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Assessment and Characterization of Rhizo-Bacteria in Petroleum–Polluted Soil in South–East, Nigeria

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Abstract
A rhizoremediation study was carried out on petroleum-polluted soil in Akiri, Imo State, Nigeria.
The assessment and characterization of bacteria capable of degrading hydrocarbons were isolated from the rhizosphere of *Mimosa pudica, Alchornia chordifolia, Chromolaena ordorata, Chloris pilosa* and *Sida acuta*. The isolates identified include *Acinetobacter, Bacillus, Micrococcus, and Pseudomonas* spp. All the isolates grew on petroleum hydrocarbon at different growth rates. Statistical analysis showed no significant difference (P>0.05) between the rhizosphere and non-rhizosphere of total culturable heterotrophic and hydrocarbon-utilizing bacterial counts in both petroleum-polluted and unpolluted (control) soils. All the plants exhibited positive rhizosphere effects on the rhizo-bacteria. Based on these results, the rhizo-bacteria isolated can serve as seeds for bioaugmentation during remediation of crude oil polluted soil environment. The plants may be employed in rhizoremediation of oil polluted soil.
Keywords: Characterization, Rhizoremediation, Bacteria, Rhizosphere effect, Petroleum-polluted soil

1. Introduction

The accumulation of petroleum hydrocarbons in the environment can cause serious problems, affecting negatively the stability of many ecosystems and can also cause difficulties for animals and human health (Chekroun *et al*., 2014). Chemical contaminants present in the aquatic ecosystem may be immobilized and accumulated in sediments or may be subject to transformation and activation processes (Martínez-Jerónimo *et al*., 2008).

Remediation of soils containing organic pollutants can be enhanced by plants by various processes (Cunningham *et al.*, 1996). *In-situ* phytoremediation strategy exploits natural or genetically engineered plant species to accumulate toxic substances (heavy metals, radioactive compounds, organic pollutants) directly from the soil. Partial or complete degradation of organic substances have been demonstrated in some cases (White, 2001).

Most plants have symbiotic relationships with soil microorganisms. The area around plant roots, otherwise called the rhizosphere contains higher populations, greater diversities and activities of microorganisms (Brimecombe *et al*. 2007) than soil with no plants. Rhizosphere microorganisms are especially critical for plant colonization of unfavourable soils, since they can alleviate biotic and abiotic stress of plants. This has caused the emergence of a green technology which employs the symbiotic relationship between plants and their rhizo-microorganisms in the breakdown of contaminants to clean up the environment. This technique is referred to as rhizoremediation (Kuiper *et al.*, 2004). A plant can be considered to be a solar-driven biological pump and treatment system, attracting water with its root system, accumulating water-soluble pollutant in the rhizosphere and concluding with the degradation or translocation of pollutants (Liste and Alexander, 2000). In some cases, rhizosphere microbes are even the main contributors to the degradation process. Plants release exudates into the soil ecosystem that increases the microbial activity and aid the degradation of xenobiotic substances. The soluble root exudates include enzymes, amino acids, sugars and low molecular weight carbohydrates (Burken and Schnoor, 1996).

The plant rhizosphere is recognized as a niche rich in growth substrates in comparison with the surrounding bulk soil (Dunfield and Germida, 2001). Rhizospheres are dynamic microenvironments in which microbial communities have access to an elevated supply of carbon, oxygen and energy rich materials from plant roots (Clegg and Murray, 2002). Rhizospheres are also stable physically, avoiding the potentially adverse effects of naturally occurring disturbances on microbial community composition or activities (Piceno *et al*., 2000). This stimulatory rhizosphere effect has been recognized for many years and was described for the first time by Hiltner in 1904 (Kuiper *et al*., 2004). In rhizoremediation, plant roots sustain the degrading microflora by supplying them with nutrients other than pollutants, and also help in spreading the degrading microorganisms to new sites in the soil.

The aims of this research, therefore, were to enumerate, isolate, and characterize the hydrocarbon-utilizing bacterial genera associated with the rhizosphere of these plants: *Mimosa pudica, Alchornia chordifolia, Chromolaena ordorata, Chloris pilosa* and *Sida acuta* found in petroleum-polluted areas of Akiri in Oguta in Imo State, determining the rhizosphere effect ratios of these plants on their rhizobacteria as well as assessing the potentials of the associated bacteria isolated to utilize crude oil.

