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***Original Research Article***

# Phytoremediation of Crude Oil Contaminated Soil Using *Glycine max* (Merril); Through Phytoaccumulation or Rhizosphere Effect?

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## ABSTRACT

**The aim of this study was to evaluate the process which *Glycine max* (soybean) uses in the phytoremediation of crude oil contaminated soil. A screen house experiment was conducted with different amounts (25g, 50g and 75g) of crude oil-contaminated soil for 110 days. The initial and final total petroleum hydrocarbon (TPH) contents of the contaminated soils and that in the plant tissues were measured and the bacterial loads and types in the soil samples were determined at the end of the study. The soil pH, moisture and organic matter contents were also determined every 21 days for 110 days. Soil samples for the above analyses were obtained from the soils treated with the various amounts of crude oil with and without *G. max* (which served as the control). The investigation revealed that the initial TPH values of the soils were higher than the final TPH values and that there were lower TPH values in the soils with *G. max* compared to soils without *G. max*. The growth of *G. max* led to 52.48% reduction against 50.15% reduction in non-vegetated soil, 66.93% reduction against 44.57% reduction in non-vegetated soil and 49.04% reduction against 44.31% reduction in soil contaminated with 25g, 50g and 75g crude oil respectively The bacterial load, pH, moisture content and the organic matter contents of the crude oil contaminated soil were significantly affected by the growth of *G. max* at different levels of significance ($P<0.05$; $P<0.01$; $P<0.001$). The results of this study have shown that the growth of *G. max* on crude oil contaminated soil reduces the TPH level, enhances bacterial growth, improves the soil pH and improves the moisture content (for high level contamination). Thus, it is suggested *G. max* is a good candidate for remediating crude oil contaminated soil and that it remediates crude oil contaminated soils through rhizospheric effect.**

**Keywords: Phytoremediation, *Glycine max*, Crude oil contamination, Bacterial load, Rhizosphere effect**

## INTRODUCTION

The problems arising from petroleum exploration and exploitation can be solved through clean up activities. Common techniques involved in the cleaning up of soil contaminated sites are the physical, chemical and thermal processes (Frick et al. 1999). These techniques however have some adverse effects on the environment and are also expensive (Frick et al. 1999; Lundstedt 2003). Some of the techniques are costly while some are not environmentally friendly leaving recalcitrant by-products in the environment. Recently, biological techniques are being evaluated for the remediation of sites contaminated with petroleum. Such biological techniques are environmental friendly and can easily be applied (Efe and Okpali 2012; Njoku et al. 2012; Dada et al. 2015; Njoku et al. 2016).

Phytoremediation is one of the biological techniques for cleaning up polluted soils. It is a highly versatile, solar driven *in situ* pollutant extraction system for removal of ecosystem trembling contaminants from soil, water, sediments, and air. Phytoremediation potential has been widely accepted as highly stable and dynamic approach for reducing eco-toxic pollutants. It signifies highly perceptive and promising field of bioresources technology (Yadav et al. 2010). Among the different remediation techniques, phytoremediation is proposed to be efficient and cost-effective with high public acceptance and environmentally friendly aspects (Lambrechts et al. 2011; Pandey 2012; Zhang et al. 2012; Sinha et al. 2013). Comparing natural attenuation, bioaugmentation and phytoremediation Cai et al. (2016) reported that phytoremediation was the most efficient technique for cleaning up contaminated soil.

According to Pivetz (2001), plants for phytoremediation should be appropriate for the climate and soil conditions of the contaminated sites. Such plants should also have the ability to tolerate conditions of stress (Siciliano and Germida 1998). Frick et al. (1999) included *G. max* in the list of plants that can grow and remediate petroleum hydrocarbon contaminated sites. However, no information was available as the time of this study on the process which *G. max* uses in remediating crude oil contaminated soil.

