PLANT-ENDOPHYTIC BACTERIAL INTERACTIONS IN PHYTOREMEDIATION OF CRUDE OIL-POLLUTED SOIL

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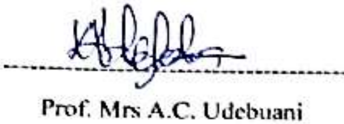
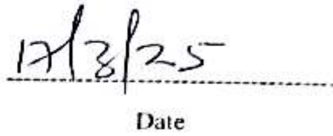
NOVEMBER, 2024

CERTIFICATION

This is to certify that this research work titled "PLANT-ENDOPHYTIC BACTERIAL INTERACTIONS IN PHYTOREMEDIATION OF CRUDE OIL-POLLUTED SOIL" was carried out by NWAJIOBI, IFEOMA JUDITH (20184141988) in partial fulfillment of the requirements for the award of Doctor of Philosophy (Ph.D.) Degree in Biotechnology in the Department of Biotechnology, School of Biological Sciences, Federal University of Technology, Owerri.

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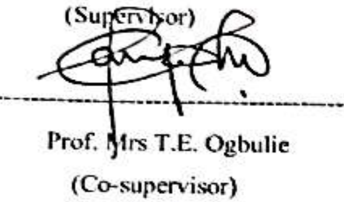

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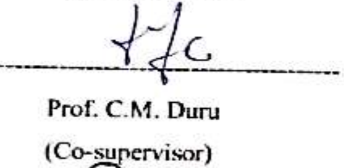
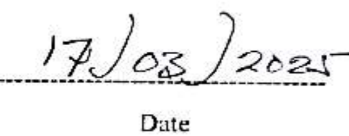

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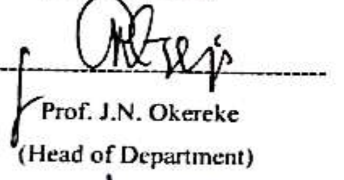
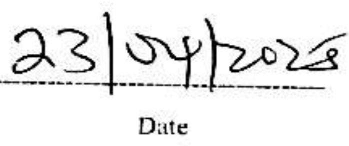

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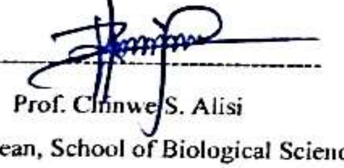
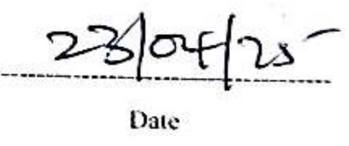

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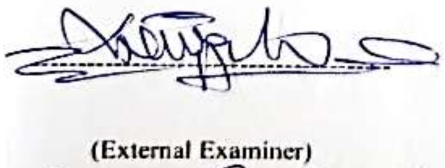
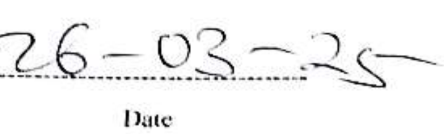

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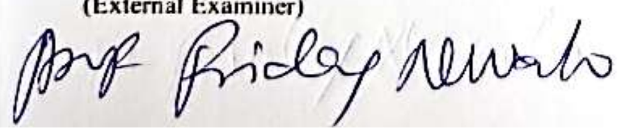

DEDICATION

This research work is dedicated to God Almighty, for His strength and support while carrying out this work and to my late father, **Hon. Augustine Nwajiobi**, who taught me never to give up.

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ABSTRACT

Endophytes reside within plant hosts asymptomatically, exhibiting continuous metabolic interactions with their hosts. The study investigated the role of plant-bacterial endophytes during phytoremediation of soil polluted with crude oil. Growth performance of *Hibiscus cannabinus* and *Zea mays* in remediation of crude oil-polluted soil samples after 90 days was analysed. Physico-chemical parameters and total petroleum hydrocarbon content of crude oil-polluted soils were analysed. Endophytic bacteria within the roots of *H. cannabinus* and *Z. mays* were isolated, tested for their bioremediation potentials and plant growth properties. Additionally, the genomic DNA of isolated endophytic bacteria were analysed for the presence of hydrocarbon degradative genes (*Alk* B genes). The 16s rRNA amplicons of gDNA of endophytic bacteria isolates were sequenced and phylogenetic tree constructed. Results of the physico-chemical parameters show the pH of the polluted soil from Egbema were acidic (4.8 to 6.6) before plant growth. Moisture content and organic carbon had mean ranges of 10.00-15.00% and 0.86-2.45% respectively. After planting using *H. cannabinus*, pH values, moisture content and organic carbon increased to a mean range value of 5.4-6.1 and 15.30-27.90% respectively while organic carbon decreased to a mean range value of 0.45-2.12%. The mean pH, moisture content and organic carbon were within the range 5.2-8.4, 5.00-28.10% and 0.24-1.84% respectively after plant growth using *Z. mays*, indicating that *Z. mays* offered greater remediation than *H. cannabinus* on oil-polluted soil. Growth performance observed during plant growth revealed that 5ml/5kg crude oil-polluted soil had the highest agronomic parameters, offering the highest crude oil remediation (46.01%) recorded using *H. cannabinus*, while 5kg Egbema-polluted soil without organic amendment showed the least remediation (4.63%). Similarly, 5ml/5kg crude oil-polluted soil showed the highest crude oil remediation (56.05%) while 5kg Egbema-polluted soil without organic amendment showed the least crude oil remediation (11.04%) after plant growth using *Z. mays.* A total of 57 endophytic bacteria were isolated from the roots of *H. cannabinus* and *Z. mays*. Six endophytic bacteria synthesized indole acetic acid (IAA), 1-aminocyclopropane-1-carboxylate (ACC) deaminase and siderophores; 14 isolates synthesized IAA and ACC; 16 isolates synthesized IAA and siderophores while 7 isolates synthesized ACC and siderophores at 10.53%, 24.56%, 28.07% and 12.28% respectively. Hydrocarbon degradation potential of isolated bacterial endophytes showed that all isolates (100%) could metabolize 2% (w/v) C_6, C_8, C_{10} and 2% (v/v) diesel as alternate sources of carbon in the absence of glucose. Moreover, genomic DNA of endophytic isolates possessed alkane monoxygenase genes responsible for hydrocarbon degradation in bacteria species. Sequenced 16s rDNA amplicons showed similarity of endophytic bacteria from the study to the genera *Proteus* and *Alcaligenes*. This study established the presence of *Proteus vulgaris*, *Proteus mirabilis* and *Alcaligenes* spp as the predominant endophytic bacteria within the roots of the phytoremediation plants *H. cannabinus* and *Z. mays.* Additionally, the presence of alkane monooxygenase genes and the ability of endophytic bacteria isolates to grow on diesel oil and n-alkanes served as an indication of the potential application of the isolates in bioremediation activities.

Key words: Endophytic bacteria, Crude oil remediation, Plant growth-promoting properties, Hydrocarbon degradative genes, *Proteus* spp.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Pollution of the environment as a result of anthropogenic activities is of major concern globally. Crude oil pollution is one of the major problems devastating even the remote areas of the world. Crude oil or petroleum is an admixture of natural hydrocarbon and polar chemical substance obtained from within earth's crust, usually under the sea (Lim, Lau, & Poh, 2016). The growing dependence on petroleum and its products have resulted in its over-exploitation, exploration and downstream processing, leading to devastating effects in the environment (Hesham, Mawad, Mostafa, & Shoreit, 2014). Often, the use of petroleum products, such as petrol, diesel and lubricating oils, have resulted in the release of various harmful chemicals, including polycyclic aromatic hydrocarbons (PAHs) and heavy metals, into the environment (Abioye, Agamuthu, & Abdul-Aziz, 2011; Hesham et al., 2014). Contaminants, such as PAHs, are not readily degradable by microorganisms within the environment due to high levels discharged or to their recalcitrance. However, certain microorganisms exists which by their associations and interactions with plants have been implicated in the degradation of these chemicals (Ma, Rajkumar, Zhang, & Freitas, 2016).

Plants are used for the removal of diverse chemical pollutants from the environment (Ma et al., 2016). This clean-up method is environmentally friendly, affordable and is a natural process of remediation. Plants absorb, degrade and compartmentalize chemical pollutants through their extensive root structure (Tiwari, Sarangi, & Thul, 2016). This cost-effective approach is less invasive and generally accepted than other methods of remediation. However, the process is slow, often extending to a period of years before removal of substantial amount of recalcitrant and persistent pollutants. Recently, microbes within plants have been harnessed for their ability to degrade and remove environmental contaminants, often as sources of carbon (Tiwari et al., 2016). Moreover, these endophytes aid host plants through the synthesis of varying phytohormones and metabolites that increase plant biomass (Ma et al., 2016). The result is an improved and more efficient remediation process.

Endophytes are microorganisms (bacterial or fungal) resident within their host plants, exhibiting continuous metabolic interactions with their hosts. Endophytic organisms exhibit varying associations with their hosts ranging from mutualistic, antagonistic and on few

occasions parasitic (Gouda, Das, Sen, Shin, & Patra, 2016). Often, there exists a symbiotic relationship between endophytes and their hosts. Plant-endophyte interactions play significant roles in the biosynthesis of diverse secondary metabolites, improve their host plants' ability to tolerate abiotic and biotic stresses, and enhance the resistance of plants to insects and pests (Chitara, Chauhan, & Singh, 2021). They also produce phytohormones including auxins, gibbrellins, and other bioactive compounds of biotechnological interest such as enzymes and drugs/drug precursors (Bhardwaj, Sharma, Jadon, & Agarwal, 2015). Alternatively, endophytes depend on their hosts for important biomolecules and nutrients necessary for normal biological activities to complete their life cycle (Chitara et al., 2021).

Bacterial endophytes have been reported to reside within most phytoremediation plants. They contain unique degradative genes which, in addition to those of the host plants, aid environmental remediation process (Hesham et al., 2014; Tiwari et al., 2016). Certain bacteria in the genera *Actinetobacter*, *Pseudomonas*, *Bacillus*, *Proteobacteria*, *Microbacteria*, *Serratia*, *Staphylococcus*, *Curtobacterium*, *Arthrobacter*, *Methylobacterium*, *Burkholderia* and *Ralstonia* have been applied in the remediation processes against chemical pollutants in association with their different plant hosts (Yousaf, Andria, Reichenauer, Smalla & Sessitsch, 2010; Wu, Xu, Liu, Guo…& Wang, 2019; Wu, Li, Xu, Liu…& Xia, 2021; Wang, Chen, Li, Ma…& Jin, 2022). Moreover, some have been reported to synthesize diverse plant growth-promoting substances such as indole acetic acid, siderophores, 1-aminocyclopropane-1-carboxylate (ACC) deaminase and could demineralize phosphates (Ma et al., 2016).

Hibiscus cannabinus L. is a non-edible plant used commercially in paper production therefore the plant is ideal for remediation studies (Abioye et al., 2011). *Zea mays* L. is a plant usually consumed as food in Nigeria and other parts of the world. Maize is a fast growing crop plant and has been used for exposure-effects studies (Abedinzadeh, Etesami, & Alikhani, 2019). Studies have shown the phytoremediation activities of *H. cannabinus* and *Z. mays* on used lubricating oil (Abioye et al., 2011), atrazine, heavy metals and wastewater (Abedinzadeh et al., 2019). However, information on the possible endophytic microbes associated with the phytoremediation plants *H. cannabinus* and *Z. mays* on exposure to crude oil is limted and therefore this study seek to assay for possible endophytic bacteria from *H. cannabinus and Z. mays,* genetically characterize them, test for possible hydrocarbon degradative genes, as well as assess the remediation potentials of bacteria endophytes on normal alkanes (C_6, C_8 and C_{10}) and diesel.

1.2 Statement of the Problem

Environmental pollution by crude oil spills and its combustion products have left devastating impacts in different parts of the world. Often, petroleum hydrocarbons penetrate the soil and groundwater, posing serious health risks, such as cancer and genetic mutations, to man and other terrestrial organisms by contamination of underground drinking water sources.

Phytoremediation is an eco-friendly and cost-effective method of remediation which involves the use of plants and rhizosphere microbes. Plants, with the aid of their extensive root systems and associated microbes, are able to take-up or transform pollutants from the environment. However, the process is slow, extending over a period of years. Recently, asymptomatic associations between plants and microorganisms (endophytes) have been found to contribute to the removal of pollutants from the environment. This novel technique serves as the bridge between phytoremediation and other remediation techniques and can prove useful in the removal of recalcitrant xenobiotics present in petroleum hydrocarbons. It is therefore imperative that endophytic bacteria that aid phytoremediation be investigated and harnessed to challenge petroleum hydrocarbon pollutants in crude oil-polluted soil.

1.3 Objectives of the Study

The main objective of the study was to investigate the potentials of plant-bacterial endophytes in phytoremediation of soil polluted with crude oil.

Specific Objectives

The specific objectives of the study include to:

(i) Determine the physico-chemical properties of crude oil-polluted and unpolluted soil samples.

(ii) Determine the total petroleum hydrocarbon (TPH) content of crude oil-polluted and unpolluted soil samples before and after plant growth experiment (PGE).

(iii) Examine the growth performance of Kenaf *(Hibiscus cannabinus)* and Maize *(Zea mays)* on crude oil-polluted and unpolluted soil samples.

(iv) Isolate and identify the endophytic bacteria in the roots of *Hibiscus cannabinus* and *Zea mays*.

(v) Determine plant growth-promoting properties (indole acetic acid, siderophores, 1-aminocyclopropane-1-carboxylate (ACC) deaminase and phosphate solubilization) of the endophytic bacterial isolates from roots of *H. cannabinus* and *Z. mays*.

(vi) Determine the bioremediation potential of isolated endophytic bacteria on hydrocarbon components of crude oil (diesel, C_6, C_8 and C_{10}).

(vii) Genetically characterize endophytic bacterial isolates from roots of *H. cannabinus* and *Z. mays*.

(viii) Determine the phylogenetic relationship existing between isolated endophytic bacteria isolated from roots of *H. cannabinus* and *Z. mays* exposed to crude-oil polluted soil and other bacteria applied in bioremediation.

(ix) Extract and characterize the hydrocarbon degradative genes of endophytic bacteria isolated from roots of *H. cannabinus* and *Z. mays* exposed to crude-oil polluted soil.

1.4 Justification of the Study

Removal of crude oil spill using plants though cheaper and environmentally acceptable is limited by time taken for substantial remediation to occur. Bacterial endophytes aid host plants in effective remediation process (Tiwari et al., 2016). *Hibiscus cannabinus* and *Zea mays* are plants with recorded phytoremediation activities (Abioye et al., 2011); although information on endophytic microbial community within these plants is scanty.

(i) Outcome of this study will establish presence of endophytic bacteria in the phytoremediation plants, *Hibiscus cannabinus* and *Zea mays*.

(ii) It will highlight possible use of identified endophytic bacteria in bioaugmentation.

(iii) Profer alternative to synthetic fertilizers in agricultural activities by exploring possible plant growth promoting ability of endophytic bacteria.

(iv) Detect phylogenetic relationship between bacterial endophytes from *H. cannabinus* and *Z. mays* applied in bioremediation activities.

(v) Sensitize farmers on the possible use of endophytes in agriculture, to reduce environmental pollution caused by synthetic chemicals.

1.5 Scope of the Study

The study encompassed assessment of physico-chemical properties of crude oil-polluted soil samples prior to and after planting, plant-growth experiment to assess the effectiveness of endophytes from *H.cannabinus* and *Z. mays* in bioremediation, plant-growth promoting activity of isolated endophytes, bacterial characterization and molecular biological screening of isolated endophytes from *H.cannabinus* and *Z. mays* to determine the phylogenetic relationship among them, and to identify different degradative genes present in endophytic bacterial isolates.

CHAPTER TWO

LITERATURE REVIEW

2.1 Crude Oil in the Environment

Environmental pollution with petroleum and petrochemical products has attracted much attention in recent times. The petroleum industry is subdivided into three major segments: the upstream process or the exploration and production segment, the downstream process or the refining and marketing segment and the midstream process or the supply segment (Walls, 2010). Upstream process involves work in the oil field or oil wells, downstream process involves processing crude oil and natural gas into usable products, while midstream process involves transportation of petroleum and its refined products and machinery involved, respectively (Walls, 2010). Occasionally, operation conditions often result to contamination of surrounding environment by oil products through accidental or deliberate seepage, and regulated discharge of wastes into rivers, coastal regions, land or air (Yavari, Malakahmad, Nasiman, & Sapari, 2015).

Large scale accidental oil spills, are responsible for significant volume of contaminants in the environment, globally. In the US, two major environmental catastrophes recorded were in 1989 and 2010, by the Exxon Valdez spill in Alaska and the British Petroleum Deepwater Horizon spill around the Gulf of Mexico (Mendelssohn, Andersen, Baltz, Caffey, …& Rozas, 2012; Spier, Stringfellow, Hazen, & Conrad, 2013). The total crude oil discharged in each case was 0.75 and 4.9 million barrels of crude oil (Table 2.1), which till date significantly impacts on the surrounding productive and vulnerable marine environment (Mendelssohn et al., 2012; Spier et al., 2013). In addition to such environmental disasters, low-level continuous seepage which results in small scale spills, exploration activities offshore, tank cleanings, and similar activities are sources of toxic contaminants, leading to diverse environmental consequences (Atlas & Hazen, 2011, Yaveri et al., 2015). However, in Nigeria, major causes of oil spill are oil exploitation and pipeline vandalization by individuals or groups, seeking government attention to correct economic marginalization and ecological disaster caused by many years of unregulated crude oil exploration and exploitation by companies in the Niger Delta (Ndimele, 2010).

Table 2.1: The highest marine spills in the world

Name of oil spill	Year	Place of oil spill	Type of fuel	Amount of oil spilled (million gallons)	Clean-up cost estimated in 2010 (US Dollars)
Gulf War	January 23, 1991	Persian Gulf, Kuwait	Crude oil	240–336	$ 540 million
Deepwater Horizon	April 20, 2010	Mexican Gulf, Mexico	Crude oil	210	$ 10 billion
Ixtoc 1 oil well	June 3, 1979	Mexican Gulf, Bay of Champeche	Crude oil	140	$ 283.9 million
Atlantic Empress oil spill	July 19, 1979	Carribean Sea, off the coast of Trinidad and Tobago	Light crude oil	9	$ 187 million
Nowruz oil field	February 10, 1983	Persian Gulf, Nowruz Field Platform	Oil	80	$161.5 million
ABT Summer	May 28, 1991	Off coast of Angola, Africa	Iranian crude oil	80	$ 163.2 million
Castillo de Bellver oil spill	August 6, 1983	Table Bay; Saldanha Bay, South Africa	Light crude oil	78.5	$ 153 million
Amoco Cadiz	March 16, 1978	Brittany coast, up to Channel Islands; Portsall, France	Light Iranian and Arabian crude oil & bunker fuel	68.7	$ 136 million
M/T Haven Tanker oil spill	April 11, 1991	Mediterranean Sea; Genoa, Italy	Crude oil	45	$ 85 million
Odyssey oil spill	November 10, 1988	North Atlantic, off the coast of Nova Scotia	North Sea crude oil	43	$ 86.7 million

Source: Lim et al. (2016).

Crude oil and its refined products such as fuel oils and lubricating oils are present in oil spills. Diverse range of hydrocarbons, nitrogenous compounds, compounds of sulphur, and heavy metals are toxicants present in crude oil which could potentially lead to acute and chronic on biota in different components of the environment (Mendelssohn et al., 2012). Remediation of these contaminants and pollutions is therefore vital. Additionally, the presence of toxic hydrocarbons in petroleum with complex structures adversely affects its cleanup and recovery. Remediation of petroleum-contaminated system could be achieved by physical, chemical or biological methods. Generally, based on the nature and volume of pollution and atmospheric conditions, one or a combination of these methods is applied (Dave & Ghaly, 2011). Each technique has its own merits and demerits. The mechanical and chemical methods are often considered as primary methods for quick cleanup and prevention of the oil spreading. However, current researches focus on alternative biological methods, as a result of attendant detrimental effects of the physical and chemical techniques (Abioye et al., 2011). Plant possess a plethora of pollutant attenuation mechanisms which facilitate their use in remediating contaminated soil and water, compared to physical and chemical approaches (Kabra, Khandare, Waghmode, & Govindwar, 2012). Phytoremediation of contaminated soils offers an environmentally acceptable, cheaper, and carbon neutral method for the removal of toxicants in the environment. In contrast to other remediation techniques, phytoremediation is non-invasive and delivers intact biologically active soil, and therefore appears attractive (Ali, Sorkhoh, Salamah, Eliyas, & Radwan, 2012).

2.1.1 Pollution and Pollutants—Sources, Generation and Effects on the Terrestrial and Aquatic Ecosystems

Environmental problems have increased exponentially in recent times due to rapid growth in human population, increased demand for several household materials and industrialization. Although development is a crucial aspect of modern living and civilization, one cannot deny that along with its merits come some demerits. Demerits of development include ways it adversely affects lives and the environment at large. Environment includes abiotic (air, soil, and climate) and biotic (man, plants, lower animals, and biosphere) components (Ana, Sridhar, Mynepalli, & Asuzu, 2010).

Demerits of development mostly result in pollution. Pollution is the introduction of contaminants into the natural environment, which alter the biochemical nature of the environment (Hou, Liu, Wang, Wang, …& Franks, 2015; Baoune, Ould, Hadj-Khelil,

Pucci, …& Polti, 2018). Pollution can be caused by chemical contaminants or energy (noise, heat or light). Toxic chemicals discharged into air, water and soil get into food chain in the environment. They eventually disturb the biochemical processes in various ecosystems leading to health abnormalities, and in some cases fatality (Baoune et al., 2018).

2.1.2 Importance of Pollutant Remediation from Environmental Matrixes

Toxic effects of oil spills on soil are of great concern. This issue comprises the focus of several research groups (El-Sheshtawy, Khalil, Ahmed, & Abdallah, 2014; Kanarbik, Blinova, Sihtmäe, Künnis-Beres, & Kahru, 2014; Gao, Wang, Guo, Hu, …& Zeng, 2015). Tang et al. (2011) reported the harmful effects on earthworm, bacteria and plants at 10.57% soil petroleum contamination. At 2% crude oil contamination, mortality rate of earthworms was reported to be 90% after 7 days, while no earthworm survived at 3% and above crude oil contamination. Likewise, 1% crude oil concentration inhibited approximately 100% bacteria. Growth inhibition was recorded in maize and wheat to be 51.3% and 48.4% respectively, on exposure to 3% crude oil concentration. Similarly, high concentrations of oil were reported to inhibit root growth (Tang, Wang, Wang, Sun, & Zhou, 2011). Increase in crude oil content from 31 mg/kg to 1000mg/kg greatly reduced the survival rate of earthworms after 14 days from 80% to 33% (Hentati, Lachhab, Ayadi, & Ksibi, 2013). Additionally, study by Ramadass et al. (2015) reported that used lubricating oil resulted in total mortality of earthworms above 3.88 g/kg soil contamination. Therefore, oil spillage on soil greatly impacts on the surrounding environments. This emphasizes vital need for efficient removal of crude oil contaminants from soil.

2.2 Fate of Crude Oil Spills

Crude oil hydrocarbons are naturally occurring substances which originated from dead and decayed organic matter deep within the sea over a period of millions of years (Atlas & Hazen, 2011). Constituents of crude oil comprises of compounds with diverse solubility, volatility, and susceptibility for biodegradation. Spilled crude oil contains aliphatic, aromatic and asphaltic fractions. Aliphatic (alkanes and alkenes) and aromatic fractions are prone to degradation, while the asphaltic fraction possessing double covalent bonds, and aromatics with highly condensed rings, are less susceptible for biodegradation (Murakami, Kitamura, Nakayama, Matsuoka, & Sakaguchi, 2008). Successful remediation of a crude oil spill involves hydrocarbon characterization, prediction of short-term and long-term fate and behaviors of the spills. Occasionally, large spills that result in substantial impacts on shorelines and wetlands,

and several smaller spills, occur yearly on wetlands and rivers, leaving acute and chronic toxicological effects on flora and fauna (Mendelssohn et al., 2012).

When an oil spill occurs in aquatic environment, it encounters various compositional changes that affect its physical and toxic properties (Mendelssohn et al., 2012). Usually, the volatile components are removed by evaporation while a low percentage undergo oxidization by UV radiation in the presence of sunlight (Yavari et al., 2015).Low molecular weight components dissolve in water and are readily degraded, while few adhere to fine particles in the water and undergo sedimentation. Substantial quantities of the oil are dispersed on the surface of the water body as small droplets, which persist until final decomposition by bacteria. Often, these droplets produce water-in-oil emulsion (mousse), which increases tenacity of oil slick (Yavari et al., 2015).

Apart from aquatic environment, crude oil spills also occur on land. Fate and degree of damage caused by crude oil hydrocarbons on plants is dependent on soil type (sand, loam, and clay) and organic matter content of soil (Lim, Lau, & Poh, 2016). Usually, when an oil spill occurs on land, volatilizable constituents with low molecular weight and high solubility such as monocyclic aromatic hydrocarbons evaporate into the atmosphere. Straight chain alkanes C_{10}–C_{16} are biodegraded by indigenous bacteria while higher molecular weight constituents such as $>C_{20}$ alkanes, polycyclic aromatic hydrocarbons (PAHs) such as naphthalene, anthracene, phenanthrene, pyrene, and their alkylated derivatives persists and are not readily biodegraded. These high molecular weight fractions penetrate soil micropores and persist in soil matrix (Lim et al., 2016). Often, they bioaccumulate resulting in mutagenicity and carcinogenicity, hence they are organic contaminants of high concern (Kumar, Arumugam, Anandakumar, Balakrishnan, & Rajavel, 2012). Ultimately, these high molecular weight constituents are removed via volatilization, photolysis, chemical or microbial degradation (Kumar et al., 2012).

2.2.1 Environmental Impact of Crude Oil Spills

Crude oil spillage impacts on both aquatic and terrestrial ecosystems negatively (Kumar et al., 2012; Lim et al., 2016). Important resources and aquatic biota resident in rivers, lakes and wetlands are threatened by oil spills. Murakami *et al.* (2008) recorded the lethal and sub-lethal effects of petroleum hydrocarbons on fish including abnormal neuron development, genetic impairment, deformities of phenotypic traits, as well as changes in normal biological activities, such as feeding, reproduction, and migration. Seabirds are also victims of hydrocarbon spills.

About 10 mL of oil slick has been reported to affect feather microstructure of seabirds, resulting in reduced thermoregulation which could be lethal (O'Hara & Morandin, 2010). Studies have demonstrated the detrimental effects of crude oil contamination on shellfish, turtles, and coastal vertebrates, such as sea ducks and otters. (O'Hara & Morandin, 2010; Camacho, Luzardo, Boada, López-Jurado, …& Orós, 2013). Another adverse effect of aquatic crude oil spills is its effect on human health. Aquatic organisms bioaccumulate high levels of petroleum hydrocarbon fractions in their tissues, which are transferred subsequently via food chain; resulting in human nutrition sources and health challenges (Camacho et al., 2013). Bioaccumulation of polycyclic aromatic hydrocarbons (PAHs) in cockle (*Cerastoderma glaucum*), oyster (*Ostrea edulis*), noble pen shell (*Pinna nobilis*), blue mussel (*Mytilus edulis*), and turbot (*Scophthalmus maximus*) have been reported in regions with petroleum spills (León, Moreno-González, González, Martínez, …& Campillo, 2013; Xiu, Pan, & Jin, 2014).

Exposure of plants in aquatic and terrestrial areas to hydrocarbons could result to chemical and physical damages. Reduced photosynthetic activity and thermoregulation as a result of fouled plant leaves, decrease in water and nutrient uptake by plant roots, and disruption of root architecture are some negative effects of oil coating of plants (Khan, Afzal, Iqbal, & Khan, 2013). Inhibition of seed germination, reduced biomass of plants, and high plant mortality has been reported after oil spill contamination (León et al., 2013). In terrestrial environment, the physical, chemical, and biological properties of soils are negatively affected by petroleum hydrocarbon spills. These compounds penetrate macro- and micro-pores in soil and thus, limit water and air transport that would be necessary for organic matter conversion (Xiu et al., 2014).

2.2.2 Crude Oil Spills Removal Techniques

Combinations of mechanical, chemical, and biological techniques are applied for the remediation of petroleum hydrocarbons. Widely applied mechanical techniques include collection and skimming, wiping, water flushing, tilling, cutting vegetation and burning (Ndimele, 2010). Mechanical removal of oil spills is often applied as an initial approach for oil cleanup in aquatic and terrestrial environments. However, they are expensive and require the use of specialized equipment (Al-Majed, Adebayo, & Hossain, 2012), therefore other techniques are considered.

In situ burning of oil, as an alternative treatment, is applied for prompt removal of thick oil film spilled on surface water or land. However, environmental conditions limit its

application. For instance, some plant communities such as needle grasses which have high sensitivity are readily damaged or destroyed by fire (León et al., 2013). Additionally, *in situ* burning threatens human health and environmental resources due to the release of smoke and the possibility of flashback and secondary fires. Thermal desorption, an *ex situ* burning technique, is growing in popularity and use. It involves use of heat to burn, decompose, or destroy contaminants in soil leaving the mineral content of the soil intact after treatment (Xiu et al., 2014).

Sorbents are oleophilic and hydrophobic substances applied in oil spill clean-up offshore and onshore. Sorbents include inorganic minerals such as clay, zeolites, silica gel; synthetic organics such as polyurethane and polypropylene; and agricultural products such as straw, mangrove barks, kenaf (Al-Majed et al., 2012; Asadpour, Sapari, Isa, & Orji, 2014). Inorganic minerals and synthetic organic products possess high sorption capacity; however, they have low retention capacity and low decomposition, respectively. Agricultural-based sorbents are less expensive, abundant, and eco-friendly. However, their limitations include low sorption capacity and low hydrophobicity (Al-Majed et al., 2012).

Application of chemical materials such as dispersants, cleaners, demulsifiers, biosurfactants, and soil oxidizers offer alternative remediation techniques. However, contrasting ideas on their effectiveness and possible toxicological effects are strong limitations to their applications (Kang, Kim, Shin, & Kim, 2010; Ndimele, 2010). For instance, Corexit 9500A oil mixture and other dispersants are toxic to aquatic species (Chase, Edwards, Qin, Wages, …& Maul, 2013). Zheng et al. (2014) reported Corexit inhibited mitochondrial functions in mammalian cells as a result of changes in intracellular oxidative balance, thus affect human health.

Biological treatment is another clean-up technique that harness the inherent ability of microorganisms and/or plants for pollution remediation (Díaz, 2010; Khan et al., 2013). *In situ* application of this technique is less expensive and causes minimal site disruption, therefore it is environmentally friendly and has greater public acceptance (Khan et al., 2013). However, biological treatment is most effective at sites with low to medium level of contamination, over a long period of time, prior to achieving optimal remedial goals.

2.2.3 Mechanisms Involved in Biological Treatments

2.2.3.1 Bioremediation Technique using Microorganisms

Some microorganisms are able to break down petroleum hydrocarbons into less complex compounds, through enzymatic reactions to produce carbon and energy needed for growth, in a process termed biodegradation (Joutey, Bahafid, Sayel, & El-Ghachtouli, 2013). Biodegradation occurs in the presence or absence of oxygen intercellularly. Anaerobic degradation utilizes Fe, Mn, sulfate, and CO_2 as electron acceptors and is relatively slow. In both aerobic and anaerobic processes, petroleum hydrocarbons act as electron donor (Suja, Rahim, Taha, Hambali, …& Hamzah, 2014).

Petroleum hydrocarbons are complex mixtures, and as a result, a single type of microorganism with distinctive enzymes may not be able to carry out complete biodegradation. A plethora of species of bacteria, archaea, and fungi are involved in the biodegradation process, as well as indigenous microbial populations (Joutey et al., 2013). Often, complete petroleum hydrocarbon degradation involves synergy between diverse microorganisms. Increased microbial activities result in improved hydrocarbon removal efficacy from a spill site, and involve various physicochemical pre-treatments (Murakami et al., 2008; Baoune et al., 2018). Application of ozone and UV radiation, chemical solvents such as acetone, oxidation, and thermal treatments have been reported to be effective in improving diffusion rate and subsequent bioavailability of contaminants in media (Ishak & Malakahmad, 2013). However, the limitations of these techniques include formation of toxic chemical residues, increased cost, and energy consumption. Alternatively, hydrocarbon degradation rate can be improved by introducing indigenous or well-adapted microorganisms to prevailing native microbes in oil contaminated soil (Khan et al., 2013). This approach, termed bioaugmentation, has several limitations for optimal operations including biotic and abiotic factors, migration and niche competition with autochthonous microorganisms (Khan et al., 2013).

In another approach, referred to as biostimulation, oxygen and essential nutrients are supplied to the contaminated site to stimulate microbial metabolic activities (Roy, Baruah, Borah, Singh, …& Bora, 2014). Nutrients such as nitrogen, phosphorus, sulfur, iron are required in proper concentrations for incorporation into microbial biomass. High nutrient application rates, however, can result in ammonia toxicity and/or eutrophication and algal growth, particularly when inorganic fertilizers are applied. Increased aeration of contaminated

sites can be achieved by techniques such as bioventing, land farming, and composting to maintain oxygen supply (Roy et al., 2014).

2.2.3.2 Bioremediation Technique using Plants

Plants possess different techniques for removal and/or degradation of organic hydrocarbons from impacted soils. Although few degradation processes occur directly in plant tissues, most degradation by plants result from rhizoremediation, which involves complex association of roots, root exudates, rhizosphere, and microbes (Cai, Zhou, Peng, & Li, 2010; Ndimele, Kumolu-Johnson, & Anetekhai, 2011; Khan et al., 2013). Specific physiology and biochemistry of plant roots, in association with the activity of rhizospheric microbes, aid plant metabolic systems remediate toxic xenobiotics.

The ability of plants to remediate is clearer, because they possess more than 100 million miles of roots per acre which offers great potential for remediating large areas of surface and depth contamination (Cai et al., 2010). In addition to root system of higher plants, diverse communities of metabolically active microorganisms exist in the soil. Microbial population density around the roots of plants is significantly higher than that of root-free soil environment due to the unique conducive habitats in the rhizospheric zone. About 40% of the photosynthetic products of plants are released as root exudates into the soil as sugars, organic acids, and aromatic compounds, rich in carbon resources for microbial growth (Khan et al., 2013). Release of root exudates initiates the chemotactic response of soil microbes towards plant roots, resulting in root colonization, which subsequently augment microbial population growth and activity for organic pollutant degradation (Cai *et al.,* 2010). Plant roots penetrate into the soil and improve soil structure, thus enhancing oxidative degradation of hydrocarbons by providing oxygen for rhizospheric microorganisms. End products of rhizospheric degradation by microbes include alcohol, acids, carbon dioxide, and water, which are less toxic than their parent compounds, and are readily assimilated (Khan et al., 2013).

In addition to promoting microbial activities, plants also discharge enzymes from roots such as dehalogenase, nitroreductase, peroxidase, and laccase that play beneficial roles in decreasing organic pollutants (Cai et al., 2010). They aid transformation of petroleum hydrocarbons by catalyzing physio-chemical reactions, as well as reducing availability of the contaminants, by binding them in the rhizospheric zone or into soil organic matter, termed phytostabilization. Little information is available on the direct uptake of hydrocarbons by roots (phytoextraction) and their subsequent sequestration within plants tissue. Only few

hydrocarbons can be taken up by plants from the soil, as a result of their high molecular weight and log $K_{ow}>4$, i.e., equilibrium constant that indicates component sorption onto soil. Hydrocarbons may experience different fates when absorbed by plant roots. Those that have low molecular weight are released into the atmosphere through transpiration processes (phytovolatilization), while non-volatilizable compounds are either sequestered in root tissues via enzymatic modification or stored in the vacuole or on plant cell walls (phytoaccumulation) (Cai et al., 2010).

2.3 Phytoremediation

Phytoremediation, otherwise known as plant-assisted bioremediation is the process of soil remediation, involving removal of toxic contaminants using plants and their roots (Lim et al., 2016). It has been defined as the use of green plants and their associated microorganisms, soil amendments and agronomic processes to remove, contain or render harmless environmental xenobiotics (Kumar et al., 2012). Plants breakdown contaminants due to the presence of root enzymes that can chemically modify and degrade these contaminants. Additionally, presence of readily degradable organic substances such as root exudates and mucilage secreted by plant root caps enhance degradation of oil contaminants.Several recent reported studies using phytoremediation are summarized in Table 2.2.

Successful implementation of phytoremediation is dependent on many factors. One of which is the addition of fertilizer as an extra nutritional source to the microorganisms and plants. Therefore, the contaminated site must be optimally fertilized to support plant growth and maximize microbial population, as well as to limit excessive fertilizer additions, which could be detrimental to the environment and also disrupt soil salinity balance. According to Ribeiro et al. (2014), choice of nutrient additions may differ among plant species, as the type of nutrient added to *Phragmites australis,* showed little to no increase in its biomass. Alternatively, Jagtap et al. (2014) observed that diesel degradation rate using *Pinus densiflora, Thuja orientalis*, and *Populus tomentiglandulosa* in 6000 mg/kg diesel contaminated soil increased from 36.9% to 75.2%, when amended with fertilizer, regardless of fertilizer concentrations added.

Table 2.2: Plants applied in phytoremediation of oil contaminated soil

Plant type	Plant species	Contaminant	Maximum removal efficiency (%)
Ornamental plant	*Mirabilis jalapa* L.	Petroleum	63.2%
Legumes, grasses	*Calopogonium mucunoides, Centrosema brasilianum, Stylosanthes capitala, Brachiaria brizantha, Cyperus aggregatus, Eleusine indica*	Crude oil	57.69% (*Eleusine indica*)
Scots pine, poplar, grass mixture, legume mixture	*Pinus sylvestris, Populus deltoides × Wettsteinii*, redfescue, *Festuca rubra, Poa pratensis, Lolium perenne,* white clover, *Trifolium repens, Pisum sativum*	Diesel	67–74% (Legume mixture)
Ditch reed, alfalfa	*Phragmites australis, Medicago sativa*	Bitumen	82% (Reed)
Non-edible plant	*Jatropha curcas*	Lubricating oil	67.3%
Crop plant, wild grasses, legume	*Triticum aestivum* L., *Secale cereale* L., *Avena sativa* L., *Hordeum vulgare, Sorghum bicolor* L. *Moench, Panicum miliaceum* L.*, Zea mays* L., *Lolium perenne* L., *Bromopsis inermis, Agropyron cristatum* L., *Agropyron tenerum* L., *Festuca pratensis* Huds., *Medicago sativa* L., *Trifolium pratense* L., *Onobrychis antasiatica* Khin.	Oil sludge	52% (Rye)
Non-edible plant	*Hibiscus cannabinus*	Lubricating oil	91.8%
Willow stand	*Salix viminalis* L.	Mineral oil & PAH	57%
Tall rescue plant	*Festuca arundinacea*	Petroleum	50%

Soybean/greenbean, sunflower/Indian mustard, mixed grasses/maize, mixed clover	*Glycine max, Phaseolus vulgaris, Helianthus annuus, Brassica juncea, Zea mays,* red clover, *Trifolium pratense*/ladino clover, *Trifolium repens*	Motor oil	100% (Mixed clover)
Alfalfa, reed	*Medicago sativa, Phragmites australis*	Bitumen	82%
Tropical pasture grass	*Brachiaria brizantha*	Crude oil	18.4%
Endophyte infected and non-infected grasses	*Festuca arundinacea* Schreb., *Festuca pratensis* Huds.	Petroleum	72% (Infected endophytic grasses)
Non-edible plants	*Dracaena reflexa* amended with 5% organic wastes	Diesel	99%
Perennial ryegrass	*Lolium perenne* L.	Diesel	57.3%
Salt marsh plants	*P. australis*	Arabian light crude oil	16%
--	*Pinus densiflora, Thuja orientalis,* and *Populus tomentiglandulosa* amended with microbialconsortium	Diesel	86.8%
Black mangrove	*Avicennia schaueriana*	Light paraffin oil	87%
Herbaceous bush	*Bassia scoparia* (L.) A. J. Scott (Chenopodiaceae)	Crude oil	Up to 57.7%

Source: Lim et al. (2016).

In laboratory-scale studies, different types of plants are being researched on for their remediation potential for oil contaminated soils. Cook and Hesterberg (2013), noted that large number of phytoremediation plants were trees and grasses, having extensive root system and greater biomass needed for rhizoremediation. For instance, Moreira et al. (2013) used *Avicennia schaueriana*, a black mangrove plant for paraffin oil remediation from 32.2 mg/g concentration of petroleum contaminated soil. After 90 days of phytoremediation, it was reported that use of *A. schaueriana* aided 87% oil removal efficiency, due to increased microbial concentrations. Moreover, use of endophyte infected grass (*Festuca arundinacea* and *Festuca pratensis*) showed 72% petroleum removal after 7 months of remediation. Endophyte infected grass was chosen in this case, as a result of its extensive root structure and shoot biomass compared to non-endophyte infected grass (Soleimani, Afyuni, Hajabbasi, Nourbakhsh, …& Christensen, 2010).

2.4 Phytoremediation Techniques

Successful phytoremediation of soil contaminated with hydrocarbons is conceptualized to occur through four different processes, namely: phytostabilization, phytodegradation, phytovolatilization and rhizodegradation (Lim et al., 2016).

2.4.1. Phytostabilization

Phytostabilization involves contaminant immobilization within the rhizospheric region, limiting hydrocarbon migration from polluted soil via erosion, leaching or spreading. Absorption and accumulation of petroleum hydrocarbons occur within the root zone, root membrane and root cells, therefore phytostabilization involves an extensive root system. Efficiency of *Salix viminalis* to remediate hydrocarbon-contaminated soils has been recorded as well as the effect of phytostabilization in low and medium petroleum contaminated soils (Lim et al., 2016). Presence of *S. viminalis* resulted in increased petroleum concentrations in rhizospheric soil, from 584 mg/kg to 1018 mg/kg after 10 h, indicating the immobilization of contaminants within the root zone. This shows that phytostabilization could reduce the pollutant concentration in the soil by containing hydrocarbon fractions in the soil.

2.4.2. Phytodegradation

In phytodegradation, petroleum hydrocarbons are broken down via metabolic activities of plants. Plants possess inherent ability to synthesize some enzymes such as dehalogenase,

nitroreductase, and laccase, which when released, catalyze pollutant degradation processes. Phytoremediation study carried out on diesel contaminated subarctic soil, showed low concentrations of diesel-range compounds in grass roots (up to 10 g/kg of dry plant tissue), while no legume roots extracts had diesel compounds. Ndimele et al. (2010) demonstrated the ability of whole and cut leaves and roots of plants to assimilate n-alkanes and liberate $^{14}CO_2$. The general pathway of conversion for alkanes in plants is summarized as:

$$\text{n-alkane} \rightarrow \text{Primary alcohols} \rightarrow \text{Fatty acids} \rightarrow \text{Acetyl-CoA} \rightarrow \text{various compounds}$$

2.4.3. Phytovolatilization

Phytovolatilization involves the absorption of petroleum hydrocarbons via plant roots, its metabolization and translocation in modified form through the plant and subsequent volatilization from the surfaces of the plant. However, this remediation method is only applicable for volatilizable oil compounds, such as trichloroethylene, and naphthalene (Kumar et al., 2012; Lim et al., 2016). This phenomenon was observed by Wiltse et al. (1998), who reported a leaf burn sensation in alfalfa plants growing in petroleum contaminated soil, which was attributed to the translocation of an unknown compound through the stem and leaves. Further study observed that this phenomenon disappeared through the progress of the experiment, indicating phytovolatilization as responsible for this effect.

2.4.4. Rhizodegradation

Rhizodegradation involves degradation of the petroleum contaminants through improved microbial activity in the rhizospheric region of soil, which is approximately 1–5 mm from the soil surface. This is a symbiotic relationship where the microorganisms supply the necessary vitamins, amino acids, and cytokinins to increase plant growth, while the plant roots provide habitat for hydrocarbon degrading microorganisms (Qixing, Zhang, Zhineng, & Weitao, 2011). Rhizodegradation is integral in phytoremediation. Muratova *et al.* (2008) observed an increase in the amount of microbial degraders in the rhizosphere from 2.4×10^6 CFU/g to 1.4×10^7 CFU/g and 4.3×10^6 CFU/g in soil using reed and alfalfa respectively. Reed plant showed a higher bitumen degradation of 82% when compared to alfalfa, having 74% degradation. Similarly, Agamuthu et al. (2010) demonstrated the presence of a large amount of hydrocarbon utilizing bacteria in the rhizospheric zone of *Jatropha curcas* at 2.4×10^7 CFU/g soil, suggesting that rhizodegradation was responsible for the phytoremediation of the oil contaminated soil. Moubasher et al. (2015) also observed that the herbaceous bush that readily grows in different soil types, *Bassia scoparia* (L.) improved remediation of petroleum contaminated sandy soil.

High tolerance of *B. scoparia* to 2–3 wt% petroleum contamination resulted in improved petroleum degradation to 57.7% after 5 months.

2.5 Phytoremediation of Crude oil in Different Environmental Matrixes

2.5.1 Phytoremediation of Crude Oil in Polluted Soils

The most common plant species used in phytoremediation of organic compounds include willows, poplar and different types of grasses. Various plants species are able to remediate petroleum hydrocarbon in polluted soils (Table 2.5).These plants can thrive to a certain degree in petroleum-contaminated soil (Yaveri et al., 2015). The four o'clock flower (*Mirabilis jalapa* L.) was demonstrated as a phytoremediator due to its tolerance for petroleum contamination. Forest tree species such as teak (*Tectona grandis*) and gmelina (*Gmelina arborea*) have also been reported to thrive in 10% w/w petroleum contaminated soil (Yaveri et al., 2015). However, higher levels of oil contamination significantly reduced biomass and height of the test plants. Branquilho (*Sebastiania commersoniana*), have also been recorded to tolerate soil petroleum contamination, with 94% crude oil removal rate. Seed germination and growth of seven species of plants, including corn (*Zea mays*), millet (*Panicum miliaceum*), sorghum (*Sorghum bicolor*), lettuce (*Lactuca sativa*), okra (*Abelmoschus esculents*), watermelon (*Citrullus lanatus*), and soybean (*Glycine max*) were observed in a laboratory study of soil contaminated with crude oil field produced water. High tolerance of sorghum, okra, millet, and corn to oil phytotoxicity was observed, compared to others (Pardue, Castle, Rodgers, & Huddleston, 2015).

Petroleum hydrocarbon bioaccumulation potential of plant is another desirable characteristic of successful phytoremediation plants. Boonsaner et al. (2011) reported high bioaccumulation levels of BTEX (benzene, toluene, ethylbenzene, and xylenes) in shoots of canna lily (*Canna indica* L.), up to 80% removal efficacy of BTEX was recorded from soil in the root zone, in 21 days. The tropical ornamental shrub, siam weed (*Chromolaena odorata* L.), demonstrated high potency for phytoaccumulation in soils polluted with crude oil and heavy metals (Ansari, Gill, Gill, Lanza, & Newman, 2014). About 80% crude oil remediation rate was observed using *C. odorata*. Physiological and morphological properties of roots in unique vegetation increase their ability to attract and harbor more microorganisms around their roots and facilitate hydrocarbon degradation (Ansari et al., 2014). Roots of mulberry (*Morus* spp.), apple (*Malus domestica*), and osage orange (*Maclura pomifera*) trees have been observed to produce flavonoids and phenolic compounds which stimulate PAH-degrading bacteria (Cai

et al., 2010). Garden balsam (*Impatiens balsamina* L.) has been reported as an ornamental plant for effective in the removal of petroleum hydrocarbon from contaminated soils (Cai et al., 2010).

In a laboratory phytoremediation study, degradation, volatilization, and bulk reduction of benzene in effluents was improved by hybrid poplar cuttings (*Populus deltoids*×*Populus nigra*) grown in flow-through reactors amended with benzene (Yaveri et al., 2015). Grasses such as annual ryegrass (*Lolium multiflorum*), bread grass (*Brachiaria brizantha*), nut grass (*Cyperus rotundus*), and mullumbimby couch (*Cyperus brevifolius*) have been identified as successful phytoremediators due to diversified, extensive, and fibrous root systems, which offer maximal root surface area. The perennial grasses, tall fescue (*Festuca arundinacea*), and perennial ryegrass (*Lolium perenne*), were applied in oil phytoremediation as a result of their extensive root systems and rapid growth on adaptation (Cook & Hesterberg, 2013).

Plants with tap root systems can penetrate deep within soil layers or the water table and remediate deeper located contaminants (Ferro, Adham, Berra, & Tsao, 2013). Deep-rooted trees such as poplars (*Populus* spp.) and willows (*Salix* spp.) have been successfully used for pollutant uptake from groundwater contaminated with petroleum hydrocarbons (Ferro et al., 2013). Poplars have also been reported to provide natural habitat for large population of oil-degrading microorganisms compared to bulk soil.

2.5.2 Phytoremediation of Crude Oil Spills in Aquatic Ecosystem

In aquatic ecosystems, such as lakes, rivers, and wetlands, different species of macrophytes thrive in or near water (emergent, submergent, or floating), that can be used as crude oil phytoremediators (Bhatia & Goyal, 2014). One advantage that makes them successful phytoremediators is their ability to grow fast. They are invasive, proliferate easily and are easily replaced with new growth after any damage caused by oil pollution (Bhatia & Goyal, 2014). Fibrous roots of selected aquatic plants provide greater surface area and denser rhizospheres for microbial colonization. Ndimele (2011) reported that fibrous root systems of water hyacinths (*Eichhornia crassipes*) successfully remediated floating petroleum hydrocarbons on surface waters. Biscuit grasses (*Paspalum vaginatum*) were also reported as able to grow in about 30 g/kg diesel-contaminated sands, thus are potential petroleum phytoremediators (Sanusi, Abdullah, & Idris, 2012). Reeds and coastal wetland plants possess strong vitality and large root surface area which aid in restoring petroleum-contaminated wetlands. Four fresh-marsh plant species, alligator weed (*Alternanthera philoxeroides*), maidencane (*Panicum*

hemitomon), common reed (*Phragmites australis*), and duck potato (*Sagittaria lancifolia*) efficiently phytoremediated South Louisiana Sweet Crude oil in polluted mesocosms (Yaveri et al., 2015).

Anaerobic degradation of crude oil usually occurs in aquatic environments, due to hypoxic and anoxic conditions of sediments or soils. Anaerobic degradation is a very slow and incomplete process. Some macrophytes are able to transport atmospheric oxygen from their shoots to their roots, thus improving aerobic respiration of rhizospheric microbes (Sanusi et al., 2012). This natural phenomenon of aquatic macrophytes make them able to oxygenate their root zones to protect themselves against phytotoxins such as Fe^{2+}, Mn^{2+}, and H_2S. Huesemann et al. (2009) reported that eelgrass (*Zostera marina*), a marine macrophyte, successfully eliminated polynuclear aromatic hydrocarbons and polychlorinated biphenyls in submerged marine sediments. Remediation processes involved include enhanced rhizospheric biodegradation via root exudates, oxygen supply, and plant enzymes. Red mangrove (*Rizophora mangle* L.) was reported to increase bacterial density in the rhizosphere ten times more than bulk sediments, via addition of oxygen into the sediments (Moreira et al., 2013).

Similarly, the aquatic weed cattails (*Typha* spp.) released elevated levels of oxygen into their rhizospheres compared to the coastal salt marsh black rushes (*Juncus roemerianus*). Difference in oxygen release intensity between the two plant species was observed to be related to the redox state of the rhizosphere (Yaveri et al., 2015). In a horizontal-vertical flow constructed wetland, cattail and bulrush (*Scirpus lacustris*) removed 99.9% of phenanthrene concentration, while black rush, a dominant coastal salt marsh plant, effectively reduced up to 15% of total petroleum hydrocarbons (TPH) concentration in contaminated sediments (Yaveri et al., 2015). In floating species, where the root system does not institute into a solid matrix, plants able to bioaccumulate and sorb pollutants from the liquid medium are considered potential phytoremediators (Rahman & Hasegawa, 2011). Generally, few studies have reported the ability of aquatic species for petroleum phytoremediation. Since most oil spills occur in aquatic environments, there is need to test the efficiency of aquatic macrophytes in phytoremediation.

2.6 Endophytes

Endophytes are microorganisms (mostly bacteria and fungi) that reside within plant tissues without showing any evidence of harm to the host, and exhibit continuous metabolic interactions with their host (White, Kingsley, Zhang, Verma, …& Kowalski, 2019a; Rao,

Kamalraj & Chelliah, 2020). The term ‘endophytes’ was coined by the German botanist, Heinrich Friedrich Link in 1809 to explain the occurrence of bacteria and fungi inside plant tissue (Verma, Kharwar & Strobel, 2009). Occurrence of microbes inside plant tissue has been reported since over 400 million years (Strobel & Daisy, 2003). Studies have established that every plant species is colonized by at least two endophytes (Kusari, Pandey & Spiteller, 2013; Wang, Wang, Wu, & Wei, 2014).

Over one hundred thousand endophytes have been reported, with more being discovered daily (Wang et al., 2014). Some endophytes live all their life cycle within their hosts, while some spend only a part of their life cycle within their host (Abreu-Tarazi, Navarrete, Andreote, Almeida, ...& Almeida, 2010). Endophytes act as reservoirs of novel bioactive secondary metabolites as a result of their microbial interactions with their hosts (Sarethy, Srivastava & Pan, 2019). Therefore, they play major roles in search for novel natural bioactive compounds, with potential use in medical practices, drug discovery, bioremediation and agriculture (Bhardwaj et al., 2015, Arora & Ramawat, 2017; Sawant, Rodrigues, & Sardessai, 2018).

2.6.1 Classification of Endophytes

Endophytic microbes can be classified by two major characteristics—time spent within their host and nature of the microorganism. Based on time spent within their host plants, endophytes are further classified as obligate and facultative endophytes. Endophytes that are vertically transmitted from parents to seedlings via seeds, or that colonize hosts as a result of activities of various host vectors are termed **obligate endophytes**. Obligate endophytes strictly rely on host plant’s metabolic activities for survival (Singh, Sharma, Kumar, Mishra, …& Kharwar, 2017). Alternatively, endophytes that live a portion of their life cycle outside host plants (biphasic) and penetrate hosts via soil rhizosphere, the atmosphere and neighbouring plants are termed **facultative endophytes** (Abreu-Tarazi et al., 2010).

Based on nature of the microorganism, endophytes can be classified into bacterial endophytes and fungal endophytes. **Bacterial endophytes** are usually applied in bioremediation, in the phytotreatment of sites polluted by crude oil, its products (diesel, petrol, lubricating oil) and its combustion products (polycyclic aromatic hydrocarbons, heavy metals). While **fungal endophytes** are applicable in medicine, for their synthesis of various unique and novel secondary metabolites useful as antibiotics, anti-fungal agents and therapeutics (Oliveira,

Gomes, Almeida, Silva, …& Cunha, 2013; Bhardwaj et al., 2015; Arora & Ramawat, 2017; Sawant, Rodrigues, & Sardessai, 2018).

Therefore, endophytic microbes comprise microorganisms originating from various sources. Endophytic microbes with larger genomes thrive readily in unstable environments, such as the soil while those with smaller genomes exist within plants, a stable environment, and are vertically transmitted (Mitter, Petric, Shin, Chain, ...& Reinhold-Hurek, 2013).

2.6.2 Method of Colonization

According to Singh *et al.* (2017), endophytes are found in different parts of the world including the Tropics, Temperate, Aquatic, Xerophytic (desert), Antarctic, Rainforest, Mangrove and coastal regions. They colonize the stems, roots, petioles, leaf segments, inflorescence of weeds, fruits, buds, seeds and dead hollow hyaline cells of plants (Goudaet al., 2016). Endophytic population in a given plant species varies, and is dependent on different components, such as host species, host developmental stage, inoculum density, genetic constituents and environmental conditions (Sharma, Pramanik & Agarwal, 2016). Endophytes are either seed transmitted or enter host plants through soil rhizosphere. Seed transmitted endophytes are passed through generations from parents to seedlings via seeds, while those that penetrate their hosts from the rhizosphere of soil enter through cracks found at the junction of lateral roots or through plant surface wounds caused by phytopathogens and later migrate into the rhizosphere. Endophytes can also penetrate hosts from root hairs and openings between epidermal cells (stomata). This is usually accompanied by their secretion of enzymes including endoglucanases, pectinases and cellulases that breakdown plant cell envelope and facilitate endorhizospheric colony. Exoglucanases aid colonization while endoglucanases breakdown high molecular weight cellulose fibres (Arora & Ramawat, 2017; Singh, Sharma, Kumar, Mishra, ...& Kharwar, 2017) and aid penetration. Cell wall degrading hydrolases including cellulases, hemicellulases, amylases and glucanases as well as lignin degradative enzymes have been found to be synthesized by a number of *Streptomyces* strains (Singh et al., 2017).

On penetration into plant host, endophytes undergo three different life stages:

a) **Neutralism/quiescent stage-** At this stage, the endophyte undergoes a latent stage or an acclimatization stage. Here, the endophyte adapts to the prevailing environmental conditions within the host; it is inactive at this stage, with no metabolic interactions with its host. Some endophytes may exist at this stage for their life time.

b) **Mutualistic stage-** At this stage, the host and the endophyte share mutual benefits from each other without any harm. This stage can be characterised by precursor molecule interaction or possible genetic recombination between the endophyte and its host.

c) **Antagonistic stage-** At this stage, the endophyte produces chemical substances that are harmful to other pathogens (antibiosis); that are competitors either to it or its host. These chemicals could also prevent attack by herbivores and other harmful conditions around hosts (Sharma, Kumar, Singh, Mishra, ...& Kharwar, 2017; Sarethy, Srivastava & Pan, 2019).

The various adaptive mechanisms aid endophytes synthesize useful bioactive compounds different from those of host plants. Microbial colonization can be detected through histological means, isolation of surface sterilized tissues and direct amplification of microbial nuclear DNA from colonized plant tissue (Singh et al., 2017).

2.6.3 Plant-endophyte Interactions and Importance

Endophytic organsims exhibit varying interactions with their host plants which could be mutualistic, antagonistic and on few occasions parasitic (Gouda et al., 2016). Often, there exists a symbiotic relationship between endophytes and their hosts. The plants benefit in terms of growth promotion and adaptability to changing environmental conditions. Plant-endophyte interactions also play significant roles in the biosynthesis of diverse secondary metabolites. They mediate interactions between host plants and their competitors, herbivores and pathogens (Elsa & Bhima, 2012), and can control food web by disrupting transfer of energy from plants to upper trophic levels. Endophytes may improve their host plants' ability to tolerate abiotic and biotic stresses, and enhance the resistance of plants to insects and pests (Kusari, Pandey & Spiteller, 2013). They also produce phytohormones including auxins, gibbrellins, and other bioactive compounds of biotechnological interest, such as enzymes and drugs/drug precursors (Bhardwaj et al., 2015).

Alternatively, endophytes depend on their hosts for important biomolecules and nutrients necessary for normal biological activities to complete their life cycles. Endophytes possess the unique ability of surviving in extremely harsh weather conditions unlike their host plants (Kaul, Gupta, Ahmed, & Dhar, 2012). This unique trait is applicable in bioremediation where endophytes aid their hosts in heavy metal uptake from metal-polluted soils.

2.7 Endophytes as Sources of Bioactive Substances

Plants and foods with high lipid content are sources of biologically active (bioactive) compounds, possessing additional nutritive properties in minute amounts (Singh et al., 2017). These compounds are products of plant-microbe interactions (endophytes), with a plethora of therapeutic activities against cancer, cardiovascular diseases, hypertension, glycaemia, thrombosis and diabetes (Atanasov, Waltenberger, Pferschy-Wenzig, Linder, ...& Uhrin, 2015; Villaescusa, Rangel-Huerta, Aguilera, & Gil, 2015). They are preferentially used as drugs these days with minimal side effects (Atanasov et al., 2015; Rao, Kamalraj & Chelliah, 2020).

Endophytes are capable of biosynthesizing new bioactive compounds different from those of host plants, as a result of possible genetic recombination and precursor molecule interactions with their host plants. These novel bioactive compounds are applied in agriculture, industry (pharmaceutical and bioremediation) and medicine. They include alkaloids, steroids, terpenoids, coumarins, peptides, polyketones, flavonoids, quinols, phenols and azadirachtin (insecticidal) (Kusari, Verma, Lamshoeft, & Spiteller, 2012).

In agriculture, these bioactive compounds are reported to enhance uptake of nutrients, fixation of atmospheric and soil nitrogen, solubility and availability of phosphate, production of plant growth hormones, in addition to inducing systemic resistance of host plants against pathogens (White et al., 2019a). While in pharmaceutical applications, these bioactive compounds serve as sources of antibiotics, anti-cancer agents, anti-viral drugs, anti-diabetic drugs and other novel compounds (Kaul et al., 2012; Sawant, Rodrigues, & Sardessai, 2018).

The current issue of multi-drug resistance (MDR) by most disease-causing pathogens, such as *Mycobacterium tuberculosis*, has prompted application of endophytes from ethno-medicinal plant origin as novel source of natural products in order to counter oxidative stress in addition to being new bioactive agents (Alvin, Kristin, Miller, & Neilan, 2014). Strobel & Daisy (2003), established that ethnobotanical profile of plants majorly in unique environmental locations, serve as rich sources of novel endophytes which synthesize unique and novel secondary metabolites. These natural products (secondary metabolites), are products of organisms in response to adverse conditions, such as presence of xenobiotics (Datta, Singh, Kumar, Dhanjal, ...& Singh, 2020), pathogenic attacks (White, Kingsley, Butterworth, Brindisi, ...& Elmore, 2019b), and changes in environmental and nutritional conditions (Alvin et al., 2014).

Endophytic fungi are mostly associated with medicinal plants. The diverse array of bioactive compounds they synthesize are exploited for curing various diseases (Kaul et al.,

2012), in agricultural practices (White et al., 2019a), production of pharmaceuticals and remediation (Sarethy, Srivastava & Pan, 2019). This is as a result of the various natural products synthesized which have unique structures and bioactivities offering a huge reservoir of bioactive compounds (Bhardwaj et al., 2015; Arora & Ramawat, 2017; Sawant, Rodrigues, & Sardessai, 2018).

2.8 Application of Endophytes

Endophytic microorganisms are applied in three major sectors—in bioremediation, in agriculture and in pharmaceuticals/medicine. Bacterial endophytes are applied in remediation of environmental contaminants including heavy metals and petroleum products, while fungal endophytes are often applied in medicine. Additionally, the two broad categories of endophytes, bacterial and fungal, secrete diverse metabolites which promote plant growth and are therefore useful in agricultural practices.

2.8.1 Endophytes in Bioremediation

Bacterial endophytes can degrade, remove and utilize petroleum hydrocarbons as carbon sources. This occurs as a result of various hydrocarbon degradative genes and plasmids therein, that aid the breakdown of polycyclic aromatic hydrocarbon (PAH) rings, resulting in n-alkane chains that are readily degradable and utilized (Yousaf, Andria, Reichenauer, Smalla, & Sessitsch, 2010; Oliveira et al., 2013). Current phytoremediation studies are focused on this area of the remediation process (Govarthanan, Mythili, Selvankumar, Kamala, …& Chang, 2016; Tiwari et al., 2016; Abedinzadeh et al., 2019; Trifi, Salem, Benzina, Fourati, …& Achouak, 2020; Chitara et al., 2021)

Bacterial endophytes also possess inherent ability which helps host plants adapt to adverse soil conditions, and improve effective phytoremediation via promotion of plant growth, metal stress alleviation, reduction of metal phytotoxicity, change in bioavailability of soil metals and metal translocation within plants (Ma, Rajkumar, Luo, & Freitas, 2011). Generally, endophytic bacteria aid phytoremediation in soils polluted with heavy metals through two major routes, the enhancement of plant growth and metal tolerance, and by altering accumulation of metals within plants tissues (Table 2.3).