2. Materials and Methods

2.1 Site description and Sample Collection

Samples were obtained from a location in Akiri in Imo State where there has been oil spill. This site could be described as a disturbed ecosystem with scanty plants growing at this location. Soil samples were collected from plants' root zones and thirty centimeters away from root zone. Pristine soil samples were collected from plants' root zones and thirty centimeters away from the root zone in the same area where there has been no known spill to serve as control. These were placed in separately marked sterile plastic bags and transported in an ice chest to the laboratory for analysis, after which the plants were taken to a plant taxonomist for identification.

2.2 Sample Processing

The roots were freed from adhering soil which is assumed to be the rhizosphere soil. Ten grams (10) from each of the samples of rhizosphere and non-rhizosphere of crude oil polluted and unpolluted soils were suspended ninety millilitres (90ml) of distilled water. The content of the flasks were serially diluted. From each dilution of 10^{-3} to 10^{-6}, aliquots (0.1ml) were plated on sterile Nutrient agar (NA) and Mineral Salt agar amended with an antifungal agent, Natamycin of quantity 21.6mg/L in duplicates.

2.3 Enumeration of bacterial counts:

The total heterotrophic bacterial count was carried out using the spread plate method on Nutrient agar. The plates were incubated at 35^{o}C for 24 to 48 hours**.**

The vapour phase transfer method was adopted for the enumeration of hydrocarbon-utilizing bacteria on the Mineral Salt agar with the following composition: NaCl 10.0g, MgSO4 0.42g, KCl 0.29g, KH_2PO_4 0.83g, $NaPO_4$ 1.25g, $NANO_3$ 0.42g, agar 15g and distilled water 1000ml as modified by Okpokwasili and Okorie (1988).

2.4 Characterization and Identification of Isolates

The bacterial isolates were examined for colonial morphology, cell micro-morphology and biochemical characteristics. Tests employed included:

Gram staining, Motility test, Catalase test, Citrate Utilization test, Indole test, Hydrogen Sulphide Production test, Methyl Red -Voges Proskauer test, Oxidase test, Sugar Fermentation test. Confirmatory identities of the bacteria were made using the Bergey's Manual of Determinative Bacteriology (Holts, 1993).

2.5 Biodegradation Test

The bacteria isolated were tested for their ability to degrade crude oil using the turbidity method as described by Ibrahim *et al.* (2008). Nutrient broth was used to culture the bacterial isolates and incubated for 24 hours at 28+2^{0}C. 0.1ml of the young culture in nutrient broth grown was inoculated into each test tube containing 9.9ml of sterile mineral salt broth and 0.1ml of crude oil. A control test tube containing 9.9ml of sterile mineral salt broth with 0.1ml of crude oil remained un-inoculated. Turbidity of the tubes was used to determine the growth of the inocula, as compared with the un-inoculated control tube.

2.6 Physicochemical analysis of soil samples

The pH and temperature of the soil samples were determined using Suntex pH MV Temp TS2 metre (MODEL SP701) in 1:1 soil solution in distilled water in accordance with the manufacturer's directions.

2.7 Statistical Analysis

The SPSS software of statistical analysis was used to analyze the results of this work. The statistical tools – One-way Analysis of Variance (ANOVA) was used to analyze the data obtained from all the plants in the various locations while Independent Student's t-test was used to analyze the polluted and unpolluted soil sample of each plant.

3. Results

3.1 Bacteriological analyses

The result of total heterotrophic counts (Figure 1) ranged from 6.91 x 10^5 cfu/g to 9.08 x 10^5 cfu/g for the polluted rhizosphere. In the unpolluted rhizosphere, it ranged from 1.92 x 10^5 cfu/g to 2.61 x 10^5 cfu/g. The counts from the non-rhizosphere ranged from 2.39x10^5cfu/g to 3.02x10^5cfu/g for the polluted soil and

$3.02x10^5$cfu/g to $3.25x10^5$cfu/g for the unpolluted soil.