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The overall goal of this investigation was to determine the technique used by *G. max* in remediation of crude oil contaminated soil. The impact of the growth of *G. max* on the total petroleum hydrocarbon, the bacterial load and the physico-chemistry (pH, moisture and organic matter contents) of soil polluted with crude oil would be investigated. Also, the amount total petroleum hydrocarbon accumulated in the tissues of the plant will be determined. The significance of the study will be useful in understanding whether the plant can be consumed after remediation activities. It will help to improve the economic value of phytoremediation and to evaluate the other uses of apart from its well documented nutritional value. It will also increase the databank of plants with the ability to clean up crude oil contaminated soil.

## MATERIALS AND METHODS

This study was carried out in the biological garden of the University of Lagos, Akoka, Lagos, Nigeria. The crude oil (Wellhead medium) was obtained from Shell Petroleum Development Company (SPDC) Port Harcourt, Nigeria while the *G. max* (TGX 1440-1E) was obtained from the Gene Bank Section of International Institute for Tropical Agriculture (IITA) Ibadan, Nigeria. The soil used is sandy loam soil and the treatments included 25 g, 50 g and 75 g crude oil mixed with 4000 g of the soil (to produce 0.63%, 1.25% and 1.88% w/w contamination respectively) filled in plastic containers. For each treatment, the control had no *G. max* grown on it. Both the treatments and control were replicated thrice. Seven seeds of *G. max* were sown into each container at 2 cm depth and the containers were moderately watered regularly to keep the soils moist.

Soil samples were collected at the surface and 15 cm depth from each container every 21 days (3 weeks) for 105 days (15 weeks). The soils from the surface and 15 cm depths were usually mixed together and the mixture used for the study of the effect of *G. max* on the pH, moisture and organic matter content of crude oil contaminated soil the above physic-chemical features. The soil samples were used in the study of the effect of *G. max* on the crude oil content of the soil were collected on the day of contamination (initial) and on 110$^{th}$ day of sowing of the seeds of *G. max* in the soils (final). Plant samples were collected 110 days after planting.

The total petroleum hydrocarbon level (TPH) in the soil samples was determined using air dried soils that were sieved through 1mm mesh. The TPH in the soil was first extracted with n-hexane by shaking with a mechanical shaker for 30 minutes as was described by Okolo et al. (2005). The soil-crude oil-n-hexane mixture was filtered into a beaker of known weight through a Whatmann No.1 filter paper. The TPH content of the filtrate was determined after heating the beaker at 40°C to a constant weight (Merkl et al. 2005). The amount of TPH lost from the soil was determined as the initial amount of TPH in the soil minus that in soil at the time of analysis. The TPH in the plant tissues was determined using ground air-dried tissues. The TPH in the plant tissues was extracted using n-hexane after grinding of the tissues following same steps as was in the case of soil samples and TPH content of the plant tissues was determined was as described by Merkl et al. (2005).

The bacterial load of the contaminated soil (with and without *G. max*) was estimated using the plate count method after serial dilution of 1g of each soil sample. The soil samples were aseptically collected from each container. The microbial population densities were determined using standard plate count method (Nwachukwu and Ugoji 1995). The identification of the isolated bacteria was done after series of biochemical test using the Berger's manual

The pH of the homogenized soils was determined following the protocols outlined by Eckerts and Sims (1995). The moisture content of the soil samples was determined according to the method of Schneekloth et al. (2002). The procedure of Miyazawa (2000) was used to determine the organic matter content of the soil samples. The effect of *G. max* on the TPH, the bacterial load, pH, moisture and organic matter contents of the soils was determined by comparing each parameter in soil with *G. max* with that in soil with *G. max*.

