MICROBIAL RESPONSE TO PLANTS' ROOTS AND HYDROCARBON CONTAMINANTS IN DIESEL-OIL IMPACTED TROPICAL SOIL

[1]Adieze, I. E.; [2]Onogbou, C. D.; [3]Ihejirika, C. E. and [1]Nwabueze, R. N.

Department of Microbiology, School of Science, Federal University of Technology, Ozverri, Imo-State

Department of Biotechnology, School of Science, Federal University of Technology, Ozverri, Imo-State

Department of Environmental Technology, School of Environmental Science, School of Science, Federal University of Technology, Ozverri, Imo-State

Abstract: Microbial response to plants' roots and contaminants in diesel-oil impacted soils were assessed in a greenhouse study. Two plants, *Pueraria* spp. and *Panicum maximum* were planted in 6% w/w polluted and control soil samples to obtain contaminated planted and uncontaminated planted pots. Contaminated control and uncontaminated control pots were also maintained. Periodically, from week zero to 8 weeks after planting (WAP), the total bacterial, fungal and hydrocarbon utilizing microbial counts of the soil samples were determined. The results showed that total bacterial and fungal counts at week zero decreased from an average of 5.8 x 10^6 CFU/g soil and 3.42 x 10^4 CFU/g soil in uncontaminated soil to 3.04 x 10^6 CFU/g soil and 2.27 x 10^4 CFU/g soil respectively, in the contaminated control samples. This could be in response to the direct toxic effects of volatile hydrocarbons, limitations in oxygen and water resulting from hydrophobic oil. At 4 WAP, the bacterial counts increased to 16.33 x 10^6 CFU/g soil and 12.67 x 10^6 CFU/g soil in the contaminated samples planted *Pueraria* spp. and *P. maximum* respectively. This could be as a result of the response of adapted species to the rhizosphere effect. At the end of the study, the percentage oil in soil were reduced by 55.63%, 64.38% and 65.45% in contaminated unvegetated sample, contaminated sample planted *Pueraria* spp. and in contaminated sample planted *P. maximum*, respectively. This study showed that microbial growth was sustained in the rhizosphere of the plant species studied. This has implications for bioremediation especially in the tropics where warm temperatures favour plants' growth and microbial activities.

Key words: Phytoremediation, plant derived hydrocarbons, rhizosphere effect and toxic volatile hydrocarbons

INTRODUCTION

Environmental pollution by petroleum hydrocarbons has become a global phenomenon of increasing importance. In order to restore contaminated sites to their initial uncontaminated useful state, a process of remediation is required. These include bioremediation -the optimization of microorganisms to remediate contaminated sites. Bioremediation of oil contaminated soil has been considered as a cost-effective technology (Margesin, 2000; Rubertoa *et al.*, 2003; Gentilia *et al.*, 2006). The success of bioremediation processes highly depends on the presence and

*Corresponding author:
Mbachu, I.A.C.

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stimulation of the activities of organisms with hydrocarbon biodegradation capabilities. These processes can supply nutrients, thus encouraging the growth of indigenous bacterial species (biostimulation), or add new species to the contaminated site (bioaugmentation), and/or alter environmental conditions to speed up the growth of bacterial colonies as seen in growing of plants in contaminated site (phytoremediation).

Phytoremediation is the *in situ* use of plants and their associated rhizosphere microorganisms to degrade, contain or render harmless, contaminants in the soil or ground water (Cunningham *et al.*, 1996). Phytoremediation can be applied to moderately contaminated soils or as a polishing step to further degrade residual hydrocarbons and to improve soil quality after the application of other remediation measures (Schnoor, 1997; Frick *et al.*, 1999). Phytoremediation of petroleum-hydrocarbon is presumed to be based on the stimulation of microbial degradation in the rhizosphere. Plants can enhance this by transporting oxygen to the root area along root channels, by lowering the water table allowing for gas phase diffusion in soil, and loosening soil aggregates. Thus providing oxygen required for substrate oxidation which is the initial step in the degradation of most hydrocarbons (Yeung *et al.*, 1997).