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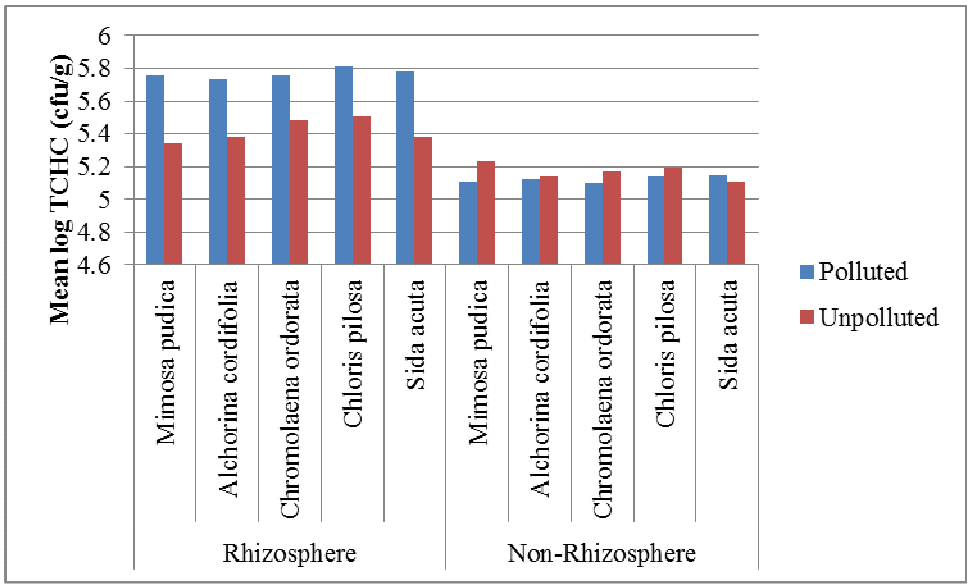

Figure 1 Distribution of heterotrophic bacteria in polluted and unpolluted rhizosphere and non- rhizosphere soil in Akiri.

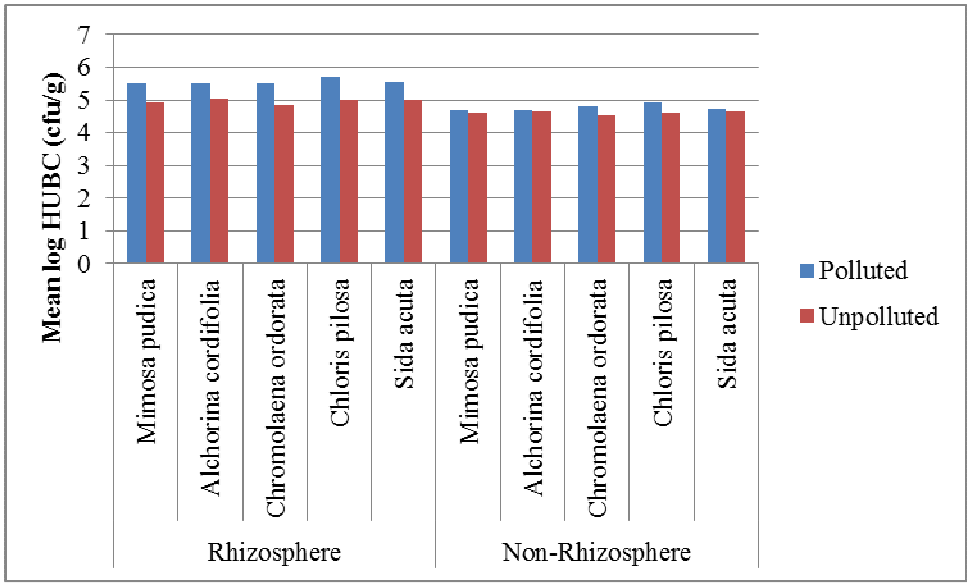

Figure 2: Distribution of Hydrocarbon-degrading bacterial in polluted and unpolluted rhizosphere and non-rhizosphere soil in Akiri.

