isolates from crude oil polluted soil.*

Isolate	Cultural Characterization	Morphological Characteristics	Gram stain	Spore stain	Coagulase	Catalase	Oxidase	Urease
1		Rod	+	+		+	+	
2	Swarming	Rod	-	-		+	-	
3		Rod	-	-		+	-	
4		Coccus	+	-		+	-	
5		Rod	+	-		+	+	+
6	Non pigmented	Rod	+	+		+	+	
7	Translucent, non pigmented	Rod	-	+		+	+	
8		Rod	-			+	-	
9	Light red	Coccus	+	+		+	+	
10	Opaque, white/creamy	Coccus	+	-	+	+	-	
11	Brownish/pink/red	Rods	+	-		+	-	
12	Chalky/velvety/purple	Rods	+	-		-	+	
13		Rod	-	+		+	-	
14	Mucoid, cream, rough	Coccobacilli	+	+		+	-	
15	Entire, low converse	Rods	+	-		+	-	
16		Rods	+	+		-	+	
17		Rods	+	+		-	+	
18		Rods	+	+		+	-	

Isolate	Cultural Characterization	Morphological Characteristics	Gram stain	Spore stain	Coagulase	Catalase	Oxidase	Urease
19		Rods	+	-		+	+	+
20		Rods	+	+		+	+	
21		Rods	+	+		-	-	
22	Green	Rods	+	-		+		
23	Cream	Rods	-	-		+	-	
24	Purple pigmented colonis	Rods	-	-		+	-	

Table 3. *Continue.*

Isolate	Citrate	Acid fast	H_2S	Motility	Glucose	Lactose	Gas prod	bacteria
1	+		-	+	+	+	+	*Bacillus*
2			+	+	+	+	+	*Pseudomonas*
3	+		+	+	+	+	+	*Protteus*
4	+		-	+	+	-	-	*Marrnococcus*
5	+		+	+	+	-	-	*Alkaligenes*
6			+	-	-	+	+	
7	+		+	-	-	-	-	*Flavobacteruim*
8	+		+	+	+	+	+	*Citrobacter*
9	-		+	-	-	-	-	*Micrococcus*
10	-		-	-	-	-	-	*staphylococcus*
11	+	+	+		-	-	-	*Gordona*
12	-	+	+	+	-	-	-	*Norcadia*
13	+		+	+	-	-	-	*Agremona*
14	+	+	+		-	-	-	*Rhodococcus*
15	+	-	-	+	+	+	+	*Listeria*
16	+		+	+	+	-	+	*sporosarcinia*
17	-	+	+	+	+	+	+	*Clostridium*
18	+		+	-	+	+	+	*Sulfidobacillus*
19		-	+	-	+	+	+	*Corynebacterium*
20	-		-	+	-	+	+	
21	+		-	+	+	+	+	*Amphibacillus*
22	+		+	+	+	+	+	*Chlorobium*
23	+		-	+	-	-	-	*Acinetobacter*
24	+		-	+		-	-	*Rhodopseubomonas*

Table 4. *Biochemical characteristics of crude oil degraders in the study.*

Isolate	Cultural Characteristics	Morphological Characteristics	Gram stain	Spore stain	Coagulast	Catalase	Oxidase	Urese
1		Coccus	+	-		+	-	
2	Non pigmented, smooth entire	Coccus	+	-		+	-	
3	Swarmy	Rods	-	+		+	-	
4	Smooth, converse, yellow	Coccus	-	-		+	-	
5	Entire, colourless, pulvinate	Rods		+		+	-	
6		Rods	-	-		+	-	
7	Irregular, non pigmented, smooth	Coccus	+	-		+	-	
8	Pigmented yellow- red	Coccus	+	-		+	-	
9	Low converse, translucent, grey	Rod s	+	-		+	-	
10		Rods	+	-		+	-	
11	Cream coloured	Rods	+	+		+	-	
12	Swarmy	Rods	-	-		+	-	
13	Creamy-orange	Rods	+	+		+	-	
14		Rods	+	-		+	-	
15		Coccoba callus	+	-		+	-	
16		Rods	+	+		+	-	
17	Opaque whitish	Cluster Coccus	+	-	+	+	-	
18	Yellowish	Coccus	+	-		+	-	
19		Rods	+	-		+	+	
20		Rods	+	-			-	
21	Circular, opaque, converse, granular	Coccus	-	-		+	-	
22		Rods	-	-		+	-	
23		Rods	+	+		-	+	
24	Yellowish	Coccus	+	-		+	-	
25	Mucoid, cream	Coccobacillus	+	-		+	-	
26		Rods	+	-		-	-	