### Statistical Analyses

Statistical analyses of the data obtained were done using Graphpad Prism 5.0 package using a 2 way ANOVA followed by Bonferroni posttests at 5%, 1% and 0.1% significance level. Correlation analyses were also carried out to determine the relationship between the different parameters

## RESULTS

### The TPH levels in the soil samples and the percentage loss of TPH

The initial TPH level for each of the treatments was significantly higher than the final TPH level (P<0.001). More TPH was lost from the soil with *G. max* than in soil without *G. max* for all the concentrations. The percentage TPH lost from the soils generally decreased with increase in the amount of crude oil added to soil. However, such was not statistically significant (P>0.05). There was a negative correlation between the percentage TPH lost from the soils and the initial and final TPH levels (p = -0.262 and p = -0.554 respectively). Also the percentage of TPH lost from the soil was negatively correlated with the organic matter content (p = -0.135) but was positively correlated with the pH (p = 0.558). No TPH was observed in the plant tissues of *G. max* from any of the contaminated soils.

**Table 1.** The TPH levels in the soil and plant samples and the percentage loss of TPH.

| Amount of Crude oil Added | Initial TPH Level (mg/kg) | Final TPH Level (mg/kg) | % TPH lost | TPH in Plant Tissue |
|---|---|---|---|---|
| 25 g | 6250± 0.00 | 3115.37± 425.44 | 50.15± 6.81 | |
| 25 g + *G. max* | 6250±0.00 | 2969.80± 563.25 | 52.48± 9.01 | 0.00 |
| 50 g | 12500±0.00 | 6552.10± 211.75 | 47.57± 1.69 | |
| 50 g + *G. max* | 12500±0.00 | 4133.87± 967.40 | 66.93± 7.74 | 0.00 |
| 75 g | 18750±0.00 | 11545.00± 106.72 | 44.31± 5.84 | |
| 75 g + *G. max* | 18750± 0.00 | 9933.47± 858.37 | 49.04± 3.54 | 0.00 |

### The Bacterial Load of the Soils at the End of Study

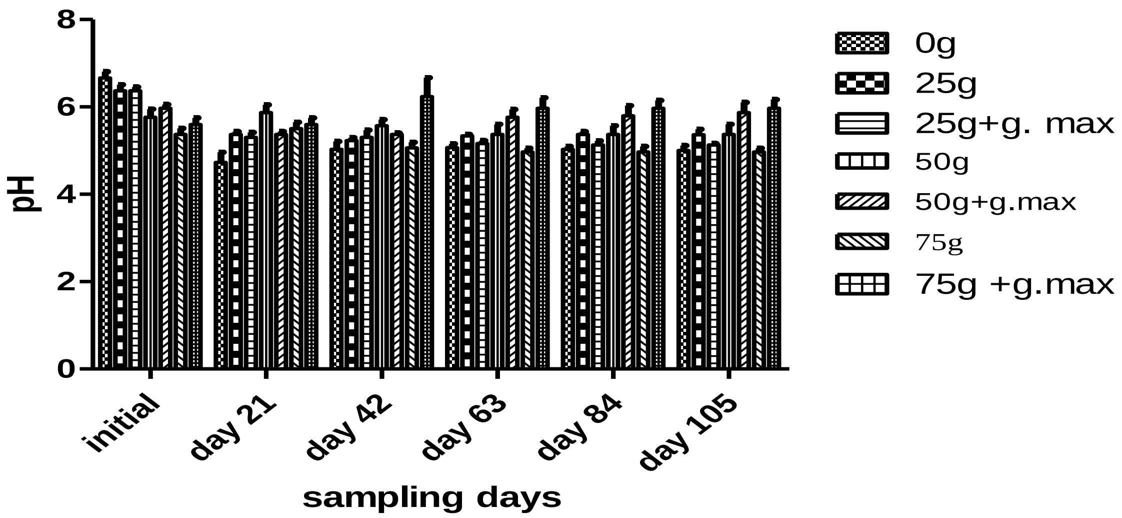
The population size and number of bacteria isolates from the soils of the different treatments are shown in table 2. The population size of the bacteria reduced with increase in the amount of crude oil added to the soil. For each amount of crude oil the growth of the *G. max* increased the population size of the bacteria. The number of isolates was highest in the soil with 50 g crude oil and no *G. max* and soil with 75 g crude oil and *G. max* (six isolates each). The least number of isolates was observed in the soil with 25 g crude oil. Some of the bacteria identified were concentration dependent