Table 2.3: Endophytic bacterial applied in phytoremediation of soils contaminated with heavy metals

Bacterial endophytes	Plant host	Heavy metal	Plant-growth promoting properties	Mechanisms
Bacillus thuringiensis GDB-1	*Alnus firma*	As, Cu, Cd, Ni, Pb and Zn	Production of IAA, siderophores, ACCD and solubilization of P	Bioremoval of Pb, Zn, As, Cd, Cu and Ni in metal amended and mine tailing extract medium; Increased biomass, chlorophyll content, nodule number and metal (As, Cu, Pb, Ni, and Zn) accumulation in *A. firma*
Pseudomonas koreensis AGB-1	*Miscanthus sinensis*	As, Cd, Cu, Pb and Zn	ND	Increased plant biomass, chlorophyll, protein content, superoxide dismutase and catalase activities, and metal uptake; however decreased malondialdehyde content in plants
Staphylococcus, Curtobacterium, Bacillus, Pseudomonas, Microbacterium, Arthrobater, Leifsonia, Paenibacillus	*Alyssum bertolonii*	Ni, Co, Cr, Cu and Zn	Production of siderophores	Had an ability to colonize plant tissues
Serratia nematodiphila LRE07, *Enterobacter aerogenes* LRE17, *Enterobacter* sp. LSE04 *Acinetobacter* sp. LSE06	*Solanum nigrum* L.	Cd	Production of IAA, siderophores, ACCD and solubilization of P	Increased Cd mobilization in soils; Stimulated plant growth and influenced Cd accumulation in plant tissues; Colonized the rhizosphere soil and some colonized plant interior tissues.
Pseudomonas sp. Lk9	*Solanum nigrum*	Cd, Zn and Cu	ND	Improved soil Fe, P and heavy metal availability, shoot dry biomass and uptake of Cd, Zn and Cu.
P. monteilii PsF84, *P. plecoglossicida* PsF610	*Pelargonium graveolens*	Cr	Production of IAA and siderophores, solubilization of P	Increased plant dry biomass, essential oil yield and chlorophyll, helped Cr(VI) sequester in roots

Rahnella sp. JN6	*Polygonum pubescens*	Cd, Pb and Zn	Production of IAA, siderophores, ACCD and solubilization of P	Showed high Cd, Pb, Zn tolerance and mobilization; Promoted plant growth and Cd, Pb, Zn uptake by rape; High level of colonization in tissue interior of rapes.
Actinobacterium	*Salix caprea*	Cd and Zn	Production of siderophores and ACCD	Enhanced plant growth and metal accumulation in leaves.
Burkholderia cepacia L.S.2.4, *Herbaspirillum seropedicae* LMG2284	*Lupinus luteus* L.	Cu, Cd, Co, Ni, Pb and Zn	ND	Bioremoval of Ni, thus reduced the metal toxicity; B. cepacia L.S.2.4 increased Ni concentration in roots, while H. seropedicae LMG2284 decreased Ni concentration in roots and shoots of Lolium perenne
Pseudomonas fluorescens VI8L1, *Bacillus pumilus* VI8L2, *P. fluorescens* II8L4, *P. fluorescens* VI8R2, *Acinetobacter calcoaceticus* II2R3	*Sedum alfredii*	Zn and Cd	Production of IAA, siderophores, ACCD and solubilization of P	Decreased Cd phytotoxicity; Improved plant growth and total Cd accumulation in host plants
Serratia marcescens LKR01, *Arthrobacter* sp. LKS02, *Flavobacterium* sp. LKS03, *Chryseobacterium* sp. LKS04	*Solanum nigrum* L.	Zn, Cd, Pb and Cu	Production of IAA, siderophores and solubilization of P	Bioaccumulation or removal of metals (Cd, Zn) in both single-ion and multi-ions systems
Bacillus sp. SLS18	*Sorghum bicolor*	Cd and Mn	Production of IAA, siderophores and ACCD	Improved plant biomass production and its total metal uptake
Pseudomonas sp. A3R3	*Alyssum serpyllifolium*	Ni	Production of IAA, siderophores, ACCD and solubilization of P;	Increased the biomass of B. juncea and Ni content in A. serpyllifolium; Showed high level of colonization in tissue interior of both plant species

			Excreted cellulase and pectinase	
B. pumilus E2S2, *Bacillus* sp. E1S2, *Bacillus* sp. E4S1, *Achromobacter* sp. E4L5 and *Stenotrophomonas* sp. E1L	*Sedum plumbizincicola*	Cd, Pb and Zn	Production of IAA, siderophores, ACCD and solubilization of P	Bacterial inoculation increased water extractable Cd and Zn contents in soil; Improved plant growth and metal uptake
Methylobacterium oryzae CBMB20, *Burkholderia* sp. CBMB40	*Lycopersicon esculentum*	Ni and Cd	ND	Biosorption considerable amount of Ni and Cd, thus reduced the metal toxicity; Promoted plant growth and reduced accumulation of Ni and Cd in roots and shoots of tomato plants
Serratia sp. LRE07	*Solanum nigrum* L.	Cd, Cr, Pb, Cu and Zn	Production of IAA, siderophores and solubilization of P	Bioaccumulation or removal of metals (Cd, Zn) in both single-ion and multi-ions systems
P. fluorescens G10, *Microbacterium* G16	*Brassica napus*	Pb, Cd, Zn, Cu and Ni	Production of IAA, siderophores, ACCD	Increased water-soluble Pb in solution and Pb-added soil; Increased biomass production and total Pb uptake
Bacillus sp. MN3-4	*Alnus firma* and *B. napus*	Pb, Cd, Zn, Ni and Cu	Production of IAA and siderophores	Exhibited bioremoval of Pb; Increased root elongation of B. napus seedlings; Reduced metal phytotoxicity and increase Pb accumulation in A. firma
Endophytes belonged to Firmicutes, Actinobacteria, Proteobacteria	*Elsholtzia splendens, Commelina communis*	Cu	Production of IAA, siderophores, ACCD and arginine decarboxylase	Increased plant dry weights and Cu content in aboveground tissue of rape

Microbacterium sp. NCr-8, *Arthrobacter* sp. NCr-1, *Bacillus* sp. NCr-5, *Bacillus* sp. NCr-9 and *Kocuria* sp. NCr-3	*Noccaea caerulescens, Thlaspi perfoliatum*	Ni	Production of IAA, siderophores and ACCD	Enhanced growth and Ni translocation in plants
Serratia nematodiphila LRE07	*Solanum nigrum* L.	Cd	ND	Promoted biomass production; Increased higher photosynthetic pigments content of leaves
Rahnella sp. JN27	*Amaranthus hypochondriacus* and *A. mangostanus*	Cd	Production of IAA, siderophores, ACCD and solubilization of P	Enhanced plant growth and Cd uptake by both plant species
Acinetobacter sp. Q2BJ2, *Bacillus* sp. Q2BG1	*Commelina communis*	Pb, Cu, Cd and Ni	Production of IAA, siderophores and ACCD	Increased plant dry weights; Increased Pb contents in above-ground tissue of rape
Ralstonia sp. J1-22-2, *Pantoea agglomerans* Jp3-3, *Pseudomonas thivervalensis* Y1-3-9	*B. napus*	Cu, Pb, Cd and Ni	Production of IAA, siderophores, ACCD and solubilization of P	Increased the biomass of rape and increased Cu contents in above-ground tissues
Burkholderia sp. SaZR4, *Burkholderia* sp. SaMR10, *Sphingomonas* sp. SaMR12 and *Variovorax* sp. SaNR1	*Sedum alfredii Hance*	Cd and Zn	ND	SaMR10 had little effect on phytoextraction, while SaMR12 and SaNR1 promoted plant growth and phytoextraction of Zn and Cd; SaZR4 only promoted Zn extraction
Endophytes belonged to Firmicutes, Proteobacteria and Actinobacteria	*Pteris vittata* and *P. multifida*	As	Production of IAA	Possessed ability of both As V reduction and As III oxidation.

Key: IAA, indole-3-acetic acid; ACCD, 1-aminocyclopropane-1-carboxylate deaminase; P, phosphorus; ND, not detected.

Source: Ma et al. (2016).

2.8.1.1. Mechanisms of Plant Growth Promotion

Abiotic and biotic stressors result in loss in yield of plants. Plants have the ability to adjust their metabolism and physiology by the synthesis of diverse proteins which act in defence against stress (Hossain, Nouri, & Komatsu, 2012). Some bacteria associated with plants demonstrated ability to aid host plants avoid or partly overcome abiotic and biotic stressors (Glick, 2010; Rajkumar, Prasad, Sandhya, & Freitas, 2013). Current studies focus on endophytic bacteria beneficial in plant growth promotion and phytoremediation of soils polluted with metals and petroleum products (Ma et al., 2016; Baoune, 2018; Proksch, 2019; He, Megharaj, Wu, Suresh, & Dai, 2020). Endophytic bacteria promote plant growth through direct or indirect means (Fig. 2.1).

2.8.1.2. Direct Plant Growth Promotion

Plant growth-promoting endophytes (PGPE) such as rhizobacteria, have various means of directly aiding proliferation of their host plants. Nitrogen fixation, mineral solubilization, synthesis of phytohormones, specific enzymes and siderophores are some of the processes through which PGPE reduce metal toxicity, and enhance the development of plants. Usually one or more of these processes are employed (Pereira & Castro, 2014; Ma, Oliveira, Nai, Rajkumar, ...& Freitas, 2015). Bacterial endophytes possessing strong nitrogen-fixing (nitrogenase) activity, play key role in plant health promotion and survive readily in soils with low nitrogen content compared to other microorganisms within the rhizosphere (Montanez, Blanco, Barlocco, Beracochea, & Sicardi, 2012; Wang, Nan, Christensen, Zhang, ...& Ma, 2018). Endophytic diazotrophs offer several advantages over rhizospheric diazotrophs as a result of the beneficial host-endophyte allelopathies. For instance, the endophytic genera *Burkholderia*, *Rahnella*, *Sphingomonas* and *Acinetobacter* isolated from the stems of *Populus trichocarpa* and *Salix sitchensis* fixed atmospheric nitrogen, providing plants with sufficient nitrogen thus enhancing growth of plant under conditions of nitrogen limitation (Ma et al., 2016; Zahoor, Irshad, Rahman, Qasim, ...& Hussain, 2017). Recent study has shown that nitrogen-fixing endophytic bacteria increase nitrogen fixation rate and nitrogen accumulation in plants living in nitrogen-poor ecosystems in long-terms (Gupta, Panwar, & Jha, 2013).

Phosphorous is a macronutrient responsible for various enzymatic processes during normal functioning of plant cells. Roles, such as glucose translocation, root development stimulation, plant and endophytic bacterial growth promotion and other physiological processes, are carried out by phosphorus in plants (Ahemad, 2015). About 75% of phosphorus

applied to soils form complexes and are therefore unavailable for uptake by plants. During stress conditions as a result of accumulation of heavy metals, some metal-resistant bacterial endophytes convert precipitated phosphates in the soil into soluble forms via acidification, chelation (PO_4^{3-}), ion exchange and release of organic acid, and mineralization of organic phosphorus in soil by extracellular acid phosphatase secretion (Ahemad, 2015), thereby increasing phosphate available to plant. Endophytic bacterial biomass can assimilate soluble phosphorus, preventing its adsorption or fixation (Ma et al., 2015). They therefore serve as phosphorus sinks through rapid microbial mobilization of phosphorus, even under phosphate-limiting condition. Subsequently, these endophytes serve as sources of phosphorus to the plant hosts on their release within plant cells.

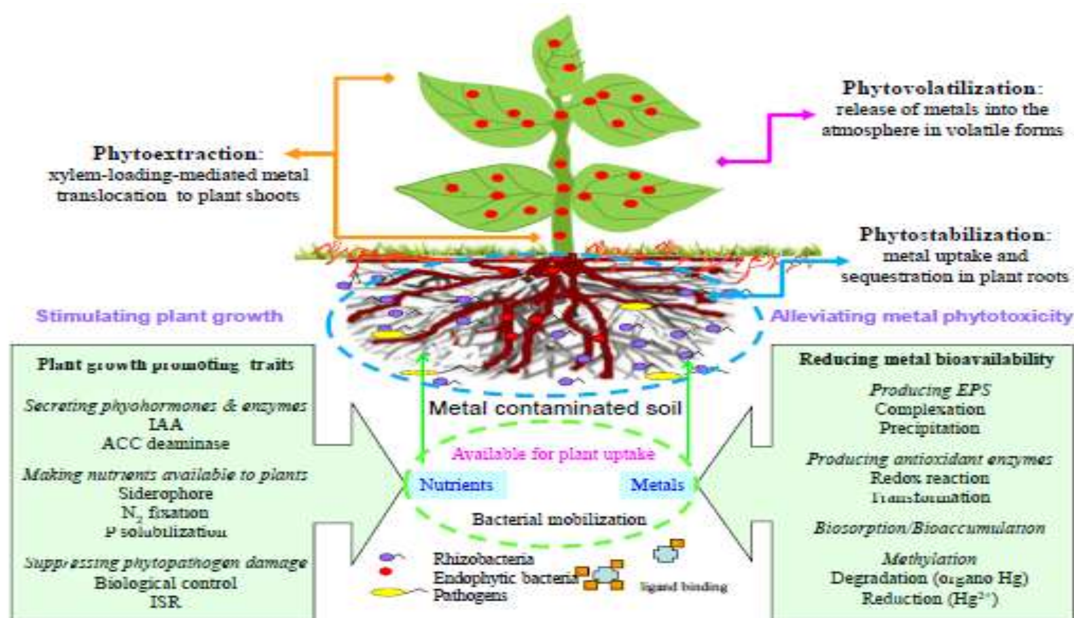


Figure 2.1: Mechanisms of plant-growth promotion in endophytic bacteria for soils contaminated with metals. Indole-3-acetic acid, IAA, 1-aminocyclopropane-1-carboxylic acid, ACC, nitrogen, N_2, phosphate, P, ISR, induced systemic resistance, ISR, extracellular polymeric substances, EPS.

Source: Ma et al. (2016).

Idriss et al. (2002) reported the benefit of extracellular phytase in aiding accessibility of phosphorus from organic compounds to plants. Maize seedlings inoculated with *Bacillus amyloliquefaciens* FZB45, a phytase secreting bacterium, exhibited significant increase in growth under phosphate limitation compared to non-inoculated controls, due to phytate-phosphate available for plant uptake. However, there is no information yet on phytase-secreting ability of bacterial endophytes.

Iron is an important mineral required by most organisms for normal physiological activities, in the form of iron-containing proteins, necessary for transpiration and as a co-factor in diverse enzymatic reactions (Ma et al., 2016). Soil iron exists as insoluble ferric ion (Fe^{3+}) in oxides, hydroxides, phosphates and carbonates which are not readily available for plant uptake. Iron chelating agents, such as siderophores, produced by microbes in plant roots, solubilize iron during iron-deficient conditions. Siderophores are organic compounds (500-1500 Da), that possess high affinity for Fe^{3+} ions. They also possess the ability to bind Fe^{2+} or other bivalent ions absorbed by plants (Rajkumar et al., 2013). Plants assimilate iron via two processes, microbial siderophores and phytosiderophores. Microbial siderophores when released, aid assimilation of iron from iron-siderophore complexes, via root mediated iron chelation (Rajkumar et al., 2013), while phytosiderophores solubilize unavailable forms of iron. Due to higher affinity of microbial siderophores for iron than phytosiderophores, plants growing in metal contaminated soils are able to accumulate high levels of iron with the aid of siderophore-producing bacteria.Therefore, bacterial siderophores, serve as major sources of phytoavailable iron in plants, during conditions of metal stress (Ma et al., 2011). Barzanti et al. (2007) reported that 83% of endophytic bacteria produce siderophores, which was induced by presence of heavy metals such as Co, Cr, Cu, Ni and Zn. In addition, reduced metal toxicity of the plant *Alyssum bertolonii* was due to increased acquisition of iron. Siderophore production by endophytic bacteria, occur as a result of iron deficiency in plant niches (Rajkumar et al., 2013). Generally, plant roots take up iron via various mechanisms including ligand exchange, directly from Fe^{3+}-siderophore complexes and/or degradation of the organic chelators such as siderophores (Ma et al., 2011).

Endophytic bacteria provide essential vitamins and growth regulators (phytohormones) to plants, thus increasing nutrients uptake and metabolism (Shi, Huang, Liu, Imran, …& Deng, 2016), by improving root growth dynamics. Studies on the role of phytohormones in plant protection from metal stress, demonstrated that endophytic colonization often increases nutrient uptake and plant biomass (Shi et al., 2016; Phetcharat & Duangpaeng, 2012). This suggests that alleviation of heavy metal stress by bacterial endophytes, is as a consequence of nutritional and biochemical benefits. Generally, five types of phytohormones are recognized, namely: indole-3-acetic acid (IAA), cytokinins, gibberellins, abscisic acid and ethylene. These are either growth inhibitors or promoters, depending on concentration of these compounds (Shi et al., 2016). Indole-3-acetic acid is an important plant auxin, involved in various physiological activities of plants, such as modification of plant development, induction of plant defense

system, and serve as a cell-cell signaling molecule (Ma et al., 2016). Downward movement of IAA establishes a concentration gradient in different parts of plant. Different concentrations of IAA in plant parts influence growth inhibition or stimulation and tissue differentiation (Persello-Cartieaux et al., 2003). Shin et al. (2016) reported that amount of IAA released by endophytes, play a vital role in modulation of plant-endophytic associations and plant development in soils contaminated with heavy metals. Moreover, endophytic bacteria beneficial to plants *in situ*, such as *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Enterobacter* and *Staphylococcus* produce phytohormones (Bacon & Hinton, 2002). However, harmful effects of the phytohormones have also been reported in maize due to significant inhibition of root elongation. Glick (2010) reported that decreased levels of IAA stimulated primary root elongation, while increased levels of bacterial IAA resulted in synthesis of lateral and adventitious roots, and reduced principal root growth. Therefore, endophytes facilitate plant development via phytohormones modification through 1-aminocyclopropane-1-carboxylate (ACC) synthesis (Persello-Cartieaux, Nussaumev, & Robaglia, 2003). Luo et al. (2011) observed that growth improvement of Cadmium hyperaccumulator, *S. nigrum* L., was induced by bacterial endophytes under cadmium stress due to synthesis of bacterial IAA and ACC deaminase.

Ethylene, a ubiquitous plant hormone, plays a vital role in plant growth and survival, in abiotic and biotic stress conditions. Ethylene is responsible for root initiation and nodule formation, cell elongation, leaf senescence, abscission and fruit ripening, in addition to translocation of auxin (Sun, Johnson, Cai, Sherameti, ...& Lou, 2010). Pathway for ethylene synthesis in higher plants is as follows: the enzyme S-adenosyl-L-methionine (SAM) synthetase catalyzes the transformation of methionine and adenosine triphosphate (ATP) to SAM; ACC synthase hydrolyzes SAM to ACC and 5-methyl thioadenosine (MTA); finally, ACC undergoes oxidization by ACC oxidase to form ethylene, carbon dioxide, and hydrogen cyanide (Glick, 2010). Heavy metal stress in plants induces ethylene production, which results in inhibited root elongation, lateral root growth and root hair formation (Viterbo, Landau, Kim, Chernin, & Chet, 2010). Endophytic bacteria alleviate stress mediated effects in plants, via enzymatic hydrolysis of ACC and subsequent reduction in ethylene synthesis by plants, during heavy metal stress conditions. The enzyme ACC deaminase, present in some bacteria and fungi, hydrolyze ACC into α-ketobutyrate and ammonia, which can be assimilated by microorganisms as nitrogen source (Viterbo et al., 2010).

2.8.1.3. Indirect Plant Growth Promotion

Most plant growth-promoting endophytes reduce plants stress through suppression of damage caused by phytopathogen (Houet al., 2015), biologically via control of pathogens or by induced systemic resistance (ISR) of plants against pathogens. Endophytic bacterial as natural biocontrol agents, have several advantages over plant growth promoting rhizobacteria (PGPR) since their interaction with plant hosts offer steady and sufficient nutrition (Hou et al., 2015; Ma et al., 2015). Most bacterial endophytes produce compounds that suppress disease-causing ability of plant pathogens, such as antibiotics, siderophores, hydrolytic enzymes such as chitinases, proteases and glucanases, and antimicrobial volatile organic compounds (VOCs) (Sheoran, Valiya, Munjal, Kundu, ...& Kumar, 2015). In most biocontrol systems, antibiotics have been reported to play significant role in suppression of diseases. The endophytic bacterial strain, *Bacillus megaterium* BP 17 and *Curtobacterium luteum* TC 10, effectively suppressed the burrowing nematode *Radopholus similis* (Thorne), via antibiotics synthesis (Aravind, Eapen, Kumar, & Ramana, 2010). Study by Bacon et al. (2012), reported that the bacterial strain *Bacillus mojavensis,* synthesized the biosurfactants C-15 surfactin that limited the maize mycotoxic fungus *Fusarium verticillioides*.

Bacterial endophytes have also been reported to be effective pathogen competitors for niche colonization and nutrient bioavailability, reducing adverse environmental impacts through nutrient loss; thus resulting in indirect increase in plant productivity (Alvin, Kristin, Miller, & Neilan, 2014). Siderophores synthesized by bacterial endophytes contain diverse chelating structural groups, such as phenolic, carboxylate and hydroxyl groups, which bind iron and other ions leading to greater plant resistance to harmful biotic and abiotic conditions (Ma et al., 2015).

Induced systemic resistance (ISR) is a state of improved resistance to a broad spectrum of pathogens at system level (Pablo et al., 2015). Induced systemic resistance (ISR) to chemically-induced diseases, as well as plant growth promoting bacteria (PGPB) is proposed to be an economically significant method in protection of the agroecosystem. ISR, induced by endophytic bacteria, has been demonstrated in many plants against different pathogens of fungal, bacterial and viral origin (Alvin et al., 2014). When plants are primed with endophytic bacteria, their defence system is induced, which the endophytes overcome in order to colonize the host. On expression of plant defense genes, ISR activates multiple potential defence mechanisms, including increased chitinases activity, β-1,3-glucanases, superoxide dismutase,

guaiacol, catalase (CAT) and peroxidases (POS) (Wan, Luo, Chen, Xiao, ...& He, 2012; Pablo, Leonard, Van, Gabriele, ...& Angela, 2015; Ma et al., 2016). Protection of cell organelles against oxidative stress, and the activity of these enzymes responsible for reactive oxygen species production may be induced as a result of the activity of bacterial endophytes (Wan et al., 2012).

2.8.2 Endophytes in Agriculture

It has been estimated that every plant contains at least two species of endophytes which could either be transmitted through seed or obtained from soil, and serve similar benefit to plants (White et al., 2019b). Endophytic microbes are often functional in that they may carry nutrients from the soil into plants, modulate plant development, increase stress tolerance of plants, suppress virulence in pathogens, increase disease resistance in plants, and suppress development of competitor plant species. Endophytic microbes have been reported to: (i) obtain nutrients in soils and transfer the nutrients to plants in the rhizophagy cycle and other nutrient-transfer symbioses; (ii) increase plant growth and development; (iii) reduce oxidative stress of their hosts; (iv) protect plants from disease; (v) deter feeding by herbivores; and (vi) suppress growth of competitor plant species. As a result of these effective functions of endophytic microbes, these symbionts could significantly reduce use of agrochemicals such as fertilizers, fungicides, insecticides, and herbicides, in the cultivation of crop plants. Endophytic microbes perform various functions in plants via various processes, depending on the microbe and plant involved.

2.8.2.1 Plant Domestication and Loss of Endophytic Microbes

Plants in natural communities maintain symbiotic associations with endophytic microbes that support growth and protect plants against biotic and abiotic stresses (Johnston-Monje, Lundberg, Lazarovits, Reis, & Raizada, 2016). Symbiotic microbes may however be lost during domestication and long-term cultivation. Long-term agrochemical use has also been reported to result in a loss of symbiotic endophytic microbes from many crop species; this led to higher incidence of diseases in these crops (White et al., 2019a). This loss of individual components of the natural endophytic community alters the functions of seed microbial community and results in seeds less capable of growth and survival than the wild type. In order to remedy the loss of essential endophytic microbes, and reduce reliance on agrochemicals in crop cultivation, there is need to reintroduce the endophytic microbes from wild relatives of crops through seed treatments (Verma, Kingsley, Bergen, English, ...& Kharwar, 2017).

2.8.2.2 Mechanisms for Endophyte-mediated Disease Suppression

Endophytes improve plant health by pathogen growth suppression and fitness (White, Kingsley, Verma, & Kowalski, 2018). Processes involved include: direct antagonism through competition with pathogens for space and nutrients; by producing antimicrobial metabolites; and through induction of systemic resistance or increasing resistance in plants against pathogens *via* upregulation of host defense genes (Hardoim, van Overbeek, Berg, Pirttilä, ...& Campisano, 2015; Irizarry & White, 2017). Studies have reported that endophytes (fungal and bacterial) provide host plants defense against pathogens and other pests, starting from germination of seed throughout the life span of the plant (Ongena & Jacques, 2008; Hardoim *et al.,* 2015; Gond, Bergen, Torres, & White, 2015). Bacterial endophytes of genus *Pseudomonas,* including *P. aeruginosa* and *P. fluorescens,* produce a variety of antifungal compounds, such as phenazine-1-carboxylic acid, 2, 4-diacetylphloroglucinol, pyrrolnitrin, pyoleutirin and volatile compounds, such as hydrogen cyanide that significantly inhibit growth of fungal pathogens (Ongena & Jacques, 2008; Mousa, Shearer, Limay-Rios, Ettinger, ...& Raizada, 2016; Bastias, Martínez-Ghersa, Ballaré, & Gundel, 2017). Species of genus *Bacillus,* synthesize a variety of biologically active molecules that are potential inhibitors of phytopathogens and thus serve as important disease control agents (Ongena & Jacques, 2008). They secrete lipopeptides that induce leakage in fungal hyphal membranes of plant pathogens reducing their virulence. This usually results in a 'quorum-quenching effect' where pathogenic fungi remain avirulent rather than causing disease. Most antifungal compounds produced by endophytes are effective against fungal membranes, inducing nutrient leakage that results in reduced virulence of the fungi (Ongena & Jacques, 2008; Mousa et al., 2016; Bastias et al., 2017).

Symbiotic endophytes improve plant resistance and protect plants against a broad spectrum of pathogens, majorly through induced systemic defense (ISR) by upregulating salicylic acid (SA), jasmonate (JA) pathways and ethylene or pathogenesis-related (PR) proteins (Bastias et al., 2017).

2.8.2.3 Endophytes alter Oxidative Stress Tolerance in Plants

Plants produce reactive oxygen species (ROS), such as superoxide ion, hydroperoxyl radicals, hydrogen peroxide, and hydroxyl radicals in response to environmental stresses (Lata, Chowdhury, Gond, & White, 2018). Release of ROS within plant tissues and cells could result in oxidative damage to plant proteins, nucleic acids, and membranes. Some endophytes are reported to induce stress tolerance to both biotic and abiotic stresses (Lata et al., 2018). During the early stages of endophytic colonization, plant defense responses are activated, producing ROS. A q-PCR analysis showed that bacteria at the early stages of colonization, caused upregulation in transcription levels of ROS-degrading genes including superoxide dismutase and glutathione reductase, that further reduced oxidative damage to plants by pathogens that induce or produce ROS (Sessitsch, Hardoim, Döring, Weilharter, ...& Woyke, 2012; Lata et al., 2018). Plants infection by fungal endophytes are reported to be responsible for higher concentrations of antioxidants (*Epichloë coenophiala*), increased tolerance to abiotic stress (*Piriformospora indica*), and drought-protective genes (*Piriformospora indica*) involved in the protection of these plants from various stressors (Sun et al., 2010; Sessitsch et al., 2012; Bastias et al., 2017).

Endophytes have also been reported to reduce oxidative stress generated in plants in soils contaminated with heavy metals (Zahoor et al., 2017). The infection of soybean by endophytic *Paecilomyces formosus* was recorded to significantly reduce lipid peroxidation, and increased production of peroxidase, polyphenol oxidase, catalase, and superoxide dismutase in Nickel-contaminated substrates (Bilal, Khan, Shahzad, Asaf, ...& Lee, 2017).

2.8.2.4 Anti-herbivory Activities of Endophytes

Some endophytes reduce herbivorous attack by insects and other herbivores by synthesizing anti-herbivory compounds within plants (Panaccione, Beaulieu & Cook, 2014; Bastias et al., 2017). Fungal endophytes species of the genus *Epichloë* (Clavicipitaceae) inhabit intercellular aerial parts of plants (leaves, culms, and seeds) and produce a variety of alkaloids that prevent feeding by herbivores. These endophytes have been applied to increase pest tolerance in commercial forage and turf grasses (Panaccione, Beaulieu & Cook, 2014; Bastias et al., 2017). However, endophytes of this group are limited to grasses and sedges. A typical example is the plant, morning glory (Convolvulaceae), which contains fungal endophytes genus *Periglandula*, known to produce ergot alkaloids that make the plant highly toxic to herbivores. Similarly, endophytic fungi of genus *Undifilum* (Pleosporaceae) contained in plants commonly referred

to as ‘locoweeds’ in the family *Fabaceae*, produce the toxic alkaloid swainsonine, a powerful anti-herbivore compound and toxin (Panaccione, Beaulieu & Cook, 2014). These examples suggest that endophytes that deter feeding by insect pests may be more common than has been currently documented. Comprehensive examination of fungal and bacterial endophytes in plants may lead to numerous additional endophytes that may be used in crops to reduce insect pest feeding or improve plant tolerance to feeding.

2.8.2.5 Transgenic Endophytes

Endophytic genomes that can modify their host transgenically may be a useful strategy and an alternative to genetic manipulation of the host plant (Li, Wu, Xing, Gao, & Zhang, 2017). Genes introduced into endophytic microbes could infer novel traits, which may be useful in bio-control of plant pathogens, growth promotion of host plants, and/or production of medicines for man or animals. For instance, the endophytic bacterium *Clavibacter xyli* subsp. *cynodontis*, that colonizes the xylem of some plant species, was modified by transgenesis to express the *Bacillus thuringiensis* gene encoding endotoxin for regulation of insects (Joutey et al., 2013; Li et al., 2017). Other examples include the endophytic *Burkholderiapyrrocinia* JK-SH007, transformed with the Bt endotoxin gene to express the insecticidal protein against stage two of *Bombyx mori* instar silkworms (Li et al., 2017); and the endophytic *Pseudomonas putida* WCS358r, transformed with an antifungal gene which on addition to wheat caused reduction of fungal populations in soil, as well as the pathogen, *Fusarium* spp (Li et al., 2017).

Future prospects in crop management may involve utilization and exploitation of transgenic modified endophytes. However, endophytic microbes are mobile; therefore limiting them to specific plants may not be easily attained or impossible.

2.8.2.6 Endobiome Interference as a Strategy to Reduce Weed Growth

Host plants and their endophytes exist in unique symbiotic relationships which can be harmful to other plants that are non-host (Bell, Hockett, Alcala-Briseno, Barbercheck, ...& Brus, 2019). Some endophytic microbes have been reported to cause growth suppression anddeath in seedlings of plants other than their adapted host (White et al., 2019b). Entry of non-adapted microbial endophytes into plant cells and tissues, resulting in plant growth repression and disruption of symbiotic activities betweenendophyte and host, is termed ‘Endobiome interference’ (White et al., 2019b). Endobiome interference is a common phenomenon in natural plant communities and might be means through which plants reduce growth of competitor plants. Endobiome interference could be applied in plant management treatment

with potential to reduce the invasive character of invasive and weedy plant species (Kowalski, Bacon, Bickford, Braun, ...& Leduc-Lapierre, 2015).

2.8.2.7 Limitations and Advances in Application of Endophytes in Agriculture

A strong limitation in the exploration and application of endophytes in crops is as a result of lack of an overall awareness of the general presence of communities of endophytic microbes in tissues of plants. The general and common assumption that most microbes on plants are either pathogenic or have non-detectable effects has led to a lack of interest in understanding the crucial roles endophytes play in plant growth promotion and health improvement. Recently, researchers have reported that endophytes are common in plants, and endophytic microbes affect plant development positively (Ricci, Tilbury, Daridon, & Sukalac, 2019). This has led to further studies to understand their usefulness and functionality.

Recent search for useful endophytes and other microbes for agricultural applications has been triggered by advent of companies with unwavering emphasis on the development and marketing of plant biostimulants including endophytes (Ricci et al., 2019). Countries such as United States of America have developed 2018 Agriculture Improvement Act (also known as the 2018 Farm Bill) for significant future advancements in products and application of endophytic microbes in agriculture.

2.8.2.8 Other Industrial Uses of Endophytes

2.8.2.8.1 As Insecticidal Agents

Synthetic insecticides, such as DDT, produce harmful by-products which are toxic, recalcitrant, and bioaccumulate in the environment. The need for safe alternative means, different from synthetic methods, has led to interest in endophytes with insecticidal properties as useful natural alternatives. Use of bioinsecticides such as peramine, a pyrrolopyrazine alkaloid, with no negative effect on mammals marked the beginning of the use insecticidal agents of endophytic origin. Peramine, isolated from the endophytic fungi *Neotyphodium coenophialum, Neotyphodium lolii, Epichole festucae* and *Epichole typhina* was discovered in the stem and leaf of tall fescue, ryegrass and other grasses (Bastias et al., 2017).

Naphthalene ($C_{10}H_8$), an insect repellent and active constituent in common mothballs, is synthesized by the fungus *Muscodor vitigenus,* which was isolated from liana (*Paullina paullinoides*). *Muscodor vitigenus*, an insect deterrent, has also shown great potency against *Cephus cinctus* (wheat stem sawfly) (Kaul et al., 2012).

2.9 *Hibiscus cannabinus* L. and *Zea mays* L.

2.9.1 *Hibiscus cannabinus*

2.9.1.1 Taxonomical Classification

H. cannabinus or kenaf (Fig. 2.2), is a cordage plant that belongs to the order *Malvales* in the family of *Malvaceae*.

Kingdom- ***Plantae*** (Plants)

Phylum- ***Tracheophyta*** (Vascular plants)

Super division- ***Spermatophyta*** (Seed plants)

Division- ***Magnoliophyta*** (Flowering plants)

Class- ***Eudicotidae*** (Dicotyledons)

Subclass-*Rosidae*

Order- ***Malvales***

Family- ***Malvaceae***

Genus- ***Hibiscus*** **L.**

Species- ***Hibiscus cannabinus*** **L.**

Plate 2.1: *Hibiscus cannabinus* L. (Kenaf)

Source: Webber & Bledsoe (2002).

2.9.1.2 Kenaf: History of Uses

Hibiscus cannabinus L., commonly known as kenaf, is a plant used since 6000 years ago mostly as cordage crop and as livestock feed. In a process known as wet-retting, the long strong bark fiber stalks are cut and tied into bundles and placed in slow moving streams or ponds to facilitate degradation and removal of the extraneous materials around the fiber strands of the bark. The fiber strands subsequently were allowed to dry and made into twine or further processed into rope or sack cloth (Fig. 2.3). The non-fibrous leafy tops are harvested as livestock feed (Webber & Bledsoe, 2002).

Plate 2.2: Kenaf fiber strands after harvesting (left) and after retting (right)

Source: Webber & Bledsoe (2002).

Production and retting of kenaf continued over thousands of years, from its first domestication in northern Africa, to subsequent introduction in India (200 years ago), Russia (1902) and China (1935) (Webber & Bledsoe, 2002). The first half of the 20th century involved research by various countries to develop improved kenaf cultivars to increase yield of kenaf bark fiber strand and ensure sufficient domestic cordage supply, including the US Department of Agriculture (USDA). The development and use of synthetic polymers for twine, rope and sacks reduced the demand for kenaf as a cordage crop. However, kenaf was selected in the early 1960s as an excellent cellulose fiber source for diverse paper products including newsprint, bond paper, and corrugated liner boards (Abioye et al., 2011). Till date, kenaf is still used in paper production.

Pulping of kenaf was reported to require less energy and processing chemicals than standard wood sources (Spaires & Kenworthy, 2001). Additionally, diverse use of kenaf has been established as a building material, adsorbent, textiles, livestock feed and fibers in new and recycled plastics (Spaires & Kenworthy, 2001; Webber & Bledsoe, 2002). In Nigeria, *H.*

cannabinus is an important species of *Hibiscus* cultivated as kitchen garden crop or used to mark farm boundaries (Falusi, 2008).

2.9.1.3 Growth Conditions

The yield and composition of kenaf can be affected by various factors including cultivar, planting date, soil fertility, photosensitivity, length of growing season, plant populations and plant maturity. Study by Falusi (2008), reported that the pink variety is usually more resistant to nematode infection than the light yellow variety. Kenaf has high crude protein content (14% to 34%) and is therefore used as livestock feed.

Typically, kenaf attains maturity 90-150 days after planting, with decrease in crude protein recorded with corresponding increase in height and maturity due to senesce of lower leaves after 90 days. Mature kenaf plants can be identified by the presence of small prickly hairs on the stem (Farusi, 2008). Introduction of organic amendment improved biomass and increased plant height of kenaf (Abioye et al., 2011) while increase in nitrogen content of soil improved plant yield (up to 168 Kg N/ha). However, very high nitrogen content resulted in reduced stalk yield (up to 224 Kg N/ha) but promoted leaf yields, while less fertile soil or less fertilizer application increased seed production (Spaires & Kenworthy, 2001). A proper nutrient balance is therefore necessary to maximize yield.

2.9.1.4 Economic Importance of Kenaf

Kenaf plant is a source of naturally occurring allelopathic chemicals that could inhibit weed seed germination and weed growth, and therefore provides a safe and cost-effective means of weed control in vegetable and agronomic crops (Spaires & Kenworthy, 2001). Research using whole-stalk kenaf as a mulch plant in vegetable production reduced weed populations and established that kenaf plant materials possess allelopathic properties. Reduced germination was recorded using extracts from stalks and leaves of kenaf from 50% to 70% in redroot pigweed (*Amaranthus retroflexus* L.) with a lesser effect on Italian ryegrass (*Lolium multiflorum* Lam.), tomato (*Lycopersicon esculentum* Mill.) and cucumber (*Cucumis sativus* L.) (Webber & Bledsoe, 2002). Kobaisy et al. (2001) analyzed the essential oil composition of the kenaf leaves and natural ability of the oil to inhibit plant (allelopathic activity) or fungal (fungicidal activity) growth. The essential oil was noted to contain 58 compounds, phytotoxic to lettuce (*Lactuca sativa* L.) and bentgrass (*Agrostis stolonifera* L.), and exhibited antifungal activity against *Colletotrichum* species (*Colletotrichum fragariae, C. gloeosporioides*, and *C. accutatum*), that cause anthracnose symptoms in plants. The major components of the essential oil included (E)-

phytol (28.16%), (Z)-phytol (8.02%), n-nonanal (5.70%), benzene acetaldehyde (4.39%), (E)-2-hexenal (3.10%), and 5-methylfurfural (3.00%). Evaluation of the leaf volatiles recorded the presence of 10 components, including ethyl alcohol, isobutyl alcohol, limonene, phellandrene, R-terpenyl acetate, citral, and four other unidentified components (Kobaisy, Tellez, Webber, Dayan, ...& Wedge, 2001).

Kenaf seeds are good source of oil (Fig. 2.4). Study by Mohamed et al. (1995), investigated the quality and quantity of oil, fatty acids, phospholipids, and sterols in seed of nine kenaf varieties, Cubano, Everglades 41, Everglades 71, GR2563, Guatemala 48, Indian, 178-18RS-10, Tainung #1, and Tainung #2. Oil content, total phospholipids and total sterol content ranged from 21.4% to 26.4% (mean, 23.7%), 3.9% to 10.3% (mean, 6.0%), 0.6% to 1.2% (mean, 0.9%) respectively. Total sterol value was similar to that reported for soybean and cottonseed oil. Palmitic, oleic, and linoleic acids were the major fatty acids with values, 20.1%, 29.2%, 45.9% total fatty acids respectively, while palmitoleic, linolenic, and stearic acids, containing 1.6%, 0.7%, 3.5% total fatty acids respectively, were the minor components. The long (C_{22}–C_{24}) and middle (C_{12}–C_{14}) chain fatty acids contents were less than 1% of the total phospholipids. Sphingomyelin, phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl serine, phosphatidyl inositol, lysophosphatidyl choline, phosphatidyl glycerol, phosphatidic acid, and cardiolipin were identified in the nine cultivars and contained 4.42%, 12.8%, 21.9%, 2.9%, 2.7%, 5.3%, 8.9%, 4.9%, 3.6% total phospholipids respectively. Phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl glycerol were the predominant phospholipids. The high oil content of kenaf seed and its similarity with cotton seed oil indicate its possible use as edible oil for human consumption. In addition to its longer shell life, the sterol emulsions could be used to reduce hypercholesterolemia. Variations among cultivars also indicate high potentials for genetic improvement in oil yield and quality.

Plate 2.3: Kenaf seed and oil extract.

Source: Webber & Bledsoe (2002).

2.9.1.5 Advances and Future Prospects

The use of kenaf has diversified from its use in cordage applications to that of a multipurpose crop with various harvestable components (bark and core of stalks, leaves, and seeds) utilized in paper production, building materials, absorbents, textiles, and as livestock feed. Recent studies have shown its potential economic and environmental benefits in the areas in phytoremediation of soil contaminated with crude oil and heavy metals (Spaires & Kenworthy, 2001; Abioye et al., 2011), as an absorbent material for toxic waste cleanup and removal of oil spills on water and as a plant-based milk alternative (Karim, Noh, Ibrahim, Ibadullah, & Saari, 2020). Kenaf also offers reduced chemicals and energy use for paper production, greater recycled paper quality, reduced soil erosion due to wind and water, replacement or reduced use of fiberglass in industrial products and increased use of recycled plastics.

2.9.2 *Zea mays*

2.9.2.1 Taxonomical Classification

Zea mays commonly called maize or corn (Fig. 2.5), is a crop plant that belongs to the order *Poales* in the family of *Poaceae*.

Kingdom- *Plantae* (Plants)

Phylum- *Tracheophyta* (Vascular plants)

Super division- *Spermatophyta* (Seed plants)

Division- *Magnoliophyta* (Flowering plants)

Class- *Liliopsida* (Monocotyledons)

Subclass-*Commenlinidae*

Order- *Poales*

Family- *Poaceae*

Sub family- *Panicoideae*

Genus- *Zea* L.

Species- *Zea mays* L.

Plate 2.4: *Zea mays* L. (Maize plant)

Source: Hossain et al. (2016).

2.9.2.2 Origin and Uses

Maize or corn (*Zea mays*) is a cereal grain which originated from Mexico and Central America about 10,000 years ago. Cultivation of maize has presently diversified from Southern Mexico to different parts of the world, including Africa, America, Asia and Europe (Hossain, Muthusamy, Bhat, Jha, ...& Kumar, 2016). Maize has become a crop of global importance, with its annual total global production (1.02 billion) exceeding that of wheat or rice. In addition to its consumption by man as food, maize serves as livestock feed and a major constituent of various industrial products including corn starch, corn oil, corn meal and corn syrup. Six major types of maize have been identified including sweet corn, flour corn, popcorn, pod corn, flint corn and dent corn. Varieties rich in sugar such as sweet corn are cultivated for human consumption while field corn varieties are cultivated for livestock feed, corn-based human foods, chemical feedstocks and production of biofuels, including ethanol (Hossain et al., 2016).

Maize serves as an important source of proteins, calories, vitamins and minerals to man. In addition to rice and wheat, maize provides about 30% of food calories to billions of people in Africa, Mesoamerica, and Asia (Shiferaw, Prasanna, Hellin, & Banziger, 2011). Presently, America and other developed countries use more maize than other parts of the world. However, the rapid increase in poultry industry, which is its major consumer, in developing countries is

predicted to increase the demand for maize production (Prasanma, 2014). Maize also serves as a model plant for biological research around the world, hence its use in various plant-growth experiments (Hossain et al., 2016).

2.9.2.3 Growth and Harvest Conditions

Maize is a warm-climate crop with shallow root system. In temperate zones, the crop is cultivated during the spring season when there is sufficient sunlight and soil moisture to facilitate crop growth. Drought conditions, mostly during silk emergence, affect pollination of maize (Hossain et al., 2016). Maize is usually mature for harvesting 3-4 months after planting date. However, the type of maize determines the harvesting period. Sweet corn is usually harvested subsequent to pollination in the **milk stage**, and prior to starch formation, while field maize is left to dry in the field. Alternatively, farmers in Nigeria harvest crops and sun-dry to avoid pest infestation and yield loss (Hossain et al., 2016). Though maize grows well in hot and wet climates, it thrives in different climatic conditions due to its ability to adapt to diverse environments therefore making the maize crop a versatile plant.

2.10 Summary of Literature Review

Studies have shown the phytoremediation activities of *Hibiscus cannabinus* and *Zea mays* on used lubricating oil (Abioye et al., 2011), atrazine (Ibrahim, Abdel-Lateef, Khalifa, & Abdel-Monem, 2013), heavy metals (Shi et al., 2016) and wastewater (Abedinzadeh et al., 2019). However, information on the endophytic microbes associated with the phytoremediation plants *H. cannabinus* and *Z. mays* on exposure to crude oil is lacking and therefore a comprehensive phytoremediation plan has not been established. This study sought to isolate endophytic bacteria from *H. cannabinus and Z. mays,* and show their remediation potentials for crude oil removal from contaminated soil. The outcome of this study was inteded to be used in bioaugmentation studies and genetic manipulations of non-pollutant degrading plants as possible phytoremediation agents.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Description of Study Site

The study site was at Egbema environment, an oil-seepage and polluted site behind NPDC (Nigerian Petroleum Development Company Limited) reservoir located at Ukwugba Obiakpu in Ohaji/Egbema Local Government Area, Imo State (Fig. 3.1a and Fig. 3.1b). Geographically, the site (Latitude 5°55′56″N, Longitude 6°76′34″E) is situated at 3 km from Onosi Creek in Obiakpu Egbema. Ohaji/Egbema is a crude oil-rich area of Imo State. It is bordered in the East by Owerri West, North by Oguta Local Government of Imo State and in the South-western region by Ogba/Egbema/Ndoni in Rivers State. Ohaji/Egbema Local Government Area comprises of seventeen (17) autonomous communities, with Ukwugba Obiakpu serving as NPDC reservoir site. The reservoir comprises of six oil wells, four of which are currently operational. Gas flaring and oil seepages are characteristics of the area with crude oil, gas and firewater pipelines criss-crossing the site.

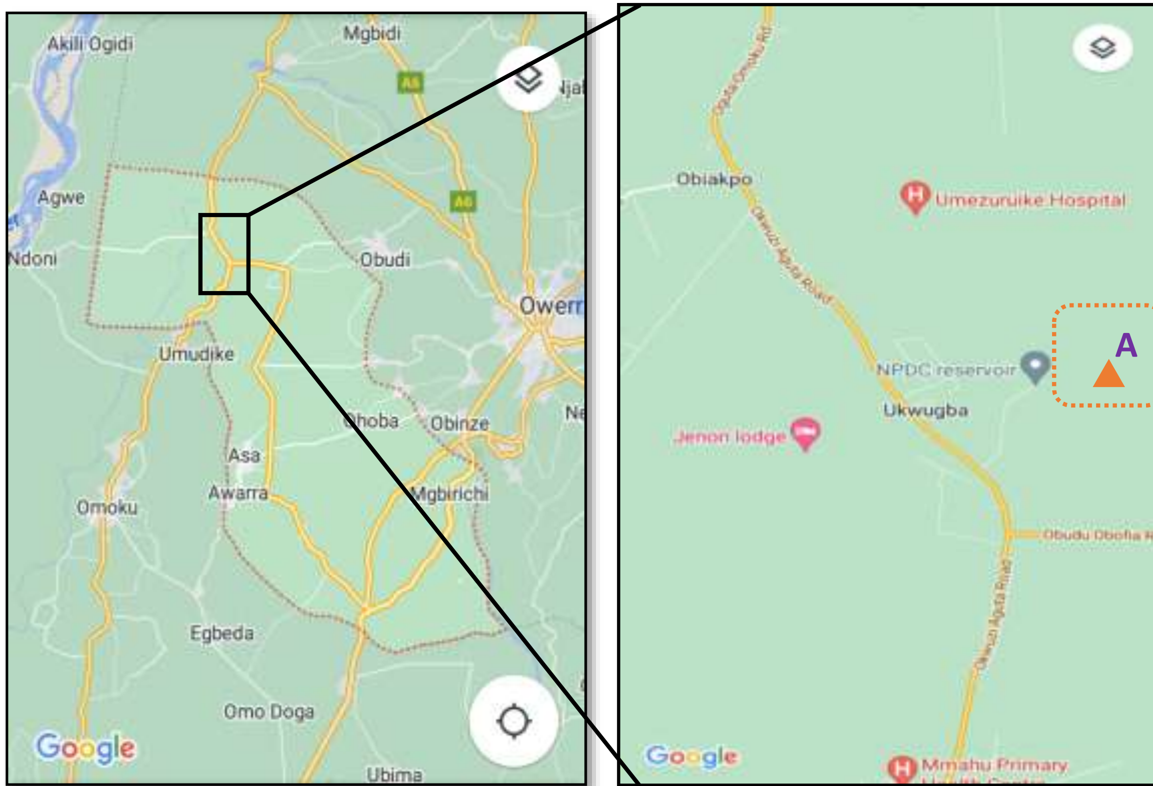


Figure 3.1a: Map of Egbema showing the study area.**Figure 3.1b**: Map of Egbema showing the study location. **Source**: Google Maps (2022).

3.1.2 Sample Collection and Procurement of Reagents

3.1.2.1 Collection of Soil Samples

Crude oil-polluted soil samples were collected from crude oil-polluted site behind NPDC at Ukwugba Obiakpu, Egbema Local Government Area, Imo State. Agricultural soil with no history of pollution was obtained from FUTO farms, School of Agricultural and Agricultural Technology (SAAT), Federal University of Technology Owerri. Soil samples were collected at the depth of 0-25 cm (top soil) using a sterilized soil auger. Soil samples were placed in sterile polyethylene bags, properly labelled, placed in an ice-chest and sent to the laboratory for analysis.

3.1.2.2 Preparation and Preservation of Soil Samples

Crude oil polluted and unpolluted soil samples for phytoremediation were air dried at room temperature for 7 days. After air-drying soil samples were sieved through 2 mm mesh size net. The fine earth was used for plant-growth while the coarse particles were discarded.

3.1.2.3 Procurement of *H. cannabinus* and *Z. mays* Seeds

The seeds of plants used for this study were procured according to the method used by Ibrahim et al. (2013) and Shi et al. (2016). Seeds of *Z. mays* were purchased from an urban market known as Ekeonuwa Market in Owerri, Imo State while *H. cannabinus* seeds were obtained from Department of Agriculture, Abia State University, Uturu, Abia State. *Z. mays* and *H. cannabinus* seeds were placed in pre-labelled air-tight containers and transported to the laboratory.

3.1.2.4 Seed Viability Test

Seed viability test was carried out using water-density procedure. *Z. mays* and *H. cannabinus* seeds were placed in a 100 ml beaker containing 50 ml of autoclaved distilled water. Viable seeds sank to the bottom of the water while non-viable seeds which floated on the surface of the water were discarded. Viable seeds were used for the plant growth experiment.

3.1.2.5 Collection, Preparation and Preservation of Banana Peels

Fresh banana peels were obtained from three fruit vendors in Ekeonuwa Market, Imo State. Banana peels were placed in sterile polyethylene bags, properly labelled and transported to the laboratory. Fresh banana peels were sun-dried for two weeks. Subsequently, dried banana peels were ground to powder using clean mortar and pestle and stored in pre-labelled air-tight containers.

3.1.2.6 Collection, Preparation and Preservation of Brewery Spent Grains

Brewery spent grains were obtained from Intafact Beverages Limited, Onitsha, Anambra State. Brewery spent grains were placed in sterile polyethylene bags, properly labelled and transported to the laboratory. Spent grains were sun-dried for seven days and stored in pre-labelled air-tight containers.

3.1.2.7 Collection of Diesel and Crude Oil

Diesel (AGO) was purchased from the Nigerian National Petroleum Corporation (NNPC) fuel station, Onitsha Road, Owerri, Imo State. Bonny light crude oil was obtained from oil well at Ukwugba Obiakpu in Egbema/Ohaji Local Government Area, Imo State. Crude oil and diesel samples were placed in properly labelled plastic containers, wrapped in black plastic bags and transported to the laboratory within 2 h of collection.

3.1.2.8 Preparation and Preservation of Diesel and Crude Oil

Crude oil and diesel were filter-sterilized using Whatman #1 filter paper. Filter-sterilized crude oil and diesel were stored in pre-labelled plastic containers. Due to its highly flammable nature, diesel was purchased 3 h prior to use. Appropriate safety measures were applied while using petroleum products.

3.1.2.9 Procurement and Preparation of Analytical Chemicals and Materials

All other chemicals and reagents to be used for the study were procured from SEGLOL Nigeria Enterprise, Ibadan, International Institute of Tropical Agriculture (IITA), Ibadan and JoeChem Ventures Nigeria, Nsukka. All chemicals were of analytical standard.

3.2 Methods

3.2.1 Study Plan

The study was divided into four (4) parts:

(i) Physico-chemical analyses of crude oil polluted and unpolluted soil samples before and after plant growth experiment (PGE).

(ii) Growth of Kenaf (*Hibiscus cannabinus*) and Maize (*Zea mays*) on crude oil-polluted and unpolluted soil samples.

(iii) Microbial studies on endophytic bacteria isolated from roots of Kenaf (*H. cannabinus*) and Maize (*Z. mays*).

(iv) Molecular characterization and screening of isolated bacterial endophytes from *H.cannabinus* and *Z. mays.*

Study Phase I:

3.2.2 Determination of Physico-chemical Properties of Crude oil Polluted and Unpolluted Soil samples

3.2.2.1 Determination of Physical Parameters of Soil Samples

3.2.2.1.1 Size Distribution and Soil Textural Class of Soil Samples

Size distribution and soil textural class were carried out using the hydrometer method (APHA, 1998). Ten (10) grams of soil was weighed into a 100 ml beaker. Distilled water (7.5 ml) and 1.5 ml of 30% H_2O_2 were added and gently stirred with a glass rod to encourage organic matter

destruction. Twenty (20) millilitres of distilled water and 10 ml of sodium hexametaphosphate solution were added. The suspension was stirred, covered and kept for 5 hours. The final volume was made up to 50 ml and stirred for 10 min. The suspension was transferred to a suspension cylinder and made up to 100 ml with distilled water. The cylinder was tightly capped and shaken severally to disperse soil particles. The hydrometer was immediately placed in the suspension and results taken exactly after 40 sec (S_1 and B_1) and 2 h (S_2 and B_2) respectively. Blank was prepared without any soil sample and room temperature recorded in °F.

$$\text{Correction factor (CF)} = (\text{Room temp in °F} - 68) \times 0.2 \quad \ldots \quad (3.1)$$

$$\text{Percent (\%) Silt} + \text{Clay} = \frac{(S_1 - B_1) + \text{CF}}{\text{Weight of sample (g)}} \times 100 \quad \ldots \quad (3.2)$$

$$\text{Percent (\%) Clay} = \frac{(S_2 - B_2) + \text{CF}}{\text{Weight of sample (g)}} \times 100 \quad \ldots \quad (3.3)$$

$$\text{Percent (\%) Sand} = 100 - (\text{Silt} + \text{Clay}) \quad \ldots \quad (3.4)$$

Where:

S_1 and B_1 = Hydrometer readings of sample and blank at 40 sec.

S_2 and B_2 = Hydrometer readings of sample and blank after 2 h.

Soil texture was determined by plotting the relative percentages on a nomograph (Figure 3.2). The point of intersection of the three relative percentages gave the soil textural class.

3.2.2.1.2 Determination of Soil Moisture Content

Moisture content was determined using the method outlined in APHA (1998). A porcelain crucible was placed in an oven at temperature of 105 ℃ and left for two (2) hours. It was left to cool to room temperature in a desiccator. The empty crucible was weighed (A). Ten (10) grams of soil was added to the crucible and weighed (B). The crucible plus soil were placed for a minimum of 12 h in the oven at 105 ℃. It was left to cool to room temperature in a desiccator and weighed again (C). The moisture content was calculated thus:

$$MC\ (\%) = \frac{(\text{B} - \text{C}) \times 100\%}{(\text{C} - \text{A})} \quad \ldots \quad (3.5)$$

$$Mcf = \frac{100 + \text{MC (\%)}}{100} \quad \ldots \quad (3.6)$$

Where:

A = Empty crucible weight.

B = Sample + crucible weight.

C = Heated sample + crucible weight.

Mcf = Moisture correction factor.

MC = Moisture content.

3.2.2.1.3 Determination of Bulk Density

Soil bulk density was determined using the Core method of Grossman and Reinsch (APHA, 1998). Soil sample was collected using the coring device. The sampler was pressed carefully into the soil to avoid compressing the soil in the confined space of the sampler. Thereafter the sampler and its content were carefully removed to preserve the natural structure and packing of the soil. The inner cylinder was separated from the outer cylinder with the undisturbed soil retained. Soil extending beyond each end of the sample holder was trimmed with a straight edge knife. Soil volume was noted as the volume of the sample holder. The soil was transferred to a pre-weighed crucible and weighed. The crucible and soil was placed in an oven at 105 ºC and weighed until a constant weight was obtained. The bulk density was calculated as:

$$Bulk\ density\ \left(\frac{\mathrm{g}}{\mathrm{cm}^3}\right) = \frac{\mathrm{Ms}}{\mathrm{Vt}} \quad \ldots \quad (3.7)$$

Where:

Ms = Mass of oven dried soil (g)

Vt = Total volume of sample (cm^3) assumed to be equal to the volume of the cylinder and was calculated from the formular

$$\mathrm{Vt} = \pi \mathrm{r}^2 \mathrm{h} \quad \ldots \quad (3.8)$$

Where:

π = 22/7

r = radius of cylinder

h = height of cylinder

3.2.2.1.4 Determination of Particle Density

Particle density was determined according to the method of Grossman and Reinsch (2002). An empty 100 mL volumetric flask without its stopper was weighed and its mass recorded to the nearest 0.1 g. Twenty-five grammes (25 g) of oven-dried and sieved soil sample was then carefully introduced into the volumetric flask. The flask without stopper and the soil were then weighed and the mass recorded. About 50 mL of distilled water was then added to the soil in the flask. The flask and its contents were gently heated on a hot plate; with 10 sec swirling for every minute to prevent foaming. The soil-water mixture was heated for 10 min to remove air bubbles. Thereafter the flask was removed from the hot plate and the mixture allowed to cool; it was then capped and allowed to stand for 24 hr. After 24 hr the flask was un-capped and distilled water added till the 100 mL mark. This was weighed without the stopper. The particle density was calculated as:

$$Particle\ Density = \frac{\text{Mass of oven} - \text{dried soil}}{\text{Volume of dry soil}} \quad \ldots \quad (3.9)$$

Soil porosity was then calculated using the equation:

$$Porosity = \left(1 - \left(\frac{\text{bulk density}}{\text{particle density}}\right)\right) \times 100 \quad \ldots \quad (3.10)$$

3.2.2.2 Determination of Chemical Parameters of Soil Samples

Soil-water suspension of ratio 1:2 (1:5 for only electrical conductivity) was prepared and shaken for one hour on a shaker. It was then filtered through Whatman #1 filter paper and the filtrate used for analysis. The procedure for water analysis was then applied (APHA, 1998).

3.2.2.2.1 Determination of pH

The pH of water samples was determined using the method of APHA (1998). Fifty (50) ml of filtered water sample was pipetted into a 100 ml conical flask. The calibrated pH meter was then used to read the pH of the solution. This was performed in triplicate.

3.2.2.2.2 Determination of Electrical Conductivity

The electrical conductivity was measured using a temperature-compensated electronic switch-gear meter according to APHA (1998) specifications. The meter was placed in soil-water suspension (1:5) and allowed to stabilize for some minutes before readings were taken. The result was read off directly in µS/cm at 20ºC.

3.2.2.2.3 Determination of Calcium Content of Soil Samples

Calcium was estimated by versenate titration method using ammonium purpurate (murexide) indicator (APHA, 1998). Filtered soil-water (5ml) was put into a 100 ml beaker after which 1 ml of NaOH solution and a pinch of murexide indicator were added. The resulting solution was tirated against 0.01N standard versenate (EDTA-disodium salt) solution till the colour changes from pink to purple.

$$\text{Ca}^{2+}\ mg/l = \frac{N \times V}{ml \text{ of sample}} \times \frac{1000}{1} \times 20.04 \qquad \ldots \qquad (3.11)$$

Where:

N = Normality of EDTA solution (0.01N).

V = Volume of EDTA solution used in titration.

3.2.2.2.4 Determination of Magnesium Content of Soil Samples

According to APHA (1998) guidelines, magnesium was estimated by versenate titration method. First, magnesium and calcium content of soil-water samples were determined, then calcium content was subtracted from both to get the magnesium content. Five (5) milliliters of filtered soil-water was put into a 100 ml beaker. One (1) millilitre of NH_4OH/NH_4Cl buffer was then added with 3-4 drops of eriochrome black T indicator. The resulting solution was titrated against 0.01N standard versenate (EDTA-disodium salt) solution till the colour changed from wine red to sky blue.

$$\text{Ca}^{2+} + \text{Mg}^{2+} me/l = \frac{N \times V}{ml \text{ of sample}} + \frac{1000}{1} \qquad \ldots \qquad (3.12)$$

Where:

N = Normality of EDTA solution (0.01N).

V = Volume of EDTA solution used in titration.

Mg^{2+} me/L = (Ca^{2+} + Mg^{2+}) – Ca^{2+} ... (3.13)

Mg^{2+} mg/L = Mg^{2+} me/L x 12.16 ... (3.14)

Ca^{2+}mg/L = Ca^{2+} me/L x 20.04 ... (3.15)

3.2.2.2.5 Determination of Sodium

Sodium in soil-water samples was determined by flame photometric method (APHA, 1998). The flame photometer was calibrated with standard solutions of sodium (10, 25, 50 and 75 ppm). The filtered soil-water sample was aspirated and the readings recorded. The concentrations obtained were multiplied by equivalent weight of sodium (23) to convert them into mg/L.

3.2.2.2.6 Determination of Potassium

Potassium in soil-water samples was determined by flame photometric method (APHA, 1998). The flame photometer was calibrated with standard solutions of potassium (10, 25, 50 and 75 ppm). The filtered soil-water sample was aspirated and the readings recorded. The concentrations obtained were multiplied by equivalent weight of potassium (39) to convert them into mg/L.

3.2.2.2.7 Determination of Ammonical and Nitrate Nitrogen

This was determined using the method described in APHA (1998). Fifty (50) ml of filtered soil-water was put into 250 ml Kjeldhal tube and set up on nitrogen distillation unit. About 0.2 g of magnesium oxide (MgO) was added and the solution distilled immediately. The distillate (30 ml) was collected in 2% boric acid and 0.2 g of Devarda's alloy was added. This was distilled again and the distillate collected in 10 ml of 2% boric acid solution. First distillate was used for ammonia and second distillate was used nitrate nitrogen.The distillate/solution was then titrated against 0.01N H_2SO_4 solution until pink colour colour started appearing. A blank with pure water was run for each set of samples.

$$\mathrm{NH_4} - N \text{ or } \mathrm{NO_3} - N(mg/l) = \frac{(\mathrm{S} - \mathrm{B}) \times \mathrm{N} \times 14}{\text{Sample volume } (ml)} \times 1000 \quad \ldots \quad (3.16)$$

Where:

S = Volume of acid used against sample.

B = Volume of acid used against blank.

N = Normality of acid.

