The Synergistic Approach/ Action of Plants and Rhizobacteria in Crude Oil Contaminated Soil Remediation in Nigeria.

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Abstract: The synergistic approach of plants and rhizobacteria in crude oil contaminated soil in three different locations were carried out. The presence of heterotrophic bacteria and hydrocarbon-utilizing bacteria isolated from the polluted and pristine rhizosphere and non-rhizosphere soils of the plants were compared. The polluted rhizosphere of total culturable heterotrophic bacterial count gave a range of $0.98x10^6$cfu/g to $1.37x10^6$cfu/g. The pristine rhizosphere count ranged from $4.11x10^5$cfu/g to $7.55x10^5$cfu/g. The polluted non-rhizosphere gave ranged from $2.39x10^5$cfu/g to $3.28x10^5$cfu/g. The pristine non-rhizosphere had a range of $2.90x10^5$cfu/g to $3.97x10^5$cfu/g. The polluted rhizosphere counts for hydrocarbon-utilizing bacteria ranged from $1.60x10^5$cfu/g to $6.91x10^5$ cfu/g. The pristine rhizosphere gave a range of $1.85x10^5$cfu/g to $3.38x10^5$cfu/g. In the polluted non-rhizosphere, the range was from $1.02x10^5$cfu/g to $1.42x10^5$cfu/g. A range of $6.05x10^4$cfu/g to $9.75x10^4$cfu/g was obtained from the pristine non-rhizosphere. There was no significant difference ($P>0.05$) between the rhizosphere and non-rhizosphere of total heterotrophic and hydrocarbon-utilizing bacterial counts in both polluted and pristine soils. All the plants exhibited positive rhizosphere effects on the rhizobacteria. Hydrocarbon-utilizers were identified as *Acinetobacter, Arthrobacter, Alcaligenes, Bacillus, Corynebacterium, Flavobacterium, Micrococcus, Serratia and Pseudomonas* spp. All the isolates grew on petroleum hydrocarbon at different growth rates. Based on these results, the organisms 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.
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1. Introduction

The usage of petroleum hydrocarbon products has increased soil contamination. This is one of the major environmental problems in Nigeria and globally. Research efforts have been devoted to develop new, low-cost, low-technology, eco-friendly treatments capable of reducing and even eliminating pollution in the atmosphere, the hydrosphere and soil environments (Rao et al., 2010). To investigate the countermeasure to remediate soils contaminated with oils, bioremediation provide such an effective and efficient strategy to speed up the clean-up processes. Bioremediation of contaminated soil is low cost, causes less interference with the soil structure and has a higher public acceptance than other approaches including soil thermal desorption and soil leaching treatment (Tang et al, 2010).

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 (Zhou et al., 2011). Partial or complete degradation of organic substances have been demonstrated in some cases (White, 2001). The use of plants to extract, sequester or detoxify pollutants is therefore known as phytoremediation (Gurska, 2009). Plants frequently do not possess complete metabolic degradation pathway for pollutants, and even more toxic by-products may be produced.

Most plants have symbiotic relationships with soil microorganisms. For example, root nodule bacteria that have symbiotic relationships with legumes are involved in Nitrogen fixation. The area around plant roots, known as the rhizosphere contains higher populations, greater diversities and activities of microorganisms than soil with no plants (Nicholas et al., 1997).

This synergistic approach of using plants and their rhizobacteria in remediation of oil polluted soil is known as rhizoremediation (Kuiper et al., 2004). Application of the synergistic action of plants and their rhizobacteria in crude oil contaminated soil remediation have been demonstrated as an appropriate and more practical alternative to clean-up of

petroleum hydrocarbon in the contaminated environments.

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 xenobiobiotic substances. The soluble root exudates include enzymes, amino acids, sugars and low molecular weight carbohydrates (Burken and Schnoor, 1996).

The objective of this study therefore was to isolate rhizosphere-inhabiting indigenous oil-degrading bacteria in plants growing in crude oil-polluted areas.

2. Materials and Methods

2.1 Study Site Description

Polluted rhizosphere and non-rhizosphere soil samples were collected from the three different crude oil polluted sites in Imo and Rivers States, Nigeria. These sites could be described as recovering ecosystems with few plants growing at these locations. Unpolluted rhizosphere and non-rhizosphere soil samples were collected from the same areas where there has been no known crude oil pollution which served as the control.

2.2 Collection of Soil Samples

Plants of the same species were collected from the crude oil polluted and unpolluted sites in separately in marked sterile plastic bags. The plants were pulled out slowly to avoid breaking their roots. Non-rhizosphere (bulk) soil samples of crude oil polluted and unpolluted sites were collected at a distance of thirty centimeters (30cm) from the plants' roots as described by Ukaegbu-Obi and Mbakwem-Aniebo (2014) in marked sterile plastic bags and transported in an ice chest to the laboratory for analyses. The plants were taken to a Plant taxonomist for identification.