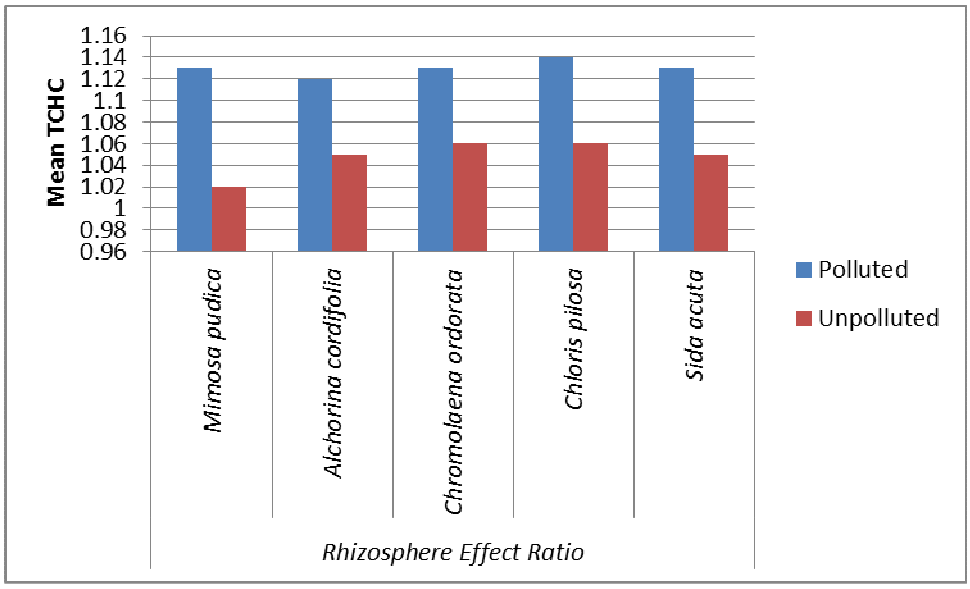

Figure 3. Rhizosphere effect ratio on Culturable heterotrophic counts for polluted and unpolluted soil samples

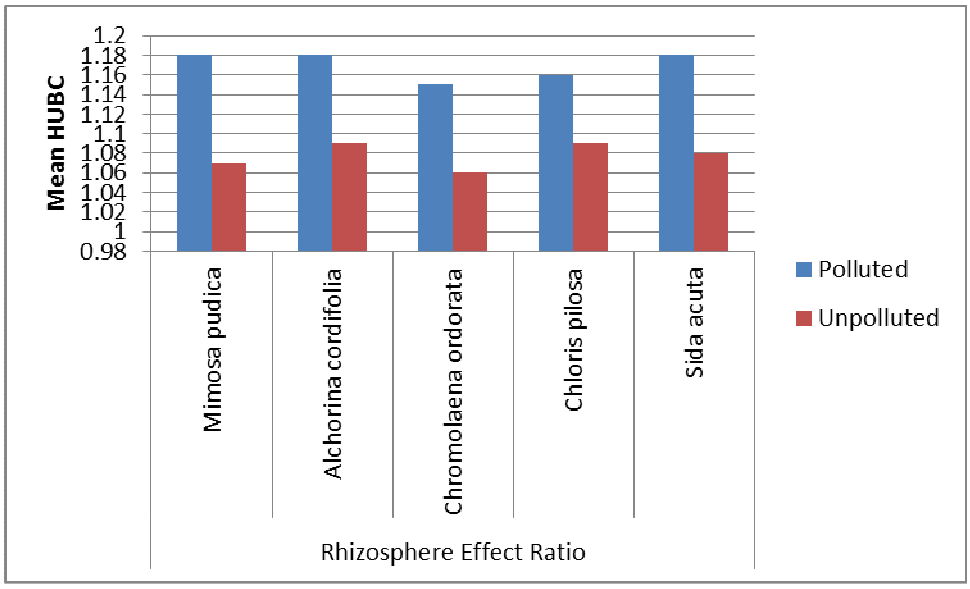

Figure 4 Rhizosphere effect ratio on Hydrocarbon-degrading bacterial counts for polluted and unpolluted soil samples