3.2.2.2.8 Determination of Sulphate

This was determined using the method described in APHA (1998). Twenty (20) ml of filtered soil-water sample was pipetted into a 100 ml conical flask. One (1) millilitre of 5% BaCl solution and 20 ml of standard Na_2SO_4 solution were added. The solution was swirled gently and absorbance read using a spectrophotometer at 450 nm. Blank was also prepared in the same manner without the water sample. The sulphate content of water sample was calculated using the formula:

$$SO_4^{2-}(mg/l) = \frac{A_T}{A_S} \times \frac{\text{concn of std soln}}{1} \times \frac{1000}{\text{vol of sample used}} \quad \ldots \quad (3.17)$$

Where:

A_T = Absorbance of sample

A_S = Absorbance of blank

3.2.2.2.9 Determination of Bicarbonate

The biocarbonate content of soil-water suspension was determined titrimetrically following the methods described in APHA (1998). Ten millilitres (10 ml) of soil-water suspension was pipetted into a 100 ml conical flask and 2 drop of phenolphthalein indicator added. Presence of carbonate was noted when the solution changed to pink colour. Mixture was titrated against 0.01N H_2SO_4 until the mixture turned colourless and volume was denoted as “A”. Further titration was carried out in order to test for bicarbonate by adding two drops of methyl orange indicator. The solution turned yellow and was titrated against 0.01N H_2SO_4 until colour changed to red. New volume was denoted as “B”. The bicarbonate content of the water sample was calculated using the formula:

$$HCO_3^- mg/l = \frac{N \times (B - A)}{ml \text{ of sample}} \times \frac{1000}{1} \times 61 \quad \ldots \quad (3.18)$$

Where:

N = Normality of acid

A = Volume of titrant against phenolphthalein indicator

B = Volume of titrant against methyl orange indicator

61 = Equivalent weight of HCO_3.

3.2.2.2.10 Determination of Organic Matter

Organic matter content of soil samples was determined according to Walkley and Black method (APHA, 1998). One gramme (1 g) of soil sample was weighed into a 500 ml conical flask and 10 ml of 1N $K_2Cr_2O_7$ solution added to it. Concentrated H_2SO_4 (20 ml) was then added and mixed by swirling gently for 1 min. This was then allowed to stand for 30 min. Blank was prepared similarly as above but without soil samples. After 30 min, 200 ml of distilled water, 3 drops of diphenylamine indicator and 0.2 g of sodium fluoride were sequentially added. The solution was then titrated against 0.5N ferrous ammonium sulphate (FAS) solution. Colour of solution changed from dull green to turbid blue and finally to brilliant green.

$$\text{Organic matter (\%)} = \frac{(B - S) \times N}{\text{Weight of soil}} \times 0.67 \quad \ldots \quad (3.19)$$

Where: B = Volume of FAS used for blank titration

S = Volume of FAS used for sample titration

N = Normality of FAS from blank titration

3.2.2.2.11 Determination of Organic Carbon

This was determined using the method of Walkely and Black (1934) as described in APHA standards of 1998. One (1) gramme of sediment was weighed into a 500 ml conical flask. Ten (10) millilitres of 1N $K_2Cr_2O_7$ and 20ml of concentrated H_2SO_4 were added. The flask was swirled carefully and allowed to stand for 30 minutes. Distilled water (200 ml) followed by 10 ml of H_3PO_4 were slowly added. A millilitre of diphenylamine indicator was then added and titrated against 0.5N Ferrous ammonium sulphate solution until green colour starts appearing

indicating the end point. This was replicated thrice. Blank titration was also made in the same manner, but without sediment to standardize the dichromate. Organic carbon was calculated using the formula:

$$\text{Organic Carbon (\%)} = \frac{10(B - S) \times 0.39 \times \text{mcf}}{B \times W} \qquad \ldots \qquad (3.20)$$

Where:

B = ml of ferrous ammonium sulphate solution used for blank.

S = ml of ferrous ammonium sulphate solution used for sample.

Mcf = moisture correction factor.

W = sample weight (g).

0.39 = conversion factor (including a correction factor for a supposed 70% oxidation of organic carbon).

3.2.2.2.12 Determination of Total Nitrogen

Total nitrogen in soil samples was determined according to Kjeldahl's method (APHA, 1998). Soil sample (1 g) was first placed in a digestion tube, 7 g of catalyst (K_2SO_4 and $CuSO_4$, ratio 9:1) and concentrated H_2SO_4 (20 ml) were added. Blank was prepared without soil samples. The mixture was covered and digested at 420 °C until the solution turned light green. The tube was removed and allowed to cool. Distilled water (60 ml) was then added and the mixture transferred to the distillation unit. Twenty-five millilitres (25 ml) of 4% boric acid was introduced into a 250 ml conical flask and placed under the condenser. The digestion tube containing the digested sample was then connected to the distillation apparatus. Sixty millilitres (60 ml) of 40% NaOH was introduced into the digested sample and the steam supply valve turned on to initiate distillation. This was heated for 5 min until all ammonia had passed over into the boric acid. About 120 ml of the distillate was collected and 1ml of indicator (100 ml of 0.1% methyl red and 200 ml of 0.2% bromocresol green) added. The mixture was titrated against 0.1N H_2SO_4 until the solution changed from green to pink.

$$\text{\% Nitrogen} = \frac{(B - A) \times 0.1 \times 14.00}{\text{Weight of sample}} \times 100 \qquad \ldots \qquad (3.21)$$

Where: A = Volume of 0.1N H_2SO_4 used in balnk titration

B = Volume of 0.1N H_2SO_4 used in sample titration

14 = Atomic weight of nitrogen

3.2.2.2.13 Determination of Phosphorus

Phosphorus in soil-water samples was determined using the APHA (1998) method. Twenty-five (25) ml of filtered soil-water sample was pipetted into a 100 ml conical flask and 2 drops of phenolphthalein indicator added. Five (5) millilitres of vanadate-molybdate reagent was added. The solution was swirled and left to stand for 10 minutes. Blank was prepared in the same manner without the water sample. The absorbance of the solution was measured using a spectrophotometer at 880 nm. Phosphorus content of soil-water was calculated using the formula:

$$\text{P}\ (mg/l) = \frac{\text{R} \times 50}{25} \qquad \ldots \qquad (3.22)$$

Where: R = Spectrophotometer reading

3.2.3 Assessment of Total Petroleum Hydrocarbon (TPH) of Crude Oil-Polluted and Unpolluted Soil Samples Before and After Plant Growth Experiment (PGE)

3.2.3.1 Extraction of Petroleum Hydrocarbons from Soil Samples

The total petroleum hydrocarbon of soil samples were solvent extracted using the closed vessel microwave heating method (Oliveira et al., 2013). Five (5) grammes of soil sample were transferred into the extraction vessel and 30 ml of acetone-hexane (1:1 v/v) added. The extraction vessel was closed and heated according to the microwave program and subsequently allowed to cool to room temperature. Sodium sulphate (5g) and solvent mixture were then added to the sample and swirled gently. The extraction mixture was then filtered through ashless filter paper and stored for gas chromatography-mass spectrophotometry (GC-MS) analysis.

3.2.3.2 Gas Chromatography-Mass Spectrophotometry Analysis and Conditions

Gas chromatography-mass spectrophotometry analysis was performed using Agilent Intuvo 9000 GC system coupled with detector system 5977B MSD with split/splitless injector. A DB-

5MS (5% phenyldimethylsiloxane) fused silica capillary column 30 m, 320µm i.d., 0.25 µm of film thickness was used with helium gas (99.999%) purity as carrier gas at flw rates f 1.2ml min^{-1}. Inlet temperature was set at 300 ℃, MS Source at 230 ℃ and MS Quad at 150 ℃. The oven temperature was programmed as follows: 50 ℃ for 2 min, increased to 250 ℃ at 20 ℃ min^{-1} and hold for 3 min. Data were acquired by GCMSD/Enhanced MassHunter Software and processed using GCMSD Data analysis software incorporated with 2017 version of NIST Library. One microlitre (1 µl) of the sample extract and standard were injected in splitless mode into the GC system using Agilent Automated Liquid Sampler (ALS) G4513A. Soil samples were analyzed prior to and after plant-growth experiment.

Study Phase II:

3.2.4 Growth of *H. cannabinus* and *Z. mays* on Crude Oil Polluted and Unpolluted Soil

3.2.4.1 Raising of Seedlings

Seedlings were raised from viable *H. cannabinus* seeds on a nursery bed (1×3 m^2) containing sandy-loamy soil at the nursery section of the teaching and research farm, School of Agriculture and Agricultural Technology (SAAT), FUTO. Nursery beds were kept moist by sprinkling 200 ml of tap water onto the nursery beds daily. Seedlings were raised for 2 weeks in the nursery. Thereafter, seedlings of similar heights were transplanted and subjected to treatment.

3.2.4.2 Preparation of Crude Oil Amended Soil and Treatment

Five kilogrammes of air-dried soil samples were introduced into pots. Test samples were amended with varying concentrations of crude oil (Table 3.1). The second batch of samples was treated with soil from Egbema, an oil seepage and polluted site (Table 3.2). Control was set up with no contaminant. All soil samples were amended with dried and ground banana peels and brewery spent grains except the negative control test samples. *H. cannabinus* and *Z. mays* plants of about 20 cm height, from the nursery, were planted in control as well as treated pots. The plants were grown for 90 days, watered 200 mL of tap water daily. Each treatment was conducted in triplicate.

3.2.4.3 Exposure of *H. cannabinus and Z. mays* to Different Concentrations of Crude Oil

Plants were allowed to grow for 90 days according to the method described by Tiwari et al. (2016). Three (3) plants, healthy and uniform in height of *H. cannabinus* and *Z. mays* (Table

3.1), were transplanted in each pot (25 cm x 20 cm x 20 cm, l x b x h). Treatments included: A1) Control pots with no contaminant amended with banana peels and brewery spent grains (positive control) A2) Control pots with no contaminant and no amendment (negative control) A3) Planted pots amended with composite with 5 mL/5Kg crude oil A4) Planted pots amended with composite with 10 mL/5Kg crude oil A5) Planted pots amended with composite with 25 mL/5Kg crude oil A6) Planted pots amended with composite with 50 mL/Kg crude oil. All pots were covered with aluminium foil to avoid possible photodegradation and evaporation of volatile constituents of crude oil from soil. Each treatment was carried out in triplicate. Planting was done in a greenhouse with constant aeration. Water temperature of 30 °C and natural photoperiod (12 hr light: 12 hr dark) were maintained daily.

3.2.4.4 Experimental Design

Table 3.1: Plant-Growth Design for Crude Oil-Polluted Soil Samples

Groups	Number of plants	Composite amendment	Exposure concentration of crude oil (mL/Kg)
A1 (+ve control)	3	Amended	5 Kg unpolluted soil
A2 (-ve control)	3	Not-amended	5 Kg unpolluted soil
A3	3	Amended	5 mL/5Kg
A4	3	Amended	10 mL/5Kg
A5	3	Amended	25 mL/5Kg
A6	3	Amended	50 mL/5Kg

Table 3.2: Plant-Growth Design for Egbema-Polluted Soil Samples

Groups	Number of plants	Composite amendment	Exposure concentration of crude oil (mL/Kg)
B1 (+ve control)	3	Amended	5 Kg polluted soil
B2 (-ve control)	3	Not-amended	5 Kg polluted soil
B3	3	Amended	1 Kg polluted soil + 4 Kg agricultural soil)
B4	3	Amended	2.5 Kg polluted soil + 2.5 Kg agricultural soil

3.2.4.5 Determination of growth parameters of *Hibiscus cannabinus* and *Zea mays* During Phytoremediation

Growth parameters of *H. cannabinus* and *Z. mays* including number of leaves, leaf width and plant height were recorded at 0, 15, 30, 45, 60 and 90 days after planting (DAF). Length of roots and shoot, fresh weight of below-ground and above-ground (2 cm above ground level) parts of test and control plants were recorded immediately after harvesting of plants.

Study Phase III:

3.2.5 Isolation and Identification of Endophytic Bacteria from Roots of Plants

3.2.5.1 Isolation of Endophytes from Roots of *H. cannabinus* and *Z. mays*

Isolation of bacterial endophytes was carried out as described by Govarthanan et al. (2016) and Oliveira et al. (2013) with slight modifications. Whole plants were uprooted and washed thoroughly with tap water and rinsed thrice in sterile distilled water. Roots were surface sterilized to ensure isolation of strictly endophytes and limit external contamination. Briefly, roots were soaked with 95% ethanol for 1 min, 3% sodium hypochlorite solution for 3 min and 70% ethanol for 30 sec respectively. Thereafter, roots were rinsed five times with sterile distilled water. To ensure no external microbial contamination and complete surface

sterilization, the final rinse water was spread plated on Nutrient Agar (NA) to detect any microbial growth. No microbial growth indicated complete surface sterilization. Roots of surface sterilized plants were aseptically cut off with sterile scalpel. One gram of surface-sterilized plant roots was ground in a sterile mortar and pestle with 10ml saline (0.85% NaCl). Serial dilutions were prepared and spread on plates of Nutrient Agar (NA) and Tryptic Soy Agar (TSA). Cycloheximide (100 mg/L) was added to culture media to suppress fungal growth (Oliveira et al., 2013). Plates were incubated at 37 °C for 72 h and observed for bacterial growth. Colonies with different morphological characteristics were sub-cultured onto a fresh medium for 3 days and purified by repeated sub-culturing on fresh medium. Bacterial isolates were stored at 4 °C for further characterization.

3.2.5.2 Identification of Isolated Endophytes

3.2.5.2.1 Phenotypic Characterization

Phenotypic characterization was done as described by Cheesbrough (2006). To identify the isolated endophytes, gram staining was done according to standard microbiological protocol. The colonies were distinguished through visual observations of colony morphology. Individual colonies were identified through specific biochemical test: motility test, catalase test, oxidase test and starch hydrolysis test, as follows:

3.2.5.2.2 Motility Test

A needle containing bacteria colony was stabbed straight in and straight out of the center of the medium. Inoculated medium was cultured for 24 h at 37 °C. Turbidity in culture medium was regarded as a positive test. Non-motile organisms only grow along the line of inoculation. Organisms with flagellum that can swim away from stab mark indicated positive test (Cheesbrough, 2006).

3.2.5.2.3 Catalase Test

Pure isolated bacterial colony was added to a clean sterile glass slide. A drop of 3% hydrogen peroxide (H_2O_2) was added. Formation of a bubble indicated the presence of the enzyme catalase (Cheesbrough, 2006). The equation for the reaction is as follows:

$$2H_2O_2 \xrightarrow{catalase} 2H_2O + O_2 \quad \ldots \quad (3.23)$$

3.2.5.2.4 Oxidase Test

Few drops of oxidase test reagent were added to a strip of filter paper (Whatman No. 1) and a loopful of bacterial colony streaked onto the reagent with a platinum loop or wooden applicator stick. Purple or violet colouration in 30 sec indicated a positive result. Colourless test or formation of light pink/purple colouration after 30 sec was regarded as a negative result (Cheesbrough, 2006).

3.2.5.2.5 Starch Hydrolysis Test

Agar was dissolved in distilled water and autoclaved at 121 °C for 15 min. Plates were aseptically poured and allowed to solidify. A single streak line of each isolated bacteria colony was made on plates and incubated upside down at 37 °C for 24 h. Iodine solution was added to plates. Formation of a clear zone around bacterial growth showed starch hydrolysis; which was regarded as a positive result. Presence of dark brown or blue/black zone depicted negative result indicating that starch has not been hydrolysed (Cheesbrough, 2006).

3.2.6 Assessment of Plant Growth-Promoting Properties of Endophytic Bacterial Isolates

3.2.6.1 Determination of Indole Acetic Acid (IAA)

This was determined according to the method of Gordon and Weber as described by Govarthanan et al. (2016) with slight modifications. Endophytic isolates were grown in Dworkin and Foster (DF) minimal medium, supplemented with 1 mg/ml tryptophan under shaking condition (200 rpm) at 30 °C for 48 h incubation. Cells were centrifuge at 8,000 X g for 15 min under room temperature. One millilitre of bacterial supernatant was added to 4 ml of Salkowski reagent (1 ml of 0.5 M $FeCl_3$, 50 ml of 35% $HClO_4$), mixed and allowed to react under dark condition at room temperature for 30 min. Blank consisted of 1 ml un-inoculated DF minimal medium with Salkowski reagent. Appearance of pink colour in the tubes indicated IAA production.

3.2.6.2 Determination of Phosphate Solubilization

This was done using Pikovskaya's agar medium as described by Tiwari et al. (2016). Pure cultures were streaked on Pikovskaya's agar medium and incubated at 37 °C. After 3 days of incubation, formation of a clear zone around the bacterial colony indicated a positive solubilization of phosphate mineral.

3.2.6.3 Siderophore Assay

This was determined following Schwyn and Neilands procedure by Chrome Azurol S (CAS) method (Tiwari et al., 2016). Pure bacterial isolates were inoculated onto CAS agar plates and incubated at 37 °C for 48 h. Colour change from blue to orange indicated siderophore production. Control consisted of CAS agar plates without inoculation.

3.2.6.4 Determination of 1-aminocyclopropane-1-carboxylate (ACC) Deaminase

The ACC deaminase activity was determined using the method of Praburaman et al. (2015). DF minimal medium (3 mM ACC, 4 g KH_2PO_4, 6 g Na_2HPO_4, 0.2 g $MgSO_4$, 0.001 g $FeSO_4$, 10 mg H_3BO_3, 10 mg $MnSO_4$, 70 mg $ZnSO_4$, 50 mg $CuSO_4$) was spread on agar plates and allowed to dry for 10 min. Bacterial isolates were inoculated and incubated for 48 h at 37 °C in a rotary shaker (200 r.p.m.). Growth of colonies was a positive indication for ACC deaminase production.

3.2.7 Assessment of the Bioremediation Potential of Endophytic Bacterial Isolates on Different n-Alkanes and Diesel

3.2.7.1. Hydrocarbon Degradation by Endophytic Bacterial Isolates

This was determined using the method described by Yousaf et al. (2010) with slight modification. Each isolate was streaked onto Nutrient Agar (NA) and Tryptic Soy Agar (TSA) amended with 2% (v/v) diesel and n-alkanes (C_6, C_8, and C_{10}). Control treatment contained isolate grown on 10% NA and TSA amended with 0.2% (w/v) glucose. Inoculated plates were incubated at 37 °C for 2 days. Control treatment was incubated for 2 days at the same temperature. Growth of strains on agar plates was regarded as a positive result.

Study Phase IV:

3.2.8 Genetic Characterization and Screening of Isolated Endophytes from Roots of *H.cannabinus* and *Z. mays*

3.2.8.1 Extraction of Genomic DNA of Bacterial Endophytes

A total of 24 endophytic bacterial isolates were selected for molecular analysis. Criteria for selection were based on isolates that tested positive for two or more plant-growth promoting properties; indole-3-acetic acid (IAA) production, synthesis of 1-aminocyclopropane-1-

carboxylate (ACC) deaminase and siderophore production. Additionally, one isolate each was selected to represent each sample in the plant growth experiment.

The genomic DNA of each sample of bacterial isolate was extracted using the CTAB method as reported by Oliveira et al. (2013). Bacterial culture was spinned to remove culture materials. Pellets were re-suspended in 1 ml of lysis buffer (10 mL of 1M Tris-HCl, pH 8, 10 mL of 5M NaCl, 10 mL of 0.25M EDTA pH 8, 1% SDS, 0.2mg/ml Proteinase K). This was vortexed and incubated at 55 ℃ until cells were completely lysed. One hundred (100) microlitre of saturated KCl solution was added and mixed by inversion. This was left to incubate on ice for 5 min to encourage precipitation of most polysaccharides and some proteins along with the insoluble SDS. The lysate was centrifuged at 14000 rpm for 15 min, the supernatant was collected in a fresh tube and extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The clear supernatant was transferred into a new tube and DNA precipitated by addition of an equal volume of isopropanol. This was mixed by inversion and incubated at room temperature for 10 min. Thereafter DNA was recovered by centrifugation at 15000 X g for 20 min, with the resulting pellet washed in 70% ethanol. The pellet was allowed to dry at room temperature and re-dissolved in 100 µl of autoclaved distilled water. Then 10µg/ml of RNase A was added and incubated at 37 ℃ for 60 mins. Extracts were stored at -20 °C for further analysis.

3.2.8.2 Agarose Gel Electrophoresis of Extracted DNA

The quality of extracted genomic DNA was determined by electrophoresing a 5-µl aliquot measures of each DNA sample across a 1% agarose (1% TBE) gel containing 1µl ethidium bromide each and scoring for either presence or absence of DNA fragmentation (Oliveira et al., 2013).

3.2.8.3 Quantification and Qualification of Extracted DNA

The genomic DNA concentration and purity of extracted DNA were assessed by spectrophotometry using the Nanodrop-Spectrophotometer. Spectra 230, 260 and 280 nm were recorded for every sample. DNA purity was determined by calculating the absorbance ratio $A_{260/280}$. Pure DNA has a ratio of 1.8 ± 0.2. Organic contamination was assessed by calculating the absorbance ratio $A_{260/230}$ (Oliveira et al., 2013).

3.2.8.4 Amplification, Purification and Sequencing of 16S rDNA Products

The DNA coding region for the 16S rDNA gene was amplified by polymerase chain reaction (PCR) using universal primers 27f and 1492r (Table 3.3) as described by Govarthanan et al. (2016). The PCR cocktail mix and PCR program are shown in Table 3.4 and Table 3.5 respectively.The reaction product (amplicons) were analysed by agarose gel electrophoresis. The band of interest were excised from the gel, purified using a commercially available Clean-up Kit and sequenced using Sanger dideoxy sequencing method (Tiwari et al., 2016). The analysed sequences were compared against the available sequence at NCBI GenBank (www.ncbi.nlm.nih.gov/Blast) nucleotide database using BLAST-n. Aligned sequence regions were detected using Mega X and phylogenetic tree constructed using neighbor-joining distance method using Mega X software version 11.

The primer sequence is as shown in Table 3.3.

Table 3.3: 16s rDNA Primers and sequences

Primer Number	*Name*	*Primer Sequence*	*Molecular Weight*	*Reference*
Primer 1	27f	**- 5' – AGA GTT TGA TCC TGG CTC AG – 3'**	1500 bp	Govarthanan et al. (2016)
Primer 2	1492r	**- 5' – CGG TTA CCT TGT TAC GAC TT – 3'**	1500 bp	Govarthanan et al. (2016)

Table 3.4: PCR cocktail mix for one sample

10× PCR buffer	2.5 µL
50mM $MgCl_2$	1.0 µL
5pMol forward primer	1.0 µL
5pMol reverse primer	1.0 µL
$DMSO_4$	1.0 µL
2.5mM DNTPs	2.0 µL
Taq 5u/µL	0.06 µL
DNA sample	3.0 µL
H_2O	13.44 µL
	25 µL

Table 3.5: PCR program

36 Cycles					
Initial denaturation	Denaturation	Annealing temperature	extension	Final Extension	Hold temperature
94˚C 5 min	94 ˚C 30 sec	56 ˚C 30 sec	72 ˚C 45 sec	72 ˚C 7 min	10 ˚C ∞

3.2.8.5 Assay of Hydrocarbon Degradative Genes of Bacterial Endophytes

This was determined using the methods described by Cebron et al. (2008) and Yousaf et al. (2010). Genomic DNA of endophytic bacterial isolates was subjected to PCR amplification to detect the presence of genes that code for the enzyme alkane monooxygenase (*Alk* B genes). Presence of *Alk* B genes were analysed for using *Alk* BF (**5' – AAC TAC MTC GAR CAY TAC GG – 3'**) and *Alk* BR (**5' – TGA MGA TGT GGT YRC TGT TCC– 3'**) primers presented in Table 3.6 (Yousaf et al., 2010). The PCR cocktail mix and PCR program are shown in Table 3.4 and Table 3.5 respectively. Amplification conditions were as follows: initial denaturation at 94 ºC for 5 min, 36 cycles of denaturation at 94 ºC for 30 sec, annealation at 56 ºC for 30 sec, elongation at 72 ºC for 45 sec and final elongation at 72 ºC for 7 min and hold at 10 ºC forever. The reaction products (amplicons) were visualized on ethidium bromide stained 1.5% agarose electrophoresis gels with 50 bp DNA ladder used. Amplicons were scored for the presence or absence of bands.

Table 3.6: Alkane degradative primers and sequences

Primer Number	*Name*	*Primer Sequence*	*Molecular Weight*	*Reference*
Primer 1	*alk*B F	**- 5' – AAC TAC MTC GAR CAY TAC GG – 3'**	550bp	Yousaf et al. (2010)
Primer 2	*alk*B R	**- 5' – TGA MGA TGT GGT YRC TGT TCC– 3'**	550bp	Yousaf et al. (2010)
Primer 3	*alk*B1 F	**- 5' – TAC GGG CAC TTC GCG ATT GA –3'**	550bp	Yousaf et al. (2010)
Primer 4	*alk*B1 R	**- 5' – CGC CCA GTT CGA MAC GAT GTG– 3'**	550bp	Yousaf et al. (2010)

3.2.8.6 Phylogeny

Nucleotide sequences obtained were aligned using Clustal W algorithm. Phylogenetic tree of isolates was constructed using the Neighbor-Joining method using Mega X software version 11. Bootstrap was set at 1000 bp and p-distance model was applied. The nucleotide sequences identified in this study were compared against other bacteria sequences at National Center for Biotechnology Information (NCBI) GenBank (www.ncbi.nlm.nih.gov/Blast) nucleotide database using BLAST-n. Additional 30 nucleotide sequences of related Proteus species and 3 closely related genera were downloaded from available GenBank database of NCBI and incorporated during construction of the phylogenetic tree (Bolivar et al., 2021).

3.2.9 Statistical Analyses

Data collected for all parameters were subjected to statistical analysis using Statistical Package for Social Science (SPSS) version 20. One-way analysis of variance (ANOVA) was used to test for differences between samples. Duncan Multiple Range Test (DMRT) was used to test for differences between the different samples and 95% significance level ($\alpha = 0.05$) was adopted.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Physico-chemical Parameters of Crude oil Polluted and Unpolluted Soil

The result of the physical parameters of varying concentratios of crude oil-polluted and unpolluted soil samples prior to plant growth (PGE I) is shown in Table 4.1a. Percentage silt, clay and sand content of the soil were highest in 50ml/5kg (17.90%), 25ml/5kg (12.90%) and 10ml/5kg (76.50%) samples respectively and lowest in 10ml/5kg (11.00%), unpolluted soil with organic amendment (11.40%) and 50ml/5kg (69.70%) samples respectively. Soil textural class was recorded as sandy loam across all samples. Soil porosity was highest in unpolluted organic amended soil (58.92%) and lowest in sample 25ml/5kg (44.43%). Moisture content of soil samples was observed to be 5.00% in 5ml/5kg, 10ml/5kg, 25ml/5kg and 50ml/5kg samples and 2.50% in unpolluted soil with organic amendment and unpolluted soil without organic amendment. Sample 50ml/5kg had the highest recorded soil bulk density (1.42g/cm^3) and particle density (2.71g/cm^3) while the least bulk density (1.10g/cm^3) and particle density (2.38g/cm^3) were observed in unpolluted soil with organic amendment and 10ml/5kg.

Values of the physical parameters of crude oil-polluted soil from Egbema before plant growth II are shown in Table 4.1b. Egbema-polluted soil with organic amendment had the highest values for percentage silt (44.76%) and clay (45.50%) while the least values for percent silt (14.90%) and clay (20.60%) were recorded in 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil. The highest and least percent sand was recorded in 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil (64.50%) and Egbema-polluted soil with organic amendment (9.80%) respectively. Soil textural clas was observed as silty clay for Egbema-polluted soil with organic amendment and Egbema-polluted soil without organic amendment and sandy clay loam for 1kg unpolluted soil + 4kg Egbema-polluted soil and 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil. Soil porosity and moisture content were highest in 1kg unpolluted soil + 4kg Egbema-polluted soil (44.21%) and 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil (15.50%) respectively and lowest in Egbema-polluted soil with organic amendment for both soil porosity (39.28%) and soil moisture content (10.00%).

Table 4.1a: Physical Parameters of Crude Oil-polluted and Unpolluted Soil Samples before Plant Growth

Soil Sample Code /Parameters	A1	A2	A3	A4	A5	A6
Size Distribution						
Silt (%)	13.00±0.10[b]	13.00±0.17[b]	17.60±0.17[d]	11.00±0.00[a]	17.00±0.10[c]	17.90±0.17[e]
Clay (%)	11.40±0.10[b]	11.40±0.10[b]	10.60±0.10[a]	12.50±0.10[c]	12.90±0.10[d]	12.40±0.10[c]
Sand (%)	75.60±0.10[d]	75.60±0.10[d]	71.80±0.20[c]	76.50±0.10[e]	70.10±0.10[b]	69.70±0.10[a]
Soil Texture	Sandy loam	Sandy loam	Sandy loam	Sandy loam	Sandy loam	Sandy loam
Porosity (%)	58.92±7.77[d]	58.92 ±4.34[d]	47.14±4.28[c]	46.22±0.75[b]	44.43±1.13[a]	47.60±1.58[c]
MC (%)	2.50±0.05[a]	2.50±0.02[a]	5.00±0.20[b]	5.00±0.15[b]	5.00±0.50[b]	5.00±0.10[b]
BD (g/cm^3)	1.10±0.20[a]	1.10±0.10[a]	1.30±0.10[bcd]	1.28±0.02[bc]	1.40±0.01[cd]	1.42±0.04[d]
PD (g/cm^3)	2.68±0.02[d]	2.68±0.04[d]	2.46±0.02[b]	2.38±0.01[a]	2.52±0.04[c]	2.71±0.01[d]

Key: A1 = 5 Kg unpolluted agricultural soil + organic amendment; A2 = 5 Kg unpolluted agricultural soil; A3, A4, A5 and A6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively; MC = Moisture Content; BD = Bulk Density; and PD = Particle Density.

± Standard deviation

Values with the same superscript are not statistically different

Table 4.1b: Physical Parameters of Crude Oil-polluted Soil from Egbema before Plant Growth

Soil Sample Code /Parameters	B1	B2	B3	B4
Size Distribution				
Silt (%)	44.70±0.10^{c}	44.70±0.10^{c}	26.10±0.10^{b}	14.90±0.10^{a}
Clay (%)	45.50±0.10^{b}	45.50±0.10^{b}	20.70±0.20^{a}	20.60±0.10^{a}
Sand (%)	9.80±0.00^{a}	9.80±0.00^{a}	53.20±0.10^{b}	64.50±0.00^{c}
Soil Texture	Silty clay	Silty clay	Sandy clay loam	Sandy clay loam
Porosity (%)	39.28±0.85^{a}	39.29±0.08^{a}	44.21±0.60^{c}	41.51±0.70^{b}
MC (%)	10.00±0.20^{a}	10.00±0.30^{a}	12.50±0.10^{b}	15.50±0.10^{c}
BD (g/cm^3)	1.70±0.02^{c}	1.70±0.01^{c}	1.35±0.02^{a}	1.55±0.03^{b}
PD (g/cm^3)	2.80±0.01^{c}	2.80±0.02^{c}	2.42±0.01^{a}	2.65±0.02^{b}

Key: B1 = 5 Kg polluted soil + organic amendment; B2 = 5 Kg polluted soil; B3 = 1 Kg polluted soil + 4 Kg unpolluted soil + organic amendment; B4 = 2.5 Kg polluted + 2.5 Kg unpolluted soil + organic amendment respectively; MC = Moisture Content; BD = Bulk Density; and PD = Particle Density.

± Standard deviation

Values with the same superscript are not statistically different

Egbema-polluted soil with organic amendment recorded the highest bulk density (1.70g/cm^3) and particle density (2.80g/cm^3) while 1kg unpolluted soil + 4kg Egbema-polluted soil recorded the least bulk density (1.20g/cm^3) and particle density (2.42g/cm^3).

Table 4.2a showed the result of the physical properties of varying concentrations of crude oil polluted and unpolluted soil after phytoremediation (PGE I) using *H. cannabinus.* The highest percentage silt, clay and sand were recorded in 50ml/5kg (15.13%), 25ml/5kg (11.67%) and unpolluted soil without organic amendment (79.80%) respectively while the least values obtained were 9.84% (unpolluted soil without organic amendment), 9.10% (unpolluted soil with organic amendment) and 73.90% (25ml/5kg) for silt, clay and sand respectively. Soil textural class remained sandy loam for all soil samples after PGE I. The highest soil porosity and moisture content were 52.10% (unpolluted soil with organic amendment) and 10.00% (5ml/5kg, 10ml/5kg, 25ml/5kg and 50ml/5kg) while the least values obtained were 39.15% (50ml/5kg) and 5.00% (unpolluted soil with organic amendment and unpolluted soil without organic amendment). Bulk density was highest and lowest in 50ml/5kg (1.57g/cm^3) and unpolluted soil without organic amendment (1.10g/cm^3) respectively while particle density was highest and lowest in 25ml/5kg (2.68g/cm^3) and unpolluted soil without organic amendment (2.05g/cm^3) respectively after PGE I using *H. cannabinus.*

Physical parameters of crude oil-polluted soil from Egbema after phytoremediation (PGE II) using are *H. cannabinus* is shown in Table 4.2b. Egbema-polluted soil without organic amendment had the highest recorded percent silt (43.80%) and clay (40.80%) while 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil had the least recorded values of 17.20% and 14.50% for percent silt and clay respectively. Percent sand was highest in 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil (68.30%) and lowest in Egbema-polluted soil without organic amendment (15.40%). Soil textural class was clay (Egbema-polluted soil with organic amendment), silty clay (Egbema-polluted soil without organic amendment) and sandy loam (1kg unpolluted soil + 4kg Egbema-polluted soil and 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil) after plant growth indicating remediation of 1kg unpolluted soil + 4kg, Egbema-polluted soil and 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil. Soil porosity and moisture content were highest in 1kg unpolluted soil + 4kg Egbema-polluted soil (44.18%) and Egbema-polluted soil without organic amendment (27.90%) and lowest in Egbema-polluted soil without organic amendment (36.14%) and 1kg unpolluted soil + 4kg Egbema-polluted soil (15.30%). The highest bulk density and particle density were 1.82g/cm^3 and 2.85 g/cm^3 observed in sample Egbema-polluted soil without organic amendment while the least bulk

density and particle density were 1.20g/cm^3 and 2.15g/cm^3 recorded in 1kg unpolluted soil + 4kg Egbema-polluted soil.

The result of the physical properties of different concentration of crude oil-polluted soil and unpolluted soil after phytoremediation (PGE I) using *Z. mays* are shown in Table 4.3a. Sample 10ml/5kg had the highest percent silt (12.60%) and clay (16.60%) while the lowest percent silt (9.70%) and clay (9.50%) were recorded in unpolluted soil without organic amendment and unpolluted soil with organic amendment respectively. Percent sand was highest (80.10%) and lowest (70.80%) in unpolluted soil without organic amendment and 10ml/5kg respectively. The textural classes of soil samples were loamy sand (unpolluted soil with organic amendment, 5ml/5kg and 50ml/5kg) and sandy loam (unpolluted soil without organic amendment, 10ml/5kg and 25ml/5kg). Soil porosity was highest (55.43%) and least (38.46%) in unpolluted soil without organic amendment and 5ml/5kg respectively. Highest moisture content of 15.00% was recorded in sample 5ml/5kg while the least moisture content of 5.00% was recorded in unpolluted soil with organic amendment and unpolluted soil without organic amendment. The highest and least bulk densities of 1.60g/cm^3 and 1.15g/cm^3 were observed in samples 5ml/5kg and unpolluted soil without organic amendment. Additionally the highest particle density 2.65g/cm^3 and least particle density 2.50g/cm^3 were observed in samples 25ml/5kg and 10ml/5kg respectively.

Table 4.2a: Physical Parameters of Crude Oil-polluted and Unpolluted Soil Samples after Plant Growth using *H. cannabinus*

Soil Sample Code /Parameters	A1	A2	A3	A4	A5	A6
Size Distribution						
Silt (%)	13.50±0.00^{b}	9.84±0.15^{a}	14.70±0.10^{e}	13.80±0.20^{c}	14.43±0.06^{d}	15.13±0.06^{f}
Clay (%)	9.10±0.10^{a}	10.40±0.10^{b}	10.70±0.01^{cd}	10.90±0.10^{e}	11.67±0.06^{f}	10.77±0.06^{de}
Sand (%)	77.40±0.10^{e}	77.80±0.10^{f}	74.60±0.10^{c}	75.30±0.10^{d}	73.90±0.10^{a}	74.10±0.10^{b}
Soil Texture	Sandy loam	Sandy loam	Sandy loam	Sandy loam	Sandy loam	Sandy loam
Porosity (%)	52.10±0.00^{c}	46.30±0.00^{b}	45.50±0.00^{b}	41.34±0.00^{ab}	43.28±0.00^{ab}	39.15±0.00^{a}
MC (%)	5.00±0.00^{a}	5.00±0.00^{a}	10.00±0.00^{b}	10.00±0.0^{b}	10.00±0.00^{b}	10.00±0.00^{b}
BD (g/cm^{3})	1.25±0.01^{b}	1.10±0.00^{a}	1.38±0.00^{c}	1.49±0.00^{d}	1.52±0.00^{e}	1.57±0.00^{f}
PD (g/cm^{3})	2.61±0.00^{c}	2.05±0.00^{a}	2.53 ±0.00^{b}	2.54±0.00^{b}	2.68±0.00^{d}	2.58±0.00^{c}

Key: A1 = 5 Kg unpolluted agricultural soil + organic amendment; A2 = 5 Kg unpolluted agricultural soil; A3, A4, A5 and A6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively; MC = Moisture Content; BD = Bulk Density and PD = Particle Density.

± Standard deviation

Values with the same superscript are not statistically different

Table 4.2b: Physical Parameters of Crude Oil-polluted Soil from Egbema after Plant Growth using *H. cannabinus*

Soil Sample Code /Parameters	B1	B2	B3	B4
Size Distribution				
Silt (%)	30.50±0.00[c]	43.80±0.20[d]	23.60±0.10[b]	17.20±0.26[a]
Clay (%)	37.90±0.10[c]	40.80±0.30[d]	15.50±0.10[b]	14.50±0.10[a]
Sand (%)	31.60±0.10[b]	15.40±0.10[a]	60.90±0.17[c]	68.30±0.20[d]
Soil Texture	Clay	Silty clay	Sandy loam	Sandy loam
Porosity (%)	36.81±1.41[a]	36.14±0.13[a]	44.18±1.45[c]	40.17±1.54[b]
MC (%)	25.40±0.20[c]	27.90±0.10[d]	15.30±0.20[a]	20.60±0.10[b]
BD (g/cm^3)	1.75±0.02[c]	1.82±0.01[d]	1.20±0.02[a]	1.40±0.03[b]
PD (g/cm^3)	2.77±0.03[c]	2.85±0.01[d]	2.15±0.02[a]	2.34±0.01[b]

Key: B1 = 5 Kg polluted soil + organic amendment; B2 = 5 Kg polluted soil; B3 = 1 Kg polluted soil + 4 Kg unpolluted soil + organic amendment; B4 = 2.5 Kg polluted + 2.5 Kg unpolluted soil + organic amendment respectively; MC = Moisture Content; BD = Bulk Density and PD = Particle Density.

± Standard deviation

Values with the same superscript are not statistically different

Table 4.3a: Physical Parameters of Crude Oil-Polluted and Unpolluted Soil Samples after Plant Growth using *Z. mays*

Soil Sample Code /Parameters	F1	F2	F3	F4	F5	F6
Silt (%)	11.20±0.02[d]	9.70±0.02[a]	11.30±0.01[d]	12.60±0.10[e]	10.93±0.10[c]	10.70±0.10[b]
Clay (%)	9.50±0.02[a]	10.20±0.10[b]	12.50±0.02[c]	16.60±0.10[e]	9.50±0.10[a]	13.50±0.10[d]
Sand (%)	79.30±0.01[d]	80.10±0.02[f]	76.20±0.10[c]	70.80±0.10[a]	79.60±0.02[e]	75.80±0.20[b]
Soil Texture	Loamy sand	Sandy loam	Loamy sand	Sandy loam	Sandy loam	Loamy sand
Porosity (%)	48.41±1.03[b]	55.43±0.43[c]	38.46±2.06[a]	43.53±1.25[a]	41.51±0.53[a]	42.31±0.55[a]
MC (%)	5.00±0.10[a]	5.00±0.20[a]	15.00±0.20[c]	10.00±0.30[b]	10.00±0.10[b]	10.00±0.20[b]
BD (g/cm^3)	1.30±0.03[b]	1.15±0.02[a]	1.60±0.05[e]	1.42±0.04[bcd]	1.55±0.01[de]	1.50±0.02[cd]
PD (g/cm^3)	2.52±0.01[a]	2.58±0.02[b]	2.60±0.01[b]	2.50±0.03[a]	2.65±0.01[c]	2.60±0.01[b]

Key: F1 = 5 Kg unpolluted agricultural soil + organic amendment; F2 = 5 Kg unpolluted agricultural soil; F3, F4, F5 and F6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively;

± Standard deviation

Values with the same superscript are not statistically different

Values of physical properties of crude oil-polluted soil from Egbema after plant growth II using *Z. mays* are shown in Table 4.3b. For the size distribution, Egbema-polluted soil with organic amendment, Egbema-polluted soil without organic amendment and 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil had the highest percent silt (35.80%), clay (38.10%) and sand (70.20%) respectively while 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil, 1kg unpolluted soil + 4kg Egbema-polluted soil and Egbema-polluted soil without organic amendment had the least percent silt (13.30%), clay (12.20%) and sand (25.70%) respectively. Soil textural class was loam for sample Egbema-polluted soil with organic amendment, clay for Egbema-polluted soil without organic amendment and sandy loam for 1kg unpolluted soil + 4kg Egbema-polluted soil and 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil. This indicated soil structure restoration for 1kg unpolluted soil + 4kg Egbema-polluted soil and 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil and nutrient depletion for sample Egbema-polluted soil without organic amendment. Moreover, addition of organic amendment improved the soil texture of sample Egbema-polluted soil with organic amendment. The highest and least values of soil porosity were 36.23% (Egbema-polluted soil without organic amendment) and 21.23% (2.5kg unpolluted soil + 2.5kg Egbema-polluted soil). Moisture content was highest in Egbema-polluted soil without organic amendment (28.10%) and least in 1kg unpolluted soil + 4kg Egbema-polluted soil (16.70%). The highest recorded values for bulk density and particle density were 1.69g/cm^3 and 2.65g/cm^3 in Egbema-polluted soil without organic amendment while the least values obtained for bulk density and particle density were 1.27g/cm^3 and 1.71g/cm^3 in 1kg unpolluted soil + 4kg Egbema-polluted soil and 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil respectively.

Chemical parameters of different concentrations of crude oil-polluted soil before phytoremediation are shown in Table 4.4a. pH values ranged from 6.70 (50ml/5kg) to 7.30 (unpolluted soil with organic amendment and unpolluted soil without organic amendment) and were within World Health Organisation (WHO) and Federal Ministry of Environment (FMEnv) Nigeria limits of 6.5 to 8.0 and 6.5 to 8.5 respectively. Electrical conductivity was highest in 5ml/5kg (67.00µS/cm) and lowest in unpolluted soil with organic amendment (30.00µS/cm). However, values were below FMEnv limit of 1000µS/cm. Calcium and magnesium content of soil samples were highest in 5ml/5kg with respective values of 25.38mg/kg and 17.28mg/kg. The least values obtained for calcium and magnesium were 11.54mg/kg and 7.85mg/kg in unpolluted soil with organic amendment and unpolluted soil without organic amendment. Sample 5ml/5kg had the highest sodium (30.99mg/kg) and potassium (35.87mg/kg) content while unpolluted soil with organic amendment had the least sodium (14.08mg/kg) and

potassium (16.30mg/kg) content. Ammonium, nitrate and sulphate levels were highest in 5ml/5kg with values 15.98mg/kg, 57.39mg/kg and 42.86 mg/kg respectively and lowest in samples unpolluted soil with organic amendment and unpolluted soil with out organic amendment with respective values of 7.26mg/kg, 26.09mg/kg and 19.48mg/kg. The highest results recorded for bicarbonate, organic carbon and organic matter were 92.31mg/kg (5ml/5kg), 0.66% (10ml/5kg) and 1.37% (10ml/5kg) respectively while unpolluted soil with organic amendment was lowest in bicarbonate (41.96mg/kg), organic carbon (0.32%) and organic matter (0.64%). The total available nitrogen was highest in unpolluted soil with organic amendment (0.077%) and lowest in sample 10ml/5kg (0.053%). Phosphorus level was highest in 5ml/5kg (0.470mg/kg) and lowest in 25ml/5kg (0.390mg/kg) before plant growth experiment (PGE).

Table 4.4b shows the chemical properties of crude oil polluted soil from Egbema before plant growth experiment. Soil samples were predominantly acidic with a range of 4.80 (Egbema-polluted soil with organic amendment) to 6.60 (1kg unpolluted soil + 4kg Egbema-polluted soil) showing severe pollution of soils. The acidic pH values were below the WHO and FMEnv standards of 6-5-8.0 and 6.5-8.5 respectively indicating need for remediation of soils. Soil electrical conductivity was highest in samples Egbema-polluted soil with organic amendment and Egbema-polluted soil without organic amendment (180.00µS/cm) and lowest in 1kg unpolluted soil + 4kg Egbema-polluted soil (85.00µS/cm). Values were within the limit of 1000µS/cm set by FMEnv Nigeria. The highest values recorded for soil calcium, magnesium and sodium were 7.83mg/kg (2.5kg unpolluted soil + 2.5kg Egbema-polluted soil), 4.53mg/kg (1kg unpolluted soil + 4kg Egbema-polluted soil) and 9.31mg/kg (2.5kg unpolluted soil + 2.5kg Egbema-polluted soil) respectively while the least values obtained were 3.45mg/kg (Egbema-polluted soil with organic amendment), 2.56mg/kg (Egbema-polluted soil with organic amendment, Egbema-polluted soil without organic amendment) and 7.15mg/kg (Egbema-polluted soil with organic amendment, Egbema-polluted soil without organic amendment) for calcium, magnesium and sodium respectively. Sample 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil recorded the highest levels of potassium (5.54mg/kg) and ammonium (6.48mg/kg) while Egbema-polluted soil with organic amendment and Egbema-polluted soil without organic amendment recorded the lowest levels of potassium (3.78mg/kg) and ammonium (5.36mg/kg).

Table 4.3b: Physical Parameters of Soil Samples from Egbema after Plant Growth using *Z. mays*

Soil Sample Code /Parameters	G1	G2	G3	G4
Silt (%)	35.80±0.10[c]	36.20±0.20[d]	23.50±0.30[b]	13.30±0.10[a]
Clay (%)	29.40±0.00[c]	38.10±0.10[d]	12.20±0.20[a]	16.50±0.00[b]
Sand (%)	34.80±0.10[b]	25.70±0.30[a]	64.30±0.10[c]	70.20±0.10[d]
Soil Texture	Loam	Clay	Sandy loam	Sandy loam
Porosity (%)	35.24±0.81[bc]	36.23±0.70[c]	34.20±0.88[b]	21.23±0.10[a]
MC (%)	26.00±0.20[c]	28.10±0.10[d]	16.70±0.20[a]	20.30±0.20[b]
BD (g/cm^3)	1.58±0.01[c]	1.69±0.02[d]	1.27±0.03[a]	1.35±0.01[b]
PD (g/cm^3)	2.44±0.02[c]	2.65±0.01[d]	1.93±0.02[b]	1.71±0.03[a]

Key: G1 = 5 Kg polluted soil + organic amendment; G2 = 5 Kg polluted soil; G3 = 1 Kg polluted soil + 4 Kg unpolluted soil + organic amendment; G4 = 2.5 Kg polluted + 2.5 Kg unpolluted soil + organic amendment respectively; MC = Moisture Content; BD = Bulk Density and PD = Particle Density.

± Standard deviation

Values with the same superscript are not statistically different

Table 4.4a: Chemical Parameters of Crude Oil-polluted and Unpolluted Soil Samples before Plant Growth

Soil Sample Code /Parameters	A1	A2	A3	A4	A5	A6	FM Env Std	WHO Std
pH	7.30 ±0.10^{c}	7.30 ±0.10^{c}	7.10 ±0.10^{b}	6.80 ±0.10^{a}	6.80 ±0.10^{a}	6.70 ±0.10^{a}	6.5-8.5	6.5-8.0
EC(µS/cm)	30.00±2.00^{a}	30.00 ±2.00^{a}	67.00±1.00^{e}	44.00±1.00^{c}	52.00 ±1.00^{d}	34.00±1.00^{b}	1000	--
Ca^{2+}(mg/Kg)	11.54±0.01^{a}	11.54 ±0.01^{a}	25.38±0.01^{e}	16.92±0.01^{c}	20.00 ±0.00^{d}	13.70±0.01^{b}	--	100-300
Mg^{2+}(mg/Kg)	7.85 ±0.01^{a}	7.85 ±0.01^{a}	17.28±0.02^{e}	11.52±0.02^{c}	13.61 ±0.01^{d}	8.90 ±0.02^{b}	20	--
Na^{+}(mg/Kg)	14.08±0.01^{a}	14.08 ±0.01^{a}	30.99±0.01^{e}	20.66±0.01^{c}	24.41 ±0.01^{d}	15.96±0.02^{b}	200	200
K^{+}(mg/Kg)	16.30±0.10^{a}	16.30 ±0.10^{a}	35.87±0.01^{e}	23.91±0.02^{c}	28.26 ±0.02^{d}	18.47±0.01^{b}	--	8.0
NH_4^{+}1(mg/Kg)	7.26 ±0.01^{a}	7.26 ±0.01^{a}	15.98±0.01^{e}	10.65±0.01^{c}	12.59 ±0.02^{d}	8.23 ±0.02^{b}	--	35
NO_3^{-}(mg/Kg)	26.09±0.01^{a}	26.09 ±0.01^{a}	57.39±0.01^{e}	38.26±0.02^{c}	45.22 ±0.02^{d}	29.58±0.01^{b}	50	--
SO_4^{2-}(mg/Kg)	19.48±0.01^{a}	19.48 ±0.01^{a}	42.86±0.01^{e}	28.57±0.02^{c}	33.77 ±0.02^{d}	22.08±0.01^{b}	100	250
HCO_3^{-}(mg/Kg)	41.96±0.02^{a}	41.96 ±0.02^{a}	92.31±0.01^{e}	61.54±0.01^{c}	72.75 ±0.02^{d}	47.55±0.01^{b}	--	100
OC (%)	0.32 ±0.01^{a}	0.32 ±0.01^{a}	0.32 ±0.01^{a}	0.66±0.02^{b}	0.32±0.01^{a}	0.64 ±0.01^{b}	--	--
OM (%)	0.64 ±0.01^{a}	0.64 ±0.01^{a}	0.69 ±0.01^{b}	1.37±0.02^{c}	0.65±0.01^{a}	1.28 ±0.02^{c}	--	--
N (%)	0.077±0.001^{d}	0.077±0.001^{d}	0.063±0.002^{c}	0.053±0.001^{a}	0.063±0.002^{c}	0.060±0.001^{b}	--	--
P(mg/Kg)	0.400 ±0.000^{b}	0.400±0.000^{b}	0.470±0.01^{d}	0.430±0.020^{c}	0.390±0.01^{ab}	0.400±0.010^{b}	--	0.01-0.19

Key: A1 = 5 Kg unpolluted agricultural soil + organic amendment; A2 = 5 Kg unpolluted agricultural soil; A3, A4, A5 and A6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively; EC = Electrical Conductivity; Ca^{2+} = Calcium; Mg^{2+} = Magnesium; Na^{+} = Sodium; K^{+} = Potassium; NH_4^{+} = Ammonium; NO_3^{-} = Nitrate; SO_4^{2-} = Sulphate; HCO_3^{-} = Biocarbonate; OC = Organic carbon; OM = Organic matter; N = Nitrogen and P = Phosphorus.

± Standard deviation

Values with the same superscript are not statistically different

Table 4.4b: Chemical Parameters of Crude Oil-polluted Soil from Egbema before Plant Growth

Soil Sample Code /Parameters	B1	B2	B3	B4	FMEnv Std	WHO Std
pH	4.80 ±0.10[a]	4.80 ±0.10[a]	6.60 ±0.20[c]	5.90 ±0.10[b]	6.5-8.5	6.5-8.0
EC (µS/cm)	180.00±2.00[c]	180.00±2.00[c]	85.00 ±1.00[a]	126.00±2.00[b]	1000	--
Ca^{2+} (mg/Kg)	3.45 ±0.01[a]	3.46 ±0.01[a]	5.32 ±0.01[b]	7.83 ±0.01[c]	--	100-300
Mg^{2+} (mg/Kg)	2.56 ±0.02[a]	2.56 ±0.01[a]	4.53 ±0.01[c]	3.96 ±0.02[b]	20	--
Na^{+} (mg/Kg)	7.15 ±0.02[a]	7.15 ±0.02[a]	7.88 ±0.02[b]	9.31 ±0.01[c]	200	200
K^{+} (mg/Kg)	3.78 ±0.02[a]	3.78 ±0.02[a]	4.11 ±0.01[b]	5.54 ±0.01[c]	--	8.0
NH_4^{+} (mg/Kg)	5.36 ±0.01[a]	5.36 ±0.01[a]	6.15 ±0.02[b]	6.48 ±0.01[c]	--	35
NO_3^{-} (mg/Kg)	54.93 ±0.02[c]	54.93 ±0.02[c]	29.32 ±0.01[a]	37.40 ±0.02[b]	50	--
SO_4^{2-} (mg/Kg)	42.61 ±0.01[c]	42.61 ±0.01[c]	23.56 ±0.01[b]	21.91 ±0.01[a]	100	250
HCO_3^{-} (mg/Kg)	35.19 ±0.01[b]	35.19 ±0.01[b]	25.29 ±0.02[a]	38.67 ±0.01[c]	--	100
OC (%)	2.45 ±0.02[c]	2.45 ±0.02[c]	0.86 ±0.01[a]	1.79 ±0.01[b]	--	--
OM (%)	3.88 ±0.01[c]	3.88 ±0.01[c]	1.54 ±0.02[a]	2.62 ±0.01[b]	--	--
N (%)	0.081 ±0.001[b]	0.081 ±0.001[b]	0.078 ±0.001[a]	0.085 ±0.001[c]	--	--
P (mg/Kg)	0.250 ±0.010[a]	0.250 ±0.010[a]	0.290 ±0.010[b]	0.320 ±0.010[c]	--	0.01-0.19

Key: B1 = 5 Kg polluted soil + organic amendment; B2 = 5 Kg polluted soil –organic amendment; B3 = 1 Kg polluted soil + 4 Kg unpolluted soil + organic amendment; B4 = 2.5 Kg polluted + 2.5 Kg unpolluted soil + organic amendment. EC = Electrical Conductivity; Ca^{2+} = Calcium; Mg^{2+} = Magnesium; Na^{+} = Sodium; K^{+} = Potassium; NH_4^{+} = Ammonium; NO_3^{-} = Nitrate; SO_4^{2-} = Sulphate; HCO_3^{-} = Biocarbonate; OC = Organic carbon; OM = Organic matter; N = Nitrogen and P = Phosphorus.

± Standard deviation

Values with the same superscript are not statistically different

Nitrate and sulphate values were greatest in Egbema-polluted soil with organic amendment with values 54.93mg/kg and 42.61mg/kg respectively and lowest in 1kg unpolluted soil + 4kg Egbema-polluted soil (29.32mg/kg) and 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil (21.91mg/kg) for nitrate and sulphate respectively. The highest values recorded for soil bicarbonate was 38.67mg/kg (2.5kg unpolluted soil + 2.5kg Egbema-polluted soil) while the least value was 25.29mg/kg (1kg unpolluted soil + 4kg Egbema-polluted soil). Egbema-polluted soil with organic amendment and Egbema-polluted soil without organic amendment had the highest soil organic carbon (2.45%) and organic matter (3.88%) content while 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil (1.79%) and 1kg unpolluted soil + 4kg Egbema-polluted soil (1.54%) were lowest for organic carbon and matter respectively. Total available nitrogen and phosphorus were highest in 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil with respective values of 0.085% and 0.320mg/kg and lowest in 1kg unpolluted soil + 4kg Egbema-polluted soil (0.078%) and Egbema-polluted soil with organic amendment (0.250mg/kg) respectively.

The result of the chemical properties of varying concentrations of crude oil polluted soil after phytoremediation (PGE I) using *H. cannabinus* are presented in Table 4.5a. Soil pH values generally increased with range 7.60 (unpolluted soil without organic amendment) to 8.20 (unpolluted soil with organic amendment) which were within the acceptable limits indicating soil remediation. Electrical conductivity reduced with range 18.50µS/cm (unpolluted soil without organic amendment) to 29.80µS/cm (10ml/5kg). Calcium, magnesium and sodium levels were greatest in 25ml/5kg (14.94mg/kg), 10ml/5kg (7.95mg/kg) and 25ml/5kg (16.59mg/kg) respectively and lowest in unpolluted soil without organic amendment (6.30mg/kg), 50ml/5kg (4.61mg/kg) and unpolluted soil without organic amendment (9.84mg/kg) respectively. The highest recorded values of potassium, ammonium and nitrate were observed in 10ml/5kg (16.04mg/kg), 5ml/5kg (7.82mg/kg) and 5ml/5kg (25.21mg/kg) while the least values recorded were 10.11mg/kg (50ml/5kg), 5.72mg/kg (unpolluted soil without organic amendment) and 17.25mg/kg (unpolluted soil without organic amendment) after plant growth respectively. Sulphate and bicarbonate were highest in 5ml/5kg (29.63mg/kg) and 25ml/5kg (38.74mg/kg) and lowest in unpolluted soil without organic amendment (13.89mg/kg) and unpolluted soil without organic amendment (32.03mg/kg) respectively. Sample 50ml/5kg had the highest recorded organic carbon (0.45%) and organic matter (0.95%) while unpolluted soil without organic amendment had the least organic carbon (0.15%) and organic matter (0.50%). The highest levels for available

nitrogen were observed in 5ml/5kg and 10ml/5kg (0.051%) while the least values were recorded in 50ml/5kg (0.032%). After plant growth, phosphorus was highest in 25ml/5kg (0.380mg/kg) and lowest in unpolluted soil without organic amendment (0.150mg/kg).

Values of the chemical parameters of crude oil-polluted soil from Egbema after phytoremediation (PGE II) using *H. cannabinus* are shown in Table 4.5b. Increase in pH values were observed with 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil having the highest pH values of 6.10 while Egbema-polluted soil without organic amendment had the least pH values for all samples. Although there was an increase in pH values, results were however still below WHO and FMEnv standards of 6.5-8.0 and 6.5-8.5 respectively. Electrical conductivity reduced across all samples with a range of 74.00µS/cm (1kg unpolluted soil + 4kg Egbema-polluted soil) to 130µS/cm (Egbema-polluted soil with organic amendment). Samples 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil and 1kg unpolluted soil + 4kg Egbema-polluted soil had the highest calcium (7.83mg/kg) and magnesium (2.50mg/kg) content respectively while Egbema-polluted soil without organic amendment had the least calcium (1.81mg/kg) and magnesium (1.22mg/kg) values. The highest values obtained for sodium and potassium were 4.25mg/kg (Egbema-polluted soil with organic amendment) and 3.59mg/kg (2.5kg unpolluted soil + 2.5kg Egbema-polluted soil) while the least values recorded after plant growth were 3.45mg/kg (1kg unpolluted soil + 4kg Egbema-polluted soil) and 2.78mg/kg) (1kg unpolluted soil + 4kg Egbema-polluted soil). Ammonium, nitrate and sulphate levels after PGE II were highest in Egbema-polluted soil with organic amendment (4.80mg/kg), Egbema-polluted soil with organic amendment (48.28mg/kg) and Egbema-polluted soil without organic amendment (28.13mg/kg) respectively and lowest in 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil (4.14mg/kg), 1kg unpolluted soil + 4kg Egbema-polluted soil (25.36mg/kg) and 1kg unpolluted soil + 4kg Egbema-polluted soil (16.72mg/kg) respectively. Bicarbonate level was highest in 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil (25.28mg/kg) and lowest in Egbema-polluted soil without organic amendment (20.20mg/kg). Egbema-polluted soil without organic amendment had the highest organic carbon (2.12%) and organic matter (3.51%) recorded after PGE II while sample had the least organic carbon (0.45%) and organic matter (0.64%). Available nitrogen and phosphorus were highest in Egbema-polluted soil without organic amendment (0.074%) and 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil (0.280mg/kg) and lowest in 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil (0.056mg/kg) and Egbema-polluted soil with organic amendment (0.210mg/kg).

Chemical parameters of different concentrations of crude oil-polluted and unpolluted soil samples after phytoremediation (PGE I) using *Z. mays* are presented in Table 4.6a. pH values of all soil samples were recorded as 8.40 except the unpolluted agricultural soil without organic amendment (7.10). Test pot for 10ml/5kg had the highest recorded electrical conductivity values (27.00µS/cm) and calcium content (16.38mg/kg) while 50ml/5kg had the least values for both electrical conductivity (14.70µS/cm) and calcium (5.65mg/kg). The highest values obtained for magnesium, sodium and potassium were 7.08mg/kg, 12.67mg/kg and 14.68mg/kg respectively all in sample 10ml/5kg while the least values obtained were 3.85mg/kg, 6.90mg/kg and 7.99mg/kg respectively all in 50ml/5kg sample. Ammonium, nitrate and sulphate levels were greatest in 10ml/5kg (6.54mg/kg), 10ml/5kg (23.48mg/kg) and 10ml/5kg (17.53mg/kg) respectively and lowest in 50ml/5kg with respective values 3.56mg/kg, 12.78mg/kg and 9.56mg/kg. Bicarbonate levels were highest in 5ml/5kg (37.48mg/kg) and lowest in 50ml/5kg (20.55mg/kg). The highest values recorded for organic carbon and organic matter were 0.42% (50ml/5kg) and 0.86% (50ml/5kg) while the least values obtained were 0.10% (unpolluted soil with organic amendment) and 0.21% (unpolluted soil with organic amendment) respectively. The highest values observed for available nitrogen and phosphorus after plant growth were 0.054% (10ml/5kg) and 0.35mg/kg (10ml/5kg) respectively while the least values observed were 0.028% (50ml/5kg) and 0.20mg/kg (unpolluted soil without organic amendment) respectively.

Table 4.5a: Chemical Parameters of Crude Oil-polluted and Unpolluted Soil Samples after Plant Growth using *H. cannabinus*

Soil Sample Code /Parameters	A1	A2	A3	A4	A5	A6	FMEnv Std	WHO Std
pH	8.20 ±0.10^{d}	7.60 ±0.10^{a}	8.00 ±0.10^{c}	7.80 ±0.10^{b}	7.80 ±0.10^{b}	7.60 ±0.10^{a}	6.5-8.5	6.5-8.0
EC(µS/cm)	23.40 ±0.10^{b}	18.50 ±0.20^{a}	27.40 ±0.10^{d}	29.80 ±0.10^{e}	25.60 ±0.10^{c}	20.10 ±0.10^{a}	1000	--
Ca^{2+}(mg/Kg)	9.23 ±0.02^{c}	6.30 ±0.02^{a}	11.43 ±0.02^{d}	14.94 ±0.01^{f}	13.15 ±0.01^{e}	7.61 ±0.02^{b}	--	100-300
Mg^{2+}(mg/Kg)	6.09 ±0.02^{c}	5.37 ±0.01^{b}	7.08 ±0.01^{e}	7.95 ±0.01^{f}	6.23 ±0.02^{d}	4.61 ±0.02^{a}	20	--
Na^{+}(mg/Kg)	11.35 ±0.02^{c}	9.84 ±0.02^{a}	13.66 ±0.02^{d}	15.46 ±0.02^{e}	16.59 ±0.02^{f}	10.24 ±0.01^{b}	200	200
K^{+}(mg/Kg)	12.03 ±0.02^{c}	10.20 ±0.10^{b}	15.79 ±0.01^{e}	16.04 ±0.02^{f}	13.23 ±0.01^{d}	10.11 ±0.01^{a}	--	8.0
NH_4^{+}(mg/Kg)	5.96 ±0.02^{b}	5.72 ±0.02^{a}	7.82 ±0.02^{e}	7.67 ±0.02^{d}	6.95 ±0.01^{c}	5.94 ±0.02^{b}	--	35
NO_3^{-} (mg/Kg)	20.46 ±0.02^{c}	17.25 ±0.01^{a}	25.21 ±0.01^{f}	24.84 ±0.01^{e}	22.19 ±0.01^{d}	19.44 ±0.01^{b}	50	--
SO_4^{2-} (mg/Kg)	14.37 ±0.01^{b}	13.89 ±0.01^{a}	29.63 ±0.10^{f}	25.31 ±0.01^{e}	23.07 ±0.02^{d}	16.15 ±0.01^{c}	100	250
HCO_3^{-} (mg/Kg)	35.62 ±0.02^{c}	32.03 ±0.02^{a}	41.44 ±0.02^{f}	39.65 ±0.01^{e}	38.74 ±0.01^{d}	32.13 ±0.01^{b}	--	100
OC (%)	0.22 ±0.02^{b}	0.15 ±0.01^{a}	0.38 ±0.01^{e}	0.32 ±0.02^{d}	0.28 ±0.01^{c}	0.45 ±0.01^{f}	--	--
OM (%)	0.53 ±0.02^{ab}	0.50 ±0.01^{a}	0.55 ±0.02^{b}	0.89 ±0.02^{c}	0.51 ±0.01^{a}	0.95 ±0.02^{d}	--	--
N (%)	0.047±0.001^{c}	0.042±0.001^{b}	0.051 ±0.002^{d}	0.051±0.001^{d}	0.049±0.001^{c} ^{d}	0.032 ±0.001^{a}	--	--
P(mg/Kg)	0.300 ±0.020^{b}	0.150 ±0.010^{a}	0.370 ±0.020^{c}	0.310±0.010^{b}	0.380 ±0.020^{c}	0.360 ±0.010^{c}	--	0.01-0.19

Key: A1 = 5 Kg unpolluted agricultural soil + organic amendment; A2 = 5 Kg unpolluted agricultural soil; A3, A4, A5 and A6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively; EC = Electrical Conductivity; Ca^{2+} = Calcium; Mg^{2+} = Magnesium; Na^{+} = Sodium; K^{+} = Potassium; NH_4^{+} = Ammonium; NO_3^{-} = Nitrate; SO_4^{2-} = Sulphate; HCO_3^{-} = Biocarbonate; OC = Organic carbon; OM = Organic matter; N = Nitrogen and P = Phosphorus.