2.3 Bacterial counts and isolation

The total culturable heterotrophic bacterial (TCHB) count was determined using the spread plate method on nutrient agar (Oxoid) according to Chikere *et al*. (2009). Soil suspensions were prepared by 10 fold serial dilutions with 1 g of soil and then 10^{-3} to 10^{-6} dilutions were spread on the plates in duplicates. The colony forming units of heterotrophs were counted after incubation at 28°C for 18 h. Hydrocarbon utilizing bacteria (HUB) were enumerated as adopted from Hamamura *et al*. (2008) using mineral salts medium with crude oil supplied by the vapour phase transfer. Isolated colonies were further purified by subculturing and identified using biochemical tests and microscopy.

2.4 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* (Holt, J.G. (1994).

2.5 Screen Test for Hydrocarbon-Utilization by Bacterial Isolates

The bacterial isolates were tested for their ability to utilize crude oil using the turbidity method as described by Ukaegbu-Obi and Mbakwem-Aniebo (2014). The bacterial isolates were cultured in nutrient broth and incubated at $28+2^0$C for 24 hours. Aliquot (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 plus 0.1ml of crude oil remained uninoculated. The tubes were incubated at room temperature for 7 days. The growth of the inocula was determined by visual observation of the mineral salt broth turbidity, as compared with the uninoculated control tube.

2.6 Statistical Analysis

The statistical tools – One-way Analysis of Variance (ANOVA) was used to analyze the data obtained from the plants while Independent Student's t-test was used to analyze the polluted and unpolluted soil sample of each plant.

3. Results

Plant-assisted bioremediation (rhizoremediation) stands out as a potential tool to inactivate or completely remove xenobiotics from the polluted environment. Therefore, it is of key importance to find an adequate combination of plant species and microorganisms that together enhance the clean-up process.

The results of the enumeration of the total culturable heterotrophic bacterial counts of the polluted and pristine rhizosphere and bulk soil are shown in Figures 1-6. The total culturable heterotrophic bacteria counts for polluted rhizosphere ranged from $0.98x10^6$cfu/g - 1.37×10^6 cfu/g, the pristine rhizosphere (control), ranged from $4.20x10^5$ cfu/g - 7.55×10^5 cfu/g; the polluted non-rhizosphere, ranged from 2.56×10^5 cfu/g - 3.12×10^5cfu/g while the pristine non-rhizosphere, ranged from 3.10×10^5cfu/g - 4.12×10^5 cfu/g.

The polluted rhizosphere counts for hydrocarbon-utilizing bacteria ranged from $1.60x10^5$cfu/g to $6.91x10^5$ cfu/g. The pristine rhizosphere gave a range of $1.85x10^5$cfu/g to $3.38x10^5$cfu/g. In the polluted non-rhizosphere, the range was from $1.02x10^5$cfu/g to $1.42x10^5$cfu/g. A range of $6.05x10^4$cfu/g to $9.75x10^4$cfu/g was obtained for pristine non- rhizosphere.

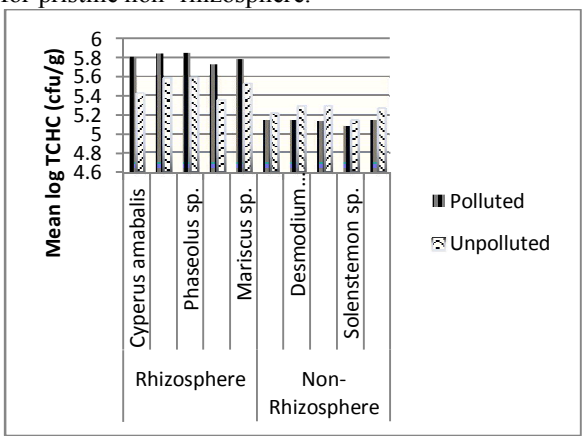

Figure 1. Total heterotrophic bacterial counts of polluted and pristine rhizosphere and non-rhizosphere soils at location 1.

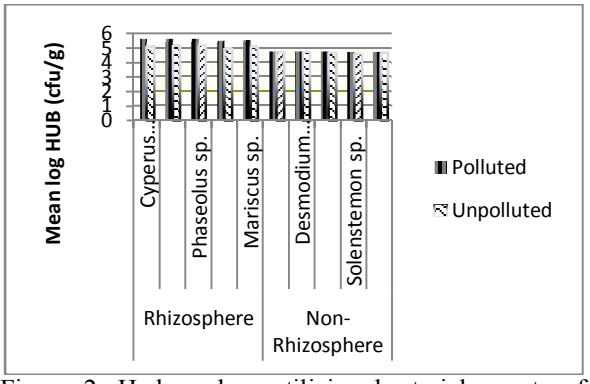

Figure 2. Hydrocarbon utilizing bacterial counts of polluted and pristine rhizosphere and non-rhizosphere soils at location 1.