Microbes are also stimulated by organics and sources of carbon and nitrogen from plant root exudates. It has been established that bacteria with the ability to degrade a wide range of crude oil components exist ubiquitously in the environment and do appear to respond quite rapidly to the presence of petroleum (Lee and Levy, 1991; Abu and Ogiji, 1996; Adieze *et al.*, 2003). This inherent characteristic impacts a large assimilatory potential to most soils.

To date however, not many studies have been carried out on phytoremediation in the tropics. Thus this work assessed the microbial response to two tropical plants (*Pueraria* spp.) a legume and Guinea grass (*Panicum maximum*) used in the remediation of diesel oil impacted soil.

The two plants are common in the oil rich South Eastern Nigeria. The grass was also chosen for the large surface area provided for microbial growth by their extensive, widely branched fibrous root system while the legume was considered because of their ability to fix nitrogen. Both plants also passed the initial seed germination test in weathered 6% (w/w) diesel oil contaminated soil.

MATERIALS AND METHODS

Plant Selection Criteria

Criteria for pre-selection of potential phytoremediation species were based on frequency of occurrence and species densities in re-vegetating contaminated sites, ease of propagation (availability and handling of seeds, seed germination in contaminated soil, vegetative propagation), and root structures.

Soil collection and preparation

The soil sample was collected from the top 0-25 cm of a garden soil at the School of Agriculture and Agricultural technology, Federal University of Technology, Owerri. The soil was air dried, and passed through a 2 mm sieve to ensure uniformity. After sieving, the soil texture was typically

sandy-loam. The soil characteristics are shown in Table I below.

Table I: Soil Characteristics

pH	6.63
Phosphorous (ppm)	15.26
Total Nitrogen (%)	0.063
Organic Carbon (%)	0.723
Organic matter (%)	1.46

Diesel oil was thoroughly mixed with the air dried sieved soil to obtain a final concentration of 6% (w/w) oil in soil. The diesel-laded soil was allowed for a period of two weeks to ensure removal of toxic volatile components of diesel before planting at week zero.

Plant materials and pot experiment set up

Two plants were used in this study *Pueraria* spp. a legume and *Panicum maximum* (Guinea grass). Young seedlings of the plants were obtained from a fallow patch of land, and used for the study.

The soil samples used for the study (contaminated and uncontaminated) were potted in chemically clean plastic pots. Each pot contained 1 kg of soil planted with *Pueraria* spp., *Panicum maximum* or un-vegetated. Six replicates of each treatment (the contaminated planted pots, uncontaminated planted pots, contaminated control pots and uncontaminated control pots) were set up and incubated for 8 weeks after planting (WAP). These ensured the comparism of microbial response in vegetated and un-vegetated treatments, and between contaminated and uncontaminated samples.

The pots were incubated under greenhouse conditions and their water contents were kept near field capacity by watering three times a week.

Determination of microbial counts and species present in treated soil samples

Microbial populations in the soil samples were assayed by standard plate count technique. Ten fold serial dilution of soil sample suspension was prepared, and aliquots 0.1 ml of dilutions 10^{-5} to 10^{-7} were plated in duplicate on nutrient agar (Oxoid) plates supplemented with fulcin (500 mg/l) an antifungal agent, and on Sabouraud's dextrose agar (SDA) plates supplemented with the antibiotics streptomycin (5 µg/ml) by spread plate technique for the determination of aerobic heterotrophic bacterial counts and total fungal counts, respectively. The plates were incubated at 30±2°C respectively for 1 - 2 days for bacterial counts, and 5 – 7 days for fungal counts after which colonies in the duplicate plates were counted and average counts recorded and used for the calculation of colony forming units per gram (CFU/g) of soil. This was done at various times during the study.

