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ASSESSMENT OF THE TOTAL PETROLEUM HYDROCARBON CONTENT OF AGRICULTURAL SOIL POLLUTED WITH DIFFERENT VOLUME OF CRUDE OIL DURING PLANT-MICROBE INTERACTION

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ABSTRACT

The effectiveness of plants in interaction with indigenous organisms in environmental clean –up was evaluated. The agricultural soil used for the study was polluted with 100ml, 200ml, 400ml and 800ml of Bonny light crude oil [100%]. Pre and post Microbial examination of the polluted soil identified the indigenous flora present in the soil to be Penicillum sp Aspergillus fumigatus, Aspergillus niger, Candida sp, Pseudomonas fluorescence, Acinetobacter baumanni, Bacillus mycoides, Klebsiella sp., Staphylococcus aureus and Escherichia coli though the absence of S aureus and E. coli was evident during the latter. Vigna unguiculata var unguiculata, Mucuna pruriens, Zea mays and Telfairia occidentalis were the test plant used. Gas chromatographic (GC) analysis revealed the total petroleum hydrocarbon (TPH) of polluted soil on comparison with the value of 10,380 kg/ mg for control sample, to be low. The high TPH obtained from samples polluted with higher concentration depicts that the numbers of plants to be cultivated for remediation could be a determining factor for a faster clean-up. Statistical analysis using analysis of variance (ANOVA) model of SPSS software however, showed there was no significant difference in the degradation of crude oil in samples that are in the green house or field.

KEY WORDS: *Bonny light crude oil, agricultural soil, crop plants, micro-organisms, Total Petroleum Hydrocarbon (TPH).*

INTRODUCTION

Our environment is constantly polluted by various organic and inorganic substances of which crude oil is one of them. Crude oil, according to Atlas (1981), describes a broad range of natural hydrocarbon – based substances and refined petroleum products, each having a different chemical composition. As a result, each type of crude oil and refined product has distinct physical properties that affect the way oil spreads and breaks down, the hazard it may pose to marine and human life, and the likelihood that it will pose a threat to natural and man-made resources (USEPA, 2006).

For example, light refined products, such as gasoline and kerosene spread on water surfaces and penetrate porous soils quickly. Fire and toxic hazards are high, but the products evaporate quickly and leave little residue. Alternatively, Sivasubamaniam *et al.* (2005) reported that heavier refined oil products pose a lesser fire and toxic hazard and do not spread on water as readily, they are more persistent, and may present a greater remediation challenge. The rate at which oil spill spread will determine its effect on the environment. Thus, oil spill has done a tremendous damage to our environment affecting the overall inhabitants.

In Nigeria, crude oil contaminated soil have adversely affected the vegetation as well as the health of animals and human in Oil producing areas of the country (Ogbulie and Iwuala, 2006). There is therefore, the need for treatment of these contaminated soils. The treatments normally used include, flushing contaminants out of the soil using water, chemical solvents, or air; destroying the contaminants by incineration, adding materials to the soil to encapsulate the contaminants and prevent them from spreading (USEPA, 2006). These methods however, are cost effective and less environmentally friendly (Ogbulie and Iwuala, 2006) hence, the need to clean up using biological entities in a less costly and environmentally friendly approach.

The principle behind the use of natural existing organisms in the soil to clean up contamination is that these microorganisms (bacteria, fungi and yeast) like other living creatures require nutrients such as nitrogen, phosphate, carbon, water and environment to grow and survive (Sivasabramaniam *et al*., 2005), as such, when these conditions are present, some of the microorganisms will break down organic contaminants, using them as a source of carbon for energy and growth. Soil bacteria such as *Pseudomonas putida, Bacillus subtilis* etc. use petroleum hydrocarbons as food and energy source, changing them into less toxic substances like CO_2, water and fatty acids. These microorganisms are abundant in association with plants. This is because, naturally, as the plant roots grow into the soil, they release root exudates, which serves as food for microorganisms, thus attracting them to the site of the release (Lines-Kelly, 2005). The activities of these microbes lead to the detoxification of organic contaminants in the soil, hence a process known as rhizoremediation (Morgan *et al.,* 2005); a plant-microbe interaction involving the use of microorganism within the rhizosphere (area around the root) to remediate polluted soil or sediment (Kuiper *et al.,* 2004; Jussila, 2006). This research therefore evaluates the fate of crude oil in polluted soil during plant-microbe interaction.