Table 4. *Continue.*

Isolate	Citrate	Acid fast	H_2S	Motility	Glucose	Lactose	Gas prod	Mexelred
1	+		+		-	-	-	*Arthrobacter*
2	+		-		-	+	-	*Flavobacterium*
3	+		+	+	-	+	-	*Pseudomonas*
4	+		-	+	+	+	+	*Marinococcus*
5	+		-	+	-	+	-	*Agromonas*
6	+		-	+	+	+	+	*Citrobacter*
7	+		-	+	+	+	+	*Agrobacterium*
8	+		-		+	+	+	*Micrococcus*
9	+		-	+	+	+	+	*Listeria*
10	+		-		-	+	-	*Gordona*
11	+		-	+	+	+	+	*Bacillus*
12	+		-	+	+	+	+	*Proteus*
13	+		-	+	-	+	-	*Sporosarcinia*
14	+	+	+		-	-	-	*Norcardia*
15	+		-	-	-	+	-	*Lactobacillus*
16	+		+		+	+	-	*Sulfidobacillus*
17	-				+	+	-	*Staphylococcus*
18				+	+	+	-	*Enterococcus*
19	+		+	-	+	+	+	*Corynebacterium*
20	+		+	+	+	+	+	*Chlorobium*
21	+		-		+	+	-	*Bradyrhizobia*
22	+		-		+	-	-	*Acinetobacter*
23	+		+-		+	+	+	*Clostridium*
24	+		-		-	+	-	*Planococcus*
25	-	+						*Rhodococcus*
26	+							*Amphibacillus*

Table 5. *Microbial succession observed in the study.*

THB	HUB	HUAB	FE
Escherichia	*Escherichia*	*Bacillus*	*Trichosporum* sp
Citrobacter	*Citrobacter* sp	*Clostridium*	*Aspergillus terreus*
Pseudomonas	*Pseudomonas*	*Plannococcus*	*Aspergillus oryzae*
Burkholderia	*Burkholderia*	*Amphibacillus*	*Acremonium kilenis*
Salmonella sp	*Salmonella* sp	*Kurthia*	*Aspergillus flavies*
Shigilla sp	*Shigella* sp	*Jonesia*	*Mucor* sp
Bacillus sp	*Marinococcus* sp		*Pennicillum* sp
Flavobacterium	*Agrobacterium* sp		*Fusarium* sp
Micrococcus sp	*Micrococcus* sp		*Candida* sp
*Agromonas*sp	*Listeria* sp		
Sporosarcinia sp	*Bacillus* sp		
Clostridium sp	*Sporosacinia* sp		
Sulfidobaccllus sp	*Staphylococcus* sp		
Amphiacillus sp	*Bardyrhizobia* sp		
Gorgona sp	*Enterococcus* sp		
Norcardia sp	*Planococcus* sp		
Rhodococcus sp	*Flavobacterium* sp		
Chlorobium sp	*Amphibacillus* sp		
Rhodopseudomonas sp	*Agromonas* sp		
Acinetobacter sp	*Sulfidobacilus* sp		
	*Rhodococcus*sp		
	Chlorobium sp		
	Rhodopseudomonas sp		
	Acinetobacter sp *Lactobacillus* sp		

THB: Total Heterotrophic Bacteria; HUB: Hydrocarbon Utilizing Bacteria; HUAB: Hydrocarbon Utilizing Anaerobic Bacteria; FE: Fungi Encountered