Aliquots (0.1 ml) of dilutions 10^{-3} to 10^{-5} of soil samples' suspensions were plated in duplicate onto the mineral salts medium. The medium contained 10.0 g NaCl; 0.42 g $MgSO_4.7H_2O$; 0.29 g KCl; 0.83 g KH_2PO_4; 1.25 g K_2HPO_4; 0.42 g $NaNO_3$; 15 g agar per litre of deionized water (Okpokwasili and

Amanchukwu, 1988). Sterile filter paper (Whatman No.1) saturated with diesel oil were aseptically placed unto the covers of the inoculated inverted plates and then incubated for 3-7 days at 30±2 °C. Hydrocarbons from the diesel oil saturated filter paper were supplied in vapour phase to the surface of the agar plate as sole source of carbon. Colonies were counted from duplicate plates and the average counts were recorded and used for the calculation of colony forming units per gram (CFU/g) soil. This was done for the various treatments at various times during the study.

Isolation and identification of hydrocarbon utilizing bacteria

Colonies of hydrocarbon utilizing species were isolated from the mineral salts agar plates of samples, based on their colonial characteristics. The isolates were purified by streaking on nutrient agar plates for bacteria species and on SDA plates for fungal species, and then respectively transferred onto nutrient agar and SDA slants in Bijou bottles and stored at 4 °C in a refrigerator for further studies.

Bacterial species isolated from the mineral salt agar plates and stored on nutrient agar slants in a refrigerator were examined for their biochemical and morphological characteristics. The colonial appearance of the isolates were examined and noted. Gram staining described by Gerhardt *et al.* (1981) was performed to determine the cellular morphologies and Gram reactions of the isolates. Other tests performed include motility test, catalase test, citrate utilization test, indole test, oxidase test, oxidative fermentative (O/F) utilization of glucose, urease test, methyl red (MR) test and Voges-Proskauer (VP) test.

Fungal isolates from stored SDA slants were subcultured to obtain pure cultures. They were then characterized and identified using both cultural characteristics and microscopy after lactophenol staining technique according to Fawole and Oso (2004) and Barnett and Hunter (1972).

Determination of residual diesel oil in soils

Residual diesel oil in contaminated soil samples were determined by the methods of Adieze *et al.* (2003).

RESULTS AND DISCUSSION

The total bacterial and fungal counts at week zero decreased from an average of 5.8×10^6 CFU/g soil and 3.42×10^4 CFU/g soil in uncontaminated soil to 3.04×10^6 CFU/g soil and 2.27×10^4 CFU/g soil in the contaminated unplanted samples (Figures 1 and 2). This could be in response to the direct toxic effects of volatile hydrocarbons, limitations in oxygen and water resulting from hydrophobic nature of oil.

At the week 4 of study, the bacterial counts increased to 16.33×10^6 CFU/g soil and 12.67×10^6 CFU/g soil in the contaminated samples planted with *Pueraria* spp. and *P. maximum* respectively (Figure 1). This increase could be as a result of the response of adapted species to the presence of carbon as well as to the rhizosphere effect, since the increase in the vegetated contaminated soil was more than that in unvegetated contaminated and vegetated uncontaminated soil samples. This result supports those of Bossert and

Bartha (1984) which reported that contamination of soil generally increase total microbial activities and Gunther *et al.* (1996) which reported that disappearance of pollutants in the rhizosphere is accompanied by higher values of microbial plate counts and soil respiration rates for the vegetated systems. They concluded that n-alkanes are part of the cuticular plant waxes and that plant derived HC are continuously released into the rhizosphere, making rhizosphere microbial communities to be partly acclimated to alkane degradation.

The increase in bacteria count was more in *Pueraria* spp. than that in *P. maximum*, it could be that rhizosphere of *Pueraria* spp. supports more microbial growth. *Pueraria* spp. is a leguminous species and would have added nitrogen to the soil. Adieze *et al.* (2012) reported that *Centrosema sp.* and *Pueraria sp.* are legumes whose synthesised nitrogen can sustain good microbial activity in their rhizosphere, while the extensive fibrous root of the *P. maximum* and its exudates will also favour stimulation and growth of microbes in its rhizosphere. This they reported may have affected degradation of soil pollutants and plant growth. Gunther *et al.* (1996) however reported that besides nutrient supply by exudates, plant impact on microclimatical and environmental soil parameters e.g. moderate temperature and moisture conditions from a vegetation cover is obvious, and stimulates the fate of aliphatic HC in the rhizosphere.