**Table 2.** The bacterial load of the crude oil contaminated soils.

| Treatment | Population size ( $x10^2$) | Number of isolate | Isolates |
|---|---|---|---|
| 25 g | 27.73 ± 0.20 | 2 | *Pseudomonas* sp, *Pseudomonas lacidororans* |
| 25 g + *G. max* | 28.00 ± 0.19 | 5 | *Shewamella* sp, *Pseudomonas* sp, *Micrococcus luteus, Acinetobacter iwoffi, Pseudomonas lacidororans* |
| 50 g | 26.80 ±0.19 | 6 | *Shewamella* sp, *Pseudomonas* sp, *Micrococcus luteus, Alcaligenes entrophis, Bacillus* sp 1, *Bacillus* sp 11 |
| 50 g + *G. max* | 27.50 ±0.14 | 5 | *Shewamella* sp, *Pseudomonas* sp, *Pseudomonas lacidororans, Achromotobacter xylosoxidans, Acinetobacter iwoffi,* |
| 75 g | 25.73± 0.74 | 5 | *Shewamella* sp, *Bacillus lincheniformis, Pseudomonas putida* 1, *Enterococcus* sp, *Psuedomonas vesicularis, Pseudomonas, putida* 11 |
| 75 g + *G. max* | 26.17 ±0.14 | 6 | *Shewamella* sp, *Psuedomonas vesicularis, Pseudomonas, putida* 11, *Pseudomonas putida* 1, *Enterococcus* sp, *Bacillus lincheniformis,* |