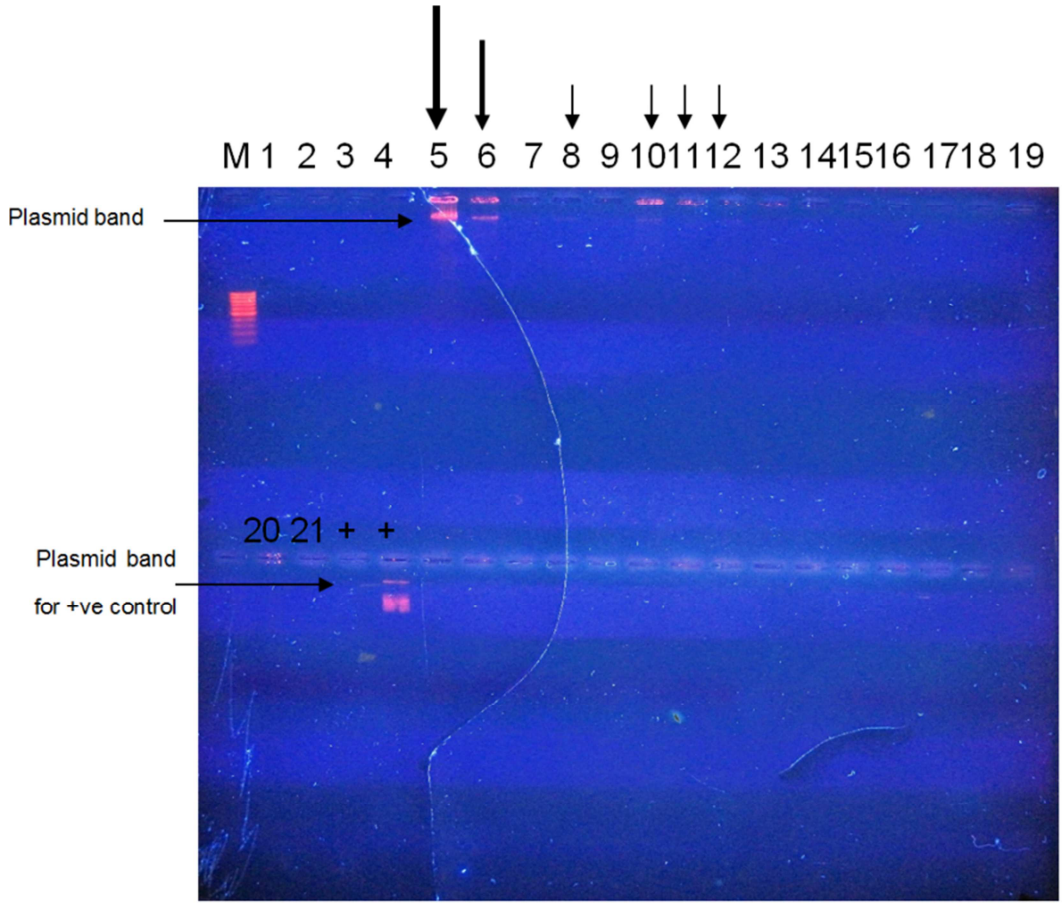

Figure 4. *Electrophoretic separation profile of plasmid DNAs from strains of Agromonas, Agrobacterium, Listeria, Sporosarcinia, Lactobacillus and Staphylococcus spp. Lines 5, 6, 8, 10, 11 and 12 bear plasmid of different band sizes and intensity.*