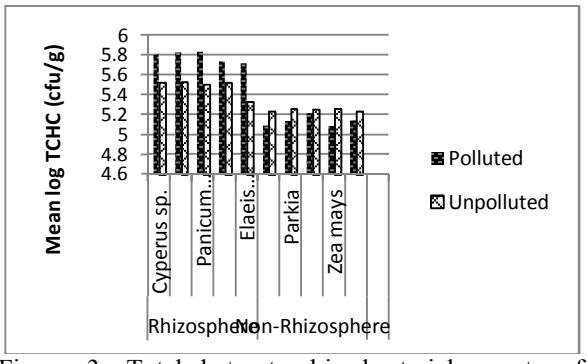

Figure 3. Total heterotrophic bacterial counts of polluted and pristine rhizosphere and non-rhizosphere soils at location 2.

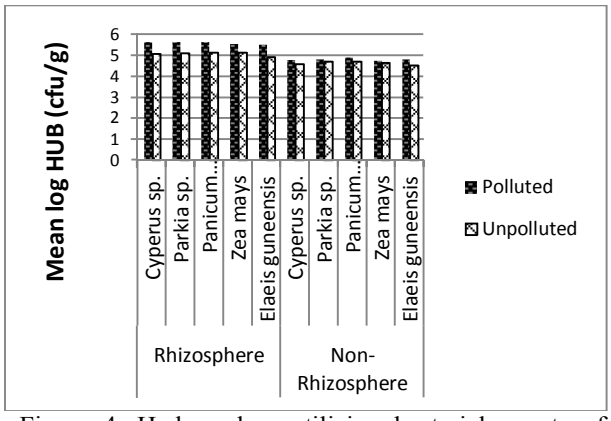

Figure 4. Hydrocarbon utilizing bacterial counts of polluted and pristine rhizosphere and non-rhizosphere soils at location 2.

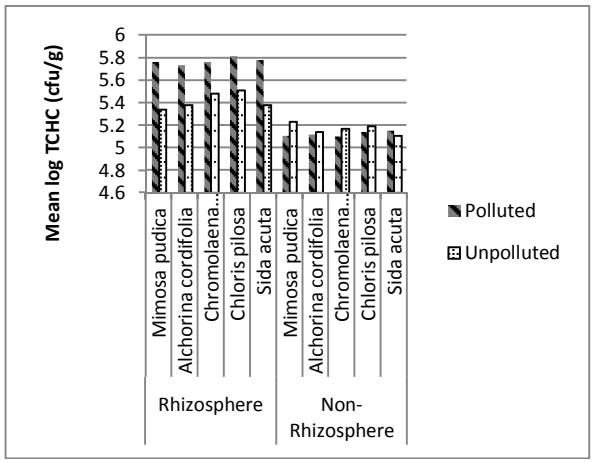

Figure 5. Total heterotrophic bacterial counts of polluted and pristine rhizosphere and non-rhizosphere soils at location 3.

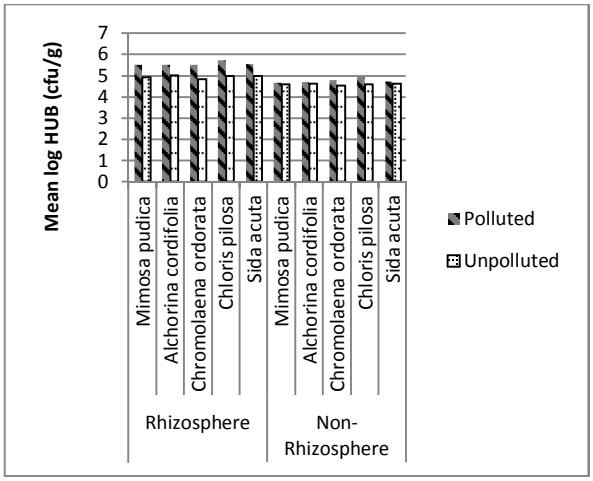

Figure 6. Hydrocarbon utilizing bacterial counts of polluted and pristine rhizosphere and non-rhizosphere soils at location 3.