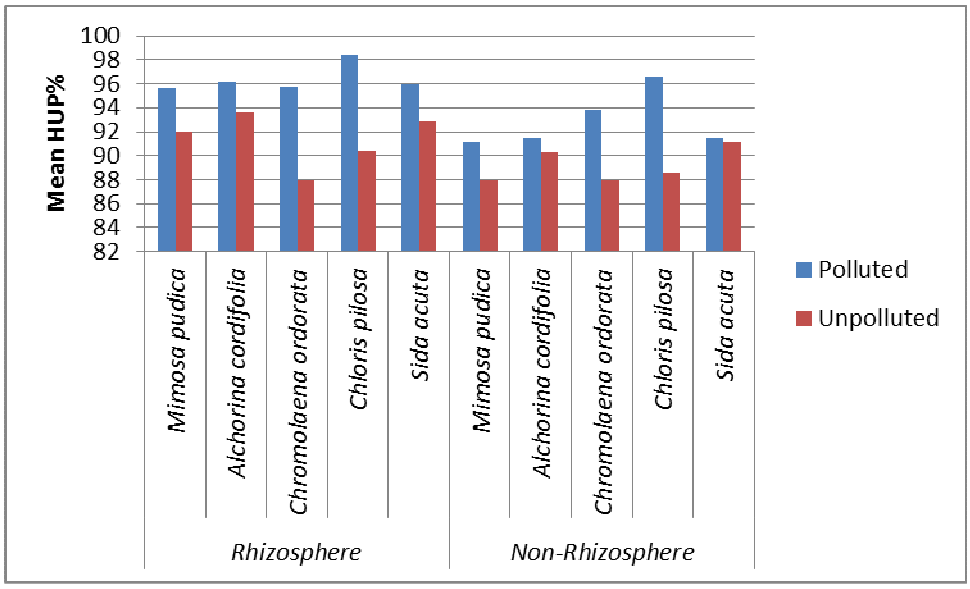

Figure 5. The percentage of hydrocarbon-utilizers in polluted and unpolluted rhizosphere and non-rhizosphere soil samples.

3.2 Physicochemical parameters.

3.2.1 Temperature Record

The temperature values recorded during this study were observed to be more supportive to mesophiIic microorganisms. The values ranged from 25^0C to 27^0C (Table 1).

3.2.2 pH Reading

The pH values obtained during this study period for the polluted rhizosphere and non-rhizosphere were slightly acidic while the pH values for the unpolluted rhizosphere and non-rhizosphere soils were almost neutral (Table 2).

Table 1. Temperature Values of Polluted and Unpolluted Rhizosphere and Non-Rhizosphere Soils.

Plant Species	Temperature (^{O}C)			
	Rhizosphere		Bulk	
	polluted	unpolluted	polluted	unpolluted
Mimosa pudica	25.0	26.3	25.4	26.5
Alchornia cordifolia	25.1	26.5	25.4	26.7
Chromolaena odorata	25.0	26.8	25.4	26.1
Chloris pilosa	25.1	26.5	25.5	26.4
Sida acuta	25.3	26.4	25.5	26.7

Table 2. pH Values of Polluted and Unpolluted Rhizosphere and Non-Rhizosphere Soils.

Plant Species	pH			
	Rhizosphere		Bulk	
	Polluted	unpolluted	polluted	unpolluted
Mimosa pudica	6.40	7.10	5.99	7.00
Alchornia cordifolia	6.25	7.02	6.10	7.03
Chromolaena odorata	5.84	6.95	5.82	6.99
Chloris pilosa	5.67	7.03	5.54	6.97
Sida acuta	5.50	6.92	5.80	7.02

Table 3: Screen Test for the Utilization of Petroleum Hydrocarbon by the Bacterial Isolates

Isolate code	Growth in medium	Bacterial isolate
DSA 1A	++	*Bacillus* sp.
DRA 4B	++	*Bacillus* sp.
DRA 5A	+++	*Pseudomonas* sp.
CSA 3A	+	*Acinetobacter* sp.
CRA 2B	+	*Micrococcus* sp.

Key: Heavy growth = +++ Moderate Growth = ++ Scanty Growth = +

4 Discussion

Soils containing diverse organic pollutants, including organic solvents, pesticides, explosives and petroleum can be remediated by the use of plants and their interaction with root microorganisms which provide nutrients in the rhizosphere leading to an increased microbial activity, survival of plants and degradation of toxic pollutants (Mirsal, 2004).