Figure 3 shows that the hydrocarbon utilizing microbial counts increased from 0.9×10^4 CFU/g and 0.8×10^4 CFU/g in contaminated control and contaminated planted with grass to 6.7×10^4 CFU/g and 12.2×10^4 CFU/g soil 4 WAP. The counts however dropped to 8.2×10^4 CFU/g in contaminated planted with grass but increased to 10.2×10^4 CFU/g soil. This could be as a result of residual metabolizable HC in the contaminated control soil 8 WAP.

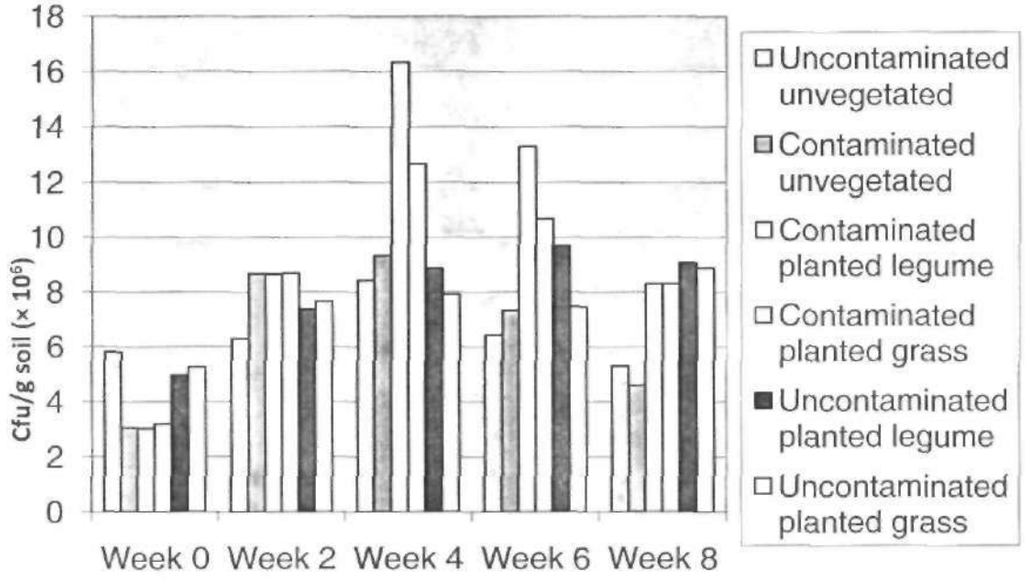

Figure 1: Total Bacterial Count

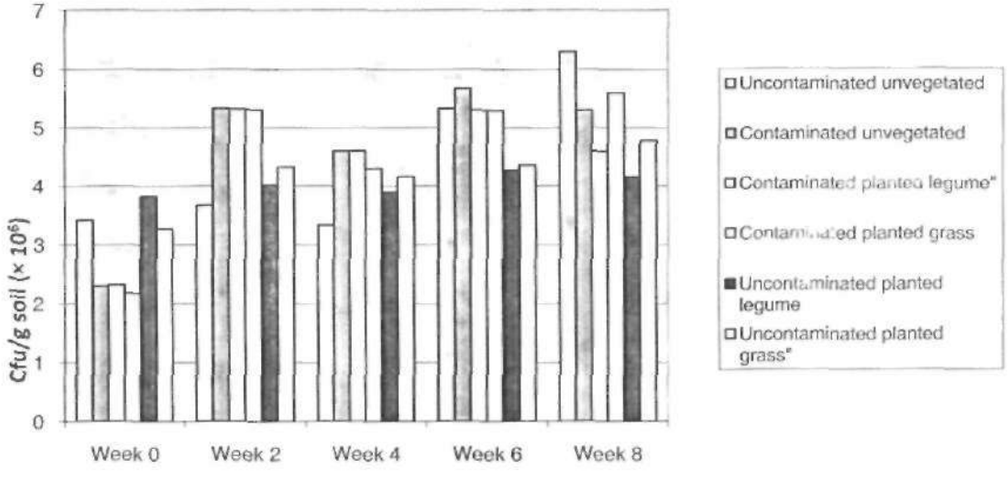

Figure 2: Fungal Count

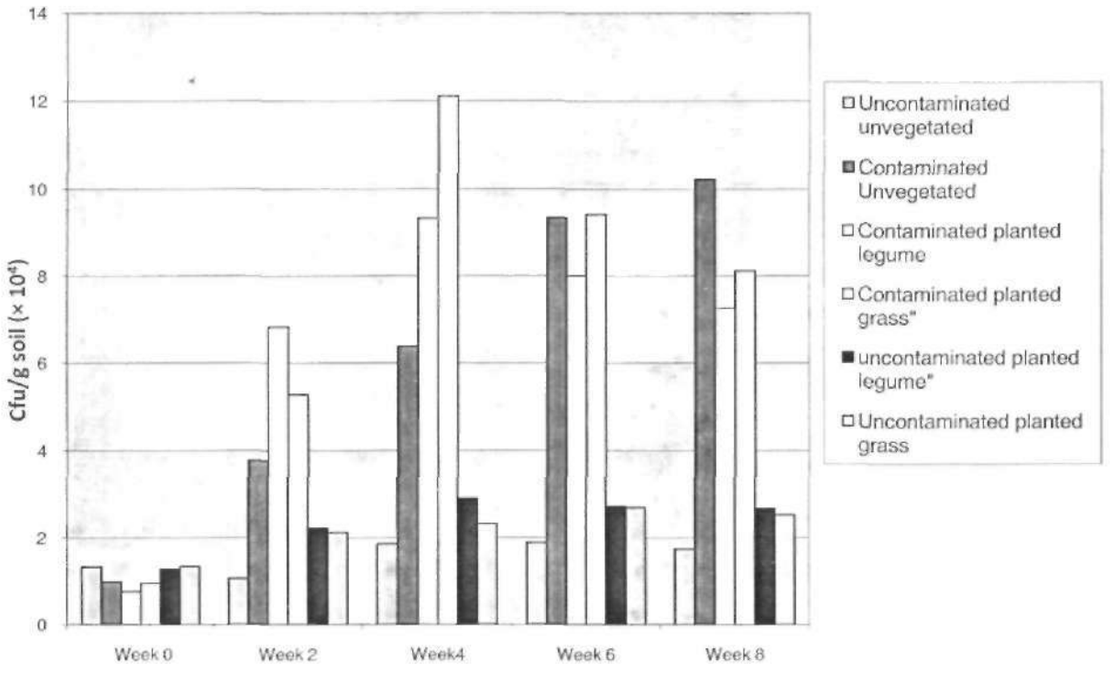

Figure 3: Hydrocarbon utilizing microbial count

Table 2: Isolated microbial species and their presence in treated soil samples between the 4th and 8th week.

	Week 4				Week 6				Week 8			
Soil treatments	A		C	D	A	B	C	D	A	B	C	D
Pseudomonas spp.	+	+	+	+	+	+	+	+	+	+	+	+
Staphylococcus spp.	+	-	-	+	+	-	+	+	+	+	+	+
Alkaligenes spp.	+	+	+	+	+	-	+	+	+	+	+	+
Bacillus spp.	+	+	+	+	+	+	+	+	+	+	+	+
Micrococcus spp.	+	+	+	+	+	-	-	-	+	-	-	-

Penicillium spp.	+	+	+	+	+	+	+	+	+	+	+	+
Rhizopus spp.	+	+	+	+	+	+	+	+	+	-	-	-
Aspergillus spp.	+	+	+	+	+	-	+	+	+	-	+	+
Fusarium spp.	+	+	+	+	+	+	+	+	+	+	+	+

Key : A = Uncontaminated soil; B= Contaminated control; C= Contaminated soil + legume; Contaminated soil + grass; + = Presence; - = Absent.