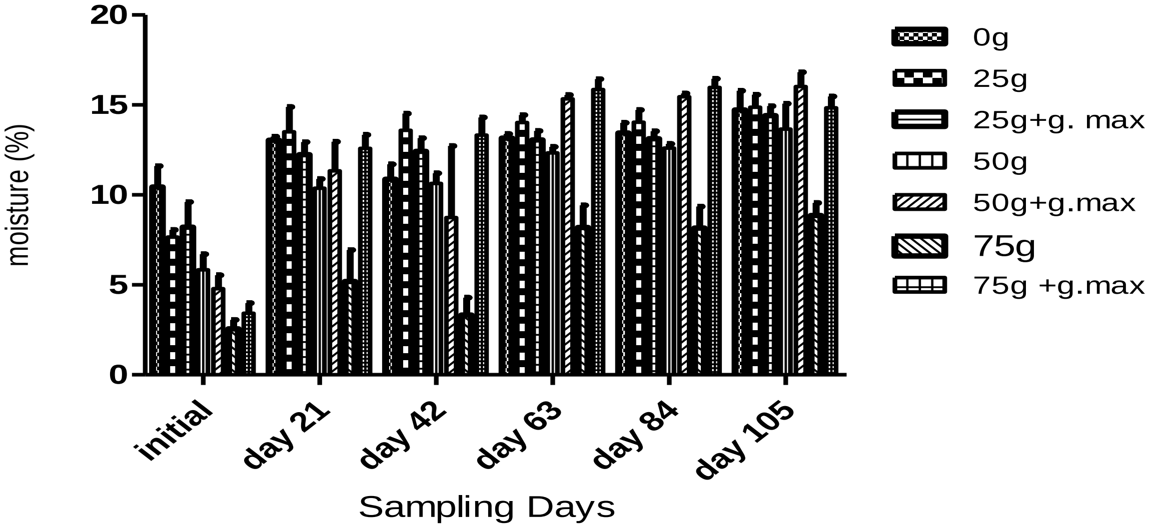
### The pH of the Contaminated Soil

The pH of the soils contaminated with the various amounts of crude shown in figure 1. In soils without *G. max*, the pH generally decreased with increase in the amount of crude oil added into the soil and the pH also reduced with increase in the study period and became steady after 63 days of study. For the soil contaminated with 25 g, the growth of *G. max* generally reduced the pH of the soil compared to the soil without *G. max*. However in the cases of soils with 50g and 75 g crude oil the growth of *G. max* generally led to increase in the soil pH. The growth of *G. max* in soil contaminated with 75 g crude oil significantly increased the soil pH ($p<0.001$) from day 42 of study (plant growth). For soils contaminated with 25 g and 50 g crude oil, growth of *G. max* had no significant effect of the soil pH ($p>0.05$). There was a positive correlation (p = 0.969) between the pH of the 25 g crude soil contaminated with *G. max* and that without *G. max*. For the soils contaminated with 50 g and 75 g crude oil, the pH of the soil with *G. max* and that of the soil without *G. max* had negative correlation (p = -0.397 for 50 g crude oil contaminated soil and P = -0.812 for 75 g crude oil contaminated soil). The pH of the soil was positively correlated with the moisture content of the soil (p = 0.714) and organic matter content of the soil (p = 0.370)



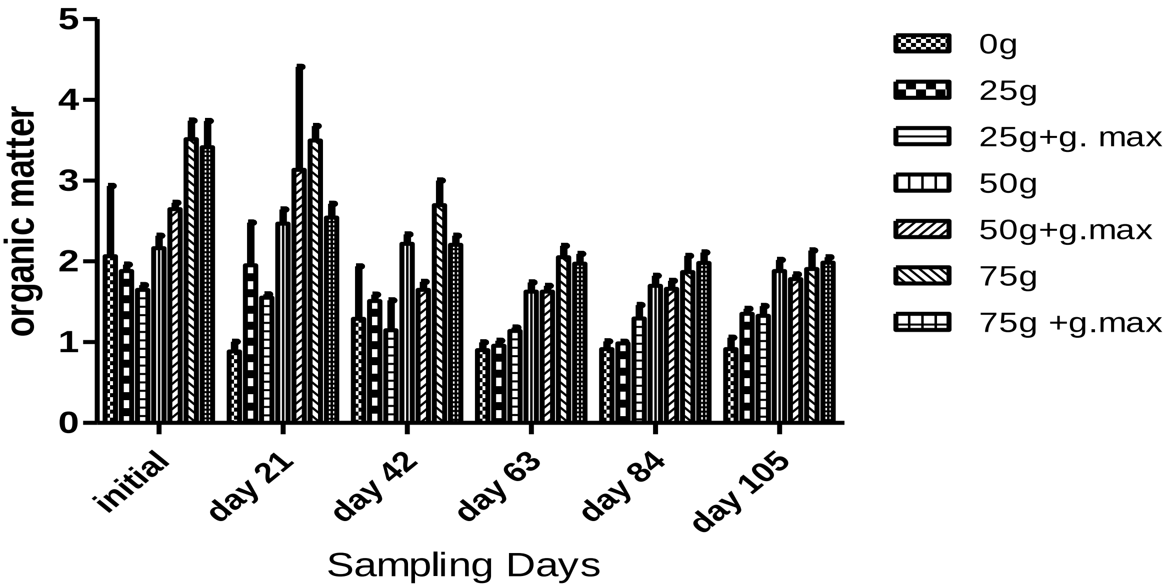

**The moisture content of soil**

The moisture content of the soils contaminated with various amounts of crude oil shown in figure 2. The moisture level decreased with increase in the amount of crude oil added to the soil and increased with the sampling days. The growth of the *G. max* in soil contaminated with 25 g crude oil generally led to reduction of the soil moisture content. The reverse was the case of the soil contaminated with 75 g crude oil where the growth of *G. max* led to significant increase of the soil moisture (($P<0.001$). For soil contaminated with 50 g crude oil, the growth of *G. max* in the first 42 days led to reduced soil moisture content after which it led to increased soil moisture level. There was a positive correlation between the moisture contents of the contaminated soils with and that of the contaminated soil without *G. max.*




**The organic matter content of Crude oil contaminated soil**

The organic matter content generally decreased with the sampling days but increased with the amount of crude oil added to the soil. Except for few cases (in soil contaminated with 50 g crude oil at days 0 and 21), the growth of *G. max* led to the reduction of the organic matter content of the soils. No significant effect of the *G. max* growth was noticed in any of the levels of contamination (P>0.05). The organic matter contents of the contaminated soils with and without *G. max* were positively correlated to each other. The organic matter content of the soil was negatively correlated with the moisture of the soil (p= -0.317).