MATERIALS AND METHODS

Seeds of four annual indigenous crops including two annual forage leguminous crop, vegetable cowpea (*Vigna unguiculata var unguiculata*) and velvet bean *Mucuna pruriens*; a cereal, maize (*Zea mays*) and a vegetable crop, fluted pumpkin (*Telfaira occidentalis*) were used for the study. These plant seeds were collected from different locations in the South Eastern part of the country. The crude

oil used was Bonny light Crude oil and was collected in sterile containers from Akiri in Oguta, Imo State, Nigeria; whereas the soil sample for microbial analysis was aseptically collected from an agricultural soil using surface sterilized soil auger and containers (Ogbulie *et al*, 2011). Soil samples were microbiologically analyzed using spread plate technique on standard media to isolate naturally existing microbial floras as described by Cheesbrough (1994). Appearance of the bacterial and fungal genera on the media was examined for morphological details and identification of bacterial isolates was as described Holt (1984).

All the seeds used for these analyses were surface sterilized and allowed to germinate before planting by incubation on pre-sterilized wet sponges (Yee *et al*, 1998). Each pot for the analysis was surface sterilized as described by Yee *et al.* (1998) and filled with 450gram of soil into which 300ml of sterile water was added after planting (Ogbulie *et al,* 2010). Watering of plants was carried out every 48h (Yee *et al*, 1998). Two different approaches including planting on plastic pots with drainage holes in a green house and in the field were adopted. After 28 days of plant growth, different volumes of crude oil together with 50ml of sterile water were added to both the potted plants in the greenhouse and in the field as described by Ogbulie *et al.*, (2011). Thereafter, no additional water was added during the remaining period of the experiment.

The difference in the concentration of each pollutant in polluted soils in the green house and in the field for the remediation process was determined to show the extent of crude oil degradation by the corresponding plants and isolates. This was carried out by collecting the soil samples and subjecting them to gas chromatography.
The total petroleum hydrocarbon content (TPH) was determined using the gas chromatograph (GC) with GC recorder interfaced with a HP Pentium III computer. Soil samples from both the greenhouse and the field were collected using cellophane bags, surface sterilized using the method of Yee *et al* (1998). The analysis was carried out in three phases as i) Extraction of crude oil from soil samples; ii) Sample clean-up/ separation and iii) Gas chromatographic analysis.

The soil samples were dried, crushed and sieved using 0.5mm sieve. About 2.0g $\pm$ 0.1 of samples were weighed into a clean extraction container. Ten millilitres of pentane (extraction solvent) was added into the samples and mixed thoroughly, and allowed to settle. The mixtures were filtered into clean solvent rinsed extraction bottles using filter paper fitted into Buchner funnels. Extracts were thereafter concentrated to 2ml and transferred for separation. One millilitre of Bonny light raw crude oil used in polluting the soil samples was diluted with 10ml of solvent. The diluted sample was concentrated to 2ml and then transferred for separation.

This was carried out by placing 1cm of moderately packed glass wool at the bottom of 10mm, I.D x 250mm long chromatographic column. Then slurry of 2g activated silica gel in 10ml of methylene chloride (CH_3Cl) was prepared and placed

into the column. Sodium sulphate was also added to the top of the column and the column was rinsed with additional 10ml of methylene chloride. The column was, however, pre-eluted with 20ml of pentane by allowing it to flow through the column at a rate of about 2minutes until the liquid in the column was just above the sulphate layer. Then 1ml of the extracted sample was immediately transferred into the column and the extraction bottle rinsed with 1ml of pentane and added to the column as well. Furthermore, the stop-cock of the column was opened and the eluant collected with 10ml graduated cylinder. Prior to exposure of the sodium sulphate layer to air, pentane was added to the column in 1-2 ml increments. Then accurately measured volume of 8-10ml of the eluant was collected. This was labeled "aliphatic**".** Following recovery of aliphatic fractions, however, and just prior to exposure of the sodium sulphate layer again, the column was eluted with 1:1 mixture of acetone and methylene chloride in 1-2ml increments. Another 10ml of the eluant was accurately measured and collected again and was labeled "aromatics". The aromatic fraction was, however, concentrated to 1ml for GC analysis.