± Standard deviation

Values with the same superscript are not statistically different

Table 4.5b: Chemical Parameters of Crude Oil-polluted Soil from Egbema after Plant Growth using *H. cannabinus*

Soil Sample Code /Parameters	B1	B2	B3	B4	FMEnv Std	WHO Std
pH	5.40 ±0.10^{b}	5.10 ±0.10^{a}	5.80 ±0.20^{c}	6.10 ±0.10^{d}	6.5-8.5	6.5-8.0
EC(µS/cm)	130.00±2.00^{d}	105.00 ±1.00^{c}	74.00 ±1.00^{a}	88.00 ±2.00^{b}	1000	--
Ca^{2+}(mg/Kg)	2.13 ±0.01^{b}	1.81 ±0.01^{a}	2.91 ±0.01^{c}	3.38 ±0.02^{d}	--	100-300
Mg^{2+}(mg/Kg)	1.67 ±0.02^{b}	1.22 ±0.02^{a}	2.50 ±0.01^{d}	2.06 ±0.01^{c}	20	--
Na^{+}(mg/Kg)	4.25 ±0.01^{d}	4.04 ±0.01^{c}	3.45 ±0.01^{a}	3.82 ±0.01^{b}	200	200
K^{+}(mg/Kg)	3.50 ±0.10^{c}	3.16 ±0.10^{b}	2.78 ±0.01^{a}	3.59 ±0.10^{c}	--	8.0
NH_4^{+}(mg/Kg)	4.80 ±0.02^{c}	4.68 ±0.01^{b}	4.16 ±0.01^{a}	4.14 ±0.01^{a}	--	35
NO_3^{-}(mg/Kg)	48.28 ±0.02^{d}	45.60 ±0.02^{c}	25.36 ±0.01^{a}	32.15 ±0.02^{b}	50	--
SO_4^{2-}(mg/Kg)	36.61 ±0.01^{d}	26.43 ±0.01^{c}	15.00 ±0.10^{a}	18.87 ±0.01^{b}	100	250
HCO_3^{-}(mg/Kg)	23.95 ±0.02^{c}	20.20 ±0.01^{a}	21.54 ±0.01^{b}	25.28 ±0.01^{d}	--	100
OC(%)	1.58 ±0.02^{c}	2.12 ±0.02^{d}	0.45 ±0.01^{a}	0.67 ±0.02^{b}	--	--
OM(%)	3.11 ±0.01^{c}	3.51 ±0.01^{d}	0.64 ±0.02^{a}	1.95 ±0.01^{b}	--	--
N(%)	0.069 ±0.001^{c}	0.074 ±0.001^{d}	0.063 ±0.002^{b}	0.056 ±0.001^{a}	--	--
P(mg/Kg)	0.210 ±0.020^{a}	0.230 ±0.010^{b}	0.260 ±0.020^{bc}	0.280 ±0.020^{c}	--	0.01-0.19

Key: B1 = 5 Kg polluted soil + organic amendment; B2 = 5 Kg polluted soil; B3 = 1 Kg polluted soil + 4 Kg unpolluted soil + organic amendment; B4 = 2.5 Kg polluted + 2.5 Kg unpolluted soil + organic amendment. EC = Electrical Conductivity; Ca^{2+} = Calcium; Mg^{2+} = Magnesium; Na^{+} = Sodium; K^{+} = Potassium; NH_4^{+} = Ammonium; NO_3^{-} = Nitrate; SO_4^{2-} = Sulphate; HCO_3^{-} = Biocarbonate; OC = Organic carbon; OM = Organic matter; N = Nitrogen and P = Phosphorus.
± Standard deviation

Values with the same superscript are not statistically different

Table 4.6a: Chemical Parameters of Crude Oil-polluted and Unpolluted Soil Samples after Plant Growth using *Z. mays*

Soil Sample Code /Parameters	F1	F2	F3	F4	F5	F6	FM Env Std	WHO Std
pH	8.40 ±0.10^{b}	7.10 ±0.10^{a}	8.40 ±0.10^{b}	8.40 ±0.10^{b}	8.40 ±0.10^{b}	8.40 ±0.10^{b}	6.5-8.5	6.5-8.0
EC(μS/cm)	19.60±0.10^{c}	16.50 ±0.10^{b}	26.80±0.10^{e}	27.00±0.10^{e}	23.40 ±0.10^{d}	14.70±0.10^{a}	1000	--
Ca^{2+}(mg/Kg)	7.54 ±0.02^{b}	8.57 ±0.01^{c}	10.31±0.01^{e}	16.38±0.01^{f}	9.00 ±0.01^{d}	5.65 ±0.02^{a}	--	100-300
Mg^{2+}(mg/Kg)	5.13 ±0.02^{b}	4.33 ±0.01^{c}	7.02 ±0.01^{e}	7.08 ±0.01^{f}	6.13 ±0.02^{d}	3.85 ±0.02^{a}	20	--
Na^{+}(mg/Kg)	9.20 ±0.02^{b}	7.04 ±0.01^{c}	12.58±0.02^{e}	12.67±0.01^{f}	10.99 ±0.01^{d}	6.90 ±0.01^{a}	200	200
K^{+}(mg/Kg)	10.65±0.02^{b}	11.31 ±0.01^{c}	14.58±0.01^{e}	14.68±0.01^{f}	12.72 ±0.01^{d}	7.99 ±0.01^{a}	--	8.0
NH_4^{+}(mg/Kg)	4.75 ±0.01^{b}	3.57 ±0.01^{a}	6.49 ±0.01^{d}	6.54 ±0.01^{e}	5.67 ±0.01^{c}	3.56 ±0.01^{a}	--	35
NO_3^{-}(mg/Kg)	17.04±0.02^{c}	15.66 ±0.02^{b}	23.30±0.01^{e}	23.48±0.02^{f}	20.35 ±0.01^{d}	12.78±0.01^{a}	50	--
SO_4^{2-} (mg/Kg)	12.7±0.01^{c}	10.30 ±0.01^{b}	17.40±0.01^{e}	17.53±0.02^{f}	15.19 ±0.02^{d}	9.56 ±0.01^{a}	100	250
HCO_3^{-} (mg/Kg)	27.41±0.01^{c}	24.49±0.01^{b}	37.48±0.01^{e}	32.76±0.01^{f}	32.72 ±0.01^{d}	20.55±0.01^{a}	--	100
OC (%)	0.10 ±0.02^{a}	0.25 ±0.01^{c}	0.30 ±0.02^{d}	0.20 ±0.02^{b}	0.24 ±0.02^{c}	0.42 ±0.02^{e}	--	--
OM (%)	0.21 ±0.01^{a}	0.36 ±0.02^{b}	0.61 ±0.01^{e}	0.42 ±0.02^{c}	0.48 ±0.02^{d}	0.86 ±0.02^{f}	--	--
N (%)	0.038±.001^{b}	0.030 ±0.002^{a}	0.053±.001^{d}	0.054±.001^{d}	0.046 ±0.002^{c}	0.028±.001^{a}	--	--
P(mg/Kg)	0.250±.010^{c}	0.200 ±0.010^{a}	0.220±.010^{b}	0.350±.020^{e}	0.300±0.010^{d}	0.290±.010^{d}	--	0.01-0.19

Key: F1 = 5 Kg unpolluted agricultural soil + organic amendment; F2 = 5 Kg unpolluted agricultural soil; F3, F4, F5 and F6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively; EC = Electrical Conductivity; Ca^{2+} = Calcium; Mg^{2+} = Magnesium; Na^{+} = Sodium; K^{+} = Potassium; NH_4^{+} = Ammonium; NO_3^{-} = Nitrate; SO_4^{2-} = Sulphate; HCO_3^{-} = Biocarbonate; OC = Organic carbon; OM = Organic matter; N = Nitrogen; P = Phosphorus.
± Standard deviation

Values with the same superscript are not statistically different

Table 4.6b shows the result of the chemical properties of crude oil-polluted soil from Egbema after phytoremediation (PGE II) using *Z. mays*. The highest pH value 6.90 was obtained in 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil while the least pH value 5.20 was obtained in Egbema-polluted soil without organic amendment. All samples were below WHO standard of 6.5-8.0 except 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil (6.90). This indicates that 90 days was not sufficient to remediate polluted soil from Egbema. Moreover, introduction of unpolluted agricultural soil (1kg unpolluted soil + 4kg Egbema-polluted soil, 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil) as composites offered greater remediation than sole remediation of crude oil polluted soil. The highest values recorded for soil electrical conductivity and calcium were 118µS/cm (Egbema-polluted soil with organic amendment) and 3.14mg/kg (2.5kg unpolluted soil + 2.5kg Egbema-polluted soil) respectively. Magnesium, sodium and potassium levels of soil samples were highest in 1kg unpolluted soil + 4kg Egbema-polluted soil (2.31mg/kg), 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil (5.27mg/kg) and 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil (3.62mg/kg) respectively. Sample Egbema-polluted soil without organic amendment had the least recorded values of 1.08mg/kg, 2.60mg/kg and 2.03mg/kg for magnesium, sodium and potassium after plant growth. Sample Egbema-polluted soil with organic amendment had the highest ammonium (4.13mg/kg), nitrate (36.54mg/kg) and sulphate (31.10mg/kg) levels while 1kg unpolluted soil + 4kg Egbema-polluted soil had the least ammonium (3.41mg/kg), nitrate (22.10mg/kg) and sulphate (13.54mg/kg) levels after PGE II. The highest and least bicarbonate values were recorded in 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil (22.33mg/kg) and 1kg unpolluted soil + 4kg Egbema-polluted soil (18.28mg/kg) respectively. Organic carbon was greatest in Egbema-polluted soil without organic amendment (1.84%) and lowest in 1kg unpolluted soil + 4kg Egbema-polluted soil (0.24%) while organic matter was highest in Egbema-polluted soil without organic amendment (3.03%) and lowest in 1kg unpolluted soil + 4kg Egbema-polluted soil (0.55%). Egbema-polluted soil without organic amendment (0.065%) and 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil (0.26mg/kg) were highest in available nitrogen and phosphorus respectively while samples 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil (0.052%) and Egbema-polluted soil with organic amendment (0.20mg/kg) had the least recorded values for nitrogen and phosphorus.

Table 4.6b: Chemical Parameters of Crude oil-polluted from Egbema samples after Plant Growth using *Z. mays*

Soil Sample Code /Parameters	G1	G2	G3	G4	FMEnv Std	WHO Std
pH	5.70 ±0.10[b]	5.20 ±0.10[a]	6.30 ±0.20[c]	6.90 ±0.10[d]	6.5-8.5	6.5-8.0
EC(µS/cm)	118.00±1.00[d]	96.00 ±2.00[c]	63.00 ±1.00[a]	71.00 ±1.00[b]	1000	--
Ca^{2+}(mg/Kg)	2.08 ±0.01[b]	1.66 ±0.01[a]	2.85 ±0.01[c]	3.14 ±0.02[d]	--	100-300
Mg^{2+}(mg/Kg)	1.59 ±0.01[b]	1.08 ±0.02[a]	2.31 ±0.02[d]	1.83 ±0.02[c]	20	--
Na^{+}(mg/Kg)	3.25 ±0.01[b]	2.60 ±0.01[a]	3.73 ±0.02[c]	5.27 ±0.02[d]	200	200
K^{+}(mg/Kg)	2.41 ±0.01[b]	2.03 ±0.02[a]	2.95 ±0.01[c]	3.62 ±0.01[d]	--	8.0
NH_4^{+}1(mg/Kg)	4.13 ±0.01[d]	3.52 ±0.02[c]	3.41 ±0.01[a]	3.48 ±0.01[b]	--	35
NO_3^{-}(mg/Kg)	36.54 ±0.02[d]	30.08 ±0.01[c]	22.10 ±0.02[a]	25.11 ±0.01[b]	50	--
SO_4^{2-}(mg/Kg)	31.10 ±0.01[d]	22.39 ±0.02[c]	13.54 ±0.01[a]	16.26 ±0.01[b]	100	250
HCO_3^{-}(mg/Kg)	21.55 ±0.01[c]	18.92 ±0.01[b]	18.28 ±0.02[a]	22.33 ±0.01[d]	--	100
OC (%)	1.31 ±0.01[c]	1.84 ±0.01[d]	0.24 ±0.01[a]	0.42 ±0.01[b]	--	--
OM (%)	2.84 ±0.01[c]	3.03 ±0.01[d]	0.55 ±0.02[a]	1.58 ±0.01[b]	--	--
N (%)	0.054 ±0.001[a]	0.065 ±0.001[b]	0.053 ±0.002[a]	0.052 ±0.002[a]	--	--
P(mg/Kg)	0.200 ±0.020[a]	0.240 ±0.010[b]	0.250 ±0.020[b]	0.260 ±0.020[b]	--	0.01-0.19

Key: G1 = 5 Kg polluted soil + organic amendment; G2 = 5 Kg polluted soil; G3 = 1 Kg polluted soil + 4 Kg unpolluted soil + organic amendment; G4 = 2.5 Kg polluted + 2.5 Kg unpolluted soil + organic amendment. EC = Electrical Conductivity; Ca^{2+} = Calcium; Mg^{2+} = Magnesium; Na^{+} = Sodium; K^{+} = Potassium; NH_4^{+} = Ammonium; NO_3^{-} = Nitrate; SO_4^{2-} = Sulphate; HCO_3^{-} = Bicarbonate; OC = Organic carbon; OM = Organic matter; N = Nitrogen; P = Phosphorus.

± Standard deviation

Values with the same superscript are not statistically different

4.1.2 Total Petroleum Hydrocarbon Content of Soil Samples before and after Plant Growth

Tables 4.7a, 4.7b and 4.7c showed the result of the total petroleum hydrocarbon (TPH) content of the unpolluted agricultural soil before plant growth experiment (PGE). A total of 27 peaks were detected with retention time ranging from 6.815 to 11.788 min. Peak 1 recorded the highest percentage area of 11.98% followed by peak 6 (7.94%) while peak 12 (1.87%) had the least percentage area for the sample.

The result of TPH analysis of unpolluted agricultural soil after 3 months PGE are shown in Tables 4.8a, 4.8b and 4.8c. About 31 peaks were detected in the sample with the greatest and least retention time of 22.797 and 5.980 min respectively. The highest percent area was recorded in peak 6 (11.96%) while the least area of 1.34% was observed in peak 12. The compound having the highest molecular weight (519.07g/mol) was reported in peak 20 while that having the least molecular weight (150.17g/mol) was observed in peak 31.

Total petroleum hydrocarbon content of 5mL/5Kg polluted soil before PGE are shown in Tables 4.9a and 4.9b. Twenty-one peaks were recorded in the sample with retention time ranging from 5.860 to 22.734 min. The least area 1.65% (RT: 9.711 min) and highest area 27.44% (RT: 5.860) were recorded in peaks 8 and 1 respectively.

Table 4.10 show the TPH result of 5mL/5Kg polluted soil after 3 months PGE. A decrease in number of peaks from 21 to 12 peaks was observed. Peak 1 (RT: 6.809 min) had the highest area of 19.73% while the least area of 5.03% was observed in peak 2 (RT: 8.800). Retention time recorded for the sample was from 6.809 to 13.041 min.

Table 4.7a: Petroleum Hydrocarbon Content of Unpolluted Agricultural Soil before Plant Growth

Peak	Retention Time	Area (%)	Name of Compound	Molecular Weight (g/mol)	Molecular Formular
1	6.815	11.98	Decamethyl-cyclopentasiloxane	370.77	$C_{10}H_{30}Si_5O_5$
2	8.286	2.28	Tris(tert-butyldimethylsilyloxy) arsane	468.70	$C_{18}H_{45}AsO_3Si_3$
3	8.795	4.88	2-Methyl-7-phenylindole	207.28	$C_{15}H_{13}N$
4	8.915	2.89	Hexamethyl-cyclotrisiloxane	222.46	$C_6H_{18}O_3Si_3$
5	9.047	2.14	Decamethyl tetrasiloxane	310.69	$C_{10}H_{30}O_3Si_4$
6	9.150	7.94	Methyltris(trimethylsiloxy) silane	310.69	$C_{10}H_{30}O_3Si_4$
7	9.373	2.69	Diethyl bis(trimethylsilyl) ester	296.58	$C_{10}H_{28}O_4Si_3$
8	9.470	5.06	2-Methyl-7-phenylindole	207.28	$C_{15}H_{13}N$
9	9.568	3.71	Tris(tert-butyldimethylsilyloxy) arsane	468.70	$C_{18}H_{45}AsO_3Si_3$
10	9.659	3.41	Methyltris(trimethylsiloxy) silane	310.69	$C_{10}H_{30}O_3Si_4$

Table 4.7b: Petroleum Hydrocarbon Content of Unpolluted Agricultural Soil before Plant Growth Cont'd

Peak	Retention Time	Area (%)	Name of Compound	Molecular Weight (g/mol)	Molecular Formular
11	9.825	4.18	Benzothiophene-3-carboxylic acid	178.21	$C_9H_6O_2S$
12	9.905	1.87	2-[bis(methylthio)methylene]-1-phenyl hydrazide		
13	9.974	4.82	2-Methyl-3-phenyl-1H-indole	207.27	$C_{15}H_{13}N$
14	10.094	2.33	2-Methyl-3-phenyl-1H-indole	207.27	$C_{15}H_{13}N$
15	10.174	5.88	5-Bromo-1-methylindole-2-carboxylic acid	254.08	$C_{10}H_8BrNO_2$
16	10.266	2.72	N-[4-(trimethylsilyl)phenyl]-acetamide	207.34	$C_{11}H_{17}NOSi$
17	10.346	2.55	1,4-Bis(trimethylsilyl)benzene	222.47	$C_{12}H_{22}Si_2$
18	10.420	3.92	4-tert-Butylphenol	150.22	$C_{10}H_{14}O$
19	10.580	2.03	Tris(trimethylsilyl) ester	342.49	$C_9H_{27}AsO_3Si_3$
20	10.638	2.01	2-Methyl-7-phenylindole	207.28	$C_{15}H_{13}N$

Table 4.7c: Petroleum Hydrocarbon Content of Unpolluted Agricultural Soil before Plant Growth Cont'd

Peak	Retention Time	Area (%)	Name of Compound	Molecular Weight (g/mol)	Molecular Formular
20	10.638	2.01	2-Methyl-7-phenylindole	207.28	$C_{15}H_{13}N$
21	10.758	5.66	2,4-dimethyl-Benzo[h]quinoline	207.27	$C_{15}H_{13}N$
22	10.998	3.10	2-Methyl-7-phenylindole	207.28	$C_{15}H_{13}N$
23	11.107	2.34	1,2-Bis(trimethylsilyl) benzene	222.47	$C_{12}H_{22}Si_2$
24	11.319	2.14	Tris(trimethylsilyl) ester	342.49	$C_9H_{27}AsO_3Si_3$
25	11.610	2.41	Tris(trimethylsilyl) ester	342.49	$C_9H_{27}AsO_3Si_3$
26	11.702	2.42	Tris(tert-butyldimethylsilyloxy) arsane	468.70	$C_{18}H_{45}AsO_3Si_3$
27	11.788	2.62	9-oxo-9H-fluorene-4-carboxylic acid	224.21	$C_{14}H_8O_3$

Table 4.8a: Petroleum Hydrocarbon Content of Unpolluted Agricultural Soil after Plant Growth

Peak	Retention Time	Area (%)	Name of Compound	Molecular Weight (g/mol)	Molecular Formular
1	5.980	7.23	Decamethyl-cyclopentasiloxane	370.77	$C_{10}H_{30}Si_5O_5$
2	6.809	9.73	Decamethyl-cyclopentasiloxane	370.77	$C_{10}H_{30}Si_5O_5$
3	8.623	1.82	Decamethyl-tetrasiloxane	310.69	$C_{10}H_{30}O_3Si_4$
4	8.784	2.34	2,4-dimethyl-Benzo[h]quinoline	207.27	$C_{15}H_{13}N$
5	8.909	1.41	1,2-Benzisothiazol-3-amine	150.20	$C_7H_6N_2S$
6	9.035	11.96	Dodecamethyl-cyclohexasiloxane	444.92	$C_{12}H_{36}O_6Si_6$
7	9.138	5.34	Dodecamethyl-cyclohexasiloxane	444.92	$C_{12}H_{36}O_6Si_6$
8	9.361	1.72	(9-oxo-9,10-dihydroacridin-4-yl)acetic acid	253.25	$C_{15}H_{11}NO_3$
9	9.459	3.15	2-Methyl-7-phenylindole	207.28	$C_{15}H_{13}N$
10	9.562	2.41	2,4-dimethyl-Benzo[h]quinoline	207.27	$C_{15}H_{13}N$

Table 4.8b: Petroleum Hydrocarbon Content of Unpolluted Agricultural Soil after Plant Growth Cont'd

Peak	Retention Time	Area (%)	Name of Compound	Molecular Weight (g/mol)	Molecular Formular
11	9.648	1.99	(9-oxo-9,10-dihydroacridin-4-yl)acetic acid	253.25	$C_{15}H_{11}NO_3$
12	9.728	1.34	Thymol	150.22	$C_{10}H_{14}O$
13	9.814	3.04	Benzothiophene-3-carboxylic acid	178.21	$C_9H_6O_2S$
14	9.968	2.13	2-Methyl-3-phenyl-1H-indole	207.27	$C_{15}H_{13}N$
15	10.088	1.57	Tris(tert-butyldimethylsilyloxy) arsane	468.70	$C_{18}H_{45}AsO_3Si_3$
16	10.168	3.84	5-Bromo-1-methylindole-2-carboxylic acid	254.08	$C_{10}H_8BrNO_2$
17	10.254	1.57	Hexamethyl-cyclotrisiloxane	222.46	$C_6H_{18}O_3Si_3$
18	10.340	1.41	2,4-dimethyl-Benzo[h]quinoline	207.27	$C_{15}H_{13}N$
19	10.414	2.67	2-Methyl-7-phenylindole	207.28	$C_{15}H_{13}N$
20	10.597	5.88	Tetradecamethyl-cycloheptasiloxane	519.07	$C_{14}H_{42}O_7Si_7$

Table 4.8c: Petroleum Hydrocarbon Content of Unpolluted Agricultural Soil after Plant Growth Cont'd

Peak	Retention Time	Area (%)	Name of Compound	Molecular Weight (g/mol)	Molecular Formular
21	10.758	3.45	5-Methyl-2-phenyl-1H-indole	207.27	$C_{15}H_{13}N$
22	10.998	2.03	2-Ethylacridine	207.27	$C_{15}H_{13}N$
23	11.107	2.67	N-[4-(trimethylsilyl)phenyl]-acetamide	207.34	$C_{11}H_{17}NOSi$
24	11.610	1.41	1,4-Bis(trimethylsilyl)benzene	222.47	$C_{12}H_{22}Si_2$
25	11.753	4.01	Hexadecamethyl-cyclooctasiloxane	593.20	$C_{16}H_{48}O_8Si_8$
26	12.972	2.58	Tris(tert-butyldimethylsilyloxy) arsane	468.70	$C_{18}H_{45}AsO_3Si_3$
27	14.712	1.67	Methyltris(trimethylsiloxy) silane	310.69	$C_{10}H_{30}O_3Si_4$
28	16.583	2.06	4-tert-Amylphenol	164.24	$C_{11}H_{16}O$
29	18.173	2.87	3,5-Bis-trimethylsilyl-2,4,6-cyclo-heptatriene-1-one	250.48	$C_{13}H_{22}OSi_2$
30	19.570	1.42	Thymol	150.22	$C_{10}H_{14}O$
31	22.797	3.29	2'-hydroxypropiophenone	150.17	$C_9H_{10}O_2$

Table 4.9a: Petroleum Hydrocarbon Content of 5mL/5Kg Polluted Soil before Plant Growth

Peak	Retention Time	Area (%)	Name of Compound	Molecular Weight (g/mol)	Molecular Formular
1	5.860	27.44	Decamethyl-cyclopentasiloxane	370.77	$C_{10}H_{30}Si_5O_5$
2	6.793	2.22	Decamethyl-cyclopentasiloxane	370.77	$C_{10}H_{30}Si_5O_5$
3	6.981	2.57	Dodecane	170.33	$C_{12}H_{26}$
4	8.097	3.05	2,4'-Dimethoxy-2'-(trimethylsilyl)oxychalcone		
5	8.315	3.19	Methylvinyl(1-cyclopentylethoxymethylvinylsilyloxy) undecycloxy-silane		
6	8.572	1.80	2-Methyl-7-phenylindole	207.28	$C_{15}H_{13}N$
7	9.018	14.66	Dodecamethyl-cyclohexasiloxane	444.92	$C_{12}H_{36}O_6Si_6$
8	9.711	1.65	6-(dimethylamino)-3,3-bis[4-dimethylamino)phenyl]-1(3H)-Isobenzofuranone	415.53	$C_{26}H_{29}N_3O_2$
9	9.797	2.94	7-chloro-2,3-dihydro-3-(4-N,N-dimethylamino benzylidene)-5-phenyl-1H-1,4-benzodiazepin-2-one	401.90	$C_{24}H_{20}ClN_3O$
10	9.842	5.70	2-(6-Ethoxy-4-methyl-quinazolin-2-ylamino)-5-methyl-pyrimidine-4,6-diol	327.34	$C_{16}H_{17}N_5O_3$

Table 4.9b: Petroleum Hydrocarbon Content of 5mL/5Kg Polluted Soil before Plant Growth Cont'd

Peak	Retention Time	Area (%)	Name of Compound	Molecular Weight (g/mol)	Molecular Formular
11	10.226	3.43	Tetradecamethyl-cycloheptasiloxane	519.07	$C_{14}H_{42}O_7Si_7$
12	10.592	7.79	Tetradecamethyl-cycloheptasiloxane	519.07	$C_{14}H_{42}O_7Si_7$
13	11.067	2.38	Dimethyl(dimethyl(2-methylphenoxy) silyloxy)octadecyloxy-silane		
14	11.742	5.32	Hexadecamethyl-cyclooctasiloxane	593.20	$C_{16}H_{48}O_8Si_8$
15	12.961	4.14	Octadecamethyl-cyclononasiloxane	667.40	$C_{18}H_{54}O_9Si_9$
16	14.672	2.30	1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-Hexadecamethyl-octasiloxane	577.20	$C_{16}H_{48}O_7Si_8$
17	16.537	1.87	1-(4,7-dihydro-2-methyl-7-oxopyrazolo[1,5-d] pyrimidin-5-yl)-formic acid	207.19	$C_9H_9N_3O_3$
18	18.128	2.01	Tetracosamethyl-cyclododecasiloxane	889.8	$C_{24}H_{72}O_{12}Si_{12}$
19	19.524	1.86	3,5-bis(1,1-dimethylethyl)-1,2-benzenediol	222.32	$C_{14}H_{22}O_2$
20	21.035	1.71	1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-Hexadecamethyl-octasiloxane	577.20	$C_{16}H_{48}O_7Si_8$
21	22.734	1.96	6-(4-ethoxyphenyl)-3-methyl-4-oxo-4,5,6,7-tetrahydro-1H-indole-2-carboxylic acid	397.47	$C_{23}H_{27}NO_5$

Table 4.10: Petroleum Hydrocarbon Content of 5mL/5Kg Polluted Oil after Plant Growth

Peak	Retention Time	Area (%)	Name of Compound	Molecular Weight (g/mol)	Molecular Formular
1	6.809	19.73	Decamethyl-cyclopentasiloxane	370.77	$C_{10}H_{30}Si_5O_5$
2	8.800	5.03	Decamethyl-tetrasiloxane	310.69	$C_{10}H_{30}O_3Si_4$
3	9.149	12.14	Hexadecamethyl-heptasiloxane	533.10	$C_{16}H_{48}O_6Si_7$
4	9.470	7.79	Tris(tert-butyldimethylsilyloxy) arsane	468.70	$C_{18}H_{45}AsO_3Si_3$
5	9.567	5.23	(9-oxo-9,10-dihydroacridin-4-yl)acetic acid	253.25	$C_{15}H_{11}NO_3$
6	9.819	6.05	2-Ethylacridine	207.27	$C_{15}H_{13}N$
7	9.905	5.83	N-[4-(trimethylsilyl)phenyl]-acetamide	207.34	$C_{11}H_{17}NOSi$
8	9.979	8.33	2-Ethylacridine	207.27	$C_{15}H_{13}N$
9	10.179	9.42	5,12-dihydro-5,12-ethanonaphthacene-6-carbonitrile		
10	10.426	6.66	Diethyl bis(trimethylsilyl) ester	296.58	$C_{10}H_{28}O_4Si_3$
11	10.763	7.66	Tris(tert-butyldimethylsliloxy) arsane	468.70	$C_{18}H_{45}AsO_3Si_3$
12	13.041	6.11	N-methyl-1-adamantaneacetamide	207.31	$C_{13}H_{21}NO$

The results of total petroleum hydrocarbon analysis of 10mL/5Kg polluted soil before plant growth are shown on Table 4.11a and 4.11b. Eighteen peaks were detected in the sample having retention time with a range of 6.821-11.793 min. The highest and least percentage areas recorded for the sample were 16.61% (6.821 min) and 3.34% (9.379 min) in peaks 1 and 4 respectively. In addition to aromatic hydrocarbons, benzo(a)anthracene-7-carbonitrile (peak 10), a polycyclic aromatic hydrocarbon of environmental concern was also detected in the sample havaing an area of 7.99% and retention time of 10.180 min.

The total hydrocarbon content of 10mL/5Kg polluted soil after 3 months PGE are shown in Tables 4.12a and 4.12b. Eighteen peaks were also recorded in the sample with retention time ranging from 6.809 to 11.610 min. The greatest percentage area (17.63%) was observed in peak 1 (6.809 min) while the least percentage area (2.89%) was observed in peak 2 (8.475 min).

Tables 4.13a and 4.13b show the TPH content of 25mL/5Kg polluted soil before PGE. A total of eighteen peaks were recorded for the sample with the highest percentage area (15.52%) observed in peak 1 (6.810 min) while peak 2 (3.02%) had the least percentage area. Highest retention time was observed in peak 18 (11.696 min) while the least retention time was recorded in peak 1 (6.810 min).

Table 4.11a: Petroleum Hydrocarbon Content of 10mL/5Kg Polluted Soil before Plant Growth

Peak	Retention Time	Area (%)	Name of Compound	Molecular Weight (g/mol)	Molecular Formular
1	6.821	16.61	Decamethyl-cyclopentasiloxane	370.77	$C_{10}H_{30}Si_5O_5$
2	8.795	4.21	1,2-Benzoisothiazol-3-amine	150.20	$C_7H_6N_2S$
3	9.150	9.75	1-phenyl-2-(2-phenyl-4H-1-benzopyra-4-yl)-ethanone	324.38	$C_{23}H_{16}O_2$
4	9.379	3.34	Tris(trimethylsilyl) ester	342.49	$C_9H_{27}AsO_3Si_3$
5	9.470	6.21	Tris(tert-butyldimethylsilyloxy) arsane	468.70	$C_{18}H_{45}AsO_3Si_3$
6	9.568	4.39	1,4-bis(trimethylsilyl)benzene	222.47	$C_{12}H_{22}Si_2$
7	9.659	3.89	Diethylbis(trimethylsilyl)ester	296.58	$C_{10}H_{28}O_4Si_3$
8	9.825	5.77	Diethylbis(trimethylsilyl)ester	296.58	$C_{10}H_{28}O_4Si_3$
9	9.968	5.09	Thymol	150.22	$C_{10}H_{14}O$

Table 4.11b: Petroleum Hydrocarbon Content of 10mL/5Kg Polluted Soil before Plant Growth Cont'd

Peak	Retention Time	Area (%)	Name of Compound	Molecular Weight (g/mol)	Molecular Formular
10	10.180	7.99	Benz(a)anthracene-7-carbonitrile	253.30	$C_{19}H_{11}N$
11	10.260	3.83	N-[4-(trimethylsilyl)phenyl]-acetamide	207.34	$C_{11}H_{17}NOSi$
12	10.420	4.71	1,2-Bis(trimethylsilyl)benzene	222.47	$C_{12}H_{22}Si_2$
13	10.643	3.51	3,5-bis-trimethylsilyl-2,4,6-cycloheptatrien-1-one	250.48	$C_{13}H_{22}OSi_2$
14	10.763	4.69	9-oxo-9H-fluorene-4-carboxylic acid	224.21	$C_{14}H_8O_3$
15	11.004	3.68	N-[4-(trimethylsilyl)phenyl]-acetamide	207.34	$C_{11}H_{17}NOSi$
16	11.113	3.59	Hexamethyl-cyclotrisiloxane	222.46	$C_6H_{18}O_3Si_3$
17	11.610	3.49	Methyltris(trimethylsiloxy) silane	310.69	$C_{10}H_{30}O_3Si_4$
18	11.793	5.26	1,4-Bis(trimethylsilyl)benzene	222.47	$C_{12}H_{22}Si_2$

Table 4.12a: Petroleum Hydrocarbon Content of 10mL/5Kg Polluted Soil after Plant Growth

Peak	Retention Time	Area (%)	Name of Compound	Molecular Weight (g/mol)	Molecular Formular
1	6.809	17.63	Decamethyl-cyclopentasiloxane	370.77	$C_{10}H_{30}Si_5O_5$
2	8.475	2.89	Methyltris(trimethylsiloxy) silane	310.69	$C_{10}H_{30}O_3Si_4$
3	8.795	4.67	1,2,4-benzenetricarboxylic acid	210.14	$C_9H_6O_6$
4	9.150	9.33	Dodecamethyl-cyclohexasiloxane	444.92	$C_{12}H_{36}O_6Si_6$
5	9.476	6.16	N-[4-(trimethylsilyl)phenyl]-acetamide	207.34	$C_{11}H_{17}NOSi$
6	9.568	4.34	2,4-dimethyl-benzo[h]quinoline	207.27	$C_{15}H_{13}N$
7	9.653	3.90	2,4-dimethyl-benzo[h]quinoline	207.27	$C_{15}H_{13}N$
8	9.819	5.88	Benzothiophene-3-carboxylic acid	178.21	$C_9H_6O_2S$
9	9.968	5.56	2-(n-pentyl)oxybenzylidene acetophenone	294.40	$C_{20}H_{22}O_2$

Table 4.12b: Petroleum Hydrocarbon Content of 10mL/5Kg Polluted Soil after Plant Growth Cont'd

Peak	Retention Time	Area (%)	Name of Compound	Molecular Weight (g/mol)	Molecular Formular
10	10.094	3.89	Tris(trimethylsilyl) ester	342.49	$C_9H_{27}AsO_3Si_3$
11	10.174	8.19	N3,N3,N6,N6-tetramethyl-9H-carbazole-3,6-diamine	253.35	$C_{16}H_{19}N_3$
12	10.266	4.02	Methyltris(trimethylsiloxy) silane	310.69	$C_{10}H_{30}O_3Si_4$
13	10.420	4.83	9-oxo-9H-fluorene-4-carboxylic acid	224.21	$C_{14}H_8O_3$
14	10.758	6.42	Tris(tert-butyldimethylsilyloxy) arsane	468.70	$C_{18}H_{45}AsO_3Si_3$
15	10.998	3.00	Tris(trimethylsilyl) ester	342.49	$C_9H_{27}AsO_3Si_3$
16	11.112	3.15	N-[4-(trimethylsilyl)phenyl]-acetamide	207.34	$C_{11}H_{17}NOSi$
17	11.318	2.91	9-oxo-9H-fluorene-4-carboxylic acid	224.21	$C_{14}H_8O_3$
18	11.610	3.23	Tris(tert-butyldimethylsilyloxy) arsane	468.70	$C_{18}H_{45}AsO_3Si_3$

Table 4.13a: Petroleum Hydrocarbon Content of 25mL/5Kg Polluted Soil before Plant Growth

Peak	Retention Time	Area (%)	Name of Compound	Molecular Weight (g/mol)	Molecular Formular
1	6.810	15.52	Decamethyl-cyclopentasiloxane	370.77	$C_{10}H_{30}Si_5O_5$
2	8.795	4.84	6,8-difluoro-4-hydroxyquinoline-3-carboxylic acid	225.15	$C_{10}H_5F_2NO_3$
3	9.047	4.17	Hexamethyl-cyclotrisiloxane	222.46	$C_6H_{18}O_3Si_3$
4	9.150	10.04	Dodecamethyl-cyclohexasiloxane	444.92	$C_{12}H_{36}O_6Si_6$
5	9.379	3.02	Tris(tert-butyldimethylsilyloxy) arsane	468.70	$C_{18}H_{45}AsO_3Si_3$
6	9.476	6.32	Hexamethyl-cyclotrisiloxane	222.46	$C_6H_{18}O_3Si_3$
7	9.568	5.00	2,4-dimethyl-benzo[h]quinoline	207.27	$C_{15}H_{13}N$
8	9.659	3.62	Tris(tert-butyldimethylsilyloxy) arsane	468.70	$C_{18}H_{45}AsO_3Si_3$
9	9.825	5.93	3,3-Diisopropoxy-1,1,1,5,5,5-hexamethyl trisiloxane	324.63	$C_{12}H_{32}O_4Si_3$

Table 4.13b: Petroleum Hydrocarbon Content of 25mL/5Kg Polluted Soil before Plant Growth Cont'd

Peak	Retention Time	Area (%)	Name of Compound	Molecular Weight (g/mol)	Molecular Formular
10	9.974	5.87	Tris(tert-butyldimethylsilyloxy) arsane	468.70	$C_{18}H_{45}AsO_3Si_3$
11	10.094	3.19	Tris(tert-butyldimethylsilyloxy) arsane	468.70	$C_{18}H_{45}AsO_3Si_3$
12	10.174	7.78	5,12-Dihydro-5,12-ethanonaphthacene-6-carbonitrile		
13	10.420	4.85	2-Methyl-7-phenylindole	207.28	$C_{15}H_{13}N$
14	10.638	3.53	2-Methyl-7-phenylindole	207.28	$C_{15}H_{13}N$
15	10.758	5.72	N-[4-(trimethylsilyl)phenyl]-acetamide	207.34	$C_{11}H_{17}NOSi$
16	11.004	3.86	9-oxo-9H-fluorene-4-carboxylic acid	224.21	$C_{14}H_8O_3$
17	11.610	3.48	Tris(tert-butyldimethylsilyloxy) arsane	468.70	$C_{18}H_{45}AsO_3Si_3$
18	11.696	3.26	Tris(trimethylsilyl) ester	342.49	$C_9H_{27}AsO_3Si_3$

The results of TPH analysis of 25mL/5Kg polluted soil after 3 months plant growth are shown in Tables 4.14a and 4.14b. A reduction in total number of peaks (17 peaks) was observed in the sample. The highest percentage area (17.31%) was observed in peak 1 (6.804 min), followed by peak 3 (11.39%) while the lowest percentage area (3.32%) was observed in peak 16 (11.610min). Values of retention ranged from 6.804 to 11.794min for the sample.

The TPH analysis results of 50mL/5Kg polluted soil prior to plant growth experiment are shown in Table 4.15a, 4.15b, 4.15c and 4.15d. A total of 45 peaks were observed in the sample with retention time of range 6.821min (peak 1) to 18.191min (peak 2). About 14 polycyclic aromatic hydrocarbons were identified in the sample with 3 confirmed as carcinogenic substances. The least percentage area of 0.71% (11.742min) was detected in peak 40 while the highest percentage area of 8.25% (6.821min) was recorded in peak 1.

The total hydrocarbon contents of 50mL/5Kg polluted soil after 3 months plant growth are shown in Tables 4.16a, 4.16b and 4.16c. Total number of peaks reduced to 27 while retention time increased with range 5.923-26.408min. The highest and lowest percentage area for the sample were detected in peaks 1 (18.53%) and 17 (1.36%). Additionally, polycyclic aromatic hydrocarbons reduced from 14 to 4 while n-alkanes (nonadecane and decane) were observed.

Table 4.14a: Petroleum Hydrocarbon Content of 25mL/5Kg Polluted Soil after Plant Growth

Peak	Retention Time	Area (%)	Name of Compound	Molecular Weight (g/mol)	Molecular Formular
1	6.804	17.31	Decamethyl-cyclopentasiloxane	370.77	$C_{10}H_{30}Si_5O_5$
2	8.795	3.76	2,4-dimethyl-benzo[h]quinoline	207.27	$C_{15}H_{13}N$
3	9.150	11.39	Dodecamethyl-cyclohexasiloxane	444.92	$C_{12}H_{36}O_6Si_6$
4	9.276	3.42	9-oxo-9H-fluorene-4-carboxylic acid	224.21	$C_{14}H_8O_3$
5	9.367	3.69	Decamethyl-tetrasiloxane	310.69	$C_{10}H_{30}O_3Si_4$
6	9.470	6.55	2-[bis(methylthio)methylene]-1-phenyl hydrazide		
7	9.568	4.96	3,3-Diisopropoxy-1,1,1,5,5,5-hexamethyl trisiloxane	324.63	$C_{12}H_{32}O_4Si_3$
8	9.825	6.30	Decamethyl-tetrasiloxane	310.69	$C_{10}H_{30}O_3Si_4$
9	9.825	5.93	2-[bis(methylthio)methylene]-1-phenyl hydrazide		

Table 4.14b: Petroleum Hydrocarbon Content of 25mL/5Kg Polluted Soil after Plant Growth Cont'd

Peak	Retention Time	Area (%)	Name of Compound	Molecular Weight (g/mol)	Molecular Formular
10	10.174	7.36	5-Bromo-1-methylindole-2-carboxylic acid	254.08	$C_{10}H_8BrNO_2$
11	10.260	3.48	Tris(trimethylsilyl) ester	342.49	$C_9H_{27}AsO_3Si_3$
12	10.420	4.10	3,5-Bis(1,1-dimethylethyl)-1,2-benzenediol	222.32	$C_{14}H_{22}O_2$
13	10.638	3.94	2,3-dihydro-N-hydroxy-4-methoxy-3,3-dimethyl-indole-2-one	207.23	$C_{11}H_{13}NO_3$
14	10.758	6.31	Tris(trimethylsilyl) ester	342.49	$C_9H_{27}AsO_3Si_3$
15	11.004	3.76	Tris(tert-butyldimethylsilyloxy) arsane	468.70	$C_{18}H_{45}AsO_3Si_3$
16	11.610	3.32	Hexamethyl-cyclotrisiloxane	222.46	$C_6H_{18}O_3Si_3$
17	11.794	4.44	2-Methyl-7-phenylindole	207.28	$C_{15}H_{13}N$

Table 4.15a: Petroleum Hydrocarbon Content of 50mL/5Kg Polluted Soil before Plant Growth

Peak	Retention Time	Area (%)	Name of Compound	Molecular Weight (g/mol)	Molecular Formular
1	6.821	8.25	Decamethyl-cyclopentasiloxane	370.77	$C_{10}H_{30}Si_5O_5$
2	8.274	1.51	1,2-Benzisothiazol-3-amine	150.20	$C_7H_6N_2S$
3	8.349	0.86	[3-(4-Methoxyphenyl)-4,5-dihydro-1,2-oxazol-5-yl] methanol	207.23	$C_{11}H_{13}NO_3$
4	8.475	1.12	6,8-difluoro-4-hydroxyquinoline-3-carboxylic acid	225.15	$C_{10}H_5F_2NO_3$
5	8.629	1.60	2,4-dimethyl-benzo[h]quinoline	207.27	$C_{15}H_{13}N$
6	8.795	3.15	1-Methyl-2-phenyl-1H-indole	207.27	$C_{15}H_{13}N$
7	8.921	1.65	2,4-dimethyl-benzo[h]quinoline	207.27	$C_{15}H_{13}N$
8	9.053	3.49	2-Methyl-7-phenylindole	207.28	$C_{15}H_{13}N$
9	9.156	6.57	3,5-Bis-trimethylsilyl-2,4,6-cycloheptatrien-1-one	250.48	$C_{13}H_{22}OSi_2$
10	9.270	1.69	Decamethyl tetrasiloxane	310.69	$C_{10}H_{30}O_3Si_4$
11	9.322	0.72	1,2,4-Benzenetricarboxylic acid	210.14	$C_9H_6O_6$
12	9.373	2.59	Tris(trimethylsilyl) ester	342.49	$C_9H_{27}AsO_3Si_3$

Table 4.15b: Petroleum Hydrocarbon Content of 50mL/5Kg Polluted Soil before Plant Growth Cont'd

Peak	Retention Time	Area (%)	Name of Compound	Molecular Weight (g/mol)	Molecular Formular
13	9.470	4.77	1-Methyl-2-phenyl-1H-indole	207.27	$C_{15}H_{13}N$
14	9.573	2.49	Decamethyl tetrasiloxane	310.69	$C_{10}H_{30}O_3Si_4$
15	9.653	2.49	9-oxo-9H-fluorene-2-carboxylic acid	224.21	$C_{14}H_8O_3$
16	9.734	1.00	Hexamethyl-cyclotrisiloxane	222.46	$C_6H_{18}O_3Si_3$
17	9.819	3.28	Tris(tert-butyldimethylsilyloxy) arsane	468.70	$C_{18}H_{45}AsO_3Si_3$
18	9.899	1.85	2-Methyl-7-phenylindole	207.28	$C_{15}H_{13}N$
19	9.968	4.10	Tris(tert-butyldimethylsilyloxy) arsane	468.70	$C_{18}H_{45}AsO_3Si_3$
20	10.094	2.28	N,N-Dimethyl-4-nitroso-3-(trimethylsilyl)aniline	222.36	$C_{11}H_{18}N_2OSi$
21	10.174	5.14	3-(4-nitrophenyl)-1-phenyl-2-propen-1-one	253.25	$C_{15}H_{11}NO_3$
22	10.260	2.62	9-oxo-9H-fluorene-2-carboxylic acid	224.21	$C_{14}H_8O_3$
23	10.340	2.10	Tris(tert-butyldimethylsilyloxy) arsane	468.70	$C_{18}H_{45}AsO_3Si_3$
24	10.420	2.90	9-oxo-9H-fluorene-4-carboxylic acid	224.21	$C_{14}H_8O_3$

Table 4.15c: Petroleum Hydrocarbon Content of 50mL/5Kg Polluted Soil before Plant Growth Cont'd

Peak	Retention Time	Area (%)	Name of Compound	Molecular Weight (g/mol)	Molecular Formular
25	10.563	1.79	Methyltris(trimethylsiloxy) silane	310.69	$C_{10}H_{30}O_3Si_4$
26	10.643	2.05	Hexamethyl-cyclotrisiloxane	222.46	$C_6H_{18}O_3Si_3$
27	10.758	4.32	6,8-difluoro-4-hydroxyquinoline-3-carboxylic acid	225.15	$C_{10}H_5F_2NO_3$
28	10.953	1.28	Methyltris(trimethylsiloxy) silane	310.69	$C_{10}H_{30}O_3Si_4$
29	10.998	2.26	1,1,1,3,5,5,5-Heptamethyltrisiloxane	221.50	$C_7H_{21}O_2Si_3$
30	11.027	1.17	Tris(trimethylsilyl) ester	342.49	$C_9H_{27}AsO_3Si_3$
31	11.113	2.06	1-Methyl-2-phenyl-1H-indole	207.27	$C_{15}H_{13}N$
32	11.158	0.92	Decamethy tetrasiloxane	310.69	$C_{10}H_{30}O_3Si_4$
33	11.193	1.26	2-Methyl-7-phenylindole	207.28	$C_{15}H_{13}N$
34	11.273	0.78	2,4-dimethyl-Benzo[h]quinoline	207.27	$C_{15}H_{13}N$
35	11.319	1.59	5-Methyl-2-phenylindolizine	207.27	$C_{15}H_{13}N$

Table 4.15d: Petroleum Hydrocarbon Content of 50mL/5Kg Polluted Soil before Plant Growth Cont'd

Peak	Retention Time	Area (%)	Name of Compound	Molecular Weight (g/mol)	Molecular Formular
36	11.433	1.36	1-Methyl-2-phenyl-1H-indole	207.27	$C_{15}H_{13}N$
37	11.467	0.96	6,8-difluoro-4-hydroxyquinoline-3-carboxylic acid	225.15	$C_{10}H_5F_2NO_3$
38	11.610	1.90	4-tert-butylphenol	150.22	$C_{10}H_{14}O$
39	11.696	1.86	Diethyl bis(trimethylsilyl) ester	296.58	$C_{10}H_{28}O_4Si_3$
40	11.742	0.71	Diethyl bis(trimethylsilyl) ester	296.58	$C_{10}H_{28}O_4Si_3$
41	11.793	1.69	2-Methyl-7-phenylindole	207.28	$C_{15}H_{13}N$
42	11.925	0.72	2,4-dimethyl-Benzo[h]quinoline	207.27	$C_{15}H_{13}N$
43	12.291	1.14	Tris(tert-butyldimethylsilyloxy) arsane	468.70	$C_{18}H_{45}AsO_3Si_3$
44	12.497	0.75	Decamethyl tetrasiloxane	310.69	$C_{10}H_{30}O_3Si_4$
45	18.191	1.22	Hexamethyl cyclotrisiloxane	222.46	$C_6H_{18}O_3Si_3$

Table 4.16a: Petroleum Hydrocarbon Content of 50mL/5Kg Polluted Soil after Plant Growth

Peak	Retention Time	Area (%)	Name of Compound	Molecular Weight (g/mol)	Molecular Formular
1	5.923	18.53	Decamethyl-cyclopentasiloxane	370.77	$C_{10}H_{30}Si_5O_5$
2	6.798	1.82	Decamethyl-cyclopentasiloxane	370.77	$C_{10}H_{30}Si_5O_5$
3	7.016	9.91	Nonadecane	268.50	$C_{19}H_{40}$
4	7.313	2.18	2,6-dimethyl-Undecane	184.36	$C_{13}H_{28}$
5	8.108	2.94	Phosphonoacetic acid	140.03	$C_2H_5O_5P$
6	8.326	2.86	1,7-Di(3-ethylphenyl)-2,2,4,4,6,6-hexamethyl-1,3,5,7-tetraoxa-2,4,6-trisilaheptane	448.8	$C_{22}H_{36}O_4Si_3$
7	8.681	2.70	Decane	142.28	$C_{10}H_{22}$
8	9.030	8.76	Dodecamethyl-cyclohexasiloxane	444.92	$C_{12}H_{36}O_6Si_6$
9	9.802	3.35	7-Chloro-2,3-dihydro-3-(4-N,N-dimethylaminobenzylidene)-5-phenyl-1H-1,4-benzodiazepin-2-one	401.90	$C_{24}H_{20}ClN_3O$
10	9.848	3.80	6-(dimethylamino)-3,3-bis[4-(dimethylamino) phenyl]-1(3H)-isobenzofuranone	415.53	$C_{26}H_{29}N_3O_2$

Table 4.16b: Petroleum Hydrocarbon Content of 50mL/5Kg Polluted Soil after Plant Growth Cont'd

Peak	Retention Time	Area (%)	Name of Compound	Molecular Weight (g/mol)	Molecular Formular
11	10.111	2.77	Tetradecamethyl-cycloheptasiloxane	519.07	$C_{14}H_{42}O_7Si_7$
12	10.151	2.58	Tetradecamethyl-cycloheptasiloxane	519.07	$C_{14}H_{42}O_7Si_7$
13	10.592	5.51	Tetradecamethyl-cycloheptasiloxane	519.07	$C_{14}H_{42}O_7Si_7$
14	11.072	2.49	N1,N1,N4-Tris(tert-butyldimethylsilyl) succinamide	458.90	$C_{22}H_{50}N_2O_2Si_3$
15	11.656	3.08	Hexadecamethyl-cyclooctasiloxane	593.20	$C_{16}H_{48}O_8Si_8$
16	11.748	3.21	Hexadecamethyl-cyclooctasiloxane	593.20	$C_{16}H_{48}O_8Si_8$
17	12.944	1.36	Octadecamethyl-cyclononasiloxane	667.40	$C_{18}H_{54}O_9Si_9$
18	12.972	2.01	Octadecamethyl-cyclononasiloxane	667.40	$C_{18}H_{54}O_9Si_9$
19	14.683	2.32	Eicosamethyl-cyclodecasiloxane	741.54	$C_{20}H_{60}O_{10}Si_{10}$

Table 4.16c: Petroleum Hydrocarbon Content of 50mL/5Kg Polluted Soil after Plant Growth Cont'd

Peak	Retention Time	Area (%)	Name of Compound	Molecular Weight (g/mol)	Molecular Formular
20	16.554	2.08	6-(4-ethoxyphenyl)-3-methyl-4-oxo-4,5,6,7-tetrahydro-1H-Indole-2-carboxylic acid	397.47	$C_{23}H_{27}NO_5$
21	18.145	2.47	Hexadecamethyl-cyclooctasiloxane	593.20	$C_{16}H_{48}O_8Si_8$
22	19.541	2.75	Eicosamethyl-cyclodecasiloxane	741.54	$C_{20}H_{60}O_{10}Si_{10}$
23	21.057	2.24	1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-heptasiloxane	503.07	$C_{14}H_{42}O_6Si_7$
24	22.757	2.59	6-(4-ethoxyphenyl)-3-methyl-4-oxo-4,5,6,7-tetrahydro-1H-Indole-2-carboxylic acid	397.47	$C_{23}H_{27}NO_5$
25	24.165	2.31	6-(4-ethoxyphenyl)-3-methyl-4-oxo-4,5,6,7-tetrahydro-1H-Indole-2-carboxylic acid	397.47	$C_{23}H_{27}NO_5$
26	25.355	2.41	6-(4-ethoxyphenyl)-3-methyl-4-oxo-4,5,6,7-tetrahydro-1H-Indole-2-carboxylic acid	397.47	$C_{23}H_{27}NO_5$
27	26.408	1.45	1,4-bis(trimethylsilyl) benzene	222.47	$C_{12}H_{22}Si_2$

4.1.2.1 Total Petroleum Hydrocarbon Degradation of Soil samples

Table 4.17a showed the results of the total petroleum hydrocarbon of the soil samples polluted with crude oil prior to and after plant growth experiment (PGE). Total petroleum hydrocarbon (TPH) ranged from 21.5671 to 179.5799µg/mL before the plant growth experiment and ranged from 11.7158 to 171.2630µg/mL after plant growth experiment. Total percent remediation recorded was lowest in Egbema-polluted soil without organic amendment (4.63%) and highest in 5mL/5Kg crude oil-polluted soil (46.01%).

The results of the total petroleum hydrocarbon of crude oil polluted soil samples before and after plant growth experiment (PGE) are shown in Table 4.17b. Prior to PGE, total petroleum hydrocarbon (TPH) ranged from 21.5671 to 179.5799µg/mL, while values recorded for TPH ranged from 10.1119 to 159.7454µg/mL after PGE. The least total percent remediation was 11.04% (Egbema-polluted soil without organic amendment) while the highest value was 56.05% (5mL/5Kg crude oil-polluted soil).

Table 4.17a: Total Petroleum Hydrocarbon Degradation of Soil prior to and after PGE using *H. cannabinus*

S/N	Soil Sample Code	TPH before PGE (µg/mL)	TPH after PGE (µg/mL)	% Remediation
1	A1	21.5671± 0.015	11.7158±0.003	45.68%
2	A2	21.5671± 0.015	19.0589±0.110	11.63%
3	A3	32.3306± 0.025	17.4560±0.020	46.01%
4	A4	46.9172± 0.208	32.2811±0.160	31.20%
5	A5	80.4671± 0.031	45.6140±0.060	43.13%
6	A6	94.1905± 0.015	89.4031±0.350	5.08%
7	B1	179.5799± 0.021	164.1112±0.250	8.61%
8	B2	179.5799± 0.021	171.2630±0.050	4.63%
9	B3	77.4659±0.025	46.8459±0.060	39.53%
10	B4	98.3665± 0.031	70.8826±0.120	27.94%

Key: A1 and A2 = 5 Kg unpolluted agricultural soil + organic amendment and –organic amendment respectively; A3, A4, A5 and A6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively; B1 and B2 = 5 Kg polluted soil + organic amendment and –organic amendment respectively; B3 and B4 = 1 Kg polluted soil + 4 Kg unpolluted soil + organic amendment and 2.5 Kg polluted + 2.5 Kg unpolluted soil + organic amendment respectively.

Table 4.17b: Total Petroleum Hydrocarbon Degradation of Soil prior to and after PGE using *Z.mays*

S/N	Soil Sample Code	TPH before PGE (µg/mL)	TPH after PGE (µg/mL)	% Remediation
1	F1	21.5671± 0.015	10.1119± 0.021	53.11%
2	F2	21.5671±0.015	16.6354± 0.020	22.87%
3	F3	32.3306± 0.025	13.6487± 0.015	56.05%
4	F4	46.9172± 0.208	26.5865± 0.025	43.33%
5	F5	80.4671± 0.031	39.3604± 0.021	51.09%
6	F6	94.1905± 0.015	80.2111± 0.021	14.84%
7	G1	179.5799± 0.021	149.4848± 0.020	16.75%
8	G2	179.5799± 0.021	159.7454± 0.020	11.04%
9	G3	77.4659±0.025	43.0983± 0.021	44.36%
10	G4	98.3665± 0.031	62.2205± 0.021	36.75%

Key: F1 and F2 = 5 Kg unpolluted agricultural soil + organic amendment and –organic amendment respectively; F3, F4, F5 and F6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively; G1 and G2 = 5 Kg polluted soil + organic amendment and –organic amendment respectively; G3 and G4 = 1 Kg polluted soil + 4 Kg unpolluted soil + organic amendment and 2.5 Kg polluted + 2.5 Kg unpolluted soil + organic amendment respectively.

4.1.3 Changes in Growth Parameters of *Hibiscus cannabinus and Zea mays*

The results for the total number of leaves observed in *H. cannabinus* during plant growth (PGE I) are shown in Figure 4.1a. The highest and least number of leaves recorded for *H. cannabinus* were 23 leaves (unpolluted agricultural soil with organic amendment) and 2 leaves (day 7). The test pot containing 50ml/5kg crude oil-polluted soil recorded the least number of leaves after a 90-day plant growth.

Figure 4.1b shows the results of the total number of leaves recorded for *H. cannabinus* during plant growth II. Number of leaves ranged from 2 leaves (day 7) to 31 leaves (day 91) after plant growth experiment. The test pot containing Egbema-polluted soil without organic amendment had the least number of leaves after 90 days.

The total number of leaves of *Z. mays* during plant growth I are shown in Figure 4.1c. Unpolluted agricultural soil with organic amendment had the highest number of leaves (13 leaves), followed by 5ml/5kg crude oil-polluted soil (12 leaves). However, the least number of leaves was recorded on day 7.

The results of the total number of leaves plant growth II using *Z. mays* are shown in Figure 4.1d. Plants in the pot for Egbema-polluted soil without organic amendment died after day-70 of plant growth with a general decline in the number of leaves across all groups. All plants in this experimental group were recorded as dead after day-84.

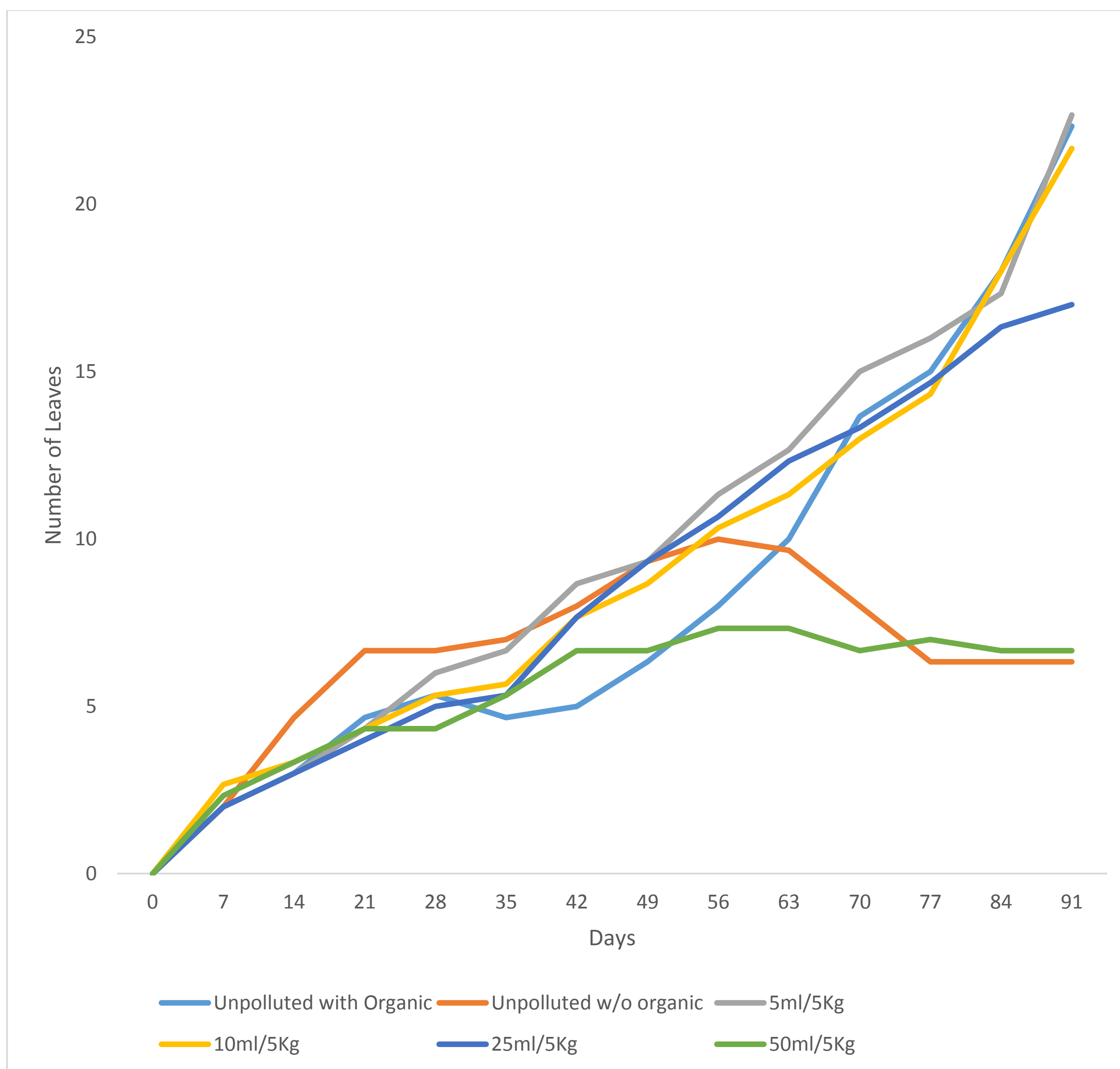


Figure 4.1a: Number of leaves of *H. cannabinus* during plant-growth I

Key: A1 (Light blue) = 5 Kg unpolluted agricultural soil + organic amendment; A2 (Orange) = 5 Kg unpolluted agricultural soil –organic amendment; A3 (Gray), A4 (Yellow), A5 (Dark blue) and A6 (Green) = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively.