## DISCUSSION

The result of the impact of the growth of *G. max* on the total petroleum hydrocarbon content of crude oil contaminated soil which we observed in this study showed clearly that there is a loss in the concentration of petroleum hydrocarbon in soil at the end of the experiment. This corroborates with the work of Efe and Elenwo

(2014) which revealed that *Axonopus sp*. and its associated microorganisms are capable of reducing the concentration of petroleum hydrocarbon in oil impacted soil. Similar reduction was also reported by Basumatary et al. (2012) on the effect of *Cyperus rotundus* on crude oil contaminated soil. In addition, the work of Budhadev et al. (2014) also showed that *Mimosa pudica* could decrease 31.7% of crude oil contaminants in low fertilizer level (200N, 100P, 100K) and 24.7% in high fertilizer level (240N, 120P, 120K). The findings of this study also conform to the reports of Aprill and Sims (1996) who reported that the extent of PAH disappearance was consistently greater in planted units compared to unplanted controls, indicating that phytoremediation enhances the removal of these compounds from contaminated soil. Furthermore, the work of Efe and Okpali (2012) revealed that the combined effect of *Axonopus sp.*, *Cyperus sp.* and oil amendments accounted for 59% reduction in hydrocarbon. All these show that plants are good agents for remediation of crude oil polluted soils. From this study, there is a confirmation that *G. max* has the potential of enhancing the removal of TPH from crude oil polluted soil.

Plants use different mechanisms to enhance remediation of crude oil which may be degradation, rhizospheric effect, containment and transfer of volatile components. The possible mechanism used by the *G. max* in this study to enhance the removal of TPH from the soil could be one or combination of those stated by earlier researchers. The presence of a pollutant in plant tissues used for remediation shows that such plant uses accumulation as a mechanism for cleaning up soils contaminated with such pollutants. However with the results of non-availability of the petroleum hydrocarbons in the *G. max* tissues suggests that accumulation is not a possible mechanisms used by *G. max* in remediating soils contaminated with crude oil. Hence it could have been achieved by activities outside the plant tissues. For instance, Ndimele (2010) stated that plant can have direct effect on pollutants or stimulate the rhizospheric microbes to degrade pollutant by providing them with enhanced growth conditions through exudate secretion. Plants can also provide co-metabolites needed by microbes in the degradation of petroleum. This idea can be affirmed by the non availability of petroleum hydrocarbon in the tissue of the plant which we observed in this study.

Typically, plants can stimulate microbe (bacteria and fungi) bioactivity about 10 – 100 times higher in the root zone by the secretion of bio-enhancing compounds including amino acids, carbohydrates, polysaccharides, flavonoids, and phenols. The plant-excreted root exudates facilitate soil microbes in bulk by providing a carbon and nitrogen source (Yadav et al. 2010). These could be attributed to be the cause of more bacteria cell observed in the soils with *G. max* when compared with those without *G. max*. According to Yadav et al. (2010), plants apart from secreting organic compounds which facilitate the growth and activities of rhizospheric microorganisms, also release certain enzymes capable of disintegrating organic contaminants in soils. According to Liljeroth and Baath (1998), microbial proliferation in the rhizosphere occurs in response to the input of organic compounds exuded by the roots. Plants support hydrocarbon-degrading microbes that assist in phytoremediation in the root zone through their ‘rhizosphere effects’ (Nie et al. 2009). In the view of Omotayo et al. (2012) more nutrients in soil can account for more microbial load, thus the more bacterial load in the soils with *G. max* can be linked to more nutrients available in such soils. Contaminants in soil and groundwater are mainly degraded by bacteria and fungi. Microorganisms produce natural catalysts (enzymes) which degrade organic compounds forming $CO_2$, methane ($CH_4$), water and mineral salts (ICSS 2006). The combination of the activities of plants and rhizospheric microbes therefore helps in increasing the efficiency of phytoremediation.