The bacterial counts of polluted and unpolluted rhizosphere and non- rhizosphere are shown in Figures 1 and 2. It was observed that the total heterotrophic bacterial counts were higher in the polluted rhizosphere than in the polluted non-rhizosphere of all the plants. This was the same for hydrocarbon-utilizing bacteria; the counts were also higher in the polluted rhizosphere than the polluted non-rhizosphere. Lynch (1990) stated that on a per gram basis, rhizosphere soil has 10–100 times more microbes than unvegetated soil. This expresses the rhizosphere effect of the plants on the bacteria.

The low counts of heterotrophic bacteria (Figure 1) recorded in this study for most petroleum-polluted soils compared to that of unpolluted soils agreed with the previous report by Jensen (1975), Umanu *et al.* (2013) and Ukaegbu-Obi and Mbakwem-Aniebo, (2014) which could be attributed to the toxic effect of petroleum-pollution. Microbial community structure has been recommended as a biological indicator of heavy metal stress (Yao *et al*., 2003).

In Figure 2, hydrocarbon utilizing bacteria were found to be higher in petroleum-polluted soils than the unpolluted soils. This finding collaborates with the reports of Hubert *et al* (1999), Michalcewicz (1995), Ukaegbu-Obi and Mbakwem-Aniebo (2014). These higher populations of petroleum degraders recorded could be due to the stimulatory effect of additional carbon and energy source in the form of pollutant. The contamination of crude oil results in an immediate change in the bacterial community structure, an increasing abundance of hydrocarbon-degrading microorganisms and a rapid rate of oil degradation, which suggests the presence of a pre-adapted, oil-degrading microbial community and sufficient supply of nutrients (Coulon *et al*., 2006; Hamamura *et al*., 2006; Tang *et al*., 2010).

The values of rhizosphere effect ratios (RER) of the Culturable heterotrophic and hydrocarbon-utilizing bacteria of the polluted samples were higher than in the unpolluted sample (Figures 3 and 4). It has been speculated that when a chemical stress is present in the soil, plants respond either by increasing or changing exudation to the rhizosphere, which modifies the composition or activity of the rhizosphere microflora. Soderberg *et al.* (2004) observed that root exudates support growth of the microbial community in the rhizosphere and will result not only in an increased population density, but also in a community structure distinct from that in the bulk soil.

The pH values were more acidic in the polluted rhizosphere and non-rhizosphere than the unpolluted rhizosphere and non-rhizosphere. This may be as a result of the time interval between time of spill (April, 2009) and time sampled (June, 2009). This shows that the acidic components of the crude oil were still present in the soil. The pH values for polluted rhizosphere and non-rhizosphere ranged from pH 5.50 to pH 6.40 and pH 5.54 to pH 6.10 respectively. The unpolluted rhizosphere and non-rhizosphere pH values recorded were ranged from pH 6.92 to pH 7.10 and pH 6.97 to pH 7.03 respectively (Table 2). The result in this study agrees with the findings of Dibble and Bartha (1979) who observed an optimal pH of 7.8, in the range of 5.0 to 7.8, for the mineralization of oily sludge in soil. The pH values recorded in this study, therefore, would support biodegradation of hydrocarbons.

The constant warm temperatures of the tropics favor plant growth during the entire year in contrast to temperate zones. Microbial activity and thus bioremediation is greatly enhanced due to more intense temperatures, higher humidity and solar radiation (Prado- Jatar *et al.,* 1993). Maximum degradation rates of organics have been found

to occur at 30 and 40^{0}C, that is temperatures in tropical savannah soils (Rivera-Cruz *et al*., 2004).

Results of the distribution of hydrocarbon-utilizing bacteria in the polluted rhizosphere and non-rhizosphere soil of the plants show that the polluted rhizosphere soil of the plants stimulated the development of higher counts (cfu/g soil) of these organisms as compared to the non-rhizosphere soil (Figure 5). The percentage of hydrocarbon-utilizers was also higher in the rhizosphere soil, than in the non-rhizosphere soil. Diab (2008) made similar observation; he reported that, the percentage of the oil degraders were higher in the rhizosphere soil than in the non-rhizosphere. The above result confirm the ability of plant roots in symbiotic relationship with rhizomicrobes to neutralize or to remove the toxic effects of the oil pollutants, probably through the plant exudates, nutrients, oxygen supply and other materials to the microbes.