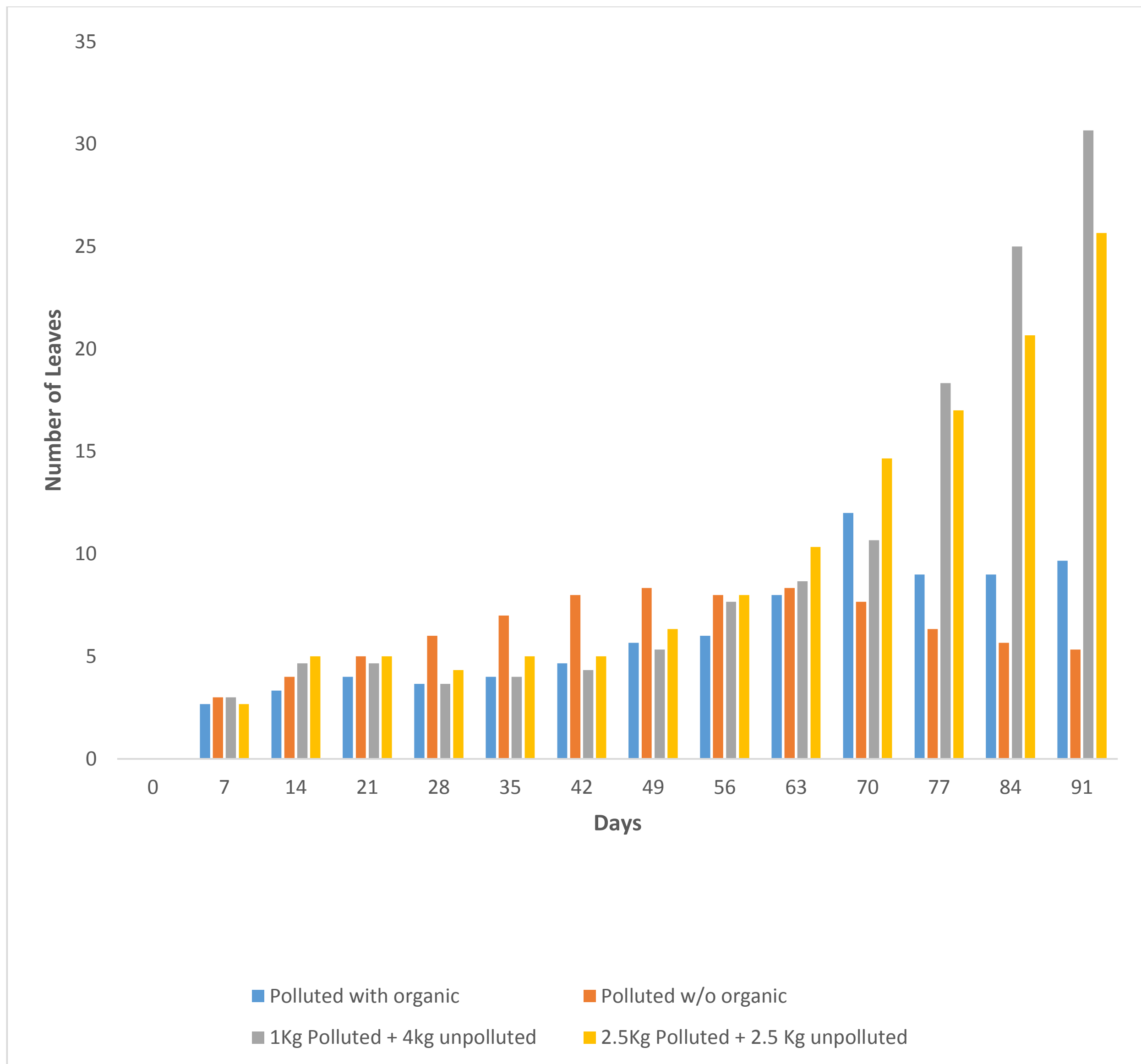


Figure 4.1b: Number of leaves of *H. cannabinus* during plant-growth II

Key: B1 (Blue) = 5 Kg polluted soil + organic amendment; B2 (Orange) = 5 Kg polluted soil–organic amendment; B3 (Gray) = 1 Kg polluted soil + 4 Kg unpolluted soil + organic amendment; B4 (Yellow) = 2.5 Kg polluted + 2.5 Kg unpolluted soil + organic amendment.

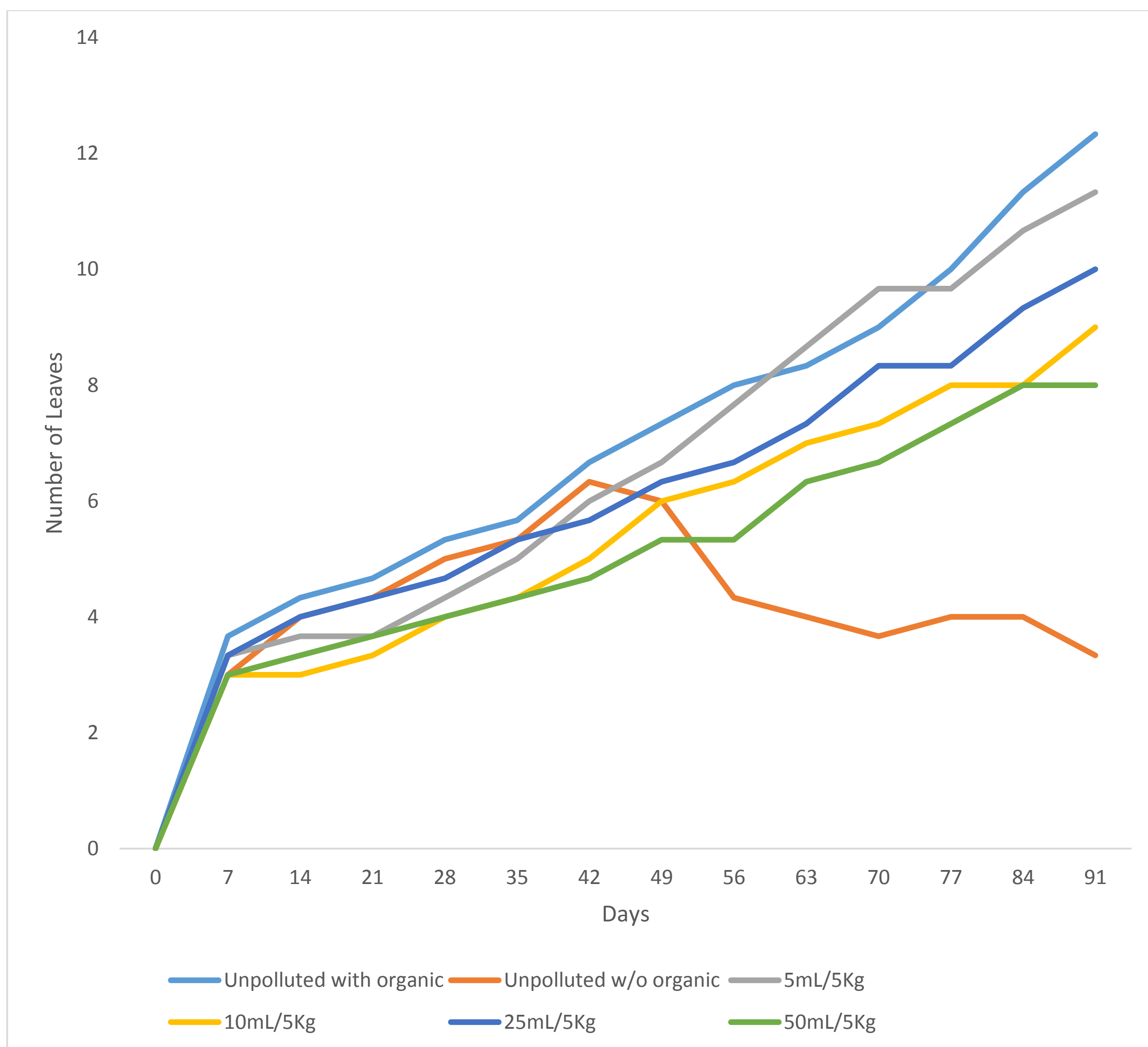


Figure 4.1c: Number of leaves of *Z. mays* during plant-growth I

Key: F1 (Light blue) = 5 Kg unpolluted agricultural soil + organic amendment; F2 (Orange) = 5 Kg unpolluted agricultural soil –organic amendment; F3 (Gray), F4 (Yellow), F5 (Dark blue) and F6 (Green) = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively.

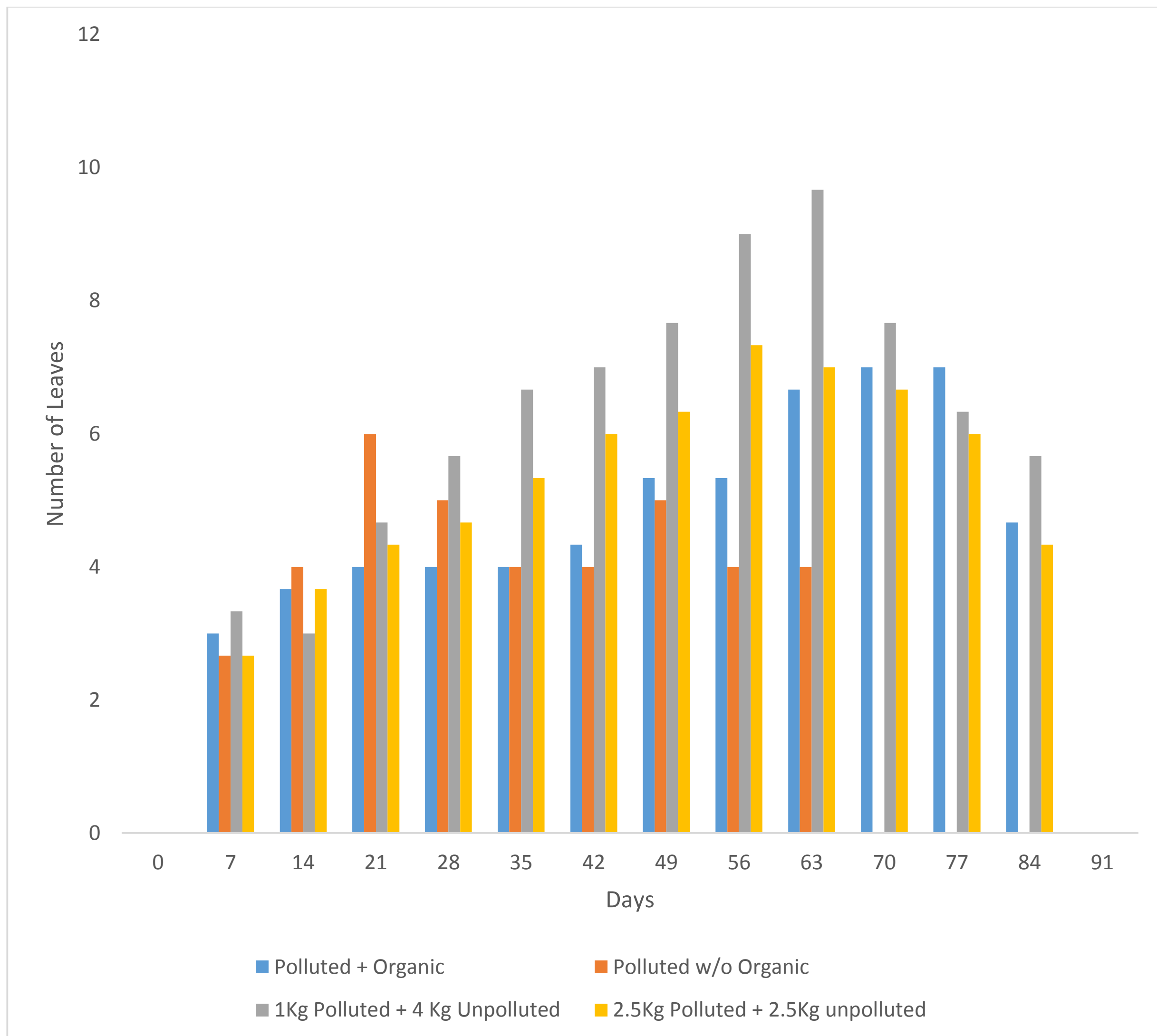


Figure 4.1d: Number of leaves of *Z. mays* during plant-growth II

Key: G1 (Blue) = 5 Kg polluted soil + organic amendment; G2 (Orange) = 5 Kg polluted soil–organic amendment; G3 (Gray) = 1 Kg polluted soil + 4 Kg unpolluted soil + organic amendment; G4 (Yellow) = 2.5 Kg polluted + 2.5 Kg unpolluted soil + organic amendment.

4.1.3.1 Fresh weights of plants after phytoremediation

Table 4.18a showed the results of the fresh weights of the roots and shoots of *H. cannabinus* after the 90-day phytoremediation. Sample 5ml/5kg crude oil-polluted soil recorded the highest fresh weight while 50ml/5kg crude oil-polluted soil recorded the least fresh weight for the roots, shoots and total fresh weight respectively.

The results of the fresh weights of the roots and shoots of *Z. mays* after the 90-day phytoremediation are shown in Table 4.18b. The highest fresh weight was recorded for 5ml/5kg crude oil-polluted soil while unpolluted agricultural soil without organic amendment recorded the least fresh weight for the roots, shoots and total fresh weight respectively.

4.1.3.2 Heights of plants after phytoremediation

Table 4.19a show the total heights, height of shoots and length of roots of *H. cannabinus* after the 90-day phytoremediation. Sample 5ml/5kg crude oil-polluted soil had the highest total plant height 137 cm after phytoremediation of crude oil polluted soil samples, closely followed by samples unpolluted agricultural soil with organic amendment (134.00 cm) and 10ml/5kg crude oil-polluted soil (132.00 cm) respectively. Samples 25ml/5kg crude oil-polluted soil and 50ml/5kg crude oil-polluted soil were observed to have total mean plant heights of 125.00 cm and 86.00 cm respectively while the unpolluted agricultural soil without organic amendment had the least mean total plant height (74.53 cm) observed. The 1kg unpolluted soil + 4kg Egbema-polluted soil had the greatest total plant height 144.0 cm after 90 days of plant growth on Egbema-polluted soil. Total plant heights of 130.0 cm and 126.0 cm were recorded in 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil and Egbema-polluted soil with organic amendment respectively while Egbema-polluted soil without organic amendment had the least recorded total heights of plants (69.0 cm).

The results of the total heights, height of shoots and length of roots of *Z. mays* after the 90-day phytoremediation are shown in Table 4.19b. The highest height of 179 cm was observed for unpolluted agricultural soil with organic amendment while the least plant height (23 cm) was observed in Egbema-polluted soil without organic amendment after 70 days.

Table 4.18a: Fresh weight of above- and below-ground parts of *H. cannabinus* after 90-day plant growth

Soil Sample Code /Parameters	A1	A2	A3	A4	A5	A6	B1	B2	B3	B4
Total Fresh Weight (g)	102.20 ±0.03	19.23 ±0.04	119.33 ±0.02	60.93 ±0.02	106.28 ±0.01	6.08 ±0.00	27.12 ±0.03	7.59 ±0.01	101.99 ±0.02	69.27 ±0.02
Fresh Weight of Roots (g)	13.94 ±0.02	10.02 ±0.02	25.19 ±0.01	8.77 ±0.01	23.43 ±0.02	2.52 ±0.01	6.36 ±0.02	2.71 ±0.02	16.96 ±0.01	16.55 ±0.02
Fresh Weight of Shoots (g)	88.26 ±0.01	9.21 ±0.02	94.14 ±0.01	52.16 ±0.01	82.85 ±0.03	3.56 ±0.01	20.76 ±0.01	4.88 ±0.01	85.03 ±0.02	52.72 ±0.00

Key: A1 and A2 = 5 Kg unpolluted agricultural soil + organic amendment and –organic amendment respectively; A3, A4, A5 and A6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively; B1 and B2 = 5 Kg polluted soil + organic amendment and –organic amendment respectively; B3 and B4 = 1 Kg polluted soil + 4 Kg unpolluted soil + organic amendment and 2.5 Kg polluted + 2.5 Kg unpolluted soil + organic amendment respectively.

± Standard deviation

Table 4.18b: Fresh weight of above- and below-ground parts of Z. *mays* after 90-day plant growth

Soil Sample Code /Parameters	F1	F2	F3	F4	F5	F6	G1	G2	G3	G4
Total Fresh Weight (g)	250.99 ±0.02	215.84 ±0.02	243.54 ±0.01	226.94 ±0.01	119.01 ±0.01	24.12 ±0.02	53.66 ±0.02	23.52 ±0.04	155.84 ±0.02	120.79 ±0.02
Fresh Weight of Roots (g)	31.21 ±0.01	26.05 ±0.02	33.87 ±0.02	25.39 ±0.02	15.91 ±0.01	9.67 ±0.01	11.50 ±0.03	5.43 ±0.02	15.44 ±0.02	13.51 ±0.01
Fresh Weight of Shoots (g)	219.78 ±0.01	189.79 ±0.01	209.67 ±0.01	201.55 ±0.01	103.10 ±0.02	14.45 ±0.03	42.16 ±0.01	18.09 ±0.02	140.40 ±0.01	107.28 ±0.01

Key: F1 and F2 = 5 Kg unpolluted agricultural soil + organic amendment and –organic amendment respectively; F3, F4, F5 and F6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively; G1 and G2 = 5 Kg polluted soil + organic amendment and –organic amendment respectively; G3 and G4 = 1 Kg polluted soil + 4 Kg unpolluted soil + organic amendment and 2.5 Kg polluted + 2.5 Kg unpolluted soil + organic amendment respectively.

± Standard deviation

Table 4.19a: Heights of *H.cannabnius* after 90-day plant growth

Soil Sample Code /Parameters	A1	A2	A3	A4	A5	A6	B1	B2	B3	B4
Total Height of Plant (cm)	134.00 ±1.00	74.53 ±0.25	137.00± 2.00	132.00± 0.90	125.00± 1.00	86.00 ±0.30	126.00± 2.00	69.00 ±0.10	144.00± 1.00	130.00± 2.00
Height of above-ground parts (cm)	126.00± 1.20	68.03 ±0.15	127.00± 1.80	122.00± 1.00	117.50± 0.10	80.00± 0.10	118.00± 2.10	62.00 ±0.10	132.00± 1.00	120.00± 1.00
Length of Roots (cm)	8.00± 0.20	6.50± 0.40	10.00± 0.20	10.00± 0.10	7.50± 0.90	6.00± 0.20	8.00± 0.10	7.00± 0.20	12.00± 2.00	10.00± 3.00

Key: A1 and A2 = 5 Kg unpolluted agricultural soil + organic amendment and –organic amendment respectively; A3, A4, A5 and A6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively; B1 and B2 = 5 Kg polluted soil + organic amendment and –organic amendment respectively; B3 and B4 = 1 Kg polluted soil + 4 Kg unpolluted soil + organic amendment and 2.5 Kg polluted + 2.5 Kg unpolluted soil + organic amendment respectively.

± Standard deviation

Table 4.19b: Heights of Z.*mays* after 90-day plant growth

Soil Sample Code /Parameters	F1	F2	F3	F4	F5	F6	G1	G2	G3	G4
Total Height of Plant (cm)	179.00± 3.00	29.33± 1.53	172.00± 2.00	111.00 ±1.00	79.00± 2.00	26.00± 1.00	33.00± 1.00	23.00± 2.00	125.00± 0.50	118.00± 1.00
Height of above-ground parts (cm)	166.00± 2.00	23.33± 1.04	160.00± 1.00	100.00 ±0.50	70.00± 1.00	18.00± 1.00	23.00± 0.50	16.00± 1.00	113.00± 1.00	107.00± 2.00
Length of Roots (cm)	13.00± 1.00	6.00± 0.50	12.00± 1.00	11.00± 0.50	9.00± 1.00	8.00± 0.00	10.00± 0.50	7.00± 1.00	12.00± 0.50	11.00± 1.00

Key: F1 and F2 = 5 Kg unpolluted agricultural soil + organic amendment and –organic amendment respectively; F3, F4, F5 and F6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively; G1 and G2 = 5 Kg polluted soil + organic amendment and –organic amendment respectively; G3 and G4 = 1 Kg polluted soil + 4 Kg unpolluted soil + organic amendment and 2.5 Kg polluted + 2.5 Kg unpolluted soil + organic amendment respectively.

± Standard deviation

4.1.4 Culture Based Description of Endophytic Bacteria Identity

4.1.4.1 Phenotypic characterization of endophytic bacteria using morphological characteristics

Figure 4.2a showed the log of total endophytic bacterial count of isolates from the roots of *H. cannabinus* using nutrient agar. The highest bacterial count was recorded in NA3A (6.332 CFU/g) followed by NA8B and NA6B with values 6.079 CFU/g respectively while the least bacterial count was observed in sample NA2A (4.875 CFU/g). The morphology of endophytic bacteria isolated from the roots of *H. cannabinus* using nutrient agar are shown in Appendix IVa. All isolates were of various sizes, ranging from 0-1 mm to 5-10 mm and creamy in colour. The isolates were predominantly round, regular or irregular in shape respectively and were either raised or flat.

The log of the total endophytic bacterial count of isolates from the roots of *Z. mays* using nutrient agar are presented in Figure 4.2b. Sample NA2B had the greatest endophytic bacterial count of 6.305 CFU/g while the lowest bacterial count was recorded in sample NA4A. The morphological characteristics of endophytic bacteria isolated from the roots of *Z. mays* using nutrient agar are presented in Appendix IVb. The isolates were creamy in colour and had different size ranges 0-2 mm to 4-6 mm respectively. The endophytic bacterial isolates were either regular or irregular in shape and were either raised or flat.

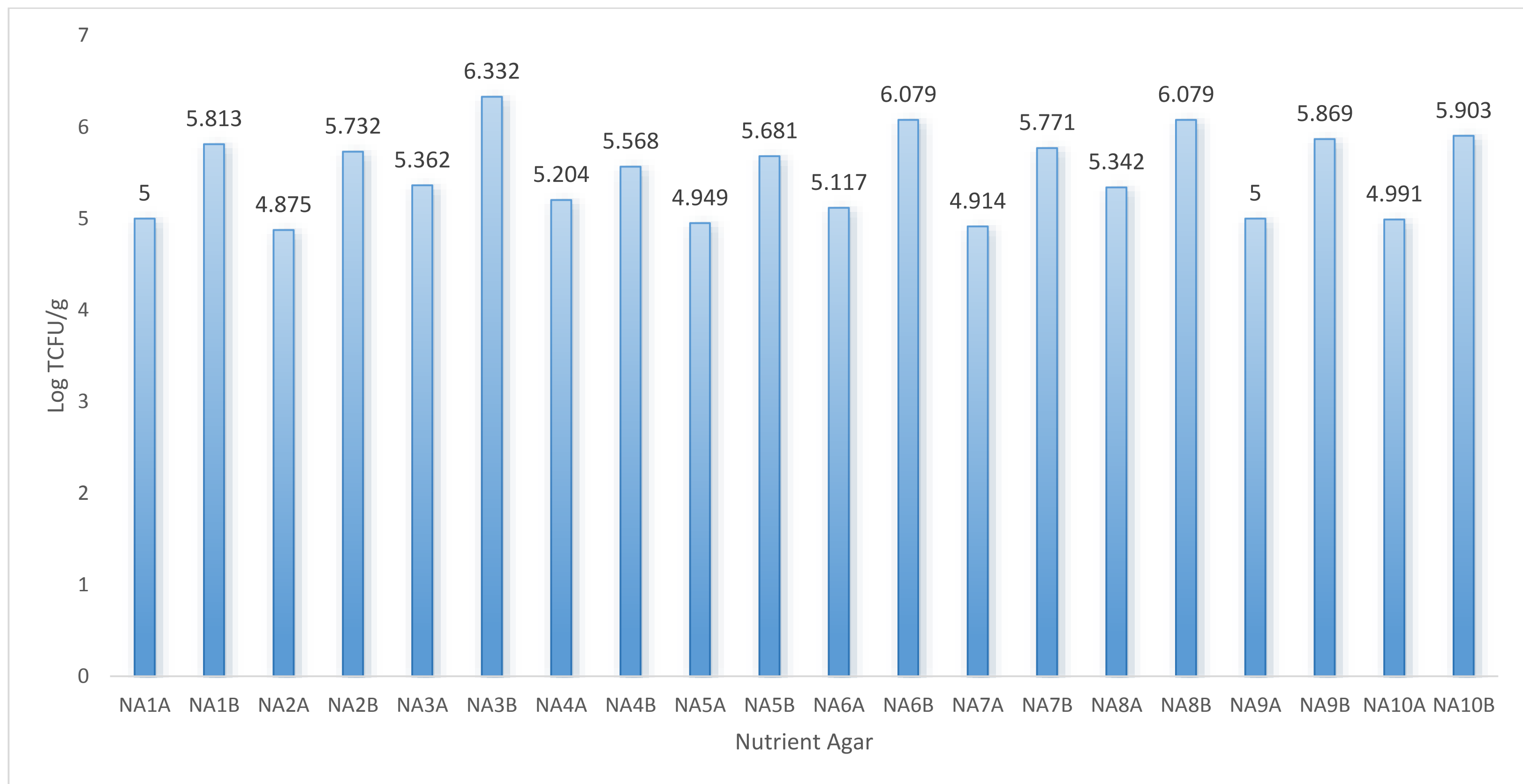


Figure 4.2a: Total bacterial count and morphology of endophytic bacteria isolated from *H. cannabinus* roots using nutrient agar (NA)

Key: NA = Nutrient Agar; A and B = 1st and 2nd replicate; 1 = 5 Kg unpolluted agricultural soil + organic amendment; 2 = 5 Kg unpolluted agricultural –organic amendment; 3, 4 5 and 6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively; 7 = 5 Kg polluted soil + organic amendment; 8 = 5 Kg polluted soil + organic amendment –organic amendment; 9 = 1 Kg polluted soil + 4 Kg unpolluted soil + organic amendment; 10 = 2.5 Kg polluted + 2.5 Kg unpolluted soil + organic amendment.

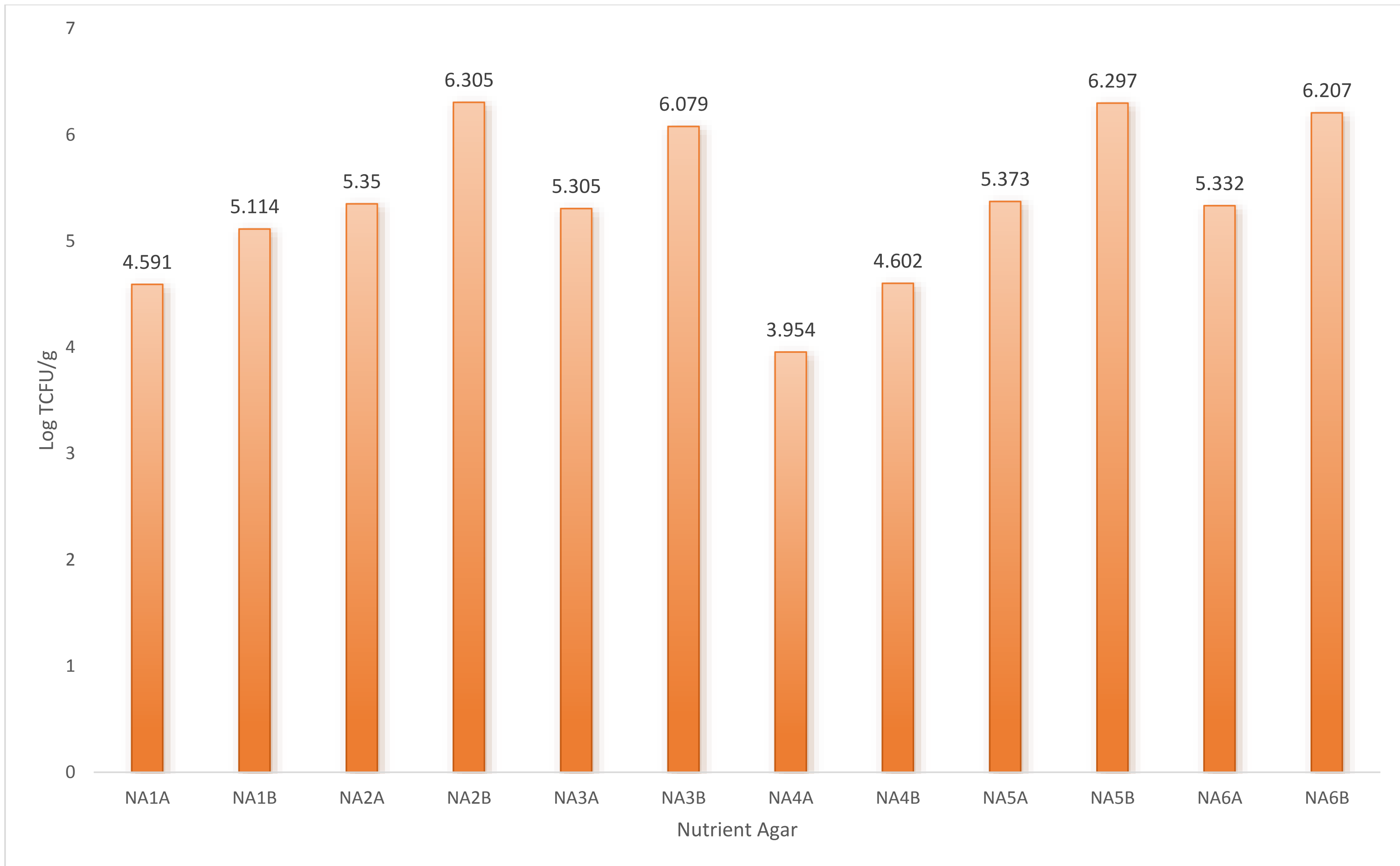


Figure 4.2b: Total bacterial count and morphology of endophytic bacteria isolated from *Z. mays* roots using nutrient agar (NA)

Key: NA = Nutrient Agar; A and B = 1st and 2nd replicate; 1 = 5 Kg unpolluted agricultural soil + organic amendment; 2 = 5 Kg unpolluted agricultural –organic amendment; 3, 4, 5 and 6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively.

Figure 4.2c showed the log of total bacterial count of endophytic bacteria isolated from the roots of *H. cannabinus* using tryptic soy agar. The total endophytic bacterial count was highest in TSA2B (5.447 CFU/g) followed by TSA9B (5.342 CFU/g). The least endophytic bacterial count 3.602 CFU/g was observed in TSA6A. Morphology of endophytic bacteria isolated from the roots of *H. cannabinus* using tryptic soy agar are shown in Appendix IVc. All isolates were of various sizes, ranging from 0-1 mm to 5-10 mm and creamy in colour. The isolates were predominantly round, regular or irregular in shape respectively and were either raised or flat.

The logarithmic value of the total endophytic bacterial count of isolates from the roots of *Z. mays* using tryptic soy agar are presented in Figure 4.2d. Highest endophytic bacterial count for this group (5.903 CFU/g) was observed in TSA3B while the least endophytic count of 3.903 CFU/g was recorded in TSA4A. The morphological characteristics of endophytic bacteria isolated from the roots of *Z. mays* using tryptic soy agar are presented in Appendix IVd. The isolates were creamy in colour and had different size ranges 0-2 mm to 4-6 mm respectively. The endophytic bacterial isolates were either regular or irregular in shape and were either raised or flat.

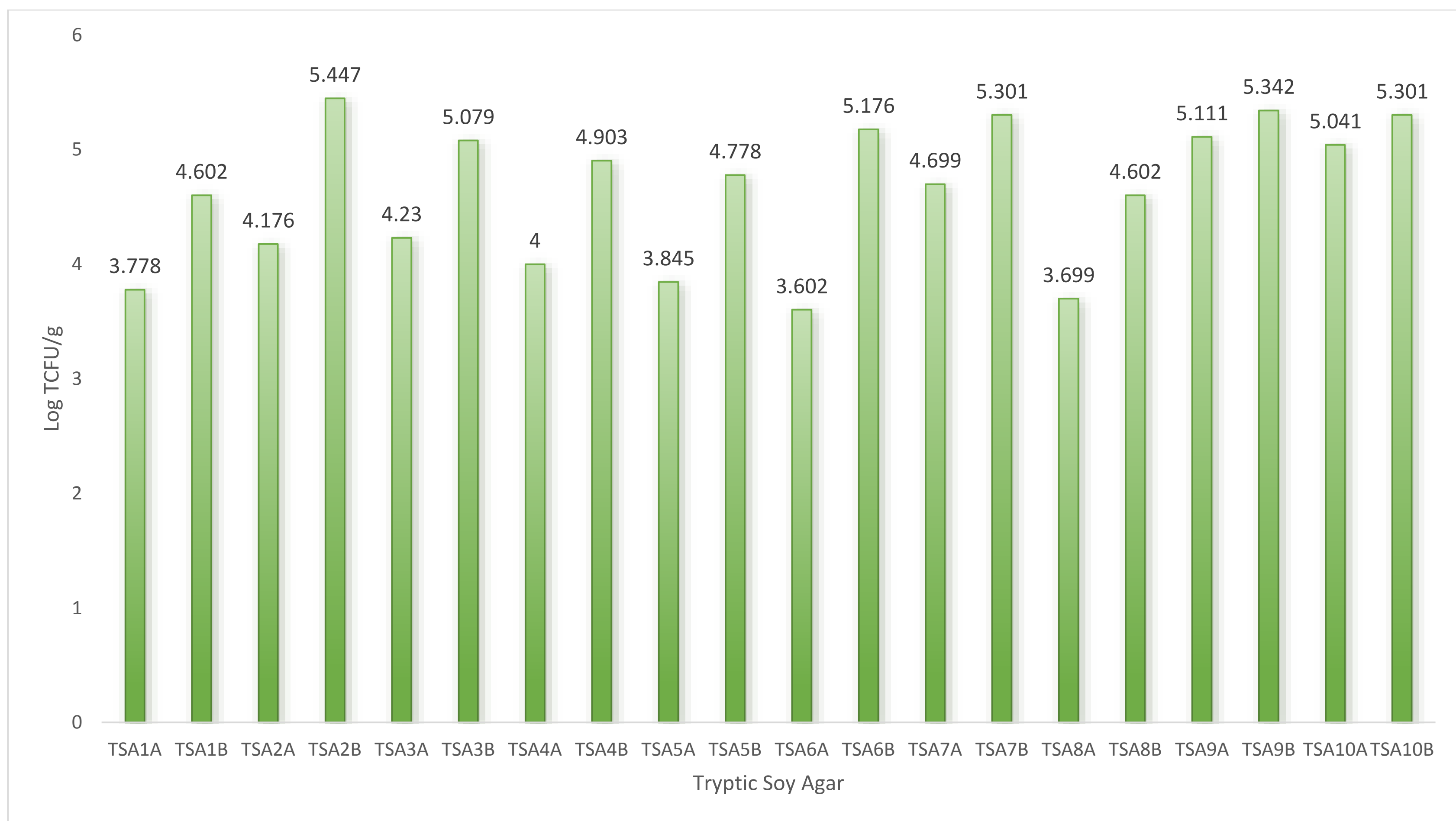


Figure 4.2c: Total bacterial count and morphology of endophytic bacteria isolated from *H. cannabinus* roots using tryptic soy agar (TSA)

Key: TSA = Tryptic Soy Agar; A and B = 1st and 2nd replicate; 1 = 5 Kg unpolluted agricultural soil + organic amendment; 2 = 5 Kg unpolluted agricultural – organic amendment; 3, 4, 5 and 6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively; ; 7 = 5 Kg polluted soil + organic amendment; 8 = 5 Kg polluted soil + organic amendment –organic amendment; 9 = 1 Kg polluted soil + 4 Kg unpolluted soil + organic amendment; 10 = 2.5 Kg polluted + 2.5 Kg unpolluted soil + organic amendment.

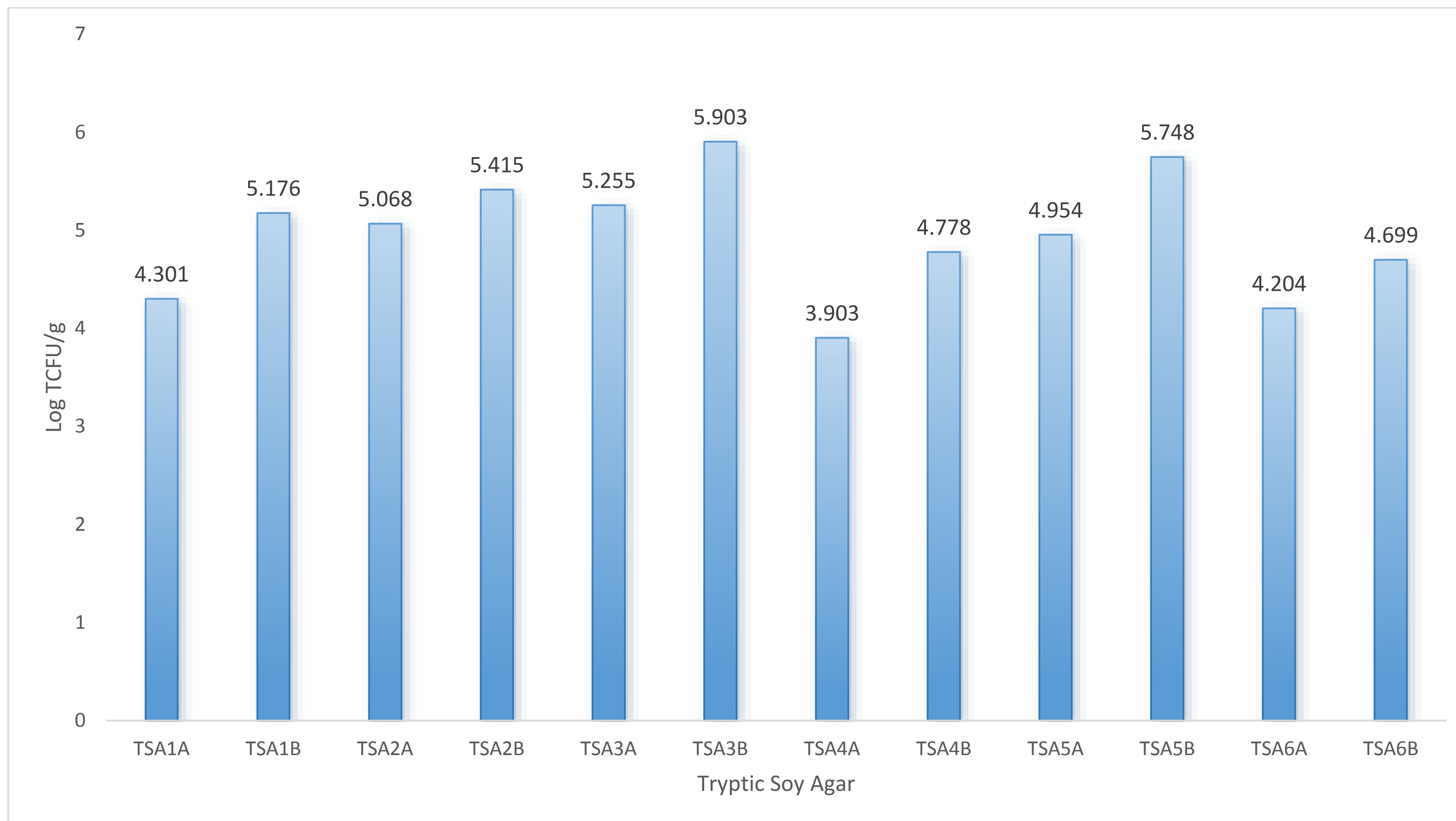


Figure 4.2d: Total bacterial count and morphology of endophytic bacteria isolated from *Z. mays* roots using tryptic soy agar (TSA)

Key: TSA = Tryptic Soy Agar; A and B = 1st and 2nd replicate; 1 = 5 Kg unpolluted agricultural soil + organic amendment; 2 = 5 Kg unpolluted agricultural – organic amendment; 3, 4, 5 and 6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively.

4.1.4.2 Phenotypic characterization of endophytic bacteria using biochemical tests

The phenotypic characterization of endophytic bacteria from the roots of *H. cannabinus* using nutrient agar are presented in Appendix Va. Isolated endophytes were tested for amylase, catalase, oxidase and motility tests. About 85% of the isolates were positive for amylase test while catalase, oxidase and motility tests were recorded positive in 60%, 25% and 20% of isolated endophytes respectively.

The results of the phenotypic characterization of endophytic bacteria from the roots of *Z. mays* using nutrient agar are presented in Appendix Vb. Ten, seven, one and three isolates were positive for amylase, catalase, oxidase and motility tests at 83.3%, 58.3%, 8.3% and 25.0% respectively.

Table Appendix Vc shows the result of phenotypic identification of endophytic bacteria isolated from *H. cannabinus* roots using tryptic soy agar. Isolated endophytic bacteria tested were a total of 16; 15 isolates were positive for amylase test, while 13, 4 and 4 isolates tested positive for catalase, oxidase and motility tests respectively.

The results of the phenotypic characterization of endophytic bacteria from the roots of *Z. mays* using tryptic soy agar are shown in Appendix Vd. Nine bacteria isolates were tested for amylase catalase oxidase and motility. Five isolates were recorded positive for amylase and catalase tests, a single isolate was positive for oxidase test, while no isolate tested positive for motility.

4.1.5 Plant Growth-Promoting Properties of Endophytes

The results of the plant-growth promoting properties of endophytic bacteria isolated from the roots of *H. cannabinus* using tryptic soy agar are shown in Table 4.20a. About 85% of the isolates tested positive for indole acetic acid (IAA) while 15% and 55% tested positive for ACC deaminase and siderophore production respectively. No isolate tested positive for phosphate solubilization.

Table 4.20a: Plant-growth properties of endophytic bacteria isolated from *H. cannabinus* roots using Nutrient Agar

Sample Number	Sample Code	IAA	ACC	Siderophore	Phosphate Solubilization
1	NA1A	+	+	-	-
	NA1B	+	+	-	-
2	NA2A	+	-	+	-
	NA2B	+	-	-	-
3	NA3A	-	-	+	-
	NA3B	+	+	+	-
4	NA4A	-	-	-	-
	NA4B	+	-	+	-
5	NA5A	+	-	-	-
	NA5B	+	-	-	-
6	NA6A	+	-	+	-
	NA6B	+	-	+	-
7	NA7A	+	-	+	-
	NA7B	+	-	-	-
8	NA8A	+	-	-	-
	NA8B	-	-	+	-
9	NA9A	+	-	-	-
	NA9B	+	-	+	-
10	NA10A	+	-	+	-
	NA10B	+	-	+	-

Key: NA = Nutrient Agar; A and B = 1st and 2nd replicate; 1 = 5 Kg unpolluted agricultural soil + organic amendment; 2 = 5 Kg unpolluted agricultural –organic amendment; 3, 4, 5 and 6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively; ; 7 = 5 Kg polluted soil + organic amendment; 8 = 5 Kg polluted soil + organic amendment –organic amendment; 9 = 1 Kg polluted soil + 4 Kg unpolluted soil + organic amendment; 10 = 2.5 Kg polluted + 2.5 Kg unpolluted soil + organic amendment. IAA = Indole-3-acetic acid; ACC = 1-aminocyclopropane-1-carboxylate deaminase.

Plant-growth promoting (PGP) properties of endophytic bacteria isolated from *Z. mays* roots using nutrient agar are presented in Table 4.20b. The enzyme ACC deaminase was synthesized by 50% of the isolates while 42% bacterial isolates produced both indole acetic acid and metal chelating siderophores was produced by 42% of endophytic isolates respectively. Phosphate was not solubilized by any of the isolated endophytic bacteria from the study.

The results of the plant-growth promoting properties of endophytic bacteria isolated from the roots of *H. cannabinus* using tryptic soy agar are presented in Table 4.20c. Of the 16 isolates tested, 12 isolates presented positive results for IAA production, 6 isolates synthesized ACC deaminase and two isolates produced siderophore. Calcium phosphate was not solubilized by any of the isolates.

Table 4.20d shows the plant-growth promoting properties of bacterial endophytes isolated from *Z. mays* roots using tryptic soy agar. All isolates produced IAA, 9% synthesized ACC deaminase, 20% produced siderophore while no isolate solubilized phosphate.

Table 4.20b: Plant-growth properties of endophytic bacteria isolated from *Z. mays* roots using Nutrient Agar

Sample Number	Sample Code	IAA	ACC	Siderophore	Phosphate Solubilization
1	NA1A	-	-	+	-
	NA1B	+	+	-	-
2	NA2A	-	-	+	-
	NA2B	-	+	+	-
3	NA3A	-	-	-	-
	NA3B	+	+	-	-
4	NA4A	+	+	-	-
	NA4B	+	-	-	-
5	NA5A	-	-	-	-
	NA5B	-	+	-	-
6	NA6A	-	-	+	-
	NA6B	+	+	+	-

Key: NA = Nutrient Agar; A and B = 1st and 2nd replicate; 1 = 5 Kg unpolluted agricultural soil + organic amendment; 2 = 5 Kg unpolluted agricultural –organic amendment; 3, 4, 5 and 6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively. IAA = Indole-3-acetic acid; ACC = 1-aminocyclopropane-1-carboxylate deaminase.

Table 4.20c: Plant-growth properties of endophytic bacteria isolated from *H. cannabinus* roots using Tryptic Soy Agar

Sample Number	Sample Code	IAA	ACC	Siderophore	Phosphate Solubilization
1	TSA1A	+	+	-	-
2	TSA2A	+	-	-	-
	TSA2B	+	-	-	-
3	TSA3A	-	+	-	-
	TSA3B	+	+	+	-
4	TSA4A	-	-	-	-
	TSA4B	+	-	-	-
5	TSA5A	-	-	-	-
	TSA5B	+	-	-	-
6	TSA6A	-	-	-	-
	TSA6B	+	-	-	-
7	TSA7A	+	-	-	-
8	TSA8B	+	-	-	-
9	TSA9A	+	+	-	-
	TSA9B	+	+	+	-
10	TSA10A	+	+	-	-

Key: TSA = Tryptic Soy Agar; A and B = 1st and 2nd replicate1 = 5 Kg unpolluted agricultural soil + organic amendment; 2 = 5 Kg unpolluted agricultural –organic amendment; 3, 4, 5 and 6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively; ; 7 = 5 Kg polluted soil + organic amendment; 8 = 5 Kg polluted soil + organic amendment –organic amendment; 9 = 1 Kg polluted soil + 4 Kg unpolluted soil + organic amendment; 10 = 2.5 Kg polluted + 2.5 Kg unpolluted soil + organic amendment. IAA = Indole-3-acetic acid; ACC = 1-aminocyclopropane-1-carboxylate deaminase.

Table 4.20d: Plant-growth properties of endophytic bacteria isolated from *Z. mays* roots using Tryptic Soy Agar

Sample Number	Sample Code	IAA	ACC	Siderophore	Phosphate Solubilization
1	TSA1A	+	+	+	-
	TSA1B	+	-	+	-
2	TSA2A	+	-	-	-
	TSA3B	+	-	-	-
3	TSA3A	+	+	+	-
4	TSA4A	+	-	-	-
	TSA4B	+	-	-	-
5	TSA5A	+	-	-	-
	TSA5B	+	-	+	-

Key: TSA = Tryptic Soy Agar; A and B = 1st and 2nd replicate; 1 = 5 Kg unpolluted agricultural soil + organic amendment; 2 = 5 Kg unpolluted agricultural –organic amendment; 3, 4 and 5 = 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively. IAA = Indole-3-acetic acid; ACC = 1-aminocyclopropane-1-carboxylate deaminase.

4.1.6 Bioremediation Potential of Endophytes using Different n-Alkanes and Diesel

Endophytic bacteria isolated from *H. cannabinus* roots using nutrient agar were grown on media amended with different n-alkanes (C_6, C_8 and C_{10}) and diesel. The results are shown in Table 4.21a. All isolates showed growth on the different media after 18 h of incubation.

The results of the bioremediation activities of endophytic bacteria isolated from roots of *Z. mays* using nutrient agar are shown in Table 4.21b. All endophytic bacteria isolated showed growth (100%) for all n-alkanes and diesel amended culture media after 18 h incubation period.

Table 4.21c show the results of the bioremediation properties of endophytic bacteria isolated from *H. cannabinus* roots using tryptic soy agar amended with hexane (C_6), octane (C_8), decane (C_{10}) and diesel respectively. Positive results were recorded for all bacterial isolates from the study indicating their hydrocarbon degradation potential.

The bioremediation potential of bacterial endophytes isolated from roots of *Z. mays* using TSA with various hydrocarbon amendments are shown in Table 4.21d. All isolates were able to grow on the differently amended media after 18 h incubation.

Table 4.21a: Bioremediation activities of endophytic bacteria isolated from *H. cannabinus* roots using Nutrient Agar

Sample Number	Sample Code	Hexane (C_6)	Octane (C_8)	Decane (C_{10})	Diesel
1	NA1A	+	+	+	+
	NA1B	+	+	+	+
2	NA2A	+	+	+	+
	NA2B	+	+	+	+
3	NA3A	+	+	+	+
	NA3B	+	+	+	+
4	NA4A	+	+	+	+
	NA4B	+	+	+	+
5	NA5A	+	+	+	+
	NA5B	+	+	+	+
6	NA6A	+	+	+	+
	NA6B	+	+	+	+
7	NA7A	+	+	+	+
	NA7B	+	+	+	+
8	NA8A	+	+	+	+
	NA8B	+	+	+	+
9	NA9A	+	+	+	+
	NA9B	+	+	+	+
10	NA10A	+	+	+	+
	NA10B	+	+	+	+

Key: NA = Nutrient Agar; A and B = 1st and 2nd replicate; 1 = 5 Kg unpolluted agricultural soil + organic amendment; 2 = 5 Kg unpolluted agricultural –organic amendment; 3, 4, 5 and 6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively; ; 7 = 5 Kg polluted soil + organic amendment; 8 = 5 Kg polluted soil + organic amendment –organic amendment; 9 = 1 Kg polluted soil + 4 Kg unpolluted soil + organic amendment; 10 = 2.5 Kg polluted + 2.5 Kg unpolluted soil + organic amendment.

Table 4.21b: Bioremediation activities of endophytic bacteria isolated from *Z. mays* roots using Nutrient Agar

Sample Number	Sample Code	Hexane (C_6)	Octane (C_8)	Decane (C_{10})	Diesel
1	NA1A	+	+	+	+
	NA1B	+	+	+	+
2	NA2A	+	+	+	+
	NA2B	+	+	+	+
3	NA3A	+	+	+	+
	NA3B	+	+	+	+
4	NA4A	+	+	+	+
	NA4B	+	+	+	+
5	NA5A	+	+	+	+
	NA5B	+	+	+	+
6	NA6A	+	+	+	+
	NA6B	+	+	+	+

Key: NA = Nutrient Agar; A and B = 1st and 2nd replicate; 1 = 5 Kg unpolluted agricultural soil + organic amendment; 2 = 5 Kg unpolluted agricultural –organic amendment; 3, 4, 5 and 6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively.

Table 4.21c: Bioremediation activities of endophytic bacteria isolated from *H. cannabinus* roots using Tryptic Soy Agar

Sample Number	Sample Code	Hexane (C_6)	Octane (C_8)	Decane (C_{10})	Diesel
1	TSA1A	+	+	+	+
2	TSA2A	+	+	+	+
	TSA2B	+	+	+	+
3	TSA3A	+	+	+	+
	TSA3B	+	+	+	+
4	TSA4A	+	+	+	+
	TSA4B	+	+	+	+
5	TSA5 A	+	+	+	+
	TSA5B	+	+	+	+
6	TSA6A	+	+	+	+
	TSA6B	+	+	+	+
7	TSA7A	+	+	+	+
8	TSA8B	+	+	+	+
9	TSA9A	+	+	+	+
	TSA9B	+	+	+	+
10	TSA10A	+	+	+	+

Key: TSA = Typtic Soy Agar; A and B = 1st and 2nd replicate; 1 = 5 Kg unpolluted agricultural soil + organic amendment; 2 = 5 Kg unpolluted agricultural –organic amendment; 3, 4, 5 and 6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively; ; 7 = 5 Kg polluted soil + organic amendment; 8 = 5 Kg polluted soil + organic amendment –organic amendment; 9 = 1 Kg polluted soil + 4 Kg unpolluted soil + organic amendment; 10 = 2.5 Kg polluted + 2.5 Kg unpolluted soil + organic amendment.

Table 4.21d: Bioremediation activities of endophytic bacteria isolated from *Z. mays* roots using Tryptic Soy Agar

Sample Number	Sample Code	Hexane (C_6)	Octane (C_8)	Decane (C_{10})	Diesel
1	TSA1A	+	+	+	+
	TSA1B	+	+	+	+
2	TSA2A	+	+	+	+
	TSA2B	+	+	+	+
3	TSA3A	+	+	+	+
4	TSA4A	+	+	+	+
	TSA4B	+	+	+	+
5	TSA5A	+	+	+	+
	TSA5B	+	+	+	+

Key: TSA = Typtic Soy Agar; A and B = 1st and 2nd replicate; 1 = 5 Kg unpolluted agricultural soil + organic amendment; 2 = 5 Kg unpolluted agricultural –organic amendment; 3, 4 and 5 = 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively.

4.1.7 Genetic Characterization of Endophytes

4.1.7.1 Agarose gel electrophoresis of extracted endophytic DNA

Plate 4.1 show the gel picture of the gDNA extracted from the selected endophytic bacteria. Extracted gDNA were clear, with distinct bands showing high output of extracted DNA.

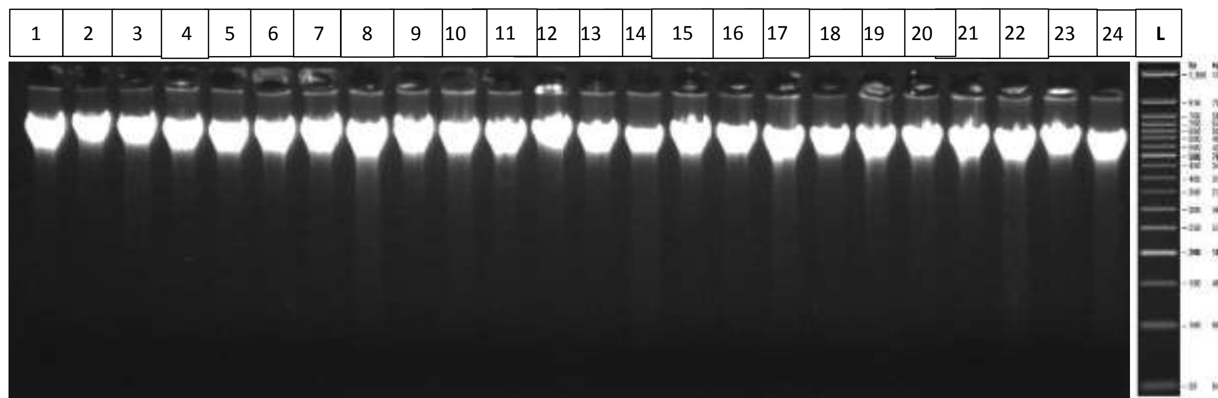


Plate 4.1: Agarose gel picture of genomic DNA from selected endophytic bacteria

KEY:
L=50bp DNA ladder; **1,2,3,4,5** represent NA1A, NA1B, NA2B, NA4A, NA4B;**6,7,8,9,10** represent NA5A, NA5B, NA6A, NA7B, NA8A; **11,12,13,14,15** represent NA8B, NA9A, NA10B, NA11A, NA11B; **16,17,18,19,20** represent NA12B, NA13B, NA14A, NA14B, NA15A;**21,22,23,24** represent NA15B, NA16A, NA16B and TSA 3 respectively.

4.1.7.2 Quantification and qualification of extracted endophytic DNA

The UV-Spectrophotometric readings of the 24 extracted gDNA of bacterial endophytes are shown in Table 4.22. Extracted gDNA were of high quality ranging from 1.85 to 1.94. This result was within the accepted range for pure DNA, 1.8±0.2. The quantities of extracted gDNA from the endophytic pure cultures were also high, ranging from 182 to 904 ng/µL.

Table 4.22: Quantification of genomic DNA of selected endophytes

Sample Code	Nucleic Acid (ng/µL)	A_{260nm}	A_{280nm}	$A^{260}/_{280}$
NA1A	504.0	10.080	5.334	1.89
NA1B	182.0	3.640	1.971	1.85
NA2B	435.3	8.707	4.623	1.88
NA4A	396.9	7.939	4.229	1.88
NA4B	305.2	6.105	3.244	1.88
NA5A	401.2	8.024	4.251	1.89
NA5B	409.7	8.193	4.337	1.89
NA6A	390.0	7.800	4.020	1.94
NA7B	557.4	11.148	5.937	1.88
NA8A	395.4	7.908	4.232	1.87
NA8B	353.6	7.072	3.742	1.89
NA9A	406.6	8.132	4.320	1.88
NA10B	311.8	6.236	3.340	1.87
NA11A	187.3	3.746	2.013	1.86
NA11B	456.6	9.131	4.835	1.89
NA12B	323.2	6.465	3.435	1.88
NA13B	402.3	8.046	4.291	1.88
NA14A	250.7	5.013	2.691	1.86
NA14B	389.7	7.794	4.134	1.89
NA15A	325.2	6.503	3.483	1.87
NA15B	992.3	19.845	10.740	1.85
NA16A	391.8	7.836	4.166	1.88
NA16B	362.4	7.249	3.888	1.86
NA20A	241.8	4.835	2.606	1.86

4.1.7.3 Detection of alkane monooxygenase genes of bacteria endophytes

The amplification results of alkane degradative genes (Alk B and Alk B1) of the extracted gDNA from selected bacterial endophytes are shown in Plate 4.2. About 46% of the genomic DNA (gDNA) of the endophytic bacterial isolates possessed alkane monooxygenase genes (Plate 4.2) except sample NA11B (well 15) which showed no band. Out of the 24 gDNA of isolates analyzed in this study (Figure 4.3), 11 isolates contained genes 550 bp in size (45.8%) the *Alk* B genes, followed by 5 isolates possessing 400 bp genes (20.8%). The least gene sizes identified in the study were 350 bp (1 isolate) and 250 bp (6 isolates) with relative abundance 4.2% and 25% respectively. About 1000 bp genes were identified in 8 isolates while 916 bp genes were observed in 10 isolates representing 33.3% and 41.7% of analyzed gDNA respectively. Out of the 24 gDNA analyzed, 12 gDNA of endophytic bacteria were isolated from *H. cannabinus* while 11 gDNA of bacterial endophytes were isolated from *Z. mays*.

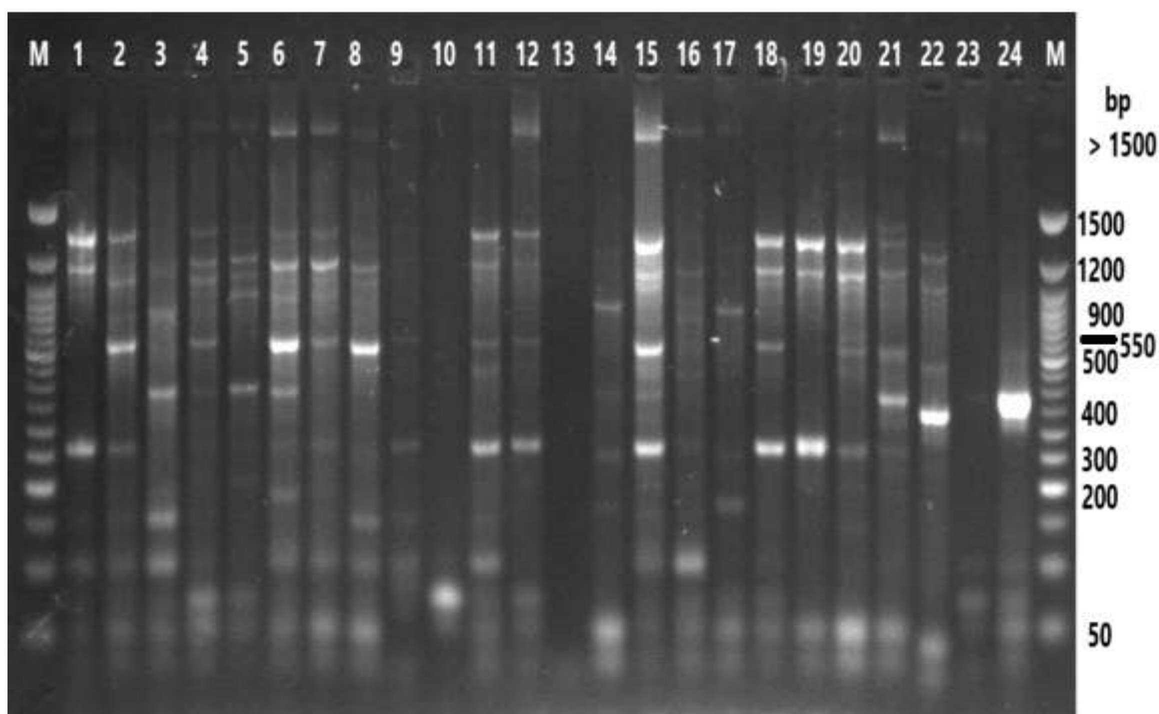


Plate 4.2: Amplification profiles of alkane degradative genes of selected extracted gDNA using Alk B primer

KEY:
M=50bp DNA ladder; **1,2,3,4,5** represent NA1A, NA1B, NA2B, NA4A, NA4B;**6,7,8,9,10** represent NA5A, NA5B, NA6A, NA7B, NA8A; **11,12,13,14,15** represent NA8B, NA9A, NA10B, NA11A, NA11B; **16,17,18,19,20** represent NA12B, NA13B, NA14A, NA14B, NA15A;**21,22,23,24** represent NA15B, NA16A, NA16B and TSA 3 respectively.

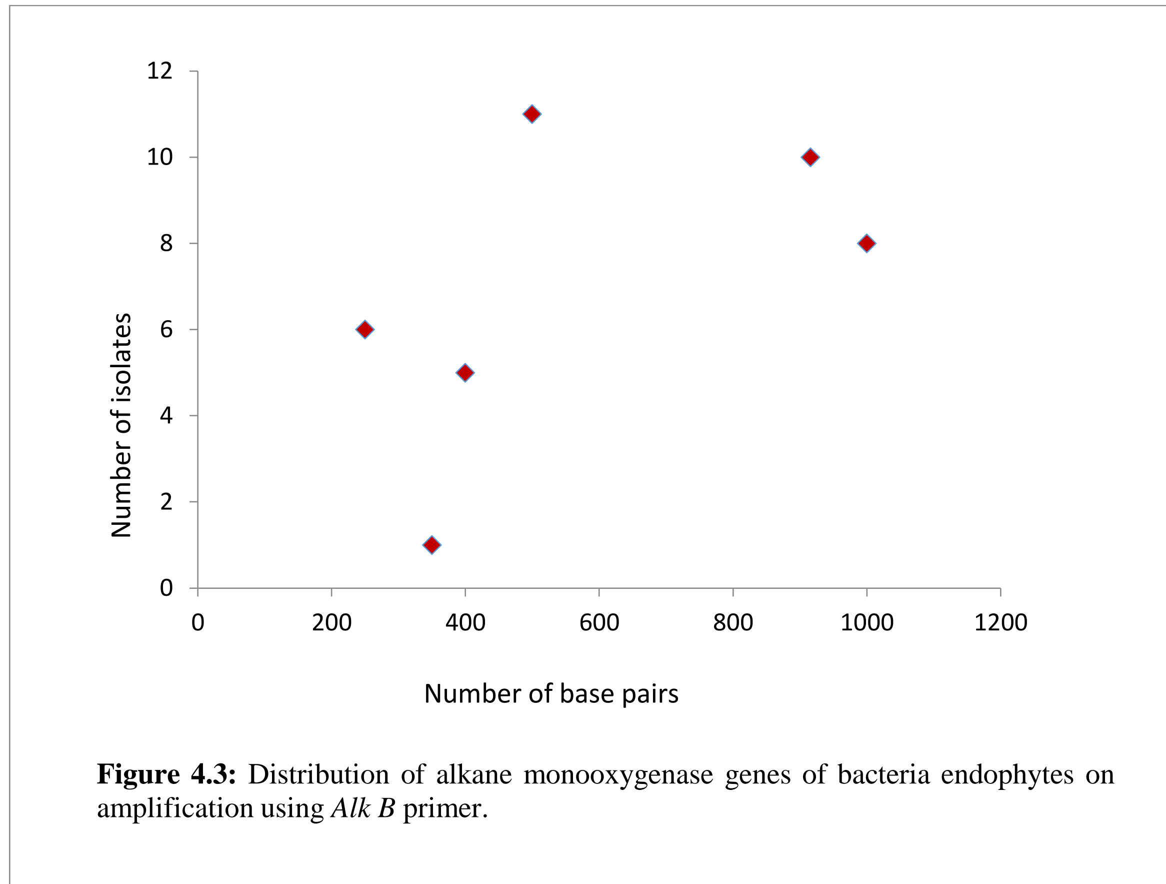


Figure 4.3: Distribution of alkane monooxygenase genes of bacteria endophytes on amplification using *Alk B* primer.