The higher bacterial load in soils with *G. max* compared to the soils without *G. max* could be due to the impacts have on microbial density and this is similar to the findings of Kirkpatrick et al. (2008) who reported that the presence of sudan grass resulted in significantly more total hydrogen-degrading microorganisms per pot when grown in soil with a TPH-C:TN ratio of 11:1 as compared to the control. According to Kirkpatrick et al. (2008) increased plant root growth in a crude oil-contaminated soil and a concomitant increase in petroleum-degrading microbial numbers in the rhizosphere have the potential to enhance phytoremediation. This may be one of the possible causes of higher loss of TPH from the soils with *G. max* compared to those without *G. max.* Significant improvement of microbial activities due to plant growth promotes the restoration of ecosystems (Nwaichi et al. 2015). According to ICSS (2006), the growth in the bacteria population density accelerates the degradation speed thus more TPH was observed to be lost from the soils with *G. max* against the soils without *G. max*.

Some of the bacteria we identified in this study have been reported to have the ability to degrade petroleum oil. For instance, Ezeji et al. (2007) listed that the major bacteria genera implicated in crude oil degradation in both soil and aquatic environments comprise mainly *Pseudomonas,* Omotayo et al. (2012) also showed that the following bacterial isolates are hydrocarbon utilizers; *Achromobacter, Athrobacter, Actinomycetes, Flavobacterium, Micrococcus and Nocardia. Micrococcus sp., Corynebacterium sp., Bacillus sp., Enterobacter sp., Pseudomonas sp., Alcaligenes sp., Flavobacterium sp., Moraxella sp., Aeromonas sp., Acinetobacter sp., Aspergillus sp. and Penicillium sp.* Frick et al. (1999) also listed some microorganisms which have the ability to degrade petroleum. Some of the bacteria which we identified in this study had been shown by previous studies to be hydrocarbon utilizers or that have the ability to degrade petroleum. Plants growth has also been shown the influence these positively. Therefore the higher microbial density in the soil with *G. max* compared to the soils without *G. max* could be attributed to the favouring conditions brought about by the plant growth. One of such as we observed in this study is the reduction in soils acidity.

According to Pilon-Smits (2005), the ability of plants to grow quickly is one of the factors that favour phytoremediation. The ability of plants to clean up polluted soils (media) depends largely on the bioavailability of the pollutant(s). This in turn depends on environmental conditions (moisture, oxidation state and temperature), biological activity (microbial community) and soil properties (soil organic matter and soil pH), (Pinto et al. 2014). Omotayo et al. (2012) also noted that effective degradation of crude oil would require simultaneous action of several metabolically versatile microorganisms with favourable environmental conditions such as pH, temperature and availability of nutrients. The oil-degrading ability of microorganisms in tropical soil has been reported to depend on the adequacy of certain environmental factors such as temperature, nutrients, moisture, pH, oxygen, the viscosity of oil, and coarseness of the affected soil (Antai and Mgbomo 1989; Ijah and Okang 1993).

The impact of plant growth on soil pH and the importance of soil pH on bioremediation of pollutants have been stated by some previous studies. Efe and Elenwo (2014) showed that the growth of *Axonopus sp*. in the crude oil impacted soils reduced the acidity of hydrocarbon content in soil. This conforms with the finding we observed in this study where the growth of *G. max* led to increased soil pH. The increased pH in the soil due to the growth of the *G. max* may lead to the soil conditions being better for bacterial growth. According to Sung et al. (1986) and Phung et al. (1988), bacteria thrive better in neutral condition than acidic condition hence the more bacterial load noticed in soils with *G. max* compared to soils without *G. max*. Thus it can be stated that the growth of *G. max* in crude oil contaminated soils reduces the acidity of such soil and make them better for bacterial growth and activities. This could be the reason for the positive correlation between the pH and percentage of TPH lost from the soil observed in this study.