This was the last phase of the analysis and was carried out by transferring the concentrated aromatic fractions into labeled gas vials with Teflon rubber crimp caps. One microlitre of the concentrated sample was injected by means of hypodermic syringe through a rubber septum into the column. The separation then occurs as the vapour constituent partition between the gas and the liquid phase. The samples were however, automatically detected as it emerges from the column at a constant flow rate by the FID detector whose response is dependent on the composition of the vapour. The difference in the concentration of each pollutant in polluted soils in the green house and in the field for the different remediation processes was therefore determined.

All the TPH data obtained in the study were statistically subjected to analysis of variance (ANOVA) using SPSS software for Windows Evaluation Version and different comparisons were as well made using T-test.

RESULTS AND DISCUSSIONS

The consortia of microorganisms present in the soil under study (before pollution), were identified to be *Aspergillus fumigatus, Aspergillus niger, Penicillium* sp, *Candida* sp*, Pseudomonas fluorescense, Acinetobacter baumanni, Bacillus mycoides, Klebsiella* sp., *Staphylococcus aureus* and *Escherichia coli*. However after pollution, the disappearance of *S. aureus* and *E. coli* was observed (Table 1).

The result of the level of total petroleum hydrocarbon used as a marker to determine the fate of crude oil in polluted soil is as shown in Tables 2. Gas chromatographic (GC) analysis carried out revealed the total petroleum hydrocarbon (TPH) of soil polluted with 100ml. 200ml, 400ml and 800ml of crude oil for green house and field samples to be generally low on comparison with the TPH value of 10,380 kg/ mg for control sample.

Comparatively, different volumes of crude oil were used in polluting plants on soil consisting of the ten (10) indigenous microbial consortia (study soil) to ascertain variations in degradation, based on volume of crude oil and location as a determining factor. Using SPSS software, several comparisons were made with analysis of variance (ANOVA) based on the concentrations used and TPH values obtained (Figures 1- 5) and different interactions regarding the location of study were compared using T test (Fig. 6-10). The result however showed that the mean TPH of all the plants in the field (location) for 100ml, 200ml, 400ml and 800ml were significantly higher ($p>0.05$) than that planted in the green house. In other words, the results at different locations showed that the level of crude oil removed from the soil samples were higher in green house samples than the field samples. Generally, comparing the effects of individual plants per different concentrations of crude oil used per location, there were significant differences in the rates at which the plants in the green house degraded crude oil of 200ml, 400ml and 800ml level of pollution., except in 100ml level., where *T. occidentalis, M. pruriens* and *V. unguiculata* var *unguiculata* (54.75mg/kg, 57.16mg/kg and 55.66mg/kg) though similar but significantly higher ($p>0.05$) than that of *Z. mays* (12.57mg/kg). Furthermore, in 100ml level of pollution of field samples, *M. pruriens* and *V. unguiculata* var *unguiculata* (59.40mg/kg and 57.53mg/kg) shared significantly similar scores, but higher than that of *Z. mays* (50.42mg/kg) and lower ($p<0.05$) than the TPH of *T. occidentalis* (77.87mg/kg). By contrast however, for 400ml pollution level for samples in the field, the rate of uptake of crude oil by *T. occidentalis* (255.66mg/kg) and *M. pruriens* (289.98mg/kg) were different but significantly higher than those of *Z.* mays (263.24mg/kg) and *V. unguiculata* var *unguiculata* (266.97mg/kg) which had similar effects. Nevertheless, Fig 6-10 shows that in the field, *T. occidentalis* showed a much better remediation effect followed by *Z. mays*, *M. pruriens* and *V. unguiculata* var *unguiculata* in that order, whereas *Z. mays* showed better promise in the green house.