4.1.7.4 Amplification of 16s rDNA amplicons

The 16s rRNA amplification results of 18 randomly selected endophytic bacterial gDNA are shown in Plates 4.3a and 4.3b. All selected gDNA amplified using 16s rRNA primer except sample NA4B (well 5). Genomic DNA of samples NA5A (well 6), NA6A (well 7), NA9A (well 11), and NA11B (well 14) gave high intensity bands while other samples amplified showing bands of low intensity.

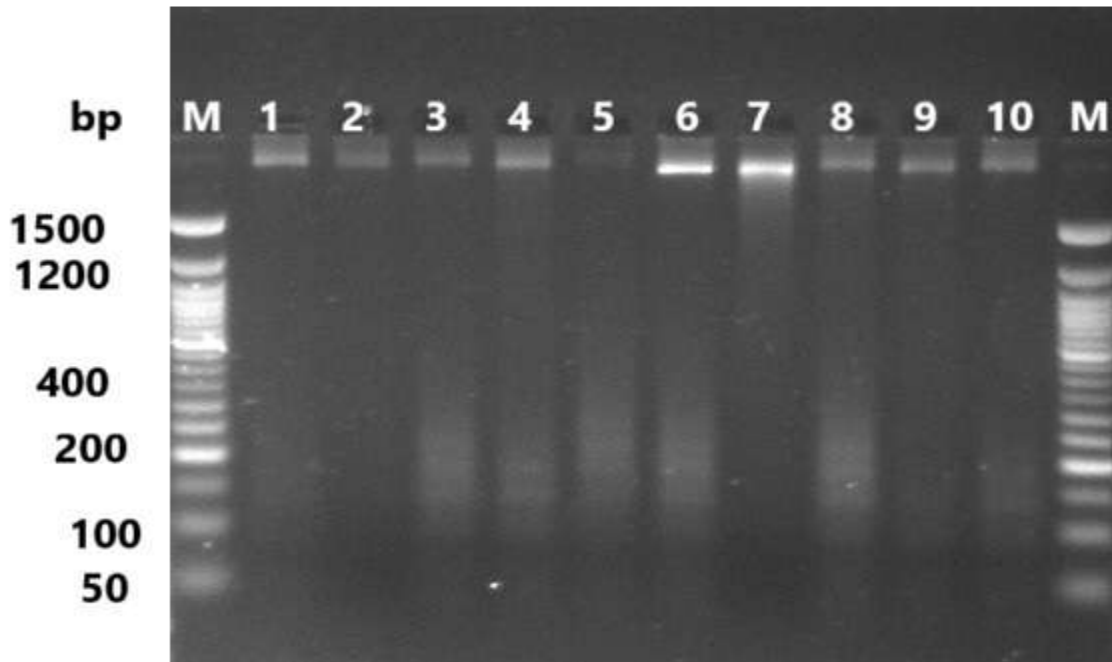


Plate 4.3a: Amplification profiles of 16s rRNA genes of selected extracted gDNA using 2f and 1492r primers

KEY:
M=50bp DNA ladder; **1**, **2**, **3, 4, 5** represent NA1A, NA1B, NA2B, NA4A, NA4B; **6, 7, 8, 9, 10** represent NA5A, NA6A, NA7B, NA8A, NA8B respectively.

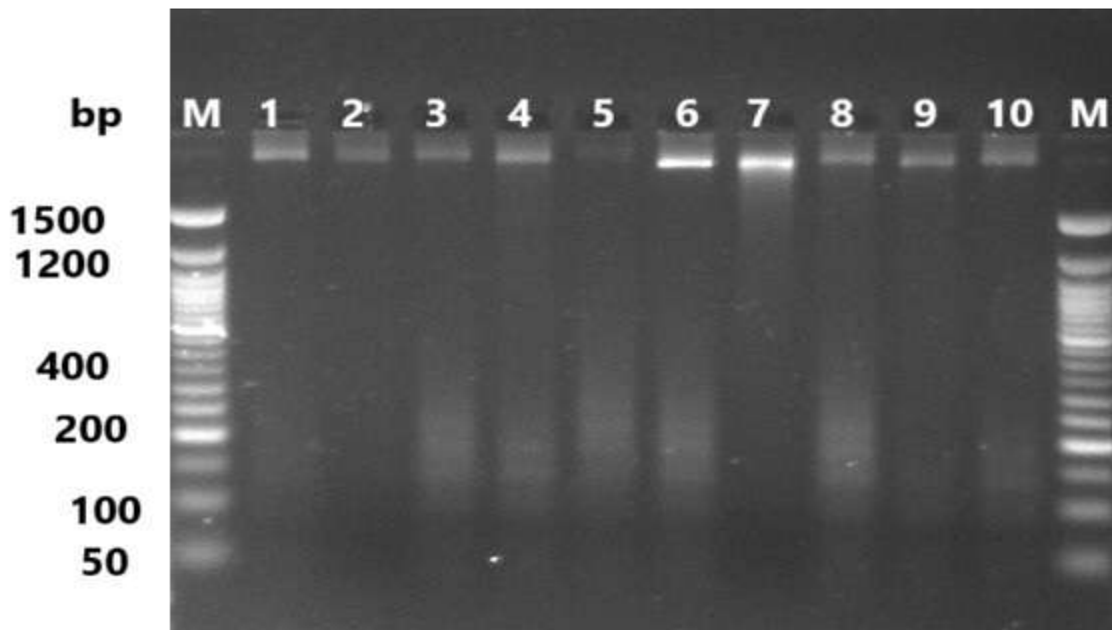


Plate 4.3b: Amplification profiles of 16s rRNA genes of selected extracted gDNA using 2f and 1492r primers

KEY:
M=50bp DNA ladder; **11, 12, 13, 14, 15** represent NA9A, NA10B, NA11A, NA11B, NA12B; **16, 17, 18** represent NA13B, NA15A, NA16A respectively.

4.1.7.5 Nucleotide sequencing and blasting of 16s rDNA amplicons

Nucleotide BLAST results of 16s rDNA amplicons of selected gDNA are shown in Table 4.23. *Proteus mirabilis* had the highest recorded identity (94.59%) while *Alcaligens faecalis* had the least recorded identity (71.65%). E-values recorded for *Alcaligens* spp. (NA9A) and *Proteus vulgaris* (NA15A) were zero. Moreover *Proteus* spp. and *Alcaligens* spp. were identified in 5 and 3 isolates respectively.

4.1.7.6 Genomic diversity analysis of endophytic bacteria isolates

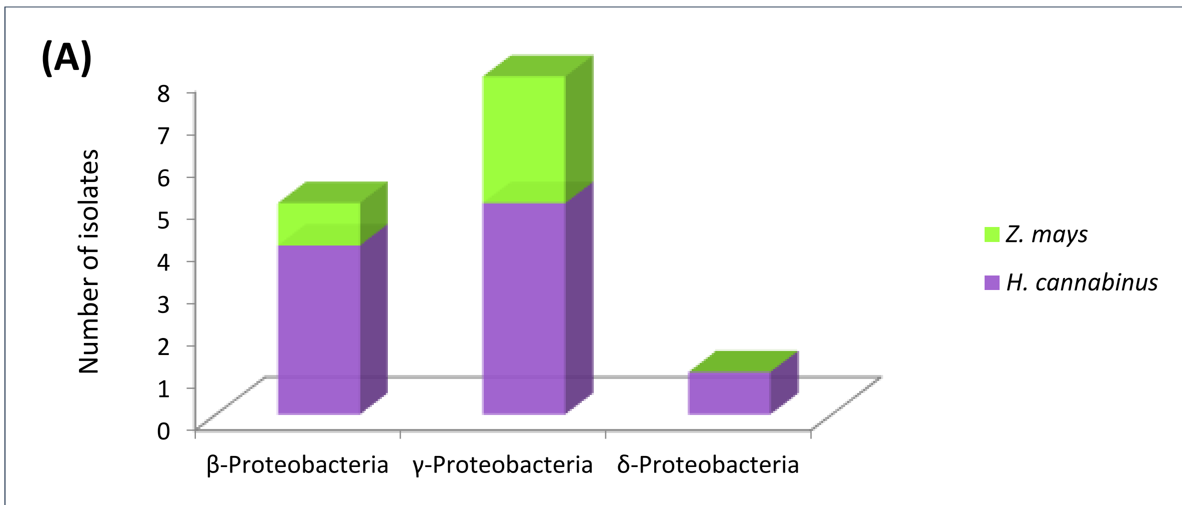
Diversity analysis of the 16s rDNA region of the genomic DNA of bacterial isolates are shown in Figure 4.4. All endophytic bacteria isolated in the study belonged to phylum *Proteobacteria*. Of the 5 isolates annoted β-*proteobacteria*, 4 isolates were from *H. cannabinus* roots while 1 isolate was from the roots of *Z. mays*. The most predominant class, γ-*Proteobacteria* contained 5 and 3 isolates from *H. cannabinus* and *Z. mays* roots respectively while a single isolate belonging to the class δ-*Proteobacteria* was from roots of *H. cannabinus* (Figure 4.4a). *Hibiscus cannabinus* offered a higher diversity as it contained bacteria from class β-*Proteobacteria*, γ-*Proteobacteria* and δ-*Proteobacteria* while *Z. mays* harboured only bacteria from class β-*Proteobacteria* (1 isolate) and γ-*Proteobacteria* (3 isolates).

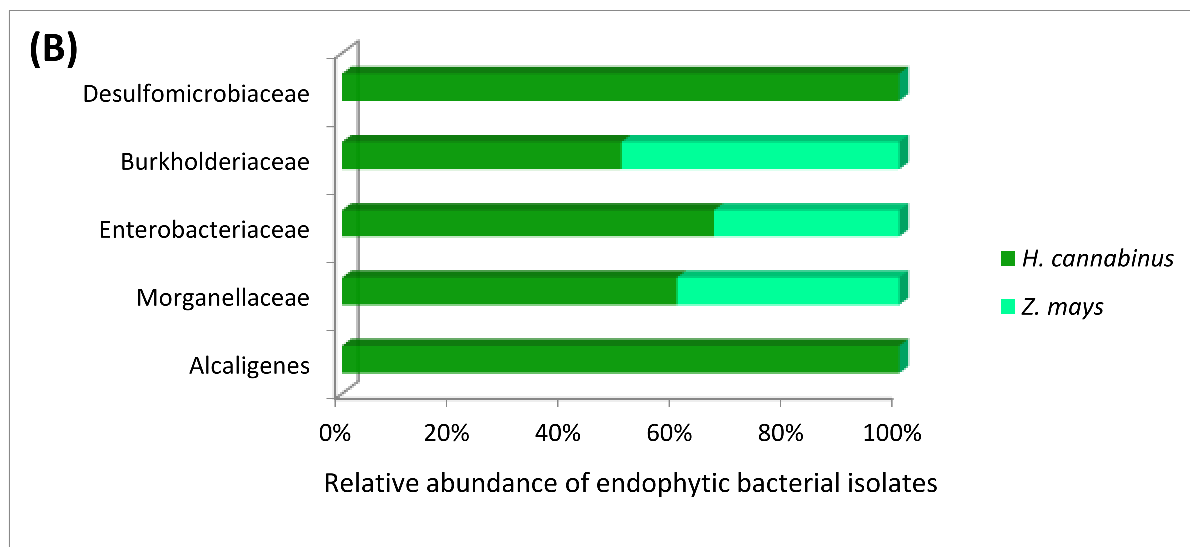
The diversity of endophytic bacterial isolates in this study was spread across orders *Burkholderiales*, *Enterobacterales* and *Desulfovibrionales*. The order *Enterobacterales* (8 isolates) was the most predominant, with 5 isolates from *H. cannabinus* and 3 isolates from *Z. mays* while the least abundant order was the *Desulfovibrionales* (1 isolate). A wide family distribution was observed in this study of the endophytic community within *H. cannabinus* and *Z. mays*. The family *Morganellaceae* recorded the highest distribution (5 isolates), followed by families *Alcaligenes* and *Enterobacteriaceae* (3 isolates each), *Burkholderiaceae* (2 isolates) while the least distribution was observed in the family *Desulfomicrobiaceae* (Figure 4.4b). In addition, *Proteus* with a relative abundance of 35.7% was the most abundant genus, followed by the genus *Alcaligenes*, relative abundance 21.4% (Figure 4.4c). Two isolates each were identified as the genera *Enterobacter* and *Burkholderia* (relative abundance 14.3%) while the genera with the least recorded relative abundance of 7.1% belonged to *Desulfomicrobium* and *Shimwella* (1 isolate each).

Table 4.23: Nucleotide Blast Results of 16s rRNA amplicons of PCR products

Sample Code	Ascension No	E-Value	% identification	Name of Organism**
NA1B	KT366027.1	2.00E-137	80.39	*Alcaligenes aquatilis*
NA2B	MZ424630.1	2.00E-35	71.65	*Alcaligenes faecalis*
NA4A	FJ492809.2	1.00E-14	89.47	*Shimwellia pseudoproteus*
NA4B	MT020362.1	7.00E-17	84.62	*Enterobacter cloacae* subsp *dissolvens*
NA5A	KY914487.1	1.00E-38	94.59	*Proteus mirabilis*
NA7B	JN622128.1	8.00E-09	82.43	*Burkholderia* spp.
NA8A	MG836238.1	0.026	78.26	*Desulfomicrobium baculatum*
NA8B	MZ363637.1	1.00E-49	76.65	*Proteus vulgaris*
NA9A	KP877536.1	0.0	89.82	*Alcaligenes* spp.
NA10B	PP967390.1	6.00E-49	92.41	*Proteus mirabilis*
NA11B	HQ697282.1	1.00E-07	80.68	*Enterobacter cloacae*
NA12B	OP704180.1	2.00E-20	76.14	*Burkholderia* spp.
NA15A	MG596968.1	0.0	89.48	*Proteus vulgaris*
NA16A	OP755983.1	3.00E-123	80.98	*Proteus vulgaris*

****organism closely identified with on NCBI Genebank Database.**





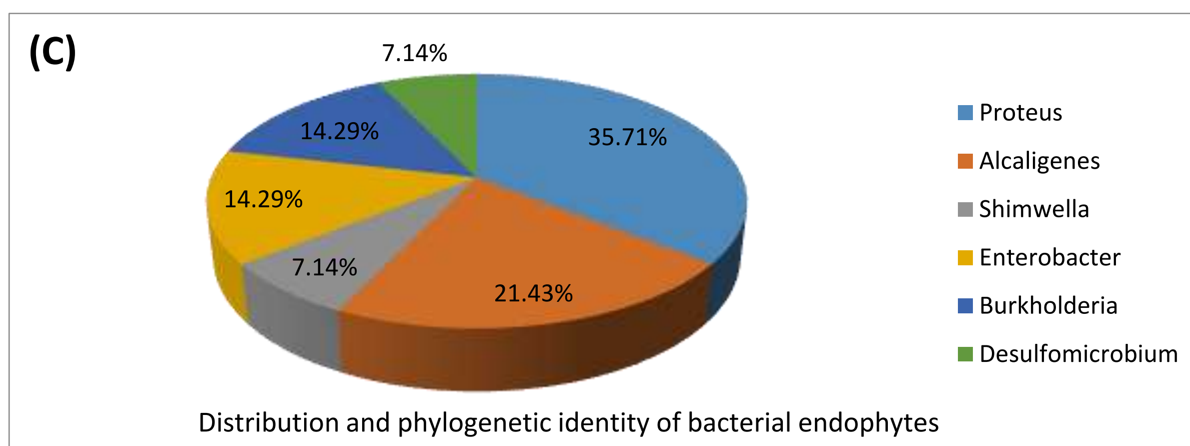


Figure 4.4: Diversity and distribution of endophytic bacteria isolated from roots of *H. cannabinus* and *Z. mays*. **(A)** Diversity at Class level **(B)** Diversity at Family level **(C)** Distribution at Genus level.

4.1.7.7 Phylogenetic tree of isolated endophytic bacterial sequences

Figure 4.5 shows the phylogenetic tree of the sequenced nucleotides of pre-selected endophytic bacterial isolates. *Proteus* spp. was the most predominant bacteria genus identified in 5 isolates (35.71%) while the least predominant bacteria identified were *Desulfomicrobium* and *Shimwellia* (7.14%) in one isolate respectively. Bacteria of genus *Alcaligens* were identified as 3 isolates (21.43%) while the genera *Burkholderia* and *Enterobacter* (14.29%) were identified as 2 of the isolates respectively.

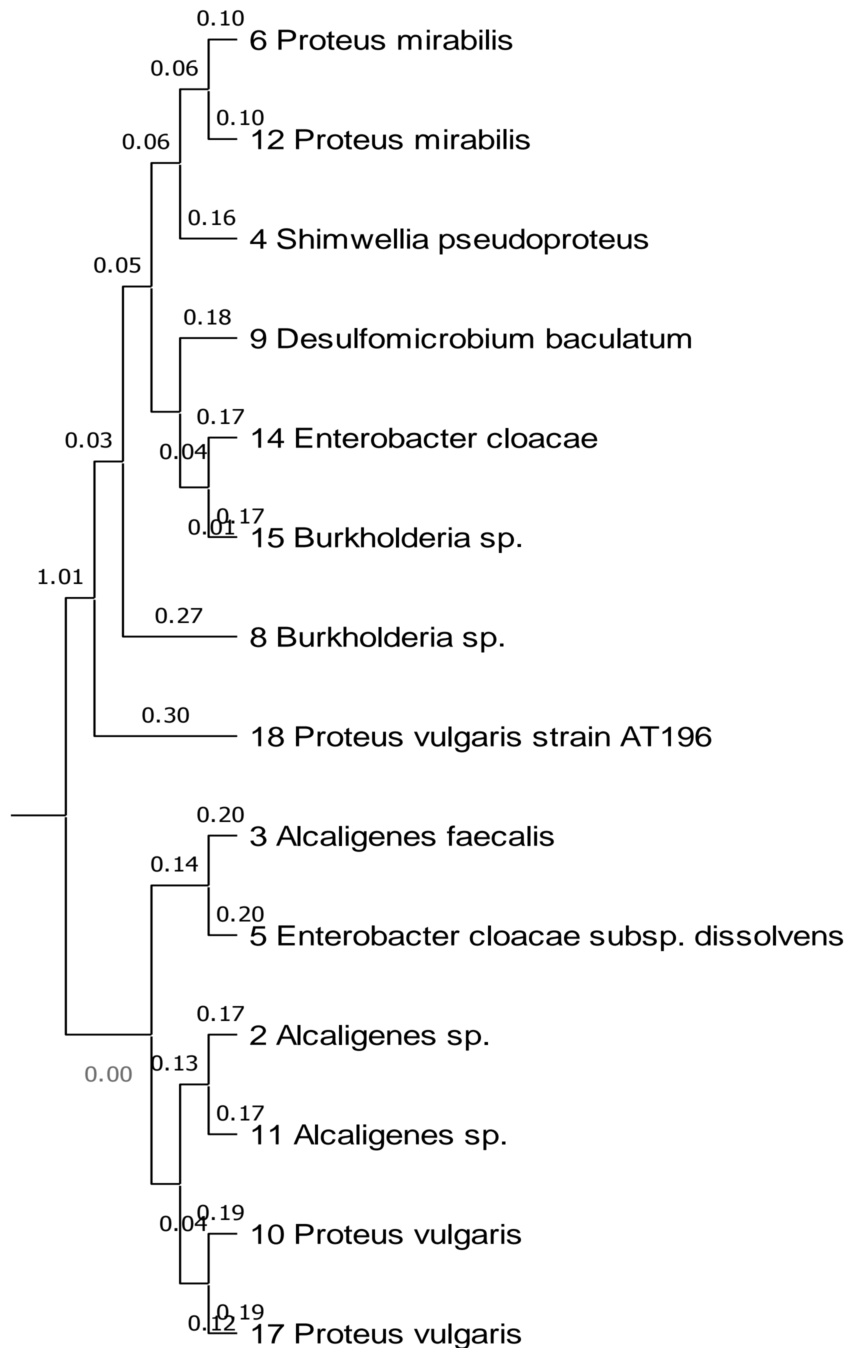


Figure 4.5: Dendrogram showing the phylogenetic relationship of 16s rRNA nucleotide sequences endophytic bacteria isolated from *H. cannabinus* and *Z. mays* roots. The phylogenic tree was constructed using neighbour-joining distance method. Bootstrap value was set at 1000 and the *p*-distance model was adopted.

4.2 Discussion

4.2.1 Physico-chemical Assessment of Crude oil-polluted and Unpolluted Soil Samples before and after Plant Growth

The choice of Egbema for this study was based on the fact that recent reports have shown that soil in Egbema axis has high acidity, low nitrogen, exchangeable bases, phosphorus and organic matter content (Okorie, Chinyere, Ifeanyi & Lazarus, 2020).The need to enhance soil fertility and quality for improved health conditions, sustainable agricultural output and food security was reported necessary in Egbema (Nwaogu & Onyeze, 2020; Okorie et al., 2020).

Moisture content observed in the study was within the range of 2.50% to 15.50% before plant growth. After 90 days of plant growth, moisture content increased to mean range of 5.0% to 28.10% showing soil remediation. Similar to the findings in this study, Edori and Iyama (2017), observed moisture to be in the range of 16.66-21.07% in their study of abattoirs. Additionally, Oliveira et al. (2013) reported moisture content within the range 17.74% to 40.10% in their study. Moisture content of soil shows their water retention characteristics. This serves as an indication of soil type, soil texture and soil health. Sandy soils have low moisture retention capacity, clay soils have very high moisture retention capacity while loamy and humus soils have average moisture content.

The soils of the study were mainly acidic with the polluted soil from Egbema the most acidic (4.80-6.60) prior to plant growth experiment (PGE). However, after PGE, an increase in pH was observed with range 5.10-8.40. The pH results obtained from the study were similar to that reported by Okorie et al. (2020) in their study of soil samples from Egbema, with range 4.8 to 5.4. Additionally, Edori and Iyama (2017) reported acidic pH values (4.59-4.99) in their study of selected abattoirs in Rivers State. Microbial activity, nutrients solubility and availability depend on soil pH. In highly acidic soils, micronutrients are not readily available to plants than in neutral soils. Hence the poor growth of test plants in Egbema soil (*H. cannabinus* and *Z. mays*).

Electrical conductivity (EC) in this study had mean range values of 30µS/cm to 180µS/cm before plant growth and 18.50µS/cm to 130.0µS/cm after plant growth. Okafor (2023) reported electrical conductivity of 80.79-84.32µS/cm and 93.95-108.25µS/cm before and after three months of phytoremediation of crude oil-polluted soil using *Phaseolus vulgaris* (beans). The

results obtained in our study were higher than the values of 592µS/cm reported by Apakama et al. (2017) and lower than values reported by Edori and Iyama (2017), with mean range of 208µS/cm to 404µS/cm. However, EC values obtained in this study were within the Federal Ministry of Environment (FMEnv) acceptable limit of 1000 µS/cm.

Soil calcium content ranged from 3.45-20.00 mg/Kg prior to PGE and 1.66-16.38 mg/Kg after PGE. Apakama et al. (2017) reported lower results of calcium (0.892 ppm) than those recorded in the study. Calcium content of soil samples was lower than the WHO stipulated standard (100-300 mg/Kg) showing the unavailability of calcium for plant growth. This could be as a result of nutrient depletion or the high acidic levels of the soil which alter nutrient availability. Values obtained for magnesium in this study ranged from 2.56-17.28 mg/Kg before plant growth. However, a reduction after plant growth was observed with values obtained for magnesium ranging from 1.08 mg/Kg to 7.95 mg/Kg. Similar to the findings of our study, Apakama et al. (2017) reported the magnesium content of Egbema-polluted soil in their study as 1.56-4.77 ppm. Additionally values recorded in the study were within the FMEnv standard of 20 mg/Kg.

Sodium content of soil was high in polluted soil samples (15.96-30.99 mg/Kg) compared with samples from Egbema (7.15 mg/Kg). Low values obtained in the Egbema soil could result in low growth rate of *H. cannabinus* (62 cm) and *Z. mays* (23 cm) as sodium serves as an important macronutrient necessary for plant growth. Potassium in soils is not readily available for plant uptake due to their ability to form complexes. Potassium levels obtained in this study had mean range values of 3.78-35.81 mg/Kg before PGE and 2.78-16.04 mg/Kg after PGE. Results obtained in this study were higher than values (0.02mg/Kg) reported by Ezeji and Chukwudi (2021) in their phytoremediation study of used motor oil using cowpea. Higher potassium levels obtained in the study could be as a result of the agricultural soil with no history of pollution. Hence nutrient availability was maximum in crude oil polluted soil samples compared to soil samples from Egbema.

Values obtained for ammonium in this study ranged from 5.36mg/Kg to 15.98mg/Kg prior to plant growth and reduced to a mean range of 3.52-7.82mg/Kg after plant growth. Results obtained in this study were within WHO limit of 35mg/Kg. Prior to PGE, soil nitrate levels in this study had mean range 26.09-57.39mg/Kg while values reduced to a mean range of 12.78-36.54mg/Kg. Values obtained in this study before PGE were slightly higher than FMEnv limit of

50mg/Kg. High values could be as a result of crude oil pollution of soils as studies have shown that petroleum contains acid anhydrides including nitrates and sulphates.

Sulphate levels in the study ranged from 19.48-42.86mg/Kg before PGE and 9.56-28.13mg/Kg after PGE. Although values obtained in this study were not up to FMEnv standard of 100mg/Kg, high values obtained are indication of poor soil health due to crude oil pollution. Alternatively, reduction in sulphate levels in the study are as a result of the phytoremediation attempt by the study plants *H. cannabinus* and *Z. mays*. The bicarbonate content of soils in the study recorded a mean range value of 25.29-92.31mg/Kg prior to plant growth and 18.28-41.44mg/Kg after plant growth. However, values were within the WHO standard of 100mg/Kg. High bicarbonate levels are indicative of the high anion content of the soil as indicated by high nitrate and sulphate values, as well as the acidic pH levels in this study.

The soil organic carbon (OC) is the amount of carbon in the given soil. Organic carbon in the study ranged from 0.32 to 2.45% prior to PGE and 0.10-2.12% after PGE. Similar to the range obtained in this study, Ezeji and Chukwudi (2021) reported organic carbon in their study as 0.72%. However, Okorie et al. (2020) reported higher findings of organic carbon 3.0-24.9% (average 11.74%) in their study on Egbema soils. Differences observed could be as a result of sampling location and seasonal variations. Organic matter content of soil samples in the study ranged from 0.64% to 3.88% prior to PGE. After PGE, organic matter slightly reduced to mean range of 0.50-3.51%. Study by Edori and Iyama (2017) recorded higher organic matter of range 22.33-29.58% than those observed in this study. The differences in the values could be as a result of study location as abattoirs are characterized by dumping of animal wastes and animal blood which are potential sources of soil organic matter.

Total nitrogen observed in the study before growth of plant ranged from 0.053-0.085% and reduced to 0.030-0.065% after plant growth. Higher nitrogen values (4.34%) were reported by Ezeji and Chukwudi (2021) in their study of motor oil contaminated soil. Phosphorus observed in the study prior to PGE had mean range values of 0.25-0.47mg/Kg and reduced to mean range values of 0.20-0.37mg/Kg after plant growth. Ezeji and Chukwudi (2021) reported similar results (0.02mg/Kg) in their study. Alternatively, Edori and Iyama reported higher values of 0.66-1.34mg/Kg in their study. In addition, values obtained obtained were slightly higher than the WHO standard of 0.01-0.19mg/Kg.

4.2.2 Hydrocarbon Remediation of Crude Oil-Polluted and Unpolluted Soil

Bioremediation of soil contaminated with petroleum hydrocarbons improved soil texture and decreased organic carbon as a result of increased microbial activity and removal of recalcitrant hydrocarbons (Liu, Wu, Gao, Yi, & Duan, 2021). Soils contaminated with hydrocarbons are harmful to microorganisms, decreasing their number, distribution and activity. Furthermore, there are decreased soil nutrients available to plants in conditions of decreased microbial population (Liu et al., 2021).

In our study, the total hydrocarbon content of soils prior to and after plant growth I showed that 5ml/5kg crude oil-polluted soil and 50ml/5kg crude oil-polluted soil had the highest (46.01%) and least (5.08%) percentage remediation respectively. The 5ml/5kg crude oil-polluted soil having the highest number of leaves, total fresh weight and total plant height offered the highest percentage of crude oil remediation when compared to other samples. Barati et al. (2017) reported 21.76% and 20.36% TPH remediation in their study using barley and oats amended with poultry biochar while Prematuri et al. (2020) recorded 38% remediation in 40 g/Kg using plants of the Aster family. Alternatively, Abdallah et al. (2022) observed a higher crude oil degradation percentage (79%) using *Acacia siberiana* than those recorded in our study after six months of phytoremediation. Our findings show a significant relationship between plant biomass and the remediation ability of *H.* cannabinus and Z*. mays*.

The 1kg unpolluted soil + 4kg Egbema-polluted soil offered the highest percentage of remediation (39.53%) using PGII while 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil and Egbema-polluted soil with organic amendment had the respective percentage of remediation of 27.94% and 8.61%. Egbema-polluted soil without organic amendment had the lowest percentage of remediation (4.63%) across all samples analyzed. The low values recorded in Egbema-polluted soil without organic amendment correlate with the low biomass yield obtained for the sample. Our findings show that the high pollution index of Egbema soil negatively impacted plant growth and therefore remediation ability was significantly hindered. However, the high percentage remediation values obtained for 1kg unpolluted soil + 4kg Egbema-polluted soil (39.53%) and 2.5kg unpolluted soil + 2.5kg Egbema-polluted soil (27.94%) show the synergistic effect of augmenting Egbema polluted soil with unpolluted soil, offering higher crude oil remediation potential compared to the un-augmented samples (Egbema-polluted soil with organic amendment

and Egbema-polluted soil without organic amendment) with percentage remediation of 8.61% and 4.63% respectively.

4.2.3 Growth effects of crude oil pollution on plant fresh weights

Fresh weights of unpolluted, crude oil polluted and Egbema-polluted soil are shown in Table 4.18a and 4.18b. The highest reduction in total fresh weight 94.05% was observed in 50ml/5kg crude oil-polluted soil having the highest crude oil contamination as compared to the control (unpolluted agricultural soil with organic amendment) for both above- and below-ground parts. Mean fresh weight was reduced by 81.92% and 95.97% for roots and shoots respectively compared to the control. For Egbema polluted soil (PGII), the highest reduction in total fresh weight 92.57% was observed in Egbema-polluted soil without organic amendment compared to the unpolluted agricultural soil with organic amendment. The total mean fresh weight of 1kg unpolluted soil + 4kg Egbema-polluted soil (101.99 g) was approximately equal to control unpolluted agricultural soil with organic amendment (102.20 g); indicating the positive effects of bioaugmentation and biostimulation in remediation activities. On average, shoot fresh weight was more reduced than root fresh weight in crude oil polluted soil while root fresh weight was more reduced than shoot fresh weight in Egbema polluted soil.

Petroleum hydrocarbon in soil results in decreased uptake of water and nutrients, root growth, plant growth and subsequently biomass yield (Palmroth, Pichtel & Puhakka, 2002; Merkl, Schultze-Kraft & Infante, 2004; Brandt et al., 2006). Root and shoot dry weight of *Festuca arundinacea* decreased by 29.70% and 53.50% when grown on soil contaminated with pyrene and phenanthrene respectively (Cheema et al., 2009). The study by Liste and Felgentreu (2006) revealed that in soil contaminated with petroleum hydrocarbon (1517 mg/kg TPH), the shoot and root of ryegrass decreased by 38.90 and 52.60% after a 95-day plant growth respectively. Nutrient availability in crude oil-contaminated soil is relatively low (Wenzel, 2009), therefore the addition of organic fertilizer serves to improve the growth of plants in these soils (Lin & Mendelssohn, 1998). In our study, Fresh weights of control without organic amendment and Egbema-polluted soil without organic amendment were reduced (81.18% and 92.57% respectively) indicating additional nutrient content in organic amendments and their effect on plant yield compared to control. Wang et al. (2012) revealed that the root and shoot dry weight of alfalfa and ryegrass increased significantly in compost-amended soil compared to un-amended soil contaminated with

pyrene after 90 days. Additionally, the report by Amadi et al. (1993) revealed the growth increase in maize grown on poultry manure-amended soils contaminated with crude oil compared to contaminated soil without manure amendment. The 5ml/5kg crude oil-polluted soil had the highest shoot, root and total mean fresh weight across all treatments. Barati et al. (2017) revealed that greater root biomass was related to more elaborate root exploration of soil resulting in higher microbial population and activity necessary for hydrocarbon degradation. Therefore, while plant height and shoot weight are strong indicators of plant health, higher shoot weight does not often imply a more efficient remediation process, rather, higher root weight is associated with greater petroleum hydrocarbon remediation (Banks et al., 2003). Results obtained in our study revealed that the plant with the highest root fresh weight (25.19 g) offered the greatest remediation (46.01%).

4.2.4 Growth effects of crude oil pollution on plant heights

Results obtained in our study showed 35.82% reduction in total plant height for 5ml/5kg crude oil-polluted soil compared to control (unpolluted agricultural soil with organic amendment). For the highest exposure concentration (50ml/5kg crude oil-polluted soil), the above-ground parts (shoots) recorded a 36.51% reduction while the below-ground parts (roots) recorded a 25.00% reduction when compared with the control. Other studies have shown the negative effect of crude oil-polluted soils on plant growth (Merkl, Schultze-Kraft & Infante, 2005; Thompson et al., 2008). Shanker et al. (2005) revealed that plant height reduction was a result of growth reduction of roots and reduced translocation of nutrients to aerial parts of plants which affected shoot cell metabolism. Similar to the findings of our study, Martin et al. (2014) recorded a decrease in the shoot height of sunflowers grown on soil contaminated with crude oil. Additionally, Barati et al. (2017) reported a 34.75% and 37.25% decrease in the shoot height of barley and oats at 8% soil TPH level. According to Chirakkara and Reddy (2015), the addition of fertilizers (organic and inorganic) has a positive effect on plant growth in contaminated soils through biostimulation. Our results showed that the addition of organic amendments (banana peel and brewery spent grains) significantly affected the total plant height of unpolluted agricultural soil with organic amendment (positive control) compared to unpolluted agricultural soil without organic amendment (negative control) after 90 days of plant growth. The total height of 1kg unpolluted soil + 4kg Egbema-polluted soil was 1.07 times that of the control (unpolluted agricultural soil with organic amendment). The results of our study show a correlation between the values observed for the total number of leaves

and total fresh weight of plants showing a significant relationship between the number of leaves, fresh weight of plants and heights of plants (biomass production).

4.2.5 Phenotypic Characteristics of Endophytic Bacteria

Phenotypic characteristics of an organism are the observable traits of the organism resulting from the interaction of its genomic makeup and environmental factor. Phenotypic identification of microorganisms involves morphological identification and biochemical tests. Out of the 57 bacterial isolates tested 46 isolates (80.70%) were gram positive while 7 isolates (12.28%) were gram negative. Thirty-nine isolates were gram positive rods while 7 isolates were gram positive cocci. Results were higher than the report of Kumar et al. (2020) that observed 59.3% (19 isolates) as gram positive and 40.6% (13 isolates) as gram negative respectively for bacterial endophytes isolated from *Oryza sativa*.

Motility in microorganisms is detected by the presence of diffuse growth away from the line of inoculation. Microorganisms possessing flagella can swim away from the stab mark or inoculation line producing a diffuse growth while non-motile organisms only grow along the line of inoculation. Eleven endophytic bacterial isolates were motile out of the 57 isolates tested representing 19.30% sample size while 80.70% (46 isolates) tested negative.

Hydrogen peroxidase (H_2O_2), an end product of the aerobic degradation of sugar is toxic to bacterial cells. Therefore, most aerobic bacteria synthesize the enzyme catalase or peroxidase for the breakdown of hydrogen peroxide into water and oxygen, protecting cellular components. About 65% (37 isolates) of endophytic bacteria in the study synthesized catalase while 35.09% (20 isolates) could not. This was observed by the formation of bubbles of oxygen on introduction of various isolates to H_2O_2.

Indophenol oxidase, in the presence of atmospheric oxygen, oxidizes phenylenediamine reagent (yellow) to indophenol (dark purple). Organisms possessing cytochrome C oxidase enzymes test positive for oxidase test. From the study, 11 bacterial isolates (19.30%) tested positive while 46 isolates (80.70%) tested negative.

Starch, a macromolecule which serves as a major source of carbon for most microbes, cannot diffuse or penetrate bacterial cell wall as a result of its large size. Bacterial cells secrete the hydrolysis enzyme amylase which breakdown starch to glucose for easier penetration into cells.

The importance of starch to microbial cells is shown by the high number of isolates that could synthesize amylase (82.46%). Forty-seven bacterial endophytes isolated in this study tested positive for amylase synthesis while a minority of 10 isolates (17.54%) could not synthesize amylase and therefore would depend on other carbon sources for the nutrient.

No endophytic bacteria isolated from the study tested positive for amylase, catalase, oxidase and motility tests; 5 isolates synthesized amylase, catalase and oxidase (8.77%); 2 isolates were positive for amylase, oxidase and motility (3.51%); 8 isolates tested positive for amylase, catalase and motility (14.03%) while a single isolate tested positive for catalase, oxidase and motility (1.75%). Thirty-two isolates were positive for amylase and catalase (56.14%); eight isolates tested positive for amylase and oxidase (14.03%); eleven for amylase and motility (19.30%); six for catalase and oxidase (10.53%); eight for catalase and motility (14.03%) while two isolates tested positive for oxidase and motility respectively.

4.2.6 Plant Growth-Promoting Properties of Endophytic Bacteria

Endophytes with plant growth-promoting abilities enhance host plant development by fixing nitrogen, solubilizing minerals, synthesizing diverse phytohormones, enzymes and siderophores (Ma et al., 2015; Baoune et al., 2018).

Indole-3-acetic acid is a phytohormone responsible for plant development modification induction of defense system of plants cell-cell signaling and other physiological benefits to plants (Ma et al., 2016). IAA can serve as a growth inhibitor or promoter depending on its concentration. Moreover, decreased IAA levels stimulate elongation of primary root while increased levels result in lateral and adventitious root synthesis and subsequent reduction of primary root growth (Glick, 2010). Bacterial endophytes aid plant development via phytohormone modification through ACC synthesis. From the study, 43 isolates (75.44%) out of the 57 isolated endophytic bacteria tested positive for IAA synthesis, while 15 isolates (24.56%) tested negative. The findings were similar to those reported by Abedinzadeh, Etesami & Alikhani (2019) and Etesami et al., (2014); where 45 isolates (75%) and 43 isolates (75.4%) presented positive results for IAA production respectively. The result of the study however, was higher than those reported by Silva et al. (2020) and Wu et al.(2021) that reported IAA levels of 24.8% (32 isolates) and 39.4% (15 isolates) respectively.

Phosphorus, an essential macronutrient, aid cellular phosphorylation of glucose in the glycolytic pathway. Translocation of glucose, stimulation of root development, plant growth promotion are cellular functions of phosphorus in plants (Ahemad, 2015). Application of phosphorus to soil is hindered by its ability to form complexes resulting in its unavailability for plant uptake. Bacterial endophytes secrete phosphatase an enzyme responsible for the conversion of precipitated and complex phosphorus in soil into soluble forms increasing phosphate availability to plants. Endophytes, therefore, serve as phosphorus sinks even under phosphate limiting conditions and release them to plants when needed. No isolated bacterial endophytes from the study solubilized phosphorus. Similarly, Silva et al. (2020) reported that no endophytic bacteria isolate in their study utilized $AlPO_4$ as phosphate source while only 4 out of 149 (0.03%) isolates utilized $Ca_3(PO_4)_2$ as phosphate source. However, Wu et al. (2021) and Etesami et al. (2014) reported that 36.8% and 42.1% of isolated bacterial endophytes in their studies could solubilize phosphate respectively. The inability of isolates in this study to solubilize phosphate was probably because phosphate solubilization was difficult to detect in the predominant genus isolated in the study (*Proteus*) as those that presented positive results in other studies were majorly from the genera *Enterobacter* and *Bacillus* (Etesami et al., 2014; Kumar et al., 2020; Wu et al., 2021).

Iron is a trace element required in haeme synthesis, transpiration and a co-factor in enzymatic reactions within plants. Soil iron is however very reactive, forming insoluble ferric ion (Fe^{3+}) complexes unavailable for uptake by plants (Wu et al., 2021). Siderophores are iron chelating proteins possessing high affinity for Fe^{3+}, Fe^{2+} and other bivalent ions (Rajkumar et al., 2013). Microbial siderophores serve as major sources of phytoavailable iron in plants than phytosiderophores due to their higher affinity for iron than phytosiderophores (Ma et al., 2011). About 38.60% (22 isolates) of isolated bacterial endophytes from the study secreted siderophore and tested positive for siderophore production while 35 isolates (61.40%) tested negative. Abedinzadeh, Etesami & Alikhani (2019) in their study reported similar findings (49.9%). However, study by Etesami et al. (2014) and Kumar et al. (2020) showed 56.14% and 56.2% siderophore production in isolated bacterial endophytes from roots of *Trifolium alexandrinum* and *Oryza sativa* respectively.

1-aminocyclopropane-1-carboxylate deaminase is an enzyme synthesized by some bacteria and fungi for the hydrolysis of ACC to α-ketobutyrate and ammonia (Glick, 2010). Moreover, the

enzyme functions in downward regulation of ethylene during conditions of plant stress. Ethylene is a phytohormone which regulates both growth and senescence in plants, depending on its concentration, time of application and plant species. Environmental stress conditions such as exposure to pollutants induce ethylene production, resulting in root elongation, inhibition lateral root growth and root hair formation which possess risk to plants (Viterbo et al., 2010). ACC deaminase hence functions in plant stress alleviation. Seventeen endophytic bacteria isolated in the study could synthesize ACC deaminase of the 57 bacterial isolates (29.3%) while 40 isolates (70.17%) tested negative. Similarly, Abedinzadeh, Etesami & Alikhani (2019) reported that 56.5% of isolated bacterial endophytes from their study synthesized ACC deaminase. Additionally, Etesami et al. (2014) reported ACC deaminase present in 63.16% of isolated endophytes in their study.

Out of the 57 isolated bacterial endophytes in the study, 6 isolates synthesized IAA, ACC and siderophores; 14 isolates synthesized IAA and ACC; 16 isolates synthesized IAA and siderophores while 7 isolates synthesized ACC and siderophores at 10.53%, 24.56%, 28.07% and 12.28% respectively.

4.2.7 Bioremediation Potential of Endophytes using Different n-Alkanes and Diesel

Endophytes possessing bioremediation potentials are applied in bioremediation studies to aid pollutant assimilation and degradation by plants (Weyens et al., 2009; Wu et al., 2021). All endophytic bacterial isolates (100%) in this study grew on Nutrient Agar (NA) and Tryptic Soy Agar (TSA) amended with 2% (v/v) n-alkanes C_6, C_8 and C_{10} as single carbon source in the absence of glucose under 24 h incubation. In addition, all bacterial isolates in the study significantly grew on 2% (v/v) diesel oil. Similar to the findings in this study, Baoune et al. (2018) observed 98% hydrocarbon utilization activity of endophytic *Streptomyces* spp isolated from their study plants. Olga et al. (2018) reported that about 92.8% and 75.9% of isolated endophytic bacteria from *Helidonium majus* degraded n-alkane fractions (C_9 to C_{14}) of diesel oil and waste oil. Pawlik et al. (2017) recorded the ability of 90% of all isolates in their study to grow on diesel oil. Additionally, n-hexadecane was utilized by 16.67% and 28.57% of endophytic bacteria isolates from *L. corniculatus* and *O. biennis* respectively (Pawlik et al., 2017). Fresh diesel has been reported to elicit detrimental effect on plants which limit their proliferation (Cruz et al., 2014). This study revealed the ability of *Proteus* spp to utilize diesel oil and n-alkanes (C_6, C_8 and C_{10}). Wu et al.

(2019) in a similar study, revealed the ability of *Bacillus safensis* ZY16 from *Chloris virgata* in degradation of long chain n-alkanes (n-undecane, n-hexadecane and n-octacosane) and diesel oil under hypersaline conditions. Alternative to the findings of this study, Iqbal et al. (2019) reported growth of identified bacterial isolates on diesel while none of the strains could grow on agar plates amended with single benzene derivatives—phenol, xylene and toluene. This reveals the different growth preferences of bacterial endophytes for carbon sources, probably as a result of the different hydrocarbon degradative enzymes contained within which aid pollutant up-take and utilization.

4.2.8 Genetic Diversity of Bacterial Endophytes

Crude oil is an admixture of low molecular weight components C_8-C_{16}, high molecular weight constituents >C_{20} alkanes, polycyclic aromatic hydrocarbons and their alkylated derivatives that penetrate soil micropores and persist in the environment (Lim et al., 2016; Tiwari et al., 2016). Due to their recalcitrance they bioaccumulate in living organisms resulting in mutagenicity and carcinogenicity, hence they are organic contaminants of high concern (Kumar et al., 2012). Most studies on hydrocarbon-degradative bacterial communities have been focused on hydrocarbon contaminated soils and sediments (Mahjoubi et al., 2019; Olajide & Adeloye, 2023). Few studies exist on hydrocarbon degradative endophytic bacteria community within phytoremediation plants (Yousaf et al., 2010; Mitter et al., 2020) Endophytic bacterial isolates from this study were from the class β-Proteobacteria, γ-Proteobacteria and Firmicutes with the phylum Proteobacteria most predominant. Wu et al. (2021) in their study identified endophytic bacteria from phyla Proteobacteria, Actinobacteria, Firmicutes, Bacteriodetes and Tenericutes. Similar to this study, they reported the phylum Proteobacteria as the predominantly isolated endophytic bacteria in *Phragmites australis* and *Chloris virgeta*. Pawlik et al. (2017) reported the phyla α-Proteobacteria, γ-Proteobacteria and Actinobacteria as endophytic bacteria community within *Lotus corniculatus* and *Oenotherna biennis*. In contrast to the findings in this study, γ-Proteobacteria was the most predominant phylum in their study. *Rhizobium* and *Rhodoccus* were the most predominant genera in *O. biennis* while *Pseudomonas* spp was the most predominant genus in *L. corniculatus* unlike those identified in this study, *Proteus* and *Alcaligenes*. Bacteria from the genera *Pseudomonas* and *Bacillus* were the most prevalently isolated endophytes in other endophytic studies (Yousaf et al., 2010; Oliveira et al., 2013; Etesami et al., 2014; Wu et al., 2021). The difference observed is probably as a result of the difference in plant species, climatic predisposition or pollution factor,

as most endophytes isolated were from plants grown on agricultural farmlands or those applied to heavy metal remediation studies.

Bacteria from the genus *Proteus* are generally Gram positive motile and rod-shaped indole positive, catalase positive and oxidase negative microorganisms found in water, soil and intestinal tracts of humans (Mohammed et al., 2016). Phenotypic identification of isolated endophytic bacteria in this study showed that isolates were predominantly rod-shaped, catalase positive and oxidase negative organisms which could synthesize indole. Endophytic *Proteus* spp have also been identified in remediation studies involving different plants. Similar to the findings of this study, Pondei et al. (2018) isolated endophytic indole synthesizing *Proteus* spp from roots of *Chromoleana odoratum* growing in petroleum contaminated sites of the Niger Delta, Nigeria. Additionally, study by Wang et al. (2022) revealed rods of oxidase and catalase positive bensulfuron methyl-degrading endophytic *Proteus* spp isolated from *Echinochloa crus-galli*.

Previous reports on *Proteus* and *Alcaligenes* have been focused on hydrocarbon contaminated sediments and sites (Mahjoubi et al., 2019; Mitter et al., 2020; Olajide & Adeloye, 2023). To the best of our knowledge, this study serves as the first report on endophytic *Proteus* and *Alcaligenes* from *H. cannabinus* and *Z. mays*. Omotayo et al. (2017) reported *Alcaligenes faecalis* with hydrocarbon degrading activity in the rhizosphere of *Paspalum vaginatum* that degraded 90.5% crude oil in 24 days. Mahjoubi et al. (2019) observed *Alcaligenes aquatilis* with hydrocarbon degradation (56% n-alkanes, 70% phenanthrene) isolated from hydrocarbon polluted sediments of Bizerte Coast, Northern Tunisia.

4.2.9 Hydrocarbon Degradative Genes of Bacterial Endophytes

Bacterial endophytes can degrade, remove and utilize petroleum hydrocarbons as carbon sources. This occurs as a result of various hydrocarbon degradative genes therein, that assist host plants in pollutant detoxification and degradation (Yousaf et al., 2010; Oliveira et al., 2013). Alkane monooxygenase, a hydrocarbon degrading enzyme has been reported as the major mechanism endophytic bacteria degrade petroleum hydrocarbon (Wu et al., 2019; Iqbal et al., 2019). Out of the 24 bacteria genome screened in our study, 23 genes (95.83%) showed the presence of *Alk* B genes. Pawlik et al. (2017) reported 5 out of 26 isolated bacterial genome in their study possessed *Alk* B genes representing 19.23% of their study sample. Alternatively, study by Mitter et al. (2020) revealed 16 isolates (5.06%) possessed *Alk* B genes from the total of 316 isolates analyzed.

Presence of *Alk* B genes, indicative of the enzyme alkane monoxygenase, offer a means endophytic bacterial isolates assisted *H. cannabinus* and *Z. mays* in the utilization of petroleum hydrocarbons and growth on crude-oil contaminated soil.

Endophytes have been reported to show higher expression levels of the alkane degradative gene (*Alk* B) than rhizobacteria (Datta et al., 2020). The findings of this study agree with this as amplicons of different sizes were detected on amplification with *Alk B* primers (Figure 3). Generally, hydrocarbon degrading bacterial endophytes resident within specific plant host depend on plant species and their response to varying physico-chemical soil properties when compared to rhizospheric bacteria (Yousaf et al., 2010; Olga et al., 2018; Mitter et al., 2020). Our study revealed a high copy number of *Alk* B genes compared to the 16S rDNA. Mitter et al. (2020) suggested that high values recorded were probably due to the presence of multiple alkane monooxygenase in each strain. Plant root exudates also determine bacterial community within the endosphere of plants hence significantly affect phytoremediation (Phillips et al., 2008; Mitter et al., 2020). The findings of this study suggest that the root endospheres of *H. cannabinus* and *Z. mays* encourage bacteria diversity by providing conducive habitats for bacteria communities possessing alkane monooxygenase genes.

4.2.10 Plant-Endophytic Bacterial Interactions in Phytoremediation of Crude Oil-Polluted Soil

A plethora of interactions between the study plants--*H. cannabinus* and *Z. mays*--and their endophytic bacteria contributed to the remediation of petroleum hydrocarbons in this study. The study plants through their root systems assimilated and translocated crude oil hydrocarbons from the soil matrix into their intercellular spaces, where the endophytic bacteria utilized the hydrocarbons as carbon sources with the aid of the enzyme alkane monooxygenase. This study has shown that this symbiotic relationship aid in effective remediation of crude oil hydrocarbons from the soil at tolerable concentration of 25 ml/Kg crude oil contamination.

In addition, the ability of endophytic bacterial isolates in this study to synthesize the enzyme ACC deaminase, synthesize metal chelating siderophores and produce indole-3-acetic acid indicate their ability to support plant growth during conditions of adverse environmental conditions created by crude oil pollution. Presence of the enzyme, ACC deaminase, in endophytic bacterial isolates in this study, indicate their ability to alleviate heavy metal stress conditions

associated with crude oil pollution via reduction in ethylene biosynthesis (Zahoor et al., 2017). Moreover, ammonia, a hydrolysis product of ACC deaminase can readily be utilized as a nitrogen source by the study plants (Phetcharat & Duangpaeng, 2012).

Soil iron is not readily available for plant uptake as a result of their existence as insoluble ferric ion (Fe^{3+}) complexes. Iron-chelating bacterial siderophores present in this study possess the ability to solubilize soil iron from their complexes increasing its bioavailability to the test plants. These plant-growth promoting activities established in this study likely served as mechanisms plant-bacterial endophytes assisted phytoremediation of petroleum hydrocarbons in the study.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

To the extent of this study, it is evident that the acidic pH results of the polluted soil samples imply pollution of soil from Egbema/Ohaji and indicate need for remediation. After planting *H. cannabinus*, pH values increased while moisture content and organic carbon decreased showing remediation activity of the plant. However, *Z. mays* offered greater remediation than *H. cannabinus* on oil-polluted soil samples. Morphological changes observed during plant growth showed that group A3 (5ml/5Kg polluted soil + organic amendment) had the highest number of leaves, total fresh weight and fresh weight of roots, offering the highest crude oil remediation (46.01%) recorded using *H. cannabinus*, while group B2 (polluted soil without organic amendment) showed the least remediation (4.63%); indicating a correlation between increase in biomass and percentage remediation. Similarly, group F3 (5ml/5Kg polluted soil + organic amendment) with the highest biomass (123.54g) showed the highest crude oil remediation (56.05%) while group G2 (polluted soil without organic amendment) showed the least crude oil remediation (11.04%) after plant growth using *Z. mays.* Samples with organic amendment offered additional nutrients resulting in an increase in petroleum degradation in amended soils compared to un-amended soils. A total of 57 endophytic bacteria were isolated from the roots of *H. cannabinus* and *Z. mays*. Six endophytic bacteria synthesized indole acetic acid (IAA), 1-aminocyclopropane-1-carboxylate (ACC) deaminase and siderophores; 14 isolates synthesized IAA and ACC; 16 isolates synthesized IAA and siderophores while 7 isolates synthesized ACC and siderophores at 10.53%, 24.56%, 28.07% and 12.28% respectively. The high frequency of plant growth-promoting properties (IAA, ACC deaminase and siderophore) recorded from endophytic bacteria isolated from *H. cannabinus* and *Z. mays* show the mechanisms employed by bacterial endophytes in aiding plant host during adverse environmental conditions. The PGP traits also show potentials of these bacteria in agricultural improvement in preference to synthetic fertilizers. Diversity analysis revealed *Alcaligenes*, *Proteus*, *Enterobacter*, *Burholderia*, *Desulfomicrobium*, and *Shimwella* as endophytic bacteria community within the roots of the phytoremediation plants *H. cannabinus* and *Z. mays* grown on crude oil polluted soil from Egbema Nigeria. In addition, isolated endophytic bacteria possessed genes coding for the enzyme alkane monooxygenase (*Alk* B genes) responsible

for hydrocarbon degradation in bacterial species. The bioremediation potentials of the isolated bacterial endophytes against n-alkanes C_6, C_8, C_{10} and crude oil product diesel, were also established indicating the possible use of the isolates as bioaugmentation agents in remediation of crude oil hydrocarbons from oil contaminated soil.

Finally, this study established a significant relationship between isolated endophytes and hydrocarbon (diesel) degradation. It also established a significant relationship between hydrocarbon degradative genes (alkane monooxygenase genes) and crude oil degradation. Hence, the study established a significant relationship between isolated endophytes and crude oil degradation.

5.2 Recommendations

Endophytic bacteria isolates from this study can be applied in the bioremediation of crude oil-polluted soil in the Egbema axis. A broader application of these isolates in in agriculture and in the phytotreatment of the Niger-Delta region of Nigeria is highly recommended based on the plant gowth-promoting properties possessed by the isolates in this study and their bioremediation potential against diesel a crude oil distillation product and n-alkanes.

5.3 Contributions to Knowledge

- This study established the presence of *Proteus vulgaris*, *Proteus mirabilis* and *Alcaligenes* spp as predominant endophytic bacteria within the roots of the phytoremediation plants *H. cannabinus* and *Z. mays.*
- Isolated endophytic bacteria contained genes coding for the enzyme alkane monooxygenase (Alk B genes) responsible for hydrocarbon degradation in bacterial species.
- The bioremediation potentials of the isolated bacterial endophytes against n-alkanes C_6, C_8, C_{10} and crude oil product diesel, were also established indicating the possible use of the isolates as bioaugmentation agents in remediation studies.

- Moreover, ability of bacterial isolates to synthesize plant growth-promoting siderophores, 1-aminocyclopropane-1-carboxylate (ACC) deaminase and indole acetic acid indicate remediation potentials and possible use of isolated bacteria from the study in preference to synthetic fertilizers and during conditions of environmental stress due to crude oil pollution.

5.4 Further Studies

- Field trails involving the application of isolated endophytes in remediation of crude oil polluted soils.

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APPENDIX I

PLANT GROWTH EXPERIMENT

Plate 4.1: Nursery of seedlings before transplanting

Plate 4.2: *Hibiscus cannabinus* after 3 months of plant-growth experiment (PGE)

Plate 4.3: Roots of *H. cannabinus* and *Z. mays* after 3 months plant-growth experiment (PGE)

APPENDIX II

PICTURES OF BIOCHEMICAL TESTS

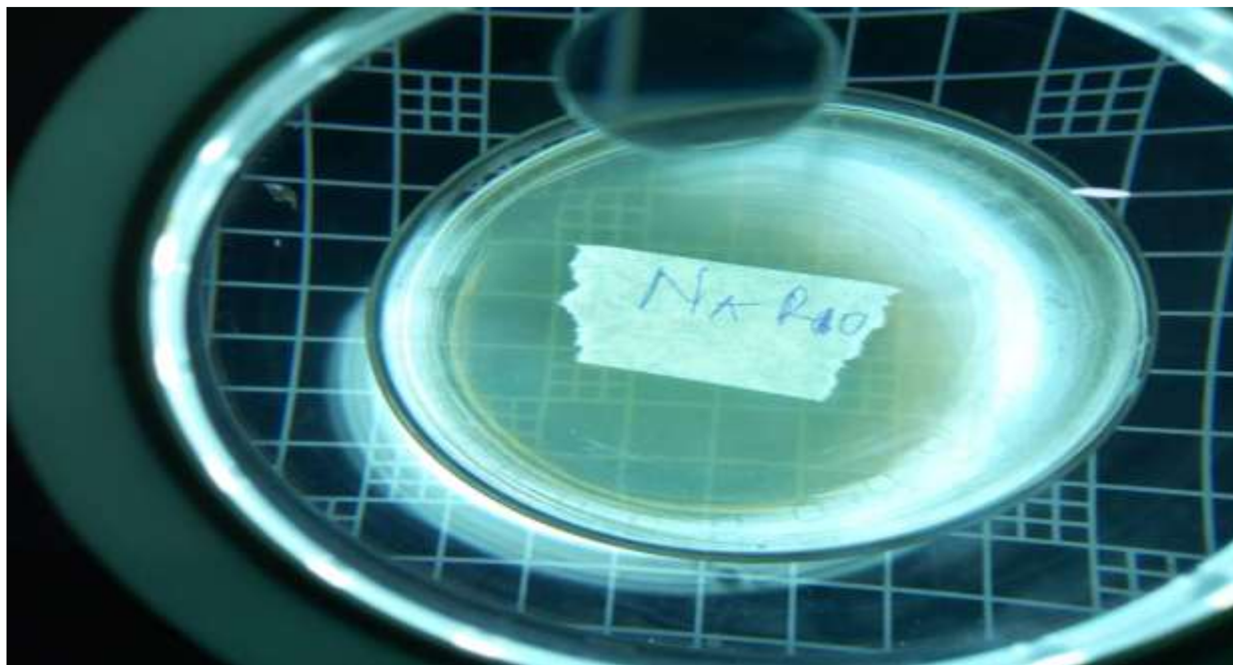

Plate 4.4a: Culture plate of last rinse water (NA R10) with no bacterial growth indicating strict isolation of endophytic bacteria

Plate 4.4b: Culture plate of last rinse water (NA R4) with no bacterial growth indicating strict isolation of endophytic bacteria

Plate 4.5: Positive and negative motility test of endophytic bacteria**Plate 4.6a:** Endophytic

bacterial isolates showing positive and negative starch hydrolysis

Plate 4.6b: Endophytic bacterial isolates showing starch hydrolysis tests

APPENDIX III

PLATES SHOWING PLANT-GROWTH PROMOTING PROPERTIES OF ENDOPHYTES

Plate 4.7: Endophytic bacterial isolates showing indole acetic acid (IAA) production

Plate 4.8: Negative phosphate solubilization of endophytic bacteria

Plate 4.9: Endophytic bacterial isolates showing siderophore synthesis

Plate 10a: Aminocyclopropane-1-carboxylate deaminase activity of endophytic bacteria (nutrient agar)

Plate 10b: Aminocyclopropane-1-carboxylate deaminase activity of endophytic bacteria (nutrient agar)

APPENDIX IV

MORPHOLOGICAL CHARACTERIZATION OF ENDOPHYTIC BACTERIAL ISOLATES

Table 4.8a: Total bacterial count and morphology of endophytic bacteria isolated from *H. cannabinus* roots using nutrient agar (NA)

Sample Number	Sample Code	Colour	Size (mm)	Shape	Elevation	TCFU/g
1	NA_1 10^{-2}	creamy	2-4	Round	Flat	1.00×10^5
		creamy	5-8	Irregular	Flat	
	NA_2 10^{-3}	creamy	0-1	Round	Flat	6.50×10^5
		creamy	2-4	Irregular	Raised	
2	NA_1 10^{-2}	creamy	1-3	Regular	Flat	7.50×10^4
	NA_2 10^{-3}	creamy	1-3	Round	Flat	5.40×10^5
		creamy	5-8	Irregular	Flat	
3	NA_1 10^{-2}	creamy	0-3	Round	Flat	2.30×10^5
		creamy	2-4	Irregular	Flat	
	NA_2 10^{-3}	creamy	0-3	Round	Flat	2.15×10^6
		creamy	2-4	Irregular	Flat	
4	NA_1 10^{-2}	creamy	0-4	Regular	Flat	1.60×10^5
		creamy	6-8	Irregular	Flat	
	NA_2 10^{-3}	creamy	1-4	Regular	Flat	3.70×10^5
		creamy	5-7	Irregular	Flat	
5	NA_1 10^{-2}	creamy	2-4	Regular	Raised	8.90×10^4
	NA_2 10^{-3}	creamy	2-4	Regular	Raised	4.80×10^5
		creamy	3-5	Irregular	Raised	
6	NA_1 10^{-2}	creamy	0-1	Regular	Flat	1.31×10^5
		creamy	2-4	Irregular	Flat	
	NA_2 10^{-3}	creamy	1-4	Regular	Raised	1.20×10^6
		creamy	2-5	Irregular	Flat	
7	NA_1 10^{-2}	creamy	1-2	Regular	Raised	8.20×10^4
		creamy	5-10	Irregular	Flat	
	NA_2 10^{-3}	creamy	0-1	Regular	Flat	5.90×10^5
8	NA_1 10^{-2}	creamy	0-1	Regular	Flat	2.20×10^5
		creamy	2-4	Irregular	Flat	
	NA_2 10^{-3}	creamy	0-1	Regular	Flat	1.20×10^6
		creamy	2-4	Irregular	Flat	
9	NA_1 10^{-2}	creamy	1-2	Regular	Flat	1.00×10^5
		creamy	2-4	Irregular	Raised	
	NA_2 10^{-3}	creamy	0-1	Irregular	Raised	7.40×10^5
		creamy	2-4	Regular	Flat	
10	NA_1 10^{-2}	creamy	2-4	Round	Flat	9.80×10^4
		creamy	4-6	Irregular	Flat	
	NA_2 10^{-3}	creamy	0-1	Regular	Flat	8.00×10^5
		creamy	1-3	Regular	Flat	

Key: 1 and 2 = 5 Kg unpolluted agricultural soil + organic amendment and –organic amendment respectively; 3, 4, 5 and 6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively; 7 and 8 = 5 Kg polluted soil + organic amendment and –organic amendment respectively; 9 and 10 = 1 Kg polluted soil + 4 Kg unpolluted soil + organic amendment and 2.5 Kg polluted + 2.5 Kg unpolluted soil + organic amendment respectively.