Table 1: Screen Test for the Utilization of Petroleum Hydrocarbon by the Bacterial Isolates

Isolate Code	Growth In Bacterial Isolate Crude Oil Medium	
PRO 1A	++	*Arthrobacter* sp.
PSO 2B	++	*Corynebacterium* sp.
PSO 3A	++	*Alcaligenes* sp.
PRO 5A	++	*Acinetobacter* sp.
NSO 1B	++	*Pseudomonas* sp.
NSO 2A	+	*Flavobacterium* sp.
NRO 3A	+	*Arthrobacter* sp.
NRO 3A	+	*Serratia* sp.
NRO 4B	++	*Pseudomonas* sp.
NRO 5B	+	*Corynebacterium* sp.
PRU 1A	++	*Flavobacterium* sp.
PSU 2B	++	*Micrococcus* sp.
PRU 3A	++	*Alcaligenes* sp.
PSU 3A	++	*Bacillus* sp.
PRU 3B	+++	*Pseudomonas* sp.
PRU 1B	+	*Arthrobacter* sp.
NSU 4B	+	*Arthrobacter* sp.
NRU 5B	++	*Acinetobacter* sp.
PSA 1A	++	*Bacillus* sp.
PRA 4B	++	*Bacillus* sp.
PRA 5A	+++	*Pseudomonas* sp.
NSA 3A	+	*Acinetobacter* sp.
NRA 2B	+	*Micrococcus* sp.

4 Discussions

Field trials have shown that remediation of petroleum contaminated sites can be enhanced by cultivation of plants. To date, a great variety of grasses, legumes and fast growing trees with high transpiration rates such as poplars, alder or willow have been applied for phytoremediation. These plants provide large surface area for root soil contact due to their expansive root system. Roots also provide ideal attachment sites for microbes and food supply/exudates consisting of amino acid, organic acids, sugars, enzymes and complex carbohydrate (Tesar et al., 2002).

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 (Figures 1, 3 and 5) recorded in this study for most crude oil-contaminated soils compared to that of pristine soils agreed with the previous reports by 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.

Unlike the result of total culturable heterotrophic count, in the non-rhizosphere soil, the mean counts of the hydrocarbon-utilizing bacteria were higher in the contaminated non-rhizosphere soil for all the plants (Figures 2, 4 and 6). This finding was also reported by Leahy and Colwell (1990) and Ukaegbu-Obi and Mbakwem-Aniebo (2014) who observed that hydrocarbon-utilizing bacteria and fungi were readily isolated from soil and also that the application of oil or oily waste to soil resulted in increased numbers of hydrocarbon-utilizing bacteria and fungi. This phenomenon of selective enrichment causes the numbers of microorganisms that can utilize the compound of interest to increase within the community (Leahy and Colwell, 1990). The addition of crude oil results in an immediate change in bacterial community structure, 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 (Hamamura et al., 2008).

The rhizosphere (both polluted and pristine) had higher frequencies of occurrence of bacterial isolates than the non-rhizosphere. This result may indicate the fact that vegetation influences some specific degradative groups of bacteria already present in the polluted sites than the total microbial diversity. Juhason *et al*. (2007) reported that in their field experiment phytoremediation increased the number of phenol-degrading bacteria in semi-coke (processed oil shale solid wastes) as well as metabolic diversity of microbial community. Exudates derived from plants can help to stimulate the survival and action of bacteria, which subsequently results in more efficient degradation of pollutants (Kuiper et al,. 2004).

The bacterial isolates obtained from different rhizosphere and non-rhizosphere of polluted and pristine soils in this study were identified to be of the following genera: *Acinetobacter, Alcaligenes, Arthrobacter, Bacillus, Corynebacterium, Flavobacterium, Micrococcus and Pseudomonas* and *Serratia*. 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 bacterial isolates utilized crude oil at different rates with *Pseudomonas* sp. having the highest growth rate followed by *Bacillus* sp., *Acinetobacter* sp., *Alcaligenes* sp., *Micrococcus* sp., and *Corynebacterium* sp., that had moderate growth rate and *Arthrobacter sp., Flavobacterium sp., Micrococcus* sp., *Serratia sp.* having scanty growth rates (Table 1). Some isolates may withstand toxic components of the oil and thrive, others may be inhibited. Other investigators (Ibrahim et al., 2008) have made similar observations. The result also showed that some bacteria isolated from uncontaminated sites had the potential to utilize crude oil but these potentials were not as high as their counterparts isolated from contaminated sites.

Conclusion

The use of plants for rehabilitation of crude oil contaminated environments is an emerging area of interest because it provides an ecologically sound and safe method for restoration and remediation. A clever solution is to combine the advantages of microbe-plant symbiosis within the plant rhizosphere into an effective cleanup technology as this may be a promising strategy to remediate more contaminated sites.

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