The symbiotic relationship between leguminous plants (*Vigna unguiculata* and *Mucuna pruriens*) used in this study and micro-organisms within the rhizosphere aided in the reduction of the TPH indicated by the low concentration of TPH obtained in comparison to the control sample. This supports the findings of Jussila (2006), who reported that the high aromatic tolerance of *R. galegae* and the viability of *Galega* plants with rhizobia in oil- polluted soils proved the legume system to be a promising method for the rhizoremediation of oil contaminated soil. Further analysis in this study showed that the interaction between the plants and microorganisms (rhizoremediation) encouraged crude oil removal from the treated soil faster than individual application (Ogbulie and Njoku, 2011). This collaborates the reports made by Kuiper *et al* (2001, 2004), Motoyama *et al* (2005), Jussila (2006) and Juhanson *et al* (2007) who attributed this success to the ability of these legumes to habour large numbers of bacterial and certain fungal species on their highly branched root systems. Plant root in this case was

upheld as a substitute for the tilling of soil to improve aeration and to incorporate additives, thus increasing microbial population and metabolic activities within the rhizosphere. These additives, usually termed the root exudates was reported ((Joner *et al,* 2002; Lines-Kelly, 2005; Juhanson *et al*, 2007) to contain mostly organic acids, sugars and amino acids which promotes microbial metabolic activity in the root zone and in turn enhances bioavailability of toxic compounds for microbes. This however, increases microbial density and diversity in contaminated environment. In other words, exudation of nutrients by plant roots creates a nutrient-rich environment in which microbial activity is stimulated. This study therefore corroborates with the reports made by Anderson *et al* (1996), Kuiper *et al* (2004) and Chaudhry *et al* (2005) that the success of this beneficial process depends on the composition of root exudates, rhizosphere competence of the root colonizing microbes, the plant species, root type, plant age, soil type, environmental factors and the pollutants.

The isolation of *Aspergillus fumigatus, Aspergillus niger, Penicillium* sp. and *Candida albicans* from the sample which persisted even after treatment supports the findings of Sutherland and da Silva *et al* (2003) who reported the degradation of polycyclic aromatic hydrocarbons (PAHs) by *Aspergillus niger* and *Penicillium janthinellum* among others. It also lend more weight to the studies made by Nkwelang *et al* (2008) on the diversity, abundance and succession of hydrocarbon utilizing microorganisms in the tropical soil polluted with oil sludge. They also isolated bacterial as *Pseudomonas* sp., *Bacillus* sp., *Acinetobacter* sp. and fungal genera as *Aspergillus* sp, *Penicillium* sp, *Candida* sp., *Mucor*, *Rhizopus* sp., *Sporobolomyces*. The report also showed that *Pseudomonas* sp., *Bacillus* sp., *Aspergillus* sp., and *Penicillium* sp were present in the polluted soil throughout the experimental period (Nkwelang *et al,* 2008).

Samples with non-leguminous plants (*Z. mays* – a cereal) and a vegetable crop (*T. occidentalis*) also had varying low TPH values when compared to the control sample. The varying TPH values observed could also be as a result of the impact of rhizosphere organisms on the plants which according to Campbell and Greaves (1990) has growth-stimulating and growth-inhibiting properties. It could also be as stated earlier, due in part to the exudation of certain compounds by plant roots which enhance the growth of specific pollutant-degrading microbes in the rhizosphere whose proliferation may also be non-selective (da Silva *et al*, 2006). Furthermore, Siciliano *et al* (2003) showed that the hydrocarbon-degrading potentials of rhizosphere soil increased through changes in the composition of a microbial community towards those containing the degradative gene. On the other hand, Jussila (2006) reported that some plant species seems to select and enrich microbial strains in the rhizosphere soil and this is also in line with the observations made from this study.