Table 4.8b: Total bacterial count and morphology of endophytic bacteria isolated from *Z. mays* roots using nutrient agar (NA)

Sample Number	Sample Code	Colour	Size (mm)	Shape	Elevation	TCFU/g
1	$NA_1\ 10^{-2}$	creamy	0-2	Regular	Flat	3.90×10^4
		creamy	2-4	Irregular	Flat	
	$NA_2\ 10^{-3}$	creamy	0-2	Regular	Flat	1.30×10^5
		creamy	4-6	Irregular	Flat	
2	$NA_1\ 10^{-2}$	creamy	1-2	Regular	Flat	2.24×10^5
		creamy	2-4	Irregular	Flat	
	$NA_2\ 10^{-3}$	creamy	1-2	Regular	Flat	2.02×10^6
		creamy	2-4	Irregular	Flat	
3	$NA_1\ 10^{-2}$	creamy	0-2	Regular	Flat	2.02×10^5
		creamy	2-5	Irregular	Flat	
	$NA_2\ 10^{-3}$	creamy	0-3	Regular	Flat	1.20×10^6
		creamy	4-6	Irregular	Flat	
4	$NA_1\ 10^{-2}$	creamy	1-2	Regular	Flat	9.00×10^3
		creamy	3-5	Irregular	Flat	
	$NA_2\ 10^{-3}$	creamy	1-2	Regular	Flat	4.00×10^4
		creamy	3-5	Irregular	Flat	
5	$NA_1\ 10^{-2}$	creamy	0-2	Regular	Flat	2.36×10^5
		creamy	2-4	Irregular	Flat	
	$NA_2\ 10^{-3}$	creamy	0-2	Regular	Flat	1.98×10^6
		creamy	2-4	Irregular	Flat	
6	$NA_1\ 10^{-2}$	creamy	0-2	Regular	Raised	2.15×10^5
		creamy	3-6	Irregular	Flat	
	$NA_2\ 10^{-3}$	creamy	0-2	Regular	Raised	1.61×10^6
		creamy	4-6	Irregular	Flat	

Key: 1 and 2 = 5 Kg unpolluted agricultural soil + organic amendment and –organic amendment respectively; 3, 4, 5 and 6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively.

Table 4.8c: Total bacterial count and morphology of endophytic bacteria isolated from *H. cannabinus* roots using tryptic soy agar (TSA)

Sample Number	Sample Code	Colour	Size (mm)	Shape	Elevation	TCFU/g
1	TSA_1 10^{-2}	creamy	3-5	Regular	Raised	6.00×10^3
	TSA_2 10^{-3}	creamy	3-4	Regular	Raised	4.00×10^4
2	TSA_1 10^{-2}	creamy	2-4	Regular	Flat	1.50×10^4
		creamy	4-6	Irregular	Flat	
	TSA_2 10^{-3}	creamy	0-4	Regular	Flat	2.80×10^5
		creamy	2-5	Irregular	Flat	
3	TSA_1 10^{-2}	creamy	2-4	Regular	Raised	1.70×10^4
		creamy	3-5	Irregular	Flat	
	TSA_2 10^{-3}	creamy	2-4	Regular	Raised	1.20×10^5
		creamy	3-5	Irregular	Flat	
4	TSA_1 10^{-2}	creamy	2-5	Irregular	Flat	1.00×10^4
	TSA_2 10^{-3}	creamy	2-6	Irregular	Flat	8.00×10^4
5	TSA_1 10^{-2}	creamy	2-4	Regular	Flat	7.00×10^3
		creamy	3-5	Irregular	Flat	
	TSA_2 10^{-3}	creamy	1-2	Regular	Flat	6.00×10^4
		creamy	2-3	Irregular	Flat	
6	TSA_1 10^{-2}	creamy	0-1	Regular	Flat	4.00×10^3
	TSA_2 10^{-3}	creamy	0-1	Regular	Flat	1.50×10^5
		creamy	3-5	Irregular	Raised	
7	TSA_1 10^{-2}	creamy	2-4	Regular	Raised	5.00×10^4
	TSA_2 10^{-3}	creamy	0-3	Regular	Raised	2.00×10^5
8	TSA_1 10^{-2}	creamy	1-2	Irregular	Flat	5.00×10^3
	TSA_2 10^{-3}	creamy	1-2	Irregular	Flat	4.00×10^4
9	TSA_1 10^{-2}	creamy	0-4	Regular	Raised	1.29×10^5
		creamy	2-5	Irregular	Flat	
	TSA_2 10^{-3}	creamy	1-4	Regular	Flat	2.20×10^5
		creamy	2-5	Irregular	Flat	
10	TSA_1 10^{-2}	creamy	1-3	Regular	Flat	1.10×10^5
	TSA_2 10^{-3}	creamy	1-3	Regular	Flat	2.00×10^5

Key: 1 and 2 = 5 Kg unpolluted agricultural soil + organic amendment and –organic amendment respectively; 3, 4, 5 and 6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively; 7 and 8 = 5 Kg polluted soil + organic amendment and –organic amendment respectively; 9 and 10 = 1 Kg polluted soil + 4 Kg unpolluted soil + organic amendment and 2.5 Kg polluted + 2.5 Kg unpolluted soil + organic amendment respectively.

Table 4.8d: Total bacterial count and morphology of endophytic bacteria isolated from *Z. mays* roots using tryptic soy agar (TSA)

Sample Number	Sample Code	Colour	Size (mm)	Shape	Elevation	TCFU/g
1	TSA_1 10^{-2}	creamy	0-2	Regular	Raised	2.00×10^4
		creamy	4-6	Irregular	Flat	
	TSA_2 10^{-3}	creamy	0-2	Regular	Flat	1.50×10^5
		creamy	4-7	Irregular	Flat	
2	TSA_1 10^{-2}	creamy	0-2	Regular	Raised	1.17×10^5
		creamy	2-6	Irregular	Flat	
	TSA_2 10^{-3}	creamy	1-2	Regular	Raised	2.60×10^5
		creamy	2-6	Irregular	Flat	
3	TSA_1 10^{-2}	creamy	0-2	Regular	Flat	1.80×10^5
	TSA_2 10^{-3}	creamy	0-4	Regular	Flat	8.00×10^5
4	TSA_1 10^{-2}	creamy	2-3	Irregular	Flat	8.00×10^3
	TSA_2 10^{-3}	creamy	2-3	Irregular	Flat	6.00×10^4
5	TSA_1 10^{-2}	creamy	0-2	Regular	Raised	9.00×10^4
		creamy	2-3	Irregular	Flat	
	TSA_2 10^{-3}	creamy	0-2	Regular	Raised	5.60×10^5
		creamy	2-3	Irregular	Flat	
6	TSA_1 10^{-2}	creamy	1-2	Regular	Flat	1.60×10^4
		creamy	3-6	Irregular	Flat	
	TSA_2 10^{-3}	creamy	3-6	Irregular	Flat	5.00×10^4

Key: 1 and 2 = 5 Kg unpolluted agricultural soil + organic amendment and –organic amendment respectively; 3, 4, 5 and 6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively.

APPENDIX V

PHENOTYPIC CHARACTERIZATION OF ENDOPHYTIC BACTERIA USING BIOCHEMICAL TESTS

Table 4.1: Phenotypic identification of endophytic bacteria isolated from *H. cannabinus* roots using Nutrient Agar through biochemical tests

Sample Number	Sample Code	Gram Stain	Amylase	Catalase	Oxidase	Motility
1	$NA_1\ 10^2A$	+ rod	+	+	-	-
	$NA_1\ 10^2B$	+ rod	+	+	-	-
2	$NA_2\ 10^3A$	+ rod	+	+	-	+
	$NA_2\ 10^3B$	+ rod	+	-	-	-
3	$NA_1\ 10^2A$	+ rod	+	-	-	-
	$NA_1\ 10^2B$	- rod	+	+	+	-
4	$NA_1\ 10^2A$	- rod	+	-	+	-
	$NA_1\ 10^2B$	+ rod	+	+	-	-
5	$NA_2\ 10^3A$	+ rod	+	+	-	-
	$NA_2\ 10^3B$	+ rod	+	+	-	-
6	$NA_2\ 10^3A$	+ rod	-	-	+	-
	$NA_2\ 10^3B$	+ rod	+	-	-	-
7	$NA_1\ 10^2A$	- rod	+	+	+	-
	$NA_1\ 10^2B$	+ cocci	-	-	-	-
8	$NA_1\ 10^2$	+ cocci	-	+	-	-
	$NA_2\ 10^3$	+ rod	+	-	-	-
9	$NA_2\ 10^3A$	+ rod	+	+	-	+
	$NA_2\ 10^3B$	- rod	+	-	+	-
10	$NA_1\ 10^2$	- rod	+	+	-	+
	$NA_2\ 10^3$	- rod	+	+	-	+

Key: 1 and 2 = 5 Kg unpolluted agricultural soil + organic amendment and –organic amendment respectively; 3, 4, 5 and 6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively; 7 and 8 = 5 Kg polluted soil + organic amendment and –organic amendment respectively; 9 and 10 = 1 Kg polluted soil + 4 Kg unpolluted soil + organic amendment and 2.5 Kg polluted + 2.5 Kg unpolluted soil + organic amendment respectively.

Table 4.2: Phenotypic identification of endophytic bacteria isolated from *Z. mays* roots using Nutrient Agar through biochemical tests

Sample Number	Sample Code	Gram Stain	Amylase	Catalase	Oxidase	Motility
1	$NA_1\ 10^2A$	+ rod	+	-	-	+
	$NA_1\ 10^2B$	+ rod	+	+	-	+
2	$NA_1\ 10^2$	+ cocci	-	-	-	-
	$NA_2\ 10^3$	+ rod	+	+	-	-
3	$NA_2\ 10^3A$	+ rod	+	-	-	-
	$NA_2\ 10^3B$	+ cocci	-	-	-	-
4	$NA_1\ 10^2A$	+ rod	+	+	-	-
	$NA_1\ 10^2A$	+ rod	+	+	-	+
5	$NA_2\ 10^3A$	+ rod	+	-	-	-
	$NA_2\ 10^3B$	+ rod	+	+	-	-
6	$NA_1\ 10^2A$	+ rod	+	+	-	-
	$NA_1\ 10^2B$	- rod	+	+	+	-

Key: 1 and 2 = 5 Kg unpolluted agricultural soil + organic amendment and –organic amendment respectively; 3, 4, 5 and 6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively.

Table 4.3: Phenotypic identification of endophytic bacteria isolated from *H. cannabinus* roots using Tryptic Soy Agar through biochemical tests

Sample Number	Sample Code	Gram Stain	Amylase	Catalase	Oxidase	Motility
1	$TSA_1\ 10^2$	+ rod	+	+	-	-
2	$TSA_1\ 10^2A$	+ rod	+	+	-	-
	$TSA_1\ 10^2B$	- rod	+	-	+	+
3	$TSA_1\ 10^2A$	- rod	+	+	+	-
	$TSA_1\ 10^2B$	+ rod	+	+	-	-
4	$TSA_2\ 10^3A$	+ rod	+	+	-	-
	$TSA_2\ 10^3B$	+ rod	+	+	-	-
5	$TSA_2\ 10^3A$	+ rod	+	+	-	-
	$TSA_2\ 10^3B$	- rod	-	+	+	-
6	$TSA_2\ 10^3A$	+ rod	+	+	-	+
	$TSA_2\ 10^3B$	- rod	+	+	+	+
7	$TSA_1\ 10^2A$	+ rod	+	+	-	-
8	$TSA_2\ 10^3$	+ rod	+	-	-	-
9	$TSA_1\ 10^2A$	+ rod	+	+	-	-
	$TSA_1\ 10^2B$	+ rod	+	+	-	-
10	$TSA_1\ 10^2$	+ rod	+	-	-	+

Key: 1 and 2 = 5 Kg unpolluted agricultural soil + organic amendment and –organic amendment respectively; 3, 4, 5 and 6 = 1 ml/Kg, 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively; 7 and 8 = 5 Kg polluted soil + organic amendment and –organic amendment respectively; 9 and 10 = 1 Kg polluted soil + 4 Kg unpolluted soil + organic amendment and 2.5 Kg polluted + 2.5 Kg unpolluted soil + organic amendment respectively.

Table 4.4: Phenotypic identification of endophytic bacteria isolated from *Z. mays* roots using Tryptic Soy Agar through biochemical tests

Sample Number	Sample Code	Gram Stain	Amylase	Catalase	Oxidase	Motility
1	TSA_1 10^2A	+ rod	+	-	-	-
	TSA_1 10^2B	+ rod	-	-	+	-
2	TSA_1 10^2A	+ cocci	-	+	-	-
	TSA_1 10^2B	+ rod	+	+	-	-
3	TSA_1 10^2	+ rod	+	-	-	-
4	TSA_1 10^2A	+ cocci	-	-	-	-
	TSA_1 10^2B	+ rod	+	+	-	-
5	TSA_1 10^2A	+ cocci	-	+	-	-
	TSA_1 10^2B	+ rod	+	+	-	-

Key: 1 and 2 = 5 Kg unpolluted agricultural soil + organic amendment and –organic amendment respectively; 3, 4 and 5 = 2ml/Kg, 5ml/Kg and 10 ml/Kg crude-oil polluted soil samples + organic amendment respectively.

APPENDIX VI

NUCLEOTIDE SEQUENCES OF ISOLATED ENDOPHYTIC BACTERIA

Sequence 1-2f>

GGGTAACGGGAGAAAGTGGTTCTGGGTTCAGGAAGGCGGAAAAGGGAACCGGAAA
GGTTGGTCCTGGGTCCGGAAAAGGGGAACAAGGGAAACGGAAGGTTTTGTCCCGGC
TACTCGAAGGGGGGAAAAAGAAAGGATATGTTTTATGT

Sequence 1-1492r>

AGCCAACCTTACGGTTACGTTGTACGACTTCCCGGGCCCGGGACAAAACCCAAGGG
TAACTTGGGACCCCTTACCGGGGCCGGGAACAAACTCCAAGGGTTCCTTGGGAACA
ATTTCCGGAGCCGGGAACAAAACCCAAG

Sequence 2-2f>

GGTACCCATTAAAGGTTTGATATGGGGTTTAGGGAGGGGGAAAAAGGAACAGAAGG
ATTTTTTCCCTGGTTCAGGAAAAGTATCAAAAAAATTCACTTGATTTTTTTTTTCTTTT
CCAAAAAATGAAAAAAACCGCATATGTTTTTTTTTATAAAGCAGGGAACCTTCGGGC
CTTGGACTATTTGAGTGGTCGTTATCGGATTAGCTAGTGGGGGGGGTAAAGCCTACC
AAGGCAACGGATCCGTAGCTGGTTGAGAGGAAGACCAGCCACACTGGGAGTGGGAC
ACGGCCCAGGACTCCTACGGGAGGGGCGGGGGGGAATTATGTAAAATGGGGGAAA
CCGGATCAGCCTCCCGCGGGTGCGAGGAGGCTTGGGGTTGTAAAGACTTTTGTCAGG
GAAAGAAATCGCCCGGAATAATACCTCGGGGGGGTGACGGTACCTGGAAGAAAAA
GCACCGTGAAACAACGTGCCAGGGGGCGCGGATATACGTAGGTTGCAAGCGTTTTC
GAAAATTACTGGCGAAAAGGGTGGGCGGGGGGTTCGGAAAAAATAGAGGTGAATC
CAAGGGCTTTAACCTGGGAATGATTTTTTACCTGCCGGGGCTAAAGGTGTCAGAGGG
GTGGTGAAATTCTTCGTTGTACCATTGAAAAGGGTAAAATATTGCGGAGGAACACC
GATGGGTAAGTCAGGCTCCCTTGGAAAAAATAATTCCCCCTATGCACCCAAGCGTGT
GAAGCAAACAGGTATAGATAACCCCTGGTTATCCCCCCCCCCCTAAACGAAGTTCA
ACTAACCTTTTTTGGGGGTCCTTTCGGGGCCTTAAGTAGCCTCACCATAAAACGGTT
GAAGATTTTACCCCTCTTCGGGGTAGTACAGGGTCCCAAAGATTAAAATCCTCCCAA
GATAATTTTTTGGGGGAA

Sequence 2-1492r>

ACAACACCATAGGGTTACCTTTGGTACGACTTCCCGGGCCCGGGACCAAACCCAAG
GGTTCCCTTTGTTCCCCTTTCCCGGGCCCGGAACCAACCCCACGGGTCCTTGTACAC
GAAATCCGGAGCCCAGACAAAAACCTAACAGCAC

Sequence 3-2f>

GAAACCCCGAAGAAGTGGTACCCTGGGCTCCAAAGGGCGGGACAAGGGACACCCC
GGAGTTTTTCCCCGGCCCCCAAAAACCAAAAAAAAAGCACCCCCTTTTTTTTCCTCC
TCCCCCAAAAAAAGAAAAAAAAAAACCCCCATTTTTTTTTTTGGGTAGGGGAAATCCC
AAAAACTCTCCCTTTGGTGTGTTCCGATATGGGAATAGCTAGGGGGGGGGGGAAAC
GCCTCACTTGGCCGAGGTCCGAAAGGGGGTCGAAAACATAACCCCCCCCCTGGGAC
TGAAACCCGCCCAAAACTCCAGGGGGGGCGGTGGGGGGAATTTTGGAAAGGGGGG
AAAACAGAAACACCCTCCGGCGGGGAAAGAAGGCTTCGGGGTGAAAAGCCTTTTTG
GGAGGGGAGAAAGGGTTCCCCTAAACGGGGAGGGGTGAGGGTGCCCGGAGAAAAA
AACCCCCGGCAAAACACCGGGCAAGGACCGGGGAAATACGGGGGGGGAAAGCCTT
AATCGGAATTACGGGGCAAAAGGGGGCGCGGGCGTTCCAAAAAAAAAATGGGAAA
CCCCCGGGCTTCCCCTGGGAACTGCTTTTTTAATTGCGGGCAGAGTTGGAAAAGGGG
GGAAAATTCCCCGGGAAGCAG

Sequence 3-1492r>

GCAAAACCCAACGGTTACCTTGTTACGACTTACCGGGCCCGGGAACCAAACCCAAC
GGTAACCTGGTAACAACTTCCGGGGCCCAGGACCAAACCCAAGGGTACCCTGGTA

Sequence 4-1492r>

GCAAACCCTAAGGGTTAACTTTGGAACGACTTCCCGGGCCCAGGACAAAACCCAAC
GGTTACTTGGTTACGATTTCCCGGGCCAAGAACAAACCCCAAGGTTTCCTTTGTACA
CCCTTCCGGGCCCCGAAACCAACCTAAACCTACTTCTTGAAAACTTTTTAAAAAATC
CAAATCAGAAACTA

Sequence 5-2f>

AGGAAAACAGGGGGGAAGTTTTGGAACCAGGGGGTCGGGAAAATGCCAACAAGGG
ACCCCCCAAAGTTTTGTCCCGGGCCCGAGAAAGGTATAACGAGAAACCCCCAAATT
TTTCCTCTCCCCCCGCAAAACCCAAAAAAGACACCCCATGTTTTTTTCCCCCACAGG
GGGGAGCTCCCTAGCCCCTTTGCCCTATTGGTGTGCCCCCCATGTGGGTAAGCGGGT
TGGGGGGGGGGGGACCGGGGGCCTCCTTGTGGGGAACGAAGACTCCAAGAATGG
GGGGTGGGGAGAAAGGGACCCCACCCCCCCCACTGGGGGACTGCGGGACACCCCG
GCCCCCCACACCCCCCCCAAGGAGGAAGGGCAGCGGGGGGGAAAAAATTTTGGAA
ATGGGGGGGCAAAAACTCCTTGGTAGCCCCCTGTCGCCGGTGGTGTTATAAAAAAA
AACCCTCCTGGGGTTGTGAAAAGGCTTTTTTTCCCGCGGAAGGAAGAAAGATTTAAT
GGTTCATAAAAGCTTTGTTAGTTTTTTGGTGTTCCCCCCGAAAAAAAAAAGCCGCCG
GTAAAAACCGCGGCGAAAAGCCGGCGGGGAATAATAGAGGAGGGGCGGGAGGTTT
TACGAAAAAAAATTTGGGGGGAAAAAAAGCGTGGGGGGGGGGGGTTAAAAAACGA

AAGGTGTAAAAACCCGCGGAGCTACATGTGGGAAATCCGCCTTAAAAAACTAGGGA
AACAAGAGTCGTGGAAGAGGGGGGGAAAAATCTAGCGGGTGGTAGGGGGAAAACC
CGCGAA

Sequence 5-1492r>

GGAAAAATCTTAAAGGTAACCTTTGTTTCCGACTTCCCGGGCCCGGAAACCAACCTC
ACGGTTATCTGGTAACGAGTCCCGCGACCCAAACCAAACTCTACGACGTATTTGGCG
AAAAATTCCAATCTACGATTACAACCCTTTCCGACTTCATGGAATCGAGTGCAGACT
CCAATCCGGACTACGACGACTTTATGAAATCCCCTTGCACTCGCAAGGTAGCA

Sequence 6-2f>

GGAAACAGGTAAGAAGGTTTTTTCTTGGTTCAGGAAAAGCGGAAAAGGGAACCCAA
AAAGGTTTGGCCGGGGTCTAGGAAGCCGTAAAAAGGAAACCGGAAGGTTTTTTCTT
GATTCTAGGGGAGAGGGGGGATAAACCCGTATGATG

Sequence 6-1492r>

AGGGCAACCCTACGGTAACCTTGGGAACGATTTCCTGGGCCCGGGAAAAACCCTAA
GGGTAACTTGGTACCGCTTTCCGGGGCCGGGACCAAACCCTAAGGTTACCTTGTCGA
AAATTTCCGAACCCCGGATAAAACCCTTTCCGACTTTCTGGAACCGAGTTGCAGACT
CCAATCCGGACTACGACAGACTTTATGAGTTCCGCTTGCTCTCGGGAGGTCGCTTCT
CTTTGTATCTGCCATTGGAACACGGGGTGGAG

Sequence 7-1492r>

GGCAAAACTCCTAGGGTTACTTTGGTACGCCTTCCCGGGCCCGGGGCCAACCCTAAG
GGTACCTTGGTAACGCTTTCCCGGGCCCGGGCCAAACCCCAAAGGTTCCTTGGTAAA
ACTTTCCGGGGCCAAGACAAAACCTTAAGGG

Sequence 8-2f>

GACACCCAGAAGGGGTTTGAACCCGGGGGCCAGGAAGGGGGAAAAAGGGAACCCC
AAGGGTTTGACCCGGGGCCCGGAAAAAGGCAAAAAAGGGAAAACGTAAATTTTTCC
CCCGCCCAGGGAGAAACAAAAAAAGAAAGCGGAAAATA

Sequence 8-1492r>

CCAAACTAGTTACTTGTCCGATTTCCTGGGCCCGGACCAAACCTAAGGGTAACTTTG
TCCACTTTCCCGGGCCGGGGACAAAACCCAAGGTTTCCTTTGTCGCAATTTTCTGGG
CCGGGATTACAACCTATTCTTACTTCTTGCAACCTATTTGGAGACTGGAACCAGCAC
TACGATCGGTTTTCTGAAATTGGCTCCCCCTCGCGGAATGCCTACC

Sequence 9-2f>

GAAACCGAAAAAGGTTGGGAACCGGGGCCCAGGAAAGGGGAAAAAGGGGGAACCC
AAAAAGTTGTGCCCTGGGGCTGGAGAGAACGGAAAAAGGGAAACTCCCAGATTTTT
TCCCGAGACCAGAAAAAAAGGAAAAATAAAACCGTATAGCGT

Sequence 9-1492r>

CAAAACATAAGGATAACGTGGTAACGATTTACGGGGGCAGGGCCCAACCCTAAGGG
TACCCTGGTACCGCTTTCCGGGGCCAGGGCCAAACCCCAAGGGTACCTTGGTGAAA
CTTTCCGGGGCCCGGAAACAAACCTAAGGGACCTCTTGTAGCCGATCCGGGGACCC
CAATCCGACCTACGACTTACTTTATGAGAACCCGCTTGCCCCTCGAGAGACCCCATT
C

Sequence 10-2f>

GTCCCCCAAAAGGTTTGTAATTTGGGTTCGGGAAAAGTCGAAAAAGGAAAACGTAG
AGTTTTGCCCTGGGTCAGAGAAAAACAAAAAAAGGAAACGCTATATTTTTTTTATG
TTAAAAAAAAACGGAAAATAAAGCGTATGATGTTTATCCCCCAAGCAGGATCTCTT
AGGAACTTGCCCTATCTGTTTGTACCCATATGGAATTAGCTTGGAGGTGGGGTACTG
TTCTATCTAGGCGAAGATCTCTAGCGGGACGAGGAGGATGACAGCCACGCTGGGAT
GAAACACGGCCCCGACTCCTAGGGGAGGGAAGCGGTGGGGTTTGTGGATAATGGGC
GCAAGTATGATGCCGCCTGGTGGGGGGAAGAGGAAGCTCGTAGGGTGAAAGAAATT
TTTAGCGGGGAGGAAGGTGTAAGTATAAACCCTTTATCATTTGGGTTACCGGAGAAA
AACCCCCGTCTATCTGCGTGCAGCAGGGGGGAAAAAAAAAAGGTGCACTTTTAATG
GGATTTAGGGGG

Sequence 10-1492r>

AAAAAACCCTTAAGGGTTACCTTGGTTACGACTTACGGGGCCAAGGACCAAACCTA
AAGGTAACCTGGTTCCGCTTTCCGGGGCCAGGACCAAAACCCAGGGGTTATTTTGTG
GAGCTTTCCGGACCCCGAACAAAAACCGAACCGACTTTAGGGAAACGAGCGCAGAC
TCCAATCCGGAACTAAGAC

Sequence 11-2f>

AAGGTAACAGATAAAGTGGATCTTGGTTCAGGTAGTCCGAAAAGGGTACCCAAAAG
TTTTTGCCCGGCTCCAGAAAAATCTATCGGAAAACCAGAGAAGTTTTGGGATAACTA
CTCGAAAGAGTGGCTAATACAGCATACGTCCTATAGGAGAAAGAGGGGGACCTTCG
GGCCTTGTGCTATTGGAGTGGCCCATATCGGATTAGCTAGTTGGTGGGGTAAAGGCC
TACCAAGGCAACGATCCCTATCTGGTTTGAGAGGACGACCAGCCACACTGTGACTG
AGACACGGGCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGTACAATGGGGG
AAACCCTGATCCAGCCATCCCGCGTGTGCGATAAAGGCCTTCGGGTTGTAAAGCACT
TTTGTCAGGAAAGAAATCGCCCGGGATAATACCTCTTGTGGATGAGGGTACCTGAA
GAATAAACACCGGCTATCTCCGTGCCACCAGCCGCGGTAATACGTAGGGTGCAAGC
GTTTATCGCAATTACTGGGCGTAAAGCGTGCGCAGGGGGGTTCGTAAAGAAAGATGT
GAAATCCCCCGGGTCAACCTTGGAACTGTGTTTTAACTATCGGGCTATAGTGTGTTA
CAGGGGGGTGGAATTCTCCGTGTAGCGCTGAGATGTGTAAAGATGTGGAGGAACAC
CGATGGGGAAGGCGGGGCCCTGGAAAAAAACTGACGCTCTGCGCGAAAACGTGGG
GAGCAAAAAAGATTATATAACCCTGGGAGTCCCCCGCCCTTAAACGATGTTCACTAT
GTTGTTGGGGCCTTCGGGGCCTTAGTATCGCGACAAAAACCGGGAAATGACCCCCC
GGGGGGAAACGGCCCCACAATTATAAAATCTTAAAGATTTTTTGGGGGCCCCCCCA

AACGGGGGGTGATGTGGATTTTTTTTTTGGGGCGGGGAAAAAACTTTTTCTACCC
CTTGGGGGTCGGGAAACCCAAAAAAATTTTGGGTGTGGCCCCCAGAAAACCGAAA
AAAAGGGGGTGTTTGGGGTGGCGTCCCTCCCCTGGCGGGAAAAGATTGTGGTTAAA
AACCCCCCAAAAAGAGCCAACCCCTTTGTTTTTTTTGTGTGTCACAAAGGAGCGC

Sequence 11-1492r>

AAGCCAACCCTTTAGGGTAACCTGGGTCCGACTTCCTGGGCCAGGGACAAACCCCA
AGGGTAACTTGGTAACGCTTACCGGGGCCGGGGCCAAACCCCAAGGGTGTCTTGGC
CGCGATTTCCGAACCCGGAAATACAACCTAATCCGACTTCTTGAAGCCGAGCGGAA
AACTAAAACCCGAACTACGA

Sequence 12-2f>

GTATCCCGTTAAGATTTTGATCTGGGTTCAGGAAGGCGGAAAAAGGGTACCCGAGA
GGTTGGTCCTGGGTCCGGGAAACCGTGAAAAGGAAACCGGAAGGTTTTTCCCGTA
CCTACTGGACGGGAGGATTATCCCGTATGTGTATAATT

Sequence 12-1492r>

AAAACTCCTAGGGTTACTGGTAACGATTTACGGGGCCCGGGAAAACCCTAAAGGTA
CCCTGGTGCCGCTTTCCGGGGCCCGAACCAAACCCTAAAGTTTATTTGGCGAAACTT
TCTGAGCCACGATTACAACCTTTTCCGACTTCATGGAGTCGAGTTGCAGACTCCAAT
CCGGACTACGACAGACTTTATGAGTTCCGCTTGCTCTCGAGAGATCACTTTCTCTTTG
TATCTGCCATTGTAGCACGTGTTTAGCCC

Sequence 13-2f>

GGAAAAAGGAAGAATTGGAACTGGGTCTAGAAATCGAAAAAGGTTTTCGAAAATTT
TTTTCCGGGTCCGGAGAAGCGTGAACAGGGAAAAGGAATGTTTTTGCTCGACCCTAC
TGGACCGGAATATTTTTCGTATTTTTTCTTCGGACAGGCGAAGAAACTAAAGATTTC
TTTGTA

Sequence 13-1492r>

AAAAACCCTATAGGGTTACTGTTTACGAATTCCGGGACCAGAAAACACCCATACGC
GAATCCTTGTCGCTTCGCGGGGCCCGAACAAAAACCTAAGGCGTATTTGTCGAAGAT
TTCTGGACTACGATTACTAATCGATTCCGACTTC

Sequence 14-2f>

GGAAAACGATTAAAAGTGGATCTGGCTTCAGGAAGCGGAAAAGGGAACCCGAAAG
GTTGGGCCCGGGTCAGGTGAACCCAAAAAAGGAAACCCGAGAGTTTTTCCCGTCTC
CACGGGGCGGAAAAAGTACATGGTTTTTTTATACGAACCTGGGGGGACATTCTCGCA
TTTTTTTATTATGTTTT

Sequence 14-1492r>

AAAAAACCCTTAGATTACTTTGTTCGATTTCCTGGGCCAGGGAAAAAACCTAAGGGT
ACCCTGGGACCGCTTACCGGGGCCCGGACCAAAACCCAAGGGTTCCTTGGCAGAAA
TTTCCGGGGCCGGGATAACAACCTATCCTGACTTTTGGAAACGAAGTGCGGAACCCA
ATCCCGGACTAAGACTGACTTTTAGGAGAACCGCTTGCTCCCCGCGAGATCGGCTTC
CTCTTTTGTATCAGCCATTGGAATATTTGTGTTGAAACCCC

Sequence 15-2f>

AGGGAACCGGAAGAAGTTGGTCCTGGGTTCAGGAAGGGGGAACAAGGGAACCCGA
AAGGTTGGGCCCGGGTCCGGAGAGGCGGAAAAAGGGAACCGGGGGGTTTGTCCCG
GCTCAAGGGGGGGGAAAAAAGAAAAACCGTTTTTTTTTTGGGCCGGGGGGGGGGG
GGGGAAGCCTTCTTGT

Sequence 15-1492r>

GGAAAAACCTAGGGGTTACCTTGTTAGGATTTACTGGGCCCGGGAAAAAACCCAAG
GGTACCTTGGTACCGCTTTCCGGGGCCCGGGCCAAACCCCAAGGGTTCCTTGGCCGC
CATTTCCGAAGCCCGGATCACAATCTAGGCTTTTTTTTGTAAACCTAGCGGGGACTC
CAATCCCGGACTTAGGATTTATTTTTTTGAGACCGGCTTGCCCCTCGCGAGATCGCTT
TCCCTTTGTACCCGGCCATTGGACGACGGGGGGAAGCCCAACCCCGAAAGGACCAA
GAGGACTTGACGTCAGCCCCCCACCTTCCTCGGGTTTATCACAGGGAGACTCATAAG
AGTTC

Sequence 16-2f>

GTAACAGGTTAGGGTTGGATCTGGGTTCAGGAAGGCGGAAAAGGGAACCGGGGGG
GTTTGTCCCGGGCCCGGGGAGGGGGAAAAAGGGAAACGGGAGGTTTTTCCTGGCC
CGGGGGAGGGGAAAAAAGGAAAACAGGTGTTTTTTTA

Sequence 16-1492r>

AAGCAAATCCTTAGGGGTAACCTGGTACGGATTTCCCGGGCCCGGGCCAAAACCCA
AGGGTAACTTGGGAAGCCTTTCCCGGGCCCGGGCCCAAACCCACAGGTTCCTGGGT
ACCCCTTTCCGGGGGCAGAACCAAACCCTTAGGGTTTCTTGGTTC

Sequence 17-2f>

AGAACCACAAGAGGTGGATCAGGCCAGAGAGCGAAAGGGTAACGAAAGTTTGGCC
CGGCGTGGAAAACTAAAAGAACCACACGTTAGTGGTGCCCGGTACTGAAAACGTAA
ATAAAACCCCATGATTCTACTTCCAAAGCAGGGGCTCTTCGGACCTTGCGCTATCGG
ATGAACCCATATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGAT
CTCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGGAGACACGGCCCAGA
CTCCTACGGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGGATGC
AGCCTTGCGGGGTGTATGAAGAAGGCCTTAGGGTTGAAAAGTACTTTCCGGGGGGG
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Sequence 17-1492r>

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APPENDIX VII

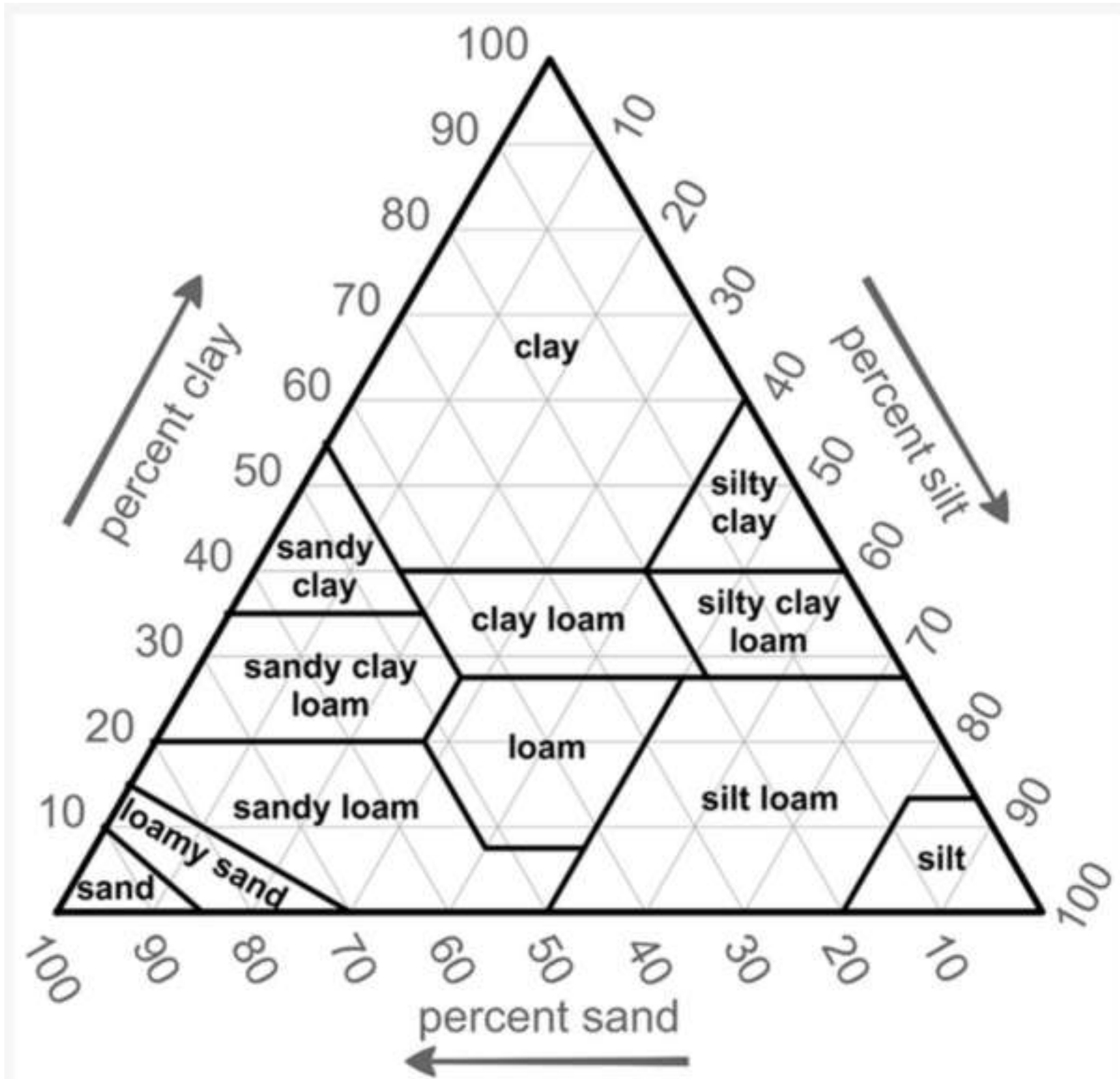

Figure 3.2: The USDA soil textural triangle (APHA, 1998)

CHROMATOGRAMS OF ANALYSED SOIL SAMPLES

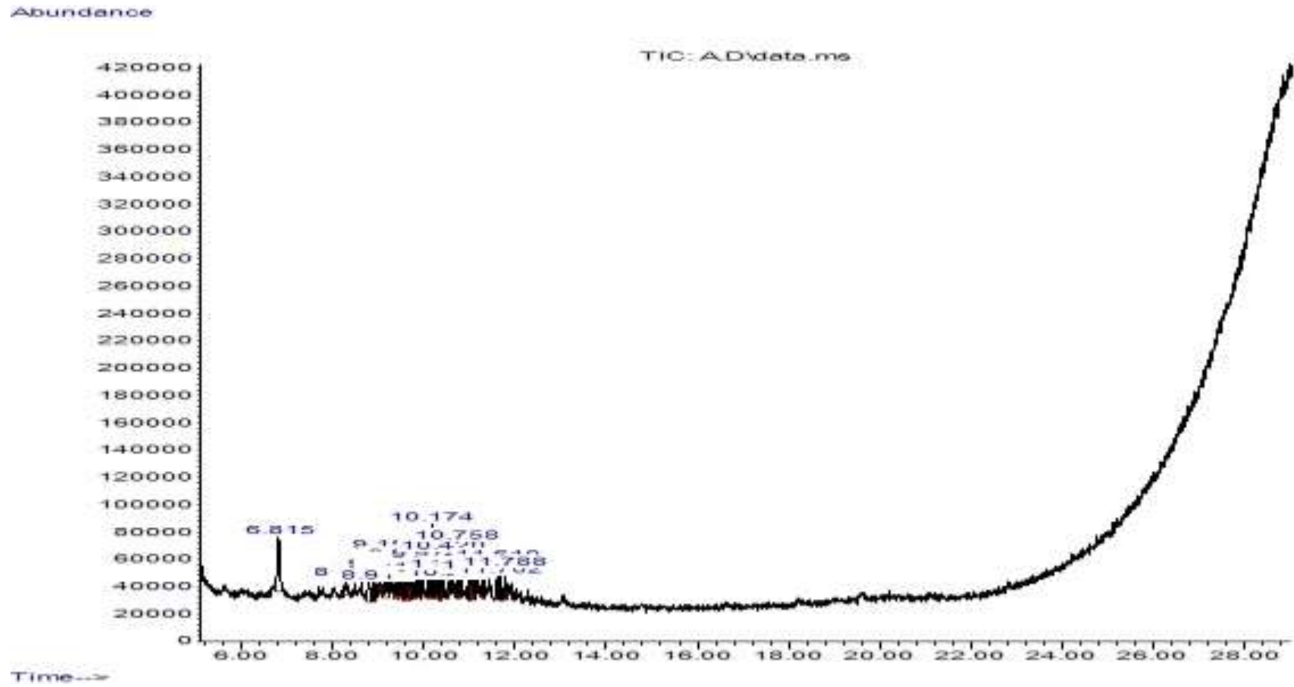

Figure 4.1a: Chromatogram of unpolluted agricultural soil before PGE using *H. cannabinus* (sample A).

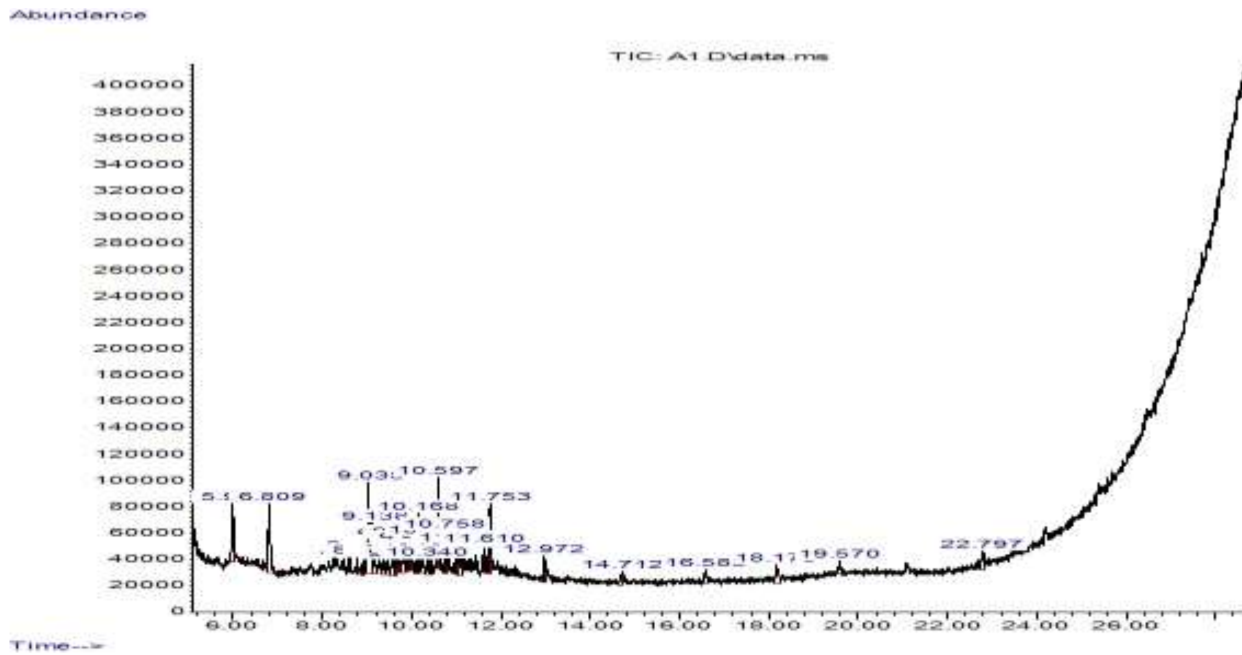

Figure 4.1b: Chromatogram of unpolluted agricultural soil after PGE using *H. cannabinus* (sample A1).

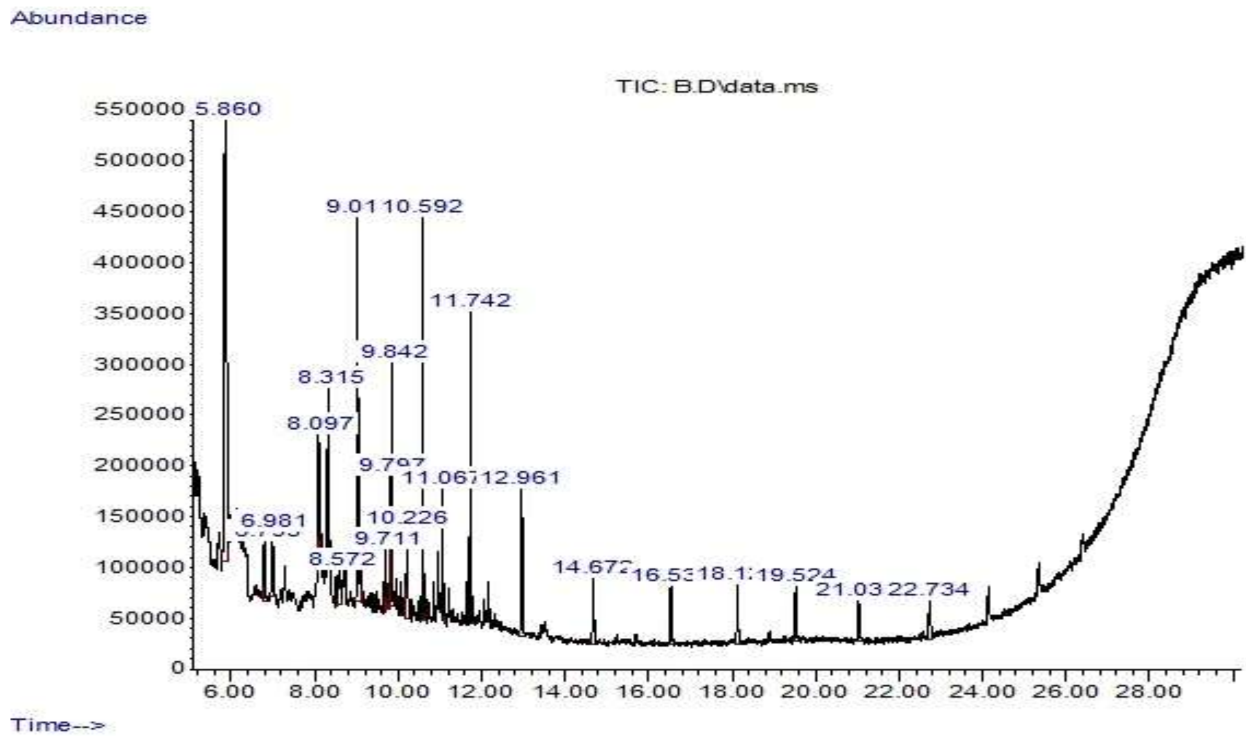

Figure 4.2a: Chromatogram of 5ml/Kg polluted soil before PGE using *H. cannabinus* (sample B).

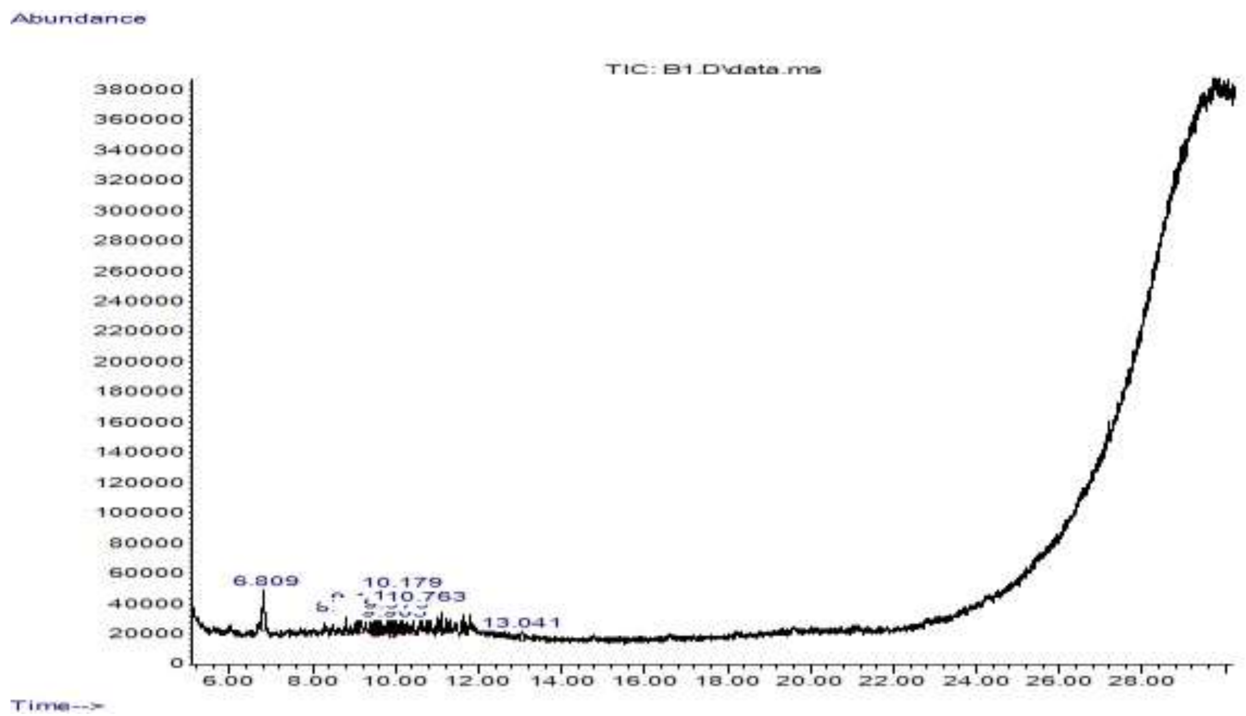

Figure 4.2b: Chromatogram of 5ml/Kg polluted soil after PGE using *H. cannabinus* (sample B1).

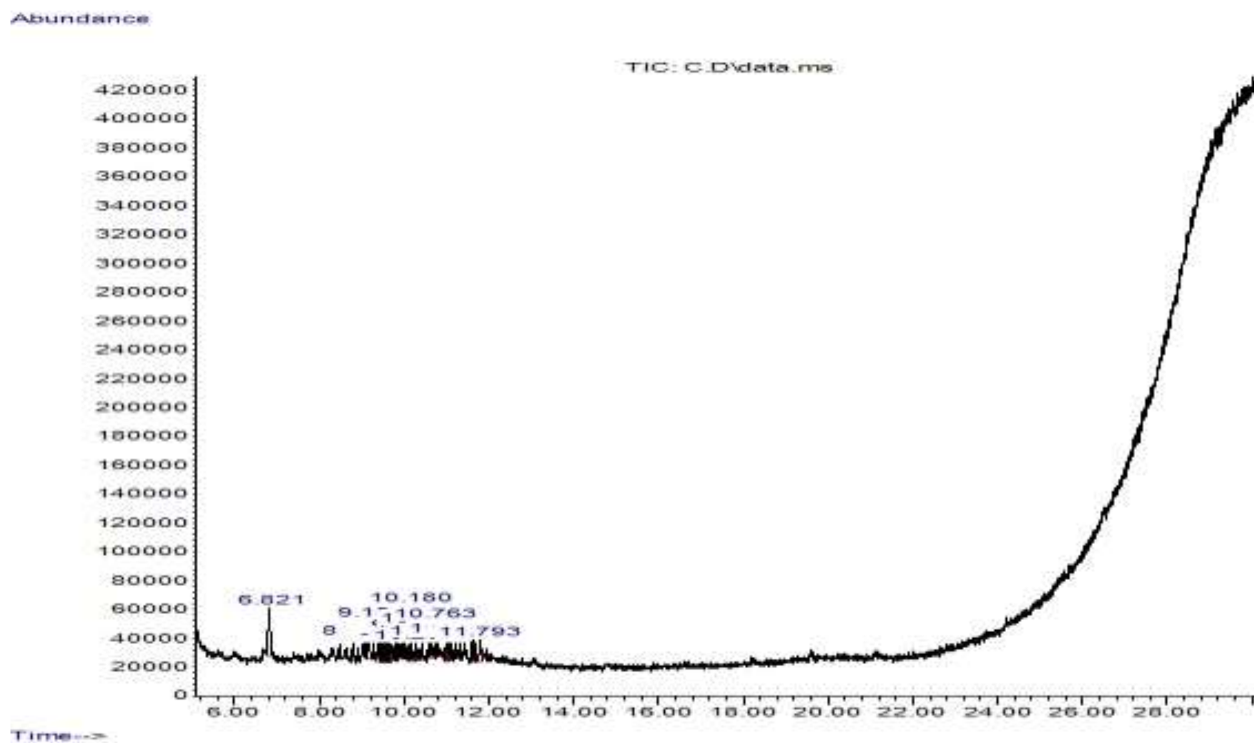

Figure 4.3a: Chromatogram of 10ml/Kg polluted soil before PGE using *H. cannabinus* (sample C).

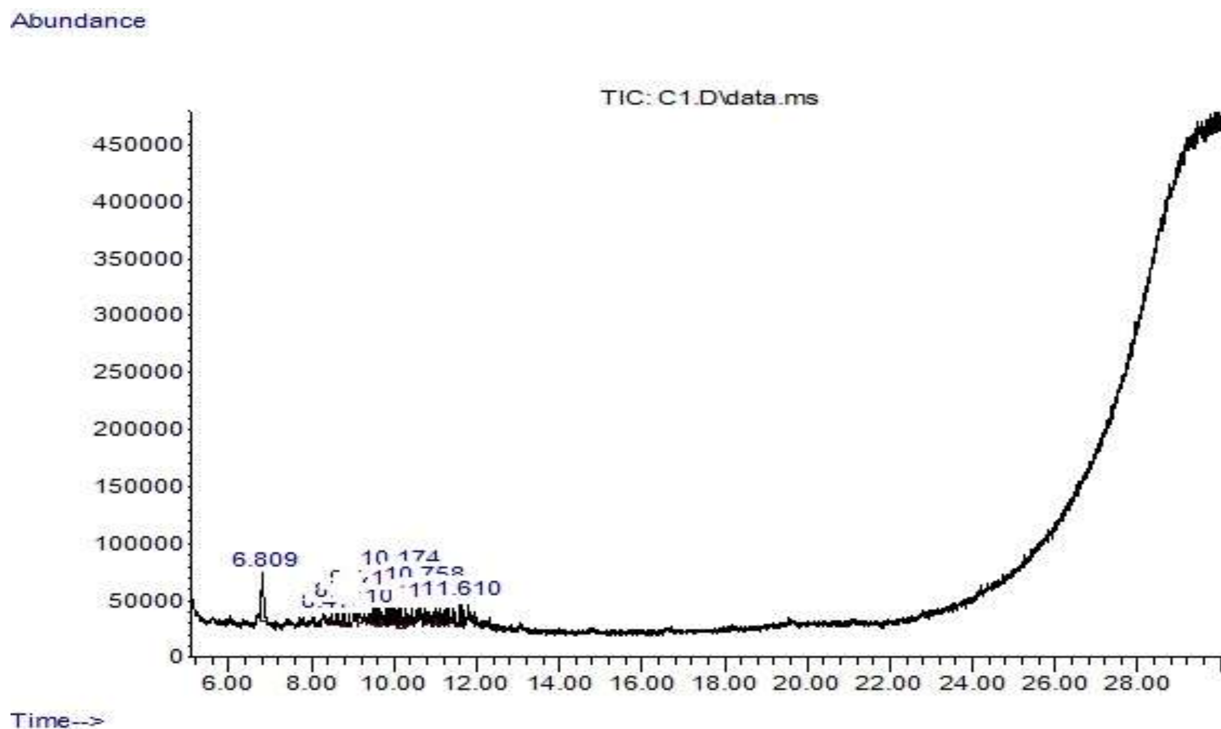

Figure 4.3b: Chromatogram of 10ml/Kg polluted soil after PGE using *H. cannabinus* (sample C1).

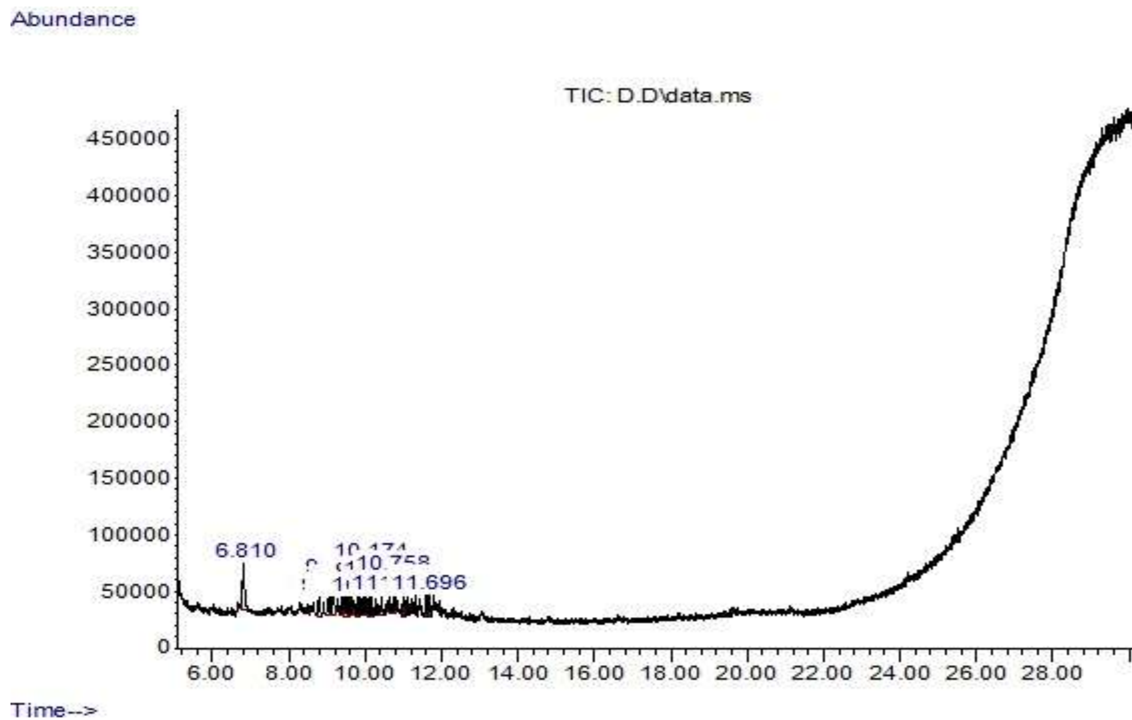

Figure 4.4a: Chromatogram of 25ml/Kg polluted soil before PGE using *H. cannabinus* (sample D).

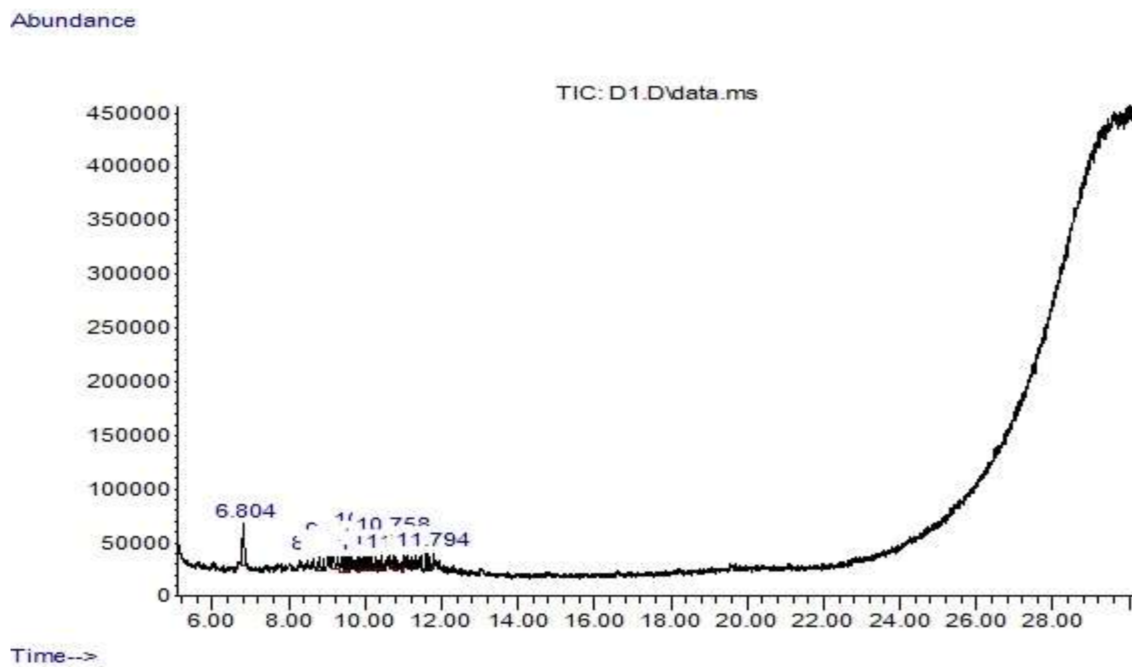

Figure 4.4b: Chromatogram of 25ml/Kg polluted soil after PGE using *H. cannabinus* (sample D1).

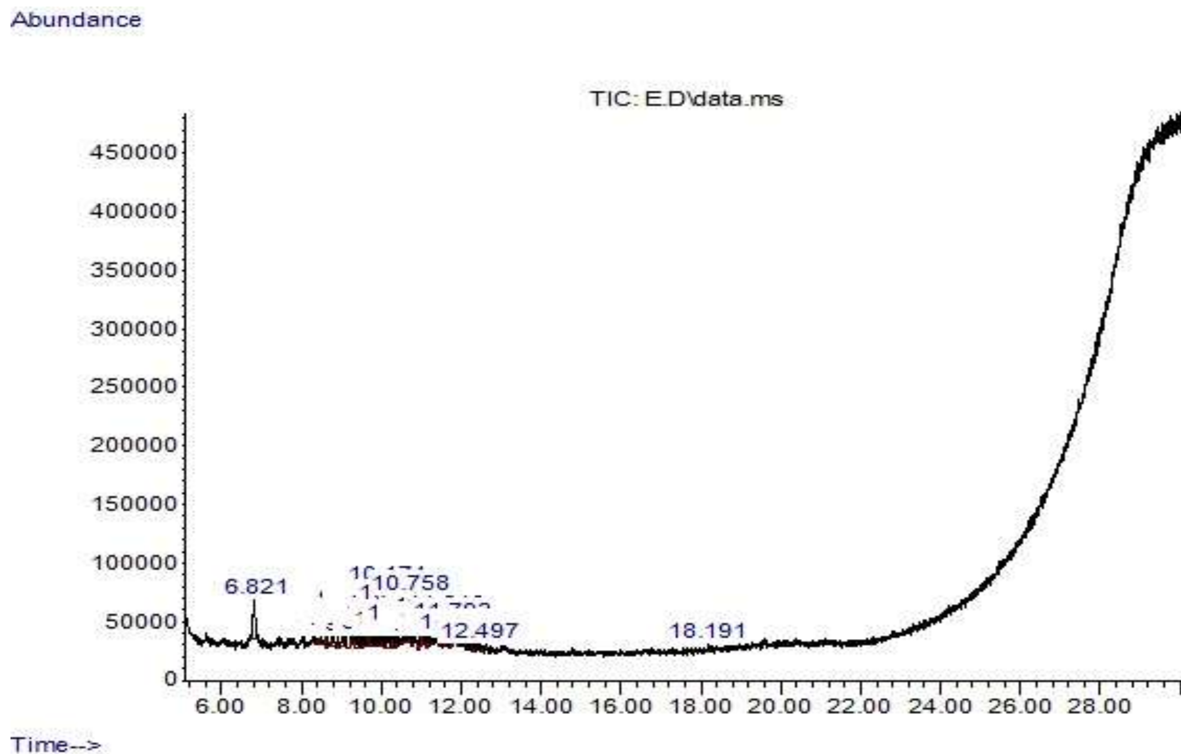

Figure 4.5a: Chromatogram of 50ml/Kg polluted soil before PGE using *H. cannabinus* (sample E).