4. Discussion

This study looked at the effects and potentials of rhizosphere microorganisms in the degradation of petroleum products in the following sites; four single plant plots, a mixed plant species plot, two oil wells plots and a non-rhizosphere plot. The field samples were collected from Kpopie and Korokoro communities of Ogoni, Rivers State, Nigeria. Pollution in this area is high and chronic, and spillage of petroleum product is more than 50 years old. Table 1 shows the physicochemical parameters of soil samples from the hydrocarbon-contaminated field soils of Ogoni. Most of the soil studied were acidic. Samples A and B had low Cu^{2+} content (Trace) while sample D (Mixed Plant species plot) had as high as 72 mg/kg. Cu^{2+} content of samples G, H and Control fell within the normal range of 15 – 30 mg/kg. All soils of study had high Pb^{2+} and Fe^{2+} content. There was significant difference between the physicochemical parameters over the various soil samples $P < 0.028 < 0.05$. Also significant differences exist between the various physicochemical parameters within the various soil samples, P-value = $0.00 < 0.05$. Table 2 shows the hydrocarbon status of the field samples as well as the microbial population changes during rhizoremediation. The TPH of soil from the field study of kpopie and korokoro fluctuated between 56 and 4, 650. 13 mg /kg. Significant difference existed between the plots. In all, the soil plot containing mixed plant species had the lowest TPH concentration of 56mg /kg, while soil sample E, oil well 3, korokoro without vegetation had the highest TPH concentration. There was no significant difference between TPH of the mixed population and that of the control. It is clear that there has been co-metabolism whereby the different plant species had given rise to a net high mineralization rate. This is in concordance with studies of Reilley *et al.,* [29]; Aprill, and Sims [2], Daane *et al.,* [9]; Kirk *et al.,* [13] and Siciliano *et al.,* [30]. It is also evident that sample D, the mixed plant species plot also gave the lowest PAH concentration of 0.314. This value is close to the PAH concentration of the control sample, Results obtained in table 2 shows that microbial abundance and activity have a corresponding effect on hydrocarbon degradation. There was more reduction of TPH in the mixed plant species plots of both experimental (2.88 mg/kg) and field site (56 mg/kg), and in the PAH (0.314mg/kg) in the field study. This finding agrees with studies of Yateem *et al.,*[37], in their study; they recorded that the total hydrocarbons in a diesel contaminated soil decreased faster in the presence of roots and nutrients compared to that in a non-vegetated soil.

Table 2 shows that specific local plants promote increased net hydrocarbon degradation. Table 4 shows that, of the 26 crude oil degrading bacteria isolated, 19 were Gram-Positive.

Although some studies have shown that oil-polluted soils are dominated by Gram-negative bacteria ([17]; [15]). The dominant culturable hydrocarbon utilizing bacteria in both experimental and field plots of this study were made up of both Gram positive and Gram-negative bacteria; *Gordona, Rhodococcus, Corynebacterium, Norcardia, Arthrobacter Spp.* etc. This corroborates with the findings of Quatrini *et al.,* [26], who isolated *Rhodococcus, Gordona* and *Norcardia* strains as the dominant hydrocarbon degraders from a hydrocarbon-contaminated mediterrenean shoreline. Grass root system in the two studies undertaken demonstrated higher degradation potential than the other plants of study. According to Aprill, and Sims [2] grass root system has the maximum root surface area per m3 of the soil than any plant type and may penetrate the soil to a depth of 3m.

Results of Bogan*et al.,* [4];Margesim, and Schinner [19], and Hamamura [10] demonstrated the prevalence of Actino-bacteria in hydrocarbon polluted soils from different geographical locations, their various studies also demonstrated the presence of multiple hydrocarbon catabolic genes in this group of bacteria. This study however recorded a mixture of microbial consortium involving both Gram positive and Gram-negative bacteria, and aerobic and anaerobic bacteria. The bacterial population dynamics and counts observed during crude oil degradation could also be associated with the degradation patterns of the total petroleum hydrocarbon (TPH) and polycylic aromatic hydrocarbon (PAH) in the different plots.

All bacteria isolated from this study are indigenous and all survived petroleum hydrocarbon contamination, they adapted to oil-contaminated soil easily as reported by other authors ([28]; [31]). This was evident in samples collected from the chronic contaminated soils of Ogoniland. Figure 4 illustrates that some of the bacteria isolated from this study contained plasmid DNA that could be responsible for crude oil degradation; regrettably this study did not carry out amino acid sequencing/comparison. Plasmid bands were found in 6 organisms; *Agromonas, Agrobacterium, Listeria, Sporosarcinia, Lactobacillus, Staphylococcus* spp. This study is in concordance with the study of Kiyohara, and Nagao [14] who recorded phenanthrene degradation genes in plasmids of different strains of *Comamanas testosteroni, Beijerinckia* and *Alkaligene faecalias* AFK2. Plasmid bands were not recorded in organisms isolated pre-contamination. This is in agreement with studies by many authors that maintain that microorganisms' genes; Pp alk B, Rh *alk* B1, and Rh *alk* B2 genes etc., borne on plasmid DNA are responsible for petroleum-oil biodegradation ([36]; [19]).