TABLE 1 Prevalence of isolates from soil under study before and after pollution

isolates	Before pollution	After pollution
Aspergillus fumigatus,	+	+
Aspergillus niger,	+	+
Penicillium sp,	+	+
Candida albicans,	+	+
Klebsiella sp.,	+	+
Bacillus mycoides	+	+
Pseudomonas fluorescens	+	+
Ainetobacter baumani	+	+
Staphylococcus aureus	+	-
Escherichia coli	+	-

Positive sign (+) is indicative of the presence of an organism before and after pollution; Negative sign (-) indicates no growth/ inability of the organism to survive in polluted soil.

TABLE 2. TPH concentration after rhizoremediation using indigenous isolates on different crude oil concentrations at different locations.

Plant samples/ conc in ml	**TPH values (mg/kg) at different locations**	
	Greenhouse	**Field**
100ml		
T. occidentalis	54.75	77.87
M. pruriens	57.16	59.40
V. unguiculata	55.66	57.53
Z. mays	12.57	50.42
200 ml		
T. occidentalis	109.92	125.68
M. pruriens	155.0	139.21
V. unguiculata var unguiculata	128.71	122.21
Z. mays	26.26	157.73
400ml		
T. occidentalis	325.15	255.66
M. pruriens	366.50	289.98
V. unguiculata	146.02	266.97
Z. mays	37.96	263.24
800ml		
T. occidentalis	665.67	472.03
M. pruriens	667.05	574.66
V. unguiculata	552.00	613.37
Z. mays	47.82	497.85

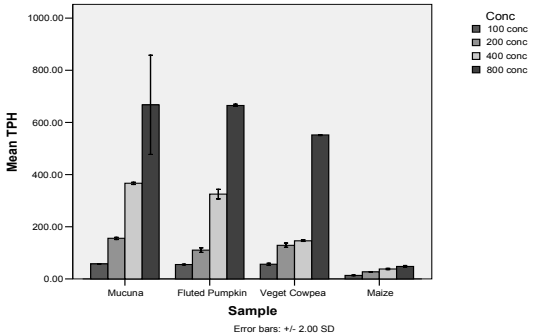

FIG 1. Comparative analysis of the effect of individual plant samples per in reducing the tph level of different concentration of crude oil during rhizoremediation in green house at 5% significant difference and 95% confident limit.

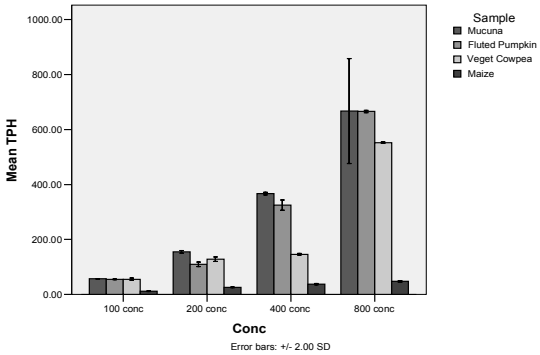

FIG 2. Comparative analysis of the effect of all the plant samples in reducing the tph level of each concentration of crude oil during rhizoremediation in green house at 5% significant difference and 95% confident limit.

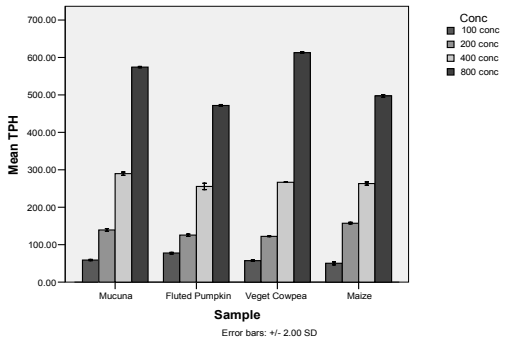

FIG 3. Comparative analysis of the effect of individual plant samples in reducing the TPH level of different concentration of crude oil during Rhizoremediation in the field at 5% significant difference and 95% confident limit.