Murotova *et al*. (2003) explained that the success of phytoremediation of hydrocarbon contaminated soil is connected with the plant's capacity to enhance microbial activity in the rhizosphere. The efficiency of this process is often connected with higher number of degrader microorganisms and their degradative activities in the rhizosphere of plants. The unpolluted rhizosphere results of hydrocarbon-utilizers percentage in this study showed higher values also than unpolluted non-rhizosphere. The values of the hydrocarbon-utilizers percentage in polluted rhizosphere and non-rhizosphere are within the reported frequencies for soil bacteria as reviewed by Leahy and Colwell (1990). The fraction of the total heterotrophic community represented by the hydrocarbon-utilizing bacteria and fungi is highly variable. This suggests that the unpolluted sites (which served as control) which had no known history of pollution as at the time of sampling may have had a prior exposure to hydrocarbons which could be either from anthropogenic sources (especially since these areas are oil producing areas and experience steady petroleum exploration) or natural sources such as plant derived hydrocarbons. A number of reports, reviewed by Atlas, (1981) and Leahy and Colwell,(1990) have shown that the numbers of hydrocarbon-utilizing microorganisms and their proportion in the heterotrophic community increased upon exposure to petroleum or other hydrocarbon pollutants and that the levels of hydrocarbon-utilizing microorganisms generally reflect the degree of contamination of the ecosystem.

It is generally accepted that bulk soil and rhizosphere microbial community structure is determined by the local native microbial community, impacted by the soil effects and vegetation (Juhanson *et al*., 2008). The composition of the microbial population in the rhizosphere depends on the composition of the exudates as well as on the plant species, root type, plant age, soil type and history of soil (Kuiper *et al.,* 2004). The bacterial isolates obtained from the different rhizosphere and non-rhizosphere of polluted and unpolluted soils in this study were identified to be of the following genera: *Bacillus*, *Acinetobacter, Alcaligenes, Arthrobacter, Corynebacterium, Flavobacterium, Micrococcus, Pseudomonas* and *Serratia* (Table 4*)*. This agrees with the findings of Tesar *et al*. (2002) who reported that a broad phylogenetic range of bacteria including species/strains of *Achromobacter, Acidovorax, Alcaligenes, Arthrobacter, Bacillus, Corynebacterium, Flavobacterium, Micrococcus, Mycobacterium, Norcadia, Pseudomonas, Rhodococcus, Sphinogomonas* and *Xanthomonas* have been identified in the breakdown of hydrocarbons. The isolates were predominantly rod shaped bacteria (88.89% of the isolates were rods and 11.11% cocci).

The bacterial isolates utilized crude oil at different rates with *Pseudomonas* sp. having the highest growth rate followed by *Bacillus* sp. that had moderate growth rate and *Acinetobacter* sp. and *Micrococcus* sp. having scanty growth rates (Table 3)

The biodegradation of petroleum and other hydrocarbons in the environment is a complex process, whose quantitative and qualitative aspects depend on the nature and amount of the oil or hydrocarbons present, the ambient and seasonal environmental conditions and the composition of autochthonous microbial community and their adaptive response to the presence of hydrocarbons.

The results of this study indicate that hydrocarbon-utilizing bacteria can be found in the rhizosphere *Mimosa pudica, Alchorinia cordifolia, Chromolaena ordorata, Chloris pilosa* and *Sida acuta*. These bacteria are not restricted to petroleum contaminated soil though. Qualitative differences in root exudates among these plants also induced the increase of bacterial population in the rhizosphere. Thus, rhizoremediation is a promising technology for the clean-up of petroleum-contaminated soils, especially in the tropics where climatic conditions favour plant growth and microbial activity and where financial resources can be limited.

5. Conclusion

This symbiotic technology should therefore be adopted as it is environmentally friendly and aesthetically pleasing, less expensive, self-sustaining and can be used for a wide variety of pollutants. Furthermore,

rhizoremediation generates less secondary waste and fewer air emissions. This is a welcome development especially now that our environment suffers a lot from pollution.