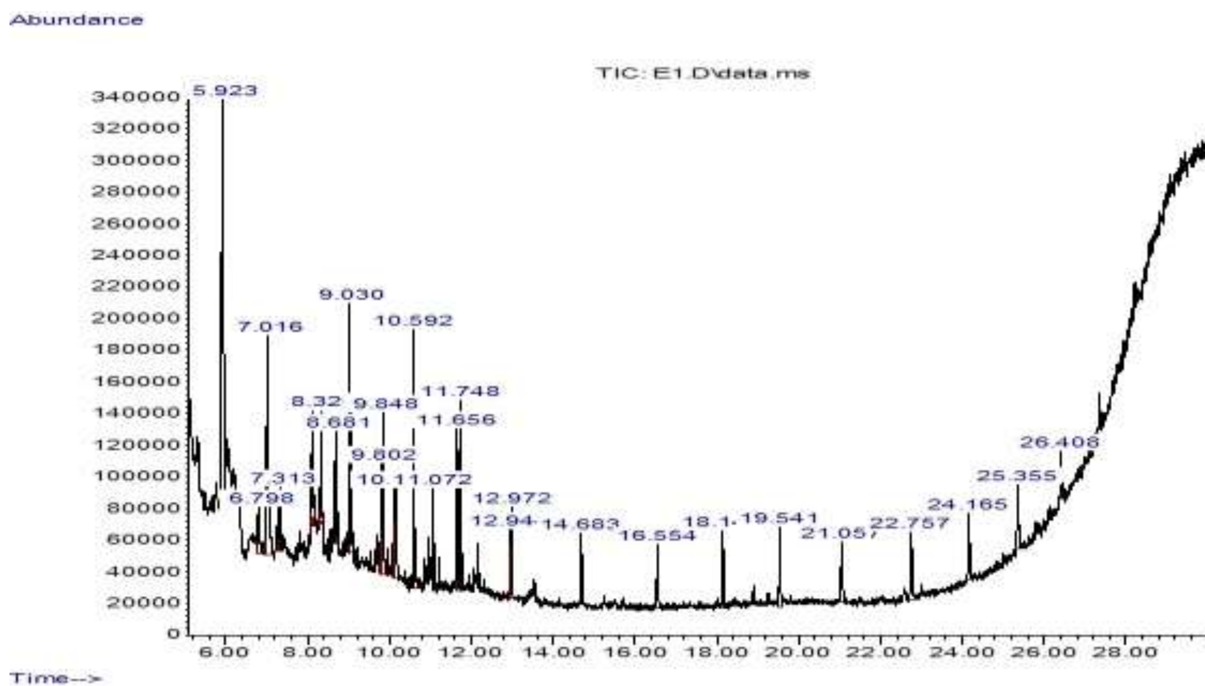

Figure 4.5b: Chromatogram of 50ml/Kg polluted soil after PGE using *H. cannabinus* (sample E1).

Oneway

[DataSet1] C:\Users\HP\Desktop\BTC STAT\PGE II using H. cannabinus INPUT.sav

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Na+ (mg/kg)	B1	3	7.1500	.02000	.01155	7.1003	7.1997	7.13	7.17
	B2	3	7.1500	.02000	.01155	7.1003	7.1997	7.13	7.17
	B3	3	7.8800	.01000	.00577	7.8552	7.9048	7.87	7.89
	B4	3	9.3100	.01000	.00577	9.2852	9.3348	9.30	9.32
	B21	3	4.2500	.02000	.01155	4.2003	4.2997	4.23	4.27
	B22	3	4.0400	.01000	.00577	4.0152	4.0648	4.03	4.05
	B23	3	3.4500	.01000	.00577	3.4252	3.4748	3.44	3.46
	B24	3	3.8200	.01000	.00577	3.7952	3.8448	3.81	3.83
	Total	24	5.8813	2.14221	.43728	4.9767	6.7858	3.44	9.32
Ca2+ (mg/kg)	B1	3	3.4500	.01000	.00577	3.4252	3.4748	3.44	3.46
	B2	3	3.4600	.01000	.00577	3.4352	3.4848	3.45	3.47
	B3	3	5.3200	.01000	.00577	5.2952	5.3448	5.31	5.33
	B4	3	7.8300	.01000	.00577	7.8052	7.8548	7.82	7.84
	B21	3	2.1400	.01000	.00577	2.1152	2.1648	2.13	2.15
	B22	3	1.8100	.01000	.00577	1.7852	1.8348	1.80	1.82
	B23	3	2.9100	.01000	.00577	2.8852	2.9348	2.90	2.92
	B24	3	3.3800	.01000	.00577	3.3552	3.4048	3.37	3.39
	Total	24	3.7875	1.85822	.37931	3.0028	4.5722	1.80	7.84
Mg2+ (mg/kg)	B1	3	2.5600	.02000	.01155	2.5103	2.6097	2.54	2.58
	B2	3	2.5600	.01000	.00577	2.5352	2.5848	2.55	2.57
	B3	3	4.5300	.01000	.00577	4.5052	4.5548	4.52	4.54
	B4	3	3.9600	.02000	.01155	3.9103	4.0097	3.94	3.98
	B21	3	1.6700	.02000	.01155	1.6203	1.7197	1.65	1.69

	B22	3	1.2200	.01000	.00577	1.1952	1.2448	1.21	1.23
	B23	3	2.5000	.01000	.00577	2.4752	2.5248	2.49	2.51
	B24	3	2.0600	.02000	.01155	2.0103	2.1097	2.04	2.08
	Total	24	2.6325	1.06167	.21671	2.1842	3.0808	1.21	4.54
	B1	3	3.7800	.02000	.01155	3.7303	3.8297	3.76	3.80
	B2	3	3.7800	.02000	.01155	3.7303	3.8297	3.76	3.80
	B3	3	4.1100	.01000	.00577	4.0852	4.1348	4.10	4.12
	B4	3	5.5400	.01000	.00577	5.5152	5.5648	5.53	5.55
K+ (mg/kg)	B21	3	4.2500	.01000	.00577	4.2252	4.2748	4.24	4.26
	B22	3	4.0400	.02000	.01155	3.9903	4.0897	4.02	4.06
	B23	3	3.4500	.01000	.00577	3.4252	3.4748	3.44	3.46
	B24	3	3.8200	.01000	.00577	3.7952	3.8448	3.81	3.83
	Total	24	4.0963	.60504	.12350	3.8408	4.3517	3.44	5.55
	B1	3	54.9300	.02000	.01155	54.8803	54.9797	54.91	54.95
	B2	3	54.9300	.02000	.01155	54.8803	54.9797	54.91	54.95
	B3	3	29.3200	.01000	.00577	29.2952	29.3448	29.31	29.33
	B4	3	37.4000	.02000	.01155	37.3503	37.4497	37.38	37.42
NO3- (mg/kg)	B21	3	48.2800	.02000	.01155	48.2303	48.3297	48.26	48.30
	B22	3	45.6000	.10000	.05774	45.3516	45.8484	45.50	45.70
	B23	3	25.3600	.01000	.00577	25.3352	25.3848	25.35	25.37
	B24	3	32.1500	.01000	.00577	32.1252	32.1748	32.14	32.16
	Total	24	40.9963	11.04086	2.25371	36.3341	45.6584	25.35	54.95
	B1	3	42.6100	.01000	.00577	42.5852	42.6348	42.60	42.62
	B2	3	42.6100	.01000	.00577	42.5852	42.6348	42.60	42.62
	B3	3	23.5600	.01000	.00577	23.5352	23.5848	23.55	23.57
SO42- (mg/kg)	B4	3	21.9100	.01000	.00577	21.8852	21.9348	21.90	21.92
	B21	3	36.6100	.01000	.00577	36.5852	36.6348	36.60	36.62
	B22	3	26.4300	.01000	.00577	26.4052	26.4548	26.42	26.44
	B23	3	15.0000	.10000	.05774	14.7516	15.2484	14.90	15.10

	B24	3	18.8700	.01000	.00577	18.8452	18.8948	18.86	18.88
	Total	24	28.4500	10.28536	2.09949	24.1069	32.7931	14.90	42.62
HCO3- (mg/kg)	B1	3	35.1900	.01000	.00577	35.1652	35.2148	35.18	35.20
	B2	3	35.1900	.01000	.00577	35.1652	35.2148	35.18	35.20
	B3	3	25.2900	.02000	.01155	25.2403	25.3397	25.27	25.31
	B4	3	38.6700	.01000	.00577	38.6452	38.6948	38.66	38.68
	B21	3	23.9500	.02000	.01155	23.9003	23.9997	23.93	23.97
	B22	3	20.2000	.02000	.01155	20.1503	20.2497	20.18	20.22
	B23	3	21.5500	.01000	.00577	21.5252	21.5748	21.54	21.56
	B24	3	25.2800	.02000	.01155	25.2303	25.3297	25.26	25.30
	Total	24	28.1650	6.76255	1.38040	25.3094	31.0206	20.18	38.68
NH4+ (mg/kg)	B1	3	5.3600	.01000	.00577	5.3352	5.3848	5.35	5.37
	B2	3	5.3600	.01000	.00577	5.3352	5.3848	5.35	5.37
	B3	3	6.1500	.02000	.01155	6.1003	6.1997	6.13	6.17
	B4	3	6.4800	.01000	.00577	6.4552	6.5048	6.47	6.49
	B21	3	4.8000	.02000	.01155	4.7503	4.8497	4.78	4.82
	B22	3	4.6800	.01000	.00577	4.6552	4.7048	4.67	4.69
	B23	3	4.1600	.01000	.00577	4.1352	4.1848	4.15	4.17
	B24	3	4.1400	.01000	.00577	4.1152	4.1648	4.13	4.15
	Total	24	5.1413	.82365	.16813	4.7935	5.4890	4.13	6.49
OC (%)	B1	3	2.4500	.02000	.01155	2.4003	2.4997	2.43	2.47
	B2	3	2.4500	.02000	.01155	2.4003	2.4997	2.43	2.47
	B3	3	.8600	.01000	.00577	.8352	.8848	.85	.87
	B4	3	1.7900	.01000	.00577	1.7652	1.8148	1.78	1.80
	B21	3	1.5800	.02000	.01155	1.5303	1.6297	1.56	1.60
	B22	3	2.1200	.01000	.00577	2.0952	2.1448	2.11	2.13
	B23	3	.4500	.02000	.01155	.4003	.4997	.43	.47
	B24	3	.6700	.01000	.00577	.6452	.6948	.66	.68
	Total	24	1.5463	.76316	.15578	1.2240	1.8685	.43	2.47

	B1	3	3.8800	.01000	.00577	3.8552	3.9048	3.87	3.89
	B2	3	3.8800	.01000	.00577	3.8552	3.9048	3.87	3.89
	B3	3	1.5400	.02000	.01155	1.4903	1.5897	1.52	1.56
	B4	3	2.6200	.01000	.00577	2.5952	2.6448	2.61	2.63
OM (%)	B21	3	3.1100	.01000	.00577	3.0852	3.1348	3.10	3.12
	B22	3	3.5100	.02000	.01155	3.4603	3.5597	3.49	3.53
	B23	3	.6400	.01000	.00577	.6152	.6648	.63	.65
	B24	3	1.9500	.02000	.01155	1.9003	1.9997	1.93	1.97
	Total	24	2.6413	1.12717	.23008	2.1653	3.1172	.63	3.89
	B1	3	.0810	.00100	.00058	.0785	.0835	.08	.08
	B2	3	.0810	.00100	.00058	.0785	.0835	.08	.08
	B3	3	.0780	.00100	.00058	.0755	.0805	.08	.08
	B4	3	.0850	.00100	.00058	.0825	.0875	.08	.09
N (%)	B21	3	.0690	.00100	.00058	.0665	.0715	.07	.07
	B22	3	.0740	.00100	.00058	.0715	.0765	.07	.08
	B23	3	.0630	.00100	.00058	.0605	.0655	.06	.06
	B24	3	.0560	.00100	.00058	.0535	.0585	.06	.06
	Total	24	.0734	.00960	.00196	.0693	.0774	.06	.09
	B1	3	.2500	.01000	.00577	.2252	.2748	.24	.26
	B2	3	.2500	.01000	.00577	.2252	.2748	.24	.26
	B3	3	.2900	.01000	.00577	.2652	.3148	.28	.30
	B4	3	.3200	.01000	.00577	.2952	.3448	.31	.33
P	B21	3	.2100	.01000	.00577	.1852	.2348	.20	.22
	B22	3	.2300	.02000	.01155	.1803	.2797	.21	.25
	B23	3	.2600	.02000	.01155	.2103	.3097	.24	.28
	B24	3	.2800	.01000	.00577	.2552	.3048	.27	.29
	Total	24	.2613	.03505	.00716	.2464	.2761	.20	.33

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
	Between Groups	105.545	7	15.078	70954.664	.000
Na+ (mg/kg)	Within Groups	.003	16	.000		
	Total	105.548	23			
	Between Groups	79.417	7	11.345	113452.929	.000
Ca2+ (mg/kg)	Within Groups	.002	16	.000		
	Total	79.419	23			
	Between Groups	25.920	7	3.703	14811.686	.000
Mg2+ (mg/kg)	Within Groups	.004	16	.000		
	Total	25.924	23			
	Between Groups	8.416	7	1.202	5657.924	.000
K+ (mg/kg)	Within Groups	.003	16	.000		
	Total	8.420	23			
	Between Groups	2803.688	7	400.527	269261.730	.000
NO3- (mg/kg)	Within Groups	.024	16	.001		
	Total	2803.712	23			
	Between Groups	2433.119	7	347.588	259879.242	.000
SO42- (mg/kg)	Within Groups	.021	16	.001		
	Total	2433.141	23			
	Between Groups	1051.832	7	150.262	601047.086	.000
HCO3- (mg/kg)	Within Groups	.004	16	.000		
	Total	1051.836	23			
	Between Groups	15.600	7	2.229	12734.908	.000
NH4+ (mg/kg)	Within Groups	.003	16	.000		
	Total	15.603	23			
OC (%)	Between Groups	13.391	7	1.913	7652.207	.000

	Within Groups	.004	16	.000		
	Total	13.395	23			
OM (%)	Between Groups	29.218	7	4.174	19642.664	.000
	Within Groups	.003	16	.000		
	Total	29.222	23			
N (%)	Between Groups	.002	7	.000	300.804	.000
	Within Groups	.000	16	.000		
	Total	.002	23			
P	Between Groups	.025	7	.004	20.786	.000
	Within Groups	.003	16	.000		
	Total	.028	23			

Post Hoc Tests

Multiple Comparisons

Dependent Variable		(I) Values	(J) Values	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
Na+ (mg/kg)	LSD	B1	B2	.00000	.01190	1.000	-.0252	.0252
			B3	-.73000*	.01190	.000	-.7552	-.7048
			B4	-2.16000*	.01190	.000	-2.1852	-2.1348
			B2 1	2.90000*	.01190	.000	2.8748	2.9252
			B22	3.11000*	.01190	.000	3.0848	3.1352
			B23	3.70000*	.01190	.000	3.6748	3.7252
			B24	3.33000*	.01190	.000	3.3048	3.3552
		B2	B1	.00000	.01190	1.000	-.0252	.0252
			B3	-.73000*	.01190	.000	-.7552	-.7048
			B4	-2.16000*	.01190	.000	-2.1852	-2.1348
			B21	2.90000*	.01190	.000	2.8748	2.9252
			B22	3.11000*	.01190	.000	3.0848	3.1352
			B23	3.70000*	.01190	.000	3.6748	3.7252
			B24	3.33000*	.01190	.000	3.3048	3.3552
		B3	B1	.73000*	.01190	.000	.7048	.7552
			B2	.73000*	.01190	.000	.7048	.7552
			B4	-1.43000*	.01190	.000	-1.4552	-1.4048
			B21	3.63000*	.01190	.000	3.6048	3.6552
			B22	3.84000*	.01190	.000	3.8148	3.8652
			B23	4.43000*	.01190	.000	4.4048	4.4552
			B24	4.06000*	.01190	.000	4.0348	4.0852
		B4	B1	2.16000*	.01190	.000	2.1348	2.1852
			B2	2.16000*	.01190	.000	2.1348	2.1852

	B3	1.43000*	.01190	.000	1.4048	1.4552
	B21	5.06000*	.01190	.000	5.0348	5.0852
	B22	5.27000*	.01190	.000	5.2448	5.2952
	B23	5.86000*	.01190	.000	5.8348	5.8852
	B24	5.49000*	.01190	.000	5.4648	5.5152
B21	B1	-2.90000*	.01190	.000	-2.9252	-2.8748
	B2	-2.90000*	.01190	.000	-2.9252	-2.8748
	B3	-3.63000*	.01190	.000	-3.6552	-3.6048
	B4	-5.06000*	.01190	.000	-5.0852	-5.0348
	B22	.21000*	.01190	.000	.1848	.2352
	B23	.80000*	.01190	.000	.7748	.8252
	B24	.43000*	.01190	.000	.4048	.4552
B22	B1	-3.11000*	.01190	.000	-3.1352	-3.0848
	B2	-3.11000*	.01190	.000	-3.1352	-3.0848
	B3	-3.84000*	.01190	.000	-3.8652	-3.8148
	B4	-5.27000*	.01190	.000	-5.2952	-5.2448
	B21	-.21000*	.01190	.000	-.2352	-.1848
	B23	.59000*	.01190	.000	.5648	.6152
	B24	.22000*	.01190	.000	.1948	.2452
B23	B1	-3.70000*	.01190	.000	-3.7252	-3.6748
	B2	-3.70000*	.01190	.000	-3.7252	-3.6748
	B3	-4.43000*	.01190	.000	-4.4552	-4.4048
	B4	-5.86000*	.01190	.000	-5.8852	-5.8348
	B21	-.80000*	.01190	.000	-.8252	-.7748
	B22	-.59000*	.01190	.000	-.6152	-.5648
	B24	-.37000*	.01190	.000	-.3952	-.3448

			B1	-3.33000*	.01190	.000	-3.3552	-3.3048
			B2	-3.33000*	.01190	.000	-3.3552	-3.3048
			B3	-4.06000*	.01190	.000	-4.0852	-4.0348
		B24	B4	-5.49000*	.01190	.000	-5.5152	-5.4648
			B21	-.43000*	.01190	.000	-.4552	-.4048
			B22	-.22000*	.01190	.000	-.2452	-.1948
			B23	.37000*	.01190	.000	.3448	.3952
			B2	-.01000	.00816	.238	-.0273	.0073
			B3	-1.87000*	.00816	.000	-1.8873	-1.8527
			B4	-4.38000*	.00816	.000	-4.3973	-4.3627
		B1	B21	1.31000*	.00816	.000	1.2927	1.3273
			B22	1.64000*	.00816	.000	1.6227	1.6573
			B23	.54000*	.00816	.000	.5227	.5573
			B24	.07000*	.00816	.000	.0527	.0873
			B1	.01000	.00816	.238	-.0073	.0273
			B3	-1.86000*	.00816	.000	-1.8773	-1.8427
			B4	-4.37000*	.00816	.000	-4.3873	-4.3527
Ca2+ (mg/kg)	LSD	B2	B21	1.32000*	.00816	.000	1.3027	1.3373
			B22	1.65000*	.00816	.000	1.6327	1.6673
			B23	.55000*	.00816	.000	.5327	.5673
			B24	.08000*	.00816	.000	.0627	.0973
			B1	1.87000*	.00816	.000	1.8527	1.8873
			B2	1.86000*	.00816	.000	1.8427	1.8773
			B4	-2.51000*	.00816	.000	-2.5273	-2.4927
		B3	B21	3.18000*	.00816	.000	3.1627	3.1973
			B22	3.51000*	.00816	.000	3.4927	3.5273
			B23	2.41000*	.00816	.000	2.3927	2.4273
			B24	1.94000*	.00816	.000	1.9227	1.9573

	B1	4.38000*	.00816	.000	4.3627	4.3973
	B2	4.37000*	.00816	.000	4.3527	4.3873
	B3	2.51000*	.00816	.000	2.4927	2.5273
B4	B21	5.69000*	.00816	.000	5.6727	5.7073
	B22	6.02000*	.00816	.000	6.0027	6.0373
	B23	4.92000*	.00816	.000	4.9027	4.9373
	B24	4.45000*	.00816	.000	4.4327	4.4673
	B1	-1.31000*	.00816	.000	-1.3273	-1.2927
	B2	-1.32000*	.00816	.000	-1.3373	-1.3027
	B3	-3.18000*	.00816	.000	-3.1973	-3.1627
B21	B4	-5.69000*	.00816	.000	-5.7073	-5.6727
	B22	.33000*	.00816	.000	.3127	.3473
	B23	-.77000*	.00816	.000	-.7873	-.7527
	B24	-1.24000*	.00816	.000	-1.2573	-1.2227
	B1	-1.64000*	.00816	.000	-1.6573	-1.6227
	B2	-1.65000*	.00816	.000	-1.6673	-1.6327
	B3	-3.51000*	.00816	.000	-3.5273	-3.4927
B22	B4	-6.02000*	.00816	.000	-6.0373	-6.0027
	B21	-.33000*	.00816	.000	-.3473	-.3127
	B23	-1.10000*	.00816	.000	-1.1173	-1.0827
	B24	-1.57000*	.00816	.000	-1.5873	-1.5527
	B1	-.54000*	.00816	.000	-.5573	-.5227
	B2	-.55000*	.00816	.000	-.5673	-.5327
	B3	-2.41000*	.00816	.000	-2.4273	-2.3927
B23	B4	-4.92000*	.00816	.000	-4.9373	-4.9027
	B21	.77000*	.00816	.000	.7527	.7873
	B22	1.10000*	.00816	.000	1.0827	1.1173
	B24	-.47000*	.00816	.000	-.4873	-.4527
B24	B1	-.07000*	.00816	.000	-.0873	-.0527

			B2	-.08000*	.00816	.000	-.0973	-.0627
			B3	-1.94000*	.00816	.000	-1.9573	-1.9227
			B4	-4.45000*	.00816	.000	-4.4673	-4.4327
			B21	1.24000*	.00816	.000	1.2227	1.2573
			B22	1.57000*	.00816	.000	1.5527	1.5873
			B23	.47000*	.00816	.000	.4527	.4873
		B1	B2	.00000	.01291	1.000	-.0274	.0274
			B3	-1.97000*	.01291	.000	-1.9974	-1.9426
			B4	-1.40000*	.01291	.000	-1.4274	-1.3726
			B21	.89000*	.01291	.000	.8626	.9174
			B22	1.34000*	.01291	.000	1.3126	1.3674
			B23	.06000*	.01291	.000	.0326	.0874
			B24	.50000*	.01291	.000	.4726	.5274
		B2	B1	.00000	.01291	1.000	-.0274	.0274
			B3	-1.97000*	.01291	.000	-1.9974	-1.9426
			B4	-1.40000*	.01291	.000	-1.4274	-1.3726
			B21	.89000*	.01291	.000	.8626	.9174
Mg2+ (mg/kg)	LSD		B22	1.34000*	.01291	.000	1.3126	1.3674
			B23	.06000*	.01291	.000	.0326	.0874
			B24	.50000*	.01291	.000	.4726	.5274
		B3	B1	1.97000*	.01291	.000	1.9426	1.9974
			B2	1.97000*	.01291	.000	1.9426	1.9974
			B4	.57000*	.01291	.000	.5426	.5974
			B21	2.86000*	.01291	.000	2.8326	2.8874
			B22	3.31000*	.01291	.000	3.2826	3.3374
			B23	2.03000*	.01291	.000	2.0026	2.0574
			B24	2.47000*	.01291	.000	2.4426	2.4974
		B4	B1	1.40000*	.01291	.000	1.3726	1.4274
			B2	1.40000*	.01291	.000	1.3726	1.4274

		B3	-.57000*	.01291	.000	-.5974	-.5426
		B21	2.29000*	.01291	.000	2.2626	2.3174
		B22	2.74000*	.01291	.000	2.7126	2.7674
		B23	1.46000*	.01291	.000	1.4326	1.4874
		B24	1.90000*	.01291	.000	1.8726	1.9274
		B1	-.89000*	.01291	.000	-.9174	-.8626
		B2	-.89000*	.01291	.000	-.9174	-.8626
		B3	-2.86000*	.01291	.000	-2.8874	-2.8326
	B21	B4	-2.29000*	.01291	.000	-2.3174	-2.2626
		B22	.45000*	.01291	.000	.4226	.4774
		B23	-.83000*	.01291	.000	-.8574	-.8026
		B24	-.39000*	.01291	.000	-.4174	-.3626
		B1	-1.34000*	.01291	.000	-1.3674	-1.3126
		B2	-1.34000*	.01291	.000	-1.3674	-1.3126
		B3	-3.31000*	.01291	.000	-3.3374	-3.2826
	B22	B4	-2.74000*	.01291	.000	-2.7674	-2.7126
		B21	-.45000*	.01291	.000	-.4774	-.4226
		B23	-1.28000*	.01291	.000	-1.3074	-1.2526
		B24	-.84000*	.01291	.000	-.8674	-.8126
		B1	-.06000*	.01291	.000	-.0874	-.0326
		B2	-.06000*	.01291	.000	-.0874	-.0326
		B3	-2.03000*	.01291	.000	-2.0574	-2.0026
	B23	B4	-1.46000*	.01291	.000	-1.4874	-1.4326
		B21	.83000*	.01291	.000	.8026	.8574
		B22	1.28000*	.01291	.000	1.2526	1.3074
		B24	.44000*	.01291	.000	.4126	.4674
		B1	-.50000*	.01291	.000	-.5274	-.4726
	B24	B2	-.50000*	.01291	.000	-.5274	-.4726
		B3	-2.47000*	.01291	.000	-2.4974	-2.4426

			B4	-1.90000*	.01291	.000	-1.9274	-1.8726
			B21	.39000*	.01291	.000	.3626	.4174
			B22	.84000*	.01291	.000	.8126	.8674
			B23	-.44000*	.01291	.000	-.4674	-.4126
		B1	B2	.00000	.01190	1.000	-.0252	.0252
			B3	-.33000*	.01190	.000	-.3552	-.3048
			B4	-1.76000*	.01190	.000	-1.7852	-1.7348
			B21	-.47000*	.01190	.000	-.4952	-.4448
			B22	-.26000*	.01190	.000	-.2852	-.2348
			B23	.33000*	.01190	.000	.3048	.3552
			B24	-.04000*	.01190	.004	-.0652	-.0148
		B2	B1	.00000	.01190	1.000	-.0252	.0252
			B3	-.33000*	.01190	.000	-.3552	-.3048
			B4	-1.76000*	.01190	.000	-1.7852	-1.7348
			B21	-.47000*	.01190	.000	-.4952	-.4448
			B22	-.26000*	.01190	.000	-.2852	-.2348
K+ (mg/kg)	LSD		B23	.33000*	.01190	.000	.3048	.3552
			B24	-.04000*	.01190	.004	-.0652	-.0148
		B3	B1	.33000*	.01190	.000	.3048	.3552
			B2	.33000*	.01190	.000	.3048	.3552
			B4	-1.43000*	.01190	.000	-1.4552	-1.4048
			B21	-.14000*	.01190	.000	-.1652	-.1148
			B22	.07000*	.01190	.000	.0448	.0952
			B23	.66000*	.01190	.000	.6348	.6852
			B24	.29000*	.01190	.000	.2648	.3152
		B4	B1	1.76000*	.01190	.000	1.7348	1.7852
			B2	1.76000*	.01190	.000	1.7348	1.7852
			B3	1.43000*	.01190	.000	1.4048	1.4552
			B21	1.29000*	.01190	.000	1.2648	1.3152

	B22	1.50000*	.01190	.000	1.4748	1.5252
	B23	2.09000*	.01190	.000	2.0648	2.1152
	B24	1.72000*	.01190	.000	1.6948	1.7452
	B1	.47000*	.01190	.000	.4448	.4952
	B2	.47000*	.01190	.000	.4448	.4952
	B3	.14000*	.01190	.000	.1148	.1652
B21	B4	-1.29000*	.01190	.000	-1.3152	-1.2648
	B22	.21000*	.01190	.000	.1848	.2352
	B23	.80000*	.01190	.000	.7748	.8252
	B24	.43000*	.01190	.000	.4048	.4552
	B1	.26000*	.01190	.000	.2348	.2852
	B2	.26000*	.01190	.000	.2348	.2852
	B3	-.07000*	.01190	.000	-.0952	-.0448
B22	B4	-1.50000*	.01190	.000	-1.5252	-1.4748
	B21	-.21000*	.01190	.000	-.2352	-.1848
	B23	.59000*	.01190	.000	.5648	.6152
	B24	.22000*	.01190	.000	.1948	.2452
	B1	-.33000*	.01190	.000	-.3552	-.3048
	B2	-.33000*	.01190	.000	-.3552	-.3048
	B3	-.66000*	.01190	.000	-.6852	-.6348
B23	B4	-2.09000*	.01190	.000	-2.1152	-2.0648
	B21	-.80000*	.01190	.000	-.8252	-.7748
	B22	-.59000*	.01190	.000	-.6152	-.5648
	B24	-.37000*	.01190	.000	-.3952	-.3448
	B1	.04000*	.01190	.004	.0148	.0652
	B2	.04000*	.01190	.004	.0148	.0652
B24	B3	-.29000*	.01190	.000	-.3152	-.2648
	B4	-1.72000*	.01190	.000	-1.7452	-1.6948
	B21	-.43000*	.01190	.000	-.4552	-.4048

			B22	-.22000*	.01190	.000	-.2452	-.1948
			B23	.37000*	.01190	.000	.3448	.3952
		B1	B2	.00000	.03149	1.000	-.0668	.0668
			B3	25.61000*	.03149	.000	25.5432	25.6768
			B4	17.53000*	.03149	.000	17.4632	17.5968
			B21	6.65000*	.03149	.000	6.5832	6.7168
			B22	9.33000*	.03149	.000	9.2632	9.3968
			B23	29.57000*	.03149	.000	29.5032	29.6368
			B24	22.78000*	.03149	.000	22.7132	22.8468
		B2	B1	.00000	.03149	1.000	-.0668	.0668
			B3	25.61000*	.03149	.000	25.5432	25.6768
			B4	17.53000*	.03149	.000	17.4632	17.5968
			B21	6.65000*	.03149	.000	6.5832	6.7168
			B22	9.33000*	.03149	.000	9.2632	9.3968
			B23	29.57000*	.03149	.000	29.5032	29.6368
NO3- (mg/kg)	LSD		B24	22.78000*	.03149	.000	22.7132	22.8468
		B3	B1	-25.61000*	.03149	.000	-25.6768	-25.5432
			B2	-25.61000*	.03149	.000	-25.6768	-25.5432
			B4	-8.08000*	.03149	.000	-8.1468	-8.0132
			B21	-18.96000*	.03149	.000	-19.0268	-18.8932
			B22	-16.28000*	.03149	.000	-16.3468	-16.2132
			B23	3.96000*	.03149	.000	3.8932	4.0268
			B24	-2.83000*	.03149	.000	-2.8968	-2.7632
		B4	B1	-17.53000*	.03149	.000	-17.5968	-17.4632
			B2	-17.53000*	.03149	.000	-17.5968	-17.4632
			B3	8.08000*	.03149	.000	8.0132	8.1468
			B21	-10.88000*	.03149	.000	-10.9468	-10.8132
			B22	-8.20000*	.03149	.000	-8.2668	-8.1332
			B23	12.04000*	.03149	.000	11.9732	12.1068

	B24	5.25000*	.03149	.000	5.1832	5.3168
	B1	-6.65000*	.03149	.000	-6.7168	-6.5832
	B2	-6.65000*	.03149	.000	-6.7168	-6.5832
	B3	18.96000*	.03149	.000	18.8932	19.0268
B21	B4	10.88000*	.03149	.000	10.8132	10.9468
	B22	2.68000*	.03149	.000	2.6132	2.7468
	B23	22.92000*	.03149	.000	22.8532	22.9868
	B24	16.13000*	.03149	.000	16.0632	16.1968
	B1	-9.33000*	.03149	.000	-9.3968	-9.2632
	B2	-9.33000*	.03149	.000	-9.3968	-9.2632
	B3	16.28000*	.03149	.000	16.2132	16.3468
B22	B4	8.20000*	.03149	.000	8.1332	8.2668
	B21	-2.68000*	.03149	.000	-2.7468	-2.6132
	B23	20.24000*	.03149	.000	20.1732	20.3068
	B24	13.45000*	.03149	.000	13.3832	13.5168
	B1	-29.57000*	.03149	.000	-29.6368	-29.5032
	B2	-29.57000*	.03149	.000	-29.6368	-29.5032
	B3	-3.96000*	.03149	.000	-4.0268	-3.8932
B23	B4	-12.04000*	.03149	.000	-12.1068	-11.9732
	B21	-22.92000*	.03149	.000	-22.9868	-22.8532
	B22	-20.24000*	.03149	.000	-20.3068	-20.1732
	B24	-6.79000*	.03149	.000	-6.8568	-6.7232
	B1	-22.78000*	.03149	.000	-22.8468	-22.7132
	B2	-22.78000*	.03149	.000	-22.8468	-22.7132
	B3	2.83000*	.03149	.000	2.7632	2.8968
B24	B4	-5.25000*	.03149	.000	-5.3168	-5.1832
	B21	-16.13000*	.03149	.000	-16.1968	-16.0632
	B22	-13.45000*	.03149	.000	-13.5168	-13.3832
	B23	6.79000*	.03149	.000	6.7232	6.8568

			B2	.00000	.02986	1.000	-.0633	.0633
			B3	19.05000*	.02986	.000	18.9867	19.1133
			B4	20.70000*	.02986	.000	20.6367	20.7633
		B1	B21	6.00000*	.02986	.000	5.9367	6.0633
			B22	16.18000*	.02986	.000	16.1167	16.2433
			B23	27.61000*	.02986	.000	27.5467	27.6733
			B24	23.74000*	.02986	.000	23.6767	23.8033
			B1	.00000	.02986	1.000	-.0633	.0633
			B3	19.05000*	.02986	.000	18.9867	19.1133
			B4	20.70000*	.02986	.000	20.6367	20.7633
		B2	B21	6.00000*	.02986	.000	5.9367	6.0633
			B22	16.18000*	.02986	.000	16.1167	16.2433
			B23	27.61000*	.02986	.000	27.5467	27.6733
			B24	23.74000*	.02986	.000	23.6767	23.8033
SO42- (mg/kg)	LSD		B1	-19.05000*	.02986	.000	-19.1133	-18.9867
			B2	-19.05000*	.02986	.000	-19.1133	-18.9867
			B4	1.65000*	.02986	.000	1.5867	1.7133
		B3	B21	-13.05000*	.02986	.000	-13.1133	-12.9867
			B22	-2.87000*	.02986	.000	-2.9333	-2.8067
			B23	8.56000*	.02986	.000	8.4967	8.6233
			B24	4.69000*	.02986	.000	4.6267	4.7533
			B1	-20.70000*	.02986	.000	-20.7633	-20.6367
			B2	-20.70000*	.02986	.000	-20.7633	-20.6367
			B3	-1.65000*	.02986	.000	-1.7133	-1.5867
		B4	B21	-14.70000*	.02986	.000	-14.7633	-14.6367
			B22	-4.52000*	.02986	.000	-4.5833	-4.4567
			B23	6.91000*	.02986	.000	6.8467	6.9733
			B24	3.04000*	.02986	.000	2.9767	3.1033
		B21	B1	-6.00000*	.02986	.000	-6.0633	-5.9367

			B2	-6.00000*	.02986	.000	-6.0633	-5.9367
			B3	13.05000*	.02986	.000	12.9867	13.1133
			B4	14.70000*	.02986	.000	14.6367	14.7633
			B22	10.18000*	.02986	.000	10.1167	10.2433
			B23	21.61000*	.02986	.000	21.5467	21.6733
			B24	17.74000*	.02986	.000	17.6767	17.8033
		B22	B1	-16.18000*	.02986	.000	-16.2433	-16.1167
			B2	-16.18000*	.02986	.000	-16.2433	-16.1167
			B3	2.87000*	.02986	.000	2.8067	2.9333
			B4	4.52000*	.02986	.000	4.4567	4.5833
			B21	-10.18000*	.02986	.000	-10.2433	-10.1167
			B23	11.43000*	.02986	.000	11.3667	11.4933
			B24	7.56000*	.02986	.000	7.4967	7.6233
		B23	B1	-27.61000*	.02986	.000	-27.6733	-27.5467
			B2	-27.61000*	.02986	.000	-27.6733	-27.5467
			B3	-8.56000*	.02986	.000	-8.6233	-8.4967
			B4	-6.91000*	.02986	.000	-6.9733	-6.8467
			B21	-21.61000*	.02986	.000	-21.6733	-21.5467
			B22	-11.43000*	.02986	.000	-11.4933	-11.3667
			B24	-3.87000*	.02986	.000	-3.9333	-3.8067
		B24	B1	-23.74000*	.02986	.000	-23.8033	-23.6767
			B2	-23.74000*	.02986	.000	-23.8033	-23.6767
			B3	-4.69000*	.02986	.000	-4.7533	-4.6267
			B4	-3.04000*	.02986	.000	-3.1033	-2.9767
			B21	-17.74000*	.02986	.000	-17.8033	-17.6767
			B22	-7.56000*	.02986	.000	-7.6233	-7.4967
			B23	3.87000*	.02986	.000	3.8067	3.9333
HCO3- (mg/kg)	LSD	B1	B2	.00000	.01291	1.000	-.0274	.0274
			B3	9.90000*	.01291	.000	9.8726	9.9274

		B4	-3.48000*	.01291	.000	-3.5074	-3.4526
		B21	11.24000*	.01291	.000	11.2126	11.2674
		B22	14.99000*	.01291	.000	14.9626	15.0174
		B23	13.64000*	.01291	.000	13.6126	13.6674
		B24	9.91000*	.01291	.000	9.8826	9.9374
		B1	.00000	.01291	1.000	-.0274	.0274
		B3	9.90000*	.01291	.000	9.8726	9.9274
		B4	-3.48000*	.01291	.000	-3.5074	-3.4526
	B2	B21	11.24000*	.01291	.000	11.2126	11.2674
		B22	14.99000*	.01291	.000	14.9626	15.0174
		B23	13.64000*	.01291	.000	13.6126	13.6674
		B24	9.91000*	.01291	.000	9.8826	9.9374
		B1	-9.90000*	.01291	.000	-9.9274	-9.8726
		B2	-9.90000*	.01291	.000	-9.9274	-9.8726
		B4	-13.38000*	.01291	.000	-13.4074	-13.3526
	B3	B21	1.34000*	.01291	.000	1.3126	1.3674
		B22	5.09000*	.01291	.000	5.0626	5.1174
		B23	3.74000*	.01291	.000	3.7126	3.7674
		B24	.01000	.01291	.450	-.0174	.0374
		B1	3.48000*	.01291	.000	3.4526	3.5074
		B2	3.48000*	.01291	.000	3.4526	3.5074
		B3	13.38000*	.01291	.000	13.3526	13.4074
	B4	B21	14.72000*	.01291	.000	14.6926	14.7474
		B22	18.47000*	.01291	.000	18.4426	18.4974
		B23	17.12000*	.01291	.000	17.0926	17.1474
		B24	13.39000*	.01291	.000	13.3626	13.4174
		B1	-11.24000*	.01291	.000	-11.2674	-11.2126
	B21	B2	-11.24000*	.01291	.000	-11.2674	-11.2126
		B3	-1.34000*	.01291	.000	-1.3674	-1.3126

			B4	-14.72000*	.01291	.000	-14.7474	-14.6926
			B22	3.75000*	.01291	.000	3.7226	3.7774
			B23	2.40000*	.01291	.000	2.3726	2.4274
			B24	-1.33000*	.01291	.000	-1.3574	-1.3026
		B22	B1	-14.99000*	.01291	.000	-15.0174	-14.9626
			B2	-14.99000*	.01291	.000	-15.0174	-14.9626
			B3	-5.09000*	.01291	.000	-5.1174	-5.0626
			B4	-18.47000*	.01291	.000	-18.4974	-18.4426
			B21	-3.75000*	.01291	.000	-3.7774	-3.7226
			B23	-1.35000*	.01291	.000	-1.3774	-1.3226
			B24	-5.08000*	.01291	.000	-5.1074	-5.0526
		B23	B1	-13.64000*	.01291	.000	-13.6674	-13.6126
			B2	-13.64000*	.01291	.000	-13.6674	-13.6126
			B3	-3.74000*	.01291	.000	-3.7674	-3.7126
			B4	-17.12000*	.01291	.000	-17.1474	-17.0926
			B21	-2.40000*	.01291	.000	-2.4274	-2.3726
			B22	1.35000*	.01291	.000	1.3226	1.3774
			B24	-3.73000*	.01291	.000	-3.7574	-3.7026
		B24	B1	-9.91000*	.01291	.000	-9.9374	-9.8826
			B2	-9.91000*	.01291	.000	-9.9374	-9.8826
			B3	-.01000	.01291	.450	-.0374	.0174
			B4	-13.39000*	.01291	.000	-13.4174	-13.3626
			B21	1.33000*	.01291	.000	1.3026	1.3574
			B22	5.08000*	.01291	.000	5.0526	5.1074
			B23	3.73000*	.01291	.000	3.7026	3.7574
NH4+ (mg/kg)	LSD	B1	B2	.00000	.01080	1.000	-.0229	.0229
			B3	-.79000*	.01080	.000	-.8129	-.7671
			B4	-1.12000*	.01080	.000	-1.1429	-1.0971
			B21	.56000*	.01080	.000	.5371	.5829

	B22	.68000*	.01080	.000	.6571	.7029
	B23	1.20000*	.01080	.000	1.1771	1.2229
	B24	1.22000*	.01080	.000	1.1971	1.2429
	B1	.00000	.01080	1.000	-.0229	.0229
	B3	-.79000*	.01080	.000	-.8129	-.7671
	B4	-1.12000*	.01080	.000	-1.1429	-1.0971
B2	B21	.56000*	.01080	.000	.5371	.5829
	B22	.68000*	.01080	.000	.6571	.7029
	B23	1.20000*	.01080	.000	1.1771	1.2229
	B24	1.22000*	.01080	.000	1.1971	1.2429
	B1	.79000*	.01080	.000	.7671	.8129
	B2	.79000*	.01080	.000	.7671	.8129
	B4	-.33000*	.01080	.000	-.3529	-.3071
B3	B21	1.35000*	.01080	.000	1.3271	1.3729
	B22	1.47000*	.01080	.000	1.4471	1.4929
	B23	1.99000*	.01080	.000	1.9671	2.0129
	B24	2.01000*	.01080	.000	1.9871	2.0329
	B1	1.12000*	.01080	.000	1.0971	1.1429
	B2	1.12000*	.01080	.000	1.0971	1.1429
	B3	.33000*	.01080	.000	.3071	.3529
B4	B21	1.68000*	.01080	.000	1.6571	1.7029
	B22	1.80000*	.01080	.000	1.7771	1.8229
	B23	2.32000*	.01080	.000	2.2971	2.3429
	B24	2.34000*	.01080	.000	2.3171	2.3629
	B1	-.56000*	.01080	.000	-.5829	-.5371
	B2	-.56000*	.01080	.000	-.5829	-.5371
B21	B3	-1.35000*	.01080	.000	-1.3729	-1.3271
	B4	-1.68000*	.01080	.000	-1.7029	-1.6571
	B22	.12000*	.01080	.000	.0971	.1429

			B23	.64000*	.01080	.000	.6171	.6629
			B24	.66000*	.01080	.000	.6371	.6829
		B22	B1	-.68000*	.01080	.000	-.7029	-.6571
			B2	-.68000*	.01080	.000	-.7029	-.6571
			B3	-1.47000*	.01080	.000	-1.4929	-1.4471
			B4	-1.80000*	.01080	.000	-1.8229	-1.7771
			B21	-.12000*	.01080	.000	-.1429	-.0971
			B23	.52000*	.01080	.000	.4971	.5429
			B24	.54000*	.01080	.000	.5171	.5629
		B23	B1	-1.20000*	.01080	.000	-1.2229	-1.1771
			B2	-1.20000*	.01080	.000	-1.2229	-1.1771
			B3	-1.99000*	.01080	.000	-2.0129	-1.9671
			B4	-2.32000*	.01080	.000	-2.3429	-2.2971
			B21	-.64000*	.01080	.000	-.6629	-.6171
			B22	-.52000*	.01080	.000	-.5429	-.4971
			B24	.02000	.01080	.083	-.0029	.0429
		B24	B1	-1.22000*	.01080	.000	-1.2429	-1.1971
			B2	-1.22000*	.01080	.000	-1.2429	-1.1971
			B3	-2.01000*	.01080	.000	-2.0329	-1.9871
			B4	-2.34000*	.01080	.000	-2.3629	-2.3171
			B21	-.66000*	.01080	.000	-.6829	-.6371
			B22	-.54000*	.01080	.000	-.5629	-.5171
			B23	-.02000	.01080	.083	-.0429	.0029
OC (%)	LSD	B1	B2	.00000	.01291	1.000	-.0274	.0274
			B3	1.59000*	.01291	.000	1.5626	1.6174
			B4	.66000*	.01291	.000	.6326	.6874
			B21	.87000*	.01291	.000	.8426	.8974
			B22	.33000*	.01291	.000	.3026	.3574
			B23	2.00000*	.01291	.000	1.9726	2.0274

	B24	1.78000*	.01291	.000	1.7526	1.8074
	B1	.00000	.01291	1.000	-.0274	.0274
	B3	1.59000*	.01291	.000	1.5626	1.6174
	B4	.66000*	.01291	.000	.6326	.6874
B2	B21	.87000*	.01291	.000	.8426	.8974
	B22	.33000*	.01291	.000	.3026	.3574
	B23	2.00000*	.01291	.000	1.9726	2.0274
	B24	1.78000*	.01291	.000	1.7526	1.8074
	B1	-1.59000*	.01291	.000	-1.6174	-1.5626
	B2	-1.59000*	.01291	.000	-1.6174	-1.5626
	B4	-.93000*	.01291	.000	-.9574	-.9026
B3	B21	-.72000*	.01291	.000	-.7474	-.6926
	B22	-1.26000*	.01291	.000	-1.2874	-1.2326
	B23	.41000*	.01291	.000	.3826	.4374
	B24	.19000*	.01291	.000	.1626	.2174
	B1	-.66000*	.01291	.000	-.6874	-.6326
	B2	-.66000*	.01291	.000	-.6874	-.6326
	B3	.93000*	.01291	.000	.9026	.9574
B4	B21	.21000*	.01291	.000	.1826	.2374
	B22	-.33000*	.01291	.000	-.3574	-.3026
	B23	1.34000*	.01291	.000	1.3126	1.3674
	B24	1.12000*	.01291	.000	1.0926	1.1474
	B1	-.87000*	.01291	.000	-.8974	-.8426
	B2	-.87000*	.01291	.000	-.8974	-.8426
	B3	.72000*	.01291	.000	.6926	.7474
B21	B4	-.21000*	.01291	.000	-.2374	-.1826
	B22	-.54000*	.01291	.000	-.5674	-.5126
	B23	1.13000*	.01291	.000	1.1026	1.1574
	B24	.91000*	.01291	.000	.8826	.9374

			B1	-.33000*	.01291	.000	-.3574	-.3026
			B2	-.33000*	.01291	.000	-.3574	-.3026
			B3	1.26000*	.01291	.000	1.2326	1.2874
		B22	B4	.33000*	.01291	.000	.3026	.3574
			B21	.54000*	.01291	.000	.5126	.5674
			B23	1.67000*	.01291	.000	1.6426	1.6974
			B24	1.45000*	.01291	.000	1.4226	1.4774
			B1	-2.00000*	.01291	.000	-2.0274	-1.9726
			B2	-2.00000*	.01291	.000	-2.0274	-1.9726
			B3	-.41000*	.01291	.000	-.4374	-.3826
		B23	B4	-1.34000*	.01291	.000	-1.3674	-1.3126
			B21	-1.13000*	.01291	.000	-1.1574	-1.1026
			B22	-1.67000*	.01291	.000	-1.6974	-1.6426
			B24	-.22000*	.01291	.000	-.2474	-.1926
			B1	-1.78000*	.01291	.000	-1.8074	-1.7526
			B2	-1.78000*	.01291	.000	-1.8074	-1.7526
			B3	-.19000*	.01291	.000	-.2174	-.1626
		B24	B4	-1.12000*	.01291	.000	-1.1474	-1.0926
			B21	-.91000*	.01291	.000	-.9374	-.8826
			B22	-1.45000*	.01291	.000	-1.4774	-1.4226
			B23	.22000*	.01291	.000	.1926	.2474
			B2	.00000	.01190	1.000	-.0252	.0252
			B3	2.34000*	.01190	.000	2.3148	2.3652
			B4	1.26000*	.01190	.000	1.2348	1.2852
OM (%)	LSD	B1	B21	.77000*	.01190	.000	.7448	.7952
			B22	.37000*	.01190	.000	.3448	.3952
			B23	3.24000*	.01190	.000	3.2148	3.2652
			B24	1.93000*	.01190	.000	1.9048	1.9552
		B2	B1	.00000	.01190	1.000	-.0252	.0252

	B3	2.34000*	.01190	.000	2.3148	2.3652
	B4	1.26000*	.01190	.000	1.2348	1.2852
	B21	.77000*	.01190	.000	.7448	.7952
	B22	.37000*	.01190	.000	.3448	.3952
	B23	3.24000*	.01190	.000	3.2148	3.2652
	B24	1.93000*	.01190	.000	1.9048	1.9552
B3	B1	-2.34000*	.01190	.000	-2.3652	-2.3148
	B2	-2.34000*	.01190	.000	-2.3652	-2.3148
	B4	-1.08000*	.01190	.000	-1.1052	-1.0548
	B21	-1.57000*	.01190	.000	-1.5952	-1.5448
	B22	-1.97000*	.01190	.000	-1.9952	-1.9448
	B23	.90000*	.01190	.000	.8748	.9252
	B24	-.41000*	.01190	.000	-.4352	-.3848
B4	B1	-1.26000*	.01190	.000	-1.2852	-1.2348
	B2	-1.26000*	.01190	.000	-1.2852	-1.2348
	B3	1.08000*	.01190	.000	1.0548	1.1052
	B21	-.49000*	.01190	.000	-.5152	-.4648
	B22	-.89000*	.01190	.000	-.9152	-.8648
	B23	1.98000*	.01190	.000	1.9548	2.0052
	B24	.67000*	.01190	.000	.6448	.6952
B21	B1	-.77000*	.01190	.000	-.7952	-.7448
	B2	-.77000*	.01190	.000	-.7952	-.7448
	B3	1.57000*	.01190	.000	1.5448	1.5952
	B4	.49000*	.01190	.000	.4648	.5152
	B22	-.40000*	.01190	.000	-.4252	-.3748
	B23	2.47000*	.01190	.000	2.4448	2.4952
	B24	1.16000*	.01190	.000	1.1348	1.1852
B22	B1	-.37000*	.01190	.000	-.3952	-.3448
	B2	-.37000*	.01190	.000	-.3952	-.3448

			B3	1.97000^{*}	.01190	.000	1.9448	1.9952
			B4	$.89000^{*}$	.01190	.000	.8648	.9152
			B21	$.40000^{*}$	.01190	.000	.3748	.4252
			B23	2.87000^{*}	.01190	.000	2.8448	2.8952
			B24	1.56000^{*}	.01190	.000	1.5348	1.5852
		B23	B1	-3.24000^{*}	.01190	.000	-3.2652	-3.2148
			B2	-3.24000^{*}	.01190	.000	-3.2652	-3.2148
			B3	$-.90000^{*}$	.01190	.000	-.9252	-.8748
			B4	-1.98000^{*}	.01190	.000	-2.0052	-1.9548
			B21	-2.47000^{*}	.01190	.000	-2.4952	-2.4448
			B22	-2.87000^{*}	.01190	.000	-2.8952	-2.8448
			B24	-1.31000^{*}	.01190	.000	-1.3352	-1.2848
		B24	B1	-1.93000^{*}	.01190	.000	-1.9552	-1.9048
			B2	-1.93000^{*}	.01190	.000	-1.9552	-1.9048
			B3	$.41000^{*}$	.01190	.000	.3848	.4352
			B4	$-.67000^{*}$	.01190	.000	-.6952	-.6448
			B21	-1.16000^{*}	.01190	.000	-1.1852	-1.1348
			B22	-1.56000^{*}	.01190	.000	-1.5852	-1.5348
			B23	1.31000^{*}	.01190	.000	1.2848	1.3352
N (%)	LSD	B1	B2	.00000	.00082	1.000	-.0017	.0017
			B3	$.00300^{*}$	.00082	.002	.0013	.0047
			B4	$-.00400^{*}$	.00082	.000	-.0057	-.0023
			B21	$.01200^{*}$	.00082	.000	.0103	.0137
			B22	$.00700^{*}$	.00082	.000	.0053	.0087
			B23	$.01800^{*}$	.00082	.000	.0163	.0197
			B24	$.02500^{*}$	.00082	.000	.0233	.0267
		B2	B1	.00000	.00082	1.000	-.0017	.0017
			B3	$.00300^{*}$	.00082	.002	.0013	.0047
			B4	$-.00400^{*}$	.00082	.000	-.0057	-.0023

	B21	.01200*	.00082	.000	.0103	.0137
	B22	.00700*	.00082	.000	.0053	.0087
	B23	.01800*	.00082	.000	.0163	.0197
	B24	.02500*	.00082	.000	.0233	.0267
B3	B1	-.00300*	.00082	.002	-.0047	-.0013
	B2	-.00300*	.00082	.002	-.0047	-.0013
	B4	-.00700*	.00082	.000	-.0087	-.0053
	B21	.00900*	.00082	.000	.0073	.0107
	B22	.00400*	.00082	.000	.0023	.0057
	B23	.01500*	.00082	.000	.0133	.0167
	B24	.02200*	.00082	.000	.0203	.0237
B4	B1	.00400*	.00082	.000	.0023	.0057
	B2	.00400*	.00082	.000	.0023	.0057
	B3	.00700*	.00082	.000	.0053	.0087
	B21	.01600*	.00082	.000	.0143	.0177
	B22	.01100*	.00082	.000	.0093	.0127
	B23	.02200*	.00082	.000	.0203	.0237
	B24	.02900*	.00082	.000	.0273	.0307
B21	B1	-.01200*	.00082	.000	-.0137	-.0103
	B2	-.01200*	.00082	.000	-.0137	-.0103
	B3	-.00900*	.00082	.000	-.0107	-.0073
	B4	-.01600*	.00082	.000	-.0177	-.0143
	B22	-.00500*	.00082	.000	-.0067	-.0033
	B23	.00600*	.00082	.000	.0043	.0077
	B24	.01300*	.00082	.000	.0113	.0147
B22	B1	-.00700*	.00082	.000	-.0087	-.0053
	B2	-.00700*	.00082	.000	-.0087	-.0053
	B3	-.00400*	.00082	.000	-.0057	-.0023
	B4	-.01100*	.00082	.000	-.0127	-.0093

			B21	.00500*	.00082	.000	.0033	.0067
			B23	.01100*	.00082	.000	.0093	.0127
			B24	.01800*	.00082	.000	.0163	.0197
		B23	B1	-.01800*	.00082	.000	-.0197	-.0163
			B2	-.01800*	.00082	.000	-.0197	-.0163
			B3	-.01500*	.00082	.000	-.0167	-.0133
			B4	-.02200*	.00082	.000	-.0237	-.0203
			B21	-.00600*	.00082	.000	-.0077	-.0043
			B22	-.01100*	.00082	.000	-.0127	-.0093
			B24	.00700*	.00082	.000	.0053	.0087
		B24	B1	-.02500*	.00082	.000	-.0267	-.0233
			B2	-.02500*	.00082	.000	-.0267	-.0233
			B3	-.02200*	.00082	.000	-.0237	-.0203
			B4	-.02900*	.00082	.000	-.0307	-.0273
			B21	-.01300*	.00082	.000	-.0147	-.0113
			B22	-.01800*	.00082	.000	-.0197	-.0163
			B23	-.00700*	.00082	.000	-.0087	-.0053
P	LSD	B1	B2	.00000	.01080	1.000	-.0229	.0229
			B3	-.04000*	.01080	.002	-.0629	-.0171
			B4	-.07000*	.01080	.000	-.0929	-.0471
			B21	.04000*	.01080	.002	.0171	.0629
			B22	.02000	.01080	.083	-.0029	.0429
			B23	-.01000	.01080	.368	-.0329	.0129
			B24	-.03000*	.01080	.013	-.0529	-.0071
		B2	B1	.00000	.01080	1.000	-.0229	.0229
			B3	-.04000*	.01080	.002	-.0629	-.0171
			B4	-.07000*	.01080	.000	-.0929	-.0471

	B21	.04000*	.01080	.002	.0171	.0629
	B22	.02000	.01080	.083	-.0029	.0429
	B23	-.01000	.01080	.368	-.0329	.0129
	B24	-.03000*	.01080	.013	-.0529	-.0071
	B1	.04000*	.01080	.002	.0171	.0629
	B2	.04000*	.01080	.002	.0171	.0629
	B4	-.03000*	.01080	.013	-.0529	-.0071
B3	B21	.08000*	.01080	.000	.0571	.1029
	B22	.06000*	.01080	.000	.0371	.0829
	B23	.03000*	.01080	.013	.0071	.0529
	B24	.01000	.01080	.368	-.0129	.0329
	B1	.07000*	.01080	.000	.0471	.0929
	B2	.07000*	.01080	.000	.0471	.0929
	B3	.03000*	.01080	.013	.0071	.0529
B4	B21	.11000*	.01080	.000	.0871	.1329
	B22	.09000*	.01080	.000	.0671	.1129
	B23	.06000*	.01080	.000	.0371	.0829
	B24	.04000*	.01080	.002	.0171	.0629
	B1	-.04000*	.01080	.002	-.0629	-.0171
	B2	-.04000*	.01080	.002	-.0629	-.0171
	B3	-.08000*	.01080	.000	-.1029	-.0571
B21	B4	-.11000*	.01080	.000	-.1329	-.0871
	B22	-.02000	.01080	.083	-.0429	.0029
	B23	-.05000*	.01080	.000	-.0729	-.0271
	B24	-.07000*	.01080	.000	-.0929	-.0471
B22	B1	-.02000	.01080	.083	-.0429	.0029

	B2	-.02000	.01080	.083	-.0429	.0029
	B3	-.06000*	.01080	.000	-.0829	-.0371
	B4	-.09000*	.01080	.000	-.1129	-.0671
	B21	.02000	.01080	.083	-.0029	.0429
	B23	-.03000*	.01080	.013	-.0529	-.0071
	B24	-.05000*	.01080	.000	-.0729	-.0271
	B1	.01000	.01080	.368	-.0129	.0329
	B2	.01000	.01080	.368	-.0129	.0329
	B3	-.03000*	.01080	.013	-.0529	-.0071
B23	B4	-.06000*	.01080	.000	-.0829	-.0371
	B21	.05000*	.01080	.000	.0271	.0729
	B22	.03000*	.01080	.013	.0071	.0529
	B24	-.02000	.01080	.083	-.0429	.0029
	B1	.03000*	.01080	.013	.0071	.0529
	B2	.03000*	.01080	.013	.0071	.0529
	B3	-.01000	.01080	.368	-.0329	.0129
B24	B4	-.04000*	.01080	.002	-.0629	-.0171
	B21	.07000*	.01080	.000	.0471	.0929
	B22	.05000*	.01080	.000	.0271	.0729
	B23	.02000	.01080	.083	-.0029	.0429

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

Na+ (mg/kg)

	Values	N	Subset for alpha = 0.05						
			1	2	3	4	5	6	7
Duncan[a]	B23	3	3.4500						
	B24	3		3.8200					
	B22	3			4.0400				
	B21	3				4.2500			
	B2	3					7.1500		
	B1	3					7.1500		
	B3	3						7.8800	
	B4	3							9.3100
	Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Ca2+ (mg/kg)

	Values	N	Subset for alpha = 0.05						
			1	2	3	4	5	6	7
Duncan[a]	B22	3	1.8100						
	B21	3		2.1400					
	B23	3			2.9100				
	B24	3				3.3800			
	B1	3					3.4500		
	B2	3					3.4600		
	B3	3						5.3200	
	B4	3							7.8300
	Sig.		1.000	1.000	1.000	1.000	.238	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Mg2+ (mg/kg)

	Values	N	Subset for alpha = 0.05						
			1	2	3	4	5	6	7
Duncan[a]	B22	3	1.2200						
	B21	3		1.6700					
	B24	3			2.0600				
	B23	3				2.5000			
	B1	3					2.5600		
	B2	3					2.5600		
	B4	3						3.9600	
	B3	3							4.5300
	Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

K+ (mg/kg)

	Values	N	Subset for alpha = 0.05						
			1	2	3	4	5	6	7
Duncan[a]	B23	3	3.4500						
	B1	3		3.7800					
	B2	3		3.7800					
	B24	3			3.8200				
	B22	3				4.0400			
	B3	3					4.1100		
	B21	3						4.2500	
	B4	3							5.5400
	Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

NO3- (mg/kg)

	Values	N	Subset for alpha = 0.05						
			1	2	3	4	5	6	7
Duncan[a]	B23	3	25.3600						
	B3	3		29.3200					
	B24	3			32.1500				
	B4	3				37.4000			
	B22	3					45.6000		
	B21	3						48.2800	
	B1	3							54.9300
	B2	3							54.9300
	Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

SO42- (mg/kg)

	Values	N	Subset for alpha = 0.05						
			1	2	3	4	5	6	7
Duncan[a]	B23	3	15.0000						
	B24	3		18.8700					
	B4	3			21.9100				
	B3	3				23.5600			
	B22	3					26.4300		
	B21	3						36.6100	
	B2	3							42.6100
	B1	3							42.6100
	Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

HCO_3^- (mg/kg)

	Values	N	Subset for alpha = 0.05					
			1	2	3	4	5	6
Duncan[a]	B22	3	20.2000					
	B23	3		21.5500				
	B21	3			23.9500			
	B24	3				25.2800		
	B3	3				25.2900		
	B1	3					35.1900	
	B2	3					35.1900	
	B4	3						38.6700
	Sig.		1.000	1.000	1.000	.450	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

NH_4^+ (mg/kg)

	Values	N	Subset for alpha = 0.05					
			1	2	3	4	5	6
Duncan[a]	B24	3	4.1400					
	B23	3	4.1600					
	B22	3		4.6800				
	B21	3			4.8000			
	B1	3				5.3600		
	B2	3				5.3600		
	B3	3					6.1500	
	B4	3						6.4800
	Sig.		.083	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

OC (%)

	Values	N	Subset for alpha = 0.05						
			1	2	3	4	5	6	7
Duncan[a]	B23	3	.4500						
	B24	3		.6700					
	B3	3			.8600				
	B21	3				1.5800			
	B4	3					1.7900		
	B22	3						2.1200	
	B2	3							2.4500
	B1	3							2.4500
	Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

OM (%)

	Values	N	Subset for alpha = 0.05						
			1	2	3	4	5	6	7
Duncan[a]	B23	3	.6400						
	B3	3		1.5400					
	B24	3			1.9500				
	B4	3				2.6200			
	B21	3					3.1100		
	B22	3						3.5100	
	B1	3							3.8800
	B2	3							3.8800
	Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

N (%)

	Values	N	Subset for alpha = 0.05						
			1	2	3	4	5	6	7
Duncan[a]	B24	3	.0560						
	B23	3		.0630					
	B21	3			.0690				
	B22	3				.0740			
	B3	3					.0780		
	B1	3						.0810	
	B2	3						.0810	
	B4	3							.0850
	Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

P

	Values	N	Subset for alpha = 0.05					
			1	2	3	4	5	6
Duncan[a]	B21	3	.2100					
	B22	3	.2300	.2300				
	B1	3		.2500	.2500			
	B2	3		.2500	.2500			
	B23	3			.2600	.2600		
	B24	3				.2800	.2800	
	B3	3					.2900	
	B4	3						.3200
	Sig.		.083	.097	.394	.083	.368	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.