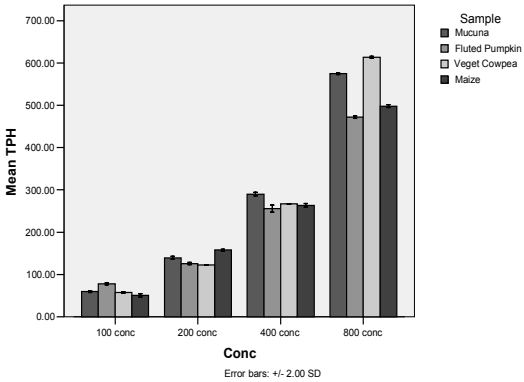

FIG 4. Comparative analysis of the effect of all the plant samples in reducing the TPH level of each concentration of crude oil during Rhizoremediation in the field at 5% significant difference and 95% confident limit.

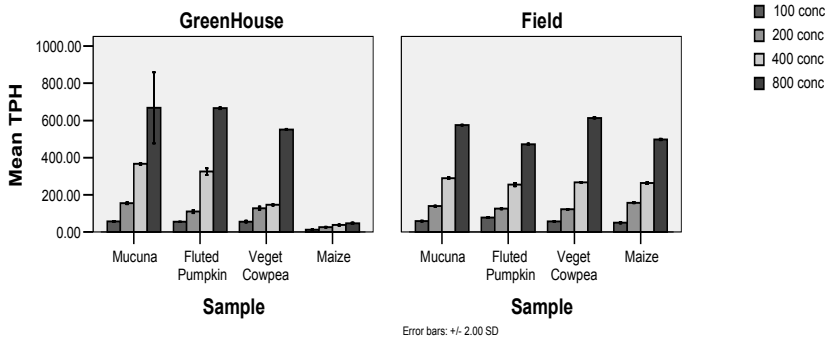

FIG 5. Ttest comparing the performance of each plant sample in Rhizoremediation studies based on location of the study.

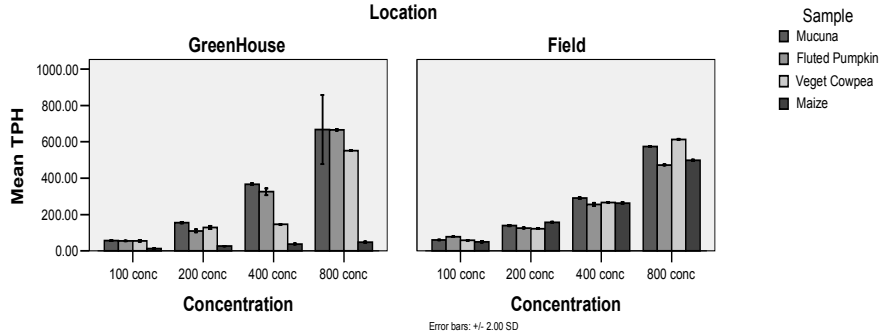

FIG 6. Ttest comparing individual volume of crude oil assimilated per plant sample in Rhizoremediation studies based on location of the study.

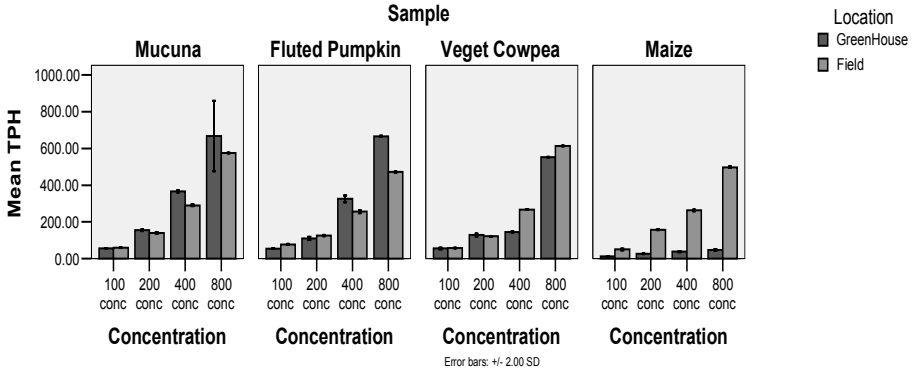

FIG 7. Ttest comparing the performance of each plant sample in Rhizoremediation studies both in the field and green house.