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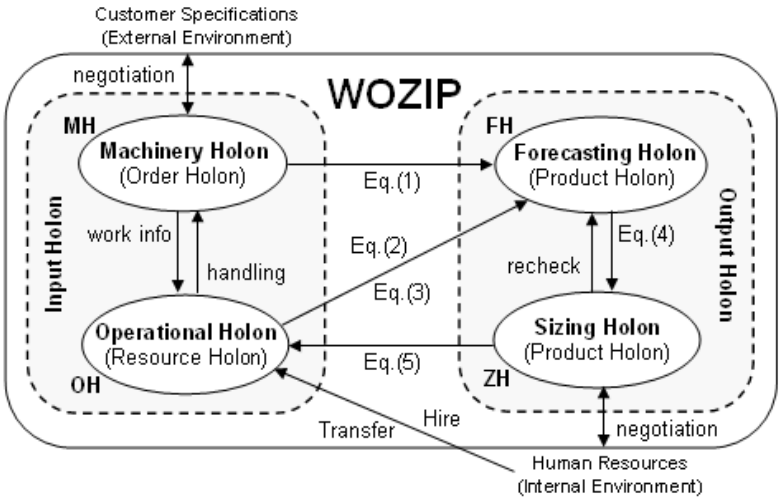

Figure 1. Architecture of WOZIP

Table 1. Datasheet of Mock-up Test

Number of Machines, $N_M = 10$ F = forecast rate
Maximum Utilisation, $U_{max} = 0.80$ Y = actual rate
Smoothing Constant, $\alpha = 0.30$

Year	Month	Rate						No. of Workers	Remarks
		Utilisation		Disturbance		Idling			
		F	Y	F	Y	F	Y		
1	Jan	0.80	0.75	0.06	0.04	0.15	0.20	**9.25 (9)**	
	Feb	0.79	0.70	0.05	0.07	0.17	0.23	**8.84 (9)**	
	Mar	0.76	0.66	0.06	0.09	0.18	0.25	**8.38 (8)**	lower δ_t
	Apr	0.73	0.60	0.07	0.11	0.20	0.26	**7.93 (8)**	
	May	0.69	0.55	0.08	0.09	0.22	0.28	**7.43 (7)**	
	Jun	0.65	0.49	0.08	0.12	0.24	0.28	**6.76 (7)**	decreasing U_t
	Jul	0.60	0.43	0.09	0.10	0.25	0.30	**6.18 (6)**	
	Aug	0.55	0.39	0.10	0.08	0.27	0.30	**5.42 (5)**	
	Sep	0.50	0.34	0.09	0.05	0.28	0.32	**4.65 (5)**	increasing χ_t
	Oct	0.45	0.30	0.08	0.04	0.29	0.32	**3.76 (4)**	
	Nov	0.41	0.25	0.07	0.08	0.30	0.33	**2.95 (3)**	
	Dec	0.36	0.23	0.07	0.12	0.31	0.30	**2.31 (2)**	
2	Jan	0.32	0.28	0.09	0.15	0.31	0.26	**2.03 (2)**	
	Feb	0.31	0.33	0.11	0.18	0.29	0.24	**2.25 (2)**	
	Mar	0.32	0.41	0.13	0.20	0.28	0.22	**2.77 (3)**	higher δ_t
	Apr	0.34	0.45	0.15	0.18	0.26	0.20	**3.57 (4)**	
	May	0.38	0.53	0.16	0.17	0.24	0.16	**4.26 (4)**	
	Jun	0.42	0.62	0.16	0.12	0.22	0.14	**5.13 (5)**	increasing U_t
	Jul	0.48	0.69	0.15	0.16	0.19	0.12	**5.94 (6)**	
	Aug	0.54	0.74	0.15	0.20	0.17	0.09	**6.99 (7)**	
	Sep	0.60	0.82	0.17	0.17	0.15	0.06	**8.15 (8)**	decreasing χ_t
	Oct	0.67	0.87	0.17	0.12	0.12	0.04	**9.24 (9)**	
	Nov	0.73	0.90	0.15	0.12	0.10	0.03	**10.06 (10)**	
	Dec	0.78	0.90	0.14	0.15	0.08	0.03	**10.77 (11)**	

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