The assessment of hydrocarbon utilizing bacterial species in the various treated soil samples during the study revealed five prominent species (*Pseudomonas* spp., *Staphylococcus* spp., *Alkaligenes* spp., *Bacillus* spp. and *Micrococcus* spp.). These isolates were present in most of the treated soil samples except *Staphylococcus* spp. and *Micrococcus* spp. While *Staphylococcus* spp. tends to be susceptible to unfavourable conditions in contaminated control soil samples and contaminated soil samples planted with legumes in the 4th week of study, the *Micrococcus* spp. showed susceptibility to the contaminated soil samples in the 6th and 8th week. This may mean that *Staphylococcus* spp. was sensitive to the toxic light hydrocarbon fractions, *Micrococcus* spp. may have been eliminated by toxic metabolic intermediates produced during the biodegradation. Infiltration of fossil fuel into the soil was observed as preventing evaporative losses of volatile hydrocarbons. National Academy of Science, (1985) reported that this can increase the toxicity of contaminants to microorganisms which may limit the microbial activity temporarily. Of the four fungal species isolated, all were present throughout the study except *Aspergillus* spp. that were absent in contaminated control on the 6th and 8th week, and *Rhizopus* spp. that were absent in all the contaminated samples on the 8 week of study. The absent species may have been out grown by species that adapted better in the polluted soils and in the rhizosphere of the plant species in polluted soil samples. They may also have been sensitive to toxic metabolic intermediates which may have built up during the biodegradation process. This result shows that the hydrocarbon utilizing species play prominent roles at various times during biodegradation processes. This implies that their individual activities can be optimized if the stage of their activity is identified. This will have implication for enhanced biodegradation.

Table 3: Residual diesel oil in soil (%) and percentage reduction

Treatments	Week 0	Week 8	% Reduction
Contaminated unplanted	5.86	2.60	55.63
Contaminated planted *Pueraria* spp.	5.84	2.08	64.38
Contaminated planted *P. maximum*	5.79	2.00	65.45

At the end of the study, gravimetric analysis showed that the percentage oil in soil were reduced to 2.60, 2.08 and 2.00 in Contaminated unvegetated soil sample, Contaminated sample planted with *Pueraria* spp. and in Contaminated soil sample planted with *P. maximum* (i.e. 55.63%, 64.38% and 65.45% reduction in oil) respectively.

Contaminated soil sample planted with *P. maximum* has the highest percentage oil reduction, 65.45%. This could be that the strains of bacteria in rhizosphere of *P. maximum* are fast degraders of hydrocarbon than those found in *Pueraria* spp. even though *Pueraria* spp. has higher count. This study collaborate the report by Adieze *et al.* (2012) that *Panicum maximum* has promising potentials for the phytoremediation of petroleum-contaminated tropical soils, given its continual growth and development of a plant's growth indices as biomass in the presence of crude oil contaminant. This supports reports that microbial population stimulated by vegetation can accelerate the removal of hydrocarbon contaminants from soil.

CONCLUSION

The results showed that microbial species were sustained in the rhizosphere region of the two plant species studied. The plant species were successfully established on polluted soils, hence the plant-microbial interaction in the rhizosphere provided enhanced breakdown of the hydrocarbons in the vegetated soil. The hydrocarbon utilizing species play prominent roles at various times during biodegradation processes as shown by their presence or absence during the study. This implies that their individual activities can be optimized if the stages of their activities are identified. This will have implication for enhanced biodegradation.

There is therefore the need to identify and screen plants that will sustain identified good microbial species in their rhizosphere and grow well in contaminated soils for use in phytoremediation. This is important in the tropics, where the constant warm temperatures favour plants growth enhancing microbial activities and bioremediation.

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