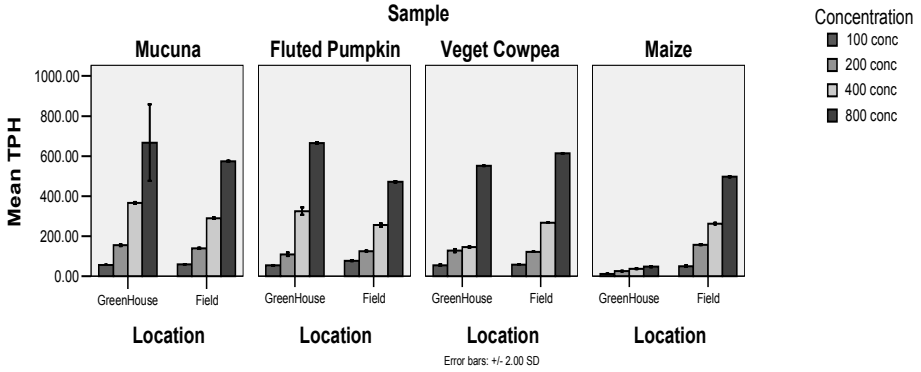

FIG 8. Ttest comparing different interactions between each study plant during Rhizoremediation studies in two locations

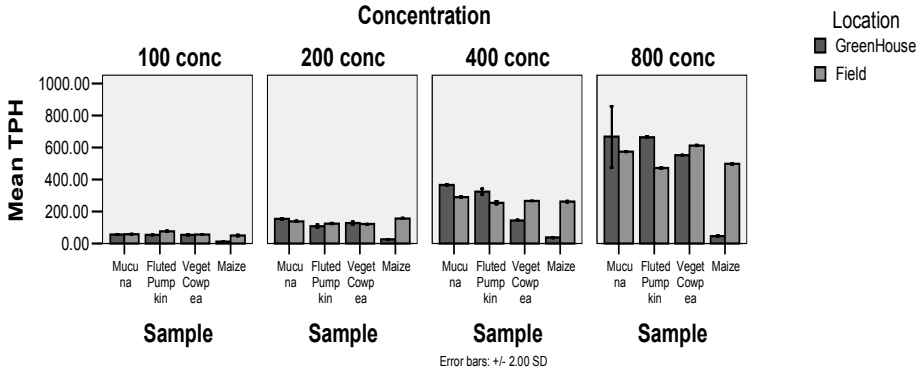

FIG 9. Ttest comparing the performance of each plant per concentration per location in Rhizoremediation process.

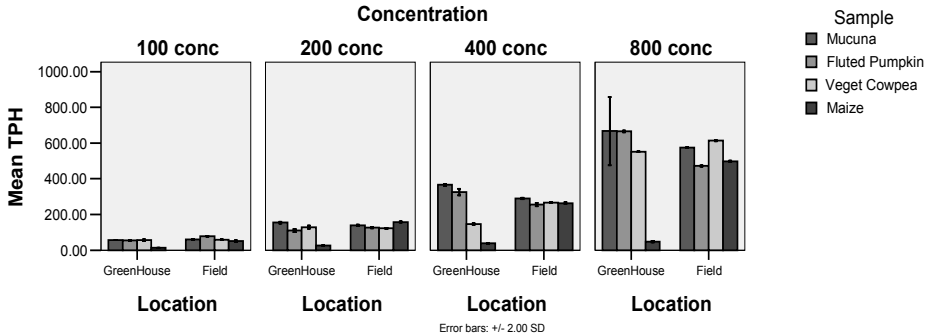

FIG 10. Ttest comparing the performance of all the study plants per concentration per location in Rhizoremediation process

CONCLUSION

Conclusively, rhizoremediation which is a plant-microbe interaction within the rhizosphere to enhance remediation appears to be an aesthetically pleasing, low-cost, minimal maintenance, *in situ* treatment for crude oil pollution in surface soil. From this study therefore, rhizoremediation has obviously shown promise, based on the use of wildtype microbes in their native environment to degrade pollutants.

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