Soil pH is an important factor that controls various physicochemical reactions. The growth and activity of soil microorganisms are very much dependent on the soil pH (Kalita and Devi 2012). The soil pH regulates the solubility, mobility, and the availability of the ionized forms of contaminants (JRB Associates Inc. 1984). While the oil may have had some direct impact in lowering the pH (Okoro et al. 2011), the growth of *G. max* and subsequent removal of TPH possibly countered the effect of crude oil on the soil pH hence more pH value for soils with *G. max* compared to soils without *G. max* as we reported in this study. The increased microbial load and decomposition activities in the vegetated soils could be the reason for the reduced pH value in some soils with *G. max* compared with those without *G. max*. This could be due to high release of acidic products. The positive correlation between the pH and the percentage of TPH lost from the soil suggests that increasing soil pH favours crude oil degradation. This is in agreement with some earlier reports that raising soil pH towards neutral favours the multiplication of hydrocarbon utilizing bacteria and thus favours bioremediation of petroleum contaminated soil

The lower moisture content in the soil with *G. max* could be due to transpiration through the leaves and greater drainage of water because of the roots penetrating and loosening the soil thereby creating pores in the soil which encourage drainage (Njoku et al. 2012). As was noted by (Njoku et al. 2014), the continuous wetting of the soil during the period of the study could be the cause of the higher moisture content of the soils at the end of

the study than at the beginning of the study. According to Ayotamuno et al. (2006), appropriate soil moisture level is a good factor for bioremediation petroleum polluted soils.
The reduction in organic matter content of the vegetated soils when compared with that in the non-vegetated soils are similar to the observations made by Ayotamuno et al. (2004) and Njoku et al. (2012). As was stated by Njoku et al. (2012), this could be as a result of organic matter removal by plants. Organic matter content should normally increase following the addition of carbonaceous substances, hydrocarbon fuels or condensates. The reduction of the organic matter content with the sampling days may indicate that significant decomposition of the petroleum hydrocarbons has taken place with different factors of decomposition enhancing the process Okoro et al. (2011). This is similar to what other researchers like Njoku et al. (2012) had reported earlier. Going by the views of Okoro et al. (2011), the general lower organic matter level in the soils with *G. max* compared to those without the plant conforms to more decomposition of crude oil taking place in the soil with *G. max* than the soil without *G. max*. This causes loss of organic matter. Both the plant and the associated microbes could have utilized the organic matter for their growth and activities leading to their lower values compared to the soils without *G.max* where there would not have been any utilization by the plant. Furthermore, as was opined by Njoku et al. (2008), organic matter is the major source of plant nutrients like phosphorus and nitrogen hence the use of such for growth and development of *G. max* during the period of the study could have led to the more reduction of the organic matter in the vegetated soil compared to the non-vegetated soil as we noticed in this study. In addition, the relationship between amount of TPH lost from the soil and the organic matter content of the soil could be due to the loss of organic carbon from the soil. Generally, the lower values of organic matter with respect to the days of sampling can also be linked to the use of the organic matter by the plants and microbes. As the days increased, the utilization increased hence the more TPH that was lost as we observed.

## CONCLUSIONS

The results obtained from this study have affirmed that *Glycine max* has the potential to reduce the concentration of hydrocarbon in crude oil impacted soil. It also showed that the growth of *G. max* in crude oil contaminated soil can influence the bacterial load, the pH, moisture content and organic matter content. From the results obtained we can infer that remediation of crude oil contaminated soil by *G. max* occurs due to the combined activities of the plant and rhizospheric microbes rather than phytoaccumulation. Further studies are recommended to understand the molecular and genetic mechanisms used by *G. max* in remediating crude oil contaminated soils

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