Endophytic organisms were found to be in abundance in this study in the 10^6 range. VanAken*et al.* [35], suggested that endophytes may have potential for remediation of environmental soil containing explosives. Also, Siciliano*et al.,*[30] identified 34 bacterial strains as having potential to enhance bioremediation of contaminated environmental soil. Daane *et al.* [9] observed that plasmid DNA contain degradative genes capable of degrading the most complex, the most recalcitrant and the most synthetic molecules. This study has shown that rhizoremediation can contribute to the restoration of polluted sites and is in consonance with reports from several authors [27].

5. Conclusion

This study has been able to use specific local Nigerian plants like elephant grass, carpet grass, palm tree, Indian bambo and fern to promote the rhizoresssmediation of crude oil-polluted soils.

Effective indigenous microorganism (EIM) that gave rise to the net mineralization of polycyclic aromatic hydrocarbons (PAHs) and total petroleum hydrocarbon (TPH) include members of the Genera *Flavobacterium, Pseudomonas, Sporosarcinia, Listeria, Bacillus, Proteus, Lactobacillus, Staphylococcus, Bradyrhizobia, Enterococcus, Planococcus, Rhodococcus Clostridium, Amphibacillus, Kurthria, Jonesia* and *Agromonas*. DNA of *Agromonas, Agrobacterium, Listeria, Sporosarcinia, Lactobacillus,* and *Staphylococcus* contain plasmids that could be responsible for the rhizoremediation of crude oil-polluted soil.

Endophytic communities took part in the net mineralization of crude oil – *Pseudomonas* and *Arthrobacter* spp. Anaerobic microbial communities also contributed to the overall degradation potential of this study. The anaerobes encountered include; *Bacillus, Clostridium, Amphibacillus, Kurthria* and *Jonesia* Spp. A mixed population of the above mentioned plants gave rise to increased net hydrocarbon degradation in the studies conducted. Both bacterial abundance and plant community composition impacted on hydrocarbon degradation potential.

Composition of microbial community in a rhizosphere differs both quantitatively and qualitatively. The total petroleum hydrocarbons (TPH) in the crude oil contaminated soisl decreased faster in the presence of roots and nutrients compared to non-vegetated soils and decreased even faster in the soil plots containing mixed plant species.

The study recorded more mineralization of polyaromatic hydrocarbons than the straight chain hydrocarbons. The rhizosphere microorganisms encountered in this study were able to mineralize the following components of crude oil; naphthalene, acenapthalene, acenaphthylene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo (b) fluoranthene, indeno(1,2,3-cd) pyrene, benzo(k) fluoranthene, benzo (B)fluorathene, debenzo (a, h) anthracene, bezo (g, h, l,) perylene.

Studies of microbial consortia are of great importance for best understanding of biodegradation process, the ability to determine the alkane degradative composition of a microbial soil community should also help develop appropriate bioremediation strategies.

Recommendation

i Rhizoremediation, a combination of phytoremediation

and bioaugmentation which makes use of rhizosphere microorganisms is recommended as the best treatment option for hydrocarbon clean-up.

ii A mixture of specific plant, population abundance of effective indigenous microorganism could be developed as an appropriate technology to tackle environmental problem and mitigate the effects of ecological disasters in Nigeria.

iii Serious efforts should be made to avert the devastating effect of crude oil on cassava staple food in Nigeria.

iv Government should sponsor research work so that studies as this will have enough fund to carry out more intensive genetic studies on organisms that bear plasmids in order to characterize and sequence the genes responsible for hydrocarbon degradation.

v Efforts should be made to collect and store (bank) these rhizosphere microorganisms, so that they could be manipulated for efficient degradation. These can be interesting tools to further improve and develop rhizoremediation into a widely accepted technique.

vi Studies are required to decipher the particular genes borne on the plasmids demonstrated from these isolates in order to develop them genetically and make room for more cost-effective rhizoremediation schemes.

vii Metabolically active microbial communities that played role in hydrocarbon degradation in this study are numerous, in addition to routine microbiological methods, this study recommends that funding will allow for molecular methods of identification e.g., metagenomics analysis which promises enormous potential for identifying novel enzymes and pathways involved in the biodegradation of poorly and inefficiently degraded pollutants, in order to capture more of these organisms.

viii It is recommended that a follow-up study be undertaken to ascertain the roles played by each microorganism in the clean-up exercise since this study has shown that a net effect of the activities of all interacting microorganisms were responsible for the crude oil-degradation.

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